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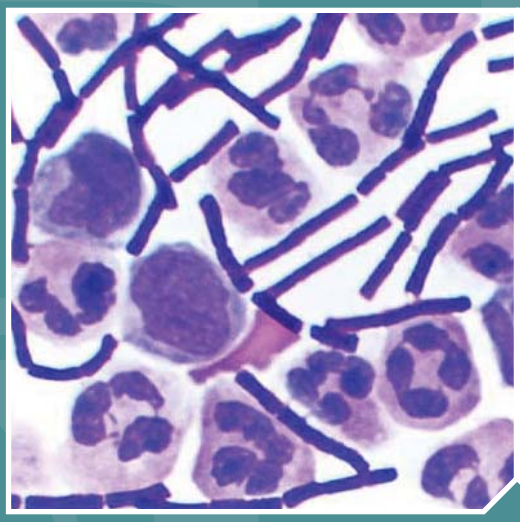
United States
Environmental Protection
Agency

Literature Review of Protocols for Processing Soils Contaminated with *Bacillus anthracis* Spores



Office of Research and Development
National Homeland Security Research Center

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U.S. Environmental Protection Agency
Office of Research and Development
National Homeland Security Research Center
Threat and Consequence Assessment Division

Disclaimer

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This document has been subjected to the Agency's review and has been approved for publication. Summaries of the literature found through this review are highlighted in this report. However, any research that was not conducted under EPA's stringent Quality Assurance Requirements could not be evaluated for accuracy, precision, representativeness, completeness, or comparability of the results and therefore no assurance can be made regarding the quality of the conclusions extracted from these publications. The contents of this document reflect the views of the contributors and do not necessarily reflect the views of the Agency. Mention of trade names or commercial products in this document or in the literature referenced in this document does not constitute endorsement or recommendation for use. Questions concerning this document or its application should be addressed to the EPA Task Order - Contract Officer Technical Representative:

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List of Acronyms and Abbreviations

°C	Degrees Celsius
µg	Microgram(s)
µm	Micrometer(s)
ABA	Anthrax Blood Agar ™
ATCC	American Type Culture Collection
AZ dust	Arizona Test Dust
BHI	Brain heart infusion (medium)
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
CEI	Cereus Ident Agar™
CFU	Colony forming unit(s)
ChrA	R & F® *anthracis* chromogenic agar
cm	Centimeter(s)
CTAB	Cetyltrimethylammonium bromide
DDAB	Didecyldimethylammonium bromide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
fg	Femtogram(s)
FITC	Fluorescein isothiocyanate
FL	Florida
g	Gram(s)
GABRI	Ground anthrax *Bacillus* refined identification
GEq	Genomic equivalents
GFP	Green fluorescent protein
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hr	Hour(s)
HSGS	High specific gravity separation
IgG	Immunoglobulin G
IM-ECL	Immunomagnetic-electrochemiluminescent
IMS	Immunomagnetic separation
ITS	Internally transcribed spacer (region)
l	Liquid medium
L	Liter(s)
LB	Luria broth
LC/MS	Liquid chromatography/mass spectrometry
LOD	Limit of detection
LRN	Laboratory Response Network

MATH	Microbial adherence to hydrocarbons
MEP	Mannitol-egg yolk-polymyxin B agar
min	Minute(s)
mL	Milliliter(s)
MN loam	Minnesota loam
N	Normal(ity)
NaOH	Sodium hydroxide
ND	Not determined
Nonidet P-40	Igepal, octylphenyl-polyethylene glycol
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline amended with 0.5% TWEEN® 20
PC-PLC	Phosphatidylcholine-specific phospholipase C
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction and denaturing gradient gel electrophoresis
PLET	Polymyxin B, lysozyme, ethylenediaminetetraacetic acid, thallous acetate agar
PVPP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RBMS	Reference background matrix soil
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism (DNA analysis)
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RV-PCR	Rapid viability polymerase chain reaction
s	Solid agar medium
SASP	Small acid-soluble proteins
SBA	Sheep blood agar
SBD	Synthetic building debris
SDS	Sodium dodecyl sulfate
SHMP	Sodium hexametaphosphate
TFA	Trifluoroacetic acid
Triton® X-100	4-(1,1,3,3-Tetramethylbutyl)phenylpolyethylene glycol
TSA II	Trypticase® soy agar with 5% sheep blood
TSB	Trypticase® soy broth
TWEEN® 20	Polyethylene glycol sorbitan monolaurate
TWEEN® 80	Polysorbate 80
UV	Ultraviolet

List of Trademarks

Trademarked Name	Registration Holder
Anthrax Blood Agar™	ABA, Heipha, Germany
Bacto™	Bacto Laboratories Pty Ltd, Mt. Pritchard, NSW, Australia
BioRobot™	Qiagen, Valencia, CA
Cereus Ident Agar ™	Heipha, Eppelheim, Germany
Chelex®	Bio-Rad, Life Science Division, Hercules, CA
Nonidet™	Shell Chemical Co, Houston, TX
NucliSENS®	bioMérieux, Inc, Durham, NC
Nycodenz®	PROGEN Biotechnik, Heidelburg Germany
PickPen®	Bio-Nobile, San Diego, CA
PowerVac	Mo Bio Laboratories, Inc., Carlsbad, CA
PrepMan®	Applied Biosystsems, Foster City, CA
R & F®	R&F Laboratories, Downers Grove, IL
Sephadex®	Amersham Biosciences AB Corporation, Uppsala, Sweden
Stomacher®	Seward Limited, West Sussex, UK
Triton®	Dow Chemical Company, Midland, MI
Trypticase®	Becton, Dickinson and Company, Franklin Lakes, NJ
TWEEN®	ICI Americas, Bridgewater, NJ
Waring®	Conair Corporation, Stamford, CT

Executive Summary

Bacillus anthracis (*B. anthracis*) spores are small, light in weight, and persistent. Many organizations have sought to identify and quantify the presence of *B. anthracis* spores within the environment. However, due to the number of other organisms and impeding chemical constituents within soil, identifying virulent *B. anthracis* within soil is a difficult task. Regardless of the detection assay, the initial sample must be processed efficiently to ensure that debris, chemical components, and biological impurities do not obstruct downstream detection. Without appropriate sample processing, the most sensitive detection assay will be ineffective. Therefore, the objective of this project was to consolidate information regarding sampling and processing protocols that have been investigated in the literature for multiple soil types. Open literature searches were performed to collect and summarize over 100 pertinent documents, focusing primarily on data gleaned in the last decade, regarding the processing of soils contaminated with *B. anthracis*.

Soil sample processing protocols can be divided into two general types: direct and indirect. For indirect processing, spores and soil particles are separated prior to downstream detection. Direct processes utilize a soil sample without first separating the spores from the bulk sample. Direct and indirect processing steps each have associated advantages and disadvantages. Indirect sample separation steps increase the proportion of target spores within the final detected sample; however, spore loss prior to detection also increases. For directly processed samples, there is potential for background organisms to overwhelm detection technologies and prevent target spores from being observed.

There are two requirements for successful indirect isolation of *B. anthracis* from soil samples: dissociate the spores from the soil particles and physically separate the free spores from the soil particles. Adding an aqueous carrier medium to a soil sample creates a sample slurry for easier manipulation. While water has been utilized, chemical additives have often been included to aid spore-soil dissociation. While some authors found that the carrier medium (or spore extraction solution) was the most important factor influencing the extraction efficiency of spores from wipes, others stated that the presence of a detergent in the aqueous carrier medium consistently improved the separation of spores from soil particles over buffer or water alone. No consensus on an optimum aqueous carrier medium could be determined from the reviewed literature. Additional research focusing on the aqueous carrier medium for processing multiple soil types under uniform dissociation and separation conditions is needed.

Centrifugation, high specific gravity separation (HSGS), immunomagnetic separation (IMS), filtration, and settling have been used by various researchers to physically separate spores from soil. To some authors, the utility of IMS for environmental samples was concerning, while others presented several advantages to using IMS including ease of use, utility for large numbers of samples, and shorter processing times compared to conventional protocols. Filtration showed promise in being able to rapidly separate spores from diverse matrices. Future work that combines an optimized aqueous carrier medium with the filtration may further increase recovery rates.

Direct soil processing falls under two principle types: culturing on *B. anthracis* selective agar and bulk DNA extraction. Researchers have sought a *B. anthracis* specific agar medium that deters background organisms and other non-*anthracis Bacillus* species and yet allows *B. anthracis* propagation and identification. Several selective media for *B. anthracis* have been developed: mannitol-egg yolk-polymyxin B agar (MEP), R & F® *anthracis* chromogenic agar (ChrA) and Cereus Ident Agar ™ (CEI). Additionally, anthrax Blood Agar™ (ABA) is a nutrient medium containing sheep blood and supplements to inhibit many fast-growing organisms. The medium that showed the most promise in the literature was Modified Polymyxin B, lysozyme, ethylenediaminetetraacetic acid, thallous acetate agar (PLET) which includes antibiotics and lysozyme to inhibit the growth of other *Bacillus* species.

Numerous kits are available from vendors that are specific for DNA extraction from soil samples. There are many advantages to using a commercial kit for bulk DNA extraction. Unfortunately, due to the difference in study designs and tested soil conditions, it is difficult to determine an overall optimum DNA extraction kit from the currently available data. An extraction kit optimization study using multiple soil types and uniform detection conditions is needed to elucidate an ideal DNA extraction kit for multiple soils.

As shown through this literature review, an optimized soil processing protocol with a known recovery rate and associated confidence intervals is needed. A reliable processing protocol would allow for multiple technicians and laboratories to produce high quality, uniform results in the event of a *B. anthracis* release. Recovery rates and confidence intervals would aid downstream human health and consequence decisions.

1.0 Introduction

Soil is a complex matrix with multiple components and a plethora of microbial activities. *Soil* as defined by the US Department of Agriculture's National Resources Conservation Service is comprised of solids, liquid, and gases that occur on the land surface and is characterized by layers that are distinguishable from the initial material or by the ability to support rooted plants in a natural environment (1). The properties of a soil fluctuate with time as weather patterns and plant growth cycles directly affect soil conditions. For this reason, pH, soluble salts, organic mass, flora, fauna, temperature, moisture, and the number of microorganisms all change with the seasons and over extended periods of time (1). There are numerous types and conditions of soils around the globe, each with specific components and compounds. The US Department of Agriculture has supplied soil with its own taxonomic classification system, which designates the following categories listed in decreasing rank: order, suborder, great group, subgroup, family, and series. All 12 orders and 60 of the 64 suborders of soil are present within the surface area of the United States and its territories (1).

One gram of soil reportedly contains up to 10 billion microorganisms and thousands of different species (2). In addition, chemical constituents of soil: organics, humic acids, etc., can interfere with the chemistry involved in downstream microbiological detection assays (3-10). An understanding of the environmental distribution of bacterial pathogens and their fate over time in nature is needed for multiple applications, including the determination of risk to wildlife, livestock, and humans in any given area, and distinguishing between natural and anthropogenic sources during an epidemic. However, due to the number of organisms and impeding chemical constituents within soil, identifying a single virulent organism within a soil sample is a difficult task.

Exposure of humans to *Bacillus anthracis* (*B. anthracis*) has been historically associated with agricultural contact with infected animals. The most common route of exposure for humans is through cutaneous exposure, while naturally occurring ingestion and inhalational exposures are rare. As a Gram–positive spore-forming pathogen, *B. anthracis* spores can survive extreme heat and drought for extensive periods. Global trade of goods and products has dispersed the organism worldwide. Currently there are endemic anthrax foci on all continents except Antarctica. Thus, *B. anthracis* is a naturally occurring organism in many soil environments (11). Close relatives of *B. anthracis* can be collocated in the soil environments (12), making detection of *B. anthracis* in soils even more complicated. There have been multiple reviews detailing the various detection assays for *B. anthracis* (13-15); however, the added complexity of processing soil samples for microbiological assessment is often neglected. The report herein will compile soil sampling and processing information acquired from research conducted within the last decade.

1.1 Characteristics of *B. anthracis*

There are a number of theories regarding the lifecycle of *B. anthracis* in soil. The predominant theory is that *B. anthracis* is an obligate pathogen with little propagation occurring directly within soil, but rather, the soil acts as a holding site from which the hosts may ingest or inhale the spores (16, 17). Within the classic *B. anthracis* lifecycle (Figure 1), vegetative *B. anthracis* propagates in a host to concentrations in the millions of bacteria per milliliter of blood, producing toxins that kill the host (18). Sporulation is initiated when predators (or other events) open a carcass, allowing the bodily fluids to drain from the infected carcass, vegetative cells are placed into the surrounding environment, and nutrients are depleted (19-23). There is a high level of uncertainty regarding the factors leading to the initial case or cases of an anthrax epizootic. Spores can persist in soil for years (18, 24), and yet there can be decades between outbreaks; there is no clear understanding of the dormancy period. However, once a spore encounters suitable environment, it will germinate, proliferate, and start the cycle again.

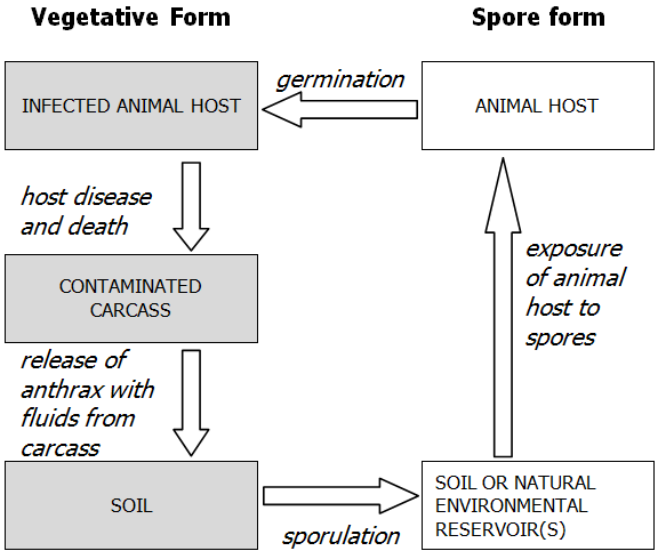

Figure 1. Classic *B. anthracis* natural lifecycle.

Figure adapted from (17).

Within the classic theory, vegetative *B. anthracis* does not survive in the environment (25), and multiplication does not occur at carcass sites (18). However, there are other hypotheses that explain persistence of *B. anthracis*. Some propose that *B. anthracis* spores can germinate and multiply vegetatively, in the rhizosphere of grass (*Festuca arundinaceae*) (26) or in the gut of earthworms (*Eisenia fetida*) (17, 27). Evidence of *B. anthracis* spores germinating, replicating, and re-sporulating in co-culture with the soil-dwelling amoeba *Acanthamoeba castellanii* and *Hartmannella vermiformis* under simulated moist soil environments has been presented within the open literature (28). Other work postulates that soil biofilms may play a role in the *B. anthracis* lifecycle (29) or that bacteriophage infection of *B. anthracis* may restore saprophytic functionality necessary for replication and survival (17, 27). Each of these hypotheses remains controversial.

Regardless of how *B. anthracis* spores came to a soil, it is generally accepted that some soils are more prone to harboring spores than others, and weather conditions influence the occurrence of environmental anthrax cases. *B. anthracis* is most often found in dry conditions with soils that are high in organic deposits and calcium and are relatively alkaline (above pH 6) (21, 22, 30). Louis Pasteur conjectured that oral cavity trauma experienced during drought conditions increases the chance of a grazing animal to acquire anthrax from spores retained within the soil (31).

*S*pores are formed as a survival mechanism when *B. anthracis* vegetative cells experience nutrient-limiting conditions. Spores are metabolically dormant and extremely resistant to environmental stresses (32, 33). *B. anthracis* spores have a diameter of approximately 1 – 1.5 µm (34). The spore is composed of a series of concentric layers; the innermost layer is the core, surrounded by a peptidoglycan layer called the cortex and two protein layers known as the spore coat and the exosporidium (outermost layer) (35). Each layer aids in protecting the viability of the spore (35). The chromosome, along with tightly bound small acid-soluble proteins (SASPs), are found at the center of the spore core (36). High levels of calcium dipicolinic acid and the SASPs protect the core DNA from ultraviolet (UV) degradation, while the core membrane and the cortex work together to keep the core dry (35, 37). The coat protects the core from foreign materials entering, while the exterior exosporidium surface-proteins interact with the environment (35, 38). Interestingly, *Bacillus* spp. directly purified from natural soil environments have been shown to have higher intrinsic UV resistance than laboratory strains, suggesting that sporulation physiology may play a role in determining spore UV resistance (39).

Fully virulent *B. anthracis* includes two large plasmids, pX01 and pX02. The pX01 plasmid contains three genes (*pag, lef,* and *cya)* which code for three proteins (protective antigen, lethal factor, and edema factor, respectively) that make up the anthrax toxin (13). The pX02 plasmid carries the proteins required for encapsulation through the *cap A, cap B,* and *cap C* genes (40). Encapsulation is important for virulence; however, the mechanisms by which encapsulation contributes to virulence have not been determined (41).

1.2 Persistence of *B. anthracis* Spores in Soil

Studies of laboratory-stored soils have shown that *B. anthracis* can remain viable for extensive periods. Sinclair et al. (42) compiled a literature review of persistence of category A agents in the environment and found several soil studies in which virulent *B. anthracis* remained viable in soil samples for up to 68 years (43-46). The multiple protective layers surrounding individual spores allow them to survive harsh environmental conditions for periods ranging from decades to centuries, during which time spores are thought to migrate within the soil following the flow of water (47). Hendriksen and Hansen (48) found vertical dispersal of *B. thuringiensis* in a field to be significant. Over 50% of the *B. thuringiensis* spores within the topsoil migrated deeper into the soil over a five-year period. However, the same study determined horizontal dispersion after seven years to be limited. Similarly, Manchee et al. (49) described viable *B. anthracis* dispersed on Gruinard Island to be within the top 10 cm of soil after 40 years, while the horizontal dispersal pattern had not changed significantly from the original release locations (21).

Blackburn et al. (50) developed an ecological niche modeling tool to predict the geographical distribution of *B. anthracis* across the continental United States. The study depicts a significant corridor of increased *B. anthracis* presence running north to south from Canada to Mexico. Griffin et al. (51) were able to confirm the existence of *B. anthracis* isolates within a similar transect of North American soils. Historically, the identified areas follow cattle trails (50). In many instances, recent anthrax cases are associated with old graves of anthrax stricken animals and adequate soil conditions (21, 51, 52). Many researchers have sought to identify and quantify the presence of *B. anthracis* within the environment. However, due to the number of background organisms and impeding chemical constituents within soil, identifying *B. anthracis* within soil is a difficult task.

1.3 Purpose

A complete method for detection of *B. anthracis* spores in soil would likely include details regarding soil collection, transport, processing, analysis, and quality assurance standards for each step. A detailed method would allow for multiple technicians and laboratories to produce high quality uniform results in the event of a wide area *B. anthracis* release. A fully developed method would be useful for determining the presence of *B. anthracis* spores, their viability, and the extent of contamination. Multiple protocols have been developed either to separate spores from soil samples before microbiological assessment or to directly extract bulk DNA to identify the initial organism(s) present within the soil. However, these studies have never been integrated to determine the overall breadth of knowledge regarding the processing efficiency. Therefore, the objective of this project is to consolidate information acquired from previous research, focusing primarily on data gleaned in the last decade, regarding the processing of soils contaminated with *B. anthracis*.

This review is intended to provide a summary of sampling and processing protocols that have been investigated in the literature for multiple soil types. Open-literature searches of PubMed, Google Scholar, and the Battelle Library using the search criteria "*Bacillus anthracis*," "soil," and "soil microbiology" were used to collect nearly 100 pertinent documents. In addition, a reference list was supplied by EPA during the project. The table in the Appendix A outlines a brief synopsis of each applicable study including the organism strain, soil type, sample processing protocol, DNA extraction protocol, detection assay, and limit of detection (LOD) determined during the various studies discussed within this review. Detailed discussions of the study results are presented hereafter.

2.0 Current State of the Science

Multiple reviews have focused on various *B. anthracis* detection assays (13-15). However, previous reviews have not included an in-depth discussion of various soil sample processing protocols for microbiological assessment. Regardless of detection assay, the initial sample must be processed efficiently to ensure that debris, chemical components, and biological impurities do not obstruct microbiological detection. Without appropriate sample processing, the most sensitive detection assay will be ineffective. As pointed out in a review by Lim et al. (53) there is a need for a universal sample processing protocol to separate, concentrate, and purify target agents from any sample type. Recovery efficiency is a critical factor in determining an ideal processing protocol. A careful balance must be attained between ensuring that the maximum number of spores and a minimum amount of debris and chemical constituents are retained in the final sample. In addition, spore viability may be of concern, especially in the cases where confirmatory culturing or sample archiving is required. Unfortunately, recovery efficiency data are lacking for many processing protocols. While there are many *B. anthracis* detection assays, few of these assays can be utilized directly with an environmental soil sample. Therefore, sample processing protocols are used to isolate and concentrate spores from a bulk soil sample. Soil sample processing protocols can be subdivided into two general types: indirect and direct. For indirect processing, spores and soil particles are separated prior to downstream detection. Conversely, direct processes utilize a soil sample without first separating the spores from the bulk sample.

2.1 Indirect Processing: Separating *B. anthracis* from Soil

Because spores have the potential to adhere to large soil aggregates (39), there are two requirements for successful isolation of *B. anthracis* from soil samples: dissociate the spores from the soil particles and separate the free spores physically from the soil particles. Protocols for spore purification from soil particles involve steps to accomplish both of these objectives. The most common types of processing protocols can be broken down into three steps with the first two working together to disrupt spore-soil interactions. The three processing steps are: (1) introduce an aliquot of soil to an aqueous carrier medium; (2) mix the soil with the liquid to aid in chemical and physical disassociation of spores from soil aggregates; and (3) separate and concentrate spores away from soil particulates (Figure 2). In some cases, additional steps are taken to concentrate and purify the final spore sample further.

Figure 2. Indirect soil processing flow diagram.

2.1.1 Aqueous carrier media

The hydrophobic exosporidium of *B. anthracis* interacts with solid soil particles and requires treatment prior to efficient spore recovery (54, 55). Adding an aqueous carrier medium to a soil

sample creates a sample slurry that can be manipulated easily. While deionized water has been utilized, chemical additives (buffers, chelating agents, surfactants, salts, emulsifiers) are often included to aid spore-soil dissociation. Chelating agents (e.g., ethylenediaminetetraacetic acid [EDTA], Chelex® 100) and surfactants (e.g., Triton™ X-100, TWEEN® 20, TWEEN® 80, sodium dodecyl sulfate [SDS]) promote desorption of spores from soil particles, whereas salt solutions (sodium chloride, aluminum sulfate) form a complex and precipitate extracellular DNA and humic acids present within the soil (56). In a study conducted by DaSilva et al. (57), the carrier medium (or spore extraction solution) was the most important factor influencing the efficiency of extracting spores from wipes.

Within the reviewed studies, there were many different aqueous media used to separate spores from soil samples. The most common type of carrier medium was a buffered solution or a buffer solution with a surfactant (Appendix A). As previously mentioned, recovery efficiency data are lacking in many studies. Table 1 outlines 10 studies that included recovery efficiency information. Among these 10 studies were 14 soil types and 12 aqueous carrier media. Studies in which recovery efficiency data were lacking or which looked at aqueous carrier media for matrices other than soil (58, 59) are briefly summarized in Appendix A.

Triton™ X-100, TWEEN® 20, TWEEN® 80, and Nonidet™ P-40 are nonionic detergents used to disrupt hydrophobic interactions between the spores and soil particles. Dragon and Rennie (60) compared Nonidet™ P 40 to Triton™ X-100, and concluded that Triton™ X-100 was the better detergent for separating spores from soil particles. However, no statistical results were presented to support this conclusion. Rastogi et al. (61) noted that a pre-study experiment showed no statistical difference in spore recovery between Triton™ X-100, TWEEN® 20, and TWEEN® 80; however, the results were not detailed within the report. Da Silva et al. (57), in a study assessing spore separation from wipes, concluded that the extraction solution (carrier solution) PBS was the worst of those tested but the addition of TWEEN® 80 significantly improved recovery efficiencies. While no study provided statistical evidence for an optimized aqueous carrier medium, the individual studies each concluded that the addition of a surfactant aided spore recovery when compared to PBS or sucrose solutions alone (57, 60).

Dabiré et al. (62) compared a weak 0.1 Normal (N) sodium hydroxide (NaOH) solution to sterile deionized water. The NaOH solution was meant to disrupt aggregates of sandy clay and clay soils through chemical interaction to release the *Pasteuria penetrans* spores. The basic solution increased the recovery rates but not by a significant amount. Similarly, two other studies tested the efficiency of a weak salt solution (0.08% sodium chloride). Santana et al. (63) reported acceptable results while Ehlers et al. (64) found that deionized water alone yielded better recovery rates from tropical soil samples.

As Table 1 demonstrates, spore recovery efficiency varied depending on the soil type and aqueous carrier medium. Hong-Geller et al. (65) noted differences between strains of *B. anthracis* with avirulent Sterne strain being more easily separated from wipes than the virulent Ames strain. A number of other parameters not detailed within the table may also have influenced the overall extraction efficiency (i.e., sample age, sample amount, dissociation protocol, detection assay). Determination of an optimum aqueous carrier medium from the available information is therefore difficult.

Table 1. Spore Separation Aqueous Carrier Media (Percent Recovery)

Reference	Soil Type	Species	Detection Assay	Sterile deionized water	Triton™ X-100 in water	Tween® 80 in water	Nonidet™ P-40 in water	PBS	Tween 20 in PBS	Tween 80 in PBS	Sucrose†	Triton X-100 in sucrose†	Tween 20 in sucrose†	NaCl solution	NaOH
Dabiré *et al.*, 2001 (62)	10 g sandy clay, clay	*Pasteuria penetrans*	Malassez counting chamber	50, 22	-	-	-	-	-	-	-	-	-	-	55, 23
Dragon and Rennie, 2001 (60)	2.5 g field soil, wallow soil, potting soil	*B. anthracis*	PLET plates	0.2, 6, 13	0.2, 6, 13	-	0.2, 6, 13	-	-	-	1, 6, 23	4.5, 6, 28	4.0, 6, 28	-	-
Ehlers *et al.*, 2008 (64)	Tropical soil	Mixed community	Microscopy direct counts	10.6	-	-	-	-	-	-	-	-	-	4.6	-
Marston *et al.*, 2008(66)	TX soil, AZ dust	*B. anthracis*	PLET and ChrA plates	-	-	-	-	-	0.5 - 7.7	-	-	-	-	-	-
Santana *et al.*, 2008 (63)	1 g Venezuelan soils	*B. thuringiensis*	LB plates	-	-	-	-	-	-	-	-	-	-	60	-
Fitzpatrick *et al.*, 2010 (67)	5 g USA soils	*Coxiella burnetii*	PCR	-	-	-	-	4.3	-	-	-	-	-	-	-
Hong-Geller *et al.*, 2010 (65)	Swabs and wipes off surfaces	*B. anthracis* Sterne, Ames	qPCR	-	-	-	-	-	Sterne: 62-90 Ames: 2-75	-	-	-	-	-	-
Bradley *et al.*, 2011 (68)	1.0 g AZ dust, MN loam, potting soil, sand	*B. anthracis* Sterne	PLET plates	-	-	-	-	-	29, 17, 17, 51*	-	-	5, 3.7, 9, 5.8	-	-	-
Da Silva *et al.*, 2011 (57)	No soil, wipes	*B. anthracis* Sterne	LB plates	40-80	-	75-100	-	3-10	-	90-100	-	-	-	-	-
Isabel, 2012 (69)	0.2 g garden soil	*B. atrophaeus*	qPCR	-	-	-	-	51	-	-	-	-	-	-	-

AZ dust – Arizona test dust
LB – Luria broth
MN loam – Minnesota loam
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PLET – Polymyxin B, lysozyme, ethylenediaminetetraacetic acid, thallous acetate agar
qPCR – Quantitative polymerase chain reaction
SBA – Sheep blood agar
- –Not Tested

*TWEEN® 20 in PBS used with automatic immunomagnetic separation (IMS) procedure.
† - Sucrose at 1.22 g mL-1

2.1.2 Spore-soil disassociation

Microbial cells are tightly bound to soil colloids with clay and organic matter posing particular challenges in spore-soil separation (10). In an experiment conducted by Nicholson et al. (39), 99% of the natural spores present in a sandy test soil were associated with the soil aggregates and not within the aqueous carrier medium, indicating that additional steps are needed to dissociate the spores from the soil. Chemical additives to the aqueous carrier medium are used to help disassociate spores from soil; however, physical means are also utilized. Physical agitation has taken the form of manual shaking, gentle agitation, use of Stomacher® laboratory blending paddle, use of blenders, vortexing, sonication, and/or bead beating.

Dabiré et al. (62) noted that more energetic dispersion protocols yielded greater spore recovery efficiencies. Dissociation of large soil aggregates was suggested as the primary cause for the increased spore recoveries. Other studies have confirmed that more energetic dispersion protocols aid in overall recovery rates. Da Silva et al. (57) determined that vortexing was statistically superior to sonication for separating *B. anthracis* from wipe samples. Similarly, Courtois et al. (70) saw enhanced homogenization using a Waring blender over sonication or chemical treatment alone. Lindahl and Bakken (71) noted that ultrasonication treatment and shaking were inferior dispersion protocols when compared to using a Waring® blender. Even with significant physical disruption, spore-soil interactions are powerful and may be only slightly interrupted by physical agitation (39). An estimated 35% - 55% of the spores remained with large stable aggregates following total soil disruption with agate marbles (62).

2.1.3 Physical separation of spores from soil

After spore-soil disassociation, spores can be separated physically from soil particles. While some protocols do not require debris-free sample material for downstream detection assays (culture, direct DNA extraction followed by molecular detection), many assays have higher sensitivities with purified samples. High and low speed centrifugation, high specific gravity separation (HSGS), immunomagnetic separation (IMS), filtration, and extended settling times have each been utilized with varying success (Figure 3).

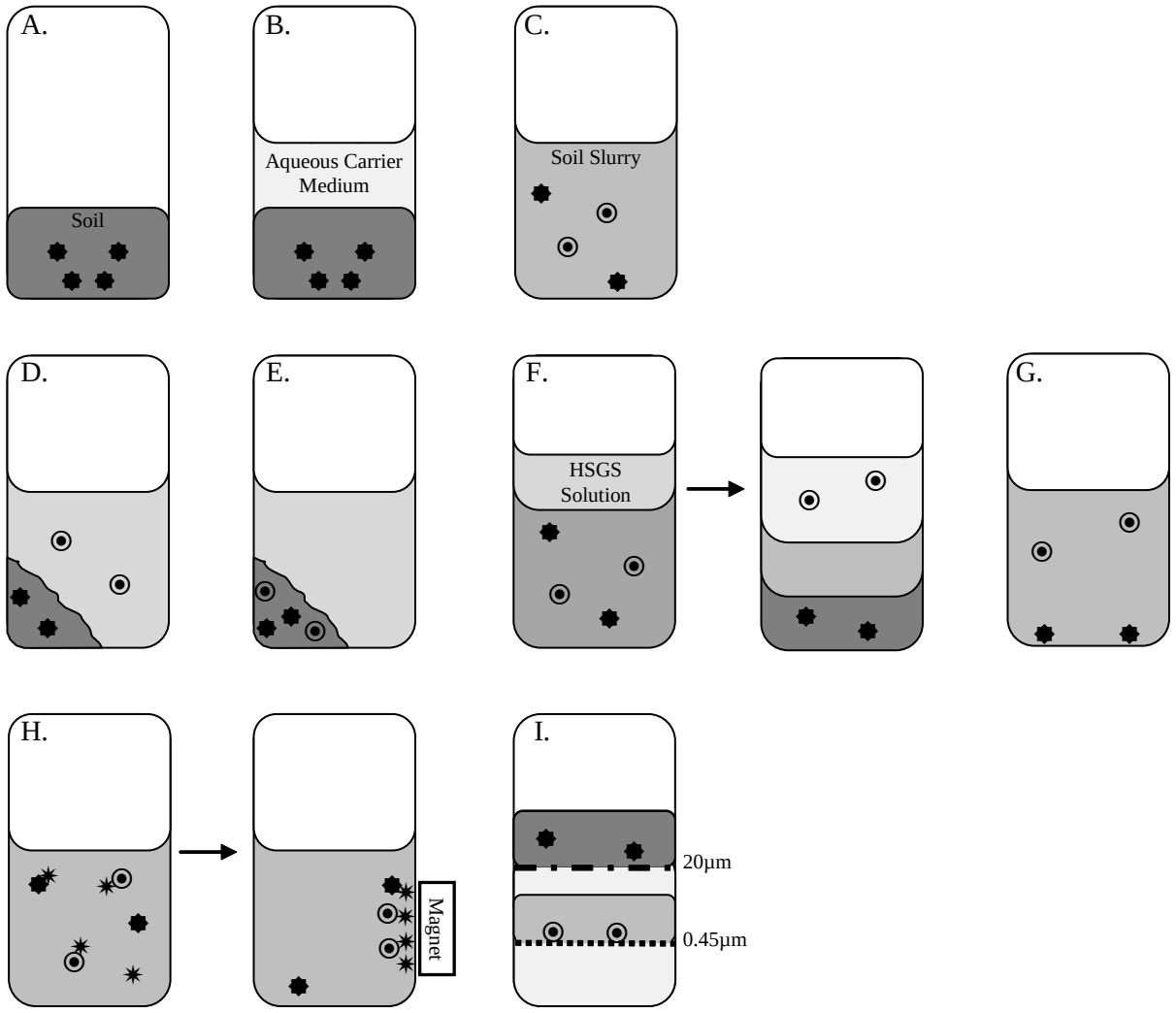

Figure 3. Indirect soil processing steps. A. Initial soil sample with soil-bound spores (✹). B. Soil sample with added aqueous carrier medium. C. Soil slurry with soil-bound spores and dissociated spores (⦿). D – I. Separation and Concentration methods; Density Separation via: D. Low-speed centrifugation; E. High-speed centrifugation; F. High specific gravity separation; G. Settling. H. Affinity capture using antibody-labeled magnetic beads (✶). I. Filtration with 20 µm and 0.45 µm pore size filters.

2.1.3.1 Density Separation

Low-speed centrifugation precipitates only dense soil particles leaving the more buoyant free dissociated spores within the supernatant. Spores remaining bound to soil particles after dissociation steps are removed with the soil particles. Spores within the supernatant can be detected directly or concentrated through additional steps. Fitzpatrick et al. (67) and Roh et al. (67, 72) used low speed centrifugation (123 × g and 2900 × g, respectively) to separate soil particles from the microbial cell fraction before DNA extraction. However, neither study specifically targeted *B. anthracis* within the soil samples. Fitzpatrick et al. (67) recovered less than 7% of the *Coxiella burnetii* present within the sandy soil samples, while Roh et al. (72)

concluded that separation of cells prior to DNA extraction (indirect DNA extraction) yielded a lower quantity of higher quality DNA extracts when compared to directly extracted soil samples. As part of the isolation steps of the GABRI (ground anthrax *Bacillus* refined identification) protocol, low speed centrifugation (657× g) of the soil sample is combined with incubation of the supernatant (54°C for 20 min) prior to plating on agar (73). Using GABRI (followed by DNA extraction and PCR), *B. anthracis* was isolated from 16/20 soil samples, but specific performance data for the protocol were not available (73). A slightly modified version of the GABRI method which used a 2000 rpm centrifugation speed, an incubation temperature of 64°C for 20 min, and addition of 50 μg/μL of Fosfomycin to the supernatant, was able to isolate *B. anthracis* from 100% of spiked and naturally contaminated soil samples in the study (74).

In contrast to low-speed centrifugation, high-speed centrifugation precipitates free spores along with other microorganisms or soil particles present in the initial suspension. Therefore, high-speed centrifugation is typically used to wash away humic acids and extracellular DNA within a soil sample before further analysis (7). Seven studies herein utilized a high-speed centrifugation step to aid in pre-washing the soil samples (5, 7, 65, 75-78). A maximum 1 g aliquot of soil was utilized in these studies. In all but one study (77), soil particles were not separated from the spores before lysis and DNA extraction. Jain et al. (77) found that additional soil pre-washing before DNA extraction diminished PCR inhibition. Conversely, Gulledge et al. (7) determined that pre-washed soil samples were not significantly different from soil samples placed directly into the extraction kit process.

A settling period following vigorous shaking has been used in combination with other separation procedures. In one study (79), a settling time was included after a vortexing step to separate 45 g of dense sand particles from the freed *Bacillus atrophaeus* subsp. *globigii* spores within a phosphate buffered saline amended with 0.5% TWEEN® 20 (PBST) solution. The supernatant was then withdrawn before concentrating the spores with high-speed centrifugation. Therefore, only spores dissociated from the sand by physical and chemical means and suspended in the collected supernatant were carried through to DNA extraction.

The studies discussed in this review used four types of HSGS solutions: sucrose solutions (40, 60, 68, 80, 81), Nycodenz® density gradient medium (64, 70, 82-84), sodium bromide solution (39), and two-phase liquid systems (85, 86). Irrespective of gradient medium, HSGS utilizes differences in specific gravity to separate *B. anthracis* from other organisms and soil components. Depending upon the sub-species, *B. anthracis* ranges in density from 1.162 - 1.184 g mL^{-1} (87) and is concentrated in the upper layers of most density gradient solutions post-centrifugation. Sucrose and Nycodenz® solutions are utilized at densities of 1.22 and 1.3 g mL^{-1}, respectively, allowing spores to concentrate within the uppermost layer following centrifugation. Two-phase liquid systems and sodium bromide include a wider range of liquid densities within a single centrifugation tube (1.0 - 1.3 g mL^{-1}) (39, 85). The spore-rich layer in these solutions is midway within the tube; the uppermost layers with lower density cell debris must be removed prior to spore collection. The added step of removing the uppermost layer significantly reduced the spore yield within the final sample. Nicholson et al. (39) determined that the addition of sodium bromide HSGS decreased indigenous spore yields from 2% - 4% to less than 0.1%. However, even with the added step, Agarwal et al. (88) were able to recover 9% - 20% of *B.*

anthracis Sterne within garden soil and over 50% from sand samples using a two-phase liquid HSGS protocol.

The utility of Nycodenz® HSGS for recovering *B. anthracis* is unknown. Multiple researchers have used Nycodenz® HSGS to separate bacterial cells from soil. However, no studies found for this review used it to target spores specifically. Rather, the Nycodenz® density gradient medium was used to prepare soil samples for total indigenous DNA extraction. Furthermore, there are conflicting efficiency results for Nycodenz® HSGS. Lindahl and Bakken (71) recovered 24% - 42% of the total indigenous cells within loam soil samples using Nycodenz® HSGS, while Courtois et al. (70) determined that 85% of the cells quantified by direct microscopy counts were lost after Nycodenz® HSGS separation.

Two comparative studies concluded that HSGS with 1.22 g mL^{-1} sucrose was the most effective protocol for spore separation, though yields were not high (40, 60). Ryu et al. (40) found a minimum LOD of 10^6 spores g^{-1} in Korean soils when spores were heat-lysed and detected through PCR. In a similar study conducted by Dragon and Rennie (60), an LOD of approximately 40 spores g^{-1} was determined for *B. anthracis* American Type Culture Collection (ATCC) 4229 spores spiked (2-8 $x10^5$ spores) into field and wallow soils and extracted using HSGS with 1.22 mg mL^{-1} sucrose and Triton X-100 solution. *B. anthracis* spores were detected via culture after being spiked in field soil, wallow soil, and potting soil with recoveries of approximately 4.5%, 5-8%, and 28%, respectively (60). In a third study, HSGS was evaluated using Arizona test dust, Minnesota loam, potting soil, and sand spiked with10^4-10^6 *B. anthracis* Sterne 34F2 spores g^{-1}. However, results were variable (68). The highest recoveries from culture shown by the Bradley et al. (68) study were 9% (10^4 spores g^{-1}), 5.8% (10^5 spores g^{-1}), 5% (10^6 spores g^{-1}), and 3.7% (10^4 spores g^{-1}) of the spores spiked into potting soil, sand, Arizona test dust, and Minnesota loam, respectively.

2.1.3.2 Affinity Capture

Bradley et al. (68) went on to compare sucrose HSGS to automated IMS. IMS utilizes antibodies bound to magnetic beads to capture and concentrate *B. anthracis*. Following the addition of the aqueous carrier medium and spore-soil dissociation, paramagnetic beads conjugated with polyclonal *B. anthracis* antibodies are added to the soil sample suspension. Any spores present within the sample bind to the antibodies. A magnetic rod is used to transfer the paramagnetic beads with the antibody-bound spores to tubes with PBST solution. The PBST solution allows the spores to be concentrated, washed, and released from the beads within a final sample tube. In the final sample tube, the spores can be verified and quantified through a variety of assays, including culture and PCR.

Bradley et al. (68) compared automated IMS recovery efficiencies for four different soil types (Arizona test dust, Minnesota loam, potting soil, and sand). For all tested soils, the minimum LOD was 10^2 spores g^{-1} of soil. Recoveries ranged from 17% - 51% among the four soils with the Minnesota loam and potting soil being the most recalcitrant. The study did note that there were a few microorganisms other than *B. anthracis* detected after culture with sand and potting soil, and the authors hypothesized that aggregates containing magnetic soil particles and

microorganisms were transferred through to the final sample. The Centers for Disease Control and Prevention (CDC) Division of Bioterrorism and Preparedness Response tested antibody specificity using time-resolved fluorescence. Results indicate that the *B. anthracis* antibody can differentiate between closely related and nonrelated bacterial strains (only *B. anthracis* spores were tested, not vegetative cells) (68). In an effort to improve the selectivity of IMS-treated soil samples, Chenau et al. (89) directly extracted SASP-B from the spores for highly sensitive liquid chromatography-tandem mass spectrometry detection. While selectivity was improved, the added processing/detection steps decreased overall sensitivity to a LOD of 7×10^4 spores g^{-1} soil.

Yitzhaki et al. (90) were able to increase the adsorption of *B. anthracis* to immunoglobulin G (IgG) labeled magnetic beads significantly with the addition of didecyldimethylammonium bromide (DDAB) in pure laboratory standards. However, they also conjectured that adsorption efficiencies would decrease by 20% - 40% for environmental samples. While IMS adsorption efficiencies for environmental samples may be of concern, IMS does have the advantage of being rapid. Fisher et al. (91) developed a rapid IMS-lateral flow protocol for identification of *B. anthracis* in liquid samples within approximately 40 minutes. Bruno and Yu (92) also noted that IMS was attractive for detecting *B. anthracis* in soil due to its simplicity, speed, and utility for large numbers of samples. "Liquid-phase" immunoassays have been used for spore capture of *B. anthracis* from dust by adding anti-*B. anthracis* antibodies to spore suspensions, incubating, and further processing the sample as described by Hang et al. (93).

2.1.3.3 Filtration

Dabiré et al. (62) and Isabel et al. (62, 69) utilized filtration to separate dissociated spores from soil samples. Using a series of sieves to separate a soil sample into different particle size fractions (>200 µm, 50 - 200 µm, 20 - 50 µm, and 0 - 20 µm), Dabiré et al. (62) concentrated *Pasteuria penetrans* spores into the 0 - 20 µm sample fraction. However, a significant number of spores were also associated with larger clay aggregates. Isabel et al. (69) used dual syringe filters to establish rapid filtration separation-based sample processing. Their protocol utilized a 5-µm pore-sized filter to separate spores from a variety of matrices including soil, dust, silica, and bentonite and an additional 0.45 µm pore-sized filter to concentrate the freed spores. On average for all matrices tested, 68% and 51% of the *B. atrophaeus* spores were recovered using the capture filtration step only (0.45 µm pore-sized filter) and the dual filter protocol, respectively.

2.2 Direct Processing: DNA Extraction of Bulk Soils and Selective Culture Media

Direct processing protocols include direct culturing of soil and bulk DNA extraction. It has been said that clinical identification of *B. anthracis* is not a problem; it is the presence of organic and inorganic compounds and extraneous bacterial flora (particularly other spore- forming *Bacillus* species) in environmental samples that interferes with *B. anthracis* detection and identification (75). While selective media have been used to isolate other *Bacillus* species from soil (94) and DNA extraction has been evaluated for isolation of *B. anthracis* from other matrices such as food, powders, and clinical samples (95, 96) or for other bacterial organisms in soil (97), direct processing of *B. anthracis* in soil requires more research. Extensive testing must be done to

develop a selective culture medium that allows differentiation between *B. anthracis* and other *Bacillus* spp. In addition, DNA obtained directly from soil samples must be purified carefully and DNA signature specificity must be carefully selected to ensure species selectivity.

2.2.1 Selective culture media

Although culturing is time consuming and laborious for large sample sets, there are times when it is critical to determine the quantity of viable *B. anthracis* within a sample or to assess the antimicrobial susceptibility of an environmental strain (98, 99). Soils abound with diverse species of microorganisms. Researchers have sought a *B. anthracis* specific agar-based medium that deters background cultures and other *Bacillus* species, yet allows *B. anthracis* to flourish. Sheep or horse blood is often included within a *B. anthracis* selective medium to evaluate hemolysis. *B. anthracis* is non-hemolytic, and the agar will remain red surrounding the cultures. Conversely, the near-neighbor bacterium *B. cereus* is hemolytic and produces an enzyme that lyses red blood cells and changes the appearance of the agar surrounding *B. cereus* growth. This review found six culture media selective for *B. anthracis* within the open literature (Table 2).

Table 2. *B. anthracis* Selective Culture Media

Reference	Medium (*State*)	Incubation Temperature °C	Incubation Time (hr)	Remarks
Bradley *et al.*, 2011 (68)	TSA II (*s*) and PLET (*s*)	35	24-48	PLET CFU were within 72% - 77% of the number of CFU found on non-selective TSA II plates. PLET agar was recommended for recovery of *B. anthracis* from unknown soils. Recoveries ranged from 1% - 51% depending on the soil and separation protocol.
Dragon and Rennie, 2001 (60)	SBA (*s*), PLET (*s*) and PLET supplemented with 5% defibrinated horse blood (*s*)	37	24-48	SBA recovered significantly more spores of *B. anthracis* ATCC 4229 than PLET medium. PLET allowed a few non-*anthracis Bacillus* strains to grow. Supplemented PLET allowed more non-*anthracis* test strains to germinate and grow. However, except for *B. subtilis* and *B. pumilus*, the non-*anthracis* strains could be differentiated from *B. anthracis*. Recoveries ranged from 4% - 28% depending on the soil and separation protocol employed.
Fasanella *et al.*, 2013(74)	TSMP	37	24 - 48	Authors stated that TSMP has the same efficacy as PLET for isolating *B. anthracis*. No recovery efficiencies were recorded.
Juergensmeyer *et al.*, 2006 (100)	ChrA (*s*)	35-37	24, 48	Due to a mutation in *B. anthracis*, the activity of PC-PLC is reduced compared to other *Bacillus* species. Therefore, colonies of other *Bacillus* species turn teal after 24 hr, and colonies of *B. anthracis* turn teal only after 48 hr allowing for species level discrimination. No recovery efficiencies were recorded.
Jula *et al.*, 2007 (101)	PLET (*s*) and SBA (*s*)	37	24	Spores were concentrated using a 0.45 µm filter. The deposit on the filter was heat treated to lyse vegetative cells prior to plating. Approximately 1/3 of the *B. anthracis*-like colonies on the PLET agar were actually *B. anthracis*. No recovery efficiencies were recorded.
Luna *et al.*, 2005 (102)	MEP (*s*) and ChrA (*s*)	30, 35	24, 24-48	Suspected *B. anthracis* isolates were cultured on the MEP agar or ChrA to aid in distinguishing between *B. anthracis* and *B. anthracis*-like organisms. No recovery efficiencies were recorded.
Luna *et al.*, 2009 (98)	Antibiotic amended PLET (*l* or *s*)	30	24, 48, 72, 96	Selectivity of PLET was improved with sulfamethoxazole, trimethoprim, polymyxin B, and lysozyme, and can select for *B. anthracis* in agricultural, environmental, and forensic investigations of *B. anthracis* isolates. No recovery efficiencies were recorded.
Marston *et al.*, 2008 (66)	SBA (*s*), PLET (*s*) and ChrA (*s*)	37	24-48	PLET agar is more sensitive than ChrA agar. Recovery ranged from 0.5% - 8% depending on the soil.
Tomaso *et al.*, 2006 (99)	CEI (*s*) and ABA (*s*)	37	24	Non-anthracis spp. turn turquoise on CEI agar, whereas *B. anthracis* does not. ABA contains supplements to inhibit fast growing environmental organisms and sheep blood to allow hemolytic differentiation between *Bacillus* spp. Percent recovery on ABA and CEI was 72% and 71%, respectively.

Vahedi *et al.*, 2009 (103)	PLET (*s*) and SBA (*s*)	37	24-48	Spores were concentrated using a 0.45 µm filter. The deposit on the filter was heat treated to lyse vegetative cells prior to plating. Confirmatory biochemical tests were conducted with *all B. anthracis*-like colonies. No recovery efficiencies were recorded.

ABA – Anthrax Blood Agar™
CEI – Cereus Ident Agar™
ChrA – R & F® *anthracis* chromogenic agar
CFU – Colony forming units
l – Liquid medium
MEP – Mannitol-egg yolk-polymyxin B agar
PC-PLC – Phosphatidylcholine-specific phospholipase C
PLET – Polymixin B, lysozyme, ethylenediaminetetraacetic acid, thallous acetate agar
SBA – Sheep blood agar
s – Solid agar medium
TSMP- Columbia blood agar with trimethoprim, sulfamethoxazole, methanol, and polymyxin
TSA II – Trypticase® soy agar with 5% sheep blood

A compounding difficulty for spore culturing is the existence of superdormant spores of *Bacillus* species (32, 33). Superdormant spores require elevated concentrations of germination compounds and/or extended incubation periods before they germinate. While most spores germinate within minutes once exposed to adequate growth conditions, naturally occurring superdormant spores may require hours to days before germination occurs (104). Therefore, even after a suitable processing or culturing protocol for most spores is employed, any superdormant spores present within a sample might not germinate. Previous work has indicated that *B. anthracis* superdormant spores might react in a manner similar to *B. cereus* and *B. megaterium* superdormant spores. However, no studies were found that specifically outline how to process soil-borne superdormant spores (33). Mannitol-egg yolk-polymyxin B agar (MEP) has been used as a selective and differential medium (102). *B. anthracis* colonies on MEP are colorless with a weak lecithinase production giving an opaque zone just beneath the colony, whereas other organisms turn yellow with mannitol fermentation and are translucent without lecithinase production. While MEP can distinguish *B. anthracis* from a number of *Bacillus* species, MEP is not sufficiently reliable (102).

R & F® *anthracis* chromogenic agar (ChrA) has also been used to distinguish *B. anthracis* from other *Bacillus* species (66, 100). ChrA includes the substrate 5-bromo-4-chloro-3-indoxylcholine phosphate, which converts to a water-insoluble blue dye in the presence of phosphatidylcholine-specific phospholipase C (PC-PLC). Among *Bacillus* species, only *B. anthracis, B. cereus*, and *B. thuringiensis* produce PC-PLC. For *B. cereus* and *B. thuringiensis*, the color change occurs within 24 hours, whereas for *B. anthracis,* the color change is seen only after 48 hours due to a nonsense mutation that reduces PC-PLC activity and eliminates its hemolytic activity (100) . Juergensmeyer et al. (100) tested ChrA on spiked soil, sewage, paper, cloth, and blood samples. Selective ingredients within the ChrA reduced the number of background soil flora capable of growing on the ChrA to approximately 10^3 colony forming units (CFU) g^{-1}. The color changing properties of *B. anthracis* colonies on the ChrA allowed them to be distinguished easily among the remaining background flora. *B. anthracis* colonies are harder to identify when *B. cereus* and *B. thuringiensis* growth is overwhelming (100). Luna et al. (102) suggested that either MEP agar or ChrA could be added to the Laboratory Response Network (LRN) protocol to help reduce the number of suspected *B. anthracis* positive environmental samples requiring confirmational testing (102).(The LRN, established by the CDC, is tasked with maintaining an integrated network of laboratories that can respond to bioterrorism, chemical terrorism and other public health emergencies.)

Tomaso et al. (99) examined the utility of Cereus Ident Agar™ (CEI) and Anthrax Blood Agar™ (ABA). CEI contains a chromatogenic substrate similar to ChrA. Only the turquoise coloration of non-anthracis spp. can be used to discriminate *B. anthracis* from its near-neighbors (99). ABA is a nutrient medium containing sheep blood and supplements to inhibit many fast growing organisms. The hemolysin gene of *B. cereus* has been found within *B. anthracis* strains on a few occasions, so hemolytic morphology is not a definitive assessment (99). *B. anthracis* could be identified appropriately 71% and 72% of the time on CEI and ABA, respectively, when tested against 92 environmental *B. anthracis* isolates and 132 other *Bacillus* spp. (99).

Polymyxin B, lysozyme, ethylenediaminetetraacetic acid, thallous acetate (PLET) is another selective medium described in the literature. Bradley et al. (68) compared PLET agar to

Trypticase® soy agar amended with 5% sheep red blood cells (TSA II) and determined the two media to be comparable. After overnight growth, the PLET CFU were within 72% - 77% of the TSA II CFU counts indicating adequate germination on the selective medium compared to the non-selective medium. The overall recommendation was to use PLET agar for *B. anthracis* recovery from unknown soil samples (68). However, little analytical support was given for this suggestion. In a comparison of PLET to ChrA, Marston et al. (66) found that PLET was more sensitive and more selective against other *Bacillus* and non-*Bacillus* species than ChrA. However, PLET and ChrA had similar *B. anthracis* recovery rates for the bacteria when it was spiked into Texas soil and Arizona test dust. Jula et al. (101) and Vahedi et al. (101, 103) used selective PLET agar to differentiate *B. anthracis* colonies from other organisms. In each study, they found that PLET was not specific for *B. anthracis*. After confirmatory biochemical testing of multiple *B. anthracis*-like colonies, *B. cereus, B. circulans, B. megaterium, B. subtilis* and *B. sphaericus* were all found on the original formulation of PLET agar. Only approximately 33% of the *B. anthracis*-like colonies tested by Jula et al. (101) were in fact *B. anthracis* colonies.

Researchers have sought to improve the original 1966 formulation of PLET medium for better selectivity (60, 98). In 2001, Dragon and Rennie (60) compared non-selective sheep blood agar (SBA) to PLET and PLET amended with 5% defibrinated horse blood. Results demonstrated that although the original PLET was more selective than PLET amended with horse blood, SBA recovered significantly more *B. anthracis* than PLET. These findings led Dragon and Rennie (60) to conclude that although PLET is selective for *B. anthracis*, PLET is not an ideal recovery medium and may underestimate the number of spores within a sample. In 2009, Luna et al. (98) sought to improve the utility of the original PLET medium further with the addition of lysozyme (150,000 units L^{-1}) and the antibiotics sulfamethoxazole (38 μg mL^{-1}), trimethoprim (2 μg mL^{-1}), and polymyxin B (15,000 units L^{-1}). The modified PLET medium was tested against 283 environmental isolates, including 23 isolates of *B. anthracis,* and could be used in a liquid broth or solid agar state. The additional antibiotics and lysozyme within the medium inhibited the growth of other *Bacillus* species and delayed the appearance of resistant *B. cereus*. Work-safety regulations in some countries prevent the use of PLET due to the high concentrations of toxic thallium acetate (1.9 mg/L) within its composition (98, 99). Based upon the breadth of data known regarding the specificity of modified PLET medium, modified PLET medium is the most promising selective culture medium for *B. anthracis* documented within the literature.

2.2.2 Direct DNA extraction from bulk soils

Prior to performing PCR analysis, DNA must be extracted from the sample. For direct DNA extraction, a small amount of soil (0.1 g - 10 g) is added to a DNA extraction buffer. Cells from all organisms present in a sample are lysed through both chemical and physical means. DNA-identifying reactions are used to seek, amplify, and detect the DNA segments of interest within the total mass of extracted DNA. The DNA extraction protocol influences the quantity and quality of template DNA available.

DNA can be extracted directly from bulk soils or from spores already removed from the soil. Delmont et al. (82) and Roh et al. (72, 82) found that direct DNA extraction produced over 33 times more DNA per gram of soil than indirect HSGS separation and over 100 times more DNA per gram of soil than low-speed centrifugation separation. While indirect DNA extraction had a

reduced concentration of DNA, the overall quality of DNA was increased compared to direct extraction protocols. Lombard et al. (56) estimated that as much as 40% of the total microbial DNA contained within a soil sample is lost during direct DNA extraction, and an additional 30% can be lost during downstream purification procedures. The initial soil conditions also have an effect on the quality and quantity of the DNA extracts. Zhou et al. (10) found that as the carbon content increased within the bulk soil sample, so too did the DNA yield; while Sjöstedt et al. (9) noted that organic content is directly proportional to humic acids, known PCR inhibitors. Therefore, appropriate measures must be taken to reduce PCR inhibitors in soil DNA extracts.

2.2.2.1 DNA Extraction Kits

Numerous kits are available from vendors that are specific for DNA extraction from soil samples. In addition, extraction kits commercialized for other sample types have been used for environmental soils. Herein, details including cost, time requirements, sample size, and LOD of 28 extraction kits and one manual protocol utilizing liquid nitrogen are presented (Appendix B).

There are two critical steps to cellular DNA extraction: cell lysis and DNA separation. The components of most kits are proprietary, but there are a few general types of lysis and DNA separation protocols. Many extraction kits utilize a combination of chemical disruption (detergents) and physical agitation (bead beating) for effective lysis of cellular membranes and release of spore DNA. Kuske et al. (105) found that 40 freeze-thaw cycles with liquid nitrogen were not sufficient to lyse *B. atrophaeus* spores, but a combination of chemical and physical agitation showed promising lysing efficiency. Once released, DNA is often bound to silica filters or magnetic beads for purification. Humic acids, polysaccharides, and urea show solubility properties equivalent to DNA and are often co-extracted, especially at higher pHs (3, 106). Washing steps are utilized to reduce the presence of co-extracted compounds post-lysis before purified DNA is concentrated in an elution buffer. In particular, polyvinylpyrrolidone (PVPP) is used to adsorb inhibiting phenols, including humic acids (106). The final elution buffer often contains Tris and EDTA to protect the extracted DNA from nuclease activity over time (106).

2.2.2.2 Comparison of DNA Extraction Kits for Soil Samples

While there are a multitude of commercial extraction kits available for soil samples, determining the overall best kit is difficult. This literature search found only three studies that directly compared two or more extraction kits for analyzing *B. anthracis* in environmental soil samples. Gulledge et al. (7) demonstrated the utility of a PLET enrichment step, but concluded that no one kit from the five tested was superior. Bradley et al. (68) determined that the QIAamp® DNA Blood Mini Kit (QIAGEN; Valencia, CA) was more efficient for Arizona test dust, while the UltraClean® Soil DNA Isolation Kit (MO BIO Laboratories; Carlsbad, CA) was more efficient for potting soil. The most comprehensive comparison looked at six commercial DNA extraction kits and three soil types: sand, clay, and loam. In this assessment, Dineen et al. (6) determined that the FastDNA® SPIN Kit for Soil (Qbiogene; Solon, OH) yielded significantly higher amounts of spore DNA from each of the three tested soil types.

Other researchers have sought an optimum extraction kit for detecting other organisms from soil. Whitehouse et al. (107) compared extraction kits for *Francisella tularensis* in multiple soils. *F. tularensis* is a non-sporulating gram-negative organism and is easier to lyse than *B. anthracis*. Whitehouse et al. (107) concluded that of the five commercial kits assessed, the UltraClean® Soil DNA Isolation Kit outperformed the other kits in the quantity and quality of purified *F. tularensis* DNA, having an LOD of 20 CFU g^{-1} of soil in all three tested soil types. The next best was the PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories; Carlsbad, CA) with an LOD calculated at 100 CFU g^{-1} for all tested soil types. Interestingly, PCR inhibition was seen only in samples extracted from the commercial potting soil with the QIAamp DNA Stool Mini Kit (QIAGEN; Valencia, CA). A study by Fitzpatrick et al. (67) analyzed the effect of combining extraction kits using *Coxiella burnetii* in sandy soil by comparing the QIAamp DNA Stool Mini Kit to the QIAamp DNA Mini Kit (QIAGEN; Valencia, CA) and the UltraClean® Soil DNA Isolation Kit when used singly and in sequence. Results showed that utilizing two kits in series nearly eliminated the presence of inhibition within final PCR reactions; however, the additional kit also reduced the overall DNA yield. Using *C. burnetii* spiked soil samples, they saw a maximum genomic equivalent yield of 4.3% using the QIAamp DNA Stool Mini Kit alone. The addition of a second extraction kit reduced the yield to less than 2%, demonstrating a significant trade-off between DNA purity and DNA yield.

Two studies were found that compared DNA extraction kits using spiked household powders. Though these studies did not utilize soil as a sample matrix, the sample media do provide insight into the ability of the extraction kits to eliminate inhibition. Dauphin et al. (76) compared five commercial kits using *B. anthracis* Ames spores in baking soda, talcum powder, and cornstarch. Of the five tested kits, the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories; Carlsbad, CA) yielded the only DNA extract without viable spores, thereby significantly reducing the risk to laboratory personnel. In a similar study setup, Rose et al. (108) spiked multiple household materials with *B. globigii*. Their assessment found the PrepFilter™ Forensic DNA Extraction Kit (Applied Biosystems; Foster City, CA) to be the best kit for extracting DNA from powder samples; however, when including the sampled liquids and solids, the best overall kit was the UltraClean® Microbial DNA Isolation Kit.

The most commonly used commercial extraction kits for soil samples found in the literature search were the UltraClean® Soil DNA Isolation Kit and the Powersoil® DNA Isolation Kit, both produced by MO BIO Laboratories (Carlsbad, CA). Both kits require approximately 90 minutes for bead-beating lysis followed by a silica spin filter to concentrate the extracted DNA. While the UltraClean® Soil DNA Isolation Kit can process a larger quantity of soil (1.0 g versus 0.25 g), the primary difference between the two kits is the presence of an Inhibitor Removal Technology® within the Powersoil® DNA Isolation Kit. In addition, each kit has a large volume companion that uses the same technology to process 10 g samples. Whitehouse et al. (107) compared the technologies for two kits; the UltraClean® Soil DNA Isolation Kit with a sample volume of 0.1 g of soil and the PowerMax® Soil DNA Isolation Kit with a sample volume of 10 g of soil. The UltraClean® Soil DNA Isolation Kit outperformed the PowerMax® Soil DNA Isolation Kit; however, the differences were minimal (107). The soil conditions apparently have a pronounced effect on the quality and quantity of extracted DNA.

Care should be taken when using different lots of DNA extraction kits. Bushon et al.(109), studied variability in DNA extraction of *B. anthracis*, *F. tularensis*, and *Vibrio cholerae* using three different lots of the MO BIO Powersoil® DNA extraction kits and found significant differences between the lots for all three organisms. The authors suggested that if different lots of extraction kits are to be used, the lots should be checked for consistency, quality control measures should be used, and new standard curves should be run with each new lot (109).

2.2.3 Enrichment Steps

Enrichment steps have been added to processing protocols to help improve recovery of spores from samples that contain a low density of spores (5, 7, 9, 110). Addition of an enrichment medium to the sample allows both germination of spores and growth of vegetative cells. As nutrients are depleted, spore-forming bacteria begin sporulation, while the proportion of vegetative cells and other non-spore forming bacteria decreases or are killed (110). Incubation and heat treatment can be used other kill remaining vegetative cells (110). Patel et al. (110) evaluated the recovery of *B. thuringiensis* spores from 58 soil samples that included enrichment with glucose yeast extract salt medium as part of sample processing and were able to recover 55-75% of the *B. thuringiensis* spores from the samples. The use of selective enrichment agar significantly lowered the detection limits in three studies (5, 7, 9). In particular, Gulledge et al. (7) found that a PLET enrichment step lowered the detection limits by as much as six orders of magnitude. The relatively new process of rapid-viability PCR (RV-PCR) also incorporates an enrichment step between two PCR reactions to determine the presence of germinated *B. anthracis* spores rapidly within a collected sample (111, 112). Currently, no soil samples have been analyzed using RV-PCR; however, optimization of this assay for soil could help reduce the time required to determine both the quantity and viability of *B. anthracis* in soil.

2.3 Purification Protocols

Because endospores of *B. anthracis* are highly resistant to unfavorable environmental conditions in comparison to vegetative cells (34, 60), purification protocols such as heat treatment and treatment with ethanol are used to help improve recovery of spores from soil and may be used during either direct or indirect processing of the sample. Heat treatment is a method of purification that has been used as part of the soil processing protocol to kill off vegetative cells in soil samples while leaving viable spores (7, 77, 101, 103). Dry heat treatment (incubation in a dry oven at 80 °C) of soil samples containing *B. thuringiensis* was evaluated by Santana et al. (63), who found that isolation of *B. thuringiensis* from soil was improved after a five-hour dry-heat treatment, although a more recent study by Patel et al. (110) was not able to achieve similar results. *Bacillus* spores have been shown to be resistant to ethanol, so ethanol has alternatively been used for removing vegetative cells from the sample (60). Dragon and Rennie (60) compared spore stock samples of *B. anthracis*, vegetative *B. cereus*, and vegetative *Pseudomonas aeruginosa* treated with both heat (incubation for 20 min in 63 °C water bath) and 50% ethanol and found that both treatments were equally effective in removing vegetative cells from the stock while maintaining viability of the spores.

3.0 Conclusions

Developing an ideal protocol for processing soil samples before microbiological assessment is challenging. As evident through this review, a significant amount of work has been done to ascertain the most efficient protocol for processing soil samples for *B. anthracis* detection. Direct and indirect protocols for sample processing were reviewed in detail. Direct processing utilizes bulk sample aliquots without first separating spores from soil particles, while indirect processing uses multiple steps to separate spores from other organisms and particles prior to detection. Direct and indirect DNA processing steps have associated advantages and disadvantages.

As described, multiple indirect soil processing protocols have been used to separate *B. anthracis* from soil particles. Indirect sample separation steps increase the proportion of target spores within the final detected sample; however, spore loss prior to detection also increases. The presence of a detergent in the aqueous carrier medium was consistently found to improve the separation of spores from soil particles. However, no consensus on an optimum aqueous carrier medium could be determined from among the reviewed works. Future research focusing on the aqueous carrier medium for processing multiple soil types under uniform dissociation and separation conditions would help fill this gap.

Spore/soil separation is a critical step in determining the overall recovery efficiency of indirect processing protocols. IMS is an attractive option for separating *B. anthracis* in soil due to its simplicity, speed, and utility for large numbers of samples, but continued work on IMS and its ability to bind *B. anthracis* selectively at low concentrations is needed. The overall utility of HSGS as a separation protocol needs to be determined before HSGS is applied within large-scale projects. Although novel dual syringe filtration has shown promise for being able to separate spores rapidly from diverse matrices, future work that combines an optimized aqueous carrier medium with the dual filter steps may be needed to increase recovery rates further.

Direct soil processing falls under two principal types: culturing *B. anthracis* on selective agar and bulk DNA extraction. When samples are directly processed, there is a potential for background organisms to overwhelm the detection assay and prevent target spores from being observed. Researchers have sought a *B. anthracis*-specific medium that deters background cultures and other *Bacillus* species and yet allows *B. anthracis* propagation and identification. Several *B. anthracis*-selective media have been developed. Based upon the amount of specificity testing, modified PLET medium was identified as the most promising selective culture medium for *B. anthracis* documented in the literature. The use of selective enrichment agar during sample processing might improve recovery of spores from soil samples with low spore density. To date no studies have utilized modified PLET agar as an enrichment step prior to *B. anthracis* detection. Future recovery efficiencies could be dramatically increased with such an effort.

There are commercial kits available to extract DNA directly from bulk soil samples, and allow for automated processing, reducing human exposure within the laboratories. While there are numerous advantages to using a commercial kit for sample processing, unfortunately, due to the difference in study designs, it is difficult to determine an overall optimum DNA extraction kit from the currently available data. An optimized soil DNA extraction kit is needed; there has yet

to be a soil DNA extraction study that compares multiple soil extraction kits uniformly across multiple soil types to determine their overall DNA recovery.

The type of sample processing employed, direct or indirect, depends upon the desired downstream applications (71). For DNA detection assays, direct bulk DNA extraction with suitable DNA purification steps may be more appropriate. However, indirect processing might be more appropriate if viability testing is required.

Regardless of whether direct or indirect processing protocols are employed, the overall recovery rates and confidence intervals are critical pieces of information for downstream human health and consequence decisions. As shown through this review, an optimized soil processing protocol with a known recovery rate and associated confidence intervals is needed. Calculations for recovery rates should be included in future studies. A reliable processing protocol would allow for multiple technicians and laboratories to produce high quality uniform results in the event of a *B. anthracis* release.

4.0 Quality Assurance

This literature review was conducted under an approved quality assurance and quality control plan. The only minor deviation from the QA/QC plan was a change to the title of the report.

5.0 References

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Appendix A – Table of Reviewed *B. anthracis* Soil Studies and their Design Elements

Appendix A: Table of Reviewed *B. anthracis* Soil Studies and their Design Elements

First Author, Year	Organism(s)	Soil Type	Soil Amt. (g)	Soil Processing	Aqueous Carrier Solution	Spore/Soil Disassociation	Spore/Soil Separation	Lysis and DNA Extraction Protocol	Detection Assay	Spore Spike (spore g^{-1} soil)	LOD (spores g^{-1} soil)	% Recovery	Remarks
Agarwal, 2002 (88)	*B. anthracis* Sterne	Sterile garden soil, sterile sand	Not known	Indirect	PBS with polyethylene glycol 4000	Vigorous homogenization	Centrifugation into two phases of the polymer system	None	Immunofluorescence microscopy with fluorescein isothiocyanate (FITC)-conjugated antibody against formalin-inactivated spores of *B. anthracis* Sterne	10^3 - 10^7	soil: 14000 sand: 5600	Soil: 9 - 20 Sand: 51 - 59	Agarwal attributed recovery differences between sand and garden soil are to flocculation/adsorption of spores to soil particles. Sonication or other mechanical disruption may aid in disrupting this bond.
Balestrazzi, 2009 (3)	*B. subtilis*	Medium textured loamy sand	5	Direct	None: direct lysis	None	None	2% SDS, 1% cetyltrimethylammonium bromide (CTAB), 60 °C 2% SDS, 4% CTAB, 60 °C 2% SDS, 1% CTAB, 60 °C, liquid N_2 grind 3% SDS, 1.2% PVP, microwave thermal shock microwave thermal shock, 3% SDS, 1.2% PVP, liquid N_2 grind	PCR southern blot and PCR for *swrAA* gene	10^4 - 10^8	2000 2000 2000 >2 x 10^8 2 x 10^8	ND	Microwave based approaches were not effective and led to ~tenfold less spore disruption.
Beyer, 1999 (4)	*B. anthracis*	Former tannery sites	100	Direct	Trypticase ® soy broth (TSB) enrichment medium	None	None	Invitrogen Easy-DNA™ Kit	PCR-Enzyme linked immunosorbent assay (ELISA) for pX01, pX02, and chromosome	0.01 - 1	0.1	ND	Samples are enriched twice before DNA extraction.
Bielawaska-Drózd, 2008 (75)	*B. anthracis* 34F2, 211	Sandy, forest, wetland	0.1	Direct	TSB enrichment medium	None	None	PLET enrichment nested PCR PLET enrichment PCR-ELISA 0.1 g soil boiled in TSB, centrifuged and washed in distilled water	Nested PCR targeting *pag* and *cap* genes	10 - 10^8	10 10 - 100 ND	ND	Compared three spore isolation protocols for *B. anthracis* in soil: (1) double incubation in TSB followed by DNA extraction and a nested PCR amplification, (2) non-selective pre-enrichment in TSB followed by DNA extraction and PCR-

												ELISA, (3) thermal protocol where soil is boiled for 10 minutes (min) before DNA extraction. Protocols 1 and 2 gave the best results, and no observed differences were found between the three types of soil evaluated.
Bradley, 2011 (68)	*B. anthracis* Sterne 34F2	Sand, AZ dust, potting soil, MN loam	1	Indirect	PBS: Tween® 20, Sucrose: Triton™ X-100 solution	End-over-end mixing	IMS sucrose HSGS (1.22 g mL^{-1})	AIMS, UltraClean® Soil DNA Isolation Kit AIMS, QIAamp DNA Blood Mini Kit	PLET culture	10 - 10^4	10^2 – 10^3 10^4 - 10^7	IMS - sand: 51 AZ dust: 29 Potting soil: 17 MN loam: 17 HSGS-sand: 5.8 AZ dust: 5 Potting soil: 9 MN loam: 3.7	Optimization of the automatic IMS protocol revealed that separation of the *B. anthracis* from soil was best accomplished by preprocessing the soil slurry samples by sonicating and vortexing (three min each) to disrupt clumps, filtering through a 30 µm pore size filter, allowing the slurry to settle, and removing the liquid from the top of the sediment and placing it in the IMS tray.
Bruno, 1996 (92)	*B. anthracis* Sterne, Ames, Vollum	Dark brown soil, light yellowish sandy soil	0.1	Indirect	PBS	Not described	IMS	None	Immunomagnetic-electro-chemiluminescent (IM-ECL) detection with polyclonal goat antiserum	0 - 10^6	Sterne: 100 Ames: 10^4 Vollum : 10^5	ND	Though there was a loss of sensitivity once soil was added, the authors still liked IM-ECL because of its speed, simplicity, and ease of use for large sample sets.
Chenau, 2011 (89)	*B. anthracis* Sterne and 4 others, nine other *Bacillus* species	Soil, milk	0.01	Indirect	4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid/bovine serum albumin (HEPES/BSA) solution	Vortex	IMS – Immuno globulin G (IgG) labeled beads	Immunocapture step followed by 80% trifluoroacetic acid (TFA) protein extraction, neutralization, and digestion	Immunocapture – Liquid Chromatography/ Mass Spectrometry (LC/MS) targeted at SASP-B proteins	10^3 - 10^8	$7x10^4$	ND	Only one soil matrix was tested to show proof of principle.

Cheun, 2003 (5)	*B. anthracis* Pasteur II	Commercial peat soil, nine field samples	1	Indirect	70% ethanol	Gentle shaking	None	Wash, no enrichment TSB, FastDNA® SPIN Kit for Soil Wash, single enrichment TSB, FastDNA® SPIN Kit for Soil Wash, double enrichment TSB, FastDNA® SPIN Kit for Soil	Nested and real-time PCR targeting *pag*, *capA*, and *sap* genes	$1 - 10^3$	1000 10 1	ND	1 g of soil contains 10^3 - 10^6 spores of different microbes, therefore it would be difficult to identify one *B. anthracis* spore g^{-1}. Soil samples are usually heat-treated to kill nonsporulated bacterial cells, but this study found that heat treatment generated false positives.
Courtois, 2001 (70)	*B. thuringiensis*	Sandy loam	5	Indirect & direct	Indirect: 0.5 g soil into 0.05 M pyrophosphate, 0.9% NaCl, or water, Direct: none	Indirect: Waring® blender homogenization Direct: none	Indirect: HSGS – Nycodenz®, Direct: none	Direct extraction: manual chemical lysis with and without bead-beating Indirect extraction: Nycodenz® HSGS followed by chemical lysis	PCR targeting 16S rRNA and dot-blot analysis	None	ND	ND	Bacteria present in a soil sample depend upon the chemical and physical properties of the soil. Percentage of bacteria extracted was not affected by the buffer; however, 85% of the cells detected by microscopy cell counts in the original soil suspensions were lost post Nycodenz® HSGS. Homogenization was enhanced over sanitation or chemical treatments using a Waring® blender.
Da Silva, 2011 (57)	Green fluorescent protein (GFP)-labeled *B. anthracis* Sterne	Wipes - rayon, cotton, polyester	None	Direct	water, water with Tween® 80, PBS, PBS with Tween® 80	Vortex or sonicate	None	None	Direct culture on LB agar plates	2×10^5	ND	3-100	The addition of Tween® 80 to the carrier medium significantly improved the overall recovery efficiency. Vortexing physically separated the spores from the wipe material better than sonicating. Extraction efficiency was dependant on the extraction solution and wipe selected.

Dabiré, 2001 (62)	*Pasteuria penetrans*	Sandy clay, clay	10	Indirect	Distilled water or NaOH	End-over-end mixing	Sieve bank (200, 50, 20 µm)	None	Malassez counting chamber microscopy	10^6	ND	75 – 87	Increasing the energy during washing steps increased the % recovery of the inoculated spores.
Dauphin, 2009 (76)	*B. anthracis*	Baking soda, corn starch, talcum powder	0.025	Indirect	PBS	Vortex	Low speed centrifugation - supernatant used for DNA extraction	NucliSENS® Isolation Kit QIAamp DNA Blood Mini Kit UltraClean® Microbial DNA Isolation Kit	pX01, pX02 chromosome	10^1 - 10^6	10^6 10^6 10^7	ND	Spores were spiked into 0.025 g of soil and washed before using each kit. The UltraClean® Kit had no viable spores in the extraction product.
Delmont, 2011 (82)	All soil organisms	Park grass silty clay, loam	Indirect : 60 Direct: 0.5	Indirect & direct	Indirect: 60 g soil into 0.9% NaCl, Direct: none	Indirect: Waring® blender homogenization Direct: none	Indirect: Nycodenz® HSGS, Direct: none	Indirect: processed sample into FastPrep Lysing system Direct: 0.5g soil into MP Biomedical FastPrep system	PCR targeting the intergenic spacer region between 16S and 23S ribosomal sequences	None	ND	ND	Although the direct extraction protocol is less time consuming and uses less soil, indirect DNA extraction reduces the proportion of eukaryotic sequences and increases the DNA length of the recovered DNA strands.
Dineen, 2010 (6)	*B. cereus* T strain	Sand, clay, loam	0.1 - 1.0	Direct	None: kit	None	None	Powersoil® DNA Isolation Kit SoilMaster™ DNA Extraction Kit EZNA® Soil DNA Kit ZR Soil Microbe DNA Kit™ FastDNA® SPIN kits for Soil IT 1-2-3™ Platinum Path Sample Purification Kit	qPCR targeting the phosphatidylinositol-specific phospholipase C gene of B. cereus (*P1-PLC*)	10^7 - 10^9	10^7 - 10^8 for sand and loam, 10^7 or below for clay	11-35	The selection of an appropriate kit depends upon the initial soil conditions and the downstream applications. The FastDNA® Spin Kit gave the highest yield of DNA while the EZNA® Soil DNA and PowerSoil® DNA Kits were more efficient at removing inhibitors.
Dragon, 2001 (60)	*B. anthracis* ATCC 4229	Potting soil, field soil, wallow soil	2.5	Indirect & direct	Deionized water or sucrose solution	Shaken by hand	Low speed centrifugation - supernatant used for sucrose HSGS (1.14 - 1.22 g mL^{-1})	HSGS, heat treatment HSGS, ethanol treatment Deionized water, heat treatment Deionized water, ethanol treatment	PLET culture, SBA culture	Unknown	40 for both carrier solutions	PS: 28, field: 6, wallow: 4.5	"Although PLET is selective for *B. anthracis*, it is not an optimal recovery medium and may miss anthrax spores in a sample." Ethanol purification proved as effective as heat

													purification.
Ehers, 2008 (64)	Mixed community	Ferralsol - tropical soil high in iron and aluminum	10	Indirect	Water or 0.8% NaCl solution	Waring® blender homogenization	Nycodenz® HSGS	None	Quantified by acridine orange direct counts	3.7×10^9	ND	water: 10.6 NaCl: 4.6	Water carrier solution with pH amendment to 7.5 gave the greatest soil bacteria yield after gradient separation; however, water without pH modification gave highest soil species richness. Using 0.8% NaCl with pH amendment gave the best purity. The selection of extraction protocol for soil samples should depend on the purpose of the study.
EPA, 2012 (79)	*B. globigii*	Sand	Indirect : 45, Direct: 0.25	Indirect & direct	Indirect: PBS -Tween® 20 Direct: none	Indirect: vigorous mixing Direct: none	Indirect: supernatant from sand settling high-speed centrifuged to precipitate spores, Direct: none	Indirect: 45 g PBST wash, 0.25 g Powersoil® DNA Isolation Kit Direct: 0.25 g Powersoil® DNA Isolation kit	qPCR targeting *recF* gene	10^6	10^4 10^6	<1	Significant difference between 0.25 g and 45 g soil sample aliquots. Presumably, the 45 g samples included a much higher concentration of spores; therefore, DNA was above the LOD.
Fasanella, 2012 (73)	*B. anthracis*	Soil from contaminated farm	7.5	Indirect	Sterile distilled water with 0.5% Tween® 20	Shaken	Low-speed centrifugation	Supernatant incubated 54 ºC for 20 min. Phosphomycin tryptose soya broth added to supernatant.	Plated on trimethoprim sulfamethoxazole polymixin5% sheep blood agar	None	ND	ND	Ground anthrax *Bacillus* refined identification (GABRI) protocol used to recover *B. anthracis* from Bangladesh soils at outbreak site.
Fasanella, 2013 (74)	*B. anthracis*	Soil from contaminated farm and garden soil spiked with *B. anthracis*	7.5	Indirect	Sterile distilled water with 0.5% Tween® 20	Vortexing for 30 min	Centrifugation at 2000 rpm for 5 min. Supernatant incubated at 64 ºC for 20 min.	Tryptose Phosphate Broth with 50 µg/µL Fosfomycin added to supernatant.	Plated on Columbia blood agar with trimethoprim sulfamethoxazole, methanol, polymixin	Spiked samples spiked with 500 spores per 7.5 g sample	ND	ND	The modified GABRI method was able to isolate *B. anthracis* from 100% of both naturally contaminated and artificially contaminated soil samples.

Fisher, 2009 (91)	*B. anthracis* ATCC 14185	Milk, water	10 mL	Indirect	PBS	None	IMS	None	Lateral-flow immune-chromatographic device for visualization of various antigens	10^6 CFU mL^{-1}	5 x 10^5 CFU mL^{-1}	85-95	Not a soil protocol, but rather a fluid milk /water protocol for food testing.
Fitzpatrick, 2010 (67)	*Coxiella burnetii*	20 soils from across U.S.	5	Indirect	PBS	Vortex	Low-speed to separate soil followed by high-speed centrifugation of supernatant to concentrate spores	UltraClean® Soil DNA Isolation kit QIAmp DNA Minikit QIAamp DNA Stool Minikit	PCR for *IS1111* gene from *C. burnetii*	800 - 10^6	ND	Max 4.3	*C. burnetii* is Gram-negative. However, the kits compared are relevant for *B. anthracis* detection. Combining two kits eliminated any seen inhibition; however, combining kits also reduced DNA (maximum yield was 4.3%) yield. The precipitated spores from the high-speed centrifugation were used to compare DNA extraction kits.
Frostegard, 1999 (106)	*B. anthracis* Sterne vegetative cells	Five French sandy, clay soils 1 Australian sandy clay	0.2	Direct	None: direct in situ lysis	Waring® blender grinding, sonication, vortexing	None	*In situ* freeze thaw with DNA extraction in buffer ranging in pH from 6.0 - 10.0	Dot blot hybridization	10^7 - 10^9	ND	ND	For all soils tested, DNA yield increased with pH of the buffer. However, larger amounts of humic materials were released at higher pH as well.
Griffin, 2009 (51)	*B. anthracis*	U.S. soils	0.25	Direct	None	None	None	1 g UltraClean® Soil DNA Isolation Kit 0.25 g Powersoil® DNA Isolation Kit	PCR targeting the *rpoB* gene for *Bacillus* genus, PCR targeting *rpoB* gene specific for *B. anthracis*	None	170 4	ND	LOD study done with cells not spores.

Gulledge, 2010 (7)	*B. anthracis* Pasteur and Sterne	FL sand, TX sand, and commercial garden soil (Peat)	0.1 - 0.5	Indirect & direct	Pretreatment solution: sodium pyrophosphate, EDTA, Tris-Cl	Vortex	None	UltraClean® Soil DNA Isolation Kit	Hybridization and PCR for *capC*, *pag*, and *lef* genes	$10 - 10^7$	10^6	ND	Overnight enrichment with PLET broth lowered the detection limits of four of the five protocols by several logs (2 - 6 log_{10}). No significant difference between the untreated and pretreated soils (direct kit lysis and indirect wash before kit lysis). No one kit gave superior DNA recovery, and soil type and organic load should be considered before selecting the appropriate kit.
								SoilMaster™ DNA Extraction Kit			10^6 sand, Peat>10^7	
								FastDNA® SPIN Kit for Soil			10^7	
								BioRobot™ M48 Workstation			>10^7	
								PLET enrichment, UltraClean® Soil DNA Isolation Kit			10^5 sands, Peat>10^7	
								PLET enrichment, SoilMaster™ DNA Extraction Kit			100	
								PLET enrichment, FastDNA® SPIN Kit for Soil			100	
								PLET enrichment, MagNA Pure® LC			>10^7	
								PLET enrichment, BioRobot M48 Workstation			100	
Hang, 2008 (93)	*B. anthracis* Sterne	Office vacuum dust		Indirect	PBS with Tween® 20 and BSA	Vortex	IMS	Liquid-phased immunoassay	Sandwich and liquid-phased immunoassay	$10^3 - 10^7$ Spores mL^{-1}	4 x 10^4 spores mL^{-1}	ND	Spores were spiked into wipe samples after removing dust from the wipe. Brain heart infusion (medium) (BHI) broth induced spore germination within five minutes.
Hong-Geller, 2010 (65)	*B. anthracis* Sterne and Ames *Yersinia pestis* A1122 and CO92	Swabs and wipes off glass, stainless steel, vinyl and plastic	None	Indirect	PBS - Tween® 20	Vortex	None	FastDNA® spin kit for soil	qPCR targeting pX01	10^7	ND	Sterne: >90 ; Ames 2-75	No significant difference was found between swab and wipe for *B. anthracis*. Sterne spores were easier to recover than Ames spores. Spores were recovered with higher efficiency from hydrophilic surfaces.

Irenge, 2010 (113)	304 bacterial strains, 37 *B. anthracis* strains, Ames, Sterne, Vollum, Delta-Sterne (soil spikes, Ames)	14 soils	2	Direct	None	None	None	PowerMax® Soil DNA Isolation Kit	qPCR targeting phosphate (*ptsI*) and adenylosuccinate synthetase (*purA*) genes	10^4 - 10^7	25 fg	ND	Sought to find *B. anthracis* specific primers.
Isabel, 2012 (69)	*B. atrophaeus*	23 common powders including garden soil	0.2	Indirect	PBS	Mixing	Filtration (5 µm)	BD GenePhm Lysis Kit	qPCR targeting the *atpD* gene	5000	5000	51	Assessed the utility of a syringe prefilter and wash protocol. Developed the DARE procedure - dual-filter for applied recovery of microbial particles from environmental and powdery samples. One filter is used to separate spores from soil, and the next filter is used to concentrate spores.
Jacobsen, 1992 (97)	*Pseudomonas cepacia*	Sandy loam	50	Indirect	Chelex® 100 in buffered solution	Manual and orbital shaker	Low speed centrifugation	Manual DNA extraction	Dot blot, southern blotting, hybridization	2.5 x 10^7 CFU g^{-1}	ND	ND	An early study looking at non-sporulating Gram-negative *Pseudomonas* and how to extract it from soil samples.
Jain, 2011 (77)	*B. anthracis*	Field soil, talcum powder	0.1	Indirect	PBS with Triton™ X-100	Vortex	Low speed centrifugation	Spore pellet 100 °C heat lysis	Real-time LAMP detecting *pag* gene	20 - 10^8	50	ND	Real-time LAMP detection was 2,000 times more sensitive than traditional PCR in this analysis
Juergensmeyer, 2006 (100)	*B. anthracis* multiple strains	Soil, sewage, blood, paper, cotton	Not known	Direct	Water	Vortex	Settle	None	Cultured on ChrA plates	10^7	10 - 10^3	ND	ChrA can distinguish between *B. cereus, B. thuringiensis* and *B. anthracis* with the rate of color change in the colonies after 48 hours.
Jula, 2007 (101)	*B. anthracis*	668 Iranian soils	Not known	Direct	Distilled water	Mixed	None	Freeze thaw lysis	PLET and blood agar culture	None	ND	ND	21 of the 668 soils contained virulent *B. anthracis* isolates. Spores in settled supernatant were filtered to concentrate.

Kane, 2009 (112)	*B. globigii* ATCC 9372	AZ dust on wipes	0.5	Indirect	pH 9.5 buffer with Tween® 80	Vortex	Filtration	Heat-treated to lyse vegetative cells. Spores heat lysed in PCR plate 95 °C for 20 min before PCR. Incubated in TSB for 16 hrs	qPCR *recF* gene	10^2 - 10^4	200	ND	Inhibition at 10^3 and 10^4 spores with 0.5 g of AZ dust. Protocol able to detect only germinated spores. Samples filtered to concentrate spores, not to separate soil from the spores.
Kuske, 2006 (12)	*B. anthracis, Francisella tularensis, Y. pestis, Clostridium perfringens*	129 U.S. soil samples	0.5	Direct	None	None	None	Bead beating ethanol precipitation with spin Sephadex® G-200 column cleanup	PCR targeting *pag* gene	None	ND	ND	0.1 pg template DNA represents 17-46 genomic equivalents (GEq), no work done to determine the extraction LOD; extracted 0.2-146 µg of DNA g^{-1} soil.
Leishman, 2010 (59)	*B. anthracis*	Water, whole milk, orange juice	3 mL	Indirect	Hexadecane solution	Vortex	Spores separate in hexadecane layer due to hydrophobic properties	None	Microbial adherence to hydrocarbons (MATH) with culturing on TSA plates	10^3 Spores mL^{-1}	ND	5	Hexadecane separation protocols were not effective.
Lindhal, 1996 (83)	*B. subtilis, Escherichia coli*	Gamma sterilized agricultural clay loam	6 or 60	Indirect	20 g soil into 0.05 M pyrophosphate pH 8.0 solution or water	Waring® blender homogenization	Nycodenz® HSGS	Physical disruption and chemical disruption of cells from soil particles	Fluorescent microscopy enumeration by acridine orange direct counting	10^9	ND	24 – 42	Method of cell-soil disruption depends on the purpose of the cell extraction. Pyrophosphate solution more efficient than water.
Luna, 2009 (98)	283 species, 162 *B. cereus* group (23 *B. anthracis* strains), 50 other *Bacillus* species	5 from FL, 5 from TX	0.5	Direct	Modified PLET broth	Vortex	None	None	Modified PLET agar with antibiotics	10^4	ND	ND	Modified PLET selectivity against *Bacillus* species. Selectivity against Bacillus species 100% at 24 hours (hr) and 96.8% at 48 hr at 30 °C.
Maarit Niemi, 2001 (78)	Environmental	Clay top soil, sandy soil	1	Indirect	Crombach buffer	Stomacher homogenization	High-speed centrifugation	Five DNA extraction protocols with varying amounts of SDS and guanidine isothiocyanate and a MoBio Soil DNA Isolation Kit	PCR-denaturing gradient gel electrophoresis (DGGE) targeting 16S rDNA V3 variable region	None	ND	ND	Different isolation and purification protocols resulted in different bacterial profiles from a soil sample.

Marston, 2008 (66)	16 *B. anthracis* strains	TX soil, AZ dust	1	Indirect	PBS -Tween® 20	Vortex	Settling time - supernatant cultured	None	Culture on PLET and ChrA	10^7	ND	0.5 - 7.7	7.7% of the spiked spores were recovered from the TX soil sample using PLET and ChrA, while only 0.5% was recovered from AZ dust. Overall, PLET is more sensitive and selective than ChrA.
Naclerio, 2009 (54)	*B. subtilis*	pyroclastic topsoil	Not known	Direct	Buffered peptone-water	Vigorous vortexing	None	None	Vegetative cells lysed by heat and before culturing on LB plates	10^{10}	ND	ND	Soil column experiments were conducted to ascertain the interaction between *B. anthracis* and soil. Key finding was that exosporium does not play a role in *B. anthracis* spore retention with the studied soil type.
Nicholson, 1999 (39)	Environmental *B. anthracis*	Three Sonoran desert soils	100	Indirect	Chelex® 100 in buffer	Vortex	Low speed centrifugation with supernatant filtration - some samples further processed with NaBr HSGS (1.0 - 1.5 g mL^{-1})	None	Culture on nutrient sporulation medium (NSM)	None	ND	1.4-4.3 post Chelex® cleaning; <1 post NaBr HSGS	Authors suggested that a majority of the spores within the tested soils were unrecoverable as the spores remained attached to the large soil particles. Their HSGS protocol significantly reduced spore yields.
Panning, 2007 (96)	*B. cereus, B. anthracis* Sterne	50 environmental and clinical samples	0.1	Direct	None	None	None	Pre-extraction: 100 µL sample with Gentra™ systems cell lysis solution Lysozyme and Proteinase K Gentra Puregene® Blood Kit QIAamp DNA Mini Kit Viral RNA Mini Kit MagAttract® DNA Mini M48 Kit MagAttract Viral RNA M48 Kit	qPCR for *pag* gene of pX01	200 - 2 x 10^5 CFU mL^{-1}	200 CFU mL^{-1}	ND	The study concluded that in light of the sensitivity and safety seen, the QIAamp Viral RNA Mini Kit and the MagAttract DNA Mini M48 Kit were optimal for spore DNA extraction in low and high throughput settings, respectively.

Parachin, 2010 (85)	Environmental soil organisms	Garden soil	5	Indirect	BactXtractor-M or BactXtractor-H	Vortex homogenization	2-phase liquid HSGS	Manual DNA extraction or MO BIO PowerMax® Soil DNA Isolation Kit	Nonspecific 16S-rRNA amplification	None	ND	ND	The environmental DNA extracted after gradient flotation was comparable in yield and purity to the direct commercial PowerSoil® Kit extracts.
Patel 2013(110)	*B. thuringiensis*	53 soil samples from diverse geographical regions in India	1	Indirect	Enrichment with sterile glucose yeast extract salt	Shaker and heat treatment	Low speed centrifugation	None	Luria-Bertani broth agar plates	None	ND	55-75%	The enrichment protocol recovered a higher percentage of spores than treatment of the samples with heat and sodium acetate treatments performed as described by (63) and (94), respectively.
Pillai, 1991 (80)	*Rhizobium leguminosarum*	Pima clay loam, brazito sandy loam	1	Indirect	Calcium chloride solution	Vortex	Sucrose HSGS (1.33 g mL^{-1})	No specific DNA extraction - cell solution directly added to PCR for heat lysis	PCR targeting the Tn5 insertion mutant	10^7 - 10^8	1 - 10 CFU	ND	Spores were not tested in this study.
Pote, 2010 (84)	Environmental	Lake sediments	100	Indirect	2% sodium hexametaphosphate (SHMP)	Vortex	Low-speed centrifugation, supernatant filtration, high-speed centrifugation and pellet Nycodenz® HSGS	PowerMax® Soil DNA Isolation Kit	DNA quantified through spectrophotometry	None	ND	ND	The first supernatant fraction following SHMP wash with low-speed centrifugation and supernatant filtration is sufficient to quantify and extract bacterial cells. Their protocol included low-speed centrifugation, supernatant filtration, high-speed centrifugation, and final cell pellet separation with Nycodenz® HSGS.
Rastogi, 2009 (61)	Plasmid-free strain of *B. anthracis*	Carpet, ceiling tile, concrete, steel, wallboard, wood	1.7 cm^2	Direct	Bacto™ buffered peptone water with Tween® 80	Sonicate and vortex	None	None	Culture on tryptic soy agar plates	10^6 - 10^8	ND	25	Study sought the decontamination effects of chlorine dioxide gas and vaporous hydrogen peroxide. A pre-study experiment showed that Tween® 80, Tween® 20, and Triton™ X-100 showed no statistical difference in spore

												recovery.
Roh, 2006 (72)	Environmental	German soil, sediment, activated sludge	0.1	Indirect & direct	Indirect: 0.1 g soil into buffer at pH 8.0, buffer with surfactant; or Chelex® 100 Direct: none	Indirect: 1 - 10 hr shake or homogenized in blender, Direct: none	Indirect: low speed centrifugation, Direct: none	Indirect: manual DNA extraction Direct: manual extraction microwave lysis, bead beating, freeze-thaw lysis, or SoilMaster™ DNA Isolation Kit	PCR targeting various phylogenic groups and restriction enzyme digestions	None	ND	ND	0.1 g sample size insufficient for indirect extraction protocols as shown through ~hundredfold increase of DNA yield for direction extraction.
Rose, 2011 (108)	*B. globigii*	Biological wash powder, skimmed milk powder, flour, talcum powder, spackling powder	0.1 mL	Direct	None	None	None	Instagene™ Matrix UltraClean® Soil DNA Isolation Kit Extract-N-Amp™ Plant and Seed Kit IT 1-2-3™ QFlow Kit QuickGene DNA Tissue Kit S PrepFilter™ Forensic DNA Extraction Kit MasterPure™ Complete DNA and RNA Purification Kit	PCR for Bg B-type SASP gene	10^8 - 10^{10}	ND	ND	Study sought to find a single DNA extraction protocol for liquids, solids, and powders in a BSL3 setting. The Ultraclean® Microbial DNA Isolation Kit was statistically best overall, and the PrepFilter™ Kit was best for the tested powders.
Ryu, 2003 (40)	13 *Bacillus* species including 4 *B. anthracis* strains	Random soil collected in Korea	0.1	Indirect	Indirect: sterile water, 10% Triton™ X-100 in PBS or 1.22 g mL^{-1} sucrose plus Triton™ X-100 in PBS	Suspended and centrifuged multiple times	Low speed centrifugation	Soil slurries incubated for 20 min in germination buffer, heat lysed during initial denaturation step of PCR	Multiplex PCR targeting *pag, cap*, and *sap* genes	10^4 - 10^8	10^6 - >10^8	ND	Sucrose/Triton™ X-100 proved to be a simple and effective protocol as it was the only one that gave results at 10^6 spores g^{-1}. Hypothesized that *B. anthracis* adheres to a variety of solid matrices with hydrophobic interactions; therefore, solutions with non-ionic detergent and a high concentration of sucrose disrupt hydrophobic interactions and lift the freed spores. Sensitivity of

												germination treatment was reduced compared to pure spore solutions. qPCR system showed identification of *B. anthracis* at 10^4 spore g^{-1} in three hr of arrival at the laboratory.
Saikaly, 2007 (55)	*B. atrophaeus* spores and cells	Synthetic building debris (SBD), leachate	0.5	Direct	None	None	None	PowerSoil® DNA Isolation Kit	qPCR targeting 16S-23S rRNA ITS region and *recA* gene	10^1 - 10^7 Bg spores	leachate 10^1, SBD 10^2	ND	Amplification efficiency for *recA* in SBD was 87% for the *B. atrophaeus* spores.
Santana, 2008 (63)	*B. thuringiensis*	Venezuelan soils	1	Direct	Dry heat followed by saline solution	Vortexing	None	None	Spread plate LB agar	None	ND	60	Isolation of *B. thuringiensis* from soil better with a dry preheat step.
Sjöstedt, 1997 (9)	*B. anthracis* Sterne and Pasteur	Litter, swamp, meadow, cultivated soil	0.1	Direct	None	None	None	Manual freeze thaw, phenol/chloroform and glass milk beads	PCR *cap* and *lef* genes, southern blot confirmation	10^7	10^3 - 10^4	ND	Detection was seen only after enrichment in Heart Infusion Broth due presumptively to inhibiting compound within the soil samples.
Stratilo, 2012 (81)	Environmental	Soil from Wood Buffalo National Park	2.5	Indirect	Sucrose solution	Shaken by hand	Low speed centrifugation - supernatant used for sucrose HSGS (1.14 - 1.22 g mL^{-1})	Suspected colonies were processed with PrepMan® sample preparation reagent	PLET culture	None	ND	ND	Processing protocol followed steps from Dragon and Rennie(60).
Tims, 2004 (58)	*B. anthracis* Ames	Talcum powder, corn starch, powder sugar, baking soda	0.001	Direct	PBS	Five min incubation	None	None	Biosensor assay	10^5	3.2 x 10^5	ND	Samples were spiked with 10^5 spores and tested.
Travers, 1987 (94)	*B. anthracis, B. thuringiensis*	WY soil	0.5	Direct	Sodium acetate buffered LB broth	Shaker and heat treatment	None	None	Culture on LB agar plates	10^6	<100	ND	While *B. thuringiensis* was the target of this study, *B. anthracis* was also removed from the soil samples.
Vahedi, 2009 (103)	Environmental	Iranian soil	Not known	Indirect	PBS	Overnight incubation	Settling time - supernatant concentrated	Heat inactivation, freeze thaw lysis, centrifugation	PLET cultures followed by PCR targeting *B. anthracis* chromosome, protective antigen, and capsule	None	ND	ND	Soil samples were cultured and positive cultures were confirmed with PCR. Samples were filtered to concentrate spores in the settled

												supernatant.
Whitehouse, 2007 (107)	*F. tularensis*	Silt loam, clay, potting soil	0.1 - 10	Direct	None	None	None	Gentra Puregene® DNA Purification Kit QIAmp DNA Stool Mini Kit SoilMaster® DNA Extraction Kit UltraClean® Soil DNA Isolation Kit PowerMax® Soil DNA Isolation Kit	PCR for *fopA* gene	10 - 10^5 spores	2 x 10^2 - 2 x 10^4 500 10^2 - 10^3 20 100	ND	UltraClean® and PowerMax® Soil DNA Isolation Kits were the most consistent and sensitive kits for extracting *F. tularensis* from soil.
Wielinga, 2011 (95)	*B. anthracis, B. thuringiensis*	corn meal, whey powder, wheat flour, soybean flour, corn grain, Irish milk	0.1	Direct	None	None	None	NucliSENS® lysis buffer and NucliSENS® DNA Magnetic Extraction Reagents	Lysis buffer-soil slurry cultured on BHI agar; qPCR for *B. anthracis* and *B. thuringiensis*	3 x 10^6	ND	<1 – 60	Sampling matrix can influence the DNA extraction efficiency.
Yitzhaki, 2006 (90)	*B subtilis, B. thuringiensis, B. anthracis*	None	None	Indirect	PBS	Sonicate or shake	IMS	None	Electron microscopy and flow cytometry	Unknown	ND	40 - 90	Cationic surfactant aided in linking the spores to the silica magnetic particles (increasing from 40 to 90% with the addition of DDAB). Overall adsorption to the magnetic particles was low.
Zhou, 1996 (10)	*Pseudomonas sp.* strain B13	Loam, sandy loam, sandy clay loam	5	Direct	None	None	None	Direct manual lysis with CTAB extraction buffer, SDS and proteinase K	PCR targeting 16S rRNA, restricted fragment length polymorphism (RFLP), southern blotting	Unknown	ND	27 - 80	Significant correlation was observed between crude DNA yield and soil organic carbon content, as the carbon content increased so too did the DNA yield.

AZ dust – Arizona Test Dust
BHI – Brain heart infusion medium
BSA – Bovine serum albumin
CFU – Colony forming units
ChrA – R & F® *anthracis* chromogenic agar
CTAB – Cetyltrimethylammonium bromide
DDAB – Didecyldimethylammonium bromide
DNA – Deoxyribonucleic acid
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme linked immunosorbent assay
EPA – U.S. Environmental Protection Agency
fg – Femtogram(s)
FITC – Fluorescein isothiocyanate
FL - Florida
GEq – Genomic equivalents
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSGS – High specific gravity separation
IgG – Immunoglobulin G
IM-ECL – Immunomagnetic-electrochemiluminescent
IMS – Immunomagnetic separation
ITS – Internally transcribed spacer region
LB – Luria broth
LC/MS – Liquid chromatography-mass spectrometry
LOD – Limit of detection
MN loam – Minnesota loam
ND – Not determined
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PCR-DGGE – Polymerase chain reaction and denaturing gradient gel electrophoresis
PLET – Polymyxin B, lysozyme, ethylenediaminetetraacetic acid, thallous acetate
PVPP – Polyvinylpyrrolidone
qPCR – Quantitative polymerase chain reaction
rDNA – Ribosomal deoxyribonucleic acid

RFLP – Restriction fragment length polymorphism (DNA analysis)
RNA – Ribonucleic acid
rRNA – Ribosomal ribonucleic acid
RV-PCR – Rapid viability-polymerase chain reaction
SASP – Small acid-soluble proteins
SBA – Sheep blood agar
SBD – Synthetic building debris
SDS – Sodium dodecyl sulfate
SHMP – Sodium hexametaphosphate
TFA – Trifluoroacetic acid
TSA – Trypticase® soy agar
TSB – Trypticase® soy broth

Appendix B – Table of Commercial DNA Kits Used for Direct Soil Analysis

Appendix B: Table of Commercial DNA Kits Used for Direct Soil Analysis

Manufacturer	Kit	Protocol	Sample Mass (g)	Cost / Sample ($)	Processing Time (hr)	Consumables Additional Reagents	Additional Equipment	Reference	Organism	Soil Type	PCR target gene	LOD (CFU g^{-1})	% Recovery	Notes
Applied Biosystems, Foster City, CA	PrepFilter™ Forensic DNA Extraction Kit	Detergent lysis, magnetic bead DNA concentration	0.1	4.3	3	Ethanol, Isopropanol	Magnetic stand	Rose *et al.*, 2011 (108)	*Bacillus globigii*	Household powders	B-type SASP gene	ND	ND	Statistically best kit overall for dry powders.
BD Molecular Diagnostics Franklin Lakes, NJ	GeneOhm™ Lysis Kit	Bead beating lysis, heat lysis	0.2		0.2	Tubes	Heat block	Isabel *et al.*, 2012 (69)	*B. atrophaeus*	Garden soil	*atpD*	5000	51	
bioMérieux Inc., Durham, NC	NucliSENS® Isolation Kit	Guanidine thiocyanate/Triton™ X-100/Tris HCl lysis, silica bead DNA concentration	0.2	9.4	3	Ethanol, Acetone	None	Dauphin *et al.*, 2009 (76)	*B. anthracis* Ames	Baking soda	pX01, pX02, chromosome	10^6		Viable spores were seen in the final extracts; however, kit gave the greatest total yield of *B. anthracis* DNA.
										Corn starch		10^6	ND	
										Talcum		10^7		
								Weilinga *et al.*, 2011 (95)	*B. anthracis, B. thuringiensis*	Multiple food matrices	pX01, pX02, chromosome	ND	<1 - 60	Sampling matrix influences the DNA extraction efficiency.
Bio-Rad Laboratories, Hercules, CA	InstaGene™ Matrix	Heat lysis, no DNA concentration - inhibitors are bound	0.1	0.5	0.5	None	None	Rose *et al.*, 2011(108)	*B. globigii*	Household powders	B-type SASP gene		ND	
Epicentre® Madison, WI	SoilMaster® DNA Extraction Kit	Hot detergent lysis, inhibitor removing resin-filled spin columns and DNA concentration	0.1	3.9	2	Ethanol	None	Roh *et al.*, 2006 (72)	Environmental	German soil, sediment, activated sludge	16S rRNA	ND	ND	Direct extraction resulted in eDNA fragments of about only 12kb in size due to significant shearing throughout the process.
								Whitehouse and Hottel, 2007 (107)	*Francisella tularensis*	Silt loam	*fopA*	100		Kit removed inhibitors from all three soil types tested.
										Clay		1000	ND	
										Potting soil		1000		
								Dineen *et al.*, 2010 (6)	*B. cereus* T-strain	Sand	*P1-PLC*	10^7	0.59	Loam extracts required dilution to dilute inhibition.
										Clay		10^8	0.00	
										Loam		10^8	0.01	
								Gulledge *et al.*, 2010 (7)	*B. anthracis* Pasteur	FL sand	*capC, pag, and lef*	10^6		Inhibition seen at concentrations greater than 10^7 spores g^{-1} soil.
										TX sand		10^6	ND	
										Garden soil		ND		

Manufacturer	Kit	Protocol	Sample Mass (g)	Cost / Sample ($)	Processing Time (hr)	Consumables Additional Reagents	Additional Equipment	Reference	Organism	Soil Type	PCR target gene	LOD (CFU g^{-1})	% Recovery	Notes
Epicentre® Madison, WI	MasterPure™ Complete DNA and RNA Purification Kit	Hot detergent lysis, DNA precipitation concentration	0.1	1.45	0.5	None	None	Rose *et al.*, 2011 (108)	*B. globigii*	Household powders	B-type SASP gene		ND	
			0.003					Luna *et al.*, 2003 (114)	*B. anthracis* Pasture	Flour, baking soda, talcum powder, cornstarch	chromosome *BaS13*	4000	ND	These results were found after germination, heat shock, sonication and autoclaving prior to DNA extraction.
FujiFilm Corporation, Tokyo, Japan	QuickGene® DNA Tissue Kit S and QuickGene-Mini80	Detergent lysis, vacuum filter DNA concentration	0.1		0.5	Ethanol	None	Rose *et al.*, 2011 (108)	*B. globigii*	Household powders	B-type SASP gene	ND	ND	
Idaho Technology Salt Lake City, UT Now Biofire Diagnostics	IT 1-2-3™ Sample Purification Kits	Bead beating lysis, DNA binds to magnetic beads, inhibition wash, DNA concentration	0.5	11.25	0.25	None	PickPen® 1-M	Dineen *et al.*, 2010 (6)	*B. cereus* T-strain	Sand	*P1-PLC*	10^7	0.06	
										Clay		ND	ND	
										Loam		10^7	0.00	
								Rose *et al.*, 2011 (108)	*B. globigii*	Household powders	B-type SASP gene	ND	ND	
MO BIO Laboratories Carlsbad, CA	UltraClean® Soil DNA Isolation Kit	Bead beating lysis, silica spin filter DNA concentration	1	3.78	1.5	Ethanol	Vortex adapter, PowerVac® manifold	Whitehouse and Hottel, 2007 (107)	*F. tularensis*	Silt loam	*fopA*	20		Kit removed inhibitors from all three soil types tested.
										Clay		20	ND	
										Potting soil		20		
								Dauphin *et al.*, 2009 (76)	*B. anthracis* Ames	Baking soda	pX01, pX02, chromosome	10^6		At a concentration of 10^6 spores mL^{-1} no viable spores were seen in the final extract, and the final extract have very clean DNA.
										Corn starch		10^6	ND	
										Talcum powder		10^7		
								Griffin *et al.*, 2009 (51)	*Bacillus* species	N-S US transect	*rpoB*	170	ND	
								Fitzpatrick *et al.*, 2010 (67)	*Coxiella burnetii*	Light sandy soil	*IS1111*			
								Gulledge *et al.*, 2010 (7)	*B. anthracis* Pasteur	FL sand	*capC, pag, and lef*	ND		Inhibition seen at concentrations greater than 10^7 spores g^{-1} soil.
										TX sand		10^5	ND	
										Garden soil		10^6		
								Bradley *et al.*, 2011 (68)	*B. anthracis* Sterne	AZ dust	LRN primer/probe	10^2		Better at extracting DNA from potting soil than AZ dust.
										Potting soil		10^3	ND	
								Rose *et al.*, 2011 (108)	*B. globigii*	Household powders	B-type SASP gene	ND	ND	Statistically best kit overall across multiple sample types among the kits evaluated in this study.

Manufacturer	Kit	Protocol	Sample Mass (g)	Cost / Sample ($)	Processing Time (hr)	Consumables Additional Reagents	Additional Equipment	Reference	Organism	Soil Type	PCR target gene	LOD (CFU g^{-1})	% Recovery	Notes
MO BIO Laboratories Carlsbad, CA	PowerMax® Soil DNA Isolation Kit	Bead beating lysis, Inhibitor Removal Technology ®, silica spin filter DNA concentration	10	20.3	0.5	None	50 mL centrifuge	Pote *et al.*, 2010 (84)	Environmental	Lake sediments	None	ND	ND	Extracted DNA was only quantified though spectrophotometry.
								Whitehouse and Hottel, 2007 (107)	*F. tularensis*	Silt loam	*fopA*	100		Kit removed inhibitors from all three soil types tested.
										Clay		100	ND	
										Potting soil		100		
								Irenge *et al.*, 2010 (113)	*B. anthracis; B. cereus*	Unknown	*pstI* and *purA*	4	ND	LOD for PCR was 25 fg (corresponded to Ct values of 35.85–38.33). Lowest soil spike concentration 10^4 spore g^{-1}.
								Parachin *et al.*, 2010 (85)	Environmental soil organisms	Garden soil	16S rRNA region	ND	ND	The environmental DNA extracted after gradient flotation was comparable in yield and purity to the direct commercial PowerSoil® Kit extracts.
MO BIO Laboratories Carlsbad, CA	Powersoil® DNA Isolation kit	Bead beating lysis, Inhibitor Removal Technology ®, silica spin filter DNA concentration	0.25	4.44	1.5	Ethanol	Vortex adapter, PowerVac manifold	Maarit Niemi *et al.*, 2001 (78)	Environmental	Clay top soil, sandy soil	16S rRNA V3 variable region	ND	ND	
								Griffin *et al.*, 2009 (51)	*Bacillus* species	Gulf coast soils	*rpoB*	4	ND	LOD for PCR was 25 fg (Ct values of 35.85–38.33*)*.
								Dineen *et al.*, 2010 (6)	*B. cereus* T-strain	Sand	*P1-PLC*	10^7	5.28	
										Clay		10^9	0.00	
										Loam		10^7	0.22	
								EPA, 2012 (79)	*B. globigii*	Sand		10^6	ND	0.25 g of sand were directly extracted using the kit.
												10^4	ND	45 g of sand were washed and the remaining pellet was processed through the kit.
								Saikaly *et al.*, 2007 (55)	*B. atrophaeus*	SBD	*recA*	10^2	87	Saikaly *et al.* added a heat incubation step before the PowerSoil® kit protocol, 70 °C for 20 min with solution C1.
										SBD	*16S ITS* region	10^1	104	
										Leachate	*16S ITS* region	10^1	97	
None	Manual	Freeze-thaw lysis, DNA precipitation	5		6	All	All	Balestrazzi *et al.*, 2009 (3)	*B. subtilis*	Loamy sand	*swrAA*	10^4	99	Spores were much harder to lyse than cells.

Manufacturer	Kit	Protocol	Sample Mass (g)	Cost / Sample ($)	Processing Time (hr)	Consumables Additional Reagents	Additional Equipment	Reference	Organism	Soil Type	PCR target gene	LOD (CFU g^{-1})	% Recovery	Notes
Omega Bio-Tek Norcross, GA	EZNA® Soil DNA Kit	Bead beating lysis, heat lysis, inhibitor removal reagent, silica spin filter DNA concentration	1	1.98	2.5	Tubes, isopropanol, ethanol	None	Dineen *et al.*, 2010 (6)	*B. cereus* T-strain	Sand	*P1-PLC*	10^7	0.39	
										Clay		10^7	0.00	
										Loam		10^7	0.30	
Qbiogene Solon, OH Now MP Biomedicals	FastDNA® SPIN Kits for Soil	Bead beating lysis, silica spin filter DNA concentration	0.5	4.81	2	Tubes, ethanol	FastPrep bead beater	Cheun *et al.*, 2003 (5)	*B. anthracis*	Garden soil	*pag, capA, and sap*	10^3	ND	After two rounds of soil sample enrichment the LOD decreased to 10 spores g^{-1} soil.
								Dineen *et al.*, 2010 (6)	*B. cereus* T-strain	Sand	*P1-PLC*	10^7	17.24	Loam extracts required 100X dilution to reduce inhibition. Highest recovery rates.
										Clay		10^7	11.54	
										Loam		10^7	2.80	
								Gulledge *et al.*, 2010 (7)	*B. anthracis* Pasteur	FL sand	*capC, pag, and lef*	ND		Inhibition seen at concentrations greater than 10^7 spores g^{-1} soil.
										TX sand		10^7	ND	
										Garden soil		10^7		
								Hong-Geller *et al.*, 2010 (65)	*B. anthracis* Sterne and Ames	Swabs and wipes off of glass, stainless steel, vinyl, and plastic	pX01	ND	Sterne: >90 Ames: 2 - 75	Ames spore DNA was more difficult to recover than the Sterne spore DNA.
								Delmont *et al.*, 2011 (82)	All soil organisms	Park grass soil	ribosomal spacer region	ND	ND	Extracted soil to determine soil metagenome. *Bacillus* species found after bead beating at 18 - 21 cm depth.
QIAGEN Valencia, CA	Gentra Puregene® Yeast/Bacteria Kit	Detergent lysis, alcohol DNA precipitation	1	1	3	None	None	Whitehouse and Hottel, 2007 (107)	*F. tularensis*	Silt loam	*fopA*	2000		
										Clay		20000	ND	
										Potting Soil		200		
QIAGEN Valencia, CA	Gentra Puregene® Blood Kit	Detergent lysis, alcohol DNA precipitation	1	1	3	None	None	Panning *et al.*, 2007 (96)	*B. cereus, B. anthracis* Sterne	Environmental samples	*pag*	10^3 CFU mL^{-1}	ND	Panning used a lysozyme, proteinase K, and heat pre-extraction cleanup protocol prior Kit DNA extraction.
QIAGEN Valencia, CA	QIAamp DNA Stool Mini Kit	Hot detergent lysis, inhibitor removing resin-filled spin columns and DNA concentration	0.5	3.84	1	Ethanol	None	Whitehouse and Hottel, 2007 (107)	*F. tularensis*	Silt loam	*fopA*	500		Inhibition was seen in the potting soil samples- no inhibition from silt loam or clay soils.
										Clay		500	ND	
										Potting soil		500		

Manufacturer	Kit	Protocol	Sample Mass (g)	Cost / Sample ($)	Processing Time (hr)	Consumables Additional Reagents	Additional Equipment	Reference	Organism	Soil Type	PCR target gene	LOD (CFU g^{-1})	% Recovery	Notes
QIAGEN Valencia, CA	QIAamp DNA Stool Mini Kit and MagAttract DNA Stool Mini Kit	Hot detergent lysis, InhibitEX® adsorption of PCR inhibitors, silica spin column DNA concentration	0.5	7.1	1.5	Ethanol	BioRobot M48 workstation	Gulledge *et al.*, 2010 (7)	*B. anthracis* Pasteur	FL sand	*capC, pag,* and *lef*	10^5		Inhibition seen at concentrations greater than 10^7 spores g^{-1} soil.
										TX sand		10^4	ND	
										Garden soil		ND		
QIAGEN Valencia, CA	QIAamp DNA Blood Mini Kit	Enzyme lysis, silica spin filter DNA concentration	1	2.64	1	Ethanol	None	Panning *et al.*, 2007 (96)	*B. cereus, B. anthracis* Sterne	Environmental samples	*pag*	2000 CFU mL^{-1}	ND	
								Dauphin *et al.*, 2009 (76)	*B. anthracis* Ames	Baking soda	pX01, pX02, chromosome	10^7		The final extracts contained clean DNA and some viable spores.
										Corn starch		10^6	ND	
										Talcum Powder		10^8		
								Bradley *et al.*, 2011 (68)	*B. anthracis Sterne*	AZ dust	LRN primers/ probes	10^2	ND	Better at extracting DNA from AZ dust than potting soil.
										Potting soil		10^3		
QIAGEN Valencia, CA	QIAamp DNA mini Kit	Enzyme lysis, silica spin filter DNA concentration	0.25	2.92	1	Ethanol	None	Panning *et al.*, 2007 (96)	*B. cereus, B. anthracis* Sterne	Environmental samples	*pag*	10^4 CFU mL^{-1}	ND	Panning used a lysozyme, protease K, and heat pre-extraction cleanup protocol prior Kit DNA extraction.
QIAGEN Valencia, CA	QIAamp Viral RNA mini Kit	Enzyme lysis, silica spin filter DNA concentration	0.25	4.4	1	Ethanol	None	Panning *et al.*, 2007 (96)	*B. cereus, B. anthracis* Sterne	Environmental samples	*pag*	10^3 CFU mL^{-1}	ND	Panning used a lysozyme, protease K, and heat pre-extraction cleanup protocol prior Kit DNA extraction.
QIAGEN Valencia, CA	MagAttract DNA Mini M48 Kit	Enzyme lysis, Magnetic Bead separation and concentration	0.25	3.26	1.5	Ethanol	BioRobot M48 workstation	Panning *et al.*, 2007 (96)	*B. cereus, B. anthracis* Sterne	Environmental samples	*pag*	10^3 CFU mL^{-1}	ND	Panning used a lysozyme, protease K, and heat pre-extraction cleanup protocol prior Kit DNA extraction.
QIAGEN Valencia, CA	MagAttract Viral RNA M48 Kit	Enzyme lysis, Magnetic Bead separation and concentration	0.25	3.68	1.5	Ethanol	BioRobot M48 workstation	Panning *et al.*, 2007 (96)	*B. cereus, B. anthracis* Sterne	Environmental samples	*pag*	10^5 CFU mL^{-1}	ND	Panning used a lysozyme, protease K, and heat pre-extraction cleanup protocol prior Kit DNA extraction.
Roche Indianapolis, IN	MagNA Pure LC DNA Isolation Kit III	Soil prewash and centrifuge, bead-beat lysis, magnetic bead technology	0.5	2.19	1.5	None	MagNA Pure LC System	Gulledge *et al.*, 2010 (7)	*B. anthracis* Pasteur	FL sand	*capC, pag,* and *lef*	ND		Inhibition seen at concentrations greater than 10^7 spores g^{-1} soil.
										TX sand		ND	ND	
										Garden soil		ND		
Sigma-Aldrich, St. Louis. MO	Extract-N-Amp™ Plant and Seed Kit	Liquid N_2 lysis, no DNA concentration	1	2.1	0.25	PCR grade water	None	Rose *et al.*, 2011 (108)	*B. globigii*	Household powders	B-type SASP gene	ND	ND	Kit did not perform well with the tested media.
Zymo Research Irvine, CA	ZR Soil Microbe DNA Kit™	Bead beating lysis, silica spin filter DNA concentration	0.25	3.05	1.5	Tubes	None	Dineen *et al.*, 2010 (6)	*B. cereus* T-strain	Sand	*P1-PLC*	10^7	0.04	Only 1 of 3 clay extracts were detected at 10^7, 3 of 3 detected at 10^8.
										Clay		ND		
										Loam		10^7	0.02	

AZ dust – Arizona test dust
CDC – Centers for Disease Control and Prevention
DNA – Deoxyribonucleic acid
EPA – U.S. Environmental Protection Agency
LOD – Limit of detection
LRN – Laboratory Response Network
ND – Not determined
PCR – Polymerase chain reaction
RBMS – Reference background matrix soil
RNA – Ribonucleic acid
rRNA – Ribosomal ribonucleic acid
SASP – Small acid-soluble proteins
SBD – Synthetic building debris

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