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Evaluation of Analytical Methods for Detection of *Bacillus anthracis* Surrogate Spores: Compatibility with Real-World Maritime Environmental Samples Collected from USCG Assets and Facilities

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DRAFT REPORT

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Evaluation of Analytical Methods for Detection of *Bacillus anthracis* Surrogate Spores: Compatibility with Real-World Maritime Environmental Samples Collected from USCG Assets and Facilities

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Disclaimer

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Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the Nation's land, air, and water resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, EPA's research program is providing data and technical support for solving environmental problems today and building a science knowledge base necessary to manage our ecological resources wisely, understand how pollutants affect our health, and prevent or reduce environmental risks in the future.

The Center for Environmental Solutions and Emergency Response (CESER) within the Office of Research and Development (ORD) conducts applied, stakeholder-driven research and provides responsive technical support to help solve the Nation's environmental challenges. The Center's research focuses on innovative approaches to address environmental challenges associated with the built environment. We develop technologies and decision-support tools to help safeguard public water systems and ground water, guide sustainable materials management, remediate sites from traditional contamination sources and emerging environmental stressors, and address potential threats from terrorism and natural disasters. CESER collaborates with both public and private sector partners to foster technologies that improve the effectiveness and reduce the cost of compliance, while anticipating emerging problems. We provide technical support to EPA regions and programs, states, tribal nations, and federal partners, and serve as the interagency liaison for EPA in homeland security research and technology. The Center is a leader in providing scientific solutions to protect human health and the environment.

This report focuses on the evaluation of analytical methods for the U.S. Coast Guard (USCG) preparedness to respond to anthrax contamination incidents. This work was coordinated and managed by the EPA's Homeland Security Research Program (HSRP) under the Department of Homeland Security (DHS) funded Analysis for Coastal Operational Resiliency (AnCOR) project.

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Acronyms and Abbreviations

AnCOR	Analysis for Coastal Operational Resiliency
ASTM	American Society for Testing and Materials, now ASTM International
B. anthracis	*Bacillus anthracis*
Ba	*Bacillus anthracis*
BHIB	Brain Heart Infusion Broth
BSC	Biological Safety Cabinet
Btk T1B2	*Bacillus thuringiensis* Subsp. *kurstaki* T1B2
°C	Degree(s) Celsius
CBR	Chemical, Biological, Radiological
CDC	Centers for Disease Control and Prevention
CESER	Center for Environmental Solutions and Emergency Response
CFU	Colony Forming Unit(s)
cm	Centimeter(s)
Ct	Threshold Cycle
dH_2O	Distilled Water
DHS S&T	U.S. Department of Homeland Security Science and Technology Directorate
DNA	Deoxyribonucleic Acid
EPA	U.S. Environmental Protection Agency
ERLN	Environmental Response Laboratory Network
ft	Feet
g	Gram(s)
h	Hour
HDPE	High-Density Polyethylene
HSMMD	Homeland Security and Materials Management Division
HSPD	Homeland Security Presidential Directive(s)
HSRP	Homeland Security Research Program
ID	Identification
in	Inch(es)
IT	Interagency Team
L	Liter(s)
µL	Microliter(s)
MCE	Methyl Cellulose Ester
MGAL	Marine Grade Aluminum
min	Minute(s)
mL	Milliliter(s)
ModG	Modified G
MRST	Maritime Response Security Team
MSKID	Marine Grade Aluminum and 50% Nonskid
NSF	National Strike Force
NTC	No Template Control
ORD	Office of Research and Development
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.05% Tween 20
PBSTE	Phosphate Buffered Saline with 0.05% Tween 20 and 30% Ethanol
PC	Positive Control

PCR	Polymerase Chain Reaction
PE	Performance Evaluation
pg	Picogram
PMP	Paramagnetic Particle
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
QMP	Quality Management Plan
qPCR	Quantitative PCR
RBM	Response Boat Medium
RBS	Response Boat Small
rcf	Relative Centrifugal Force
RH	Relative Humidity
rpm	Revolution(s) per Minute
RV-PCR	Rapid Viability-PCR
sec	Second
SOP	Standard Operating Procedure
SS	Sponge Stick
T&E II	Testing and Evaluation II Program
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
USCG	United States Coast Guard
VFC	Vacuum Filter Cassette

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Executive Summary

The U.S. Environmental Protection Agency (EPA) is the primary federal agency responsible for the protection and decontamination of indoor/outdoor structures and water infrastructure vulnerable to chemical, biological, and radiological (CBR) terrorist attacks. Under the Homeland Security Research Program (HSRP) of the EPA Office of Research and Development (ORD) conducts research to develop methods and technologies able to rapidly and cost-effectively remediate areas affected by CBR terrorist attacks. On the National Response Team, EPA, and the U.S. Coast Guard (USCG), working along with other federal agencies, provide technical assistance, resources, and coordination of preparedness, planning, response, and recovery activities for emergencies involving hazardous substances, pollutants and contaminants, oil, and weapons of mass destruction in natural and technological disasters, and other environmental incidents of national significance. In such instances, EPA and USCG provide Federal On-Scene Coordinators and coordinate preparedness for and response to hazard incidents that occur in the inland zone and the coastal zone, respectively. The USCG installations, facilities, and assets, due to their unique roles and responsibilities in national security, could also be targets of CBR terrorism attacks. Therefore, under the Department of Homeland Security (DHS) Science and Technology Directorate (S&T) funded Analysis for Coastal Operational Resiliency (AnCOR) project, the EPA-HSRP is providing support to the USCG in its efforts to be better prepared to respond to bioterrorism incidents. EPA and USCG have formed an Interagency Team (IT) to support research under the AnCOR project of which this task was a part.

EPA-HSRP has developed extensive protocols for sampling, analysis, and decontamination to respond to biological contamination incidents; however, the response to any contamination incident is specific to the affected site and surrounding environment. The coastal zone facilities and assets of the USCG—including small and large boats and other vehicles in diverse geographical areas and maritime environmental conditions—can pose complex and unique challenges for adapting existing methods or developing new ones for sampling, analysis, and decontamination to respond to biological contamination incidents. The performance of the methods may, in part, depend on the outdoor surfaces and materials being sampled and analyzed. The USCG bases and ports, by nature of their mission and location, may have unique surfaces and/or environments that could affect sampling and analysis methods. The diversity of surfaces at a USCG base that would be impacted during bioagent remediation necessitate proactive sample collection approaches to define the ongoing extent of contamination, the effectiveness of completed decontamination, and the need for waste disposal. The purpose of this project was twofold: 1) to evaluate the microbiological plate culture and EPA's Rapid Viability Polymerase Chain Reaction (RV-PCR) analytical methods included in the EPA-ORD Protocol for Detection of *Bacillus anthracis* (*Ba*) from Environmental Samples During the Remediation Phase of an Anthrax Incident for their compatibility with detection of *Ba* surrogate spores in real-world maritime environmental samples collected from the USCG coastal zone assets and their immediate surroundings; and 2) to understand difficulties associated with processing and analyzing those samples and identify capability gaps in this mission space.

Determination of contamination status of USCG facilities and assets is necessary to make decisions regarding safety and deployability. This report provides data and information that can be used to inform sampling operations and strategies following an outdoor biological contamination incident impacting a USCG base. Ultimately, it is desired that these findings will facilitate recovery following a large-scale biological incident.

Two sampling campaigns were successfully completed at Base Portsmouth, one on 04 November 2020

and one on 26 March 2021, to collect samples of residual inert and biological deposits on representative nonporous and porous maritime asset surfaces (e.g., aluminum on boats, nonskid tread on decks of watercraft, touchscreens, concrete piers) and surrounding grounds and infrastructure and materials (e.g., soil, vegetation, gravel). Established and commonly used EPA methods for sponge stick wipes (57 samples), vacuum filter cassettes (VFCs, 48 samples), and grab samples (48 samples) for bulk material collection were utilized. Samples were then transported to the laboratory and spiked with target spore loads of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) T1B2 barcoded spores. Spores were then recovered and processed by microbiological plate culture (culture method) and RV-PCR.

Overall, for the samples collected (sponge sticks, VFCs, and grab samples), the culture method resulted in 10 false positive results, as determined by PCR screening, and the RV-PCR method resulted in 0 false positives. Overall, there were 19 false negative results for the culture method and 26 false negative results for RV-PCR. An abundance of background microbial load compared to the spiked target spore load and particulates within samples contribute to false negative results. Samples with high microbial background load can mask the identification of target colonies on agar plates and lead to RV-PCR signal suppression. Particulates within samples can reduce the amount of sample volume processed and increase sample process times during filtration steps, particularly for vegetation and soil grab samples. The results of the performance of both the culture and the RV-PCR analytical methods are presented and discussed. Also, briefly presented are potential mitigation suggestions for sample types that are difficult to process.

In overall conclusions, both the culture and the RV-PCR methods are valuable methods and can give similar results for relatively clean samples. The culture method generally takes longer time to provide sample analysis results. The background microbial flora in complex environmental samples can overwhelm culture plates and obscure colony morphology of the target biothreat agent, leading to false negative results with the culture method. Additionally, background microbial flora with a similar or identical morphology to the target biothreat agent can be present within samples, triggering PCR screening of colonies and possibly repeated PCR screening (to minimize risk of false negatives) if presumptive morphology is present in large numbers. The RV-PCR method can provide rapid results, which is of high significance in a wide-area incident involving multiple cities and environments. It is akin to a biological indicator, it gives a positive or negative result and there is no iterative or repeat analysis on sample aliquots, giving the method a clear end of analysis without the need for multiple follow-up PCR screenings. RV-PCR constitutes a small laboratory footprint and requires less culture media, resulting in relatively less BSL-3 waste. The method, however, needs to be less labor-intensive and use of automated liquid handling and DNA extraction is essential. Complex environmental samples such as soil, grass, and other grab samples, are difficult to analyze using the current sample processing methods to recover spores. To mitigate this problem, a major emphasis needs to be placed on development of improved and high-throughput sample processing methods for such complex samples.

1.0 INTRODUCTION

1.1 Background

On the National Response Team, the U.S. Environmental Protection Agency (EPA) and the U.S. Coast Guard (USCG) work along with other federal agencies, providing technical assistance, resources and coordination on preparedness, planning, response, and recovery activities for emergencies involving hazardous substances, pollutants and contaminants, oil, and weapons of mass destruction in natural and technological disasters, and other environmental incidents of national significance. EPA and USCG provide Federal On-Scene Coordinators and coordinate preparedness for and response to hazard incidents in the inland zone and coastal zone, respectively.

Since the 2001 terrorist attacks and the Amerithrax (Anthrax) incidents, EPA has also been fulfilling its homeland security responsibilities, as assigned by various Presidential Directives, by expanding its original leadership role in environmental protection, decontamination, and cleanup during the contaminations caused by hazardous chemical, biological, and radiological (CBR) substances to include CBR terrorism incidents. As a result, EPA has been a focal point for many resources, including research and development products, to respond to CBR incidents and protect human health and the environment. USCG was also involved in the responses to the 2001 terrorist attacks and the Amerithrax (Anthrax) incidents (USCG, 2015). Especially, USCG through its National Strike Force (NSF) was extensively engaged in the 9/11 terrorist attack response, as well as in supporting the Amerithrax incidents cleanup at the Capitol Hill and other response locations. In particular, the NSF provided tactical entry teams, specialized equipment, management support, and a deputy incident commander for the anthrax response emergency phase. USCG continues to enhance and expand their capabilities to respond to bioterrorism incidents and effectively protect human health, and coastal zone assets and facilities (Maritime Environmental Response Mission).

EPA's Homeland Security Research Program (HSRP) provides science and technology-based solutions needed to effectively respond to and recover from natural or man-made disasters, including bioterrorism incidents. Under the Department of Homeland Security (DHS) Science & Technology Directorate (S&T) funded Analysis for Coastal Operational Resiliency (AnCOR) project, the EPA-HSRP is providing support to the USCG in its efforts to be better prepared to respond to bioterrorism incidents. EPA-HSRP has developed extensive protocols for sampling, analysis, and decontamination to respond to biological contamination incidents; however, the response to any contamination incident is specific to the affected site and surrounding environment. The coastal zone facilities and assets of the USCG—including small and large boats and other vehicles in diverse geographical areas and maritime environmental conditions—can pose complex and unique challenges for adapting existing methods or developing new ones for sampling, analysis, and decontamination to respond to biological contamination incidents.

Following a biohazard contamination incident such as release of *Bacillus anthracis (Ba),* accurate sample analysis results help determine the extent and magnitude of contamination, which informs responders for selection of decontamination strategies and helps determine the success of decontamination. Finally, sample analysis helps the responsible authorities make reoccupancy decisions.

The focus of this task order was to evaluate the gold-standard microbiological plate culture and EPA's Rapid Viability Polymerase Chain Reaction (RV-PCR, Létant et al., 2011) analytical methods and associated sample processing procedures for their compatibility with detection of *Ba* surrogate spores in

the real-world maritime environmental samples collected from the USCG coastal zone assets and their immediate surroundings. Both of these analytical methods are described in detail in the EPA-ORD Protocol for Detection of *Bacillus anthracis* from Environmental Samples During the Remediation Phase of an Anthrax Incident (EPA, 2017) and both methods were evaluated using barcoded *Bacillus thuringiensis* subsp. kurstaki (*Btk*) as the contaminant. *Btk* is a commonly used surrogate for *Ba,* and the genetic barcode insert (termed T1B2) allowed for differentiation of naturally occurring *Btk* and the spores applied for this study.

This study also assesses the challenges associated with processing and analyzing samples collected from USCG facilities and assets, and identifies analytical method capability gaps. Results from this study will help the USCG to recover rapidly following a biological contamination incident and return assets to duty after successful decontamination. The outcome of this study will provide data and information to improve sampling operations and strategies that will facilitate recovery following a large-scale biological incident.

1.2 Objective

The overall objective of this task was to gather and generate data useful for EPA and USCG decision-makers and other first responders regarding analytical method performance and impact of interferences on *Btk* spore detection sensitivity that can lead to more effective planning and execution for the recovery of a USCG base following a biological incident.

1.3 Scope

This task had three steps. First, in collaboration with the EPA and USCG, multiple surface types and bulk (grab) sample types expected to be encountered in a wide-area biological agent contamination incident involving a USCG base (and that were prevalent and available for sampling), were prioritized and selected (e.g., vessel surfaces, vessel washdown water, pier surfaces, wide-area base surfaces). Second, these sample types were collected using various sampling methods (sponge stick wipes, vacuum filter cassettes (VFC), bulk [grab] samples) from the USCG installation at Portsmouth, VA. Lastly, the collected samples were returned to Battelle's laboratory, spiked with barcoded *Btk* T1B2 spores, and analyzed using existing EPA methods for both culture and RV-PCR to determine the impact of the sample matrix on the recovery and analysis of spores from various sample matrices.

2.0 MATERIALS AND METHODS

2.1 Target Maritime Surface/Material Sampled

The maritime sample type (surface or material sampled) and number of samples sets collected for the applied sampling method are provided in Table 1. A sample set is defined as one sample type and one associated sampling method, the three sampling methods used were: 1) surface sampling using sponge stick (SS) wipes, surface sampling using 37-mm VFCs, and grab (bulk sample of material collected) sampling. At least nine replicates for each sample set were collected for subsequent laboratory analysis, from which three replicates were spiked with 0 spores, three with 300 spores, and three with 3,000 spores. The total number of sample sets collected (including field blanks) is shown in Table 1.

Table 1. Maritime Sample Type (Surface or Material) and Number of Sample Sets Collected per Sampling Method.

Sample Types	Number of Sets Collected for each Sampling Method		
	SS Wipe	37-mm VFC	Grab
Aluminum on response boats	2	--[a]	--
Nonskid tread	2	2	--
Touch screens (on-board)	2	--	--
Concrete on piers	--	2	--
Wash water, small vessels	--	--	1 (+1) [b]
Gravel	--	--	1
Soil	--	--	1 (+1) [c]
Vegetation	--	--	1 (+1) [c]
Field blanks	2	1	2
Total	**7**	**5**	**5**

[a] Sample purposely not collected.

[b] Extra sample set (at least nine replicate samples, three samples for each of three spore spike levels) collected and analyzed.

[c] Extra sample collected as back-up, but not analyzed.

2.1.1 Aluminum on Response Boats

Response Boat Small II (RBS, 29-ft) and Response Boat Medium (RBM, 45-ft) were the primary focus of the sampling effort when the target surface was exterior aluminum. The aluminum was a marine grade aluminum (samples designated [MGAL]), type 5086, which is the typical metal used in the construction of small and medium response boats for the USCG. A representative image of the aluminum surface sampled is shown in Figure 1.

Figure 1. Representative Area of Aluminum (Cabin Roof) Sampled on an RBS II (White Sampling Template Shown).

2.1.2 Nonskid Tread

Nonskid tread (designated NSKD) adhered to surfaces of an RBM is depicted in Figure 2. The same nonskid tread is also used on RBS vessels. The nonskid (antislip) tread was 3M™ Safety-Walk™ Coarse Tapes and Treads. The tread appears to be similar to the tread used in other EPA decontamination and surface sampling research. Per the manufacturer product description, the product consists of large abrasive particles (24-grit aluminum oxide) bonded by a tough, durable polymer to a dimensionally stable plastic film. The reverse side is coated with a pressure-sensitive adhesive covered by a removable protective liner.

Figure 2. Representative Nonskid Tread Adhered to Aluminum Surfaces Sampled on an RBM.

2.1.3 Touchscreens (On-board)

Glass touchscreen displays of various response boats (small and medium) and a Maritime Response Security Team (MRST) 36-ft Zodiac Hurricane boat were sampled. A typical touchscreen measured 12 x 12 inches (in) and two or three such screens were present on a single boat. Consequently, touch screens from three or four boats were sampled to collect the minimum of nine replicate samples per sample set for subsequent analysis. A representative on-board touchscreen in an RBS is shown in Figure 3.

Figure 3. Representative Touchscreen Sampled (12 in x 12 in Screen on an RBS).

2.1.4 Concrete Piers

Concrete piers along the dock for RBM and RBS vessels and the piers for the large ships were sampled. There was no known difference in the concrete of the two piers sampled, but they were selected to purposely collect samples from two distinct locations on Base Portsmouth. Photographs depicting the piers for the ships and a close-up of the concrete are provided in Figure 4.

Figure 4. Representative Concrete Pier Surface Sampled.

2.1.5 Wash Water (Small Vessels)

Response boats are routinely washed by the USCG with freshwater from a hose at the base pier after sea excursions. All exterior surfaces are rinsed (no detergent added), and the water runs off and empties into the sea via drain ports (deck drains and scuppers) located throughout the boat. The wash water represents a composite grab sample that could be readily and rapidly obtained with little additional equipment or training. A vessel wash water sample was collected from the bow of an RBS and stern deck of an RBM. The washdown water would flow over various surfaces, primarily the aluminum and nonskid tread, but included glass windows in the case of the RBS. An example area of the deck surface washed on an RBM is depicted in Figure 5.

Figure 5. Representative Deck Surface (Primarily Nonskid Tread with Some Aluminum) of an RBM Washed for the Collection of Wash Water Samples.

2.1.6 Gravel

Gravel is common throughout the base for unpaved roads, but highly trafficked areas exist. An example gravel parking lot is shown in Figure 6.

Figure 6. Gravel Lot on Base Portsmouth as the Source of the Gravel Samples.

2.1.7 Soil

Soil type will vary across USCG bases based on geographical location and can also vary within a specific base. Two soil types were selected for collection at Base Portsmouth: a loam type sample representative of the grounds that support growth of grass and a sand type collected in close proximity to the shoreline. The primary sample selected for completion of the test matrix was the loam soil, and the sand was retained as an extra. Representative sources of the soil samples collected on the grounds and shoreline at Base Portsmouth are shown in Figure 7.

Figure 7. Representative Sources of the Soil Samples Collected on the Grounds (Left, Loam) and Shoreline (Right, Sand) at Base Portsmouth.

2.1.8 Vegetation

Like soil, the vegetation on a USCG base will depend on the geographical location and can vary within a specific base. For this study, grass was selected as representative vegetation to sample. Similar to the rationale for soil collection , the grass selected as the primary vegetation sample for collection and analysis was representative for Base Portsmouth and regularly mowed as part of grounds upkeep (Figure 8). The grass was beginning to grow for the season but had not yet been mowed. A tall saltmarsh grass (*Spartina alterniflora*) growing along the shoreline was sampled as an extra sample set.

Figure 8. Representative Sources of the Vegetation (Left, Grass; Right, Saltmarsh Grass) Samples Collected on the Grounds and Shoreline at Base Portsmouth.

2.1.9 Field Blanks

Field blanks were collected as controls by handling the sampling media in the same manner as surface samples except that the sampling media did not contact a surface or material. For example, the sponge stick wipes were removed from their original manufacturer's packaging and immediately placed (not contacting a surface) in the receptacle for packaging, and the bag was sealed, and shipped to the laboratory. For the washdown water blank, the non-sterile water used to wash the boat was collected from the end of the freshwater supply hose and collected in a 1-L sterile bottle.

2.2 Sampling Methods

Note that personnel conducting the sampling were not required to wear full personal protective equipment (nitrile gloves were worn) as the sampling was not performed to collect a *Ba* target or to establish a field sampling method.

2.2.1 Sponge Stick Sampling Method

3M sponge sticks™ prewetted with a neutralizing buffer (3M, St. Paul, MN Part number SSL10NB), shown in Figure 9, were purchased for sample collection per established EPA sampling methods (Rose et al., 2011, EPA, 2013, and Tufts et al., 2014) and the Center for Disease Control and Prevention's (CDC's) Anthrax Surface Sampling Guide (CDC, 2021). The sponge sticks were used to sample a 10 in x 10 in (645 square centimeter (cm^2)) area (defined by a template overlaying the target surface) following the sampling pattern (30 linear passes over the area in a vertical, horizontal, and diagonal pattern) defined in the EPA sampling method. The Work Instruction to collect sponge stick samples is provided in Appendix A.

Figure 9. Prewetted Sponge Stick from 3M Used for Surface Sampling.

2.2.2 Vacuum Filter Cassette Sampling Method

VFCs, 37-millimeter (mm)-diameter, 0.8 micrometer (µm) pore mixed cellulose ester (MCE) membrane (SKC, Inc. Eighty Four, PA, Part No. SKC 225-3-01), were used for surface sample collection per established EPA sampling methods (Calfee, 2013). An assembled and disassembled VFC are shown in Figure 10. The VFCs were used to sample a 12 in x 12 in (929 cm^2) area (defined by a template overlaying the target surface) over a 5-minute (min) (300-second (sec)) sampling duration following the sampling pattern (50 linear passes over the area in a vertical S-pattern followed by 50 linear passes in a horizontal S-pattern, with each pass being ~3-sec duration) defined in the EPA sampling method (Calfee et al., 2013). The EPA-specified ≥5 liters/minute (L/min) sampling rate was used. The Work Instruction to collect VFC samples is provided in Appendix B.

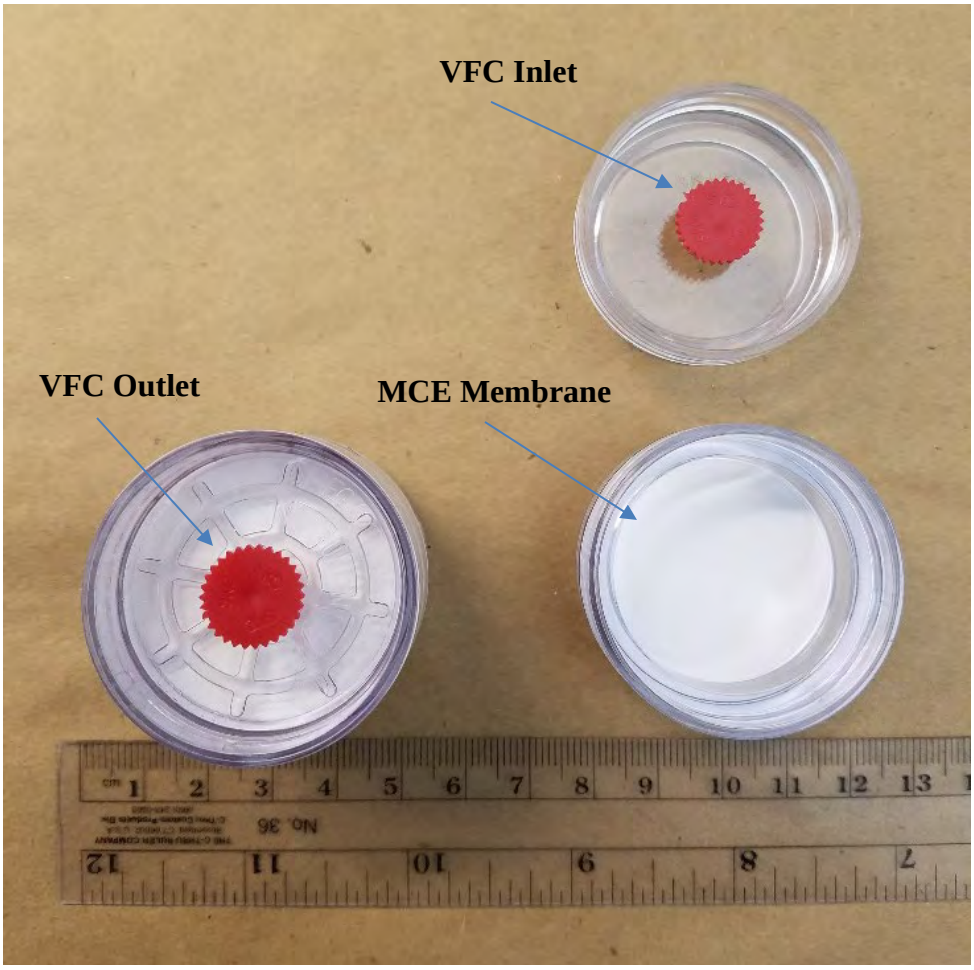

Figure 10. Vacuum Filter Cassettes (37-mm Diameter), Assembled (Left) and Disassembled (Right) for Surface Sampling.

2.2.3 Grab Sampling Method

The grab sampling method was specific to the material being sampled or collected. Four (4) grab sampling methods were employed, one each for: 1) boat wash water runoff, 2) gravel, 3) soil, and 4) vegetation.

2.2.3.1 Boat Wash Water Runoff Sampling Method

The RBS and RBM wash water runoff collection were new methods developed on this project and were specific to the boat type from which the sample was collected. The primary target for the one required sample was the RBS, which had stern and bow scuppers or drain ports. The stern drains were too close to the waterline and thus a collection container could not be placed low enough to catch the runoff. The stern scuppers on the RBS were ~30 to 60 centimeters (cm) (~1 to 2 feet (ft)) above the water line and allowed adequate room to position a collection container. Figure 11 shows a close-up view of the starboard stern scupper/drain port. The water collection apparatus developed, shown in Figure 12, comprises a 1-L sterile bottle (like those bottles used for other grab sampling methods) secured with hose clamps at the end of an extendable pole. (The pole used was one that comes with a bristle brush from West Marine). The Work Instruction to collect wash water samples is provided in Appendix C.

Figure 11. RBS Starboard Stern Scupper.

Figure 12. Wash Water Runoff Collection Apparatus.

2.2.3.2 Gravel Sampling Method

Gravel sampling was performed per established EPA method for sampling rail ballast (Serre and Oudejans, 2017). The method entailed one operator donning gloves and randomly grabbing handfuls of stones from the source and placing them into sterile 1-L bottles to the half-full line. Bottles were capped with their lids and sealed with parafilm. Each bottle of gravel represented a single sample replicate. The Work Instruction to collect gravel samples is provided in Appendix D.

2.2.3.3 Soil Sampling Method

The soil sampling method entailed one operator donning gloves and using a small garden hand spade to remove (by scraping) the top 1 to 2 in of the soil, then scooping the soil into a 1-L sterile bottle. Two (2) 1-L sterile bottles were filled with the soil to collect a composite soil sample. Note, no field measurements of soil temperature, moisture content, or pH were taken. Bottles were capped with their lids and sealed with parafilm. The Work Instruction to collect soil samples is provided in Appendix E. The soil was mixed in the laboratory, and a fixed quantity was used to analyze from each sample replicate.

2.2.3.4 Vegetation Sampling Method

Vegetation sampling, specifically grass, was performed as described in Mikelonis et al., 2020. The method entailed one operator donning gloves, grabbing a handful of grass, clipping the grass just above the soil and then placing the grass into a 1-L sterile bottle. Two (2) 1-L sterile bottles were filled with the grass to collect a single sample replicate. If the grass length exceeded the height of the bottle, the grass was folded to fit within the bottle. Bottles were capped with their lids and sealed with parafilm. The Work Instruction to collect grass samples is provided in Appendix F.

2.3 Sampling Representative Maritime Surfaces/Materials

Samples of representative maritime surfaces and grab materials were collected in two sampling campaigns conducted at Base Portsmouth in Portsmouth, VA. Campaign #1 occurred on a clear, sunny day, 04 November 2020, from approximately 0900 to 1800 hours (h). Early morning temperature and relative humidity (RH) were 20°C and 50% to a mid-day high temperature of 23°C and RH 43% and an end-of-day condition of 17°C and 65% RH. Sampling Campaign #2 occurred on a mostly cloudy morning from 0800 to 1200 h on 26 March 2021. Early morning conditions were 22°C and 80% RH with the temperature rising to 25°C and RH dropping to 66% by noon. Rain occurred on 25 March 2021. In all sampling events, no dew or unevaporated rain was present and surfaces or materials were dry when they were sampled. As discussed above, three traditional sampling methods: sponge sticks, VFCs, and grab were used to collect samples from surfaces and materials commonly found at USCG bases. EPA has established sampling protocols for these methods, which were summarized in Work Instructions for the field team to execute. A summary of the samples collected is provided in Table 2.

Table 2. Maritime Sample Type (Surface or Material) and Number of Samples Collected per Sampling Method.

Sample Types	Surface/Material Source Description	Sampling Campaign Date	Air T / RH (°C) / (%)	Samples Using Each Method		
				SS Wipe	37-mm VFC	Grab
Aluminum on response boats	#1, RBS washed	11/04/2020	21 / 46	12	--	--
	#2, RBS not-washed	11/04/2020	20 / 50	12	--	--
Nonskid tread	RBM	11/04/2020	20 / 36	12	12	--
	RBS	11/04/2020	23 / 43	12	12	--
Touch screens (on-board)	RBS, RBM, MRST	11/04/2020	21 / 36	9	--	--
	RBS, RBM, MRST	3/26/2021	22 / 80	11	--	--
Concrete on piers	Base (Large Vessels)	11/04/2020	19 / 54	--	12	--
	Base (Response Boats)	11/04/2020	21 / 51	--	12	--
Wash water, small vessels	RBS	3/26/2021	25 / 66	--	--	1[(a)]
	RBM	3/26/2021	25 / 66	--	--	1[(a)]
Gravel	Base Lot	11/04/2020	17 / 65	--	--	12
Soil	Base Grounds	3/26/2021	28 / 69	--	--	1[(b)]
	Shoreline	3/26/2021	28 / 69	--	--	1[(b)]
Vegetation (Grass)	Base Grounds	3/26/2021	23 / 73	--	--	10
	Shoreline	3/26/2021	22 / 79	--	--	10
Field blanks	N/A	11/04/2020 and 3/26/2021	15 / 72 25 / 69	1 1	1	1[(c)] 1[(d)]

[(a)] Composite sample totaling ~10-L by collecting ten (10) ~1-L bottles of wash water runoff.

[(b)] Composite sample totaling ~2-L by collecting two (2) ~1-L bottles of soil from a single area.

[(c)] Comprised an empty 1-L bottle sealed with no contents.

[(d)] Comprised three (3) 1-L bottles filled with fresh water from the hose (at the dock) used to wash down the boats.

2.3.1 Surfaces Sampled with Sponge Sticks

2.3.1.1 Sponge Sticks – Aluminum of Small Boat

Exterior, exposed aluminum surfaces of two different RBSs were sampled during the first sampling campaign in November 2020 to collect the two independent sample sets. Samples from the RBSs were collected on the roof of the cabin. Both boats had been out to sea on exercises before sampling. The RBS for Sample Set #1 was washed down with freshwater before sponge stick wipes were collected; the RBS for Sample Set #2 was not washed with freshwater before sponge stick wipes were collected. Consequently, Sample Set #2 contained grime and sea salt spray residue. An image depicting the sampling location and sample collection is provided in Figure 13. A representative image of the sponge stick after sample collection from the RBS is provided in Figure 14.

Figure 13. Sponge Stick Sampling Location of Aluminum Surfaces from the RBS.

Figure 14. Sponge Stick After Sampling Aluminum Surface of the RBS.

2.3.1.2 Sponge Sticks – Nonskid Tread

Exterior and exposed nonskid tread surfaces of an RBM (Sample Set #1) and an RBS (Sample Set #2) were sampled during the first sampling campaign in November 2020. The sampling location and sample collection are shown in Figure 15. Images of the sponge stick after sample collection from the RBS are provided in Figure 16.

Figure 15. Sponge Stick Sampling Location of Nonskid Tread from the RBS and RBM.

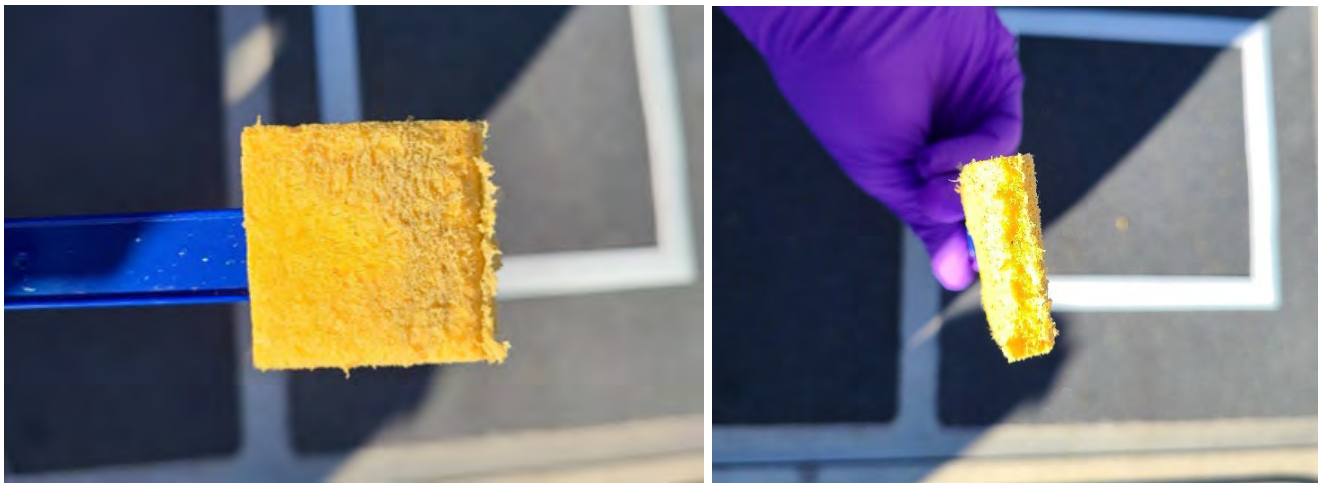

Figure 16. Sponge Stick After Sampling Nonskid Tread from the RBS and RBM.

2.3.1.3 Sponge Sticks – On-Board Touchscreens

An image depicting the sampling location and sample collection on-board an RBM is provided in Figure 17. An image of the sponge stick after sample collection from the RBS is provided in Figure 18.

Figure 17. Sponge Stick Sampling of On-Board Touchscreens from the RBM.

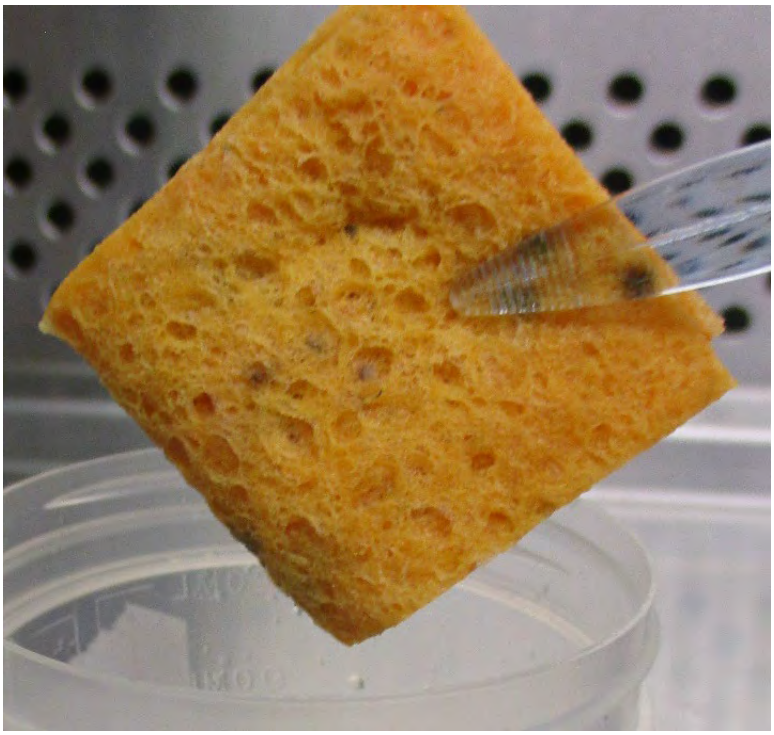

Figure 18. Sponge Stick After Sampling Touchscreen Surface on the RBS.

2.3.2 Surfaces Sampled with Vacuum Filter Cassettes

2.3.2.1 Vacuum Filter Cassettes – Nonskid Tread

An image depicting the sampling location and sample collection is provided in Figure 19. An image of the VFC after sample collection from the RBM is provided in Figure 20.

Figure 19. Location of VFC Sampling of Nonskid Tread from the RBM.

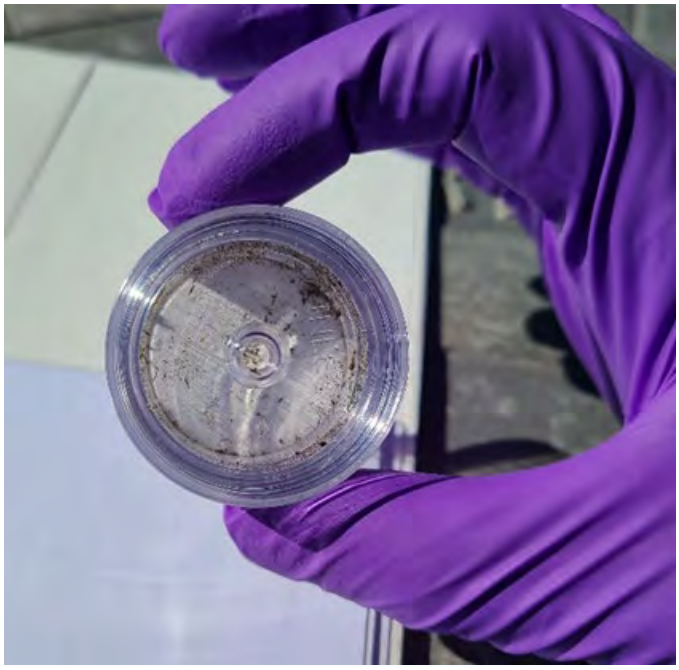

Figure 20. VFC After Sampling Nonskid Tread Surface of the RBM.

2.3.2.2 Vacuum Filter Cassettes – Concrete Pier

Images depicting the sampling locations and sample collection are provided in Figure 21. An image of the VFCs after sample collection from the concrete pier are provided in Figure 22.

Figure 21. Location of VFC Sampling of Concrete Piers.

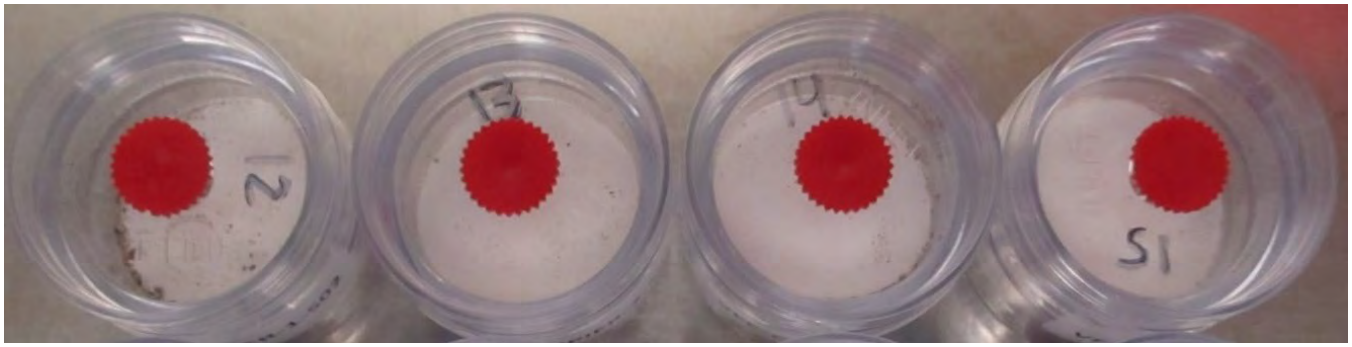

Figure 22. VFCs After Sampling Concrete Pier.

2.3.3 Materials Sampled Using a Grab Method

The materials sampled and the sampling methods for the four grab samples are described below.

2.3.3.1 Grab - Vessel Wash Water

Boats are commonly washed with a fresh water source (no detergent added) available at the dock. The washdown was performed by a USCG staff member and thus considered representative of an actual washdown using the freshwater supply at the dock and associated 5/8-in-diameter garden hose with an adjustable brass spray nozzle. The specifications of the nozzle were not known, but it appeared to be similar to a heavy-duty adjustable brass spray nozzle (Dramm Model # 14033591; Home Depot) that has been used to apply the water for washdown by EPA during other decontamination applications and sampling method development projects. For those studies, a target flow rate of 4 ± 1 L/min operating at a source pressure of 30 pounds per square in gauge (psig) was used; by observation, a similar volumetric flow rate was used for the vessel washdown during sample collection. The spray nozzle setting was arbitrary and adjusted to produce a small (estimated as <30-cm-diameter) cone at 1-m distance. Nozzle-

to-surface distance was also variable, but was estimated to have typically ranged from 0.5 to 1.5 m. The fill rate of the 1-L bottles was somewhat variable, but the bottles typically filled at an estimated rate of at least 2 L/min at peak flow (a 1-L bottle was filled within 30 sec.). The washdown did not follow a scripted pattern or established method/protocol. The washdown was performed in a manner so that the water preferentially flowed to the drain from which the sample was collected. The flow and force of the water was such that the wash water flow was directed toward the drain. The washdown was focused on the collection side of the boat. An estimated area of 4 square meters (m^2) was washed in a <5 min period. The exterior surface area covered by the washdown of the RBS was glass windows, aluminum roof and deck, and nonskid tread on the deck. The exterior surface area covered by washdown of the RBM was comprised mostly of nonskid tread with some exposed aluminum. This approach was adequate to create a water stream flowing from the scupper of the RBS and side drains of the RBM that allowed for collection (the flow was high enough to prevent rinse water from adhering to the outer surface of the boat and flowing into the sea). A photograph depicting water washdown of the RBS is provided in Figure 23, and the washdown water sampling method is shown in Figure 24.

Figure 23. RBS Washdown that Generated the Wash Water for Collection.

Figure 24. Vessel Washdown Water Nontraditional Sampling Method Applied to an RBS Scupper.

2.3.3.2 Grab – Gravel

Images depicting grab sample collection of gravel and a close-up of the source gravel are provided in Figure 25. A representative sample of gravel in a 1-L Nalgene bottle is shown in Figure 26. Gravel was sampled by collection into a 1-L Nalgene bottle to the ½ full mark, ~900 g.

Figure 25. Collection of Gravel Grab Sampling on the Base Grounds and a Close-up of the Gravel.

Figure 26. Representative Sample of Gravel in a 1-L Nalgene Bottle.

2.3.3.3 Grab – Soil

Images of soil sample collection from the grounds of Base Portsmouth are provided in Figure 27. The completed bulk sample of soil collected in a filled 1-L bottle is shown in Figure 28.

Figure 27. Grab Sampling Images Depicting Collection of Soil on the Base Grounds (Left) and of Sand Along the Shoreline (Right).

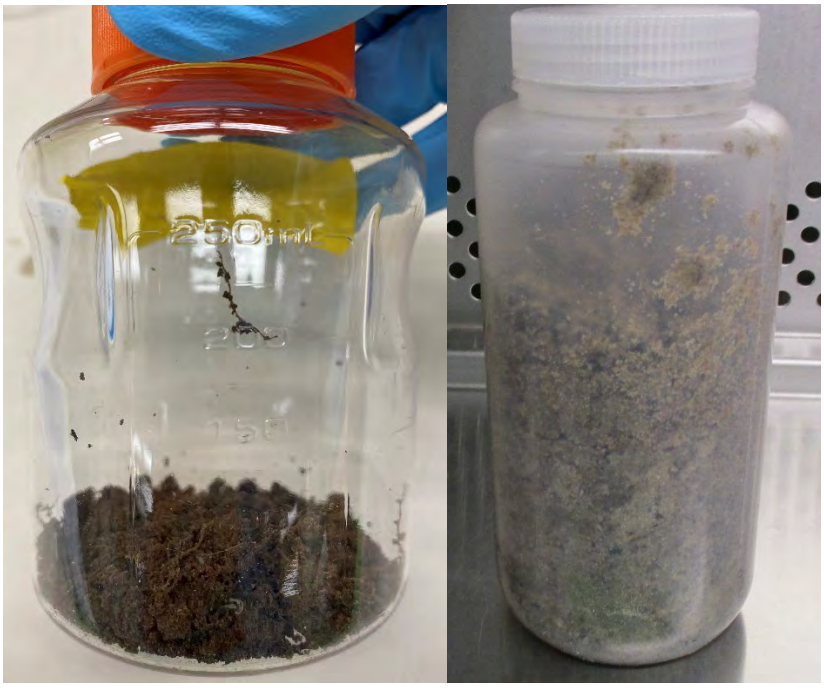

Figure 28. Collected Soil Sample in a 250-mL or 1-L Bottle (Left, Loamy Soil; Right, Sandy Soil).

2.3.3.4 Grab – Vegetation (Grass)

An image of a grass sample being collected from the grounds of Base Portsmouth is provided in Figure 29. The completed bulk sample of grass collected in a 1-L bottle is shown in Figure 30.

Figure 29. Grab Sampling Images Depicting Collection of Grass on the Base Grounds (Left) and at the Shoreline (Right).

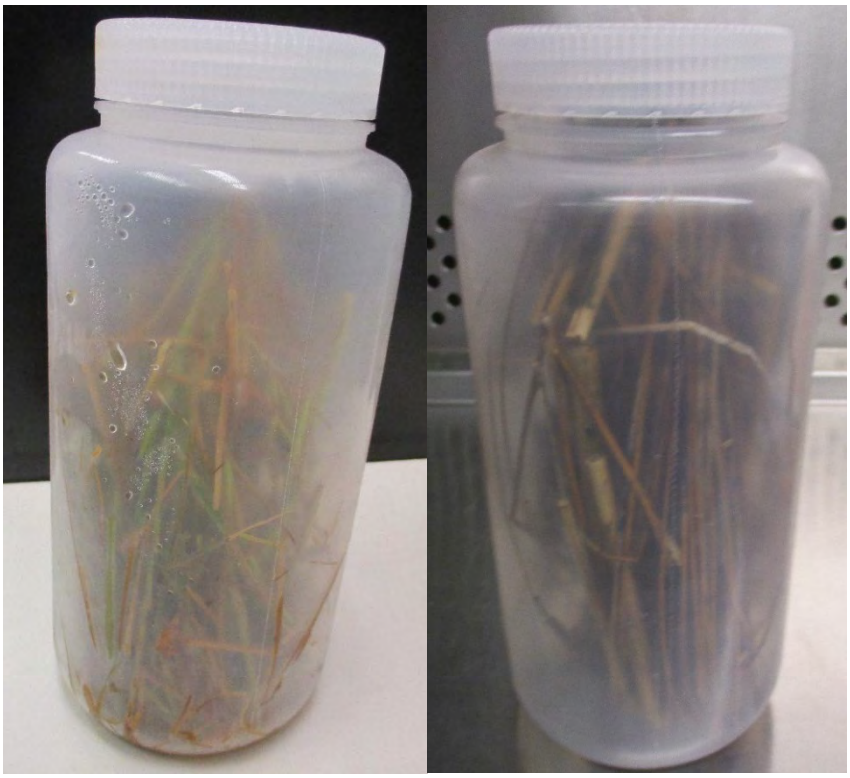

Figure 30. Collected Grass Sample in a 1-L Bottle (Left, Base Ground Grass; Right, Shoreline Grass).

2.4 Test Matrix

Each of the collected surface samples described in Sections 2.3.1 and 2.3.2 was processed to recover spiked *Btk* T1B2 spores, and the recovered spore suspension was analyzed to quantify and identify recovered *Btk* T1B2 spores to assess the EPA-provided culture and RV-PCR methods.

The completed test matrices for traditional sampling methods: sponge sticks, VFCs, and grab samples are provided in Table 3. In total, 135 collected samples were analyzed, comprising 54 sponge sticks, 36 VFCs, and 45 grab samples. Nominally, triplicate samples for each of two *Btk* T1B2 spore loading levels (300 and 3,000 colony forming units [CFU]) and triplicate samples of unspiked (0 CFU load) samples were analyzed for each sample type. Additional spikes and blanks were included as controls and are discussed in the results section.

Following sample processing, the recovered sample volume was split nominally in half, and therefore the total target spores available listed in Table 3 were divided by two to represent the number of *Btk* T1B2 spores available for each of the two analytical methods (culture and RV-PCR), as described in the "U.S. EPA Protocol for Detection of *Bacillus anthracis* in Environmental Samples During the Remediation Phase of an Anthrax Incident, Second Edition" (EPA, 2017). The method details are discussed in further detail in Sections 2.6.3 to 2.6.5. Negative controls that were handled only within the analytical laboratory were included to assess the potential for sample cross-contamination. Field blank samples were collected to serve as a baseline to represent the expected best-case performance of the method because of the absence of potentially competing or interfering grime or flora.

Culture and RV-PCR analytical methods were used to detect and/or quantify recovered *Btk* T1B2 spores from spiked samples and subsequently recovered in the sample extracts. Trypticase Soy Agar (TSA) was the primary medium used for all culture analyses.

Table 3. Sample Analysis Test Matrix for All Collected Real-World Maritime Samples.

Sample Type	Sample ID	Sampling Method	Replicate Samples per *Btk* T1B2 Spore Spike	Target Spore Load	Analytical Method (CFU)[(a)]	
					Culture	Molecular
Small Boat Marine Grade Aluminum	SBMGAL-1	SS	3, 3, 3	0, 300, 3,000	TSA	RV-PCR
Small Boat Marine Grade Aluminum	SBMGAL-2	SS	3, 3, 3	0, 300, 3,000	TSA	RV-PCR
Touchscreen	TCHSCRN-1	SS	3, 3, 3	0, 300, 3,000	TSA	RV-PCR
Touchscreen	TCHSCRN-2	SS	3, 3, 3	0, 300, 3,000	TSA	RV-PCR
Nonskid Tread	NSKID-1	SS	3, 3, 3	0, 300, 3,000	TSA	RV-PCR
Nonskid Tread	NSKID-2	SS	4, 4, 4	0, 300, 3,000	TSA	RV-PCR
Nonskid Tread	NSKID-1	VFC	4, 4, 4	0, 300, 3,000	TSA	RV-PCR
Nonskid Tread	NSKID-2	VFC	4, 4, 4	0, 300, 3,000	TSA	RV-PCR
Concrete Pier	CONPIER-1	VFC	4, 4, 4	0, 300, 3,000	TSA	RV-PCR
Concrete Pier	CONPIER-2	VFC	4, 4, 4	0, 300, 3,000	TSA	RV-PCR
Boat Washdown Water	SBWASH-1	Grab	3, 3, 3, 3	0, 300, 3,000, 30,000	TSA	RV-PCR
Boat Washdown Water	SBWASH-2	Grab	3	30,000	TSA	RV-PCR
Gravel	GRAVEL-1	Grab	3, 3, 3	0, 300, 3,000	TSA	RV-PCR
Soil	SOIL-1	Grab	3, 3, 3, 3	0, 3,000, 30,000, 300,000	TSA	RV-PCR
Vegetation (Grass)	GRASS-1	Grab	3, 3, 3, 3	0, 300, 3,000, 30,000	TSA	RV-PCR

[(a)] Nominally half of the target quantity of spores loaded were available for each of the two analytical methods.

2.5 Overall Method Implementation

The traditional procedures used to spike/recover/analyze the sponge sticks, VFCs, grab and nontraditional methods are shown as they occur in chronological order, as depicted graphically in the process flow diagram of Figure 31.

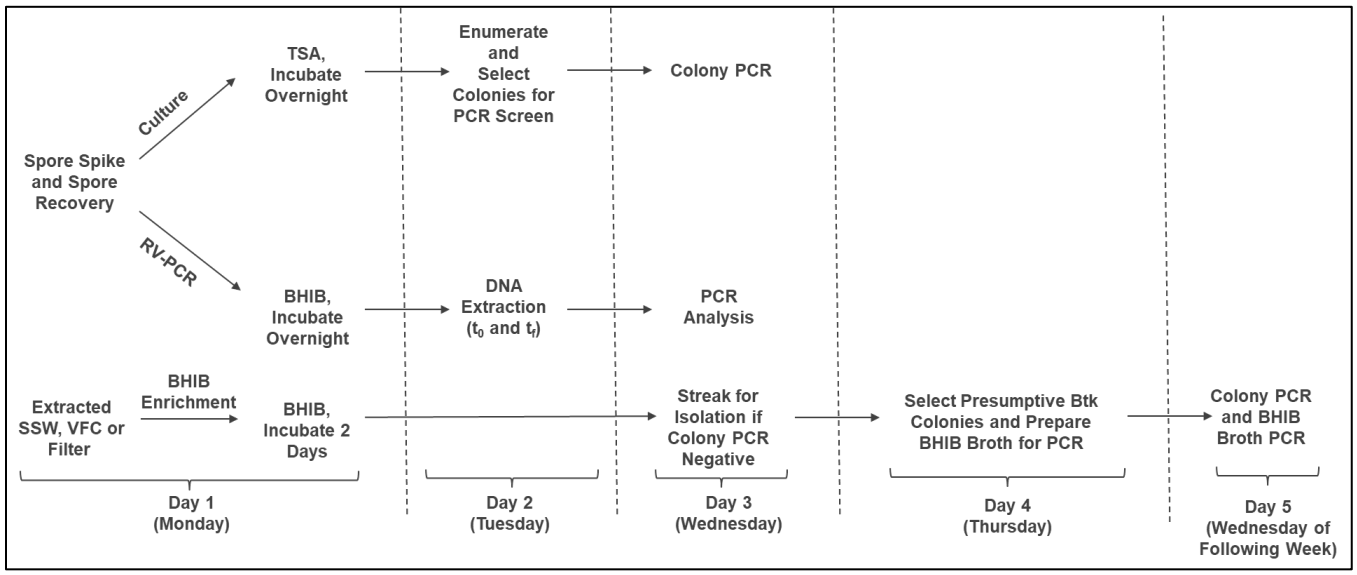

Figure 31. Process Flow Chart Depicting Key Process Steps in Chronological Order.

The methods implemented, in the form of Work Instructions followed by the analytical staff, are provided in Appendices H through N. These Work Instructions also complement the microbiological methods described in Section 2.6, and emphasize glove-changing schedules that were implemented to minimize cross-contamination. Work Instructions were reviewed, as needed, with the EPA Project Team to ensure consistency with published methods.

The above process workflow was used to analyze a batch of 16 samples per trial, with 1 trial conducted per week. For each weekly trial, the test samples (e.g., sponge sticks or VFCs) were spiked using *Btk* T1B2 spores suspended in water or PBST per "Work Instruction for Spiking with *Bacillus thuringiensis kurstaki* (*Btk*) HD-1 T1B2 Spores" in Appendix H. The spores spiked onto test samples were recovered following the "Work Instruction for *Bacillus thuringiensis kurstaki* (*Btk*) T1B2 Spore Recovery from Maritime Samples – Sponge Sticks, Vacuum Cassettes, and Grab Samples," process described in Appendix I. The recovered suspension volume was then split equally between the culture method and RV-PCR. The culture aliquot was plated onto TSA media and incubated overnight as described in the "Work Instruction for Culture of *Bacillus thuringiensis kurstaki* (*Btk*) T1B2 Spores Recovered from Sponge Stick Wipes, Vacuum Filter Cassettes, and Grab Samples" process in Appendix J. The T_0 RV-PCR aliquot was stored frozen while the recovered spores enriched overnight, then the T_f aliquot was removed, and the DNA was extracted from both T_0 and T_f aliquots per the "Work Instruction for Manual DNA Extraction and Purification from *Bacillus thuringiensis kurstaki* (*Btk*) T1B2 Spores" process described in Appendix K. The extracted DNA was then analyzed using the real-time PCR assay described in Section 2.6.5.4 and per the "Work Instruction for Real Time PCR Analysis for *Bacillus thuringiensis kurstaki* (*Btk*) T1B2 DNA" process described in Appendix L. The real-time PCR assay was also used to confirm or refute presumptive *Btk* T1B2 colonies selected from the culture analysis per the "Work Instruction for Selecting Presumptive *Bacillus thuringiensis kurstaki* (*Btk*) T1B2 Colonies for qPCR Confirmation" process described in Appendix M. Selected samples for which the culture was a nondetect were further analyzed using an enrichment per the "Work Instruction for BHIB Enrichment for Culture" process described in Appendix N.

2.6 Microbiological Methods

Bacillus thuringiensis subsp. *kurstaki* (*Btk*) with the T1B2 genetic barcode (Buckley et al., 2012) was selected as the surrogate for *Ba* in the current study because it is physically and genetically similar to *Ba* (Tufts et al., 2014 and Greenberg et al., 2010) and has been used previously for outdoor testing research conducted by EPA, and is planned to be used in future outdoor release testing by EPA. Use of *Btk* with the T1B2 barcode makes it distinguishable from wild-type/naturally occurring *Btk* at the molecular level and provides a level of resolution for the study so that naturally occurring *Btk* did not confound the PCR results.

Traditional sample processing and analytical methods (both a culture and RV-PCR analytical method) were conducted as described in the EPA Protocol (EPA, 2017), with modification to incubation temperature, culture media and real-time PCR assay to optimize detection of *Btk* T1B2.

Following are sections that summarize specific procedures and steps applied to conduct the study.

2.6.1 Spore Stock

A single spore stock of *Btk* with T1B2 barcode was used as the biological test agent for the entire study. *Btk* is commonly used as a biopesticide, the T1B2 barcoded version was produced to allow for differentiating environmental *Btk* spores from those used in a test event (Buckley et al., 2012). The *Btk*

T1B2 strain was handled as a Risk Group I agent following the Biosafety in Microbiological and Biomedical Laboratories guidelines and Battelle biosafety work practices for such agents and was reviewed by the Institutional Biosafety Committee for compliance with recombinant organisms. A spore bank was produced using sporulation broth as follows and used as needed for the duration of the study.

An isolate of *Btk* T1B2 was provided by EPA and streaked for isolation on TSA, then incubated overnight at 30 ± 2 degrees Celsius (°C). An isolated colony was then used to inoculate 50 milliliter (mL) aliquots of nutrient broth and incubated overnight at 30 ± 2°C with shaking at 200 revolutions per minute (rpm). Modified G (ModG) (500 mL) of sporulation broth (see Appendix G, Table 1 for formulation details) was inoculated with 50 mL of the overnight *Btk* T1B2 culture, and then incubated in a 3-L Fernbach flask at 30 ± 2°C with shaking at 200 rpm. The culture was observed via wet mount microscopy every 1 to 3 days for sporulation. Following 5 days of incubation, the ModG culture reached > 99% sporulation.

The sporulated culture was centrifuged at 10,000 relative centrifugal force (rcf) for 12 min in multiple 250-mL bottles. After removing and discarding the supernatant, the resulting pellets were resuspended to a total volume of approximately 100 mL with sterile distilled water (dH_2O), transferred into a sterile glass vessel, and heat shocked at 60 to 65°C for 1 h in a water bath with gentle agitation. (Note: a control flask with a thermometer was used to ensure the desired temperature was achieved and maintained during the heat shock step). The spores were then washed twice by repeated centrifugations at 10,000 rcf for 12 min using 100-mL dH_2O per wash. After the final centrifugation, the spores were resuspended to a total volume of 100 mL in sterile dH_2O. The spore bank was assigned a unique lot number and stored refrigerated at 2 to 8°C. Spore bank concentration was determined by spread plating serial dilutions onto TSA, followed by 30 ± 2°C overnight incubation and enumeration of CFU.

2.6.2 Spiking Samples

On the day of sample processing for spore recovery, *Btk* T1B2 spore stock was vortex-mixed and diluted using sterile dH_2O or phosphate buffered saline with 0.05% Tween 20 (PBST) and used to directly spike the samples at two spore spike levels (300 CFU or 3,000 CFU). For washdown and vegetation samples, a third spore spike level of 30,000 CFU was incorporated. For soil samples, the 300 CFU spore level was omitted, and samples were processed with 3,000 CFU, 30,000 CFU and 300,000 CFU. Three replicates of each sample were used for each spore spike level, including zero spore spike level. Each spiking stock was spread plated onto TSA on the day of testing to calculate the actual concentration of spores spiked in CFU/mL.

Each sponge stick was positioned in a specimen cup so that the dirty side was facing up and *Btk* T1B2 spores were directly spiked onto the surface of each sponge stick (the sides of the sponge that could contact the specimen cup wall were not spiked; see Figure 32). For VFCs, the final spiking stock concentrations were directly applied over the surfaces of collected particulates and filter. Gravel was spiked on the top layer of ~900 grams (g) of gravel. Sponge sticks, VFCs, gravel, and soil were spiked with 100-µL volume total in a dropwise fashion; washdown samples were spiked with 100-µL volume; and vegetation samples were spiked with 500-µL volume and larger droplet size to enable downward movement of the spiking stock into the grass grab sample. Spiking procedure is further outlined in Appendix H.

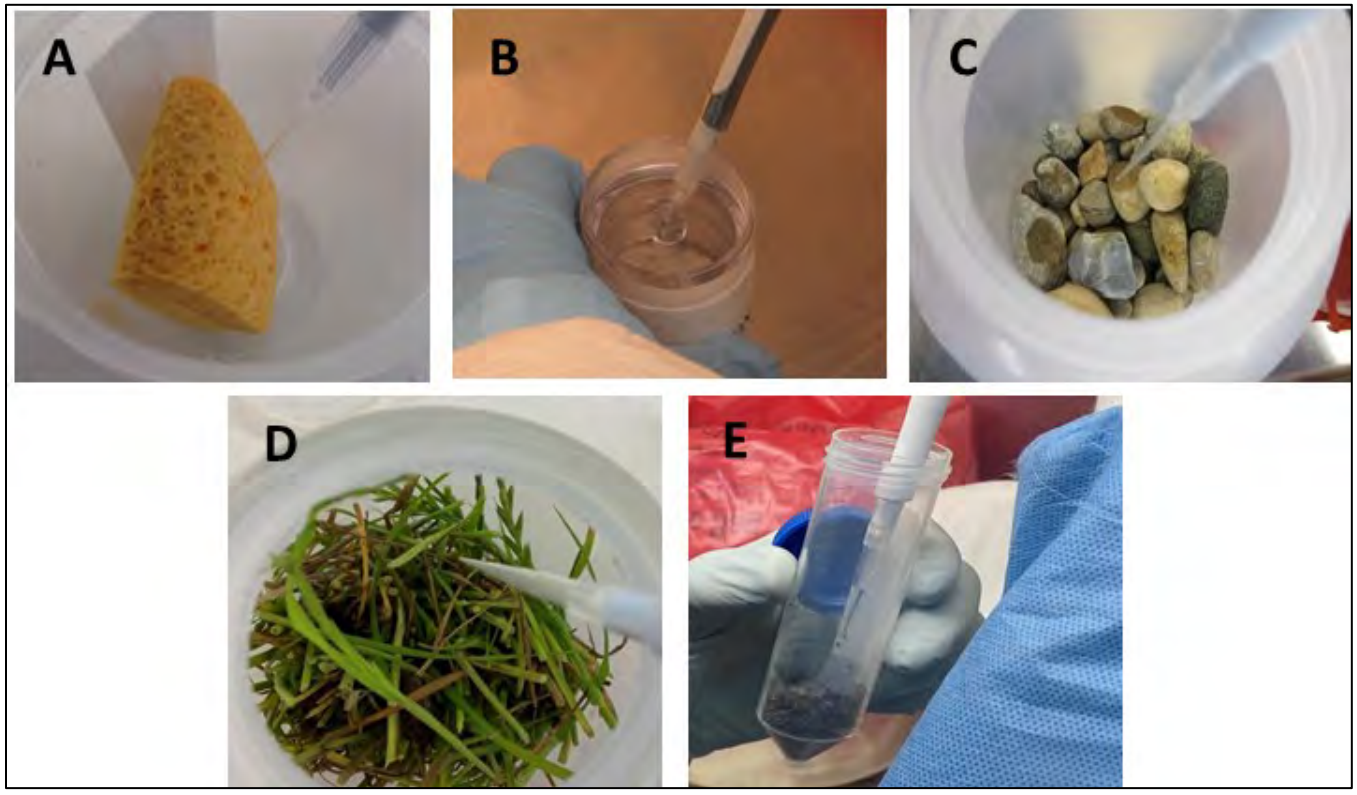

Figure 32. Sponge Stick (A), Vacuum Filter Cassette (B), Gravel (C), Vegetation (D), and Soil (E) Being Spiked with *Btk* T1B2 Suspension

2.6.3 Sample Processing for Spore Recovery

Throughout the spore recovery procedure, gloves were changed between handling samples to limit the likelihood of cross-contamination. Spore recovery methods were summarized as work instructions for the laboratory staff to execute (Appendix I).

2.6.3.1 Sponge Sticks

Following sample collection, samples were stored at 2 to 8°C until sample spiking and spore recovery. The remaining sponge stick handle was removed, and the sponge stick was unfolded, transferred aseptically to a Stomacher bag (Seward, Bohemia, NY) containing 90 mL cold (2 to 8°C) Phosphate Buffered Saline with 0.05% Tween 20 and 30% ethanol (PBSTE), then homogenized for 1 min at 260 rpm in a Stomacher 400. Each sample then sat for 10 min to allow foam to settle before removing the sponge. Absorbed liquid was expelled from the sponge into the Stomacher bag and the sponge was removed. The suspension (~90 mL) was gently mixed by pipetting up and down three times with a sterile 50-mL pipet, then the suspension was split into two (2) 50-mL sterile conical tubes and centrifuged at 3,500 rcf for 15 min in a swinging bucket rotor at 4°C with the brake off. To concentrate the sample, ~65 mL of supernatant was removed and the remaining ~25 mL of supernatant was used to suspend the pellets. The suspension was split in half and used for culture-based analysis as described in Section 2.6.4 and RV-PCR analysis as described in Section 2.6.5.

2.6.3.2 Vacuum Filter Cassettes

Following sample collection, samples were stored at 2 to 8°C until sample spiking and spore recovery. Spore recovery using 5 mL of PBSTE was added to the conical tube containing the nozzle and tubing and set aside. Six (6) mL total of PBSTE was used to rinse and recover particulates collected within the VFC by adding 2 mL of PBSTE in three successive rinse steps. Following the second rinse step, the filter was transferred to the 2-ounce (oz.) cup containing rinsate. The nozzle and tubing containing 5 mL PBSTE was sonicated in a sonicating bath for 1 min, then vortexed for 2 min and combined with filter rinsate in the 2-oz. cup. The 2-oz. cup containing filter and 11 mL of PBSTE was sonicated in a sonicating bath for 3 min. As much suspension as possible, typically ~ 8 mL, was transferred to a 15-mL conical tube, and the suspension was split in half and used for culture-based analysis as described in Section 2.6.4 and RV-PCR analysis as described in Section 2.6.5.

2.6.3.3 Grab Samples (Wash Water, Gravel, and Vegetation)

Following sample collection, samples were stored at 2 to 8°C until they were spiked. Spores were recovered by adding 500 mL of sterile PBST to the 1-L bottles containing gravel or vegetation and shaking the bottle vigorously with one hand on the bottom and the other on the top using an over the shoulder back-and-forth motion for 2 min. The sample was allowed to settle for 30 sec and then the eluent was poured into a clean sterile 500 mL container. The washdown water or eluent from gravel and vegetation was mixed vigorously by hand for 30 sec; then, the liquid was poured into a 0.45-µm filter funnel (MicroFunnel™ Filter, Pall Corporation, Washington, NY, Cat. 4804) to the 100 mL gradation line. If the volume passed through the filter without becoming clogged, an additional 100-mL aliquot and 50-mL aliquot was added for a total of 250 mL for gravel and a total of 500 mL for washdown or vegetation eluate. If a 100-mL or 50-mL aliquot took longer than 10 min to pass through the filter, no further volume was added. At 30-min post-sample addition, if the sample did not completely pass through, the remaining volume in the filter unit was carefully removed. The total volume vacuum filtered was documented. The filter membrane was then removed using sterile forceps and transferred to a 50-mL conical tube so that it was positioned in the bottom half of the tube with the inlet side of the membrane facing the center of the tube. Then, 10 mL of PBSTE was added and vortex-mixed at maximum speed on a platform vortex for 10-sec bursts for 2 min to dislodge spores. The suspension in tubes was allowed to settle for 2 min, then transferred to a 50-mL conical tube. An additional 10 mL of PBSTE was added to the 50-mL tube containing the membrane and vortexed as described for the first 10 mL, then pooled with the first 10 mL for each sample. This 20-mL pooled volume was vortex-mixed, allowed 30 sec of settling time, and then split in half for culture-based analysis as described in Section 2.6.4 and RV-PCR analysis as described in Section 2.6.5.

2.6.3.4 Grab Samples (Soil)

Soil collected from the field was homogenized by manual shaking and then parsed into 50-mL conical tubes, each sample containing 10 g of soil. Following spiking of soil with target spore load of *Btk* T1B2, 40 mL of PBST was added to each soil sample and vortex-mixed for 30 sec, followed by bath sonication for 10 min. The samples were then manually mixed for 2 min. Each sample was then spun at 1,000 rcf for 5 min in a swinging bucket centrifuge and the supernatant was transferred to a clean 50 mL tube, leaving ~2.5 mL of supernatant in the pelleted soil. The supernatant and pellet were heat shocked at 70 ± 2°C for 1 h. The supernatant was split in half, with ~18 mL available for culture analysis and ~18 mL available for RV-PCR.

Sterile soil was processed alongside the field samples as a control. The sterilization process was completed by spreading soil onto a Pyrex glass dish and autoclaving twice at 121°C for 45 min with soil cooling to room temperature in between.

Moisture analysis (based on ASTM International (ASTM) D 2216) and pH analysis (based on EPA Method 9045D) were performed on the unsterilized and sterilized soil.

Moisture analysis: Soil (~5 g) was placed in pre-weighed aluminum dish using Sartorius Balance (R200D, Sartorius Instruments, McGraw Park, Illinois). Heated in oven at 105°C overnight (Thermodyne Furnace, 30400, Barnstead Thermolyne Corporation, Ramsey, MN). Transferred heated samples into a desiccator to cool to room temperature, then weighed the soil again.

$$W=[(M_{cws}-M_{cs})/(M_{cs}-M_c)]x100$$

where: W = water content (%); M_{cws} = mass of container and wet sample (g); M_{cs} = mass of container and dry sample (g); and M_c = mass of container (g).

pH analysis: Soil (~14 g) was transferred to 50-mL tube and 15 mL of reagent grade water was added and stirred for 10 min. The samples were centrifuged at 9,000 rpm for 20 min, then supernatant was decanted and pH was measured using a Thermo Scientific Orion Dual Star pH/ISE Benchtop instrument.

2.6.4 Culture Method

Culture-based microbiological analysis was performed on each sample by filtering the recovered spore suspension through 0.45-µm MicroFunnel filters (Pall Corporation, Washington, NY, Cat. 4804), then placing the filters onto solid bacterial growth media (TSA) or spread plating 0.1 mL of the recovered extract onto TSA. A modification to the EPA Protocol (EPA, 2017) used in the current study was that sample analysis proceeded directly to filter-plate or 0.1 mL spread plating of undiluted samples without dilutions, since spike levels were at or near the method detection limit. Work instructions for the culture method are detailed in Appendix J.

For MicroFunnel filter analysis, each filter was prewetted with 5 mL of PBST, 10 mL of PBST was added to each MicroFunnel filter to suspend aliquots, and then 1-mL to 8-mL aliquots of the extract were applied and vacuum filtered. The walls of each MicroFunnel filter were rinsed with 10 mL of PBST and filtered through the MicroFunnel filter, then the filter membrane was removed and placed onto TSA media.

Colonies with a typical *Btk* T1B2 morphology following overnight incubation at 30 ± 2°C were counted to determine percent spore recovery. Typical *Btk* T1B2 morphology on TSA is 2 to 5 mm in diameter, flat or slightly convex with edges that are irregular, and has a ground-glass appearance.

Two different microbiologists enumerated colonies over the course of the project, all of whom were trained by the project's lead microbiologist to consistently identify presumptive *Btk* T1B2 based on colony morphology. The lead microbiologist periodically reviewed the enumeration results to help ensure consistency and integrity, which is an important consideration and factor in the application of the method because the culture analysis was subjective to the assessment of colony morphology. Colonies identified during culture analysis are reported as presumptive *Btk* T1B2.

A small subset of presumptive *Btk* T1B2 were screened using a real-time PCR assay targeting the T1B2 barcode. A portion of a single colony or up to 10 colonies were pooled and suspended in 100 µL of PCR-grade water, heated for 5 min at 95 ± 2°C, centrifuged at 14,000 rpm for 2 min and the supernatant was analyzed in triplicate. An average threshold cycle (Ct) value of ≤ 40 was recorded as a positive result. The work instruction for colony PCR is located in Appendix M.

For Brain Heart Infusion Broth (BHIB) enrichment, the spores that remained on the filter or sponge were enriched following spore recovery within the 50 mL conical tube or specimen cup by adding 25 mL of BHIB, then incubated at 30 ± 2°C for 24 to 48 h. If *Btk* T1B2 colony morphology was not observed on TSA plates from culture analysis (spread plate or MicroFunnel Filters), turbid BHIB was then streaked onto three TSA plates for isolation and incubated overnight at 30 ± 2°C. Colonies with *Btk* T1B2 morphology that were isolated on these streak plates were screened using a real-time PCR assay targeting the T1B2 barcode. If colonies with *Btk* T1B2 morphology were not isolated on streak plates, an aliquot of the BHIB suspension (50 µL) was pelleted by centrifugation at 12,000 rcf for 2 min, supernatant was discarded, and the pellet was suspended in 100 µL of PCR-grade water. The suspended pellet was lysed at 95 ± 2°C for 5 min, then screened using real-time PCR assays. An average Ct value of ≤ 40 was recorded as a positive result. The work instruction for BHIB enrichment is located in Appendix N.

2.6.5 RV-PCR Method

2.6.5.1 Further Sample Processing for RV-PCR

Following filtration of half (12.5 mL for sponge stick; ~5 mL for VFC; 10 mL for wash water, gravel, and vegetation; or ~17 mL for soil samples) of recovered extract through the Whatman™ Autovial™ filter vials (with polyvinylidene difluoride membrane, Whatman, Marlborough, MA Cat. AV125NPUAQU), two buffer washes were performed according to the EPA Protocol (EPA, 2017). The first wash was 12.5 mL of cold (4°C) high salt buffer (10X PBS) followed by 12.5 mL of cold (4°C) low salt wash buffer (1X PBS). The top portion of the manifold was then removed and placed into a capping tray with pre-filled luer lock caps to seal the filter vials. Five (5) mL of cold (4°C) BHIB was then added to each filter vial, the vials were capped, and then vortex-mixed for 10 min on a setting of 7. Images of the manifold and capping tray are provided in Figure 33. Following the vortex step, the broth culture was mixed by pipetting up and down ~10 times and before incubating, a 1-mL aliquot was transferred to a screw cap tube and stored at -20°C as the time zero (T_0) aliquot. The capped filter vials were then incubated overnight for ~16 h (time final, T_f) in an incubator shaker set to 30 ± 2°C at 230 rpm. Note that the EPA Protocol specified 9 h or longer (EPA, 2017); the 16-h incubation allowed for a standard work schedule to be maintained rather than the overnight shift that would have been required by a 9-h incubation.

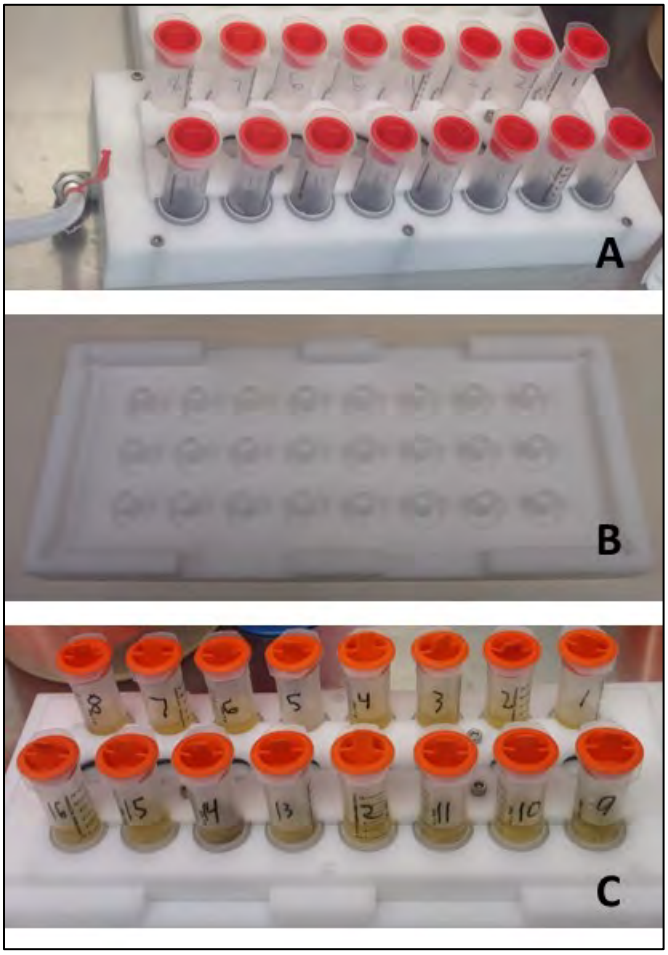

Figure 33. Manifold Containing 16 Filter Vials (A); Capping Tray (B); and Capped Filter Vials Containing BHIB (C).

Following overnight incubation (~16 h) of the filter vials, the vials were mixed on the platform vortex for 10 min with speed set to 7. Following the vortex step, the culture suspension was mixed by pipetting up and down ~10 times, and a 1-mL aliquot was transferred to screw cap tubes and labeled as the T_f aliquot. These processing steps are described in Appendix I.

2.6.5.2 DNA Extraction and Purification

Prior to extraction of deoxyribonucleic acid (DNA), the lysis buffer with antifoam reagent and the alcohol wash was added according to the manufacturer's instructions in the Magnesil Blood Genomic, Max Yield System Kit (Promega, Madison, WI, Cat. MD1360) and a heat block was preheated to 80°C. All screw-capped, 1-mL aliquots were thawed and centrifuged at 14,000 rpm (18,188 rcf) for 10 min (4°C), and 800 µL of the supernatant from each tube was removed and discarded. To extract the DNA, 800 µL of lysis buffer was added to each tube and the samples were mixed by vortexing on high (~1,800 rpm) in 10-sec pulses for a total of 60 sec. Each tube was then vortex-mixed for 10 sec at low speed directly before the lysate was transferred to a 2-mL labeled Eppendorf tube. The lysate tube was then incubated at room temperature for 5 min. Uniformly resuspended paramagnetic particles (PMPs) (600 µL) were added to each lysate tube and the samples were mixed by vortexing. After vortexing each T_0 and T_f tube for 10 sec (high, ~1,800 rpm), the samples were incubated at room temperature for 5 min.

The samples were then placed on the magnetic stand with the hinged side of the tube facing toward the magnet after briefly resuspending the particles by vortexing. The magnetic rack was then inverted to ensure all PMPs were contacting the magnet. After 10 sec, the tubes were opened, and the liquid removed without disturbing the PMPs. Lysis buffer (360 µL) was then added to each T_0 and T_f tube, the tube was capped and vortexed for 10 sec. The tubes were then placed on the magnetic stand and inverted again. The supernatant was then removed, and 360 µL of salt wash solution was added to each tube. The tubes were capped and vortexed for 10 sec, placed on the magnetic stand, and inverted. The supernatant was removed without disturbing the PMP pellet. The pelleted PMPs were washed a second time with 360 µL of salt wash solution.

After removal of the second salt wash supernatant, 500 µL of alcohol wash (70% ethanol) was added to each tube. The tubes were vortexed for 10 sec, placed on the magnetic stand, and inverted. The supernatant was then removed, and two more alcohol washes were conducted for a total of three 500-µL alcohol washes. A fourth alcohol wash was then conducted using 500 µL of 70% ethanol. After the supernatant from the wash was removed, all tubes were opened and allowed to air-dry for 2 min. The open tubes were then heated at 80°C in a heat block inside a Biological Safety Cabinet (BSC) until the PMPs were dry (~20 min). DNA was then eluted from the PMPs by the addition of 200 µL of elution buffer to each T_0 and T_f tube. The tubes were then closed, vortexed for 10 sec, and incubated in the heat block for 80 sec. The tubes were then vortexed another 10 sec and incubated in the heating block for 1 min. The vortexing and heating was repeated four times for a total of five times. The tubes were then removed from the heating block and incubated at room temperature for at least 5 min. Each tube was briefly vortexed and then centrifuged at 2,000 rpm (371 rcf) at 4ºC for 1 min. The tubes were then vortexed and placed on the magnetic stand for at least 30 sec. The eluate was collected (~80 to 90 µL) and transferred to clean labeled 1.5-mL tubes on a cold block. The tubes were centrifuged at 14,000 rpm (18,188 rcf) at 4°C for 5 min to pellet any particles remaining with the eluted DNA. The supernatant was carefully removed and transferred to a new 1.5-mL tube using a new tip for each tube. The T_0 and T_f DNA extracts were stored at 4ºC until RV-PCR analysis or at -20ºC if the analysis could not be performed within 24 h. The work instruction for DNA purification is Appendix K.

2.6.5.3 *Btk* T1B2 DNA Preparation

Genomic DNA of *Btk* T1B2 was extracted for use as a positive control for RV-PCR based analysis. The *Btk* T1B2 vegetative cell culture that DNA was extracted from originated from the spore stock used for spike/recovery tests. DNeasy Blood & Tissue Kit (Part No. 69504, Qiagen, Germantown, MD) was used following the manufacturer provided Gram-positive bacteria protocol to extract *Btk* DNA. The resulting DNA was quantified by Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Waltham, MA, Cat. P11496). The purified DNA was assigned a unique lot number, dispensed as multiple aliquots, stored frozen at below -20°C, and used as needed as the positive control for PCR analysis.

2.6.5.4 Real-Time PCR Assay

The specific tag 2 primer sequences from Buckley et al. (2012) were paired with a TaqMan probe designed using the PrimerQuest Tool (Integrated DNA Technologies, Coralville, IA). When comparing amplification of *Btk* T1B2 control DNA using the specific tag 2 primers described in Buckley et al. (2012)—coupled with SYBR Green chemistry with the TaqMan PCR assay combining the specific tag 2 primers and the TaqMan probe designed using the PrimerQuest Tool—the sensitivity was similar; however, the SYBR Green assay had amplification in no template control wells, therefore analysis was performed using the TaqMan assay (Table 4).

Table 4. *Btk* T1B2 TaqMan PCR Assay Primers and Probe Sequences.

***Btk* T1B2 Oligo**	**Sequence**	**Length**	**T_m (°C)**	**GC (%)**
Forward Primer	GGT ACA AGC AAC GAT CTC CAG AAT	24	64.5	45.8
Probe	6FAM-CGC CGA CGC TTT ACA TAC TAT GAG AGG-MGBNFQ	27	67.5	51.9
Reverse Primer	TGA AGG TTA ATT AGC GCA TTT GAA	24	62.0	33.3
Amplicon	GGT ACA AGC AAC GAT CTC CAG AAT TCG CCG ACG CTT TAC ATA CTA TGA GAG GCA CCT TAA GGT GTC TTT TCT TTT TGG ACA TTA CAT CCA TTT TGT TTT TCC ACC TTA TTT CAA ATG CGC TAA TTA ACC TTC A	133	71.3	39.1

The PCR assay Master Mix was prepared using the recipe provided in the formulations and recipes appendix (Appendix G, Table 2). Each sample DNA extract was assayed in triplicate reactions. Controls consisted of four positive control wells containing 50 picograms (pg) of DNA extracted from *Btk* T1B2 and four no template controls (NTCs) were also included with each assay. An Applied Biosystems 7500 Fast Real-Time PCR Instrument (Waltham, MA) was used for PCR assay development and testing. Thermocycler conditions with a fast ramp rate were:

- Stage 1: 1 cycle at 50°C for 2 min
- Stage 2: 1 cycle at 95°C for 2 min
- Stage 3: 45 cycles at 95°C for 3 sec followed by 60°C for 30 sec

The work instruction for real-time PCR is Appendix L.

2.7 Data Reduction and Analysis

2.7.1 Percent Recovery of Presumptive Btk T1B2 Colonies

The percent recovery ($E_{recovery}$) of *Btk* T1B2 spores from each spiked sample was calculated by dividing the number of presumptive *Btk* T1B2 CFU recovered ($N_{recover}$) from the sample by the actual number of *Btk* T1B2 spores spiked (N_{spike}), as determined by stock suspension titer for each test, then multiplied by 100. $N_{recover}$ is a product of the presumptive *Btk* T1B2 spore concentration ($C_{recover}$) (CFU/mL) and the total volume of extract used to recover the spores ($V_{extract}$) (mL). Mathematically, the percent recovery is expressed as follows:

$$E_{recovery}(\%) = \frac{C_{recover} * V_{extract}}{N_{spike}} * 100\%$$

Further, the number of presumptive *Btk* T1B2 spores present in the volume of recovered suspension plated onto spread plates or via MicroFunnel filter membrane was divided by the suspension volume analyzed to yield a presumptive *Btk* T1B2 spore concentration ($C_{recover}$) (CFU/mL). The recovered suspension volume ($V_{extract}$) was used to determine *Btk* T1B2 CFU recovered from the sample. The percent recovery was calculated for all volumes plated. The reported percent recovery was determined using the below rules:

1) Report the percent recovery from the aliquot that has between 20 to 80 CFU on MicroFunnel Filter membranes.

2) Report the higher-volume aliquot percent recovery if the CFU counted from both aliquots is less than 20.

3) Report the higher-volume aliquot percent recovery if the CFU counted from both aliquots is between 20 to 80.

4) Report the lower-volume aliquot percent recovery if the background microbial flora on the high-volume aliquot produces numerous colonies or a lawn of growth, thus complicating the identification of Btk T1B2 colonies.

5) Report the percent recovery from the spread plate that has between 25 and 250 CFU. Note, since spike levels were at or near the method detection limit for samples processed in this study, 10^{-1} and 10^{-2} dilutions were not spread plated as described in EPA Protocol (EPA, 2017).

The number of CFU is reported as presumptive *Btk* T1B2 colonies. PCR analysis of presumptive colonies is required to positively confirm the presence of *Btk* T1B2. To perform this task, a portion of the presumptive colony was collected into 100 µL of PCR-grade water in microcentrifuge tubes. The colony suspension was then heated for 5 min on a heat block at 95°C. The lysate was cooled and then centrifuged at 14,000 rpm (18,188 rcf) for 2 min, and the supernatant was analyzed using the real-time PCR assay targeting the *Btk* T1B2 target. The work instruction for colony PCR is located in Appendix M.

2.7.2 RV-PCR Method

The Ct values for the T_0 and T_f timepoints as well as the delta Ct value (ΔCt) were reported. The ΔCt is generated by subtracting the average Ct (from triplicate reactions) value generated by the T_f aliquot from the average Ct (triplicate reactions) value generated by the T_0 aliquot. A $\Delta Ct \geq 9$ value indicates that viable *Btk* T1B2 spores were detected in the sample if the following criterion was met:

The ΔCt must be greater than or equal to 9 for the *Btk* T1B2 target: $(\Delta Ct = Ct\ (T_0) - Ct\ (T_{final}) \geq 9)$

Additional criteria exist for the positive confirmation of a sample if analyzing samples obtained from an actual incident, but for this study the above criterion was used.

2.7.3 Presentation of Results

The method employed to recover *Btk* T1B2 spores was consistent with current EPA methods, as described in Section 2.6.4. In the instance of an actual biological agent release, the entire suspension of spores recovered from samples would be analyzed using either a culture method or a RV-PCR method. In the study performed and reported here, however, the recovered suspension was split as described in Sections 2.6.4 and 2.6.5, so that approximately half of the suspension was used for culture analysis and the other half for RV-PCR analysis. Consequently, neither the culture nor the RV-PCR method processed the total quantity of spores available in the suspension for analysis. Rather, each split sample suspension had a maximum of nominally half the actual spiked spore quantity available for their respective analyses. Therefore, in the presentation of results in tables and figures, unless explicitly noted otherwise, column headers or axis labels denote the nominal maximum number of recovered spores available in the sample for its respective analysis, which was half of the target spore load.

3.0 RESULTS AND DISCUSSION

As described in the previous section, all results presented in plots have an x-axis title and labels of 0, 150, and 1,500 CFU representing the nominal number of spores available for analysis. Similarly, the summary results in the tables contain the same nominal quantity of spores available, and the determined quantity of spores applied to the surface sampler substrate being assessed. This convention of presenting the results was considered the most accurate and consistent representation and allowed for the most unambiguous discussion and interpretation of results across all sample types and analytical methods, recognizing that the samples were originally spiked with target quantities of *Btk* T1B2 spores of 0, 300, and 3,000 CFU, but recovered suspensions from processed samples were split into approximately equal volume for the two analyses.

Note that the spores available for analysis represent the maximum number of spores available (assumes 100% spore recovery from the filter and no physical losses associated with processing of samples); it is not an absolute indication of the analytical method limit of identification. Rather, it is a measure of the method's end-to-end performance to detect *Btk* T1B2.

3.1 Sponge Stick Sample Analysis Results

3.1.1 Sponge Stick Sample Culture Analysis

A summary of the average and standard deviation values of the measured recovery of presumptive *Btk* T1B2 spores from sponge sticks that were used to wipe maritime surfaces and then spiked (inoculated) in the laboratory with a target of 300 or 3,000 CFU is presented in Table 5. The nominal quantity of spores available for analysis of 150 and 1,500 CFU represents one-half the target spore load applied to the surfaces and the determined number of spores available represents one-half the measured number of *Btk* T1B2 spores based on the *Btk* suspension titer and volume applied on the day of spiking. The spore recovery percentage of presumptive *Btk* colonies recovered as determined by culture analysis using TSA plates are plotted in Figure 34 through Figure 36. The quantity of presumptive *Btk* T1B2 colonies for each sponge stick sample used in the percent recovery calculations is reported in Table 5.

Table 5. Presumptive *Btk* T1B2 Spores Recovered from Laboratory-Spiked Sponge Sticks that Previously Sampled Different Maritime Surfaces.

Surface Type (Sample ID)	Sample Replicates	Spores Available for Analysis (CFU)		Spore Recovery (CFU) (X ± σ)[(c)]	Spore Recovery (%) (X ± σ)[(d)]
		Nominal[(a)]	Determined[(b)] (X ± σ)		
Small Boat Aluminum (SBMGAL-1)	3	0	0	12 ± 1.0	N/A
	3	150	87 ± 34	33 ± 8.6	40 ± 5.3
	3	1,500	870 ± 340	440 ± 110	52 ± 7.3
Small Boat Aluminum (SBMGAL-2)	3	0	0	17 ± 13	N/A
	3	150	87 ± 34	37 ± 21	50 ± 42
	3	1,500	870 ± 340	410 ± 79	50 ± 8.9
Nonskid Tread (NSKID-1)	3	0	0	18 ± 6.8	N/A
	3	150	87 ± 34	94 ± 68	99 ± 40
	3	1,500	870 ± 340	330 ± 40	41 ± 12
Nonskid Tread (NSKID-2)	4	0	0	130 ± 180	N/A
	4	150	130 ± 55	100 ± 92	120 ± 150
	4	1,500	1,300 ± 550	500 ± 170	39 ± 7.8
On-Board Touchscreen (TCHSCRN-1)	3	0	0	5.4 ± 5.0	N/A
	3	150	140 ± 55	280 ± 310	170 ± 170
	3	1,500	1,400 ± 550	390 ± 360	32 ± 27
On-Board Touchscreen (TCHSCRN-2)	3	0	0	0	N/A
	3	150	150 ± 130	39 ± 42	25 ± 9.0
	3	1,500	1,400 ± 550	610 ± 930	26 ± 27

[(a)] Nominally one-half of the target spore load on the surface and assuming 100 % recovery of spores.

[(b)] Based on the spiking suspension titer measured per trial, 100 % recovery, and one-half of extract used for culture analysis.

[(c)] Presumptive *Btk* T1B2 colonies based on morphology, and one-half of extract used for culture analysis.

[(d)] Calculated using the actual spore loading applied during spiking and total presumptive *Btk* T1B2 spores recovered on each sponge stick sample.

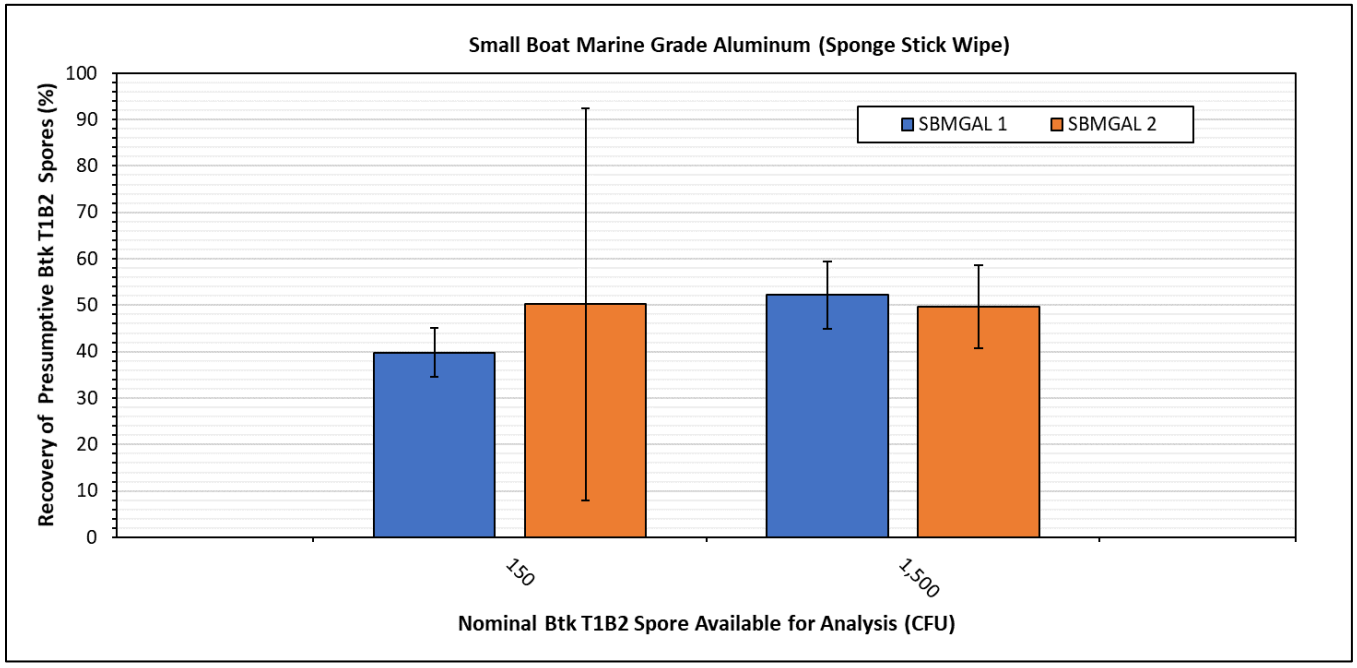

Figure 34. Presumptive *Btk* T1B2 Spore Recovery from Sponge Sticks Spiked with *Btk* T1B2 Spores After Having Sampled Small Boat Aluminum Surfaces

Average ± One Standard Deviation of N = 3 Replicates. Sponge Stick Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores.

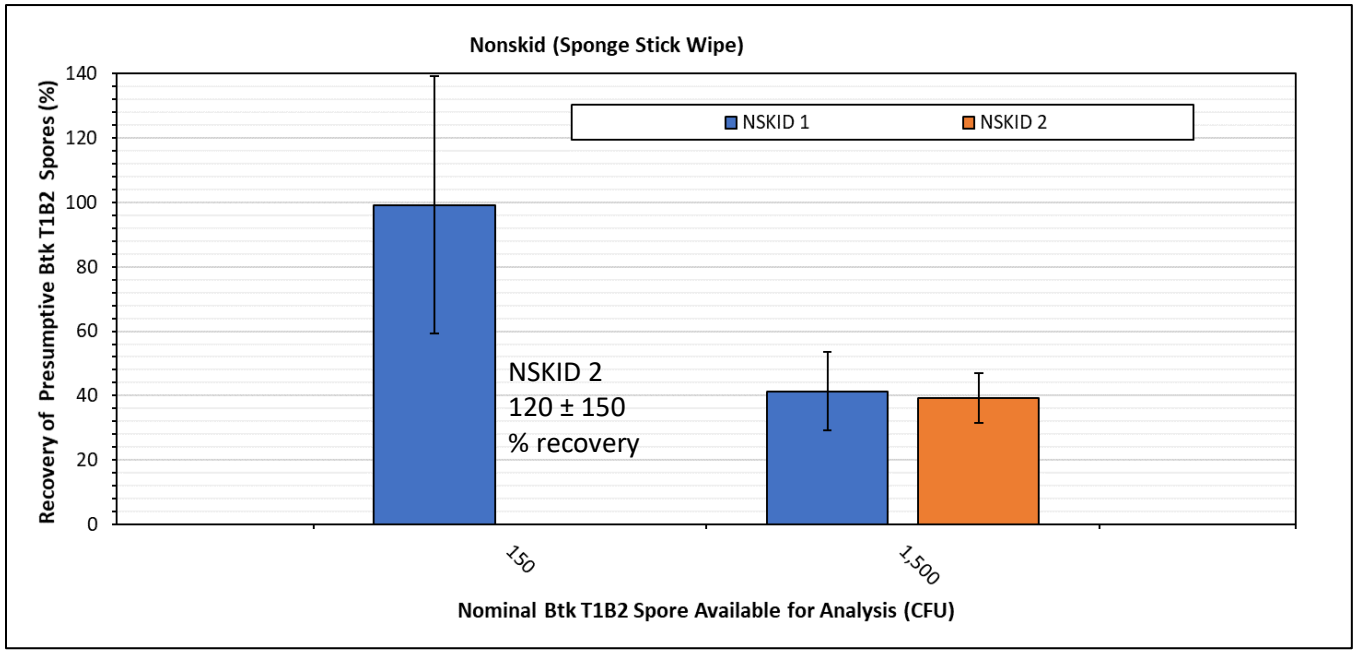

Figure 35. Presumptive *Btk* T1B2 Spore Recovery (%) from Sponge Sticks Spiked with *Btk* T1B2 Spores After Having Sampled Nonskid Tread Surfaces

Average ± One Standard Deviation of N = 3 Replicates. Sponge Stick Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores.

For nonskid 2, 150 CFU nominal spore load percent recovery values are not plotted in Figure 35 because percent recovery of 120 ± 150 % is not an accurate reflection of target spores recovered. The percent recovery values, particularly for low spore level samples, are inflated from the presence of background microorganisms with *Btk* morphology.

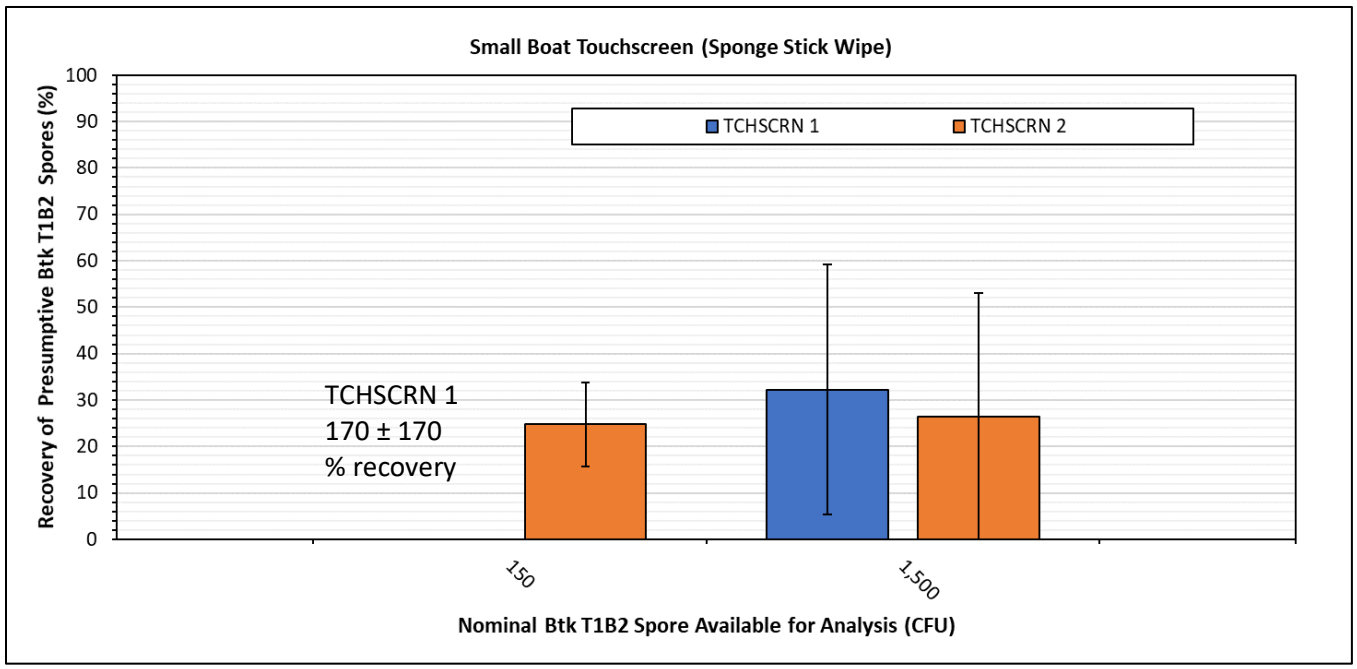

Figure 36. Presumptive *Btk* T1B2 Spore Recovery (%) from Sponge Sticks Spiked with *Btk* T1B2 Spores After Having Sampled On-Board Touchscreen Surfaces

Average ± One Standard Deviation of N = 3 Replicates. Sponge Stick Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores.

For touch screen, 150 CFU nominal spore load percent recovery recovery values are not plotted in Figure 36 because percent recovery of 170 ± 170 % is not an accurate reflection of target spores recovered. The percent recovery values, particularly for low spore level samples, are inflated from the presence of background microorganisms with *Btk* morphology.

The images in Figure 37 through Figure 39 show examples of culture plates with 2-mL or 8-mL volumes on TSA at all three spike levels: 0 CFU spike, 300 CFU spike, and 3,000 CFU spike. Presumptive colonies were present on zero spike samples, although the number of presumptive colonies increased as the spike level was increased.

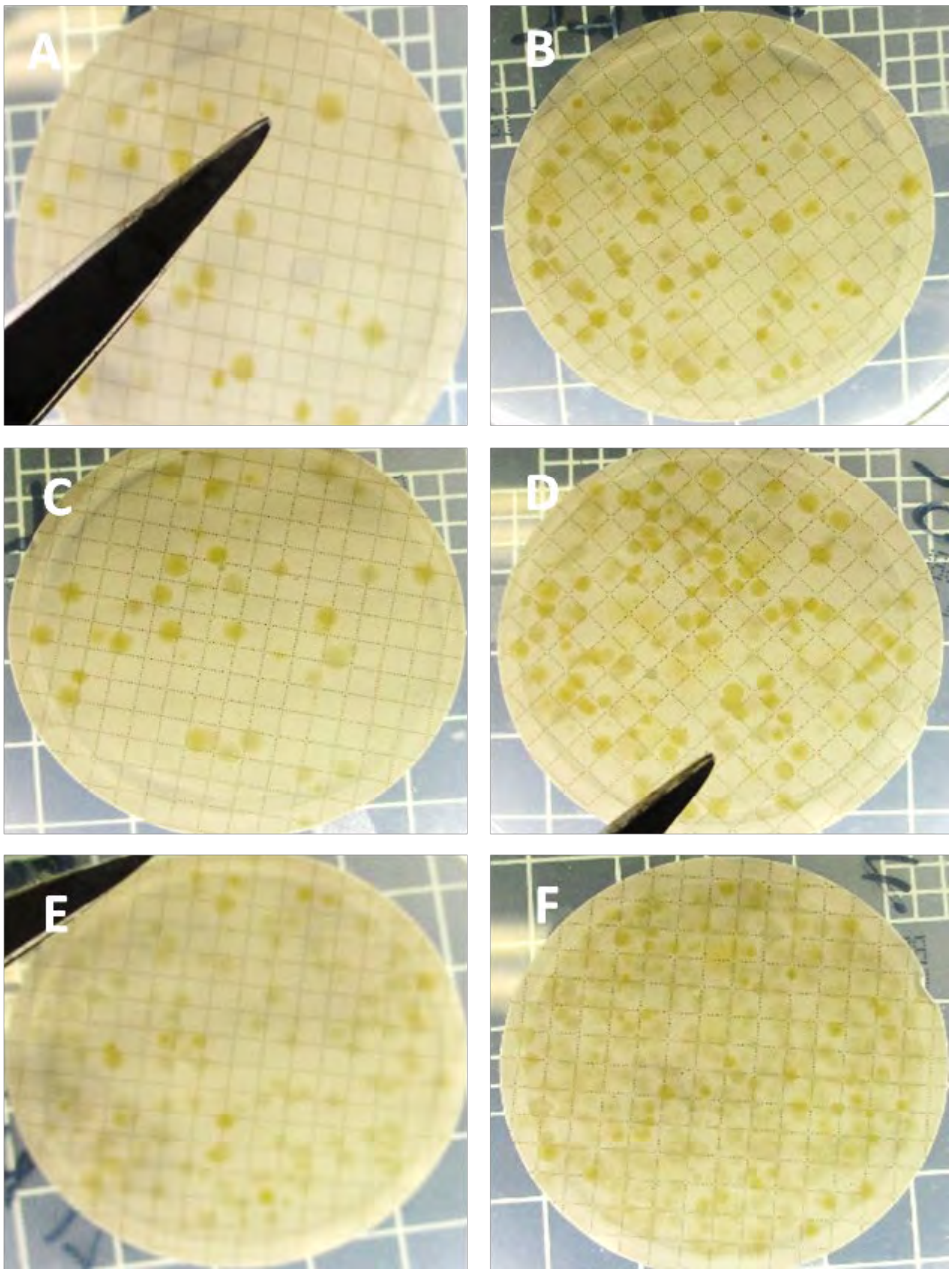

Figure 37. Culture Images of Spore Recovery from Small Boat 2 Marine Grade Aluminum Surface Sampled Using Sponge Stick and Plated on TSA.

Presumptive Btk colonies were observed in zero spore spike samples (Average <1 CFU/mL).
Image Descriptions: (A) Zero Spore Spike, 2 mL; (B) Zero Spore Spike, 8 mL; (C) 300 Spore Spike, 2 mL; (D) 300 Spore Spike, 8 mL; (E) 3,000 Spore Spike, 2 mL; (F) 3,000 Spore Spike, 8 mL.

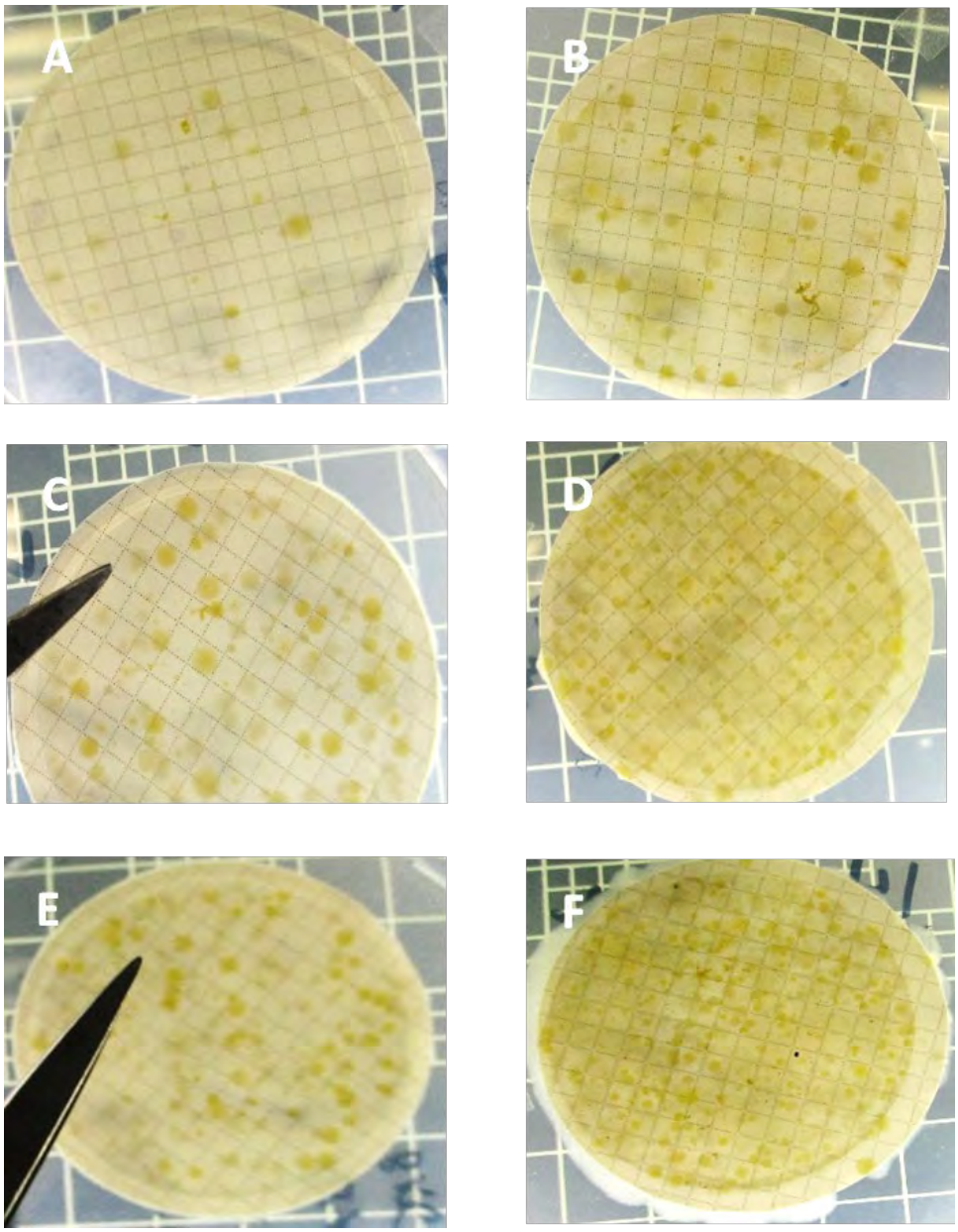

Figure 38. Culture Images of Spore Recovery from Nonskid Boat 1 Surface Sampled Using Sponge Stick and Plated on TSA.

Presumptive Btk colonies were observed in zero spore spike samples (Average < 3 CFU/mL when plating 2 mL volume for Boat 1, < 15 CFU/mL when plating 2 mL volume for Boat 2).
Image Descriptions: (A) Zero Spore Spike, 2 mL; (B) Zero Spore Spike, 8 mL; (C) 300 Spore Spike, 2 mL; (D) 300 Spore Spike, 8 mL; (E) 3,000 Spore Spike, 2 mL; (F) 3,000 Spore Spike, 8 mL

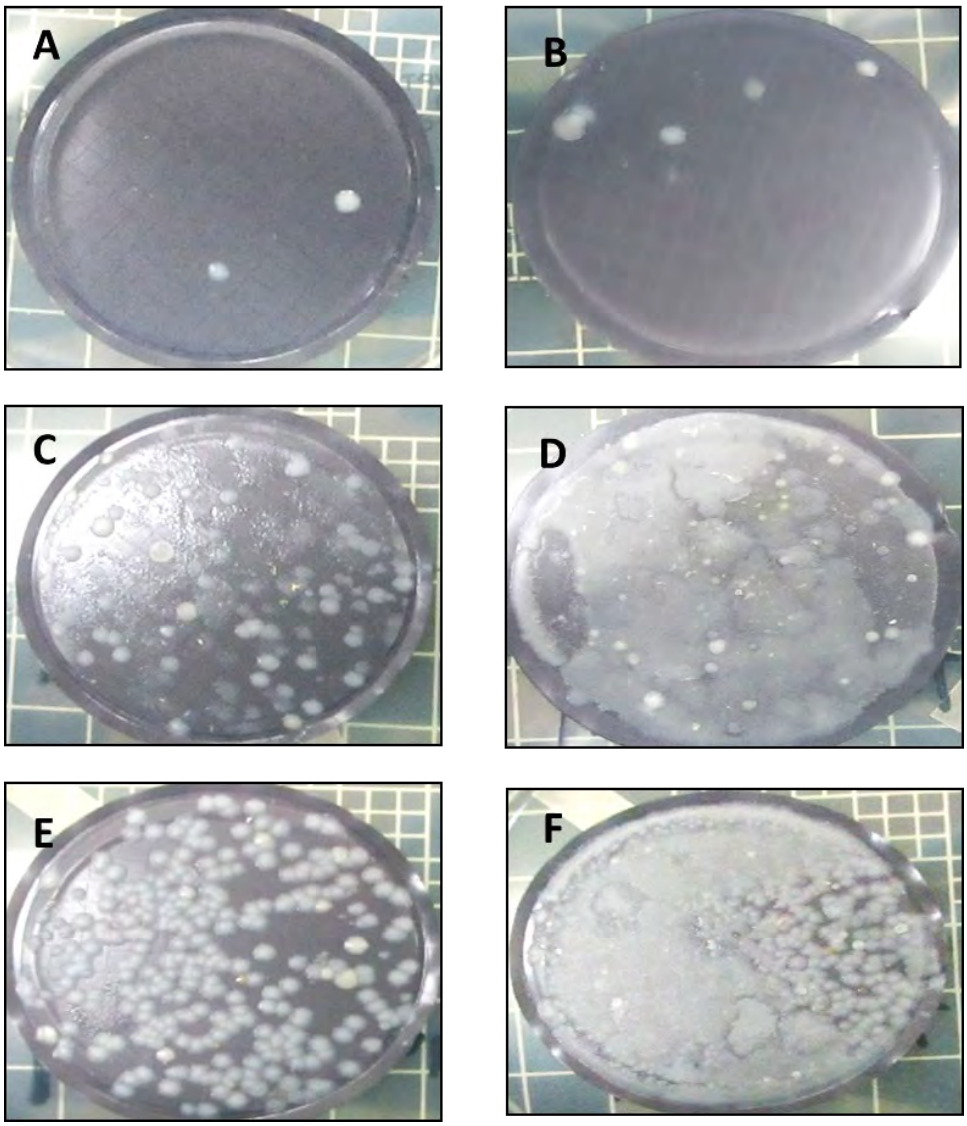

Figure 39. Culture Images of Spore Recovery from Touch Screen Boat 2 Surface Sampled Using Sponge Stick and Plated on TSA.

Presumptive Btk colonies were not observed in zero spore spike samples.
Image Descriptions: (A) Zero Spore Spike, 2 mL; (B) Zero Spore Spike, 8 mL; (C) 300 Spore Spike, 2 mL; (D) 300 Spore Spike, 8 mL; (E) 3,000 Spore Spike, 2 mL; (F) 3,000 Spore Spike, 8 mL

3.1.2 Colony Confirmation by PCR

Based on the colony morphology, presumptive *Btk* T1B2 colonies were identified for all surfaces (52 of 57 samples) sampled using sponge sticks with the following exceptions: 1) on-board touchscreen from Boat 1 (one replicate); 2) on-board touchscreen from Boat 2 (three replicates); and 3) nonskid tread from Boat 2 (one replicate). The presence of presumptive *Btk* T1B2 morphology did not mean that the *Btk* T1B2 spores were recovered from the surfaces. Nineteen (19) samples with inert and biological deposits were 0-spike samples, meaning they were not inoculated with *Btk* T1B2 spores, yet presumptive *Btk* colonies were still isolated from the spore recovery. Colonies with morphology indistinguishable from *Btk* T1B2 were present on the culture plates, as indicated by a negative PCR result for presumptive *Btk* T1B2 colonies. The confirmation of target *Btk* T1B2 was assessed by colony PCR from the initial

culture plates, colony PCR from BHIB enrichment culture from the sponge stick samples, or PCR of an aliquot of the BHIB enrichment culture from the sponge stick samples. Results from PCR confirmatory testing are shown in Table 6. A total of six sample replicates were false positive samples; however, the colony PCR Ct values were 37, 37, 35, 32, and 38. By comparison, the eight spiked field blank or laboratory spike samples had a Ct value of 21.2 ± 0.6. The two other false positive samples had Ct values of 37 and 39 for the BHIB enrichment culture from sponge samples. The cause of these high Ct value false positive samples is either low level contamination between samples or potentially nonspecific PCR amplification.

Table 6. Summary of the Accuracy of Identification of Presumptive *Btk* T1B2 Colonies by PCR Confirmation from Spiked Sponge Sticks Used to Sample Different Maritime Surfaces.

Surface Type (Sample ID)	Nominal Spore Load (CFU)	Culture Replicates Presumptive Positive[a]	Colonies from Initial Culture Plates PCR-Screened (# PCR +) [b]	Colony PCR Ct (X ± σ) [c]	Colonies from BHIB Streak Plates PCR-Screened (# PCR +) [d]	BHIB PCR-Screened (# PCR +) [e]	BHIB PCR Ct (X ± σ) [f]
Small Boat Aluminum (SBMGAL-1)	0	3 of 3	9 (0)	N/A	0	2 (1)	37.2
	150	3 of 3	7 (2)	22.2 ± 0.4	1 (0)	0	N/A
	1,500	3 of 3	12 (3)	22.8 ± 0.8	0	0	N/A
Small Boat Aluminum (SBMGAL-2)	0	3 of 3	4 (1)	37.2	0	0	N/A
	150	3 of 3	12 (3)	22.7 ± 1.7	0	0	N/A
	1,500	3 of 3	12 (3)	22.5 ± 1.0	0	0	N/A
Nonskid Tread (NSKID-1)	0	3 of 3	6 (2)	36.0 ± 1.0	0	2 (0)	N/A
	150	3 of 3	12 (2)	22.9 ± 1.0	0	1 (0)	N/A
	1,500	3 of 3	12 (3)	23.3 ± 1.4	0	0	N/A
Nonskid Tread (NSKID-2)	0	3 of 4	21 (1)	32.1	0	1 (0)	N/A
	150	4 of 4	27 (3)	25.7 ± 4.6	1 (0)	1 (0)	N/A
	1,500	4 of 4	31 (4)	22.6 ± 1.3	1 (0)	0	N/A
On-Board Touchscreen (TCHSCRN-1)	0	2 of 3	11 (1)	38.1	0	1 (1)	39.1
	150	3 of 3	21 (3)	23.6 ± 3.2	0	0	N/A
	1,500	3 of 3	21 (3)	20.9 ± 0.6	0	0	N/A
On-Board Touchscreen (TCHSCRN-2)	0	0 of 3	0	N/A	0	0	N/A
	150	3 of 3	28 (3)	20.8 ± 3.1	0	0	N/A
	1,500	3 of 3	30 (3)	20.5 ± 0.8	0	0	N/A

[a] Presumptive *Btk* T1B2 was present on initial culture plates.

[b] Number of colonies PCR-screened from initial plating, with number of PCR positive replicates in parentheses.

[c] Colony PCR Ct values for positive samples (Ct value of ≤ 40).

[d] Number of colonies PCR-screened from BHIB streak plates, with number of PCR positive replicates in parentheses.

[e] Number of samples with PCR screening of BHIB enrichment culture, with number of PCR positive replicates in parentheses.

[f] BHIB enrichment culture PCR Ct values for positive samples (Ct value of ≤ 40).

3.1.3 Sponge Stick Sample RV-PCR Analyses

A summary of the average and standard deviation of the RV-PCR ΔCt values for the detection of *Btk* T1B2 spores recovered from sponge sticks that were used to wipe maritime surfaces and then spiked (inoculated) with *Btk* T1B2 spores in the laboratory with a target of 300 or 3,000 CFU is presented in Table 7. The nominal quantity of spores available for analysis represents one-half the target spore load applied to the surfaces, and the determined number of spores available represents one-half the measured number of *Btk* T1B2 spores applied based on the *Btk* suspension titer and volume applied on the day of spiking. Sample replicates with an RV-PCR ΔCt value ≥ 9 are RV-PCR positive, indicating that viable *Btk* T1B2 spores were recovered. The RV-PCR ΔCt results are plotted in Figure 40 through Figure 42. The plots all depict the ΔCt threshold value of 9 as a dashed line with an area shaded in red representing a negative detection result, and an area of green representing a positive detection result.

Table 7. RV-PCR Analyses of Spiked Sponge Sticks that Were Used to Sample Different Maritime Surfaces for Detection of *Btk* T1B2 Spores.

Surface Type (Sample ID)	Number of Replicates	Spores Available for Analysis (CFU)		ΔCt[c] (X ± σ)	RV-PCR Replicates Positive[d]
		Nominal[a]	Determined[b]		
Small Boat Aluminum (SBMGAL-1)	3	0	0	0.4 ± 2.5	0
	3	150	87 ± 34	16.9 ± 5.7	3
	3	1,500	870 ± 340	21.1 ± 5.1	3
Small Boat Aluminum (SBMGAL-2)	3	0	0	0 ± 0	0
	3	150	87 ± 34	15.6 ± 2.2	3
	3	1,500	870 ± 340	22.2 ± 0.9	3
Nonskid Tread (NSKID-1)	3	0	0	1.4 ± 1.3	0
	3	150	87 ± 34	15.3 ± 8.0	3
	3	1,500	870 ± 340	14.9 ± 4.6	3
Nonskid Tread (NSKID-2)	4	0	0	0.6 ± 2.6	0
	4	150	130 ± 55	11.3 ± 2.6	3
	4	1,500	1,300 ± 550	17.2 ± 2.0	4
On-Board Touchscreen (TCHSCRN-1)	3	0	0	4.6 ± 4.1	0
	3	150	140 ± 55	21.1 ± 3.2	3
	3	1,500	1,400 ± 550	23.1 ± 2.4	3
On-Board Touchscreen (TCHSCRN-2)	3	0	0	0 ± 0	0
	3	150	150 ± 130	20.1 ± 3.5	3
	3	1,500	1,400 ± 550	23.0 ± 2.1	3

[a] Nominally one-half of the target spore load on the surface and assuming 100% recovery of spores.

[b] Based on the spiking suspension titer measured per trial, 100 % recovery, and one-half of extract used for culture analysis.

[c] PCR assay for T1B2 Barcode Gene Target.

[d] Number of replicates with a RV-PCR ΔCt value ≥ 9.

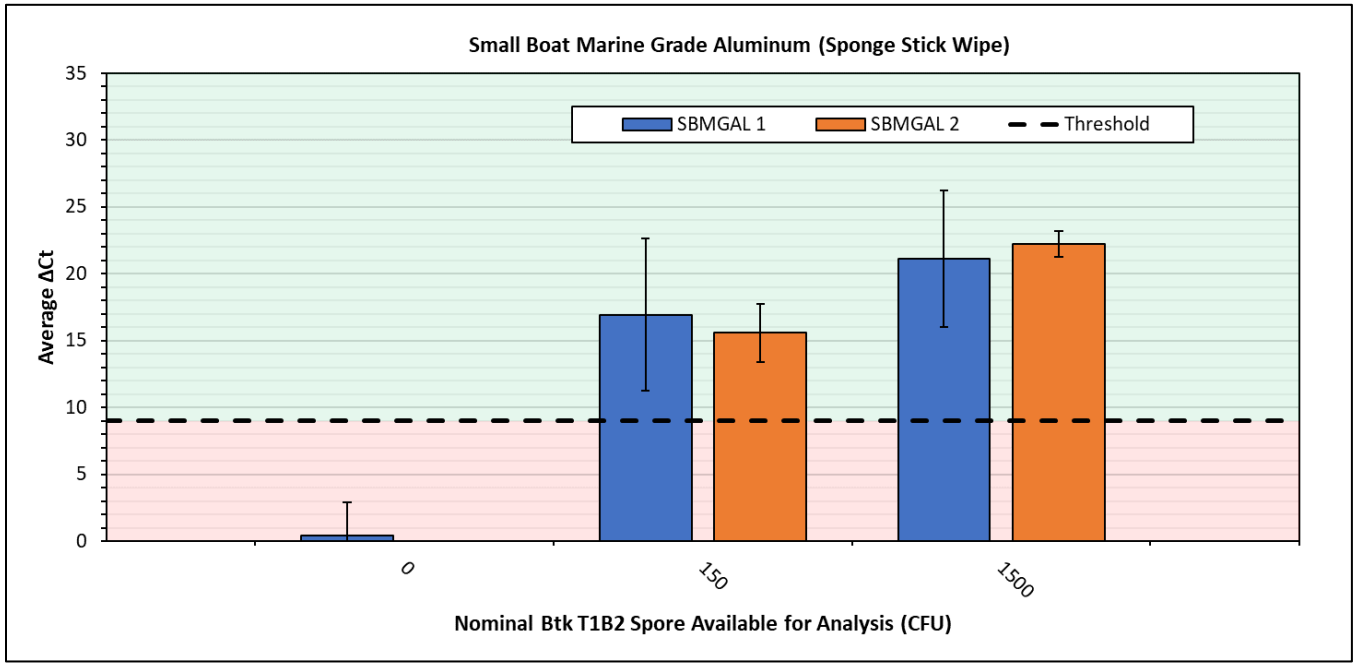

Figure 40. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from Sponge Sticks Spiked with *Btk* T1B2 Spores After Having Sampled Nonskid Tread Surfaces.

Average ± One Standard Deviation of N ≥ 3 Replicates. Sponge Stick Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores. Positive Result Equals ΔCt ≥ 9.

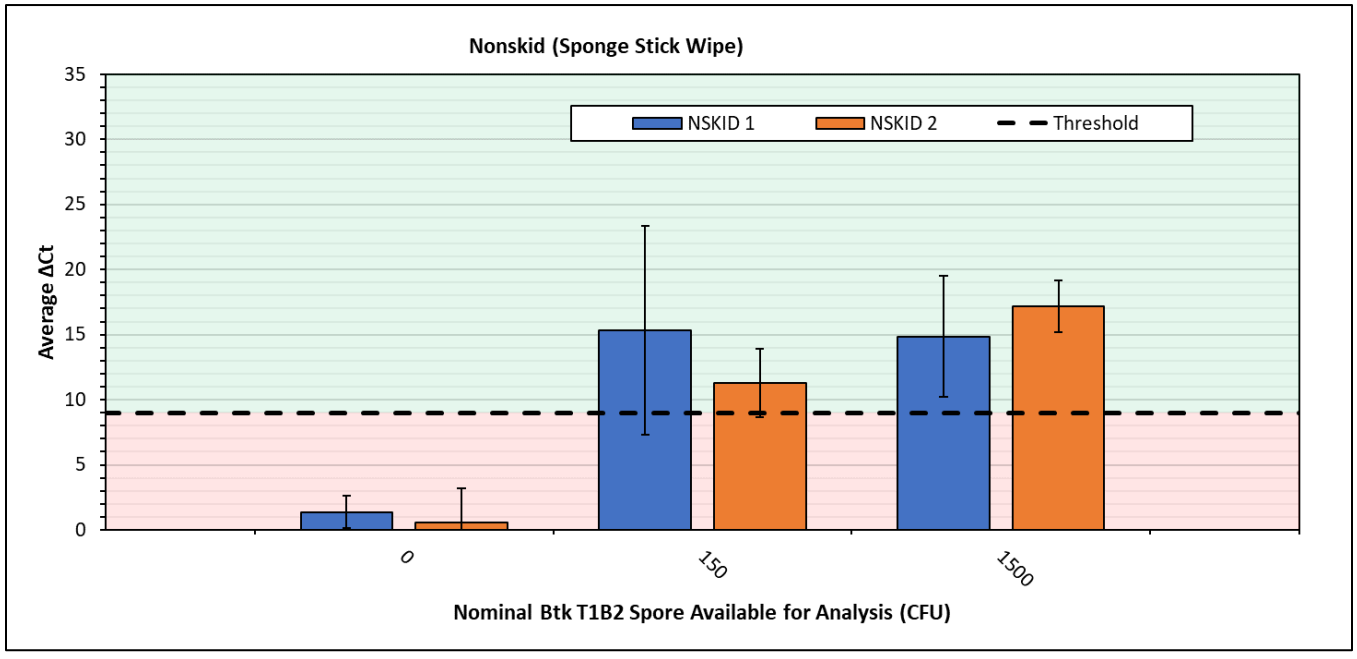

Figure 41. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from Sponge Sticks Spiked with *Btk* T1B2 Spores After Having Sampled Nonskid Tread Surfaces.

Average ± One Standard Deviation of N ≥ 3 Replicates. Sponge Stick Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores. Positive Result Equals ΔCt ≥ 9.

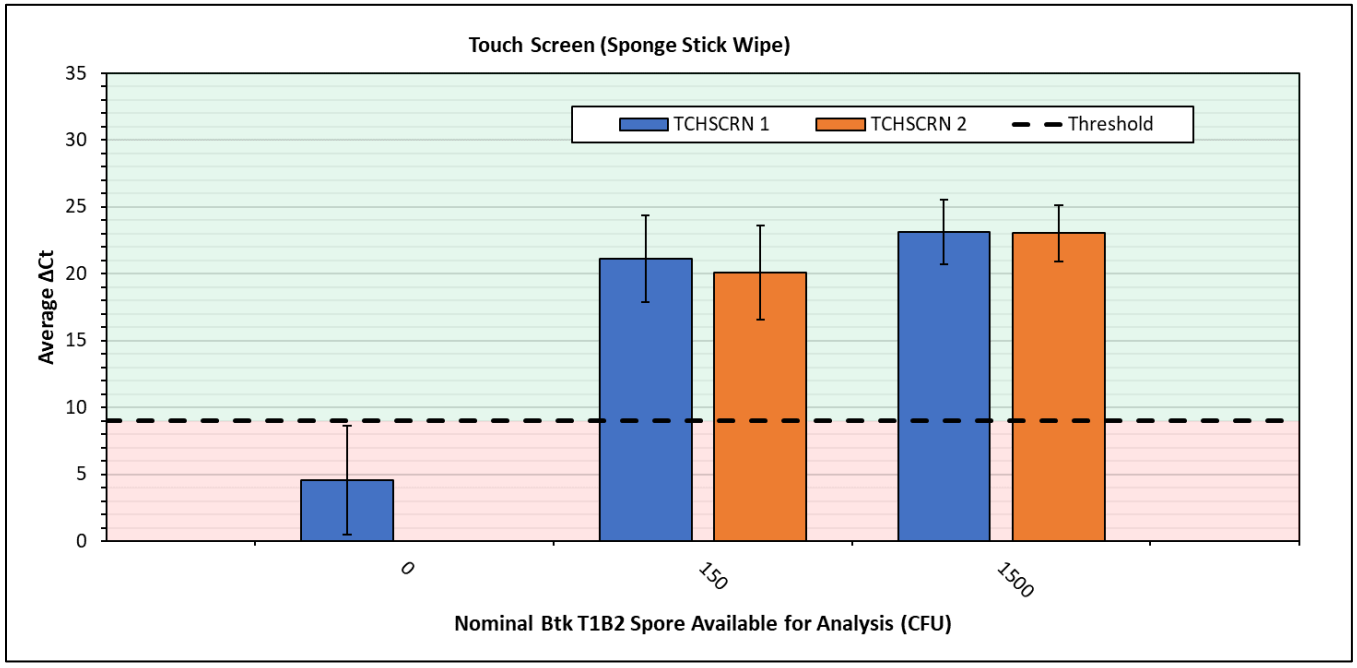

Figure 42. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from Sponge Sticks Spiked with *Btk* T1B2 Spores After Having Sampled On-Board Touchscreen Surfaces; Positive Response Equals ΔCt ≥ 9.

Average ± One Standard Deviation of N = 3 Replicates. Sponge Stick Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores. Positive Result Equals ΔCt ≥ 9.

3.1.4 Analytical Method Comparison of Sponge Stick Samples

Culture analysis identified presumptive *Btk* T1B2 colonies for most samples, even zero spike samples, indicating that background microbial flora included wild-type *Btk* or another organism that had a morphology indistinguishable from *Btk* T1B2 on TSA plates. Three exceptions occurred where presumptive *Btk* T1B2 colonies were not present: 1) on-board touchscreen from Boat 1 (one replicate); 2) on-board touchscreen from Boat 2 (three replicates); and 3) nonskid tread from Boat 2 (one replicate). Colony PCR from initial culture plates; colony PCR of colonies isolated from BHIB enrichment culture of the sponge stick samples; and/or PCR analysis of the BHIB enrichment culture was therefore required to confirm or refute the presence of *Btk* T1B2. To compare the two methods, culture with PCR confirmation and RV-PCR results were assessed to determine which method may be more likely to detect viable spores that have been spiked onto sponge sticks that contain outdoor interferents.

PCR screening of presumptive *Btk* T1B2 colonies was negative in some cases, indicating that background microbial flora with colony morphology indistinguishable from the morphology of *Btk* T1B2 were present on TSA culture plates; and hence, present in the samples collected in the field. It is possible that wild-type/naturally occurring *Btk* and their presence led to an inflation in presumptive spore recovery values by the culture method. Presumptive culture identification by colony morphology, colony identification confirmed by PCR, and RV-PCR results are shown in Table 8.

For culture analysis of sponge stick samples, 48 of 57 (84 %) that had been used to collect inert and biological deposits were determined to be true positive or true negative. A true positive is defined as a sample spiked with *Btk* T1B2 spores that was confirmed positive by PCR. A true negative is defined as a sample that was not spiked with *Btk* T1B2 spores and tested negative in PCR confirmatory screening. Six samples were false positive and three samples were false negative using the culture method. For the

six false positive samples, colony PCR Ct values were ≤ 40, each measuring between 32 to 38, or a BHIB culture PCR Ct value of 37. For comparison, colony PCR Ct values of *Btk* T1B2 isolated from field blanks or laboratory blanks (new sponge spiked with *Btk* T1B2 spores) was 21.2 ± 0.6. For the three false negative samples, a minimum of 26 presumptive *Btk* colonies were available for colony PCR; however, only one colony from each of these samples was screened. If more colonies were screened for these samples, it is possible the sample would have been confirmed positive. The EPA Protocol specifies that one to three colonies from MicroFunnel filters and a minimum of three colonies from spread plates should be PCR-screened for target confirmation (EPA, 2017). All field blank and laboratory blank controls were true negatives (19 of 19).

For RV-PCR analysis of sponge stick samples, 56 of 57 (98 %) that had been used to collect inert and biological deposits were determined to be true positives or true negatives. A true positive is defined as a sample spiked with *Btk* T1B2 spores that had a ΔCt of ≥ 9. A true negative is defined as a sample that was not spiked with *Btk* T1B2 spores and had a ΔCt of < 9. The one false negative sample had been spiked with 300 CFU target spore load and had a ΔCt of 8.7. One laboratory blank sample was a false positive with a ΔCt of 9.8.

Table 8. Analytical Method Comparison Displaying Culture Presumptive, Culture ID with PCR Confirmation, and RV-PCR Replicates Positively Identified (N = 3) for Surfaces Sampled with Sponge Sticks.

Sample Surface	Actual Spike Level (CFU)	Presumptive Culture Result	Culture PCR Confirmation	Culture % Recovery	RV-PCR Result	RV-PCR ΔCt
Marine Grade Aluminum, Boat 1	0	Positive	Negative	N/A	Negative	-1.92
	0	Positive	Positive	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	3.1
	250	Positive	Negative	33.8	Positive	22.8
	150	Positive	Positive	44	Positive	16.5
	120	Positive	Positive	41.7	Positive	11.5
	2,500	Positive	Positive	45.1	Positive	26.9
	1,500	Positive	Positive	52	Positive	18.9
	1,200	Positive	Positive	59.6	Positive	17.5
Marine Grade Aluminum, Boat 2	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	0	Positive	Positive	N/A	Negative	0
	250	Positive	Positive	27.6	Positive	14.6
	150	Positive	Positive	24	Positive	14.1
	120	Positive	Positive	99	Positive	18.1
	2,500	Positive	Positive	39.5	Positive	21.3
	1,500	Positive	Positive	53.6	Positive	23.1
	1,200	Positive	Positive	56	Positive	22.3
Touch Screen, Boat 1	0	Positive	Positive	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	7.9
	0	Negative	Negative	N/A	Negative	5.8
	150	Positive	Positive	42.2	Positive	24.8
	340	Positive	Positive	111.6	Positive	19.9
	340	Positive	Positive	367.1	Positive	18.7
	1,500	Positive	Positive	51.2	Positive	23.9
	3,400	Positive	Positive	44.1	Positive	20.4
	3,400	Positive	Positive	1.43	Positive	25.1
Touch Screen, Boat 2	0	Negative	Negative	N/A	Negative	0
	0	Negative	Negative	N/A	Negative	0
	0	Negative	Negative	N/A	Negative	0
	110	Positive	Positive	30.6	Positive	22.2
	180[(a)]	Positive	Positive	86.7	Positive	21.9
	600	Positive	Positive	29.2	Positive	16
	1,100	Positive	Positive	18.6	Positive	21.2
	1,800[(a)]	Positive	Positive	27.6	Positive	22.6
	6,000	Positive	Positive	56.1	Positive	25.3

[(a)]Stock enumeration plate outside 25 – 250 CFU range.

Table 8. Analytical Method Comparison Displaying Culture Presumptive, Culture ID with PCR Confirmation, and RV-PCR Replicates Positively Identified (N = 3) for Surfaces Sampled with Sponge Sticks (Cont.)

Sample Surface	Actual Spike Level (CFU)	Presumptive Culture Result	Culture PCR Confirmation	Culture % Recovery	RV-PCR Result	RV-PCR ΔCt
Nonskid, Boat 1	0	Positive	Negative	N/A	Negative	0
	0	Positive	Positive	N/A	Negative	1.7
	0	Positive	Positive	N/A	Negative	2.4
	250	Positive	Negative	134.4	Positive	9.3
	150	Positive	Positive	107.3	Positive	12.3
	120	Positive	Positive	55.8	Positive	24.42
	2,500	Positive	Positive	27.3	Positive	12
	1,500	Positive	Positive	48.8	Positive	20.2
	1,200	Positive	Positive	47.9	Positive	12.4
Nonskid, Boat 2	0	Positive	Negative	N/A	Negative	-2.5
	0	Positive	Positive	N/A	Negative	3.8
	0	Positive	Negative	N/A	Negative	1.1
	0	Negative	Negative	N/A	Negative	0
	250	Positive	Negative	135.2	Positive	9.5
	120	Positive	Positive	322.9	Positive	13.1
	340	Positive	Positive	17.2	Negative	8.7
	360	Positive	Positive	9.6	Positive	14
	2,500	Positive	Positive	38.7	Positive	18.7
	1,200	Positive	Positive	48	Positive	15.9
	3,400	Positive	Positive	41.3	Positive	19
	3,600	Positive	Positive	29.3	Positive	15

Results for all samples processed, including positive and negative controls, are shown in Table 9. Culture method correctly determined 67 of 76 samples (88%) with six false positive samples; however, all six of these samples had colony PCR Ct values between 32 to 38 or BHIB enrichment culture PCR of 37. Colony PCR of *Btk* T1B2 from the eight spiked field blank or laboratory spike (new sponge stick spiked with *Btk* T1B2 spores) controls had a Ct value of 21.2 ± 0.6, suggesting that Ct values between 32 to 38 might be caused by low level contamination or potentially nonspecific PCR amplification. The RV-PCR method correctly determined 74 of 76 samples (97%), with only one false positive laboratory blank with a ΔCt value of 9.78 and one false negative sample from a 300-target spore load sample that had a ΔCt value of 8.7.

For culture analysis, the marine grade aluminum surfaces sampled with sponge sticks had two false positive samples with Ct values of 37 when PCR analysis was performed on colonies and BHIB enrichment culture of the processed sponge stick samples and one false negative sample. The false negative sample had 33 presumptive colonies and only one was PCR-screened, so it is possible that the sample would have been confirmed positive if more colonies were screened. RV-PCR results matched the expected outcomes in all cases.

For culture analysis, the touch screen surfaces sampled with sponge sticks had one false positive sample with a Ct value of 39 when PCR analysis was performed on BHIB enrichment culture of the processed sponge stick samples. The same sample replicate had a Ct value of 38 for colony PCR. For all eight of the spiked field blank or laboratory spike (new sponge stick spiked with *Btk* T1B2) controls, colony PCR of *Btk* T1B2 colonies resulted in a Ct value of 21.2 ± 0.6. Therefore, a Ct value of 38 for a colony PCR reaction may have been caused by low level contamination or potentially nonspecific PCR amplification. RV-PCR results matched the expected outcome in all cases.

For culture analysis, the nonskid tread surfaces sampled with sponge sticks had three false positive samples. The first had a colony PCR Ct value of 37, and the BHIB enrichment of the extracted sponge stick was PCR negative. The second had a colony PCR Ct value of 35 with four presumptive *Btk* colonies pooled, and the third had a colony PCR Ct value of 32 for a pool of 10 presumptive *Btk* colonies. Colony PCR of the eight spiked field blank or laboratory spike samples had a Ct value of 21.2 ± 0.6, therefore Ct values between 32 to 37 may have been caused by low level contamination or potentially nonspecific PCR amplification. There were two false negative samples for culture analysis, although only one presumptive colony each was PCR-screened with 26 or 28 well-isolated colonies available for PCR screen. However, the BHIB enrichment cultures of the processed sponge stick samples were also negative for both replicates.

Table 9. Analytical Method Comparison Displaying Culture ID with PCR Confirmation and RV-PCR for Surfaces Sampled with Sponge Sticks.

Surface Type	Culture Method (PCR Confirmation)			RV-PCR Method		
	True Positive or True Negative	False Positive Sample	False Negative Sample	True Positive or True Negative	False Positive Sample	False Negative Sample
Small Boat Aluminum 1	7	1	1[(a)]	9	0	0
Small Boat Aluminum 2	8	1	0	9	0	0
Nonskid 1	6	2	1[(b)]	9	0	0
Nonskid 2	10	1	1[(b)]	11	0	1[(d)]
Touchscreen 1	8	1	0	9	0	0
Touchscreen 2	9	0	0	9	0	0
Controls	19	0	0	18	1[(c)]	0
Totals	**67**	**6**	**3**	**74**	**1**	**1**

Positive PCR threshold of 40 for colony PCR and BHIB enrichment culture PCR. The definition for Positive Ct threshold per the EPA Protocol (EPA, 2017) is ≤ 40.

[(a)] Only PCR-screened 1 colony from membrane plate, 33 were presumptive.

[(b)] Only PCR-screened 1 colony from membrane plate, 28 and 26 were presumptive on 2-mL plate.

[(c)] ΔCt value of 9.78 of laboratory blank, may be caused by cross-contamination that occurred during DNA extraction or nonspecific amplification.

[(d)] ΔCt value of 8.67.

3.1.5 Analysis of Controls

For sponge sticks, there were a total of eight zero spike sponge sticks that served as reagent control laboratory blanks, three zero spike sponge sticks that were opened in the field that served as field blanks, three (3) 300-CFU spiked field blanks, three (3) 3,000-CFU spiked field blanks, one (1) 300-CFU spiked sponge stick, and one (1) 3,000-CFU spiked sponge stick that was handled in the laboratory only (laboratory spike).

All controls performed as expected except for one zero spore spike sponge stick that was RV-PCR positive with a ΔCt of 9.8 (laboratory blank). For all eight of the spiked field blank or laboratory spike (new sponge stick spiked with *Btk* T1B2) samples where colony PCR was performed, the average Ct value was 21.2 ± 0.6.

3.2 Vacuum Filter Cassette Sample Analysis Results

3.2.1 Vacuum Filter Cassette Sample Culture Analysis

A summary of the average and standard deviation values of the measured recovery of presumptive *Btk* T1B2 spores from VFCs that were used to sample maritime surfaces and then spiked (inoculated) in the laboratory with a target of 300 or 3,000 CFU is presented in Table 10. The nominal quantity of spores available for analysis of 150 and 1,500 CFU represents one-half the target spore load applied to the surfaces, and the determined number of spores available represents one-half the measured number of *Btk* T1B2 spores applied based on the *Btk* suspension titer and volume applied on the day of spiking. The spore recovery percentage of presumptive *Btk* colonies recovered was determined by culture analysis using TSA plates. The spore recovery percentages of presumptive *Btk* colonies recovered as determined by culture analysis using TSA plates is plotted in Figure 43 and Figure 44.

Table 10. Presumptive *Btk* T1B2 Spores Recovered and Associated Spore Recovery (%) from Laboratory-Spiked Vacuum Filter Cassettes that had Previously Sampled Different Maritime Surfaces.

Surface Type (Sample ID)	Sample Replicates	Spores Available for Analysis (CFU)		Spore Recovery (CFU) (X ± σ)[c]	Spore Recovery (%) (X ± σ)[d]
		Nominal[a]	Determined[b] (X ± σ)		
Nonskid Tread (NSKID-1)	4	0	0	140 ± 94	N/A
	4	150	110 ± 72	180 ± 56[e]	200 ± 92
	4	1,500	1,100 ± 720	330 ± 190	47 ± 52
Nonskid Tread (NSKID-2)	4	0	0	11 ± 19	N/A
	4	150	110 ± 72	46 ± 42	71 ± 97
	4	1,500	1,100 ± 720	150 ± 70	16 ± 4.7
Concrete Pier (CONPIER-1)	4	0	0	60 ± 39	N/A
	4	150	110 ± 72	110 ± 110	100 ± 88
	4	1,500	1,100 ± 720	220 ± 120	22 ± 8.1
Concrete Pier (CONPIER-2)	4	0	0	75 ± 62	N/A
	4	150	110 ± 72	54 ± 31	62 ± 47
	4	1,500	1,100 ± 720	120 ± 76	11 ± 3.2

[a] Nominally one-half of the target spore load on the surface and assuming 100% recovery of spores.

[b] Based on the spiking suspension titer measured per trial, 100 % recovery, and one-half of extract used for culture analysis.

[c] Presumptive *Btk* T1B2 colonies based on morphology, and one-half of extract used for culture analysis.

[d] Calculated using the actual spore loading applied during spiking and total presumptive *Btk* T1B2 spores recovered on each VFC sample.

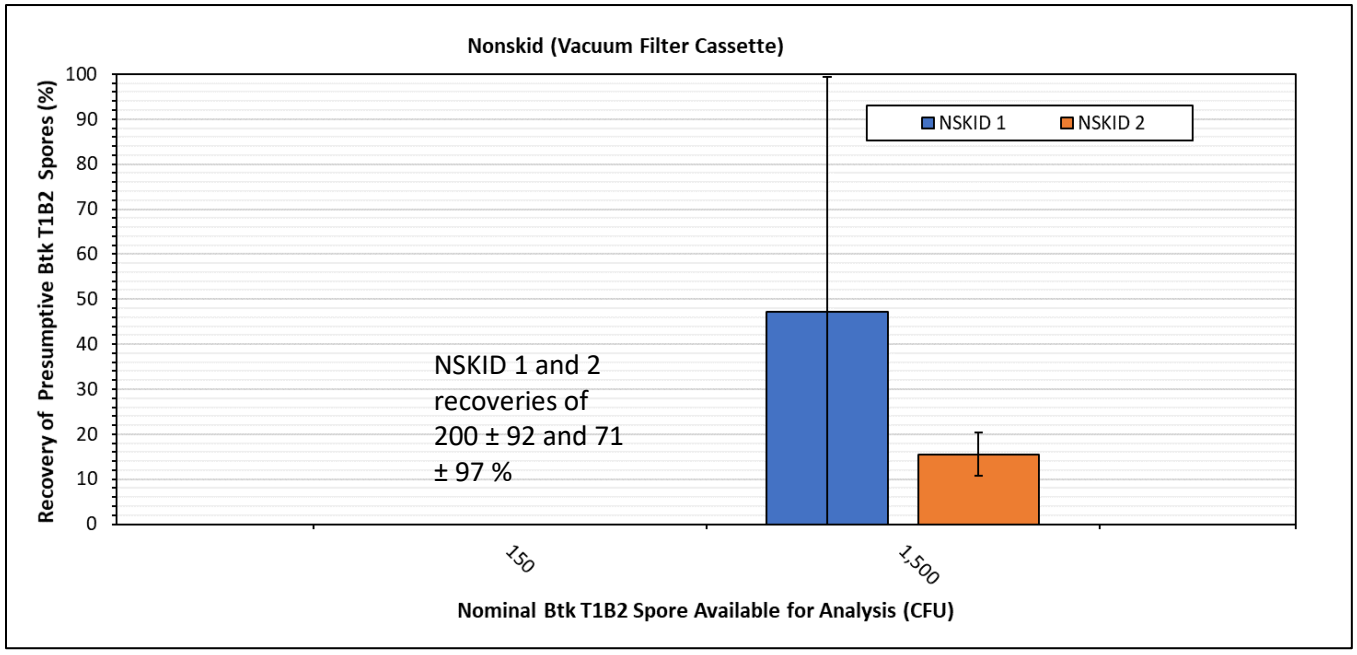

Figure 43. Presumptive *Btk* T1B2 Spore Recovery (%) from VFCs Spiked with *Btk* T1B2 Spores After Having Sampled Nonskid Tread Surfaces.

Average ± One Standard Deviation of N = 4 Replicates. VFCs Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores.

For nonskid 1 and 2, 150 CFU nominal spore load percent recovery values were not plotted in Figure 43 because percent recovery of 200 ± 92 % and 71 ± 97 % is not an accurate reflection of target spores recovered. The percent recovery values, particularly for 150 CFU nominal spore level samples are inflated from the presence of background microorganisms with *Btk* morphology.

For Concrete Pier 1, 150 CFU nominal spore load percent recovery values were not plotted in Figure 44 because percent recovery of 100 ± 88 % is not an accurate reflection of target spores recovered. The percent recovery values, particularly for 150 CFU nominal spore level samples, are inflated from the presence of background microorganisms with *Btk* morphology.

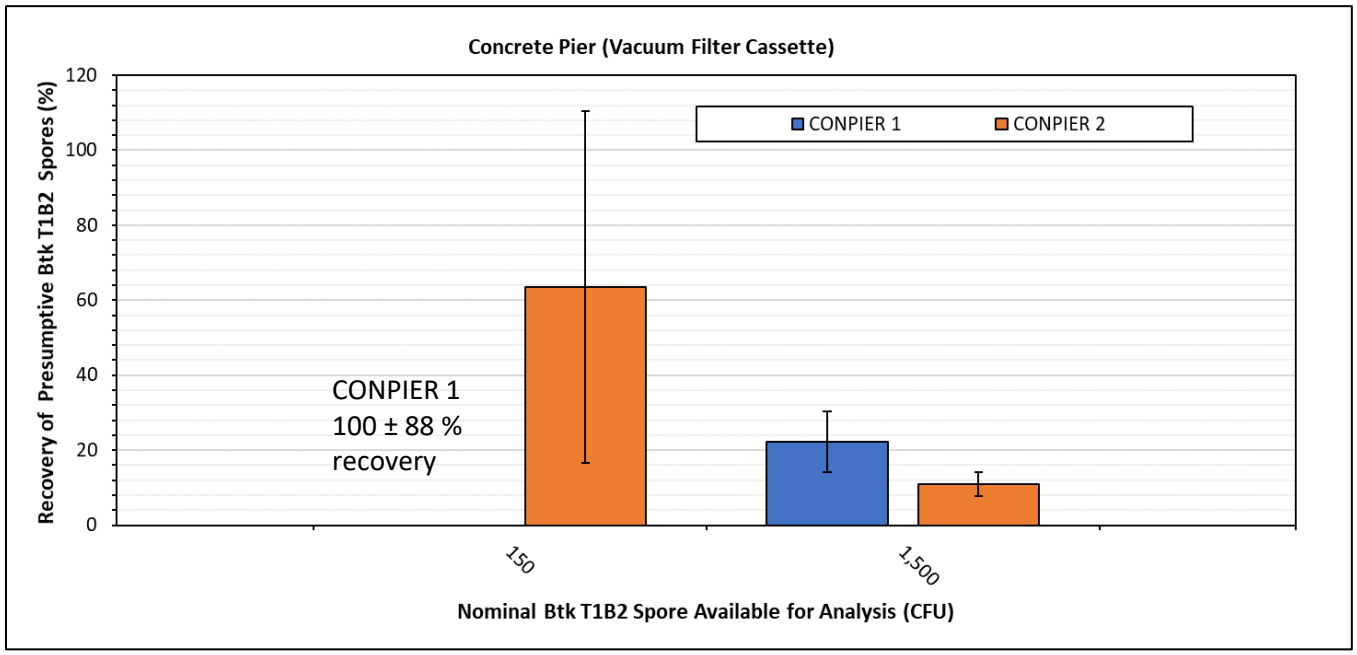

Figure 44. Presumptive *Btk* T1B2 Spore Recovery (%) from VFCs Spiked with *Btk* T1B2 Spores After Having Sampled Concrete Pier Surfaces.

Average ± One Standard Deviation of N = 4 Replicates. VFCs Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores.

The images in Figure 45 and Figure 46 show examples of culture plates with 1 mL or 3 mL volumes on TSA at all three spike levels: 0 CFU spike, 300 CFU spike, and 3,000 CFU spike. Presumptive colonies were present on zero spike samples, although the number of presumptive colonies increased as spike level increased.

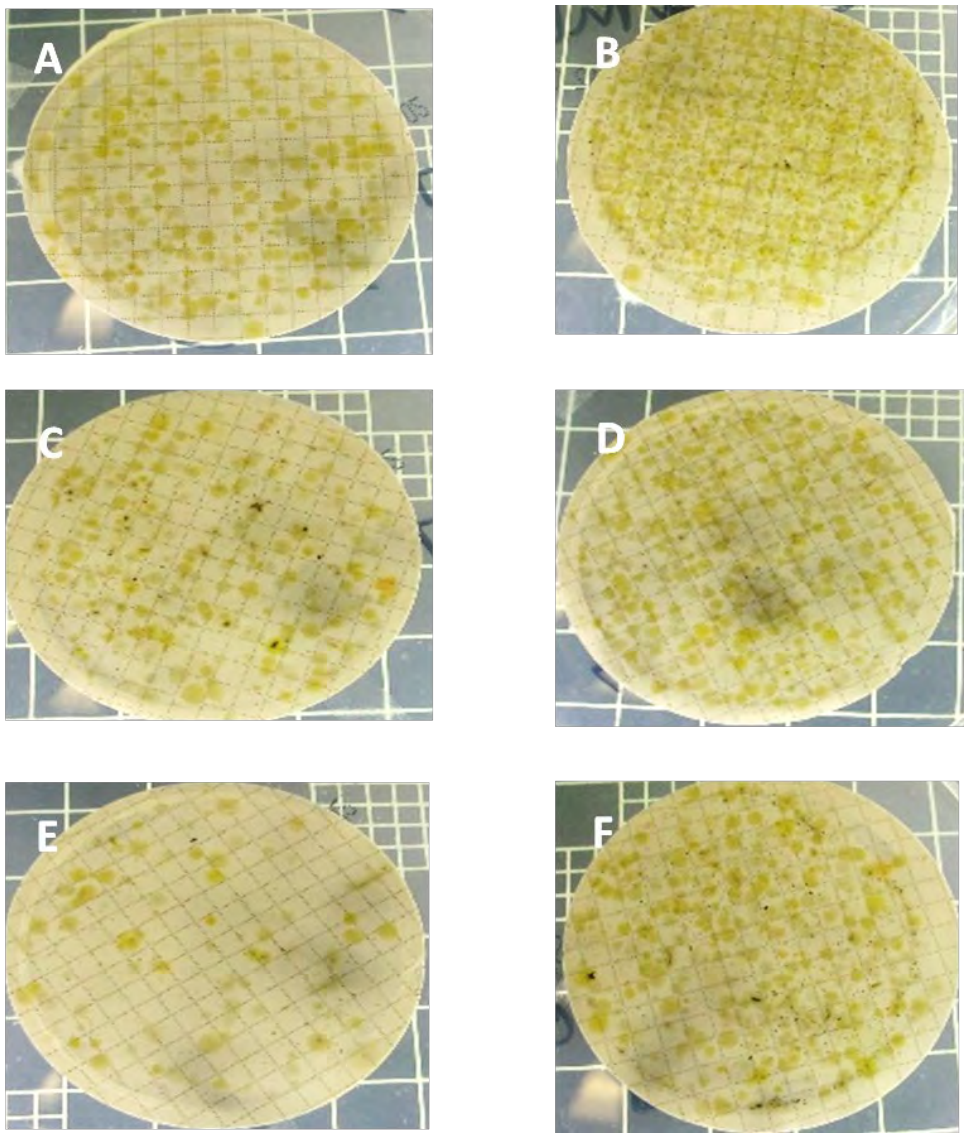

Figure 45. Culture Images of Spore Recovery from Nonskid 1 Surface Sampled Using Vacuum Filter Cassette and Plated on TSA.

Presumptive Btk colonies were observed in zero spore spike samples (Average ~55 CFU/mL when plating 1 mL volume for Boat 1, < 3 CFU/mL when plating 1 mL volume for Boat 2).
Image Descriptions: (A) Zero Spore Spike, 1 mL; (B) Zero Spore Spike, 3 mL; (C) 300 Spore Spike, 1 mL; (D) 300 Spore Spike, 3 mL; (E) 3,000 Spore Spike, 1 mL; (F) 3,000 Spore Spike, 3 mL

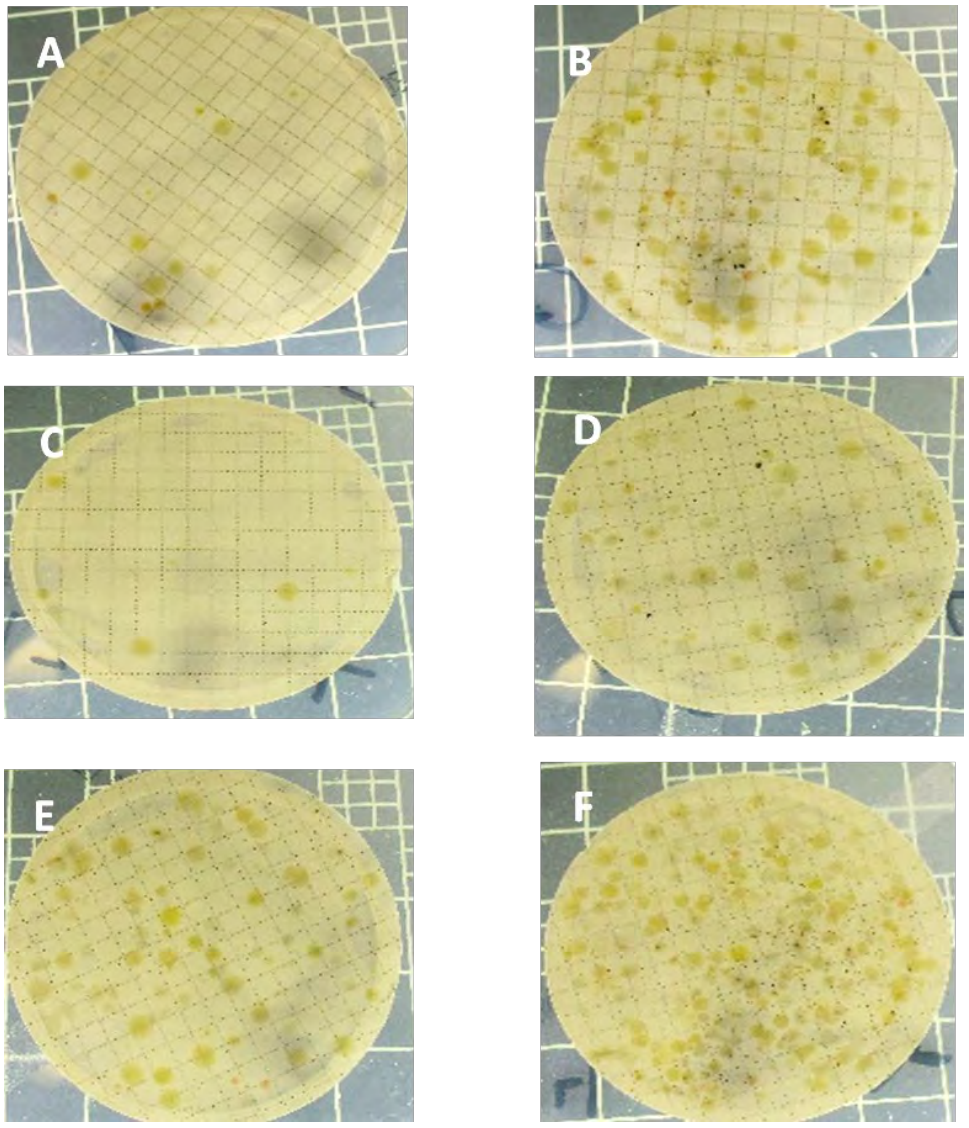

Figure 46. Culture Images of Spore Recovery from Concrete Pier 1 Surface Sampled Using Vacuum Filter Cassette and Plated on TSA.

Presumptive Btk colonies were observed in zero spore spike samples (Average ~10 CFU/mL when plating 1 mL volume for Pier 1, ~13 CFU/mL when plating 1 mL volume for Pier 2).
Image Descriptions: (A) Zero Spore Spike, 1 mL; (B) Zero Spore Spike, 3 mL; (C) 300 Spore Spike, 1 mL; (D) 300 Spore Spike, 3 mL; (E) 3,000 Spore Spike, 1 mL; (F) 3,000 Spore Spike, 3 mL

The VFCs were not grossly loaded with particulates when viewed prior to spore recovery, as shown in Figure 47.

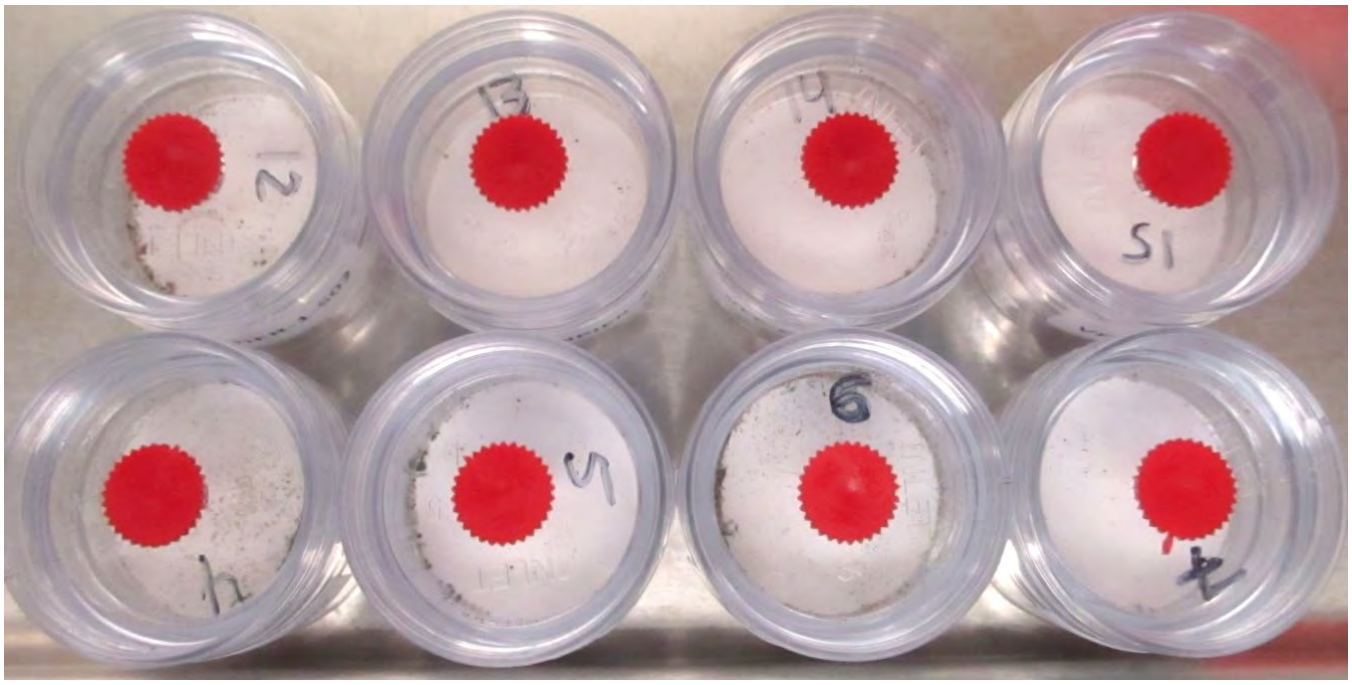

Figure 47. Vacuum Filter Cassette Following Sampling of Surfaces.

Image Descriptions: Top Row: VFCs collected from concrete pier; Bottom Row: VFCs collected from nonskid tread.

3.2.2 Colony Confirmation by PCR

Based on the colony morphology, presumptive *Btk* T1B2 colonies were identified for all surfaces (46 of 48 samples) sampled using VFCs except for two replicates sampled from nonskid tread on Boat 2. The presence of presumptive *Btk* T1B2 morphology did not mean that *Btk* T1B2 spores were recovered from the surfaces. Sixteen (16) of these samples with inert and biological deposits were 0-spike samples, meaning they were not inoculated with *Btk* T1B2 spores, yet presumptive *Btk* colonies were still isolated from the spore recovery. Colonies with morphology indistinguishable from *Btk* T1B2 were present on the culture plates, as indicated by a negative PCR result for presumptive *Btk* T1B2 colonies. The confirmation of target *Btk* T1B2 was assessed by colony PCR from the initial culture plates, colony PCR from BHIB enrichment of the vacuum membrane, or PCR of an aliquot of the BHIB enrichment culture from the vacuum membrane sample. Results from PCR confirmatory testing are shown in Table 11. There was a total of five sample replicates that were false positive samples; however, the colony PCR Ct values were 32, 34 and 37. By comparison, the 11 spiked field blank or laboratory spike samples had a Ct value of 21.2 ± 1.4. The other false positive samples had Ct values of 38.3 (laboratory blank) and 40 for BHIB enrichment culture PCR. Ct values between 32 and 37 for colony PCR and Ct values between 38 and 40 for BHIB enrichment culture PCR may have been caused by low level contamination or potentially nonspecific PCR amplification.

Table 11. Summary of the Accuracy of Identification of Presumptive *Btk* T1B2 Colonies by PCR Confirmation from Spiked VFCs Used to Sample Different Maritime Surfaces.

Surface Type (Sample ID)	Nominal Spore Load (CFU)	Culture Replicates Presumptive Positive[a]	Colonies from Initial Culture Plates PCR-Screened (# PCR +) [b]	Colony PCR Ct (X ± σ) [c]	Colonies from BHIB Streak Plates PCR-Screened (# PCR +) [d]	BHIB PCR-Screened (# PCR +) [e]	BHIB PCR Ct (X ± σ) [f]
Nonskid Tread (NSKID-1)	0	4 of 4	31 (0)	N/A	3 (0)	4 (1)	40.0
	150	4 of 4	31 (0)	N/A	3 (0)	4 (1)	37.6
	1,500	4 of 4	31 (3 of 4)	25.6 ± 2.6	0	1 (1)	31.1
Nonskid Tread (NSKID-2)	0	2 of 4	11 (0)	N/A	1 (0)	3 (0)	N/A
	150	4 of 4	25 (2 of 4)	24.3 ± 1.2	1 (0)	2 (2)	34.7 ± 0.2
	1,500	4 of 4	31 (4 of 4)	23.0 ± 1.9	0	0	N/A
Concrete Pier (CONPIER-1)	0	4 of 4	32 (2 of 4)	33.6 ± 1.4	3 (0)	3 (0)	N/A
	150	4 of 4	25 (2 of 4)	24.2 ± 0.8	1 (0)	1 (1)	34.3
	1,500	4 of 4	31 (3 of 4)	25.2 ± 2.3	1 (1)	1 (1)	23.3
Concrete Pier (CONPIER-2)	0	4 of 4	31 (1 of 4)	37.3	2 (0)	3 (0)	N/A
	150	4 of 4	31 (3 of 4)	24.5 ± 2.0	0	1 (1)	31.7
	1,500	4 of 4	31 (4 of 4)	22.5 ± 1.2	0	0	N/A

[a] Presumptive *Btk* T1B2 was present on initial culture plates.

[b] Number of colonies PCR-screened from initial plating, with number of PCR positive replicates in parentheses.

[c] Colony PCR Ct values for positive samples (Ct value of ≤ 40).

[d] Number of colonies PCR-screened from BHIB streak plates, with number of PCR positive replicates in parentheses.

[e] Number of samples with PCR screening of BHIB enrichment culture, with number of PCR positive replicates in parentheses.

[f] BHIB enrichment culture PCR Ct values for positive samples (Ct value of ≤ 40).

3.2.3 Vacuum Filter Cassette Sample RV-PCR Analysis

A summary of the average and standard deviation values of the RV-PCR ΔCt values for the detection of *Btk* T1B2 spores recovered from VFCs that were used to sample maritime surfaces and then spiked (inoculated) with *Btk* T1B2 spores in the laboratory with a target of 300 or 3,000 CFU are presented in Table 12. The nominal quantity of spores available for analysis represents one-half the target spore load applied to the surfaces and the determined number of spores available represents one-half the measured number of *Btk* T1B2 spores applied based on the *Btk* suspension titer and volume applied on the day of spiking. Sample replicates with a RV-PCR ΔCt value ≥ 9 are RV-PCR positive, indicating that viable *Btk* T1B2 spores were recovered. The plots depict an area shaded in red that is the region of a negative detection result and an area of green that is a positive detection result, delineated by the *Btk* T1B2 barcode target ΔCt value ≥ 9. The RV-PCR ΔCt results are plotted in Figure 48 and Figure 49. The plots all depict the ΔCt threshold value of 9 as a dashed line.

Table 12. RV-PCR Analyses of Spiked Vacuum Filter Cassette that Were Used to Sample Different Maritime Surfaces for Detection of *Btk* T1B2 Spores.

Surface Type (Sample ID)	Number of Replicates	Spores Available for Analysis (CFU)		ΔCt[(c)] (X ± σ)	RV-PCR Replicates Positive[(d)]
		Nominal[(a)]	Determined[(b)]		
Nonskid Tread (NSKID-1)	4	0	0	1.0 ± 1.2	0
	4	150	110 ± 72	4.3 ± 4.3	1
	4	1,500	1,100 ± 720	10.5 ± 2.0	3
Nonskid Tread (NSKID-2)	4	0	0	0.0 ± 0.0	0
	4	150	110 ± 72	12.5 ± 1.3	4
	4	1,500	1,100 ± 720	16.7 ± 3.9	4
Concrete Pier (CONPIER-1)	4	0	0	0.0 ± 0.0	0
	4	150	110 ± 72	7.4 ± 8.4	2
	4	1,500	1,100 ± 720	15.9 ± 5.0	3
Concrete Pier (CONPIER-2)	4	0	0	0.0 ± 0.0	0
	4	150	110 ± 72	15.0 ± 4.4	4
	4	1,500	1,100 ± 720	16.6 ± 4.8	4

[(a)] Nominally one-half of the target spore load on the surface and assuming 100% recovery of spores.

[(b)] Based on the spiking titer measured each test trial, 100% recovery efficiency, and one-half of extraction used for RV-PCR analysis.

[(c)] PCR assay for T1B2 Barcode Gene Target.

[(d)] Number of replicates with a RV-PCR ΔCt value ≥ 9.

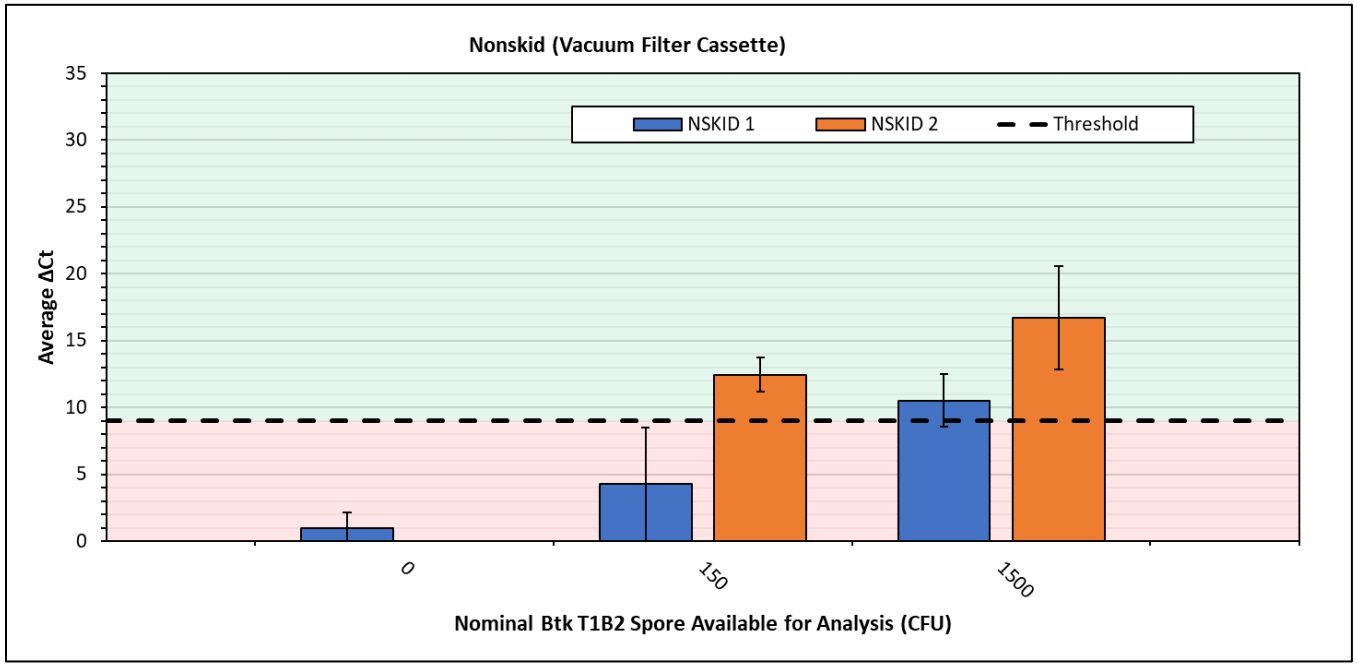

Figure 48. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from VFCs Spiked with *Btk* T1B2 Spores After Having Sampled Nonskid Tread Surfaces.

Average ± One Standard Deviation of N = 4 Replicates. VFCs Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores. Positive Result Equals ΔCt ≥ 9.

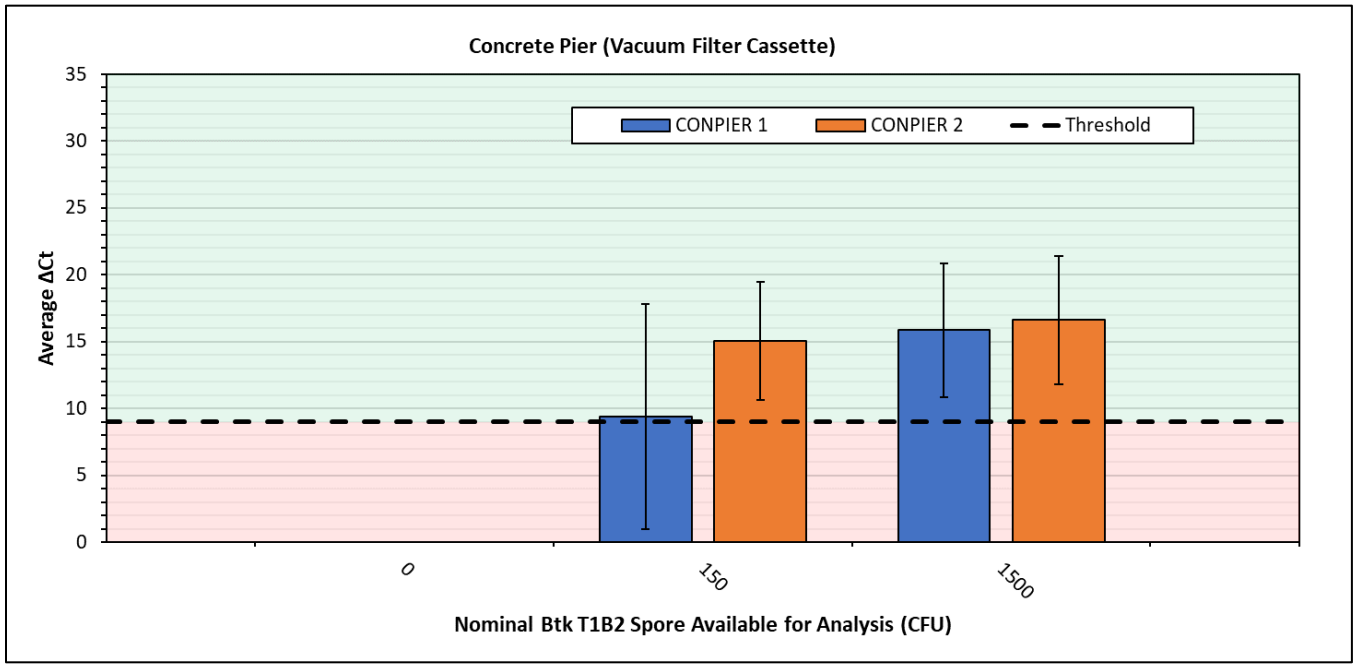

Figure 49. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from VFCs Spiked with *Btk* T1B2 Spores After Having Sampled Nonskid Tread Surfaces.

Average ± One Standard Deviation of N = 4 Replicates. VFCs Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores. Positive Result Equals ΔCt ≥ 9.

3.2.4 Analytical Method Comparison of VFC Samples

Culture analysis identified presumptive *Btk* T1B2 colonies for all samples, except for two replicates sampled from nonskid tread on Boat 2, indicating that background microbial flora included wild-type *Btk* or another organism that had a morphology indistinguishable from *Btk* T1B2 on TSA plates. Colony PCR from initial culture plates, the colony PCR of colonies isolated from BHIB enrichment culture of the VFC samples, and/or PCR analysis of the BHIB enrichment culture was therefore required to confirm or refute the presence of *Btk* T1B2. To compare the two methods, culture with PCR confirmation and RV-PCR results were assessed to determine which method may be more likely to detect viable spores that have been spiked onto VFCs that contain outdoor interferents.

PCR screening of presumptive *Btk* T1B2 colonies was negative in some cases, indicating that background microbial flora with colony morphology indistinguishable from the morphology of *Btk* T1B2 were present on TSA culture plates; and hence, present in the samples collected in the field. It is possible that wild-type/naturally occurring *Btk* and their presence led to an inflation in presumptive spore recovery values by the culture method. Presumptive culture identification by colony morphology, colony identification confirmed by PCR, and RV-PCR results are shown in Table 13.

For culture analysis of VFC samples, 40 of 48 (83%) that had been used to collect inert and biological deposits were true positives or true negatives. A true positive is defined as a sample spiked with *Btk* T1B2 spores that was confirmed positive by PCR. A true negative is defined as a sample that was not spiked with *Btk* T1B2 spores and was negative for PCR confirmatory screening. Four (4) samples were false positive and four samples were false negative using the culture method. For the four false positive samples, colony PCR Ct values were ≤ 40, each measuring between 32 to 37, or a BHIB culture PCR Ct value of 40. For comparison, colony PCR Ct values of *Btk* T1B2 isolated from field blanks or laboratory blanks (new VFC spiked with *Btk* T1B2 spores) was 21.2 ± 1.4. Twenty-two (22) of 23 field blank and laboratory blank controls were true negatives, with one false positive with a BHIB culture Ct value of 38.

For RV-PCR, 41 of 48 samples (87%) that had been used to collect inert and biological deposits were determined as true positives or true negatives. A true positive is defined as a sample spiked with *Btk* T1B2 spores that had a ΔCt of ≥ 9. A true negative is defined as a sample that was not spiked with *Btk* T1B2 spores and had a ΔCt of < 9. All seven nontrue sample results were false negative samples; five were spiked with a 300-CFU target spore load and two were spiked with a 3,000-CFU target spore load. ΔCt values for the false negative samples ranged from 0 to 8.8.

Table 13. Analytical Method Comparison Displaying Culture Presumptive, Culture ID with PCR Confirmation, and RV-PCR Replicates Positively Identified (N = 4) for Surfaces Sampled with Vacuum Cassettes.

Surface Type	Actual Spike Level (CFU)	Presumptive Culture Result	Culture PCR Confirmation	Culture % Recovery	RV-PCR Result	RV-PCR ΔCt
Nonskid, Boat 1	0	Positive	Positive	N/A	Negative	1.9
	0	Positive	Negative	N/A	Negative	2.1
	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	410	Positive	Negative	126.1	Negative	1.5
	120	Positive	Negative	275	Negative	6.6
	250	Positive	Positive	114.4	Positive	9.1
	97[(a)]	Positive	Negative	348.1	Negative	0
	4,100	Positive	Positive	11.5	Positive	10.4
	1,200	Positive	Positive	29.3	Positive	12.8
	2,500	Positive	Positive	23.3	Positive	10.8
	970[(a)]	Positive	Positive	153.2	Negative	8
Nonskid, Boat 2	0	Positive	Negative	N/A	Negative	0
	0	Negative	Negative	N/A	Negative	0
	0	Negative	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	410	Positive	Positive	10.2	Positive	11.4
	120	Positive	Positive	18.3	Positive	13.8
	250	Positive	Positive	39.6	Positive	11.3
	97[(a)]	Positive	Positive	264.6	Positive	13.3
	4,100	Positive	Positive	8.6	Positive	19.6
	1,200	Positive	Positive	16.5	Positive	19.6
	2,500	Positive	Positive	18.9	Positive	16.2
	970[(a)]	Positive	Positive	22.3	Positive	11.4
Concrete Pier 1	0	Positive	Negative	N/A	Negative	0
	0	Positive	Positive	N/A	Negative	0
	0	Positive	Positive	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	410	Positive	Positive	126.1	Negative	7.3
	120	Positive	Positive	24.4	Positive	20.4
	250	Positive	Positive	48.4	Negative	0
	97[(a)]	Positive	Negative	269.2	Positive	10
	4,100	Positive	Positive	12.3	Positive	18.6
	1,200	Positive	Positive	19.3	Positive	20.1
	2,500	Positive	Positive	30.4	Negative	8.8
	970[(a)]	Positive	Positive	33.4	Positive	15.9

[(a)] Stock enumeration plate outside 25 – 250 CFU range.

Table 13. Analytical Method Comparison Displaying Culture Presumptive, Culture ID with PCR Confirmation, and RV-PCR Replicates Positively Identified (N = 4) for Surfaces Sampled with Vacuum Cassettes (Cont.)

Surface Type	Actual Spike Level (CFU)	Presumptive Culture Result	Culture PCR Confirmation	Culture % Recovery	RV-PCR Result	RV-PCR ΔCt
Concrete Pier 2	0	Positive	Negative	N/A	Negative	0
	0	Positive	Positive	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	410	Positive	Positive	20.6	Positive	11.6
	120	Positive	Positive	33.6	Positive	21.5
	250	Positive	Positive	74.8	Positive	13.4
	97[(a)]	Positive	Positive	153.2	Positive	13.7
	4,100	Positive	Positive	10.7	Positive	14.3
	1,200	Positive	Positive	7.9	Positive	23.4
	2,500	Positive	Positive	9.8	Positive	16.2
	970[(a)]	Positive	Positive	19	Positive	12.6

[(a)] Stock enumeration plate outside 25 – 250 CFU range.

Results for all samples processed, including positive and negative controls, are shown in Table 14. The culture method correctly determined 62 of 71 samples (87%) with five false positives; however, all five of these samples had colony PCR Ct values between 32 to 37, or a Ct of 38 to 40 for BHIB PCR. Colony PCR of *Btk* T1B2 from the 11 spiked field blank or laboratory spike (new vacuum cassette spiked with *Btk* T1B2 spores) controls had a Ct value of 21.2 ± 1.4, suggesting that Ct values between 32 and 37 might be caused by low level contamination or potentially nonspecific PCR amplification. The culture method also had four false negative replicates. One of the four false negative sample replicates had only one presumptive *Btk* colony screened of 47 that were available on the 1-mL MicroFunnel membrane plate. The EPA Protocol specifies that one to three colonies from MicroFunnel filters and a minimum of three colonies from spread plates should be PCR-screened for target confirmation (EPA, 2017). For the other four false negative replicates, 8 to 10 colonies were PCR-screened in addition to PCR screening of BHIB enrichment culture PCR; therefore, if additional colony PCR screenings were performed, the false negative results may not have changed.

The RV-PCR method correctly determined 64 of 71 samples (90%) with zero false positive samples and seven false negative samples, five of which were at the 300-target spore load.

For culture analysis, nonskid tread surfaces sampled with VFCs had one false positive sample that had a Ct value of 40 when PCR analysis was performed on a BHIB enrichment culture of the processed VFC sample, and the colony PCR was negative. There were three false negative samples at the 300 CFU spike level with only the fourth replicate confirmed positive, although the BHIB culture PCR Ct was high (37.6). RV-PCR results had zero false positives and three false negatives at the 300 CFU level and one false negative at the 3,000 CFU level.

For culture analysis, concrete pier surfaces sampled with VFCs had three false positive samples with Ct values of 32, 34, and 37 for colony PCR. For all 11 spiked field blanks or laboratory spikes (new vacuum cassette spike with *Btk* T1B2 spores), controls resulted in a Ct value of 21.2 ± 1.4. Therefore, a Ct value between 32 and 37 may have been caused by low level contamination or potentially nonspecific

PCR amplification. Additionally, analysis of BHIB enrichment culture PCR of the processed vacuum membrane was negative for all three of these false positive samples. There was one false negative sample at the 300 CFU spike level for culture analysis. RV-PCR results had two false negative samples at the 300 CFU level and one at the 3,000 CFU level.

Table 14. Analytical Method Comparison Displaying Culture ID with PCR Confirmation and RV-PCR for Surfaces Sampled with Vacuum Filter Cassettes.

Surface Type	Culture Method			RV-PCR Method		
	True Positive or True Negative	False Positive Sample	False Negative Sample	True Positive or True Negative	False Positive Sample	False Negative Sample
Nonskid 1	8	1	3[a]	8	0	4
Nonskid 2	12	0	0	12	0	0
Concrete Pier 1	9	2	1[b]	9	0	3
Concrete Pier 2	11	1	0	12	0	0
Controls	22	1	0	23	0	0
Totals	**62**	**5**	**4**	**64**	**0**	**7**

Positive PCR threshold of 40 for colony PCR and BHIB enrichment culture PCR. The definition for Positive Ct threshold per the EPA Protocol (EPA, 2017) is ≤ 40.

[a] One replicate spiked with 97 CFU, which is the low end of the acceptance range for a sample spiked with *Btk* T1B2 spores.

[b] One replicate spiked with 97 CFU, which is the low end of the acceptance range for a sample spiked with *Btk* T1B2 spores.

3.2.5 Analysis of Controls

For VFCs, there were a total of seven zero spike VFCs that served as reagent control laboratory blanks, four zero spike VFCs that were opened in the field that served as field blanks, four (4) 300-CFU spiked field blanks, four (4) 3,000-CFU spiked field blanks, two (2) 300-CFU spiked VFCs, and two (2) 3,000-CFU spiked VFCs that were handled in laboratory only (laboratory spike).

All controls performed as expected except for one zero spike vacuum cassette that was culture positive with a BHIB culture PCR Ct value of 38. For all 11 of the spiked field blank or laboratory spike (new VFC spiked with *Btk* T1B2) samples on which colony PCR was performed, the average Ct value was 21.2 ± 1.4.

3.2.6 Considerations for Culture Analysis False Positive Results for Sponge Sticks and VFCs

Regarding false positive results for colony PCR and BHIB enrichment PCR, it is important to establish limit of detection thresholds for distinguishing a positive PCR response from a negative PCR response. These thresholds will vary depending on the PCR assay used for detection and should be established experimentally, although a Ct value threshold of 40 is the norm for any PCR assay used for biothreat detection and was therefore used for this study. For culture PCR analysis, a Ct value threshold of ≤ 40 was used to establish positive results for colony PCR and BHIB enrichment PCR. Comparatively, for RV-PCR, a ΔCt value threshold of ≥ 9 was used to establish positive results. For example, if the T_0 aliquot was undetected, a value of 45 (total number of PCR cycles run) was assigned; and if the T_f aliquot resulted in a Ct value of ≤ 36, the sample was positive for RV-PCR ($T_0 - T_f = \Delta Ct$). A baseline equivalent to the T_0 used for RV-PCR is not included in BHIB enrichment PCR analysis, and its absence may have led to more false positive results for culture method compared to RV-PCR method.

3.3 Grab Sample Analysis Results

For grab samples, 500 mL of PBST was added to sample. However, particulates such as soil and other debris limit filtration onto MicroFunnel filter membranes (0.45 µm), slowing processing speed and reducing the total volume that can be analyzed, were present as shown in Table 15.

Table 15. Volume of Recovered Suspension Concentrated onto MicroFunnel Filter.

Grab Sample Type	PBST Volume (mL)	Volume of Suspension Processed through MicroFunnel Filter, 0.45 µm (X ± σ)
SBWASH 1 and 2	500	500 ± 0
Gravel	500	250 ± 0
Soil	40	N/A
Vegetation	500	131.5 ± 42.1

The water content of the soil was 26.18 ± 0.01% with a pH of 6.615 ± 0.002, and the sterilized soil water content was 0.97 ± 0.00%, with a pH of 5.621 ± 0.002.

3.3.1 Grab Sample Culture Analysis

A summary of the average and standard deviation values of the measured recovery of presumptive *Btk* T1B2 spores from spiked wash water, gravel, soil, and vegetation (grass) samples is presented in Table 16. The nominal quantity represents one-half the target spore load applied to the grab sample, and the determined number of spores available represents one-half the number of presumptive *Btk* T1B2 spores spiked, assuming the full sample volume was processed.

The spore recovery percentage of presumptive *Btk* colonies recovered as determined by culture analysis using TSA plates is plotted in Figure 50 through Figure 53.

The percent recovery of presumptive *Btk* T1B2 spores for grab samples was highly variable and unreliable with percent recoveries > 100% for all surfaces analyzed with less than 15,000 spore load with few exceptions (gravel with 1,500 CFU, and soil samples) due to the presence of high background microbial growth.

Table 16. Presumptive *Btk* T1B2 Spores Recovered and Associated Spore Recovery (%) from Laboratory-Spiked Grab Samples.

Grab Sample Type	Sample Replicates	Spores Available for Analysis (CFU)		Spore Recovery (CFU) (X ± σ)[c]	Spore Recovery (%) (X ± σ)[d]
		Nominal[a]	Determined[b] (X ± σ)		
Wash Water-1	3	0	0	850 ± 490	N/A
	3	150	90 ± 0	1,200 ± 330	1,400 ± 370
	3	1,500	900 ± 0	1,300 ± 670	150 ± 74
	3	15,000	30,000 ± 0	4,700 ± 770	16 ± 2.6
Wash Water-2	0	N/A	N/A	N/A	N/A
	3	15,000	30,000 ± 0	7,100 ± 420	24 ± 1.4
Gravel	3	0	0	1,400 ± 1,900	N/A
	3	150	180 ± 0	370 ± 270	200 ± 150
	3	1,500	1,800 ± 0	1,600 ± 910	88 ± 51
Soil	3	0	0	760 ± 340	N/A
	3	1,500	2,200 ± 0	840 ± 100	38 ± 4.6
	3	15,000	22,000 ± 0	1,800 ± 350	8.2 ± 1.6
	3	150,000	220,000 ± 0	11,000 ± 7,000	5.1 ± 3.2
Sterile Soil	1	0	0	0	0
	1	1,500	2,200 ± 0	930	42
	1	15,000	22,000 ± 0	7,600	35
	1	150,000	220,000 ± 0	46,000	21
Vegetation (Grass)	3	0	0	6,700 ± 4,100	N/A
	3	150	55 ± 0	9,900 ± 2,700	18,000 ± 4,900
	3	1,500	550 ± 0	12,000 ± 4,400	2,200 ± 810
	3	15,000	30,000 ± 0	12,000 ± 2,600	40 ± 8.7

[a] Nominally one-half of the target spore load applied to the grab sample type and assuming 100% recovery of spores.
[b] Based on the spiking suspension titer measured per trial, 100 % recovery, and one-half of extract used for culture analysis.
[c] Presumptive *Btk* T1B2 colonies based on morphology and one-half of suspension used for culture analysis.
[d] Calculated using the actual spore loading applied during spiking and total presumptive *Btk* T1B2 spores recovered from each sample.

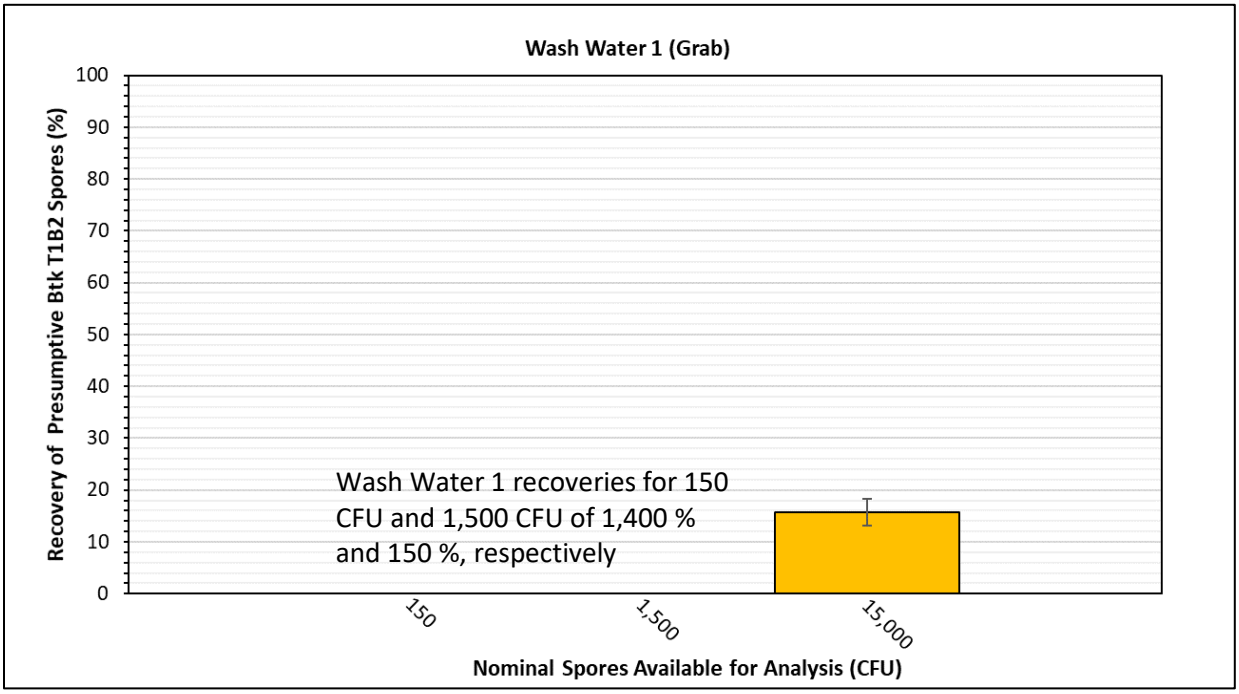

Figure 50. Presumptive *Btk* T1B2 Spore Recovery Percentage (Average ± One Standard Deviation of N = 3 Replicates) from Wash Water Grab Samples Spiked with *Btk* T1B2 Spores.

Wash Water 1 percent recovery values for 150 and 1,500 CFU nominal spore load are not plotted in Figure 50 because percent recovery values of 1,400 ± 370 % and 150 ± 74 % are not an accurate reflection of target spores recovered. The percent recovery values are inflated from the presence of background microorganisms with *Btk* morphology.

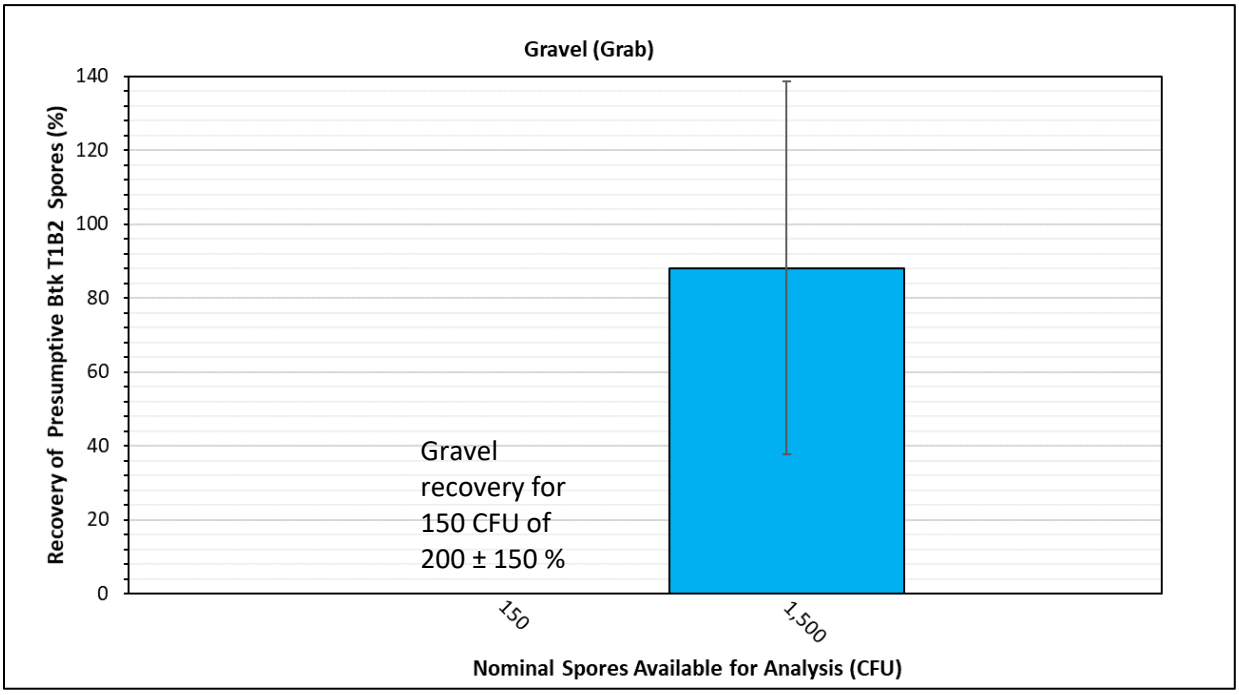

Figure 51. Presumptive *Btk* T1B2 Spore Recovery Percentage (Average ± One Standard Deviation of N = 3 Replicates) from Gravel Grab Samples Spiked with *Btk* T1B2 Spores.

Gravel percent recovery values for 150 CFU nominal spore load are not plotted in Figure 51 because percent recovery of 200 ± 150 % is not an accurate reflection of target spores recovered. The percent recovery values are inflated from the presence of background microorganisms with *Btk* morphology.

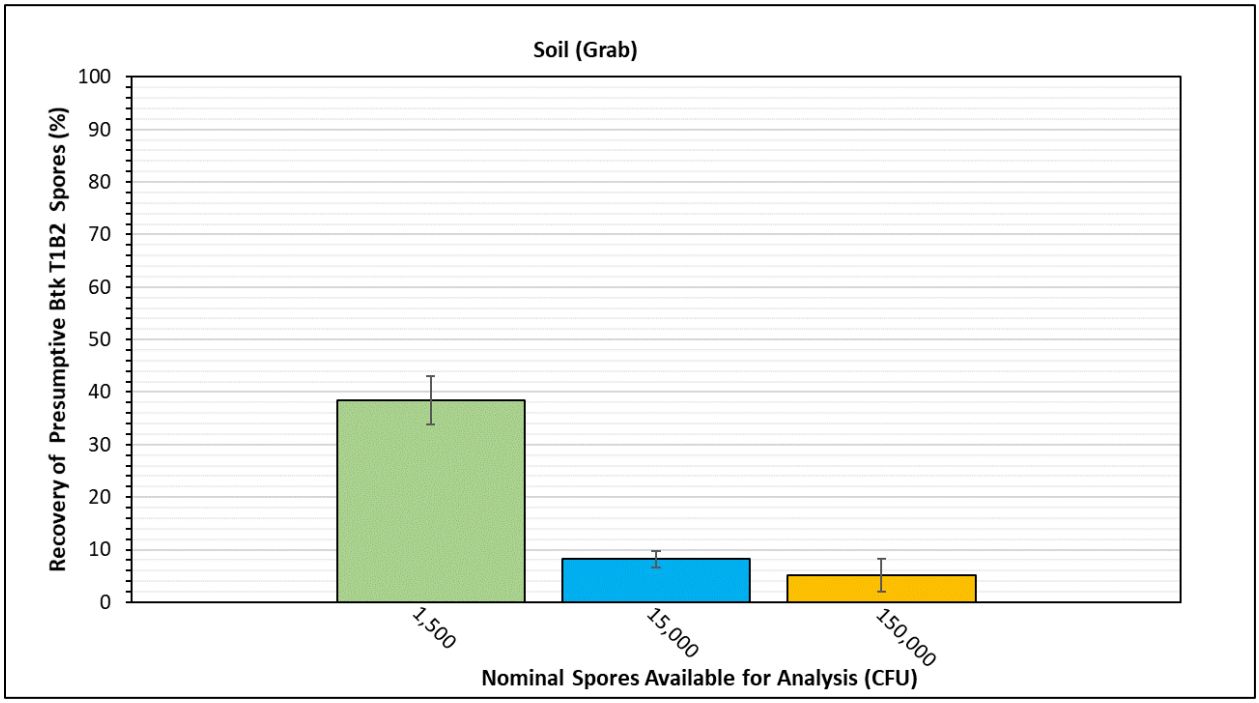

Figure 52. Presumptive *Btk* T1B2 Spore Recovery Percentage (Average ± One Standard Deviation of N = 3 Replicates) from Soil Grab Samples Spiked with *Btk* T1B2 Spores.

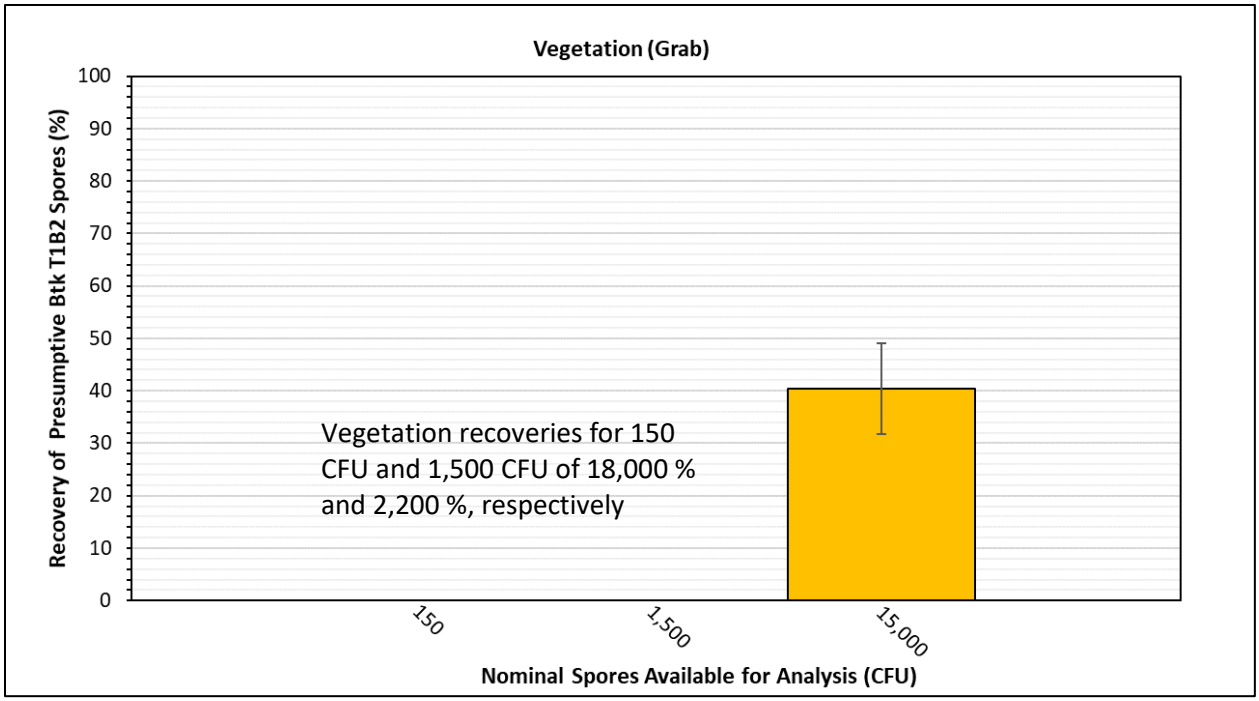

Figure 53. Presumptive *Btk* T1B2 Spore Recovery Percentage (Average ± One Standard Deviation of N = 3 Replicates) from Vegetation Grab Samples Spiked with *Btk* T1B2 Spores.

Vegetation percent recovery values for 150 and 1,500 CFU nominal spore loads are not plotted in Figure 53 because percent recoveries of 18,000 % and 2,200 % are not an accurate reflection of target spores recovered. The percent recovery values are inflated from the presence of background microorganisms with *Btk* morphology.

For grab samples, representative images of the culture plates are shown in Figure 54 through Figure 57. Presumptive *Btk* colonies were isolated from all nonsterile sample types, including the zero spore spike samples, resulting in unreliable spore recovery values.

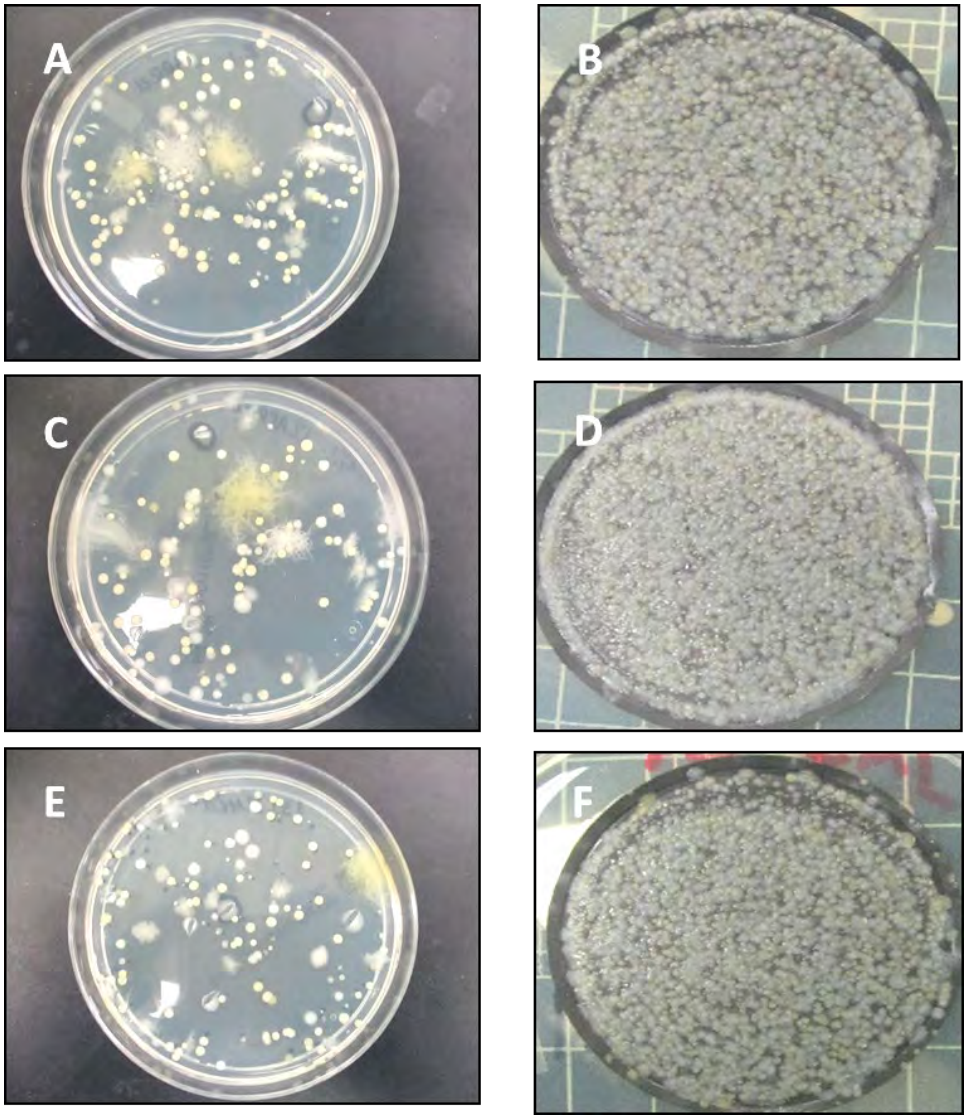

Figure 54. Culture Images of Spore Recovery from Washdown Grab Samples Plated on TSA.

Presumptive Btk colonies were observed in zero spike samples and 2 mL volumes plated were overwhelmed with background growth.
Image Descriptions: (A) Zero Spore Spike, 0.1 mL; (B) Zero Spore Spike, 2 mL; (C) 300 Spore Spike, 0.1 mL; (D) 300 Spore Spike, 2 mL; (E) 3,000 Spore Spike, 0.1 mL; (F) 3,000 Spore Spike, 2 mL

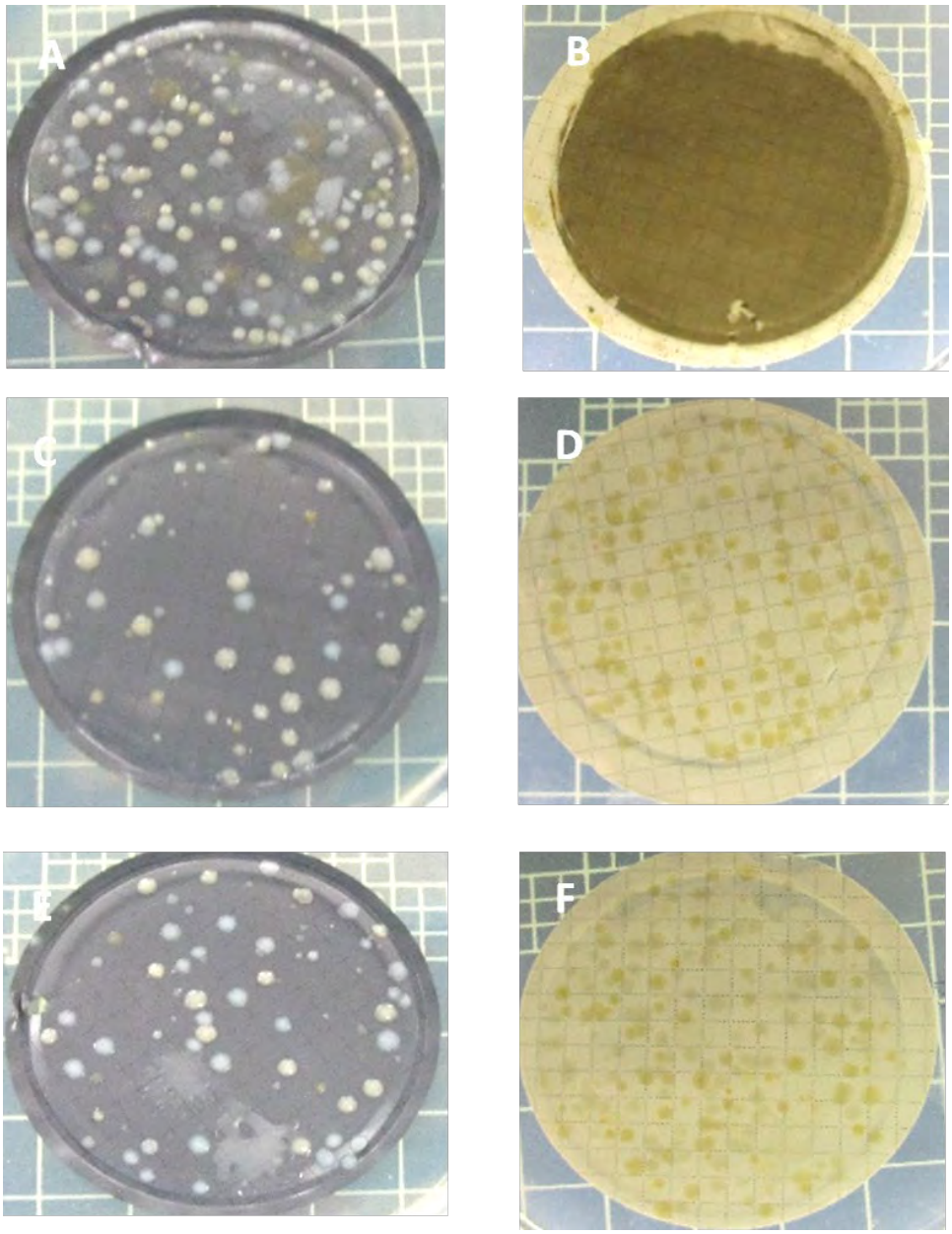

Figure 55. Culture Images of Spore Recovery from Gravel Grab Samples Plated on TSA.

Image Descriptions: (A) Zero Spore Spike, 1 mL; (B) Zero Spore Spike, 4 mL; (C) 300 Spore Spike, 1 mL; (D) 300 Spore Spike, 4 mL; (E) 3,000 Spore Spike, 1 mL; (F) 3,000 Spore Spike, 4 mL

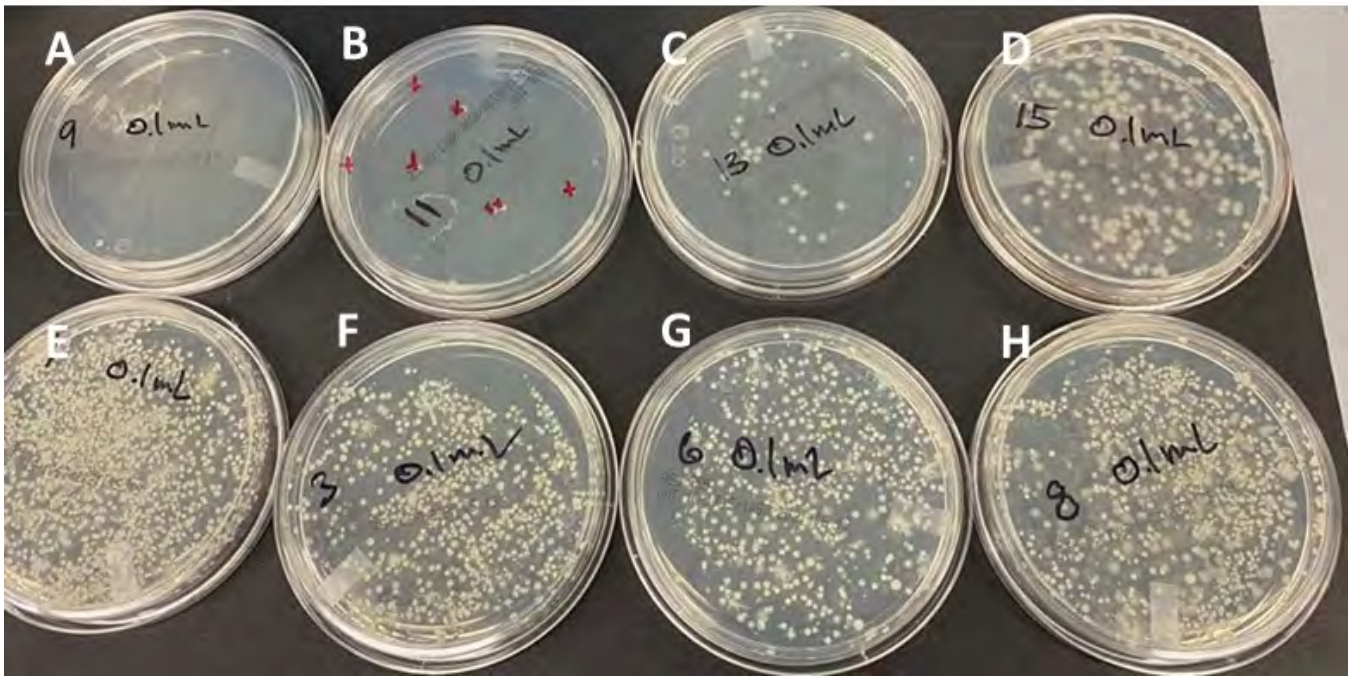

Figure 56. Culture Images of Spore Recovery from Soil Grab Samples Plated on TSA.

Presumptive Btk colonies were observed in zero spike samples. Image Descriptions: (A) Zero Spike Sterile Soil, 0.1 mL; (B) 3,000 Spike Sterile Soil, 0.1 mL; (C) 30,000 Spike Sterile Soil, 0.1 mL; (D) 300,000 Spike Sterile Soil, 0.1 mL; (E) Zero Spike Nonsterile Soil, 0.1 mL; (F) 3,000 Spike Nonsterile Soil, 0.1 mL; (G) 30,000 Spike Nonsterile Soil, 0.1 mL; (H) 300,000 Spike Nonsterile Soil, 0.1 mL.

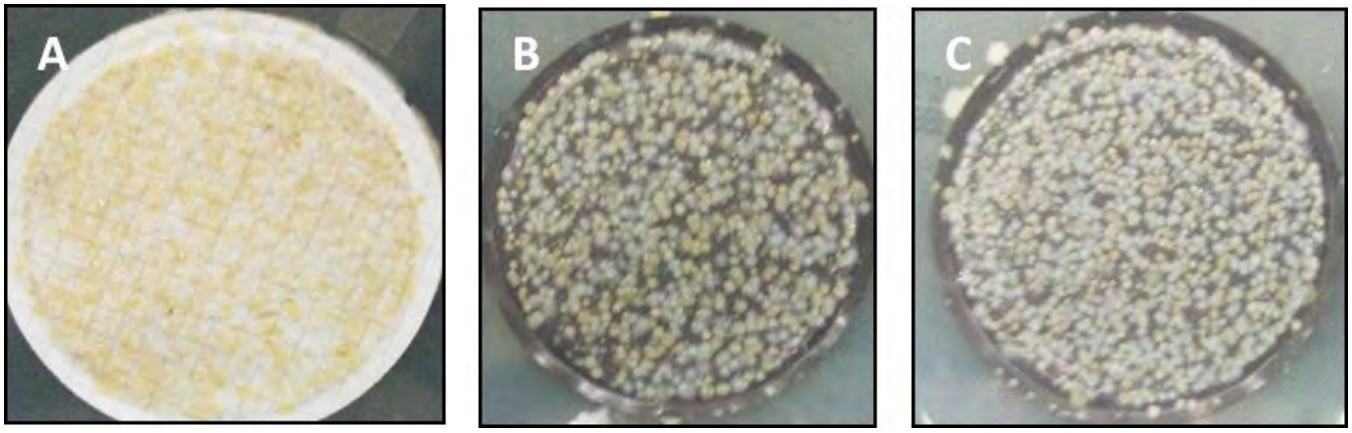

Figure 57. Culture Images of Spore Recovery from Vegetation Grab Samples Plated on TSA.

Presumptive Btk colonies overwhelmed zero spike samples. Image Descriptions: (A) Zero Spike, 2 mL; (B) 300 Spike, 2 mL; (C) 3,000 Spike, 2 mL.

3.3.2 Colony Confirmation by PCR

Based on the colony morphology, presumptive *Btk* T1B2 colonies were identified for all grab samples (48 of 48 samples). Background microbial flora levels were high, even more so than observed for sponge stick and vacuum filter cassette samples due to the larger surface area sampled and subsequent concentration alongside target organism onto the filter membrane. Twelve of these samples with inert and biological deposits were 0-spike samples, meaning they were not inoculated with *Btk* T1B2 spores, yet presumptive *Btk* colonies were still isolated from the spore recovery. Colonies with morphology indistinguishable from the morphology of *Btk* T1B2 were present on the culture plates, as indicated by a negative PCR result for presumptive *Btk* T1B2 colonies. The confirmation of target *Btk* T1B2 was assessed by colony PCR from the initial culture plates, colony PCR from the BHIB enrichment culture, or PCR of an aliquot of the BHIB enrichment culture. Results from PCR confirmatory testing are shown in Table 17.

For all grab samples (wash water, gravel, soil, and vegetation) spiked at the 300 *Btk* T1B2 spore level, only one replicate of the gravel samples was positive by culture analysis as confirmed by colony PCR. At the 3,000 CFU target spore load level, all three wash water sample replicates, two gravel sample replicates, one soil replicate, and one vegetation sample replicate were confirmed positive by colony PCR. Additionally, one 3,000 CFU replicate was confirmed positive by PCR of the BHIB culture enriched soil pellet, although the PCR Ct value was 38.7.

Since detection limits were relatively high for grab samples compared to sponge stick and vacuum filter cassette samples, a 30,000 CFU target spore load was added to the test matrix for wash water and vegetation. Additionally, a 300,000 CFU target spore load was added to the test matrix for soil. At the 30,000 CFU target spore load level, all replicates were confirmed positive by colony PCR for sample types tested (wash water, vegetation, and soil) with Ct values < 21. Soil samples were processed with target spore loads of 300,000 CFU and were confirmed culture positive by PCR with Ct values < 21.

Table 17. Summary of the Accuracy of Identification of Presumptive *Btk* T1B2 Colonies by PCR Confirmation from Spiked Grab Samples.

Grab Samples	Nominal Spore Load (CFU)	Culture Replicates Presumptive Positive[a]	Colonies from Initial Culture Plates PCR-Screened (# PCR +) [b]	Colony PCR Ct (X ± σ) [c]	Colonies from BHIB Streak Plates PCR-Screened (# PCR +) [d]	BHIB PCR-Screened (# PCR +) [e]	BHIB PCR Ct (X ± σ) [f]
Wash Water-1	0	3 of 3	30 (0 of 3)	N/A	30 (0)	3 (0)	N/A
	150	3 of 3	30 (0 of 3)	N/A	20 (0)	3 (0)	N/A
	1,500	3 of 3	30 (3 of 3)	23.3 ± 1.2	10 (0)	3 (2)	38.6 ± 0.7
	15,000	3 of 3	30 (3 of 3)	18.2 ± 0.7	0	0	N/A
Wash Water-2	15,000	3 of 3	30 (3 of 3)	18.9 ± 0.5	0	0	N/A
Gravel	0	3 of 3	30 (0 of 3)	N/A	3 (0)	0	N/A
	150	3 of 3	28 (1 of 3)	22.4	2 (0)	0	N/A
	1,500	3 of 3	30 (2 of 3)	23.1 ± 0.6	1 (0)	0	N/A
Soil	0	3 of 3	27 (0 of 3)	N/A	0	3 (0)	N/A
	1,500	3 of 3	33 (1 of 3)	22.1	2 (0)	3 (2)	38.1 ± 0.6
	15,000	3 of 3	33 (3 of 3)	20.4 ± 0.5	3 (0)	3 (3)	37.0 ± 0.6
	150,000	3 of 3	33 (3 of 3)	20.4 ± 1.0	0	3 (3)	34.6 ± 1.9
Sterile Soil	0	0 of 1	0	N/A	0	1 (0)	N/A
	1,500	1 of 1	11 (1 of 1)	19.7	0	1 (1)	20.5
	15,000	1 of 1	11 (1 of 1)	21.8	0	1 (1)	21.2
	150,000	1 of 1	10 (1 of 1)	19.8	1 (1)	1 (1)	20.2
Vegetation	0	3 of 3	30 (0)	N/A	0	3 (0)	N/A
	150	3 of 3	30 (0)	N/A	2 (0)	3 (0)	N/A
	1,500	3 of 3	30 (1 of 3)	29.0	2 (0)	2 (0)	N/A
	15,000	3 of 3	30 (3 of 3)	19.7 ± 0.8	0	0	N/A

[a] Presumptive *Btk* T1B2 was present on initial culture plates.

[b] Number of colonies PCR-screened from initial plating, with number of PCR positive replicates in parentheses.

[c] Colony PCR Ct values for positive samples (Ct value of ≤ 40).

[d] Number of colonies PCR-screened from BHIB streak plates, with number of PCR positive replicates in parentheses.

[e] Number of samples with PCR screening of BHIB enrichment culture, with number of PCR positive replicates in parentheses.

[f] BHIB enrichment culture PCR Ct values for positive samples (Ct value of ≤ 40).

Data suggest the limit of detection is lower for the culture method when PCR is performed on BHIB enrichment culture of the soil pellet compared to colony PCR screen of isolated colonies for the culture method. However, the Ct values generated from BHIB enrichment are ≥ 36 for 3,000 and 30,000 target spore spike levels. The BHIB enrichment culture PCR data in Table 18 suggest that soil chemical components (inhibitors) are not interfering with PCR analysis of the enriched soil pellet, with Ct values of 20.2 to 21.2 in sterile soil and suppressed Ct values of 33.7 to 38.9 in nonsterile soil. Therefore, the Ct value suppression seen in nonsterile soil is likely due to growth competition from the background microbial flora present in the soil.

Table 18. PCR Analysis of BHIB Enrichment of Soil Pellet.

Sample Type	PCR (Ct Values)			
	0 Spores (X ± σ)	3,000 Spores (X ± σ)	30,000 Spores (X ± σ)	300,000 Spores (X ± σ)
Nonsterile Soil	Not Detected	38.9 ± 1.6	37.0 ± 0.6	33.7 ± 0.6
Sterile Soil	Not Detected	20.5	21.2	20.2

3.3.3 Grab Sample RV-PCR Analysis

A summary of the average and standard deviation of the RV-PCR ΔCt values for the detection of *Btk* T1B2 spores recovered from grab samples is presented in Table 19. Sample replicates with a RV-PCR ΔCt value ≥ 9 are considered positive, indicating that viable *Btk* T1B2 spores were recovered.

The RV-PCR ΔCt results are plotted in Figure 58 through Figure 61. The plots all depict the ΔCt threshold value of 9 as a dashed line, with an area shaded in red representing a negative detection result, and an area of green representing a positive detection result. Wash water grab samples were RV-PCR negative at the 300 CFU and 3,000 CFU target spore load levels. The 30,000 CFU target spore load level was added since detection limits were high for this grab sample type. All replicates processed at the 30,000 CFU target spore load were RV-PCR positive, with an average ΔCt value of 12.2. Gravel grab samples were RV-PCR negative for two of three replicates at the 300 CFU and 3,000 CFU target spore load levels. The positive replicate ΔCt values were 10.4 (300 CFU) and 9.8 (3,000 CFU).

Soil grab samples were RV-PCR negative for two of three replicates at the 3,000 CFU target spore load level. The ΔCt for the RV-PCR positive replicate at the 3,000 CFU target spore load was 10.4. All replicates at the 30,000 CFU and 300,000 CFU target spore load were RV-PCR positive. The average ΔCt value of the 30,000 CFU target spore load was 13.3, and the average ΔCt value of the 300,000 CFU target spore load was 16.6.

Vegetation grab samples were RV-PCR negative at the 300 CFU and 3,000 CFU target spore load levels. The 30,000 CFU target spore load level was added since detection limits were high for this grab sample type. All replicates processed at the 30,000 CFU target spore load were RV-PCR positive with an average ΔCt value of 12.6.

Table 19. RV-PCR Analyses of *Btk* T1B2 Spores Spiked Grab Samples.

Grab Sample Type	Number of Replicates	Spores Available for Analysis (CFU)		ΔCt[c] (X ± σ)	RV-PCR Replicates Positive[d]
		Nominal[a]	Determined[b]		
Wash Water-1	3	0	0	0.0 ± 0.0	0
	3	150	90 ± 0	0.0 ± 0.0	0
	3	1,500	900 ± 0	7.0 ± 0.8	0
	3	15,000	30,000 ± 0	12.2 ± 0.6	3
Wash Water-2	3	0	0	Not Tested	Not Tested
	3	15,000	30,000 ± 0	14.7 ± 0.8	3
Gravel	3	0	0	0.7 ± 1.3	0
	3	150	180 ± 0	7.5 ± 2.6	1
	3	1,500	1,800 ± 0	6.9 ± 3.1	1
Soil	3	0	0	0.6 ± 1.0	0
	3	1,500	2,200 ± 0	8.7 ± 1.5	1
	3	15,000	22,000 ± 0	13.3 ± 1.2	3
	3	150,000	220,000 ± 0	16.6 ± 0.6	3
Sterile Soil	1	0	0	0	0
	1	1,500	2,200 ± 0	25.0	1
	1	15,000	22,000 ± 0	24.8	1
	1	150,000	220,000 ± 0	24.5	1
Vegetation (Grass)	3	0	0	0.0 ± 0.0	0
	3	150	55 ± 0	0.0 ± 0.0	0
	3	1,500	550 ± 0	1.6 ± 1.5	0
	3	15,000	30,000 ± 0	12.6 ± 1.3	3

[a] Nominally one-half of the target spore load on the surface and assuming 100% recovery of spores.

[b] Based on the spiking titer measured each test trial, 100% recovery efficiency, and one-half of extraction used for RV-PCR analysis.

[c] PCR assay for T1B2 Barcode Gene Target.

[d] Number of replicates with a RV-PCR ΔCt value ≥ 9.

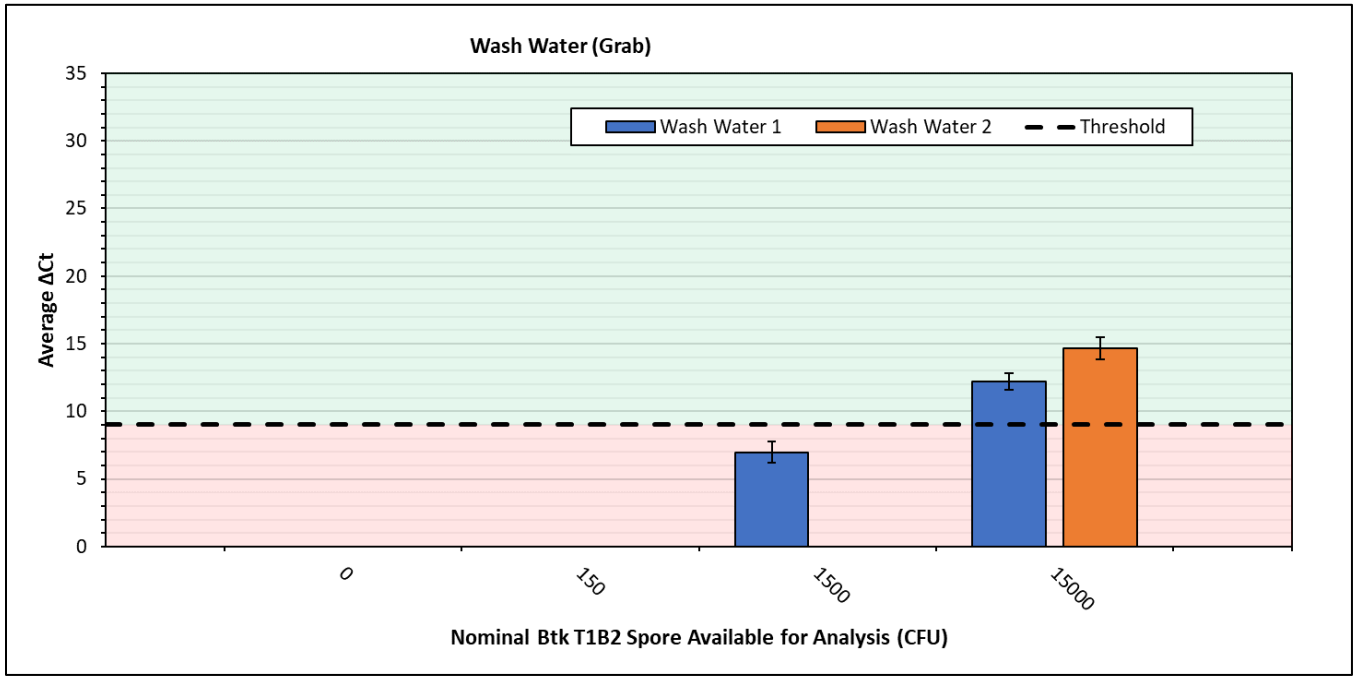

Figure 58. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from Vessel Wash Water Grab Samples.

Average ± One Standard Deviation of N = 3 Replicates. Vessel Washdown Water Spiked with Nominal 300, 3,000, or 30,000 Btk T1B2 Spores. Positive Response Equals ΔCt ≥ 9.

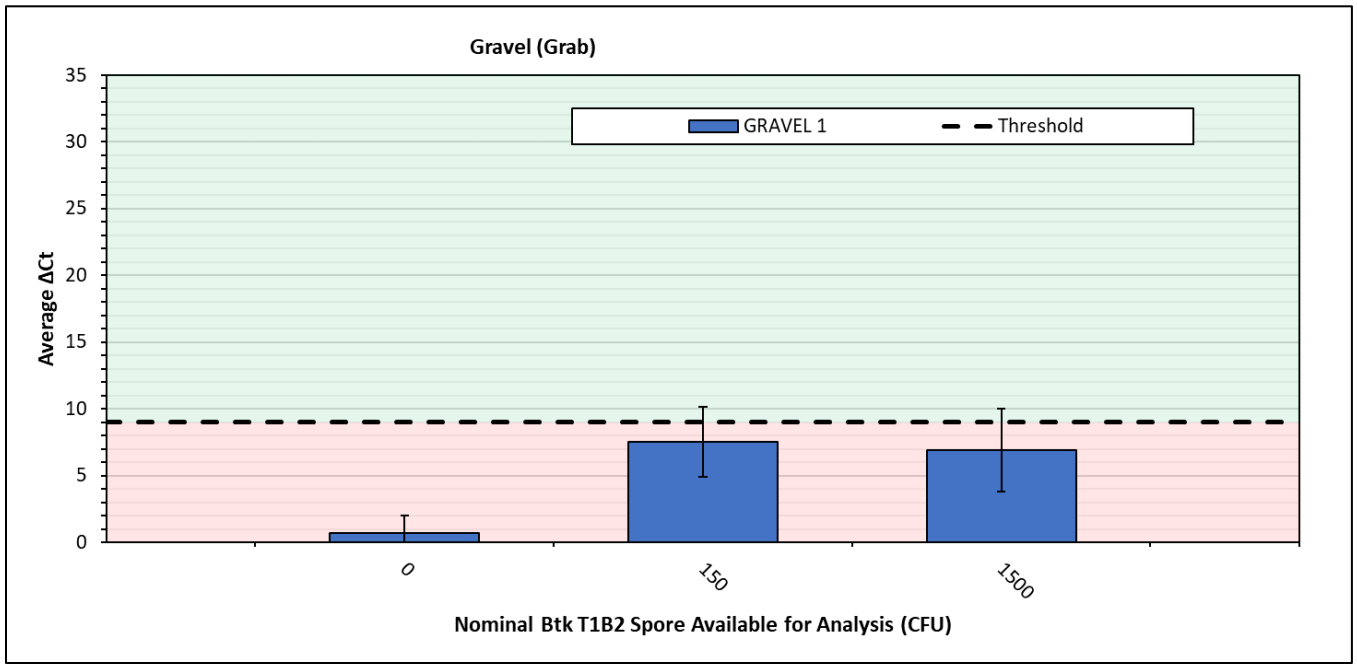

Figure 59. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from Gravel Grab Samples.

Average ± One Standard Deviation of N = 3 Replicates. Gravel Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores. Positive Response Equals ΔCt ≥ 9.

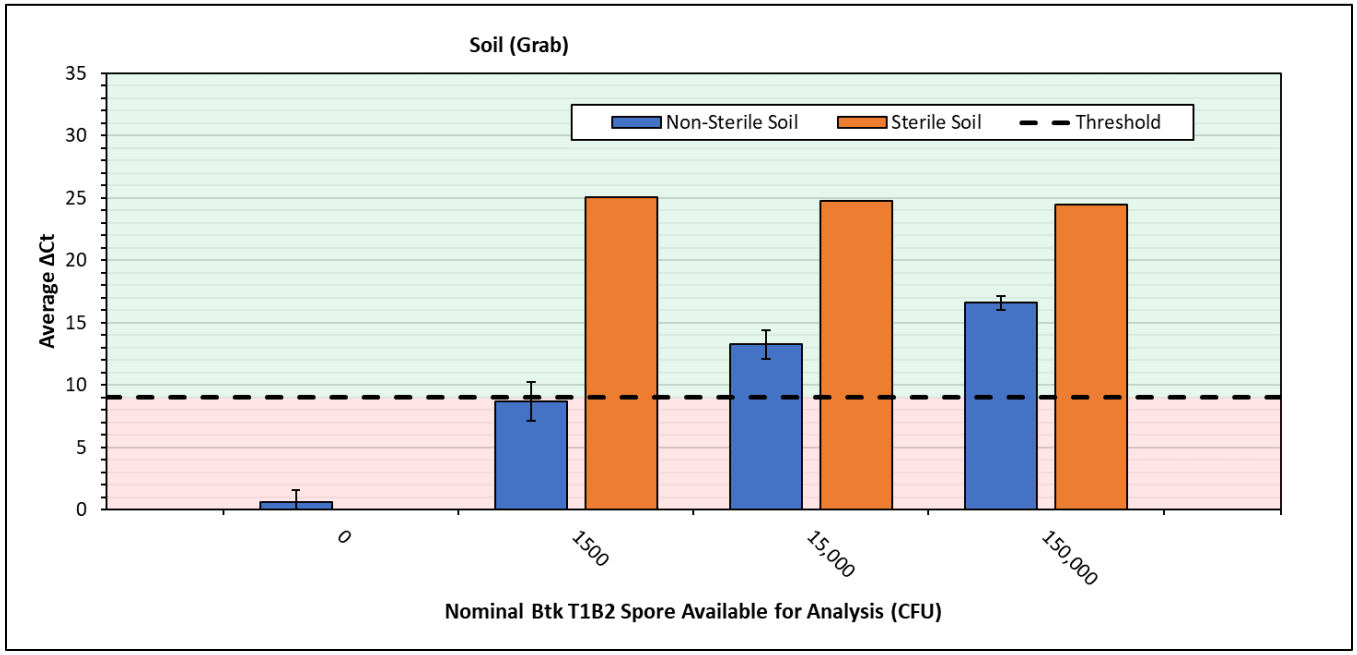

Figure 60. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from Soil Grab Samples.

Average ± One Standard Deviation of N = 3 Replicates. Soil Spiked with Nominal 3,000, 30,000, or 300,000 CFU of Btk T1B2 Spores. Positive Response Equals ΔCt ≥ 9.

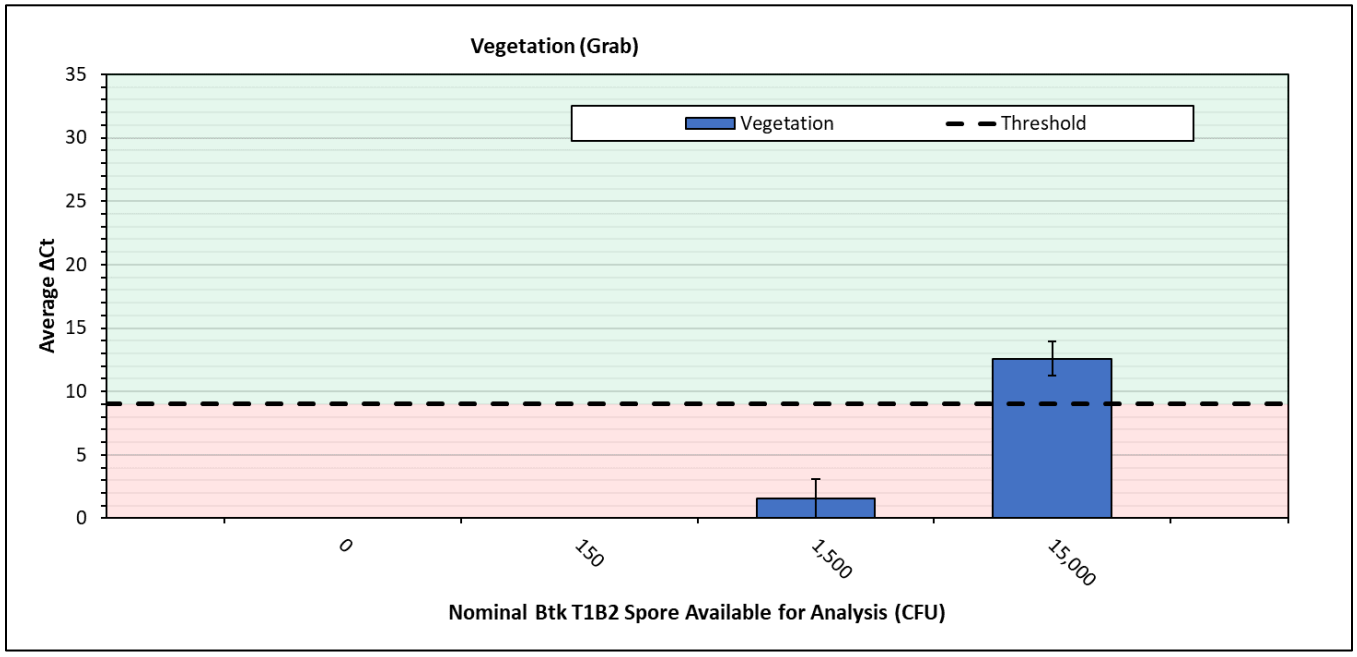

Figure 61. RV-PCR Analysis of *Btk* T1B2 Spores Recovered from Vegetation (Grass) Grab Samples.

Average ± One Standard Deviation of N = 3 Replicates. Vegetation (Grass) Spiked with Nominal 300 or 3,000 CFU of Btk T1B2 Spores. Positive Response Equals ΔCt ≥ 9.

3.3.4 Analytical Method Comparison for Grab Samples

Culture analysis identified presumptive *Btk* T1B2 colonies for all grab field samples, indicating that background microbial flora included wild-type *Btk* or another organism that had a morphology indistinguishable from *Btk* T1B2 on TSA plates. PCR confirmation was performed on either colonies from initial culture plates, colonies isolated from BHIB enrichment culture of the grab samples, and/or the BHIB enrichment culture to confirm or refute the presence of *Btk* T1B2. To compare the two methods, culture with PCR confirmation and RV-PCR results were assessed to determine which method may be more likely to detect viable spores that have been deposited onto wash water, gravel, soil, or vegetation containing outdoor interferents.

PCR screening of presumptive *Btk* T1B2 colonies were negative in some cases, indicating that background microbial flora with morphology indistinguishable from that of *Btk* T1B2 were present on TSA culture plates, therefore present in the samples collected in the field. It is possible that wild-type/naturally occurring *Btk* led to an inflation in presumptive spore recovery values by the culture method. Presumptive culture identification by colony morphology, colony identification confirmed by PCR, and RV-PCR results are shown in Table 20.

For culture analysis of field grab samples, 36 of 48 (75%) were true positives or true negatives. A true positive is defined as a sample spiked with *Btk* T1B2 spores that was confirmed positive by PCR. A true negative is defined as a sample that was not spiked with *Btk* T1B2 spores and was negative for PCR confirmatory screening. All 12 nontrue sample results were false negative using the culture method, meaning they were not confirmed positive by PCR following colony PCR or PCR of BHIB culture. All 16 field blank and laboratory blank controls were true negatives.

For RV-PCR analysis of field grab samples, 30 of 48 (63%) were true positives or true negatives. A true positive is defined as a sample spiked with *Btk* T1B2 spores that had a ΔCt of ≥ 9. A true negative is defined as a sample that was not spiked with *Btk* T1B2 spores and had a ΔCt of < 9. All 18 nontrue sample results were false negative samples, eight were spiked with 300 CFU target spore load and ten were spiked with 3,000 CFU target spore load. ΔCt values for the false negative samples ranged from 0 to 8.3.

Table 20. Analytical Method Comparison Displaying Culture Presumptive, Culture ID with PCR Confirmation, and RV-PCR Replicates Positively Identified (N = 4) for Grab Samples.

Sample	Actual Spike Level (CFU)	Presumptive Culture Result	Culture PCR Confirmation	Culture % Recovery	RV-PCR Result	RV-PCR ΔCt
Washdown 1	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	180[(a)]	Positive	Negative	1,611.00	Negative	0
	180[(a)]	Positive	Negative	1,555.00	Negative	0
	180[(a)]	Positive	Negative	944.00	Negative	0
	1,800[(a)]	Positive	Positive	96.3	Negative	7.29
	1,800[(a)]	Positive	Positive	233.33	Negative	7.57
	1,800[(a)]	Positive	Positive	114.81	Negative	6.07
	60,000	Positive	Positive	18.3	Positive	11.54
	60,000	Positive	Positive	13.2	Positive	12.72
	60,000	Positive	Positive	15.6	Positive	12.44
Washdown 2	60,000	Positive	Positive	25.1	Positive	14.74
	60,000	Positive	Positive	22.3	Positive	13.84
	60,000	Positive	Positive	23.9	Positive	15.42
Washdown Field Blanks	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	4.42
	180[(a)]	Positive	Positive	19.44	Positive	24.85
	600	Positive	Positive	90	Positive	13.15
	1,800[(a)]	Positive	Positive	31.67	Positive	24.83
	6,000	Positive	Positive	26.5	Positive	25.06
Gravel	0	Positive	Negative	N/A	Negative	2.2
	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	360	Positive	Negative	97.2	Negative	6.7
	360	Positive	Negative	133.3	Negative	5.4
	360	Positive	Positive	377.8	Positive	10.4
	3,600	Positive	Positive	30	Positive	9.8
	3,600	Positive	Negative	113.3	Negative	3.6
	3,600	Positive	Positive	121.1	Negative	7.2
Gravel Lab Blank	0	Positive	Negative	N/A	Negative	0
	360	Positive	Positive	11.1	Positive	12.2
	3,600	Positive	Positive	24.4	Positive	18.2

[(a)] Stock enumeration plate outside 25 – 250 CFU range.

Table 20. Analytical Method Comparison Displaying Culture Presumptive, Culture ID with PCR Confirmation, and RV-PCR Replicates Positively Identified (N = 4) for Grab Samples (Cont.)

Sample	Actual Spike Level (CFU)	Presumptive Culture Result	Culture PCR Confirmation	Culture % Recovery	RV-PCR Result	RV-PCR ΔCt
Soil	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	1.7
	4,400	Positive	Positive	42.4	Negative	8.3
	4,400	Positive	Positive	39.4	Positive	10.4
	4,400	Positive	Negative	33.3	Negative	7.4
	44,000	Positive	Positive	7.6	Positive	14.2
	44,000	Positive	Positive	10	Positive	13.7
	44,000	Positive	Positive	7	Positive	11.9
	440,000	Positive	Positive	1.4	Positive	16.9
	440,000	Positive	Positive	6.8	Positive	15.9
	440,000	Positive	Positive	7.1	Positive	16.9
Sterile Soil	0	Negative	Negative	N/A	Negative	0
	4,400	Positive	Positive	42.4	Positive	25
	44,000	Positive	Positive	34.6	Positive	24.8
	440,000	Positive	Positive	20.7	Positive	24.5
Vegetation	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	0	Positive	Negative	N/A	Negative	0
	110	Positive	Negative	16,094.30	Negative	0
	110	Positive	Negative	23,579.60	Negative	0
	110	Positive	Negative	14,424.20	Negative	0
	1,100	Positive	Negative	2,335.10	Negative	0
	1,100	Positive	Negative	1,393.90	Negative	3.1
	1,100	Positive	Positive	2,998.10	Negative	1.7
	60,000	Positive	Positive	50.3	Positive	12.6
	60,000	Positive	Positive	36.7	Positive	13.9
	60,000	Positive	Positive	34.2	Positive	11.2
Vegetation Control (PBST)	0	Negative	Negative	N/A	Negative	0
	110	Positive	Positive	6.9	Positive	22.1
	1,100	Positive	Positive	15.7	Positive	22.3

Results for all samples processed, including positive and negative controls, are shown in Table 21. For culture, 52 of 64 samples (81%) were determined as true positives or true negatives using the culture method. All 12 nontrue sample results were false negative using the culture method, meaning they were not confirmed positive by PCR following colony PCR or PCR of BHIB culture. All 16 field blank and laboratory blank controls were true negatives. The RV-PCR method correctly determined 46 of 64 samples (72%), with zero false positives and 18 false negatives.

Many of the grab samples had a high load of presumptive *Btk* colonies, including the zero spore spike samples. Therefore, an important consideration for the culture method is establishing a maximum

number of presumptive colonies that should be screened using PCR. The method currently lists that a minimum of three colonies be screened from spread plates or 1 – 3 colonies from membrane filter plates. For samples with a high number of background organisms with presumptive *Btk* morphology, a maximum number of colonies or a method for pooling multiple colonies or pooling and mixing growth from culture plates would need to be established.

For wash water surface culture analysis, all three 300 CFU target spore load level samples were false negatives. For RV-PCR, all 300 CFU and 3,000 CFU target spore load level samples were false negatives.

For gravel samples, two of three 300 CFU target spore load level samples were false negative for both culture and RV-PCR. At the 3,000 CFU target spore load level, one of three replicates was false negative for culture and two of three replicates were false negative for RV-PCR.

For soil samples, one of three replicates was false negative for culture and two of three replicates were false negative for RV-PCR at the 3,000 CFU spike level. All replicates were positive at the 30,000 CFU and 300,000 CFU target spore load levels for both culture and RV-PCR.

For vegetation culture analysis, all three 300 CFU target spore load level and two of three 3,000 CFU target spore load level samples were false negatives. For RV-PCR, all 300 and 3,000 CFU target spike level samples were false negatives.

Table 21. Analytical Method Comparison Displaying Culture ID with PCR Confirmation and RV-PCR Replicates for Grab Samples.

Sample Type	Culture Method			RV-PCR Method		
	True Positive or True Negative	False Positive Sample	False Negative Sample	True Positive or True Negative	False Positive Sample	False Negative Sample
SBWASH 1	9	0	3	6	0	6
SBWASH 2	3	0	0	3	0	0
GRAVEL	6	0	3	5	0	4
SOIL	11	0	1	10	0	2
VEGETATION	7	0	5	6	0	6
CONTROLS	16	0	0	16	0	0
Totals	**52**	**0**	**12**	**46**	**0**	**18**
Positive PCR threshold of 40 for colony PCR and BHIB culture PCR. The definition for Positive Ct threshold per the EPA Protocol (EPA, 2017) is ≤ 40.						

3.3.5 Analysis of Controls (Grab)

Water that was used for washdown of the small boat was analyzed as a field blank control at the zero spike, 300 CFU target spike and 3,000 CFU target spike levels. Sample replicates for each condition above were determined as true positives or negatives for both culture and RV-PCR analysis methods. A true positive or true negative indicates that the result matches the expected outcome, for example, if a sample spiked with *Btk* T1B2 was positive, it was a true positive. Gravel purchased from a home improvement store was included as a laboratory blank control at the zero spike, 300 CFU target spike and 3,000 CFU target spike levels. Each condition was determined as a true positive or negative (for both culture and RV-PCR analysis methods). Soil that was sterilized by autoclave treatment was included as a control at the zero spike, 3,000, 30,000, and 300,000 CFU target spike levels. Each condition was determined as a true positive or negative (for both culture and RV-PCR analysis methods). As a vegetation control, 500 mL of PBST was included at the zero spike, 300 CFU target spike and 3,000 CFU target spike levels and processed alongside vegetation samples. Each condition was determined as a true positive or negative for both culture and RV-PCR analysis methods.

4.0 QUALITY ASSURANCE/QUALITY CONTROL

Quality assurance (QA)/quality control (QC) procedures were performed in accordance with the Scientific, Testing, Research, and Modeling, Support (STREAMS III) Program Quality Management Plan (QMP). The QA/QC procedures and results are summarized below.

4.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, water baths, refrigerators/freezers) used at the time of the evaluation was verified as being certified, calibrated, or validated.

4.2 QC Results

QC efforts conducted during testing included positive and negative controls for both spread plate samples and qPCR. In addition, *Btk* spike suspensions were quantified to verify either CFU/mL titer or target spike concentrations.

Positive and negative control results were within the target requirements for the qPCR. Applied Biosystems 7500 Fast system performance was assessed according to internal standard operating procedures (SOPs) and maintained at regular intervals—monthly (optical and background calibration), every 6 months (dye calibration), and annually (RNase P calibration). For culture analysis, the PC spore stock maintained a single morphological appearance consistent with *Btk* T1B2 throughout the study, as determined at the beginning of each trial. Media and reagents used for culture analysis were screened (negative controls) and had no growth, showing that reagents used were not the source of contamination.

4.3 Operational Parameters

Micropipettes, thermometers, and timers used were calibrated against a traceable standard at regular intervals (every 6 months or annually) and used only within the acceptable calibration interval established by internal SOPs.

4.4 Audits

4.4.1 Performance Evaluation Audit

Performance evaluation audits were conducted to assess the quality of the results obtained during these experiments. Table 22 summarizes the PE audits that were performed; the equipment was verified to be within an acceptable tolerance range.

Table 22. Performance Evaluation Audits

Measurement	Audit Procedure	Allowable Tolerance	Actual Tolerance
Volume of liquid from micropipettes	Gravimetric evaluation	± 10%	Passed calibration as found/as returned
Time	Compared to independent clock	± 2 sec/h	Passed calibration as found/as returned
Temperature	Compared to independent calibrated thermometer	± 2°C	Passed calibration as found/as returned

4.4.2 Technical Systems Audit

A technical system audit was conducted on laboratory procedures under STREAMS Task Orders in January 2021 and July 2021 to ensure that tests were being conducted in accordance with the appropriate QAPP and QMP.

4.4.3 Data Quality Audit

At least 10% of data acquired during the evaluation were audited. Data were reviewed from November 9, 2020 through May 17, 2021. A QA auditor traced the data from the initial acquisition, biologic plate counts, PCR ΔCt calculation, data reduction and statistical analysis, to final reporting to ensure the integrity of reported results. All calculations performed on the data undergoing the audit were verified. No issues were noted with the data collection and reporting process, and all calculations were performed accordingly.

4.5 QA/QC Reporting

Each assessment and audit was documented in accordance with the QAPP and QMP. For these tests, no findings were noted during the TSA or in the data quality audit, and no follow-up corrective action was necessary. QA/QC procedures were performed in accordance with the QAPP.

4.6 Data Review

Records and data generated in the evaluation received a QC/technical review before they were utilized in calculating or evaluating results and prior to incorporation in this report.

5.0 SUMMARY OF METHOD OBSERVATIONS AND EXPERIENCES

While implementing the method, key observations and experiences were noted that will be useful to understand and/or take into consideration for future iterations or versions.

5.1 Sample Processing Considerations

Sponge sticks, VFCs, wash water, and grab samples each have unique processing procedures, complicating the analyses by requiring different protocols and equipment. Samples will need to be batched according to sample type and transported to a laboratory with the necessary equipment. Sponge sticks require the use of a Stomacher 400 and swinging bucket centrifuge, VFCs require a bath sonicator, and wash water and grab samples require a filter manifold for concentrating the sample volume onto a filter membrane.

For gravel ballast sample processing, the 500 mL bottles (Daigger Item # EF2247C) specified in the extraction protocol for gravel ballast (Serre and Oudejans, 2017) are high-density polyethylene (HDPE) and are not autoclavable. The bottles become misshapen when autoclaved at 121°C gravity cycle for 15 min, Figure 62 shows rounding of the bottom of a bottle postautoclaving that could lead to sample spills during processing. These bottles should be sterilized by irradiation or another method, or a replacement product that can be autoclaved should be considered.

Figure 62. Autoclaving HDPE Bottles Compromises their Structure

Grab sample suspension/rinsate can lead to clogged MicroFunnel membranes, limiting the total volume that can be analyzed per sample.

For soil samples with a 0.25 g/mL ratio of soil to extraction buffer, limit of detection may not improve even if more volume were available for analysis for either method (culture or RV-PCR) because the high background microbial flora that survive the heat shock and are recovered alongside target spores and the particulates lead to clogging of the filter. Data suggest for the soil sample, plating ≥ 0.1 mL suspension

will produce an overwhelming level of background microbial growth. The sterile soil sample spiked with 3,000 CFU target spore level had < 10 colonies per 0.1 mL plate, so plating dilution series would make detection of spores less likely. Filtering 20 mL through the filter vial for the RV-PCR method averaged 41 min, with an additional ~15 min for each of the two subsequent washes with 10X PBS and 1X PBS, making this a time-consuming process.

5.2 Method Qualitative Assessment

Given our experiences running analytical methods, there are pros and cons for both methods.

5.2.1 Culture Method

The strengths of the culture method are that it allows for a quantifiable measure of target; isolation of target organism; and confirmatory PCR screening of colonies that provides a definitive result. Weaknesses of the culture method are that background microbial flora can overwhelm culture plates and obscure colony morphology, leading to false negative results. Additionally, background microbial flora with a similar or identical morphology can be present within samples, triggering PCR screening of colonies and possibly repeated PCR screening (to minimize risk of false negatives) if presumptive morphology is present in large numbers.

5.2.2 RV-PCR Method

The strengths of the RV-PCR method are that it is akin to a biological indicator, it gives a positive or negative result and there is no iterative or repeat analysis on sample aliquots, giving the method a clear end of analysis without the need for multiple follow-up PCR screenings. The method can provide rapid results, which is of high significance in a wide-area incident involving multiple cities and environments. RV-PCR constitutes a small laboratory footprint and requires less culture media, resulting in relatively less BSL-3 waste. The weaknesses of the RV-PCR method are that it does not allow for quantification of target; target organism is not isolated for banking (unless additional streak plating is performed using the T_f aliquot); DNA purification steps are time-consuming; each sample is split into T_0 and T_f aliquots resulting in two DNA purification extractions per sample and six PCR reactions per assay (may be improved if automated DNA extraction is performed, and multiplex assays are available and validated); and the presence of background microbial flora could compete with target organism growth during enrichment, suppressing signal due to interferent and potentially leading to false negative results.

5.2.3 Time/Cost Estimates

The sample analyses were performed in laboratory analysis batches of 16 samples using a single manifold system for RV-PCR. The 16 samples were the maximum that was deemed reasonable to process considering a normal 8:00 AM to 5:00 PM workday, without overtime and/or a night shift that may be used by the EPA's Environmental Response Laboratory Network (ERLN) if actual emergency response samples were being processed. A single batch was completed over four to five consecutive days of operation, starting with sample control spiking and spore recovery and culturing on Day 1 (refer to Figure 31 in Section 2.7). (Note: had these been actual samples collected postbiological release, the spiking activity would, obviously, not be performed by the ERLN). Day 2 consisted of culture colony counting from agar plates, presumptive colony selection for PCR screening, and nucleic acid extraction for RV-PCR. Day 3 involved PCR analysis of colonies from culture plates and T_0 and T_f aliquots for RV-PCR. For samples that were not confirmed positive by colony PCR, additional streak plates of the enriched filter or sponge stick were performed on Day 4 and Day 5 for the culture method. If incubation time for RV-PCR was reduced to 9 h and a night shift performed nucleic acid extraction, results could

be completed in the next day after spore recovery. If additional enrichment of the sponge stick or filter is not performed for the culture method, indicated as optional in the EPA Protocol for initial and clearance stages, results could be completed the next day after spore recovery. The text below is quoted from page 53 (EPA, 2017).

> *Note: "For faster sample analysis results during the initial stages of an incident (e.g., incident characterization) and during post-decontamination/clearance phase, it is recommended that the remainder of all suspensions (e.g., undiluted, 10^{-1} and 10^{-2} dilutions) be filtered using an additional MicroFunnel™ and plated as described above, instead of proceeding with enrichment in TSB."*

Estimated staff time to spike 16 samples with *Btk* spores and then process and analyze them was approximately 56 h of labor and $1,500 of consumables. The estimation of 56 h of staff time was distributed as follows:

- 8 h for activities related specifically to the spiking of the materials being assessed, which was a requirement of the study, but not an activity that would be performed had these been actual field samples. This task included time to prepare the stock suspensions, enumerate stock suspensions, spike the samples, and prepare associated documentation.
- 10 h for spore recovery.
- 14 h for culture analysis.
- 24 h for RV-PCR analysis.

If the EPA method had been followed without any changes (most notably the samples would not be split for analysis and either the culture only or the RV-PCR method only would have been used), a batch of 16 samples would take an estimated 34 h of labor and $1,000 of consumables to perform culture analysis (with PCR confirmation of at least three colonies per sample). To process the same number of samples, an estimated 40 h of labor and $1,200 of consumables would be required using RV-PCR analysis. Each of these methods can generate results within two days for analysis of the recovered spore suspension; additional time would be required for enrichment of the sponge stick or filter. The labor required for nucleic acid extraction for 16 samples is ~14 h; exploring options to reduce this labor or using an automated sample processing or/and automated nucleic acid extraction procedure may increase throughput and reduce sample cost. Thus, there is no significant time or cost advantage for one method over the other as per the methods with modifications used in this effort.

5.3 Culture Processing Considerations

Background growth and grime interfere with target *Btk* T1B2 morphology identification on culture plates. Presumptive *Btk* T1B2 colonies need to be PCR-screened to confirm or refute the presence of the target organism. The method defines that a minimum of three presumptive colonies (for this project, *Btk* T1B2) are screened to confirm the presence of the target organism. The method should define a maximum number of colonies that should be screened; otherwise, all presumptive colonies would need to be screened.

5.3.1 BHIB Enrichment Culture Analysis

The culture method detailed in the EPA Protocol (EPA, 2017) instructs users to streak turbid BHIB enrichment culture in triplicate on solid media plates; then, if any colonies with target morphology are isolated, to PCR screen those target colonies. A similar protocol is also used by the CDC-LRN. PCR on

a 50-µL aliquot of the BHIB culture is performed only if a colony with target morphology is not observed on streak plates. In previous evaluation of BHIB enrichment culture, data showed that the target organism was not isolated from sponge sticks and VFCs when streaked from turbid enrichment broth for nonfield blank samples due to the significant presence of the competing background organisms in those samples; however, the target organism was present in enrichment broth for many VFC samples, as determined by PCR analysis of an aliquot of broth (Calfee et al., 2019). Given these results, it would be preferred to perform PCR on enrichment broth, and only streak additional plates from the enrichment broth if attempting to isolate the target organism from BHIB enrichment following a positive PCR identification.

BHIB enrichment for sponge sticks samples is not as effective as enrichment for 47-mm filter membrane samples, which may be because a 25-mL volume of BHIB does not completely cover the sponge within a specimen cup and/or because the recovery of spores from VFCs is not as efficient as from sponge sticks; thus, more spores remain on the VFC membrane than on the sponge.

5.4 RV-PCR Processing Considerations

The RV-PCR method requires great care and diligence to implement effectively. Most notably, the method requires changing gloves between samples for each step, which is onerous and time-consuming. However, this added measure is critical when analyzing samples from the field collected after an incident, as the samples and associated results are high-value and high-impact—they will support key decisions in the response and impact response timelines, credibility, and cost. During the RV-PCR method, when applying vacuum to the filter vial manifold, the filtrate is pooled in the manifold reservoir and contacting the bottom of the filter vials near the vacuum source. It is recommended to increase the depth of the bottom section of the manifold so that the filtrate does not pool and contact the bottom of filter vials.

Recovered sample suspensions with high particulate loads can clog the RV-PCR filter vials. As a result, the below two rules were applied to expedite sample processing and the inclusion of buffer washes:

- At 15-min, post-sample addition to the filter vial. If the sample did not pass through the filter vial, a reduced volume of high and low salt wash buffer (5 mL) was added, rather than omitting one or both entirely.
- At 1-h, post-sample addition to the filter vial. If the sample did not pass through the filter vial, the high and low salt wash steps were omitted.

The RV-PCR manifold, capping tray, and manifold incubator rack for holding manifold/capping trays in the shaker incubator are custom-manufactured equipment. Scaling up sample processing would need to consider supply chain and time required to manufacture custom parts.

5.4.1 Biological Safety Level 3 Considerations

Transfer of the RV-PCR manifold/capping tray method into a BSL-3 laboratory may present sample handling challenges, but they are expected to be manageable with proper training of experienced staff. The filter vials are sealed via a capping tray with a compression luer cap that does leak on occasion and are arranged in a tray with little space between vials, making physical wiping of the vials with decontaminant a challenge. Direct contact between the metal capping tray and plastic bags during shaking incubation and platform vortex mixing can lead to bag tears. Therefore, packaging of manifold within durable (8-mil thickness recommended) bags or use of a biocontainment box with absorbent is recommended for incubation and platform vortex mixing to avoid select agent release during these steps.

Considerations for proper containment and effective implementation of containment by properly trained and experienced staff are expected to work in such an environment.

5.4.2 Suggestions to Improve RV-PCR Throughput

5.4.2.1 Nucleic Acid Extraction

For a set of 16 samples, the nucleic acid extraction procedure takes ~14 h of labor and consumes 624 1-mL micropipette tips, 96 200-µL micropipette tips, and 80 2-mL microcentrifuge tubes in addition to the nucleic acid kit consumables. PCR analysis of a 50-µL aliquot of the T_f BHIB from filter vials using thermolysis instead of nucleic acid extraction procedure may produce results similar to the T_0 and T_f aliquots that were extracted using the nucleic acid extraction procedure, with less labor, consumables and biohazardous waste generated. As per the EPA Technical Lead for this effort, however, considering the post-2001 Amerithrax response queries from the U.S. Government Accountability Office, stringent DNA extraction procedures were warranted to minimize PCR inhibition and were included in the EPA Protocol (EPA, 2017). The RV-PCR process of washing filter vials with 10X and 1X PBS may decrease PCR inhibitors to a level that reduces the risk of a false negative sample. Alternatively, a different DNA purification technique could be utilized. Bushon et al. 2021 found that a Qiagen DNA purification kit reduced processing times compared to the Promega purification procedure utilized in this study.

5.5 Sponge Stick Sample Analysis

5.5.1 Biological Safety Level 3 Considerations

Transfer of the sponge stick method into a BSL-3 laboratory will present sample handling challenges.

- Stomacher 400 equipment footprint fills the depth of a Class II BSC and is a high energy homogenization process in a nonrigid container (Stomacher bag) that is subject to puncture from particulates recovered from heavily soiled surfaces, and leakage may occur.
- Stomacher bag stands are available to hold bags upright to prevent tipping and spillage, but the stand fills the depth of a Class II BSC.
- Transferring volume from the Stomacher bag to and from tubes is subject to dripping and spillage.

5.5.2 Sponge Stick Method Considerations

The sponge stick method uses 90 mL of buffer to extract a sponge. The next step following stomaching is to reduce the volume by centrifugation. Percent recovery could potentially be gained by reducing the volume used for stomaching.

5.6 Vacuum Filter Cassette Sample Analysis

Recovery efficiencies are low for this sampling method, possibly due to poor removal of spores from surfaces or poor recovery from the vacuum cassette. A vortex mix rather than bath sonication may improve recovery of spores from the VFC filter. The bubble and cavitation energy of a bath sonicator may not transfer through plastic tubes/2 oz. cups, and the signal may be damped by racks or distributed nonuniformly. A previous program assessing sonication in the recovery of spores from soil samples did not improve spore recovery (Silvestri, 2016).

5.7 Grab Sample Analysis

The approach used for this project for analysis of washdown or grab samples was to concentrate the collected eluate (250 mL) onto a 47-mm mixed ester cellulose filter membrane (0.45 µm), then recover the spores from the filter membrane using 20 mL of extraction buffer (PBSTE) with agitation for culture and RV-PCR analysis methods. The concentration of suspension onto this 47-mm filter and subsequent removal of the spores may reduce recovery of spores for both culture and RV-PCR analysis. Culturing the 47-mm filter directly onto solid media for the culture approach or broth enrichment of this filter may improve detection limits.

Additionally, grab samples can contain particulates that clog the filter membrane, limiting the total volume of sample that can be processed. A centrifugation step, rather than membrane filtration, for concentration should be considered to allow more sample volume to be processed. For vegetation samples, the membrane filter clogged following filtration of 132 ± 42 mL of the 500 mL available. Bushon et al. 2021 demonstrated that results obtained using a centrifugation step were comparable to membrane filtration and took less time.

5.8 Difficult-to-Analyze Sample Types and Recommendations

Grab samples had fewer true positive/true negative (true results) samples for both culture (75 %) and RV-PCR (63 %) methods. All of the nontrue grab sample results were false negative, meaning the samples were spiked with *Btk* T1B2 spores, and the samples were not confirmed positive by PCR. For each of the grab sample types, background microorganisms recovered alongside target spores are the main contributor to false negative samples, either masking the presence of target colonies or outcompeting the target organism for nutrients. To reduce the impact of background microorganisms, 30% ethanol was a component in the recovery buffer for wash water, gravel, and vegetation to inhibit growth or reduce viability of vegetative organisms without affecting the *Btk* spores. For soil samples, a heat shock (70°C) was incorporated to reduce the viability of vegetative organisms. Background microorganisms with presumptive *Btk* morphology were still recovered at levels of 30 to 100 CFU/mL (wash water 1), 8.8 to 180 CFU/mL (gravel), 105 to 167 CFU/mL (vegetation) and 23 to 57 CFU/mL (soil) for zero spike samples. Total background microorganisms for these samples were 1,490 to 1,800 CFU/mL (wash water 1), 80-262 CFU/mL (gravel), 334-750 CFU/mL (vegetation), and 8,040 to 23,160 CFU/mL (soil) for zero spike samples. By comparison, the 300 CFU spike level samples would have a maximum of 15 CFU/mL following concentration of the 500 mL spore recovery volume to 20 mL for wash water, gravel, and vegetation or 7.5 CFU/mL for the soil samples.

Particulates in grab samples can reduce the maximum total volume concentrated by filtration, particularly for vegetation and soil samples. However, particulates will vary in field samples, so it is expected to be a problem to overcome for all grab samples. For vegetation samples, 132 ± 42 mL of 500 mL total volume was processed through the MicroFunnel filter (47 mm, with 0.45 µm pore size). The RV-PCR filter vials are also susceptible to clogging or reduced flow rates from particulates within grab samples. The soil samples averaged 41 min of filtration time for 20 mL of spore recovery to pass through the filter vial, and additional time was required for each of the two filter vial wash steps.

Reduction of background microorganisms prior to culture could be explored using chemical treatment, perhaps a higher concentration of ethanol, and/or heat shock for all grab sample types. Another possibility is to evaluate selective broth media for enrichment rather than BHIB, which is a nutrient rich nonselective medium.

Removal of particulates by centrifugation would be a method to explore for reducing the impact of filter clogging. Post-centrifugation, the supernatant could be concentrated onto a filter, and the particulates could be resuspended in a buffer or media for detection of target spores separately or recombined with the supernatant following the filtration of the supernatant to maximize sample capture onto filter membranes and shorten filtration times.

6.0 CONCLUSIONS

Samples of residual inert and biological deposits on representative maritime asset surfaces (e.g., aluminum on boats, nonskid tread on decks of boats, touchscreens, and concrete piers) and surrounding grounds and infrastructure and materials (e.g., soil, vegetation, and gravel) were collected using established EPA methods for sponge stick wipes, VFCs, and grab samples for bulk material collection. The ambient background of inert and biological material would also be present if sampled following a biological contamination incident such as anthrax. Two sampling campaigns were successfully completed at the USCG Base Portsmouth, VA (one on 04 November 2020 and one on 26 March 2021).

Overall, for samples with maritime inert and biological deposits (sponge sticks, VFCs, and grab), the culture method resulted in 10 false positive results, and the RV-PCR method resulted in 0. Overall, there were 19 false negative results for the culture method and 26 false negative results for RV-PCR for samples with maritime inert biological deposits (sponge sticks, VFCs, and grab).

Samples with high microbial background load can mask the identification of target colonies on agar plates for the culture method and lead to RV-PCR signal suppression. Particulates within samples can reduce the amount of sample volume processed and increase sample processing times during filtration steps, particularly for vegetation and soil grab samples. Isolation of background microorganisms with target morphology from environmental samples complicates culture analysis, and requires PCR confirmation, which can delay sample analysis results.

Biological decontamination response scenarios will be widely varied and as demonstrated by this project, require multiple different sample types to determine extent of contamination, decontamination efficacy, and clearance phase monitoring. Given all these variables, there may be instances where both the culture methods and RV-PCR methods described here are applicable for use. In general, our summary of the culture method is that it is labor- and reagent/material-intensive, but very straightforward for laboratory staff to accomplish. Our observation of the RV-PCR method is that it has the potential to be much more streamlined, with less labor and fewer laboratory consumables. However, to deal with the low contamination levels and relatively high background in the samples included in this project, the RV-PCR method required labor intensive steps—such as sequential mixing and wash steps, DNA purification, and custom manifolds and equipment—which may negate some of the advantages. Our data demonstrated that one example of a scenario where the culture method may be preferable would be for grab samples (75% correct results for culture compared to 63% for RV-PCR) and one example of a scenario when the RV-PCR methods would be preferable would be sponge sticks (98% correct results for RV-PCR compared to 84% for culture). The bottom line is that each response will have to be considered on its own merits, and both methods will likely be used in various situations.

It would be valuable to assess ways to simplify both analytical methods to improve turnaround time and reduce the amount of biohazardous waste generated. To reduce iterative analysis with the culture method, a maximum number of colonies screened from a sample should be defined. Otherwise, field samples with high background microbial loads with presumptive target morphology would need an indefinite number of colonies screened. In addition, spread plating multiple dilutions in triplicate is useful in establishing a quantitative measure of the presumptive colonies, but for detection purposes, triplicate plating may not be necessary and reducing to single or duplicate replicates could reduce the total number of plates (media) consumed. For the RV-PCR method, a reduction in processing steps could be evaluated in a sample complexity-dependent manner to determine which steps are crucial for detection. Additionally, use of automated sample processing and nucleic extraction can further expedite sample analysis using the RV-PCR method with more accuracy.

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APPENDIX A: WORK INSTRUCTION FOR SURFACE SAMPLING USING CELLULOSE SPONGE STICKS

1.0 References

1.1 Miscellaneous Operation Procedure (MOP) 6583 *"Assembly of 3M Sponge Stick™ Kits."* Prepared by Arcadis, Inc. for the National Homeland Security Research Center

1.2 CDC NIOSH: Surface sampling procedures for *Bacillus anthracis* spores from smooth, non-porous surfaces

1.3 CDC NIOSH: Instructor Guide for Anthrax Surface Sampling

2.0 Surface Preparation

Note: This procedure utilizes pre-prepared 3M sponge stick sampling kits, which are assembled in accordance with Reference 1.1. For information on assembly, refer to this document.

All preparation and sampling will be done in two-person teams consisting of a **sampler** and an **assistant**.

2.1 Before beginning, the **sampler** and **assistant** will don a new pair of gloves overtop of their primary personal protective equipment (PPE). The **assistant** need only don a new pair at the start of the first sample. A glove change for the **assistant** is not necessary provided that the gloves remain unsoiled throughout their use.

2.2 Next, the **sampler** will obtain a clean 10" x 10" Teflon® sampling template from the **assistant** and place it over the desired area, if necessary, using pieces of tape on the outside edges to secure the template in place.

2.2.1 If the surface is unable to accommodate a 10" x 10" sampling area, measure out an area equivalent to 100 in^2. The same sampling procedure will remain unchanged for the alternate test area.

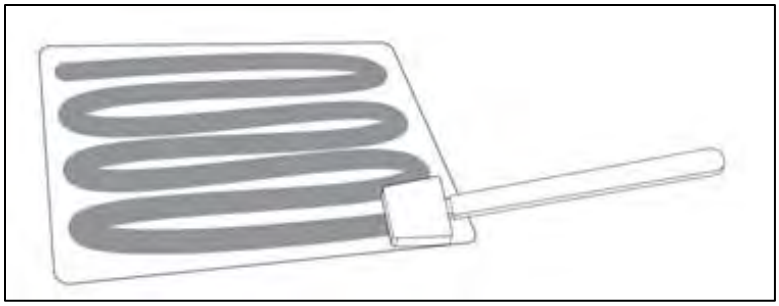
3.0 Sampling

3.1 When ready to sample, the **assistant** will retrieve one pre-made sponge stick kit and open the outer overpack bag, as well as the bag containing the sponge stick. Care will be taken to ensure that the **assistant** does not touch the sponge or its handle. The **assistant** will then present the open sponge stick bag to the **sampler**.

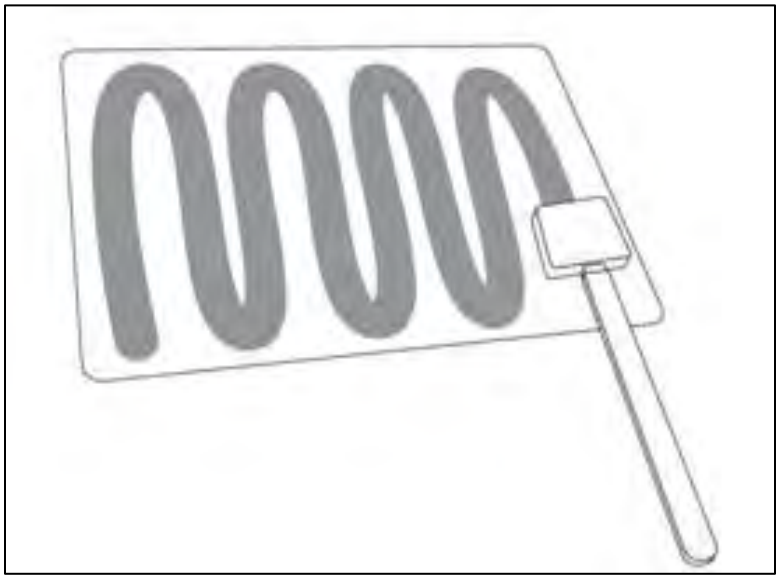
3.2 The **sampler** will remove the sponge stick by grasping the handle above the thumb stop, making sure not to touch below the stop.

3.3 In total, the **sampler** will make four passes over the area inside of the template.

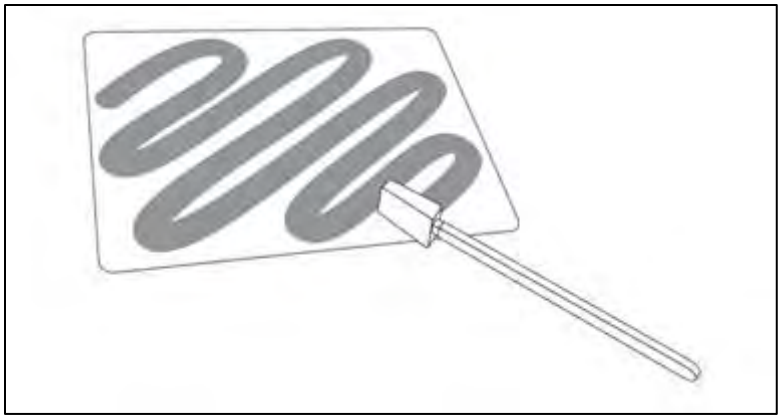
3.3.1 **Pass 1:** Starting in a corner of the template, place the sponge flat on the surface against one of its widest faces and apply gentle, but firm pressure. Ensure that the entire face of the sponge is in contact with the surface. While keeping the sponge pressed down, move the sponge horizontally, in an overlapping S-pattern over the entire area of the template. Covering the entire area will take approximately eight passes of the sponge.



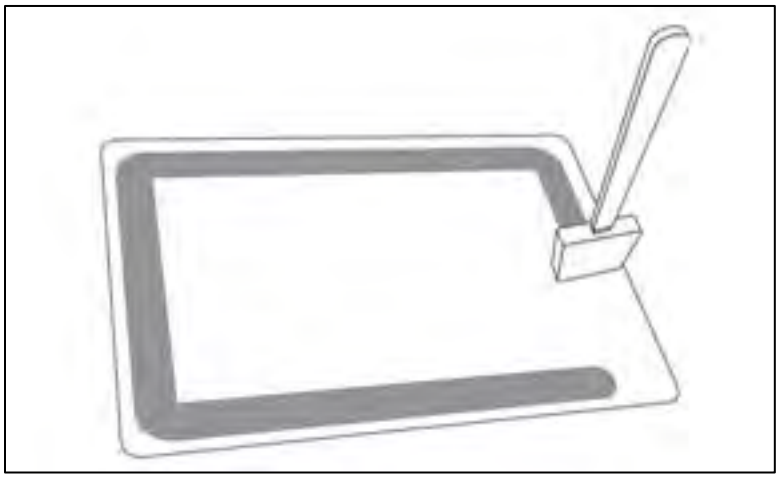
3.3.2 **Pass 2:** Turn the sponge over (to the opposite wide face) and in a similar fashion, use a vertical, overlapping S-pattern to cover the entire surface. Covering the entire area will again take approximately eight passes of the sponge.



3.3.3 **Pass 3:** Turn the sponge so that one of the narrow faces of the sponge will be flat against the surface. Starting in a corner of the template area, use a similar overlapping S-pattern to cover the surface, diagonally toward the opposite corner. Strokes will be 45˚ to the previous two patterns. Upon reaching the center of the surface, flip the sponge over (to the opposite narrow face) and continue sampling the remaining half of the surface using the same technique. Covering the entire area will take approximately 14 passes of the sponge.



3.3.4 **<u>Pass 4:</u>** Finally, using the full width of the sponge tip, wipe the perimeter of the sampling area once, in a circular pattern.



3.4 While the **sampler** is working, the **assistant** will document the layout of the sampling location, which can be done using pictures, drawings, comments, or a combination of the three.

4.0 <u>Sample Recovery</u>

4.1 When the **sampler** has finished, the **assistant** will open a specimen container and hold it out for the **sampler**.

4.2 Without handling or taking the cup from the assistant, the **sampler** will press the sponge into the bottom of the cup and break off the head by moving the handle back and forth until it separates.

4.2.1 The sampler should take care to not touch below the thumb stop on the sponge stick while attempting to break off the head.

4.3 Once the head has been separated into the cup, the **assistant** will then secure the lid, label the container accordingly, and seal the lid with a strip of parafilm.

4.4 The **assistant** will place the cup into a resealable secondary container (one secondary container will hold all the samples from one location).

4.5 The overpack bag and stomacher bag (if included) from each kit will be retained and packed back into their resealable secondary containment (bag, bin, etc.).

4.5.1 Attempt to remove excess air from the samples and secondary containers to aid in repackaging.

4.6 While the **assistant** is packing, the **sampler** will move the template to the next sampling location and then discard their outer glove gloves.

4.7 Steps 2.1 through 4.6 will be repeated for each sample taken.

5.0 Storage and Shipping

Note: The following procedure will be performed prior to storing samples, moving samples offsite, or shipping samples to the laboratory.

5.1 Wipe the internal surfaces of any sample shipping or storage container with a bleach or disinfectant wipe.

5.2 When all samples from a location have been collected, sealed, and placed in secondary containment, wipe down the outside of the secondary containment with bleach or a disinfectant wipes and then move the secondary containers into storage, along with any corresponding overpack and stomacher bags.

5.2.1 Attempt to remove as much excess air from within containers as possible.

5.3 If shipping the samples, place enough cold packs or water ice among the secondary containers to ensure that samples remain cold for the duration of the trip to the processing laboratory.

5.4 Any samples that will not be shipped/moved the day of collection, will be retained in a refrigerator or freezer until they can be shipped.

5.5 Samples will be processed within 48 hours of collection.

APPENDIX B: WORK INSTRUCTION FOR SURFACE SAMPLING USING VACUUM CASSETTE FILTERS

1.0 References

1.1 Miscellaneous Operating Procedure (MOP) 3164: "*Procedure for 37MM Cassette and Trace Evidence Filter Vacuum Sampling of Large and Small Coupons.*" Prepared by Arcadis, Inc. for the National Homeland Security Research Center

2.0 Flow Check

Note: This procedure utilizes pre-prepared 37-mm vacuum cassette sampling kits, which were assembled in accordance with Reference 1.1 above. For information on assembly of these kits refer to this document.

All preparation and sampling will be done in two-person teams consisting of a **sampler** and an **assistant**.

2.1 Start the sampling pump.
2.2 Using a length of Tygon® tubing (approximately 3 ft long), attach a 37-mm cassette, denoted "Flow Check" to the pump.
2.3 Attach a rotameter or equivalent flow measuring device upstream from the cassette and adjust the pump until a flow of 5 ± 0.5 liters per minute is achieved.
2.4 Record the rotameter value as well as the setpoint/flow readout value on the pump.
    2.4.1 This value will be considered representative of the flow through cassettes being used for sampling.
2.5 When pump adjustment is complete, remove the vacuum cassette and leave the length of Tygon tubing attached for later sampling.

3.0 Sampling Preparation

3.1 Before beginning, the **sampler** and **assistant** will don a new pair of gloves over top of their primary personal protective equipment (PPE). At a minimum, the **sampler** will always don a new pair of gloves before beginning a new sample; however, the **assistant** need not change gloves provided they remain unsoiled.
3.2 Next, the **sampler** will obtain a clean 12" x 12" template from the **assistant** and place it over the desired area, if necessary, using pieces of tape on the outside edges to secure the template in place.
    3.2.1 If the surface is unable to accommodate a 12" x 12" sampling area, measure an alternate area equivalent to 144 in^2. The same sampling procedure will be used for the alternate test area.
3.3 When ready to proceed, the **assistant** will open a pre-made cassette kit and remove the sealed cassette bag from within.
3.4 When able, the **assistant** will record the bag collection ID on the sampling log sheet, ensuring that the sample ID matches the current location.

3.5 The **assistant** will then open the cassette bag, and hold it so that the **sampler** can remove the cassette and any components within.

3.5.1 The **sampler** may need to assemble the cassette filters by removing the red plugs, attaching the front nozzle, and attaching a back-PVC adaptor. Ensure that the red plugs are kept clean and saved for later use.

3.6 When the cassette is ready, the **assistant** will ensure that the length of Tygon tubing used in Step 2.2 is attached to the sampling pump and will remove any previously used PVC adaptors from the free end. The free end will then be handed to the **sampler**.

3.7 The **sampler** will connect this end to the vacuum cassette filter using the downstream PVC adaptor on the cassette.

3.7.1 After attaching the tubing, the **sampler** will ensure that the orientation of the cassette is correct, and all fittings are appropriately attached.

3.8 At this time, the **assistant** will obtain a stopwatch in order to time the sampling procedure.

4.0 Sampling

4.1 Each cassette sample will consist of two portions, each lasting 150 seconds for a total of 300 seconds of sampling. For the first portion, the **sampler** will perform horizontal S-strokes across the width of the template at an approximate rate of 3 seconds per pass for a total of approximately 50 passes covering the entire area. During the second portion, the **sampler** will perform vertical S-strokes across the area at a similar rate, for an additional 50 passes covering the entire area. Figure B-1 illustrates the sampling pattern for each portion.

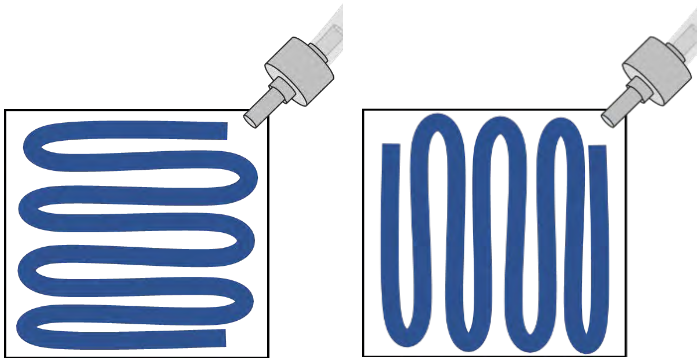


Figure B-1. Mock illustration of the first (left) and second (right) portion vacuum cassette sampling patterns.

4.2 When ready to begin sampling, the **sampler** will position the cassette nozzle in the corner of the template and turn on the pump. Upon initiating flow, the **sampler** will begin vacuuming over the area, using horizontal S-strokes.

4.2.1 The Tygon nozzle should be kept angled such that the tapered end of the nozzle is flush with the surface.

4.3 As soon as the **sampler** begins sampling, the **assistant** will start the stopwatch and monitor the progress of the **sampler**.

4.4 While the **sampler** is working, the **assistant** will document the layout of the sampling location, which can be done using pictures, drawings, comments, or a combination of the three.

4.5 When the first portion (150 seconds) has elapsed, the **assistant** will prompt the **sampler** to change direction of sampling and will continue to monitor progress.

4.5.1 It may be necessary for the **assistant** to call out intermediate times during the sample portions (i.e., halfway, 75 seconds, 30 seconds left) in order to ensure that the **sampler** is keeping a good pace and covering the entire area in the allotted time. At conclusion of the first portion, the **sampler** should return to a corner of the template before continuing with the vertical passes.

4.6 At the conclusion of the second portion (300 seconds), the **assistant** will prompt the **sampler** to stop the pump and conclude the sample.

5.0 Sample Recovery

5.1 After completion of the sample, the **sampler** will remove the nozzle from the cassette and retain it in their hand, while holding the cassette with the opposite hand.

5.1.1 The **sampler** will keep the nozzle only in one hand, designated the "nozzle" hand. To avoid cross contamination, the **sampler** will not swap the nozzle between hands.

5.2 The **assistant** will retrieve a conical tube from the cassette kit, remove its lid, and present the open end to the **sampler**.

5.3 The **sampler** will place the nozzle (adaptor end down) into the tube, while continuing to hold the cassette with their opposite hand.

5.4 The **assistant** will secure the cap on the tube and place it inside the additional, labeled bag inside the cassette kit.

5.5 Next, the **sampler** will use their "nozzle" hand to remove the cassette from the tubing connecting it to the pump. When removed, the **sampler** will hold out the cassette to the **assistant**.

5.5.1 The Tygon tubing will be retained for the next sample in the set; however, the upstream PVC adaptor will be discarded. A new section of Tygon tubing will be used with each new sample set (i.e., new sample location).

5.6 The **assistant** will retrieve the two cassette end plugs, take the cassette from the **sampler**, and use the plugs to seal the ends of the cassette. The cassette will then be placed in the same labeled bag as the conical tube.

5.7 The **assistant** will immediately seal the bag, wipe the outside with a bleach or disinfectant wipe, and place it back into its original secondary containment (i.e. the cassette kit bag).

5.7.1 Attempt to remove as much air as possible before sealing.

5.8 The secondary containment will be resealed by the **assistant** and wiped down with a bleach or disinfectant wipes.

5.8.1 Attempt to remove as much air as possible before sealing.

5.9 The final, double contained sample will be placed into a resealable storage bin/bag.

5.10 The **sampler** will discard their outer gloves.

5.11 Steps 3.1 through 5.9 will be repeated for each sample collected, until the sample matrix is finished.
5.12 If the sample collection is finished for the location, perform a post-set flow check of the pump, following the procedure in Steps 2.1 through 2.5.
5.13 Record the post-set flow in the appropriate data sheet, as well as the set point/readout flow from the pump.

6.0 Storage and Shipping

Note: The following procedure will be performed prior to storing samples, moving samples offsite, or shipping samples to the laboratory.

6.1 Wipe the internal surfaces of any shipping or storage container with a bleach or disinfectant wipe.
6.2 When all samples from a location have been collected, sealed, and placed in secondary containment, wipe down the outside of each secondary containment with bleach or disinfectant wipes and then move the containers into the storage/shipping container.
    6.2.1 Attempt to remove as much excess air from within containers as possible.
6.3 If shipping the samples, place enough cold packs or water ice among the secondary containers to ensure that samples remain cold for the duration of the trip to the processing laboratory.
6.4 Any samples that are not shipped the day of collection, will be retained in a refrigerator or freezer until they can be shipped.
6.5 Samples will be processed within 48 hours of collection.

APPENDIX C: WORK INSTRUCTION FOR WATER WASHDOWN COLLECTION

1. Don clean nitrile gloves.
2. Remove bottles from Ziploc bag (retain bag) and then the lids from the five sterile bottles.
3. Start tap water flow used for boat washdown and allow to run for at least 15 sec to flush the hose before sample collection.
4. Don a second pair of clean nitrile gloves (over the first pair).
5. Target flow rate of 4 ± 1 L/min operating at a source pressure of 30 psig should be determined by filling a 4-L vessel and adjusting flow accordingly.
6. Set spray nozzle setting to produce a small (estimated to < 30-cm-diamter) cone at 1-m distance. Nozzle-to-surface distance will be variable, but attempt to maintain distance of 0.5 to 1.5 m.
7. Note: The washdown does not need to follow a scripted pattern or establish method/protocol, but should be performed in a manner so that the water preferentially flows to the drain from which the sample was collected. The washdown focuses on the collection side of the boat. An estimated area of 4 m^2 should be washed in a < 5-min period. The exterior surface area covered by the washdown of the RBS should cover glass windows, aluminum roof and deck, and non-skid tread on the deck.
8. Fill the 1-L bottles with the washdown water using the pole/bottle holder for sampling from the drains/scuppers on the side of the watercraft. Fill all five bottles consecutively.
9. Turn off water.
10. Doff outer nitrile gloves and don a clean second pair.
11. Secure lids on the bottles and apply parafilm around the lid/bottle interface to help seal and secure the lid.
12. Place each bottle in a Ziploc bag (retain bag).
13. Place bottles in cooler and pack the cold packs around them.
14. Doff all gloves.
15. Fill remaining void volume in cooler with bubble wrap. Seal cooler by wrapping with supplied duct tape.
16. Record the following information on this form:
    a. Date: _____
    b. Start/Stop Time: _____
    c. Operator Name: _____
    d. Air Temperature/Relative Humidity (using supplied logger): _____

Notes/Comments:_____

_____

_____

_____

_____

APPENDIX D: WORK INSTRUCTION FOR GRAVEL SAMPLING

Materials:

- 1-L Nalgene bottle (Thermo Scientific Item# 2187-0032)
- 14” x 10” secondary containment bag
- Nitrile gloves

Field Collection (Hot Zone):

1. At the sampling location, **sampler** and **support** person each don a new pair of gloves.
2. **Support** person obtains the appropriate sample kit, containing one wide-mouth, 1-L, Nalgene bottle; check and record sample number.
3. **Support** person opens the overpack bag, maneuvers Nalgene bottle to the bag opening, and opens Nalgene lid using one hand to hold the bottle through the bag, and the other to twist and remove the lid.

   Note: **Support** person should maintain the lid in one hand and bottle in the other hand throughout the collection. Do not place lid or bottle down and do not remove bottle from overpack bag.
4. While **support** person holds bottle and lid, **sampler** person collects gravel at a depth of 0 to 10 cm using gloved hands.
5. **Sampler** person drops each gravel piece into the bottle without touching the bottle.
6. **Samper** person collects enough gravel to fill the 1-L bottle to the 1/2 full mark.
7. **Support** person places the cap back on the bottle, allows the bottle to drop to the bottom of the overpack bag, and secures the overpack bag opening.
8. **Support** person stores the bottle/bag containing the sample.
9. **Support** person and **sampler** person doff outer pair of gloves.

Field Sampling Kit Preparation:

All materials needed for collection of each sample will be prepared in advance using aseptic technique. A sample kit for a single ballast sample will be prepared as follows:

1. Using a permanent marker, mark the 1/2 full level (83 mm (3.25”) from the bottom) on each 1-L Nalgene bottle.
2. One 1-L, sterile, Nalgene bottle and one 14” x 10” overpack bag will be uniquely labeled as specified in the sample analysis plan.
3. Two additional labels will be added to the overpack bag (these labels will be affixed to the laboratory extraction sample bottle and containment bag upon sample processing).
4. The sterile, labeled, 1-L Nalgene bottle will be added to the overpack bag.
5. Each prepared bag is one sampling kit.

Reference:

Boehm, A. B., J. Griffith, C. McGee, T. A. Edge, H. M. Solo-Gabriele, R. Whitman, Y. Cao, M. Getrich, J. A. Jay, D. Ferguson, K. D. Goodwin, C. M. Lee, M. Madison and S. B. Weisberg (2009). "Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands." J Appl Microbiol 107(5): 1740-1750.

APPENDIX E: WORK INSTRUCTION FOR SOIL SAMPLE COLLECTION

1. Don fresh gloves over base pair. (If multiple samples are to be taken per site, don multiple gloves over base pair.)

3. Open the zip-top bag containing a pre-sterilized, 1-L sterile bottle.

4. Open the bottle, placing the cap open side down on a sterile surface or holding it carefully in your hand. Avoid touching the inside of the cap.

5. Using a small garden hand spade, remove (by scraping) the top 1 to 2 inches of the soil then scoop the soil into the 1-L sterile bottle. Two, 1-L sterile bottles need to be filled with the soil to collect a composite soil sample. Cap bottles with lid and seal with parafilm.

6. Upon return to the laboratory, mix the soil and use a fixed quantity for each sample replicate to analyze.

Figure E-1. Soil collection using hand spade and bottle (and completed soil sample – far right).

7. Wipe down tube with an alcohol wipe, taking care to avoid the label.

8. Place sample bottle into the prepared overpack bag and seal.

9. Wipe down the large zip-top bag with a bleach wipe.

10. Place the zip-top bag into a second zip-top bag for transport.

APPENDIX F: WORK INSTRUCTION FOR VEGETATION SAMPLING

Materials:

- 1-L Nalgene bottle (Thermo Scientific Item# 2187-0032)
- 14” x 10” secondary containment bag
- Nitrile gloves
- Shears

Field Collection (Hot Zone):

1. At the sampling location, **sampler** and **support** person each don a new pair of gloves.
2. **Support** person obtains the appropriate sample kit containing one wide-mouth, 1-L, Nalgene bottle; check and record sample number.
3. **Support** person opens the overpack bag, maneuvers Nalgene bottle to the bag opening, and opens Nalgene lid using one hand to hold the bottle through the bag, and the other to twist and remove the lid.

   Note: **Support** person should maintain the lid in one hand and bottle in the other hand throughout the collection. Do not place lid or bottle down and do not remove bottle from overpack bag.
4. While **support** person holds bottle and lid, the **sampler** person collects vegetation by grabbing a handful of grass and clipping the grass just above the soil using gloved hands.
5. **Sampler** person then places the grass into a 1-L sterile bottle, without touching the bottle.
6. **Samper** person collects enough vegetation to fill two 1-L bottles for each replicate sample.
7. If the grass length exceeds the height of the bottle, the grass should be folded to fit within the bottle.
8. **Support** person places the cap back on the bottle, allows the bottle to drop to the bottom of the overpack bag, and secures the overpack bag opening.
9. **Support** person stores the bottle/bag containing the sample.
10. **Support** person and **sampler** person doff outer pair of gloves.

Field Sampling Kit Preparation:

All materials needed for collection of each sample will be prepared in advance using aseptic technique. A sample kit for a single ballast sample will be prepared as follows:

1. One (1) 1-L, sterile, Nalgene bottle and one 14” x 10” overpack bag will be uniquely labeled as specified in the sample analysis plan.
2. Two (2) additional labels will be added to the overpack bag (these labels will be affixed to the laboratory extraction sample bottle and containment bag upon sample processing).
3. The sterile, labeled 1-L Nalgene bottle will be added to the overpack bag.
4. Each prepared bag is one sampling kit.

APPENDIX G: WORK INSTRUCTION FOR FORMULATIONS OF RECIPES USED IN BIOLOGICAL TEST METHODS

Spore Production

Table 1. Components of Modified G Sporulation Medium

Ingredient	Amount/L
Yeast Extract	2.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCl_2 \bullet 2H_2O$	0.03 g
$CuSO_4 \bullet 5H_2O$	0.005 g
$FeSO_4 \bullet 7H_2O$	0.0005 g
$MgSO_4 \bullet 7H_2O$	0.2 g
$MnSO_4 \bullet H_2O$*	0.06 g
$ZnSO_4 \bullet 7H_2O$	0.005 g
K_2HPO_4	0.5 g
dH_2O	1000 mL

*$MnSO_4 \bullet H_2O$ substituted for $MnSO_4 \bullet 4H_2O$. If $MnSO_4 \bullet 4H_2O$ is used, add 0.05 g.

Table 2. Real-Time PCR Assay Conditions

Component	Volume for One Reaction (µL)
TaqMan Fast Advanced Master Mix (Applied Biosystems, Cat. 4444556)	12.5
Platinum Taq Polymerase (Invitrogen, Cat. 10-966-034)	0.1
Btk T1B2 Forward Primer (25 µM)	1.0
Btk T1B2 Reverse Primer (25 µM)	1.0
Btk T1B2 Probe (2 µM)	1.0
PCR Grade Water	4.4
Template	5.0
Total Volume	25

APPENDIX H: WORK INSTRUCTION FOR SPIKING WITH *BACILLUS THURINGIENSIS KURSTAKI (Btk)* HD-*1 T1B2* SPORES

I. PURPOSE/SCOPE

To spike Sponge Stick Wipes (SSW), Vacuum Filter Cassettes (VFC), and Grab (GRB) samples for spore recovery testing.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.
Bacillus thuringiensis kurstaki (*Btk*) HD-1 T1B2 Stock (2×10^8 CFU/mL)	In house	HD1.T1B2.120919	TBD	2-8 °C
Sterile Deionized (DI) Water				RT
TSA				2-8 °C
1.5- or 2-mL Tubes	Eppendorf		N/A	RT
Sterile Forceps	N/A	N/A	N/A	RT
VFC	SKC	18109-7E1-219	N/A	RT
Sponge Stick	3M			RT
Specimen Cup	N/A			RT

Equipment

Item	Manufacturer	Serial Number	Thermometer/Rees #	Calibration Due
Biosafety Cabinet (BSC)	The Baker Company		N/A	
Micropipette Type:L1000	Rainin		N/A	
Micropipette Type:L200	Rainin		N/A	
Micropipette Type:L10 or L20	Rainin		N/A	
Refrigerator	Fisher			

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette filter tips
- Biohazard bags

Performed by: _____ Date: _____

III. PROCEDURE

A. Decontaminate the BSC with DNA Erase; bleach and isopropanol prior to use.

B. Name SSW, VFC, and GRB samples as follows:

1. Label each sample with sample ID per the following:

i. AAA-BBB-CCC-DDD

1. AAA = Sample #
2. BBB = Sample Type
3. CCC = Location
4. DDD = Spore Spike Level

ii. Electronically populate table below with sample names to be prepared on each day from the Sample Log.

Sample #	Sample Type	Location	Filter Vial Type	Spore Spike Level (CFU)	Sample ID
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					

C. Spike Samples

1. Prepare Spiking Stocks

i. Fill in information from stock tube.

Organism	Lot	Prep date	Concentration
Btk HD-1 T1B2	HD1.T1B2.120919	12/09/2019	2 X 10^8 cfu/mL

Performed by: _____ Date: _____

ii. Target stock concentrations:

Concentration	Total Spores per 100 µL
3.0 X 10^6 cfu/mL	300,000
3.0 X 10^5 cfu/mL	30,000
3.0 X 10^4 cfu/mL	3,000
3.0 X 10^3 cfu/mL	300

iii. Prepare dilutions of stock in sterile DI water. Vortex stock on high for 30 seconds prior to preparing dilutions.

Show calculations:

Dilution 1: (2.0 X 10^8 cfu/mL)*(X)=(3.0 X 10^7 cfu/mL)(1 mL) → 150 µL of stock into 850 µL H_2O

Dilution 2: (3.0 X 10^7 cfu/mL)*(X)=(3.0 X 10^6 cfu/mL)(1 mL) → 100 µL of Dilution 1 into 900 µL H_2O

Dilution 3: (3.0 X 10^6 cfu/mL)*(X)=(3.0 X 10^5 cfu/mL)(1 mL) → 100 µL of Dilution 2 into 900 µL H_2O

Dilution 4: (3.0 X 10^5 cfu/mL)*(X)=(3.0 X 10^4 cfu/mL)(1.5 mL) → 150 µL of Dilution 3 into 1,350 µL H_2O

Dilution 5: (3.0 X 10^4 cfu/mL)*(X)=(3.0 X 10^3 cfu/mL)(1.5 mL) → 150 µL of Dilution 4 into 1,350 µL H_2O

Dilution 6: (3.0 X 10^3 cfu/mL)*(X)=(3.0 X 10^2 cfu/mL)(1.5 mL) → 150 µL of Dilution 5 into 1,350 µL H_2O

2. Spike Sponge Sticks

i. Position sponge in specimen cup so that the dirty side is facing up. Change forceps between samples.

ii. Prior to spiking sponges, vortex the stock for 30 seconds.

iii. Per sponge, transfer a 120-µL aliquot of the appropriate Stock tube (Dilution 4 for 3,000 CFU and Dilution 5 for 300 CFU) into a 1.5-ml tube.

iv. Place ten (10) 5-µL droplets onto each side of the sponge stick (20 5-µL droplets total), being as careful as possible to avoid having spiked surfaces contact the specimen cup wall. Position sponge as shown in Figure H-1. The same pipet tip can be used to place all 20 droplets; dispose of the 120 µL aliquot once each sponge has been spiked.

v. Seal the specimen cup and process immediately using spore recovery Work Instruction (WI) or store @ 2 to 8 °C.

2-8 °C Start time: _____ Date/Initials: _____

Performed by: _____ Date: _____

3. Spike VFCs
    i. Wipe each cassette with 10% bleach solution or bleach wipes followed by a clean Kimwipe and discard wipes into an autoclavable biohazard bag.
    ii. Prior to spiking filters, vortex the stock for 30 seconds.
    iii. Per VFC, transfer a 120-µL aliquot of the appropriate Stock tube (Dilution 4 for 3,000 CFU and Dilution 5 for 300 CFU) into a 1.5-ml tube.
    iv. Remove the red plug and apply 20 5-µL droplets onto each filter as shown in Figure H-2. The same pipet tip can be used to place all 20 droplets; dispose of the 120-µL aliquot once each VFC has been spiked.
    v. Seal the VFC and process immediately using spore recovery WI or store @ 2 to 8 °C.

    2-8 °C Start time: _____ Date/Initials: _____

4. Spike Wash Water Grab Samples
    i. Wipe 1-L container with 10% bleach solution or bleach wipes.
    ii. Prior to spiking samples, vortex the stock for 30 seconds.
    iii. Per wash water grab sample, transfer a 100-µL aliquot of the appropriate Stock tube (Dilution 3 for 30,000 CFU, Dilution 4 for 3,000 CFU and Dilution 5 for 300 CFU) into a 1.5-ml tube containing 400 µL sterile H_2O, for 500 µL volume total. Mix by vortex.
    iv. Apply twenty (20) 25-µL droplets into the wash water liquid and pipette tip submerged slightly into wash water. The same pipet tip can be used to add all 20 droplets.
    v. Seal the container and process within 1 hour of spiking or store @ 2 to 8 °C.

    2-8 °C Start time: _____ Date/Initials: _____

5. Spike Gravel Grab Samples
    i. Wipe a 1-L container with 10% bleach solution or bleach wipes.
    ii. Prior to spiking samples, vortex the stock for 30 seconds.
    iii. Per gravel grab sample, transfer a 120-µL aliquot of the appropriate Stock tube (Dilution 4 for 3,000 CFU and Dilution 5 for 300 CFU) into a 1.5-ml tube.
    iv. Apply twenty (20) 5-µL droplets onto the surface of the gravel (top layer) as shown in Figure H-3. The same pipet tip can be used to place all 20 droplets; dispose of the 120-µL aliquot once each gravel grab sample has been spiked.
    v. Seal the 1-L container and process immediately using spore recovery WI or store @ 2 to 8 °C.

    2-8 °C Start time: _____ Date/Initials: _____

Performed by: _____ Date: _____

6. Spike Grass Grab Samples
    i. Wipe a 1-L container with 10% bleach solution or bleach wipes.
    ii. Prior to spiking filters, vortex the stock for 30 seconds.
    iii. Per grass grab sample, transfer a 100-µL aliquot of the appropriate Stock tube (Dilution 3 for 30,000 CFU, Dilution 4 for 3,000 CFU and Dilution 5 for 300 CFU) into a 1.5-ml tube containing 400 µL sterile H_2O, for 500 µL volume total. Mix by vortex.
    iv. Apply twenty (20) 25-µL droplets onto the surface of the grass to cover as many grass surface areas as possible as shown in Figure H-4. The same pipet tip can be used to place all 20 droplets.
    v. Seal the 1-L container and process within 1 hour of spiking or store @ 2 to 8 °C.

    2-8 °C Start time: _____ Date/Initials: _____

7. Spike Soil Grab Samples
    i. Wipe a 50-mL container with 10% bleach solution or bleach wipes.
    ii. Prior to spiking samples, vortex the stock for 30 seconds.
    iii. Per soil sample, transfer a 100-µL aliquot of the appropriate Stock tube (Dilution 2 for 300,000, Dilution 3 for 30,000, or Dilution 4 for 3,000 CFU) onto the 10-g soil aliquot in a dropwise fashion to distribute the spike throughout the soil sample. Mix by vortex.
    iv. Seal the 50-mL container and process within 1 hour of spiking or store @ 2 to 8 °C.

    2-8 °C Start time: _____ Date/Initials: _____

8. Enumerate stock
    i. Spread 100-µL aliquots of Dilutions 5 and 6 onto TSA in triplicate.
    ii. Incubate plates
        1. Invert the plates and incubate them at 30°C ± 2°C for 18 to 24 hours. *Btk* produces flat or slightly convex, 2 to 5 mm colonies, with edges that are slightly irregular and have a “ground glass” appearance.

        Incubation start Date/Time: _____ Initials: _____

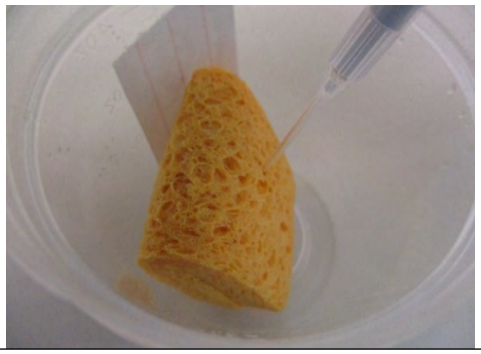
        Incubation end Date/Time: _____ Initials: _____

Performed by: _____ Date: _____

iii. Plate counts

1. Record counts in the table below.

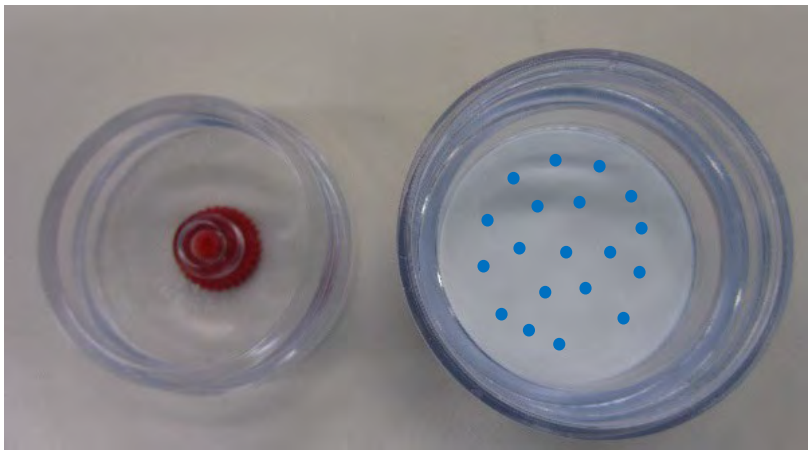
Dilution #	Media Type	Volume/ (Dilution on Plate)	Plate Counts			Average Counts	CFU/mL
			Plate 1	Plate 2	Plate 3		
5 (3.0 X 10^3 cfu/mL)	TSA	100 µL/ (10^{-1})					
6 (3.0 X 10^2 cfu/mL)	TSA	100 µL/ (10^{-1})					



Position with folded side up or stick side up. Do not spike the sides of the sponge that could contact the specimen cup wall.

Place ten (10) 5-µL evenly dispersed droplets on each side for a total of twenty (20) 5-µL droplets.

Figure H-1. Spiking Diagram for Sponge Sticks



Place twenty (20) 5-µL drops evenly dispersed onto filter.

Figure H-2. Spiking Diagram for VFC

Performed by: _____ Date: _____

Figure H-3. Spiking of Gravel Grab Samples

Figure H-4. Spiking of Grass Grab Samples

Reviewed by: _____ Date: _____

Performed by: _____ Date: _____

APPENDIX I: WORK INSTRUCTION FOR *BACILLUS THURINGIENSIS KURSTAKI (Btk) T1B2* SPORE RECOVERY FROM MARITIME SAMPLES – SPONGE STICKS, VACUUM CASSETTES, AND GRAB SAMPLES

I. PURPOSE/SCOPE

To recover *Bacillus thuringiensis kurstaki* (*Btk) T1B2* spores from Sponge-Stick Wipes (SSW), Vacuum Cassette Filters (VFC), and Grab (GRB) samples.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials and Date
Extraction Buffer with Tween 20 + 30% Ethanol	In-house			2-8 ºC	
Phosphate Buffered Saline with 0.05% Tween 20 (PBST)	Teknova			RT	
Stomacher Lab Blender Bags	Seward		N/A	RT	
Stomacher Bag Racks	Seward	BA6096	N/A	RT	
10X PBS	Teknova			2-8 ºC	
1X PBS (pH 7.4)	Teknova			2-8 ºC	
Brain Heart Infusion Broth (BHIB)	BD			2-8 ºC	
Conical Tubes 15-mL			N/A	RT	
Conical Tube 50-mL	Falcon		N/A	RT	
Screw Top Flask 250 mL	Corning		N/A	RT	
0.45-µm Filter Vials	Whatman		N/A	RT	
2-mL Tubes				RT	
Sterile Forceps	Unomedical		N/A	RT	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer / Rees #	Calibration Due	Initials/ Date
Biosafety Cabinet (BSC)	The Baker Company	57553 57544	N/A		
Micropipette Type: L1000	Rainin		N/A		
Incubator Shaker	New Brunswick	590644988			
Refrigerator					
Swinging Bucket Centrifuge	Beckman Coulter	X59221	N/A	N/A	
Stomacher	Seward	40142	N/A	N/A	
Sonicator Bath	Bransonic	RNC010140514E	N/A	N/A	
Water Bath					

N/A = Not Applicable

Other Supplies and Equipment

- Forceps
- Biohazard bags
- Bleach
- 5-mL, 25-mL, and 100-mL serological pipets
- Pipette aid
- Ziplock bags
- Stainless Steel SureSeal Cassette Opener, SKC Cat. 225-13-5A

Filters – Electronically update this table with sample names from the Sample Log

Sample #	Sample Type	Location	Filter Vial Type	Spore Spike Level (CFU)	Sample ID
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					

III. PROCEDURE

A. Sample Processing: Spore Recovery for Sponge-Stick Samples

Note: Process samples from negative control to high inoculation level. Change gloves when working from an inoculated sample to a sample containing a lower inoculation level, or if contamination of gloves is suspected. Pre-aliquot reagents from the kit to prevent contamination of reagents between runs.

1. Prior to sample processing, prepare the following items:

- Fill sample tube rack with 50-mL screw cap conical tubes and label as appropriate; two 50-mL conical tubes are required per sample.
- In a BSC, attach the vacuum manifold to the vacuum trap, waste container (with 400 mL of bleach), and vacuum source. Attach the filter vials to the manifold, using outer rows first. Verify that all filter vials are completely pushed down. Place a red pull tab tapered plug in each filter vial.
- Document filter vial and sample tube labels.
- Extraction Buffer **with** Tween 20 + Ethanol (1,500 mL) will be needed per set of 16 samples (90 mL per sample).
- High salt wash buffer (10x PBS) (225-mL aliquot) in a 250-mL screw capped bottle per set of 16 samples (12.5 mL per sample).

- Low salt wash buffer (1x PBS) (225-mL aliquot) in a 250-mL screw capped bottle per set of 16 samples (12.5 mL per sample).

2. Add 90 mL cold (4°C) Extraction Buffer **with** Tween 20 + Ethanol to each Stomacher bag.

3. Using sterile forceps, remove the remaining portion of the sponge-stick handle and unfold the sponge.

4. Aseptically add a sponge-stick to a Stomacher bag. Open one bag at a time; close and seal bag prior to moving to the next sample. **Note: Save specimen cup for broth enrichment of sponge.**

5. Place an unsealed bag containing a sample into the Stomacher so the sponge rests evenly between the homogenizer paddles and stomach each sample for 1 min at 260 rpm (Figure I-1). Open the door of the Stomacher and remove the bag. Reseal bag.

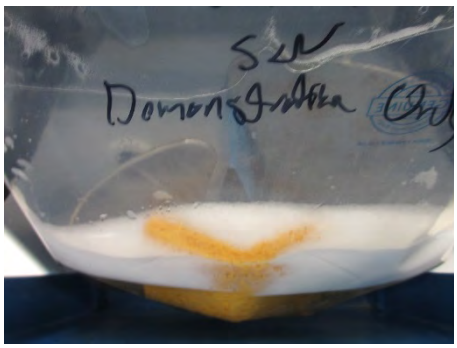


Figure I-1. Sponge is opened and centered between paddle positions.

6. Stomach all sponges; removal of bag from Stomacher begins the settle time. Allow bags to sit for 10 min to allow elution suspension foam to settle.

7. Grab the sponge from the outside of the bag with hands. With the bag closed, move the sponge to the top of the bag while using hands to expel liquid from the sponge.

8. Open the bag, remove sponge, and place into a labeled specimen cup using sterile forceps. Store sponge at 2 to 8°C until enrichment in BHIB (See WI #7 Appendix N: Work Instruction for BHIB Enrichment for Culture).

9. Follow steps described above for each sample, changing forceps between samples.

10. Gently mix the suspension in the Stomacher bag up and down three times with a sterile 50-mL pipet. Remove half of the suspension volume (~45-46 mL) and place it in a 50-mL screw cap centrifuge tube (Aliquot 1). Place the remaining suspension (~45-46 mL) into a second 50-mL tube (Aliquot 2). Adjust the suspension volumes so that volume is equal in both tubes.

11. Process the suspension for each sample, as described above.

12. Place 50-mL tubes into sealing centrifuge buckets and decontaminate centrifuge buckets before removing them from the BSC.

13. Centrifuge tubes at 3,500 × g with the brake off for 15 min in a swinging bucket rotor at 4°C.

14. Each sample has two pelleted aliquots (Aliquot 1 and Aliquot 2). Using a sterile 50-mL pipet, remove the supernatant from Aliquot 1 and discard it in an autoclavable leak-proof biohazard container. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the bottom of the tube. Stop pipetting when meniscus reaches the 5-mL gradation level of on 50-mL Falcon tube, leaving ~2 to 3 mL in each tube. Next, using the same pipet, remove 20 mL of supernatant from Aliquot 2 and add it to Aliquot 1 pellet. Discard the remaining supernatant from Aliquot 2 into an autoclavable leak-proof biohazard container.

15. Vortex Aliquot 1 (containing ~22 mL of supernatant) for 30 sec to resuspend the pellet, then transfer entire volume to Aliquot 2.

16. Vortex Aliquot 2 for 30 sec to resuspend the pellet. This pooled suspension of ~25 mL will be used for culture and RV-PCR analytical methods. Record total volume for each sample in Table I-1.

Table I-1. Volume of Sample Recovered from Sponge Sticks.

Sample Number	Sample ID	Total Volume Recovered from Sponge-Stick	Recorded by:
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			

17. Transfer 11 mL of the pooled extract and store on ice or in refrigerator until processed on same day using WI #4 Appendix J: Work Instruction for Culture of Recovered Spores.

18. Place manifold and Whatman Autovial filter vials with red caps in BSC. Label all filter vials. Record filter vial labels.

19. Vortex each RV-PCR aliquot and allow 3 to 5 min of settle time to avoid loading large particulates into filter vial. Transfer 12.5 mL of the pooled suspension volume from each

tube to the corresponding labeled filter vial by lifting red cap slightly. Change serological pipets and gloves between samples.

20. Complete filtration of liquid through filter vials. Turn off vacuum pump.

Note 1: At 15 min post-sample addition, if sample has not completely passed through the filter vial, a reduced volume of high salt wash buffer will be added (5 mL) to avoid prolonged filtering delays. It is desired to add a lower volume of each salt wash buffer (10X and 1X) than to omit one or both entirely.

Note 2: At 1 hour post-sample addition, if sample has not completely passed through the filter vial, the high salt and low wash steps will be omitted.

Sample #	Sample ID	Sample Addition		Volume of Wash Buffers		Recorded by:
		Start Time[1]	End Time[2]	10X	1X	
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						

[1] Record the time of adding the final sample to filter vial.
[2] Record end time for samples that have clogging and meet the criteria in Notes 1 and 2 above.

21. Proceed to RV-PCR processing section (Section E) below, with filter vial manifold.

B. Sample Processing: Spore Recovery for VFC Samples

Note: Process samples from negative control to high inoculation level. Change gloves when working from an inoculated sample to a sample containing a lower inoculation level, or if contamination of gloves is suspected. Pre-aliquot reagents to prevent contamination of reagents between runs.

1. Prior to sample processing, prepare the following items:

- Fill sample tube rack with 15-mL screw cap conical tubes and label as appropriate, each containing 11 mL sterile Extraction Buffer **with** Tween 20 + Ethanol.

- One labeled 2-oz sterile cup with lid per sample, sterilized by autoclave (gravity cycle, 121°C for 15 min).
- In a BSC, attach the vacuum manifold to the vacuum trap, waste container (with 250 ml of bleach), and vacuum source. Attach the filter vials to the manifold, using outer rows first. Verify that all filter vials are completely pushed down. Place a red pull tab tapered plug in each filter vial.
- Document filter vial and sample tube labels.

2. For each 37-mm filter cassette, prepare one 15-mL conical tube containing 11 mL of sterile Extraction Buffer **with** Tween 20 + Ethanol and label one 2-oz sterile cup.

3. In the BSC, remove the conical tube containing the nozzle and the cassette from the containment bags and wipe the outside of the conical tube with a disinfectant and place it into a rack. Aseptically add 5 mL of Extraction Buffer **with** Tween 20 + Ethanol (from the 11 mL of a pre-measured aliquot of PBST + Ethanol [PBSTE]) to the conical tube containing the nozzle and tubing and set aside.

4. Remove the band from around the cassette using sterile scissors. Wipe each cassette with 10% bleach solution or bleach wipes followed by a clean Kimwipe and discard wipes into an autoclavable biohazard bag.

5. Remove the red plug from the inlet of the cassette; the plug on the back side should be kept in place. Using a pipette dispense 2 mL of Extraction Buffer **with** Tween 20 + Ethanol from the tube now containing the 6 mL into the cassette and replace plug. Roll the cassette around to allow the liquid to touch all surfaces of the inside of the cassette. If there is a large quantity of particulate matter, more Extraction Buffer **with** Tween 20 + Ethanol may be required. Particulate matter should be dampened enough to prevent aerosolization.

6. Using the cassette tool, pry open the top section of the cassette, using care not to spill the Extraction Buffer **with** Tween 20 + Ethanol inside the cassette and set aside, plug side down as shown in Figure I-2. Set the bottom portion containing the filter aside carefully (filter side up), and using a pipette rinse the walls of the cassette with 2 mL of Extraction Buffer **with** Tween 20 + Ethanol. Transfer the rinsate using the same pipette to the appropriately labeled 2-oz sterile cup.

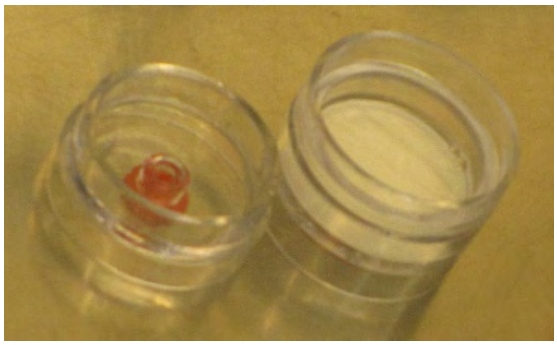


Figure I-2. Vacuum Cassette with Top Section Removed

7. Using the cassette tool, remove the middle section of the cassette (this piece is holding the filter in place) and place on top of top section as shown in Figure I-3. Using sterile forceps aseptically remove the filter without picking up the support filter underneath. Place the filter in the 2-oz cup with the rinsates.

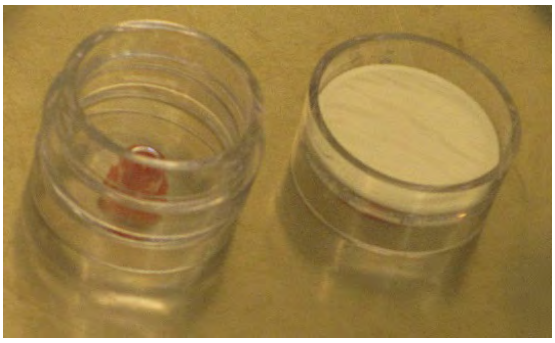


Figure I-3. Vacuum Cassette with Top and Middle Sections Removed

8. Use the remainder of the 6 mL Extraction Buffer **with** Tween 20 + Ethanol to rinse walls of the middle and top sections (configuration shown in Figure I-3, image on left) of the cassette and transfer rinsate to 2-oz cup. Discard the cassette sections, support filter, plugs, and transfer pipette in an autoclavable biohazard bag.

9. Disinfect the outside of the 2-oz cup with 10% bleach solution and place in tray. Decontaminate the BSC with 10% bleach solution and don a fresh pair of gloves in between samples. Repeat procedure described above for each 37-mm filter cassette.

10. Seal the conical tubes containing 5 mL Extraction Buffer **with** Tween 20 + Ethanol, tubing, and nozzle with Parafilm. Place the rack of conical tubes into the sonicating bath to a level that allows at least 1 inch (~2.5 cm) of tube to be above the water line. Place a weight on top of the tubes to prevent them from floating or tipping over. Sonicate for 1 min and remove tubes from the sonicating bath. Dry and disinfect each tube with a 10% bleach solution.

11. Vortex the conical tubes 2 min using platform vortex at Setting 10 (high setting), then transfer the 5 mL Extraction Buffer **with** Tween 20 + Ethanol to the appropriate 2-oz cup. To transfer volume, use 1-mL micropipette to remove volume collected in the tubing nozzle, then use pipette tip to remove nozzle from the 15-mL conical tube. Before disposing of nozzle, depress pipette piston to expel any remaining extract volume from the nozzle into the 15-mL conical tube (See Figure I-4).

Figure I-4. Nozzle Removal Using 1-mL Pipette

12. Seal all of the 2-oz cups with Parafilm. Place the rack of 2-oz cups in the sonicating bath and cover with a weight on top of the cups to prevent them from floating or tipping over. There should be 1 inch (~2.5 cm) between the level of the water and the cup lids. Sonicate for 3 min without heat. Remove rack from the bath and dry each cup with a Kimwipe and place in the BSC. Place cups in a sealable plastic lidded box.

13. Using a 10-mL serological pipet, transfer as much suspension as possible from each 2-oz cup to a 15-mL conical tube. Record total volume for each sample in Table I-2. **Note: Save 2-oz cups containing filter.** Store at 2 to 8 °C until enrichment in Trypticase Soy Broth on same day (See WI #7 Appendix N: Work Instruction for BHIB Enrichment for Culture).

Table I-2. Volume of Sample Recovered from VFC

Sample Number	Sample ID	Total Volume Recovered from VFC	Volume Available per Analytical Method (Total Volume ÷ 2)	Recorded by:
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				

14. Vortex each sample, then allow 3 to 5 min settle time to avoid transferring large particulates into filter vial and cause clogging. Transfer half (~5 mL) volume of each sample to corresponding labeled filter vial. Change serological pipets between each sample.

15. Store the remaining half (~5 mL) of the pooled extract for microbiological analysis (WI #4 Appendix J: Culture of Recovered Spores). Store aliquot on ice or in refrigerator until processed on same day. Change serological pipets between each sample.

16. Complete filtration of liquid through filter vials. Turn off vacuum pump.

Note 1: At 15 min post-sample addition, if sample has not completely passed through the filter vial, a reduced volume of high salt wash buffer will be added (5 mL) to avoid prolonged filtering delays. It is desired to add a lower volume of each salt wash buffer (10X and 1X) than to omit one or both entirely.

Note 2: At 1 hour post-sample addition, if sample has not completely passed through the filter vial, the high salt and low wash steps will be omitted.

Sample #	Sample ID	Sample Addition		Volume of Wash Buffers		Recorded by:
		Start Time[1]	End Time[2]	10X	1X	
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						

[1] Record the time of adding the final sample to filter vial.
[2] Record end time for samples that have clogging and meet the criteria in Notes 1 and 2 above.

C. Spore Recovery for Grab Samples (Wash Water, Gravel Vegetation - See Section D for Soil Samples)

Note: Process samples from negative control to high inoculation level. Change gloves when working from an inoculated sample to a sample containing a lower inoculation level, or if contamination of gloves is suspected. Pre-aliquot reagents to prevent contamination of reagents between runs.

1. Prior to sample processing, prepare the following items:

- In a BSC, attach vacuum manifold to waste container containing appropriate amount of bleach for a final concentration of 1% NaOCl after collecting all waste fluids.
- In a BSC, attach the RV-PCR vacuum manifold to the vacuum trap, waste container (with 500 mL of bleach), and vacuum source. Attach the filter vials to the manifold, using outer rows first. Verify that all filter vials are completely pushed down. Place a red pull tab tapered plug in each filter vial.
- Document filter vial and sample tube labels.

2. Add 20 mL of Extraction Buffer with PBSTE to a MicroFunnel unit with 0.45-µm GN-6 Metricel membrane (Pall ID: 4800 or equivalent); this filtration unit will be referred to as the filter unit. Apply vacuum after PBSTE completely passes through membrane, turn off vacuum, and apply the membrane to a Trypticase Soy Agar plate using sterile forceps. This sample will serve as a negative control. Incubate this control at 30°C overnight and check for sterility.

Start time _____ End time _____ Sterility (Yes/No) _____

3. Weigh grab samples before and after extraction and record weights below.

Sample Number	Sample ID	Pre-Extraction Weight (Grams)	Post-Extraction Weight (Grams)	Recorded by:
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				

4. Carefully add 500 mL of sterile Phosphate Buffered Saline with 0.05% Tween 20 (PBST) to each 1-L sample bottle. Parafilm seal lid and place into secondary bag with absorbent.
5. Shake vigorously for 2 min by hand.
    a. Grasp 1-L sample bottle with one hand on bottom of bottle, the other hand around the bottle near the top. Hold bottle over shoulder and shake vigorously back and forth.
6. Allow sample to settle for 30 sec.
7. Pour off eluent into clean, labeled, 500-mL container. Ensure sample labels for each collection bottle match their respective eluent bottle.
8. Vigorously mix 0.5 L grab eluate aliquots by hand for 30 sec. Allow 30 sec of settle time.
9. Pour mixed grab eluate into filter unit to the 100-mL gradation line.
10. Apply vacuum until entire 100 mL passes through membrane. Once complete, break vacuum pressure, then close valve.
11. Repeat Steps 8 through 10 five times with an additional 100 mL of grab eluate, for a total of 500 mL of grab eluate (250 mL for gravel) onto a single 47-mm filter.

    Note: If filter becomes clogged, less than 500 mL of sample (250 mL for gravel) will be processed. Record volume filtered in the table below. At 30 min post-sample addition, if sample has not completely passed through the filter, the remaining volume in the filter unit will be removed.

Sample #	Sample ID	Filtration Start Time	Filtration End Time	Total Volume Filtered	Recorded By:
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					

12. Remove the filter membrane using sterile forceps and transfer to a 50-mL conical tube. Position the membrane in the bottom half of the conical tube with the inlet side of the membrane facing the center of the tube. Avoid placing the filter into the conical portion of the tube.

13. Repeat Steps 5 through 12 for all samples.

14. Add 10 mL of PBSTE to 50-mL conical tubes containing membrane filters.

15. Vortex at maximum speed on platform vortex in 10-sec bursts for 2 min to dislodge spores.

16. Let tubes settle for 2 min, then transfer volume to a clean 50-mL conical tube.

17. Repeat extraction of each membrane filter by adding another 10 mL of PBSTE to the 50-mL conical tube with membrane.

18. Repeat Steps 15 and 16, transferring volume to the same 50-mL conical tube per sample for a total recovered pooled spore recovery volume of 20 mL.

Note: Save filter membrane. Store at 2 to 8°C until enrichment in BHIB on same day (See WI #7 Appendix N: Work Instruction for BHIB Enrichment for Culture).

19. Vortex each 20-mL sample, then allow 30 sec of settle time to avoid transferring large particulates into RV-PCR filter vial. Transfer half (10 mL) volume of each sample to corresponding labeled RV-PCR filter vial.

20. Store the remaining half (10 mL) of the pooled spore recovery volume for microbiological analysis (WI #4 Appendix J: Work Instruction for Culture of Recovered Spores). Store aliquot on ice or in refrigerator until processed on same day.

21. Complete filtration of liquid through RV-PCR filter vials. Turn off vacuum pump.

Note 1: At 15 min post-sample addition, if sample has not completely passed through the filter vial, a reduced volume of high salt wash buffer will be added (5 mL) to avoid prolonged filtering delays, It is desired to add a lower volume of each salt wash buffer (10X and 1X) than to omit one or both entirely.

Note 2: At 1 hour post-sample addition, if sample has not completely passed through the filter vial, the high salt and low wash steps will be omitted.

Sample #	Sample ID	Sample Addition		Volume of Wash Buffers		Recorded by:
		Start Time[1]	End Time[2]	10X	1X	
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						

[1] Record the time of adding the final sample to filter vial.
[2] Record end time for samples that have clogging and meet the criteria in Notes 1 and 2 above.

D. Spore Recovery for Soil Samples

Note: Process samples from negative control to high inoculation level. Change gloves when working from an inoculated sample to a sample containing a lower inoculation level, or if contamination of gloves is suspected. Pre-aliquot reagents to prevent contamination of reagents between runs.

1. Prior to sample processing, prepare the following items:

- Set one water bath to 75 to 80 C, and a second to 70 to 75°C (optional).
- In a BSC, attach the RV-PCR vacuum manifold to the vacuum trap, waste container (with 500 mL of bleach), and vacuum source. Attach the filter vials to the manifold, using outer rows first. Verify that all filter vials are completely pushed down. Place a red pull tab tapered plug in each filter vial.
- Document filter vial and sample tube labels.
- Weigh 10 ± 0.1 g soil aliquots in 50-mL tubes.

Sample #	Sample ID	Sample Weight (g)	Weighed/Recorded by:
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			

2. Add 40 mL of PBST to each 10-g soil sample. Parafilm tubes.

3. Vortex samples for 30 sec.

4. Sonicate samples for 10 min in bath sonicator.

5. Manually mix each sample for 2 min.

6. Spin at 1,000 x g for 5 min.

7. Transfer supernatant to a clean 50-mL tube (leaving ~2.5 mL of supernatant with each pellet). Save pellet aliquots for heat shock.

8. Measure pH of supernatant using pH strips. Parafilm tubes.

Sample #	Sample ID	Volume Recovered per Sample (mL)	Sample pH	Recorded by:
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				

9. Heat shock supernatant and pellets at 70 ± 2°C for 1 hour with intermittent mixing of tubes. One-hour heat time starts when pilot tube containing 40 mL of PBST reaches 70 ± 2°C. Once pilot tube reaches temperature, turn down water bath to avoid heating above 70 ± 2°C, or transfer to second water bath set to 70°C. Post-heat shock and allow samples to cool to ambient temperature. Measure temperature of pilot tube to assess return to ambient temperature.

Batch 1: Heat Start Time _____ Heat End Time _____

Time Ambient Temperature Reached _____

Batch 2: Heat Start Time _____ Heat End Time _____

Time Ambient Temperature Reached _____

10. Split supernatant in half, ~20 mL for culture and ~20 mL for RV-PCR analysis.

11. Vortex each ~20-mL RV-PCR aliquot, then allow 30 sec of settle time to avoid transferring large particulates into RV-PCR filter vial. Transfer 12.5 mL volume of each sample to corresponding labeled RV-PCR filter vial. Add additional volume to filter vials if clogging does not occur, up to full 20 mL.

12. Store culture aliquot (~20 mL) and pellet at 2 to 8°C for microbiological analysis (WI #4 Appendix J: Culture of Recovered Spores).

13. Complete filtration of liquid through RV-PCR filter vials. Turn off vacuum pump.

Note 1: At 15 min post-sample addition, if sample has not completely passed through the filter vial, a reduced volume of high salt wash buffer will be added (5 mL) to avoid

prolonged filtering delays. It is desired to add a lower volume of each salt wash buffer (10X and 1X) than to omit one or both entirely.

Note 2: At 1 hour post-sample addition, if sample has not completely passed through the filter vial, the high salt and low wash steps will be omitted.

Sample #	Sample ID	Sample Addition		Volume of Wash Buffers		Recorded by:
		Start Time[1]	End Time[2]	10X	1X	
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						

[1] Record the time of adding the final sample to filter vial.
[2] Record end time for samples that have clogging and meet the criteria in Notes 1 and 2 above.

E. RV-PCR Sample Processing: Buffer Washes and Broth Culture

1. Place into BSC: a Ziplock bag with orange caps (one per filter vial), 10-mL serological pipets and cold (4°C) 10X PBS in 250-mL screw cap bottle.

2. Transfer 12.5 mL of cold (4°C) **high salt wash buffer (10x PBS)** to each filter vial using a 10-mL serological pipet. Change pipet and gloves between each sample.

3. Complete filtration of liquid through the filter vials.

4. Place into the BSC: 10-mL serological pipets and cold (4°C) 1X low salt wash buffer in 250-mL screw cap bottle.

5. Transfer 12.5 mL cold (4°C) **low salt wash buffer (1x PBS)** to each filter vial using a 10-mL serological pipet. Change pipet and gloves between each sample.

6. Complete filtration of liquid through filter vials. Turn off vacuum pump.

7. Using an Allen wrench, unscrew the top of the manifold and break the seal on manifold using a plate sealer to separate the top of the manifold.

8. Using a tray preloaded with caps, move the top of the manifold with the filters still in place and firmly press down, capping the bottoms of the filters. Repeat pressing down on each filter vial to ensure a good seal.

9. Place bleach soaked wipes onto the manifold to soak up the filtered waste and disinfect for 20 min.

10. Place into the BSC: 5-mL serological pipets, 1,000-µL pipet, 1,000-µL tips, cold (2-8°C) BHIB aliquoted in 50-mL conical tubes, sharps container, and orange caps.

11. Pipet 5 mL of **cold BHIB** into each filter vial using a 5-mL serological pipet. Use a new pipet for each filter vial. Dispose of the red cap and place the orange cap firmly into the top of the filter. Change gloves between each sample.

12. Record the time of the BHIB addition; this represents T_0. Bleach wipe the filter vial.

Time of BHIB addition: _____

13. Place the rack of capped filter vials in a plastic bag, seal, double bag, and bleach the bag.

14. Vortex the filter vials for 10 min on the platform vortexer, Setting 7.

Start Time:_____ End Time:_____ Speed:_____

15. Place 2-mL screw cap tubes for T_0 aliquots onto ice in the BSC.

16. After vortexing, transfer filter vials to the BSC. Remove bag.

17. Uncap one filter vial at a time and open the corresponding 2-mL tube. Using a 1-mL pipette or serological pipet (if filter deteriorated), gently pipet up and down ~10X to mix. Transfer 1 mL from each vial to the corresponding pre-chilled (on ice) 2-mL screw cap tube for T_0. Cap the tube and place it back onto ice. Wipe the filter vial with a bleach soaked laboratory wipe. Change gloves between each sample. After transferring the T_0 aliquots for all samples, place the filter vial rack in a transfer container, seal, and bleach the container. Store the T_0 aliquot at -20 °C overnight.

T_0 -20°C Storage Start Time: _____ End time: _____
Initial/Date:_____

18. Transfer the filter vial rack to the shaker incubator. Secure the rack. Incubate at 30 ± 2°C at 230 rpm, overnight (i.e., 16 hours from the addition of BHIB to the filter vials). These samples are referred to as the T_f samples. Following incubation record turbidity observation and volume remaining in the table below.

Start Time:_____ End Time:_____ Speed:_____
Temperature:_____

Sample #	Sample ID	Turbid (Yes/No)	Volume Remaining (mL)	Recorded by:
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				

19. Proceed to WI #3 Appendix K: Work Instruction for DNA Purification to process T_0 and T_f samples

IV. Technical Review

Performed by: _____ Date:_____

APPENDIX J: WORK INSTRUCTION FOR CULTURE OF *BACILLUS THURINGIENSIS KURSTAKI (Btk) T1B2* SPORES RECOVERED FROM SPONGE STICK WIPES, VACUUM FILTER CASSETTES, AND GRAB SAMPLES

I. PURPOSE/SCOPE

Culture of *Bacillus thuringiensis kurstaki (Btk)* T1B2 spores recovered from sponge stick wipes (SSW), vacuum filter cassettes (VFC), and grab (GRB) samples following the **EPA/600/R-17/213** published by the EPA July 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.
Phosphate Buffered Saline with Tween (0.05%) (PBST)	Teknova			2-8°C
Microfunnel filters	PALL			RT
Trypticase Soy Agar				2-8°C

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/Rees #	Calibration Due
Biosafety Cabinet (BSC)	The Baker Company		N/A	
Stationary Incubator			N/A	N/A
Vacuum Manifold	Gelman Sciences	N/A	N/A	N/A

N/A = Not Applicable

Other Supplies and Equipment

- Forceps
- Bleach
- 5-mL, 10-mL, and 25-mL Serological Pipettes
- Pipette Aid

Performed by: _____ Date: _____

Filters – Electronically update this table with sample names from the Sample Log

Sample #	Sample Type	Location	Filter Vial Type	Spore Spike Level (CFU)	Sample ID
1	SSW				
2	VFC				
3	GRB				
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					

Performed by: _____ Date: _____

III. PROCEDURE

Note: The following procedure is to be carried out with the extract taken from WI #2 (Appendix I – Work Instruction for Btk Spore Recovery). Process 2-3 PBST-only negative control filter funnels alongside samples.

A. Culture Method

1. Label filter funnels per sample as indicated below. For some samples, the neat sample will be spread in triplicate, as indicated below.

Sample #	Sample ID	Volume to Plate
N/A	PBST Negative Control	8 mL
1	SSW	2 mL and 8 mL
2	VFC	1 mL and 3 mL
3	GRB	0.1 mL, 1 mL, and 4 mL
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		

2. Place the filter funnels onto the vacuum manifold in a Class II BSC.

3. Add 5 mL of PBST to each filter funnel. Apply vacuum.

4. With the vacuum valve closed and the vacuum pressure released, place 10 mL of PBST into each filter cup.

5. Vortex each sample, then allow 3 to 5 minutes of settle time to avoid loading large particulates into filter funnel. For each SSW, VFC, and GRB sample, add the volume indicated in Step 1. **Save any remaining volume of culture aliquot and store at 2 to 8°C.**

6. Close the vacuum valve and release the vacuum pressure. Rinse the walls of each filter funnel using 10 mL of PBST. Apply vacuum.

7. With the vacuum valve closed and the vacuum pressure released, remove the membrane from the filter funnel and place onto TSA. Dispose of filter bases and then change glove.

Performed by: _____ Date: _____

8. Incubate plates inverted overnight at 30°C ± 2°C. **Following incubation, save culture plates to PCR screen presumptive *Btk* colonies.**

a. *Btk* produces flat or slightly convex colonies, with edges that are slightly irregular and have a "ground glass" appearance.

Incubation Start Date/Time: _____ Initials: _____

Incubation End Date/Time: _____ Initials: _____

9. Enter results into the tables below.

Sample ID	***Btk T1B2* Colonies**		**Total Colonies (All Morphologies)**	
	CFU/ 2 mL	**CFU/ 8 mL**	**CFU/ 2 mL**	**CFU/ 8 mL**
PBST Negative #1	N/A		N/A	
PBST Negative #2	N/A		N/A	

Sample ID	***Btk T1B2* Colonies**			**Total Colonies (All Morphologies)**		
	0.1 mL	**0.1 mL**	**0.1 mL**	**0.1 mL**	**0.1 mL**	**0.1 mL**

IV. Technical Review

Reviewed by: _____ Date:_____

Performed by: _____ Date: _____

APPENDIX K: WORK INSTRUCTION FOR MANUAL DNA EXTRACTION AND PURIFICATION FROM *BACILLUS THURINGIENSIS KURSTAKI (Btk) T1B2* SPORES

I. PURPOSE/SCOPE

Manual DNA extraction and purification of *Bacillus thuringiensis kurstaki (Btk) T1B2* spores from recovered surfaces.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials/Date
Lysis Buffer	Promega			RT	
PMP	Promega			RT	
Salt Wash Solution	Promega			RT	
Alcohol Wash	Promega			RT	
70% Ethanol	Inhouse			RT	
Elution Buffer	Promega			RT	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials/ Date
Biosafety Cabinet (BSC)	The Baker Company	57544	N/A		
Micropipette Type: L200	Rainin		N/A		
Micropipette Type: L200	Rainin		N/A		
Micropipette Type: L1000	Rainin		N/A		
Micropipette Type: L1000	Rainin		N/A		
Ultra-Low Freezer	Woods	X34664			
Refrigerator	Thermo Fisher	35840			
Centrifuge	Eppendorf	X58983	N/A	N/A	
Heat Block	VWR	949039	N/A	N/A	
Thermometer			N/A		

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette tips
- Biohazard bags
- Bleach
- Prepare tubes

III. PROCEDURE

A. Manual DNA Extraction and Purification

Prepare lysis buffer with anti-foam according to manufacturer's instructions in the Magnesil Blood Genomic, Max Yield System, Kit. Prepare the alcohol wash solution by adding ethanol and isopropyl alcohol according to manufacturer's instructions. Prepare 70% ethanol by adding 6 mL sterile water to 14 mL EtOH. Transfer sufficient volume of buffer to sterile, 100-mL reservoir immediately before use. ***Pre-heat heat block to 80°C prior to Section 10.4.8.***

NOTE: Process samples from negative control to high inoculation level. Change gloves when moving from an inoculated sample to a sample containing a lower inoculation level, or if contamination of gloves is suspected. Pre-aliquot reagents from the kit to prevent contamination of reagents between runs.

1. After the overnight (16 hours) incubation, remove the filter vial manifold from the shaker incubator. Thaw T_0 aliquots if they were stored at -20°C.
2. Vortex filter vials for 10 minutes on platform vortexer with speed set to 7.

   Start: _____ End:_____ Speed:_____
3. Transfer the filter vial manifold to the BSC, remove and discard bags.
4. Set up 2-mL screw cap tubes for T_f aliquots in a tube. Do not use 1.5-mL tubes. Transfer T_f aliquot screw cap tubes to the BSC.
5. Transfer the filter vial rack to the BSC. Uncap one filter vial at a time and transfer 1 mL to corresponding 2-mL tube after gently pipetting up and down ~10 to mix.
6. Centrifuge 2-mL screw cap tubes (both T_0 and T_f) at 14,000 rpm for 10 minutes (4°C).

   Start: _____ End:_____ Speed:_____
7. Remove 800 µL of the supernatant from each tube, using a 1,000-µL pipet and dispose to waste. Do not disturb the pellet.
8. Add 800 µL of lysis buffer using a 1,000-µL pipet, using a new tip for each sample. Cap the tubes and mix by vortexing on high (~1,800 rpm) in 10 second pulses for a total of 60 seconds.
9. Vortex each screw cap tube briefly (low speed, 5 to 10 seconds) and transfer the entire sample volume to a 2-mL Eppendorf tube (ensure the tubes are labeled correctly during transfer). Incubate the T_0 and T_f lysate tubes at room temperature for 5 minutes.
10. Vortex the PMPs on high (~1,800 rpm) for 30 to 60 seconds, or until they are uniformly resuspended. Keep PMPs in suspension by briefly vortexing (3 to 5 seconds) before adding to each T_0 and T_f lysate tube.
11. Uncap one tube at a time and add 600 µL of PMPs to each T_0 and T_f tube (containing 1 mL sample).

12. Vortex each T_0 and T_f tube for 5 to 10 seconds at high speed. Incubate at room temperature for 5 minutes, briefly vortex, and then place on the magnetic stand with hinged-side of the tube facing toward the magnet.

13. Invert tubes 180 degrees (upside down) turning away from you, then right side-up, then upside down toward you, then right side-up (caps up) position, allowing all PMPs to contact the magnet.

14. Check to see if any beads are in the caps and if so, repeat the tube inversion cycle again. Let the tubes sit for 5 to 10 seconds before opening. Maintain the tube layout when transferring tubes between the magnetic stand and tube rack.

15. Uncapping one tube at a time, withdraw all liquid using a 1,000-µL pipet, placing the pipet tip in the bottom of the 2-mL tube. Be sure to remove all liquid without disturbing PMPs. Use a new pipet tip to remove any residual liquid, if necessary. If liquid remains in the tube cap, remove by pipetting.

16. Uncap each tube one at a time and add 360 µL of lysis buffer using a 1,000-µL pipet. Vortex on low setting for 5 to 10 seconds, and transfer to tube rack.

17. Vortex each tube for 5 to 10 seconds (low) and place back on the magnetic stand. After all tubes are in the stand, follow tube inversion cycle, as described in Step 13.

18. Remove all the liquid as described in Step 15. Use a new tip for each T_0 and T_f tube.

Wash Steps:

19. Uncap each tube one at a time and add 360 µL of **Salt Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 to 10 seconds, and transfer to tube rack. Place tube rack back on magnetic stand. Invert as described in Step 13. Remove all the liquid as described in Step 15. Use a new tip for each T_0 and T_f tube. This is **1st Salt Wash.**

20. Uncap each tube one at a time and add 360 µL of **Salt Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 to 10 seconds, and transfer to tube rack. Place tube rack back on magnetic stand. Invert as described in Step 13. Remove all the liquid as described in Step 15. Use a new tip for each T_0 and T_f tube. This is **2nd Salt Wash.**

21. Uncap each tube one at a time and add 500 µL of **Alcohol Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 to 10 seconds, and transfer to tube rack. Place tube rack back on magnetic stand. Invert as described in Step 13. Remove all the liquid as described in Step 15. Use a new tip for each T_0 and T_f tube. This is **1st Alcohol Wash.**

22. Uncap each tube one at a time and add 500 µL of **Alcohol Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 to 10 seconds, and transfer to tube rack. Place tube rack back on magnetic stand. Invert as described in Step 13. Remove all the liquid as described in Step 15. Use a new tip for each T_0 and T_f tube. This is **2nd Alcohol Wash.**

23. Uncap each tube one at a time and add 500 µL of **Alcohol Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 to 10 seconds, and transfer to tube rack. Place tube rack back on magnetic stand. Invert as described in Step 13. Remove all the liquid as described in Step 15. Use a new tip for each T_0 and T_f tube. This is **3rd Alcohol Wash.**

24. Uncap each tube one at a time and add 500 µL of **70% Ethanol**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 to 10 seconds, and transfer to tube rack. Place tube

rack back on magnetic stand. Invert as described in Step 13. Remove all the liquid as described in Step 15. Use a new tip for each T_0 and T_f tube. This is **4th Alcohol Wash.**

25. If necessary, use a 200-µL pipet to remove remaining 70% ethanol, being careful to not disturb PMPs.

26. Open all T_0 and T_f tubes and air dry for 2 minutes.

27. Close tubes and transfer to heat block. Re-open tubes once placed on the heat block at 80°C until the PMPs are dry (~20 minutes, or until dry). Allow all the alcohol solution to evaporate since alcohol may interfere with analysis. If residual condensation is present, do not remove, leave it in place.

Start: _____ End:_____ Temperature:_____

28. DNA elution: While they are in the heating block add 200 µL of elution buffer to each T_0 and T_f tube, and close tube. Vortex for 10 seconds and place back on heating block for 80 seconds.

29. Briefly vortex the tubes (5 to 10 seconds) taking care to prevent the liquid from entering the tube cap and let the tube sit in the heating block for 1 minute. Reduce vortex speed if liquid appears to enter the tube cap lid.

30. Repeat Step 29 four more times.

31. Remove the tubes from the heating block, place them in a tube rack in the BSC, and incubate at room temperature for at least 5 minutes.

Start: _____ End:_____

32. Briefly vortex each tube (5 to 10 seconds) on low speed and centrifuge at 2,000 rpm, 4°C for 1 minute.

33. Briefly vortex each tube and place on the magnetic stand for at least 30 seconds.

34. Collect liquid from each T_0 and T_f tube and transfer ~80-90 uL to a clean, labeled, 1.5-mL tube on ice (check tube labels to ensure the correct order). Use a new tip for each tube. Visually verify absence of PMP carryover during final transfer. If magnetic bead carryover occurred, place 1.5-mL tube on magnet, collect liquid, and transfer to a clean, labeled, 1.5-mL tube.

35. Centrifuge tubes at 14,000 rpm at 4°C for 5 minutes to pellet any particles remaining with the eluted DNA; carefully remove supernatant from all samples and transfer to a new 1.5-mL tube using a new tip for each tube.

Start: _____ End:_____

36. Store T_0 and T_i DNA extract tubes at 4°C until PCR analysis. Continue to ***PCR analysis***.

Note: If PCR cannot be performed within 24 hours, freeze DNA extracts at -20°C.

Labeled:_____

Date/Time:_____ Storage Temperature:_____

Storage Location:_____

IV. Technical Review

Performed by: _____ Date:_____

APPENDIX L: WORK INSTRUCTION FOR REAL-TIME PCR ANALYSIS FOR *BACILLUS THURINGIENSIS KURSTAKI (Btk)* T1B2 DNA

I. PURPOSE/SCOPE

Real-time PCR analysis for *Bacillus thuringiensis kurstaki (Btk)* T1B2 DNA.

II. MATERIALS/EQUIPMENT

Enter material lot and expiration dates used into FORM A:

Materials

Item	Manufacturer	Product Number
TaqMan Fast Advanced PCR Mix (2x)	Applied Biosystems	4444556
Platinum Taq DNA Polymerase	Invitrogen	10966-034
Custom Primers and Probes w/ 6-FAM Reporter Dye	Applied Biosystems	Custom
PCR Grade Water	Fisher Scientific	BP2484100
Optical Plate Seal	ThermoFisher	4311971

Equipment

Item	Manufacturer	Serial Number	Thermometer/Rees #	Calibration Due
Biosafety Cabinet (BSC)	Baker Thermo Forma		N/A	
Micropipette Type: 10			N/A	
Micropipette Type: 20			N/A	
Micropipette Type: 200			N/A	
Micropipette Type: 1000			N/A	
Freezer				
Centrifuge	LabNet	K4070898	N/A	N/A
7500 Fast	Applied Biosystems	275017115	N/A	

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette tips, 96-well 0.1 mL FAST plates, optical caps, bleach, DNA erase, 70% isopropanol

Attach FORM A: Date: _____

III. PROCEDURE

A. Prepare samples for qPCR

Note: This step must be performed in the BSC outside the PCR clean room set-up area. Prepare a fresh aliquot of PCR-grade water per sample batch to use for 1:10 dilutions and NTCs.

1. ***T_0 and T_f DNA extracts*:** Label 1.5-mL tubes with the sample identifier and "10-fold dilution." Add 90 µL of PCR-grade water to the tubes.
2. Mix T_0 and T_f DNA extracts by vortexing (3 to 5 seconds), spin at 14,000 rpm for 2 minutes, and transfer 10 µL of supernatant to 1.5-mL Eppendorf tubes with 90 µL of PCR-grade water, maintaining the plate layout.

Note: No centrifugation is required if PCR analysis is conducted immediately after DNA elution.

B. Real-time PCR Analysis of DNA Extracts

1. Decontaminate the PCR workstation by treating all work surfaces with bleach solution, followed by 70% isopropanol. After decontamination, discard gloves and replace with a new clean pair.

Note: If gloves become contaminated, they should be disposed of and fresh gloves donned. Only open one tube at a time throughout the process. At no point, should more than one tube be open. Do not allow hands (gloved or otherwise) to pass over an open tube, PCR plate, or any reagent container. All used pipet tips, gloves and tubes must be discarded in a biohazard autoclave bag.

2. Determine the number of reactions that are to be run. Prepare a sufficient volume of Master Mix to allow for one extra reaction for every 10 reactions, so that there is enough Master Mix regardless of pipetting variations. *For each batch of samples, PCR Master Mix should be made for four positive controls (PCs), four NTCs, and six DNA extracts per sample (three for T_0 and three for T_f DNA extracts).* Record sample names and reaction numbers on ***FORM A.***
3. In a clean PCR-preparation hood, pipet 20 µL of Master Mix into the wells of the PCR plate. Label four wells as NTC.
4. Add 5 µL of PCR-grade water into each of the NTC wells.
5. Lightly seal the NTC wells with optical caps and cover all other wells of the plate using optical caps.
6. Vortex each sample briefly, then add 5 µL to each sample well. Lightly seal the sample wells with optical caps.
7. Vortex the PC, *B. thuringiensis kurstaki* T1B2 DNA [10 pg/1 µL or 50 pg/5 µL] by adding 5 µL to each PC well. Tightly seal the wells using optical caps.

C. Within the Post-Amplification Laboratory, load 96-well plates onto 7500 Fast.

1. Set up 7500 Fast (TaqMan)
   a. Open the 7500 Fast Software and select *New Experiment*

i. Set Experiment Properties:

1. Enter an Experiment Name
2. Select 7500 Fast (96 wells)
3. Select Quantitation – Standard Curve
4. Select TaqMan Reagents
5. Select Fast (~40 minutes to complete a run)

ii. Plate Step

1. Define the Target and Samples
    a. Define a target with designated reporter (6-FAM) and *None* as the quencher. Multiple targets can be selected if more than one target will be run on the plate.
    b. Define samples by selecting *Add New Sample* for all samples, include NTCs and standard curve concentrations as sample names.
2. Assign Targets and Samples
    a. Highlight the wells that will be used for this assay, then check the assign box to assign the target. Check appropriate task (Unknown, Standard, or Negative Control).
    b. Highlight the sample wells, then check the assign box to assign the sample.
    c. Highlight the standard curve wells, to enter the sample name, then enter a quantity for each standard under the assign target pane.
    d. Select ROX as the passive reference from the Passive Reference drop down box.

iii. Run Method

1. Under Graphical View, enter 25 µL as the reaction volume.
2. Set thermocycling conditions to match the below settings:

Temperature (°C)	**Time**	**Cycles**
50.0	*2:00*	Hold
95.0	*2:00*	Hold
95.0	*0:03*	45
60.0	*0:30*	
25 µL Total Volume		

3. Select *Save As* and assign unique plate file name and save in project folder.

iv. Start Run

1. Centrifuge the plate at 300 x g for 1 to 2 minutes at room temperature or in Labnet's MPS-1000 Mini Plate Spinner briefly. Check that the samples are at the bottom of the wells and no bubbles are at the bottom of the wells.
2. Select *Start Run*.
3. When run is complete, burn the file to a CD.
4. Remove 96-well plate from the 7500 Fast and dispose.

D. Analysis

1. Open the assay with the most current version of 7500 Fast software
   a. Select the Analysis Tab
   b. Select Plot Settings:
      i. Plot Type: ΔRn vs Cycle
      ii. Graph Type: Log
      iii. Plot Color: Well
   c. Select Options:
      i. Target: Select target that was assigned to wells
      ii. Threshold: Uncheck Auto and Auto Baseline
      iii. Show: Check Threshold, Baseline Start
   d. In Amplification Plot, set the Threshold to 0.1.
   e. In View Well Table, view Ct values for all samples. Adjust the baseline manually in the Amplification Plot so that the Baseline End is two Ct values below the lowest Ct value whole number, ignoring values to the right of the decimal. For example, if the lowest Ct value is 22.610105, the Baseline End cursor should be set to 20.
   f. After moving Baseline End, recheck the Ct values and adjust again if necessary.
2. Save file with the file extension "_Analyzed"
3. Export Results
   a. Select *Export*
   b. Check the Results option, one file
   c. Enter a unique plate file name with run date and initials
   d. Select file type, .xls (Excel)
   e. Browse File Location to save in project-specific location
   f. Select Start Export, then Close Export Tool

4. Print Report

   a. Select Print Report

   b. Check the below selections and then Print Report:

      i. Experiment Summary
      ii. Results Summary
      iii. Amplification Plot
      iv. Standard Curves
      v. Results Table (By Well)

   c. Under Analysis Setting, Select Multicomponent Plot

      i. Highlight all NTC wells, then select Print from the icon on the Multicomponent Plot

      ii. Highlight all Standard wells, then select Print from the icon on the Multicomponent Plot

      iii. Highlight all Sample wells, then select Print from the icon on the Multicomponent Plot

   d. Annotate printouts

      i. Initial and date every page

      ii. Initial, date, and error or otherwise annotate all errors and comments

      iii. Indicate which, if any, wells of the standard curve were omitted

      iv. Indicate multicomponent results for each well on the Results Table

5. Quality Control Acceptance Criteria

   a. Verify the below acceptance criteria are met

      - Amplification in PC wells
      - NTC wells have no amplification

IV. Data Calculations

Calculate the average Ct value from the replicate reactions for T_0 and T_f DNA extracts of each sample. Subtract the average Ct value of the T_f DNA extract from the average Ct value of the T_0 DNA extract to generate delta Ct value (ΔCt). If there is no Ct value for the T_0 DNA extract (i.e., the T_0 is non-detect), use 45 (total number of PCR cycles used) as the Ct value.

Performed by: _____ Date: _____

V. Technical Review

All data will receive technical review and QC review in accordance with QA. I-005.

Technical Review Initials/Date: _____

QC Review Initials/Date: _____

DNA ASSAY: 96-Well Plate Setup for Fast 7500 (FORM A)

Project: _____ **Barcode: _____**

Target: *Btk* T1B2

1. Calculate the total number of reactions per plate:

Sample wells + 4 NTC wells + 4 PCs +_____ extras = _____ total rxns/plate (Y)

2. Prepare the Master Mix by combining the following reagents in an appropriate tube according to the following calculation:

Reagent volume (X) x total rxns/plate (Y) = total volume of reagent needed

Reagent	Manufacturer	Lot No.	Exp. Date	X	Y	Total Volume (µL)
TaqMan Fast Advanced Master Mix (Cat. 4444556)	Applied Biosystems			12.5 µL		
Platinum Taq Polymerase	Invitrogen			0.1 µL		
Btk T1B2 For. Primer (25 µM)	In-House		TBD	1 µL		
Btk T1B2 Rev. Primer (25 µM)	In-House		TBD	1 µL		
Btk T1B2 Probe (2 µM)	In-House		TBD	1 µL		
PCR Grade Water				4.4 µL		
Total				20 µL		

3. Distribute 20 µL of Master Mix into each reaction well, as indicated in the plate layout, below. Loosely cover all wells containing Master Mix with caps.
4. Add 5 µL of PCR-grade water to each of the NTC Wells. Cap wells tightly.
5. Add 5 µL of PNC (Method Blank) to the corresponding wells and secure the caps.
6. Add 5 µL of Sample to the corresponding wells and secure the caps.
7. Add 5 µL of PC to the corresponding wells and secure the caps.

Positive Control Prep Date		Positive Control Lot	

8. Centrifuge the plate using Labnet's MPS-1000 Mini Plate Spinner at room temperature, and then load the plate onto the 7500 Fast.

Performed by: _____ Date: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H	PC 50 pg	PC 50 pg	PC 50 pg	PC 50 pg					NTC	NTC	NTC	NTC

Technicians	Signature	Date
Master Mix, NTC		
Samples		
Standards		
Analyst		

Reviewed By: _____ Date: _____

APPENDIX M: WORK INSTRUCTION FOR SELECTING PRESUMPTIVE *BACILLUS THURINGIENSIS KURSTAKI (Btk)* T1B2 COLONIES FOR QPCR CONFIRMATION

I. PURPOSE/SCOPE

Select and screen *Bacillus thuringiensis kurstaki (Btk)* T1B2 colonies recovered on culture plates using qPCR.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.
PCR-grade water	Teknova			RT
1-µL loop, 10-µL loop or inoculating needles			N/A	RT
1.5- or 2-mL tubes		N/A	N/A	RT

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Calibration Due
Biosafety Cabinet (BSC)	The Baker Company		
Heat Block	VWR		N/A
Thermometer			
Camera	N/A	N/A	N/A

N/A = Not Applicable

Other Supplies and Equipment

- Bleach
- 5-mL, 10-mL, and 25-mL serological pipettes

III. PROCEDURE

A. Selecting Colonies

1. Pipette 100 µL of PCR-grade water into 1.5- or 2-mL tubes.

2. Select colonies. Take pictures of colonies that are selected.

3. Use 1-µL loop, 10-µL loop, or inoculating needle to select the colony.

4. Immerse needle into PCR-grade water and rotate to dislodge cellular material.

5. Colonies from a single sample can be pooled to increase the number of presumptive colonies screened. Up to 10 colonies can be pooled within a 100-µL volume of PCR-grade water. Repeat Steps 3 and 4 to pool multiple colonies from a single sample and record the number of colonies pooled in the table below.

6. Lyse the colony suspension for 5 minutes on a heat block at 95 ± 2°C.

   Incubation Start Date/Time: _____ Initials: _____

   Incubation End Date/Time: _____ Initials: _____

7. Store lysed suspension at – 20°C for qPCR analysis.

8. Prior to qPCR analysis, thaw tubes, centrifuge at 14,000 rpm for 2 minutes. Use supernatant for qPCR.

Filters – Record Filter ID and Morphology for Selected Colonies

Tube #	Filter ID	Volume (mL)	Morphology (Btk or Background)	# of Colonies Pooled	PCR Result
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

IV. Technical Review

Reviewed by: _____ Date:_____

APPENDIX N: WORK INSTRUCTION FOR BHIB ENRICHMENT FOR CULTURE

I. PURPOSE/SCOPE

Enrich extracted sponge or filter in Brain Heart Infusion Broth (BHIB).

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials/Date
PCR-grade water	Teknova			RT	
10-µL loop or inoculating needles				RT	
1.5- or 2-mL tubes				RT	
Trypticase Soy Agar (TSA) plates				2 to 8°C	
BHIB				RT	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/Rees #	Calibration Due	Initials/ Date
Biosafety Cabinet (BSC)	The Baker Company		N/A		
Incubator	Precision				
Thermometer	Traceable	N/A	N/A	N/A	
Heat Block	VWR				
Refrigerator	Fisher	C3274822	115	8/2020	

N/A = Not Applicable

Other Supplies and Equipment

- 25-mL serological pipettes

III. PROCEDURE

A. Enrichment of Sponges and Filters

1. Add 25 mL of BHIB to each specimen cup containing the extracted sponge or filter.
2. Incubate cups at 30°C ± 2°C for 24-48 hours.

Incubation Start Date/Time: _____ Initials: _____

Incubation End Date/Time: _____ Initials: _____

3. Evaluate the BHIB enrichment for samples.

I. If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.

II. If broth is turbid, record as positive growth (G+) and proceed to Step 4.

Sample Number	Filter ID	Growth (G+) or No Growth (NG)		Recorded by:
		24 hours	48 hours	
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				

4. For samples that have not been confirmed positive by culture membrane plating, streak turbid samples onto TSA. Cap tightly and mix BHIB with growth for 30 seconds. Remove a loopful of broth with a 10-µL loop and streak triplicate TSA plates for isolation. Store enriched samples at 2 to 8°C.

5. Incubate the isolation plates and BHIB with growth at 30°C ± 2°C for a maximum of three days.

Incubation Start Date/Time: _____ Initials: _____

Incubation End Date/Time: _____ Initials: _____

6. Examine plates for *Btk* colonies.

I. If presumptive *Btk* colonies are isolated and positive identification has not already been confirmed by PCR from a representative sample, record the sample

in the table below as a colony selection sample and proceed to PCR confirmation from BHIB streak plates (Section B).

II. If **NO** presumptive *Btk* colonies are isolated and positive identification has not already been confirmed by PCR from a representative sample, record the sample in the table below as a BHIB Analysis sample and proceed to PCR confirmation of BHIB Enriched Samples (Section C).

Sample #	Filter ID	Colony Selection or BHIB Analysis	Number of Colonies Screened	PCR Result	Recorded by:
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					

B. Selecting Colonies

1. Pipette 100 µL of PCR-grade water into 1.5- or 2-mL tubes.

2. Select colonies.

3. Use 1-µL loop, 10-µL loop or inoculating needle to select the colony.

4. Immerse needle into PCR-grade water and rotate to dislodge cellular material.

5. Colonies from a single sample can be pooled to increase the number of presumptive colonies screened. Up to 10 colonies can be pooled within a 100-µL volume of PCR-grade water. Repeat Steps 3 and 4 to pool multiple colonies from a single sample and record the number of colonies pooled in the above table.

6. Proceed to Lysis and Storage (Section D)

C. PCR Confirmation of BHIB Enriched Samples

1. Transfer 50 µL of broth with growth to a microcentrifuge tube**.**
2. Centrifuge at 12,000 × g for 2 minutes.
3. Remove and discard the supernatant in an autoclavable biohazard container. Add 100 µL of PCR-grade water to the tube containing the bacterial pellet.
4. Resuspend the pellet by flicking the tube.
5. Proceed to Lysis and Storage (Section D)

D. Lysis and Storage

1. Lyse colony screen and BHIB enrichment samples for 5 minutes on a heat block at 95 ± 2°C.

   Incubation Start Date/Time: _____ Initials: _____

   Incubation End Date/Time: _____ Initials: _____
2. Store lysed suspension at -20°C for qPCR analysis or refrigerator if processed same day.
3. Prior to qPCR analysis, thaw tubes, centrifuge @ 14,000 rpm for 2 minutes. Use supernatant for PCR analysis.

IV. Technical Review

Reviewed by: _____ Date:_____

United States
Environmental Protection
Agency

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