BIODEGRADATION OF *BACILLUS ANTHRACIS* ENDOSPORES IN COMPOST

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Dedication

"We do not want you to be uniformed… about the troubles we experienced ...we were under great pressure, far beyond our ability to endure, so that we despaired of life itself… but this happened that we might not rely on ourselves but on God, who raises the dead."

-2 Corinthians 1:8-9

Abstract

Anthrax, a lethal infectious disease affecting primarily herbivores, is caused by an endospore-forming bacterium, *Bacillus anthracis* (*BA*). Endospores from *BA* are extremely stable in the environment and pose a communicable risk as a natural zoonosis and as a potential bioweapon. Composting results in microbial biodegradation of organic matter, reaching temperatures up to 70°C. I investigated the ability of compost to inactivate *BA.* Surrogate strains were sporulated over a range of temperatures to mimic natural sporulation environmental variations and tested in two separate experiments. The first, exposed four surrogate strains to temperatures mimicking a previous long-term (150 days) field-scale compost temperature profile. The second, composted *BA* endospores from two surrogate strains in laboratory scale composters for 33 days. Composting resulted in a 2 and 4 $\log_{10}$ reduction in endospore viability in oven and lab scale experiments, respectively. Results suggest composting has potential as a disposal method for anthrax related mortalities.

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ATCC	American Type Culture Collection
AVA	anthrax vaccine adsorbed
BA	*Bacillus anthracis*
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
C/N	carbon to nitrogen ratio
CCME	Canadian Council of Ministry of the Environment
CFIA	Canadian Food Inspection Agency
CFU	colony-forming unit
CWD	chronic wasting disease
d	day
DNA	deoxyribonucleic acid
DPA	pyridine-2,6-dicarboxylic acid (dipicolinic acid)
EC	electrical conductivity.
EF	edema factor
HHS	U.S. Department of Health and Human Services
L1	*Bacillus licheniformis* ATCC® 14580™
L2	*Bacillus licheniformis* JB0501-3
LD_{50}	lethal dose
LF	lethal factor
MAPK	mitogen-activated protein kinase
NSMP	nutrient sporulation medium including phosphate
NTDENR	Northwest Territories department of Environment and Natural Resources
PA	protective antigen
PBS	phosphate buffered saline
PBS-BSA	PBS containing 1% bovine serum albumin
PCR	polymerase chain reaction
RMP	rotations per minute
RNA	ribonucleic acid
SASP	small acid-soluble endospore proteins
Spore	endospore
USDA	US Department of Agriculture
USEPA	United States Environmental Protection Agency
T1	*Bacillus thuringiensis* ATCC® 33679™
T2	*Bacillus thuringiensis* 4A3
TC	total carbon
TN	total nitrogen
US	United States of America

Chapter 1

1. Anthrax disease

1.1 Outbreak history of anthrax

Anthrax is a lethal mammalian disease, capable of affecting humans and remains a continual global problem with outbreaks dating back to the middle ages (Spencer, 2003; Sternbach, 2003). Scholars believe that the fifth (grievous murrain) and sixth plagues (boils) described in the Old Testament were anthrax, which occurred about 1445 B.C. (Dragon & Rennie, 1995; Goel, 2015; Guichard *et al*., 2012). The first European pandemic, called the "Black Bane" of 1613 resulted in the death of approximately 60,000 cattle (Organization & Epizootics, 2008). The bacterium *Bacillus anthracis* that causes anthrax, was first isolated in 1863 by the French scientist Casimir-Joseph Davaine (Goel, 2015; Guichard *et al.,* 2012) and later identified by the German physician and scientist, Robert Koch in 1875 (Goel, 2015; Turnbull, 1999). In South Africa, the frequency of anthrax outbreaks peaked in 1923 with yearly estimates of 30,000 to 60,000 animal mortalities (Hugh-Jones & De Vos, 2002) In 1945, an outbreak of anthrax in Iraq killed over one million sheep (Turnbull & Shadomy, 2010) and one in Zimbabwe in 1978-1980 led to about 10,000 human infections and 182 deaths (Goel, 2015; Kobuch *et al.,* 1990). One of the largest epidemics associated with anthrax infection by inhalation occurred in 1979. Approximately, 100 individuals were infected following an accidental release of infectious *B. anthracis* endospores. These aerosolized endospores originated from a Soviet military facility in Sverdlovsk and resulted in ≈ 70 human deaths (Meselson *et al.,* 1994; Spencer, 2003; Sternbach, 2003).

Before the development of a vaccine in the 1930's, *B. anthracis* was one of the leading causes of global mortalities in cattle, goats, horses and sheep (Hugh-Jones & Blackburn, 2009; Hugh-Jones & De Vos, 2002; Logan & De Vos, 2009). Between 1979-1980, there were 179 fatal cases and over 94,000 cutaneous anthrax cases reported in Zimbabwe (Goel, 2015). In 1979, Australia experienced a month-long epidemic of anthrax that required the vaccination of approximately 50,000 cattle and 1700 sheep (Hugh-Jones, 1999; Turnbull, 1998; Turner *et al.,* 1999). Even with the availability of a vaccine, anthrax still remains a global problem, with over 40 countries reporting anthrax cases in 2000 (Hugh-Jones & De Vos, 2002). There are still areas where *B. anthracis* is considered endemic such as Africa and Asia, which have large herbivore populations that are susceptible to infection. As of 2012, outbreaks have been reported in 67 countries, with Antarctica being a notable exception. Indicating a possible environmental correlation with susceptibility of outbreaks of anthrax. As of January 2019, 81 cases of cutaneous anthrax were reported in Tanzania, Africa. This outbreak included four deaths and was thought to have originated from handling or consuming the 16 infected cattle carcasses from the outbreak of anthrax in November 2018 (WHO, 2019). As of April 2018, U.S. Department of Health and Human Services (HHS) allocated $25 million to stockpile an anthrax antitoxin, anthim®, as part of a medical countermeasure to protect against national security threats (HHS, 2018). It is clear that anthrax still remains a threat in the 21st century and as *B. anthracis* occurs naturally in soils world-wide it will undoubtedly remain a significant health threat for the foreseeable future.

1.2 Global Impact

Anthrax outbreaks have strong socioeconomic impacts globally. It has been estimated that around 1 billion people earn less than two dollars US per day and rely on livestock for a

significant portion of their livelihood (Grace, 2015; Grace *et al.,* 2012). Anthrax outbreaks have a high economic impact on livestock trade internationally but developing communities are particularly vulnerable (Grace *et al.,* 2012). These poor communities often cannot afford the costs associated with proper vaccinations and/or antibiotics or the decontamination of infected materials that arise as a result of livestock mortalities. Often these animals are multipurpose to the farmers or ranchers/shepherds. Communities rely on livestock for transportation (humans and heavy loads), assistance in working their land (tilling, seeding, pulling), fertilizer (manure), food (meat and milk products), clothing (hides), religious beliefs (ceremonial instruments and sacrifices) and as a sign of wealth (marriage dowries) (Upton, 2004).

1.2.1 Canadian Anthrax Outbreaks

In Canada, outbreaks of anthrax have occurred from Ontario to Alberta and there have been repeated outbreaks in the Mackenzie Bison Range in the Northwest Territories, Wood Buffalo National Park in Northern Alberta and in some areas of Manitoba and Saskatchewan. In Canada, cattle are the most common livestock species that develop anthrax, followed by horses and sheep. From June to September 2006, the Canadian Food Inspection Agency (CFIA, 2013) reported 806 livestock deaths across 153 sites as a result of anthrax outbreaks. These losses included 493 cattle, 254 bison, 6 horses, 33 sheep, 13 cervids, 3 pigs and 2 goats (Himsworth & Argue, 2008). The Northwest Territories department of Environment and Natural Resources (NTDENR) estimates that an anthrax outbreak in July 2012 resulted in the mortality of at least 450 bison, representing a third of the original Mackenzie Wood Bison (*Bison bison athabascae*) population (Beaumont, 2013; NTDENR, 2013). In March 2012, the bison population was estimated at 1531, but one year later it dropped to approximately 714 animals (Beaumont, 2013). Disposal

methods during this outbreak were limited due to remote locations and as a result the carcasses were covered with tarps to help prevent scavenging and allowed natural putrefaction (NTDENR, 2013). This impacted the local socioeconomic status of the Mackenzie indigenous communities as hunting tags were rescinded for the 2012-2013 hunting season. In September 2012, all harvesting of the Mackenzie bison populations was closed to allow the population to recover (NTDENR, 2013).

In October 2018, an outbreak of anthrax was reported on a farm near Fort St. John in British Columbia. There were 13 confirmed deaths on a farm of more than 150 animals (MOA, 2018). In 2006, infections were documented in two farmers that contracted a cutaneous form of anthrax during an outbreak in cattle in Saskatchewan (CDC, 2018). Cutaneous is the only form of human infection ever reported in Canada (MOA, 2018; CDC, 2018).

1.3 Causative agent of anthrax

Within the *Firmicutes* taxa, *Bacillus* is a Gram-positive, aerobic, rod-shaped bacterium (Table 1-1). This group is distinguished by their low G + C content and their ability to enter a state of dormancy by undergoing sporulation to produce endospores that can remain viable for decades within soils (Goel, 2015; Setlow, 2003). Members of the *Bacillus* group including *Bacillus cereus, Bacillus thuringiensis,* and *B. anthracis* possess very similar genomes (<1% difference), but differ dramatically in their impacts on the health of livestock and humans. Members of this genus are linked to food poisoning, serve as a biological insecticide and cause anthrax, respectively (Medini *et al.,* 2005).

Table 1-1. Differential characteristics of *Bacillus* genus (LOGAN & DE VOS, 2009).

Differential Characteristics	*B. anthracis*	*B. atrophaeus*	*B. cereus*	*B. licheniformis*	*B. mycoides*	*B. subtilis*	*B. thuringiensis*
Endospore Formation	+	+	+	+	+	+	+
Gram Positive	+	+	+	+	+	+	+
Aerobic Growth	+	+	+	+	+	+	+
Anaerobic Growth	+	-	+	+	+	-	+
Motility	-	+	+	+	-	+	+
Lecithinase Activity	+	-	+	-	+	-	+
Rhizoid colonies	-	-	-	-	+	-	-
Parasporal Crystals	-	-	-	-	-	-	+
10°C	-	+	-/+	-	-/+	-/+	-/+
20°C	+	+	+	+	+	+	+
30°C	+	+*	+*	+	+*	+*	+*
37°C	+*	+	+	+*	+	+	+
40°C	+	+	+	+	-/+	+	+
50°C	-/+	+	?	+	-	-/+	-

*Optimal Growth Temperature

Distinguishing characteristics of these bacteria arise mainly due to genes found on highly mobile plasmids. For example, the δ-endotoxins, responsible for the insecticidal properties of *B. thuringiensis* are encoded by the *cry* gene which is plasmid-associated (Hill *et al.,* 2004). *B. cereus,* which is commonly found in starch foods, is a major food-

borne pathogen capable of causing gastrointestinal disease (Stenfors Arnesen *et al*., 2008) A condition known as *B. cereus* emetic syndrome is caused by cereulide production and is also plasmid-encoded (Stenfors Arnesen *et al.,* 2008). Occasionally, *Bacillus subtilis* and *Bacillus licheniformis* are also associated with food poisoning (Medini *et al.,* 2005), but *B. anthracis*, the causative agent of anthrax, is considered to be the only obligate pathogen among the *Bacillus* genus (Logan & De Vos, 2009; Organization & Epizootics, 2008).

1.3.1 Pathogenesis

The pathogenic potential of *B. anthracis* is dependent on the presence of two plasmids (pXO1 and pXO2), encoding for a tripartite toxin and the bacterial capsule respectively, both of which are essential for evasion of the host immune system (Ala'Aldeen, 2001; Dixon *et al.,* 1999; Scorpio *et al.,* 2006; Taft & Weiss, 2008). The pXO1 plasmid is involved in the expression of lethal factor (LF) and edema factor (EF) as well as a protective antigen (PA) (Helgason *et al.,* 2000), while pXO2 encodes for the formation of the γ-linked poly-D-glutamic acid capsule (Goel, 2015; Prince, 2003). The protective antigen binds to the host cell, allowing the entry of LF and EF. The combination of PA with EF, which is a calmodulin-dependent adenylate cyclase, produces the conditions that are favorable for the development of edema. When PA combines with LF, a zinc metalloproteinase, mitogen-activated protein kinase (MAPK) is cleaved, causing the cell to lyse (Dixon *et al.,* 1999; Prince, 2003). *B. anthracis* has the ability to evade the host's immune system as the capsule is not a polysaccharide, but composed of amino acids that are not recognized by the host's immune system, inhibiting leukocyte activity (Goel, 2015; Turnbull, 1999; Ala'Aldeen, 2001). The pathogen enters macrophages where it multiplies safely eventually killing the macrophage (Goel, 2015; Twenhafel, 2010).

Although a peptide capsule is not common in bacteria, some species, including *B. subtilis*, *Bacillus megaterium*, and *B. licheniformis* synthesize glutamic acid polymers that can either constitute a capsule or be secreted into the medium. The biochemical structures of *B. anthracis* and *B. licheniformis* capsules are identical, and the same precursor, L-glutamic acid is used for capsule formation, suggesting similar biosynthetic pathways (Mock & Fouet, 2001).

1.3.2 Infectivity

The infectious agent of anthrax are the dormant endospores of *B. anthracis,* as the bacterium itself is fragile when it is in a metabolically active - vegetative state (Setlow, 2014). During nutrient deprivation, *B. anthracis* cells undergo sporulation to produce endospores. Endospores are the primary infectious form of this organism, with infection of the mammalian host occurring through ingestion, inhalation or cutaneous exposure. *Bacillus* endospores are exceptionally resistant to environmental stressors such as heat and desiccation, enabling them to remain viable in contaminated food and soil for decades if not centuries (Nicholson *et al.,* 2000). Although considered metabolically dormant, *B. anthracis* endospores respond to changes in the environment, and when conditions are favourable such as after ingestion by a suitable host, they germinate and produce vegetative cells. Grazing herbivores are particularly vulnerable to anthrax infection, but all mammals including humans are susceptible. Endospores can enter the body of mammals through cutaneous abrasions or by absorption in the respiratory or gastrointestinal tract. Grazing herbivores are particularly vulnerable to infection as abrasions in the oral cavity are common (Goel, 2015; Dragon & Rennie, 1995). The infectious dose of *B. anthracis* varies among host species and with route of infection. As few as ten inhaled *B. anthracis* endospores are thought to be sufficient to infect cattle and

sheep (Organization & Epizootics, 2008). Cattle (*Bos taurus*) and sheep (*Ovis aries*) are considered most susceptible, followed by horses (*Equus caballus*) and goats (*Capra aegagrus hircu*) (Watson and Kier, 1994). Birds and reptiles are resistant to anthrax, possibly a reflection of their role as scavengers, whereas humans exhibit an intermediate susceptibility to infection. The higher and lower body temperatures of birds and reptiles, respectively, has also been proposed to contribute to the reduced susceptibility of these hosts (Goel, 2015; Dragon *et al.,* 1999). The average body temperature of birds is 42°C as compared to 39°C in cows and goats. In Canada, cattle (*Bos taurus*) were the most common livestock species associated with anthrax outbreaks, going back to the early twentieth century (Dragon *et al.,* 1999) with bison (*Bison bison*) being the most common infected in the recent outbreaks.

In humans, 2,500- 55,000 inhaled endospores are considered the average lethal dose (LD_{50}) (Wallin *et al.,* 2007). It has been reported that as few as ten inhaled *B. anthracis* endospores can lead to infection in cattle and sheep (Watson and Kier, 1994; Smith, 1973). Upon infection through ingestion, inhalation or cutaneous exposure, macrophages take up the infectious endospores by phagocytosis, which then germinate to produce vegetative *B. anthracis* cells (Dixon *et al.,* 1999; Nicholson, 2002). Cutaneous exposure is the most common method of acquisition and often leads to black-crusted sores or eschars. The Greek word for coal is *anthrakis*, and is the origin of the term 'anthrax' (Dixon *et al.,* 1999; Goel, 2015). Inside the macrophage, *B. anthracis* proliferates releasing endotoxins that result in edema, haemorrhaging, necrosis, and septicaemia, which is frequently fatal if untreated (Dixon *et al.,* 1999; Taft & Weiss, 2008; Twenhafel, 2010). The concentration of vegetative *B. anthracis* cells in bodily fluids following death by sepsis can range from 10^7 -10^8 per mL (Dixon *et al.*, 1999). Acquisition of anthrax via

injection is also been recognized as an additional route of infection as it can be a frequent contaminant in heroin (Ringertz *et al.,* 2000; Sweeney *et al.,* 2011). Pulmonary or inhalation of endospores, although the rarest method of acquiring the anthrax disease, is the most lethal route of acquisition with a mortality of 90% as compared to 20% with cutaneous acquisition and 50% as a result of ingestion (Dixon *et al.,* 1999; Nicholson, 2002).

1.3.2 Treatment and Prevention

Historically, penicillin was the main antibiotic used to treat anthrax. However, recently increased resistance to penicillin has made quinolone antibiotics including ciprofloxacin and doxycycline the antibiotics of choice, which specifically interfere with the folding of DNA synthesis by inhibiting gyrase (Dixon *et al.,* 1999; Turnbull, 1998). Pasteur first investigated anthrax vaccines for use in sheep in 1881 (Prince, 2003). Max Sterne developed a vaccine from an endospore suspension of an avirulent, non-capsulated live strain of *B. anthracis* 34F in 1939 (Prince, 2003). Vaccination is often not practical or feasible for potential carriers such as free-ranging wood bison (Hugh-Jones & De Vos, 2002); NTDENR, 2013). Human vaccines were developed in the 1940s, using a cell-free filtrate that contained the supernatant toxins of *B. anthracis* “anthrax vaccine adsorbed” (AVA) (Dixon *et al.,* 1999).

1.3.3 Disease Manifestations

The rapid onset of disease as a result of inhalation of endospores can result in death in cattle and sheep without significant clinical manifestations of illness within a few days after infection (WHO, 2008). The rapid onset of anthrax increases the potential for covert mass mortalities within a short period. In 1997, over 78,000 cattle where vaccinated following an outbreak of anthrax in one month in Australia (Turner *et al.,* 1999). In the

10-week outbreak period in Australia, over 80 different premises were confirmed with anthrax outbreaks (Johnson, 2006). Symptoms depend on the species of infected host as well as the mode and severity of infection. Most frequently, symptoms include blood in milk, urine or feces, staggering, laboured breathing and swollen lymph nodes (Turnbull, 1998). With species that are highly susceptible to the disease, presentation may be asymptomatic with symptoms being noticeable only within a few hours before death (Turnbull, 1998). In human inhalation cases, flu-like symptoms are the initial signs of infection (Turnbull, 1998). Death usually results from tissue hypoxia, extensive liver necrosis and pleural edema (Prince, 2003). Mortalities exhibit bloating, a lack of rigor mortis and unclotted blood which is commonly discharged from oral, nasal and anal orifices (Hugh-Jones & De Vos, 2002; Organization & Epizootics, 2008). The bloody discharge from the nose, mouth or anus of infected livestock can contain 10^7 to 10^8 bacilli per mL, contaminating the surrounding environment as vegetative cells undergo sporulation upon exposure to oxygen (Dixon *et al.,* 1999). This produces a reservoir of endospores that can infect future susceptible hosts (Dragon *et al.,* 1999; Turnbull, 1998). Discharge of *B. anthracis* cells from pigs was found to be far lower than with ruminants with only 10^2 cells per mL of bodily fluid (Turnbull, 1998). Sporulation requires oxygen, which enters the carcass through natural orifices or post-mortem openings in the body cavity that may form during natural decomposition or as a result of contact with scavengers (Hugh-Jones & Blackburn, 2009).

1.4 Detection Methods

Several methods have been used to confirm the presences of *B. anthracis* during suspected anthrax outbreaks; including bacteriological tests, such as classic culturing techniques or molecular identification. The classical standard technique is to attempt to

culture *B, anthracis* from blood or biopsy samples, perform a Gram stain and extract DNA to confirm the presence of the causative bacterium using PCR (Makino & Cheun, 2003). Differentiation of *B. anthracis* from other genera is easiest based on morphological differences in colonies formed on blood agar plates. After growth at 37ºC overnight, the colonies of *B. anthracis* are 2-5 mm in diameter, grayish white and non-hemolytic (Dixon *et al.,* 1999). API 50 CH test strips used with API 20E test strips (API Laboratory Products, Plainview, N.Y.) are able to identify members of the *B. cereus* group. Microscopically. *B. anthracis* is non- motile and exhibits linked rods that stain purple upon Gram staining, are 0.5-2.5 µm in width and 1.2-10 µm long (Dixon *et al.,* 1999). When stained with methylene blue, the capsule is easily visualized under a light microscope and endospores measure 2 µm by 1 µm in size approximately (WHO, 2008).

1.5 Infectious Cycle

Weather and environment play a significant role in the occurrence of anthrax outbreaks (WHO, 2008; Grace *et al.,* 2012). Infectious endospores survive in soils and can be aerosolized as a result of soil disruption by grazing livestock, exposure of barren ground in pastures, flooding (Who, 2008; Grace *et al.,* 2012) or even lightning. Although outbreaks of anthrax can occur year round, in Canada there is a seasonal trend for outbreaks, with most outbreaks occurring mid-July to mid-August – usually during the dry, hot weather that follows a wet spring (Dragon *et al.,* 1999; Dragon & Rennie, 1995; Salb *et al.,* 2014). Other seasonal influences such as heavy rainfall further aide in the dissemination of *B. anthracis* endospores (WHO, 2008; Himsworth & Argue, 2008). For instance, the 2006 outbreak in Saskatchewan occurred under unusually wet conditions with higher than average rainfall and snow levels as precipitation levels averaged 296.6 mm as compared to 132.8 mm over the previous 5 years (Himsworth & Argue, 2008).

Heavy rainfall permits buoyant endospores to rise or be flushed from their natural soil reservoir and drainage can cause endospores to be concentrated in low-lying areas such as sloughs or bison wallows. These low-lying areas are often the last site to retain water, and upon evaporation provide the optimal conditions for exposure to infectious endospores which are inhaled by grazing ruminants (Himsworth & Argue, 2008). Behavioural practices may also contribute to renewed cycles of infection as the aerosolization of the endospores can occur as bulls roll in dirt baths. Aerosolized dust has been reported to reach volumes of 30 m^3 and drift 75 m from the site of this activity (Dragon *et al.,* 1999). Haemorrhagic exudates escape from the nose, mouth and anus of infected animals, contaminating the surrounding environment where vegetative cells undergo sporulation when exposed to air and serve as a source for the infection of susceptible hosts (WHO, 2008; Hugh-Jones & Blackburn, 2009 & Logan & De Vos, 2009). Endospores may attach externally to scavengers such as bears, wolves (Nishi *et al.,* 2007), ravens or herring gulls and endospores have been recovered from the digestive tracts of avian species (Dragon *et al.,* 1999; Hugh-Jones & De Vos, 2002). Vultures (*Cathartes aura*) ingesting infected carcasses can also spread *B. anthracis* endospores (Nicholson, 2002). Carnivores or scavengers ingesting infected carcasses can further spread endospores or become infected (Hugh-Jones & Blackburn, 2009). Blowflies and earthworms also contribute to the dissemination of endospores (WHO, 2008; Hugh-Jones & De Vos, 2002; Hendriksen & Hansen, 2002). Rain droplets may also disperse endospores and new plant growth may acquire endospores from the soil (Hendriksen & Hansen, 2002). To reduce reoccurrence of anthrax disease cycles, effective methods of containment, decontamination and disposal of contaminated carcasses, soils and other materials must be established (Nicholson, 2002).

1.6 Bioweapon

The virulence, resistance to environmental factors and multiple routes of infection highlight the threat of *B. anthracis* as a significant natural zoonosis, but endospores have also been employed as a biological weapon (WHO, 2008; Ala'Aldeen, 2001; Cole, 2010; Montville, 2005; Reuter *et al.,* 2011; Scorpio *et al.,* 2006; Setlow, 2006). The first use of anthrax as a bioweapon against livestock occurred during the First World War when the German army deliberately infected Argentinian livestock and feed destined for trade, resulting in the death of 200 mules (Logan & De Vos, 2009). During the Second World War, Japanese aircraft sprayed Chinese cities with biological agents including *B. anthracis* endospores (Johnson, 2011). In the 1940's, Gruinard Island, off the coast of Scotland was used as a site to test *B. anthracis* as a biological weapon. From 1942 to 1943, bombs filled with infectious endospores were detonated over flocks of sheep (Manchee *et al.,* 1994). Due to the extensive mortalities and level of contamination, access to the island was restricted and it became known as "Anthrax Island". It was not until over 40 years later that the island was decontaminated (Manchee *et al.,* 1994). The anthrax attack of 2001 in the United States occurred when envelopes filled with infectious endospores were distributed in the mail, resulting in 22 victims and 5 deaths (Wallin *et al.*, 2007). The cleanup of the contaminated facilities from these attacks cost hundreds of millions of dollars and took years to complete (Franco & Bouri, 2010). There remains a high level of international concern over the potential of *B. anthracis* endospores to be used as a biological weapon (Wallin *et al.*, 2007).

1.7 Sporulation and Germination

Bacillus bacteria in their metabolically active state are referred to as vegetative cells, but respond to nutrient deprivation by undergoing sporulation to produce endospores that are

metabolically dormant, resistant to heat, desiccation, radiation and chemicals (Dragon & Rennie, 1995; Goel, 2015; Stephens, 1998). Endospores are multilayered and consist of a core surrounded by an inner membrane, cortex, spore coat and depending on species, an exosporium (Setlow, 2003). The exosporium is present in *B. anthracis*, and *B. thuringiensis*, but absent in *B. subtilis* and *B. licheniformis.* When endospores come in contact with a nutrient profile that contains specific germinants, germination occurs returning the endospore to a fragile vegetative form (Setlow, 2014). Germinants for *B. anthracis* include inosine and specific isomers of alanine as germination is induced by L-alanine, but inhibited by D-alanine (Weiner *et al.,* 2003). Release of peptidoglycan fragments from other bacteria can also trigger germination, independent of the germinants described above (Shah *et al.,* 2008).

Germination is a much faster process than sporulation and reports indicate that at 37°C indicators of germination are present within 2 h and fully apparent by 6 h. In a previous study, germination was not observed at 46°C or 18°C (Organization & Epizootics, 2008), but at 37°C sporulation was apparent after 10 h, requiring as much 24 h for completion. In comparison, germination can occur within 10 min following exposure to the appropriate germinants (Organization & Epizootics, 2008).

1.8 Endospore Thermal Resistance

There are many factors that influence the thermal resistance of endospores including thermal adaptation, range of exposure temperatures, availability of specific nutrients and the moisture level at the time of sporulation (Palop *et al.,* 1999; Baril *et al.,* 2012). Adaptation as a result of previous temperature exposure can influence the thermal resistance of the both the endospores and vegetative cells of *Bacillus* species. For example, thermophilic species that grow optimally above 70°C possess heat-stable

enzymes that are absent in mesophiles or psychotrophs (Warth, 1978). Studies with various *Bacillus* species, including *B. anthracis*, have shown that increasing the temperature during sporulation correlates with increased thermal resistance of vegetative cells. Studies suggest that this relationship is linear, but inconsistencies among species, suggests that factors other than just temperature influence thermal tolerance (Raso *et al.,* 1995). Overall, little is known about the effect of sporulation temperature on the survival of *B. anthracis* in natural habitats (Nicholson, 2002). Investigating the impact of sporulation temperatures on numbers of viable endospore is crucial, as the degree of thermal resistance has important implications for decontamination protocols (Condon *et al.,* 1992; Palop *et al.,* 1999).

Increased sporulation temperatures result in endospores that are more thermal resistant (Nicholson *et al.,* 2000), but it can also influence the efficiency and duration of sporulation. For *B. anthracis and B. cereus,* reports indicate that the critical temperature for sporulation is above 9°C, as sporulation required 2 weeks at 12°C, but failed to occur at 9°C, with the number of vegetative cells declining below detectable levels at this temperature (Organization & Epizootics, 2008). The first live attenuated anthrax vaccine was developed by Pasteur using cultures grown at 42°C, and as result these cells lacked capsules (Hanna, 1998).

The specific nutrients available during sporulation directly impact the mineralization of the endospore core. Levels of divalent cations influence the formation of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) which lowers the core water content and functions to protect endospore DNA against wet and dry heat, desiccation, UV radiation and some disinfectants (Huang *et al.,* 2007; Setlow *et al.,* 2006; Setlow, 2007; Slieman & Nicholson, 2001). Endospores that lack DPA or have lower cation levels are less resistant

to heat (Gerhardt & Marquis, 1989; Paidhungat *et al.,* 2000; Setlow *et al.,* 2006; Setlow, 2007). Likewise, endospores deficient in calcium-DPA (caDPA) are unstable and tend to germinate when conditions are unfavourable for the survival of vegetative cells (Huang *et al.,* 2007). Spores incubated at higher temperatures (70 to 80°C) lose caDPA, resulting in loss of wet heat resistance due to elevated core water content (Setlow, 2014) and RNA degradation (Segev *et al.,* 2012). Thermal resistance of DNA is also conferred by saturation of α/β-type small acid-soluble endospore proteins (SASP) that bind to DNA (Melly *et al.,* 2002; Reineke *et al.,* 2013). During germination, SASP are hydrolysed to provide nutrients that promote the germination process (Setlow, 2003). The water content within the endospore core also impacts environmental fitness; a lower core water content is associated with increased resistance to moist heat (Beaman & Gerhardt, 1986; Gerhardt & Marquis, 1989; Reineke *et al.,* 2013; Setlow *et al.,* 2006; Setlow, 2014). For example, water accounts for only 25% of the weight of those endospores that exhibit the greatest heat resistance (Coleman *et al.,* 2007).

The medium in which sporulation occurs also has an impact on the thermal resistance of endospores. For example, endospores produced on solid medium are three times more wet-heat resistant than those formed in liquid culture (Rose *et al.,* 2007). The nature of the matrix that contains the infectious endospores can also influence the efficacy of decontamination. The addition of tallow significantly reduced the ability of disinfectants to inactivate endospores (Amoako *et al.,* 2013). Although endospore resistance is mainly determined by sporulation temperature, it can also be influenced by pH with the viability of endospores being the highest at a neutral pH and decreasing with increasing acidification (Hugh-Jones & Blackburn, 2009).

Endospore morphology and heat resistance appear to be influenced by temperatures at the time of sporulation and less so during vegetative growth (Baril *et al.,* 2011; Juneja *et al.,* 2010). Studies with various *Bacillus* species, including *B. anthracis* have shown that increasing temperature during sporulation linearly enhances thermal resistance (Beaman & Gerhardt, 1986), but this relationship is not observed in all *Bacillus* spp. (Raso *et al.,* 1995).

Endospores of *B. cereus* were found to differ in their response to thermal inactivation, exhibiting a linear response in thermal resistance with increasing sporulation temperature (20-45°C) (González *et al.,* 1999) and (17, 30 and 42°C) (Beaman & Gerhardt, 1986). In some strains, thermal resistance of endospores decreased at higher sporulation temperatures (35°C) (González *et al.,* 1999) and 30°C (Gonzalez *et al.,* 1999). Likewise the endospores formed by *Bacillus coagulans* at 45 ºC were more heat resistant than those formed at 55ºC (González *et al.,* 1999).

As there are cardinal temperature ranges for bacterial growth, the literature also suggests that there are cardinal temperatures for sporulation with minimum, optimal and maximum temperatures. Estimating the optimal sporulation temperature that maximizes endospore thermal resistance is vital for estimating the efficacy of decontamination procedures that could reduce the risk of outbreaks of anthrax (Baril *et al.,* 2012).

Overall, little is known about the effect of temperature during sporulation on the viability of endospores in natural habitats (Nicholson, 2002). One possible method of disposing of anthrax contaminated carcasses may be through composting as this procedure has been shown to result in the effective decomposition of beef cattle carcasses (Reuter *et al.,* 2011; Xu *et al.,* 2010, 2014). However, before such a practice can be recommended, it is necessary to assess the degree of degradation of endospores that differ in their thermal

tolerance during composting. Such an approach is crucial, as the sporulation temperature influences thermal resistance of endospores and to some extent composting relies on thermal exposure for biological inactivation (Xu *et al.,* 2009a, 2009b, 2010, 2014; Erickson *et al.,* 2009a; Van Herk *et al.,* 2004; Guan *et al.,* 2009, 2010; Reuter *et al.,* 2011; Baril *et al.,* 2012; Condon *et al.,* 1992; Palop *et al.,* 1999).

1.2. Disposal Methods Following an Anthrax Outbreak

1.2.1 Anthrax Decontamination

As one of the most resilient life forms on the planet, *Bacillus* endospores are exceptionally resistant to disinfectants, and environmental stressors such as heat, radiation and desiccation (Dixon *et al.,* 1999; Nicholson *et al.,* 2000; Reuter *et al.,* 2011). Endospore survival depends on nutrient availability, microbial competition and environmental conditions with the half-life of endospores in soil reported to range from 13 (Hendriksen & Hansen, 2002) up to 200 years (Batt & Robinson, 2014). The resistant nature of endospores also results in the need for more rigorous sterilization procedures as compared to disinfecting vegetative bacterial cells. It took the British government more than 40 years, using 280 tonnes of formaldehyde and 2,000 tonnes of seawater to successfully decontaminate Gruinard Island following its use as a testing range for anthrax biological weapons (Manchee *et al.,* 1994). Endospores are not easy to detect as they are odourless and extremely small (1 μm × 3 μm). The ease of endospore production, the virulence, resistance to environmental factors and multiple routes of infection highlight the potential for mass causalities and the generation of infectious wastes as a result of outbreaks. Consequently, in the event of an accidental or intentional *B. anthracis* outbreak, efficient technologies are required to contain and inactivate endospores that are associated with contaminated livestock, waste, or food destined for human or animal

consumption. Bio-containment and disposal methods such as rendering, incineration, deep burial with lime, or chemical treatment are techniques commonly used to dispose of materials contaminated with *B. anthracis* endospores.

1.2.2 Rendering

Rendering is a closed system used for the thermal and mechanical destruction of pathogens (133°C for 30 min, 300000 Pa). This disposal method requires transportation of infected materials to centralized, dedicated facilities, a practice that increases the risk of dissemination of infectious material (Gilroyed *et al.,* 2016; Reuter *et al.,* 2011; Turnbull, 1998). During a large disease outbreak, the amount of infectious material generated could easily overwhelm the capacity of a rendering plant (Gilroyed *et al.,* 2016). Consequently, in the event of an anthrax outbreak, technologies are required to contain and inactivate infectious endospores. Most carcasses contaminated with endospores are disposed of by incineration and or deep burial. In Canada, up until 1967, anthrax contaminated carcasses were mainly disposed of by deep burial (Dragon *et al.,* 1999). Between 1976-1991, carcasses were incinerated inside a pit prior to the pit being filled in (Dragon *et al.,* 1999). Presently, rendering is the preferred disposal method for anthrax-contaminated carcasses (CFIA, 2013), but outbreaks often occur in remote locations where rendering facilities are unavailable.

1.2.3 Incineration

Incineration is preferred over burial as burial sites can be compromised by high water tables where burial depth must be restricted to avoid flooding of the burial pit (Dragon *et al.,* 1999). Complete incineration of contaminated carcasses within a dedicated facility can ensure containment and complete destruction of endospores. However, these procedures still require transportation of infectious materials to the site of incineration.

The Northwest Territories Department of Environment and Natural Resources reported that following an anthrax outbreak of bison, carcasses were treated with formaldehyde and then incinerated on site (Nishi *et al.,* 2007). At each site, incineration of the carcasses required, 440 kg of stoker's coal, 220 kg green wood and 1400 kg of dried wood. In this process a coal bed was prepared using 12-15 bags (22 kg) of coal in which five to six logs of green wood were arranged perpendicular to the axis of the carcass. The carcass was rolled onto the coal bed, using a hand winch. The remaining wood was stacked on top and doused with 20 L of diesel fuel. Once the pyre was lit it would burn for three to four days at which point ≥ 95% of the original carcass was incinerated (Nishi *et al.,* 2007). Current guidelines accept two methods of incineration in Canada, by pyre or pit. The pyre system is structured with a criss-crossed bottom layer of sturdy wood such as fence posts or railroad ties and a middle layer of either accelerant (diesel or kerosene) soaked wood or coal (23 L of fuel per carcass) or two large bales (545 kg per carcass) with pallets stacked over the bales. The final layer of the pyre system is the carcass supine with any contaminated soil or exudates. The burn pit is about 0.5 m deep with accelerant-soaked straw or wood lining the bottom with sturdy wood (heavy timber or beams) stacked on top. The ash resulting from the combustion of these materials needs to be decontaminated by chemical treatment or subject to additional burning to ensure that all organic matter is combusted. Using combustion to dispose of anthrax-contaminated carcasses requires a lot of fuel (≈ 128 cubic feet of wood per 500 kg of carcass) (CFIA, 2013). Pyre incineration eliminates the need for transportation, but increases the time needed for complete incineration and it is difficult to confirm complete destruction of endospores. Pyre piles also pose a risk of causing wild fires. Ash is treated with disinfectant and buried as materials that are not completely incinerated may still be infectious. Public acceptance of

this practice is also an issue as the smoke negatively impacts air quality and attracts spectators to the disposal event that may cause public disturbances (Sutmoller *et al.,* 2003). In some areas incineration may not be allowed due to the risk of causing grass or brush fires within the vicinity of the disposal site (Hugh-Jones & De Vos, 2002). Where incineration is restricted due to environment conditions or immediate disposal is not possible, deep burial can be used as an alternative (CFIA, 2013).

1.2.4 Burial

Current procedures for deep burial of contaminated carcasses require a deep (2 meter) hole to be dug within a layer of clay (1 meter) and for the carcass to be covered with another layer of clay and topsoil (1 meter). The carcass and all soil placed in the pit are treated with a chemical disinfectant such as formalin (10%) or lye (CFIA, 2013). Burial requires heavy machinery such as a backhoe and consideration given to the water table and records of the burial site should be kept indefinitely (CFIA, 2013). Out-dated protocols, such as deep burial with lime, can create a natural soil reservoir of endospores and there is evidence that the alkaline environment may actually promote endospore survival and the risk of anthrax outbreaks (Dragon & Rennie, 1995). The buoyant nature of endospores also increases the risk of disease transmission during heavy rainfalls as the endospores may float to the soil surfaces (Nicholson, 2002). Anthrax burial sites can act as sources of infection for wildlife as seen in Wood Buffalo National Park where foxes and ant colonies moved endospores to the soil surface (Dragon *et al.,* 1999).

1.2.5 Chemical Treatment

In Canada, the preferred method of disposing of anthrax contaminated carcasses is incineration or rendering, but deep-burial with chemical treatment of the pit area (10% formalin or 5% sodium hydroxide) at 50 litres/square meter is also considered acceptable

(CFIA, 2013). Coating the carcass with formaldehyde, covering the head and plugging the orifices to reduce bodily discharge, can reduce the dissemination of endospores by scavengers or flies. Approximately 22 - 44 L of formaldehyde (10%) are required to dispose of each carcass (Nishi *et al.,* 2002; Nishi *et al.,* 2007).

1.2.6 Alternative Measures

Traditional disposal methods for infected livestock and contaminated manure, soil and food, such as burial, incineration, rendering, autoclaving or chemical treatment can negatively impact air or soil and are limited in terms of the number and mass of carcasses that can be disposed of. These methods often involve non-routine farm practices, potentially increasing the numbers of visitors to the outbreak site and increasing the risk of endospore dissemination (Gwyther *et al.,* 2011; Reuter *et al.,* 2011). Composting may be a viable alternative when traditional methods for the disposal of contaminated carcasses and soils are deemed inappropriate.

1.3. Composting

1.3.1 Efficient Composting

To reduce re-occurring disease cycles, effective methods of containment, decontamination and disposal of contaminated materials must be established. Composting provides an inexpensive containment method, but currently little information exists on the persistence of *B. anthracis* endospores during composting. Composting may offer a unique green solution to allow for the containment and safe disposal of contaminants arising from an anthrax outbreak (Stanford *et al.,* 2015). Composting is an inexpensive waste management method that is simple to implement and has a flexible load capacity. It is described as the controlled breakdown and stabilization of organic materials by

microorganisms in a moist aerobic environment, with a pH range of 6.5 – 9.0 within the compost matrix and carbon dioxide, heat and water as the main end products of the decomposition process. When organic matter is fully decomposed, the final product is called humus as it contains high concentrations of humic acids. Composting is also relatively easy to implement in all seasons. It does not require any external energy as the natural biological processes of microbial metabolism generates the heat that can reach temperatures as high as 70°C (Xu *et al.,* 2011; Xu *et al.,* 2009a). The efficiency of composting depends mainly on the level of microbial activity which is dictated by several parameters including temperature, moisture, carbon to nitrogen ratio (C/N), particle size and oxygen concentration (Xu *et al.,* 2010). The degree of temperature achieved during composting is related to the level of microbial activity and is an indicator of the success of the composting process. Generally mesophilic and thermophilic stages of composting refer to temperatures up to 40°C and 40-70°C, respectively (Figure 1-1).

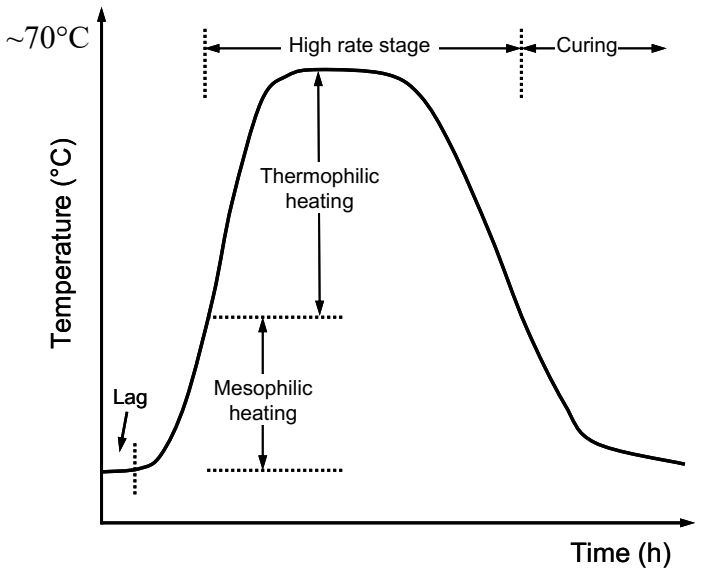

Figure 1-1. Schematic of typical composting temperature vs. time curve (Xu *et al.,* 2011; Xu *et al.,* 2009a).

Composting has the potential to be an effective on-site disposal method of infected livestock carcasses as it has been shown to reduce the numbers of viruses, protozoa, helminthes and bacteria (Gwyther *et al.,* 2011; Xu *et al.,* 2009a). Insulating the composter in cooler environments is recommended by using straw bales and perforated plastic so as to retain moisture and heat within the pile (Xu *et al.,* 2009a; Xu *et al.,* 2010). The United States Environmental Protection Agency (USEPA) and the Canadian Council of Ministry of the Environment (CCME, 2005) recommend a minimum temperature of 55°C be achieved in a static composter for a minimum of three days to ensure thermal inactivation of pathogens (Xu *et al.,* 2009a). There is little information regarding the impact of composting on the survival of endospore-forming bacteria such as *B. anthracis* (Gwyther *et al.,* 2011).

1.3.2 Moisture

Moisture is one of the crucial factors that can influence the efficiency of the composting process with a desired range of 50-60% (Xu *et al.,* 2010). To determine the moisture

content, a sample from the composter is dried in an oven at 55 ºC and the amount of evaporative water lost is estimated. An alternative field test is the simple hand 'squeeze test' where the level of moisture exuded is used as an indicator of moisture content. If the sample crumbles the composting matrix is too dry and if water drips from the hand it is too wet. Moisture levels greater than 68% may restrict the diffusion of oxygen into the structure and inhibit the composting process (Stanford *et al.,* 2000). Composting practices to maintain proper moisture levels include adding water to increase moisture or adding dry bulk organic matter such as straw to decrease moisture or mixing the composting pile to allow air circulation and subsequent desiccation.

1.3.3 Carbon and Nitrogen

The microbes involved in decomposition are directly dependent on the carbon and nitrogen sources inside compost. Materials largely composed of carbon are utilized primarily for energy, and nitrogen is critical for microbial protein synthesis. Many of the materials required to formulate the compost matrix can often be found on-site of an outbreak or acquired at little cost. Common carbon sources include wood chips, straw and sawdust or even moldy silage or plant trimmings. Manure can serve as a nitrogen source and as a source of epiphytic microbes as it naturally contains the microorganisms required for composting. For optimal composting, a carbon to nitrogen ratio (C/N ratio) between 25/1 to 30/1 is adequate with 30/1 being recommended (CCME, 2005; Xu *et al.,* 2009a; Xu *et al.,* 2010).

1.3.4 Particle Size

The particle size during composting influences airflow and therefore impacts the rate of degradation of organic matter by microbes. Composting temperatures are negatively influenced by restricted airflow. If the size of the particles is too large, air pockets may

form. If it is too small, the mixture may form aggregates upon contact with water that restrict airflow. The optimized composting process needs a balance between particle size and particle surface area. Smaller particles have larger surface areas, accelerating the microbial degradation of organic matter and thus the oxygen demand for aerobic respiration. Increased oxygen demand may require more turning events to avoid the development of anaerobic conditions. Conditions are considered ideal when the free air space amounts to 20-30% of the total volume of compost and particle sizes range from 0.32 to 5 cm.

1.3.5 Oxygen

Oxygen concentration has a direct impact on the activity of the microbial population. During composting, the microbial community requires oxygen to efficiently metabolize organic matter. Oxygen concentrations that drop below 5% cause a shift in the microbial community, promoting the growth of facultative anaerobic or anaerobic bacteria that exist with little or no oxygen. Anaerobic degradation results in much lower temperatures as compared to aerobic composting, and generates volatile fatty acids, methane and hydrogen sulfide, a gas that is toxic and has an offensive odour. Oxygen diffusion into the composting pile is influenced by moisture content and porosity. Optimally, oxygen concentration within compost should not drop below 5%. Supply of oxygen gives the most efficient aerobes a competitive advantage over anaerobes and avoids the offensive odours associated with anaerobic decomposition. Primarily three methods are used to increase the infiltration of oxygen into compost; physical turning, convective airflow and mechanical aeration. Convective airflow is the preferred method of aerating composters as it reduces the aerosols that are formed by physical turning or mechanical aeration and

reduces the risk of dissemination of contaminated material through contact (CCME, 2005).

1.3.6 Negative Aspects of Composting

There are some negative aspects when considering composting as a method of disposing of carcasses infected with anthrax. Depending on the outbreak location, carcass type and amount of matrix material needed for disposal, the land area required for composting may be large. This may prove to be a challenge in forested or remote areas where access to equipment is limited. There may also be negative environmental impacts associated with composting; including leachate, attraction of vermin or pests and the release of malodourous gases. Compared to other alternative disposal methods, composting is relatively slow, resulting in longer quarantine times and increased risk of breaches in containment barriers from wildlife. Disposal using composting also requires additional management and supervision due to the requirement of precise parameters for uniform heating of the pile as inconsistent temperatures within the pile may permit the survival of pathogens in areas where temperatures are insufficient to inactivate pathogens.

1.3.7 Counteracting the Negative Aspects of Composting

Using a closed or contained composter design, which has a flexible load capacity to accommodate diverse ecoregions where anthrax outbreaks occur, can reduce some of these negative impacts. The system can also be closely monitored and managed to ensure successful heating cycles. If properly enclosed, the leachate can be contained and scavengers and pests effectively excluded from compost. Gaseous emissions can also be modulated as they have been reported to be influenced by composting parameters such as the substrate source, moisture content and porosity (Oudart *et al.,* 2012). Additionally, introduction of endospores into the nutrient rich compost matrix may induce germination,

easing decontamination as the vegetative cells are more susceptible to inactivation during composting.

1.3.8 Benefits of Composting

There are many benefits to composting organic wastes. Composting is an environmentally friendly method, diverting waste from landfills and avoiding any potential pollution risks concerning exudates associated with anaerobic decaying materials. Following composting, the overall volume of waste materials is reduced by up to 70%, doubling the density of finished compost. Mass and volume reductions ease the handling of compost and reduce the cost associated with collection, transportation and disposal of organic waste. Composting on-site also reduces the cost and risk associated with the handling and transportation of contaminated organic wastes. Composting completes the nutrient recycling chain as beneficial soil amendments are generated from waste materials. The final compost material can benefit soil fertility, water-holding capacity, overall plant health, or potentially be sold. Low cost and simple set-up procedures require minimal training, using materials readily found on livestock operation sites, or that can be acquired with little expense. An additional aspect is that anthrax outbreaks maybe more likely to be reported if a simple onsite disposal method was available. Livestock producers have been known to report anthrax deaths as result of plant toxins or lightning in order to avoid the costs associated with a definitive diagnosis of anthrax (Dragon *et al.,* 1999). The load capacity for composting is flexible and can be scaled down to accommodate household or whole farm needs. Large scale composting (approximately 100,000 kg materials) has shown to result in the complete decomposition of beef carcasses without physical turning after approximately 150-200 days. With only a few long bones remaining after 147 days, which were degraded further following an additional cycle of composting (Reuter *et al.,*

2010). Small scale (100 kg materials) composting resulted in significant degradation of prions within a month (Xu *et al.,* 2014).

1.3.9 Pathogen Reduction

Composting reduces pathogen viability through a number of mechanisms including elevated temperature, alkaline pH and microbial competition. High temperatures produced during the composting process can kill infectious pathogens such as *Escherichia coli*, *Salmonella*, *Campylobacter* and *Giardia*. Since composting can occur year-round, composting offers an all-season biosecurity alternative for many pathogens. In 2004, when British Columbia experienced an outbreak of avian influenza, composting was implemented by the Canadian Food Inspection Agency (CFIA) to effectively contain and eliminate the mass volumes of infectious poultry waste. Composting is governed by federal and provincial regulations, while the CFIA controls the sale of all compost in Canada (CFIA, 2013).

1.3.10 Thermal Inactivation of Endospores

Dry heat inactivates endospores by DNA damage (Setlow *et al.,* 2006). The mechanisms of thermal inactivation of endospores as a result of wet heat is not entirely known but is proposed to involve the release of DPA which increases core water content and results in the denaturation of key endospore proteins (Batt & Robinson, 2014). Endospores are much more resistant to dry heat than wet heat (Batt & Robinson, 2014). One study investigated the inactivation of *B. anthracis* endospores in peptone water and ground beef at 45 and 70°C, with the results showing increased reduction of the endospores in ground-beef (Tamplin *et al.,* 2008). *B. anthracis* endospores are inactivated when boiled for 10 min or after 3 h at 140°C (Batt & Robinson, 2014). However, the biological and chemical factors responsible for inactivation, as well as the DNA damage were not further explored

(Juneja *et al.,* 2010). The effect of manure and a ground beef matrix on survivability suggests that some components within ground beef reduce the thermal survival of *B. anthracis* endospores (Juneja *et al.,* 2010; Tamplin *et al.,* 2008). Previous research investigated the viability of endospores from *B. thuringiensis* and *B. licheniformis,* after long-term (>200 days) field scale composting (Reuter *et al.,* 2011). Both of these bacteria are closely related to *B. anthracis*. Composting reduced the numbers of viable endospore, but further research is needed to assess the efficacy of this approach, as spatial variation within the pile resulted in uneven heat distribution and variability in the inactivation of *Bacillus* endospores. Investigating DNA damage is particularly important as the distinguishing characteristics among *Bacillus* species are encoded on highly variable and mobile extrachromosomal DNA elements (Helgason *et al.,* 2000; Hoffmaster *et al.,* 2004). Researchers have identified *B. cereus* strains that possess genes encoding the pXO1 anthrax toxin complex, and pXO2 capsular genes (Hoffmaster *et al.,* 2004). These *B. anthracis*-like strains were still distinguishable from *B. anthracis* by phenotypic and biochemical properties, but did exhibit increased virulence. Therefore, to determine if there is a risk of horizontal transfer of genes post- composting, research is needed to determine the extent to which *Bacillus* DNA is degraded during composting.

Composting may offer a green solution to aid biosecurity concerns following an outbreak of anthrax. An outbreak of anthrax by natural or intentional means would have devastating consequences; potentially compromising the safety and trade of Canadian livestock. The consequences associated with the resulting mass casualties would impact national political and social stability, resulting in severe economic consequences. The national livestock and food supply are vulnerable to mass contamination by anthrax endospores. High volumes of infectious material limit bio-containment and disposal

options. As livestock producers are often paid an indemnity for animal mortalities, but not for the cost associated with disposal and decontamination, composting may provide a desirable disposal alternative for infected carcasses.

Chapter 2

Anthrax has global social, economic and public health importance as outbreaks affect international trade of livestock, resulting in devastating impacts for national agriculture industries. Currently, little information exists on the persistence of *B. anthracis* endospores in contaminated food during the composting process (Juneja *et al.,* 2010; Tamplin *et al.,* 2008). My project investigated the effect of fresh cattle manure, autoclaved compost and inert matrices on endospore survivability. Previous research has indicated that undefined components within ground beef influence *B. anthracis* endospore thermal survival (Juneja *et al.,* 2010; Tamplin *et al.,* 2008).

Previously described differences in thermal resistance of endospores among *Bacillus* spp. may reflect differences in cell morphology and biochemistry (Reuter *et al.,* 2011). Within the literature, there is limited information on the long-term survival of *Bacillus* endospores in compost, but there is some indication that the extent and duration of survival differs among *Bacillus* spp. (Greenberg *et al.,* 2010). There is a lack of long-term experimental studies that have examined *Bacillus* endospore survival and even fewer studies have compared the environmental viability of endospores among different *Bacillus* species (Greenberg *et al.,* 2010). The objective of my study was to investigate the impact of thermal exposure on the inactivation of endospores from non-pathogenic *Bacillus* species as a model for the fate of virulent *B. anthracis* endospores. Previously described differences in thermal resistance of endospores among *Bacillus* species may reflect distinct variations amongst species both in cell morphology and biochemical characteristics (Reuter *et al.,* 2011).

Composting provides an inexpensive containment and disposal method, but currently little information exists on the persistence of *Bacillus* endospores during composting. This leaves the possibility that composting may effectively reduce the viability of *B. anthracis* endospores.

My thesis further explores the inactivation of endospore originating from *Bacillus* spp. My research intended to extend previous research (Reuter *et al*., 2011) by exposing *Bacillus* endospores to lab-scale composting systems under controlled lab conditions. Lab scale conditions bypass potential field-scale biosafety restrictions of composting and sample numbers, allowing a controlled examination of endospore inactivation.

2.1 Hypothesis.

1. Higher temperatures during sporulation can increase endospore thermal resistance and survivability of *Bacillus* endospores within compost.
2. Composting temperatures will reduce the viability of *Bacillus* endospores.

2.2 Objectives

The overall objective is to investigate the inactivation of *Bacillus* endospores during composting and examine the factors that are associated with reduced viability by examining the impact of temperature, matrix composition and sporulation temperature.

Chapter 3

Heat and desiccation are the predominant factors affecting viability of *Bacillus licheniformis* and *Bacillus thuringiensis* spores during simulated composting.[1]

3.1 Introduction

Bacillus anthracis is the causative agent of anthrax, a disease primarily targeting herbivores, although all mammals including humans are susceptible (Erickson and Kornacki, 2003). Released from its host, *B. anthracis* can enter a dormant state through the formation of spores which are extremely resistant to a wide range of harsh physical and chemical conditions (Setlow, 2006), remaining viable in the environment for decades (Nicholson *et al.,* 2000). In order to study *B. anthracis* spores, a number of non-pathogenic experimental surrogates have been evaluated, with spores of *B. thuringiensis* gaining acceptance as a suitable model (Greenberg *et al.,* 2010; Bishop, 2014). Previous investigations have shown that a number of infectious bacteria and viruses are killed during composting (Xu *et al.,* 2009a). More recent work using spores from *B. licheniformis* and *B. thuringiensis* as surrogates for *B. anthracis* has also suggested that composting reduces the number of viable spore (Reuter *et al.,* 2011; Stanford *et al.,* 2015). Both of these field-scale studies reported that the viability of spores during composting was correlated with compost temperature and duration of exposure to

[1] This chapter is adapted from the published manuscript: Stanford, K., Harvey, A., Barbieri, R., Xu, S., Reuter, T., Amoako, K. K., … McAllister, T.A. (2016). Heat and desiccation are the predominant factors affecting viability of *Bacillus licheniformis* and *Bacillus thuringiensis* spores during simulated composting. *Journal of Applied Microbiology, 120,* 90-98. For this manuscript, the role of Amanda Harvey included responsibilty of experimental design, setup, data collection and initial analysis.

temperatures > 55ºC, but these findings were inconclusive due to variations in temperature distribution within the compost structures examined.

*Bacillus s*pore viability has been shown to be affected by sporulation temperature (Palop *et al.,* 1999; Baril *et al.,* 2012). In compost or other matrices, survival of spores has been shown to be species and strain-dependent (Nicholson *et al.,* 2000; Byrne *et al.,* 2006), with exposure to high temperature resulting in the degradation of RNA in spores (Segev *et al.,* 2012). Ecological studies have also noted seasonality in the transmission of *B. anthracis* and that the duration of *B. anthracis* spore viability depends on soil type (Dragon *et al.,* 1999; Nicholson 2002)

In addition to varying sporulation conditions and/or environmental factors impacting spore viability, compost can also contain microenvironments (Reuter *et al.,* 2011) with site-specific variation in moisture content, temperature, microbiota and chemical constituents. This makes it difficult to ascertain which of these parameters influences spore viability. The objective of the present study was to examine the impact of sporulation temperature, matrix composition and temperature on the viability of *B. licheniformis* and *B. thuringiensis* spores.

3.2 Materials and Methods

3.2.1 Bacterial strains

Two strains of *B. licheniformis* ATCC® 14580™ (L1), JB0501-3 (L2) and two strains of *B. thuringiensis* ATCC® 33679™ (T1), and 4A3 (T2) were used. Two strains (L1, T1) were obtained from the American Type Culture Collection (ATCC) and two strains (L2, T2) were obtained from the Lethbridge Research and Development Centre culture collection and had previously been used in a field-scale composting experiment (Reuter *et al.,* 2011).

3.2.2 Spore Preparation

Stock cultures were maintained at -20°C in a modified nutrient sporulation medium including phosphate (NSMP; ATCC medium 1209) and glycerol (20%). Working cultures were prepared by streaking cells from stock cultures on nutrient agar and incubating at 37°C overnight. A single isolated colony was then inoculated into 10 mL NSMP broth and incubated at 37°C under agitation (150 RPM) for 16-18 h. From this suspension, 1 mL was inoculated into 500 mL of NSMP and incubated at 10, 25, 30 or 37°C under agitation at 150 RPM. Sporulation was monitored periodically by examining cultures directly with phase-contrast microscopy (Reuter *et al.,* 2011) or with bright field microscopy after staining the spores (Schaeffer and Fulton 1933). Once cultures showed ≥ 80% sporulation, they were stored at 4°C for up to 7 d until harvest.

Spores were harvested by centrifugation of cultures at 10,000 × g for 10 min at 4°C. The supernatant was discarded and 150 mL of phosphate buffered saline (PBS)-Tween 20 (0.05%) added to re-suspend the pellet. This washing procedure was repeated 3 times. Aliquots of each spore suspension were examined using phase-contrast and staining methods to ensure that vegetative cells and cellular debris were removed. After the final wash, pellets were suspended in 100 mL of 50% ethanol and mixed for 1 h at 100 RPM on a shaking platform. Suspensions were centrifuged, the supernatant discarded, and the pellet re-suspended in 100 mL of 0.01% PBS containing 1% bovine serum albumin (BSA, Sigma Aldrich, Oakville ON) and maintained at 4°C.

3.2.3 Preparation of compost matrix and silica beads

Fecal pats from cattle receiving a barley silage-based diet were collected and pooled. The compost matrix was prepared by mixing feces (1:10) with white spruce (*Picea glauca*) wood shavings in a mortar mixer (12S; Crown Construction Equipment, Winnipeg,

Canada; Xu *et al.,* 2013b). The compost matrix was then oven-dried at 55°C for 1 week, ground through a 4 mm screen and stored at 4°C. One week prior to inoculation with spores, 3 g of the compost matrix or silica beads (4 mm) were weighed into 50 mL Falcon tubes, prior to being autoclaved (≥121°C, ≥100 kPa) for 30 min. Sterile tubes were then capped and maintained at room temperature until inoculated with spores.

3.2.4 Incubation of spores

On day 0, 6 mL of autoclaved filtered water was added to tubes containing dried compost matrix and mixed by thoroughly shaking by hand. Spore suspensions were normalized to 8 $\log_{10}$ CFU mL^{-1} with PBS-BSA and 300 µL was inoculated into Falcon tubes containing moistened compost or silica beads for each of the *Bacillus* strains at each of the 3 sporulation temperatures (25, 30 and 37°C). Triplicate tubes were prepared for each of the time points (0, 25, 50, 100 and 150 d) examined over the experiment. Tubes were distributed across treatment and with the exception of d 0 tubes, were placed inside duplicate incubators (Isotemp, Fisher Scientific, Ottawa, ON) initially at 28°C. The incubators were manually adjusted as needed to mimic the average temperature profile of compost in a previous field-scale study (weight 85,000 kg; Xu *et al.,* 2009a; Figure 3-1).

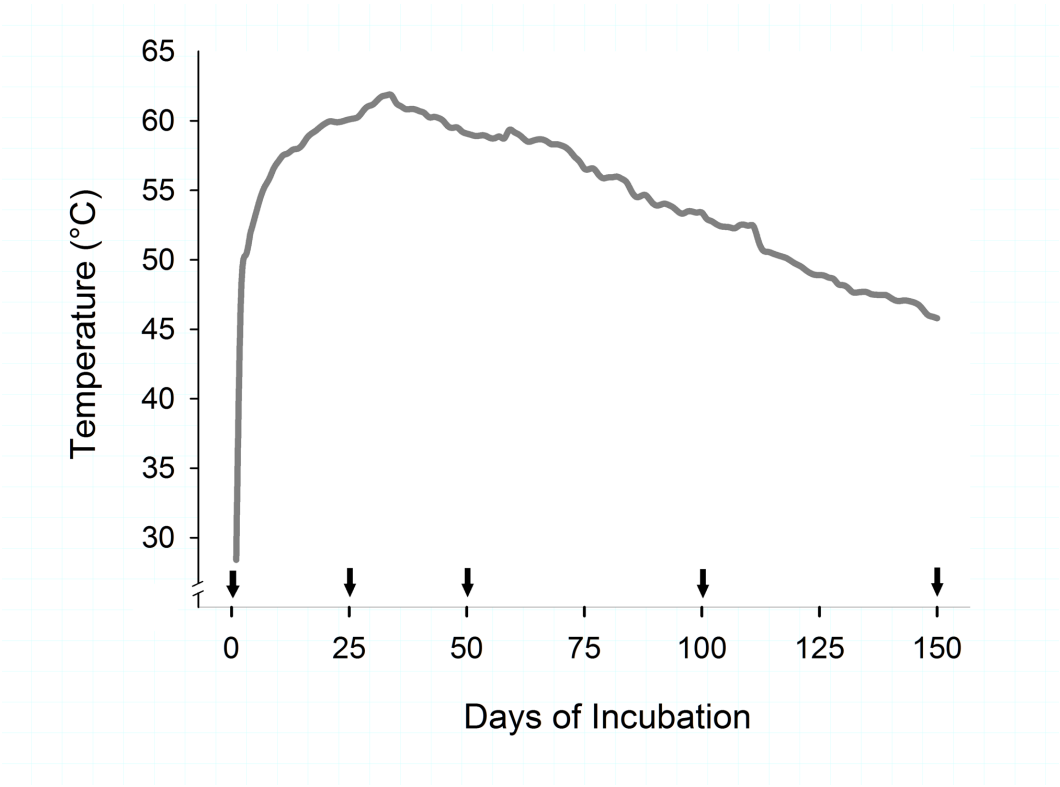

Figure 3-1 Temperature profile during incubation of *Bacillus* spores based on average temperature profile during compost Xu *et al.,* (2009a).
Arrows represent days of sampling.

Temperature of each incubator was monitored daily using a dual thermocouple digital thermometer (Digi-Sense Dual J-T-E-K, Fisher Scientific). Control tubes of compost matrix and silica beads inoculated with spores from each *Bacillus* strain sporulated at 37°C were maintained at room temperature and sampled on the same day as those within incubators.

3.2.5 Sampling and enumeration of Bacilli

Tubes were sampled on d 0, 25, 50, 100 and 150. Incubated tubes were allowed to equilibrate to room temperature, while d 0 and control tubes (maintained at room temperature) were immediately processed by adding 30 mL PBS and vortexing thoroughly. Subsamples (1.5 mL) were then transferred to microtubes and heat-treated in

a thermal mixer to kill any vegetative cells (75°C, 20 min 300 RPM). Subsequently, 1 mL aliquots were serially diluted in PBS (9 mL), plated in duplicate onto nutrient agar and incubated at 37°C for 16 h. The limit of quantification was ≥ 30 CFU for the first (10^{-1}) dilution and only plates containing 30 to 200 CFU were enumerated.

3.2.6 Confirmation of bacterial species

Motility of the germinated *Bacillus* strains was assessed using motility test medium (FDA 2001) with *Bacillus mycoides* ATCC® 6462™ as a negative control and *B. thuringiensis* var. *kurstaki* (HD-1), strain number GK 1806 as a positive control. API 20E and API 50 CHB/E tests (bioMérieux, St. Laurent, QC) were completed according to the manufacturer's instructions. Inoculum for the API 20E test strips was prepared using the saline medium provided (0.85% NaCl), with *Escherichia coli* ATCC® 25922™ and *Klebsiella pneumonia* ATCC® 700603™ included as controls. For both API tests, results were read after 24 and 48 h incubation at 37°C.

Single colonies of overnight cultures from all strains were inoculated into 50 µL of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0, Qiagen, Hilden, Germany), heat treated at 95°C for 5 min under agitation (300 RPM), centrifuged at 10,000 × g for 5 min and the supernatant used as template DNA. Bacterial DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen). Total reaction volume was 30 µL; 2 µL of template, 15 µL of 2x Mastermix (TaqMan Plus, Thermo Fisher Scientific, Burlington ON), 0.6 µL of the 16S rRNA 1492_Lab1 primer CGGTTACCTTGTTACGACTT, 3 µL CoralLoad PCR buffer (Qiagen) and 8.8 µL of PCR-grade water. PCR reaction conditions were: 1 × 95°C; 35 cycles of 30 s at 94°C, 45 s at 58°C and 1.15 min at 72°C followed by a final extension at 72°C for 10 min. PCR fragments were sequenced using both the forward and reverse primers (Eurofins Genomics, Louisville, USA). Individual forward and reverse sequences

were aligned and compared against the NCBI database using Geneious Pro 5.5 software for bacterial identity (Kearse *et al.,* 2012).

3.2.6 Statistical analyses

Numbers of *Bacillus* were log transformed prior to analysis using the mixed model procedure of SAS (SAS 9.3, SAS Institute, Cary, NC). For all statistical tests, significance was $P < 0.05$ and Falcon tube was considered the experimental unit. For mixed model analyses, strain of *Bacillus*, sporulation temperature, matrix (compost or silica beads) and day of sampling were considered fixed effects, with sampling day treated as a repeated measure and incubator a random variable. For control tubes left at room temperature, matrix, strain of *Bacillus* and sampling day were fixed effects.

3.3 Results

3.3.1 Temperatures of incubators

Temperature profiles in the incubators were adjusted to follow a rapid heating and slow, steady cooling pattern that have typically been observed in previous cattle mortality composting experiments (Figure 3-1). Temperature was near-maximal (60^{o}C) when tubes were sampled on d 25. Incubators were maintained at $\geq$ 55^{o}C for 78 of the 150 d study period. During the study, temperatures peaked at 62^{o}C on d 32, slowly declining to a low of 48^{o}C upon the final sampling on d 150.

3.3.2 Viability of spores retained at room temperature

During 150 d at room temperature, viability of *Bacillus* was strain specific and showed minor fluctuations (Table 3-1). Strains, L2 and T1 and T2 did not significantly change in spore viability during 150 d at room temperature. In contrast, viability of L1 spores remained constant only until d 100, showing a rapid 1.7 $\log_{10}$ CFU g^{-1} decline by d 150.

Table 3-1. Influence of species and day of measurement on viability of *Bacillus* spores (log_{10} CFU g^{-1}) during incubation at room temperature (means of both compost and silica bead matrices) and temperatures reflective of mortality composting (means of all sporulation temperatures, both compost and silica bead matrices).

	Species*			
Day	L1	L2	T1	T2
	At room temperature			
0	$6.6^{bc}\pm0.2$	$6.0^{b}\pm0.3$	$6.2^{b}\pm0.3$	$6.8^{bc}\pm0.3$
25	$6.9^{c}\pm0.3$	$7.1^{c}\pm0.3$	$6.5^{bc}\pm0.3$	$7.1^{c}\pm0.3$
50	$6.9^{c}\pm0.3$	$7.2^{c}\pm0.3$	$6.2^{b}\pm0.3$	$6.9^{c}\pm0.3$
100	$6.4^{bc}\pm0.4$	$5.9^{b}\pm0.3$	$6.4^{bc}\pm0.3$	$5.8^{b}\pm0.3$
150	$4.9^{a}\pm0.3$	$6.7^{bc}\pm0.3$	$6.8^{bc}\pm0.3$	$5.9^{b}\pm0.4$
	At compost temperatures			
0	$6.3^{bc} \pm 0.04$	$6.5^{b} \pm 0.04$	$6.5^{b} \pm 0.04$	$6.7^{b} \pm 0.04$
25	$7.4^{a} \pm 0.08$	$6.6^{b} \pm 0.08$	$5.9^{c} \pm 0.08$	$6.1^{c} \pm 0.08$
50	$6.6^{b} \pm 0.12$	$4.9^{d} \pm 0.12$	$4.1^{e} \pm 0.14$	$4.6^{d} \pm 0.12$
100	$5.7^{b} \pm 0.26$	$3.9^{e} \pm 0.26$	$2.1^{g} \pm 0.28$	$2.9^{e} \pm 0.26$
150	$2.0^{f} \pm 0.27$	$0.8^{h} \pm 0.26$	$0.8^{h} \pm 0.28$	$1.7^{g} \pm 0.27$

^{a,b,c,d,e,f,g,h} Means within temperature category with different superscripts differ ($P < 0.05$).
*Species *B. licheniformis* ATCC® 14580™ (L1), *B. licheniformis* JB0501-3 (L2), *B. thuringiensis* ATCC® 33679™ (T1), and *B. thuringiensis* 4A3 (T2).

3.3.3 Overall effects of matrix, strain and sporulation temperature

Incubation in compost increased the viability of spores ($P < 0.001$) compared to incubation in silica beads, although the increase was relatively modest and unlikely to be biologically significant (0.4 log_{10} CFU g^{-1}; Table 3-2). However, the number of spores in compost increased in the initial 25 d of incubation, likely as a result of germination, vegetative growth and subsequent sporulation of vegetative cells. In contrast, the numbers

of viable spores in the silica bead matrix steadily declined over the experimental period (Figure 3-2). After d 25, levels of spores in compost also steadily declined for the duration of the experiment.

Comparing species and strains of *Bacillus* over the incubation period, spores of L1 remained more viable ($P < 0.001$) as compared to the other strains. Overall viability of T1 was reduced compared to others ($P < 0.001$) during incubation at compost temperatures, while that of T2 and L2 were intermediate and similar (Table 3-2).

As expected, sporulation temperature impacted spore viability, although the yield of spores at 10°C was too low to include this sporulation temperature in the experiment. Sporulation at 25 and 30°C produced spores with a higher ($P < 0.001$) viability than those generated at 37°C (Table 3-2). Day of sampling also affected overall viability of spores ($P < 0.001$), with viability being similar at d 0 and 25 (6.5 $\log_{10}$ CFU g^{-1}), but steadily declining thereafter to an average of 1.3 $\log_{10}$ CFU g^{-1} by d 150 (Table 3-2).

Table 3-2. Main effects influencing overall viability of *Bacillus* spores during incubation at temperatures reflective of composting temperatures.

Factor		Mean spores log_{10} CFU g^{-1}	SEM
Heating matrix			
	silica beads	4.4[a]	0.07
	manure+shavings	4.8[b]	0.07
Species*			
	L1	5.6[c]	0.10
	L2	4.5[b]	0.10
	T1	3.9[a]	0.11
	T2	4.4[b]	0.10
Sporulation temperature ^{o}C			
	25	4.8[b]	0.09
	30	4.8[b]	0.09
	37	4.2[a]	0.09
Day sampled			
	0	6.5[a]	0.02
	25	6.5[a]	0.03
	50	5.1[b]	0.06
	100	3.6[c]	0.13
	150	1.3[d]	0.14

[a,b,c,d] Means within a category with different superscripts differ ($P< 0.001$).
*Species, *B. licheniformis* ATCC® 14580™ (L1), *B. licheniformis* JB0501-3 (L2), *B. thuringiensis* ATCC® 33679™ (T1), and *B. thuringiensis* 4A3 (T2).

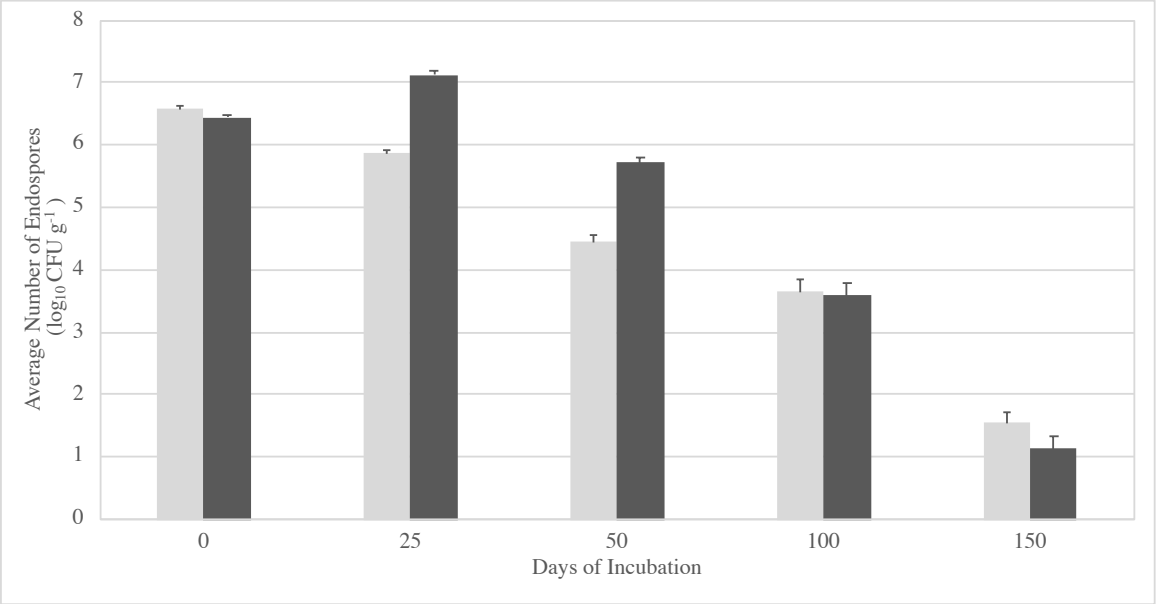

Figure 3-2. Mean numbers of viable Bacillus spores log10 CFU g-1 across all strains and sporulation temperatures in compost and silica bead matrices over 150 days of incubation. Compost matrix (dark) and silica beads (light). Error bars indicate standard error**.**

3.3.4 Species and time effects on spore survival

Although overall viability of *Bacillus* spores did not change between d 0 and 25 (Table 3-2), viability of individual species/strains incubated at compost temperatures showed marked differences during this period (Table 3-1). Of those evaluated, L2 was the only strain that exhibited similar spore viability between d 0 and 25. In contrast, L1, demonstrated significant vegetative growth (1 $\log_{10}$ increase; $P < 0.05$), while both T1 and T2 showed significant declines during this period. Viability of L1 spores at d 50 was equal to that of d 0, while viability of spores from other strains continued to steadily decrease. After 150 d of incubation, viability was the same for L2 and T1 spores (0.8 $\log_{10}$ CFU g^{-1}), with the viability of spores being 1.2 and 0.9 $\log_{10}$ higher ($P < 0.05$), in strains L1 and T2, respectively (Table 3-1).

3.3.5 Impacts of sporulation temperature, time and species/strain on spore survival

Although overall spore viability was highest for spores produced at 25 and 30°C (Table 3-2), species and strain-specific relationships for sporulation temperature and spore viability were also apparent (Figure 3-3). Vegetative growth occurred for the first 25 d for all sporulation temperatures of L1 and L2 and for T2 in the compost matrix at 30 and 37°C (Figure 3-3), although only L1 significantly increased overall spore numbers during this period (Table 3-1). Viability of L1 spores did not differ by sporulation temperature until d 150, when spores produced at 25 and 30°C were more numerous than spores produced at 37°C ($P < 0.05$; Figure 3-3A). For L2, spore viability after d 50 ranked according to sporulation temperature 25 > 30 > 37°C ($P < 0.05$; Figure 3-3B). Only T1 had spore viability patterns identical to overall trends (25 and 30 > 37°C) for the entire study period (Figure 3-3C). After d 50, T2 spores produced at 30°C had higher viability ($P < 0.01$) than spores produced at other temperatures (Figure 3-3D).

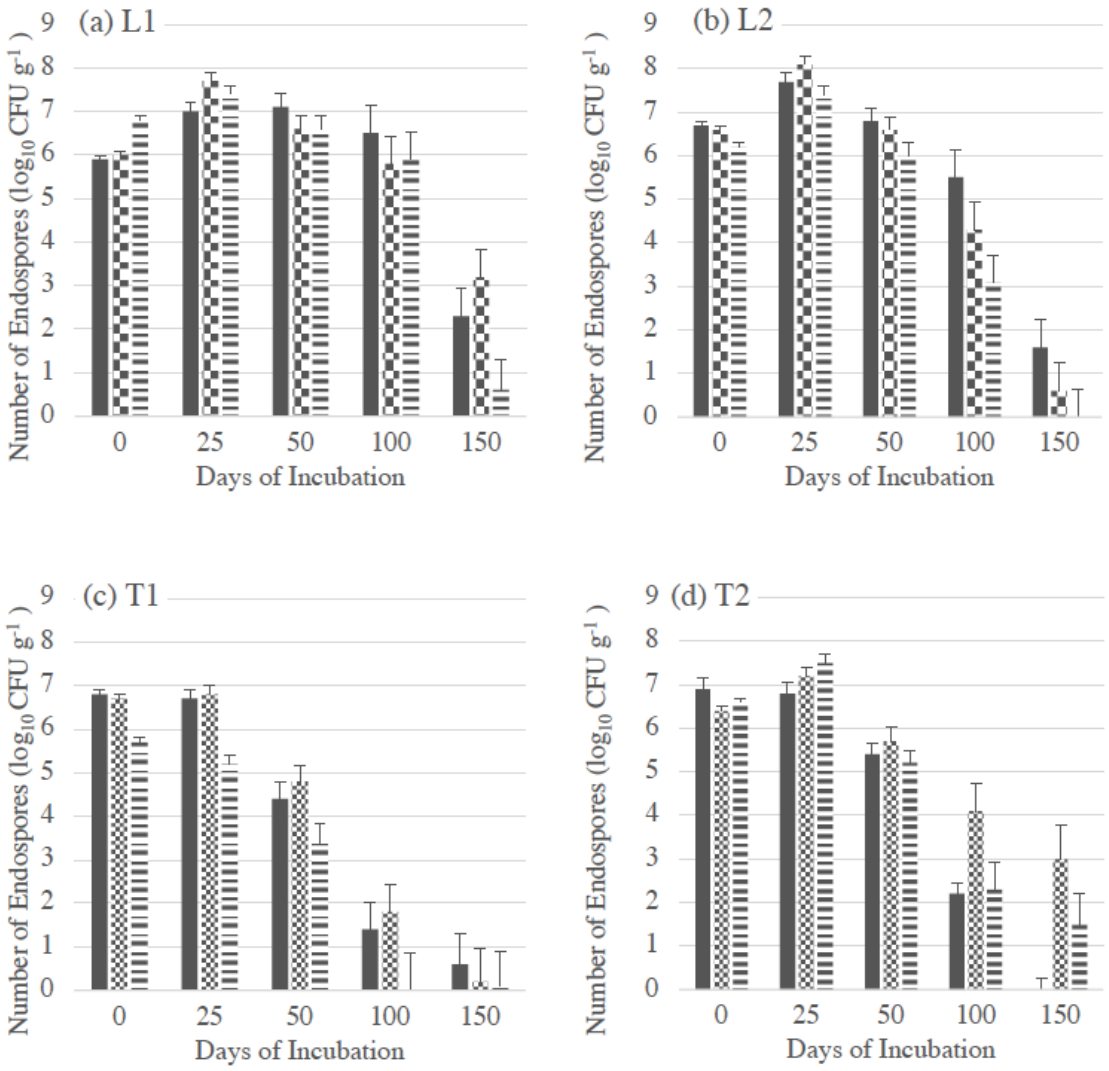

Figure 3-3. Impacts of species*, sporulation temperature and time on viability of *Bacillus* endospores incubated in compost matrix.
Sporulated at 25°C (solid), Sporulated at 30°C (checker) and Sporulated at 37°C (lines).
Species, *B. licheniformis* ATCC® 14580™ (L1), *B. licheniformis* JB0501-3 (L2), *B. thuringiensis* ATCC® 33679™ (T1), and *B. thuringiensis* 4A3 (T2).
Error bars indicate standard error

3.4 Discussion

3.4.1 Incubation Temperatures

The temperature increased to > 50°C within the first 72 h and peaked at 62°C on d 32 before gradually declining to 48°C after 150 d. Consequently, incubation temperatures used in the present study mimicked those of a large compost windrow with adequate levels of moisture and carbon to support sustained heating (Xu *et al.,* 2009a; McCarthy *et al.,* 2011), with 78 d at thermophilic temperatures (≥ 55°C). Similar to actual compost, moisture was gradually lost during incubation of the compost matrix as tubes were capped but not sealed with O-rings. Even with this development, the compost matrix would have supported prolonged thermophilic composting.

In field-scale mortality compost, no changes in spore viability were reported by Reuter *et al.,* (2011) in a windrow which sustained thermophilic temperatures for 23 d, while a separate windrow which maintained thermophilic temperatures for 111 d reduced the viability of both *B. licheniformis* and *B. thuringiensis* spores to a similar extent to that reported in my study. Accordingly, composting could be recommended for disposal of anthrax mortalities when amendments with the appropriate moisture levels and composition were readily available to support prolonged thermophilic composting. Alternatively, if composting conditions were less optimal, germinants to induce vegetative growth of *B. anthracis* may be advisable in tandem with composting. Bishop (2014) reported a 6 $\log_{10}$ reduction in *B. anthracis* spores maintained for two weeks at 22ºC after use of alanine and inosine as germinants.

3.4.2 Overall effects of matrix, sporulation temperature and strain of *Bacillus*

As both compost and silica beads were autoclaved prior to incubation with spores, differing microbiota would not have influenced spore survival. The addition of water and available nutrients in compost matrix would have initially produced an environment supportive of vegetative growth of *Bacillus* strains and the overall number of viable spores in compost increased ($P < 0.05$) by d 25 as compared to d 0 (Figure 3-2). Vegetative growth of *Bacillus* spp. has been reported in compost at temperatures > 50°C (Xiao *et al.,* 2011), but after d 50, numbers of viable spores did not differ among matrices, possibly due to incubation temperatures > 60°C or desiccation of spores in compost after prolonged incubation. Accordingly, pH and moisture along with heating are recognized triggers of spore germination (Gaillard *et al.,* 1998). The proportion of intact as compared to degraded spore RNA is also thought to influence spore germination (Segev *et al.,* 2012). The reason as to why some spores, but not others of the same strain respond to germination stimuli is unknown (Xu *et al.,* 2009b).

Generally, increased sporulation temperatures have also increased spore viability during heating (Beaman and Gerhadt 1986; Collado *et al.,* 2006; Olivier *et al.,* 2012), although effects of sporulation temperature on spore viability appear to be strain-specific (Gonzalez *et al.,* 1999). Reduced water content with increased sporulation temperature has been recognized as a key factor leading to increased thermal-resistance of spores (Melly *et al.,* 2002; Olivier *et al.,* 2012). In my study, reduced viability of strains sporulated at 37°C compared to those produced at 25 or 30°C was not expected, but was most likely due to the strains of *Bacillus* used and would be of lesser practical significance than the limited sporulation observed at 10°C.

Similar to my study, Baril *et al.,* (2012) reported a 10-fold reduction of *B. licheniformis* sporulation at 10 and 20°C compared to optimal spore production at 45°C, although these authors produced maximal concentrations of only 2.6 $\log_{10}$ CFU mL^{-1} of spores. As insufficient spores were produced for experimental examination of sporulation at 10°C, anthrax mortalities occurring at temperatures ≤ 10°C would likely result in production of fewer spores and be of lower risk for disease transmission. Sporulation of *B. anthracis* requires exposure to oxygen and is not known to occur within the carcass (Watson and Keir, 1994), although the number of spores necessary for *B. anthracis* transmission is species and route-dependant. For humans, the medium lethal dose (LD_{50}) for cutaneous anthrax may require 10 or less spores (Watson and Kier, 1994), while inhalation anthrax may require 2,500 to 55,000 spores (Ingelsby *et al.,* 2002). Data for oral transmission to grazing livestock are lacking, although ingestion of 8 $\log_{10}$ spores did not produce disease in guinea pigs, a species known to be highly susceptible to *B. anthracis* infections (Watson and Kier, 1994).

Comparing overall viability of spores by strain and species of *Bacillus*, vegetative growth between d 0 and 25 was a primary determinant. Significant vegetative growth for all sporulation temperatures during incubation at compost temperatures was demonstrated only for L1 (Table 3-1), although both L2 and T2 showed trends toward increasing spore viability during the first 25 d of incubation in the compost matrix (Figure 3-3). Consequently, L1 had higher ($P < 0.05$) overall spore viability as compared to the other types of *Bacillus* evaluated (Table 3-2). Thermophilic *Bacillus* species accounted for 87% of bacterial isolates collected from compost (Strom, 1985), with *Bacillus thermodenitrificans* and *B. licheniformis* being the predominant species in composts with temperatures > 55°C (Zhang *et al.,* 2002). *B. thuringiensis* is common in soil (Martin and

Travers, 1989) and recognized as an accurate surrogate for soil-borne *B. anthracis* (Bishop, 2014), but is not a recognized thermophile (able to grow at 55ºC; Ronimus *et al.,* 1997). Consequently, the heightened vegetative growth between d 0 and 25 of *B. licheniformis* strain L1 as compared to the two *B. thuringiensis* strains evaluated is not surprising. Like L1, all sporulation temperatures of L2 showed vegetative growth at compost temperatures between d 0 and 25, although to a lesser extent than for L1.

3.4.3 Interacting factors affecting spore survival

One of the challenges in inactivating *Bacillus* spores is the diversity of their responses based on sporulation temperature, species and strain (Gonzalez *et al.,* 1999; Melly *et al.,* 2002; Reuter *et al.,* 2011). In my study, all *Bacillus* spores arising from at least one of the sporulation temperatures were rendered non-viable after 150 d of incubation (Figure 3-3). In general, viability of spores was reduced as a result of sporulation at 37ºC, with spores from strain T2 being the exception.

During incubation, only L1 produced significant ($P < 0.05$) vegetative growth between d 0 and 25, although L1 sporulated at 25ºC tended to increase in spore viability until d 50 (Figure 3-3A). In contrast to L1, the overall viability of other *Bacillus* spores generally declined after d 25. Vegetative growth of spores has also been noted in a previous study where compost reached temperatures above 55ºC for < 7 days and the number of spores of one strain of *B. cereus* increased after 56 days of composting (Stanford *et al.,* 2015). Vegetative growth of *B. anthracis* outside of an animal host is likely insignificant (Bishop, 2014) and limited differences in spore viability among *Bacillus* evaluated in the present study regardless of species, strain, heating matrix, or sporulation temperature support the potential use of composting for disposal of anthrax mortalities. The average

5.2 $\log_{10}$ CFU g^{-1} reduction in spore viability after 150 d of exposure to compost temperatures exceeds the 97% inactivation of *B. anthracis*, *B. subtilis* and *B. thuringiensis* spores occurring after exposure of spores to 220ºC for 50 ms (Setlow *et al.,* 2014) and is equivalent to the 4.9 $\log_{10}$ CFU mL^{-1} reduction in spores of *B. cereus*, *B. coagulans* and *B. licheniformis* reported by Furukawa *et al.,* (2005) at temperatures and times used for food preservation (85ºC, 30 min).

As spores in the present study were incubated with 3 g of compost matrix or silica beads for 150 d at an average temperature of 54.4 ºC with gradual loss of moisture occurring in the case of compost matrix and minimal moisture present in the case of silica beads. Desiccation of spores may have been more severe than in field-scale mortality compost windrows which may maintain at least 60% moisture over the composting period (Reuter *et al.,* 2011). Inactivation of spores is thought to occur by two pathways: DNA damage occurring during desiccation/dry heat or damage to spore proteins during wet heating (Setlow *et al.,* 2014). An actively heating compost windrow would likely be classified as dry heat as previous studies of wet heat have incubated spores directly in water (Coleman *et al.,* 2007; Setlow *et al.,* 2014). The compost environment is also alkaline, with a pH frequently exceeding 9 upon completion of composting (Xu *et al.,* 2009a, Xu *et al.,* 2013b). This may further improve spore inactivation as an alkaline environment enhances germination of *B. thuringiensis* (Wilson and Benoit, 1993). A compost windrow in the study of Reuter *et al.,* (2011), which attained temperatures similar to those in my study may also demonstrated an approximate 5 $\log_{10}$ CFU g^{-1} reduction in numbers of *Bacillus* spores. Consequently, heat and spore desiccation may be the primary factors responsible for declining spore viability in compost as both compost and silica beads in the present study were autoclaved prior to use, negating any impacts of microbiota.

To produce disease, ingestion of *B. anthracis* spores by livestock likely requires a dose several magnitudes higher than achievable through aerosol exposure. Consequently, limited sporulation noted in the present study at low temperatures would likely reduce the risk of pathogen transmission from livestock mortalities occurring at ambient temperatures $\leq 10^{o}$C. Long term (150 d) exposure of spores to compost temperatures appears to offer reductions of spore numbers equivalent to that produced during heat preservation of foods at higher temperatures (85^{o}C) for short durations (30 min). Accordingly, sustained thermophilic compost temperatures can reduce the number of viable *Bacillus* spores by more than 99.99%, equivalent to the ≥ 5 $\log_{10}$ CFU g^{-1} reduction noted in my study. However, future studies directly evaluating the fate of *B. anthracis* in compost are required.

Chapter 4

Inactivation of *Bacillus anthracis* spores during laboratory-scale composting of feedlot cattle manure[2]

4.1 Introduction

Anthrax is a lethal mammalian disease, capable of infecting animals and humans and remains an ongoing global problem (Spencer, 2003; Sternbach, 2003). Before the development of a vaccine for the treatment of livestock in the 1870's, anthrax was one of the leading causes of worldwide mortality in cattle, goats, horses and sheep (Hugh-Jones and Blackburn, 2009). During the past 20 years, even with the availability of a vaccine, human anthrax is still a significant problem in Western Africa, Eastern Europe and Central Asia, with sporadic outbreaks continuing to occur in livestock in the United States and Canada (Levin, 2014).

The causative agent of anthrax is *B. anthracis*, a Gram-positive, aerobic, endospore forming rod-shaped bacterium (World Health Organization, 2008). Spores are the primary infectious form of *B. anthracis* with infection occurring through ingestion, inhalation or cutaneous exposure (Thappa and Karthikeyan, 2001). *Bacillus* spores are resistant to heat, desiccation, radiation and chemical treatment (Stephens, 1998) so decontamination can be a challenge. It has been reported that *Bacillus* spores can persist in soil for at least 300 years (Nicholson *et al.*, 2000). The infectious dose of *B. anthracis* spores varies among

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[2] This chapter is adapted from the published manuscript: Xu, S., Harvey, A., Barbieri, R., Reuter, T., Stanford, K., Amoako, K. K., … McAllister, T. A. (2016). Inactivation of *Bacillus anthracis* Spores during Laboratory-Scale Composting of Feedlot Cattle Manure. *Frontiers in Microbiology, 7,* 806. For this manuscript, the role of Amanda Harvey included responsibilty of experimental design, setup, data collection and initial analysis.

host species and with route of infection. As few as ten inhaled *B. anthracis* spores are sufficient to cause infection in cattle and sheep (Smith, 1973), while 500-55,000 inhaled spores is the estimated range for a lethal dose for humans (Wallin *et al.*, 2007).

In Canada, there is still a concern with repeated outbreaks of anthrax in wild bison populations in the Northwest Territories, Northern Alberta, Manitoba and Saskatchewan (Beaumont, 2013; Elkin *et al.*, 2013). Moreover, an anthrax outbreak occurred in Saskatchewan in 2006, resulting in the death of 804 livestock (Himsworth and Argue, 2008). Proper disposal of animal carcasses infected with *B. anthracis* is essential to minimize spore contamination and reduce the risk of transmission to animals and humans. In addition, the extreme virulence, environmental persistence and multiple routes of infection have also resulted in anthrax spores being employed as a biological weapon (Ala'Aldeen, 2001; Cole, 2010). Therefore, in the event of a natural or a terror related outbreak of *B. anthracis*, proper technologies are required to inactivate spores associated with contaminated livestock carcasses.

In Canada, current disposal practices approved by Canadian Food Inspection Agency (CFIA) for *B. anthracis* infected carcasses include incineration and deep burial with chemical treatment using 10% formalin or 5% sodium hydroxide (CFIA, 2013). However, Canada's vast geographical area and transportation distances frequently make incineration impractical as a disposal method. Moreover, deep burial can render the disposal site a long-term reservoir of spores with heavy rain fall and soil saturation promoting renewed transmission as viable spores migrate to the soil surface (Himsworth and Argue, 2008; Nicholson, 2002). Composting may offer a practical and economical means for the safe disposal of carcasses during an anthrax outbreak. Composting is an aerobic decomposition process whereby organic matter is degraded by the actions of

mesophilic and thermophilic bacteria and fungi. Compost is often alkaline (pH 8-10) as a result of the liberation of ammonia from the deamination of amino acids and temperatures can reach ~70ºC and exceed 55ºC for weeks or even months (Xu *et al.*, 2009a; Stanford *et al.*, 2015). These conditions have been shown to inactivate most microbial pathogens including *Listeria* (Erickson *et al.*, 2009a), Shiga-toxigenic *Escherichia coli* (Xu *et al.*, 2009a), *Salmonella* (Erickson *et al.*, 2009b), *Giardia, Cryptosporidium* (Van Herk *et al.*, 2004), and avian influenza, Newcastle disease and foot-and-mouth disease viruses (Guan *et al.*, 2009; Guan *et al.*, 2010). Even recalcitrant proteins such as the prions associated with scrapie, chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE) are degraded during composting (Xu *et al.*, 2014).

Previous research groups have previously used related spore-forming bacteria (i.e., *B. licheniformis*, *B. thuringiensis* and *B. cereus*) as surrogates for investigating the inactivation of *B. anthracis* in compost under field conditions (Reuter *et al.*, 2011; Stanford *et al.*, 2015). To further define the feasibility of composting for disposal of *B. anthracis* infected carcasses, assessment of the fate of *B. anthracis* spores in compost is required. However, such studies with *B. anthracis* can only be safely conducted under full laboratory containment, conditions that were met using specially designed laboratory-scale composters (Xu *et al.*, 2010). Recent findings have shown that sporulation temperature was a key factor influencing survival of *B. cereus* spores in cattle carcass compost (Stanford *et al.*, 2015). Therefore, the objective of my study was to assess the survival of *B. anthracis* spores generated at different sporulation temperatures (15ºC, 21ºC or 37ºC) using laboratory composters in containment.

4.2 Materials and Methods

4.2.1 Laboratory composting experiment

Passively aerated laboratory-scale composters were used as described by Xu *et al.,* (2010). These 110-L cylindrical polyethylene vessels were sealed and insulated with a 50 mm layer of polyurethane foam. For the purpose of passive aeration, an air plenum (0.1 m height) was created at the bottom of each composter using a perforated polyethylene panel with 10 mm diameter holes. Inlet and outlet air holes (25 mm) were drilled in the side, near the bottom and in the lid to enable passive aeration. Fresh feedlot manure (45 ± 0.1 kg; wet-weight basis) and white spruce (*Picea glauca*) wood shavings (4.5 ± 0.1 kg) were thoroughly mixed in a mortar mixer (12S; Crown construction equipment, Winnipeg, MB) to form a matrix with a moisture content of 76.0 ± 0.3%. The physicochemical properties of the ingredients are described in Table 4-1. Spores of *B. anthracis* and its surrogate *B. thuringiensis* were composted in a level 3 biocontainment laboratory at the CFIA in Lethbridge, AB (Figure 4-1). Prior to the compost experiment, the lab benches and floors were swab-tested to ensure they were not contaminated with *Bacillus* spores. Identical matrices without inoculation with *Bacillus* spores were composted outside of containment with samples being collected for measurement of physicochemical parameters during the composting process. Experiments inside and outside of containment were started simultaneously with four replicated composters outside containment and two replicated composters for each *Bacillus* species inside containment (Figure 4-1).

Table 4-1. Physicochemical characteristics of materials included in matrices used for laboratory composting under both non-containment and containment conditions

Parameters*	Cattle manure	Wood shavings
Moisture (%)	81.1 ± 0.5	9.6 ± 0.0
Bulk density (kg m^{-3})	854 ± 6	88 ± 1
Total carbon (%)	41.8 ± 0.9	51.4 ± 0.6
Total nitrogen (%)	2.19 ± 0.07	0.08 ± 0.00
C/N ratio	19.1 ± 0.3	662.2 ± 27.5
pH	7.99 ± 0.16	4.87 ± 0.03
EC (ds m^{-1})	1.16 ± 0.06	0.05 ± 0.00
NH_4-N (mg kg^{-1})	4237 ± 500	3 ± 1
(NO_2+NO_3)-N (mg kg^{-1})	40.1 ± 5.2	6.2 ± 0.0

*All parameters except moisture and bulk density are expressed on a dry-weight basis (w w^{-1}); Moisture and bulk density are expressed on a wet-weight basis; EC: electrical conductivity.

4.2.2 Spore preparation

The strain of *B. anthracis* Sterne was kindly provided by Dr. Elizabeth Golsteyn Thomas (CFIA) and Mr. Doug Bader (Defence Research Development Canada) with NCBI Genome Reference Sequence of NC_005945.1(pXO2-), while *B. thuringiensis* 4A3 was provided by Dr. Tim Lysyk (Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre). Spores of *B. anthracis* Sterne and *B. thuringiensis* 4A3 were prepared from triplicate overnight cultures of single discrete colonies as previously described by Shields *et al.,* (2012) and Reuter *et al.,* (2011), respectively. Briefly, 100 µl of the overnight culture was used to inoculate culture flasks containing 50 mL of tryptic soy agar (Becton Dickinson [BD], Franklin Lakes, NJ) supplemented with 5% sheep blood for *B. anthracis* or AKnumber 2 agar (BD) supplemented with 20 µg mL^{-1} $MgSO_4$

and 80 μg mL^{-1} $CaCl_2$ for *B. thuringiensis*. Flasks were incubated at either 15°C, 21°C or 37°C for 8 days until sporulation was complete. Spores were harvested by dispensing phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST; Sigma-Aldrich, St. Louis, MO) into each flask with gentle mixing in the presence of glass beads to suspend the spores. Suspensions were transferred into 50 mL Falcon tubes and centrifuged at 6,000 × g for 10 min at 4°C to pellet the spores. The supernatant was discarded, and the pellets were washed five times with PBST. After the last wash, pellets were resuspended in 50% ethanol and centrifuged as described above to remove all remaining vegetative cells. After washing, a droplet of the suspension was examined using phase-contrast microscopy to ensure that cellular debris and vegetative cells were removed. For each *Bacillus* species, spores generated at each temperature were re-suspended in PBS with 1% bovine serum albumin (BSA; Sigma-Aldrich) and then serially diluted (1:10) for estimating spore CFU as described by Shields *et al.,* (2012) and Reuter *et al.,* (2011).

			Sampling days				
			Day 0	Day 8	Day 15	Day 22	Day 33
	Species	Sporulation temperatures	Numbers of nylon bags and silica bead tubes collected				
Composter 1	*B. thuringiensis*	15 ºC	2	2	2	2	2
		21 ºC	2	2	2	2	2
		37 ºC	2	2	2	2	2
Composter 2	*B. thuringiensis*	15 ºC	2	2	2	2	2
		21 ºC	2	2	2	2	2
		37 ºC	2	2	2	2	2
Composter 3	*B. anthracis*	15 ºC	2	2	2	2	2
		21 ºC	2	2	2	2	2
		37 ºC	2	2	2	2	2
Composter 4	*B. anthracis*	15 ºC	2	2	2	2	2
		21 ºC	2	2	2	2	2
		37 ºC	2	2	2	2	2
Room Temperature Control	*B. thuringiensis*	15 ºC	4	4	4	4	4
		21 ºC	4	4	4	4	4
		37 ºC	4	4	4	4	4
	B. anthracis	15 ºC	4	4	4	4	4
		21 ºC	4	4	4	4	4
		37 ºC	4	4	4	4	4
			Total		180 bags and 60 tubes		
			Total per day		36 bags and 12 tubes		

Figure 4-1. Schematic representation of the experimental design and numbers of *B. thuringiensis* and *B. anthracis* samples collected during composting under biocontainment conditions. Duplicated composters were used for each *Bacillus* species sporulated at 15ºC, 21ºC, and 37ºC. Manure was inoculated with spore suspensions and then sealed in nylon bags prior to placing into composters. For a room temperature control, autoclaved silica beads were inoculated to achieve the same spore concentration as for inoculation of manure and then sealed in Falcon tubes. Two replicate nylon bags for each sporulation temperature and species were collected from the composters at days 0, 8, 15, 22, and 33. Room temperature controls of duplicate nylon bags and tubes (i.e., total number=4) for each sporulation temperature and species were collected on the same sampling day.

4.2.3 Nylon bag preparation

Due to the high concentration of *Bacillus* spores in feedlot manure compost matrix, generated spores were inoculated into feces collected directly from the rectum of cattle. The feces had physicochemical properties that were similar to the feedlot manure used in compost, but reduced background microbiota for the subsequent detection of *Bacillus* spores (data not shown). Feces (5.0 ± 0.1 g; wet basis) was shaped into spheres and then inoculated with *Bacillus* spore suspensions to achieve ~7.5 $\log_{10}$ CFU g^{-1} manure for each sporulation temperature and *Bacillus* species. However, a lower concentration of *B. thuringiensis* spores (i.e., ~5.5 $\log_{10}$ CFU g^{-1} manure) was used at 37°C due to reduced yield, a response that has been observed for this species when it was sporulated by others at 40°C (Ignatenko *et al.*, 1983). Inoculated manure spheres were sealed in nylon bags (5 × 10 cm; 53 μm pore size; ANKOM Technology, Macedon, NY) prior to placing into composters. For controls, 3 g of autoclaved (121°C, 20 min) silica beads (4 mm; Fisher Scientific, Ottawa, ON) were inoculated with spore suspensions to achieve the same spore concentration as in manure and then sealed in a sterile 50 mL Falcon tube. Controls of nylon bags containing inoculated manure spheres and tubes containing inoculated silica beads were both retained at room temperature during the experimental period. Duplicate control nylon bags and tubes for each sporulation temperature and *Bacillus* species were collected at each sampling day (Figure 4-1). Nylon bags were prepared on the day of compost construction and implanted into compost immediately after the compost was prepared.

4.2.4 Nylon bag implantation and sampling procedures

For each sporulation temperature and *Bacillus* species, two replicate nylon bags were placed in a larger polyester mesh bag (5 mm pore size) along with 200 g of freshly mixed

compost. As each composter was filled, four replicate mesh bags for each sporulation temperature were placed at a depth of 30 cm below the surface of the compost matrix, resulting in a total of twelve mesh bags in each composter. Single mesh bags for each sporulation temperature were collected from each composter after 8 and 15 days of composting (Figure 4-1). A total of three mesh bags were removed at each sampling time per replicate composter (Figure 4-1). After collection at day 15, each composter was emptied, and contents were mixed with water to return the compost to its original moisture level. Compost was then returned to its original composter for a second heating cycle. As the composters were refilled, the remaining mesh bags were placed in each composter at the same depth as in the first cycle. In the second composting cycle, mesh bags were collected after 22 and 33 days (Figure 4-1). Compost temperature was continuously measured at the same depth as the mesh bags were implanted (Xu *et al.*, 2010). Composters outside of containment were managed similarly, except that the manure implanted in the compost matrix was not inoculated with *Bacillus* spores. The experiment was designed in this manner as compost within the composters in containment could not be removed from the containment laboratory for chemical analysis.

Compost in each composter outside of containment was collected at day 15 after mixing and moistening, and also from each of the mesh bags for physicochemical analyses. Compost temperatures, oxygen concentration, moisture, bulk density, total carbon (TC), total nitrogen (TN), pH, electrical conductivity (EC), and mineral N (NH_4^+ and $NO_2^- + NO_3^-$) were measured (Xu *et al.*, 2010).

4.2.5 Enumeration of *Bacillus thuringiensis* and *Bacillus anthracis*

Upon removal of mesh bags from compost, nylon bags were removed from mesh bags and enclosed within a water-tight container. At each sampling day, controls of silica bead

tubes and nylon bags retained at room temperatures were also sealed within double packaged Ziplock bags (SC Johnson, Racine, WI). Subsequently, all sealed cups and bags were submerged in bleach (10%) for 30 min and retained within the containment laboratory for enumeration of *Bacillus* spores. Nylon bags of each *Bacillus* species were placed into sterile stomacher bags containing 45 mL PBS and blended for 2 min in a Stomacher 400 (Seward, Fl) at 230 rpm. A 5 mL aliquot was transferred to a 15 mL Falcon tube and incubated at 75°C for 20 min in a shaking water bath. After cooling, a 100 µl aliquot was serially diluted (10^{-1}-10^{-5}) into PBS and plated onto duplicate blood tryptic soy agar plates. For silica bead samples, 30 mL of PBS were added into each 50 mL Falcon tube containing the beads and then mixed using a serological pipet. A 5 mL aliquot was then transferred to a 15 mL Falcon tube. The tubes were heat treated, serially diluted and plated as described for nylon bags. Colonies on the plates were enumerated after incubation at 37°C for 16-18 h, and only plates that contained 30 to 300 CFU were counted. The quantification limit was set at ≥ 30 CFU in the first dilution (10^{-1}). Numbers of *B. thuringiensis* and *B. anthracis* spores were calculated as CFU per g of the original weight of manure in the nylon bags or silica beads.

4.2.6 Statistical analysis

Numbers of *B. thuringiensis* and *B. anthracis* spores were log transformed before analysis. Changes in the spore number for each *Bacillus* species and temperature profiles during biocontainment composting were analyzed using the MIXED Procedure of SAS (Version 9.2; SAS Institute Inc., Cary, NC) with time treated as a repeated measure in the model. Main effects of sampling day, sporulation temperature and their interaction were considered to be statistically significant at a probability level of < 0.05.

4.3 Results

4.3.1 Compost properties

Under biocontainment, compost temperatures for each *Bacillus* species were affected ($P < 0.05$) by composting cycle. All the composters heated rapidly, with temperatures peaking after 2 days at 72°C for composters containing *B. anthracis* and 70°C for those containing *B. thuringiensis* (Figure 4-2A). Subsequently, temperatures steadily declined, but increased again and peaked at 61°C for *B. anthracis* at day 10 and 57°C for *B. thuringiensis* at day 9. During the first composting cycle, temperature remained above 55°C for 8 and 5 days for *B. anthracis* and *B. thuringiensis*, respectively (Figure 4-2A). After mixing and moistening of compost on day 15, temperatures did not exceed 55°C and peaked at 53°C on day 17 for both *B. anthracis* and *B. thuringiensis* (Figure 4-2A), which were lower ($P < 0.05$) than the peak temperatures measured in the first cycle.

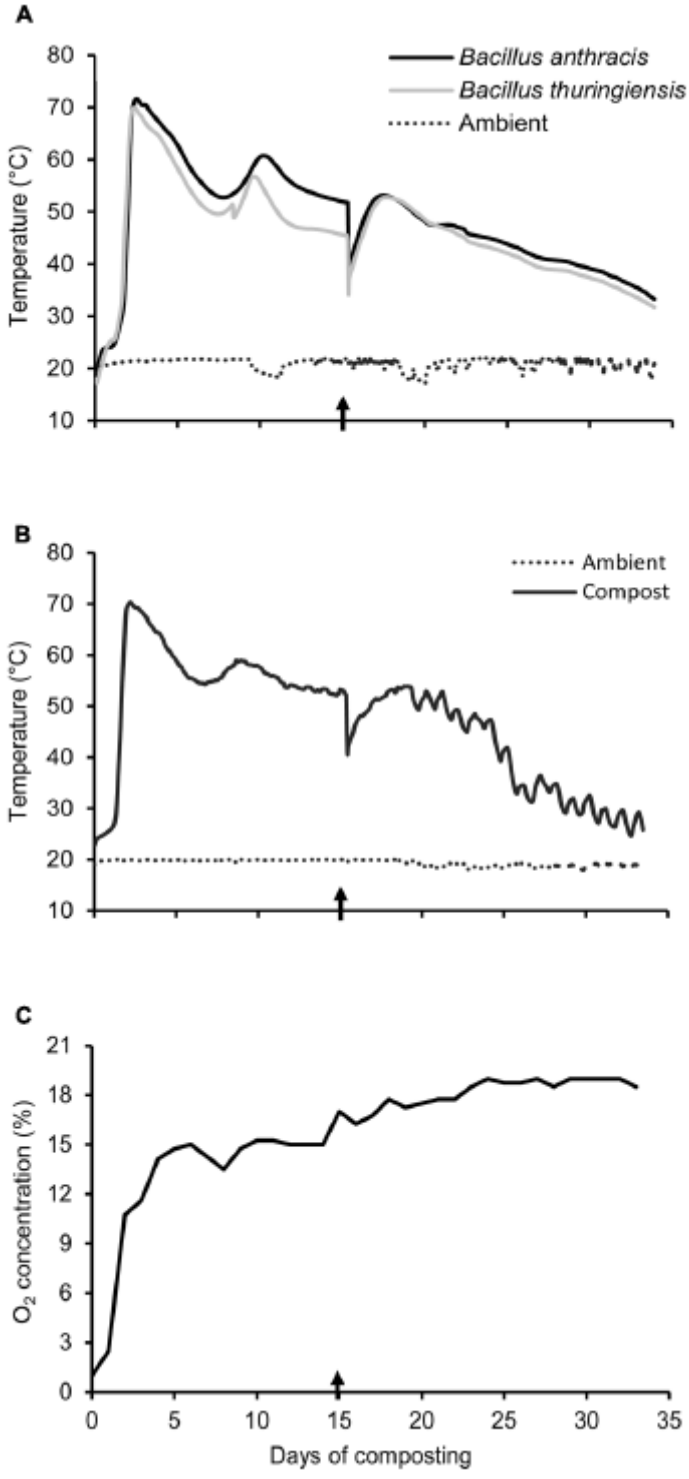

Figure 4-2. (a) Temperature during composting of feedlot cattle manure containing *B. thuringiensis* and *B. anthracis* spores under containment, and (b) temperature and (c) O_2 concentration during composting of feedlot cattle manure outside of containment. Arrows indicate the date when compost was mixed and moistened.

Compost temperature in containment (Figure 4-2A) mirrored that obtained outside of containment (Figure 4-2B), which was used to assess changes in physicochemical parameters during composting. Oxygen concentration was 1% in compost at day 0 (Figure 4-2C) and then increased to 15% after 5 days, and remained between 15% and 19% until the end of experiment (Figure 4-2C). During the two cycles of composting, moisture content consistently remained in the range of 74-77% (wet weight basis) (Table 4-2). Levels of TC, C/N ratio, NH_4^+-N, and (NO_2^- + NO_3^-)-N steadily declined over each composting cycle, whereas TN tended to increase (Table 4-2). The pH was 7.7 at the start of composting, and increased to 9.1 and 9.3 after the first and second composting cycle, respectively (Table 4-2). Both compost EC and bulk density gradually declined during the initial composting cycle. However, compost EC remained the same whereas bulk density increased over the second cycle (Table 4-2).

4.3.2 Survival of *Bacillus thuringiensis* spores

For controls, enumerated *B. thuringiensis* spores generated at all three temperatures remained consistent within the silica beads and manure at room temperature, only declining by 0-0.3 $\log_{10}$ CFU g^{-1} over 33 days (Figure 4-3). During two cycles of composting, viability of *B. thuringiensis* spores sporulated at 15°C and 21°C exhibited a similar reduction of 2.7 and 2.6 $\log_{10}$ CFU g^{-1}, respectively (Figures 4-3A and 4-3B). This reduction was more ($P < 0.05$) pronounced in the first than the second composting cycle (Figures 4-3A and 4-3B). Overall, the reduction in viability of spores generated at 37°C averaged 0.6 $\log_{10}$ CFU g^{-1} after two cycles of composting (Figure 4-3C), which was lower than ($P < 0.05$) those sporulated at either 15°C or 21°C (Figures 4-3A and 4-3B).

Table 4-2. Physicochemical changes of compost mixtures during laboratory-scale composting

Parameters*	Day 0	Day 8	Day 15 (Before mixing)	Day 15 (After mixing)	Day 22	Day 33
Moisture (%)	75.1 ± 0.6	75.2 ± 1.7	74.3 ± 1.4	77.6 ± 0.5	76.7 ± 1.1	77.0 ± 1.0
Total carbon (%)	46.1 ± 0.3	45.3 ± 0.3	44.8 ± 0.2	44.9 ± 0.5	43.7 ± 0.3	42.6 ± 0.6
Total nitrogen (%)	1.40 ± 0.02	1.57 ± 0.05	1.53 ± 0.03	1.63 ± 0.04	1.82 ± 0.03	2.20 ± 0.05
C/N ratio	33.0 ± 0.5	29.0 ± 1.2	29.4 ± 0.6	27.7 ± 0.9	24.1 ± 0.6	19.4 ± 0.6
pH	7.65 ± 0.06	8.84 ± 0.03	9.11 ± 0.03	8.92 ± 0.02	9.17 ± 0.04	9.25 ± 0.03
EC (ds m^{-1})	1.02 ± 0.05	0.63 ± 0.09	0.63 ± 0.09	0.66 ± 0.07	0.66 ± 0.07	0.66 ± 0.10
NH_4-N (mg kg^{-1})	2514 ± 15	1229 ± 296	1274 ± 198	1163 ± 64	429 ± 156	192 ± 68
(NO_2+NO_3)-N (mg kg^{-1})	33.5 ± 7.8	10.3 ± 3.4	8.4 ± 2.3	12.6 ± 9.5	1.9 ± 0.7	3.4 ± 0.8
Bulk density (kg m^{-3})	491 ± 7	481 ± 17	471 ± 19	552 ± 19	559 ± 19	573 ± 21

*All parameters except moisture and bulk density are expressed on a dry-weight basis (w w^{-1}); Moisture and bulk density are expressed on a wet-weight basis; EC: electrical conductivity.

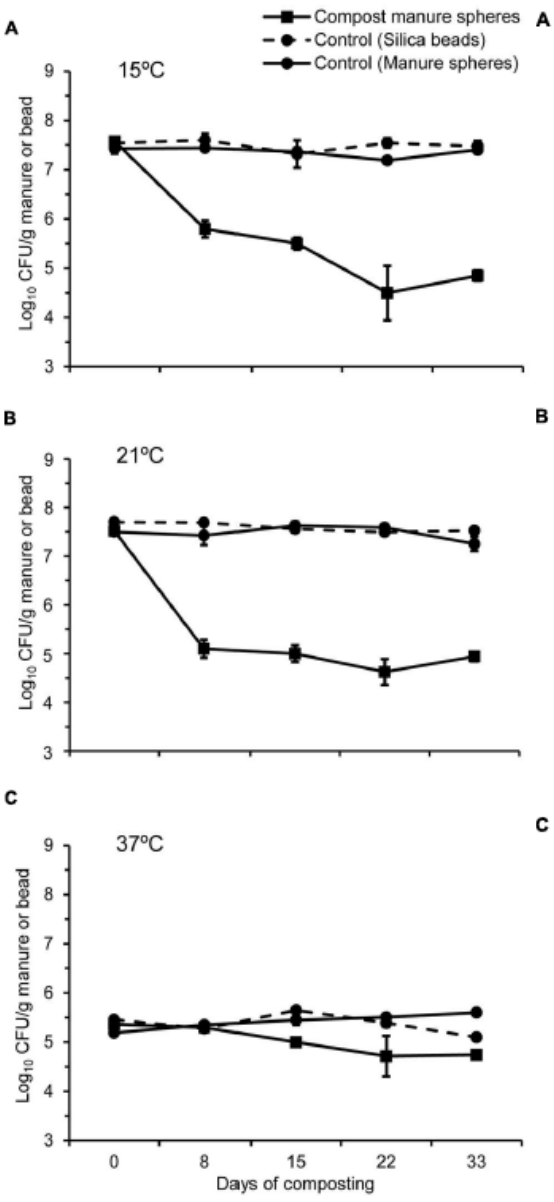

Figure 4-3. Survival of *Bacillus thuringiensis* spores (Log_{10} CFU g^{-1} manure) generated at different temperatures (a) 15ºC, (b) 21ºC and (c) 37ºC and placed in manure spheres that were composted with feedlot cattle manure for 33 days. For controls, silica beads and fresh manure spheres were inoculated with *B. thuringiensis* spores and kept at room temperature over 33 days.

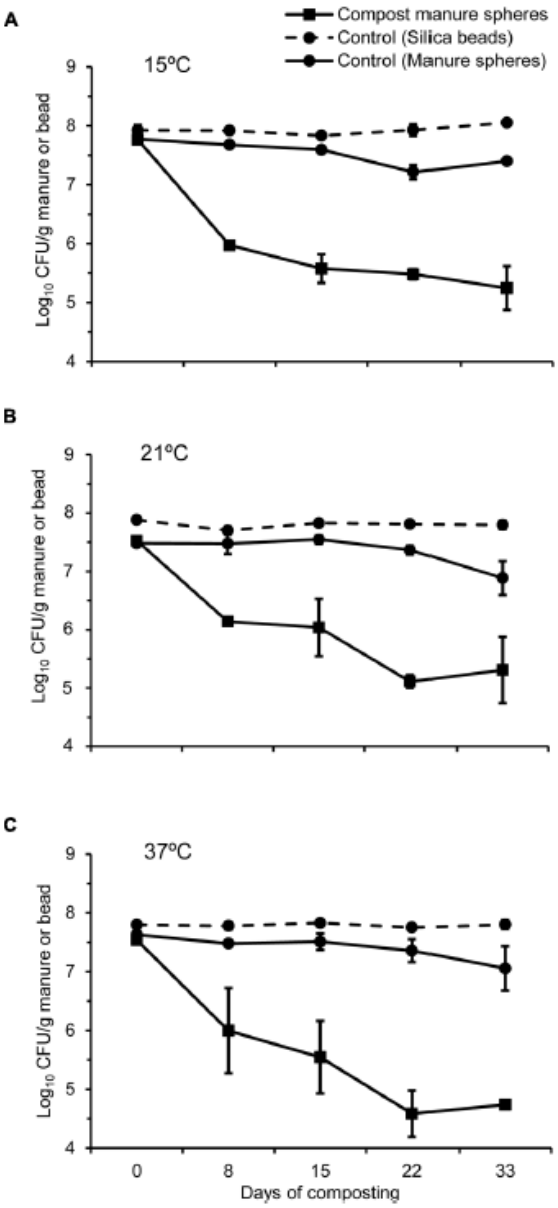

Figure 4-4. Survival of *Bacillus anthracis* spores (Log_{10} CFU g^{-1} manure) generated at different temperatures (a) 15ºC, (b) 21ºC and (c) 37ºC and placed in manure spheres that were composted with feedlot cattle manure for 33 days. For controls, silica beads and fresh manure spheres were inoculated with *B. anthracis* spores and kept at room temperature over 33 days.

4.3.3 Survival of *Bacillus anthracis* spores

Spores of *B. anthracis* generated at all three sporulation temperatures remained virtually stable in the control silica beads stored at room temperature over 33 days (Figure 4-4). In contrast, spore viability decreased by 0.4-0.6 $\log_{10}$ CFU g^{-1} in the control manure over the same time period (Figure 4-4). Composting reduced the number of viable spores compared to inoculated control samples held at room temperature. However, sporulation temperature exerted no effect on the survival of *B. anthracis* spores in compost (Figure 4-4). The numbers of viable spores generated at 15°C, 21°C and 37°C declined by 2.2, 1.5, and 2.0 $\log_{10}$ CFU g^{-1}, respectively, after the first composting cycle (Figures 4-4A, 4-4B and 4-4C). After the compost was mixed and moistened, numbers of viable *B. anthracis* spores generated at all three temperatures continued to decline, with reductions of 2.5, 2.2, and 2.8 $\log_{10}$ CFU g^{-1} at 15°C, 21°C, and 37°C, respectively (Figures 4-4A, 4-4B and 4-4C).

4.4 Discussion

4.4.1 Laboratory-scale composters

Passively aerated laboratory-scale composters (Xu *et al.*, 2010) were used to study the composting of *B. anthracis* under containment conditions. In my study, physiochemical changes in compost over the experimental period were similar to those reported in previous studies (Xu *et al.*, 2010, 2013a, 2014), demonstrating that the laboratory-scale composters were appropriate as a model for examining the ability of composting to inactivate *B. anthracis* spores. Using the same model, the degree of degradation of scrapie (Xu *et al.*, 2013a, 2014), CWD and BSE (Xu *et al.*, 2014) have been investigated,

showing a 1-3 $\log_{10}$ reduction in these prions after two cycles of composting. My findings further demonstrate a 2-3 $\log_{10}$ reduction of *B. anthracis* spores after two cycles of composting. Due to biocontainment restrictions for field-scale composting of *B. anthracis*, it was necessary to first assess the inactivation of *B. anthracis* spores within biocontained laboratory composting systems.

4.4.2 Compost Temperatures

Several techniques, alone or in combination, have been tested for the inactivation of *Bacillus* spores, including heating, radiation, UV treatment, chemicals, and high pressure (Xing *et al.*, 2014; Setlow, 2006; Roberts and Hoover, 1996). The mechanisms involved in these inactivation methods are linked to physicochemical modifications of metabolism proteins, inactivation of critical enzymes essential for spore germination, accumulative DNA damage, breakdown of spore membrane permeability barriers, and flattening and lengthening of the spores (Coleman *et al.*, 2010; Cléry-Barraud *et al.*, 2004). Composting is a natural biological process involving decomposition and stabilization of organic matter within an aerobic environment. Achieving optimal temperatures in compost is critical to reducing the viability of *Bacillus* spores. The majority of pathogens in compost are rendered non-viable if exposed to temperatures above 55°C for an extended period of time (Xu *et al.*, 2009a; Kalbasi *et al.*, 2006). Guidelines for composting from the Canadian Council of Ministers of the Environment (CCME, 2005) and the United States Environmental Protection Agency (USEPA, 1995) both suggest that the duration of exposure at or above 55°C should be at least 15 consecutive days within compost windrows and 3 consecutive days in confined or in-vessel composters. My recent findings showed that sustained thermophilic temperatures for 78 of 150 days in a laboratory oven reduced the number of viable *B. licheniformis and B. thurigiensis* spores in cattle manure

by ≥ 5 $\log_{10}$ CFU g^{-1} (Stanford *et al.*, 2016). In the present study, temperature profiles showed that compost temperature remained ≥ 55°C for an average of 7 days, resulting in 2-3 $\log_{10}$ CFU g^{-1} reduction of *B. anthracis* spores in compost. Although temperatures did not exceed 55°C in the second heating cycle, mixing and moistening of compost extended the period for inactivation of *B. anthracis* spores. However, the reduction of *B. anthracis* spores was lower in the second cycle as compared to the first cycle. Peak temperature was higher and the duration of exposure to thermophilic temperatures was longer in the first as compared to the second composting cycle, suggesting that thermophilic compost temperatures ≥ 55°C were more effective at reducing spore viability than mesophilic temperatures. This is consistent with previous results on the inactivation of *B. cereus* as a surrogate for *B. anthracis* during composting of cattle carcasses for 217 days (Stanford *et al.*, 2015).

4.4.3 Survival of *Bacillus* spores

Moreover, the duration of survival of *Bacillus* spores is lower when exposed to wet heat compared to dry heat, although the mechanisms for this phenomenon have not been fully elucidated (Nicholson *et al.*, 2000). My laboratory-scale compost remained moist (i.e., ~76% moisture content; wet weight basis) as I added water to the compost after the first cycle. This approach likely increased the inactivation of *Bacillus* spores as compared to the scenario where dehydration would lead to a reduction in composting activity. Field scale compost piles consist of a heterogeneous matrix of organic matter with significant variation in moisture content within the mass, particularly with static composting (Xu *et al.*, 2009a). The decline in spore viability may be less in regions of the pile where moisture levels are suboptimal for composting. In addition, pH has been reported to influence the heat resistance of *Bacillus* spores. Heat resistance is greatest at near

neutrality pH and decreases under acid or alkaline conditions (Palop *et al.*, 1999). Baweja *et al.,* (2008) showed that the viability of *B. anthracis* Sterne spores was reduced by exposure to either acidic or alkaline chemicals. In my study, compost pH increased by more than 1.5 units to a pH ~9, conditions that likely enhanced the inactivation of *B. anthracis* spores in compost.

4.4.4 Survival of surrogate strain

Bacillus anthracis belongs to the *Bacillus cereus* group along with *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis* with which it shares many morphological, biochemical, and genetic similarities (Maughan and Van der Auwera, 2011; Harrell *et al.*, 1995). *B. cereus*, *B. licheniformis* and *B. thuringiensis* have been previously used as surrogates for *B. anthracis* to assess the survival of *B. anthracis* spores during long term composting (i.e., ~200 days) of cattle carcasses (Reuter *et al.*, 2011; Stanford *et al.*, 2015). Recent studies have shown that *B. thuringiensis* is gaining acceptance as the most suitable model for *B. anthracis* (Greenberg *et al.*, 2010; Bishop and Robinson, 2014; Tufts *et al.*, 2014). One of the genetic differences between these two species is that *B. anthracis* lacks a gene encoding a pleiotropic regulator, which is involved in sensing the external environment, activation of exoenzyme synthesis and other functions related to spore survival within the environment (Gohar *et al.*, 2008; Bishop, 2014). However, this difference does not appear to result in differences between these two species in their ability to cope with environmental stresses, including dry heat, wet heat, and chlorination (Setlow *et al.*, 2014; Buhr *et al.*, 2012; Rice *et al.*, 2005). My results demonstrated that *B. thuringiensis* spores were inactivated in a manner similar to *B. anthracis* by composting, further supporting the use of *B. thuringiensis* spores as a model to assess the environmental robustness of *B. anthracis* spores.

4.4.5 Sporulation Temperature

To date, little is known about the effect of the sporulation temperature on survival of *Bacillus* spores during composting. Studies with various *Bacillus* species have shown that increasing the temperature during sporulation correlates with increased heat resistance of *B. cereus* (Collado *et al.*, 2006), *B. weihenstephanensis* (Baril *et al.*, 2011), *B. licheniformis* (Raso *et al.*, 1995), and *B. subtilis* (Condon *et al.*, 1992). However, this trend can be inconsistent among different strains within the same species (González *et al.*, 1999; Fernandez-Coll & Rodriguez-Toro, 1986), indicating that there are likely genotypic differences among strains that impact the heat resistance of *Bacillus* spores (Raso *et al.*, 1995; Condon *et al.*, 1992). Results in my study are consistent with Ignatenko *et al.,* (1983) who reported that a rise in sporulation temperature from 20°C to 35°C increased the thermal resistance of *B. thuringiensis* spores. For species of *B. anthracis*, Baweja *et al.,* (2008) demonstrated that *B. anthracis* Sterne spores generated at 45°C were more resistant to wet heat than those sporulated at 25°C. However, *B. anthracis* spores sporulated at temperatures as high as 37°C in my study did not exhibit superior survival during composting as compared to those that were formed at lower temperatures. The complex environmental conditions during composting likely make temperature exposure only one of many factors that influence the viability of *B. anthracis* spores and it is possible that strains of *B. anthracis* differ in responses to these environmental challenges. Investigating the composting of endospores exposed to different sporulation temperatures is crucial, as it has important implications concerning decontamination protocols under various weather conditions (Palop *et al.*, 1999; Condon *et al.*, 1992). My findings suggest that composting may be suitable for disposal of *B. anthracis* infected carcasses at geographical locations experiencing mild (15-21°C) or hot (> 30°C) ambient

temperatures. There is evidence that *B. anthracis* spores may undergo germination and growth in plant rhizosphere (Saile and Koehler, 2006) and may grow and amplify within amoebic hosts that remain viable outside of this temperature range (Dey *et al.*, 2012).

4.4.6 Composting *Bacillus*

To my knowledge, this is the first study describing the inactivation of *B. anthracis* spores as a result of composting. Limited biomass in the laboratory-scale composters resulted in thermophilic temperatures (≥ 55°C) for only 7 days during two composting cycles over a period of 33 days. This contrasts with a previous field-scale system employed for investigation of the same strain of *B. thuringiensis* spores where temperatures ≥ 55°C were recorded for 75 days out of 230 days of composting (Reuter *et al.*, 2011). These differences in duration of thermophilic temperatures were reflected in the degree of inactivation of *B. thuringiensis* spores with a 1-3 $\log_{10}$ reduction in the laboratory composters after 33 days, as compared to a ~5 $\log_{10}$ reduction within field-scale composters over 112 days (Reuter *et al.*, 2011). Therefore, it is not unrealistic to expect a more extensive inactivation of *B. anthracis* spores during field-scale composting. However, the complete inactivation of *B. anthracis* spores during composting is likely unrealistic. In practice, compost piles are affected by a number of internal and external factors including the heterogeneous nature of animal tissues and other matrix components that can result in fluctuating heat generation and distribution (Xu *et al.*, 2009a). Microbial communities that may play a role in the inactivation of spores may also differ among locations within the compost pile (Tkachuk *et al.*, 2014). Consequently, further research is required to investigate the survival of *B. anthracis* spores in the microenvironments of compost piles where thermophilic temperature conditions may be compromised.

In my study, *B. anthracis* Sterne, an attenuated non-encapsulated variant, was employed (Welkos and Friedlander, 1988). This strain does not have the pXO2 plasmid, which carries the capsule genes. However, all chromosomal genes responsible for sporulation and germination are present (Cléry-Barraud *et al.*, 2004). Thus, spore components and spore resistance of this strain are assumed to be identical to those of the wild strain (Pézard *et al.*, 1993). However, further comparisons of the difference in the survival of spores from the wild type *B. anthracis* strain and Sterne strain may be required to assess the potential use of composting for disposal of *B. anthracis* infected carcasses.

Furthermore, heat resistance of *Bacillus* spores is affected by the nature of matrix in which spores are heated (Coroller *et al.*, 2001). I showed that the numbers of viable *B. anthracis* spores in manure retained at room temperatures declined by ~0.5 $\log_{10}$ over 33 days while spores in the silica beads remained stable over the same time period. This suggests that the majority of the reduction in spore viability was associated with exposure to high temperatures, but microbial activity may have also contributed to this response. It is possible that the thermal resistance of *B. anthracis* spores originating from carcass exudate may differ from those composted in manure (Stanford *et al.*, 2015). Future studies should evaluate the survival of *B. anthracis* spores in compost in the presence of animal tissues or fluids with various levels of fat, carbohydrate or proteins. However, it had been previously shown that tissues other than ossified bones in bovine carcasses are completely degraded during field scale composting (Xu *et al.,* 2009a).

In conclusion, composting should be considered as a simple method for on-site containment of infected carcasses in the event of an anthrax outbreak. The outcomes from my study showed a 3 log inactivation of *B. anthracis* spores was achieved after one month of laboratory-scale composting. Further reductions in survival of *B. anthracis*

spores are likely possible with field-scale composting as the duration of the thermophilic period is typically much longer. Although all *B. anthracis* spores might not be completely destroyed by composting, potential for the spread of these spores at infectious doses after composting would be reduced after land application due to both dilution and inactivation effects. Therefore, carcass composting might be considered as a viable method to reduce the dissemination of *Bacillus* spores to the surrounding environment.

Chapter 5

5.1 Summary of Major Findings

The overall objective of this study was to investigate inactivation of *Bacillus* endospores exposed to compost conditions. In the first experiment, after 150 days of exposure to temperatures mimicking field-scale composting, the oven experiment showed an average of 5.2 $\log_{10}$ CFU g^{-1} reduction in spore viability. The primary factors for reduced viability appear to be temperature and spore desiccation as the impacts of compost microbiota were negated by autoclaving both compost and silica beads. The second experiment using laboratory-scale composters, compost temperature remained ≥ 55°C for an average of 7 days and again the results showed a 2-3 $\log_{10}$ CFU g^{-1} reduction of *B. anthracis* spore viability. To my knowledge, this is the first time that composting was used to inactivate *B. anthracis* endospores. The second experiment highlights a potential issue with compost, as the second cycle did not exceed 55°C, although returning moisture and oxygen extended the period of inactivation of endospores. Composting reduced endospore viability, but further research is still needed to assess the efficacy of this approach, as spatial variation within the pile resulted in uneven heat distribution and variability in the inactivation of *Bacillus* endospores.

5.2 Heat Distribution

To address the issues with potential inadequate heat distribution one could run the composting process through repetitive composting cycles. Multiple composting cycles could help ensure that at some point the endospores are exposed to adequate temperatures that would achieve inactivation. Addition of carbon and or nitrogen sources to the piles may be required to ensure that temperatures above 55°C are obtained during subsequent cycles.

Another option would be to promote the germination of the *B. anthracis* endospores to the fragile vegetative forms, by two methods. Firstly, one could ensure that carcasses are not breached so that internal organs are not exposed to oxygen. This would prevent exudate escaping from the carcass and prompt onsite composting of carcasses could help prevent sporulation. The second option would be addition of germinants to the compost setup such as alanine and inosine. If *B. anthracis* could be maintained in the vegetative state, cells could be inactivated at lower temperatures in less time (Bishop, 2014). Further investigations of these two methods are needed to address their viability and the potential of using both concurrently while composting.

5.3 Airflow methods

Disposal using composting also requires additional management and supervision due to the requirement of precise parameters for uniform heating of the pile as inconsistent temperatures within the pile may permit the survival of pathogens in areas where temperatures are insufficient to inactivate pathogens. This investigation physically turned composters to aerate the piles, but this is the not the preferred method as it risks further dissemination of contaminated materials and places workers at risk. Convection airflow could be an alternative method of promoting aeration within the pile (CCME, 2005) an approach that needs further investigation during the composting of *B. anthracis*. I propose that in mini-composters could be transported to location of the outbreak and be used to compost individual carcasses on-site. Systems such as drum composters lend themselves to convective airflow and could be tested in field evaluation experiments.

5.4 Challenges of Compost Onsite

There are some negative aspects when considering composting as a method of disposing of carcasses infected with anthrax. Depending on the outbreak location, carcass type and

amount of matrix material needed for disposal, the area required for composting may be large. This may prove to be a challenge in forested or remote areas where access to equipment is limited. Remote locations also have the challenge to implement compost as access to materials such as enough manure or wood shavings may also be limited. The difficulty and cost of transporting manure and wood shavings is not as expensive as compared to renewed disease cycles due to inadequate alternative disposal methods or the risks to personnel when transporting infected materials.

There may also be negative environmental impacts associated with composting; including leachate, attraction of vermin or pests and the release of malodourous gases. These negative aspects of compost can be mitigated with design of bio-contained mini-composters brought onsite during an outbreak. Current methods during outbreaks in remote locations, if burial or incineration is not possible, include covering carcasses with formaldehyde and /or tarps, can also result in the formation of leachate and attract scavengers as occurred during an outbreak in the July 2012 (NTDENR, 2013).

Compared to other alternative disposal methods, composting is relatively slow resulting in longer quarantine times and increased risk of breaches in containment barriers from wildlife. Although compost may not completely destroy all endospores, it mitigates the risks of handling and transporting infectious materials post-composting as compared to current disposal alternatives. Compost may be a great alternative to introduce while waiting for seasonal changes to safely permit incineration or also reduces the concentration and overall amount of infectious materials to be transported for alternative disposal methods such as rendering.

5.5 Changes to Anthrax Disease Management

In April 2013, CFIA made changes on how the agency manages certain reportable diseases. Although any suspected cases still must be reported to CFIA, the responsibilities shift to livestock producers to manage anthrax outbreaks. Producers are now responsible for costs associated with anthrax investigations, including collecting and performing anthrax tests, quarantining infected areas, cleaning and disinfection of all materials and carcasses. CFIA no longer will help cover the cost of disposal of any animal carcasses (CFIA, 2013). Current CFIA guidelines accept deep burial with preference for incineration disposal of anthrax carcasses and contaminated materials. The methods are outdated and may be restrictive due to environmental conditions. The negative air quality due to smoke from incineration (Sutmoller *et al.,* 2003) and restrictions on proximity to flammable materials such as range or heavily forested areas restrict the use of incineration (Hugh-Jones & De Vos, 2002). Burial returns *B. anthracis* endospores to their natural soil reservoir to survive for decades with the potential for new infection as a result of flooding (Nicholson, 2002) or disturbance of burial sites by humans or wildlife (Dragon *et al.,* 1999; Himsworth and Argue, 2008; Nicholson, 2002). When traditional methods for disposal are deemed inappropriate compost may be a viable alternative.

The changes by CFIA for how an anthrax outbreak is handled now leave the livestock producer to work with local veterinarians and this study shows that compost has the potential to be viable alternative, providing a simple onsite disposal method. Livestock producers will appreciate how compost is low cost and has simple set-up procedures, requires minimal training and uses materials that are readily available at most livestock operations.

5.6 Future Areas of Research - DNA Damage

Investigating DNA damage is particularly important as the distinguishing characteristics among *Bacillus* species are encoded on highly variable and mobile extrachromosomal DNA elements (Helgason *et al.,* 2000; Hoffmaster *et al.,* 2004). Researchers have identified *B. cereus* strains that possess genes encoding the pXO1 anthrax toxin complex, and pXO2 capsular genes (Hoffmaster *et al.,* 2004). These *B. anthracis*-like strains were still distinguishable from *B. anthracis* by phenotypic and biochemical properties, but did exhibit increased virulence. Therefore, to determine if there is a risk of horizontal transfer of genes post-composting, research is needed to determine the extent to which Bacillus DNA is degraded during composting.

5.7 Wild type *B. anthracis* strain

These investigations are the first to compost a strain of *B. anthracis*. The next step is to investigate the survival of endospores from the fully virulent wild type *B. anthracis* strain to compost conditions. Field-scale composting conditions with more biomass would result in a longer thermophilic period and may result in a greater reduction in endospore viability.

5.8 Interrupting Anthrax Disease Cycle

Following an anthrax outbreak, carcasses undergo rapid putrefaction while exuding poorly clotting infectious materials from all orifices into the environment. Within the rhizosphere of grasses, *B. anthracis* endospores may germinate, creating a new population of vegetative cells that persist and survive (Organization & Epizootics, 2008). Pasteur was able to provide evidence that buried carcasses that died due to anthrax served as sources of reinfection. Even earth worms assisted in this reinfection cycle when they transported endospores back to the surface (Guichard *et al.,* 2012) as spores remain viable

after passing through their digestive tract (Hendriksen & Hansen, 2002). Spring showers can flood anthrax burial sites permitting buoyant endospores to rise or be flushed to the ground surface and runoff to collect and dry in low lying areas such as sloughs or bison wallows. As these low lying areas are favourable grazing areas, this provides optimal means for re-infection of livestock attracted to the rich foliage (Himsworth & Argue, 2008). As opposed to burial or burning the carcasses, individually composting these carcasses on site using bio-contained composters would interrupt the reinfection cycle by containing and reducing the number of infectious endospores returned to the environment.

5.9 Proposed Field Compost Design

Future studies need to be completed on the feasibility of composting anthrax mortalities as rapid putrefaction makes the animal carcass fragile and prone to tearing apart upon moving. This would make moving mortalities into bio-contained-composters difficult and dangerous. I propose an alternative approach during an anthrax outbreak would be to build composters on-site, limiting the transportation and reducing the risk of opening the carcass (Figure 5-1). Building a bio-contained composter to each individual carcass has the benefit of composting the quickly decaying materials while still intact, increasing the likelihood that the materials being composted are fragile vegetative cells and not thermal resistant endospores. I propose that when conditions for carcass disposal are not optimal for incineration, that instead of burial or tarping that individual composters are designed to each carcass.

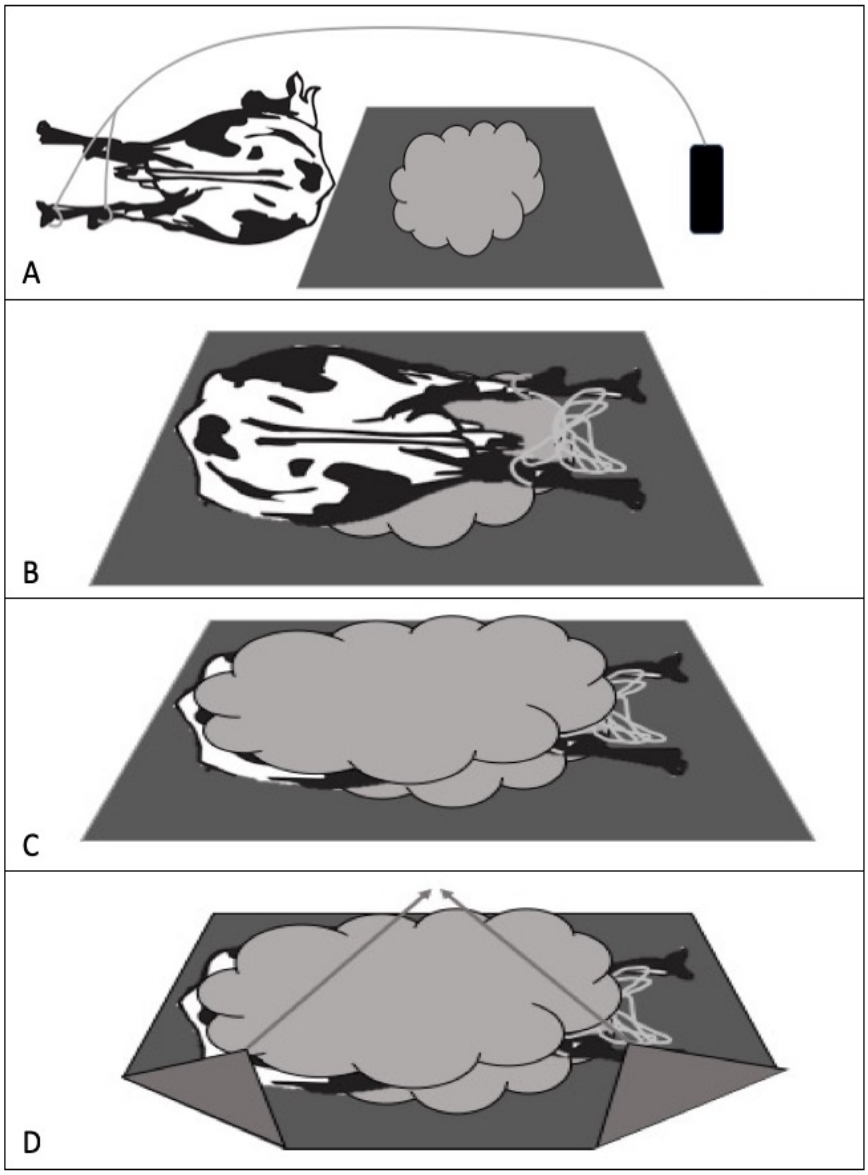

Figure 5-1. Proposed field design of composting carcass.
(A) Carcass with ropes attached to lower extremities, tarp materials with compost layer and a hand-crank winch, or come-a-long, with a land anchor.
(B) Carcass rolled onto tarps with compost layer underneath and ropes are disconnected from winch and left with carcass.
(C) More compost is added.
(D) Edges of tarp are pulled and secured to center over carcass to create biocontainment of compost and carcass.

Adjacent to each carcass a tarp is laid down with layer of pre-mixed compost and ropes are attached to lower extremities of the carcass and a hand-cranked winch or come along (Figure 5-1A). The carcass is then carefully rolled on top of the compost materials and rope materials are left with carcass (Figure 5-1B). More compost materials are added (Figure 5-1C). Finally, biocontainment of carcass is completed by turning up the corners of the tarp, closing the carcass and compost materials towards the center (Figure 5-1D). Grommets attached to the corners of the tarp can then be attached to ropes and tied to center. Future experiments will need to be conducted to work out specific design parameters for optimal composting conditions. Once the compost has been setup, specific additions such as a cage or fence will aid preventing scavengers. Conditions to be investigated include: compost materials needed, airflow, biocontainment and duration and number of compost cycles. Experiments for compost materials are to help technical staff in the field determine the optimal ratios of manure to woodchips needed for a range of carcass sizes and degree of decay. For instance, experiments on different degrees of carcass degradation may be needed to determine approximant moisture present to be able to adjust in the field. Airflow needs to be investigated, as materials may need to be added to aid passive aeration, such as PVC pipes. Preventing leakage and scavengers will be addressed in the biocontainment design experiments that address how well certain tarps will contain leakage and sizes required in the field to contain each carcass. Thermometers will monitor the compost cycle and will aid field technicians in determining when compost cycle completes. Following the first cycle of compost, the environment conditions can be readdressed to see if incineration can be considered. For instance, composting infectious carcasses will not only reduce that amount of infectious materials, but also reduced endospore viability therefore reducing the concentration of endospore

and therefore the risk of reinfection are lowered. Post-compost, these less infectious and reduced volume, can be either incinerated if environmental conditions changed (seasonal changes), composted again or these materials can be buried. An advantage of this design is this setup is bio-contained, this creates the option of transporting these bio-contained carcasses to areas permitting incineration (areas with less forest).

Future investigations of mini-onsite composters will be beneficial for disease management for rural communities but also developing countries. Developing countries need cost effective waste management alternatives to manage anthrax and other infectious disease outbreaks.

5.10 Temperature Conditions

My study was able to show that sporulation was very limited and very slow at lower temperatures, indicating that risks of sporulation are reduced when environmental conditions drop below 10°C. Another option to ensure more *Bacillus* remain in their fragile vegetative state would be to setup and manipulate carcases at night or when temperatures drop. Compost is a year-round disposal alternative, and I propose that initial composters be setup when the evenings are cooler with second cycles of composting used during winter months.

Sporulation temperatures impacted spore viability but was species dependent. In the first experiment, spores produced at 37°C had higher viability than endospores sporulated at 25°C and 30°C. In the second experiment, *B. thuringiensis* endospores that were produced at 37°C had increased survival as they had lower reductions of viability compared to spore generated at 15 or 21°C. In contrast to the endospores of *B. anthracis* that indicated sporulation temperature had no impact on survival. I would propose that similar to cardinal temperatures for growth, that *Bacillus* endospores have cardinal

sporulation ranges. On further exploration, a range can be determined for the minimum, maximum and optimal temperatures. At this optimal sporulation temperature, the endospores would have maximum increased thermal resistance. This temperature range may be correlated with the cardinal ranges for vegetative growth. As seen with my results, *B. thuringiensis* had increased survival compared to *B. anthracis*. This could be due to the fact that *B. thuringiensis* optimal growth temperature is 30°C (Table 1-1) as compared to *B. anthracis* at 37°C. Future experiments with *B. anthracis* at higher than 37°C, such as 39 up to 45°C, are warranted as these temperatures are more representative of the body temperatures of cattle and birds, respectively. As the high body temperature of birds has been one factor that has been proposed to account for the reduced susceptibility of avian species to anthrax (Goel, 2015; Dragon *et al.,* 1999). Estimating the optimal sporulation temperature that maximizes endospore thermal resistance is vital for estimating the efficacy of decontamination procedures that could reduce the risk of anthrax outbreaks (Baril *et al.,* 2012).

5.11 Conclusion

Overall, compost showed to reduce the concentration and amount of infectious materials. Furthermore, it reduces the risks associated with burial or transporting infected tissues to locations where incineration is possible. With outbreaks in British Columbia, as recent as October of 2018, anthrax remains a current threat in Canada. Current disposal methods have limitations and challenges and alternative methods need to be investigated. These studies have shown that compost is a viable option and with further investigation, future outbreaks may have the anthrax disease cycle interrupted and reduce future outbreaks with the implementation of composting.

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