COMPARISON OF THE RECOVERY OF BACILLUS ANTHRACIS AND BACILLUS THURINGIENSIS SPORES FROM POROUS MEDIA: CONSIDERING TIME AND MOISTURE CONTENT

By

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ABSTRACT

COMPARISON OF THE RECOVERY OF BACILLUS ANTHRACIS AND BACILLUS THURINGIENSIS SPORES FROM POROUS MEDIA: CONSIDERING TIME AND MOISTURE CONTENT

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Bacillus anthracis spores are agents used for biological warfare owing to high virulence, and extreme resistance to environmental stresses with negligible degradation in viability. There is an interest in understanding the persistence and subsequent health risks due to *B. anthracis* spores in the indoor environment during bioterror attacks, but neither the time-dependent behavior of *B. anthracis* spores on porous media nor the suitability between *B. anthracis* and its recommended experimental surrogate, *B. thuringiensis* spores, are well known. This work specifically focused on recovery of *B. anthracis* and *B. thuringiensis* spores from HVAC filters, a porous fomite, considering the effects of sampling time and moisture content using culture-based quantification. Effects of time significantly affected the recovery of *Bacillus* spores. Although there was an overall reduction in viability of *Bacillus* spores over time, the reduction in recovery of *B. anthracis* and *B. thuringiensis* varied remarkably. Clumping in *B. thuringiensis* spores and accelerated dehydration rates at higher moisture levels might have resulted in increased observed recovery of *B. thuringiensis*. Hence it is concluded that, *B. thuringiensis* spores is not an ideal experimental surrogate for *B. anthracis* in recovery studies. Inclusion of time and moisture emphasizes the importance of variation in recoveries and inferences between the surrogate and *B. anthracis* spores. The data generated in this study will be useful for persistence studies that measure reduction or decay but are dependent on recovery. Furthermore, this information will inform quantitative microbial risk assessment (QMRA) models that estimate the risk of exposure to *B. anthracis* spores in indoor environments.

I dedicate this thesis to Harshavardhan Nirmal, a.k.a. Jutty

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CHAPTER 1: INTRODUCTION

Bacillus endospores are formed in response to acute unfavorable environmental conditions such as starvation, chemical and thermal stresses, desiccation, pH extremes, mechanical force, and antibiotics (Setlow, 2003; Nicholson et al., 2000; Sternbach, 2003). When exposed to favorable conditions these endospores are capable of germinating. Spores of *Bacillus anthracis* maintain their viability over decades, with negligible degradation (Dragon & Rennie, 1995). The virulence and durability of *B. anthracis* spores, along with the ease and affordability of their manufacture, have made them agents of biological warfare (Inglesby et al., 1999; Inglesby et al., 2002). The detrimental effect of *B. anthracis* in biological warfare is well documented (Cole, 2010; Riedel, 2005; Riedel, 2004; Christopher et al., 1997; Block, 2001). Although the use of *B. anthracis* as a bioterror agent has been documented as early as World War I, it was not until the 2001 anthrax attacks in the United States that *B. anthracis* and bioterrorism gained global attention (Inglesby et al., 2002; Riedel, 2005, Sternbach et al., 2003; Cole, 2010). The aftermath of the 2001 anthrax attacks included economic losses, public chaos, and political turbulence of significant magnitude, because the spores were delivered in letters targeting senate and media persons and contaminating government buildings (Day, 2003). According to a PubMed search result using the keyword "anthrax"— the number of studies immediately following the 2001 anthrax attacks increased by approximately 414% during the years 2001 and 2002 compared to the year 2000.

The persistence of *B. anthracis* endospores in different environmental matrices such as soil, water, and air makes the possible exposure scenarios more minatory in cases of bioterror events (Manchee et al., 1981; Dragon et al., 2001; Minett & Dhanda, 1941; Brachman et al., 1966). Understanding the behavior of aerosolized biothreat agents over time is a major concern

of the US Environmental Protection Agency and the Department of Homeland Security. Considering the persistence of *Bacillus* endospores in air, it is also vital to quantitatively and qualitatively monitor the indoor environment over time in scenarios of intentional bioweapon releases. Because humans spend 94% of the day indoors at work, in enclosed vehicles, and during indoor recreation, etc., the quality of the air inhaled over time correlates to the quality of their health (Jenkins et al., 1992; Lai & Nazaroff, 2000). HVAC filters are part of this indoor environment, which are used in more than 70% of residential and commercial establishments in the United States, and have been used as sampling sources in cases of an intentional indoor release or an outdoor infiltration of weaponised endospores (Noris et al., 2011; Kim et al., 2008; Krauter & Biermann, 2007; Van Cuyk et al., 2012; Stanley et al., 2008; Calfee et al., 2014). Sampling these filters from different intakes/ outlets of an air handling unit within a given building could help predict the pattern of spread of pathogens used during the attack (Calfee et al., 2014).

Sampling and decontamination studies have been successful in documenting the efficacy of the techniques and materials used, but the recovery over time has been rarely considered. To our knowledge, the following recovery studies using bacterial spores have been the only studies carried out on filter media to date: Wang et al. (2001), Moritz et al. (2001), Farnsworth et al. (2006), Kim et al. (2008), Solon et al. (2012), Stanley et al., (2008); Van Cuyk et al., (2012); Burton et al. (2005), Maus et al. (2000), Mittal et al. (2011), and Calfee et al. (2014). However, only four studies to date have considered time-dependent (24 hours or greater) recovery of bacterial spores: Moritz et al. (2001) – 450 days, Mittal et al. (2011) – 210 days, Kim et al. (2008) – 28 days, and Maus et al. (2000) – 5 days.

It is interesting to find that these filter-based studies have produced some widely differing results such as: very high (90% or greater) extraction or recovery efficiencies (Wang et al., 2001; Burton et al., 2005; Farnsworth et al., 2006); high extraction efficiency but poor culturability (Wang et al., 2001); high extraction efficiency with no change or minimal loss in culturability (Maus et al., 2000; Burton et al., 2005; Farnsworth et al., 2006; Kim et al., 2008; Mittal et al., 2011); higher recoveries at high ambience moisture content (Wang et al., 2001; Moritz et al., 2001); reported high survival or proliferation of microbial spores during higher moisture content of the filters (Moritz et al., 2001; Solon et al., 2012); and no change in viability or evidence of proliferation observed in the recovery, under both high and low relative humidity (Maus et al., 2000).

All of these studies were invariably carried out using the spores of species other than *B. anthracis*: *B. subtilis, B. atrophaeus, B. golbigii,* and *B. thuringiensis*. Moritz et al. (2001) did not specify the type of bacterial species used. Although it is an accepted practice to use surrogate species in experimental research, it is important to test the similarities between the surrogate and the species of interest experimentally. Most of the commonly used surrogate spores such as *B. subtilis, B. atrophaeus,* and *B. golbigii* are approximately half the diameter and 25% the volume of *B. anthracis* spores. Furthermore, these surrogate species lack essential glycoproteins that are responsible for the formation of the outermost layer of *B. anthracis* spores, called exosporium (Hachisuka et al., 1984; Carrera et al., 2007; Greenberg et al., 2010). Therefore, use of such morphologically and phylogenetically different surrogates in some experiments would yield different results especially on porous media where the chances of infiltration are directly correlated to the size and aerodynamic properties of the spores. *Bacillus thuringiensis* spores are morphologically and genetically close to *B. anthracis* which make them ideal surrogate

candidates for experimental purposes (Carrera et al., 2007; Greenberg et al., 2010; Tufts et al., 2013). Although physical parameters of *B. thuringiensis* and *B. anthracis* spores are comparable, time-dependent recovery of either from filters has not been experimentally studied to date. Therefore, prior to using the suggested best-suited surrogate in place of *B. anthracis* spores, it is strongly warranted to test both the species using identical experimental conditions.

Quantitative studies of *B. anthracis* spores are needed in order to improve understanding for persistence modeling and risk assessment. The efficacy of decontamination and disinfection processes following bioterror attacks are evaluated by wipes and swabs and sampling of recovered spores. This information is integrated with fate-and-transport, and dose-response models within QMRA frameworks. Therefore, a robust set of recovery data of *B. anthracis* over time would prove useful for persistence and risk assessment research in the future. Furthermore, an evaluation of whether surrogate data, specifically *B. thuringiensis* spores is suitable for predicting the behavior of *B. anthracis* spores on porous media would prove useful for future.

1.1. Research Objectives

The objective of this study was to compare the recovery of *B. anthracis Sterne* (non-pathogenic surrogate) to *B. thuringiensis HD 2-61* spores from HVAC filters over a short-term period of 168 hours. This study is the first one of its kind to carry out recovery of *B. anthracis* and its surrogate *B. thuringiensis* experimentally in the same study with identical sporulation and experimental procedures. Hence, the results from this study could help in understanding the short-term, time-dependent recovery of *B. anthracis* and *B. thuringiensis* spores as well as help in future selection of surrogates and quantification techniques for experimental purposes.

Therefore, goals of this study are to:

- Quantify and understand the time-dependent recovery of spores of *B. anthracis* alongside its surrogate *B. thuringiensis* from HVAC filters
- Investigate if *B. thuringiensis* and *B. anthracis* are experimentally similar
- Investigate the effects of varied moisture content loaded on the filters on recovery for both the species

Hypotheses specific to each goal in this study, are presented in the following sub-sections.

1.1.1. Specific Objective I

To quantify the time-dependent recovery of spores of B. anthracis alongside B. thuringiensis

It is hypothesized that *Bacillus* spores will have a time-dependent reduction in recovery, from HVAC filters. Filter based recovery studies of *Bacillus* spores over time are scarce. Based on culture-based results from Kim et al. (2008) in which *B. golbigii* spores were inoculated and sampled from HVAC filters over a period of about 30 days, it is expected that there will be a reduction in the recovery of spores from HVAC filters. To achieve this objective, sample coupons of HVAC filters will be artificially loaded with *B. anthracis* and *B. thuringiensis* spores of known similar concentrations, and recovered at the same time intervals using a series of mechanical extraction processes. Amounts of recovered spores at each time point were quantified and will be represented in spores/ filter.

1.1.2. Specific Objective II

To investigate if B. thuringiensis and B. anthracis spores behave similarly in recovery studies from porous media

It is hypothesized that *B. anthracis* and *B. thuringiensis* spores will have identical time-dependent recovery from HVAC filters. Most of the published studies in the past have used the species that belonged to the *B. subtilis* family. Surrogates such as *B. atrophaeus*, *B. subtilis*, and *B. golbigii* are approximately half the diameter and 25% the volume of pathogenic *B. anthracis*. Therefore, use of such surrogates with morphological and phylogenetic differences in experiments would yield different results especially on porous media where there are chances of infiltration which is correlated to the size and aerodynamic properties of the spores. In 2010, *Bacillus thuringiensis,* was suggested to be an ideal suitable surrogate for pathogenic *B.*

anthracis sharing morphological and genetic similarities (Carrera et al., 2005; Greenberg et al., 2010; Tufts et al., 2013). Although Solon et al. (2012) studied the recovery of *B. thuringiensis* spores from HVAC filters, there is no experimental evidence to date that *B. anthracis* and *B. thuringiensis* behave identically. The results generated in Objective I will be used to achieve this objective.

1.1.3. Specific Objective III

To study the effect of different moisture content loaded on the filters on recovery

It is hypothesized that *Bacillus* spores will have a lower recovery when less water is applied to the HVAC filter. Recoveries obtained at high moisture contents on filter or fomites have resulted in greater culturability and recovery, suggesting that there could be greater detachment or poorer attachment when the sampling matrix is moist (Wang et al., 2001; Moritz et al., 2001; Nanasaki et al., 2010; Lopez et al., 2013). Therefore the sensitivity of the wet application method used in this study will be tested. Considering that HVAC systems encounter frequent conditions of fluctuating moisture levels, and higher moisture content have found to favor greater culturability of spores, this study will also inform the effect of a saturated and a lower water content applied to the filter over time. To achieve this objective, sample coupons of HVAC filters will be artificially loaded with *B. anthracis* and *B. thuringiensis* spores of known similar concentrations with two different moisture contents, and subsequently recovered and extracted over time as described in Objective I.

Summary of experimental planning and design are provided in detail in APPENDIX-A. Chapter 2 provides a comprehensive literature review of related research and motivation for this study. Results and discussion are presented in Chapters 3 and 4, respectively.

CHAPTER 2: LITERATURE REVIEW

2.1. *Bacillus anthracis* Spores

Bacillus is the genus of rod-shaped, endospore forming, Gram-positive bacteria that are ubiquitous in the environment which includes both pathogenic and non-pathogenic species. *Bacillus* species have a wide range of applications such as antibiotic production as well as in biological warfare (Katz & Demain, 1977; Spencer, 2003).

Intentional mailing of *B. anthracis* spore-laden letters sent to the senate and media offices in 2001 highlighted the need for data on *B. anthracis* to protect public health. The average number of studies on anthrax in 2001 and 2002, following the 2001 anthrax attacks increased by 414% when compared to publications in 2000, according to a PubMed search. The increased concern for *B. anthracis* spores was due to their high pathogenicity, durability, and ease of production and dissemination in the environment.

Spores are formed from the live vegetative cells as a result of starvation and adverse environmental conditions which are not favorable for germination and proliferation. They then can later germinate under favorable conditions. The robustness of the spores and their ease to spread have increased the need to understand their persistence over time. Unlike vegetative cells, spores remain viable, resist heat, cold-treatment, light, chemicals, and antibiotics (Dragon & Rennie, 1995; Dixon et al., 1999; Inglesby et al., 1999). Spores are reported to be viable after decades of isolation (Graham-Smith, 1930; Graham-Smith, 1941). For example, Redmond et al. (1998) found that samples of sugar used by the German saboteurs in 1917 during World War I contained viable spores of *B. anthracis* even after nearly 80 years of archiving.

2.2. *Bacillus anthracis* spores – a Bioweapon

Bacillus anthracis has been used in bioterrorism throughout time. In *American Scientist*, 2001, Steven M. Block wrote that "Biological weapons have been called "the poor man's atom bomb."". Several attacks and outbreak have occurred in the past in different countries leading to social chaos and political disruption.

- 1917-1918: *Bacillus anthracis* spores laden sugar samples were used by the German army to infect the livestock of Allied Nations, which resulted in death of 200 mules in Argentina during the World War I (Redmond et al., 1998; Christopher et al., 1997).
- 1932- 1945: Bioweapon research and experiments were conducted by Japan in Japanese-occupied Manchuria. *Bacillus anthracis* and other lethal pathogens were intentionally used to infect the prisoners, resulting in at least 10,000 deaths. During this period, over 11 Chinese cities were impacted by *B. anthracis* by contaminating the municipal water supplies, food, and direct spraying into the homes (Christopher et al., 1997).
- 1942: In an experiment with bioweapons during the World War II carried out by Great Britain, a large number of bombs filled with *B. anthracis* spores were detonated on Gruinard Island in Scotland. The spread of the spores were tested on 80 sheep resulting in death of all sheep due to anthrax infection. The persistent nature of the spores made the island inhabitable for decades (Manchee et al., 1981; Manchee et al., 1994).

- 1979: An anthrax outbreak in Sverdlovsk military microbiology facility resulted in death of about 86% of the people exposed to *B. anthracis* spores in less than a week's time (Meselson et al., 1994).
- 2001: Bioterror attacks by mail containing *B. anthracis* spores in the United States, killed five people and hospitalized at least 17 people. Over 10,000 people were potentially exposed to *B. anthracis* in different states in the United States. Post terror decontamination measures were highly labor intensive and expensive (Jernigan et al., 2001).

It was estimated that an aerosol release of 100 kg of *B. anthracis* spores upwind of a city size of Washington D.C. could result in nearly 3 million deaths, which is an equivalent of hydrogen bomb explosion (Inglesby et al., 1999).

2.3. Inhalation Anthrax

The use of *B. anthracis* spores as a bioterrorism agent poses a threat to human health. Exposure and infection may occur via three routes: dermal (cutaneous anthrax), ingestion (gastrointestinal anthrax), and inhalation (inhalation anthrax). The mortality rates of inhalational anthrax in humans are as high as 100% in the absence of any medical intervention (Bush et al., 2001; Jernigan et al., 2001; Borio et al., 2001; Barakat et al., 2002; Chensue, 2003). Within three months of the 2001 anthrax attacks, 22 individuals were infected with the bacteria of which eleven resulted in inhalation anthrax. A total of five patients died due to inhalation anthrax (Day, 2003). Credible and validated exposure assessment for inhalation anthrax could aid in better management of bioterror events in the future. Due to the presence of numerous uncertainties, exposure assessments for *B. anthracis* spores have been previously conducted with large numbers of assumptions in which decay of spores over time was not considered (Canter, 2005;

Hong et al., 2010). It is a major concern of the US Environmental Protection Agency and the Department of Homeland Security to understand the behavior of such aerosolized biothreat agents with time because consequences of an attack may be directly correlated to both the virulence and the persistence of these spores under ambient environmental conditions.

2.4. Indoor Air and HVAC Filters

A single exposure to a large concentration of *B. anthracis* spores in scenarios of bioterror attacks or accumulated exposures to lesser concentration of *B. anthracis* spores over time could lead to inhalation anthrax risks. The major underlying concern with *B. anthracis* spores is, in case of an intentional release, inhalational risk will be the major component of overall risk from all possible exposure routes, when a contaminated area is reoccupied (Hong & Gurian, 2012). In case of an indoor release, spores have a high capability to be reaerosolized and infect building inhabitants. It was predicted using mechanistic modeling that more than 90% of aerosolized anthrax remain aerosolized inside the building for the first 48 hours after a hypothetical indoor release (Sextro et al., 2002). Even if decontamination procedures follow immediately after an attack, there are high possibilities of anthropogenic activities and chaos following the attack which could cause reaerosolization of spores. Greater than 80% of reaerosolized *B. anthracis* spores under simulated office conditions were observed to be in human respirable range (Weis et al., 2002). Once inhaled, there is a high probability of *B. anthracis* spores to germinate into vegetative cells and multiply in the respiratory system (Cote et al., 2009; Day et al., 2011; Dixon et al., 1999).

The 1979 anthrax outbreak at Sverdlovsk was found to be the result of a missing air filter that failed to capture *B. anthracis* spores coming from the production line in the drying facility (Wilkening, 2006). This indicates that high-grade air filters are proficient units to entrap spores

from spreading into outer environment. Nearly 18% of particles in the size range of *B. anthracis* spores could be captured by the air filters in Minimum Efficiency Reporting Value (MERV) 6 category (Carrera et al., 2005; Waring &Siegel, 2008).

More than 70% of residential air handling units (AHU) are comprised of HVAC systems in the United States (Noris et al., 2011). In cases of indoor bioterror attacks or infiltration of biological weapons from outdoor events, HVAC filters have found to act as distribution conduits in the building and also as potential sampling agents to determine the spread of microorganisms in indoor environment (Van Cuyk et al., 2012; Krauter & Biermann, 2007; Noris et al., 2011; Stanley et al., 2008; Kim et al., 2008; Calfee et al., 2014). Therefore, HVAC filters may act as concentrating systems to classify the quality and quantity of the biological agent used in the attack if the extraction, recovery, quantification and survival of the bioterror agents are clearly understood over time.

2.5. HVAC Filters – Reliable Sampling Sources for Indoor Microbial Contamination

In scenarios of intentional bioterror attacks, HVAC filters could act as inexpensive sampling sources to classify the quality and quantity of the biological agent used in the attack over a wide area, because of their abundance.

A large-scale study of the aerosol composition from two buildings located in Minneapolis and Seattle, respectively carried out by Stanley et al. (2008), examined loaded filter samples from the air handing units over a period of five and three months, respectively to look for airborne microbial contamination on filters. Comparison between the recoveries of fresh and shipped samples of air filters found that, shipping time played a significant role in affecting (lowering) the culturability of *B. subtilis* spores. Survivability of the spores was also suggested to be dependent on shipping conditions and time. This study did not aim to recover any specific

microorganism, but the goal was to identify the background culturable bacterial aerosols from large public buildings. Multiple filters of different classes composed of cotton or polyester blend, synthetic fibers, or fiberglass media were used in each of the four AHUs in each building. The results showed that a total of 39 different species were detected from the filters samples from both the buildings. It was demonstrated that the most commonly found species in both the buildings despite their demographic and seasonal diversities belonged to 11 different spore forming species of *Bacillus* genus with evident indoor sources and growth promoters such as potted plants (including the soil and insecticides), food (cooked, spoilt, improperly refrigerated). Of the hardy spore-forming entrapped *Bacillus* spores, three of them (*B. cereus*, *B. subtilis*, *B. thuringiensis*) belonged to the commonly used surrogate species for *B. anthracis*. This highlights the significance that indoor ambience and human activities could promote the thriving of the hardy spore forming, *Bacillus* population once they are captured in the AHU from an indoor or an outdoor release (Stanley et al., 2008).

A study carried out by Moritz et al. (2001) aimed to identify the capability of HVAC filters to capture, proliferate, and also release the bacterial species, even though the specifics of the microorganisms used in this study were not provided in detail. The increased release of unspecified bacterial spores was observed during cold and damp season of higher relative humidity (>80%) in his work. This 15-month long experiment carried out on two HVAC handling systems aimed at studying the capabilities of the air filters in HVAC units in retaining microorganisms, and to examine if they act as a source of airborne microbial contamination. Microbial content captured by filters were measured regularly at 14-day intervals. Bacterial counts from one of the filter systems increased, which was attributed to the plausible proliferation on the filters and subsequent dissemination through the filter air under increased

relative humidity. The released bacterial population was found to fall under the respirable size range of the humans. It was recommended that by the use of a pre-heater in front of the HVAC filters, the microbial growth could be annulled by controlling the relative humidity. This study indicated that the fluctuating moisture content present in the filters over time could possibly influence the adhesive behavior of vegetative bacteria depending on the degree of wetness; however this needs further investigation (Moritz et al., 2001).

In general, a better understanding of the fate and transport of spores, time-dependent recovery, and subsequent persistence pertaining to HVAC filters could serve as a constructive tool for future risk assessment studies and clean-up strategies in indoor environments. Based on the results of previous work described herein, it was shown that, HVAC filters are capable of capturing, releasing, and possibly providing a substrate for proliferation of different bacterial species. Extending the same findings towards *B. anthracis* spores warrants further investigation in light of their resiliency and potential use in bioterrorism. The following sections discuss the previously published research studies on recovery and quantification techniques of bacterial spores from filters and porous media.

2.6. Recovery Studies

Microbial recovery studies with various types of filters, that have been performed through seeding and cultivation experiments in order to define extraction and recovery efficiencies of *Bacillus* spores on media are presented in this section.

Wang et al. (2001) observed that inoculating polycarbonate filters with *B. subtilis* endospores and subsequent extraction, under varied relative humidity (rH) conditions over time (up to 8 hours), resulted in the extraction efficiencies of the spores as high as 98 ± 1%. The extraction efficiency was enumerated from the total microbial count in the eluted fluid via

epifluorescence microscopic quantification. It was found that efficient extraction techniques of vortexing followed by ultrasonic treatment could yield high recoveries of spores from the inoculated samples. Contrast to high recovery and extraction efficiencies measured using epifluorescence microscopy, the relative culturability of these spores enumerated using plate-culturing technique were as low as 5% and 17% for rH 30% and 85% respectively. Variables such as sampling time, rH, and type of the sampler, significantly and independently affected the culturability of the *B. subtilis* endospores. Additionally the study suggested that the desiccation stresses in the spores could account for their decrease in culturability over time. It was suggested that the higher culturability could be due to higher rH and higher amounts of water passing through the unit filter area. It could be inferred from this study that variables like water content on the filters, extraction methods, and filter types could affect the quantity and quality (viability or culturability) of the spores released from the filters. Though the culturability was low, the extraction was found to be as high as 100% by epifluorescence microscopy which indicates the need for better understanding of the effect of humidity or water content on recovery and culturability (Wang et al., 2001).

In a study by Burton et al. (2005), four types of filters of organic and inorganic origins were loaded with *B. subtilis var. niger* spores whose extraction efficiencies were reported for two extraction sequences. Extraction techniques of vortexting with ultrasonic agitation and vortexing with mechanical shaking were compared in this study. Extraction sequence of vortexting followed by shaker agitation was demonstrated to be the efficient technique of the two. The samples were extracted at different time steps of 15min, 1h, and 4h. The recovery efficiencies from extraction processes ranged between 66 -123% over time for different filters. High extraction efficiencies that exceeded 100% were not explained in this study. Culturability was

measured using plate-culturing quantification, and total counts of spores extracted as well as those present on the filters were measured using optical particle counter and epifluorescence microscopy. There was no loss in culturability over time reported in this study in contrast to Wang et al. (2001). The differences in the culturability observed in both the studies were attributed to the types of filters used, and differences in experimental procedures adopted for sporulation. It was observed that the overall culturability reduced in the case of polycarbonate filters compared to other types of filters (Burton et al., 2005).

A recovery optimization study of *B. golbigii* from commonly used filters in HVAC systems was carried out by Kim et al. (2008). This work aimed at an eluting technique in which maximum possible culturability could be attained. The test filters were loaded with *B. golbigii* spores by two methods namely, manual and aerosol loading with subsequent elution by hand-shaking agitation (500 shakes). The spores were eluted using deionized-filtered water and Tween-80 solution separately which had mean recovery rates of 102% and 118%, respectively for manual loading. A slightly greater recovery was observed when eluted with Tween-80. It was hypothesized that the surfactant Tween-80, could reduce the clustering or clumping of the *B. golbigii* spores. Spores were eluted and recovered with 100% culturability which was determined using plate-culturing technique. Recoveries greater than 100% were observed for artificial as well as aerosol loading when eluted with water, which were 102% and 106%, respectively. While aerosolized tests for the vegetative bacterial cells were futile due to the fragility of the cells. It is reasonable to assume that spores are more likely to maintain their culturability than vegetative bacteria cells during filtration and elution. This contributes to the reliability of HVAC filters as sampling media for spores after an attack (Kim et al., 2008).

A relatively long-term survival study was carried out by Mittal et al. (2011) on *B. atrophaeus* spores, on HEPA filters, which are of higher efficacy for capturing smaller sizes of bacteria compared to the HVAC filters. The test filters were loaded with the spores through aerosolized dispersion with constant airflow through them over time, which aimed to simulate a real-life AHU. It was observed that, all the other microorganisms survived no more than 6 days on their filters, however the spores of *B. atrophaeus* lasted on the HEPA filters up to 210 days (in absence of nutrients) with no significant loss in its viability.

Lack of validation of the extraction methods and recoveries greater than 100% led to the recovery study of *B. thuringiensis* spores from HVAC filters by Solon et al. (2012), which aimed at determining the most suited methodology for filter elution. *Bacillus thuringiensis* spores were artificially loaded on to HVAC filters of MERV 7 (commonly used filter type in residential and commercial buildings). A series of elution techniques such as sonication, shaking, and vortexing were tested in varied combinations to understand the best order for maximum recovery. It was found that the elution processes in the sequence of sonicating – vortexing – mechanical shaking yielded the maximum recovery compared to other combinations of techniques. The results of this study suggested the possibility of growth on the filters, suspecting that the filter media could act as a substrate for the growth of the deposited spores. Growth analysis was carried out by eluting the spores with and without the presence of filters during the extraction processes. When there was a filter sample present for 32 minutes during the agitation sequence, it resulted in approximately twice the recovery of bacterial spores. The authors hypothesized that it was due to growth that an increase in spore counts was observed, however factors such as wetness of the filter, disintegration of spores from the filters over longer periods of time when soaking, chemistry between the spores and the filters' surfaces were not considered (Solon et al., 2012).

Higher recovery percentages observed in the previous works (Wang et al., 2001; Burton et al., 2005; Farnsworth et al., 2006) as well as in the above mentioned study create the need to investigate growth as a factor over time with a better and consistent quantification technique.

A study carried out by Calfee et al. (2014) aimed to compare the efficiencies in recovery between extraction-based and two vacuum-based sampling methods for *B. atrophaeus* spores from HVAC filters of MERV 8. It was found that extraction-based yield were approximately 33% higher than the vacuum-based results. It was demonstrated that in the presence of dust, there was a higher culturability of spores, though there was no statistical difference made by the presence of dust. The recoveries were normalized to that of the reference steel coupon whereby biases during recovery were eliminated (Calfee et al., 2014). In case of normalizing with the initial applied concentration of aerosolized spores, the recovery was found to be as low as 4.6%. This indicates that, in a real-life scenario of aerosolized or reaerosolized spores, it is more likely that a major fraction of the spores remain on the surface of filter media. Interestingly, the results of this study did not observe a recovery that exceeded the initial inoculation volume. In other words recoveries close to or greater than 100% were not observed. Further research is needed in this area because of contrasting recoveries observed in different studies mentioned above in comparison to this work.

Since all of the recovery studies mentioned herein invariably carried out research using agents other than *B. anthracis* spores, it is of interest to study *B. anthracis* directly because agents such as *B. atrophaeus, B. subtilis, and B. golbigii* spores are smaller in size compared to *B. anthracis* spores, therefore their transport properties in air will vary from that of *B. anthracis* (Carrera et al., 2007). Surrogate agent used by Solon et al. (2012) was although morphologically and genetically similar to *B. anthracis*, it lacks the recovery over time, and the benchmark to

compare with *B. anthracis* spores. There it is important to carry out recovery studies as a function of time using *B. anthracis* spores.

2.7. Surrogate Selection

Due to the extensive pathogenicity and the ease of dissemination, the use of *B. anthracis* spores in most research facilities is prohibited due to the requirements of minimum bio-safety levels. The virulence of the spores mandates that experiments need to be conducted under Biosafety Level (BSL) – 3 conditions. Experimental release or recovery studies of such spores outside the BSL- 3 facility are forbidden. Therefore, it is required to select non-pathogenic surrogate strains for *B. anthracis*, which could successfully mimic its behavior, on recovery or on release for experimental purposes. Studies that have identified such reliable surrogates, compared to *B. anthracis* in the past were considered for this work.

Bacillus species such *B. anthracis*, *B. thuringiensis*, and *B. cereus* belong to *B. cereus* family, and are morphologically, dimensionally and genetically comparable with variations in their pathogenicity (Greenberg et al., 2010). On the other hand, commonly used surrogates such as *B. atrophaeus*, *B. subtilis* belong to the *B. subtilis* family. Due to numerous taxonomical methods, there were reclassifications of several strains of *B. subtilis* and *B. golbigii* as *B. atrophaeus* (Fritze & Pukall, 2001). Frequent references of *B. atrophaeus* as previously known as *B. golbigii* or as *B. subtilis var. niger* could be encountered in some studies (Carrera et al., 2007; Sagripanti et al., 2007; Kim et al., 2008).

Multiple closely related spores of different species of *Bacillus* were closely observed using microscopy along with the virulent strain of *B. anthracis* in order to determine the ones with maximum closeness to the species of interest, *B. anthracis* based on the size distribution by Carrera et al. (2007). A total of 14 types of spores were assessed for morphological similarities

under transmission electron microscope, of which seven were different strains of *B. anthracis*, and the other seven belonged to the same genus, *Bacillus*. It was reported that despite a similarity in genome sequences among the seven different strains of *B. anthracis*, their lengths varied significantly. The authors suggested that the best alternatives for *B. anthracis* are *B. thuringiensis* and *B. cereus* based on their similarities in dimensional and aerodynamic properties. The most commonly used surrogates *B. atrophaeus or B. subtilis* for *B. anthracis,* used in recovery studies are different from *B. anthracis* spores as they are thinner and smaller (nearly half the size of *B. anthracis*). Besides the commonality in the structure of the *Bacillus* spores, *B. anthracis*, *B. cereus*, and *B. thuringiensis* possess an outermost layer called exosporium, which are lacking in *B. subtilis*, *B. golbigii*, or *B. atrophaeus* (Carrera et al., 2007).

Since most of the published studies reported in the previous sections performed inoculation through aerosolization, the aerosolized spores of *B. atrophaeus* or *B. subtilis* would likely differ significantly from that of *B. anthracis* when released.

In an experimental-surrogate identification study by Greenberg et al. (2010), it was reported that *B. atrophaeus* was most often used for research purposes followed by *B. cereus* and *B. subtilis* in place of *B. anthracis*. The earliest reported usage of *B. atrophaeus* was by the US government for biological warfare test studies in 1943. However, there was no basis or a concrete criterion for selecting these species as surrogates. Though this study was a review and not experimental, it was the first one of its kind to comprehensively provide a selection protocol and identify the best suited surrogate for experimental purposes (Greenberg et al., 2010). Species with genetic similarity to *B. anthracis*, morphology, pathogenicity, and response to chemical and environmental stresses, ease of culture, cost, and history of use were several benchmarks used for surrogate selection. It was suggested that the potential surrogates for *B. anthracis* could be *B.*

cereus, *B. thuringiensis*, *B. atrophaeus*, and *B. megaterium*. However, based on the genetic and morphological parallels, *B. thuringiensis* and *B. cereus* were recommended to the best suited surrogates for *B. anthracis*.

A comparative review by Tufts et al. (2013) on the behavior of *B. thuringiensis* and *B. anthracis* spores when aerosolized, reported that, aerosolized *B. thuringiensis* and *B. anthracis* are similar in behavior, however all the *B. thuringiensis* spores in a sample could not be considered as a representative of *B. anthracis* spores. The most influencing factors for the aerodynamic behavior of spores such as size, exosporium, morphology, hydrophobicity, and density were compared. It was suggested that, when prepared in similar conditions, such as sporulation media, rH, temperature, pH, sporulation time, the aerodynamic properties of *B. thuringiensis* and *B. anthracis* appear similar. The restoration of size to aspect ratio and presence of exosporium are important because they play a significant role in aerosolization. The underlying statement of this review was that, though all *B. thuringiensis* spores cannot be considered as *B. anthracis* spores owing to heterogeneity in preparation methods, if the spores are prepared in a similar manner their heterogeneity in preparation would be minimized and considered analogous.

Spores of *B. thuringiensis* were used as surrogate for *B. anthracis* in the recovery study by Solon et al. (2012) from HVAC filters. Minimal differences in the genetics and morphology between *B. anthracis* and *B. thuringiensis*, non-pathogenicity, ease to accessibility, compatibility to laboratory usage, non-clumping property of *B. thuringiensis* HD1011 strain were highlighted in this work for the selection of *B. thuringiensis*.

Therefore, from the above referred studies, based on the laboratory suitability, availability, genetic and size similarities, the spores of *B. thuringiensis* appears to be the best

candidate for the surrogate usage in the research studies. However, there is no study that compares the behavior of *B. thuringiensis* along the side of *B. anthracis* to evaluate recovery experimentally on porous media. The study by Solon et al. (2012) could provide useful information about the behavior of *B. thuringiensis* on filter media; however extrapolating it to *B. anthracis* spores requires further investigation with similar experimental conditions.

2.8. Risk Assessment Studies

Since the spores of *B. anthracis* are highly persistent in the environment, and possess very low ability for environmental attenuation and decay, there arises a need for active decontamination of the site of interest in case of any deliberate release. According to the U.S. Environmental Protection Agency (EPA), cleaning of the site is considered safe enough for reoccupancy, when there is no longer any growth of *B. anthracis* spores from all the post-remediation samples (Canter, 2005). This highly conservative practice is due to the absence of confidence in the limit of detection (LOD) for the spores. LOD represents the instrumental or environmental limit that is considered as an output during analytical methods to detect *B. anthracis*. These values serve as an input variable in risk assessment studies. Estimation of the LOD depends on recovery efficiencies with the consideration of time and experimental stages as recovery efficacy directly affects the LOD. Improving the recovery efficiency could amplify the sensitivity of the LOD (Herzog et al., 2009). Though recovery efficiency and LOD do not definitively indicate the risk, they are needed to estimate exposure concentrations and thereby interrelated and required to develop a credible and substantial risk assessment. Risk assessments so far are based on a large number of assumptions and uncertainties as well as the absence of time-varying recoveries of spores. Spores accumulated in air filters such as HVAC are considered as samples that could help in inferring the exposure and risk resulting from the

release itself (Hong et al., 2010; Hong et al., 2012). The remarkable degree of persistence of the spores and high mortality rates of inhalation anthrax has highlighted the importance of risk estimation for the long-term fate and transport of the spores. Long-term modeling strongly depends on validated short-term data that contains acceptable uncertainties and assumptions. Therefore more studies on time-dependent recovery are needed to credibly assess the LOD, fate and transport of the spores, exposure scenario and hence the risk.

2.9. Knowledge Gaps

Previous research leaves some important knowledge gaps that need to be addressed in order to understand the time-dependent recovery of spores. It is vital to carry out studies with respect to time to discretely distinguish between variability in recovery over time and the decay of spores, particularly when subsequent extrapolations to long-term scenarios will be made.

High extraction efficiencies as observed in the past studies (Wang et al., 2001; Burton et al., 2005; Farnsworth et al., 2006) provide information about the success of their respective recovery strategies. However, all of these studies have used spores belonging to the *B. subtilis* family, which are morphologically and dynamically different from *B. anthracis* (Greenberg et al., 2010). Additionally, the spores of *B. atrophaeus* or *B. subtilis* are nearly half the size of that of *B. anthracis* (Carrera et al., 2007). Due to smaller size, wet spores of *B. subtilis* or *B. atrophaeus* become denser than *B. anthracis*, thereby affecting transport properties in air or water (Greenberg et al., 2010). Use of dissimilar surrogates for recovery studies could be considerably misleading, especially on porous media where size of the spores could play an influencing factor on recovery because of entrapment within the media. Hence, it is critical to employ a surrogate that is similar in relevant properties as that of pathogenic *B. anthracis*.

Though the theoretical compatibility of *B. anthracis* and *B. thuringiensis* has been reviewed and published previously (Tufts et al., 2013; Carrera et al., 2007; Greenberg et al., 2010; Solon et al., 2012) there are no studies to date that have experimentally demonstrated the recovery of *B. anthracis* and *B. thuringiensis* from HVAC filters in parallel. Therefore, studies are needed to experimentally test the behavior of *B. anthracis* and *B. thuringiensis* concurrently to verify their theoretical compatibility.

Recovery studies that were carried out under different relative humidity (rH) conditions and moisture levels saw an increased spore recovery and culturability (Moritz et al., 2001; Wang et al., 2001). Greater culturability of *B. subtilis* spores was correlated to greater amount of water loaded per unit filter (Wang et al., 2001). Fomite to finger transfer efficiency of *B. thuringiensis* spores for porous media such as cotton and polyester also increased with increasing rH (Lopez et al., 2013). Higher transfer efficiency was attributed to the wetness of the spores facilitating them to transfer. The authors suggested that, a low rH could result in stronger adhesion between the *B. thuringiensis* spores and the non-porous fomites resulting in lesser transfer efficiency. A similar finding was reported in which, adhesion of *B. subtilis* spores increased upon drying on a non-porous surface (Nanasaki et al., 2010). Although the underlying role of moisture content over time, upon recovery is clearly not understood, further experimental work with different moisture levels could help address the gap pertaining to dryness and adhesion.

To summarize, the identified gaps from the previous studies are:

- Lack of time-dependent recovery studies of *B. anthracis* spores
- Lack of conclusive evidence that *B. anthracis* and *B. thuringiensis* spores have identical or similar behavior when identical sporulation and experimental procedures are used to measure recovery from porous media

- Lack of quantitative studies on the effect of moisture levels on recovery from porous media over time

CHAPTER 3. EVALUATING TIME-DEPENDENT VARIABILITY IN RECOVERY OF BACILLUS SPORES FROM HVAC FILTERS

3.1. Abstract

Time-dependent recovery of *B. anthraics* spores is not well understood to date. The objective of this study was to quantify and compare the recoveries of *Bacillus anthracis Sterne* and *Bacillus thuringiensis HD 2-61* spores from HVAC filters, a porous fomite, over a period of 168 hours under different moisture conditions. The results were analyzed using a general linear model (GLM) with factors of sampling time, moisture content, and species. The recovered concentrations of *B. anthracis* spores statistically decreased over time while *B. thuringiensis* spores demonstrated an increase followed by a decrease in all trials. In the GLM, main effects as well as the interaction effects of sampling time, water content, and the species, significantly affected the log-reductions. Results from supplemental tests, indicated that, the increase in the recovery of *B. thuringiensis* might have been due to individual or combined effects of clumping, moisture content, and rate of dehydration. Overall, the resulting trends in recovery of *B. anthracis* and *B. thuringiensis* spores from this study were dissimilar and indicated that *B. thuringiensis* spores could not be an ideal experimental surrogate for *B. anthracis* spores in recovery studies. The data generated in this study may be useful for persistence modeling and subsequent studies in quantitative microbial risk assessment (QMRA) to estimate the risk of exposure to *B. anthracis* spores in indoor air.

Keywords: B. anthracis, B. thuringiensis, HVAC, Anthrax, Bioterrorism

3.2. Introduction

Weaponized *Bacillus anthracis* spores gained importance as a public health issue due to fear of bioterrorism related exposures and subsequent infection. Mortality rates as high as 100% were observed in cases of inhalational anthrax, without medical intervention in humans, compared to other forms of anthrax such as cutaneous and gastrointestinal anthrax (Bush et al., 2001; Jernigan et al., 2001; Borio et al., 2001; Barakat et al., 2002; Chensue, 2003). Due to the presence of numerous uncertainties about inhalational exposure to *B. anthracis*, risk assessments have been based on a large number of assumptions where the fate of spores over time have not been considered (Canter, 2005; Hong et al., 2010). Time-dependent predictions of recovery and persistence modeling of the spread of spores in the indoor environments are important for more credible risk assessments and decontamination strategies.

Heating, ventilation and air conditioning (HVAC) systems are widely used in more than 70% of the residential air handling units in the United States (Noris et al., 2011). In cases of indoor bioterror attacks or infiltration of biological weapons from outdoor events, HVAC filters have been found to act as distribution conduits in buildings and potential sampling media to determine the spread of microorganisms (Van Cuyk et al., 2012; Krauter & Biermann, 2007; Norris et al., 2011; Sextro et al., 2002; Stanley et al., 2008; Kim et al., 2008; Calfee et al., 2014). Therefore, HVAC filters may act as pointers to classify the quality and quantity of the biological agent used in the attack if the extraction, recovery, quantification and survival of the agents are clearly understood over time.

Several studies have carried out extraction and recovery of spores of *B. anthracis* surrogates from various filter media (Wang et al., 2001; Burton et al., 2005; Farnsworth et al., 2006; Kim et al., 2008; Solon et al., 2012; Calfee et al., 2014; Moritz et al., 2001; Mittal et al.,

2011; Maus et al., 2000; Van Cuyk et al., 2012; Stanley et al., 2008); however none of them have quantitatively reported the recovery efficiency as a function of time for *B. anthracis* spores from filters. Instead studies were conducted with spores of *B. thuringiensis, B. subtilis, B. atrophaeus* and, *B. golbigii*.

Furthermore, recovery studies carried out under different relative humidity conditions found greater recovery at higher relative humidity conditions (Moritz et al., 2001; Wang et al., 2001). Humidity was also directly correlated to culturability and inversely correlated to adhesion of spores on porous and non-porous surfaces (Lopez et al., 2013; Nanasaki et al., 2010). Effect of moisture on filters should to be tested because the indoor air system is constantly subjected to fluctuating relative humidity variations in order to maintain a stable relative humidity within the building (Woloszyn et al., 2009).

Biosafety concerns mandate the need for experimental surrogates for *B. anthracis* spores. Theoretical reviews and microscopic examinations have suggested that the best suited surrogate for *B. anthracis* in aerosol and in water was *B. thuringiensis* (Greenberg et al., 2010; Tufts et al., 2013; Solon et al., 2012; Carrera et al., 2007). However, similarities between *B. anthracis* and *B. thuringiensis* spores are valid if and only if the spores are prepared and experiments are carried out identically (Tufts et al., 2013). Thus, the absence of recovery studies using both the species warrants a need to test them under identical physical and experimental conditions over time for comparison.

The goals of this study are to quantify the recoveries of *B. anthracis Sterne* strain and *B. thuringiensis HD 2-61* spores from HVAC filters over a 168-hour period with two different moisture contents. The quantified time-based recovery of spores, will be used to address, (1) the time-dependent behavior of *Bacillus* spores on HVAC filters; (2) the suitability of the surrogate

(*B. thuringiensis* spores) with respect to *B. anthracis* spores; and (3) the effect of moisture content on the recovery of *Bacillus* spores.

3.3. Materials and Methods

3.3.1. Species Used

The *B. anthracis Sterne* strain and *B. thuringiensis HD 2-61* were used for this experiment because of their physical similarities to the virulent strain of *B. anthracis*. Strains of *B. anthracis Sterne* and *B. thuringiensis* were provided by Dr. Frances Downes (Program in Public Health, Michigan State University) and Dr. Julia Bell (Department of Large Animal Clinical Sciences, Michigan State University) respectively. The strains were stored at -80^{o}C until initiation of the experiments. The experiments were carried out at different times in the lab to ensure that the species were evaluated separately and the potential for cross-contamination was eliminated.

3.3.2. Inoculum Preparation

The experiments were carried out in a biosafety level (BSL)—2 facility. Spores of each species were separately produced from vegetative cells by suspending a loopful of each strain in 5mL of Tripticase Soy Broth (TSB) medium at 37^{o}C for 24 hours. A loopful of the incubated TSB suspension was spread onto Tripticase Soy Agar (TSA) slants. The slants were incubated at 37^{o}C for 10 days. After 10 days, over 90% of the cells had formed spores. On day 11, the spore layer was removed by washing, and resuspended in 5 mL of Type 1 ultrapure water at room temperature. This spore solution is hereafter referred to as "master stock". The master stocks were quantified by plating 0.1 mL of serial dilutions onto TSA plates on the same day. Spores of *B. anthracis* and *B. thuringiensis* were differentiated based on their colonies. *Bacillus thuringiensis* spores form colonies of bigger size on TSA plates compared to *B. anthracis* spores. Master stocks were then stored at 4^{o}C to prevent germination of spores. The master stocks were

rapidly vortexed to ensure homogeneity and diluted in Type 1 ultrapure water to yield 10 mL of desired spore concentrations in the range of 10^6 to 10^7 spores/ mL. These diluted solutions, are hereafter referred to as "stock solutions", and were used to spike the HVAC filters during the inoculation process. Calculation details for preparation of master stock and stock solutions are presented in APPENDIX-B.

3.3.3. Preparation and Inoculation of HVAC Filters

A MERV 7, Hi-E40 HVAC filter (Purolator Products Air Filtration Company, Jeffersonville, IN) was cut into 1 x 1 inch pieces. These pieces were wrapped in aluminum foil to avoid contamination by dust particles, and sterilized by autoclaving at 121^{o}C for 15 minutes.

Three replicate filters were each spiked with 20 droplets of 20 µL, 0.4 mL stock solution, of *B. anthracis* spores. A volume of 0.4 mL was used in order to saturate the entire surface of the filter with the inoculum (Solon et al., 2012). The same inoculation procedures were used for the stock solution of *B. thuringiensis* spores. To measure the effects of lower water content on recovery, a separate stock solution was prepared with half the water used in the original stock solution. The same inoculation procedure described above for the first stock solution was repeated, but only 20 droplets of 10 µL, 0.2 mL, were spiked on the filters. This amount of solution covered the surface of the filters but did not saturate them. Therefore, a lower volume of inoculum with a similar concentration of spores was achieved. The exact spore concentrations applied to each filter for both the 0.4 mL or 0.2 mL inoculation volumes for both species are summarized in Table 1. Inoculated filters were gently wrapped in aluminum foil and placed inside a sterile desiccator to ensure a dust-free and moisture-free environment. Spores were recovered and quantified at 0 hour (30 minutes after application when all droplets had been

absorbed by the filter), 24, 48, and 168 hours. Thus a total of 48 filters (three filter replicates x two concentration of spores x four time points x two species), along with eight negative controls (one blank filter per time point per species) were tested.

3.3.4. Elution of Spores from Filters

Spores were recovered from the filters using elution sequences following the method described by Solon (2010). At each time point, filters were carefully unwrapped and suspended in 20 mL Type 1 ultrapure water in sterilized 50 mL centrifuge tubes. Aseptic tongs were used to transfer the filters from the aluminum foil to the centrifuge tubes. Tongs were washed with 70% ethanol solution, rinsed with deionized water, and dried after each transfer to avoid mixing of spores or contamination between the filters. Elution of spores from the filters was carried out in a three-stage process. The stages included sonication, followed by vortexing, and mechanical shaking. During sonication, filter samples suspended in 20 mL of distilled water were placed in a water bath inside a Branson Mechanical Ultrasonic Cleaner, Model 2510R-DTH operating at frequency of 42 kHz ± 6% for 15 minutes. Following sonication, solutions were vortexed in a Fischer Scientific Digital Vortex Mixer at 1000 rotations per minute for 2 minutes. The final stage of elution was mechanical shaking in a VWR Minishaker at 170 rotations per minute for 15 minutes. Quantification was done by plate-culturing serial dilutions of the eluted solutions in triplicates on TSA plates.

3.3.5. Culture-based Quantification

For all samples, a volume of 0.1 mL of the eluted solution was spread on TSA plates. The plates were incubated at 37^{o}C for about 24 hours. Plates with colony-forming units (CFUs) in the range of 25 to 250 were considered for estimating the number of CFUs per mL of the original eluted sample. It was assumed that each colony originated from an individual spore. The data were converted to the spore equivalent of spores per mL, and subsequently spores per filter based on the volume of the eluting fluid used (20 mL). At each time point, nine recovery data were obtained. Blank TSA plates and plates with Type 1 ultrapure water were also incubated as negative control plates to ensure contaminant-free elution and plating during quantification.

3.3.6. Tests for Clumping and Drying Rates

A set of supplemental experiments was carried out in order to test the probable clustering of *B. thuringiensis* spores, using surfactant Tween-20 to separate spores in the solution. The recovered CFUs from the surfactant experiments were used to qualitatively identify the presence of clumping. An additional analysis to investigate the severity of the "bump" was carried out by comparing the areas under the curve enclosed by the coordinates of the recovery points. Dehydration rates of the water content loaded on filters were also measured separately to understand the effect of moisture content on culturability.

It was previously observed that addition of surfactants during the elution process (to the eluting solution) yielded higher numbers of spores during the recovery (Kim et al., 2010). In this study, surfactant was added directly to the stock solution prior to inoculation. It was hypothesized that clumping, if any, would occur prior to inoculation, and not during drying, on the media. A concentration of 0.1% of Tween-20 was achieved in the surfactant-spores solution

(He et al., 2003; Dang et al., 2001). Inoculation volume of 0.4 mL, with a concentration of approximately 2.6 x 10^6 spores per mL, followed by elution and quantification procedures as discussed above were repeated for *B. thuringiensis* samples with and without the addition of Tween-20. The *B. thuringiensis* spores, with and without the addition of Tween-20 were also observed with a scanning electron microscope (SEM).

To observe the drying rates of the filters over 168 hours for each water content, 0.4 mL and 0.2 mL of Type 1 ultrapure water was spiked on to separate filter coupons in triplicates. The filters were wrapped and stored in desiccator at identical physical conditions. Filters were weighed over 168 hours with time intervals of 8 hours and 16 hours.

3.3.7. Scanning Electron Microscopy (SEM)

SEM was used to observe the tangible effects of autoclaving on HVAC filters, and to observe the nature of *B. thuringiensis* spores, in the presence and absence of Tween-20. Pieces of HVAC filter samples were examined in a Joel JSM-6610LV scanning electron microscope (JOEL Ltd., Tokyo, Japan). Samples of *B. thuringiensis* spores with and without the presence of Tween-20 were processed and fixed on aluminum stubs, according to SEM procedure described in APPENDIX-D. Fixed samples were examined in a Joel JSM-7500F (cold field emission electron emitter) scanning electron microscope (JOEL Ltd., Tokyo, Japan).

3.3.8. Data Treatment

The total plate counts from each triplicate plate were converted to spores recovered/ filter by multiplying by the inverse of the volume of extract plated (0.1 mL), by the dilution factor of the corresponding plate, and the volume of total extract used to elute the filter (20 mL). The calculated concentrations of recovered spores were represented in terms of spores per filter for easier interpretation. To normalize the recovery data, all the recoveries were divided by the number of applied spores (Table 1). These data were subsequently $\log_{10}$ transformed and averaged for each time point (N=9). This is hereafter referred to as "log-reduction", which was calculated using Eq. (1). In order to show the differences in the results produced by the choice of denominator, the recoveries were divided by the average recovery obtained at the 0 hour, and $\log_{10}$ transformed. By normalizing with 0 hour recovery, a 100% initial recovery rate was assumed. This is hereafter referred to as "normalized log-reduction", which was calculated using Eq. (2). Recovery rates and normalized recovery rates were computed using Eq. (3) and Eq. (4) respectively, to represent the recoveries in terms of percentages.

$$\text{Log Reduction} = \log_{10}\left(\frac{\text{Nt}}{\text{Ni}}\right) \quad \ldots. (1)$$

$$\text{Normalized Log Reduction} = \log_{10}\left(\frac{\text{Nt}}{\text{N0}}\right) \quad \ldots. (2)$$

$$\text{Recovery Rate} = \frac{\text{Nt}}{\text{Ni}} * 100\% \quad \ldots. (3)$$

$$\text{Normalized Recovery Rate} = \frac{\text{Nt}}{\text{N0}} * 100\% \quad \ldots. (4)$$

where N_t=Average number of spores recovered at time, *t*, N_i=Initial number of spores applied to the filters, N_0=Average number of spores recovered at time 0 hour.

3.3.9. Statistical Tests

Statistical analyses were performed using the general linear model (GLM) Univariate procedure of SPSS version 20 (SPSS Inc., Chicago, IL) on data sets containing: (i) the dependent variable log-reductions, at all four time steps (0, 24, 48, and 168 hours) across species (*B. anthracis* and *B. thuringiensis*), and moisture contents (0.4 mL and 0.2 mL), (N=144); (ii) log-reductions of *B. thuringiensis* spores with and without the addition of Tween-20 (N = 72) to assess for significant differences due to the addition of Tween-20 over time. The analyses included both main effects as well as all interaction effects on the response variable.

One-tailed t-tests were performed to assess for significant reduction on log-reductions between two time steps for both the species separately. A significance level (α) of 0.05 was used for all tests.

3.4. Results

Results obtained in this study are presented in the following order: (i) comparison of data treatment techniques; (ii) evaluation of log-reductions and normalized log-reductions; (iii) comparison of *B. anthracis* and *B. thuringiensis* spores; (iv) evaluation of the effect of surfactants on clumping of *B. thuringiensis* spores; and (v) comparison of log-reduction between different moisture levels.

3.4.1. Concentrations of applied and 0 hour recovered spores

Mean concentrations obtained from the 0 hour extractions and enumeration ranged from $2.27 \pm 0.167 \times 10^6$ CFU to $7.00 \pm 1.34 \times 10^5$ CFU per filter. Applied concentration and average 0 hour recovered total concentrations obtained for *B. anthracis* and *B. thuringiensis* spores at both moisture levels are summarized in Table 1. The calculated recoveries obtained during 0 hour were used to normalize the recoveries measured over time using Eq. (2) and (4). Applied concentrations of spores (Table 1) were used in Eq. (1) and (3), to obtain log ratios. The 0 hour recovery of *B. anthracis* spores was close to the applied concentration but *B. thuringiensis* spores lowered by an order of magnitude or 1-$\log_{10}$.

3.4.2. Comparison of recovery rates and normalized recovery rates

Table 2 presents the summary of t recovery rates and normalized recovery rates of *B. anthracis* and *B. thuringiensis* spores. Recovery rates of *B. anthracis* were statistically decreasing ($p = 0.008$) over time. For *B. thuringiensis*, recovery rates statistically increased ($p < 0.001$) greater than 100% at 24 hours.

As expected, normalized recovery rates for *B. anthracis* and *B. thuringiensis* at the 0 hour were the same, due to the assumption of 100% initial recovery. Because the *B. anthracis*

recovery at 0 hour was close to applied concentrations, the differences between the 0 hour recovery rates and normalized recovery rates were insignificant. However, the differences for *B. thuringiensis* were significantly different ($p<0.001$).

Due to this variation both the values were retained for comparing their respective log-reduction values.

By 168 hours, recovery for *B. anthracis* declined to 10% for 0.4 mL and 34% for 0.2 mL and recovery for *B. thuringiensis* declined to 38% for 0.4 mL and 56% for 0.2 mL. For both water levels, B. thuringiensis increased at 24 hours sampling followed by a decrease. This is discussed in the next section.

Table 1. Summary of applied and average 0 hour recovered concentrations of *Bacillus* spores

Species	**Volume of inoculum/ filter (mL)**	**Total Applied Concentration [a] (CFU)**	**Total 0 hour Recovered Concentration [a, b, c] (CFU ± SD)**
B. anthracis	0.4	1.15×10^6	$1.13 \pm 0.203 \times 10^6$
B. thuringiensis	0.4	1.09×10^6	$7.00 \pm 1.34 \times 10^5$
B. anthracis	0.2	2.29×10^6	$2.27 \pm 0.167 \times 10^6$
B. thuringiensis	0.2	2.19×10^6	$8.47 \pm 2.15 \times 10^5$

a, Total concentration of CFUs per filter
b, Average of three TSA plate counts for each of the three filter replicates. Plates were incubated at 37^{o}C for 24 hours
c, N=9

Table 2. Summary of recovery rates and normalized recovery rates over time (N=9)

Species (volume/ filter, mL)	**Recovery Rate (%)**				**Normalized Recovery Rate (%)**			
	0 h	**24 h**	**48 h**	**168 h**	**0 h**	**4 h**	**8 h**	**168 h**
B. anthracis (0.4)	98 ± 18	78 ± 18	52 ± 13	10 ± 3	100 ± 18	79 ± 18	53 ± 13	10 ± 3
B. thuringiensis (0.4)	64 ± 12	116 ± 31	79 ± 19	38 ± 2	100 ± 19	180 ± 48	123 ± 30	58 ± 3
B. anthracis (0.2)	99 ± 7	77 ± 9	54 ± 9	34 ± 5	100 ± 7	78 ± 9	54 ± 9	35 ± 5
B. thuringiensis (0.2)	39 ± 10	55 ± 7	59 ± 12	56 ± 10	100 ± 25	143 ± 18	153 ± 31	146 ± 26

3.4.3. Comparison of *B. anthracis* and *B. thuringiensis* spores

Figures 1 and 2 illustrate the comparison of the log-reductions for *B. anthracis* and *B. thuringiensis* over time, at the two moisture levels respectively. *Bacillus anthracis* and *B. thuringiensis* behaved dissimilarly over time. Recoveries of *B. anthracis* were gradually reducing, while recoveries of *B. thuringiensis* increased followed by a decrease over time. At the higher water content (0.4 mL) there was a slight overlap between the error bars of *B. anthracis* and *B. thuringiensis* reductions (Fig.1) up to the 48 hours period which was not observed at the lower water content (0.2 mL) in Fig. 2.

Figures 3 and 4 illustrate the comparison of the normalized log-reductions for *B. anthracis* and *B. thuringiensis* over time, for two moisture levels respectively. With the assumption of 100% recovery, the data indicates that *B. anthracis* is reducing over time, while *B. thuringiensis* increased at 24 hours over 100%, then decreased as their corresponding recoveries lie on either sides of the X-axis. This was observed for both moisture levels.

Overall, as a function of time *B. anthracis* reduced with respect to applied concentration of the inoculum while, *B. thuringiensis* consistently increased at 24 hours, which appears like a "bump" in all figures.

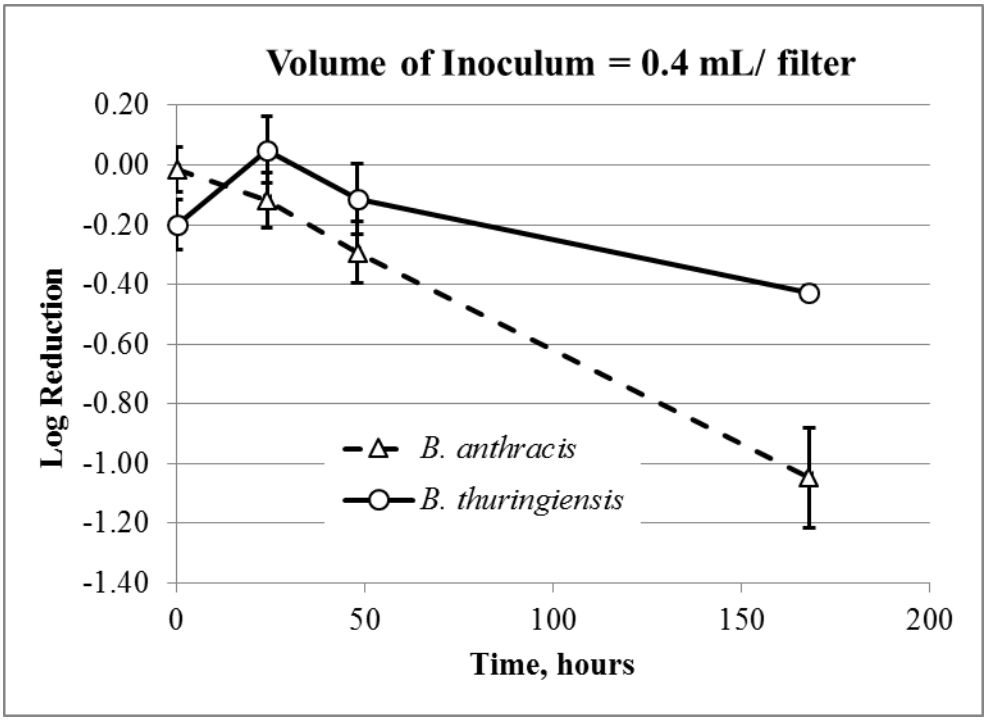

Figure 1. Log-reduction from Eq. (1) as a function of time for 0.4 mL volume of inoculation for both *B. anthracis* and *B. thuringiensis*. Error bars indicate standard deviation from the mean.

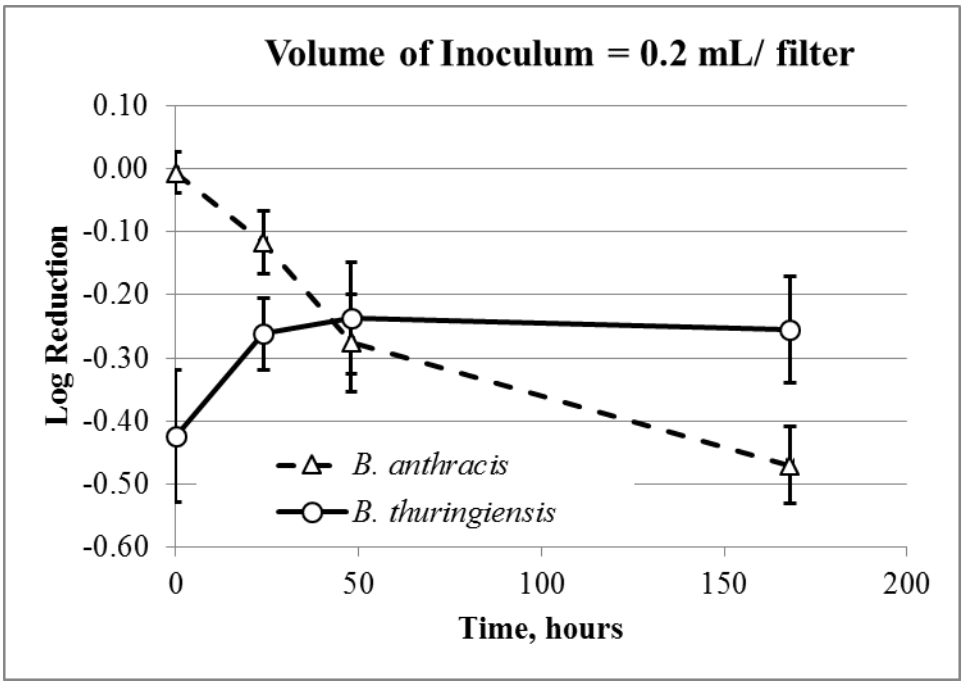

Figure 2. Log-reduction from Eq. (1) as a function of time for 0.2 mL volume of inoculation for both *B. anthracis* and *B. thuringiensis*. Error bars indicate standard deviation from the mean.

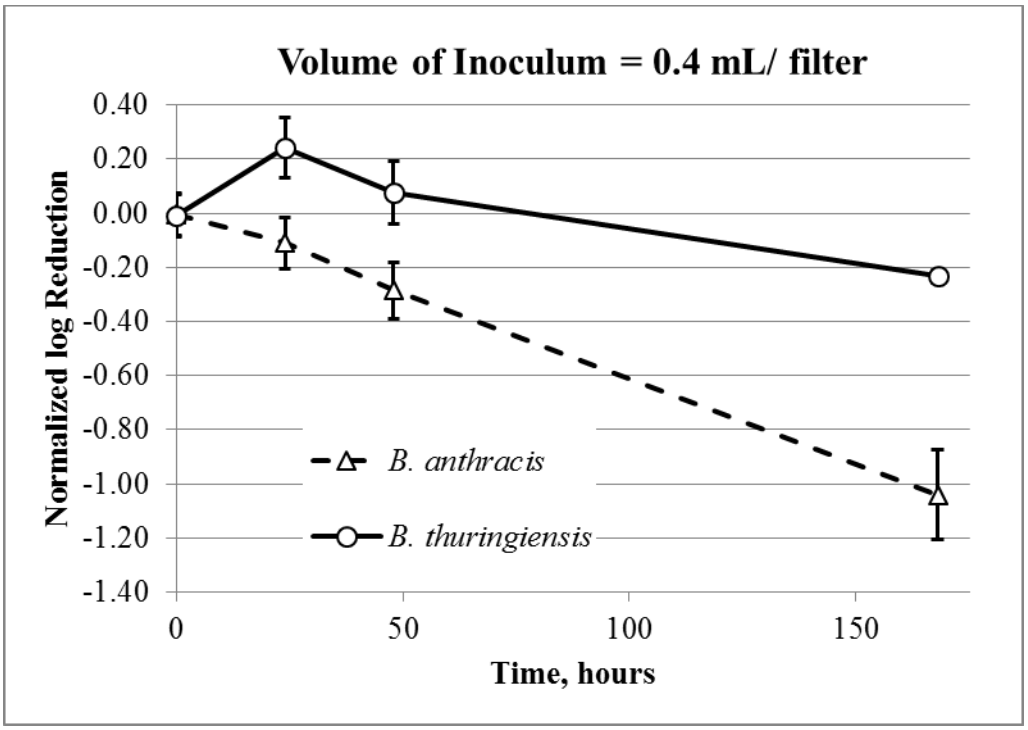

Figure 3. Normalized log-reduction from Eq. (2) as a function of time for 0.4 mL volume of inoculation for both *B. anthracis* and *B. thuringiensis*. Error bars indicate standard deviation from the mean.

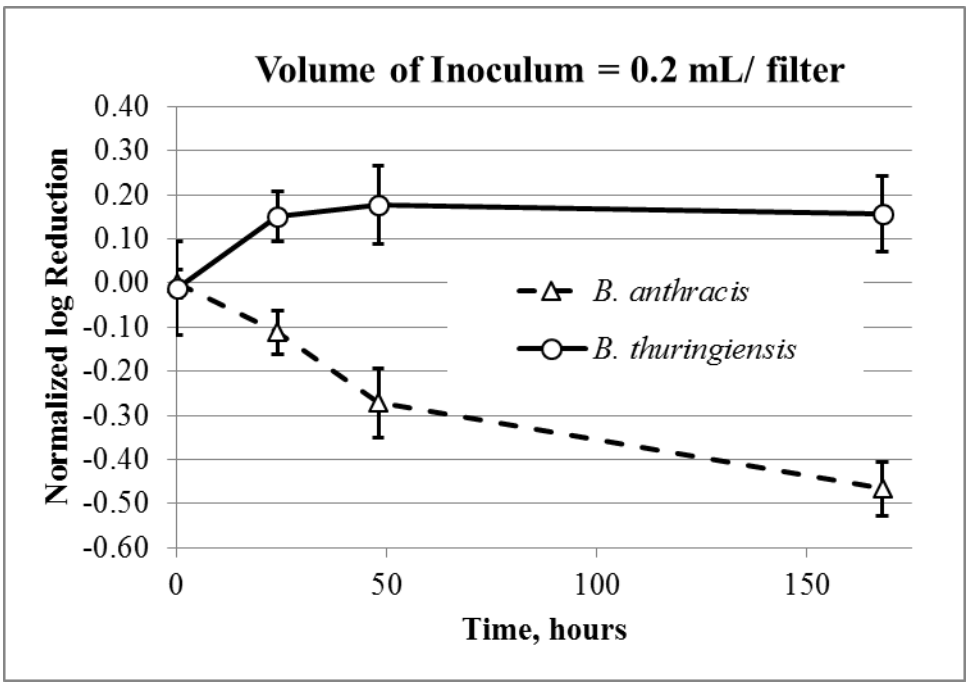

Figure 4. Normalized log-reduction from Eq. (2) as a function of time for 0.2 mL volume of inoculation for both *B. anthracis* and *B. thuringiensis*. Error bars indicate standard deviation from the mean.

3.4.4. Effect of surfactants on clumping of *B. thuringiensis* spores

The apparent bump in *B. thuringiensis* was thought to be due to clumping. Results from additional experiments with and without the addition of surfactant Tween-20 to test for clumping of *B. thuringiensis* spores revealed that there was an increase followed by a decrease in the log-reduction trend even in the presence of surfactants. The "bump" was observed in the log-reductions with and without Tween-20 (Fig. 5). A linear trend line was fit to evaluate the severity of the bump in each case. On comparing their error sum of squares (SSE) of their average plate count results, it was found that the recoveries in the presence of Tween-20 (SSE= 0.0002013) deviated less from the linear trend than in the absence of Tween-20 (SSE=0.00109). In other words, recoveries were more consistent in the presence of Tween-20.

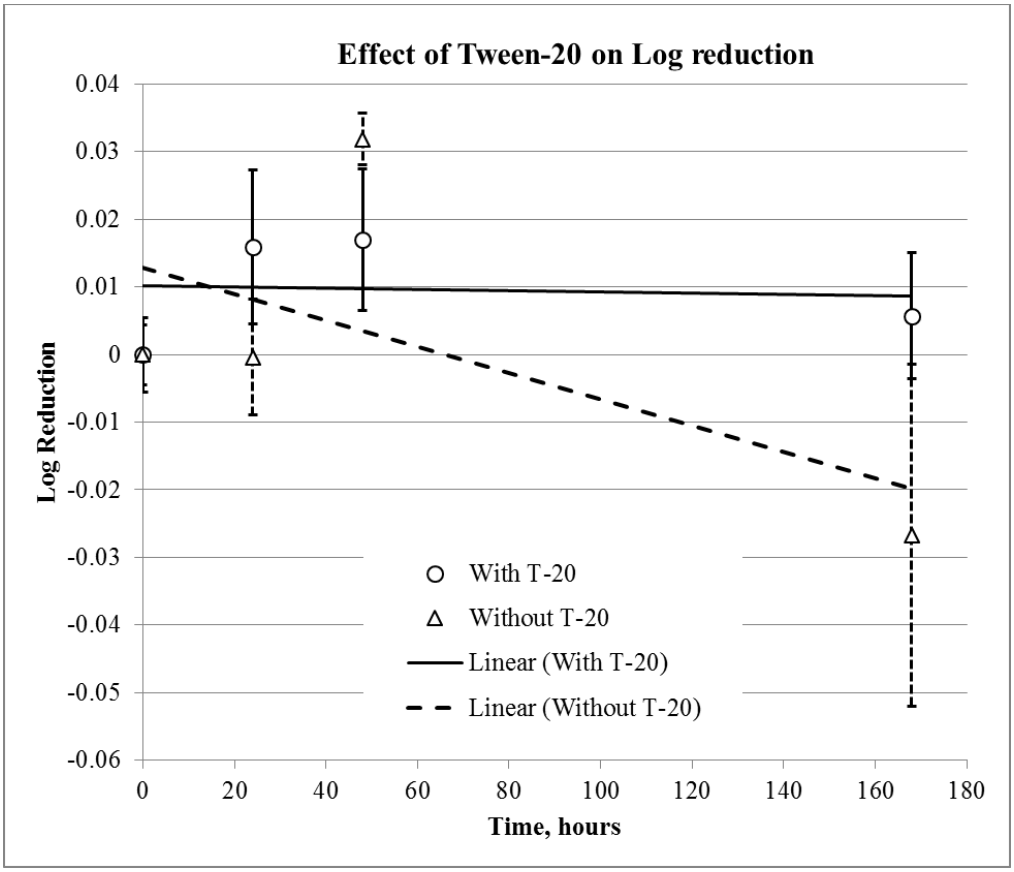

Figure 5. Log-reduction from Eq. (1) as a function of time for 0.4 mL volume of inoculation of *B. thuringiensis* spores with and without addition of Tween-20. Error bars indicate standard deviation from the mean.

The area enclosed by the coordinates of recovery over time for samples with Tween-20 was 1.472 square units, and that of coordinates of recovery over time for samples without Tween-20 was 2.610 square units. Larger area enclosed by the samples without Tween-20 indicates that the bump is more severe and pronounced compared to the samples with Tween-20. Also the standard deviation of the entire sample (N=36) with Tween-20 was 0.011, and that for the sample (N=36) without Tween-20 was 0.024. Greater standard error about the mean also indicates the larger spread in recovery values which may be due to the presence of clumps (Ilstrup, 1990).

3.4.5. Comparison of log reductions between different moisture levels

Figures 6 and 7 represent the log-reductions of the two species at two moisture levels as a function of time. Log-reductions of *B. anthracis* at both moisture levels for the first 48 hours practically coincided with each other (Fig. 6). The log-reductions for 0.4 mL samples were statistically lower than those of the 0.2mL at the 168 hours. Interestingly in Figure 7, the behavior of *B. thuringiensis* spores was different in comparison to that of *B. anthracis* spores. There was no concurrence between the data points of two moisture levels for *B. thuringiensis*. The log-reductions for 0.2 mL samples were statistically lower than that of 0.4 mL except for 168 hours, which is opposite of the observations for *B. anthracis*.

Figures 8 and 9, show the normalized log-reductions at the two moisture levels as a function of time according to Eq. (2), for *B. anthracis* and *B. thuringiensis* respectively. The results from the two moisture levels for the first 48 hours were similar for both the species. The normalized log-reductions till the 48 hours were not significantly different from one another for either species. Therefore, from the normalized log-reductions, the change in moisture levels does

not appear to cause a significant difference in recovery for about the first 50 hours of the experiment.

Overall, less water content loaded per filter did not affect the measured recovery for 48 hours for *B. anthracis* spores (Fig. 6, 8); less water content loaded per filter resulted in lesser culturability of *B. thuringiensis* spores (Fig. 7); less water content resulted in higher culturability for wither species in the 168 hours measured recovery (Fig. 6, 7).

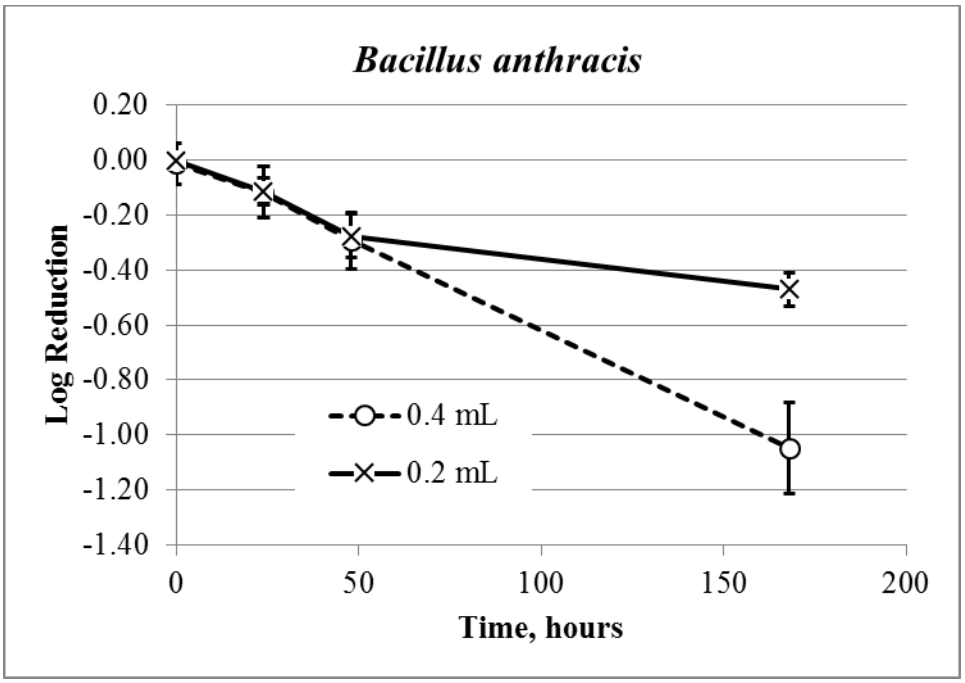

Figure 6. Log-reduction from Eq. (1) as a function of time for *B. anthracis* spores for both volumes of inoculation. Error bars indicate standard deviation from the mean.

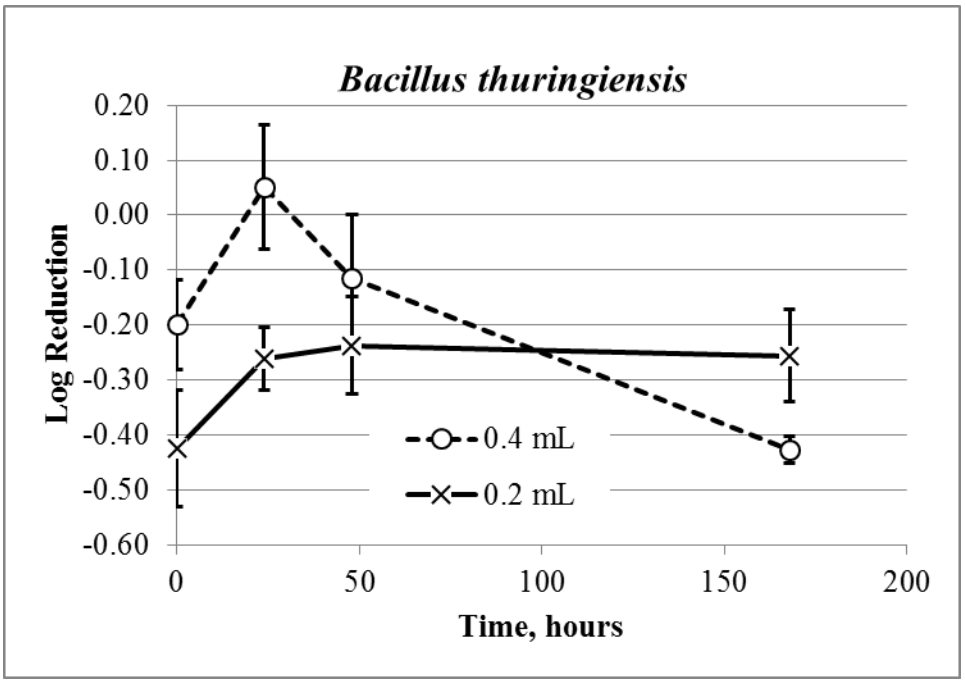

Figure 7. Log-reduction from Eq. (1) as a function of time for *B. thuringiensis* spores for both volumes of inoculation. Error bars indicate standard deviation from the mean.

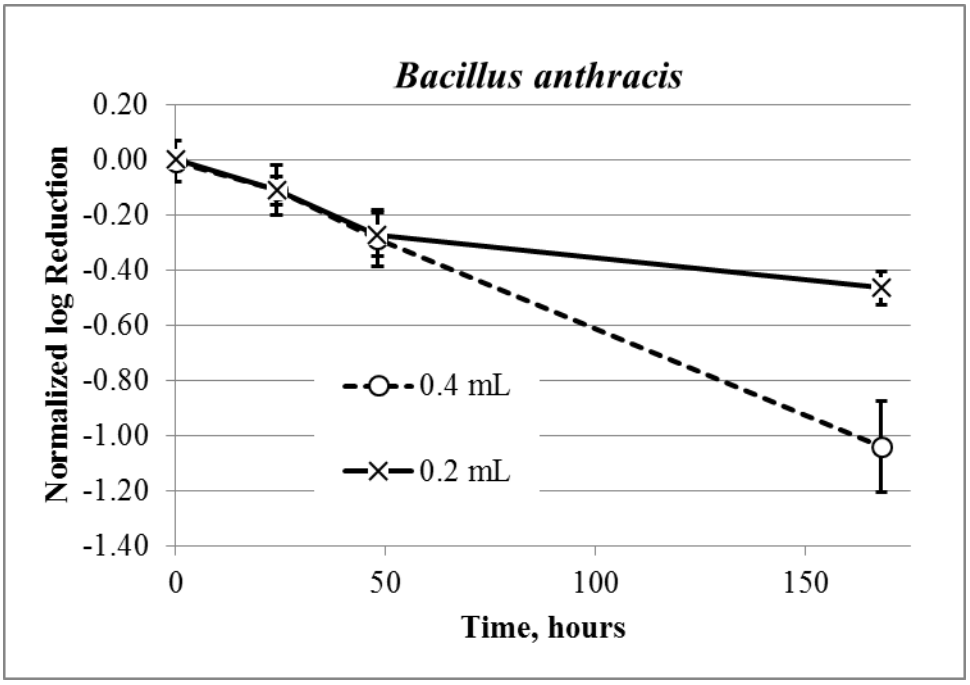

Figure 8. Normalized log-reduction from Eq. (2) as a function of time for *B. anthracis* spores for both volumes of inoculation. Error bars indicate standard deviation from the mean.

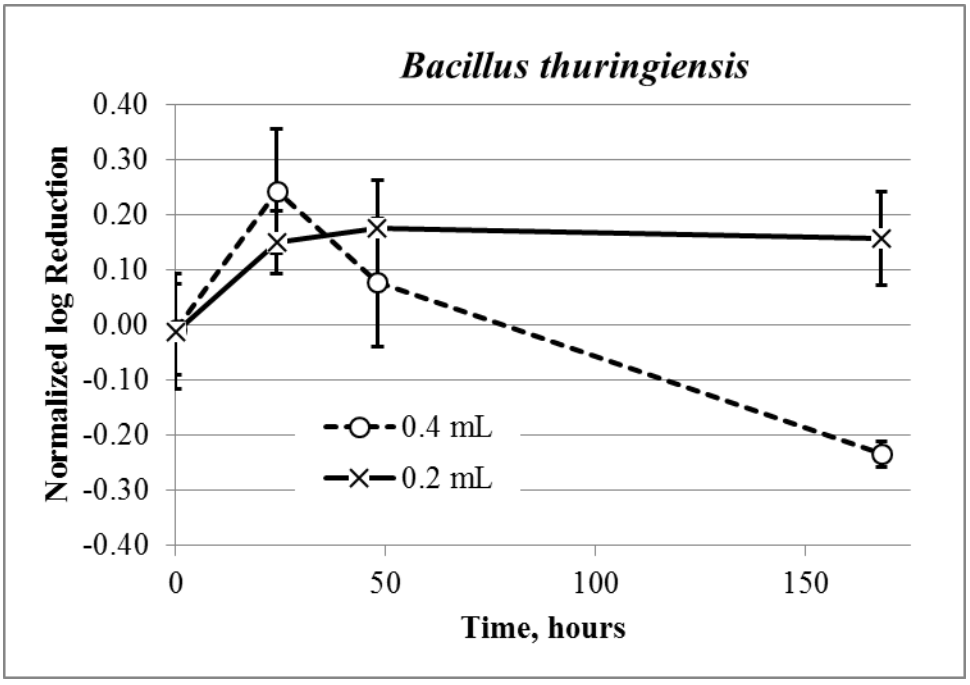

Figure 9. Normalized log-reduction from Eq. (2) as a function of time for *B. thuringiensis* spores for both volumes of inoculation. Error bars indicate standard deviation from the mean.

3.4.6. Comparison of Drying Trends of Two Water Contents

The rates of dehydration were tested using sterile water on HVAC filters with identical experimental conditions. HVAC filter coupons loaded with 0.4 mL and 0.2 mL of water weighed over time showed that, for approximately first 50 hours, the rates of drying of 0.4 mL and 0.2 mL filter samples were identical. The drying data (APPENDIX-C) indicated that both 0.4 mL and 0.2 mL samples dehydrated similarly until the 50 hours ($m_{0.4}$ = -3.938; $m_{0.2}$ = -3.704). After that, the 0.4 mL samples had an accelerated dehydration compared to 0.2 mL filters until the end of 100 hours ($m_{0.4}$ = -4.119; $m_{0.2}$ = -0.0563). Although the 0.2 mL samples were dry for a longer duration (approximately 50 hours more) than the 0.4 mL sample, the latter filter had an accelerated rate of dehydration between 50 –100 hours period (Fig. C. 1) which may have influenced the measured recovery by reducing the culturability of spores.

3.4.7. Microscopy

Porous media offers more space for attachment of spores and are likely to entrap them over time leading to lower recovery. HVAC filters were evaluated using SEM before and after autoclaving to check for any differences caused by the autoclaving process. Moistened HVAC filter coupons identical to the ones used in this study were observed using light microscope for tangible variations caused during drying over time. The results from SEM as well as light microscopy indicated that there were no discernable physical changes of the HVAC filters due to autoclaving or drying respectively.

SEM was also used to evaluate clumping. Images observed using SEM of *B. thuringiensis* spores with and without Tween-20 indicated that there was a reasonable amount of clumping or clustering present in both the samples (Fig. 10(a) & 10(b)). However, the nature of

clumps varied remarkably in both samples (Fig. 10(c) & 10(d)). In presence of Tween-20 a relatively lower number of clumps were observed of smaller size (Fig. 10(e)). Whereas, in the absence of Tween-20, clumps were dense, thickly layered, and strongly coiled to one another (Fig. 10(f)). Clumping of spores was thus confirmed to exist at a higher degree in the absence of the surfactant. Though a distinct conclusion could not be drawn between the presence and absence of surfactant on clumping, it could be evidently stated that the degree of agglomeration or clustering was greater in the absence of surfactants. This supports the results presented in Section 3.4.4 which used CFUs to evaluate clumping.

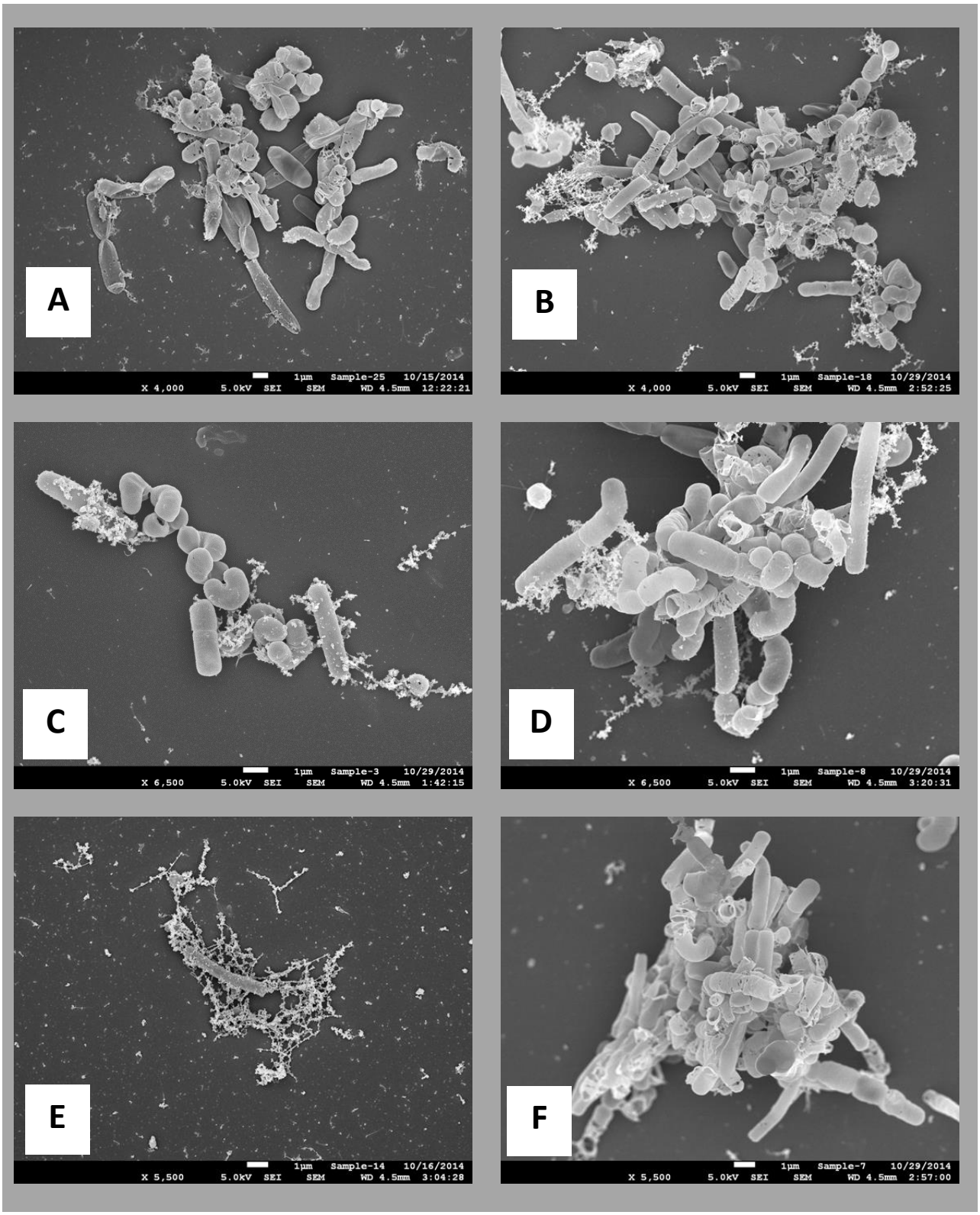

Figure 10. SEM images of *B. thuringiensis* spores (A, C, E) with addition of Tween-20 and (B, D, F) without the addition of Tween-20 (More images using SEM are presented in APPENDIX D)

3.4.8. Statistical Analyses:

Results of Univariate GLM analyses showed that the independent variables had a significant effect on the response variable (log-reduction). Main effects and interaction effects of all three independent variables, species (*B. anthracis* and *B. thuringiensis*), sampling time (0, 24, 48, and 168 hours), and moisture contents (0.4 mL and 0.2 mL) were highly significant ($p < 0.001$), with R^2 value of 0.826. The analysis of the entire data set provided statistical evidence of the significance between the log-reduction of both the species. With the inclusion of the interaction with time this significance increases, indicating that time plays a major role in the recovery of *Bacillus* spores.

There was no significant difference in log-reduction of *B. thuringiensis* due to addition of Tween-20 ($p = 0.651$). However there was a high significant difference on including the interaction between time and surfactant ($p < 0.001$), once again indicating that time strongly influences the recovery. Results from one-tailed t-tests showed that there was an overall statistical decrease in the log-reduction of *Bacillus* spores regardless of the species over time ($p < 0.001$). Results from statistical analyses are presented in APPENDIX-E.

3.5. Discussion

3.5.1. Comparison of Normalization Factors

Recovery studies in the past have used multiple normalization factors to calculate their recovery rates. Commonly used denominators in survival, inactivation, or persistence studies are the 0 hour recovery, because total duration of such tests are over long periods such as 1000 hours, 60 days, and 40 days (Masago et al., 2008; Abad et al., 1994; Tripp 1960). Some, time-independent recovery studies used the recovery from non-porous control surfaces in order to eliminate the losses or biases involved during actual sampling of porous surfaces (Calfee et al., 2013; Calfee et al., 2014; Edmonds et al., 2009). However, most recovery rates are computed by normalizing with respect to the applied concentration of spores for short-term studies (Kim et al., 2008; Krauter et al., 2005; Buttner et al., 2004; Wang et al., 2001; Burton et al., 2005). The differences in results and subsequent inferences on using different normalization techniques especially for surrogate species are highly significant (Murali & Mitchell, 2014). In the normalized recovery rates (Table 2), the projected increase in the case of *B. thuringiensis* when compared to *B. anthracis*, appears as if *B. anthracis* spores were lower when compared to *B. thuringiensis* at all time points. It is possible that such normalization might lead to an inference that *B. thuringiensis* was increasing or growing above the actual applied inoculum concentration. The recovery rates (Table 2) show that *B. anthracis* had 0 hour recovery close to 100% because the applied concentration and the 0 hour recovery for *B. anthracis* did not vary significantly. On the other hand, *B. thuringiensis* had greater than 70% loss (0.2 mL treatment) at the 0 hour recovery. This high initial decrease in recoverability of *B. thuringiensis* spores was morphed in the normalized recovery rates leading to a projected apparent rise. Hence, it is acceptable to use 0 hour recoveries or recoveries from reference coupons for normalization for a short-term study as

long as it does not differ from the applied concentration statistically. Referencing to a 100% initial recovery for persistence studies might lead to incorrect inferences about the trend of decay. Since this is a recovery study, hereafter the discussion will be pertained only to recovery rates or log-reductions based on applied concentrations.

3.5.2. Recovery of *Bacillus* spores over Time

Table 2, Figures 1 and 2 show the recovery rates and log-reductions over time for all the treatments respectively. *Bacillus anthracis* was found to gradually decrease over time while *B. thuringiensis* was found to consistently increase followed by a decrease with the appearance of a "bump" in all trials. Inherent time-dependent reduction was anticipated for the spores of both the species (Kim et al., 2008; Stanley et al., 2008). An increase after the 24 hours recovery for *B. thuringiensis* spores was found to be similar to a previously published extraction study, in which an increase in recovery of *B. thuringiensis* spores, after 32 minutes of extraction from inoculated autoclaved HVAC filters was reported, though the applied or increased percentages were unreported (Solon et al., 2012). It was suggested that the filter media by itself could act as a substrate for microbial proliferation.

Besides the above mentioned work, studies of *B. thuringiensis* on filters or porous media over time are limited for comparison purposes. Nevertheless, there are studies in which *B. thuringiensis* spores were tested for survival and germination in natural and sterile (autoclaved) soil samples. Considering the lack of filter-spores studies, studies on sterile soil samples with similar physical conditions are the closest to compare time-dependent recovery. A similar time-delayed "bump" was observed in *B. thuringiensis* spores -- on sterile Kohnan and Arakawa soil samples followed by a decrease (Akiba, 1986); and on Varna soil samples amended with proteins after 24 hours (Saleh et al., 1970). It was concluded that *B. thuringiensis* spores were capable of

germinating in soil; however the subsequent decreases were unexplained. These results have suggested that the germination and proliferation of *B. thuringiensis* spores even in sterile soil is likely.

Considering the observations from these past studies, the possible reasons for the increase in *B. thuringiensis* could be due to germination of the spores over time and/or proliferation on the filter media. However lack of nutrients, sterility and safety levels during the experiments warrant delving further into other possible intricate factors such as (1) clumping; (2) adhesion leading to aggregated detachment from the filters followed by disintegration of clumps during mechanical extraction, and (3) varying moisture content on the filters over time. Clumping and moisture were tested and addressed in this study.

3.5.2.1. Clumping in *B. thuringiensis* spores

Bacillus thuringiensis spores have the tendency to clump (Dulmage et al., 1969). Presence of peripheral appendages and exosporium as in case of *B. thuringiensis* spores, have been found to aid their capability to adhere to solid surfaces (Tufts et al., 2013; Husmark, 1993). *Bacillus thuringiensis* spores are stronger adherent to the surface and to themselves than *B. anthracis* spores due to the presence of hairy naps on their outermost layer (Tufts et al., 2013). Our results were in close relation to the results of Sugimoto et al. (1996), in which a "bump" was observed in the recovery of dried *B. stearothermophilus* spores (morphologically similar to *B. thuringiensis* spores) from aluminum strips. It was reported that the external appendages on *B. stearothermophilus* spores caused an enhanced adhesion on the aluminum surface leading to lower recovery. The inconsistency in the recovery was unexplained. Therefore, it was suspected that the combined effects of adhesion to the filters and clumping of *B. thuringiensis* might have led to disintegration of *B. thuringiensis* spores in clusters which decreased over time as the

moisture content decreased due to drying. Test for clumping revealed that irrespective of the surfactant Tween-20, a "bump" was observed in log-reduction of *B. thuringiensis* spores (Fig. 5). However, the SSE values of the mean plate counts showed that recoveries were more consistent in the presence of Tween-20. This could mean that clumping was not as pronounced as it was, prior to addition of Tween-20. Lesser clumping might have led to a more stable recovery resulting in a lower SSE when a linear model was used to evaluate the severity of the bump. Moreover, visual evidence of SEM imaging showed that there was a greater degree of clumping or clustering of *B. thuringiensis* spores in the absence of surfactants. Microscopic images of two samples were not conclusive enough to determine if clumping caused the significant increase in the recovery of *B. thuringiensis* spores. Although addition of Tween-20 did not completely eliminate clumping, it reduced clumping significantly by discretizing a large numbers of spores leading to a more consistent recovery. Hence it is could be said that, in presence of Tween-20, recoveries were more consistent, but clumping or aggregation was observed in both the samples using SEM, it is concluded that clumping could not be solely accountable for significant rise in the recovery of *B. thuringiensis* spores.

3.5.2.2. Comparison of drying rates of different moisture contents

A past recovery study of *B. subtilis* spores from polycarbonate filters reported an increased spore recovery and culturability under higher relative humidity (85%); this greater culturability of *B. subtilis* spores was attributed to greater amount of water loaded per unit filter (Wang et al., 2001).

Transfer efficiency of *B. thuringiensis* spores from porous and non-porous media such as cotton, polyester, and stainless steel to fingers, increased with increasing relative humidity. It was directly correlated that wetness of the spores facilitated better transfer. In other words, a low relative humidity could result in stronger adhesion between the *B. thuringiensis* spores and fomites resulting in lesser transfer efficiency (Lopez et al., 2013).

Adhesion of *B. subtilis* spores increased upon drying of the inoculum on sterilized stainless steel surface (Nanasaki et al., 2010). Although the underlying role of moisture content over time, upon recovery is clearly not understood, it is possible that varied moisture levels could directly or indirectly correlate to recovery of spores by affecting the spore drying and adhesion over time. Based on previously published results on recovery of spores from porous and non-porous surfaces, the adhesion of spores on the surface of the filter was expected to strengthen upon drying resulting in a lower recovery. The hypothesis was that, decreasing moisture or water content loaded per filter would result in lower recovery of spores over time. However, the observed results did not support this hypothesis completely. Log-reductions (Fig. 6 and 7) for the first 48 hours from the reduced moisture samples (0.2 mL) were statistically lower than that of higher moisture samples (0.4 mL) as expected for *B. thuringiensis;* but for *B. anthracis* spores, there were no statistical differences in the log-reduction. Also, in contrast to the hypothesis, filters with lower moisture (0.2 mL) had higher recovery in the 168 hours for *B. anthracis* and *B.*

thuringiensis spores (Fig. 6 & 7) which leads to the possibility of changes in viability or culturability.

Ishihara et al. (1994) found a strong linear correlation between the percentage of viability and percentage of free water content present in the *B. subtilis* spores, when desiccated for up to 24 hours. After 24 hours, it was not feasible to draw a correlation because the viability had reached, and stayed at 0% till the end of the experiment (Ishihara et al., 1994). This strong correlation could explain the influence of free water content to the viability of spores in this study.

Although the true mechanism of the contribution of moisture content to the recovery is unclear, since both the filters were in equal moisture content at the 168 hours, it is suggested that, the rate of dehydration may have affected the recovery rather than the degree of dehydration. Based on the dehydration results, filter samples with greater moisture content (0.4 mL) dehydrated at an accelerated pace between 50 hours- 168 hours. In the light of the drying rates and the culturable counts obtained for *B. anthracis* and *B. thuringiensis* at the 168^{th} hour, it is hypothesized that the culturability of spores may be linearly correlated to the rate of dehydration rather than to the state of being dry. This results in decline in measured recovery on speedier dehydration rates.

3.5.3. Surrogate Suitability

From our results, we conclude that *B. thuringiensis* and *B. anthracis* spores behaved dissimilarly over time on HVAC filters. Gradual and significant reduction in *B. anthracis* spores over time was not reflected in the behavior of *B. thuringiensis* spores. Effect of moisture and rate of dehydration on adhesion phenomena, presence of appendages, and clumping could account for this variability in the recovery of *B. thuringiensis* spores. Although similarities were established in the past reviews between the surrogate and *B. anthracis* spores, (Greenberg et al., 2010; Tufts et al., 2013) they were not tested experimentally to date on filter media. Bishop (2014) reported that *B. anthracis Sterne* spores and *B. thuringiensis* spores behave differently in response to the germinants in non-sterile soil microcosms. Germinated vegetative forms of *B. anthracis* strains were not able to persist in soil samples while *B. thuringiensis* spores remained persistent on germination (Bishop, 2014). From the results observed in this study, it is concluded that *B. thuringiensis* spores are not a suitable surrogate for *B. anthracis* spores on porous media (Fig. 1 and 2).

3.5.4. Summary

Based on the experimental conditions in which the tests were performed and the observed results of this study, the following conclusions are made for the time-dependent recovery of *B. anthracis* and *B. thuringiensis* spores from HVAC filters:

(i) Time had a significant effect on recovery regardless of the species used. The results of culturable counts indicate that there was an eventual statistical reduction in recovery after 168 hours in both the species when compared to the original applied concentrations of spores.

(ii) *Bacillus anthracis Sterne* and *B. thuringiensis* spores had dissimilar time-dependent behavior on HVAC filters. *Bacillus anthracis* spores consistently and statistically decreased with time whereas *B. thuringiensis* spores constantly had a 'bump' at the 24 hours or 48 hours recovery in all trials. Hence, irrespective of time, from the experimental conditions and observed results *B. thuringiensis* spores could not be an ideal experimental surrogate for *B. anthracis* spores. It is suggested that *B. anthracis Sterne* strain could be the best suitable surrogate for pathogenic *B. anthracis*.

(iii) Variation in moisture did not affect the recovery of *B. anthracis* significantly for the first 48 hours, and for *B. thuringiensis*, lower moisture content resulted in lower log-reduction until 48 hours. For both the species the log-reductions at the 168 hours reversed the expected trend for lower water content samples. Based on the results of drying tests it is suggested that the rate of dehydration could influence the recovery of spores than the degree of dehydration.

(iv) From the evidences of all tests the consistent ‘bump’ observed in *B. thuringiensis* spores might be due to individual or combined effects of clumping, moisture content, and rate of dehydration.

3.6. Concluding Remarks

Our study quantified the time-depended recovery of *B. anthracis Sterne* and *B. thuringiensis HD 2-61* spores from HVAC filters. Recoveries over different moisture content were also evaluated for the effect of water content on recovery over time. *Bacillus thuringiensis* spores constantly had a 'bump' at the 24 hours or 48 hours recovery in all trials, and *B. anthracis* statistically reduced over time. The abnormal increase in the recovery of *B. thuringiensis* could be due to individual or combined effects of clumping, moisture content, and rate of dehydration.

The results in this study are based on culturable counts. No experiments were carried out attempting to estimate the non-culturable viable spores in the sample. Regardless of the species, over time, eventually there was a significant decrease in the log-reduction. Results from preliminary experiments using real-time PCR (qPCR) quantification on recovery of *B. anthracis Sterne* spores using sporulation, inoculation, and extraction techniques identical to this study indicated that there was a significant difference between the culture-based and molecular-based recovery over time (APPENDIX-F). Since, qPCR enumerates both live as well as dead counts in the sample this difference could be attributed to spores: that are viable yet not culturable, in dormant stage, or large amounts of dead spores. Considering this high and significant difference between the two quantification methods over time as well as the remarkable durability of viability in spores over decades, it would be worthwhile to explore the true viable counts over time for persistence and risk assessments.

Our study was carried out on new, sterile filters, extending the recovery study using the presence of dust particles and commonly found debris in indoor filters are encouraged for a more realistic setup of indoors. Our results are expected to be successfully replicable under identical

sporulation and experimental procedures, and suggest that experiments with aerosolized moisture-free application are warranted to test the aerosol surrogacy of *B. thuringiensis* spores.

3.7. Acknowledgements

We thank Dr. Evangelyn Alocilja and Dr. Joan Rose for useful discussion. We thank Andrew Bruce and Carol Flegler for assistance in data collection.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

The final chapter of this thesis is devoted to discussion of the three research hypotheses and main conclusions, followed by pertinent suggestions for future work.

Three research hypotheses were formulated for this study. For all three hypotheses, the entity of interest was the amount of *Bacillus* spores recovered per filter or the log- reduction, as per the context. In the following sections of this chapter, the main findings are reviewed for each hypothesis followed by corresponding discussion.

4.1. Hypothesis I

Bacillus spores will have a time-dependent reduction in recovery from HVAC filters

This hypothesis was fully supported for both the species at both the moisture levels tested. According to the observed culture-based results, there was an overall time-dependent reduction in both the species when compared to their respective inoculated concentrations (APPENDIX-B). The reduction trends observed in this study were comparable to that observed in Kim et al. (2008). The relationship between the log-reduction of *B. anthracis* spores and time was statistically decreasing over time while in contrast, a consistently increasing yet decreasing trend was observed for *B. thuringiensis* spores over time. The results indicate that, *B. anthracis* spores had a greater reduction over time when compared to *B. thuringiensis* spores, signifying that there would be a larger fraction of *B. anthracis* spores that was capable of persisting on the filter surface over time.

It is important to regard the fact that, these results are a reflection of culture-based data and not true viability of spores. Spores are known for maintaining their viability and persistence in environmental matrices over decades. Therefore, it is warranted that studies in the future, account for spores over time beyond their culturability, highlighting their true viability.

4.2. Hypothesis II

Bacillus anthracis and B. thuringiensis spores will have identical time-dependent recovery from HVAC filters

This hypothesis was not supported by this study for the variables, time and moisture content. Regardless of the overall reduction observed in both the species, the reduction trends of *B. anthracis* and *B. thuringiensis* spores differed remarkably over time. *Bacillus anthracis* spores showed a consistently declining curve over time, whereas in case of *B. thuringiensis* the reduction curves of all trials were consistently increasing followed by a decrease leading to an appearance of a 'bump' like structure.

There has been only a handful of *Bacillus* spores' recovery work carried out to date, among which only one (to our knowledge) was using *B. thuringiensis* spores from HVAC filters (Solon et al., 2012). The authors observed a similar increase after 32 minutes of extraction from inoculated sterile HVAC filters, but the applied or increased percentages were unreported. Therefore it could be said that, a similar recovery pattern was previously observed yet, it is hard to extrapolate over time because of lack of time-dependent studies.

Similar abnormal increase was observed for *B. thuringiensis* spores in the past when recovered from sterile soil matrices under comparable physical experimental and incubation conditions. Saleh et al. (1970) and Akiba (1986) observed an increase followed by a decrease in *B. thuringiensis* spores, recovered from Varna soil samples and Kohnan, Arakawa soil samples respectively after approximately 24 hours or 48 hours.

Based on above mentioned studies, the suggested possible reasons for the increase in *B. thuringiensis* could be due to germination of the spores over time, or proliferation of spores on the filter media. But germination alone could not have accounted for an increase in *B.*

thuringiensis spores. Germination ought to be followed by proliferation in order to produce such a bump**.** However lack of nutrients, sterility and safety levels during the experiments warrant delving further into other possible intricate factors such as clumping and varying adhesion leading to aggregated detachment from the filters followed by disintegration of clumps during mechanical extraction, varying moisture content on the filters over time.

It has to be noted that clumping of *B. thuringiensis* spores has been reported since 1969 (Dulmage et al., 1969). Besides clumping, there were some published works in the past pertaining to the variation in morphology between *B. anthracis* and *B. thuringiensis* spores – it was suggested that the presence of hairy filamentous appendages on the periphery of *B. thuringiensis* spores enhance their capability to adhere on surfaces (Tufts et al., 2013; Husmark, 1993).

It is interesting to note that in a 4 week study (Sugimoto et al., 1996) using *B. stearothermophilus* (morphologically comparable to *B. thuringiensis*) and *B. subtilis* (does not possess appendages or exosporium), dried and recovered from aluminum strips, *B. subtilis* showed a consistent decline whereas *B. stearothermophilus* had an increase followed by a decrease. Presence of hairy filamentatious structures (appendages) on the periphery of *Bacillus* spores have to date, been positively correlated to the adhesion on solid surfaces (Tufts et al., 2013; Husmark, 1993; Ronner et al., 1990; Sugimoto et al., 1996; Husmark & Ronner, 1990). It is therefore convincing to suggest that, presence of appendages and diminishing moisture over time might be instrumental in the increased recovery of *B. thuringiensis* spores.

Although addition of an emulsifier such as Tween-20 to the spore solution minimized clumping to a great extent when compared to the samples in absence of Tween-20, there was evidences of clumped or clustered spores in both the samples observed using SEM. Hence it is

could be said that, in presence of Tween-20, recoveries were consistent, however as clumping or aggregation was observed in both the samples using SEM, it is concluded that clumping could not be solely accountable for significant rise in the recovery of *B. thuringiensis* spores. Therefore, significant increase in the reduction of *B. thuringiensis* spores might not have resulted solely due to clumping; however clumping may have been one of the contributory factors.

Hence, the abnormal increase in the recovery of *B. thuringiensis* spores could be due to multiple factors such as germination – which needs further true account of viability to confirm, clumping, effects of adhesion, and varying moisture levels during drying.

Overall, the complete reason contributing to the reduction trend of *B. thuringiensis* spores could not be addressed from this study, however it can be said that *B. thuringiensis* spores are not ideal experimental surrogates for *B. anthracis* spores based on this data.

4.3. Hypothesis III

Measured recovery of Bacillus spores will be lower when water content per HVAC filter is less

This hypothesis was partially supported by the study. It was anticipated that reduced amount of water loaded per filter would lead to lower recovery over time. With regard to *B. anthracis* spores, the change in water content did not make a significant difference in the reduction value up to 48 hours, while for *B. thuringiensis* spores, the hypothesis was somewhat supported until 48 hours. In both the species, the 168 hour reduction data failed to support the hypothesis. In other words, filters with lower water content had a higher recovery when compared to the filter samples with higher water content. This abnormal reverse in trend under lowered moisture content was not observed in the past. Studies such as (Moritz et al., 2001; Wang et al., 2001; Lopez et al., 2013; Nanasaki et al., 2010) reported an increase in recovery or culturability from porous and non-porous matrices at a higher relative humidity or higher water content loaded per filters.

It is suspected that, besides the primary variable for this hypothesis which is the moisture content per filter, time could also play a chief role in influencing the recovery which was not previously evaluated. Results (APPENDIX-C) from the tests that were carried out in order to test for dehydration rates of different water contents (0.4 mL and 0.2 mL) loaded per filter, led to some interesting inferences. It was found that, although there was higher amount of water per filter as in case of 0.4 mL inoculation volume, the corresponding dehydration rate after 50 hours was significantly accelerated when compared to that of the filter loaded with 0.2 mL water.

Ishihara et al. (1994) demonstrated that the viability of *B. subtilis* spores, desiccated for 96 hours, declined drastically as the percentage of free water in the spores decreased. Free water

is that fraction of water present inside the spore that is available for microbial germination and growth. Also, free water is capable of diffusing with the water macromolecules present in a given matrix, via semi-permeable outer-most layer called, exosporium. Swelling of spores when present in a solution and shrinking of spores when dried were reported in the past, which can be attributed to the free flow of water molecules through the exosporium (Abel-Santos, 2012). It is therefore reasonable to assume that, the percentage of free water present inside a spore could be interrelated to the water present outside the spore. In the study conducted by Ishihara et al. (1994), the viability of *B. subtilis* spores declined with high correlation to that of free water content in the spore up to 24 hours. But, it was not feasible to compare the rate of reduction in free water with respect to viability after the 24^{th} hour, because the viability reached 0% at the 24^{th} hour reading (Ishihara et al., 1994). It could be speculated that, the rate of ambient dehydration influences the free water of the spores, and thereby influencing viability of spores, significantly.

Therefore, besides the sole phenomena of 'being dried' or 'degree of dehydration', it is suggested that time could also play a significant role in affecting the culturable fraction of spores which are reflected in the recovery. Hence, based on the results from hypothesis I and supplemental drying tests, it is hypothesized that, higher the rates of dehydration of *Bacillus* spores could lead to lower recovery, regardless of the initial moisture content.

4.4. Conclusions

Based on the results of this work as well as previously mentioned works herein the following conclusions could be drawn:

(i) Regardless of the species, the culturable counts of *Bacillus* spores extracted from HVAC filters significantly reduced over time. Statistical tests determined that, time is the most affecting factor in the log-reduction.

(ii) Although both *B. anthracis* and *B. thuringiensis* spores reduced over time, their corresponding reduction trends were remarkably dissimilar. The abnormal increase in *B. thuringiensis* spores during 24 hours and subsequent decrease in all trials could be the result of individual or combined effects of clumping or clustering, varying moisture content, rate of drying, and delayed germination, but not limited to these.

(iii) *Bacillus thuringiensis* spores are not an ideal experimental candidate for *B. anthracis* in time-dependent studies.

(iv) Lower moisture content did not significantly lower the recovery of *B. anthracis* spores up to 50 hours ($p = 0.404$), but it did result in lower reduction for *B. thuringiensis* spores as expected up to 48 hours ($p < 0.001$). However lower moisture content yielded higher recovery for both the species at the 168 hours. It is therefore suggested from the results of supplemental tests that, lower moisture content need not lead to a lower recovery, but accelerated dehydration rates could result in a lower recovery.

4.5. Recommendation for Future Work

This study has limitations which would be worthwhile to address in future studies. Recommendations for future work are as follows:

(i) Extending the recovery study using inoculation via aerosolization of spores, in absence of moisture to test the aerosol suitability of *B. thuringiensis* as a potential surrogate for *B. anthracis* spores

(ii) Extending the recovery study using the presence of dust particle and commonly found debris in indoor filters is encouraged for a more realistic setup of indoors.

(iii) Carrying out recovery experiments with different attenuated strains of *B. anthracis* spores, and different types of filters used commercially and domestically.

(iv) Using molecular-based techniques and advanced microscopy are suggested in order to have a more credible account of true viability, along with culture-based quantification techniques.

APPENDICES

APPENDIX A: SUMMARY OF EXPERIMENTAL PLANNING AND DESIGN

Based on the objectives and hypotheses, this study, will quantify the recovery of *B. anthracis* and *B. thuringiensis* spores over duration of 168 hours from HVAC filter coupons under two different moisture conditions. Results from these experiments will be instrumental in comparing the effects of sampling time and moisture content on the recovery of the two species as well as the experimental convergence of *B. thuringiensis* spores and *B. anthracis* spores. Therefore, the independent variables in this work are: sampling time, species, and moisture content. The dependent variable is recovery which is described by log-reduction.

Table A. 1 clearly presents the timeline adopted for each task in this experiment. The timeline is represented in terms of 'day' which does not correspond to any actual calendar date. Figure A. 1, shows the schematics for a single time point. Triplicate filters, marked as 'A', 'B', and 'C', are eluted in a centrifuge tube at every time point. The elution processes are sequentially represented in the adjacent box (Fig. A. 1). These processes correspond to Days 16-18 and 23 in Table A. 1. Following the elution, plate-culturing method of quantification was used to enumerate the recovery at all time points. Triplicate plates for every filter resulted in nine recovery values at every time step. A total of 56 filters and 168 plates along with 16 negatives, a total of 184 plates were evaluated.

Table A. 1. Generalized timeline for recovery experiments: planning module for future use

Task	Day*																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Prepare BA[a] and BT[b] vegetative cells																								
Spread BA and BT cells on separate TSA slants																								
Sporulation of BA and BT (waiting period)																								
Extract the BA & BT spores from the slants using sterile Type-I water to yield master stock																								
Quantify using serial dilution plating																								
Count the plates from Day 14																								
Prepare stock solutions of desired concentrations from the master stock																								
Cut, autoclave, and store HVAC filter coupons																								
Spike 0.4 mL and 0.2 mL of BA stock solution to different filters in triplicates & store in desiccator																								

Table A. 2. (cont'd) Generalized timeline for recovery experiments: planning module for future use

Elute coupons for 0 hour extractions																							
Quantify the elution using serial dilution plating																							
Count the plates from Day 16																							
Elute coupons for 24 hour extractions																							
Quantify the elution using serial dilution plating																							
Count the plates from Day 17																							
Elute coupons for 48 hour extractions																							
Quantify the elution using serial dilution plating																							
Count the plates from Day 18																							
Elute coupons for 168 hour extractions																							
Quantify the elution using serial dilution plating																							
Count the plates from Day 23																							`
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**

- **All the experiments were carried in between 12/26/2013 to 1/24/2014*
- *a, Bacillus anthracis (BA)*
- *b, Bacillus thuringiensis (BT)*
- *Blue box represents the beginning of experiments for BT (Day 20 to Day 28), procedures identical to the beginning Day 16*

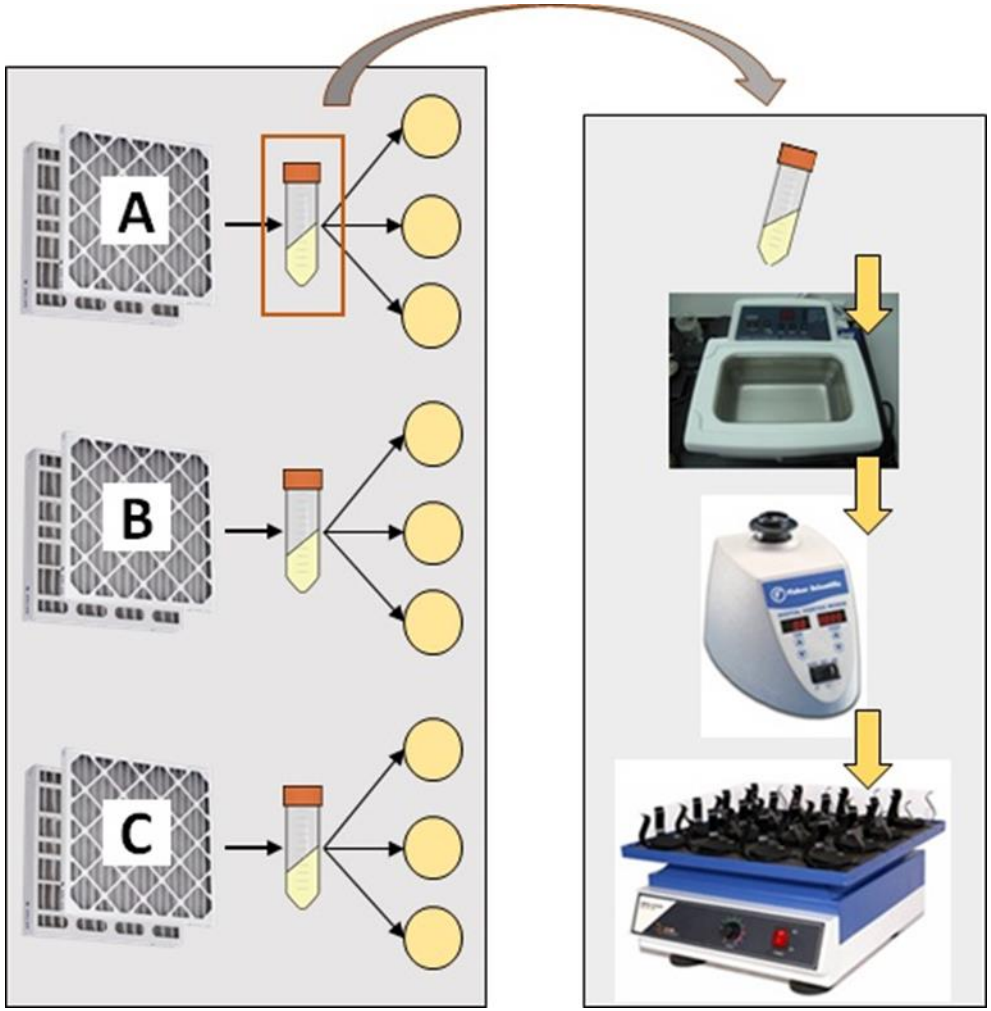

Figure A. 1. (L) Overall schematics representing sequential processes, inoculation, elution, and quantification involved in the recovery experiments for a given sampling time. (R) Series of mechanical processes, sonication, vortexing, and shaking involved in the elution stage.

APPENDIX B: APPLIED AND RECOVERY DATA COLLECTED FROM BACILLUS ANTHRACIS AND BACILLUS THURINGIENSIS SPORES AT DIFFERENT MOISTURE LEVELS OVER TIME

Table B. 1. Mean concentrations of master stocks of *Bacillus* spores

Species	Mean plate counts	Conc, spores/ mL
B. anthracis	78.67	7.87E+07
B. thuringiensis	96.67	9.67E+07

Table B. 2. Concentrations of stock solutions of *Bacillus* spores needed for 0.4 mL inoculation

Species	C_M, spores/ mL	$V_{Applied}$/ filter, mL	C_D, spores/ filter	V_1, mL	C_1, spores/ mL	V_{stock}, mL	V_{water}, mL
B. anthracis	7.87E+07	0.4	1.00E+06	10	2.50E+06	0.318	9.682
B. thuringiensis	9.67E+07	0.4	1.00E+06	10	2.50E+06	0.259	9.741

Table B. 3. Concentrations of stock solutions of *Bacillus* spores needed for 0.2 mL inoculation

Species	C_M, spores/ mL	$V_{Applied}$/ filter, mL	C_D, spores/ filter	V_1, mL	C_1, spores/ mL	V_{stock}, mL	V_{water}, mL
B. anthracis	7.87E+07	0.2	2.00E+06	10	1.00E+07	1.271	8.729
B. thuringiensis	9.67E+07	0.2	2.00E+06	10	1.00E+07	1.034	8.966

where C_M = Concentration of master stock, C_D = Desired concentration to be applied on filters, C_1 = Concentration of stock solutions, $V_{Applied}$ = Vol. of inoculant applied on each filter, V_{stock} = Vol. of stock solution in 10 mL total stock solution, V_{water} = Vol. of water in 10 mL total stock solution

Equations:

(i) $V_1 = V_{stock} + V_{water} = 10$ mL …. (5)

(ii) $C_1 = \frac{C_D}{V_{Applied}}$ …. (6)

(iii) $V_{stock} = \frac{V_1 * C_1}{C_M}$ …. (7)

(iv) $V_{water} = 10 - V_{stock}$ …. (8)

Table B. 4. Summary of plate counts and recovered concentrations of *B. anthracis* (0.4 mL)

Filter	Time (hrs)	Plates	Plate recovery	Spores recovered (Spores/ filter)
A	0	A1	58.00	1.16E+06
	0	A2	43.00	8.60E+05
	0	A3	51.00	1.02E+06
B	0	B1	58.00	1.16E+06
	0	B2	51.00	1.02E+06
	0	B3	48.00	9.60E+05
C	0	C1	78.00	1.56E+06
	0	C2	63.00	1.26E+06
	0	C3	58.00	1.16E+06
A	24	A1	41.00	8.20E+05
	24	A2	37.00	7.40E+05
	24	A3	39.00	7.80E+05
B	24	B1	38.00	7.60E+05
	24	B2	36.00	7.20E+05
	24	B3	38.00	7.60E+05
C	24	C1	63.00	1.26E+06
	24	C2	53.00	1.06E+06
	24	C3	57.00	1.14E+06
A	48	A1	32.00	6.40E+05
	48	A2	25.00	5.00E+05
	48	A3	25.00	5.00E+05
B	48	B1	26.00	5.20E+05
	48	B2	28.00	5.60E+05
	48	B3	21.00	4.20E+05
C	48	C1	44.00	8.80E+05
	48	C2	29.00	5.80E+05
	48	C3	40.00	8.00E+05
A	168	A1	30.00	6.00E+04
	168	A2	29.00	5.80E+04
	168	A3	43.00	8.60E+04
B	168	B1	85.00	1.70E+05
	168	B2	51.00	1.02E+05
	168	B3	69.00	1.38E+05
C	168	C1	66.00	1.32E+05
	168	C2	72.00	1.44E+05
	168	C3	48.00	9.60E+04

Table B. 5. Summary of plate counts and recovered concentrations of *B. thuringiensis* (0.4 mL)

Filter	Time (hrs)	Plates	Plate recovery	Spores recovered (Spores/ filter)
A	0	A1	27.00	5.40E+05
	0	A2	39.00	7.80E+05
	0	A3	28.00	5.60E+05
B	0	B1	39.00	7.80E+05
	0	B2	31.00	6.20E+05
	0	B3	29.00	5.80E+05
C	0	C1	39.00	7.80E+05
	0	C2	47.00	9.40E+05
	0	C3	36.00	7.20E+05
A	24	A1	77.00	1.54E+06
	24	A2	86.00	1.72E+06
	24	A3	89.00	1.78E+06
B	24	B1	52.00	1.04E+06
	24	B2	46.00	9.20E+05
	24	B3	46.00	9.20E+05
C	24	C1	62.00	1.24E+06
	24	C2	60.00	1.20E+06
	24	C3	50.00	1.00E+06
A	48	A1	48.00	9.60E+05
	48	A2	50.00	1.00E+06
	48	A3	56.00	1.12E+06
B	48	B1	34.00	6.80E+05
	48	B2	48.00	9.60E+05
	48	B3	30.00	6.00E+05
C	48	C1	45.00	9.00E+05
	48	C2	51.00	1.02E+06
	48	C3	26.00	5.20E+05
A	168	A1	212.00	4.24E+05
	168	A2	207.00	4.14E+05
	168	A3	218.00	4.36E+05
B	168	B1	182.00	3.64E+05
	168	B2	203.00	4.06E+05
	168	B3	196.00	3.92E+05
C	168	C1	215.00	4.30E+05
	168	C2	208.00	4.16E+05
	168	C3	199.00	3.98E+05

Table B. 6. Summary of plate counts and recovered concentrations of *B. anthracis* (0.2 mL)

Filter	Time (hrs)	Plates	Plate recovery	Spores recovered (Spores/ filter)
A	0	A1	120.00	2.40E+06
	0	A2	122.00	2.44E+06
	0	A3	109.00	2.18E+06
B	0	B1	105.00	2.10E+06
	0	B2	120.00	2.40E+06
	0	B3	113.00	2.26E+06
C	0	C1	124.00	2.48E+06
	0	C2	103.00	2.06E+06
	0	C3	104.00	2.08E+06
A	24	A1	87.00	1.74E+06
	24	A2	75.00	1.50E+06
	24	A3	78.00	1.56E+06
B	24	B1	88.00	1.76E+06
	24	B2	90.00	1.80E+06
	24	B3	103.00	2.06E+06
C	24	C1	80.00	1.60E+06
	24	C2	87.00	1.74E+06
	24	C3	105.00	2.10E+06
A	48	A1	81.00	1.62E+06
	48	A2	65.00	1.30E+06
	48	A3	62.00	1.24E+06
B	48	B1	66.00	1.32E+06
	48	B2	58.00	1.16E+06
	48	B3	64.00	1.28E+06
C	48	C1	64.00	1.28E+06
	48	C2	50.00	1.00E+06
	48	C3	43.00	8.60E+05
A	168	A1	39.00	7.80E+05
	168	A2	37.00	7.40E+05
	168	A3	37.00	7.40E+05
B	168	B1	35.00	7.00E+05
	168	B2	33.00	6.60E+05
	168	B3	34.00	6.80E+05
C	168	C1	50.00	1.00E+06
	168	C2	47.00	9.40E+05
	168	C3	40.00	8.00E+05

Table B. 7. Summary of plate counts and recovered concentrations of *B. thuringiensis* (0.2 mL)

Filter	Time (hrs)	Plates	Plate recovery	Spores recovered (Spores/ filter)
A	0	A1	39.00	7.80E+05
	0	A2	42.00	8.40E+05
	0	A3	34.00	6.80E+05
B	0	B1	64.00	1.28E+06
	0	B2	52.00	1.04E+06
	0	B3	48.00	9.60E+05
C	0	C1	35.00	7.00E+05
	0	C2	29.00	5.80E+05
	0	C3	38.00	7.60E+05
A	24	A1	71.00	1.42E+06
	24	A2	65.00	1.30E+06
	24	A3	63.00	1.26E+06
B	24	B1	53.00	1.06E+06
	24	B2	54.00	1.08E+06
	24	B3	67.00	1.34E+06
C	24	C1	47.00	9.40E+05
	24	C2	63.00	1.26E+06
	24	C3	60.00	1.20E+06
A	48	A1	60.00	1.20E+06
	48	A2	46.00	9.20E+05
	48	A3	55.00	1.10E+06
B	48	B1	70.00	1.40E+06
	48	B2	70.00	1.40E+06
	48	B3	61.00	1.22E+06
C	48	C1	80.00	1.60E+06
	48	C2	87.00	1.74E+06
	48	C3	53.00	1.06E+06
A	168	A1	65.00	1.30E+06
	168	A2	67.00	1.34E+06
	168	A3	72.00	1.44E+06
B	168	B1	57.00	1.14E+06
	168	B2	40.00	8.00E+05
	168	B3	62.00	1.24E+06
C	168	C1	54.00	1.08E+06
	168	C2	79.00	1.58E+06
	168	C3	60.00	1.20E+06

APPENDIX C: RECOVERY DATA COLLECTED FROM SUPPLEMENTARY EXPERIMENTS TO TEST FOR CLUMPING AND DRYING RATES

Table C. 1. Summary of plate counts and recovered concentrations of *B. thuringiensis* without Tween-20 (0.4 mL)

Filter	Time (hrs)	Plates	Plate recovery	Spores recovered (Spores/ filter)
A	0	A1	45.00	9.00E+05
	0	A2	54.00	1.08E+06
	0	A3	51.00	1.02E+06
B	0	B1	68.00	1.36E+06
	0	B2	67.00	1.34E+06
	0	B3	63.00	1.26E+06
C	0	C1	54.00	1.08E+06
	0	C2	66.00	1.32E+06
	0	C3	55.00	1.10E+06
A	24	A1	53.00	1.06E+06
	24	A2	52.00	1.04E+06
	24	A3	50.00	1.00E+06
B	24	B1	47.00	9.40E+05
	24	B2	48.00	9.60E+05
	24	B3	39.00	7.80E+05
C	24	C1	76.00	1.52E+06
	24	C2	85.00	1.70E+06
	24	C3	81.00	1.62E+06
A	48	A1	139.00	2.78E+06
	48	A2	156.00	3.12E+06
	48	A3	137.00	2.74E+06
B	48	B1	193.00	3.86E+06
	48	B2	184.00	3.68E+06
	48	B3	190.00	3.80E+06
C	48	C1	157.00	3.14E+06
	48	C2	184.00	3.68E+06
	48	C3	171.00	3.42E+06
A	168	A1	54.00	1.08E+06
	168	A2	64.00	1.28E+06
	168	A3	72.00	1.44E+06
B	168	B1	177.00	3.54E+05
	168	B2	161.00	3.22E+05
	168	B3	192.00	3.84E+05
C	168	C1	114.00	2.28E+05
	168	C2	N/A	N/A
	168	C3	116.00	2.32E+05

Table C. 2. Summary of plate counts and recovered concentrations of *B. thuringiensis* with Tween-20 (0.4 mL)

Filter	Time (hrs)	Plates	Plate recovery	Spores recovered (Spores/ filter)
A	0	A1	52.00	1.04E+06
	0	A2	61.00	1.22E+06
	0	A3	44.00	8.80E+05
B	0	B1	61.00	1.22E+06
	0	B2	48.00	9.60E+05
	0	B3	47.00	9.40E+05
C	0	C1	65.00	1.30E+06
	0	C2	75.00	1.50E+06
	0	C3	62.00	1.24E+06
A	24	A1	77.00	1.54E+06
	24	A2	65.00	1.30E+06
	24	A3	56.00	1.12E+06
B	24	B1	110.00	2.20E+06
	24	B2	148.00	2.96E+06
	24	B3	168.00	3.36E+06
C	24	C1	129.00	2.58E+06
	24	C2	82.00	1.64E+06
	24	C3	81.00	1.62E+06
A	48	A1	137.00	2.74E+06
	48	A2	146.00	2.92E+06
	48	A3	164.00	3.28E+06
B	48	B1	59.00	1.18E+06
	48	B2	71.00	1.42E+06
	48	B3	88.00	1.76E+06
C	48	C1	78.00	1.56E+06
	48	C2	92.00	1.84E+06
	48	C3	103.00	2.06E+06
A	168	A1	90.00	1.80E+06
	168	A2	112.00	2.24E+06
	168	A3	98.00	1.96E+06
B	168	B1	54.00	1.08E+06
	168	B2	49.00	9.80E+05
	168	B3	55.00	1.10E+06
C	168	C1	63.00	1.26E+06
	168	C2	52.00	1.04E+06
	168	C3	66.00	1.32E+06

Table C. 3. Summary of weights of filters loaded with 0.4 mL and 0.2 mL water content

Volume of water/ filter = 0.4 mL						**Volume of water/ filter = 0.2 mL**					
Date	**Time**	**Filters**	**Weight, mg**	**Filters**	**Weight, mg**	**Date**	**Time**	**Filters**	**Weight, mg**	**Filters**	**Weight, mg**
25-Aug	11:00	A, blank	330	B, blank	339	25-Aug	11:00	A, blank	345.8	B, blank	319.9
26-Aug	11:00	A	855	B	816.1	26-Aug	11:00	A	612	B	596.5
26-Aug	17:00	A	827.8	B	772.7	26-Aug	17:00	A	581.4	B	561
27-Aug	11:00	A	745.4	B	650.8	27-Aug	11:00	A	496.1	B	480.5
27-Aug	17:00	A	724.3	B	617	27-Aug	17:00	A	476.7	B	465.1
28-Aug	11:00	A	649.5	B	491	28-Aug	11:00	A	406.2	B	400.2
28-Aug	17:00	A	625.5	B	450.6	28-Aug	17:00	A	384.6	B	379.1
29-Aug	11:00	A	572.8	B	346.4	29-Aug	11:00	A	346.2	B	337.6
29-Aug	17:00	A	549.4	B	337.3	29-Aug	17:00	A	336.1	B	323.6
30-Aug	11:00	A	503.4	B	337.1	30-Aug	11:00	A	335.5	B	319.1
30-Aug	17:00	A	457.3	B	336.8	30-Aug	17:00	A	334.9	B	314.6
31-Aug	11:00	A	380.7	B	337.2	31-Aug	11:00	A	335.3	B	318.5
31-Aug	17:00	A	352	B	336.8	31-Aug	17:00	A	334.9	B	318.2
1-Sep	11:00	A	328.3	B	336.2	1-Sep	11:00	A	334.9	B	318.1
1-Sep	17:00	A	328.1	B	339.9	1-Sep	17:00	A	334.8	B	318.2
2-Sep	11:00	A	328.4	B	336.4	2-Sep	11:00	A	334.9	B	317.7
2-Sep	17:00	A	328.1	B	336.2	2-Sep	17:00	A	334.2	B	317.5
3-Sep	11:00	A	328.4	B	337.7	3-Sep	11:00	A	334.6	B	318.9
3-Sep	17:00	A	327.8	B	336.7	3-Sep	17:00	A	334.6	B	317.7

The highlighted row is the 0-hour measured weights.

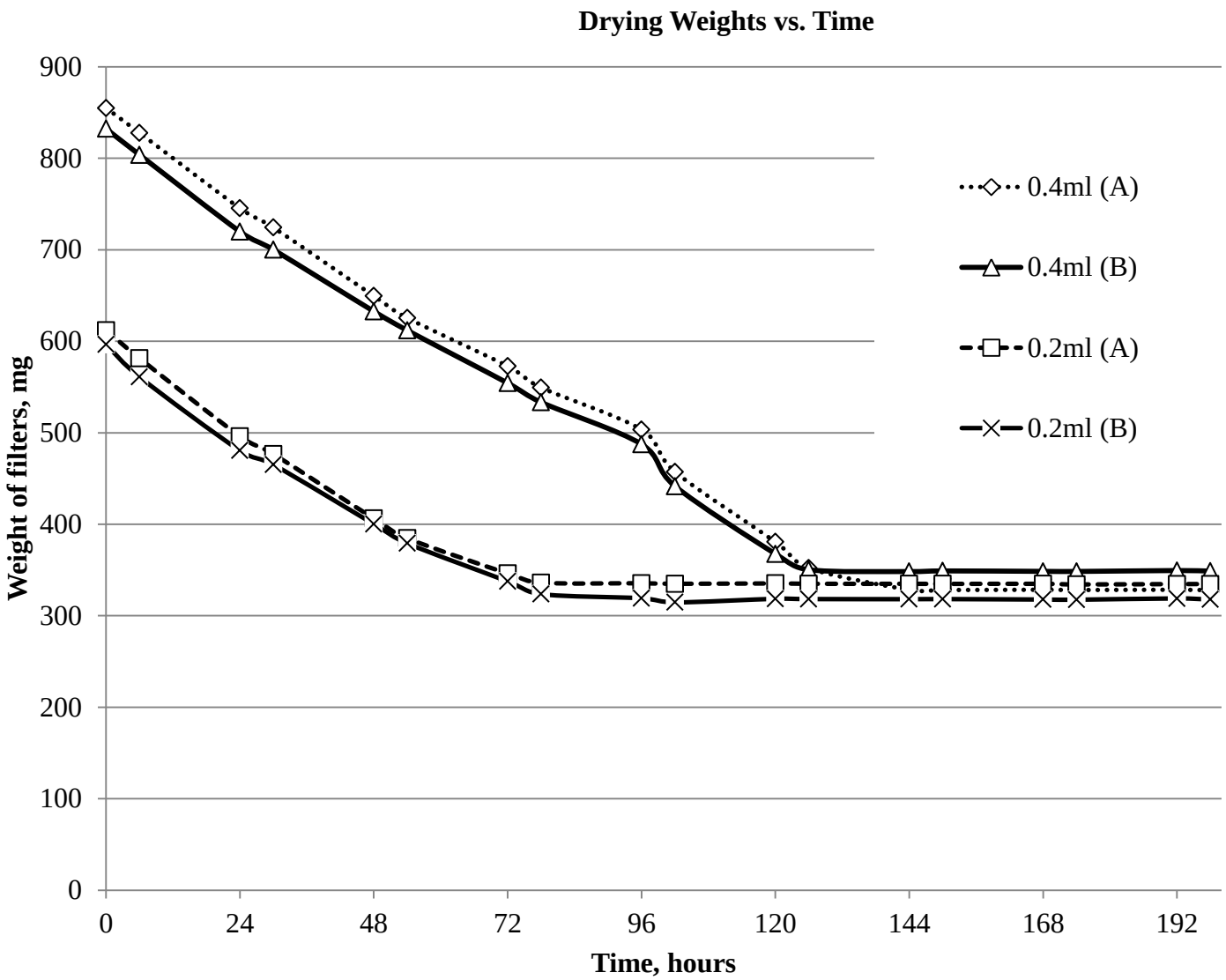

Figure C. 1. Weight of filters (mg) as a function of time for filters loaded with 0.4 mL and 0.2 mL volumes of water

APPENDIX D: SCANNING ELECTRON MICROSCOPY

Procedure for Scanning Electron Microscopy (SEM)

SEM was used to observe the tangible effects of autoclaving on HVAC filters, and to observe the nature of *B. thuringiensis* spores, in the presence and absence of Tween-20.

In order to examine the effects of autoclaving on filter samples, fresh and autoclaved HVAC filters (identical to experimental dimensions) were subjected to SEM side by side. Pieces of filter samples were mounted on aluminum stubs using high vacuum carbon tabs (SPI Supplies, Westchester, PA). The samples were coated with platinum to an approximate thickness of 8 nm in a Quorum Technologies Q150T Turbo Pimped Coater (Quorum Technologies, Laughton, East Essex, England). Samples were examined in a Joel JSM-6610LV scanning electron microscope (JOEL Ltd., Tokyo, Japan).

In order to observe the *B. thuringiensis* spores with and without the presence of Tween-20, small quantities of corresponding samples of *B. thuringiensis* spores were mixed with an equal quantity of 4% glutaraldehyde buffered with 0.1 M sodium phosphate at pH 7.4. Fixation was allowed to proceed for one-half hour at 4oC. One drop of 1% Poly-L-Lysine (Sigma Aldrich P1399) was placed on a plastic petri dish and a 12 mm round glass cover slip was placed on top of the drop and allowed to stand for 5 minutes. The coverslip was removed which previously faced down. The suspension was allowed to settle for 5 minutes. The coverslip was then gently washed with several drops of water and placed in a graded ethanol series (25%, 50%, 75%, and 95%) for 5 minutes in each step and with 5 minutes changes in 100% ethanol (Klopmparens et al., 1986). Samples were critical point dried in a Leica EM CPD300 (Leica Microsystems, Vienna, Austria) using liquid carbon dioxide as the transitional fluid. Samples were mounted on aluminum stubs using epoxy glue- System Three Quick Cure 5 (System Three Resins, Inc., Auburn, WA). Samples were coated with iridium to an approximate thickness of 5.5 nm in a

Quorum Technologies Q150T Turbo Pimped Coater (Quorum Technologies, Laughton, East Essex, England) for 60 s. Samples were examined in a Joel JSM-7500F (cold field emission electron emitter) scanning electron microscope (JOEL Ltd., Tokyo, Japan). Images obtained using SEM is presented in Figures C. 1 through C. 18.

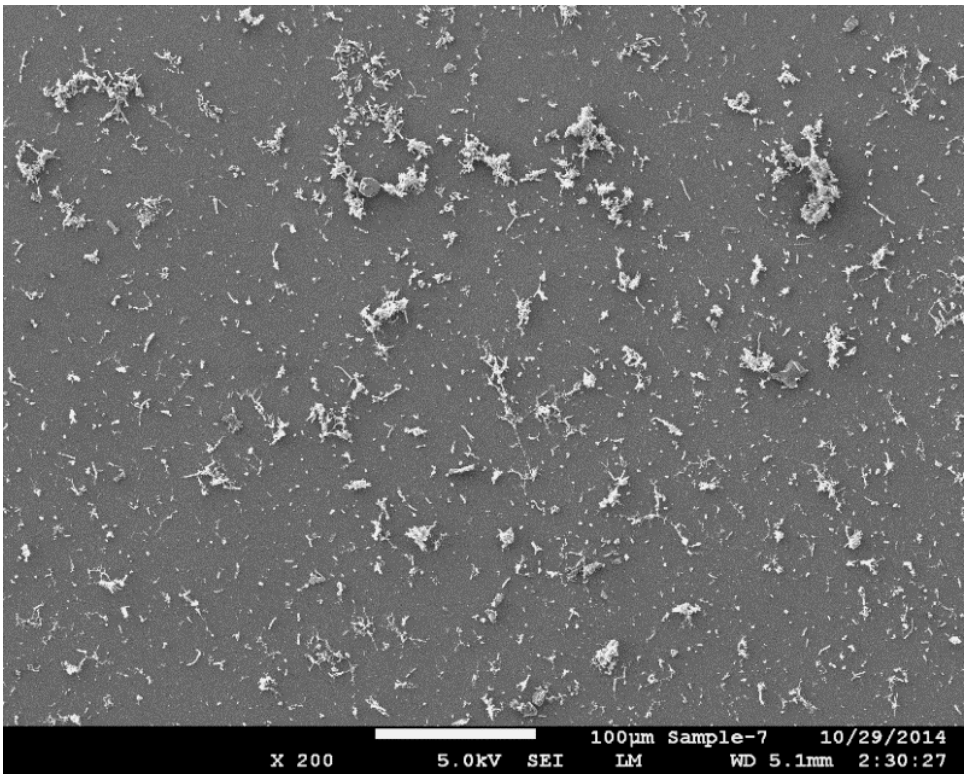

Figure D. 1. Scattered clusters of *B. thuringiensis* spores without Tween-20 (200x)

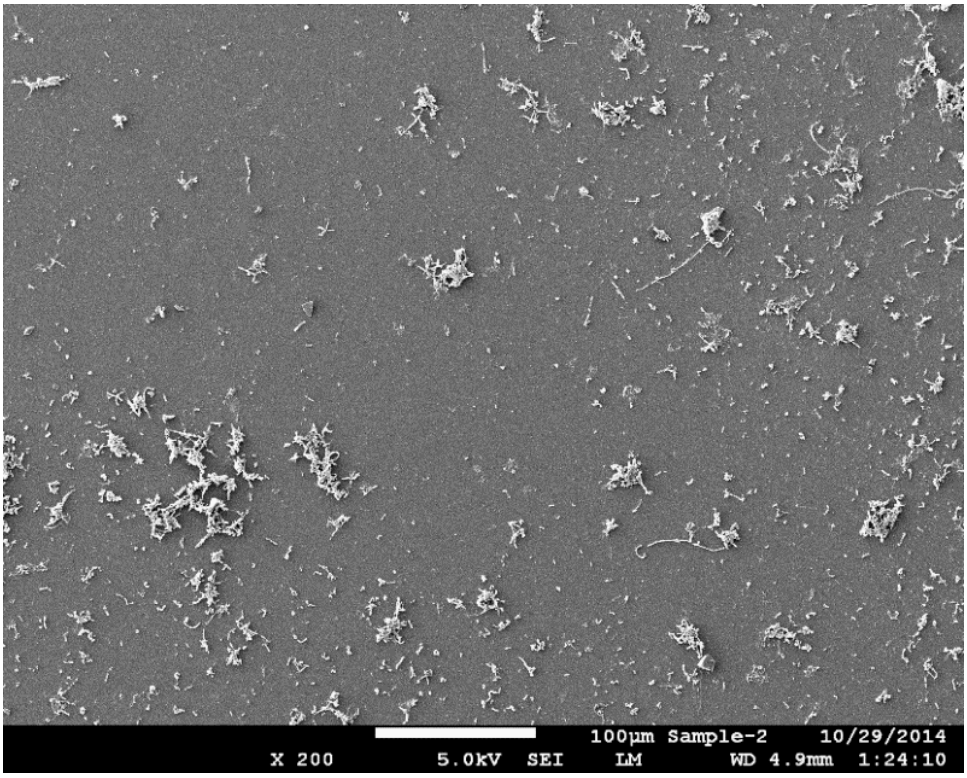

Figure D. 2. Spores of *B. thuringiensis* with Tween-20 (200x) showing lesser clustering as compared to the absence of Tween-20

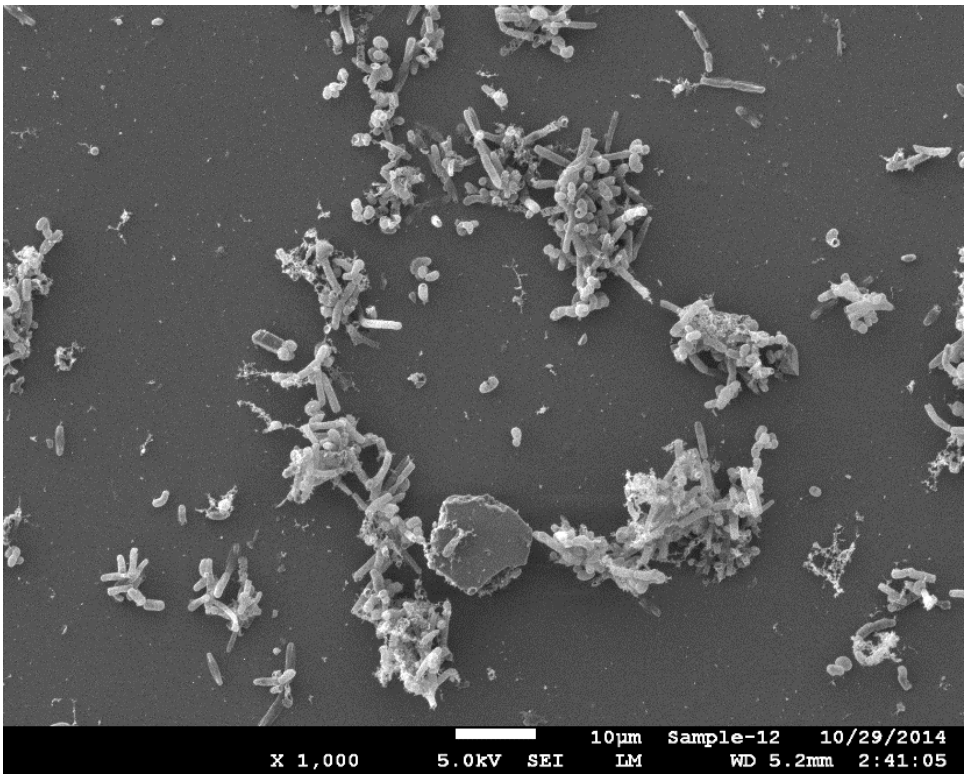

Figure D. 3. Joint clumps of *B. thuringiensis* spores without Tween-20 (1000x)

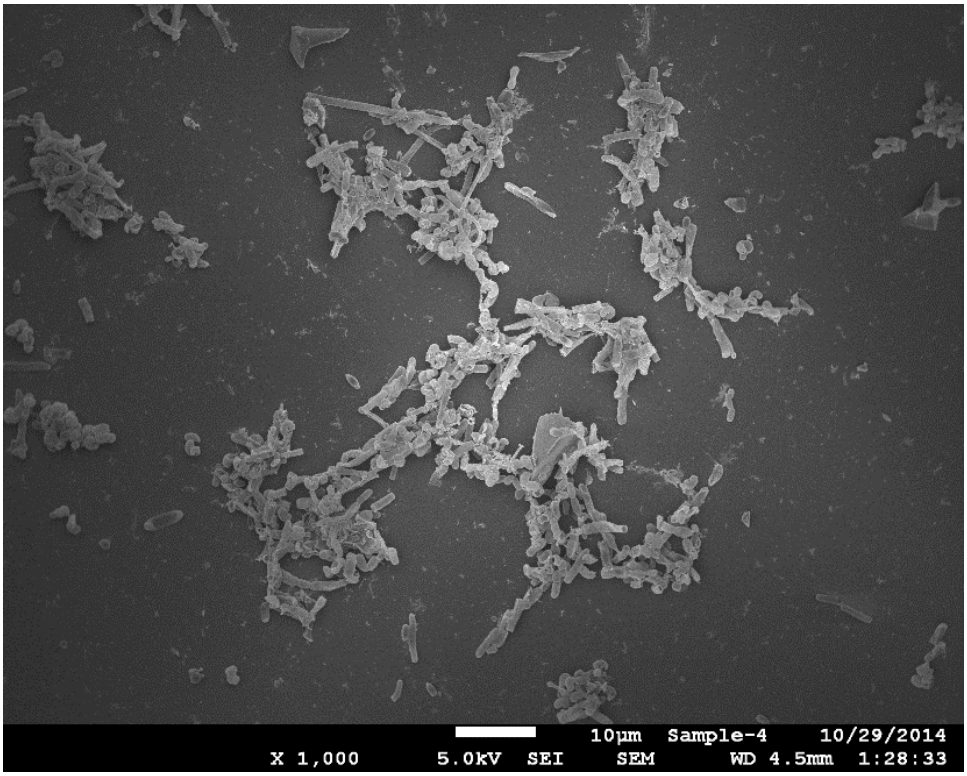

Figure D. 4. Clumps of *B. thuringiensis* spores with Tween-20 (1000x)

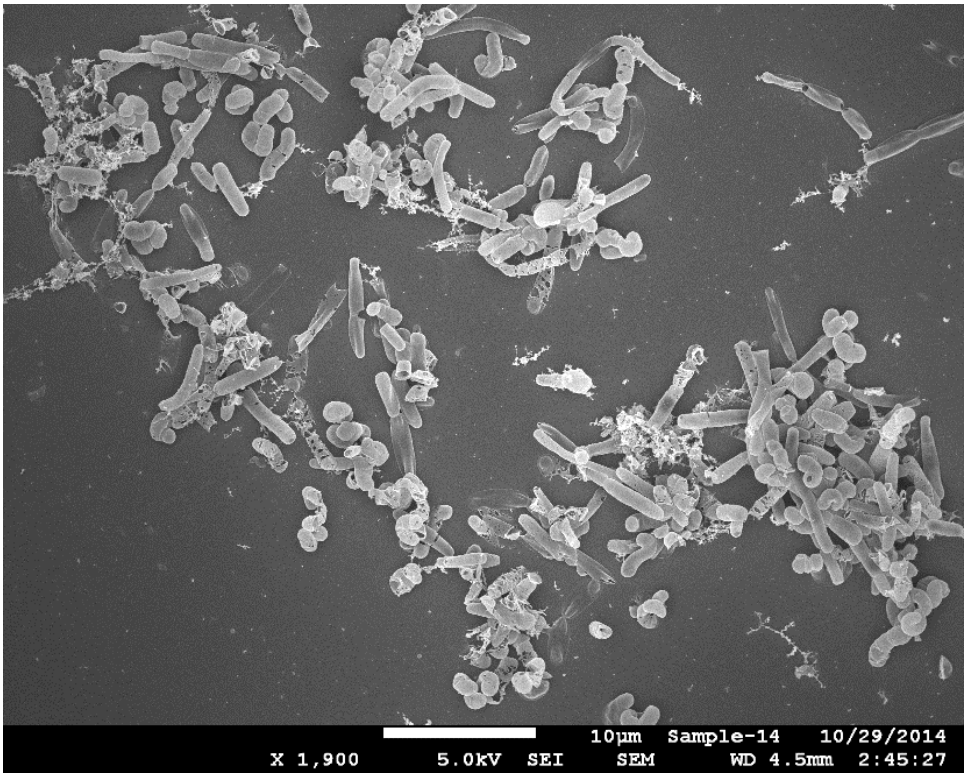

Figure D. 5. Images of *B. thuringiensis* spores without Tween-20 (1900x)

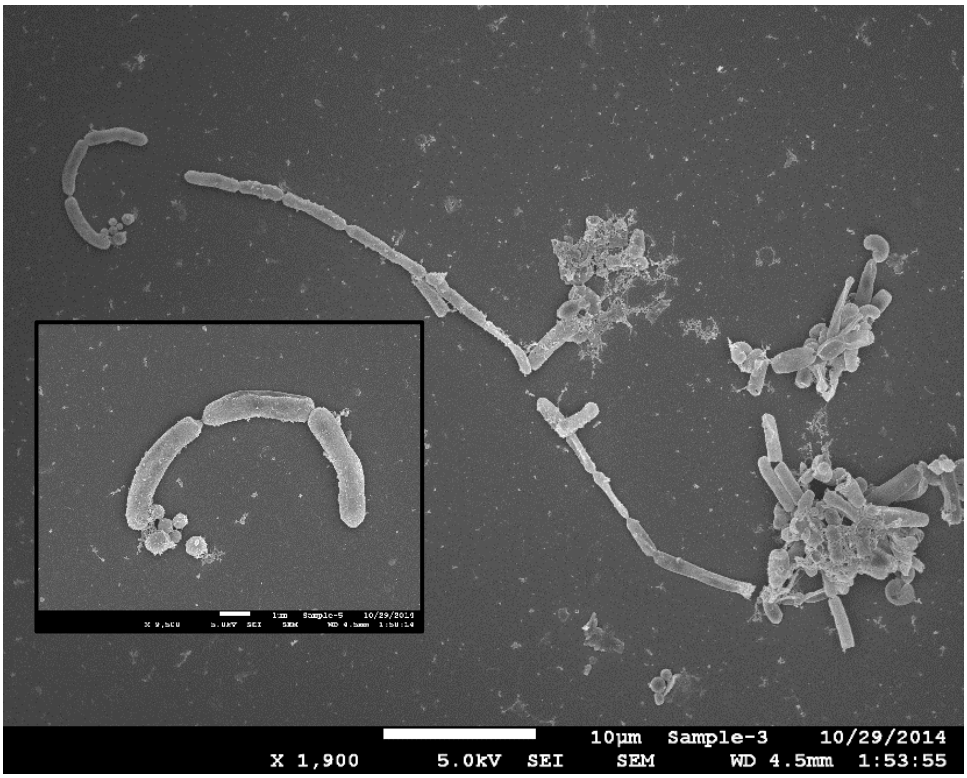

Figure D. 6. Images of *B. thuringiensis* spores with Tween-20 (1900x) showing an overall lesser degree of attachment

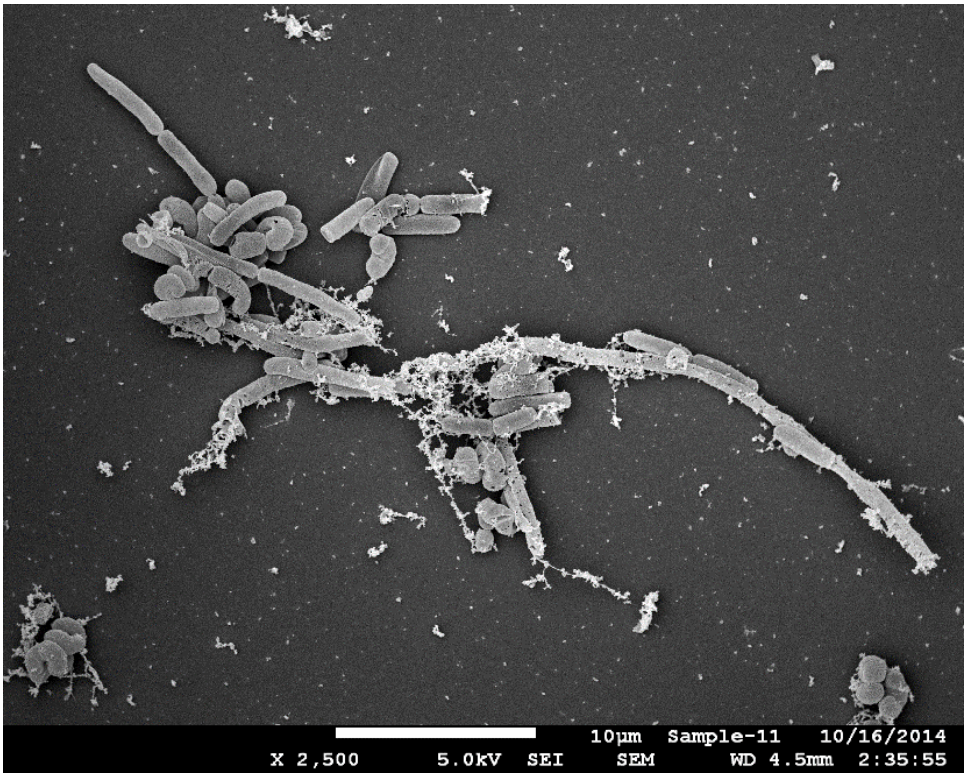

Figure D. 7. Spores of *B. thuringiensis* without Tween-20 (2500x)

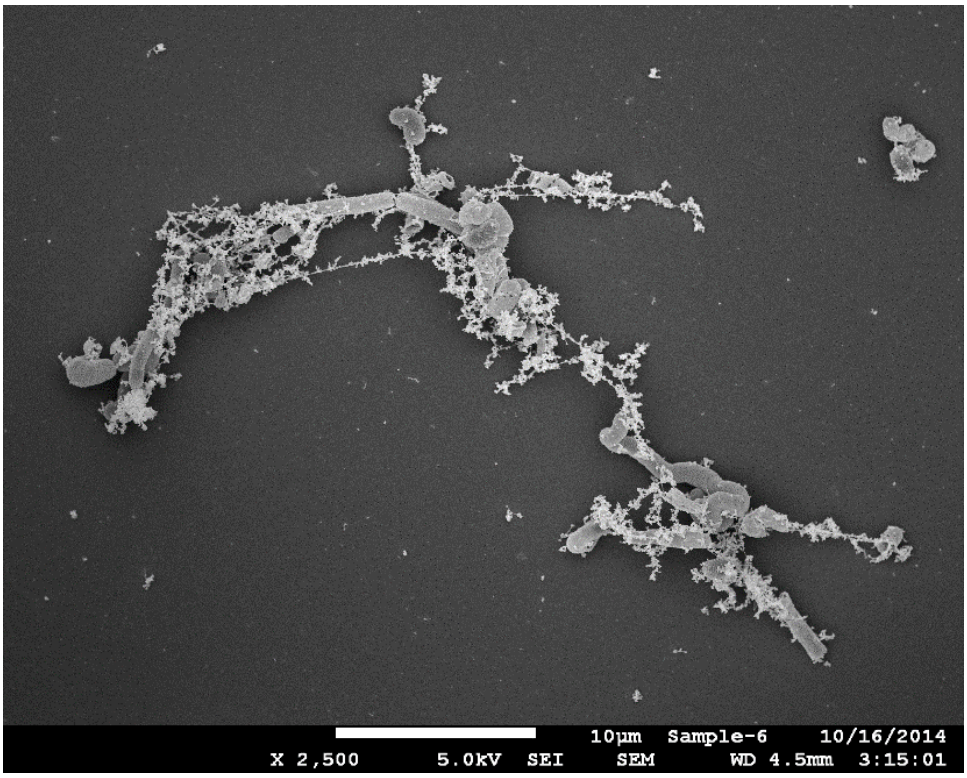

Figure D. 8. Spores of *B. thuringiensis* with Tween-20 (2500x)

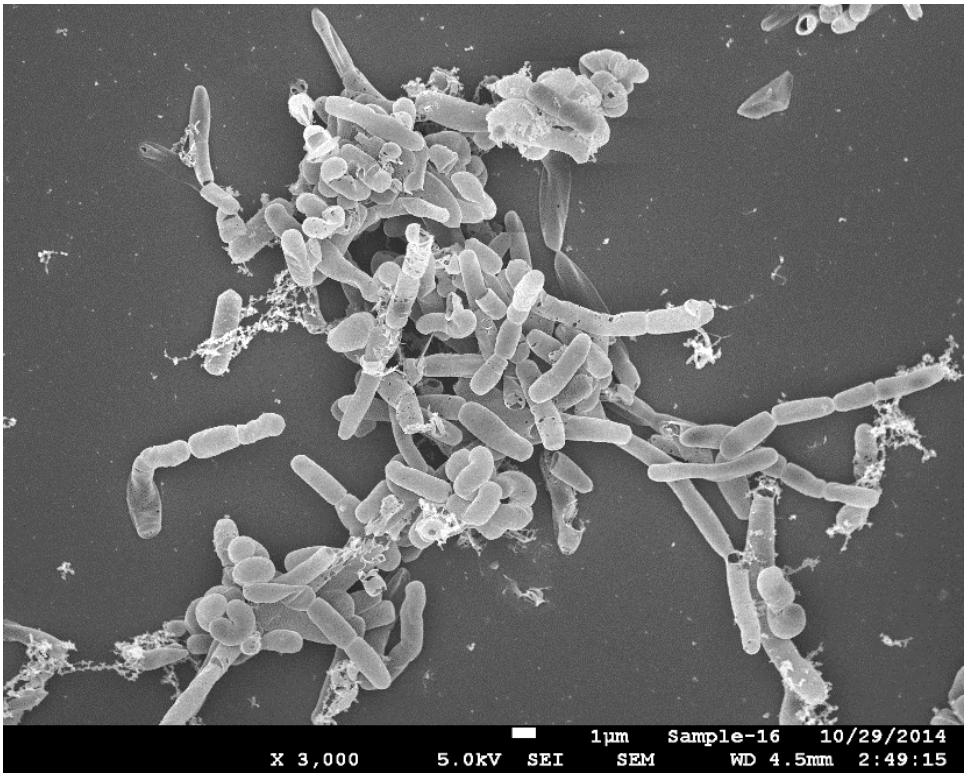

Figure D. 9. Dense clump of *B. thuringiensis* spores without Tween-20 (3000x)

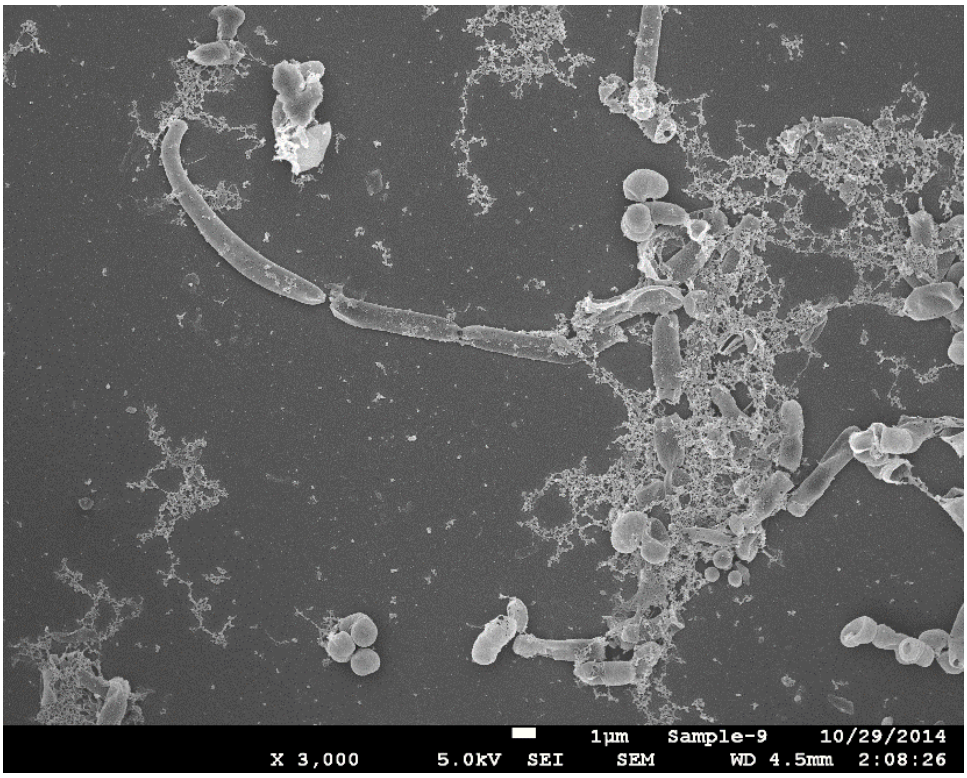

Figure D. 10. Spores of *B. thuringiensis* with Tween-20 (3000x)

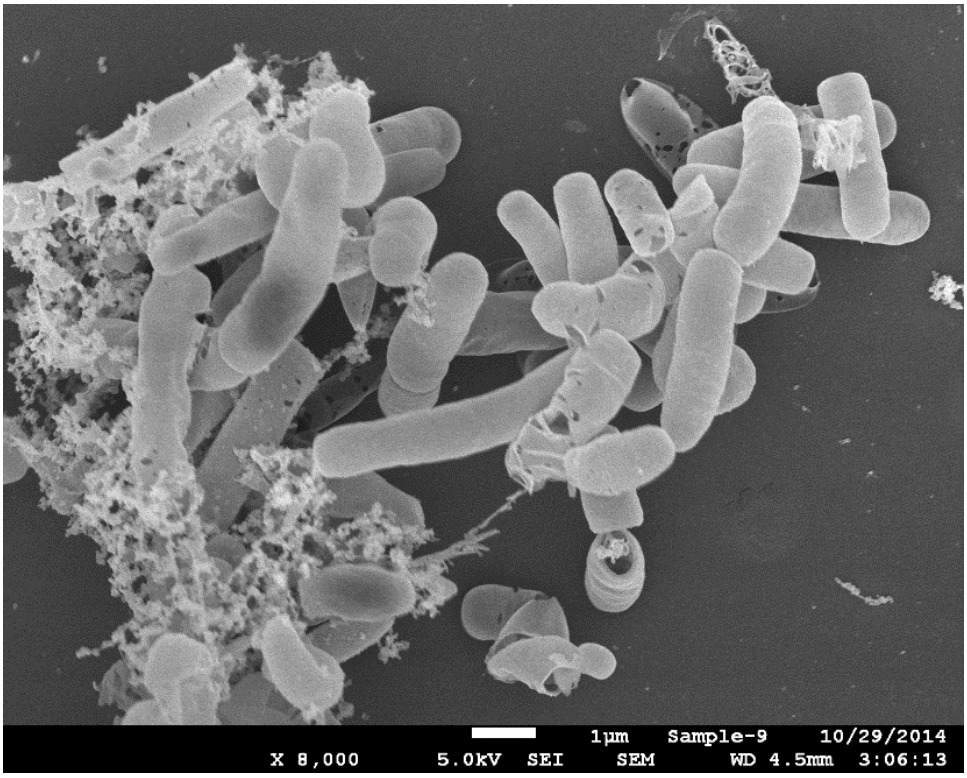

Figure D. 11. Spores of *B. thuringiensis* without Tween-20 (8000x) with strong clustering observed at higher magnifications

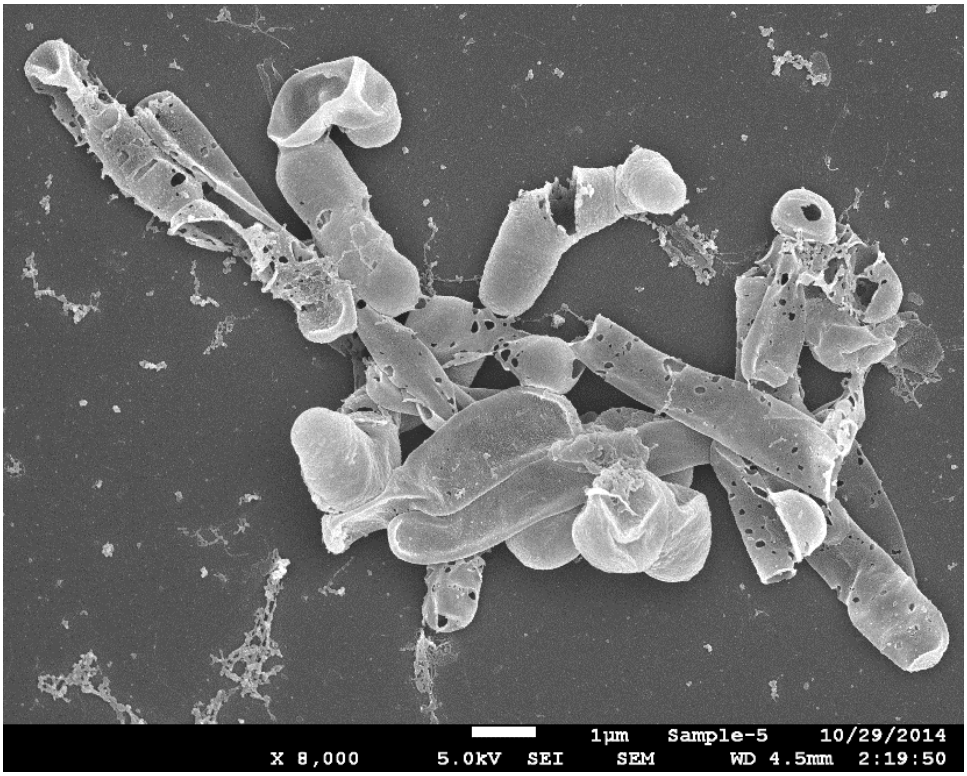

Figure D. 12. Images of *B. thuringiensis* spores with Tween-20 (8000x)

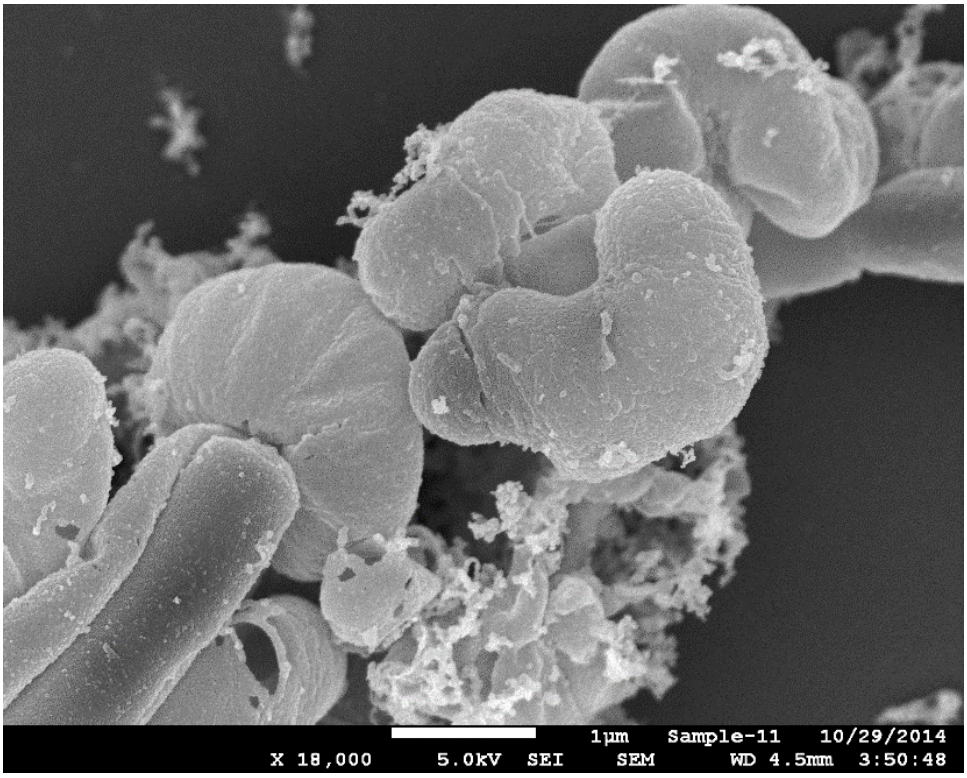

Figure D. 13. Surface of clumped *B. thuringiensis* spores without Tween-20 (18000x)

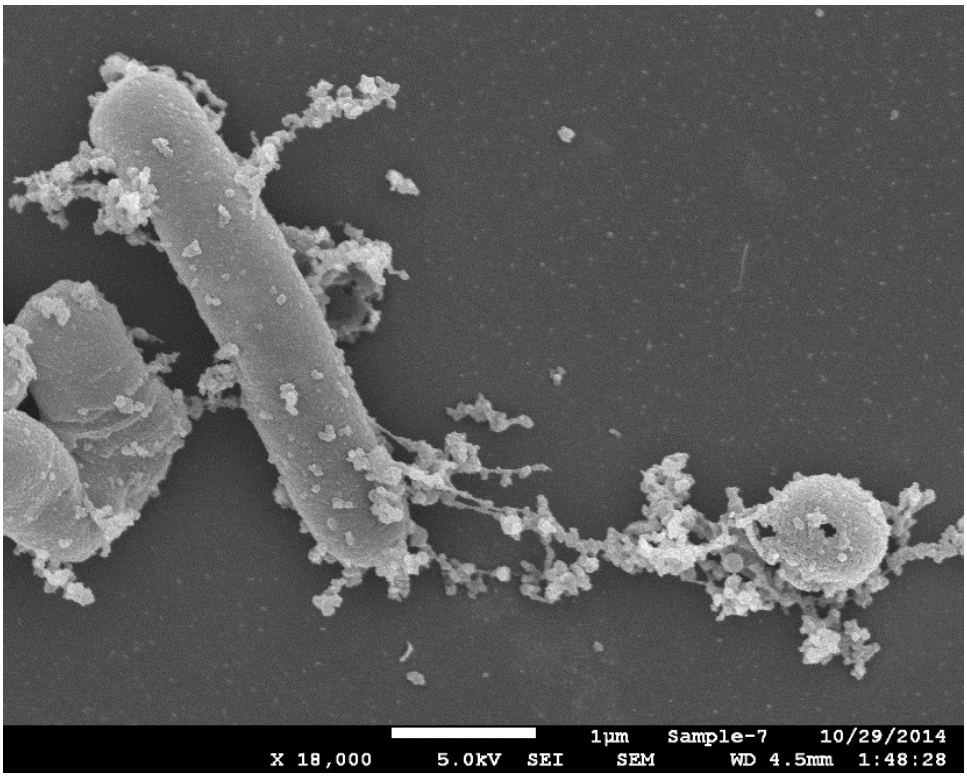

Figure D. 14. Separated spores of *B. thuringiensis* with Tween-20 (18000x)

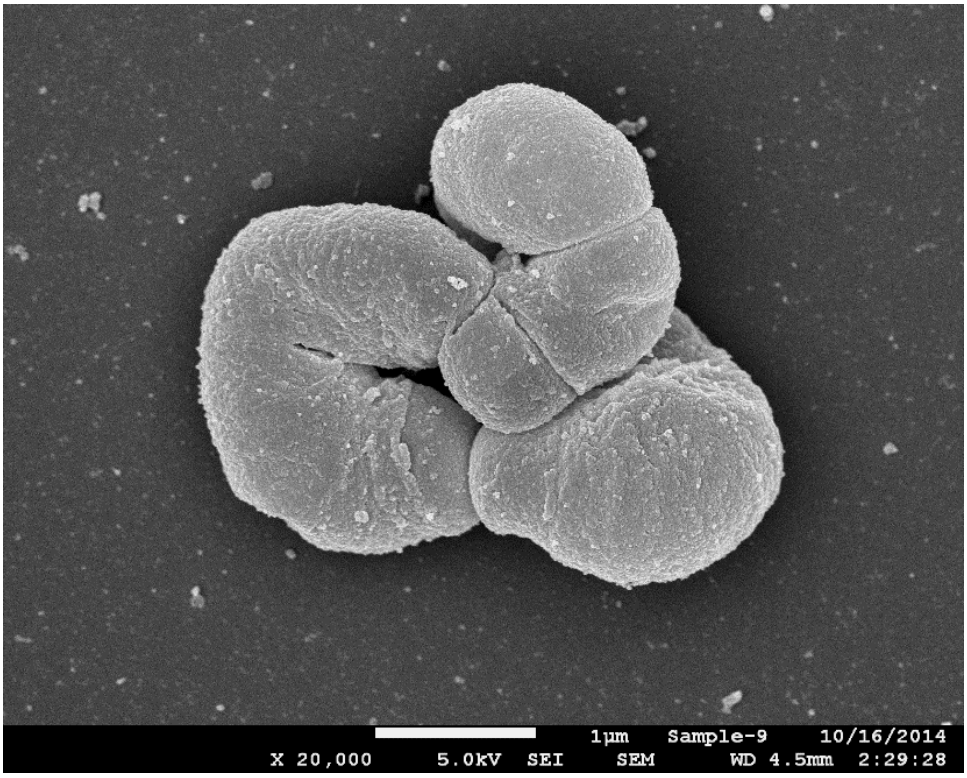

Figure D. 15. Coiled spores of *B. thuringiensis* without Tween-20 (20000x)

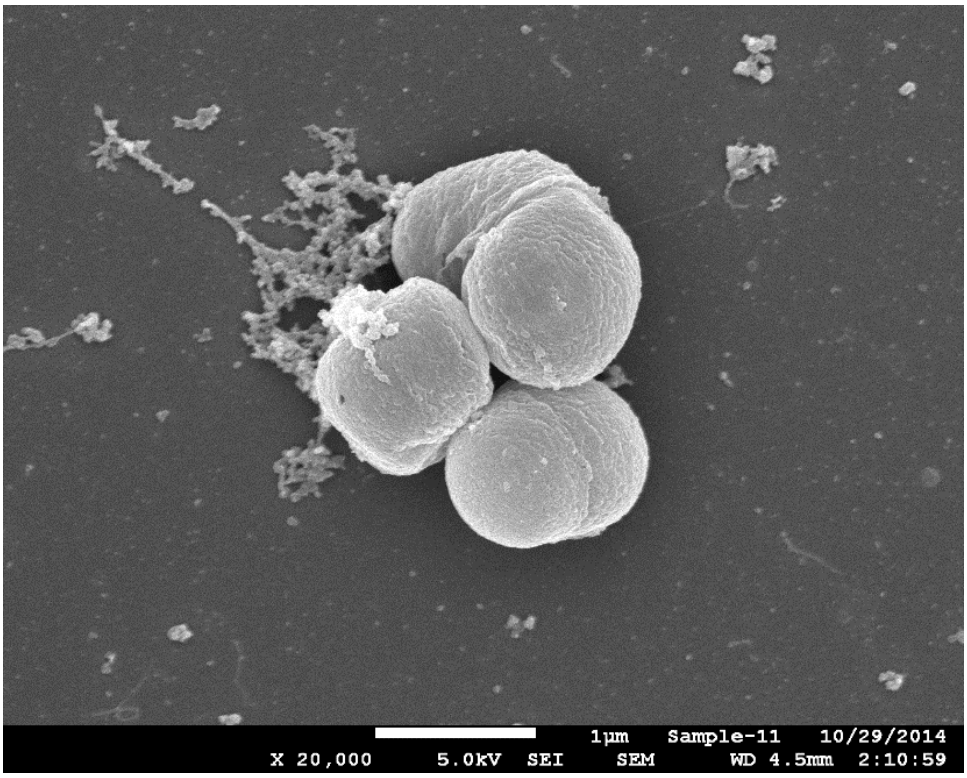

Figure D. 16. Coiled spores of *B. thuringiensis* with Tween-20 (20000x) similar to Fig. C. 16

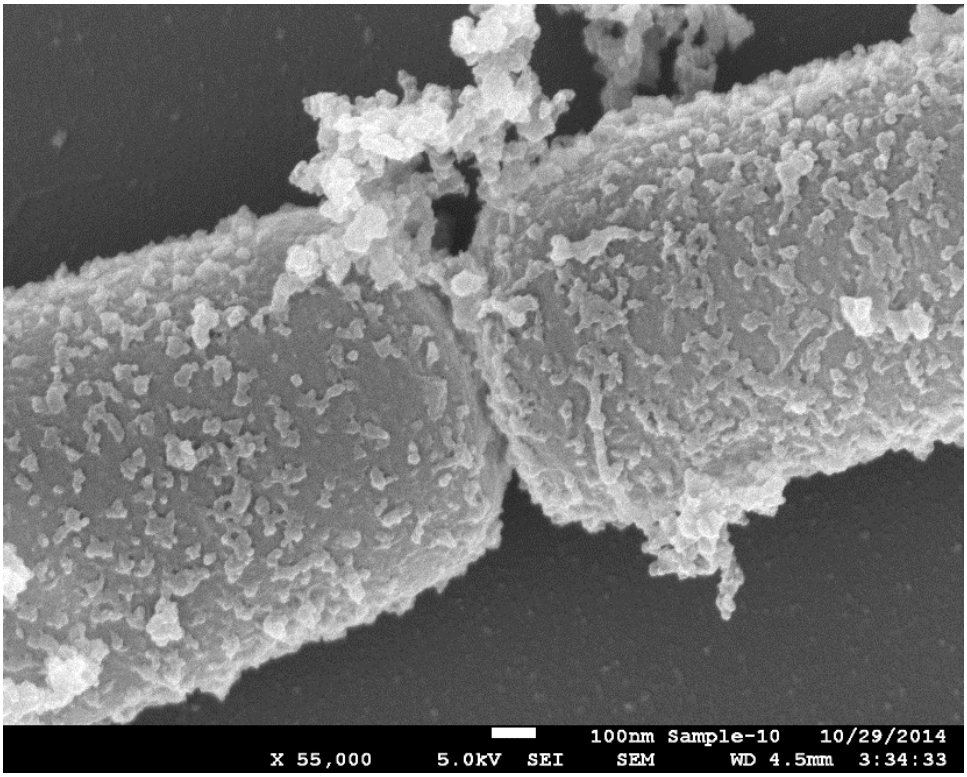

Figure D. 17. Connection between two *B. thuringiensis* spores without Tween-20 (55000x) observed at very high magnification

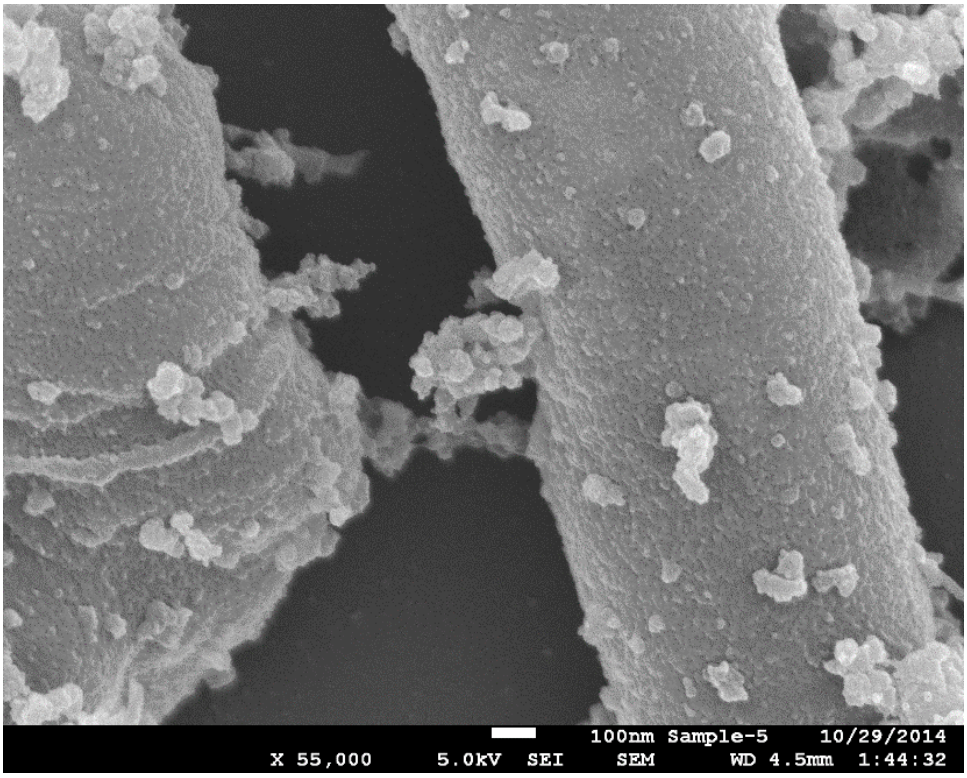

Figure D. 18. Spores of *B. thuringiensis* with Tween-20 (55000x)

APPENDIX E: STATISTICAL ANALYSES

Univariate General Linear Model Analysis on Complete Data Set (N=144)

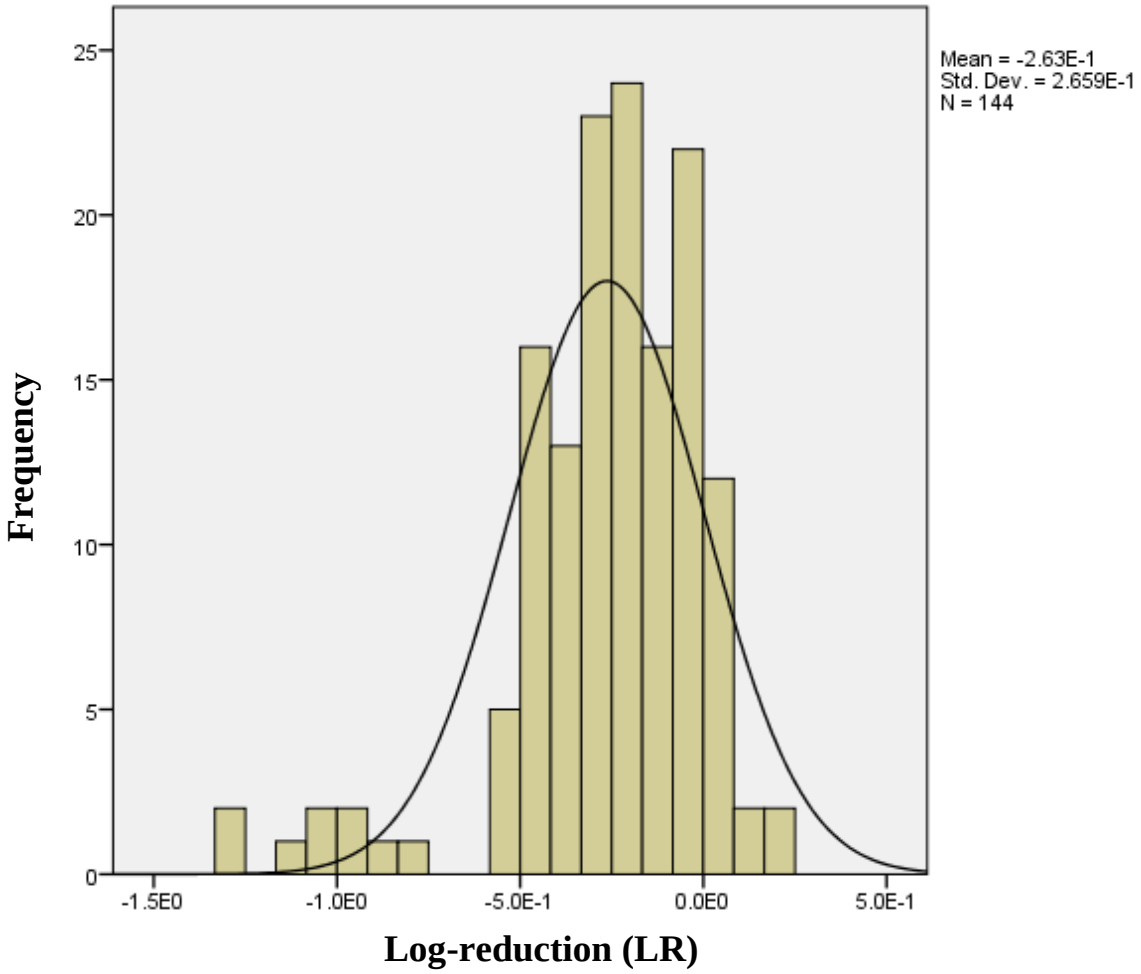

Figure E. 1. Normal distribution of the response variable, log-reduction (LR) for a combined data set of both *B. anthracis* and *B. thuringiensis* using both moisture levels at all sampling times

Table E. 1. Descriptive statistics of the dependent variable of the complete data set

Dependent Variable: TLR				
Species		**Mean**	**Std. Deviation**	**N**
B. anthracis	0.4 mL	-3.6870E-01	4.24886E-01	36
	0.2 mL	-2.1723E-01	1.86080E-01	36
	Total	-2.9296E-01	3.34482E-01	72
B. thuringiensis	0.4 mL	-1.7246E-01	1.95165E-01	36
	0.2 mL	-2.9461E-01	1.12075E-01	36
	Total	-2.3353E-01	1.69561E-01	72
Total	0.4 mL	-2.7058E-01	3.42830E-01	72
	0.2 mL	-2.5592E-01	1.57414E-01	72
	Total	-2.6325E-01	2.65918E-01	144

Table E. 2. Tests of Between-Subjects effects (Time, Species, Treatment)

Dependent Variable: TLR					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	8.354[a]	6	1.392	108.502	.000
Intercept	.916	1	.916	71.381	.000
Species* Treatment	.674	1	.674	52.510	.000
Species * Time	2.043	1	2.043	159.174	.000
Treatment * Time	1.520	1	1.520	118.427	.000
Species	.506	1	.506	39.409	.000
Treatment	.600	1	.600	46.724	.000
Time	3.983	1	3.983	310.389	.000
Error	1.758	137	.013		
Total	20.091	144			
Corrected Total	10.112	143			
a. R Squared = .826 (Adjusted R Squared = .819)					

Table E. 3. Parameter estimates for complete data set

Dependent Variable: TLR						
					95% Confidence Interval	
Parameter	**B**	**Std. Error**	**t**	**Sig.**	**Lower Bound**	**Upper Bound**
Intercept	-.346	.024	-14.288	.000	-.394	-.298
[Species=1.00] * [Treatment=1.00]	.338	.036	9.272	.000	.266	.410
[Species=1.00] * [Treatment=2.00]	.299	.032	9.347	.000	.235	.362
[Species=2.00] * [Treatment=1.00]	.313	.032	9.797	.000	.250	.376
[Species=2.00] * [Treatment=2.00]	0[a]					
[Species=1.00] * Time	-.003	.000	-11.173	.000	-.003	-.002
[Species=2.00] * Time	.001	.000	3.395	.001	.000	.001
[Treatment=1.00] * Time	-.003	.000	-10.882	.000	-.004	-.003
[Treatment=2.00] * Time	0[a]					
[Species=1.00]	0[a]					
[Species=2.00]	0[a]					
[Treatment=1.00]	0[a]					
[Treatment=2.00]	0[a]					
Time	0[a]					
a. This parameter is set to zero because it is redundant.						

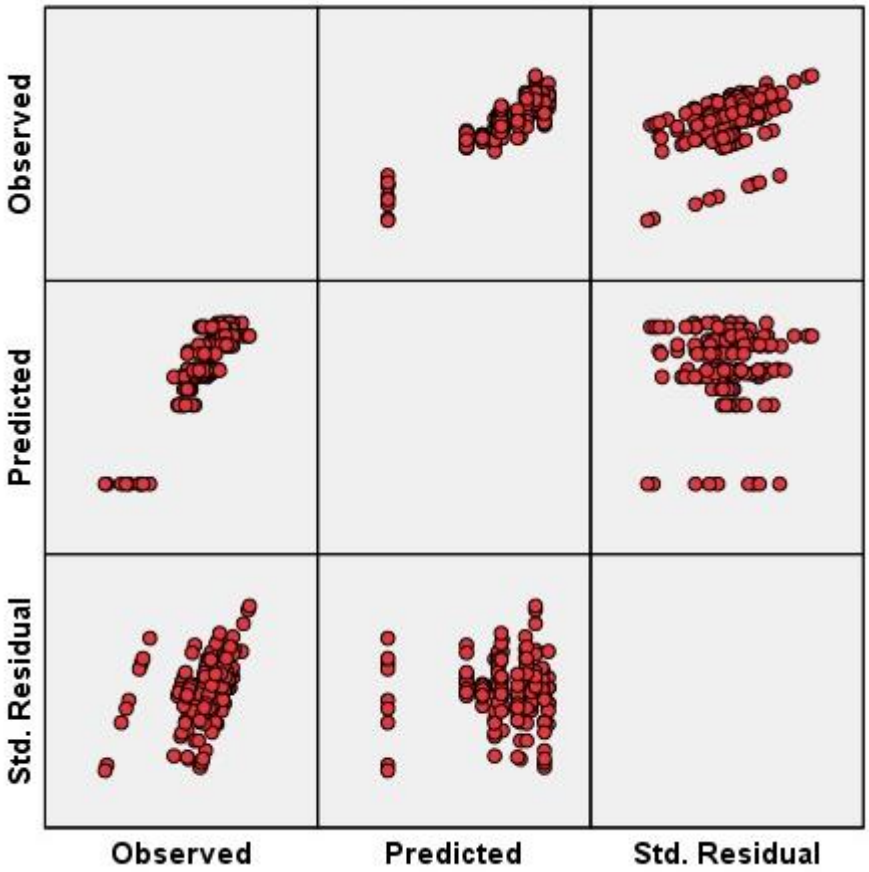

Figure E. 2. Scatter plot matrix of standard residuals, observed, and predicted values of the dependent variable (LR) of the complete data set

Univariate General Linear Model Analysis on Surfactant Data Set (N=71)

Table E. 4. Descriptive statistics of the dependent variable of surfactant data set

Dependent Variable: TLR			
Tween	**Mean**	**Std. Deviation**	**N**
No Tween	1.9673E-03	2.42890E-02	35
Yes Tween	9.6443E-03	1.15472E-02	36
Total	5.8599E-03	1.91875E-02	71

Table E. 5. Tests of Between-Subjects effects (Time, Tween-20)

Dependent Variable: TLR					
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	.006[a]	3	.002	6.805	.000
Intercept	.005	1	.005	17.256	.000
Tween	6.103E-05	1	6.103E-05	.207	.651
Time	.003	1	.003	9.681	.003
Tween * Time	.002	1	.002	7.884	.007
Error	.020	67	.000		
Total	.028	71			
Corrected Total	.026	70			
a. R Squared = .234 (Adjusted R Squared = .199)					

Table E. 6. Parameter estimates for surfactant data set

Dependent Variable: TLR						
					95% Confidence Interval	
Parameter	**B**	**Std. Error**	**t**	**Sig.**	**Lower Bound**	**Upper Bound**
Intercept	.010	.004	2.619	.011	.002	.018
[Tween=1.00]	.003	.006	.455	.651	-.009	.014
[Tween=2.00]	0[a]					
Time	-9.709E-06	4.428E-05	-.219	.827	-9.810E-05	7.868E-05
[Tween=1.00] * Time	.000	6.397E-05	-2.808	.007	.000	-5.193E-05
[Tween=2.00] * Time	0[a]					
a. This parameter is set to zero because it is redundant.						

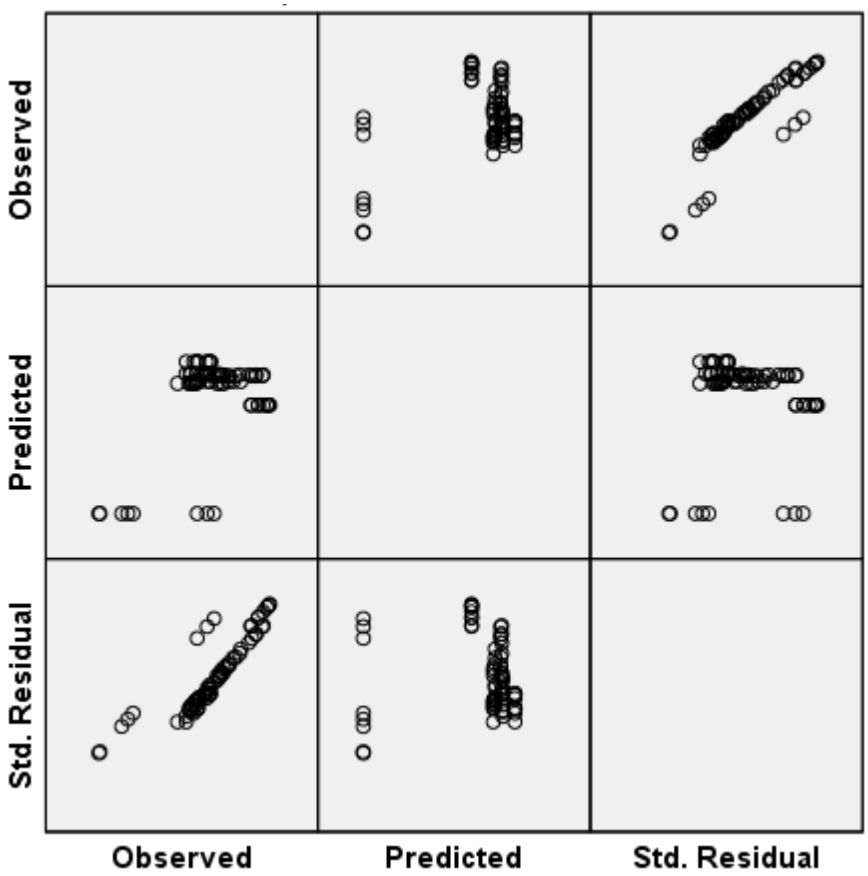

Figure E. 3. Scatter plot matrix of standard residuals, observed, and predicted values of the dependent variable (LR) of the surfactant data set

APPENDIX F: SUMMARY OF REAL-TIME PCR FOR DIFFERENT MOISTURE LEVELS TO QUANTIFY BACILLUS ANTHRACIS

Table F. 1. Forward and Reverse Primer Sequences for *B. anthracis Sterne* spores*

Position	**DNA Sequence**
Forward, 5'-3'	AGAAGTGCATGCGTCGTTCTT
Reverse, 5'-3'	GCGACCGTACTTGAATTCGAA
*Primers were obtained from Intergrated DNA Technologies®	

Table F. 2. Composition of Reaction Mixture

Component	**Volume**
DNA extracted from *Bacillus anthracis Sterne* spores solution*	5 µL
Roche Lightcycler® 480 SYBR Green I Master	10 µL
Forward primer	1 µL
Reverse primer	1 µL
RNA-ase free wáter	3 µL
*DNA was extracted using heat-inactivation method. Spore solutions were oven-heated at 100^{o}C for 30 minutes, followed by centrifugation at 11000xg for 5 minutes	

Table F. 3. Summary of Lightcycler® Operation Cycles

Operation Cycles	**Temperatures, time**
Pre-incubation	95^{o}C, 5 minutes
Amplification (45 cycles)	95^{o}C, 10 s
	55^{o}C, 20 s
	72^{o}C, 10 s
Melting curve	95^{o}C, 5 s
	65^{o}C, 1 hour
	97^{o}C, continuous
Cooling	40^{o}C, 10 s

Table F. 4. Summary of applied and recovered concentrations of *B. anthracis Sterne* spores enumerated using qPCR

Volume of water/ filter = 0.4 mL					**Volume of water/ filter = 0.2 mL**				
Applied concentration (spores/ filter)			**2.34E+09**		**Applied concentration (spores/ filter)**			**7.58E+09**	
Filter	**Time (hrs)**	**Copies/ reaction**	**Copies/ filter (spores/ filter)**	**Log reduction**	**Filter**	**Time (hrs)**	**Copies/ reaction**	**Copies/ filter (spores/ filter)**	**Log reduction**
A	0	4.91E+04	1.96E+08	-1.08E+00	A	0	1.33E+05	5.32E+08	-1.15E+00
B	0	4.52E+04	1.81E+08	-1.11E+00	B	0	1.38E+05	5.52E+08	-1.14E+00
C	0	7.16E+04	2.86E+08	-9.12E-01	C	0	1.29E+05	5.16E+08	-1.17E+00
A	24	4.39E+04	1.76E+08	-1.12E+00	A	24	1.12E+05	4.48E+08	-1.23E+00
B	24	4.85E+04	1.94E+08	-1.08E+00	B	24	1.06E+05	4.24E+08	-1.25E+00
C	24	6.52E+04	2.61E+08	-9.53E-01	C	24	1.24E+05	4.96E+08	-1.18E+00
A	48	1.94E+05	7.76E+08	-4.79E-01	A	48	3.51E+05	1.40E+09	-7.32E-01
B	48	2.73E+05	1.09E+09	-3.31E-01	B	48	5.12E+05	2.05E+09	-5.68E-01
C	48	2.51E+05	1.00E+09	-3.67E-01	C	48	2.84E+05	1.14E+09	-8.24E-01
A	168	1.95E+05	7.80E+08	-4.77E-01	A	168	3.44E+05	1.38E+09	-7.41E-01
B	168	5.45E+04	2.18E+08	-1.03E+00	B	168	2.95E+05	1.18E+09	-8.08E-01
C	168	1.71E+05	6.84E+08	-5.34E-01	C	168	3.08E+05	1.23E+09	-7.89E-01

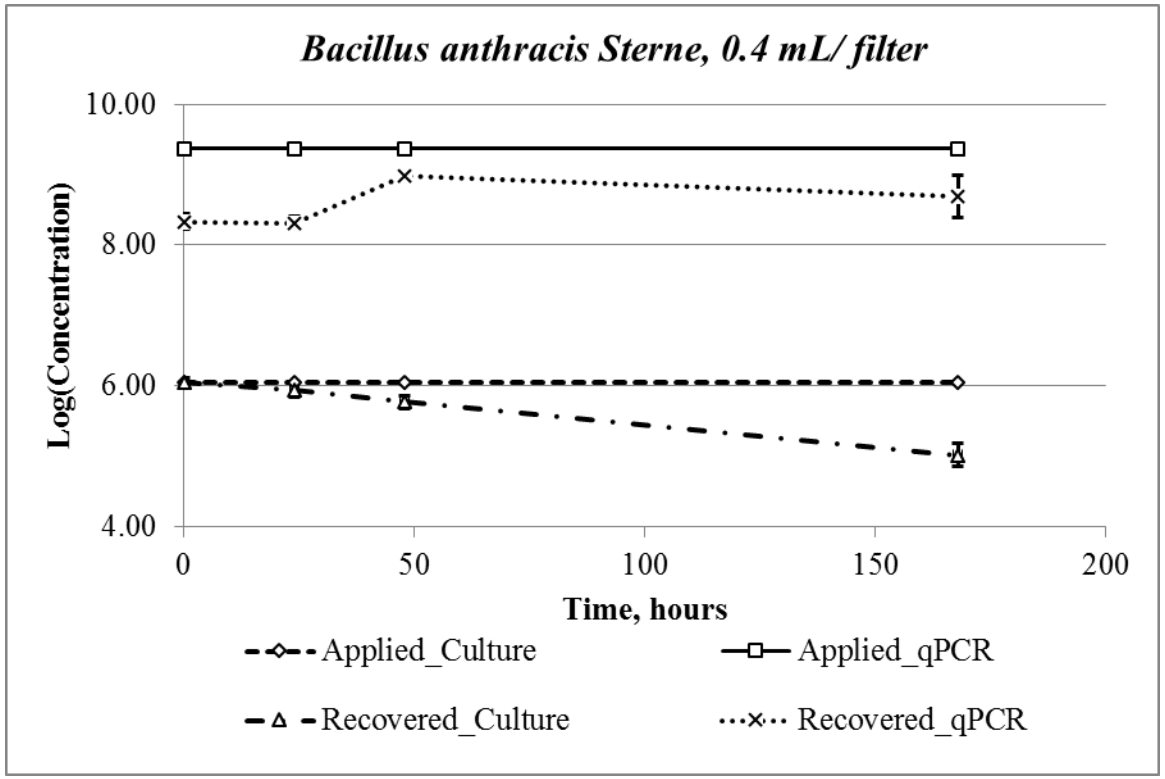

Figure F. 1. Log-concentration of *B. anthracis Sterne* spores as a function of time for 0.4 mL volume of inoculation quantified using culture-based and molecular-based techniques. Error bars indicate standard deviation from the mean.

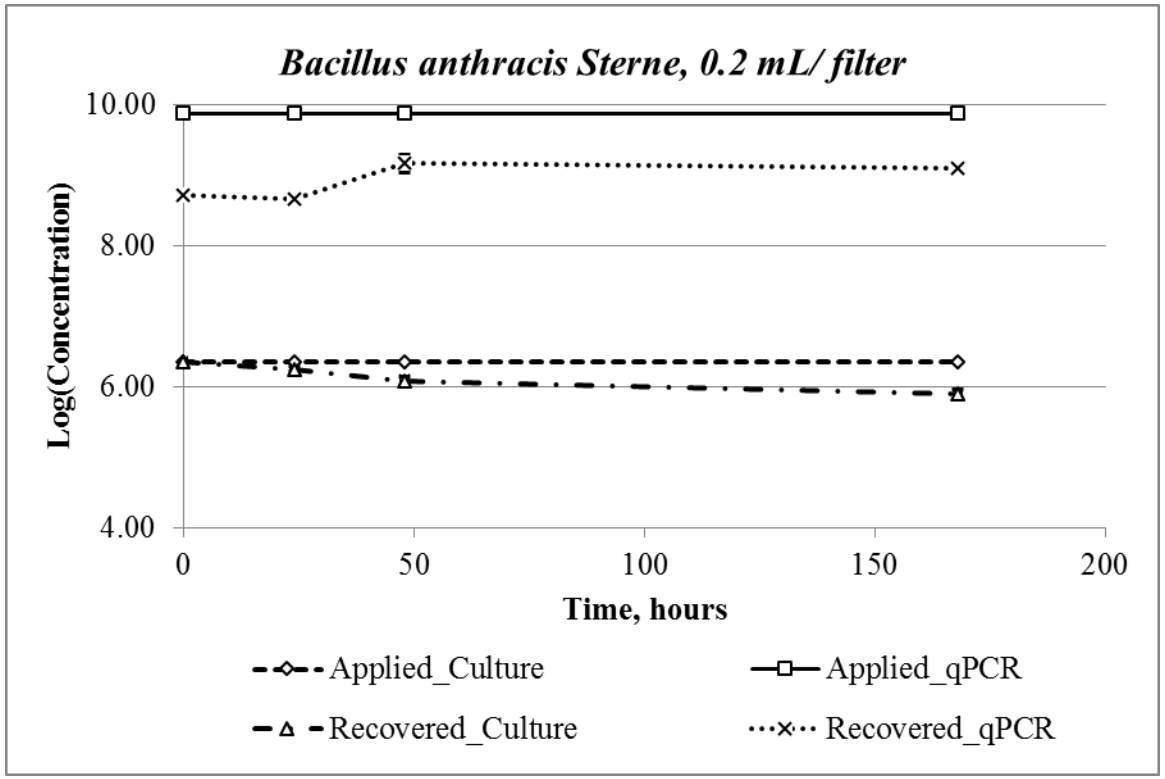

Figure F. 2. Log-concentration of *B. anthracis Sterne* spores as a function of time for 0.2 mL volume of inoculation quantified using culture-based and molecular-based techniques. Error bars indicate standard deviation from the mean

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