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Thermal Inactivation of Bacillus anthracis Spores Using Rapid Resistive Heating

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THERMAL INACTIVATION OF *BACILLUS ANTHRACIS* SPORES USING RAPID RESISTIVE HEATING

THESIS

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Abstract

Bacillus anthracis (*B.a.*) is the causative agent of anthrax. *B.a.* spores pose a threat because have been used as a bioweapon throughout history and can survive harsh conditions for prolonged periods of time. Rapid resistive heating was used for the thermal inactivation of spores in order to determine *B.a.* spore viability, to include partial and complete thermal inactivation. This microbiological study sought to obtain a correlation between exposure time, temperature, and spore viability. This information is invaluable when modeling employment effects for agent defeat weapons to destroy *B.a.* stockpiles through thermal inactivation. Partial and complete thermal inactivation of the spores were found using rapid resistive heating at short duration exposure times from 0.26 to 7 seconds at temperatures ranging from 73.5 to 888°C. Power supply pulses were accomplished by applying varying voltages ranging from 9 to 200 Volts for 12 separate power supply input times ranging from 0.1 to 10 seconds. Higher temperatures were needed to thermally inactivate the *B.a.* spores as exposure times decreased.

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THERMAL INACTIVATION OF *BACILLUS ANTHRACIS* SPORES

USING RAPID RESISTIVE HEATING

I. Introduction

Bacillus anthracis (*B.a.*) is the causative agent of anthrax. Its bacteria spores have been used as a bioweapon. This agent poses a threat to the United States and its allies, which is why the Centers for Disease Control and Prevention (CDC) categorizes *B.a.* as a Category A agent. This category is classified as the riskiest category by the CDC and includes high-priority agents. There are six total microorganisms, including *B.a.*, which are Category A agents.

There is motivation for using thermal decontamination of *B.a.* spores for agent defeat scenarios. Spore-forming microorganisms are much harder to kill than microorganisms which do not form spores (Levi, 2004). *B.a.* is a microorganism which produces spores. Spores can survive in harsh environments for several years and remain dormant until favorable conditions are met to allow growth (Milby et al.). For example, spores remained in the top soil on Gruinard Island for over 40 years after the British detonated experimental anthrax bombs on the island during World War II (U.S. Congress Office of Technology Assessment, 1993). Due to the persistance of *B.a.*, thermal inactivation is rendered one of the main methods for removal of spores.

1.2 Motivation

This microbiological study seeks to obtain a correlation between exposure time, temperature, and spore viability. This information is invaluable when modeling employment effects for agent defeat weapons to destroy *B.a.* stockpiles through thermal inactivation.

1.3 Scope

The work reported in the thesis addresses two overall goals. The first goal is to develop a resistive heating method to simulate a thermal environment of conventional munitions or agent defeat weapons on *B.a.* spores on surfaces. This technique would then be used to analyze inactivation of *B.a.* spores on surfaces by brief heat exposures in order to link the spore viability with the simulated explosive environment created by the agent defeat weapons. Achieving these goals required achieving and recording temperatures necessary to partially and completely inactivate the *B.a.* spores at various short exposure times.

1.4 Problem Statement

Determine *B.a.* spore viability, to include partial and complete thermal inactivation, using rapid resistive heating for short duration exposure times (0.26 to 7 seconds) at high temperatures ranging from 73.5 to 888°C. Apply power supply voltage pulses ranging from 9 to 200 Volts for 12 separate power supply input times ranging from 0.1 to 10 seconds to tungsten filaments that were treated with adsorbed *B.a.* spores.

1.5 Approach

Successful completion of the objectives and goals required extensive prototyping/initial testing and experimentation. Preparation and planning was needed before, during, and after the thermal inactivation testing.

Preparation for the thermal inactivation tests included acquiring spore samples and developing an appropriate heating method. The *B.a.* were hydrated, grown, harvested, and purified to make stock and diluted solutions in order to achieve the

desired spore population density when a drop was applied as a sample to a filament. Prototyping, initial testing and research was accomplished on different heating methods in order to choose the appropriate setup for the thermal inactivation testing. Possible setups included the AFIT Xenon Thermal Simulator (AXTS), clamping thin reproducible coiled tungsten wires to a power supply with alligator clips, or clamping a lamp containing a light bulb filament to a power supply with alligator clips; the lamp setup was selected for use in this study. Wire and filament configurations as well as sample compositions were also tested before the light bulb filament method was chosen. Samples were made by applying a drop from the *B.a.* solution onto each of the filaments (once the light bulb envelopes were removed and holders were made). The final step in the *B.a.* spore sample preparation process was desiccation.

For the inactivation studies, high temperature exposures up to 888°C were coupled with short time durations between 0.258 to 7 seconds to attain the simulated explosion environmental. The lamp setup allowed a reproducible experimental methodology for thermal inactivation studies. A total of 12 exposure times and 120 heat exposures were investigated. Videos of the filament were captured using a FLIR Thermacam and Pinnacle recorder involving manual changes of temperature range setting on the FLIR.

After being exposed to the heat in the thermal inactivation studies, the viability of *B.a.* spores, to include partial and complete inactivation, was determined after a series of steps. To remove the spores from the filaments, the hard surface recovery method used in this study was sonication. After nutrient agar plates were made and inspected, a *B.a.* spore drop was placed onto a plate with a plate spreader for each microdilution.

Microdilutions for each *B.a.* sample were made in deep well plates using a 12-tip pipette. After being plated onto the agar plates, the *B.a.* once again was grown in an incubator and colonies were counted after the 24-hour mark of growth and the 48-hour mark of growth (793 agar plates for 1,586 plates counted). An equation was applied to determine how many spores remain.

Heat exposure times were corrected from the power supply input times by using time temperature profiles. Time temperature profile plots are constructed by obtaining data points from reviewing each recorded FLIR video. In this study, 3,100 data points are annotated from reviewing the videos. In order to ensure exposure times are calculated accurately, the study required emissivity corrections to the FLIR using a thermocouple and accounting for the percent error of the FLIR in the time temperature profiles.

1.6 Document Structure

The following work was separated into different chapters: Chapter 2 – Background, Chapter 3 – Method of Experimentation, Chapter 4 – Results and Analysis, and Chapter 5 – Conclusions and Recommendations. Chapter two provides a literature review over concepts used in the thesis, such as microbiology, *B.a.* characteristics, hard surface recovery, thermal inactivation, and instrumentation. Next, chapter three discusses the methods of sample preparation, to include controls and prototyping/initial testing, and procedures for each of the experiments conducted. Chapter four describes how data was reduced and how analysis was performed to present the results of the conducted experiments. Finally, chapter five provides overall conclusions of the results of the conducted experiments and offers insight into ideas for forthcoming research at AFIT.

II. Background

2.1 *Bacillus anthracis* (*B.a.*) and Historical Significance

Bacillus is a very hardy genus that produces multi-resistant endospores. *B.a.* is considered to be the most dangerous species of *Bacillus* and has been used as a bioweapon several times throughout history, to most recently include the 2001 U.S. anthrax attacks. The 2001 U.S. anthrax attacks utilized a dissemination method, where letters distributed through the postal system resulted in 5 deaths (all inhalational cases) and an additional 17 non-fatal cases due to inhalational or cutaneous infection (Inglesby et al., 2002). As a result, major post offices now have the Biohazard Detection System (BDS) to mitigate the threat. Other key historical events include weaponization use of biological material by Germany in World War I, aerosolization as the method for delivery used by terrorist group Aum Shinrikyo in the 1993 Tokyo incident, and accidental release of material in 1979 by the former Soviet Union causing 68 deaths. (Takahashi et al., 2004; Inglesby et al., 2002). The hardiness of this endospore contributes to the usage of *B.a.* throughout the world with twenty countries which have investigated its use as a biological weapon (Grace, 2003). The spore resistance is contributed to by its core, cortex, and coat structures, although not contributed to the exosporium (Driks, 2003). Due to their hardiness, *B.a.* spores are multi-resistant to heat (dry or wet), desiccation, chemical, UV radiation, gamma-radiation, and ultrahigh hydrostatic pressure (Nicholson et al., 2000). *B.a.* poses a high risk to national security and public health as categorized by the Centers for Disease Control and Prevention (CDC). The following subsections will further describe *B.a.* by discussing sporulation, microorganism significance, surrogates, and spore structure.

2.1.1 *B.a.* Sporulation

B.a. is the causative agent of anthrax and produces endospores that can remain dormant under limited conditions (depletion of a readily metabolized carbon or nitrogen source) until more favorable conditions arise and vegetative cells reproduce (Setlow and Johnson, 2001; Moberly et al., 1966). "Dormant but viable spores can persist in the environment over millennial time spans in a metabolically inactive state (Nicholson et al., 2000)." Once favorable conditions arise, spores rapidly germinate and resume vegetative growth (Driks, 2003). The sporulation stages are summarized in Figure 1 (Setlow and Johnson, 2001; Zolock, 2002).

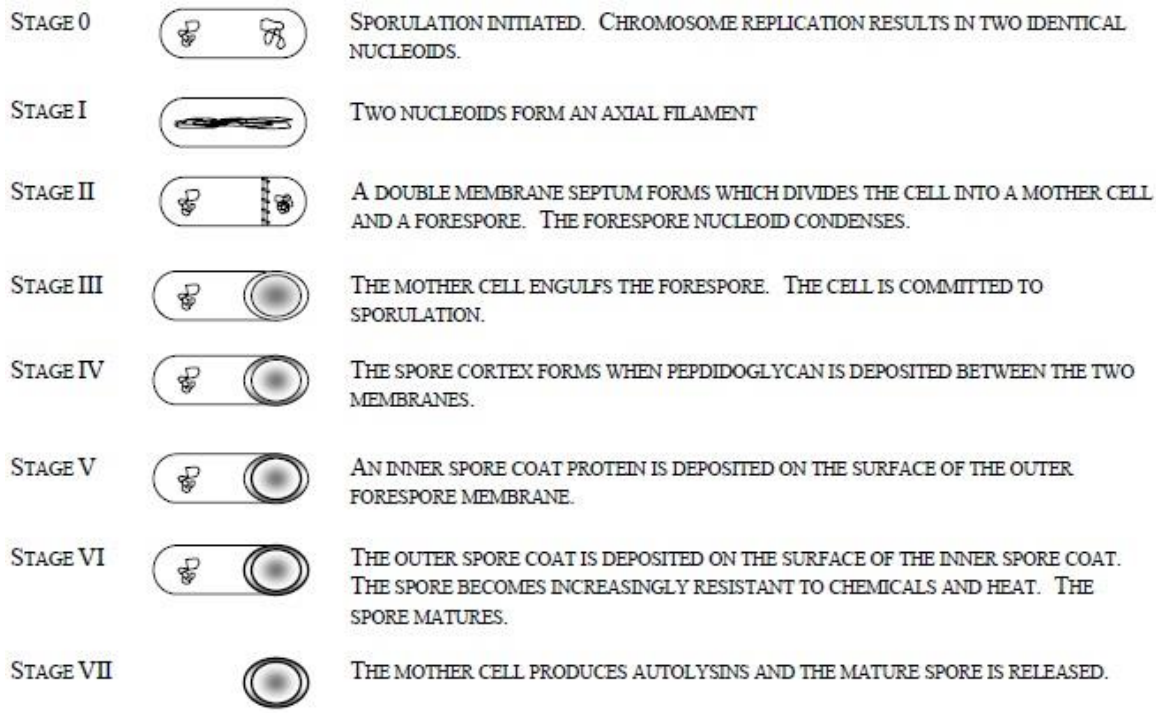


Figure 1. Sporulation Summary (from Setlow and Johnson, 2001)

2.1.2 Significance of *B.a.*

The aerobic microorganism is characterized by the CDC as a Category A agent with only 5 other such microorganisms. Category A agents pose the most risk (especially with public health and national security) amongst the A-C agent categories. Category B agents are still designated a high risk but pose a lower risk in comparison to the Category A agents which are predominately food- and water-borne pathogens having the potential to be used as bioweapons. Category A agents include three other bacteria (*Yersinia pestis* (plague), *Francisella tularensis* (tularemia), a toxin from *Clostridium botulinum* (botulism)), and two viruses (smallpox and the viral hemorrhagic fever family). The CDC further describes Category A agents as high-priority agents due to the fact that they:

- "can be easily disseminated or transmitted from person to person;
- result in high mortality rates and have the potential for major public health impact;
- might cause public panic and social disruption; and
- require special action for public health preparedness (CDC, 2007)."

2.1.3 Surrogates of *B.a.* and Spore Structure

The deadly nature of the *B.a.* suggests the use of less lethal surrogates to simulate the environment required to destroy stockpiles using agent defeat weapons. A common surrogate for *B.a.* is *Bacillus thuringiensis* (*B.t.*) which has been used to conduct thermal inactivation research. However, the research conducted to support this thesis utilizes the *B.a.* Sterne strain which is used in livestock vaccines and is the most widely used variety

of *B.a.* spores due to the ability to use it to perform research in a Biosafety Level 2 (BSL-2) laboratory.

Research done on fully virulent strains requires a BSL-3 laboratory, which is very expensive. The difference between the Sterne strain and the fully virulent strain, aside from producing sub-lethal amounts of toxin, is its composition. The Sterne strain is able to produce sub-lethal amounts of toxin because it lacks a large plasmid (pXO2), which codes for capsule formation; it contains another large plasmid (pXO1), which codes for anthrax toxin production but there is no concern about the toxic effect of the capsule (Harvill et al., 2005).

The spore structure of the genus *Bacillus* is shown in Figure 2 (Goetz, 2005; Bacon, 2009). The spore core, which is composed of deoxyribonucleic acid (DNA) and small acid-soluble proteins (SAP) in a dipicolinate matrix, is the innermost part of the spore (Driks, 2003). The spore cortex and coat are relatively complex at the molecular level and their functions are still being defined, whereas the function of the exosporium is even less defined (Driks, 2003). *B.a.* is gram-positive and rod-shaped.

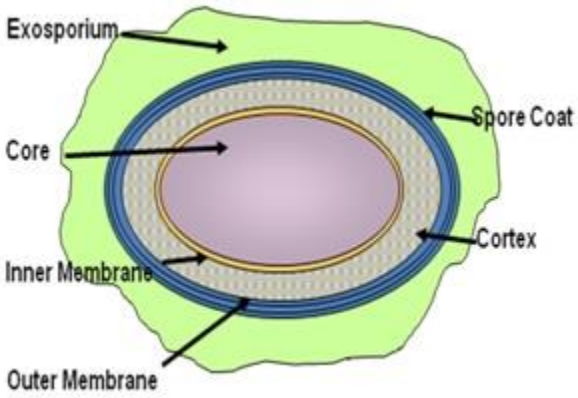


Figure 2. *Bacillus* Spore Structure (from Goetz, 2005)

2.2 Desiccation

Desiccation is the term used to describe spores that have been dried after being purified or suspended in a solution. Commercially used desiccators are available to dry spores, including ones that are vacuum-sealed. The desiccator used in this study dried spores by using humidity sponges containing indicating gel. The blue indicating gel in the humidity sponges turns pink after moisture has been absorbed. More details about the desiccation process used in this study can be found by referring to chapter 3 (section 9 and subsection 3).

Desiccated spores were used in the research study, but if the spores would have been wet (suspended in water), the water content in the core of the spore would be 28 to 50 percent of the total wet weight, while the water content of the cortex, coat, and exosporium regions would be 75 to 80 percent of the total wet weight (Nicholson et al., 2000).

2.3 Hard Surface Recovery Methods

The hard surface recovery method used in this research was sonication. Other more common methods are known to outperform sonication for the hard surface recovery of *B.a.* spores, but it was the best option for this research since coiled filament samples were used instead of the typical flat, smooth metal coupons used in literature.

A recent study specifically for the hard surface recovery of *B.a.* spores determined that vortex mixing swabs outperformed sonication extraction by greater than 25 percent (Hodges et al., 2006). Macrofoam/foam swabs were found to recover the most *B.a.* spores out of any other process for flat, smooth metal coupons, including polyester and

cotton swabs (Lewandowski et al., 2010; Buttner et al., 2004; Rose et al., 2004). The only source found which refuted the theory of macrofoam swabs performing better than other swab types had determined that of 4 swab types tested (cotton, dacron, rayon, and macrofoam) on 2 flat, smooth surfaces and 2 non-typical surfaces (not flat or smooth), "no single swab outperformed all others on all four surfaces" (Edmonds et al., 2009).

The amount of *B.a.* spores extracted from sonication for this research were remarkably high compared to literature on other studies. The higher extraction rate may be attributed to precise work done in the laboratory by performing the research in batches so that impaction from settling does not occur in tubes and microdilutions. Prototyping and initial testing confirmed that settling makes spores difficult to remove from surfaces. Refer to Table 7 in chapter 4 (section 7 and subsection 2) to see the table comparing the hard surface recoveries of *B.a.* between Grijalva and previous work in literature. Overall, the recovery of spores within this study during prototyping/initial testing was on par or better when compared to previous studies with a 41-52% recovery range and average percent spore recovery of 46.5%.

2.4 Microdilutions

Microdilutions were used to determine the spore populations in the stock solution and original filament sample solutions, the process is shown in Figure 3 (Bacon, 2009). The figure shows a hypothetical setup for a 1:10 dilution series; microdilutions for this research were done in deep-well plates. The figure explains how to quantify the spores, which includes performing six dilution factors (theoretically, the number of dilutions would need to be adjusted depending on the amount of spores in the starting solution;

seven dilution factors were used for the study), followed by plating the seven dilutions on agar dishes, and finally counting the colonies.

Figure 3 below shows a pictorial description of the general process for microdilutions and obtaining colonies on agar plates. In addition to the figure, other important features also need to be included in the protocol while performing research in order to obtain accurate data points for the number of colonies recovered. There is a need to incubate the agar plates for up to 24 hours at 37ºC, which is the optimal temperature for *B.a.* incubation. After incubation and counting colonies, incorporating the CFU/mL equation explained in Equation 2 in chapter 4 (section 6), accounts for the aliquots used (the recovery amount will be much smaller without applying this equation). Another important aspect is using the agar plates that contain 30-300 colonies, since it is assumed the amount of spores recovered is accurate to the order of magnitude when determining spore concentrations in the originally used spore suspensions (Goetz, 2005).

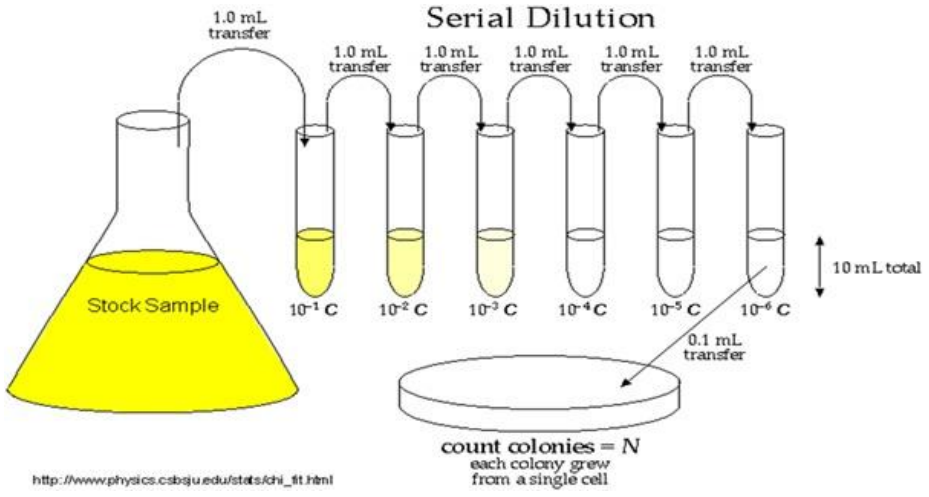


Figure 3. Process for Microdilutions (from Bacon, 2009)

2.5 Heat Inactivation

The thermal inactivation studies were conducted to simulate the environment of *B.a.* spores when conventional munitions were used as agent defeat weapons. Battelle, used *Bacillus thuringiensis* (*B.t.*) in the 1999 revision report *Thermal Deactivation of Aerosolized Bacteria* to the Defense Threat Reduction Agency (DTRA); this document was used as a guide to come up with the best temperature ranges possible for the Grijalva thermal inactivation studies.

Figure 4 below, from Bacon, 2009, shows the plot for the thermal inactivation of *Bacillus* spores from previous work (Goetz, 2005; Hawkins, 2008; Bacon, 2009). The figure is an Arrhenius plot, which delineates the thermal inactivation of *B.a.* and its surrogate *B.t.* spores and is supported mostly by research done at AFIT. The Arrhenius plot comparing the thermal inactivation of spores in this study with Battelle can be seen in chapter 4 (section 10).

It was difficult to compare the results achieved for this thesis to previous work due to the limited number of studies that performed research in the 0.1- to 10-second exposure time range. Other studies only used one exposure time and could not be placed in the literature summary below. Since Battelle used the most similar methodology for heat exposure (a single heating exposure with one long burst per spore sample [versus numerous shorter bursts as seen is some previous data such as Bacon]) and the closest times and temperatures for those heat exposures as the ones used in this thermal inactivation study, reduced data was compared to this source during analysis (Bacon, 2009).

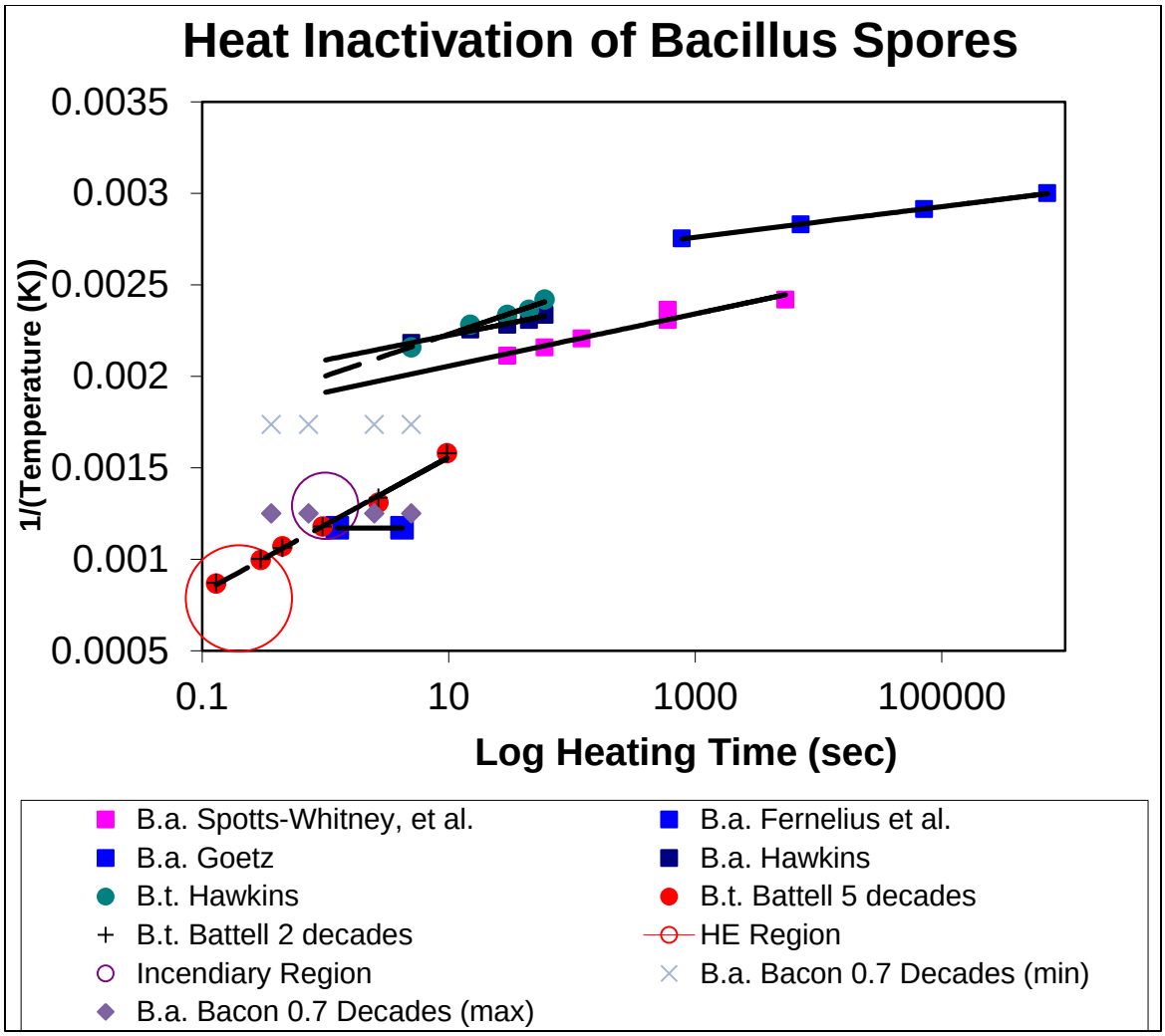


Figure 4. Literature summary of thermal treatment of *Bacillus* spores (from Bacon, 2009, which also shows work done by Goetz and Hawkins)

2.6 Instrumentation

For the thermal inactivation research, the process used was a design using rapid resistive heating; some of the instrumentation for the success of these studies were a FLIR, thermocouple, and x-ray analytical microscope. Each will be discussed in the following subsections.

2.6.1 FLIR Thermacam PM695

A FLIR Thermacam PM695, detector type Focal Plane Array (FPA), uncooled microbolometer, was used to capture the maximum temperatures during resistive heating and create time-temperature profiles through analyzing recorded infrared videos from the Pinnacle video card transfer. The FLIR can detect temperatures within three temperature ranges, with a total range between -40°C to 2000°C. The technical specifications of the FLIR are shown below in Table 1 (Bauer, 2010). Table 1 shows 2 of the 3 temperature ranges were used for the thermal inactivation studies: 0 to 500°C and 350°C to 2000°C.

Table 1. FLIR Thermacam PM695 Technical Specifications

Specification	Value
Field of View / Min focus distance	24°x18° / 0.5m
Spatial resolution (IFOV)	1.3 mrad
Thermal Sensitivity	< 0.08°C at 30°C
Image Frequency	50 / 60 Hz non-interlaced
Detector Type	Focal Plane Array (FPA), uncooled microbolometer 320 x 240 pixels
Spectral Range	7.5 to 13μm
Temperature Range 1 (not used) Temperature Range 2 (used) Temperature Range 3 (used)	-40°C to 120°C 0 to 500°C 350°C to 2000°C
Measurement Accuracy	±2°C
Operating Temperature Range	-15°C to 50°C
Storage Temperature Range	-40°C to 70°C
Weight	2.4 kg (5.3lbs.), including battery
Size	209mm x 122mm x 130mm (8.23” x 4.80” x 5.12”)
Battery System	1 rechargeable nickel-metal-hydride (NiMH) battery

Figure 5 shows the schematic of the FLIR's typical uncooled microbolometer cell, which uses a temperature-dependent resistor to absorb incoming radiation (Capper et al., 2001; Bauer, 2010). A typical uncooled microbolometer cell is measured to be about 50 μm^2 (Capper et al., 2001). A thermal camera, which produces an IR image has cells which are positioned into a 2D array and placed at the focal point of an IR-transmitting lens. The temperature-dependent resistor works by using an infrared (IR) absorbing material which detects and records changes in a bias applied to the material; the FLIR camera electronics output a corresponding temperature to each of the detected changes.

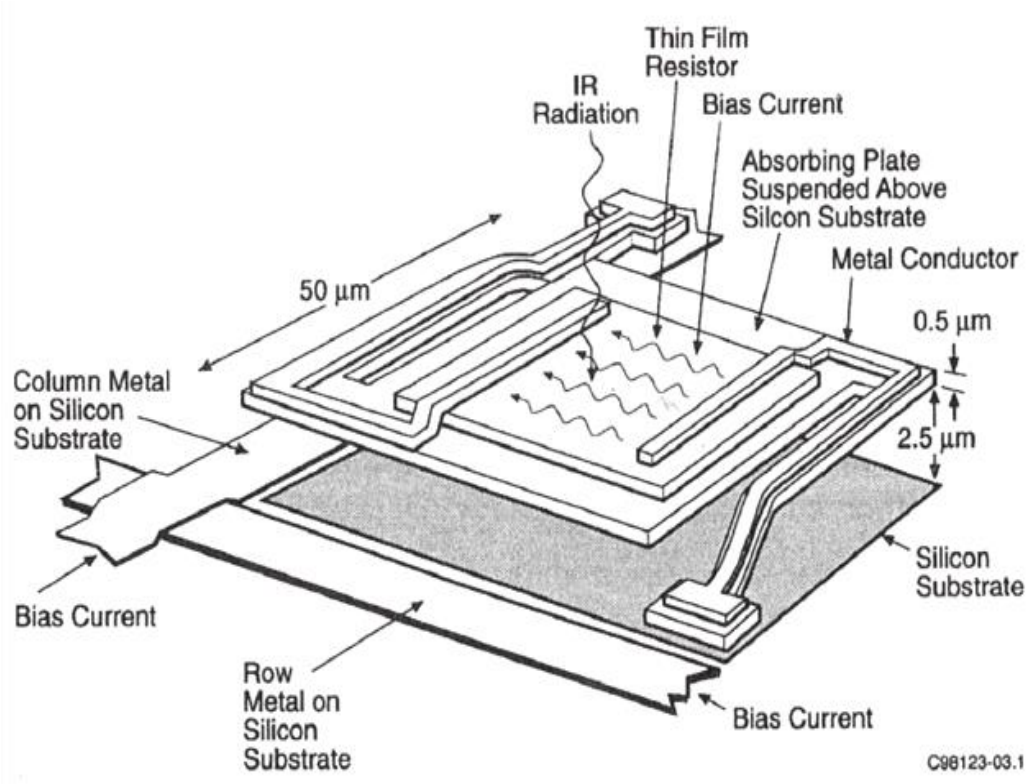


Figure 5. Typical Uncooled Microbolometer Cell (from Capper et al., 2001)

The most widely used IR absorbing material is vanadium oxide (Capper et al., 2001). Vanadium oxide application to the FLIR was described by Bauer as: "A thin layer

of vanadium oxide is applied over a silicon nitride plate that sits above a silicon baseplate through the support of two silicon nitride legs. The legs provide electrical contact between the IR absorbing plate and the electrical circuit-containing silicon substrate below. A voltage is applied across the vanadium oxide surface to provide an electrical current; this voltage is varied as IR radiation is absorbed and the camera electronics determine a corresponding temperature" (Bauer, 2010; Capper et al., 2001).

The process used in this thesis to determine the percent error of the FLIR uncooled microbolometer was time-temperature profiles at room temperature. The percent error quoted by the manufacturer was ± 2% but it was found to be higher as will be discussed in future chapters.

2.6.2 Thermocouple

The FLIR emissivity tables included the emissivity of tungsten but lacked the characteristics of a tungsten wire/filament; therefore, a thermocouple was used in this study to correct the quoted emissivity of tungsten by the manufacturer. A thermocouple works by recording the temperature of the coiled wire/filament and comparing it to the reading on the FLIR. To be a valid reading, the tip of the loop has to be in contact with the metal wire/filament throughout the entire heat exposure. Further detail is provided in chapter 3.

2.6.3 X-Ray Analytical Microscope

An x-ray analytical microscope was used to determine the composition of several different wire/filament samples. For this study, it was necessary to verify tungsten was the element found in the light bulb filament. The purity of tungsten in the light bulb

filament could be determined through the use of an x-ray analytical microscope. The specific microscope used, Horiba X-Ray Analytical Microscope XGT-7000, has the capability to detect any element except for elements below sodium on the periodic chart of the elements through the use of a spectrum flowchart. Further detail can be found in chapter 3, section 8. Appendix A shows the x-ray analytical microscope used for this thesis and Appendix B shows the x-ray analytical microscope analysis for the FEIT Electric filament used in the thermal inactivation studies.

III. Method of Experimentation

3.1 Introduction

The following chapter describes the methodology conducted for this research including hard surface recovery, thermal inactivation of *Bacillus anthracis* spores, and the rapid resistive heating thermal inactivation method through the use of a direct current (DC) power supply. The approach leverages a series of connected equipment with the setup including a laptop with appropriate software, a DC power supply, alligator clips attached to the power supply, a FLIR (forward looking infrared), a recorder, a lamp with copper leads, and a tungsten filament extracted from a light bulb by removing the light bulb envelope.

The bacteria used for this study was *Bacillus anthracis*, Sterne strain, which produces sublethal amounts of toxin and is frequently used for livestock vaccines. Microbiological techniques, such as heat-fixing and microdilutions were used for sample preparation, verification, and analysis. The methods used to prevent contamination to ensure the most sterile environment possible were autoclaving, using a 30% ethanol solution, and cleaning with methanol/ethanol/bleach. Controls were used in prototyping/initial testing for increased accuracy. The spores were grown and harvested from blood agar. A light microscope and scanning electron microscope (SEM) were also used for spore verification.

Purified and desiccated *B.a.* spores were subjected to power pulses controlled by various input voltages to determine a relationship between time, temperature, and efficacy of heating on the inactivation of the *B.a.* spores. The power supply input time

duration ranged from 0.1 seconds to 10 seconds with 17 amps. The temperatures of samples exposed to the power supply were recorded through the use of a FLIR and a video transfer recorder. The FLIR responds to emission; thus, emissivity was calibrated through the use of a thermocouple and prototyping/initial testing through the use of tungsten wire/filament samples not containing *B.a.* allowing the correction of temperature readings from the FLIR as needed. The exposure time was adjusted from the power supply input times by constructing time-temperature profiles; these time corrections ensured a more accurate time reflection of the maximum temperature achieved by each *B.a.*-encrusted tungsten filament sample. The tungsten filament from the light bulbs was characterized through the use of an x-ray analytical microscope. After exposures to a heat pulse, the *B.a.* samples were sonicated, microdilutions were performed, and aliquots were plated in nutrient agar and incubated to 37°C for 24- and 48- hours. The *B.a.* spores were analyzed by counting colony forming units (CFUs). The next chapter describes the analysis of the data including the usage of a CFU/mL equation from Goetz (Goetz, 2005) to determine the amount of spores recovered, as well as to determine where spores were lost. The following sections further describe each process in more detail.

3.2 Microorganism Source and Spore Hydration

Bacillus anthracis, Sterne strain, was the surrogate microorganism of choice to model the inactivation of lethal *B.a.* spores in response to heating profiles of various time durations and maximum temperatures. The strain was provided by thesis committee member Dr. Charles A. Bleckmann, AFIT/ENV (Air Force Institute of Technology/Systems and Engineering Management).

The gram-positive bacteria were handled in accordance with Biosafety Level 2 (BSL-2) guidelines in a BSL-2 laboratory at the Air Force Institute of Technology. Equipment, tools, and other resources were used from both the ENP (Engineering Physics) and ENV departments at AFIT. The NAPCO 1200 Class II Type A/B3 Biosafety Cabinet was the main laboratory location used for the study.

The first batch of *B.a.* was created from freeze-dried bacteria. The freeze-dried bacteria were hydrated using a few inches of Biochemical Oxygen Demand (BOD) buffer in a previously autoclaved flask. The flask was sterilized in a Tuttnauer Brinkmann 3870 autoclave at 121°C for 15 minutes. Other materials prior to contact with the *B.a.* spores were also autoclaved at the same temperature and time, while materials that had already been in contact with *B.a.* spores were autoclaved at the same temperature for an additional 15 minutes to ensure proper sterility. Sterilization indicators were used as an extra precaution to ensure the *B.a.* spores were eradicated after using the autoclave. Bleach, methanol, and ethanol were used to sterilize equipment and tools requiring repetitive and quick usage, such as the biosafety cabinet surface and the glass plate spreader. One ethanol used for this purpose was 95% Fisher Scientific denatured ethanol. Another ethanol used, but for non-lethal *B.a.* spore solutions, was 200 proof Sigma-Aldrich Ethanol, HPLC/Spectrophotometric Grade. Both bottles of ethanol were stored in a properly-labeled chemical hazards cabinet and used within health and safety compliance standards, including use of each MSDS (Material Safety Data Sheet).

3.3 Spore Growth and Harvesting

A sheep's blood agar was used to ensure optimum growth of *B.a.* A glass plate

spreader and an inoculating loop were used to make several *B.a.* plates. The *B.a.* on the blood agar plates were allowed to overcrowd in the Imperial III incubator for up to one month in larger agar plates. The temperature used to grow the *B.a.* in the incubator was 37°C since it is the optimum growth temperature for this microorganism, as is for many microorganisms. The process of plate overcrowding ensured an environment where spores would emerge and outnumber the vegetative cells.

Autoclaved tools were used to harvest the *B.a.* spores from the blood agar plates by scraping *B.a.* spores off of the blood agar plates**.** Figure 6 shows an example of *B.a.* growth on a blood agar plate with an inoculating loop. The *B.a.* from all of the agar plates were eventually combined into one culture tube and then moved into a 15-mL centrifuge tube to make a large batch for future stock solutions, tests, etc. The centrifuge tube used was a Becton Dickinson Falcon Blue Max Jr. 15-mL polypropylene conical tube. The control used was a blank blood agar plate, which was also placed in the incubator, to ensure the agar in the plates was not contaminated.

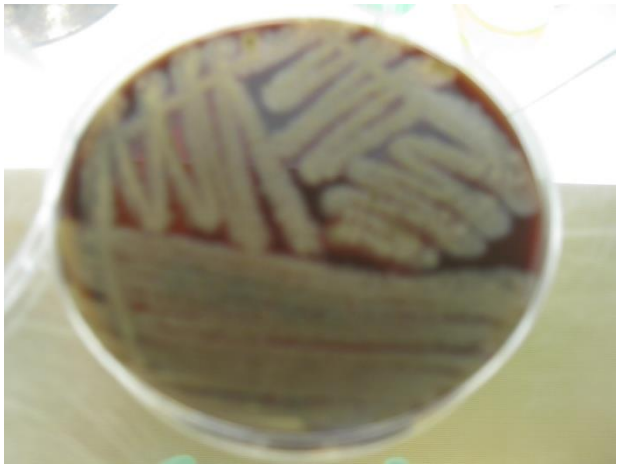


Figure 6. *B.a.* growth on Sheep's Blood agar plate using an inoculating loop

3.4 Spore Purification

The spore purification method utilized six centrifuge cycles. The Eppendorf Centrifuge 5810 R was used to centrifuge spores. The process separates the spores from the vegetative cells within the centrifuge tube, which contains all of the harvested *B.a.* from the blood agar.

Spores were purified with a 30% ethanol solution made from distilled water and 200 proof Sigma-Aldrich Ethanol, HPLC/Spectrophotometric Grade. The dilution was obtained by using a 1000-mL glass bottle and screw cap. A molarity calculation specified the values required for the desired dilution, the equation is as follows.

$$M_1V_1 = M_2V_2 \quad (1)$$

where, M_1= the original concentration of the solution (unit: M or mol/L), V_1= the original volume of the solution (unit: L), M_2= the final concentration of the solution after the dilution, and V_2= the final volume of the solution after the dilution.

Spores needed to be separated from the vegetative cells. Impurities in the harvested *B.a.* were the vegetative cells. The vegetative cells were gray in color whereas the targeted spores within the centrifuge tube were white in color. The white purified *B.a.* spores can be observed in Figure 7. Six centrifuge cycles were required to remove impurities. Each centrifuge cycle required the followed the preceding steps: the excess ethanol solution (supernatant) was poured out as waste removal, the gray impurities were scraped out with an autoclaved tool, new ethanol solution was poured into the tube until the 4-mL mark, the centrifuge tube was vortex mixed, and the centrifuge tube was placed into the centrifuge for the next 20-minute cycle. Counterbalances were used within the

centrifuge for each cycle. The settings used on the centrifuge were 4°C, 4000 rpm, and 20m.

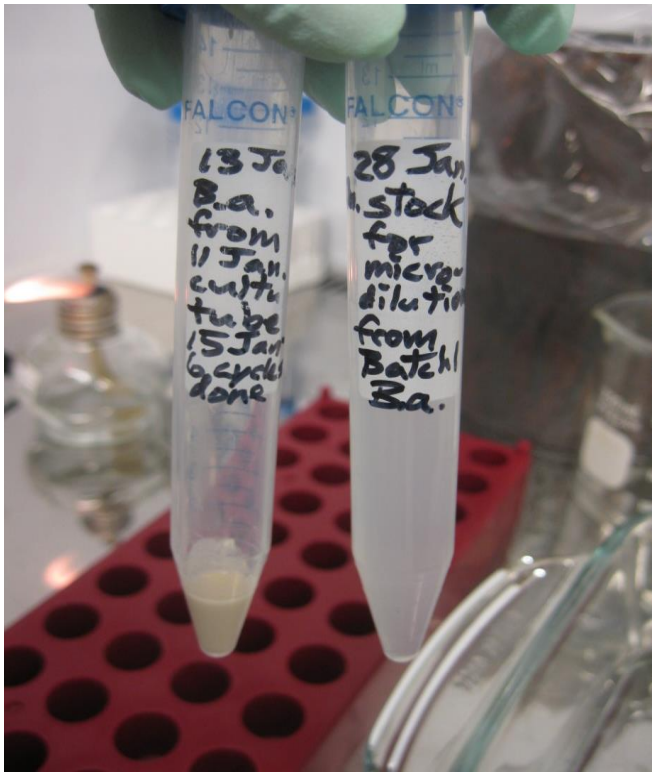


Figure 7. White *B.a.* spores after 6 centrifuge cycles on the left side; Cloudy stock solution on the right side

3.5 Stock Solution

Before a stock solution could be made from the purified spores in the centrifuge tube, spore verification was required to ensure future thermal inactivation studies were not compromised due to only using the much more heat-sensitive vegetative cells. The verification was done visually on a light microscope by using the microbiological process of heat-fixing on glass slides. Additionally, visual checks helped to guarantee the sample had not been contaminated. The heat-fix was accomplished by passing the slide through

the flame a few times to make the bacteria stick to the slide and remain intact.

The stock solution was made from the purified spores and a 30% ethanol solution. The ethanol solution was used since it does not kill off the *B.a.* spores at a low concentration for extended periods of time. A sterilized inoculating loop was used to take a scoop of purified *B.a.* spores from the centrifuge tube to another centrifuge tube containing the 30% ethanol solution. The loop was vigorously tapped against the sides of the centrifuge tube within the ethanol solution to ensure the spore glob would come off. The inoculating loop was sterilized again before taking another scoop of purified *B.a.* spores and repeating the removal process. The stock solution was vortex mixed for 30 seconds to ensure proper mixing and was continuously vortex mixed before making each subsequent dilution. Figure 7 from the previous section shows an example of the cloudy stock solution.

An experimental turbidity standard of 24 dilutions was created from microdilutions and the stock solution (1:10 dilution series) to determine the proper dilution for use in the thermal inactivation studies. After completion of the microdilutions, the 288 wells from three deep well plates were transferred to centrifuge tubes with a fixed 100-µL pipette to complete the experimental turbidity standard set of 24 dilutions, see Figure 8 for an example. After plating 100µL of each dilution, incubating at 37ºC for 24-hours, and colony counting was accomplished, conversions and a CFU/mL equation from Goetz (Goetz, 2005) were used to determine the original spore population in each of the dilutions. Knowing the original spore population in each dilution helped make a determination as to which dilution had greater than 10^8 CFU/mL

to ensure greater than 10^6 spores would be present in a 10-µL drop of *B.a.* spores on the tungsten filament for the thermal inactivation studies. It was determined that a solution which contained greater than 10^8 CFU/mL was the stock solution. The aforementioned processes and equation will be described in the upcoming sections and the next chapter, respectively.

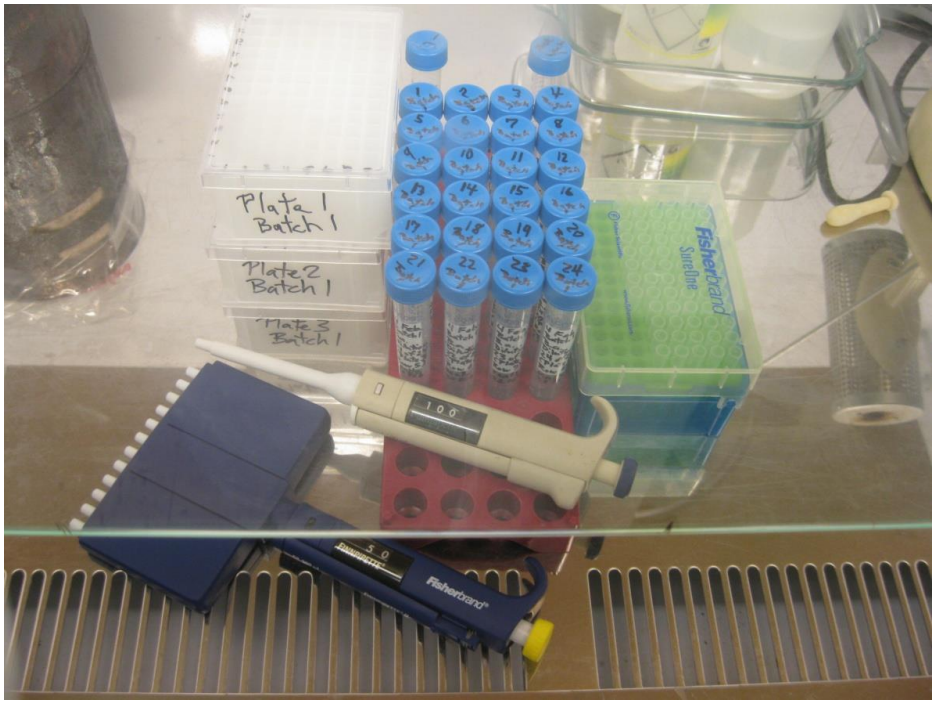


Figure 8. Experimental turbidity standard- 24 dilutions from the stock solution arising from the microdilutions in the 3 deep-well plates

The 10-µL drop was determined from prototyping/initial tests to conclude how many microliters were needed to create a thin spore layer to have uniform coverage on the tungsten filament of the light bulb. Ensuring the whole filament is thinly coated allows for better uniform heating on the filament.

Heat-fixing was used on 16 of the 24 dilutions from the experimental turbidity standard. These heat-fixed slides were also viewed under a light microscope to ensure

the *B.a.* batch was not contaminated and spores were present. After spore confirmation, centrifuge tubes were stored in a locked Frigidaire freezer. The centrifuge tubes included the purified spores, stock solution, and the 24 dilutions from the experimental turbidity standard.

The microdilution processes, such as plating and colony counting, will be described in greater detail in the upcoming sections within this chapter, while the CFU/mL equation will be further discussed in the next chapter.

3.6 Hard Surface Recovery

Prototyping/initial testing was done on different methods of hard surface recovery. The best method was determined by recovering *B.a.* spores from tungsten. Methods tested included sonication, cotton swabbing and then vortex mixing, and cotton swabbing and then centrifuging, as shown in Figure 9.

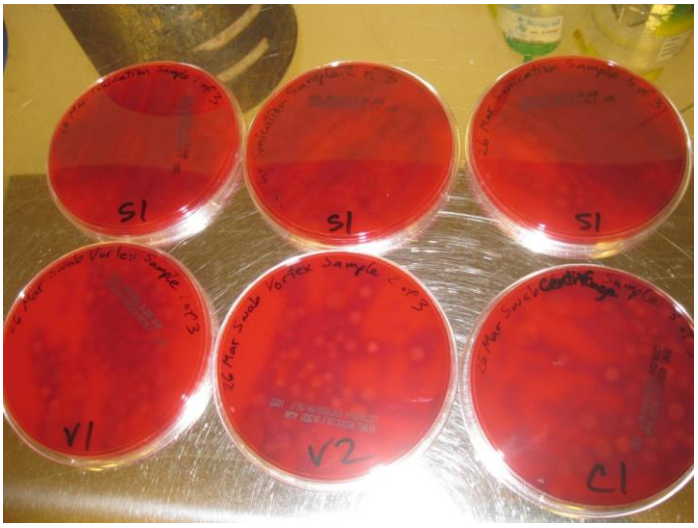


Figure 9. Three samples of sonication (S1), two samples of swabbing and vortex mixing (V1 and V2), and one sample of swabbing and centrifuging (C1)

To perform these tests, 5-µL *B.a.* samples were taken from the stock solution and placed on the six flat tungsten coupons that were previously sterilized with bleach. After visual confirmation that the spores had dried, the six samples of *B.a.*-encrusted tungsten were placed in centrifuge tubes containing 30% ethanol solution after being swabbed, if necessary. The ends of the swabs were cut to fit inside of the centrifuge tubes. Prior to swabbing the tungsten surfaces, swabs were slightly moistened with 30% ethanol solution to ensure a greater recovery of spores.

The centrifuge tubes containing the spores had three possible paths. Three centrifuge tube samples were sonicated for 60 minutes, two swabbed samples were vortex mixed for five minutes each (repeated cycles of 30 seconds on, 5 seconds off), and the last swabbed sample was run in the centrifuge for 20 minutes (again ensuring there were counterbalances). The tubes were vortex mixed using the Daigger Vortex Genie 2, which was set to vortex samples by touch. Finally, an aliquot of 100µL was plated on blood agar for each sample dilution and incubated at 37°C for 24-hours.

One blood agar plate was used as a control to ensure there was no contamination from the agar plates. Anytime a set of agar plates containing *B.a.* were placed into the incubator, a "control" agar plate such as the one above was also placed into the incubator. The precautionary step provided feedback as to whether contamination, if it was to occur, was from the agar itself or not. The step ensured other routes could be investigated if the *B.a.* spore samples were not growing properly or if different types of colonies were observed in the plates with the *B.a.* growth.

It was determined that sonication would be the best choice for use on the tungsten

filament due to more visible growth and less clumping as well as the fact that swabbing a filament would be difficult. Additionally, the *B.a.* separated better which makes it much easier to count colonies on plates (less clumping). The other methods not only had much more *B.a.* clumping but less growth at the 24-hour incubation mark as compared to sonication. Clumping was a huge issue since the amount of *B.a.* on a plate cannot be determined without the proper dilution and the ability to count colonies, which is why aliquots will be used after thermal inactivation testing and the experimentations were done in batches.

A scanning electron microscope (SEM) and light microscope were used for *B.a.* spore verification and to further ensure there was no contamination. The SEM used was the Zeiss Evo LS10, whereas the light microscope used was the Zeiss Imager.M2m. To prepare the samples on the sterilized quartz cover slip, 0.1µL from each of the *B.a.* solutions were pipetted beside the proper label on the gold-looking side of the quartz cover slip. The samples were allowed to air dry within a contained mini agar plate to ensure no contamination. Figure 10 shows the SEM preparation on the quartz cover slip from each of the hard surface recovery methods. After the quartz cover slips were visually verified to be dry, they were carefully mounted by using sterilized tweezers for placing the cover and then slight pressure was applied to allow the cover slip to stick. Figure 11 shows the mounting of the quartz cover slip for the SEM for other samples containing the stock solution and the first five dilutions from the experimental turbidity standard; *B.a.* specimens were applied to quartz cover slips that were made conductive by a thin layer of gold.

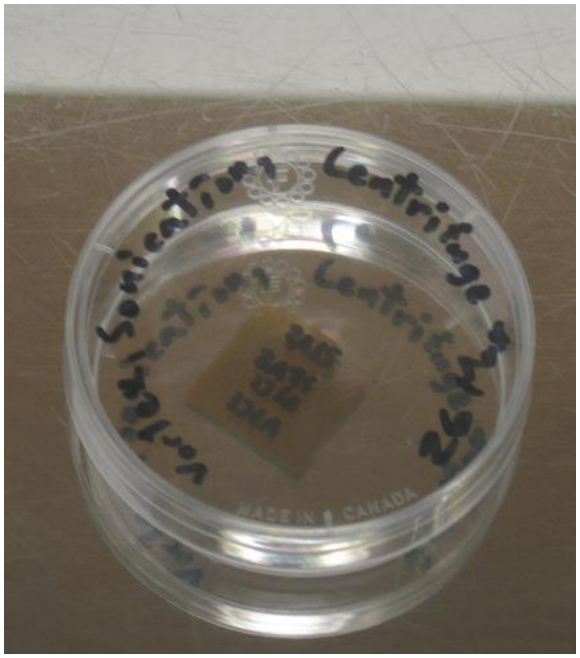


Figure 10. SEM preparation: drying quartz cover slip containing *B.a.* samples for hard surface recovery methods

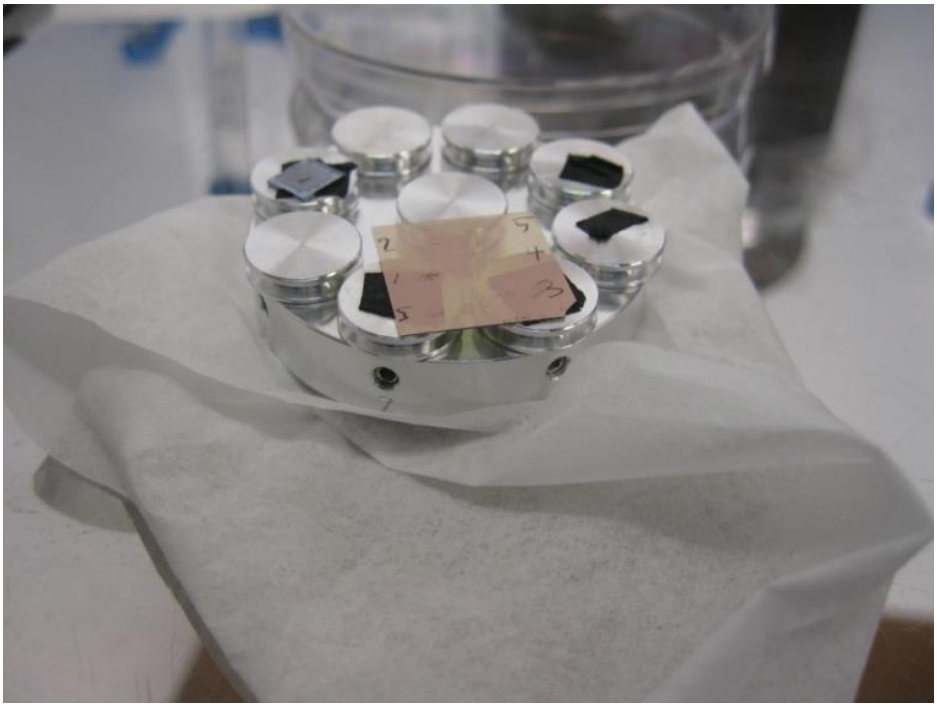


Figure 11. SEM mount: *B.a.* specimens were applied to quartz cover slips that were made conductive by a thin layer of gold

Sample SEM figures from the Zeiss Evo LS10 were received from thesis committee member, Dr. Daniel Felker (DLF). As discussed in chapter 2 (section 1, subsection 3), *B.t.* is a common surrogate of *B.a*. Figure 12 shows a *B.t.* test specimen on a tungsten filament. Figure 13 shows a *B.t.* test sample on a quartz cover slip with a thin layer of gold.

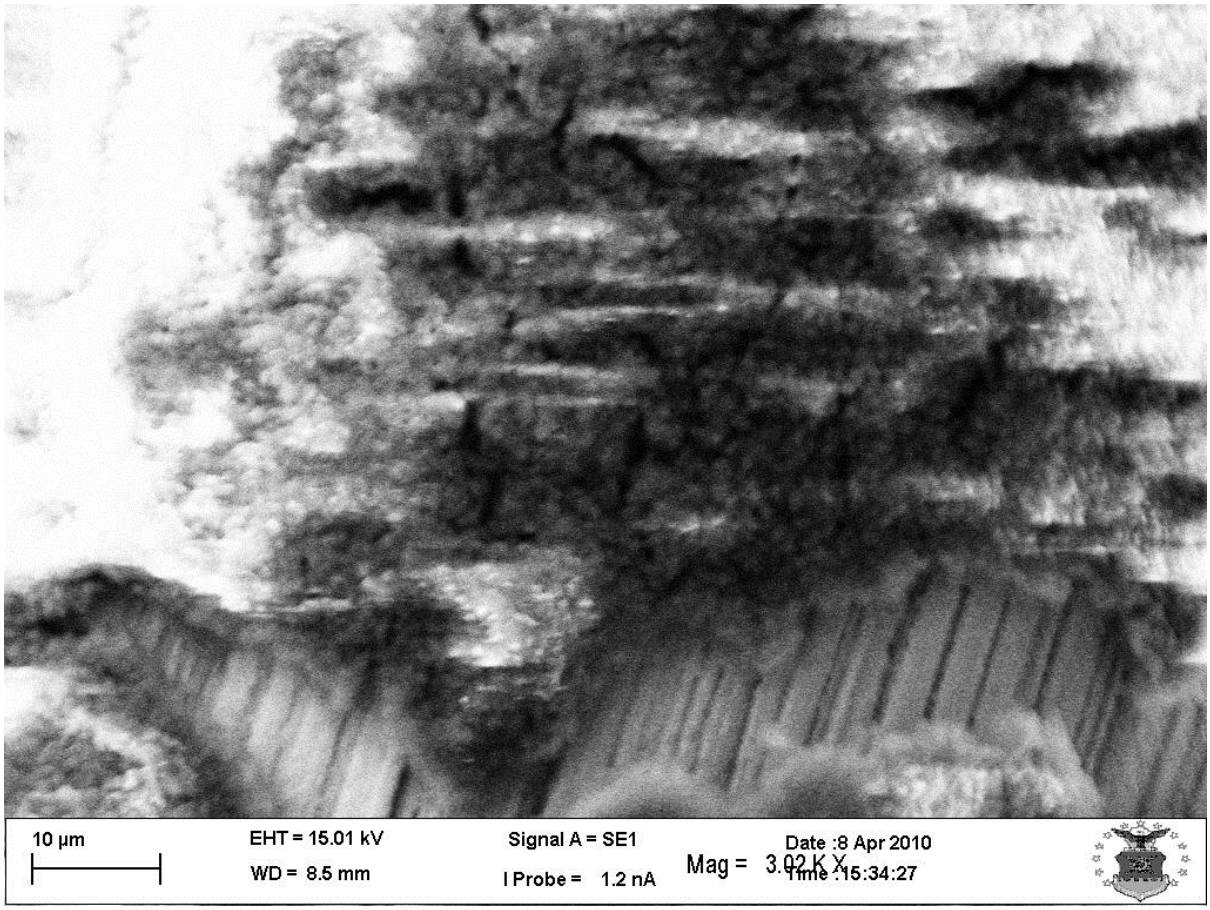


Figure 12. *B.t.* test specimen on tungsten filament (from DLF)

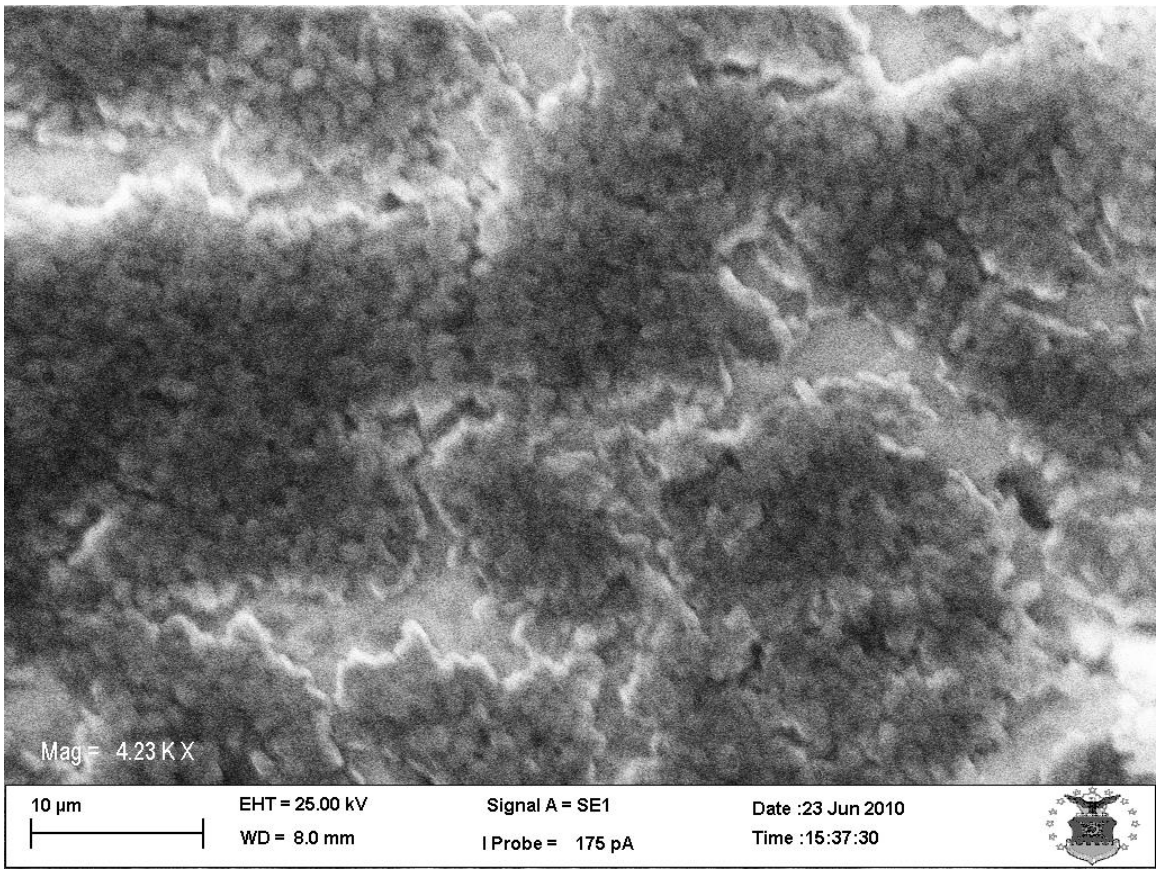


Figure 13. *B.t.* test sample on quartz cover slip with a thin layer of gold (from DLF)

3.7 Agar Preparation

Nutrient agar was used for the rest of the study. Switching from Sheep's blood agar to nutrient agar proved to be much more economical and did not seem to impact B.a. growth differently. Many flasks of nutrient agar were made for the rest of the study.

The process of making a nutrient agar required using distilled water, a 1000-mL flask, a 50-mL beaker, Fisher Scientific dehydrated nutrient agar, a rounded tool, and a scale. First, the 50-mL beaker was zeroed on the scale so that 23 grams of dehydrated nutrient agar could be slowly added with the help of the rounded tool. Once the right

amount was obtained, the dehydrated agar was added to the 1000-mL flask. Distilled water was then added to the 1000mL line on the flask and mixed with the nutrient agar using the rounded tool. Additionally, clumps were smashed and further mixed until gone. The flask was autoclaved at 121ºC for 15 minutes. After the agar was autoclaved, the agar was allowed to cool off inside of the flask to the point where the flask could be handled with personal protective equipment (PPE). During the cooling period of the autoclaved agar, foil was used to cover the top of the flask in order to keep the agar as sterile as possible.

The nutrient agar plates were inspected before use and checked for bubbles which is a common occurrence with nutrient agar plates. PPE was used to pour the agar into the empty plates. Agar plates that could not be used immediately were stored temporarily in a Fisher Scientific low temperature incubator, which is kept at 4°C. Agar plates left in the low temperature incubator for too long or too close to the cooling source were discarded during inspections due to lines forming in the agar which indicates degradation. The lines could be best seen by moving the agar plates under light. A few thousand agar plates were made to account for the prototyping/initial testing, the collection of 1586 data points, and to ensure quality agar plates were used for the thermal inactivation studies.

3.8 Purity of Tungsten

The light bulbs used for the thermal inactivation studies were 100-Watt Soft White FEIT Electric Triple Life incandescent light bulbs. The light bulbs were the most economic and effective for the study.

Since light bulb filaments have been manufactured throughout history with different elements, verification of tungsten composition in light bulb filaments was crucial to ensure the presence of tungsten without other mixed elements. For example, thorium and rhenium have previously been used in light bulb filaments or mixed in as an alloy. The filament composition was important to determine if hazardous elements were present while also validating the purity of tungsten. A Horiba X-Ray Analytical Microscope XGT-7000 was used to verify the purity of tungsten in the wire/filament samples.

Five wire/filament samples, including an oxidized filament, were tested on the microscope to validate the purity of tungsten. The samples included 4 light bulb filaments from different brands and the laboratory tungsten wire. These samples did not contain *B.a.* spores. Each sample was kept in an empty agar plate when not in use and during transportation in order to avoid contamination of the wire/filament samples. Each wire/filament sample was placed carefully on a separate piece of tape while prepping for the analysis of composition in terms of which elements are present in the sample. It was important to use tweezers to push down every part of the sample onto the tape so as to not lose the sample in the vacuum-sealed microscope during analysis. The samples were loaded into the microscope as seen below in Figure 14. Although each sample had to be analyzed separately, all of the samples could be loaded at once.

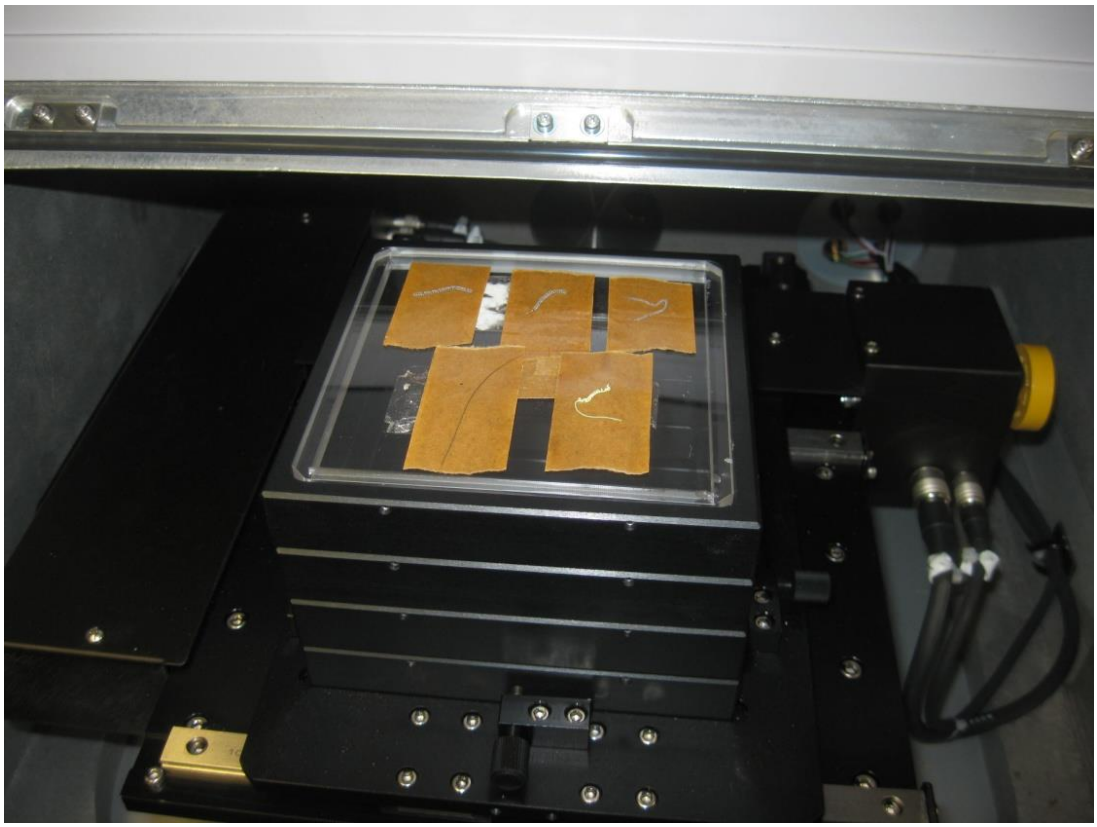


Figure 14. X-ray analytical microscope samples

Mr. Eric Taylor in the ENP department provided training and guidance on all aspects of the use of the x-ray analytical microscope (Taylor, 2010). This included usage of software flowcharts to ensure proper use of the machine and analysis of the wire/filament samples. Every sample had to be moved into position by the microscope joystick and controls. The microscope was kept at 50kV and each sample was run for ten minutes. Each run generated a graph (spectrum) for analysis purposes.

Once the graph was produced by the machine, possible elements were listed for the different peaks. The analysis began by matching the proper elements to their peaks. The vertical lines bisecting the peaks were the proper elements for the peaks and thus

showed the purity of the tunsten within the wire/filament samples. The element rhodium was always elimated since the element compromises the filament of the x-ray generator and would be constantly seen in the spectra produced by the microscope.

Figure 15 shows some of the steps and the flowchart used for the x-ray analytical microscope, including the close-up of the point on the wire/filament being analyzed and the real-time spectrum being generated. The area of analysis is indicated by the crosshair on the coiled wire/filament. Figures showing the analysis of each sample verifying the purity of tungsten, including the explanation of each sample, will be discussed in the next chapter and displayed in the appendices.

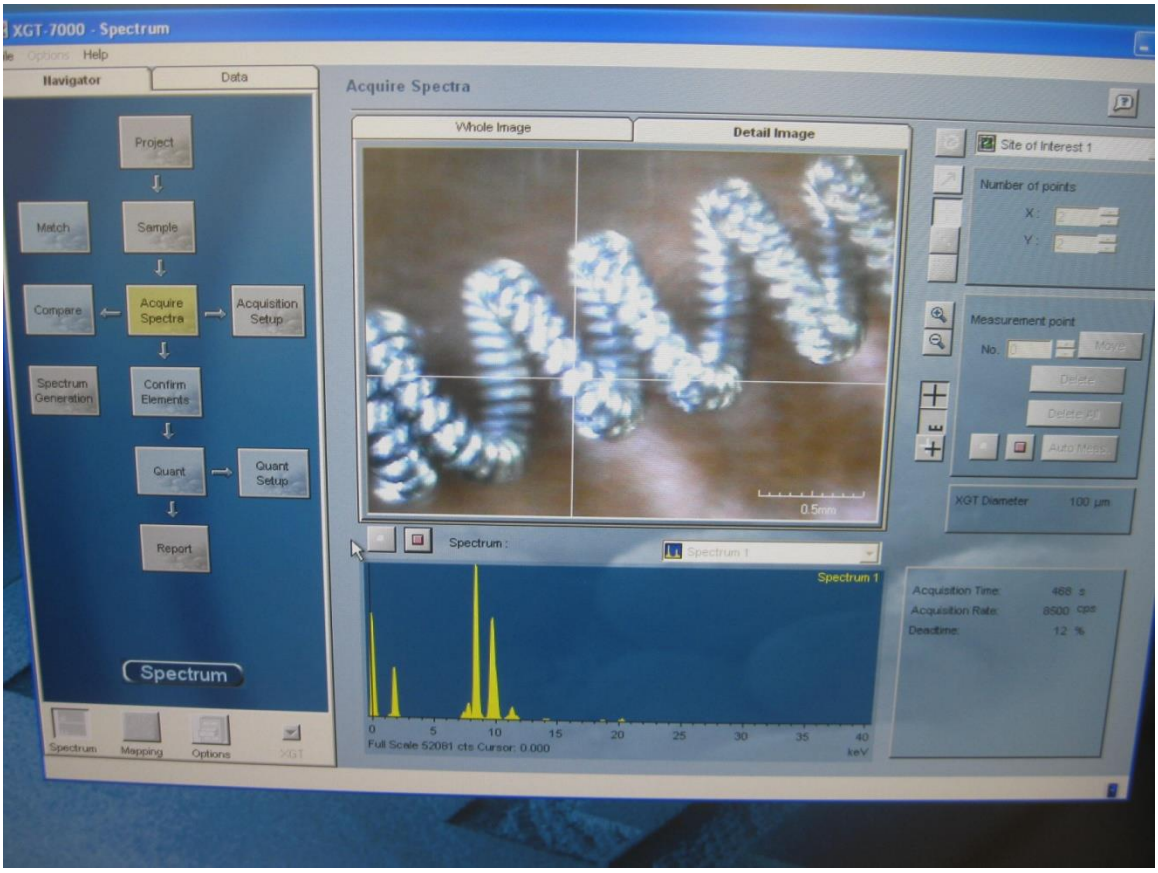


Figure 15. X-ray analytical microscope analyzing a sample

3.9 Sample Preparation for Thermal Inactivation Testing

Sample preparation followed the three steps of obtaining filaments, *B.a.* application, and desiccation. Each filament comprised of one *B.a.* sample for a total of 125 samples prepared for thermal inactivation testing. Each step is discussed in further detail in the subsequent three subsections.

3.9.1 Obtaining the Filaments

Many light bulbs were needed to obtain 125 filament samples for the application of *B.a.* spores. The 125 obtained tungsten filament samples required 3 to 4 times as many light bulbs (not including the many light bulbs used for the preliminary trials discussed in the next section). The tools used to separate the soft white bulb envelope from the screw cap and tungsten filament were a pipe cutter, wire snips, and personal protective equipment. As previously noted, the tungsten filament samples were obtained from 100-Watt FEIT Electric Soft White Triple Life incandescent light bulbs.

To begin the filament extraction process, the pipe cutter was placed at the edge of the brass base on the light bulb and slowly tightened by turning the adjusting knob at the bottom of the pipe cutter. The pipe cutter was tightened until there was enough pressure for the bulb to be held by the pipe cutter alone, which allowed the grip to be repositioned to the brass base on the light bulb and the adjusting knob on the pipe cutter. The knob was slowly tightened until the glass envelope on the light bulb was cracked off. For safety, sharp pieces of glass left protruding on the brass base were broken off using wire snips. Due to further safety concerns of broken glass over the floor, a large cardboard box was used underneath the laboratory table to ensure proper containment of the glass.

The envelope removal process yielded some broken filament samples and required inspection for cracks of still intact filament samples to ensure quality samples were used for the thermal inactivation study. Samples not passing the inspection were included in the large cardboard box containing the cracked light bulb envelopes.

The tungsten filament samples required holders in order to be properly secured. The sample holders protected the filaments, held the samples securely in place in an upright position, and allowed for *B.a.* sample application and desiccation with minimal moving of the filaments and samples in general. The customized filament sample holders were made out of cardboard and a razor knife. Larger filament holders were also created to minimize movement of the filament samples during transportation and to allow for samples to be covered to address outside contamination concerns. Figure 16 below shows the experimental cardboard sample holders.

Figure 16. Experimental cardboard sample holders

3.9.2 *B.a.* Application

Using a Fisherbrand pipette, a 10-µL drop from the *B.a.* stock solution was applied to the tungsten filament on the light bulb while on the experimental cardboard holders. The 10-µL drop contained greater than 10^6 spores. Little by little, the *B.a.* spores were applied to different parts of the filament to ensure a thin layer was spread throughout the filament. A subtle difference in color on the filament as the solution was applied allowed for the application of *B.a.* spores where needed. Each sample was allowed to visually dry while spores were applied to other filaments on the holders. A separate process was needed in order to dry the spores.

3.9.3 Desiccation

After the *B.a.* spores were applied to the tungsten filaments, the next step was to dry the spores. The process was accomplished using a Nalgene 5317-0180 Desiccator and six Humidity Sponge Category Number 3150s each time a batch of *B.a.* samples was desiccated.

The humidity sponges played a key role in the desiccation process. It was determined that 40.5 hours was needed to desiccate each set of samples in their respective handmade cardboard holders, see Figure 17. Three sets of samples were run through the desiccator after the shelving units were repositioned due to limited space. Desiccation was considered complete after all of the blue indicating gel in the 6 humidity sponges turned pink.

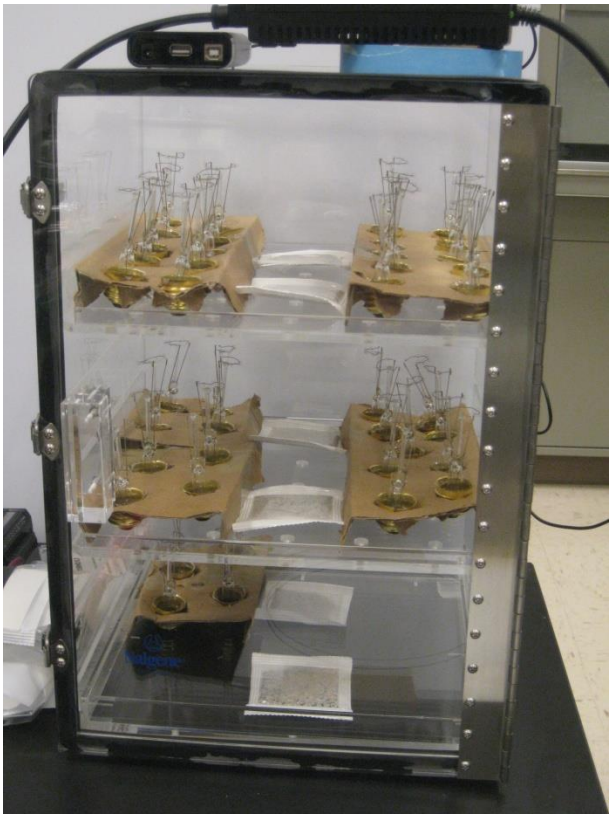


Figure 17. Nalgene 5317-0180 desiccator with samples

3.10 Thermal Inactivation Prototyping/Initial Testing

Two sets of pre-trials were conducted to increase the validity of the study while also shortening the timeline and utilizing fewer resources in the long run. The most important aspect was to ensure minimizing the waste of *B.a.*-encrusted filament samples as well as the spread *B.a.* spores across the laboratory. The first set of pre-trials was conducted to ascertain if an accurate temperature measurement could be obtained from the FLIR. Once temperatures were verified as accurate, the second set of pre-trials were accomplished to acquire a temperature range for the thermal inactivation study and to determine if high enough temperatures could be reached in short time durations.

The first set of pre-trials involved an emissivity correction for the FLIR. The FLIR responds to emission assumed to have a blackbody spectrum. *B.a.* spores were not used on these samples. The gray-body emissivity was corrected through the use of an Omega HH12A thermocouple. The FLIR emissivity table required the emissivity for tungsten to be set to 0.05 on the FLIR parameters, but did not indicate an emissivity reading for a coiled wire/filament. Occasionally a mismatch between the thermocouple and the FLIR existed which made it necessary to correct the emissivity on the FLIR parameters. The process was crucial due to the fact that temperature discrepancies of up to 128°C were possible if the emissivity would have remained unchanged at 0.05. The problem was addressed by changing the FLIR emissivity parameters until the temperatures recorded by both the thermocouple and the FLIR were similar. The thermocouple required the tip of the small loop on the end of the thermocouple to be touching the tungsten wire/filament the entire time while the current was applied to get an accurate reading. The temperature readings between the thermocouple and the FLIR were similar but not exact due to the heat capacity loss to the wire on the thermocouple. The thermocouple was limited to the 200-degree Celsius mark and would display "OL" on the screen to signify there was an overload when attempting to go past the limit. Major Michael Koehl provided help with equipment and readings in order to properly record readings on the thermocouple and the FLIR while controlling the power supply. After continually adjusting the emissivity, the emissivity parameter on the FLIR was finally set to 0.95, which is close to that of a blackbody (1.0).

Once temperature readings checked out, prototyping and initial testing on the tungsten filaments without *B.a.* spores could be achieved in order to determine profiles of different temperatures with time for varying voltage settings inputted into the computer software. It was determined there was less variance in temperature and power supply outputs when using a constant 17 amp current. To change the temperatures, the voltage inputs were varied for each time input used (0.1 to 10 seconds). The time and voltage inputs were recorded, as well as the maximum temperatures at those voltages, in order to set up a plan for the thermal inactivation testing. The FLIR limitation necessitated a plan for using different temperature ranges to run the samples. The FLIR required a setting of either the 0-500ºC or 350-2000ºC range to record temperature which made possible the issue of missing a temperature reading in the blending parts of the ranges. Additionally, usage of the 350-2000ºC range would mean that temperatures below 215°C would not be picked up by the FLIR. The issue affects time-temperature profiles while will be discussed in the next chapter.

A maximum voltage of 200V was used at the short time durations in order to achieve the highest temperature possible. Maximum temperatures reached were found to be less reproducible as the power supply reached the maximum voltage of 200V. For example, a 0.1 second input into the computer software yielded a 995-degree Celsius mark as the maximum temperature recorded. Therefore, the setup was capable of reaching the desired temperatures for the thermal inactivation testing.

3.11 Thermal Inactivation Testing Setup

The pre-trials were done to ensure an accurate measurement was obtained from the FLIR and the setup was appropriate for the thermal inactivation study, including reaching the high temperatures needed at the short time durations. The rapid resistive heating thermal inactivation method through the use of a direct current (DC) power supply will be described in this section.

The setup included a laptop with software connected to the Agilent 6030A System Power Supply, the FLIR Thermacam PM695, Pinnacle video transfer (recorder), lamp with alligator clips on the copper leads, and a FEIT Electric light bulb tungsten filament (base included). The FLIR was on a SLIK The Professional 4 tripod stand. The setup is credited to the work of Dr. Dan Felker, Major Michael Koehl, and author Crystal Grijalva. Figure 18 shows a close-up the setup for the pre-trials and thermal inactivation testing. The large device on the right side of the Pinnacle recorder is the battery supply for the FLIR, which was safely plugged in an outlet behind where the testing would be performed.

The lamp was chosen for the setup due to the copper wire leads that could be easily clamped by the alligator clips, which are attached to the direct current power supply. The lamp cord was cut using wire snips in order to expose the copper leads from the lamp cord, about one and a half inches of the flexible electrical cord insulation was also discarded. As an added safety feature, a raised glass plate was used underneath the alligator clip-copper lead connection since glass is not a conductor. A sheet of paper with

a circle drawn around the base of the lamp was used to keep the distance between the FLIR and the sample standardized between each run.

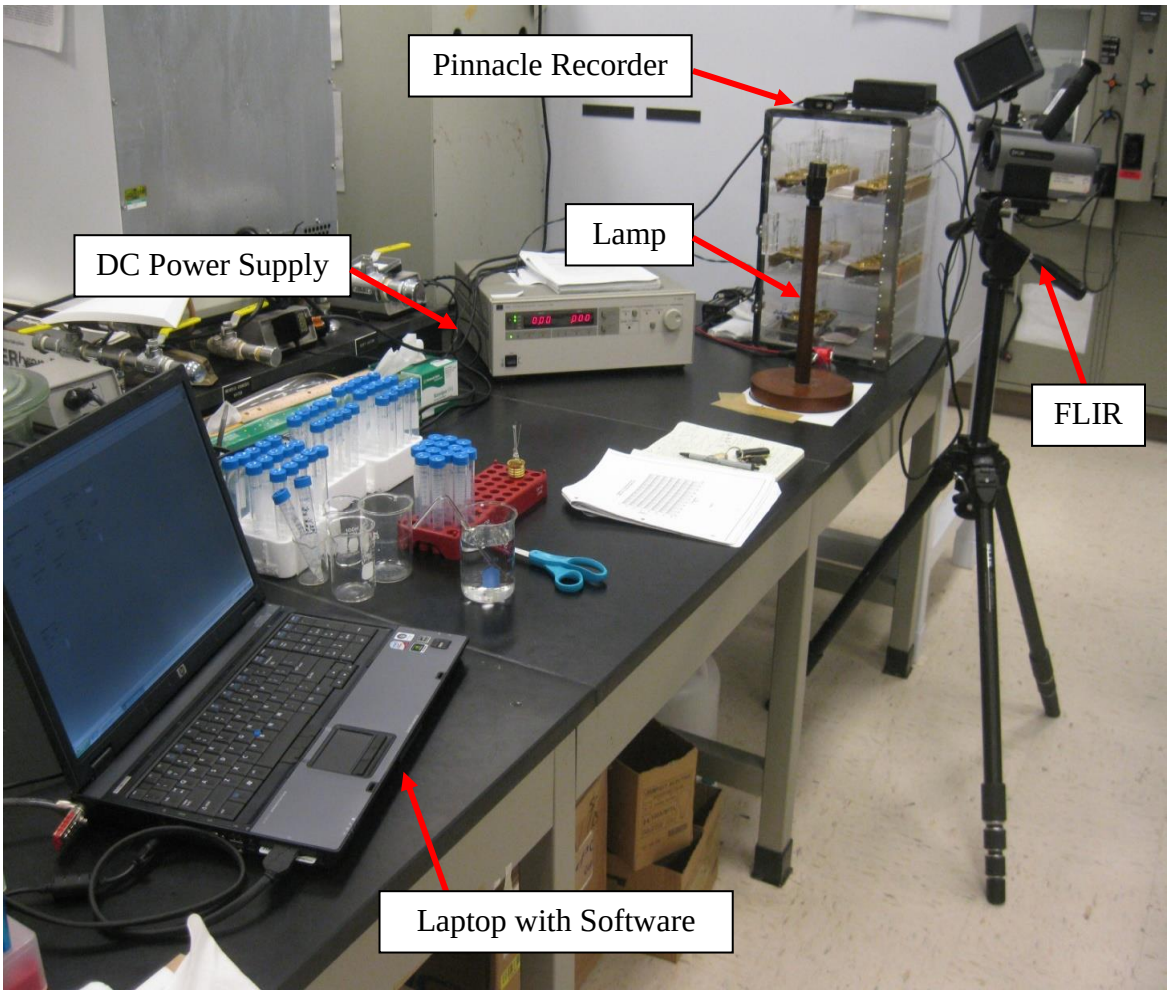


Figure 18. Setup for thermal inactivation testing

The laptop software included a program that controlled the power supply. The program had the capability to input parameters of time, voltage (volts), and amperage (amps) by the user for the output of the direct current power supply onto the samples. The inputs could then be verified by the output given by the program (only voltage and amps could be seen as outputs). The program also indicated if there was a proper

connection to the DC power supply through the usage of a green checkmark (red 'X' if there was a bad connection). The laptop program was created by Dr. Dan Felker.

3.12 Thermal Inactivation Testing

During heating of each desiccated *B.a.*-encrusted tungsten filament sample, the power supply input times ranged from 0.1 seconds to 10 seconds at the 17 amps applied, while the voltage was varied to produce different maximum temperatures (approximate time-voltage settings for desired temperature output measured in previous piloting testing). A Pinnacle video transfer (recorder) and FLIR were used to record and save infrared videos. Finally, samples were prepared for sonication in order to discover how many spores were recovered after heating. The steps are further described below and in the subsequent sections.

Battelle's 'Thermal Deactivation of Aerosolized Bacteria' (Alexander, Ogden, LeVere, Dye, & Kohler, 1999) used *Bacillus thuringiensis* (*B.t.*) in the 1999 revision report to the Defense Threat Reduction Agency (DTRA); the document was used as a guide to come up with the best temperature ranges possible for the thermal inactivation studies. *B.t.* is a common surrogate used to simulate *B.a.*. Battelle also used a similar time range (0.13 to 9.70 seconds) as the power supply input time range used in the thermal inactivation studies for this thesis (0.1 to 10 seconds), thus it was an appropriate source to use as a guide. A total of 12 exposure times were used with a temperature range of 73.5 to 888°C and a voltage range of 9 to 200V. The expectation in the thermal inactivation studies was to produce a method that could achieve temperatures that would

meet and exceed the Battelle temperatures used for loss of spore viability; the expectation was previously met during the initial testing.

The comparison in time and temperature ranges between the Battelle studies and the thermal inactivation studies from this thesis can be seen below in Table 2; the approximate temperature ranges for the Battelle data in the table are from analyzing the plots in the Battelle report. Spore recovery from the thermal inactivation studies will be analyzed, discussed, and compared to the Battelle data from Table 2 in the next sections and chapter. Although the temperature of 995°C could be achieved in the initial testing using the maximum voltage from the power supply of 200V and the power supply input time of 0.1 seconds, during the thermal inactivation studies the maximum temperature that could be reached with the desiccated *B.a.* samples at the same input time was 888°C. The difference is remarkable given the maximum temperatures are greater than 100°C apart.

Table 2. Comparison of Battelle Time Inputs/Temperature Ranges to Studies

Battelle (*B.t.*): Dwell Times/Approximate Temperature Ranges/Temperature for Loss of Spore Viability	**Thermal Inactivation Studies (*B.a.*): Power Supply Time Inputs/ Temperature Ranges/Voltage Ranges**
0.13 seconds/520-940°C/880°C	0.10 seconds/100-888°C/20-200V
0.30 seconds/500-825°C/730°C	0.30 seconds/145-853°C/20-150V
0.45 seconds/495-735°C/660°C	0.50 seconds/125-862°C/18-45V
0.95 seconds/400-700°C/575°C	0.75 seconds/111-760°C/15-50V
2.70 seconds/300-600°C/490°C	1.00 seconds/116-794°C/15-32V
9.70 seconds/200-420°C/360°C	1.50 seconds/117-631°C/15-28V
	2.00 seconds/83.6-600°C/12-25V
	2.50 seconds/149-643°C/13-26V
	3.00 seconds/119-659°C/15-26V
	5.00 seconds/104-457°C/12-22V
	7.50 seconds/101-485°C/12-22V
	10.0 seconds/73.5-487°C/9-20V

A FLIR Thermacam PM695, detector type Focal Plane Array (FPA), uncooled microbolometer, was used to monitor and record the maximum temperature of the *B.a.*-encrusted tungsten filament samples as they were exposed to the heat from the direct current power supply. The manually entered object parameters used on the FLIR Thermacam PM695 for the thermal inactivation studies can be observed below in Table 3. A ruler was used to place the FLIR 0.3 meters away from the filament sample. The tripod stand was adjusted to ensure the height of the FLIR was the same height as the filament sample attached to the lamp, thus the FLIR would only have to be adjusted by turning the FLIR from side-to-side to ensure the filament sample was centered within the line-of-sight of the FLIR. While screwing in the light bulb base for each *B.a.*-encrusted filament sample, the temperature from the FLIR had to be adjusted to temperature range -40 to 120 °C in order to see the filament and center it within the FLIR area box before readjusting to one of the desired temperature ranges of 0 to 500°C or 350 to 2000°C.

Table 3. FLIR Thermacam PM695 Object Parameters

Object Parameter	**Value**
Emissivity	0.95
Distance	0.3 meters
Ambient Temperature (T_{amb})	27°C
Humidity	78%
Reference Temperature (T_{ref})	21°C
Temperature Range 1/Filter	-40 to 120°C/Off
Temperature Range 2/Filter	0 to 500°C/Off
Temperature Range 3/Filter	350 to 2000°C/On

Analysis of maximum temperature from the FLIR was accomplished using an area function, more specifically Amax, which finds the spot within an area with the

highest temperature. The Amax rectangle was manually entered to fit around the filament sample and centered. For each new sample, the FLIR was swiveled to the right or left on the tripod to ensure the Amax rectangle was centered on the sample because the lamp was always in the same spot. See Figure 19 to see a screenshot from one of the recorded FLIR videos that shows the Amax rectangle; this sample reached 888°C as a maximum temperature at a power supply time input of 0.1 seconds.

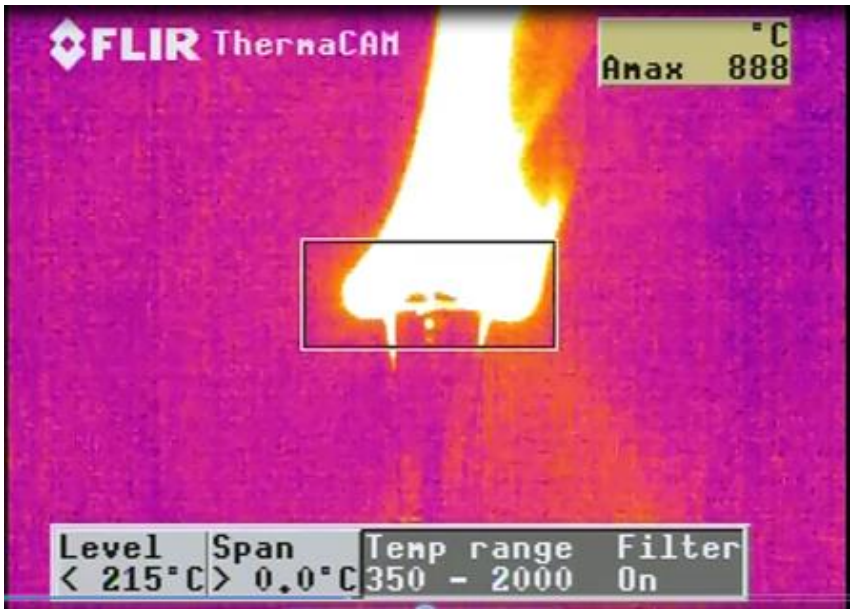


Figure 19. Screenshot from FLIR during thermal inactivation testing

A Pinnacle video transfer (recorder) was connected to the FLIR to record and save infrared videos by the use of a USB thumb drive. Figure 20 shows the recorder in action during the thermal inactivation studies as the sample starts to heat up with an orange color. The video card had a maximum recording time of 30 seconds. The infrared video was analyzed after each heat exposure to a sample in QuickTime Player on a personal laptop.

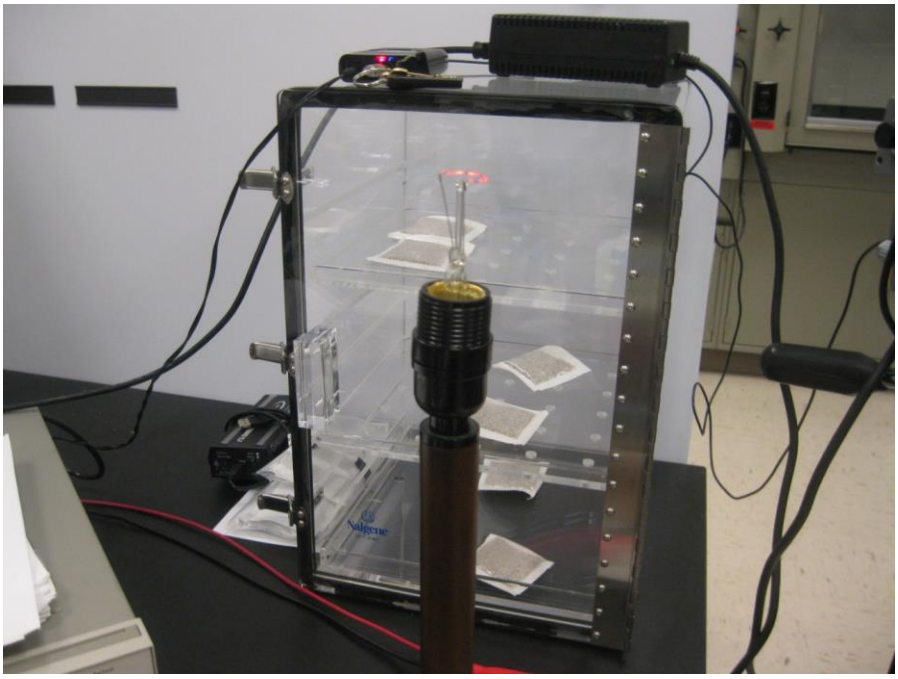


Figure 20. Thermal inactivation testing

The maximum temperature could be annotated by observing the video while also ensuring the temperature from the heated sample was properly recorded due to different temperature range inputs used for the FLIR. For example, at the temperature range of 350 to 2000°C, the FLIR cannot record temperatures below 215°C and the FLIR can also not record temperatures above 500°C in the 0 to 500-degree Celsius range.

In the general specifications, the manufacturer states the FLIR temperature measurement accuracy is ±2%. The next chapter will compare this stated accuracy to the recorded accuracy through the use of time-temperatures profiles and also determine the times at which the samples reached maximum temperature during heating as compared to the input times in the laptop program for the power supply output.

Once each sample was heated, the filament sample was snipped with scissors into a centrifuge tube and prepared for sonication. Preparation included vortex mixing each sample for 30 seconds to allow spore recovery from the edges of the centrifuge tube due to the samples sliding into the tube after being cut. After sonication, microdilutions and colony counting are the final steps to gather data for use in analysis in the next chapter using the CFU/mL equation.

3.13 Sonication

Sonication was the recovery method chosen for retrieving *B.a.* spores from the tungsten filament. The hard surface recovery method ensured better separation of the spores as compared to other recovery methods, which resulted in less clumping. The Branson 1210 sonicator was used for this process.

To maximize time and space in the sonicator, different beaker sizes were tested to ascertain how many beakers and centrifuge tubes could fit into the sonicator. Placing additional tubes into the beakers in the sonicator was the best method since the centrifuge tubes could stand more upright with more tubes inside of the beakers. Furthermore, the method ensured that the beakers stayed in place during sonication due to the additional weight. It was determined that four beakers of different sizes and 22 centrifuge tubes could fit into the sonicator while still functioning properly. The beakers used were a 50-mL that could hold three centrifuge tubes, a 100-mL that could hold five centrifuge tubes, a 150-mL that could hold six centrifuge tubes, and a 200-mL that could hold eight centrifuge tubes.

Each centrifuge tube contained one sample and one mL of 30% ethanol solution to cover the sample. Distilled water was used inside of each beaker and outside of the beakers, ensuring the samples were completely covered. Once all of the samples were properly placed in the beakers and the sonicator, the time on the sonicator was set to 60 minutes. Microdilutions immediately followed sonication of the samples as explained in the next section.

3.14 Microdilutions

In the process of performing 793 microdilutions, 1:10 dilution series were accomplished using Fisher Scientific 96-well deep well plates, Fisherbrand 12-tip pipettes, Fisherbrand pipettes, Fisherbrand SureOne pipette tips, and Thermo Scientific polystyrene clear plate covers. The deep well plates were autoclaved. Some pipette tips came sterile out of sealed boxes while the others were wiped down with methanol to become sterile. Additionally, the pipettes were wiped between samples to ensure cross-contamination did not occur.

Between four to seven dilution factors were used for the heat exposures from the thermal inactivation studies while two to four dilutions were used for the redone ones; therefore, 647 dilutions were made (not including the samples that were redone which added another 146 dilutions and will be discussed in the next chapter). Since the dimensions of the 96-well deep well plates were 8 wells by 12 wells, 12 samples could be used per deep well plate. The original spore solution samples to be diluted were the heated and sonicated filament samples in 1mL of 30% ethanol solution. As previously mentioned, microdilutions were accomplished immediately after sonication.

To set up the microdilutions, each successive deep well contained a one tenth dilution of the previous well to ensure a 1:10 dilution series. Each of the wells in the deep well plate was first filled with 450µL of 30% ethanol solution by using a twelve-tip pipette. Next, a pipette was used to obtain 50µL of the original spore solution samples from each of the vortex mixed centrifuge tubes and placed in their respective well, known as the first dilution (1:10 dilution or 10^{-1} dilution factor). Next, the twelve-tip pipette was used to mix the dilution ten times before transferring 50µL from the current well to the next well, known as the second dilution (1:100 dilution or 10^{-2} dilution factor). The pipette tips were replaced between dilutions. The mixing and transferring procedure was continued throughout the remaining dilutions. The 50µL from the last dilution was discarded. Table 4 shows the dilutions and dilution factors. Figure 21 shows the twelve-tip pipette used for the microdilutions.

Table 4. Dilutions and Dilution Factors in 1:10 Dilution Series

Dilution	**Dilution Factor**
1^{st} dilution (1:10 dilution)	10^{-1}
2^{nd} dilution (1:100 dilution)	10^{-2}
3^{rd} dilution (1:1000 dilution)	10^{-3}
4^{th} dilution (1:10,000 dilution)	10^{-4}
5^{th} dilution (1:100,000 dilution)	10^{-5}
6^{th} dilution (1:1,000,000 dilution)	10^{-6}
7^{th} dilution (1:10,000,000 dilution)	10^{-7}

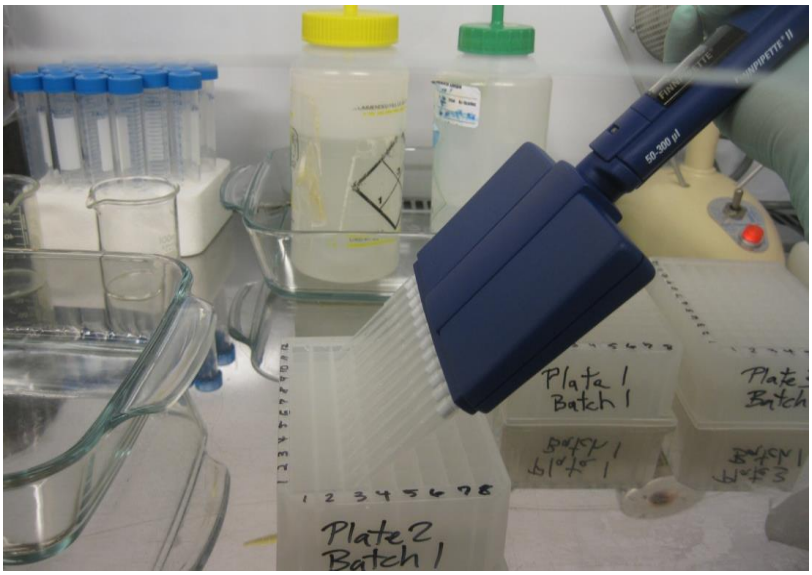


Figure 21. Twelve-tip pipette used in microdilutions

The plates were covered after finishing the microdilution process to await plating on nutrient agar. As far as the final count of the centrifuge tube samples and microdilution samples in the deep well plates: in total, there were 123 centrifuge tubes (3 were controls and the rest contained the heated and sonicated filament samples) and 16 deep well plates full of 793 diluted samples (647 from the thermal inactivation samples and 146 from the redone microdilutions). The next sections describe how each of the 793 dilutions were plated on nutrient agar and then incubated to be able to count colonies.

3.15 Plating Samples onto Nutrient Agar and Incubation for Colony Counting

Once the microdilutions were completed, each dilution was plated onto nutrient agar and incubated. A glass plate spreader was used to plate the *B.a.* spores from the dilutions onto the nutrient agar dishes. Bleach was used to clean off the glass plate spreader between plated dilutions and wiped with Kimberly-Clark Professional WypAll X60 white reinforced wipers.

All previously created nutrient agar plates were inspected before use whether or not they were freshly made or stored in the low temperature incubator set at 4°C. The inspection of the nutrient agar plates identified some unusable plates with bubbled areas or some deterioration caused by placement in the colder portion of the low temperature incubator. A set of nutrient agar plates ready for inspection and use can be seen below in Figure 22. Once the inspection was completed, 100µL of each dilution was plated onto the nutrient agar with a fixed 100µL pipette.

Figure 22. A set of nutrient agar plates ready for inspection and use

After each small set of dilutions was plated, the agar plates were incubated for total times of 24-hours and 48-hours. The Imperial III incubator was again used for bacterial growth. The temperature used to grow the *B.a.* was 37°C since that is the optimum growth temperature for this microorganism. After incubation, colonies could be counted as described in the next section of the chapter.

3.16 Counting Colonies

At the 24- and 48-hour incubation marks, the colony forming units (CFU) from each nutrient agar plate were counted. Colonies were counted using the Leica Quebec Darkfield Colony Counter as seen in Figures 23 and 24. The data from colony counting not only was important to determine if *B.a.* spores were completely inactivated, but also in the application of using the CFU/mL equation from Goetz (Goetz, 2005). This equation, which will be described in the next chapter, was used to determine and analyze the viability of spores (just as in the Battelle study) by finding out how many spores were recovered from each heated filament sample.

Colonies were counted by hand and recorded from 647 nutrient agar plates (not including the 146 redone samples). The most accurate plated dilution from the series of four to seven plated dilutions was the one containing between 30-300 CFU. These plates were counted two to three times on the dark field colony counter to ensure an accurate count both at the 24- and 48-hour marks. Accomplished in several batches, a total of 1586 data points were recorded to account for the 24- and 48-hours marks for each agar plate. To illustrate appropriate and inappropriate amounts of colonies to count, see Figures 23 and 24. Figure 23 showcases the optimal amount of CFU wanted on an agar plate (between 30-300 CFU), while Figure 24 is a prime example of too many CFU on a plate for counting (greater than 300 CFU).

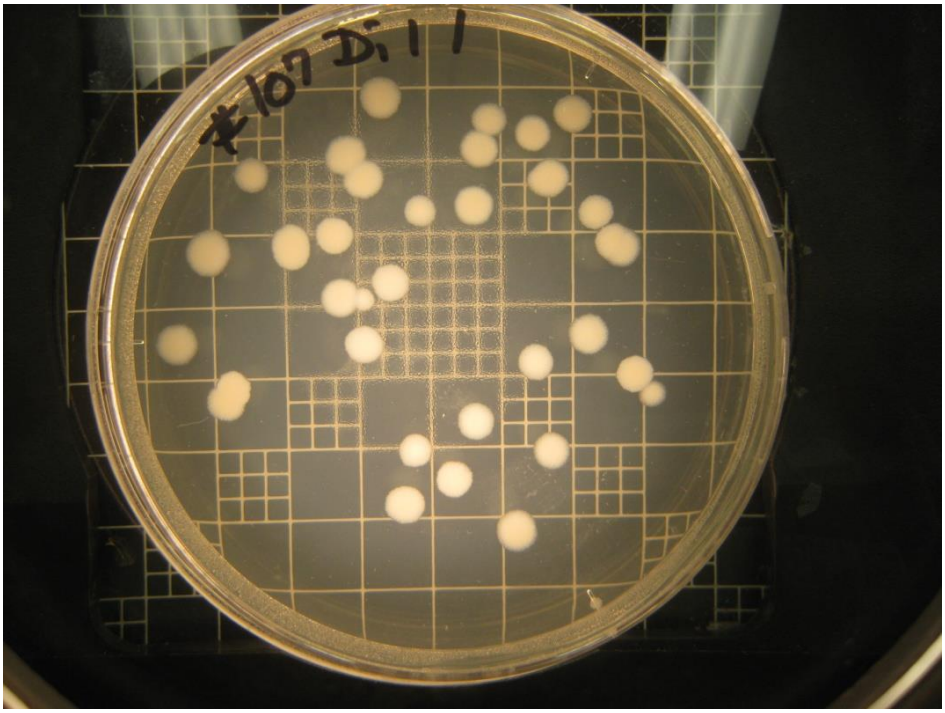


Figure 23. Example of just the right amount of CFU on a nutrient agar plate

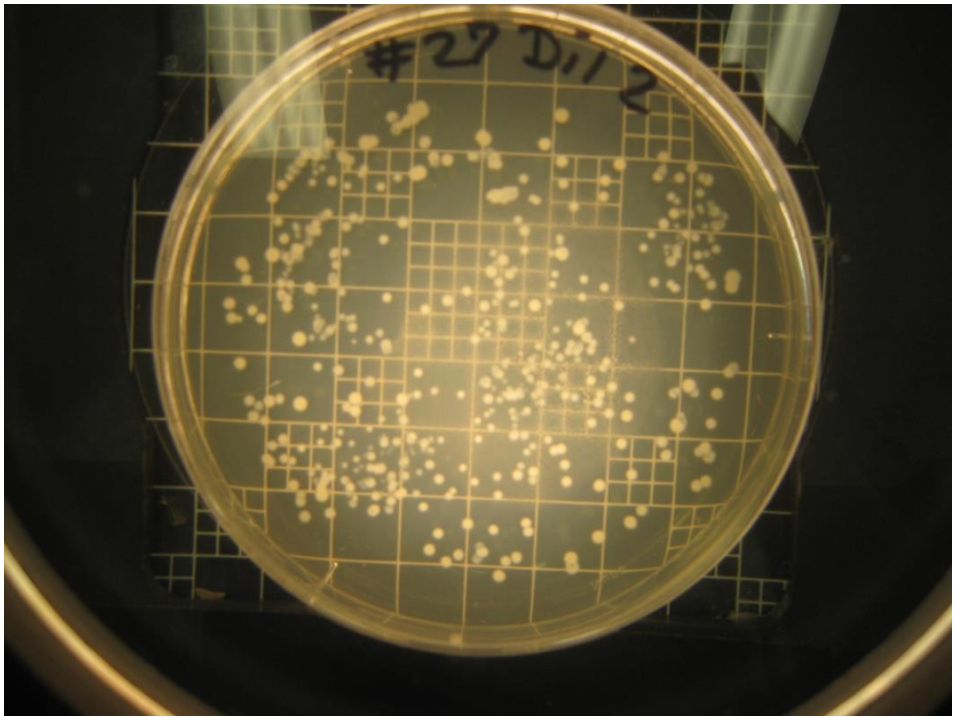


Figure 24. Example of too many CFU on a nutrient agar plate

IV. Results and Analysis

4.1 Chapter Overview

The chapter contains results and analysis of the microbiological experiments described in Chapter 3. The main goal was to simulate an environment where conventional munitions are used as agent defeat weapons on a *B.a.* stockpile or facilities and transportation vehicles containing stockpiles. The analysis from the heat exposures could help link the spore viability with the simulated explosive environment created by some agent defeat weapons. To do this, rapid resistive heating was used for the thermal inactivation studies. The setup consisted of a laptop with software connected to a direct current power supply which was also connected by alligator clips to the copper wire leads of a lamp. The lamp contained the screwed in *B.a.*-encrusted tungsten filaments (light bulbs with envelopes removed). A FLIR Thermacam PM695 and Pinnacle video transfer (recorder) were used to capture infrared videos. It is also important to note that data points from the heat exposures were acquired in several batches to prevent accumulation of *B.a.* spores in the centrifuge tubes and deep-well plates. Included in this chapter are observations and explanations about materials, processes, accuracy of equipment readings, and the *B.a.* used in the study.

As was discussed in chapter 3, a total of 12 exposure times and 100 heat exposures (20 additional filaments used for pre-trials) were used with a temperature range of 73.5 to 888°C and a voltage range of 9 to 200V. The power supply input time range was 0.1 to 10 seconds but as time-temperature profile plots will show later in this chapter, these power supply inputs produced inaccurate exposure times and were modified to

reflect corrected exposure times ranging from 0.258 to 7 seconds. Microdilutions ranged from 4 to 7 total per heat exposure (2 to 4 for redone samples) depending on the estimated *B.a.* spore viability.

Several months were needed in order to properly analyze and reduce the thermal inactivation data, which included 3,100 time-temperature profile data points from reviewing 120 FLIR heat exposure videos (20 used in pre-trials) and 1,586 data points for CFU counts at different time intervals from several batches (793 data points taken at the 24-hours mark and 793 data points taken at the 48-hour mark). The CFU data points encompassed counting colonies on agar plates from variable dilutions for each of the 120 heat exposures (20 filaments for pre-trials) and 3 controls (647 data points at each 24-hour mark) and an extra 146 data points at each 24-hour mark for the 60 redone microdilutions (redone samples) of the previously heated samples.

Taking additional readings at the 48-hour mark made it easier to count the 1,586 sets of CFUs from the agar plates due to the larger colony size. Additionally, the extra readings at the 48-hour marks ensured that if more *B.a.* spores grew on the agar plates, they could be observed after a longer time to repair from the heat exposures and sonication. Extra data points were taken only for verification of the previously collected data points at 24-hours of growth. Table 5 shows the breakdown of collected data for CFU counts before the data reduction and analysis.

Table 5. Data Point Breakdown for CFU Counts

	# Redone Samples	# Heat Exposures	# Dilutions	24-Hour Marks	48-Hour Marks
(includes 2 controls)		36	7	252	252
		47	5	235	235
(includes 1 control)		40	4	160	160
Data Points Total		**120**		**647**	**647**
	44		2	88	88
	6		3	18	18
	10		4	40	40
Extra Data Points Total	**60**			**146**	**146**
24- and 48-Hour Total				**793**	**793**
Grand Total					**1,586**

Analyzed data includes time-temperature profiles (3,100 data points), viability of spores at different times and temperatures (to include an extra plot to show partial and complete inactivation) by counting colonies which were plated from the microdilutions, x-ray analytical microscope results, spore hard surface recovery, and spore loss during thermal inactivation. The relationships between the data and analysis from this study and previous research from similar types of studies will also be discussed in this chapter; the focus will be a comparison of data between this study and Battelle's 'Thermal Deactivation of Aerosolized Bacteria' (Alexander, Ogden, LeVere, Dye, & Kohler, 1999) since Battelle's exposure times and temperatures were used as a guide for the layout of exposure times and temperatures for the purpose of this thesis.

4.2 Observations of Materials and Equipment for Thermal Inactivation

The materials used were dependent on the equipment required for the thermal inactivation studies. A major concern for the thermal inactivation studies was to find a machine or setup that had the capacity to perform at high enough temperatures (up to the 800- and 900-degree Celsius range) at controlled short time specifications for exposure times (especially including the 0.1- to 1-second range). A secondary concern was using a setup that would not readily contaminate a laboratory space or cause difficulty in sanitizing the laboratory and its equipment. A tertiary concern was to use a thin reproducible wire coil for studies. As the thickness of a wire decreases, the heat capacity also decreases which was ideal for thermal inactivation testing.

The equipment analyzed for use was the AFIT Xenon Thermal Simulator (AXTS) and a direct current power supply to be used with a wire/filament (latter used in the study). The AXTS prototype would have needed a drilled metal coupon for mounting but the AXTS was ruled out as an option at that point in time with the available set ups by Koehl and Bauer and confirmed as not being an option based on training and conversations with Koehl and Bauer (Koehl, 2009; Bauer, 2010). The prototypes for the wire filament design included clamping different thicknesses of tungsten wire to alligator clips or using light bulbs (containing tungsten filaments) in a lamp which contained copper leads that were clamped to the alligator clips. Koehl assisted with prototyping and initial testing.

Problems arose during prototyping and initial testing. The wire to be used needed to be a thin reproducible coil. Tungsten wire was deemed appropriate but during some

prototyping and initial testing the manually coiled tungsten wire would easily break at the areas where the alligator clips were clamped when the power supply produced high temperatures. The wire could not take the pressure from the clamps. Another idea to counteract this problem, mentioned by Koehl while helping in the preliminary testing, was to use a light bulb filament (which is made of tungsten) and clamp the leads from the filament straight to the alligator clips. The latter idea seemed like the best solution since the filaments were thin and reproducible. However, further prototyping/initial testing highlighted the issue of the filaments being stretched (which would cause less *B.a.* recovery due to loss during thermal inactivation testing) and a setup that was not reproducible enough.

The idea to use a light bulb filament was further modified by the author. Instead of directly applying the alligator clips to the light bulb filaments, the whole bulb (minus the envelope) was screwed into a lamp where the alligator clips were instead clamped onto cut and exposed copper wire leads from the lamp. The alligator clips and exposed copper wire leads were placed and secured on a safe non-conducting glass surface. The setup proved to be the best option during more prototyping and initial testing since the setup was reproducible and the lamp could be in the same position during all of the measurements (sheet of paper was taped to the table with a drawn circle for the lamp to stay in between). The setup also ensured a higher *B.a.* recovery could be achieved due to the filaments not being stretched/broken by the heat, pressure, and weight of the alligator clips as in the previous set of initial prototyping tests. Finally, the setup achieved the goal of producing high temperatures at low exposure times.

4.3 Analysis of Tungsten (W) Purity in Wire Filament Samples

Since light bulb wiring has been manufactured throughout history with different elements, verification of tungsten composition in light bulb filaments was crucial to ensure the presence of tungsten without other mixed elements. For example, thorium and rhenium have previously been used in light bulb filaments or mixed in as an alloy. The main concern was the ruling out of thorium composition within the filaments since it could pose possible toxicity hazards, especially when exposed to high temperatures. The method used for the verification of the composition of tungsten in the wire/filament samples was an x-ray analytical microscope.

Five wire filament samples were tested to validate the purity of tungsten, to include 4 light bulb filaments from different brands and the laboratory tungsten wire. Although the theoretically sound and reproducible light bulb filaments were viable options for the thermal inactivation testing, the presence of thorium would disqualify them for future usage in the studies. Thorium is a naturally occurring radioactive element that decays by emitting alpha particles, is dangerous in gaseous form, can easily ignite in powder form, and upon decay emits radon gas, which is unsafe in an enclosed environment. The five wire filament samples tested in the x-ray analytical microscope were an unknown light bulb brand from Walgreens, a General Electric (GE) light bulb, a FEIT Electric light bulb, a laboratory wire, and an oxidized FEIT Electric filament (used in prototyping/initial testing).

Also important in choosing the correct filament was the proper light bulb configuration. The laboratory tungsten wire was not affected because the wire could be

coiled manually and no leads were present (although there was a downfall due to breakage on the wire in the clamped areas where the alligator clips resided when high heat was subjected to the wire). All of the light bulb filaments except one had a configuration that was perpendicular to the floor when screwed into the lamp. The configuration would have been problematic for the thermal inactivation studies due to one side having a longer lead going from the light bulb base to the filament, thus giving a lag time on one side for the current to reach the *B.a.* spores on the filament. In comparison, the FEIT Electric light bulb filament instead had a configuration which was horizontal to the floor when screwed into the lamp and even lead lengths from the light bulb base to the filament; this was promising for a reproducible and accurate setup.

The FEIT Electric light bulb was eventually chosen for the thermal inactivation studies due to the purity of tungsten found in its composition, the reproducible coil (filament), and a sound process for the thermal inactivation studies due to even lead lengths (equal current for both sides). Appendix B shows the x-ray analytical microscope analysis for FEIT Electric filament used in this study. It includes the spectrum, conditions, and location of the analysis performed on the filament. Appendices C-F show the same type of analysis performed on the x-ray analytical microscope for the rest of the tungsten wire/filament samples. As can be seen in the appendices, the same characteristic peaks for tungsten were found in all 5 samples' spectra and are signified in the spectra as a "W." Alloyed metals were not detected. Rhodium is always the first peak in the spectra produced since this element compromises the filament for the x-ray

generator within the x-ray analytical microscope. Appendix F shows the oxidized form of Appendix B which was used in the pre-trials for thermal inactivation testing.

4.4 Observations with Colony Morphology

B.a. and *B.a.* spore identification was observed in several ways. It was necessary to ensure there was not any contamination and that spores were present by crowding agar plates with *B.a*. The scanning electron microscope (SEM) and light microscope were valuable in confirming the presence of the *B.a.* spores but not to the level of magnification and clarity of an atomic force microscope for characterizing surface morphology as done by Zolock (Zolock, 2002). All that was needed for this study was to confirm *B.a.* and *B.a.* spore presence. Another way to ensure proper identification of the *B.a.* was to look at colony morphology and is described more in detail below.

To further ensure the sample was not contaminated and *B.a.* was properly identified, the colony morphology was compared to that of Zolock (Zolock, 2002). Contamination with other colonies of non-related bacteria is a huge factor to consider. Although the colony was overgrown (about four to five months in the incubator), it was confirmed to be a match to the Zolock colony which was incubated for less time. The typical raised "glass-like" look was apparent in both and the growth was similar. The overgrown colony obtained in the laboratory is shown in Figure 25, while the colony produced by Zolock is shown in Figure 26.

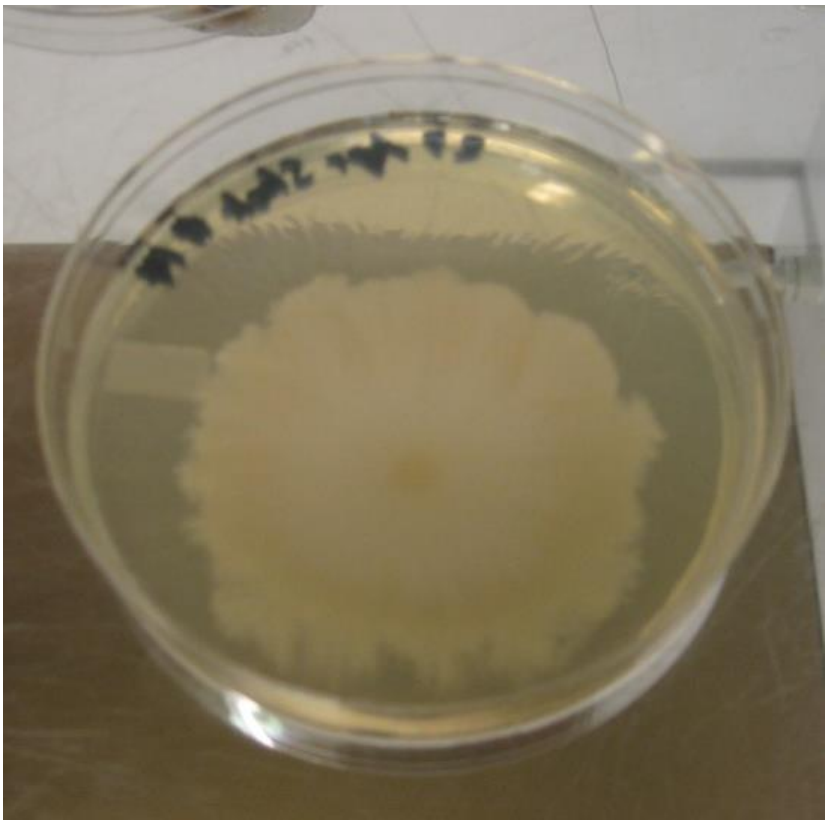


Figure 25. *B.a.* colony morphology

Figure 26. Zolock *B.a.* colony morphology (from Zolock, 2002)

4.5 Observations while Counting Colonies

At the 24- and 48-hour incubation marks, the colony forming units (CFUs) from each of the 793 agar plates were counted for a total of 1,586 data points. Some data points had to be thrown out due to excessive clumping and/or possible contamination.

Taking extra readings at the 48-hour marks ensured that if more *B.a.* spores grew on the agar plates, they could be observed after a longer time to repair from the heat exposures and sonication. Extra data points were taken only for verification of the previously collected data points at 24-hours of growth. The extra readings at the 48-hour marks made it easier to count the 1,586 sets of CFUs from the agar plates. It was rare to find extra CFUs from the 24- to 48-hour readings; there were only a couple of cases where one extra colony of growth appeared. It was beneficial to verify the CFU count at the 48-hour mark since colonies were easier to see and count due to increased colony size.

Dilutions proved important due to a typical set of dilutions starting with several colonies of growth and then eventually ending with no CFUs as more and more dilutions were plated onto agar dishes. When analyzing dilutions for selection during data reduction, typically dilutions 1 through 3 (10^{-1} through 10^{-3}, respectively) were the most likely to contain the appropriate amount of colonies (30-300 CFUs) without clumping. The first dilution can have too many colonies to count or too much clumping. Seven dilutions were initially used in the first few batches as prototyping and initial testing to ensure the appropriate amount of colony ranges were captured without too much clumping. The dilutions were scaled down to 5 and then 4 in subsequent batches due to

reaching the appropriate number of colonies for thermal inactivation data. The typical *B.a.* growth process for several dilutions is best seen by looking at the control, as seen in Figure 27.

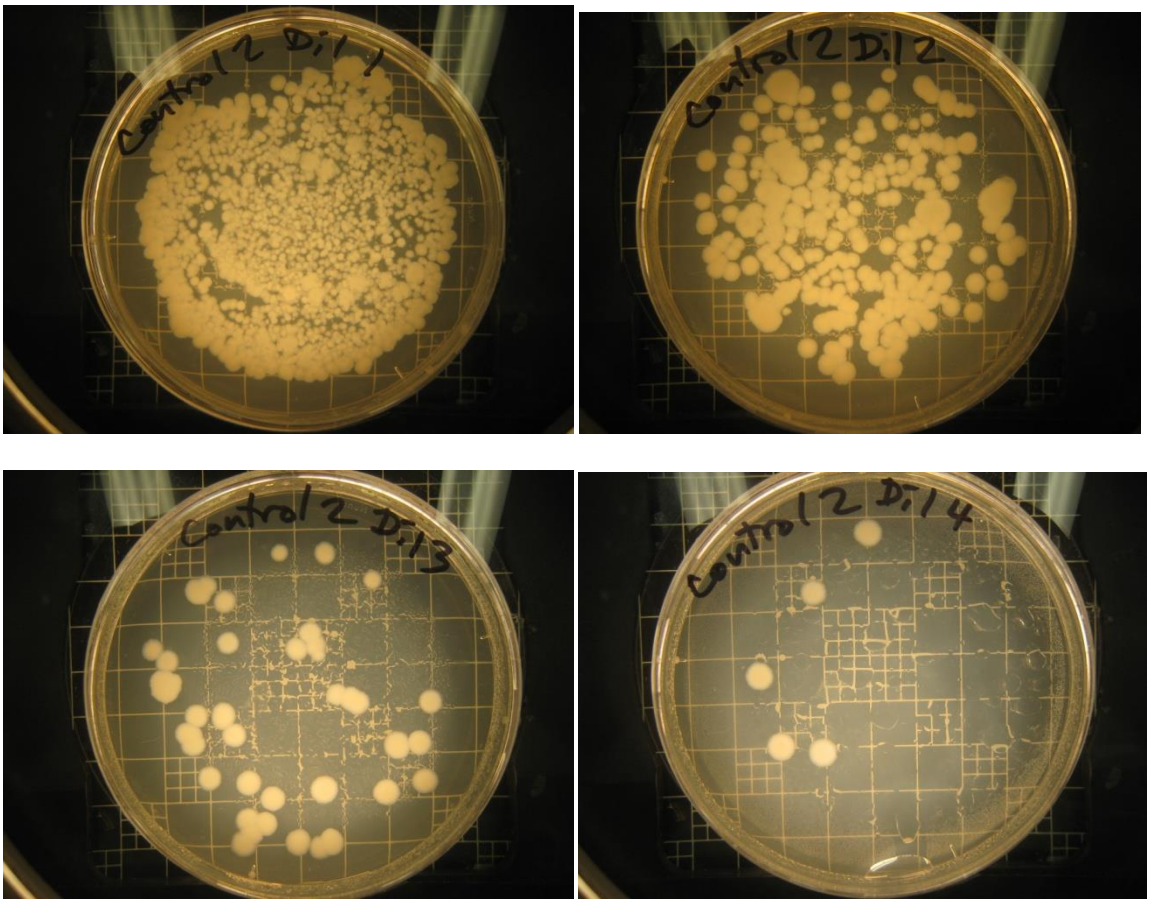


Figure 27. Typical *B.a.* growth process for several dilutions

Data points from selected dilutions (typically 1-2 agar plates) were then used to determine the spore population through the use of an equation, as described in the next section. There were dilution sets, however, that did not follow the typical dilution scheme from Figure 27. These included too much clumping at more diluted samples while not having any at the typical more concentrated dilutions, too much clumping in general, or possible contamination. These extraneous dilution sets or individual dilutions

were thrown out during the rest of the analysis for spore recovery. Figure 28 shows some examples of too much clumping and/or contamination.

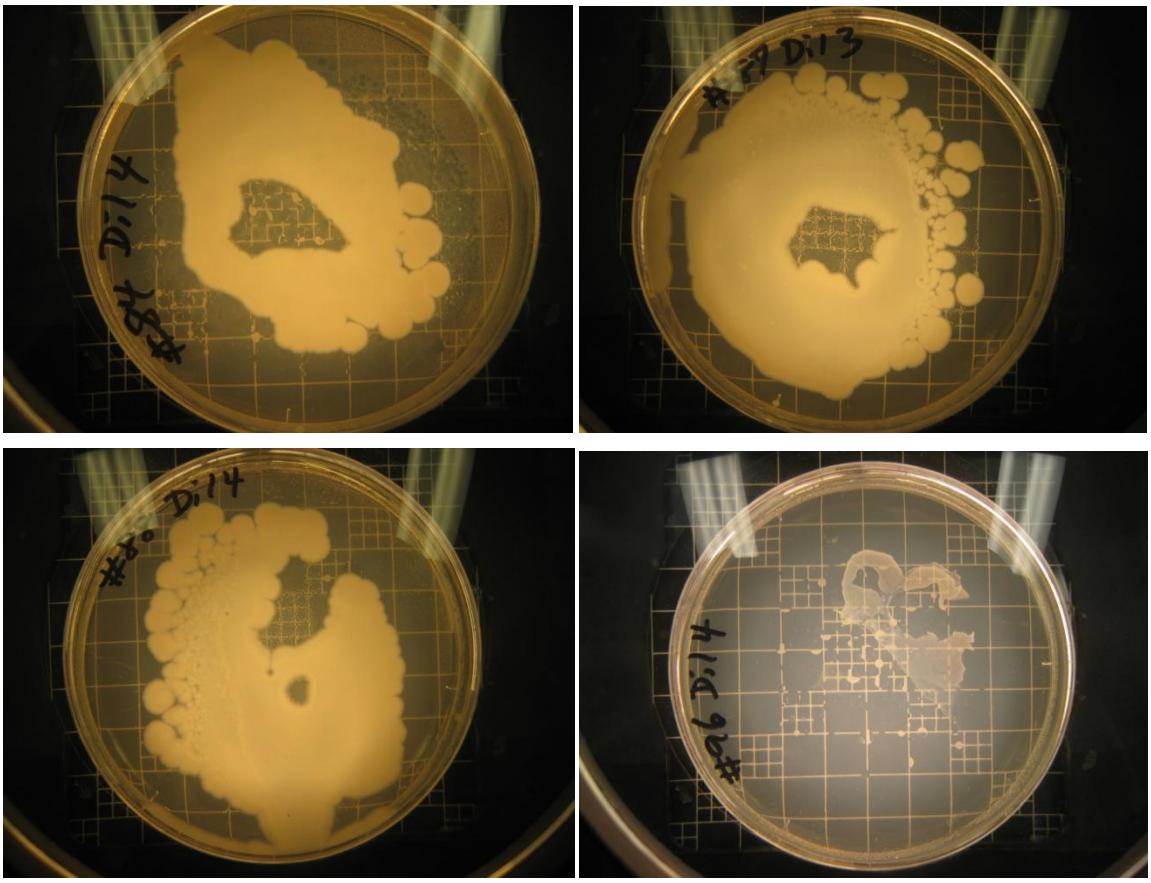


Figure 28. Examples of too much clumping and/or contamination

4.6 Determining Spore Populations

The CFU/mL equation was used to determine original spore populations (unit: CFU/mL) in the thermal inactivation and stock solutions (Goetz, 2005). Each dilution was made with 30% ethanol solutions. The information was used to determine and analyze the viability of spores after being subjected to varying temperatures at different exposure times (100 heat exposure samples). The colony number, as counted in each of

the 793 agar plates at the 24- and 48-hour marks, was divided by the dilution factor (from microdilutions) and the volume of spore solution plated, as shown below in Equation 2.

$$\frac{\text{CFU}}{\text{mL}} = \frac{\text{\# colonies}}{(\text{df})(\text{vol plated})} \tag{2}$$

where, CFU = Colony Forming Units, df = dilution factor (no unit), and vol plated = volume plated onto agar dish from microdilutions (unit: mL). For this thesis, the volume plated was 0.05mL (stock solution determination) and 0.1mL (stock solution determination and thermal inactivation spore solutions) while the dilution factors ranged from 10^{-1} to 10^{-7}. For the equation to remain valid 30-300 CFUs needed to be used; therefore, the dilution factor was chosen based on this information.

4.7 Analysis of Spore Loss

Spore loss was largely due to the hard surface recovery method of sonication (average spore loss of 53.5% and highest spore loss of 59% without application of heat) and the loss of viability from thermal inactivation (which was to be expected due to the destruction of the spores with heat). Spore loss was also contributed to ejected spores from heat exposures, screwing in the light bulb, screwing out the light bulb, and possible desiccation. During heat exposures very few viable spores grew on the agar plates located on the base of the lamp underneath the sample. While screwing in the light bulbs, spores fell onto PPE and the cavity of the light bulb base directly below the sample (the latter also during heat exposure). Other sources of spore loss, although minimal, were due to microdilutions, the plate spreader, and materials used to contain and transfer the spores.

Additional prototyping and initial testing was conducted with controls to determine spore loss due to sonication as the method for hard surface recovery of the spores. The observed *B.a.* spore loss was compared to previous work in literature for the hard surface recovery. Hard surface recovery methods were determined to be the main source of spore loss in this and various other studies.

4.7.1 Controls Used to Determine Spore Loss

Sonication was a major source of spore loss as the method for hard surface recovery for the study. Controls not only aided in determining spore loss but also helped verify stock solution, filament application, and starting spore dilution populations.

Different types of controls were used during the thermal inactivation testing to help verify spore counts and determine spore loss. Controls included nutrient agar plates that were strategically placed in several locations during a few thermal inactivation runs, two controls that were desiccated *B.a.* spore filament samples that were not heated but sonicated, and the last control that was a drop of *B.a.* spores that were not heated or sonicated. The controls will be described in more detail below.

First, up to ten nutrient agar plates were used on a few runs during the thermal inactivation testing. These agar plates were used to determine how many viable spores, if any, were ejected off of the tungsten filament during testing. The sets of agar plates were strategically placed around the base of the lamp and near the lamp, to include places above and perpendicular to those agar plates already situated. Packing tape was used, when needed, to place the 10 agar plates. See Figure 29 for a demonstration of the strategically placed agar plates. Very few colonies were found in the agar plates located

on the base of the lamp underneath the sample, although the exact numbers of viable and nonviable spores on these plates were not quantifiable. No other agar plates grew viable spores in the incubator. This proved spore loss due to ejected spores before and during the thermal inactivation testing was negligible.

One issue was that there was no method to capture the amount of spores lost directly underneath the sample in the light bulb base. Spore loss could have occurred due to screwing in or unscrewing the base of the light bulbs containing the filaments before and after the applied power pulses controlled by the input voltage. The spores could have fallen into the base of the bulb directly underneath the sample during the set up and removal of the filament. Spore loss could have also occurred during the rapid resistive heating process by extracted spores falling into the base of the bulb.

Figure 29. Nutrient agar plates used in ten places to try to capture spores during thermal inactivation testing

Next, two desiccated *B.a.*-encrusted tungsten filament samples were used as controls. The intent of the prototyping/initial testing was to determine how many spores were lost due to sonication from averaging the amount of spores recovered from the two control samples (unit: CFU/mL) and using the final control, described next, as a base to determine the percentage of spores that were lost and recovered. These two samples were not heated so that spore loss due to hard surface recovery was the main focus. To quantify the recovered spores, microdilutions (1:10 dilutions) were performed for up to seven dilution factors after sonication, followed by incubation, plating the up to seven dilutions, and finally counting the colonies (while applying the CFU/mL equation). The calculated spore loss and recovery percentages can be found in Table 6 in the next subsection.

The final control was done to ensure 10^6 CFU/mL were still appropriate as the starting spore solution (not stock solution originally applied to filaments) and serve as the base number of spores that can be quantifiably recovered. This is a double check from the originally calculated starting stock solution (10^8 CFU/mL) from the experimental turbidity standard, discussed in the last chapter, to ensure 10^6 spores were present in the 10-µL drop. To manage this control, a 10-µL drop from the stock solution was added to one mL in the centrifuge tube. Microdilutions (1:10 dilutions) were then performed for seven dilution factors, followed by incubation, plating dilutions, and finally counting the colonies (while applying the CFU/mL equation). This final control was used in conjunction with the two controls discussed above, where the average percent of spores recovered was calculated, in order to calculate the percent of spores lost due to sonication

as the method of hard surface recovery. The calculated spore loss and recovery percentages can be found in Table 6 in the next subsection.

4.7.2 Comparison of Spore Loss to Previous Work

Hard surface recovery methods were determined to be the main source of *B.a.* spore loss in this and various other studies. The hard surface material chosen for the thermal inactivation studies was a reproducible, coiled metal tungsten filament. There was no previous data found in literature using a metal filament for the hard surface recovery of *B.a.* spores. Although no filament comparison was available, other metals have been used for hard surface recovery. Smooth, flat stainless steel metal coupons are usually used as the source of hard surface materials for the recovery of *B.a.* spores. Other coupon sources used for hard surface recovery have been glass, chemical agent-resistant coating (CARC)-painted steel (non-smooth steel surface), polycarbonate, and vinyl tile (Lewandowski et al., 2010; Edmonds et al., 2009).

The highest spore loss seen between the two sonicated controls was 59% while the average spore loss was 53.5%. This is considered to be a normal fraction of spores lost when compared to literature. It is important to note that a thin layer of *B.a.* spores was applied and the data points from the heat exposures were acquired in several batches to prevent accumulation of *B.a.* spores in the bottom of the centrifuge tubes and deep-well plates. Other methods to prevent impaction and spore loss also included vortex mixing (for evenly mixed solutions) of centrifuge tubes and using pipette tips for mixing within the deep wells in the deep-well plates between the series of microdilutions. The usage of a thin *B.a.* layer on the filament, vortex mixing, and mixing throughout the study

allowed for more spore recovery especially during hard surface recovery and for the agar plating preparation.

Recent research demonstrated an average spore loss range of 52.8% to 77.2% (highest spore loss range of 57.5% to 90%) for metal surfaces. Table 6 shows the comparison between this study and previous studies for the hard surface recoveries of *B.a.* spores with metal sources. The recovery percentages of other authors in Table 6 is based on the recovery by various swabs since the efficacy of extraction by sonication was shown to be 25% less than swabs (Hodges et al., 2006). The reason for the much higher amounts of spore recovery for Edmonds et al. is a much thicker layer of spores than those of the other authors. The recovery of spores within this study was on par with previous studies, although desiccation also played into the spore percentage losses for this study.

Table 6. *B.a.* Spore Hard Surface Recoveries on Metal

Author	Metal Source	Avg % Spores Recovered	Avg % Spores Lost	Highest % Spores Lost	Recovery Range (%)	All Spore Recoveries (%)
Grijalva	Tungsten Filament	46.5	53.5	59.0	41.0-52.0	41.0, 52.0
Hodges et al., 2006	Stainless Steel	39.0	61.0	68.3	31.7-49.1	31.7, 37.8, 37.2, 40.1, 38.0, 49.1
Rose et al., 2004	Stainless Steel	22.8	77.2	90.0	10.0-30.7	10.0, 27.7, 30.7
Edmonds et al., 2009	CARC-painted steel	47.2	52.8	57.5	42.5-55.7	47.0, 42.5, 43.6, 55.7

4.8 Exposure Time Analysis using Time-Temperature Profiles

The power supply input time range was 0.1 to 10 seconds but after time-temperature profiles were analyzed and plotted, these power supply input values were inaccurate exposure times and were modified to reflect corrected FLIR exposure times ranging from 0.258 to 7 seconds. The corrected exposure times represented the length of time the *B.a.*-encrusted filament samples were subjected to the maximum temperature; more detail to follow in the first subsection. Heat exposure times were corrected because the power supply input times were affected by the time to achieve maximum temperature (lead times) and the significant cooling times. Therefore, the ideal square pulses for the time-temperature profile plots could not be achieved, although plots showed the pulses were close to this model considering the rare wavering of time within the pulses (within a few degrees at the longer power supply input times) for the lower temperature range for the FLIR; this was not the case for the higher temperature range FLIR setting. Theoretically, the heat capacity of the filament would have to be negligibly small in order to have a square wave pulse.

An alternative method of analysis with time-temperature profiles is to determine average maximum temperatures for the corrected time exposures versus the maximum temperature for the corrected time exposures. This change would account for slightly lower temperatures overall for the corrected exposure times for maximum temperature. In this study the maximum temperature was used amongst the corrected time exposures. It was more suitable to obtain the maximum temperatures versus the average maximum temperatures in this study due to the FLIR and player limitations.

Time-temperature profiles were largely affected by limitations of the FLIR, player, and the power supply. The average percent error of the power supply for the time at maximum temperature was 25.451%; the average percent error after accounting for outliers was still a staggering 20.132%. The average percent error of the FLIR (after emissivity correction) was ± 5.3% with a maximum percent error of ± 6.28%. Data reduction of the time-temperature data required several weeks since it consisted of 3,100 data points obtained from recorded FLIR videos. The data points were taken from the starting room temperature through the time it took to return to room temperature for each of the 120 heat exposures. The instrumentation limitations, observations in the data and profiles, calculations, and corrected exposure times will be discussed in the first subsection while observations and generalizations from time-temperature profiles along with four sample time-temperature profile plots will be discussed in the last subsection.

4.8.1 Corrected Exposure Times

Corrected exposure times depended on thorough analysis, to include observations, and limitations of the equipment used. Time-temperature profiles were the method used to correct exposure times. Equipment limitations included the FLIR and player.

FLIR videos recorded by the Pinnacle were analyzed after each heat exposure in QuickTime Player to ensure each sample was recorded correctly. Additionally, the player was used to manually extract the 3,100 data points from the 120 heat exposure videos (20 filaments used for pre-trials) to create the time-temperature profiles; more details on the analysis will be discussed later in this section. The data points began at the starting room temperature and continued after each heat exposure until returning to room

temperature. The temperature was annotated at the initial point before the glow started on the FLIR, where the glow started on the FLIR, and also when the glow stopped on the FLIR. During analysis, the player was found to have a limiting factor with playback. The player had the capability of viewing and measuring the temperatures by the second; however, the optimal unit for analysis would have been milliseconds. The limitation affected time-temperature profiles because it caused a need to make assumptions on exactly when the temperatures changed within the second; these assumptions also depended on how many readings were collected every second based on the capacity of the FLIR.

The FLIR also had a limitation which affected the time-temperature profiles and caused the need for further assumptions as in the player limitation, especially in the higher temperature range setting. For the vast majority of the heat exposures, the FLIR setting had to be manually set to the 350 to 2000°C range due to the need to subject *B.a.*-encrusted filament samples to higher temperatures throughout the thermal inactivation studies. The limitation was especially true for the shorter power supply input times. Data points below 215°C were not available in aforementioned FLIR setting since the FLIR cannot pick up such temperatures while in the manual setting and the FLIR was not quick enough to catch temperatures for quicker spikes leading up to the maximum temperature although it was much better for cooling temperatures after the maximum temperature. The response given for unavailable data was represented by the FLIR as "Amax <215ºC" and was appropriately annotated as <215ºC during analysis. Unfortunately, complete plots could not be made when such a FLIR setting was used

because less data points were available for extraction from the videos. Time-temperature profile plots could be created for the FLIR setting that were manually set to the 0 to 500°C range but again, many of the thermal inactivation studies were conducted in the 350 to 2000°C range. Sample plots will be discussed in the next subsection.

The corrected exposure time was calculated by using 16.28% of the maximum temperature reached. The FLIR accuracy is quoted by the manufacturer in the general specifications to be ± 2%, although this figure was measured to be closer to ± 5.3% (± 6.28% maximum) due to the variance in the temperature recorded by the FLIR during room temperature time conditions. The measured percent error at room temperature of 16.28% of the maximum temperature was used instead of 10% value recommended by committee members in calculating the corrected exposure times to properly account for the error of the FLIR. Once the range (within 16.28% of the maximum temperature) was established for each of the 100 heat exposures for the 12 power supply input times, the corrected exposure time was found for each of these *B.a.*-encrusted filament samples using the 3,100 data points. An average was then taken to determine the final corrected exposure time for each of the 12 specific power supply input times. Table 7 shows the 12 corrected exposure times at maximum temperature for the thermal inactivation studies for each of the 12 power supply input times and the standard deviation.

The next section will show sample time-temperature profile plots. It is unfortunate that most time-temperature profile plots of the 100 possible plots that could have been made could not be completed due to limitations of the FLIR and player. Other concerns will be addressed as well.

Table 7. Corrected Exposure Times at Maximum Temperature

Power Supply Input Time (s)	Corrected Exposure Time (s)	Standard Deviation (σ)	Difference in Exposure Time (s)
0.100	0.258	0.153	0.160
0.300	0.400	0.116	0.100
0.500	0.488	0.033	0.010
0.750	0.623	0.088	0.130
1.000	0.813	0.216	0.190
1.500	1.200	0.252	0.300
2.000	1.710	0.276	0.290
2.500	1.950	0.362	0.550
3.000	2.730	0.295	0.270
5.000	4.000	0.489	1.000
7.500	5.980	0.621	1.520
10.000	7.000	0.837	3.000

4.8.2 Sample Time-Temperature Profile Plots

Some sample time-temperature profile plots were made for the FLIR temperature range setting that was manually set to the 0 to 500°C range (the other FLIR setting did not allow temperatures to be recorded under 215°C). The setting gave the ability to analyze whether data points in the plots modeled a general resemblance to ideal square pulses. A square pulse was not achieved in the plots due to the times needed to achieve maximum temperature (lead times) and the times needed to cool back down to room temperature (cooling times). Although the goal of a square pulse was not achieved, time-

temperature profile plots showed that pulses were close to this model for this FLIR temperature range setting, considering the rare wavering of time within the pulses (within a few degrees at the longer power supply input times). This was not the case for the higher FLIR temperature range setting, in which most of the inactivation studies occurred. No cooling mechanism was used after the heat exposures to improve upon the theoretical square pulse plot, although cooling would not be implemented in the field and does not represent a typical environment if conventional munitions are used as agent defeat weapons. The scenario is particularly apt for spores absorbed on surfaces; thus, this situation is expected to be quite relevant to spores dispersed following a detonation explosion. As will be seen below, there was concern that some of the data could be erroneous due to the power supply being variable, lead and cooling times, and sparse data sets within each of the 12 exposure times. FLIR and play limitations were already discussed in the previous subsection. The only concern was that since the plots did not display the ideal square pulse (difficult to achieve in a laboratory setting), lead and cooling times may have affected the inactivation of the spores. Other concerns are that more data points were needed and the power supply producing different maximum temperatures when the same amount of voltage is applied.

There were 3,100 data points across 12 power supply input times and 100 total heat exposures just to calculate the 12 corrected exposure times. Keep in mind that each of the figure plots below are simplified and are only a data point in time for calculating the average time for the corrected exposure times; as described in the previous subsection, an average was taken for time based on varying voltages within a power

supply input time (12 averages total from the 3,100 data points). The figure plots below are a sampling; they are only 4 of a possible 100 plots for the 3,100 data points. Due to limitations on temperature ranges on the FLIR, not all data points are available to make proper plots. This was further compounded by the limitations of the player.

Some of the pulses appeared to be poorly formed and some evidence of power supply malfunction was seen during prototyping and initial testing. The power supply ranges were maxed out for time and voltage in attempting to achieve the desired exposure times and temperatures such that varying temperatures were achieved for identical input times and voltages.

Generalizations and calculations were created using the time-temperature profiles. Lead-time was defined as the time period required to heat up to the maximum temperature range from room temperature while cooling time was defined as the time period required to cool to room temperature from the maximum temperature range. Lead times, based on the time-temperature profile plots and the 3,100 data points, averaged out to be 1.1 seconds for the corrected exposure times ranging from 0.258 to 7 seconds, while the cooling times averaged out to be 2.2 seconds. These lead and cooling times are further confirmed in the below 4 sample time-temperature profile plots, see Figures 30 through 33.

The length of time at the maximum temperature was affected by longer cooling times (typically more so at longer power supply input times or with lower heat exposure temperatures) and longer lead times (not the case at the shortest power supply time input

intervals and tending to increase as the power supply input times increased). Figures 30 through 33 graphically show these trends. The plots also show the corrected exposure times which are denoted by the blue arrows and signify the width of the acceptable data points used when these corrected exposure times were calculated. Corrected exposure times were calculated as defined in the last subsection.

Although a square pulse could not be achieved, quick spikes to the maximum temperature range were possible at shorter power supply input times. Figures 30 and 31 both show corrected exposure times to be about 0.3 seconds each at the 0.1-second power supply input times but again these were only a couple of data points in the average calculation of the corrected exposure time as described in the last subsection.

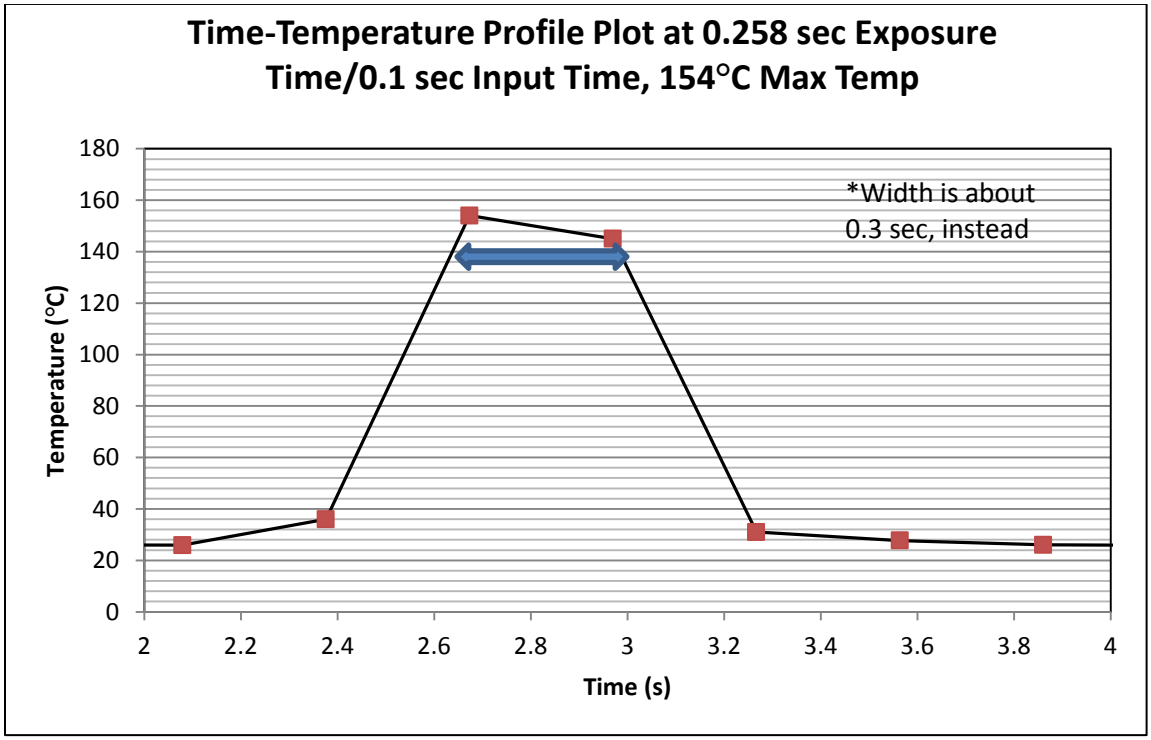


Figure 30. Sample time-temperature profile plot at 0.258s exposure time

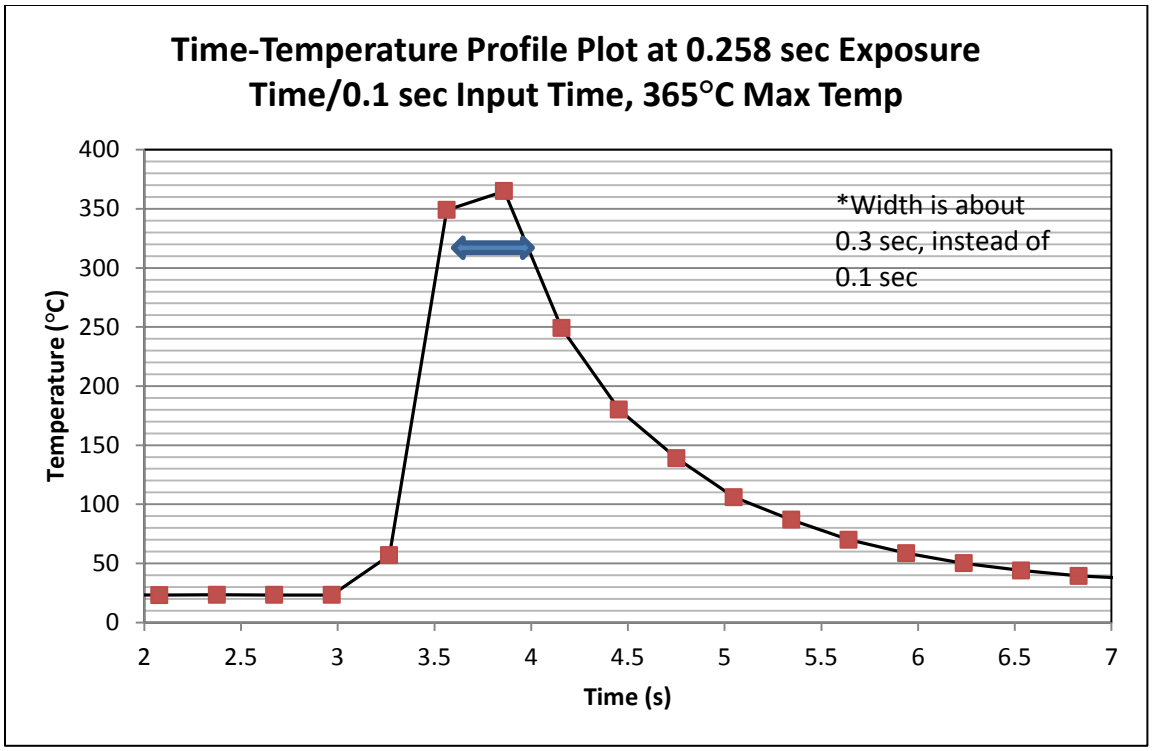


Figure 31. Sample time-temperature profile plot at 0.258s exposure time

Figures 30 and 31 show two sample time-temperature profile plots for the corrected exposure time of 0.258 seconds at the different heat exposures of 154ºC and 365ºC, respectively. An average cooling time of 1.6 seconds was shown in the plots, which were below average as compared to the 2.2-second average cooling time for all of the corrected exposure times (0.258 to 7 seconds). The next two sample time-temperature profile plots, Figures 32 and 33, are for longer power supply input times and have much longer lead times than seen in Figures 30 and 31.

Figure 32 shows the time-temperature plot for the corrected exposure time of 1.2 seconds at a heat exposure of 400ºC. Figure 33, also below, shows the corrected exposure time of 7 seconds at a heat exposure of 347ºC. The cooling times are 2.97

seconds and 2.67 seconds, respectively (both slightly above the average). The lead time for Figure 32 is below average at 0.594 seconds, while the lead time for Figure 33 is significantly above the average lead times for the time-temperature profiles at 3.564 seconds. Since the data points to the left and right of the blue arrow did not fall within 16.28% of the maximum temperature in Figures 32 and 33, they could not be used in calculations for the corrected exposure time even though it appears that the exposure time should be longer in the plots. The average lead time calculated for all of the time-temperature profiles at the corrected exposure time (7 seconds) was 3.5 seconds, so the value of 3.564 seconds is not alarming even though the average lead time calculated for all of the corrected exposure times (0.258 to 7 seconds) was 1.1 seconds.

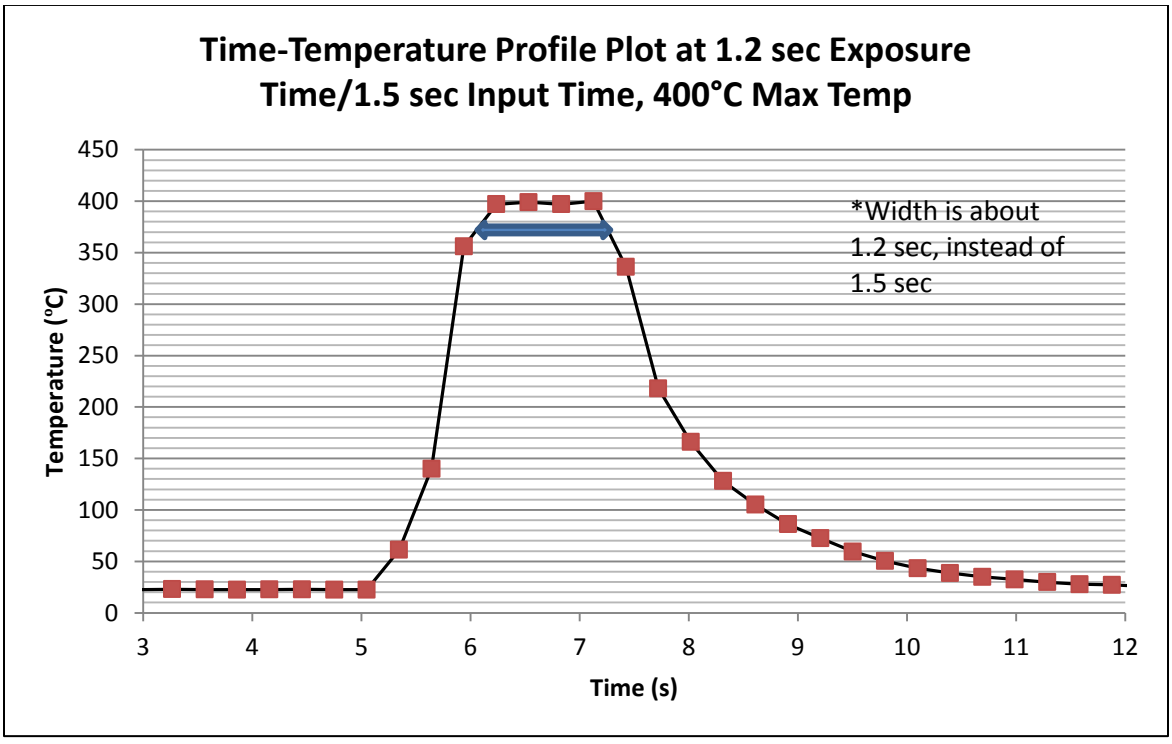


Figure 32. Sample time-temperature profile plot at 1.2s exposure time

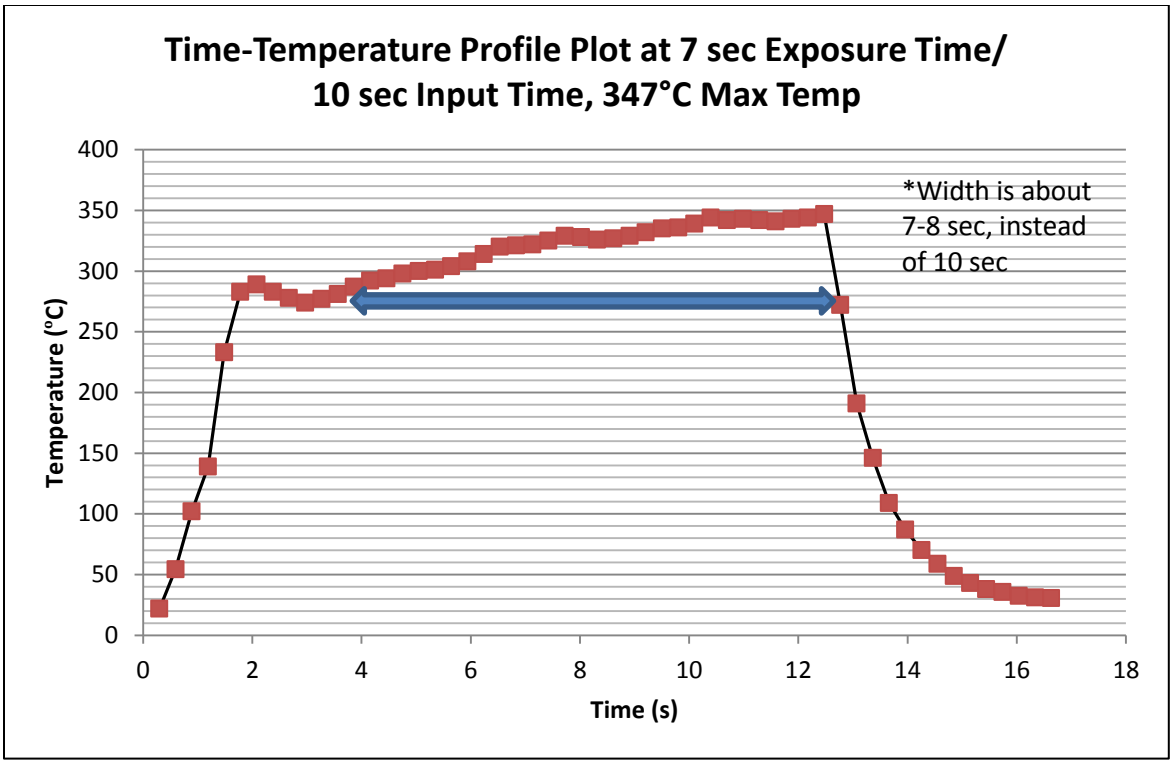


Figure 33. Sample time-temperature profile plot at 7s exposure time

Without exposure time corrections, using the 3,100 time-temperature profile data points, the times for the spore viability plots and subsequent loss of viability plots as well as the partial and complete thermal inactivation plots would not have been as accurate. The next section will discuss and plot the *B.a.* spore viability ranges for each of the 12 exposure times (100 heat exposures), while the subsequent section will compare data to previous studies in literature.

4.9 Spore Viability Ranges for 12 Exposure Times

After extensive analysis was completed using the 1,586 data points from counting colonies on 793 agar plates at the 24- and 48-hour marks (used to determine the viability of spores from the CFU/mL equation, unit: CFU/mL) and the 3,100 data points from the

time-temperature profiles (used to determine the maximum temperatures reached and corrected exposure times), proper viability ranges (unit: CFU/mL) could be established and plotted. Due to the 12 exposure times, there are 12 tables and 12 graphs in this section to show the viability of the spores at those exposure times which were subjected to varying voltages. Partial and complete inactivation is shown in the tables. A thermal inactivation (loss of viability) table and plots are found in the next section, which shows the condensed thermal inactivation outcome and is compared to the thermal inactivation outcome from Battelle's 'Thermal Deactivation of Aerosolized Bacteria' (Alexander, Ogden, LeVere, Dye, & Kohler, 1999).

Overall, the thermal inactivation setup proved effective for both partially and completely inactivating the *B.a.* spores. The viability at each maximum temperature reached for each heat exposure sample was assembled into tables and plots shown in Tables 8 through 19 and Figures 34 through 45. Partial inactivation is the highest temperature observed before no colonies could form while complete inactivation is the lowest temperature at which colonies no longer formed. Within the tables, the red denotes a loss in viability (partial inactivation) while the green denotes no viability (complete inactivation). Each table is displayed with its corresponding plot. The units included temperatures measured in degrees Celsius, the power supply input times/exposure times measured in seconds, and the viability measured in CFU/mL.

One issue is that there can be large gaps in the data between temperatures analyzed for partial versus complete inactivation due to sparse data within those exposure times; thus, it is assumed complete inactivation should be a lower temperature than the

complete inactivation quoted in this thesis and the partial inactivation should be a higher temperature than the partial inactivation quoted in this thesis. To prevent this, future research can be focused so that more than 100 heat exposures are done for the 12 exposure times or less exposure times are done for the 100 heat exposures so that research and analysis can focus on a tighter time range with several more data points. Additionally, 20 filaments were used in pre-trials to determine what maximum temperatures corresponded to from various voltage inputs for a total of at least 120 required heat exposures.

General correlations of exposure temperatures and times with viability were made. As expected, higher temperatures were needed to inactivate the *B.a.* spores as exposure times decreased. Assumptions were discussed earlier in this section in terms of temperatures needed for partial and complete inactivation. In general, viability of the spores went down as temperature increased but it was not always the case. This could be due to the loss of spores during thermal inactivation testing, hard surface recovery after thermal inactivation, microdilutions, spore impaction in tubes (minimized by doing experiments in smaller batches with vortex mixing), or agar plate spreading of the spores.

The viability (CFU/mL) was calculated as specified in chapter 4, sections 5 and 6. As will be seen in some of the tables and plots below, viability did not necessarily follow a perfect trend line. This is because spore loss was not only attributed to thermal inactivation, as specified in chapter 4, section 7; thus, *B.a.* spore recovery can be varied.

Table 8. *B.a.* Viability at 0.258 sec Exposure Time/0.1 sec Input Time

Temp (deg C)	Viability (CFU/mL)
100	2.60E+03
154	2.00E+02
365	1.00E+02
555	5.00E+02
683	2.00E+02
779	1.00E+02
814	2.00E+02
850	1.00E+02
860	1.00E+02
876	0.00E+00
880	0.00E+00
888	0.00E+00

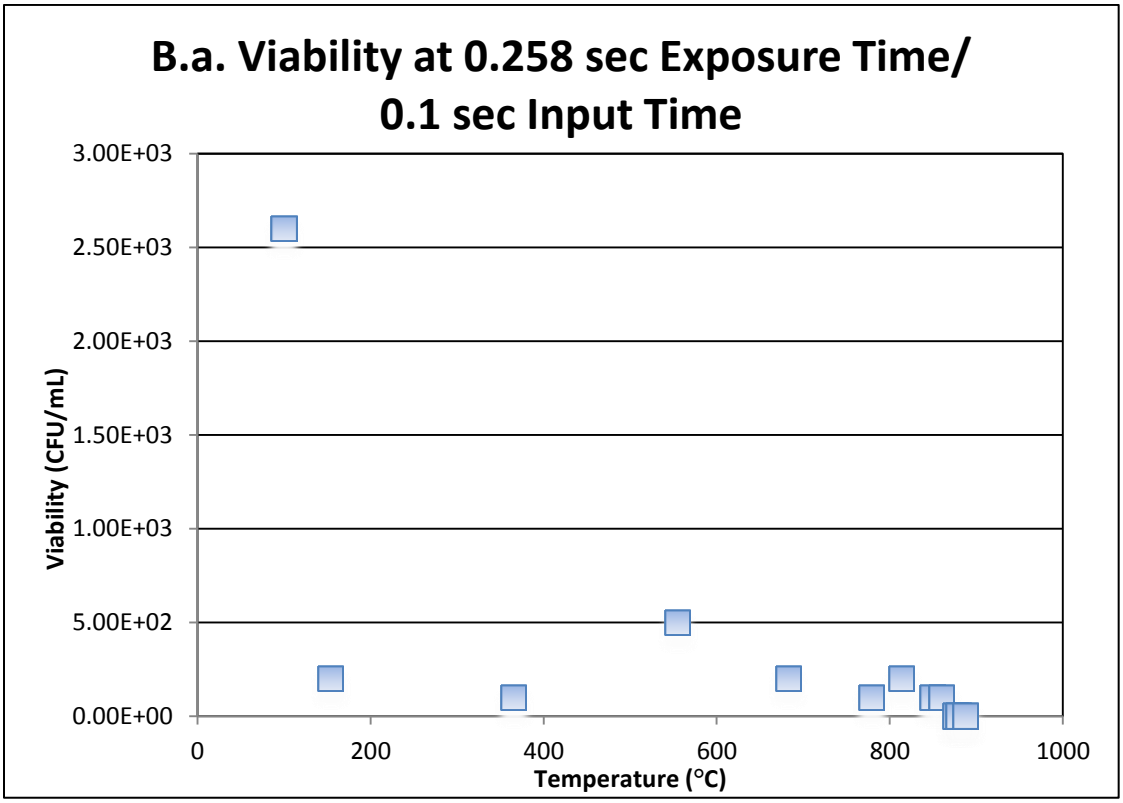


Figure 34. *B.a.* Viability at 0.258 sec Exposure Time/0.1 sec Input Time

Table 9. *B.a.* Viability at 0.4 sec Exposure Time/0.3 sec Input Time

Temp (deg C)	Viability (CFU/mL)
145	3.00E+03
159	1.00E+03
239	1.40E+03
420	7.00E+02
629	1.00E+02
674	0.00E+00
721	0.00E+00
767	0.00E+00
788	0.00E+00
819	0.00E+00
853	0.00E+00

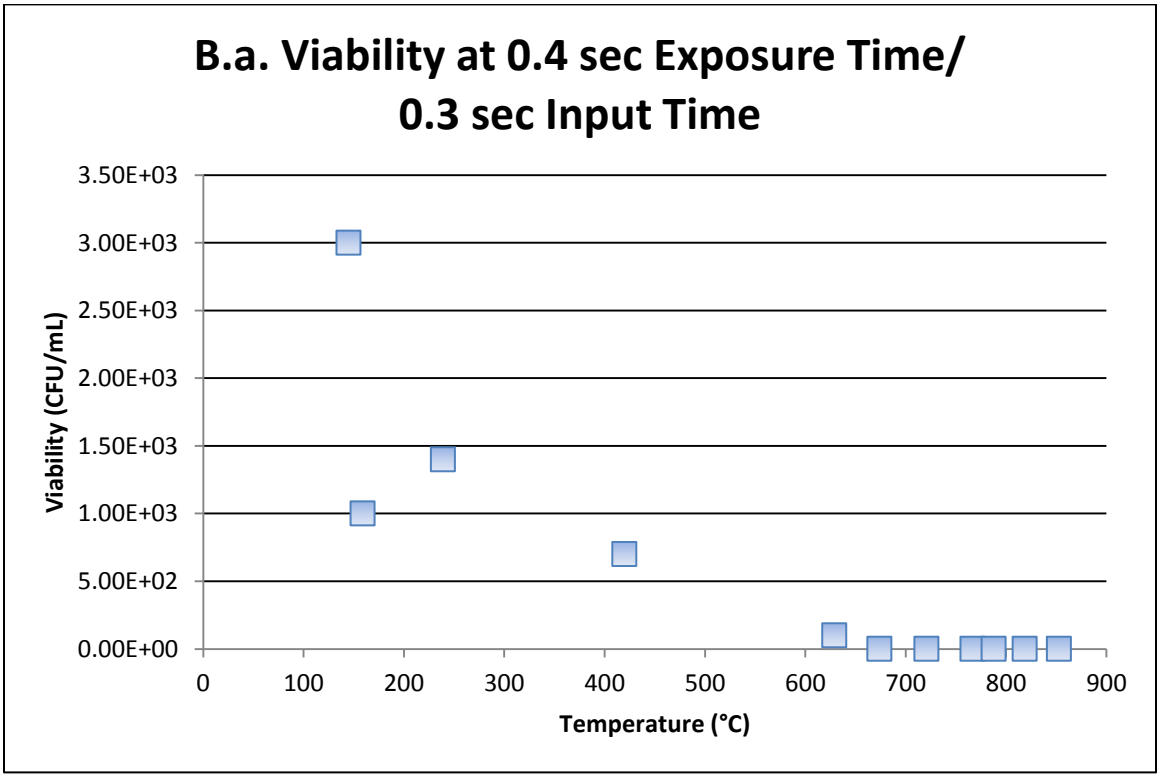


Figure 35. *B.a.* Viability at 0.4 sec Exposure Time/0.3 sec Input Time

Table 10. *B.a.* Viability at 0.488 sec Exposure Time/0.5 sec Input Time

Temp (deg C)	Viability (CFU/mL)
125	1.00E+03
185	9.00E+02
304	8.00E+02
436	1.00E+02
572	2.00E+02
618	1.00E+02
696	0.00E+00
743	0.00E+00
745	0.00E+00
773	0.00E+00
801	0.00E+00
862	0.00E+00

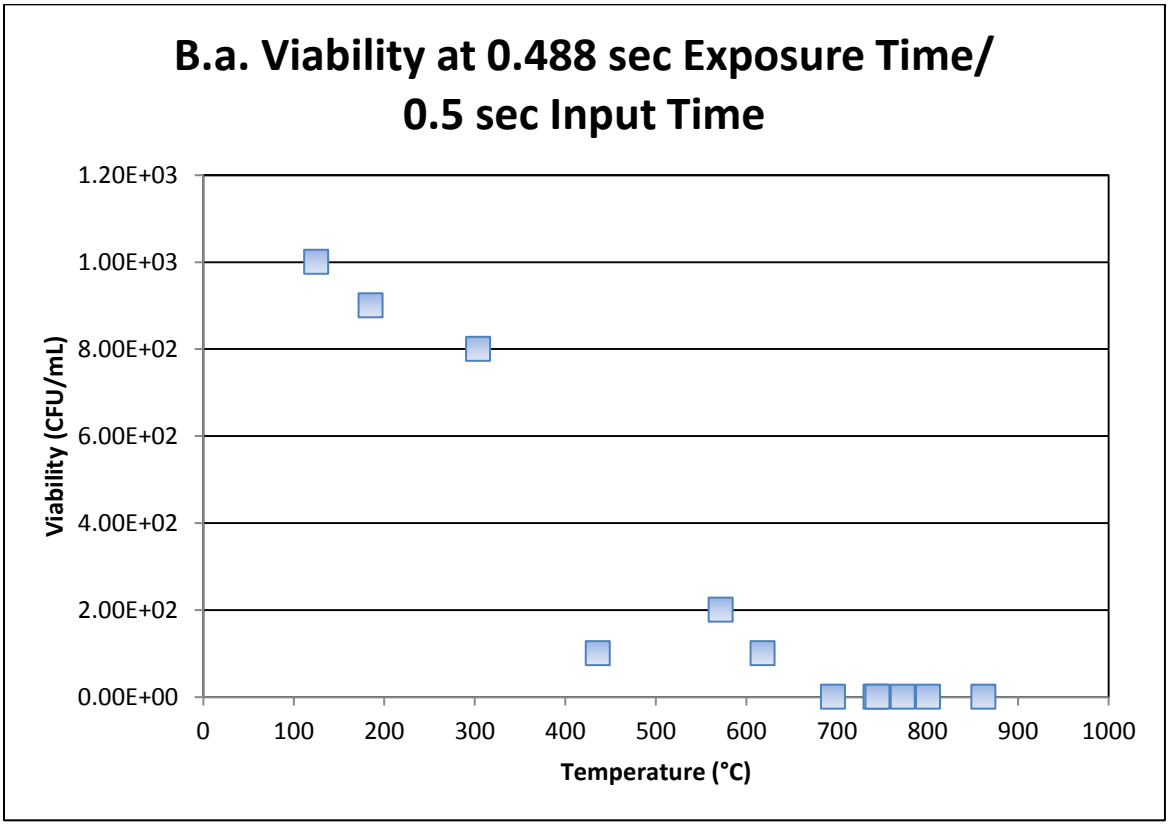


Figure 36. *B.a.* Viability at 0.488 sec Exposure Time/0.5 sec Input Time

Table 11. *B.a.* Viability at 0.623 sec Exposure Time/0.75 sec Input Time

Temp (deg C)	Viability (CFU/mL)
173	2.00E+03
261	1.00E+02
306	2.00E+02
463	3.00E+02
578	1.00E+02
611	1.00E+02
657	0.00E+00
709	0.00E+00
733	0.00E+00
754	0.00E+00
760	0.00E+00

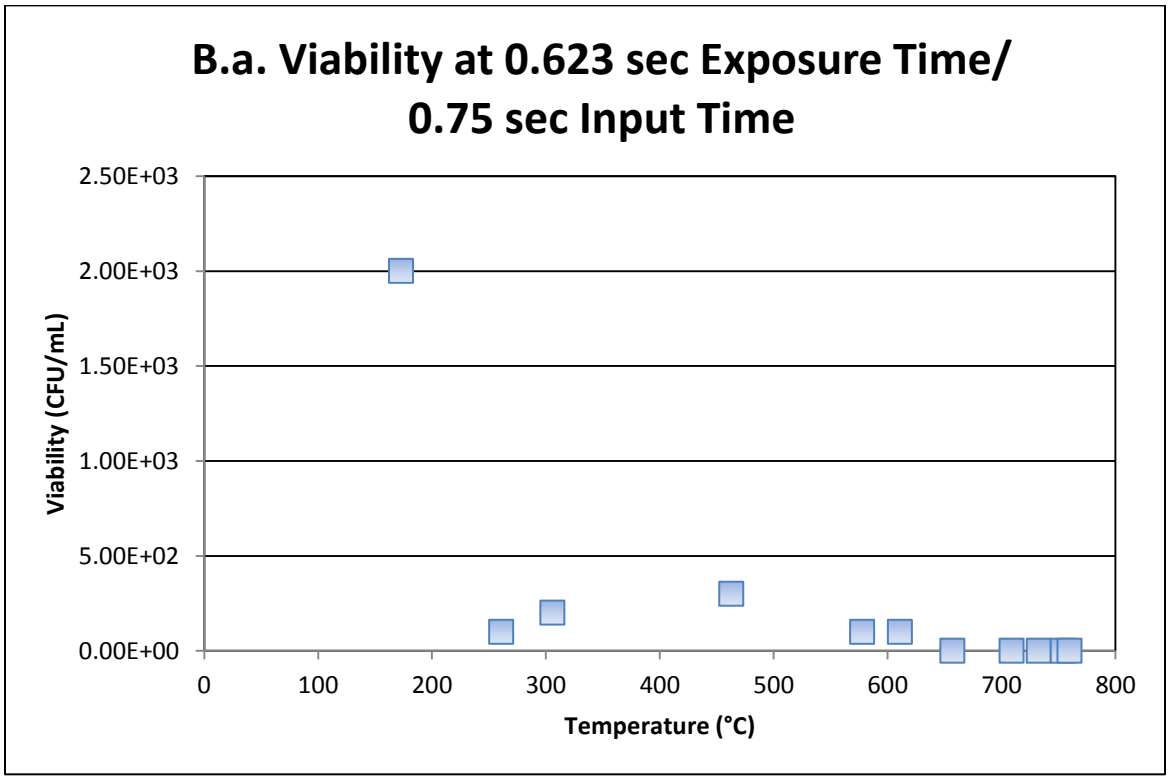


Figure 37. *B.a.* Viability at 0.623 sec Exposure Time/0.75 sec Input Time

Table 12. *B.a.* Viability at 0.813 sec Exposure Time/1.0 sec Input Time

Temp (deg C)	Viability (CFU/mL)
116	1.80E+03
246	4.00E+03
307	1.80E+03
474	2.00E+03
561	1.00E+03
570	2.00E+02
581	2.00E+02
622	0.00E+00
634	0.00E+00
791	0.00E+00
794	0.00E+00

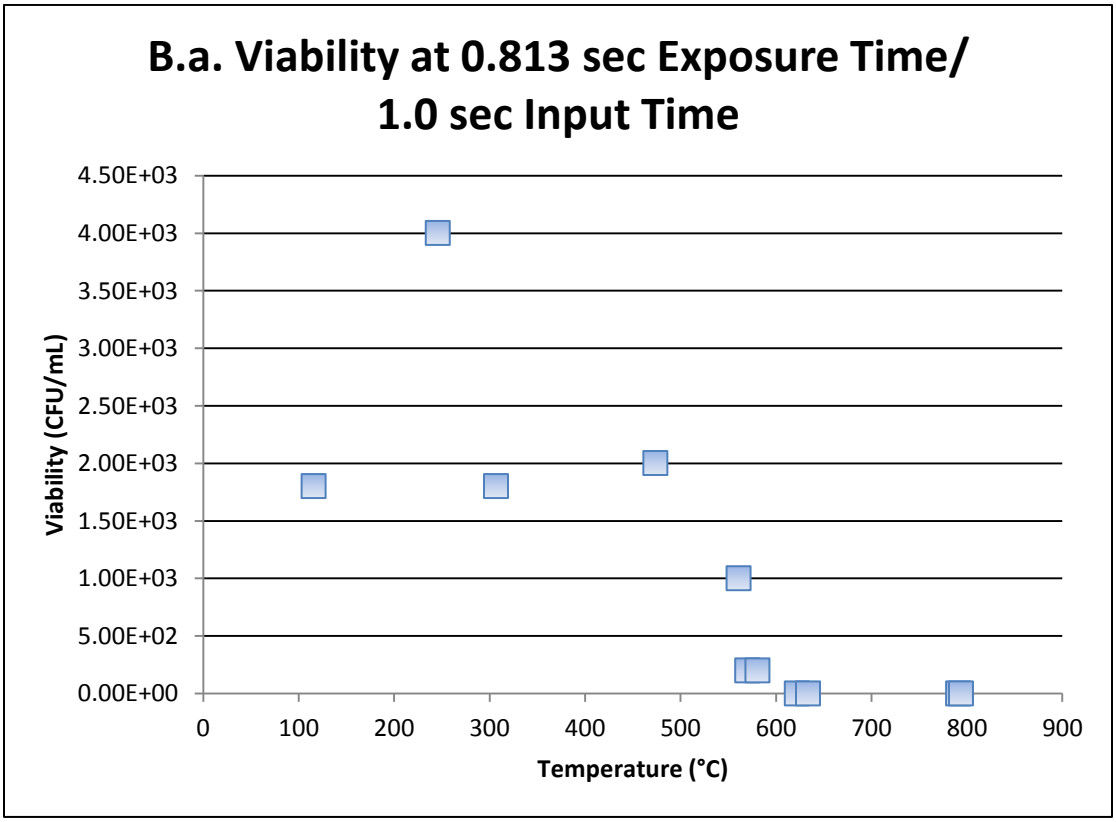


Figure 38. *B.a.* Viability at 0.813 sec Exposure Time/1.0 sec Input Time

Table 13. *B.a.* Viability at 1.2 sec Exposure Time/1.5 sec Input Time

Temp (deg C)	Viability (CFU/mL)
117	1.10E+03
192	1.00E+03
270	5.00E+02
318	3.00E+02
400	1.00E+02
545	1.00E+02
631	0.00E+00

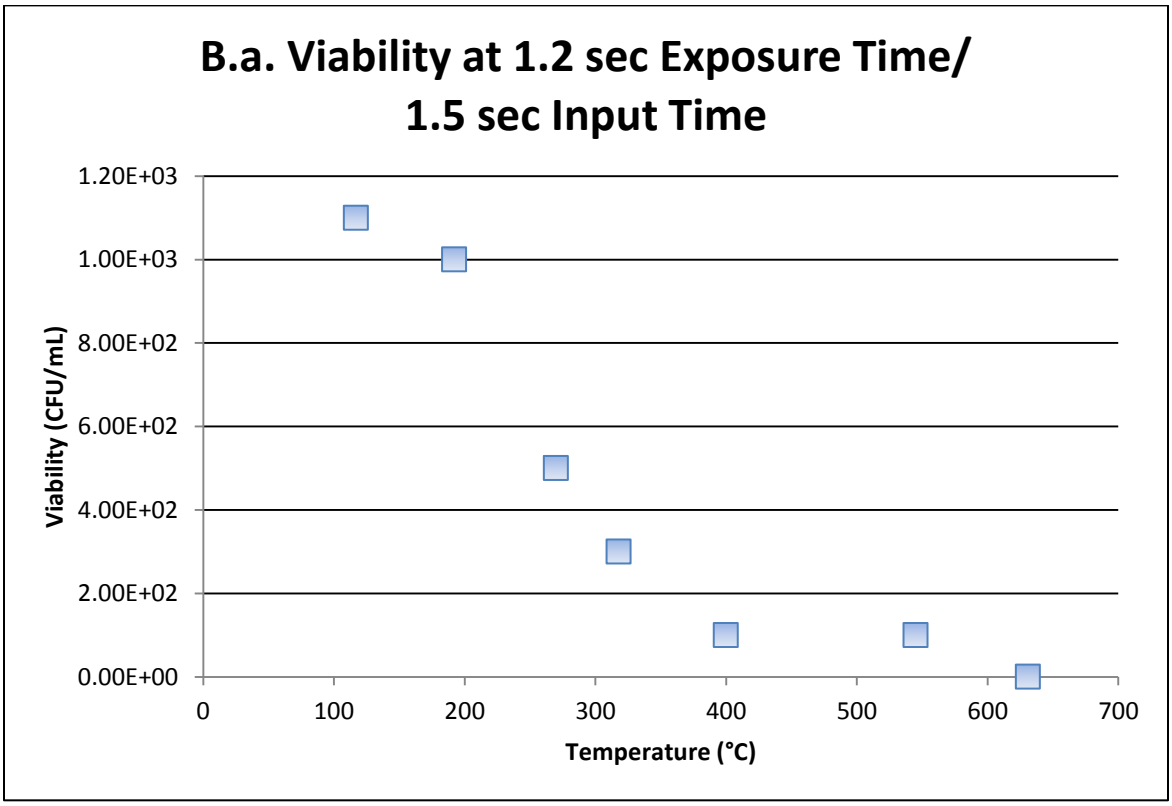


Figure 39. *B.a.* Viability at 1.2 sec Exposure Time/1.5 sec Input Time

Table 14. *B.a.* Viability at 1.71 sec Exposure Time/2.0 sec Input Time

Temp (deg C)	Viability (CFU/mL)
83.6	1.00E+03
163	1.20E+03
175	3.00E+03
250	2.00E+03
343	2.20E+03
434	2.00E+02
509	0.00E+00
600	0.00E+00

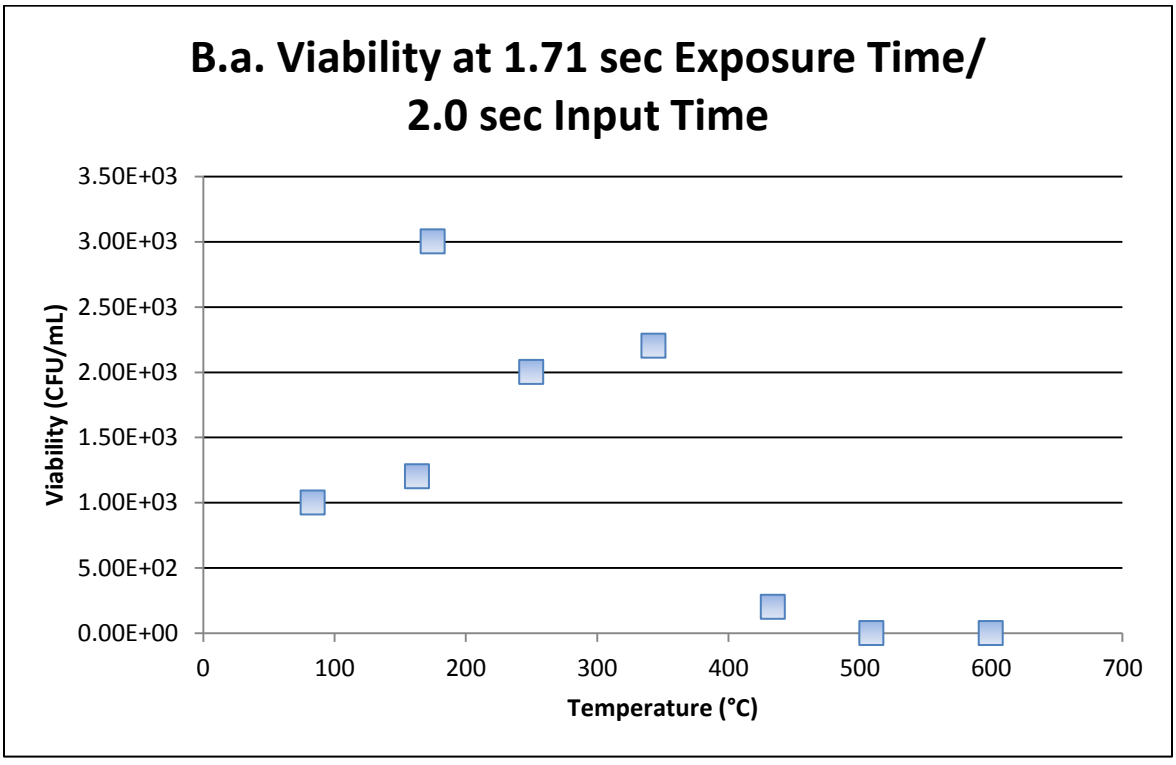


Figure 40. *B.a.* Viability at 1.71 sec Exposure Time/2.0 sec Input Time

Table 15. *B.a.* Viability at 1.95 sec Exposure Time/2.5 sec Input Time

Temp (deg C)	Viability (CFU/mL)
149	1.20E+03
178	2.00E+03
262	1.00E+02
318	8.00E+02
501	0.00E+00
519	0.00E+00
643	0.00E+00

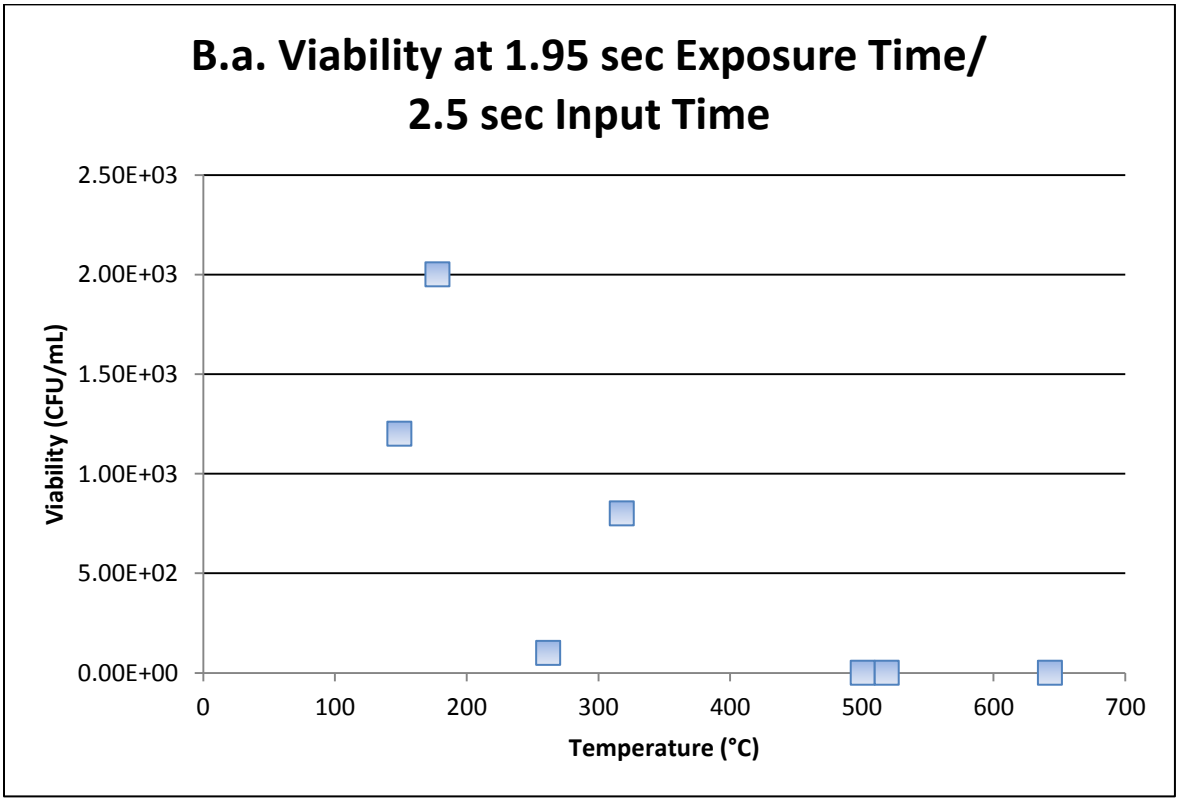


Figure 41. *B.a.* Viability at 1.95 sec Exposure Time/2.5 sec Input Time

Table 16. *B.a.* Viability at 2.73 sec Exposure Time/3.0 sec Input Time

Temp (deg C)	Viability (CFU/mL)
119	1.30E+03
245	1.00E+03
305	1.00E+02
442	0.00E+00
659	0.00E+00

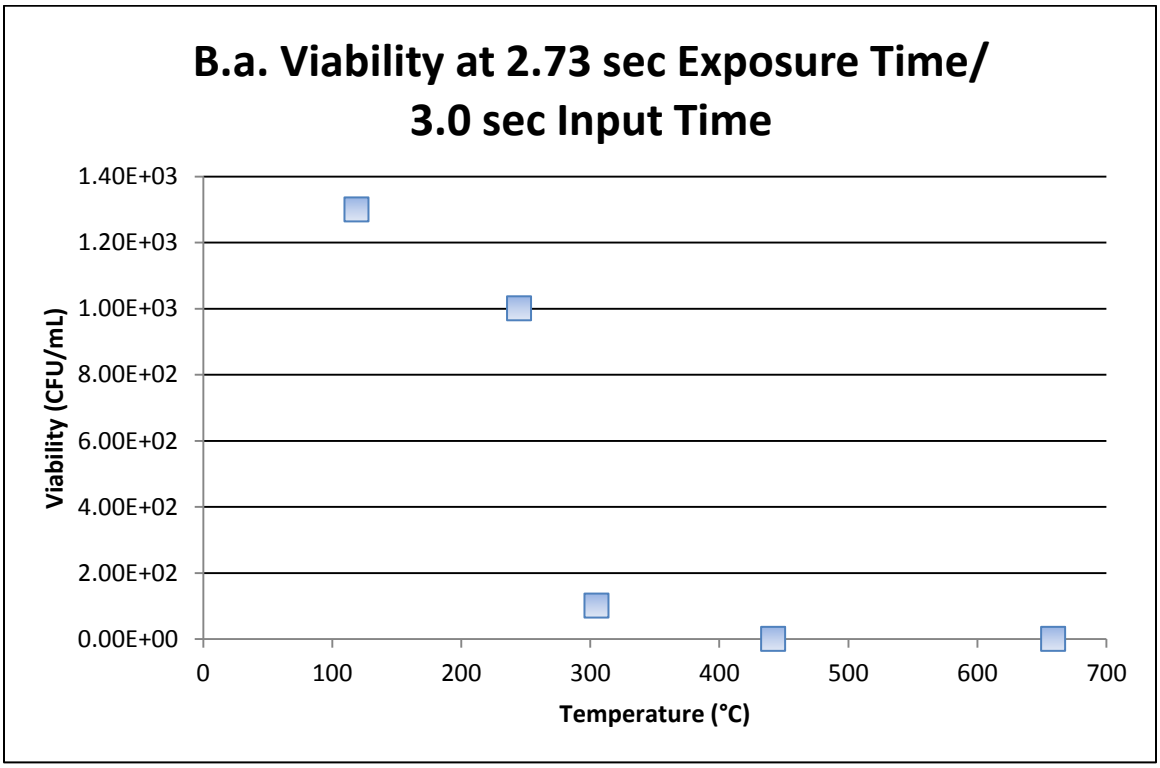


Figure 42. *B.a.* Viability at 2.73 sec Exposure Time/3.0 sec Input Time

Table 17. *B.a.* Viability at 4.0 sec Exposure Time/5.0 sec Input Time

Temp (deg C)	Viability (CFU/mL)
104	4.00E+03
178	8.00E+02
283	1.00E+02
417	0.00E+00
457	0.00E+00

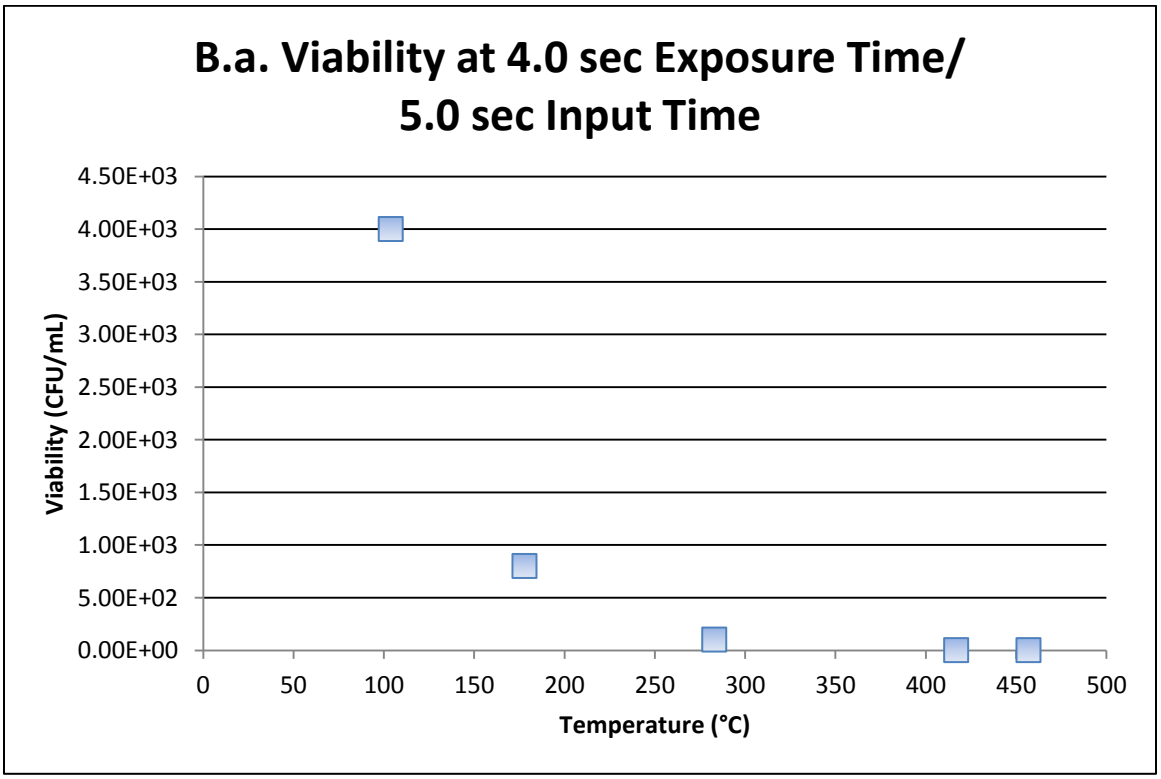


Figure 43. *B.a.* Viability at 4.0 sec Exposure Time/5.0 sec Input Time

Table 18. *B.a.* Viability at 5.98 sec Exposure Time/7.5 sec Input Time

Temp (deg C)	Viability (CFU/mL)
101	5.00E+03
167	4.00E+02
281	1.00E+02
301	0.00E+00
485	0.00E+00

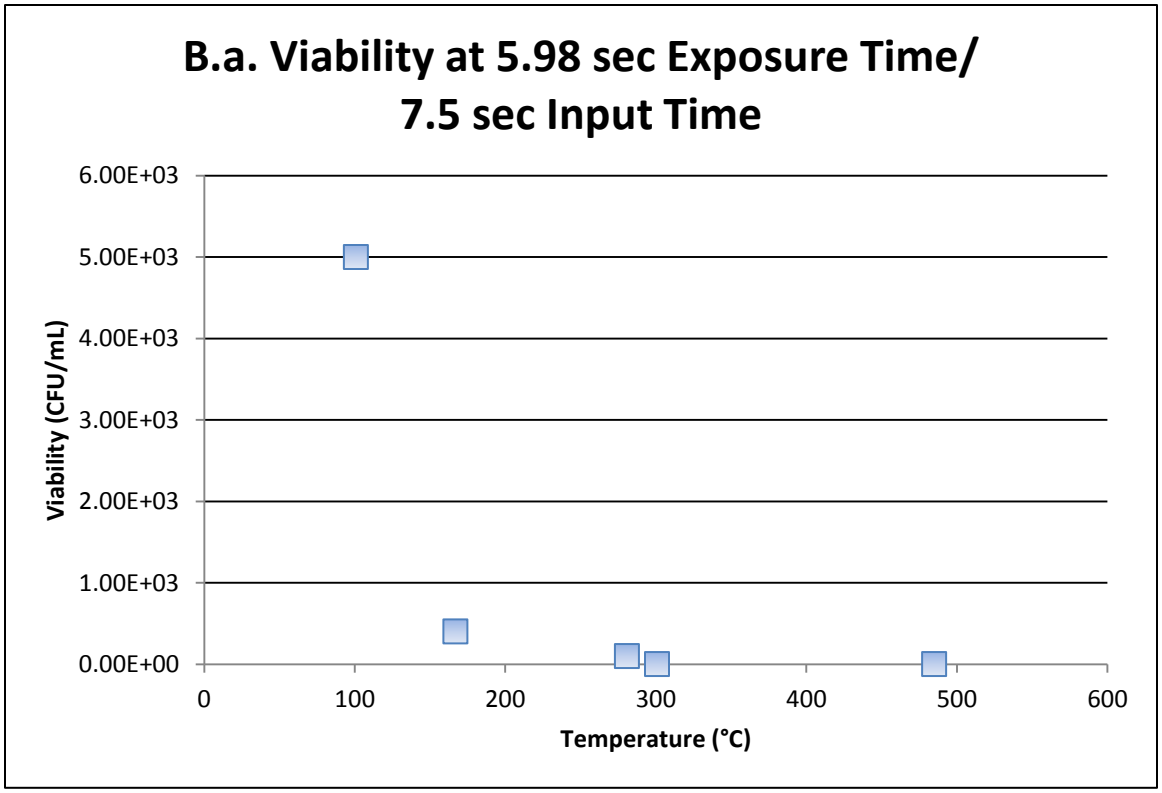


Figure 44. *B.a.* Viability at 5.98 sec Exposure Time/7.5 sec Input Time

Table 19. *B.a.* Viability at 7.0 sec Exposure Time/10.0 sec Input Time

Temp (deg C)	Viability (CFU/mL)
73.5	2.00E+03
121	3.00E+02
171	2.00E+02
271	1.00E+02
347	0.00E+00
487	0.00E+00

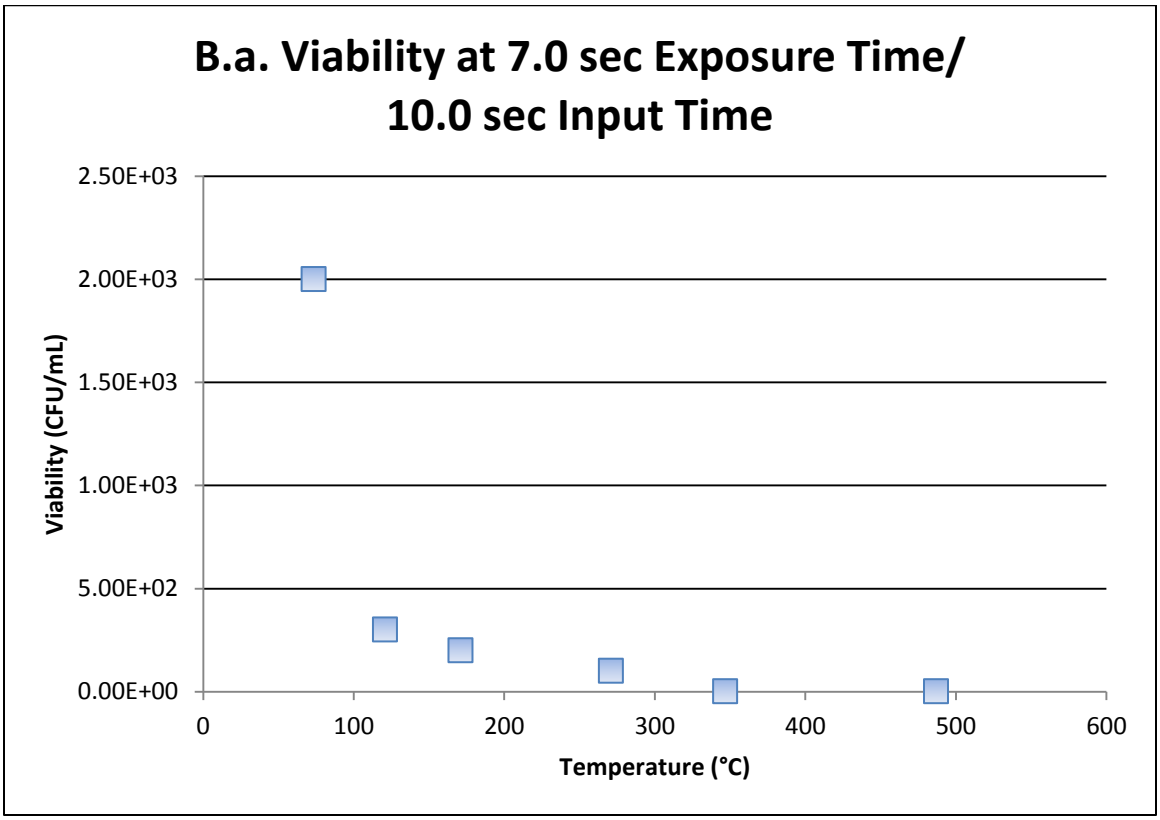


Figure 45. *B.a.* Viability at 7.0 sec Exposure Time/10.0 sec Input Time

4.10 Thermal Inactivation and Comparison to Previous Studies

The previous *B.a.* spore viability tables and plots were used to determine at what maximum temperature the spores were partially inactivated and completely inactivated at each of the 12 power supply input times/exposure times. The data was further reduced to create 1 table and 3 plots in order to observe the overall thermal inactivation outcomes which included 2 Arrhenius plots. The focus was to compare results to Battelle's 'Thermal Deactivation of Aerosolized Bacteria' (Alexander, Ogden, LeVere, Dye, & Kohler, 1999) due to that study having the closest similarity in terms of exposure times and temperatures to this study as compared to other studies. Key similarities and differences between the Grijalva and Battelle thermal inactivation studies will be further discussed, mostly reflecting upon the processes for data acquisition and analysis. Table 20 shows the loss of viability comparison between Grijalva and Battelle through displaying the temperature required to thermally inactivate spores (through partial inactivation). Figure 46 visually shows the partial and complete inactivation from this study in an Arrhenius plot; Figures 47 and 48 compare the data for thermal inactivation between Battelle and the study. Other studies will also be briefly discussed.

Table 20. *B.a.* Thermal Inactivation (Loss of Viability) Outcome

Time (sec)	Max Temp (deg C)	vs Battelle	Time (sec)	Temp (deg C)
0.258	860		0.13	880
0.4	629		0.3	730
0.488	618		0.45	660
0.623	611		0.95	575
0.813	581		2.7	490
1.2	545		9.7	360
1.71	434			
1.95	318			
2.73	305			
4	283			
5.98	281			
7	271			

The maximum temperature needed for the loss of viability of *B.a.* spores after being exposed to heat can be characterized through partial inactivation and complete inactivation. The definition for loss of viability is important to note; although different definitions exist, the general model stays the same (when *B.a.* is no longer growing to its typical levels). Battelle's definition for loss of viability is "showing a significant reduction in the ability to form colonies" which was usually the last temperature before reaching 10^1 spores according to the correlation made from analyzing all of the plots. Partial inactivation in this study was defined as the last temperature needed before no colonies formed; complete inactivation was defined by the first temperature at which colonies no longer formed. Fortunately, the temperatures needed to partially and completely inactivate the *B.a.* spores were achieved for each one of the exposure times in

this study. Figure 46 below visually shows the partial (last alive temperature) and complete (no surviving spores) inactivation from this study in an Arrhenius plot.

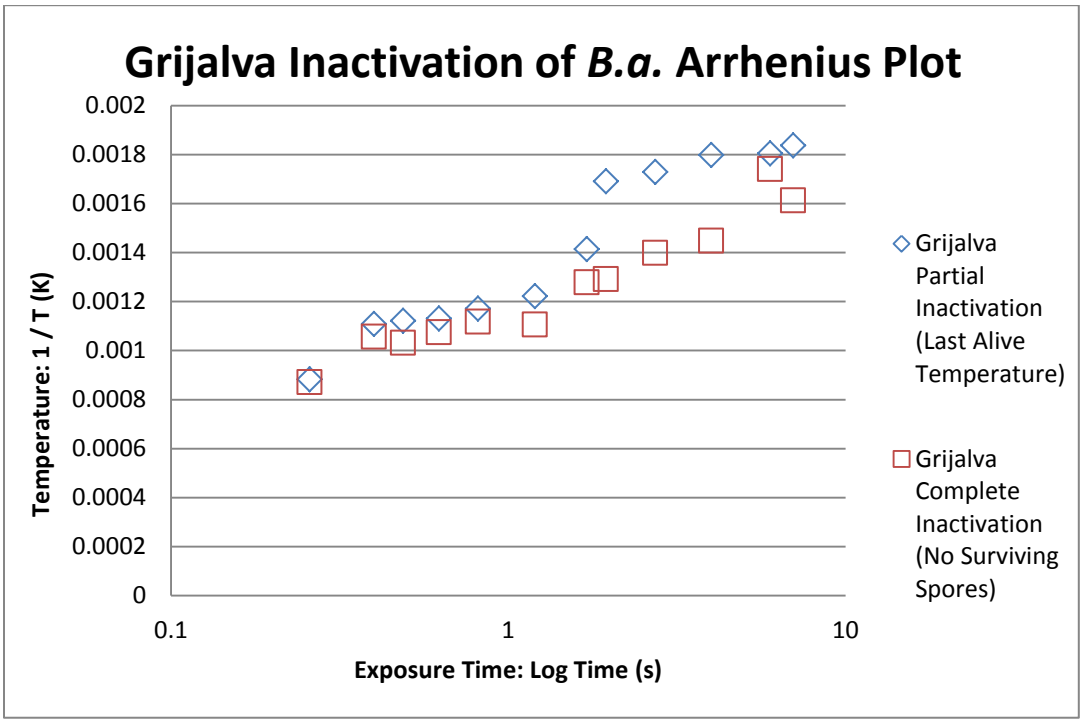


Figure 46. Grijalva Inactivation of *B.a.* Arrhenius Plot

Still, other similarities and differences remain between the Battelle data and this study. The times used for this study are the corrected exposure times from the power supply input times, as described previously in this chapter. For the comparable time ranges, thermal inactivation for Battelle was achieved through using different devices where flow rate was combined with a preheater on one device and direct injection without a preheater was used on the second device; these devices caused problems with correcting for time to reach temperature as in this study. By contrast, rapid resistive heating through a direct current power supply was used as the heat source for this study.

Time corrections to achieve the desired temperatures for both studies is also affected by Battelle aerosolizing the *B.a.* versus the *B.a.* adhering to a hard surface as in this study. The Battelle data also shows that 85% of the spores are typically accounted for within the device used but did not discuss loss of spores due to other issues post collection; it is very common, as was previously described in the chapter, for spore loss throughout the study. Another key difference was that the surrogate used for the Battelle data was *Bacillus thuringiensis* (*B.t.*) var. kurstaki while *Bacillus anthracis* (*B.a.)* Sterne strain was used as the bacterial genus and species in this study.

Figures 47 and 48 below compare the data for thermal inactivation between Battelle and this study through loss of viability plots with ones of those being an Arrhenius plot. As expected, both studies showed that higher temperatures were needed to inactivate the *B.a.* spores as exposure times decreased. Also, both data sets follow a similar trend line; therefore, this helps validate the methodology and analysis of this study when compared to that of Battelle. Although the spores in this study were thought to be completely dried through desiccation, there may have been more time and or indicator gel packets needed (greater than 40.5 hours with greater than 6 indicator gel packets). This means that the spores would have still contained some moisture and required lower temperatures for inactivation; Battelle used dry spores.

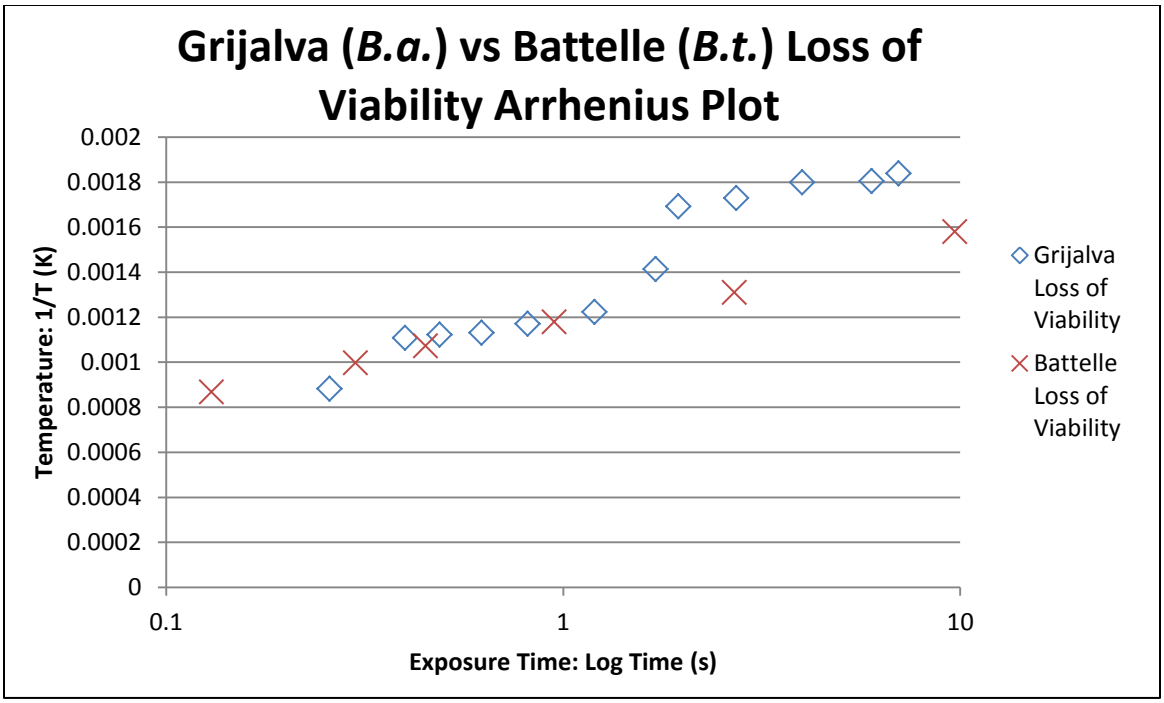


Figure 47. Grijalva (*B.a.*) vs Battelle (*B.t.*) Loss of Viability Arrhenius Plot

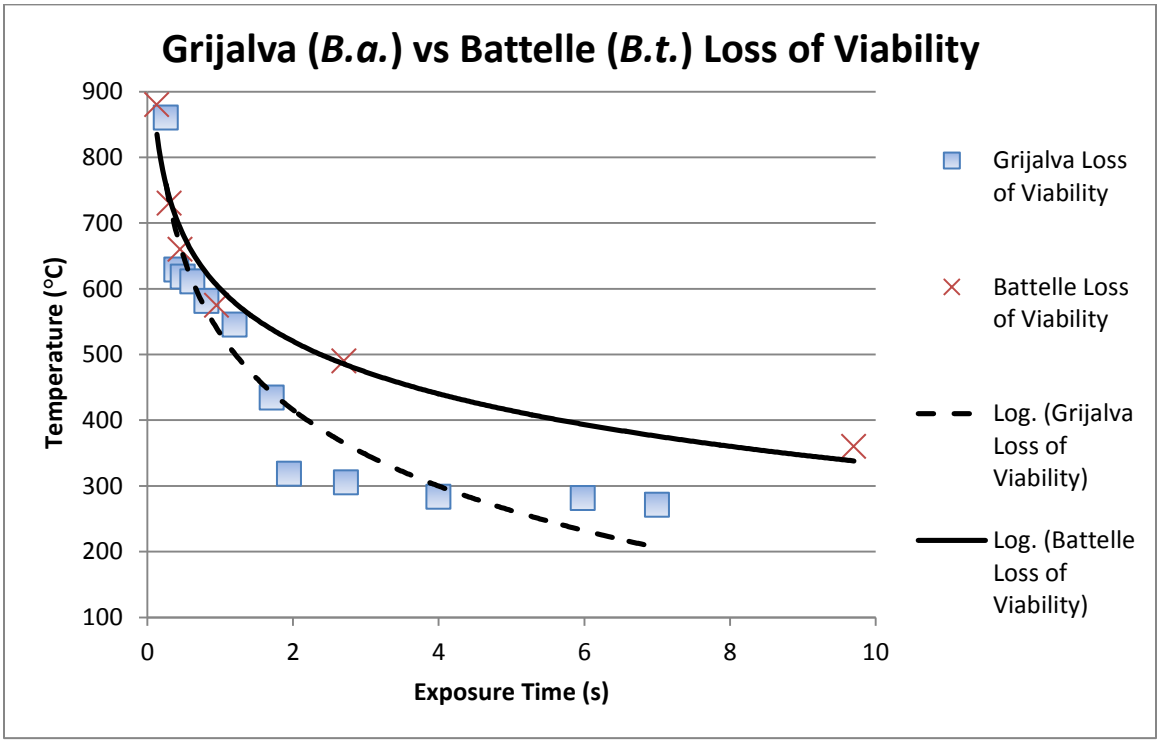


Figure 48. Grijalva (*B.a.*) vs Battelle (*B.t.*) Loss of Viability Plot

It is difficult to compare this study to literature for several reasons. Laboratories use different surrogates, setups, and methods of heating and extraction. It was also difficult to find prior literature, other than the ones mentioned in chapter 2 and this chapter, that had short exposure times, a good range of exposure times that included the short exposure times (range of 0.1 to 10 seconds), or studies that included enough statistical data points or that did not include multiple short bursts of applied temperature to achieve the high temperatures, such as in the Bacon data where laser irradiation was used for multiple heat bursts for thermal inactivation (Bacon, 2009). Although similar exposure times and temperatures are applied in some literature, the thermal inactivation of the bacteria can vary widely. For example, another study (Holwitt et al., 2000) used the exact same surrogate as Battelle and had a 350°C difference for its complete inactivation in comparison to temperature needed for Battelle's loss in viability (partial inactivation) at the 1-second exposure time; this is a huge disparity in temperature to inactivate the spores. The Battelle data and the data presented in this study were derived from one continuous burst of applying temperature for the thermal inactivation studies and both used several statistical data points to achieve the results that were presented.

V. Conclusions and Recommendations

5.1 Chapter Overview

This chapter provides an overview and summary of the thesis based on the results and analysis presented in the previous chapter. In addition, suggestions for future research related to this study will be discussed.

5.2 Conclusions

The initial goals of the thesis were met. The success of the thesis can be attributed to the materials, equipment, light bulb filament configurations and composition, and extensive prototyping and initial testing throughout the study. Additionally, the success from the thermal inactivation of *B.a.* spores using rapid resistive heating stemmed from the use of a setup which simulated an explosive environment.

The main goal was to simulate an environment where conventional munitions are used as agent defeat weapons on a *B.a.* stockpile or facilities and transportation vehicles containing stockpiles. The study could then use analysis from the heat exposures to help link the spore viability with the simulated explosive environment created by the agent defeat weapons. The most difficult aspect of the study was finding a method that could reach high enough temperatures (ranging from 800-900°C) at short exposure times (a range as low as 0.1 to 1 second). Therefore, the best success was the development of a thermal inactivation design to accomplish the required high temperatures while also achieving low exposure times. Although 888°C was the highest temperature reached during the inactivation testing with the *B.a.*-encrusted tungsten filaments (exceeded

temperature goal), temperatures reached were up to 995°C during prototyping and initial testing (without *B.a.* spores on the filaments).

The setup was created for the rapid resistive heating method for the thermal inactivation studies where the goal was to use a thin reproducible coiled wire. As the thickness of a wire decreases, the heat capacity also decreases which was ideal for thermal inactivation testing. In theory, the concept seemed like a viable path; however, the requirement to clamp the alligator clips from the power supply directly onto the wires/filaments for heat conduction caused the wires/filaments to continuously break during prototyping/initial testing as higher temperatures were applied due to the pressure of the clamps on the wire/filament. In cases where the wire/filament did not break, the coil would stretch out causing the hot coil and alligator clips to move. The coil stretching would have caused an unsafe laboratory environment due to increased risk of *B.a.* spore contamination spreading since stretching would increase *B.a.* spore loss from the coil throughout the study. Furthermore, the dynamic environment caused by the stretching would create a non-reproducible thermal inactivation setup which would decrease the validity of the results and limit future research. A light bulb filament was thin and reproducible but to counteract the breakage described above, the idea to use a light bulb filament was further modified by not removing it from the light bulb. Instead of directly applying the alligator clips to the light bulb filaments, the whole bulb (minus the envelope) was screwed into a lamp where the alligator clips were instead clamped onto cut and exposed copper wire leads from the lamp. The alligator clips and exposed copper wire leads were placed and secured on a safe non-conducting glass surface. This became

the design for the rapid resistive heating method used in the thermal inactivation studies. The setup proved to be the best option during prototyping and initial testing since the setup was reproducible and the lamp could be in the same position during all of the subsequent testing (sheet of paper was taped to the table with a drawn circle for the lamp to stay in between). The setup also ensured a higher *B.a.* recovery could be achieved due to the filaments not being stretched and broken by the heat, pressure, and weight of the alligator clips as in the previous set of initial prototyping tests. Finally, the setup achieved the goal of producing target high temperatures at low exposure times.

The next goal was to attempt to achieve and record the temperatures necessary to partially and completely inactivate the *B.a.* spores to determine a correlation between exposure time, temperature, and spore viability. In this study, higher temperatures were needed to thermally inactivate the *B.a.* spores as exposure times decreased. The thermal inactivation trend from this work is similar to previous studies. The temperatures needed to partially and completely inactivate the *B.a.* spores were achieved for each of the 12 exposure times used in the study. Most notably, *B.a.* spores at the 0.1 sec power supply input time/0.258 sec corrected exposure time were partially inactivated at 860°C and completely inactivated at 876°C. There is much more to learn about the thermal inactivation of *B.a.* spores caused by short duration heating. Only limited data exists in previous studies which characterize the time and temperature relationships for exposures due to the sparse data found on the exposure time range of 0.1 to 1 second with one continuous heat burst; therefore, using a methodology that can reach these specifications helps fill the gap in literature and gives the basis for future research at AFIT. Finally,

temperature ranges for the inactivation of spores at the 1-second exposure time even varied by a 350°C difference across the limited literature in that time range (Holwitt et al., 2000 and Alexander, Ogden, LeVere, Dye, & Kohler, 1999); this is a huge discrepancy in literature.

5.3 Recommendations for Future Work

The research included in this study investigated the thermal inactivation of *B.a.* spores using rapid resistive heating. While the goals of this research were met, avenues for improvement and scientific exploration are still available and should be pursued.

The first step in improving upon the study is to apply spore samples to the filaments that contain a higher spore population density. More robust results could be obtained for spore viability, to include partial and complete inactivation, with a higher spore count (using higher density spore solutions). The design and methodology that was tested and used in this study could be applied to more prototyping/initial testing to see where more of the spore loss occurred and to ensure enough spores can be recovered to account for the high percentage of spore loss that occurs throughout the study. Although a higher spore population density is desired, the goal is to apply a thin layer of spores to the filament in order to prevent clumping which can cause inaccurate results due to the spores not having direct contact to the heat source. When considering spore density in future studies, it is important to note that according to research (Harwood and Cutting, 1990), "Sonication is a useful and relatively simple cell breakage technique; however, it is extremely variable, even between similar samples, and it is only effective on cell suspensions of relatively low density ($< 2 \times 10^9$ cells/ml)." It is suggested that a much

higher density of spores be used in future work to handle the high spore losses found in multiple places throughout this study (as was also found in other studies in literature).

For this study, one of the issues was that were large gaps in the data between temperatures analyzed for partial versus complete inactivation due to sparse data within those exposure times; thus, it is assumed complete inactivation should be a lower temperature than the complete inactivation quoted in this thesis and the partial inactivation should be a higher temperature than the partial inactivation quoted in this thesis. To prevent this, future research can be focused so that more than 100 heat exposures are done for the 12 exposure times or less exposure times are done for the 100 heat exposures so that research and analysis can focus on a tighter time range with several more data points.

Desiccation and sonication (as the hard surface recovery method) were found to be the main contributors to spore loss in this study but further testing could provide additional data points for better insight about where spore loss occurred throughout the study. A potential beneficial modification to the methodology would be to have the machine shop build custom covers to put on top of the light bulb base so that prototyping and initial testing could be accomplished to determine how many spores are lost due to desiccation or spores falling off of the filament during thermal inactivation studies and while screwing in and out the light bulb base from the lamp before and after the studies. Hard surface recovery could also be employed on the custom covers so those spores can serve as additional data points when analyzing spore thermal inactivation.

Performing thermal inactivation testing on both wet and desiccated samples is an area of work that is worth exploring. The data excursion would help simulate a humid environment which could affect the response variable. Additionally, thermal inactivation studies would be more effective on reducing the viability of spores using wet samples. Lower temperatures across the board would be required to partially and completely inactivate the wet spore samples when compared to the desiccated spore samples. A comparison of the thermal inactivation for both the wet and desiccated samples using short exposure times with high temperatures with the setup used would increase the limited literature for this specific application. A future study for a wet environment should utilize a thin spore layer on the wet samples in order to decrease clumping and obtain more accurate thermal inactivation results.

An alternative method of analysis with time-temperature profiles is to determine average maximum temperatures for the corrected time exposures versus the maximum temperature for the corrected time exposures. This change would account for slightly lower temperatures overall for the corrected exposure times for maximum temperature. In this study the maximum temperatures were paired with the corrected time exposures. It may be more suitable to study the maximum temperatures versus the average maximum temperatures depending on the constraints of FLIR and player limitations.

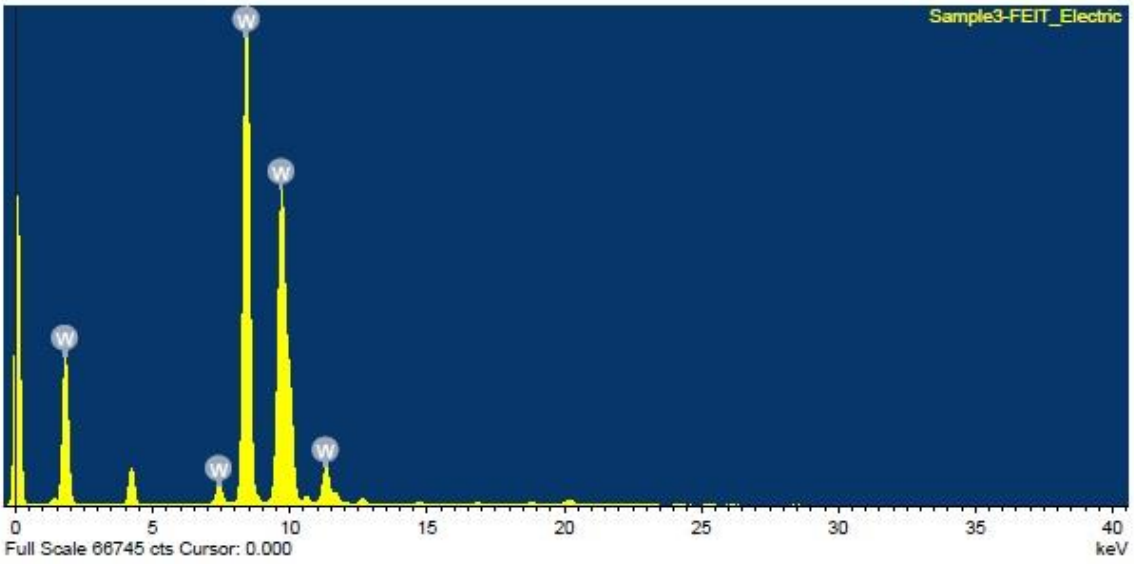
It is also highly recommended to continue the practice of extensive prototyping and initial testing. Not much discussion or evidence of prototyping and initial testing is found in many previous studies. Initial prototype testing serves to ensure goals for the study are met and that better designed methodologies and instrumentation are used to

yield better results. Microbiological studies especially need this attention to ensure spores are purified to proper levels and there is less contamination throughout the study.

Finally, if funding allows, updated equipment and software would greatly help with performance enhancement and more accurate analysis and results for the thermal inactivation study. For example, a FLIR Thermacam that can measure more frames per second, or if possible, has the capability to read all temperatures without having to manually change the temperature range setting would vastly improve temperature readings and time temperature profiles, especially due to the fact that there was also a limitation where temperatures below 215°C could not be picked up by the FLIR in the highest temperature range setting. Secondly, the amperage and voltage settings on the power supply had to be maxed out in order to achieve the highest temperatures needed at the shorter exposure times for this study; a more powerful power supply could help decrease power supply malfunction and mitigate potential issues that can be encountered from continually maxing out such a device. In addition, higher temperatures could also be achieved with a more powerful power supply but initial prototype testing would have to confirm that the setup could handle the current from the power supply. Finally, the recorder and player could also be updated to allow for a more comprehensive review of the data needed to more precisely determine maximum temperatures and more accurate time-temperature profiles for the heated *B.a.* spore samples.

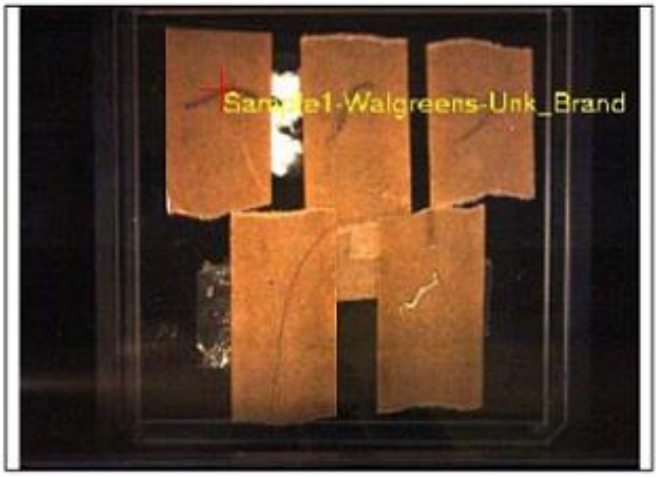
Appendix A. Horiba X-Ray Analytical Microscope XGT-7000

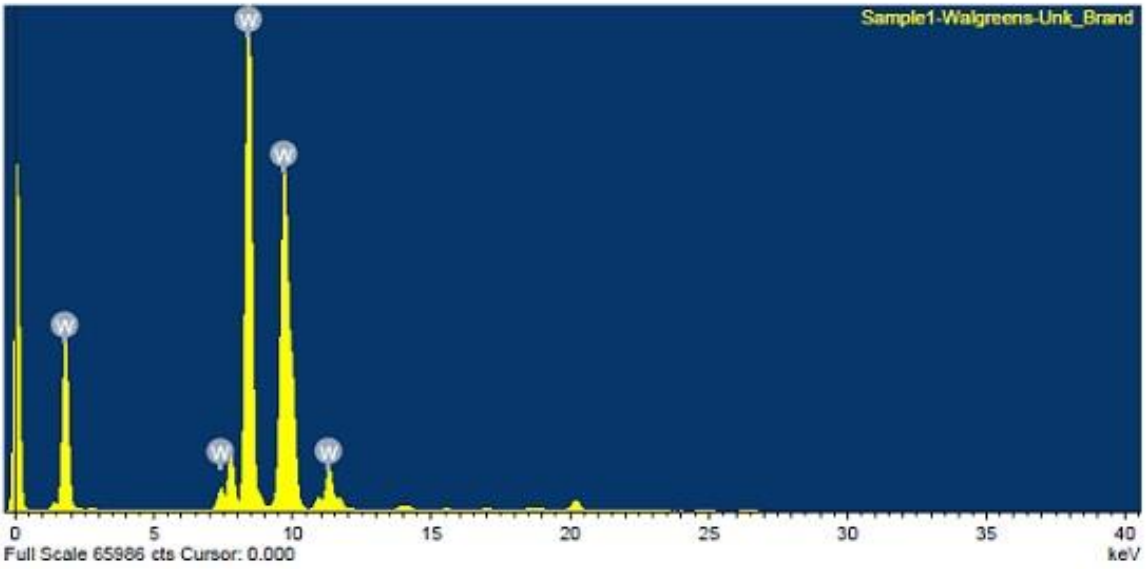
Appendix B. X-ray analytical microscope analysis for FEIT Electric filament



Spectrum Name	
Label :	Sample3-FEIT_Electric
Collected :	5/12/2010 2:17:14 PM
Live Time	600.0 [s]
X-ray tube vol.	50.00 [kV]
Current	1.000 [mA]
Process time :	2
XGT Dia.	100 [µm]

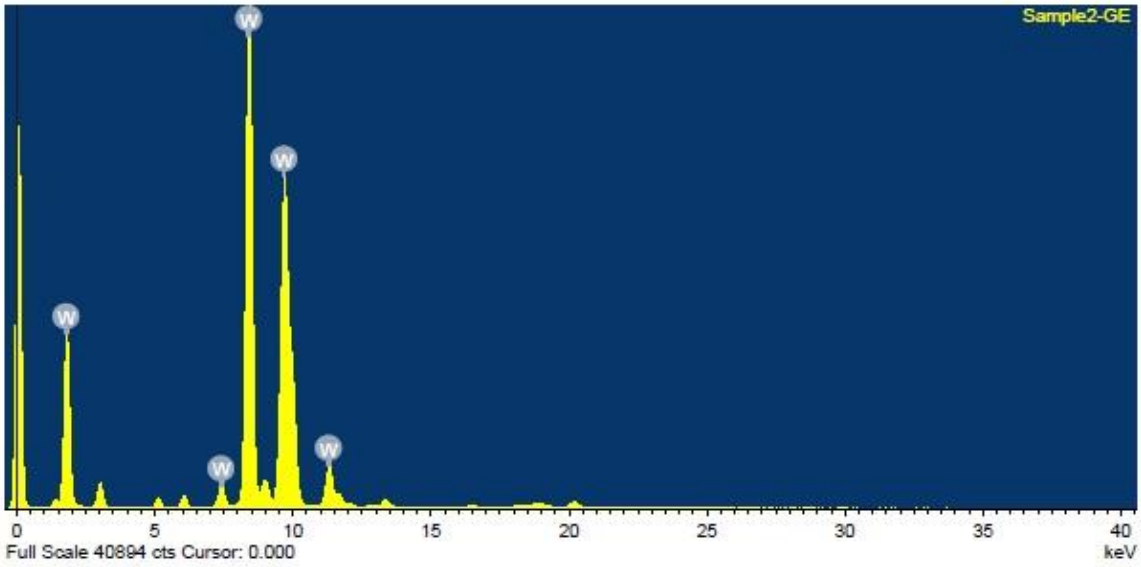
Appendix C. X-ray analytical microscope analysis for Walgreens filament





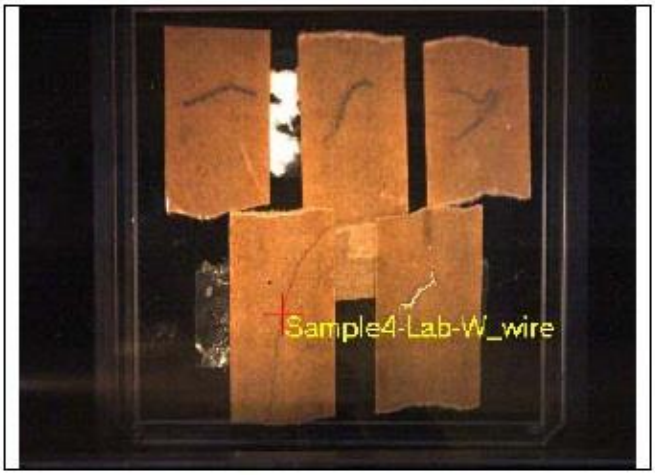
Spectrum Name	
Label :	Sample1-Walgreens...
Collected :	5/12/2010 1:46:14 PM
Live Time	600.0 [s]
X-ray tube vol.	50.00 [kV]
Current	1.000 [mA]
Process time :	2
XGT Dia.	100 [µm]

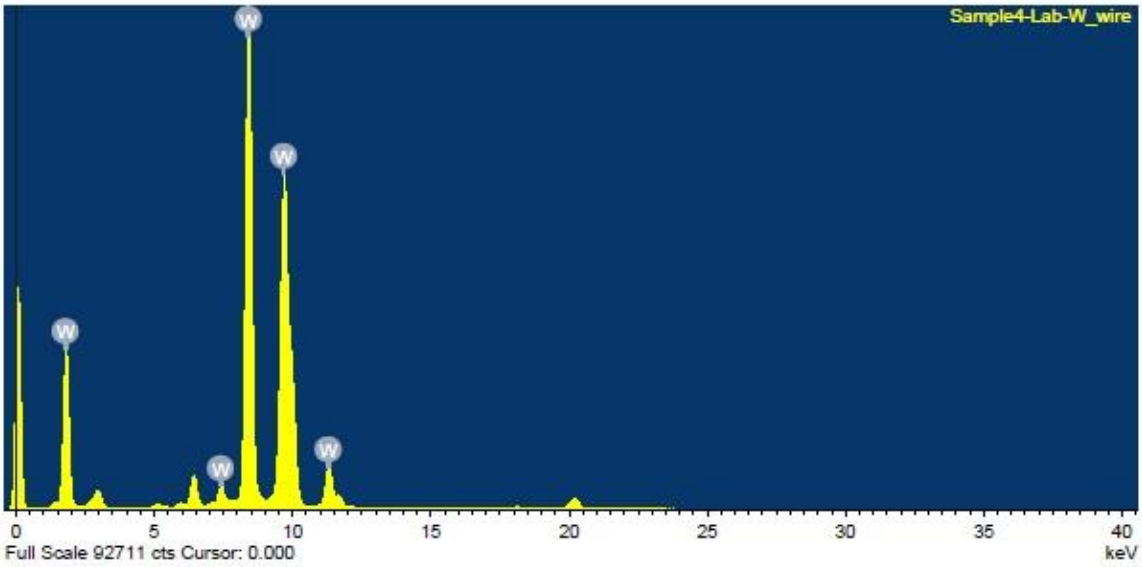
Appendix D. X-ray analytical microscope analysis for General Electric filament



Spectrum Name	
Label :	Sample2-GE
Collected :	5/12/2010 2:46:44 PM
Live Time	600.0 [s]
X-ray tube vol.	50.00 [kV]
Current	1.000 [mA]
Process time :	2
XGT Dia.	100 [µm]

Appendix E. X-ray analytical microscope analysis for laboratory wire





Spectrum Name	
Label :	Sample4-Lab-W_wire
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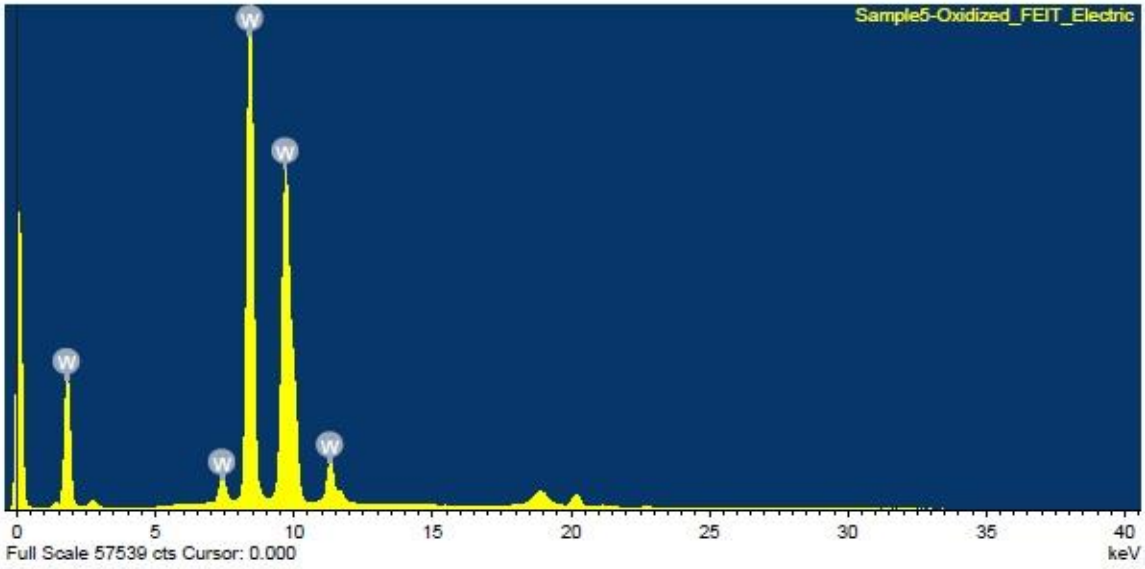
Live Time	600.0 [s]
X-ray tube vol.	50.00 [kV]
Current	1.000 [mA]
Process time :	2
XGT Dia.	100 [µm]

Appendix F. X-ray analytical microscope analysis for oxidized FEIT Electric filament



Spectrum Name	
Label :	Sample5-Oxidized_...
Collected :	5/12/2010 3:30:52 PM
Live Time	600.0 [s]
X-ray tube vol.	50.00 [kV]
Current	1.000 [mA]
Process time :	2
XGT Dia.	100 [µm]

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14. ABSTRACT

Bacillus anthracis (*B.a.*) is the causative agent of anthrax. *B.a.* spores pose a threat because have been used as a bioweapon throughout history and can survive harsh conditions for prolonged periods of time. Rapid resistive heating was used for the thermal inactivation of spores in order to determine *B.a.* spore viability, to include partial and complete thermal inactivation. This microbiological study sought to obtain a correlation between exposure time, temperature, and spore viability. This information is invaluable when modeling employment effects for agent defeat weapons to destroy *B.a.* stockpiles through thermal inactivation. Partial and complete thermal inactivation of the spores were found using rapid resistive heating at short duration exposure times from 0.258 to 7 seconds and temperatures ranging from 73.5 to 888°C. Power supply pulses were created by applying varying voltages ranging from 9 to 200 Volts for 12 separate power supply input times ranging from 0.1 to 10 seconds. Higher temperatures were needed to thermally inactivate the *B.a.* spores as exposure times decreased.

15. SUBJECT TERMS

Biological weapons, bioweapons, *Bacillus anthracis*, *B.a.*, spores, agent defeat weapons, stockpiles, heat inactivation, rapid resistive heating, thermal inactivation, *Bacillus*, anthrax, *Bacillus thuringiensis, B.t.*, weapons of mass destruction, combating weapons of mass destruction, counterproliferation, countering weapons of mass destruction, spore inactivation

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