Investigation of *Bacillus anthracis* spore survival in soils from Kruger National Park in South Africa and Etosha National Park in Namibia

By

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Submitted in fulfilment of the requirements for the degree

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2022

Declaration

I, Kamini Govender, declare that this dissertation submitted for the degree MSc (Veterinary Tropical Diseases) at the University of Pretoria, is my original work and has not been submitted by me for the purposes of a degree at another institution.

Kamini Govender

12 December 2022

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Acknowledgements

A sincere thank you to my supervisor, Prof Henriette van Heerden. I feel extremely privileged to work on and to make a scientific contribution to this incredible topic, so I want to wholeheartedly thank you for giving me this opportunity. Thank you for your continuous support throughout this project. I will always remember you for your kindness, patience and understanding. You are a true inspiration.

To my co-supervisor, Dr Ayesha Hassim, thank you for being more than just a co-supervisor. Thank you for always going above and beyond. Thank you for all your teachings, in science and in life. It was truly an honour to be mentored by you.

To Dr Wendy Turner, it was an absolute pleasure to work with you. Thank you for your superb input. Thank you for always being so encouraging and supportive.

To Mr Jawid Hassim, a huge thank you for making a major part of this project possible. Your efforts are greatly appreciated and thank you for always being available to assist. I cannot thank you enough for your contributions.

Thank you to Dr Jenny Rossouw for accommodating us and allowing this project to take place in the BSL-3 facility at the Special Bacterial Pathogens Reference Laboratory (National Institute of Communicable Diseases, NICD). I truly appreciate your kindness, consideration and hospitality. A warm thank you to Mr Herman Geyer for his technical assistance as well. I would also like to thank Dr Monica Birkhead from the Electron Microscopy unit at the NICD for her wonderful efforts and contributions to this project.

I would also like to thank Dr Sunday Ochai and Dr Charles Byaruhanga, for their assistance with the data and statistical analysis. I will always be grateful for your all time and efforts.

I want to acknowledge and express my gratitude to the following funding bodies for their contributions: The National Science Foundation, the National Research Foundation, AgriSETA and the UP postgraduate bursary programme. I will always be immensely grateful to the funders for the impact that their generosity has had on my life, both professionally and personally.

To my mum and brother, thank you for your endless sacrifices, unconditional love, and support. Mum, I am because you are.

Dedication

To my late Dad,

this one is for you.

List of abbreviations

Abbreviation	Definition
µL	Microlitre
µM	Micromoles
µm	Micrometre
ANOVA	Analysis of variance
B. anthracis	*Bacillus anthracis*
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
B. thuringiensis	*Bacillus thuringiensis*
BCL	*Bacillus* collagen like
BSL	Biosafety level
Ca	Calcium
CaM	Calmodulin
cAMP	Cyclic-adenosine monophosphate
CFU	Colony forming units
CVL	Central Veterinary Laboratory
DALRRD	Department of Agriculture, Land Reform and Rural Development
DNA	Deoxyribonucleic acid
DPA	Dipicolinc acid
DVTD	Department of veterinary tropical diseases
EF	Oedema factor
ENP	Etosha National Park
FRET	Fluorescence resonance energy transfer
g	Gram
K	Potassium
KNP	Kruger National Park
LF	Lethal factor
MAPKK	Mitogen activated protein kinase kinase
Mg	Magnesium
mL	Millilitre
MLVA	Multiple Locus Variable Number Tandem Repeat Analysis
Mn	Manganese
NA	Nutrient agar
Na	Sodium
NICD	National Institute for Communicable Diseases
nm	Nanometre
OBP	Onderstepoort Biological Products
PA	Protective antigen
PBS	Phosphate-buffered saline
PCI	Phenol:chloroform:isoamylalcohol
PET	Polymyxin B - EDTA - Thallous acetate agar
PFU	Plaque forming units
qPCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
SASP	Small acid soluble protein
SBA	Sheep blood agar
SBPRL	Special Bacterial Pathogens Reference Laboratory
SNP	Single nucleotide polymorphism
SOM	Soil organic matter
SSVO	Skukuza State Veterinary Office
VNTR	Variable Number Tandem Repeat

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ABSTRACT:

Investigation of *Bacillus anthracis* spore survival in soils from Kruger National Park in South Africa and Etosha National Park in Namibia

By

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Supervisor: Prof Henriette van Heerden

Co-supervisors: Dr Ayesha Hassim and Dr Wendy Turner

Degree: Master of Science (Veterinary Tropical Diseases)

Bacillus anthracis is a soil-borne pathogen and the aetiological agent of anthrax, which is a disease that primarily affects ungulates in enzootic regions. These enzootic regions occur where suitable soil types promote the survival of *B. anthracis* endospores despite several environmental stresses. This study focuses on the enzootic regions of Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia where the disease is unmanaged, and some pathogen dynamics have been well documented over time. In KNP and ENP, Pafuri and Okaukuejo are described as the high-incidence anthrax regions, respectively, while Skukuza (KNP) and Otjovasandu (ENP) are described as the low incidence regions. The phylogenetic distribution of *B. anthracis* strains over time differs between the parks. All isolates in ENP belong to the A-subclade while in KNP, there are isolates from both the A- and B-subclades. In KNP, the B-subclade dominated before the 1990s, but the A-subclade dominated thereafter. The main aim of this study was to investigate how the different soil types from these high- and low-incidence areas affect the survival of different *B. anthracis* strains in a laboratory experiment. A fully insulated terra-simulator with sensors which measured the soil moisture, ambient temperature, humidity, and light was designed and programmed to mimic the environmental conditions of Pafuri in real time. A pilot study using the Sterne strain was conducted initially to evaluate the experimental procedure, decontamination methods and the terra-simulator itself. Thereafter, two strains each from KNP and ENP were selected (one KNP A-, one B- and two ENP A-clade strains) as well as the Sterne strain and was inoculated into the four different soil types. The inoculated soils were incubated in the terra-simulator and monitored for 6 months. Spore counts were done at monthly intervals using classical microbiological techniques for the isolation of *B. anthracis* from soil and reported as CFU/g. In addition, soil analyses were conducted on the soil samples which looked at the soil pH, exchangeable

cations, soil density and soil organic matter. The spores were also prepared for electron microscopy to assess the differences in the nap layer of the exosporium (outermost layer of the endospore) for the strains used in this study. Overall, Pafuri soil had the best spore survival for all strains except for Sterne, followed by Otjovasandu, Okaukuejo and Skukuza. The A-strains survived better than the B-strain in all soil types while the Kruger B-strain showed a better survivability only in Pafuri. Okaukuejo and Otjovasandu supported the spore survival of the A-strains equally well, especially for the ENP endemic strains. The Sterne strain showed an overall poor environmental survivability. By 6 months, there was very little decline in the spore counts between the sampling time points, thus, the spore counts appeared to stabilise, with the exception of Sterne which continued to decline. Our findings suggested that strain type, time, relative humidity, and temperature were significant determinants of spore survival across the different soil types. Furthermore, soil characteristics such as pH, the availability of macro and micronutrients, soil organic matter, texture and moisture also influenced the spore survival across the various soil types. We identified differences in the filament lengths of the nap layers where the ENP A-strains had the longest filaments followed by the KNP A-strains and then the B-strain. We also identified appendages on *B. anthracis* spores which had only been previously described in pathogenic *B. cereus* strains. In conclusion, our findings showed that the A-strains survived better than the B-strain and that the B-strain survives best in its endemic soil type, Pafuri. The Sterne strain showed significant environmental lability when compared to the fully virulent strains. Spore survival is dependent on a number of factors including strain type and environmental determinants such as temperature, relative humidity and soil characteristics. These environmental determinants are factors that are constantly changing due to a culmination of climactic events which highlights the importance of consistent disease surveillance for this pathogen. In addition, there is a need for improved predictive modelling systems which considers the changing climate and its effects on the soil characteristics and spore survival.

1. CHAPTER ONE: INTRODUCTION

1.1. Overview

Anthrax has a long history from biblical times to modern day (Farrar, 1995). The earliest documented cases suggesting anthrax infections dates back to 1491 BC and refers to the fifth and sixth plagues of Egypt (Klemm and Klemm, 1959). Robert Koch used *Bacillus anthracis*, the aetiological agent of anthrax, as the basis for Koch's Postulates in 1877 and Louis Pasteur developed the first vaccine against anthrax in 1881 (Turnbull and Shadomy, 2010). This disease was one of the main causes of uncontrolled mortality in cattle, sheep and goats worldwide until the development of an effective veterinary vaccine (Sterne, 1937, Sterne, 1939) in addition to antibiotic therapy (Knudson, 1986) and quarantine strategies. The interest and concern with anthrax remained due to its use as a biological weapon, which was evident in the anthrax outbreaks in Sverdlovsk, Russia in 1979 (Meselson et al., 1994) and the attack in the United States of America in 2001 (Jernigan et al., 2001).

Anthrax is a zoonotic disease which typically affects domestic and wild herbivores while less susceptible hosts such as carnivores and pigs, and sometimes humans, are affected less frequently (WHO, 2008). The causative agent of anthrax is *Bacillus anthracis* which is a Gram-positive, rod-shaped, spore- and capsule-forming bacterium that is typically found in moist, alkaline, calcium rich soils (Koch, 1876, Van Ness, 1971, WHO, 2008). The *B. anthracis* spore form is resistant to a wide range of extreme weather conditions, chemicals, pressure, ultraviolet and ionisation radiation, especially when compared to the vegetative form of this bacterium (Gould, 1977, Turnbull et al., 1996). The spores were thought to be the infectious particles of this pathogen as they may remain dormant in suitable soil types for months, or even years (WHO, 2008), but the vegetative bacteria will also cause disease if ingested or comes into contact with open skin (Bischof et al., 2007).

When the spores are ingested by susceptible host animals, germination occurs and the protective antigen, edema- and lethal-factors are produced. This forms the commonly known anthrax toxin complex which is responsible for the oedematous swellings and haemorrhages that are typically seen in infected animals (Leppla, 1991, Smith et al., 1955, Stanley et al., 1961). The disease occurs acutely and is unfortunately almost always fatal. The vegetative forms undergo sporulation when oxygen and nutrient deficiencies are detected. The spores remain in the soil, especially at carcass sites and this allows the transmission cycle to continue (WHO, 2008).

Furthermore, practices such as the burial of infected carcasses allow the spores to become dormant and remain viable for decades (Turnbull et al., 1996). During World War II, *B. anthracis* spores were deliberately released on Gruinard Island (Scotland) during experimental biological warfare trials. The spores remained viable in the soil for over 40 years until the soils were successfully decontaminated (Miles et al., 1988). During the 2016 anthrax outbreak in Siberia (Russia), the source of the outbreak was suggested to be *B. anthracis* spores that were preserved in permafrost which subsequently thawed due to increasing temperatures. This caused a new infection cycle in a region where outbreaks had not been reported in over 70 years (Timofeev et al., 2019). *Bacillus anthracis* spores were also isolated from bones recovered in the Kruger National Park (South Africa) that were estimated to be 200 years old (De Vos, 1990).

Bacillus anthracis spore survival is attributed to the spore structure which consists of multiple layers of specialised concentric layers which protects the spore from desiccation and various environmental, chemical and mechanical stresses (Driks, 2009, Setlow, 2007, WHO, 2008). In addition, dipicolinic acid in the core causes dehydration of the spore and aids in heat resistance (Church and Halvorson, 1959, Powell, 1953). The outermost layer of the spore is the exosporium which consists of the basal layer and outer-nap layer (Gerhardt and Ribi, 1964). The exosporium is hypothesised to affect the spore's attachment to mammalian host cells and may aid in spore persistence in suitable soil types (Bozue et al., 2015, Williams et al., 2013). The persistence of *B. anthracis* spores is also dependent on a host of other factors which includes the initial concentration of spores in the soil, environmental conditions such as soil type and structure, rainfall, temperature, natural microbiota of the soil, vegetation and the availability of certain minerals in the soil (Carlson et al., 2018, Sterne, 1967, Titball and Manchee, 1987, Van Ness, 1971).

Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia both have enzootic and non-enzootic anthrax regions (De Vos et al., 1990, Ebedes, 1977, Hugh-Jones and De Vos, 2002, Ochai et al., 2022, Pienaar, 1967). Some anthrax dynamics in these regions have been well described over time with regards to the host species involved and outbreak seasonality (Huang et al., 2022, Hugh-Jones and De Vos, 2002, WHO, 2008), the dominant *B. anthracis* strain types (Beyer et al., 2012, Keim et al., 2000, Smith et al., 2000) and the soil types in these regions (Beugler-Bell and Buch, 1997, Hugh-Jones and De Vos, 2002, Lindeque and Turnbull, 1994, Venter, 1986). Anthrax is unmanaged in these areas as *B. anthracis* is considered to be part of the natural ecosystem (Hugh-Jones and De Vos, 2002) so this provides researchers with a unique opportunity to further study this pathogen.

In KNP, Pafuri, the northernmost part of the park, is considered to be the anthrax enzootic or high-incidence region (Hugh-Jones and De Vos, 2002, Pienaar, 1967). The greater kudu (*Tragelaphus strepsiceros*), which is a browser has been implicated as the main host species over time. This species contributed up to 75% of recorded cases from the 1960-1990's (De Vos and Bryden, 1995, WHO, 2008). In the past, large outbreaks would be experienced every 10 years and would occur during the dry season (De Vos et al., 1990, De Vos and Bryden, 1995, Pienaar, 1961). However, since 2008, a fewer number of outbreaks have been reported and these outbreaks occurred in the "wet" season but mainly affected impala (*Aepyceros melampus*), which is a mixed grazer and browser, however Huang (2022) noted that the shift from kudu to impala occurred mid-1990's (Basson et al., 2018, Huang et al., 2022). The role of blowflies (*Chrysomya* spp.) in the transmission cycle of *B. anthracis* has been hypothesised to be the cause of exposure to browsing species. It is theorised that the blowflies are able to mechanically transfer *B. anthracis* spores onto surrounding vegetation after they feed on animals that have died from anthrax (Basson et al., 2018, Braack and De Vos, 1990).

In ENP, Okaukuejo, is the central part of the park and is considered to be the anthrax enzootic or high-incidence region (Ebedes, 1977). The anthrax dynamics in ENP differs from KNP with regards to the outbreak season and host species (Huang et al., 2021, Huang et al., 2022). The outbreaks in ENP appear to be seasonal and this may be attributed to the variations arising from hosts feeding behaviours (Turner et al., 2014, Turner et al., 2016). It has been suggested that this may in turn alter the exposure rates and seasonal immune trade-offs such that other environmentally transmitted pathogens may be influenced by the presence of gastrointestinal parasites (Cizauskas et al., 2014, Havarua et al., 2014, Turner et al., 2013). In ENP, grazing and mixed-feeding herbivores are typically affected by anthrax. There is also a difference in the species which are affected during the wet and dry seasons whereby more mortalities of plains zebra (*Equus quagga*) and other herbivores are reported in the wet season while more mortalities of African elephant (*Loxodonta africana*) are reported in the dry season, with sporadic cases throughout the year (Ebedes, 1977, Lindeque and Turnbull, 1994). Of all recorded cases, 52% are plains zebras, 22% are blue wildebeest (*Connochaetes taurinus*), and only 1.7% are browsers (Havarua et al., 2014, Turner et al., 2013).

The soil types across KNP were divided into seven major classes based on the parent material from which they originated (Venter, 1986). Based on anthrax suitability models designed for KNP, three regions in the park, namely Pafuri, Shingwedzi and Letaba were favourable for spore survival and persistence (Steenkamp et al., 2018). Pafuri, the high incidence anthrax region (northern KNP) is comprised of alluvial, lithosol and smectic clay soils. Skukuza, the low incidence region anthrax (southern KNP) is comprised of mostly weakly developed shallow and solonetzic duplex soil (Venter,

1986). The genotypic analysis of *B. anthracis* strains in KNP over time showed the presence of both the A- and B-subclades. The A-subclade isolates were distributed throughout the park, while the B-subclade isolates were restricted to the north. Furthermore, isolates from both subclades were evident in the anthrax outbreaks from 1970- 1981 with the B-subclade dominating, but the B-subclade isolates have not been detected since the 1990's with the A-subclade isolates dominating recent outbreaks (Smith et al., 2000).

The ENP soils have been divided into five major groups, namely sandy soil, sandy loamy to sandy soil, sandy loamy to sandy clayey soil, soil from fluvial sediments, and saline soils (Beugler-Bell and Buch, 1997, Le Roux et al., 1988). According to Ebedes (1977) two enzootic or high incidence regions were identified in ENP, namely, central ENP (Okaukuejo) and northeastern ENP (Namutoni and Andoni). Okaukuejo, the high incidence anthrax region which is also the central region of ENP, consists of alkaline, shallow soils associated with calcrete, carbonate-rich silty loamy to sandy-loam soil (Ebedes, 1977). Otjovasandu, the low-incidence anthrax region and western region of ENP consists of mostly gritty, sandy soils with very low nutrient and organic matter contents (Beugler-Bell and Buch, 1997). Despite the different soil classifications across ENP, a relatively limited diversity compared to KNP, of 24 *B. anthracis* genotypes clustering within the A-subclade from 337 mortalities over three decades were detected in a survey (Beyer et al., 2012).

1.2. Problem statement and justification

The role of soil in the life cycle of *B. anthracis* requires much more investigation as it is a complex system with a number of contributing abiotic and biotic factors. This pathogen has been widely studied but there is a knowledge gap as to why some soil types support the survival of some *B. anthracis* strains and why others do not. Furthermore, understanding why some strains have adapted to certain soil types and understanding why these soil types support their survival may also be important to provide an improved understanding of all the factors that contribute to persistence of *B. anthracis* in certain regions. In addition, there are also questions about the role of the exosporium and its influence on spore survival.

1.3. Aim

The main aim of this study was to investigate the spore survival of *B. anthracis* strains in soils from high- and low-incidence anthrax regions in Kruger and Etosha National Parks.

1.4. Objectives

A pilot study using the non-pathogenic *B. anthracis* (Sterne $34F_2$) strain was done in the BSL-2+ laboratory at the Department of Veterinary Tropical Diseases (DVTD) before investigating the spore survival of the pathogenic strains in the Special Bacterial Pathogens Reference laboratory (SBPRL) BSL-3 facility at the National Institute for Communicable Diseases (NICD), South Africa. The purpose of the pilot study was to assess the design of the terra-simulator in a laboratory experiment and to demonstrate the safety of this study by showing the efficacy of the disinfection methods. These results were used to obtain permission for the main study from the Director of Animal Health from the Department of Agriculture, Land Reform and Rural Development (DALRRD), South Africa under the Animal Disease Act 35 (1984) section 20 addressing biosecurity. To obtain the main aim, we set out to achieve the following objectives for this study:

1. Pilot study:

   Investigate the *B. anthracis* Sterne $34F_2$ strain spore survival in soils from the high- and low-incidence anthrax regions in KNP and to evaluate the design and functionality of terra-simulator which was also linked to the Pafuri weather station in order to mimic natural weather conditions from a high-incidence anthrax region within the terra-simulator.

2. Main study:

   Investigate the A- and B-subclade *B. anthracis* strains (one KNP A- and B-, two ENP A-subclades and the Sterne $34F_2$ strain) spore survival in soils from high- and low-incidence anthrax regions from Kruger and Etosha National Parks for 6 months in the terra-simulator.

3. Investigate the spore structure of South African and Namibian *B. anthracis* strains from the A- and B-subclades using electron microscopy with focus on the exosporium.

1.5. Research questions

The following research questions will be used to investigate how the different soil types from anthrax high and low incidence regions in KNP and ENP affect the survival and exosporium of the *B. anthracis* spores in the different soil types and the differences in the spore coats.

1. Is the design of the terra-simulator sufficient for the main purpose of this study?

2. Does the spore survival of virulent *B. anthracis* strains from the A- and B-subclades differ in soils from anthrax high and low incidence regions in Kruger and Etosha National Parks?

3. Does the exosporium of southern African *B. anthracis* strains from the A- and B-subclades differ from each other?

1.6. Significance of the study

This study provides a unique research opportunity to evaluate the roles of the environmental factors and soil types in shaping the ecology and evolution of this zoonotic pathogen in Kruger and Etosha National Parks. Additionally, an improved understanding of this system may provide better management strategies, especially for the commercial and subsistence farmers in the surrounding areas.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. Introduction

Bacillus anthracis along with *Bacillus cereus* and *Bacillus thuringiensis* are the most well studied species in the *B. cereus sensu lato* group which are Gram positive, spore forming, soil borne, rod shaped bacteria and belong to the family *Bacillaceae.* Despite their genetic relatedness, these bacteria display different diseases where *B. cereus* is typically a foodborne pathogen, *B. thuringiensis* is an insect pathogen and *B. anthracis* is a mammalian pathogen (de Maagd et al., 2001, Drobniewski, 1993, Helgason et al., 1998, Koch, 1876). However, there has been evidence of genetic exchange among species whereby *B. cereus* obtained *B. anthracis* virulence plasmids and was able to cause disease (Klee et al., 2006, Klee et al., 2010).

Anthrax is primarily a bacterial disease of ungulates and is caused by *B. anthracis*, a Gram positive, spore forming bacterium commonly found in moist alkaline soils which are rich in calcium and organic matter (Dragon and Rennie, 1995, Koch, 1876, Turnbull, 2008, Van Ness, 1971). Anthrax mainly affects domestic and wild herbivores when they ingest or inhale *B. anthracis* spores from contaminated soils while grazing but episodic spill over to carnivores, pigs, other scavengers or predators and occasionally humans have also been reported when they come into contact with infected animals, animal products or any items contaminated with *B. anthracis* spores (Hugh-Jones and De Vos, 2002).

The spore forming capability of *B. anthracis* plays a pivotal role in its survival and persistence in the environment, especially in soil, for extended time periods (Gould, 1977, Sterne, 1959, Wilson and Russell, 1964, Turnbull, 2008). The spores are resistant to a wide range of adverse environmental factors (Cieslak and Eitzen Jr, 1999, Dragon and Rennie, 1995, Sterne, 1937). These spores germinate under favourable conditions and the vegetative cells are able to cause infection in the non-immune host (Lincoln et al., 1961). The vegetative cells are responsible for the formation of the toxin complex which causes the typical symptoms and lesions associated with this disease. It should also be noted that the vegetative form is typically observed in the host but may sometimes occur in the environment (Hoover et al., 1994, Lincoln and Fish, 1970). Replication of the vegetative cells may occur at the entry site of *B. anthracis* spores, or in the respiratory organs, but it depends on the route of exposure (Glomski et al., 2007). The fatal bacteraemia is then caused by further proliferation of the bacilli which is spread into the bloodstream by the lymphatic system and causes the fatal disease in non-immune hosts (Glomski et al., 2007).

Bacillus anthracis is a major a threat to the health of livestock and this can also negatively impact the economy (Turnbull, 2008). Anthrax outbreaks may have implications such as the loss of milk from infected dairy herds, loss of animals and animal products such as hides and meat. This will also result in further costs incurred to treat the herds and their environments (Hugh-Jones, 1999, Turnbull et al., 1999). Anthrax is also an important factor in the public health sector as it can be used in biowarfare and is also a life-threatening zoonotic disease (Fasanella et al., 2010, Inglesby et al., 1999, Sterne, 1967, Turnbull, 2008). It also puts all individuals handling infected animals such as caretakers, veterinary professionals, abattoir workers and farmers at high risk (Hugh-Jones, 1999, Turnbull et al., 1999).

Anthrax is globally distributed although it is endemic to certain regions of Africa, Asia, the Americas and Australia (Blackburn, 2006, Carlson et al., 2019, Hugh-Jones, 1999, Keim et al., 2000, Keim and Smith, 2002, Smith et al., 1999, Turnbull, 2008). Anthrax can be controlled and possibly eradicated in livestock by vaccination where it is necessary due to the economic impacts (Sterne, 1939, Sterne, 1967). In contrast, eradication of the disease in endemic or enzootic areas such as game reserves and national parks is more complicated as the vaccination of wildlife is not always possible. In instances where it is possible, it is often costly as the immobilisation of the animal is required along with qualified game rangers and veterinary professionals (Hugh-Jones and De Vos, 2002). Control measures such as burning or burying carcasses are sometimes put in place in nature reserves to reduce the amount of spores in the environment but such practices interfere with the natural processes of the ecosystem and natural cycling of the bacterium (WHO, 2008). Anthrax outbreaks in game reserves and national parks are seen as part of the natural environment and are typically uncontrolled or unmanaged unless endangered species are affected (Hugh-Jones and De Vos, 2002, Turnbull, 2008).

2.2. *Bacillus anthracis* aetiology

Bacillus anthracis falls into the *Firmicutes* phylum, *Bacillaceae* family and belongs to the *B. cereus sensu lato* group of bacteria. This group comprises of 11 species (Liu et al., 2017) namely; *B. anthracis, B. cereus, B. mycoides, B. pseudomycoides, B. thuringiensis, B. weihenstephanensis* (Lechner et al., 1998), *B. bingmayongensis, B. cytotoxicus, B. gaemokensis, B. manliponensis* and *B. toyonensis* (Giffel and Beumer, 1999). These bacteria share many genetic similarities but differ with regards to their clinical manifestations, host preferences, pathogenesis and phenotype (Drobniewski, 1993, Kolstø et al., 2009, Rasko et al., 2005).

2.3. Cellular and colony morphology of *Bacillus anthracis*

Bacillus anthracis is a Gram positive bacterium which has rod shaped bacilli which measure 1,0–1,5 × 3,0–10,0 µm. The bacilli are usually seen in short chains when observed from blood smears, but appears in long chains when observed from culture as seen in Figure 1 (Mock and Fouet, 2001). The thick peptidoglycan layer and secondary polymers of the cell wall prevent decolourisation during the Gram staining process and the bacteria appear purple (Bartholomew and Mittwer, 1952). Furthermore, the thick cell wall protects the bacterium from mechanical damage (Beveridge, 2001). When *B. anthracis* cells are subjected to the Giemsa stain (Barcia, 2007), the vegetative cells have distinct encapsulated, square ended 'box-shaped' bacilli (Figure 1) which occur in long chains and this differentiate it from other Gram positive bacteria. Furthermore, the lack of motility in *B. anthracis* helps to differentiate it from other members of the *B. cereus* group.

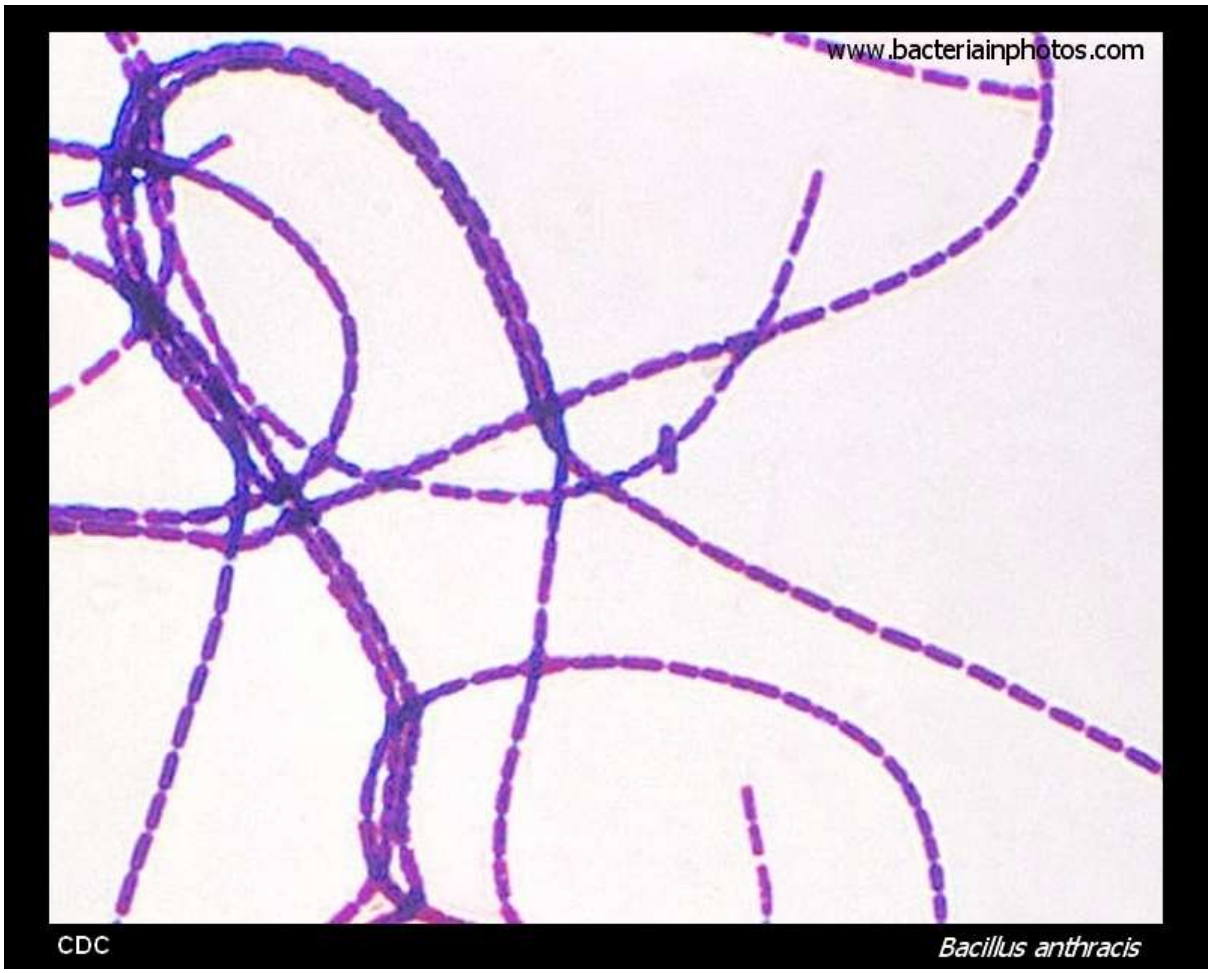


Figure 1: Typical cellular morphology of *Bacillus anthracis* with box shaped bacilli that occurs as long chain in cultures. Obtained from http://www.bacteriainphotos.com/*Bacillus*_anthracis_microscopy.

The colonies of *B. anthracis* have a grey, "ground glass" appearance (Figure 2). The edges of the colonies are usually irregular and show tangled bacterial filaments which are referred to as "medusa heads" (Figure 2). Another typical characteristic of *B. anthracis* colonies is the "spiking" that is observed when a bacteriologic loop is pulled across a colony. This results in the colony standing perpendicular to the agar surface (Parry et al., 1983). Furthermore, a clear zone of lysis is observed when *B. anthracis* cultures are inoculated with phage gamma (Parry et al., 1983, Vilas-Boas et al., 2007). The absence of haemolysis on blood agar and the sensitivity to penicillin are also defining characteristics of *B. anthracis* (Parry et al., 1983, Turnbull et al., 1991).

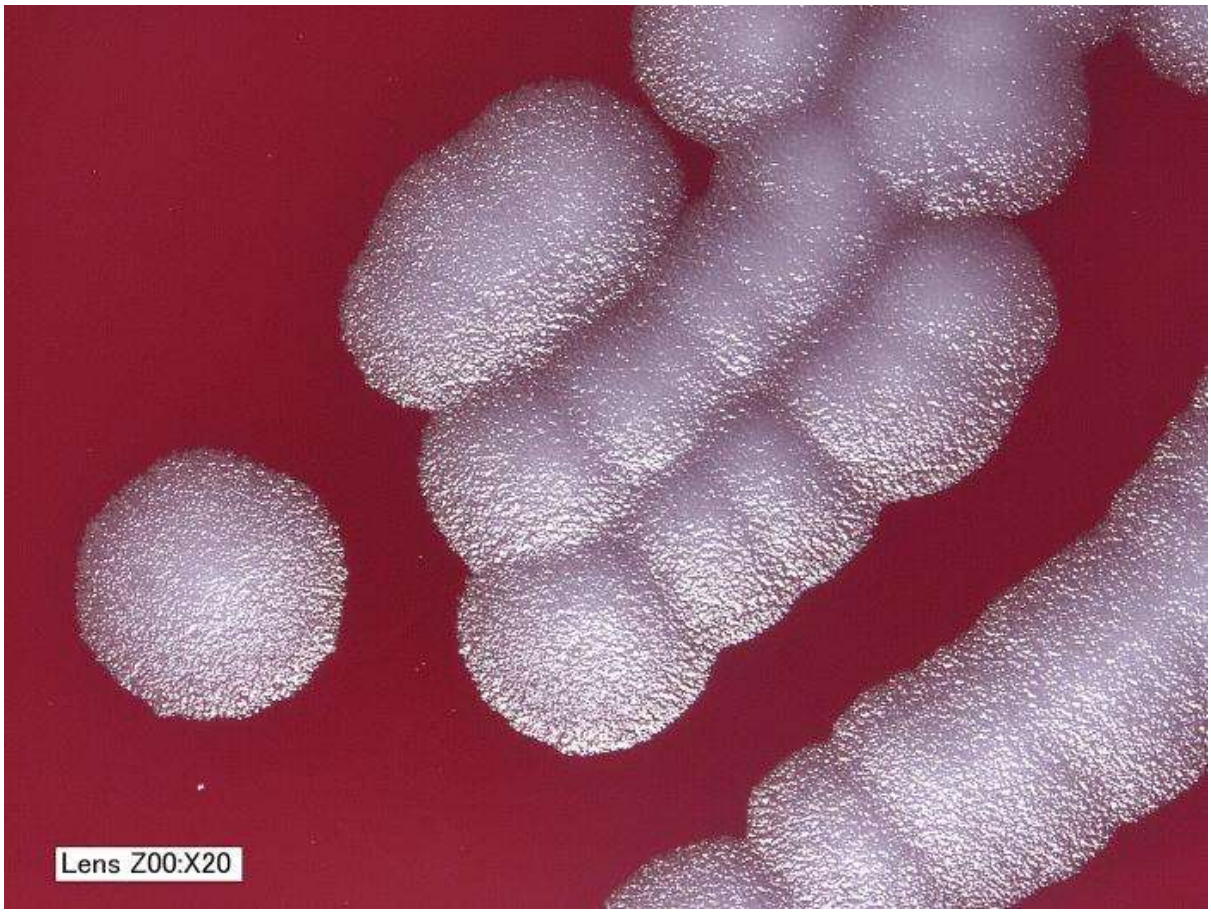


Figure 2: Typical colony morphology of *Bacillus anthracis* grown on 5% sheep blood agar. Grey-white colonies with a ground glass appearance. Obtained from https://phil.cdc.gov/details_linked.aspx?pid=15644.

2.4. *Bacillus anthracis* genome

The *B. anthracis* genome is approximately 5 megabase pairs which is comprised of the chromosome and the pXO1 and pXO2 plasmids (Ravel et al., 2009, Read et al., 2003). The nucleotide sequence of the genome has approximately 35% G + C bonds which means that the DNA has a much lower melting

temperature and higher buoyant density than other bacteria (Keim et al., 2009). In addition, a higher buoyant density than other *Bacillus* species the spore size and the resistance to heat and desiccation (Carrera et al., 2008). The plasmids code for a number of genes, but most importantly they are responsible for producing virulence factors which cause disease, namely the lethal and oedema toxins and the poly-D-glutamic acid capsule of *B. anthracis* (Leppla, 1991, Smith et al., 1955, Stanley et al., 1961).

The pXO1 plasmid is approximately 184.5 kilobase pairs, codes for the lethal factor (LF) and is a zinc metalloprotease. The edema factor (EF) is a calmodulin-dependent adenylate cyclase and the protective antigen (PA) which binds to the LF and EF to form the anthrax toxin complex (figure 3). The anthrax toxin complex is responsible for causing haemorrhage, edema, and necrosis during infection (Leppla, 1991, Okinaka et al., 1999a). The pXO2 plasmid is approximately 95.3 kilobase pairs, contains three genes which codes for the full poly-D-glutamic acid capsule biosynthesis and is vital for a successful anthrax infection as it protects vegetative *B. anthracis* cells from phagocytosis by the host's immune response (Leppla, 1995, Okinaka et al., 1999b). It is important to note that the loss of either plasmid results in an attenuated strain like the Sterne ($34F_2$) strain which will be discussed later on, therefore, both plasmids are essential for full virulence (Hanna and Ireland, 1999).

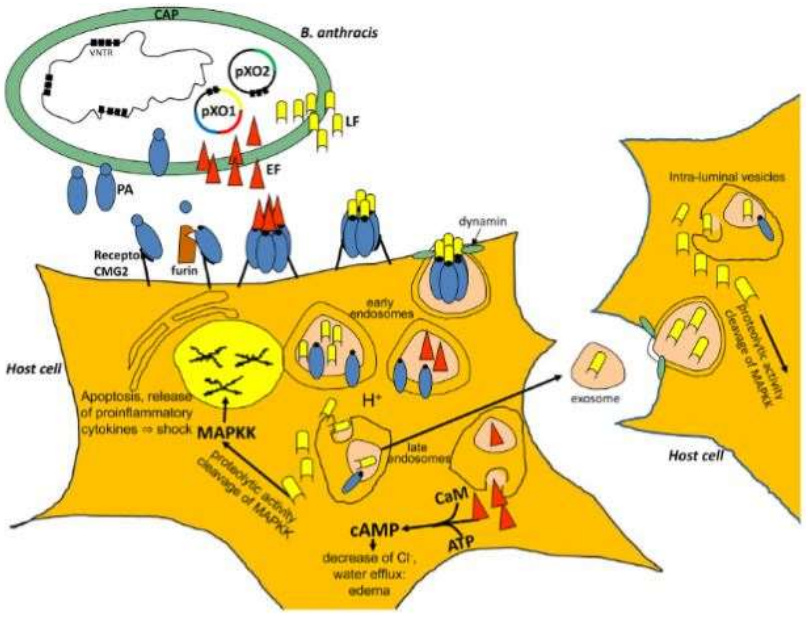


Figure 3:Overview of the molecular mechanisms of virulence of *Bacillus anthracis*. Adapted from (Pilo and Frey, 2011). Abbreviations: CAP, capsule; PA, protective antigen; EF, oedema factor; LF, lethal factor; VNTR, variable number tandem repeat; MAPKK, mitogen-activated protein kinase kinase; CaM, calmodulin; cAMP, cyclic adenosine mono phosphate.

The PA binds to host cell receptors and is cleaved by furin (Figure 3). This allows the multimerisation of the cleaved subunits to form a ring-shaped channel. The EF and LF then bind to the cleaved subunits which forms the edema toxin or the lethal toxin. This forms the PA:EF and PA:LF complexes which are then internalised by endocytosis in a dynamin-clathrin dependent mechanism. The acidic environment in the endosomes induces a conformational change in the PA which results in a channel that can be inserted into the endosomal membrane (Jiang et al., 2015). The translocation process is driven by the channel as it allows a proton gradient to be formed between the endosome and the cytosol (Krantz et al., 2006). The EF and LF are destabilised from the PA channel due to the acidic environment of the endosomes, unfold and translocate through the channel (Krantz et al., 2004). This causes large amounts of cAMP to be produced by EF, causes apoptosis, initiates the release of pro-inflammatory cytokines and LF and cleaves MAPKK (Figure 3). The toxins may be stored long term and can cause disease a long time after the initial infection takes place because part of the toxin can "hide" in intra-luminal vesicles and may then leave the infected cell in the form exosome, thus making it easier for the toxin to incorporate into non-infected cells and cause disease in new host cells (Friebe et al., 2016, Pilo and Frey, 2011, Pilo and Frey, 2018).

2.5. Forms of diseases

Bacillus anthracis survives by killing its host (Hugh-Jones and De Vos, 2002). The spores are usually ingested from the surrounding environments by animals but the spores may also be introduced to animals via breaks in the skin caused by open wounds and skin lesions (Hugh-Jones and De Vos, 2002) and by the inhalation of spores (Barandongo et al., 2018, Glomski et al., 2007). Once the *B. anthracis* spores enter the body, they are engulfed by macrophages but are not destroyed as the poly-D-glutamic acid capsule is poorly recognised by the host's immune response, therefore, they are able to germinate to produce vegetative cells. The vegetative cells proceed to multiply and express the genes on their virulence plasmids to produce the toxins and capsule responsible for causing anthrax as described above. When conditions become unfavourable for survival and multiplication, the vegetative cells sporulate and these spores are able to persist for extended periods in certain environments as they are resistant to a range of biological extremes and adverse conditions (Turnbull, 2008).

The severity of disease is dependent on the concentration of spores that were ingested, the host's susceptibility to the disease and feeding patterns of the host. Different disease presentations have been observed for animals and humans. Three different manifestations of anthrax have been observed in animals as a result of inhaling or ingesting spores; namely the peracute, acute, and subacute to chronic forms (Hugh-Jones and De Vos, 2002), whereas four have been observed in humans; namely cutaneous

(Doganay et al., 2010), gastrointestinal (Beatty et al., 2003, Ndyabahinduka et al., 1984), pulmonary (Brooksher and Briggs, 1920, Debord and Vidal, 1998) and injectional (Ringertz et al., 2000).

Peracute anthrax lasts for about two hours, usually affects ruminants such as kudu (*Tragelaphus strepsiceros*), impala (*Aepyceros melampus*), cattle, sheep and goats. Some animals only show clinical signs just before death such as blisters in the oral cavity, bleeding from the nose and anus, convulsions and paddling movements of the legs while the majority of peracute anthrax cases are found deceased without showing any indication of clinical illness (Jubb et al., 2012).

In acute anthrax, which is typically less than 72 hours and usually affects horses, donkeys and plains zebra (*Equus quagga*), a wider range of symptoms have been reported. This includes depression, loss of appetite, disturbed breathing patterns, haemorrhaging, diarrhoea, abortion and oedematous swellings of the tongue, throat and ventral body parts which is then followed by death (Jubb et al., 2012, Hugh-Jones and De Vos, 2002).

In subacute to chronic anthrax, which lasts for more than three days before death or until recovery occurs, usually affects pigs, carnivores and other animals with some level of natural immunity. The most common symptoms include oedematous swelling of the face, neck and throat which may cause asphyxia and which results in the animal having difficulty with the ingestion of food and water. The outcome of this infection ranges as it may lead to a more acute stage and the animal may succumb to the infection, or the animal may recover (Brennan, 1953, Jubb et al., 2012, Turnbull et al., 1992).

2.6. Diagnostic tools for the detection and identification of *Bacillus anthracis*

The gold standard for a laboratory confirmation of an anthrax infection is the isolation of *B. anthracis* from clinical specimens or environmental samples using presumptive and confirmatory tests. Presumptive tests are performed by directly culturing samples onto blood agar (Fellows, 1996, Tomaso et al., 2006) and/or polymyxin-lysozyme-EDTA-thallous acetate (PLET) (Knisely, 1966). If *B. anthracis* colonies are seen as described by Parry (1983) which are grey colonies with a ground glass appearance, the colonies may be Gram stained (Bartholomew and Mittwer, 1952), Giemsa stained (Barcia, 2007) or subjected to a M'Fadyean reaction (M'Fadyean, 1903). The confirmatory tests include testing for a lack of motility (Burdon and Wende, 1960), penicillin sensitivity (Barnes, 1947), gamma phage sensitivity (Buck et al., 1963) and the induction of capsule formation in blood or on bicarbonate media (Sirard et al., 1994).

The virulence factor genes, namely the protective antigen (*pag*), lethal factor (*lef*) or *cya* gene and the capsule genes are the targets used for molecular detection of *B. anthracis* (Beyer et al., 1999, Ellerbrok et al., 2002). The quantitative real-time polymerase chain reaction (qPCR) uses probes which are more sensitive and specific than conventional PCR (Beyer et al., 1996, Beyer et al., 1999), to identify the genomic DNA of *B. anthracis*. Fluorescence resonance energy transfer (FRET) hybridisation probes (Caplin et al., 1999), which are sequence-specific oligonucleotides labelled with fluorescent dyes were specifically designed for the detection and quantification of *B. anthracis* DNA which results in highly specific detection of the target (Turnbull, 2008). The protocol for real-time PCR using the LightCycler targets three genes, namely the *pag* gene, which is on the pXO1 plasmid; the *cap-C* gene, which is on the pXO2 plasmid and the *sasp* gene, which is a chromosomal marker (Turnbull, 2008). Furthermore, the advantage of using this method is the speed and efficiency at which samples can be tested (Turnbull, 2008). A PCR run which contains between 30-40 cycles can be completed in 20-30 minutes and the formation of amplification, melting and quantification curves by the LightCycler Nano SW 1.0 software allows the user to view the progression of the reaction in real time. Therefore, these molecular methods are used in combination with the microbiological methods for *B. anthracis* confirmation.

For the identification of *B. anthracis* strains from different origins, common sub-typing methods have been proven inadequate as they lack sufficient discriminatory power due to the slow rate of evolution of *B. anthracis* (Harrell et al., 1995, Keim et al., 1997, Kim et al., 2005). Short-sequence DNA repeats in prokaryote genomes called variable number tandem repeats (VNTR) were detected in various genes of *B. anthracis* including the virulence plasmids (Van Belkum et al., 1998). Multiple locus VNTR analysis (MLVA) can be used to type *B. anthracis* strains of different origins with a high enough discriminatory power as the VNTRs showed a high enough mutation rate in order to produce polymorphisms (Keim et al., 2000). With the use of eight VNTRs (MLVA8) which included six chromosomal (*vrrA*, *vrrB1*, *vrrB2*, *vrrC1*, *vrrC2* and *CG3*) and two plasmid markers (pXO1-aat and pXO2-at), 426 *B. anthracis* strains from different origins were classified to a cluster level (Keim et al., 2000).

This new typing method became the norm for studying the diversity of *B. anthracis* strains from all over the world. However, this method was further improved by Van Ert et al (2007) and Lista et al (2006) to include more markers by increasing the MLVA analysis to 15 (MLVA15) (Van Ert et al., 2007) and 25 (MLVA25) (Lista et al., 2006). Beyer et al (2012) and Thierry et al (2014) combined previously described VNTR markers to produce MLVA31 which resulted in a greater coverage of the genetic diversity of Namibian and French *B. anthracis* strains (Beyer et al., 2012, Thierry et al., 2014). Two VNTR loci – bams13 and bams30 are some of the most polymorphic tandem repeats in *B.*

anthracis and are also associated with the genes which code for the structural components of the exosporium of the spore (Lista et al., 2006, Sylvestre et al., 2003).

Canonical single nucleotide polymorphisms, termed canSNPs were described by Van Ert et al (2007) and analyses of these markers allows *B. anthracis* strains to be characterised into any of the three major lineages as these SNPs represent key phylogenetic positions (Van Ert et al., 2007). Another bacterial subtyping method, core-genome multilocus sequence typing (cgMLST) has been used alongside whole genome sequencing to provide a novel approach for standardised high resolution strain genotyping for *B. anthracis* (Abdel-Glil et al., 2021). A combination of long- and short-read next generation sequencing technologies allowed the accurate reconstruction genomes which enabled detailed in-silico genotyping of *B. anthracis* strains(Brangsch et al., 2022). A recent study completed whole genome sequencing of a human anthrax isolate using the Nanopore sequencing platform. This was done in just one day and in a high-containment facility. Such work and advances in sequencing technologies allows for a more rapid response to public health emergencies (McLaughlin et al., 2020).

2.7. Management of *Bacillus anthracis*

There are many different factors involved in managing anthrax, but the main aim is to interrupt the life cycle of the pathogen itself. Some of the factors involved in the successful management of the disease include a good surveillance system, prophylactic measures and disease regulatory actions such as vaccination, treatment with antibiotics, disinfection with formaldehyde; and proper disposal of carcasses by incineration (Turnbull, 2008). Managing anthrax in wildlife requires a different approach. For example, the anthrax control measures for South African National Parks are only employed if it negatively impacts the natural ecosystem (Hugh-Jones and De Vos, 2002). The Sterne ($34F_2$) strain anthrax vaccine was discovered by Max Sterne in the 1930s (Sterne, 1937). The Sterne strain is acapsular as it has naturally lost its pXO2 plasmid (Figure 4). Since both plasmids are required for fully virulent strain (Figure 4), this avirulent vaccine strain can still provide a sufficient protective immune response without causing severe disease as only the anthrax toxins are produced without the poly-D-glutamic acid capsule (Sterne, 1939). The Sterne vaccine is currently the most commonly utilised and effective veterinary vaccine available (WHO, 2008). The Sterne strain has also proven to have an excellent safety record as it has been used around the world, especially in endemic areas by veterinary professionals.

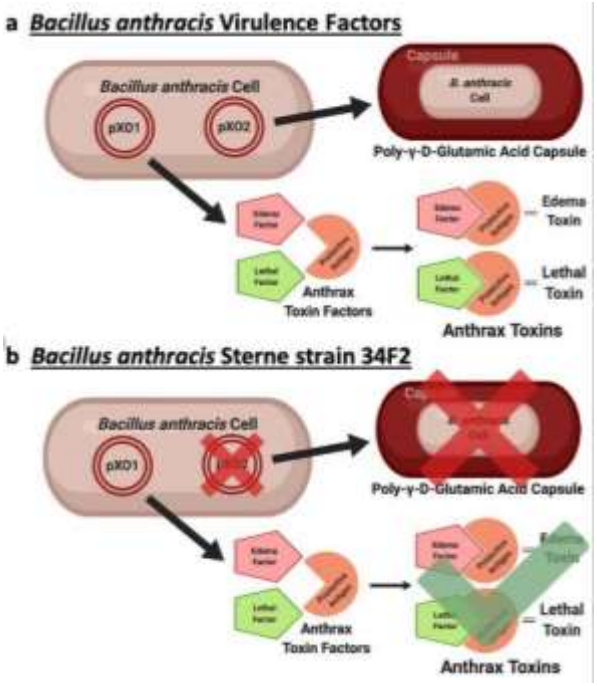


Figure 4: Illustration of the *Bacillus anthracis* Sterne strain. A) *Bacillus anthracis* which has both plasmids (pXO1 and pXO2) resulting in a fully virulent cell. B) *Bacillus anthracis* Sterne strain $34F_2$, which only has the pXO1 plasmid. Obtained from DOI:10.1038/s41541-020-0208-3.

Furthermore, anthrax is a notifiable disease in many countries, including South Africa, therefore control measures are enforced by the Department of Agriculture, Land Reform and Rural Development (formerly Department of Agriculture, Forestry and Fisheries) and the State Veterinary services. For the prevention of anthrax infections in humans, efforts are aimed at controlling the disease in livestock by vaccination as this provides a degree of protection for humans as they are not in direct contact with the pathogen in day to day life (Turnbull, 2008). Ultimately, the main goal for managing anthrax infections is to reduce the number of spores in the soil to prevent frequent recurrence of the disease (Turnbull, 2002).

2.8. Molecular characterisation of *Bacillus anthracis*

Based on the genotypic characterisation of strains of *B. anthracis* researchers have theorised that the geographic origin of *B. anthracis* is the sub-Saharan African continent, (Keim et al., 1997, Smith et al., 1999). *Bacillus anthracis* is divided into three major clades, namely, A, B and C. The A-clade strains are distributed worldwide whereas B- and C-clade strains appear to show a more limited geographic distribution (Sahl et al., 2016, Van Ert et al., 2007, Vergnaud et al., 2016). Despite being the most broadly dispersed clade, the subgroups of the A-clade strains also show specific geographic patterns.

The large A-clade is represented in Figure 5. The A-clade is further divided into four monophyletic subclades. Subclade A.Br.005/006 is basal to all other subclades and is largely restricted to southern Africa (Smith et al., 1999, Van Ert et al., 2007). The B clade (upper left part of Figure 5) has been detected in some parts of Europe (Derzelle et al., 2011, Girault et al., 2014, Van Ert et al., 2007) and South Africa (subclade B.Br 001/002 Kruger) (Smith et al., 1999, Smith et al., 2000) . Clade C has only been detected in North America (Keim et al., 2000, Van Ert et al., 2007).

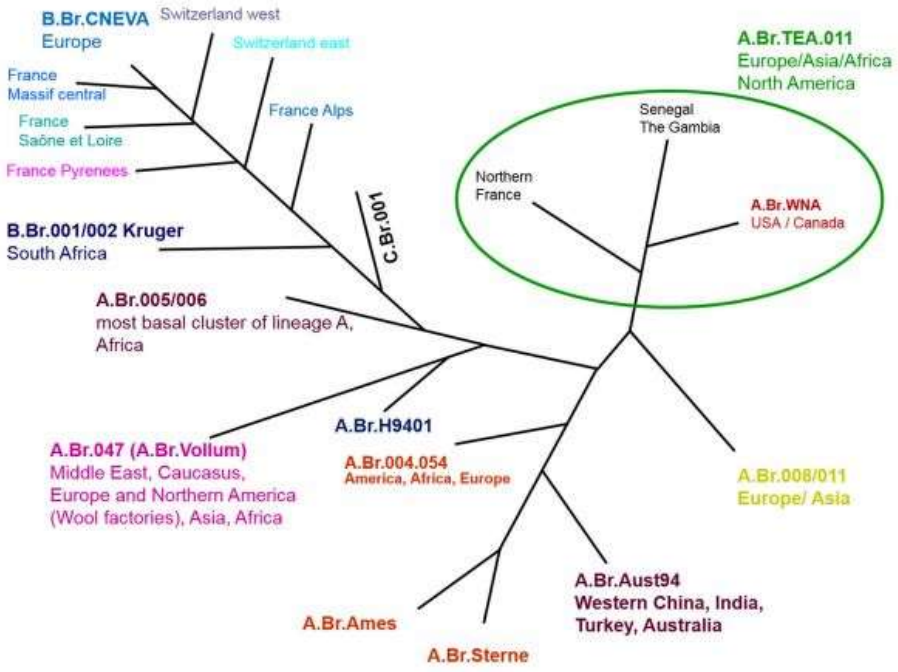


Figure 5: Summary of the most recent phylogeographic data of *Bacillus anthracis* strains. Branch lengths are not scaled. Image obtained from https://doi.org/10.1016/j.meegid.2018.06.024.

The genotypic analysis of southern African *B. anthracis* strains from Kruger National Park (KNP) indicates the presence of strains from both the A- and B-subclades of *B. anthracis* (Smith et al., 2000). The A-subclade isolates are distributed widely across KNP, while the B-subclade is restricted to northern KNP. This finding led to the understanding that the B-subclade strains are possibly less adaptable to ecological variation than the A-subclade strains (Smith et al., 2000). During the anthrax outbreaks that occurred between 1970 to 1981, strains from both subclades could be detected, however, since then, only *B. anthracis* strains from the A-subclade could be detected (Smith et al., 2000). In contrast, a survey of *B. anthracis* genetic diversity detected 24 *B. anthracis* genotypes from 337 mortalities over three decades in ENP indicates that *B. anthracis* is less diverse in Etosha National Park

(ENP) than KNP, the dominant strains are more stable over time, and all belong to the A-subclade as seen in Figure 6 (Beyer et al., 2012).

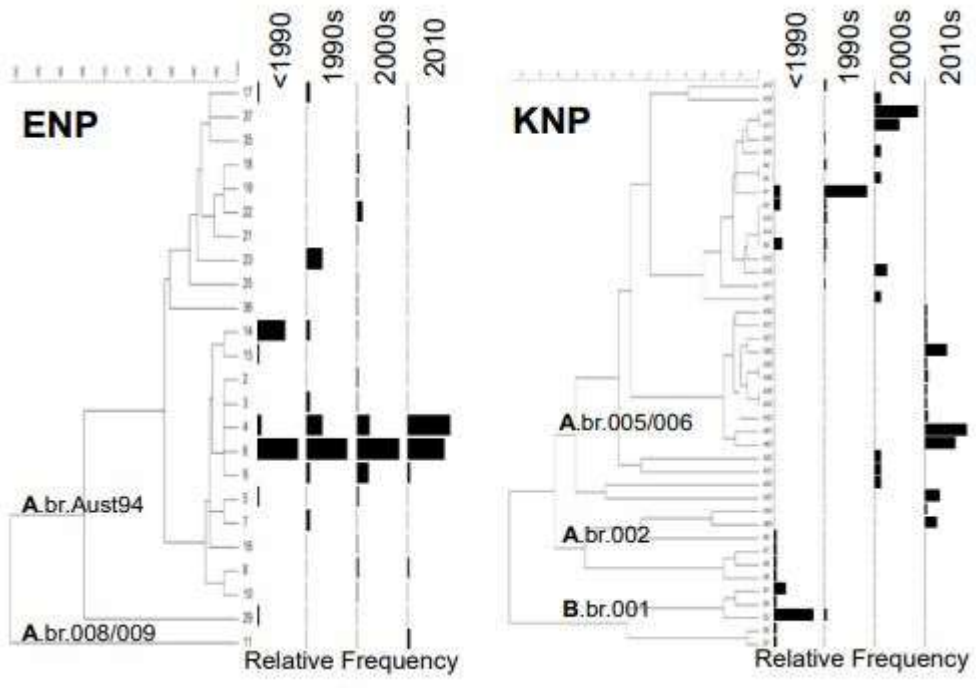


Figure 6: *Bacillus anthracis* genetic diversity in Etosha National Park (ENP, N=24 genotypes) (Beyer et al, 2012) and Kruger National Park (KNP, N=45 genotype) (Hassim A, 2017, docotoral dissertation) over 50 years, based on the 31-marker multi-locus variable number tandem repeats analysis (MLVA). The histograms show the relative frequency among strains detected by decade. The dominant strains in ENP are relatively stable over time, while in KNP show significant change over time. Unweighted pair group method with Arithmetic mean (UPMGA) image generated from MLVA data from Beyer et al. (2012) and Hassim (2017). Clade labels are based on van Ert et al (2007). Figure produced by Yen-Hua Huang.

2.9. *Bacillus anthracis* in Kruger and Etosha National Parks

In areas where *B. anthracis* is naturally present in the environment, certain environmental conditions such as calcium rich, alkaline soils with high levels of organic matter and climatic events where heavy rainfalls are followed by severe droughts triggerr anthrax outbreaks (Dragon and Rennie, 1995, Turnbull, 2008, Van Ness, 1971). As a result, this produces anthrax zones which have correlated concentrations of *B. anthracis* spores (Cieslak and Eitzen Jr, 1999). In southern Africa, KNP and ENP have been used to research the pathogen and its properties as *B. anthracis* is considered a part of the natural ecosystem in these areas (Hugh-Jones and De Vos, 2002).

There have been several anthrax cases reported annually, however large outbreaks are experienced from time to time in both KNP and ENP (Ebedes, 1977, Lindeque and Turnbull, 1994, Smith et al., 1999,

Smith et al., 2000). Both parks have anthrax endemic areas which are considered high incidence zones and anthrax non-endemic areas which are considered low incidence zones. This is advantageous as it allows the investigation disease incidence in populations that encounter high or low levels of pathogen in the environment (Lindeque and Turnbull, 1994, Smith et al., 2000, Steenkamp et al., 2018).

In KNP, anthrax outbreaks are typically observed every ten years near the end of the winter period and early in summer when water sources are scarce (De Vos et al., 1990, Hugh-Jones and De Vos, 2002). Over the three decades from 1960 to 1990, the greater kudu (*Tragelaphus strepsiceros*), a browser, contributed 75% of recorded cases over three decades (1960 to 1990) (Smith et al., 1999). However, smaller anthrax outbreaks have occurred since 2008, but mostly in the wet season which is similar to the occurrences in ENP (Basson et al., 2018). Furthermore, the implicated host species during the recent outbreaks was the impala (*Aepyceros melampus*), which is a mixed grazer and browser (Basson et al., 2018). It is hypothesized that the exposure of *B. anthracis* to browsing species may be caused by blowflies which feed on infected carcasses and then transfer spores to the surrounding vegetation (Basson et al., 2018, Blackburn et al., 2014, Braack and De Vos, 1990). Zebra (*Equus quagga*) in KNP was not a significant host in the last decade but now consists of 10% of cases in outbreaks (Huang et al., 2022).

The anthrax cases in ENP exhibit seasonality (Turner et al., 2013). The mortalities of plains zebra (*Equus quagga*) and other plains herbivores peak towards the end of the wet season while the mortalities of African elephant (*Loxodonta africana*) peak in the dry season, however, sporadic cases are reported through the year (Beyer et al., 2012, Ebedes, 1977, Lindeque and Turnbull, 1994, Turner et al., 2013). The seasonality of the outbreaks in ENP have been associated with the varying behaviours of hosts during foraging which affects their exposure rates (Havarua et al., 2014, Huang et al., 2021, Turner et al., 2014) and seasonal immune trade-offs (Cizauskas et al., 2014, Ochai et al., 2022). In ENP, grazing and mixed-feeding herbivores are more exposed to anthrax infections than browsers. Of all recorded cases, 52% are plains zebras, 22% are blue wildebeest (*Connochaetes taurinus*) which are both grazers (Turner et al., 2013) and only 1.7% are browsers (Havarua et al., 2014).

Furthermore, the contribution that scavengers and predators make to the spread of *B. anthracis* spores is also important. The spores are dispersed when the carcasses are fed on, spores are also ingested and then released in faeces (Ebedes, 1977, Pienaar, 1967). Vultures have been assumed to be an important role player in the transmission of *B. anthracis* spores over long distances and contamination of water sources (De Vos et al., 1990, Smith et al., 1999). However, it was also found that vegetative *B. anthracis* bacilli are not able to survive in the digestive tract of vultures and this could be the reason for the low spore count found in vulture faeces (Houston and Cooper, 1975, Lindeque and Turnbull, 1994).

Therefore, if vultures feed on fresh carcasses immediately, the risk of sporulation of vegetative bacilli is greatly decreased and therefore will not persist in the soil (De Vos et al., 1990). In addition, vulture guano has been shown to alter the soil chemistry and nutrient levels which results in soils that have a lower pH and higher levels of total nitrogen. This change has been hypothesised to suppress the survival of the *Firmicutes* bacteria, especially the long term survival of *Bacillus* and *Clostridia* sporeformers. (Ganz et al., 2012).

Based on anthrax suitability models designed for KNP, three regions in the park, namely Pafuri, Shingwedzi and Letaba were favourable for spore survival and persistence (Steenkamp et al., 2018). Pafuri, the high incidence anthrax region and northern region of KNP, is comprised of alluvial, lithosol and smectic clay soils. Skukuza, the low incidence anthrax region and southern region of KNP is comprised of mostly weakly developed shallow and solonetzic duplex soil (Venter, 1986).

The soils in ENP have been classified into five major groups, namely sandy soil, sandy loamy to sandy soil, sandy loamy to sandy clayey soil, soil from fluvial sediments, and saline soils (Le Roux et al., 1988). According to Ebedes (1977) two enzootic or high incidence regions were identified in ENP, namely, central ENP (Okaukuejo) and northeastern ENP (Namutoni and Andoni). Okaukuejo, the high incidence anthrax region and the central region of ENP consists of alkaline, shallow soils associated with calcrete, carbonate-rich silty loamy to sandy-loam soil (Ebedes, 1977). Otjovasandu, the low-incidence anthrax region and western region of ENP consists of mostly gritty, sandy soils with very low nutrient and organic matter contents (Beugler-Bell and Buch, 1997).

2.10. The impact of soil biology on *Bacillus anthracis*

There have been many different concepts proposed to understand why *B. anthracis* spores persist in some soil types and not others and why this occurs. The “soil capability” concept proposed by van Ness (1971) stated that *B. anthracis* survives in the soil by undergoing sporulation, growth and germination depending on the conditions and nutrients provided by the soil, however this is disputed due as it has been difficult to demonstrate vegetative bacteria in soils. Furthermore, vegetative cells may be out competed in the soil during growth phases (De Vos and Braack, 1990, Dragon and Rennie, 1995).

The “persistent spore” concept stated that the vegetative growth phase is host-dependent and ignored the roles of the soil and external environment but another variation of this concept, the “concentrator

area" concept considers the role of calcium in the survival of *B. anthracis* spores. It states that environments with high concentration of calcium or calcium sources enhance the preservation of spores (Dragon and Rennie, 1995). It was also suggested that strains from the A- and B-subclades vary in their calcium dependency, where the B-subclade strains favour soils with higher calcium concentrations and pH levels (Smith et al., 1999).

A study aimed at detecting bacterial endospores in soil by terbium fluorescence suggested that bacterial "hot spots" may exist and this be may be attributed to soil type, soil depth and soil carbon-to-nitrogen ratios (Brandes Ammann et al., 2011). Another study reported that the addition of tryptophan to soils, which is an essential amino acid, positively influences the soil bacterial species, in conjunction with both the soil total nitrogen and pH (Nwachukwu et al., 2022). There is still a huge knowledge gap as to why some soil types support spore survival and others do not, therefore, an improved understanding of all possible interactions between the *B. anthracis* spores, soil types, environmental conditions and other microorganisms is extremely necessary (Hugh-Jones and De Vos, 2002).

It was also suggested free-living soil microbes such as amoebas and other protozoa may contribute to the amplification and in turn, the persistence of *B. anthracis* spores in environments with stagnant water or moist soils. It was further hypothesised that these microorganisms may provide a favourable microenvironment which allows for a complete life cycle from spore germination to replication of vegetative bacilli, and finally, sporulation. Furthermore, this also occurs in a pXO1-dependent manner (Dey et al., 2012). The soil microbial community may also provide *B. anthracis* with additional capabilities or hinder its survival. Schuch and Fischetti (2009) reported that some bacteriophages are able to essentially protect actively growing *B. anthracis* bacilli by providing alternatives to sporulation which stimulate endosymbiosis with the soil. Additionally, another study demonstrated that *B. anthracis* readily forms biofilms and that the spores are part of the biofilm community, however, the effect of this on the ecology and evolution of *B. anthracis* remains unclear (Lee et al., 2007).

It has also been suggested that *B. anthracis* is able to germinate in the rhizosphere of plants and is able to survive in its vegetative form outside of an animal host in addition to being capable of exchanging genetic material in the environment (Saile and Koehler, 2006). In a study conducted using a native grass, *Enneapogon desvauxii* in ENP, it was demonstrated that *B. anthracis* spores increased the rate of establishment of the native grass, and an even higher rate for seeds which were exposed to blood and spores (Ganz et al, 2014). The interactions between the native grass, *B. anthracis* and soil had a significant impact on the soil bacterial taxa richness and community composition. It was hypothesised

that the increased growth rate of the grass in the presence of *B. anthracis* spores may increase host grazing and in turn result in increased transmission to hosts (Ganz et al., 2014).

2.11. *Bacillus anthracis* spores

The sporulation of *B. anthracis* may take place within 48 hours of the detection of nutrient deficiencies and these spores have a characteristic oval shape (Parry et al., 1983). Under suitable conditions, the spores are tolerant to a range of factors which include heat, cold, dehydration, ultraviolet radiation and antimicrobials (Turnbull et al., 1991). Spore persistence in the environment is also influenced by water activity, temperature, pH, the availability of nutrients and germination factors (WHO, 2008). It was shown that between 0–4 °C, *B. anthracis* did not sporulate and died in 6 days (Howie, 1949). *Bacillus anthracis* sporulation is initiated at a much slower rate as humidity decreases. Spore germination occurs within a 20–44 °C range. Spore germination time was also increased above 39 °C and below 30 °C. Davies (1960) showed that germination could not occur at a relative humidity of 80% or below even at 26 °C and 37 °C which highlights the impact of humidity on spore germination. Furthermore, it was also noted that the time for germination time was extended when humidity levels dropped below 100% (Davies, 1960).

The spores can survive in nature for extended periods due to its structure which consists of three protective layers: the spore coat, the cortex and the exosporium (Driks, 2009, Warth et al., 1963) (Figure 7). The core contains the genetic material, important enzymes and proteins such as pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) and small, acid-soluble spore proteins (SASP) required for sporulation, germination and overall spore preservation and protection (Church and Halvorson, 1959, Kornberg et al., 1968, Powell, 1953). The spore coat provides a flexible layer which can withstand the torsional strain that the chromosome experiences during germination and it also provides protection for the core. The cortex of the spore contains a peptidoglycan layer and is responsible for protecting the spore from mechanical damage and it also aids in drying out the core (Aronson and Fitz-James, 1976). The interspace is the area between the spore and the exosporium which creates the separation between these layers (Figure 8) (Gerhardt and Ribi, 1964).

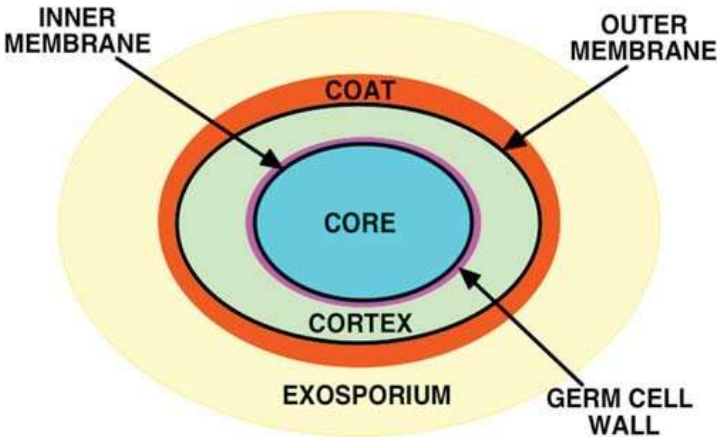


Figure 7: Schematic structure of the *Bacillus anthracis* spore. Obtained from https://doi.org/10.1128/microbiolspec.TBS-0003-2012.

The spore proteins are protected from heat inactivation by the low water content in the spores in combination with calcium cations. High levels of DPA aids in reducing the core water content, which is an essential component in spore resistance to wet heat (Gerhardt, 1989). The SASP's and DPA protects the DNA and RNA which are housed in the core from several types of damage (Gerhardt, 1989, Murrell, 1967). DPA is only synthesised during sporulation, and is taken into the developing spore (Murrell, 1967, Powell, 1953). The DPA and calcium cations remain stable while the spore is dormant but are released rapidly (approximately 1 to 2 minutes for individual spores) when germination is initiated (Magge et al., 2008, Foster and Johnstone, 1990). This release is an important signal to trigger the germination process and referred to as the stage I of germination. Stage I is then followed by stage II events, which is the hydrolysis of the spore's peptidoglycan cortex by cortex-lytic enzymes (Foster and Johnstone, 1990, Magge et al., 2008).

The exosporium is the outermost layer of the spore and it is exposed to the host. It is a loose-fitting, balloon-like layer which surrounds the spore and consists of two sublayers, the outer hair-like nap layer and the basal layer (Figure 8) (Gerhardt and Ribi, 1964, Hachisuka et al., 1966). It has been theorised that important antigens and genetic markers may be present in the exosporium (Steichen et al., 2003). In addition, the ability of *B. anthracis* spores to bind to different soil types has been attributed to certain features of the exosporium (Williams et al., 2013). It is hypothesised that the exosporium may have roles in spore germination and interactions with mammalian host cells as it is the outermost layer (Brahmbhatt et al., 2007b). It may also have a role in virulence and is being investigated as a possible target for future vaccine development (Brahmbhatt et al., 2007a, Cote et al., 2012, Cybulski Jr et al., 2008). The molecular structure and function of the exopsorium are not well described to date but the *Bacillus* collagen-like proteins (Bcl) have been identified as the main structural proteins of the

exosporium (Lai et al., 2003, Redmond et al., 2004). The exosporium consists of these Bcl proteins and a host of other glycoproteins which contribute to the structure and function of the exosporium (Bozue et al., 2015, Steichen et al., 2003, Redmond et al., 2004, Boydston et al., 2006).

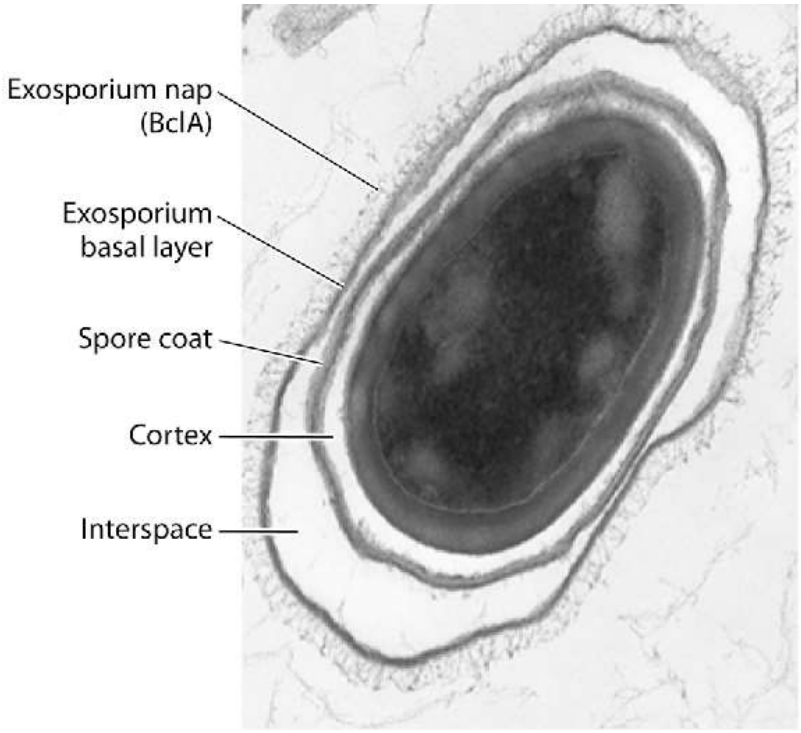


Figure 8: Transmission electron micrograph of the *Bacillus anthracis* spore. Obtained from DOI:10.1128/MMBR.00050-15.

The *Bacillus* collagen-like A protein (BclA) appears to be a structural component of the exosporium's nap layer (Steichen et al., 2003, Sylvestre et al., 2002) (Figure 8) while BclB has been associated with the basal layer of the exosporium and its integrity with regards to the formation and maintenance (Thompson, 2007). It has been hypothesised that polymorphisms in the BclA region leads to variations in filament length of the nap layer, impacting the spore's response to various environments (Sylvestre et al., 2003). Furthermore, Leski (2009) proposed that the sequence and length polymorphisms found in the *Bcl* genes of which five have been consistently identified in *B. anthracis*, may be used as a basis for the detection and fingerprinting of *B. anthracis* strains. *B. anthracis* can be identified with confidence using this method as the *Bcl* genes are able to create unique signatures for specific strains (Leski et al., 2009).

3. CHAPTER THREE: MATERIALS AND METHODS

3.1. Ethical clearances

This study was approved by the University of Pretoria's Faculty of Veterinary Science research ethics committee (REC076-20) and section 20 of the Animal Disease Act (Act No. 35 of 1984) was approved by the Director of Animal Health from the Department of Agriculture, Land Reform and Rural Development (DALRRD), South Africa (reference 12/11/1/1/6 MG and 1771KL).

3.2. Materials

The Sterne anthrax spore vaccine (Batch number 89 1) was obtained from Onderstepoort Biological Products (OBP), South Africa (SA). The sporulation agar was prepared as described in the WHO guideline (Turnbull, 2008) and the 5% sheep blood agar (SBA) was obtained from Thermo Scientific™, SA. The nutrient agar was prepared according to the manufacturer's recommendations (Thermo Scientific™, South Africa). The diagnostic confirmatory tests were completed using 10 µg penicillin disks (Oxoid™, Thermo Scientific™, SA) and Gamma phage (~10^7 phages/mL) (Barnes, 1947, Buck et al., 1963). All serial dilutions were performed 1:10 in phosphate buffered saline (PBS, pH 7.45) (Gibco®, Thermo Scientific™, SA). The Invitrogen PureLink™ Genomic DNA kit (Thermo Scientific™, SA) was used for the DNA extraction from culture and the MN NucleoBond RNA and DNA soil kit for RNA from soil (Separations, SA) was used for the total nucleic acid extraction from soil. The FastStart SYBR Green Master (Roche, SA) was used for the qPCR using the LightCycler Nano (Roche, SA). The MyTaq™ Red Mix 2x (Bioline, SA) was used for the Multiple-locus variable number tandem repeat analysis (MLVA) using the MiniAmp™ Plus Thermal Cycler (Applied Biosystems™, SA). Agarose gels were analysed using the GelDoc imaging system (Bio-Rad, SA). The fixative for the *B. anthracis* spores was 2.5% EM grade glutaraldehyde, in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, ≥98%), pH 7.0, osmotically adjusted by the addition of 0.09 M sucrose (Sigma, for molecular biology), 0.01 M magnesium chloride (Merck EMSURE®, SA), and 0.01 M calcium chloride (Merck EMSURE®, SA). Ruthenium red stain (Sigma-Aldrich, SA) and L-lysine acetate (Fluka, Sigma-Alrich, SA) was also used for the staining of spores and 1% buffered osmium tetroxide (SPI-Chem) was used for post fixation. Spores were embedded in London resin (London Resin company, Agar Scientific, United Kingdom). Sections were cut on a Leica EM-UC6 ultramicrotome. The spores were analysed on the FEI BioTwin Spirit transmission electron microscope fitted with an Olympus Quemsa CCD camera using the OSIS calibrated software. The soil analyses were conducted by NviroTek Lab for the KNP soils and the Ministry of Agriculture Water and Land Reform for the ENP soils.

3.3. The "terra-simulator"

The inoculated soil samples were incubated in a double-glazed E glass biohazard pod which reduced the interaction with spores (Figure 9). The terra-simulator was made from double-glazed (vacuum in between the glass) E glass (for the sides) and shatterproof plexiglass (for the front and base) as well as protected silicone corners for added structural integrity. All materials in the terra-simulator were also non-porous to ensure successful disinfection and sterilisation of surfaces. The soil samples were placed in re-sealable, plastic containers which ensures that the *B. anthracis* spores are self-contained. The UV lamp (Figure 10A) and light sensors were used to set night and day cycles. Each soil sample had its own soil sensor to measure temperature and humidity as its own microenvironment (Figure 10B). The ambient temperature and humidity sensors measured the terrarium's environment. A microcontroller was used to modulate the heat lamps, UV and humidity reading sensors (Figure 10C) and was linked to the TerraClient program which was specifically designed for this task (Figure 11A). It was able to mimic day-night cycles, temperature, and humidity by obtaining data from the weather station in KNP's high-incidence anthrax region, Pafuri (Figure 11B). The advantage of using programming to regulate these environmental factors was that the terrarium could be monitored and regulated remotely which made the system ideal to study virulent *B. anthracis* strains in the BSL-3 facility as it reduced the physical interaction with spores. The risk assessment for the terra-simulator and this study is provided in Appendix 1.

Figure 9: The developed terra-simulator holding the individual soil containers along with the soil, light and humidity sensors.

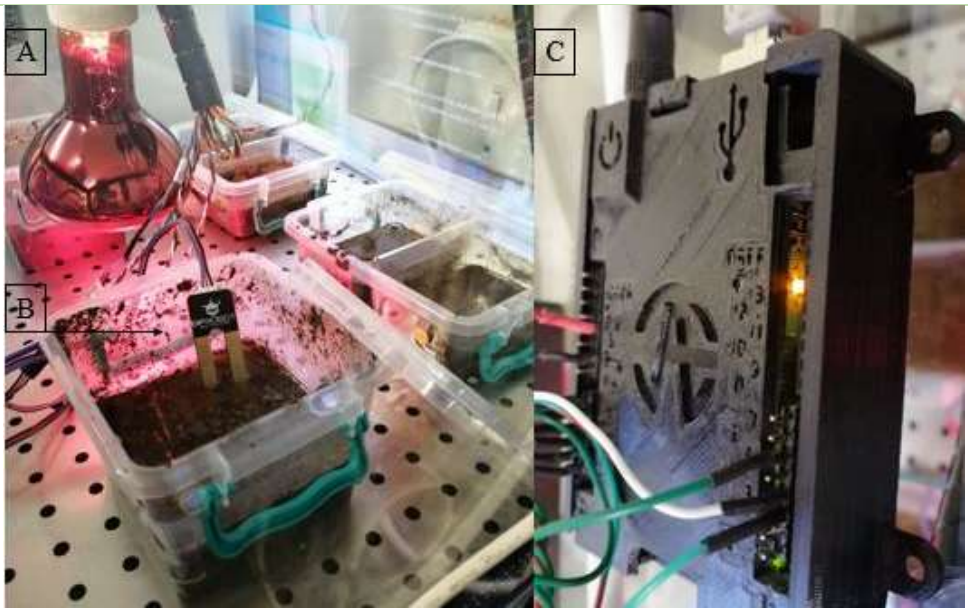


Figure 10: A: The NOMOY heat and UV lamp which provides heat and light within the terra-simulator. B: The DFROBOT soil sensors are responsible for monitoring the soil humidity to record the effect of the modulated environment on the soil. C: The microcontroller modulates the system. All the sensors (soil, heat and light) in the system are connected to the microcontroller. It reports all the recorded data to the TerraClient program which monitors the system.

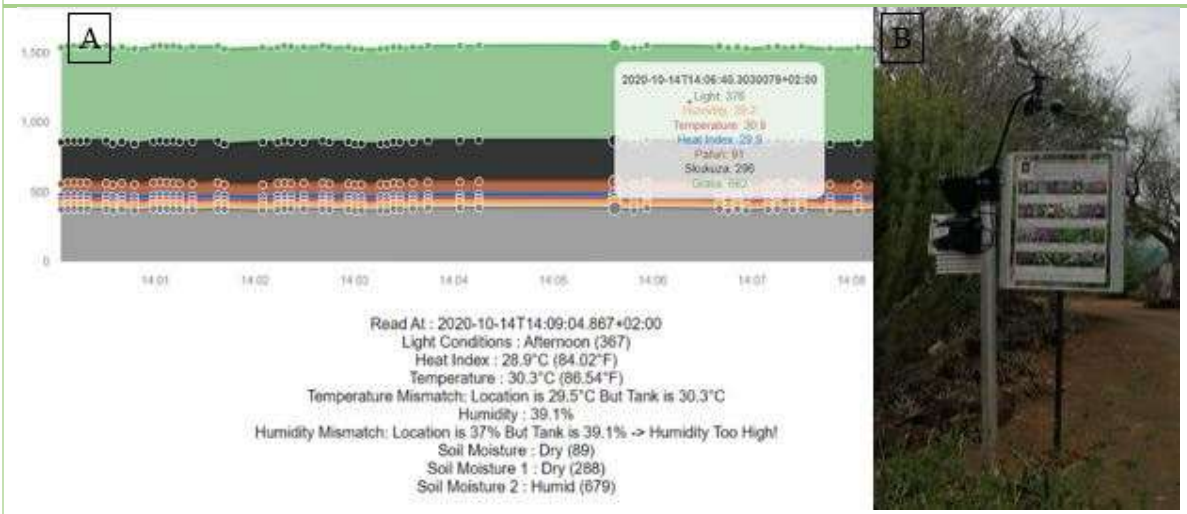


Figure 11: A: TerraClient is the program that monitors the system and records all the data logged from the various sensors in the system as well as simulating the Pafuri temperature and humidity within the tank. B: The weather station in Pafuri that TerraClient receives readings from in order to regulate conditions in the tank.

3.4. Study areas

In this study, we focused on two flagship national parks in southern Africa (Figure 12), Kruger National Park (KNP) in South Africa and Etosha National Park (ENP) in Namibia which are nearly 2000 km apart. Anthrax is an endemic disease within these parks and *Bacillus anthracis* is considered to be part of the natural ecosystems. There are also high-incidence (Pafuri in KNP and Okaukuejo in ENP) and low-incidence (Skukuza in KNP and Otjovasandu in ENP) areas which have been described in both parks. Differences exist between the parks such as the dominant *B. anthracis* strain type over time, outbreak seasonality and the hosts affected during the outbreak periods, however, this study focuses on the roles of the pathogen and the environment in the epidemiology of this disease. Therefore, comparing these two parks under these circumstances provides us with a unique opportunity to further investigate this pathogen and how spore survival changes in different soil conditions.

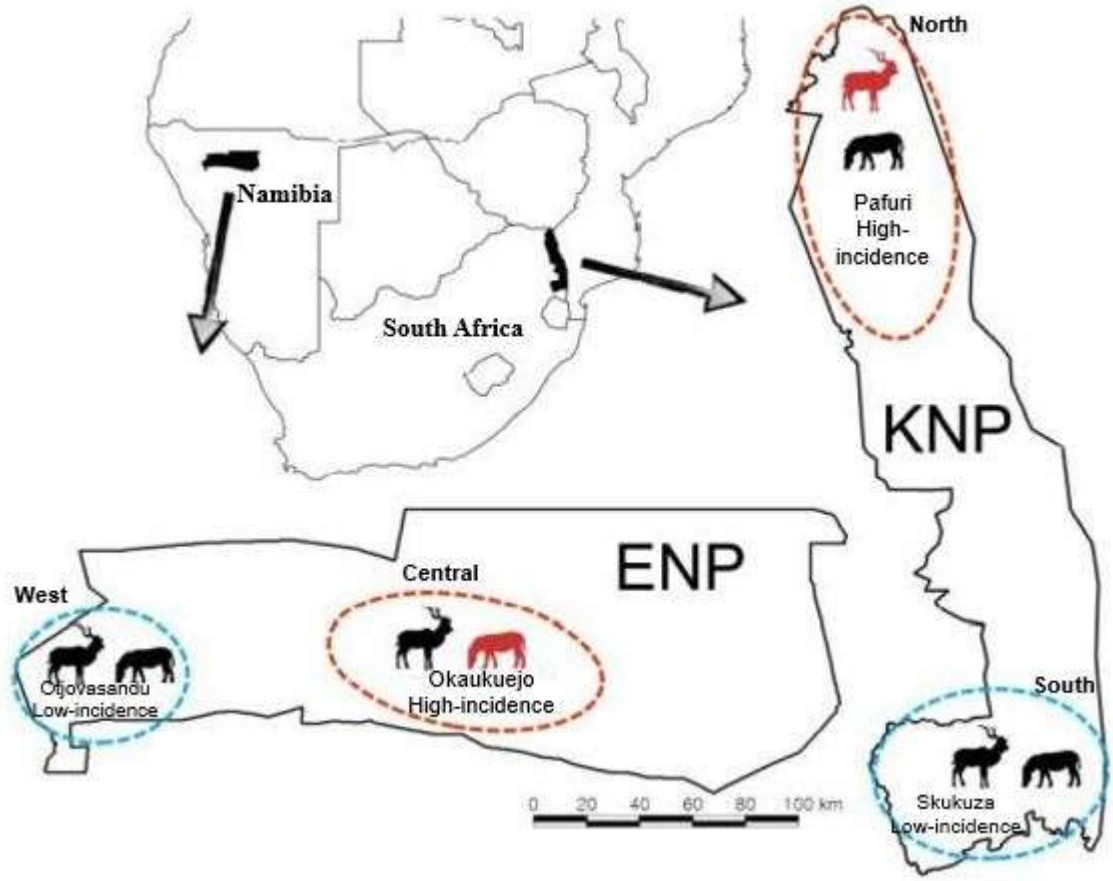


Figure 12: Kruger and Etosha National Parks (KNP and ENP) in southern Africa. This image shows the sites where anthrax outbreaks occur endemically with the high- and low-incidence regions depicted by the red and blue circles, respectively. Kudu is the most common anthrax host species in KNP, and zebra is the most common in ENP.

3.4.1. Kruger National Park

Kruger National Park is approximately 19,485 km^2 and is located in the north-eastern corner of South Africa (Figure 12). The soil across KNP is divided into seven major classes based on the major geological units within KNP (Venter, 1986). The soil types range from alluvial, persiallitic, lithosols, solonetzic, smectic clay and shallow soils (Figure 13). Pafuri is mostly alluvial, smectic clay and lithosols soil while Skukuza has mostly solonetzic and weakly developed shallow soils. KNP has varying land elevations across the park. Pafuri has lower elevations which are also surrounded by higher elevations. (Venter, 1986). The soil profiles show a tendency of becoming shallower as the rainfall levels decreases from the south to the north in KNP. Historically, the average rainfall across KNP was between 300 – 500 mm and this also differs between north and south (MacFadyen et al., 2018). There are five major rivers across KNP and they differ with regards to their landscapes (Muller and Villet, 2004). There are also temperature differences across KNP, whereby the north is warmer than the south (De Vos et al., 1990).

3.4.2. Etosha National Park

Etosha National Park is almost 23,000 km^2 and is located in northern Namibia (Figure 12). The soils of Etosha are categorised in five major groups, namely sandy soil, sandy loamy to sandy soil, sandy loamy to sandy clayey soil, soil from fluvial sediments, and saline soils (Beugler-Bell and Buch, 1997, Le Roux et al., 1988). Okaukuejo mostly consists of, carbonate-rich silty loamy to sandy-loam soil. Otjovasandu mostly consists of mostly gritty, sandy soils with very low nutrient and organic matter contents. ENP is largely flat but there are hills in the eastern and far western regions of the park (Figure 14) (Beugler-Bell and Buch, 1997, Ebedes, 1977, Turner et al., 2022). The rainfall in ENP is highly seasonal with an annual average of 355 mm in Okaukuejo (central ENP) (Turner et al., 2022). The wet season typically starts in November and ends in April. The highest rainfall levels generally occur during January and February (Le Roux et al., 1988, Turner et al., 2022). Most of the water sources available to the animals are natural artesian, contact springs or man-made boreholes (Auer, 1997). Three characteristic seasons occur across ENP, the cool dry, the hot wet and the hot dry seasons (Ebedes, 1977, Turner et al., 2022). Historically, the temperatures in ENP ranged from 6.8 – 28.4 ºC in winter and 17.4 – 32.4 ºC in summer (Ebedes, 1977, Turner et al., 2022).

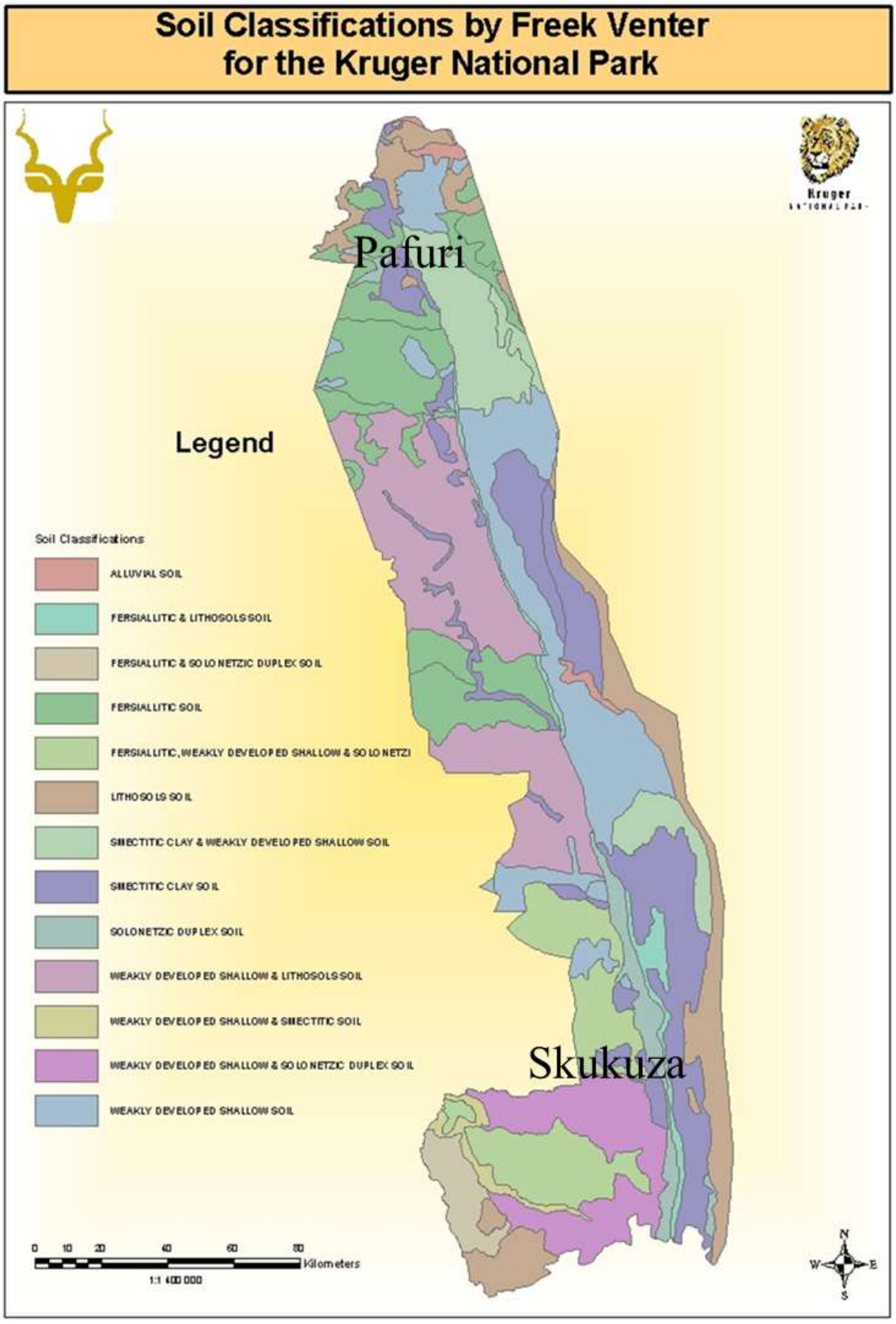


Figure 13: Map of Kruger National Park showing the soil classifications as described by Venter (1986). The soil samples were collected from northern KNP, Pafuri and southern KNP, Skukuza.

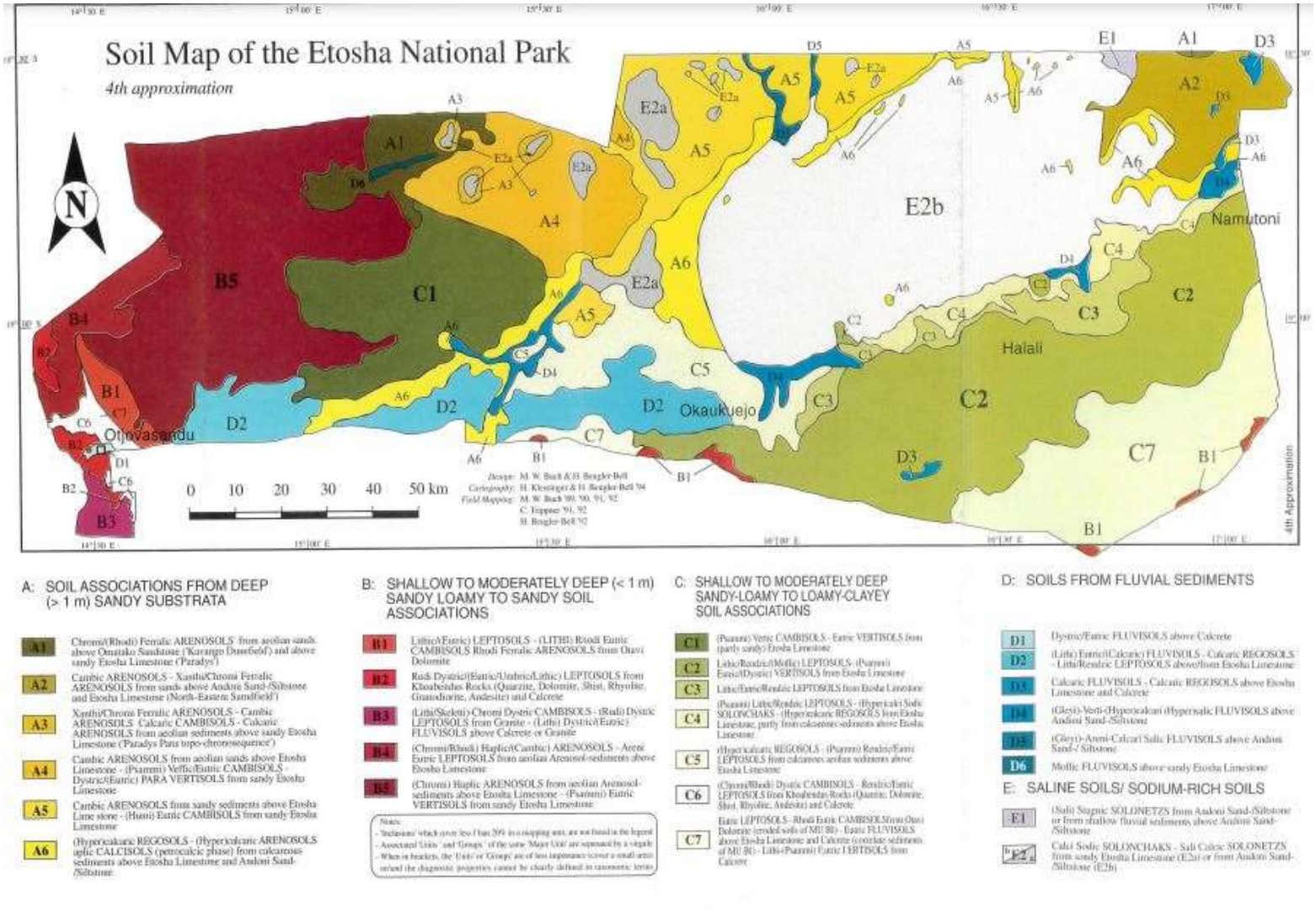


Figure 14: Soil map of Etosha National Park from Beugler-Bell and Buch (1997) showing the differences in the soil types across the park. The soil samples were collected in central ENP, Okaukuejo (C5) and western ENP, Otjovasandu (B5).

3.5. Soil sample collection

The soils used in this experiment were collected from the anthrax high-and low-incidence regions of KNP and ENP. Soils from the surface (top layer) were collected (approximately 1 kg each) from at least three different points in these areas and tested for the absence of *B. anthracis* using both the microbiological and molecular diagnostic methods as outlined by the WHO guidelines (Turnbull, 2008). A short description of the selected soils is given in Table 1. The KNP soil samples were tested by the Skukuza State Veterinary Services in KNP, South Africa and the ENP soil samples were tested by the Central Veterinary Laboratory (Windhoek) in Namibia. The Namibian soil samples, and *B. anthracis* strains were shipped to NICD-SBPRL (National Institute for Communicable Diseases – Special Bacterial Pathogens Reference Laboratory) BSL-3 laboratory as category A biohazardous samples and all necessary import permits were in place. The soils from KNP were transported to UP-DVTD (University of Pretoria – Department of Veterinary Tropical Diseases) BSL-2 plus laboratory under a red cross permit. The South African *B. anthracis* strains were already stored in the BSL-3 facility at NICD-SBPRL.

Table 1: Description and locations of the selected soils that were used for the spore survival study.

Country:	Area:	Description:	Location:	Result:
South Africa (Kruger National Park)	Pafuri	High incidence anthrax region. Dark, loamy, well-structured soil.	S 22.43239 E 31.24473	*B. anthracis* not isolated.
	Skukuza	Low incidence anthrax region. Loose, weakly developed soil.	S 24.986441 E 31.583028	*B. anthracis* not isolated.
Namibia (Etosha National Park)	Okaukuejo	High incidence anthrax region. Grey/brown chalky (carbonate), loamy soil.	S 19.143543 E 15.875657	*B. anthracis* not isolated.
	Otjovasandu	Low incidence anthrax region. Reddish/brown unstructured, loose, sandy soil.	S 19.16509 E 14.54707	*B. anthracis* not isolated.

This study was separated into two parts, part A, the pilot study which was aimed at testing the terra simulator and its functionality by looking at the *B. anthracis* Sterne strain spore survivability in soils from KNP (Pafuri and Skukuza). In addition, the pilot study also afforded us the opportunity to optimise our methodology for part B, the main study which involved the pathogenic *B. anthracis* strains which took place in the BSL-3 facility at NICD-SBPRL.

3.6. PART A: Pilot study

3.6.1. Preparing *Bacillus anthracis* Sterne ($34F_2$) strain spores

The Sterne anthrax spore vaccine (100 µL) was cultured onto 5% SBA plate and incubated overnight at 37 ºC. Mixed bacterial growth was observed; therefore, the vaccine was heat treated for 10 minutes at

65 ºC to remove contaminants while still selecting for *B. anthracis* spores. The heat-treated Sterne vaccine (100 µL) was cultured onto 5% SBA plates and incubated overnight at 37 ºC to obtain a pure culture. A single colony was cultured onto sporulation agar which contains manganese sulphate to stimulate sporulation and bacteriological agar. These plates were incubated for five days at 37 ºC. To harvest the spores, a total volume of 5 mL of PBS was used to lift the spores from the plate and the bacterial lawns were scraped with a sterile spreader. The bacterial suspension was then gently mixed with a Pasteur pipette to create a homogenous solution. The spore suspension was stored at 4 ºC after harvesting. The spore counts were performed by culturing 1:10 dilutions of the spore stock solution. A total volume of 100 µL of selected dilutions (10^0, 10^{-2}, 10^{-4} and 10^{-6}) were cultured in triplicate onto 5% SBA and incubated overnight at 37 ºC. *Bacillus anthracis* colonies were counted, and the mean number of colonies were multiplied by the dilution factors to determine the viable spore count which was reported as CFU/g.

3.6.2. Inoculation of soil samples with the *Bacillus anthracis* spore stock solution

The soil samples from Pafuri and Skukuza were divided into 100 g each and placed in separate resealable, autoclave safe plastic containers. In a Biosafety Class II cabinet, the soil samples were first moistened with 5 mL of PBS using a Pasteur pipette and then inoculated with 10 mL of each spore stock which had a final concentration of 10^6 spores/mL. The spore stock solution was also added using a Pasteur pipette and small drops were made across the entire surface. Wetting the soils before adding the spore stocks ensured that no mixing was required after the inoculation with the spore stocks to prevent contamination and possible aerosolization. The inoculated soil samples were then incubated in the terra-simulator for the duration of the experiment which was performed in the BSL2-plus facility at UP-DVTD.

3.6.3. Detection of *Bacillus anthracis* Sterne (34F_2) from inoculated soil samples

After inoculation, the soils were collected and prepared for the spore enumerations. Small amounts of soil were collected from random sections in the container to ensure that the whole contents of the container were sufficiently represented. A total 1 g of soil was collected in this manner and added to 9 mL of PBS. The samples were then heat treated at 65ºC for 10 minutes to reduce the bioburden. Serial dilutions (1:10) were prepared from the soil samples and 100 µL of selected dilutions (10^0, 10^{-2}, 10^{-4} and 10^{-6}) were cultured onto 5% SBA and incubated overnight at 37 ºC. Sterne colonies were detected at very low levels (~10 spores/g). It was decided that the initial concentration of 10^6 spores/mL should be increased.

All steps up to this point were repeated to increase the starting concentration of the spore suspension. After inoculating the soil samples with a starting concentration of 10^9 spores/mL of the spore suspension, the soil samples were collected at day 0, 14 and 28 to determine the viable spore counts. The remaining Sterne spore stock was stored at 4 ºC and served as the positive control for this experiment. Viable spore counts from the spore stock were also done in conjunction with the soil isolations. *Bacillus anthracis* colonies were counted, and the viable spore counts were reported as average CFU/g. The phage and penicillin sensitivity tests were performed on suspect *B. anthracis* colonies for confirmation. In addition, other *Bacillus* spp. were also identified morphologically following the guidelines by Parry et al (1983) and the most commonly present species were reported. The bacteriophage activity was monitored by looking for areas of lysis on the *B. anthracis* lawns on the 10^0 culture plates. This experiment took place in October 2020. The temperature and humidity values for the time period of the pilot study are provided in Appendix 2.

3.6.4. Statistical and data analysis

The spore survival of the *B. anthracis* Sterne strain from the anthrax high and low incidence regions of KNP were compared to identify differences or trends between the soil types. Statistical analyses were performed using IBM SPSS Statistics version 27 (SPSS Inc., SA). A one-way Analysis of Variance (ANOVA) was used to evaluate the *B. anthracis* Sterne spore survival in these two soil types. Post hoc comparisons were performed using Tukey honest significance difference (HSD) assuming equal variances. A P-value of <0,05 was considered significant. All the data were recorded on Excel (Microsoft Office 365). The $\log_{10}$ CFU/g Sterne spore counts in each soil type was plotted for selected time points in addition to the $\log_{10}$ total CFU which included other *Bacillus* spp that were identified on the culture plates. The relative humidity and temperature of the terra-simulator over 28 days was also plotted to show the fluctuations over time and is shown in Appendix 2.

3.6.5. Environmental monitoring for contamination around the terra-simulator

Testing for contamination around the terra-simulator was conducted where all surfaces touched such as the handles, the inside and outside perimeters of the tank and the surrounding bench tops were swabbed. The laptop and mouse that was in use was also swabbed. Nutrient agar (NA) plates were also left open in the tank for at least one hour during the environmental testing. The swabs were streaked onto NA plates. All plates were then incubated at 37ºC for 48 hours.

3.7. PART B: Main study

3.7.1. *Bacillus anthracis* strains selected

A total of eight strains were preliminarily selected for this study. Table 2 shows the year, locality and the source of the strains considered. The KNP strains selected represent the dominant strain types for those outbreak periods from both the A- and B-subclades. The ENP strains selected were based on spore phenotype with regards to persistence levels in the soil over time. All strains were stored at room temperature on sporulation agar. All strains were cultured using the microbiological techniques outlined by the WHO guidelines (WHO, 2008). The strains indicated in bold in Table 2 were chosen because they had the best growth in comparison to the other strains considered. All cultures pure and showed sensitivity to Gamma phage and penicillin.

Table 2: Information on the preliminary *Bacillus anthracis* strains considered[1] for the spore survival study, with regards to their locality, source and the year in which they were isolated

Country	Strain number	Locality	Source	Clade	Year
South Africa (Kruger National Park)	SBPRL-141	Pafuri (Hapi pan)	Soil	Kruger B	1975
	SBPRL-150	Pafuri (Hapi pan)	Animal bone	Ancient A	1990-1991
	SBPRL-191	Pafuri (Hapi pan)	Animal skin	Ancient A	1990-1991
	SBPRL-243	Pafuri (Hapi pan)	Soil	Kruger B	1970-1980
Namibia (Etosha National Park)	**WT-2**	Okaukuejo	Soil	A	2016
	WT-53	Natco	Soil	A	2016
	WT-63	Grunewald	Soil	A	2012
	WT-72	Okaukuejo	Soil	A	2010

[1] Strains indicated in bold were selected for this study

The spore stocks were prepared for SBPRL-191, SBPRL-243, WT-2, WT-53 and the Sterne strain as described above in section 3.6.2 and were used to inoculate the soils at a starting concentration of 10^9 spores/mL. The volumes for the serial dilutions were adjusted to account for the additional number of soil samples that needed to be inoculated as described below.

3.7.2. Inoculation of soil samples from Kruger and Etosha National Parks with selected *Bacillus anthracis* spore stocks

The soils from the four study areas (Pafuri, Skukuza, Okaukuejo and Otjovasandu) were placed into separate containers which had six compartments each (Figures 15 and 16). The six compartments were for each of the selected strains mentioned above, one for the Sterne strain and one for a negative control (uninoculated soil). This allowed all strains to be inoculated into all the different soils. Each compartment held approximately 100 g of soil. The soil samples were first moistened with 5 mL of PBS using a Pasteur pipette and then inoculated with 10 mL of each spore stock per compartment which was

also added using a Pasteur pipette and small drops were made across the entire surface. Wetting the soils before adding the spore stocks ensured that no mixing was required after the inoculation with the spore stocks to prevent contamination and possible aerosolization. The negative controls only received 15 mL of PBS. It was noted that there were also clear physical differences between the different soils as seen in Figures 15 and 16, especially with regards to their texture, structure and colour.

All containers were then placed in the terra-simulator (see section 3.3 for full details) where it stayed for the duration of the experiment and the soil sensors were inserted into the Sterne inoculated soils of each container. The heat lamps provided heat and maintained the day and night cycles. The conditions of the terra-simulator were maintained and monitored in relation to Pafuri's weather station by the TerraClient program and humidity was manually maintained by adding PBS to soils when the humidity levels were recorded as low. In addition, the conditions of the terra-simulator were re-programmed to repeat the temperature and humidity conditions that was experienced in the pilot study which took place in October 2020 (the temperature and humidity are indicated in Appendix 2). This was done to investigate the effect of the uncommon weather pattern (cold, with high rainfall in summer followed by extremely high temperatures with no rainfall) that occurred during the pilot study on the *B. anthracis* spores, especially the Sterne strain and took place during day 120 and 150 of the main study.

Figure 15: Soils from Kruger National Park. Pafuri on the left and Skukuza on the right.

Figure 16: Soils from Etosha National Park. Okaukuejo on the left and Otjovasandu on the right.

3.7.3. Isolation of *Bacillus anthracis* from inoculated soil samples incubated in the terra-simulator

The soils were samples from each compartment at 10 time points: day 0, 7, 14, 21, 28 (month 1) and then monthly until month 6. Before collecting the soil samples from the containers at each time point, all sensors were gently wiped with 10% F10 decontaminant and isopropanol to prevent damage to them. The containers were then sealed and wiped with 10% F10 before removing it from the terra-simulator and into a biosafety cabinet. In the biosafety cabinet, small amounts of soil were collected from random sections in the container to ensure that the whole contents of the container were sufficiently represented. A total 1 g of soil was collected and added to 9 mL of PBS. All other compartments were sufficiently covered to prevent cross-contamination. Once the soils were collected, the containers were wiped again and moved back into the terra-simulator with all sensors in place.

The collected soil samples were then heat treated at 65 ºC for 10 min to reduce the bioburden. Serial dilutions (1:10) were prepared from the soil samples and 100 µL of selected dilutions (10^0, 10^{-2}, 10^{-4} and 10^{-6}) were cultured onto 5% SBA in triplicate and incubated overnight at 37 ºC. *Bacillus anthracis* colonies were counted, and the viable spore counts were reported as average CFU/g. Other *Bacillus* spp were identified morphologically following the guidelines by Parry (1983) and the bacteriophage activity was monitored by looking for areas of lysis on the *B. anthracis* lawns on the 10^0 culture plates. The phage and penicillin sensitivity tests were performed on suspect *B. anthracis* colonies for confirmation.

3.7.4. Soil analyses of soils collected from Kruger and Etosha National Park

A sample of each soil type was sent for analyses to Nvirotek laboratory (Hartebeespoort, South Africa) for the KNP soils and the Ministry of Agriculture, Water and Land Reform (Namibia) for the ENP soils. At Nvirotek laboratory, the Mehlich 3 test was performed as it is used for the determination of macronutrients (Sodium, Na; Calcium, Ca; Magnesium, Mg; and Potassium, K) and micronutrients (Copper, Cu; Zinc, Zn; Manganese, Mn; and Iron, Fe) in soils. However, only Mn from the micronutrients will be focused on for this study. The Mehlich 3 method as described by Mehlich (1984) which is a weak acid soil extraction procedure was used for the soil analysis as it has the advantage of being applicable for a number of elements (Mehlich, 1984). In addition, the pH, soil density, soil organic matter was also tested.

The tests performed by the Ministry of Agriculture, Water and Land Reform included the soil moisture content, pH, extractable cations which shows the available Na, Ca, Mg and K, Mn, texture and particle

size analysis and the organic matter content. The soil samples were prepared by drying the soil samples at 35 ºC. The soil samples were then sieved through a 2 mm sieve and is referred to the fine earth fraction, whereas particles which are larger than >2mm is referred to as stones and gravel. The fine earth fraction was used for subsequent analysis. The soil moisture was measured in a 1:2.5 soil:water ratio suspension on a mass to volume basis. The pH was measured in a 1:2.5 soil:1M potassium chloride ratio suspension on a mass to volume basis. The extractable cations were extracted with 1M ammonium acetate at pH 7 and measurement of the available Ca, Mg, K and Na was done using inductively coupled plasma (ICP). Cation exchange capacity was measured by using 50:50 ammonium acetate (1M) and ethanol to extract Ca, Mg, K and Na from the soil and measured using atomic absorption spectrophotometry. The texture and particle size analysis were conducted by dispersing the soil with sodium hexametaphosphate/sodium carbonate. The pipette method was used to determine the percentage of silt and clay. The sand fraction was determined by sieving to collect particles that are greater than 53 microns. The organic matter content was measured using the Walkley-Black method which is a sulphuric acid-potassium dichromate oxidation process.

3.7.5. Data and statistical analysis

Descriptive statistics were performed to determine the mean, range and standard deviation of the spore counts by soil type, strain, sampling time point (month) and the environmental conditions. The effects of the different soil types, *B. anthracis* strains, temperature and relative humidity on spore counts over time (months) were also measured using a classical repeated-measures design implemented with Generalized Least Squares (GLS) regression and with ANOVA to perform a likelihood ratio test. The predictor variables were soil type (Pafuri, Skukuza, Okaukuejo and Otjovasandu), *B. anthracis* strain (Sterne, SBPRL-243, WT-2, WT-53, SBPRL-191), month (0,1,2,3,4,5,6), day and night temperature, and relative humidity. The interactions between soil type and strain, soil type and month, and strain and month were also considered. Data was recorded using Excel (Microsoft Office 365). Statistical analyses were performed using IBM SPSS Statistics version 27 (SPSS Inc., SA). A one-way Analysis of Variance (ANOVA) was used to evaluate the spore survival.

3.8. Nucleic acid extraction from soil

The Macherey-Nagel (MN) Nucleobond RNA soil kit and DNA set for Nucleobond RNA soil kit was utilised for the total nucleic acid extraction from the soils. The DNA was used for the qPCR described below in section 3.9 to confirm that *B. anthracis* could not be detected in the soils before beginning the experiment and to monitor the negative controls in the first month of the experiment in case of cross-contamination. The RNA and remaining DNA will be used for future studies on the soil microbiome.

A total volume of 200 µL of RNA later was first added to the collected soil samples and incubated at room temperature overnight to preserve the RNA in the soil. The RNA later was then removed from the samples before continuing with the extraction. The soil samples were prepared by combining 2 g of soil with the NucleoSpin Bead Tubes Type A, 3.2 mL of lysis buffer E1, 400 µL of buffer OPT and 400 µL of Phenol:Chloroform:Isoamylalcohol (PCI) (25:24:1 v/v) in a 15 mL falcon tube. The cells were then lysed by continuously vortexing the samples for 5 min at maximum speed. The samples where then centrifuged at 13000 x g for 5 min and the supernatants were transferred to clean 15 mL falcon tubes.

The binding conditions were adjusted by adding 125 µL of buffer E2 per 1 mL of supernatant and briefly vortexed. The samples were then incubated at room temperature for 2 min and centrifuged at 4500 x g for 5 min. In preparation for the column set up and equilibration, a Nucleobond RNA column (which also includes a filter) was combined with a plastic waster arranged in a clean 50 mL falcon tube. The filters were equilibrated with 12 mL of buffer EQU by adding the buffer to the upper rim of the filters and the flow through was discarded. It is important to note that steps involving the Nucleobond RNA column are performed with gravity flow.

The supernatant of each sample was then loaded onto the centre of each filter and allowed to drip through the column. The flow through was discarded as chemical waste as it contained PCI. The filters were flushed with 6 mL buffer E3 by starting at the rim of the filters. The flow through and the filters were then discarded. The columns were washed with 8 mL buffer E4 and the flow through was discarded. The columns were then transferred to clean 50 mL falcon tubes and RNA was eluted with 5 mL buffer ERNA. For DNA elution, the columns were transferred to clean 50 mL falcon tubes and eluted with 5 mL buffer EDNA.

The RNA and DNA were precipitated by adding 3.5 mL of isopropanol to each eluate and vortexing for 5 s. The precipitated RNA and DNA were then transferred into Nucleospin Finisher columns in 50 mL falcon tubes, centrifuged at 4500 x g for 4 min and the flow through was discarded. The Nucleospin Finisher columns were washed with 5 ml of buffer E5 and centrifuged at 4500 x g for 4 min. The columns were then transferred to a clean 50 mL falcon tube. The RNA and DNA were eluted by adding 80 µL RNase-free H2O to the center of the silica and centrifuged at 4500 x g for 2 min. The RNA was stored at -80°C and the DNA was stored at -20°C.

3.9. Molecular identification of *Bacillus anthracis* using real-time Polymerase Chain Reaction

The aim of this procedure was to demonstrate the presence of *B. anthracis* using FRET hybridisation probes which specifically targets *B. anthracis* DNA as a diagnostic aid to the isolation of bacterial culture from environmental and biological samples following the WHO/OIE Handbook 4th edition (Turnbull, 2008). This assay was designed to target three genes on the *B. anthracis* genome, namely the *pag* gene, which is on the pXO1 plasmid; the *cap-C* gene, which is on the pXO2 plasmid and the *sasp* gene, which is a chromosomal marker. The primer sequences are referenced from (Ellerbrok et al., 2002). We focused on the *pag* gene for this study (Table 3) as this was done for more of a confirmation purpose rather than full identification where all three genes would be required.

Table 3: A list of primers and probes required for the detection of *Bacillus anthracis* using real-time PCR

Target gene	Primer/probe	Primer/probe sequence (5' – 3')	Primer/probe concentration ([µM])
pag	BAPA-S	CGATCAAGTATATGGGAATATAGCAA	0.5
	BAPA-R	CCGGTTTAGTCGTTTCTAATGGAT	
	BAPA-FL	TGCGGTAACATTCACTCCAGTTCGA-X	0.2
	BAPA-LCRed 640	CCTGTATCCACCCTCACTCTTCCATTTTC-P	

The molecular identification of *B. anthracis* using real-time PCR was done using the Roche FastStart SYBR Green Master enzyme kit according to the manufacturer's recommendations. All reagents were completely thawed and centrifuged with a mini centrifuge before being used, and the probes were kept from exposure to bright light. All reagents, except enzymes and probes, were briefly vortexed prior to use. All reagents were kept on ice during the preparation of the master mix, including the DNA to maintain the integrity of the nucleic acids. Every run contained one positive control (Sterne strain) and one negative control (nuclease free water).

Each reaction contained 5 µL of sample DNA, (in the case of the negative control, 5 µL nuclease free water was used), 2x conc FastStart Mastermix, a final concentration of 0.5 µM of each of the primers (BAPA-S and BAPA-R) and 0.2 µM of each of the probes (BAPA-FL and BAPA-LCRed 640) to yield a final volume of 20 µL per reaction. The samples were briefly centrifuged and then placed in the LightCycler Nano where the reactions were incubated at 95 °C for 10 min. The PCR proceeded for 45 cycles of 95 °C for 10 s, 57 °C for 20 s and 72 °C for 30 s. Thereafter, one single acquisition of signals at the end of the annealing phase. Melting occurred at 95 °C for 1 s, 40 °C for 30 s and 80 °C for 1 s with continuous acquisition of signals. Cooling occurred at 40 °C for 30 s. The results were interpreted

by reading the amplification, melting and quantification curves produced by the LightCycler Nano SW 1.0 software with a cut off Ct value of 37.

3.10. Deoxyribonucleic acid extraction from the *Bacillus anthracis* culture

The Invitrogen PureLink® Genomic DNA kit following the Gram positive bacteria protocol was used for the DNA extraction. Five single colonies were selected at random from each culture plate of the inoculated soils. This resulted in a total of 200 extractions from single colonies. The DNA was used for the Multilocus Variable Number Tandem Repeat Analysis which is described below in section 3.11.

A pure, single colony from fresh culture was harvested, resuspended with 180 µL in freshly prepared lysozyme digestion buffer to lyse the cells and incubated at 37 °C for a minimum of 30 min. A total volume of 20 µL proteinase K and 200 µL genomic lysis/binding buffer was added to the samples and vortexed and then incubated at 55 °C for 30 min. After the incubation period, the microcentrifuge tubes were centrifuged to remove drops from the inside of the lid. Thereafter, 200 µL of ethanol (96–100%) was added to the sample and vortexed for 5 s to create a homogenous solution.

A total volume of 600 µL of lysate samples was then transferred to the PureLink spin columns (in a 2 mL collection tube) and centrifuged at 10000 x g for 1 min. The spin columns were then transferred to clean 2 mL collection tubes and the flow through was discarded. A total volume of 500 µL of wash buffer 1 was added to the spin columns and then centrifuged at 10000 x g for 1 min. The collection tube was replaced again and the flow through was discarded. A total volume of 500 µL of wash buffer 2 was added to the spin column and centrifuged at maximum speed for 3 min. The samples were also centrifuged for a further 1 min to ensure all the buffer is removed. The spin columns were transferred to clean 1.5 mL microcentrifuge tubes and 100 µL of genomic elution buffer was added to the centre of the spin columns, incubated at room temperature for 1 min, and then centrifuged at maximum speed for 1 min. The samples were stored at –20 °C until further analysis.

3.11. Multi-locus variable number tandem repeat analysis (MLVA) using selected markers

The *BclA* gene of *B. anthracis* is associated with bams13 as it falls within the open reading frame of the *BclA* gene which is a structural protein responsible for the nap layer of the exosporium (Lista et al., 2006, Sylvestre et al., 2003) while the *BclB* gene is associated with bams30 and is responsible for the formation and maintenance of the basal layer of the exosporium (Leski et al., 2009, Thompson, 2007). The hypothesis is that the size of the bams13 fragment (BclA region) may be responsible for the variation in filament length of the hairy nap layer of the exosporium (Sylvestre et al., 2003). Therefore,

this MLVA was focused on two VNTR loci – bams13 and bams30 which are two of the most polymorphic tandem repeats in *B. anthracis* and are associated with exosporium of the spore. Therefore, analysing these two VNTR loci allowed us to view the genetic differences with regards to the exosporium of the *B. anthracis* strains included in this study.

In addition to viewing the genetic differences, analysing these loci also afforded us the opportunity to check for cross contamination across the soil types. Furthermore, the conditions of the terra-simulator were programmed to repeat the temperature and humidity conditions that was experienced in the pilot study which took place in October 2020 (the temperature and humidity are indicated in Appendix 2). This was done to assess the effect of the drastic weather changes on all the *B. anthracis* spores and took place during day 120 and 150 of the main study.

Analysing bams13 and bams30 will also show if changes occurred at a molecular level with the spores as they were in stored on sporulation agar slants for more than 5 years before being inoculated into the soil and to see if any changes occurred after the inoculation and the temperature changes. Table 4 shows the required primer sequences and concentrations and expected size of repeat units and range of fragment sizes of the bams13 and bams30 loci.

Table 4: The primer sequences required for the Multilocus variable number tandem repeat analysis for the bams13 and bams30 markers of *Bacillus anthracis* and the expected size of the repeat units and range of fragment size

Target	Primer	Sequence 5'- 3'	Primer concentration	Size of repeat unit (bp)	Expected range of fragment size (bp)
Bams13	Bams13 F	CTAGTGCATTTGACCCTAATCTTGT	20 µM	9	337-868
	Bams13 R	AATTGAGAAATTGCTGTACCAAACT			
Bams30	Bams30 F	CAGAAAATATTGGACCTACCTTCC			268-925
	Bams 30 R	AGCTAATCACCTACAACACCTGGTA			

Each reaction contained 5 µL of sample DNA, in the case of the negative control, 5 µL nuclease free water was used, 2x conc MyTaq Red mix, PCR grade water, a final concentration of 20 µM of each of the required forward and reverse primers to yield a final volume of 15 µL per reaction. The samples were briefly centrifuged and then placed in the MiniAmp plus thermal cycler where the reactions were incubated at 95 °C for 2 min. The PCR proceeded for 40 cycles of 95 °C for 15 s, 56 °C for 1 min and 72 °C for 2 min. A final elongation cycle at 72 °C for 5 min was also included. Sterne was included as the positive control. The PCR products were run on a 3% agarose gel at 110 V for three hours or until enough separation was observed between the molecular weight markers. Two molecular weight markers

(50 bp and 100 bp) were included and were added after every eight wells on the gel along with the positive control to ensure accurate readings of band sizes and to account for warping of the gels during the run.

3.12. Transmission electron microscopy of selected *Bacillus anthracis* strains

The main aim of this procedure was to view and assess the differences in the "hairy" nap layer of the exosporium, which is the outermost layer of *B. anthracis* spores. For this exercise, we included the original spores that were stored in sporulation agar and the spores which were isolated from the ongoing soil experiment, to investigate if these storage conditions (artificial vs "natural") may affect the exosporium. Fresh *B. anthracis* cultures were prepared and plated onto sporulation agar and incubated at 37°C for 5-6 days. The cultures were observed by light microscopy after malachite green staining (Schaeffer and Fulton, 1933) and harvested when less than 5% of vegetative cells were observed.

The spores were processed for two different transmission electron microscopy techniques: negative-staining of whole cells; and conventional resin-embedding for the sectioning of spores. The spores were harvested by gently scraping the lawns using a bacteriological loop and a suspension was created with 200 µL of PBS. The spores were processed as unwashed (as described above) and washed for the negative staining and the sectioning techniques. The spores were washed by centrifuging the suspensions at 250 x g for 2 min to allow for gentle pelleting. The supernatant was gently removed and discarded. The pellet was then resuspended in 200 µL of PBS and the wash step was repeated twice. The remaining pellet was then resuspended in the primary fixative (see section 3.2) and stored at 4°C. After 24 hrs, this suspension was streaked onto 5% SBA and incubated at 37°C for at least 72 hrs to check the viability of the spores and to ensure that the spores were inactivated before removing them from the BSL-3. The spores were then negatively stained and also prepared for routine resin-embedding as described in the paragraphs below.

Prior to negative staining, 300 mesh copper grids were coated with 0.3% Formvar® in chloroform as the support film. Stabilisation of the support film with carbon was not possible. For the negative staining, the prepared grids were placed onto drops of washed or unwashed spores for several minutes, for adsorption of the spores to the support film. Excess fluid was then carefully removed using filter paper, the grids were washed in three drops of deionised, distilled water with drying between each rinse drop, then placed on a drop of 2% buffered phosphotungstic acid for several seconds and immediately removed. Grids were then air dried in labelled holders and viewed at 120 kV on a FEI Spirit BioTwin transmission electron microscope fitted with an Olympus Quemesa CCD camera with calibrated OSIS software.

For the sectioning of spores, washed and unwashed spores were routinely processed, and were also processed in a protocol developed for visualisation of capsular polysaccharides (Birkhead et al., 2018) which is described below. Routine processing involved primary fixation in 2.5% glutaraldehyde, buffer rinsing which was repeated thrice for 30 min each with gentle centrifugation for pelleting when necessary for 10 min at 500 x g, then post-fixation in 1% buffered osmium tetroxide for 1.5 hours, buffer rinsing which was repeated thrice for 30 min each with a final rinse in pure water for 5 min prior to dehydration in a graded ethanol series (10%, 30%, 50%, 70%, 90%, 100%) at 30 min intervals. Three rinses of absolute ethanol were performed before resin infiltration and embedding in London resin according to manufacturer's instructions.

An additional protocol was also utlised for sectioning as described by Birkhead (2018). The sporulating isolates were either scraped from the culture plates into 1.5 mL microcentrifuge tubes or processed whilst attached to plugs of agar taken from the plates using polytop vials. The capsular polysaccharides were stabilised and stained with 0.075 M L-lysine acetate and 0.075% ruthenium red, in a 20 min pre-fixation with 5% glutaraldehyde and 6.25% methanol-free formaldehyde in 0.1 M sodium cacodylate buffer, pH 6.8. The cells were then fixed without L lysine acetate, for a further 72 hrs (once viability testing had been completed), rinsed with chilled ruthenium red-containing buffer, post-fixed in 1% osmium tetroxide for 90 min, buffer rinsed, gradually dehydrated in a graded ethanol series, and infiltrated with and embedded in white resin. All stages were performed on ice, and all reagents contained 0.075% ruthenium red, with the exception of the two final dehydration stages (90% and 100% ethanol). All sections (70 nm) were cut on a Leica EM-UC6 ultramicrotome, and double stained with aqueous 4% uranyl acetate for 15 min followed by Reynold's lead citrate for a further 15 min.

4. CHAPTER FOUR: RESULTS

4.1. PART A: Pilot study

4.1.1. Sterne spore survival

The Sterne spores that were inoculated into the Pafuri and Skukuza soils could be detected up to days 21 and 14, respectively (Table 5). The *B. anthracis* Sterne spores showed a steady and rapid decline over the first 14 days (Figure 17). Thereafter, it could not be detected in Skukuza and persisted at low levels in Pafuri until day 21. By day 28, the Sterne spores were completely undetectable in both soils. There was an average minimum and maximum temperature of 20.35 ºC and 40.41 ºC respectively and an average overall temperature of 26.33 ºC during this time period. The remaining Sterne spore stock that was stored at 4 ºC and served as the positive control for the experiment also showed the same trend with regards to its decline (Figure 17) and only remained viable up to day 28 (Table 5).

Table 5: The total colony forming units (CFU) and viable Sterne spores[1] isolated from Pafuri and Skukuza soils at selected time points over 28 days

	Day 0	Day 14	Day 21	Day 28
Pafuri (Total CFU)	$2{,}50 \times 10^6$	$6{,}47 \times 10^4$	$6{,}03 \times 10^4$	$5{,}83 \times 10^4$
Skukuza (Total CFU)	$6{,}33 \times 10^6$	$6{,}56 \times 10^4$	$5{,}56 \times 10^4$	$4{,}86 \times 10^4$
Pafuri (Viable Sterne spores from soil)	$1{,}93 \times 10^6$	$1{,}33 \times 10^3$	$6{,}67 \times 10^2$	0
Skukuza (Viable Sterne spores from soil)	$3{,}67 \times 10^6$	$2{,}33 \times 10^3$	0	0
Sterne (control)	$1{,}59 \times 10^9$	$1{,}30 \times 10^8$	$2{,}23 \times 10^4$	$0{,}23 \times 10^1$

[1].The viable spore counts are reported as spores/g soil.

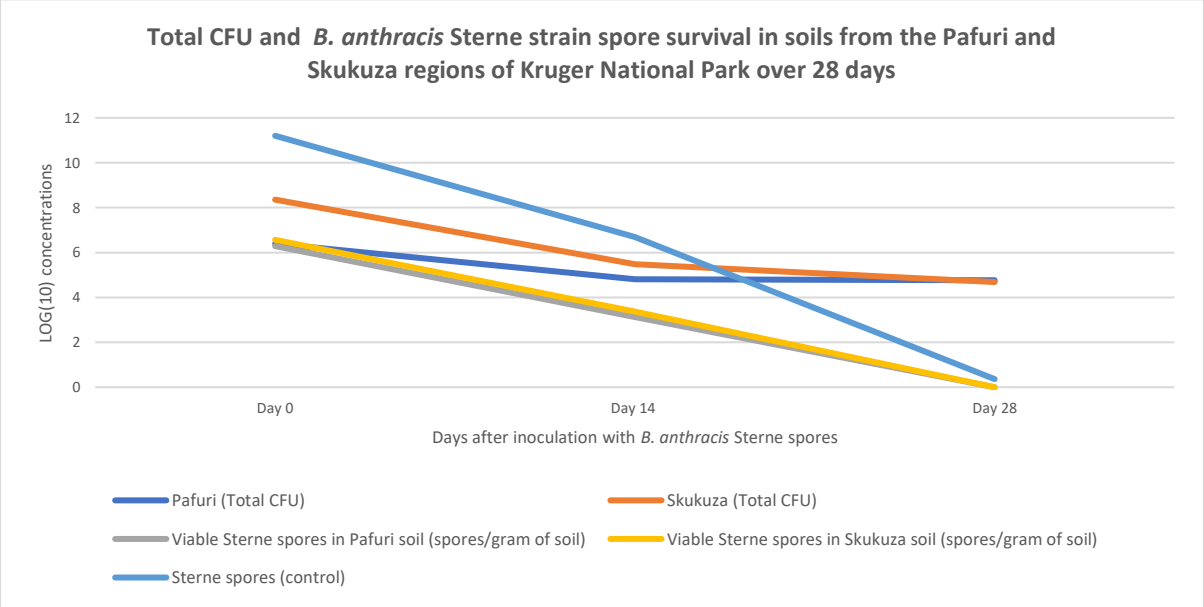


Figure 17: Graph showing the total colony forming units (CFU) and viable Sterne spores in soils from Pafuri and Skukuza in Kruger National Park over 28 days

It did appear that the spores had better survival in the Skukuza soils, however, they persisted for a longer time in the Pafuri soils as spores could be detected up to day 21 (included as an additional time point since the viable spore counts on day 14 were lower than expected but it was not included in subsequent analysis).

The total colony forming units (CFU) for both soils are also shown in Table 5 and this comprised of a variety of other *Bacillus* spp. *Bacillus cereus* was most abundantly present in the Skukuza soil while the Pafuri soil had an almost equal distribution of *B. cereus*, *B. thuringiensis* and *B. subtilis*. All sub-cultures of suspected *B. anthracis* colonies were penicillin and phage sensitive and therefore confirmed as *B. anthracis*. In addition, lytic bacteriophages were also identified on day 0 from both the Pafuri (~37 plaque forming units, pfu/mL) and Skukuza (~24 pfu/mL) soils, however this decreased from day 14 onwards as the Sterne spores began to rapidly decrease.

4.1.2. Statistical analysis

A one-way between groups ANOVA was conducted to investigate the spore survival of the *B. anthracis* Sterne strain ($34F_2$) in soils from Pafuri and Skukuza in KNP. There was a significant difference in the Sterne spore survival rates between the two soils at the $p < 0,05$ level for both Skukuza [F (2,6) = 414,724; p = 0,000118] and Pafuri [F (2,6) = 58, 207; p = 0,0000037]. Post-hoc comparisons using the Tukey HSD test indicated that the mean score for the spore survival in the Skukuza soil on day 0 (Mean (M) = 37,67; (Standard deviation (SD) = 3,055) and day 14 (M = 2,33; SD = 0,577) was significantly different from the Pafuri soil on day 0 (M = 20,00; SD = 4,359) and day 14 (M = 1,33; SD = 0,577), however, by day 28 the spores were undetectable in both soils.

4.1.3. Environmental monitoring of the terra-simulator

Growth was not observed on any of the NA plates. This shows that the terra-simulator was a sufficient containment pod including the use of resealable plastic containers. The use of 10% F10 disinfectant and 70% ethanol were also sufficient as surface disinfectants.

4.2. PART B: Main study

4.2.1. *Bacillus anthracis* viable spore counts from inoculated soils

Our findings showed that the spore counts were starting to stabilise by six months, with the highest decline seen between day 0 and day 28 (Figure 18). Between months 6 - 17 (until the termination of the

project), the decline in spore survival was minimal for all strains, except Sterne which had a continuous decline and had the lowest spore counts at the end of the project as seen in Table 6. We also saw that the Skukuza soil also had the highest decline in spore survival for all strains for this period when compared to Pafuri, Okaukuejo and Otjovasandu. There was also a clear variation across all the soils with regards to spore survival (Table 6 and Figure 18) and the general soil microbial diversity (Table 7).

The A-strains survived better than the B-strain as the spore counts of the A-strains from both South Africa (SBPRL-191) and Namibia (WT-2 and WT-53) were still high across all the soil types at the end of the project, with the exception of Skukuza as seen in Table 6. The South African B-strain (SBPRL-243) survived less well than the A-strains. The B-strain had the best survival in the Pafuri soil when compared to other soils as it had similar survival trend to the A-strains as seen in Figure 18. The results from the spore survival study also highlighted the poor survivability of the Sterne strain when compared to the other *B. anthracis* strains included in this study as Sterne had the lowest spore counts at the end of the project as seen in Table 6.

Table 6: Viable *Bacillus anthracis* spore counts at selected time points in this study

Soil	*Bacillus anthracis* strain	Spore count after inoculation on day 0 (spores/g of soil)	Spore count on day 180 (spores/g of soil)	Final spore count on day 525 (spores/g of soil)
Pafuri (KNP – High incidence anthrax region)	WT-2	$3{,}16 \times 10^7$	$1{,}25 \times 10^7$	$1{,}01 \times 10^7$
	WT-53	$3{,}31 \times 10^7$	$1{,}39 \times 10^7$	$1{,}11 \times 10^7$
	SBPRL-191	$3{,}25 \times 10^7$	$1{,}42 \times 10^7$	$1{,}19 \times 10^7$
	SBPRL-243	$3{,}27 \times 10^7$	$1{,}19 \times 10^7$	$9{,}07 \times 10^6$
	Sterne	$2{,}41 \times 10^7$	$2{,}43 \times 10^6$	$1{,}65 \times 10^5$
Skukuza (KNP – Low incidence anthrax region)	WT-2	$2{,}75 \times 10^7$	$7{,}60 \times 10^6$	$4{,}07 \times 10^6$
	WT-53	$2{,}83 \times 10^7$	$7{,}73 \times 10^6$	$4{,}37 \times 10^6$
	SBPRL-191	$2{,}71 \times 10^7$	$1{,}02 \times 10^7$	$7{,}20 \times 10^6$
	SBPRL-243	$2{,}36 \times 10^7$	$6{,}53 \times 10^6$	$3{,}21 \times 10^6$
	Sterne	$2{,}46 \times 10^7$	$3{,}03 \times 10^6$	$2{,}77 \times 10^5$
Okaukuejo (ENP – High incidence anthrax region)	WT-2	$3{,}09 \times 10^7$	$1{,}01 \times 10^7$	$8{,}17 \times 10^6$
	WT-53	$3{,}02 \times 10^7$	$1{,}02 \times 10^7$	$8{,}17 \times 10^6$
	SBPRL-191	$2{,}74 \times 10^7$	$1{,}03 \times 10^7$	$8{,}77 \times 10^6$
	SBPRL-243	$2{,}58 \times 10^7$	$5{,}20 \times 10^6$	$2{,}37 \times 10^5$
	Sterne	$2{,}53 \times 10^7$	$4{,}47 \times 10^6$	$1{,}17 \times 10^6$
Otjovasandu (ENP – Low incidence anthrax region)	WT-2	$3{,}20 \times 10^7$	$1{,}19 \times 10^7$	$1{,}00 \times 10^7$
	WT-53	$3{,}13 \times 10^7$	$1{,}23 \times 10^7$	$1{,}04 \times 10^7$
	SBPRL-191	$2{,}96 \times 10^7$	$1{,}17 \times 10^7$	$9{,}70 \times 10^6$
	SBPRL-243	$2{,}90 \times 10^7$	$8{,}63 \times 10^6$	$6{,}23 \times 10^6$
	Sterne	$2{,}87 \times 10^7$	$5{,}77 \times 10^5$	$1{,}46 \times 10^5$

In the soils from KNP, Pafuri, which is a high-incidence anthrax region, had the best spore survival for all strains from both clades, except for Sterne (Figure 18). The spores from fully virulent strains had a more stable survival in Pafuri compared to the other soil types in this study. In the Skukuza soil, which is a low-incidence anthrax region in KNP, we observed more variation in the survival trends (Figure 18). Sterne had the poorest survival from all the strains followed by SBPRL-243 which is a South African B-clade strain. The South African A-clade (SBPRL-191) also survived better in comparison to the Nambian A-clades (WT-2 and WT-53) in this soil type (Table 6). In the soils from ENP, Okaukuejo (high incidence anthrax region) and Otjovasandu (low incidence anthrax region) all A-clade strains survived equally well in these soil types (Figure 18), most notably the Namibian A-strains. We observed differences in the survival of SBPRL-243 and Sterne, where Sterne appeared to have its best survival in Otjovazandu compared to all other soil types.

Table 7 gives a summary of the basic soil microbial diversity that was observed over the duration of the experiment. Varying levels of bacteriophage activity were noted in the four soils from the anthrax high- and low-incidence regions. These lytic bacteriophages were seen on the 10^0 SBA plates during the isolations and appeared as "doughnut" rings in the heavy lawns of *B. anthracis* colonies. The final plaque forming units per ml (pfu/ml) reported here is an average of the number of bacteriophages that were identified from all the soils which were inoculated with the different *B. anthracis* strains at each time point (Day 0 – 180). We observed the highest bacteriophage activity in Pafuri (35-45 pfu/mL) and Skukuza (~24-32 plaques pfu/mL). Okaukuejo had a moderate bacteriophage activity (~10-15 pfu/mL). We did not detect any bacteriophage activity in Otjovasandu.

The negative soils did not show any bacteriophage activity, but various *Bacillus* spp. were identified across the four soil types (Table 7). Growth was also mostly observed on the 10^0 SBA plates and had a mix of *Bacillus* spp. as reported in Table 7. The numbers of colonies identified are not reported in Table 7 as it was difficult to count the exact number of colonies. The variation of *Bacillus* spp. identified also declined as time progressed during the experiment where we see only the most abundant species in the last two months (day 150 and day 180) of the experiment (Table 7). The species identified differed between parks and between the anthrax high- and low-incidence regions as seen in Table 7. *Bacillus* cereus was most commonly present in the Skukuza soil while the Pafuri soil had an almost equal distribution of *B. cereus*, *B. thuringiensis*, *B. subtilis*. We also noted that Otjovasandu had a rather poor diversity with regards to the soil bacteria that were isolated We mostly identified *B. subtilis* in the Otjovasandu soil whereas Okaukuejo had a more diverse range of *Bacillus* spp. but *B. subtilis* and *B. cereus* were most abundant.

Table 7: Soil microbial diversity across the four soil types (Pafuri, Skukuza, Okaukuejo and Otjovasandu over six months (Day 0 – 180) with focus on the bacteriophage activity (plaque forming units/mL, pfu/mL) and *Bacillus* spp. identified

Soil type	Soil microbial activity	Day 0	Day 28	Day 60	Day 90	Day 120	Day 150	Day 180
Pafuri	Bacteriophages (pfu/mL)	45	41	44	42	41	39	35
	Other *Bacillus* spp isolated	*B. cereus, B. megaterium, B. mycoides, B. pumilus, B. subtilis, B. thuringiensis.*	*B. cereus,* B megateriu m, B.mycoide s, *B. pumilus B. subtilis, B. thuringiens is*	*B. cereus,* B megaterium *, B. mycoides, B. pumilus, B. subtilis, B. thuringiensis.*	*B. cereus, B. megaterium , B. mycoides B. pumilus, B. subtilis, B. thuringiensis.*	*B. cereus, B. thuringiensis, B. subtilis.*	*B. cereus, B. thuringiensis, B. subtilis.*	*B. cereus, B. thuringiensis, B. subtilis.*
Skukuza	Bacteriophages (pfu/mL)	32	30	30	28	27	25	24
	Other *Bacillus* spp isolated	*B. cereus, B. mycoides, B. subtilis.*	*B. cereus, B. mycoides, B. subtilis.*	*B. cereus, B. mycoides, B. subtilis,*	*B. cereus, B. subtilis*	*B. cereus, B. subtilis*	*B. cereus.*	*B. cereus.*
Okaukuejo	Bacteriophages (pfu/mL)	15	12	11	11	13	10	10
	Other *Bacillus* spp isolated	*B. cereus, B. mycoides, B. pumilus, B. subtilis.*	*B. cereus, B. mycoides, B. pumilus B. subtilis.*	*B. cereus, B. mycoides, B. pumilus B. subtilis.*	*B. cereus, B. mycoides, B. subtilis.*	*B. cereus, B. mycoides, B. subtilis.*	*B. cereus, B. subtilis,*	*B. cereus, B. subtilis,*
Otjovasandu	Bacteriophages (pfu/mL)	0	0	0	0	0	0	0
	Other *Bacillus* spp isolated	*B. subtilis, B. cereus, B. pumilus*	*B. subtilis, B. cereus, B. pumilus*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*

Between day120 to 150 of the main study, the conditions of the terra-simulator were changed to repeat the temperature and humidity that was experienced in the pilot study which took place in October 2020 (the temperature and humidity are indicated in Appendix 2). This assessed the effect of the drastic weather changes from the period of the pilot study on all the *B. anthracis* spores. Sterne showed a decrease across all soil types between day 120 and day 150 as seen in Figure 18, however, the A- and B-subclade strains remained relatively stable during this change and even after day 150 when the conditions in the terra-simulator went back to mimicking Pafuri's weather in real time (Figure 18).

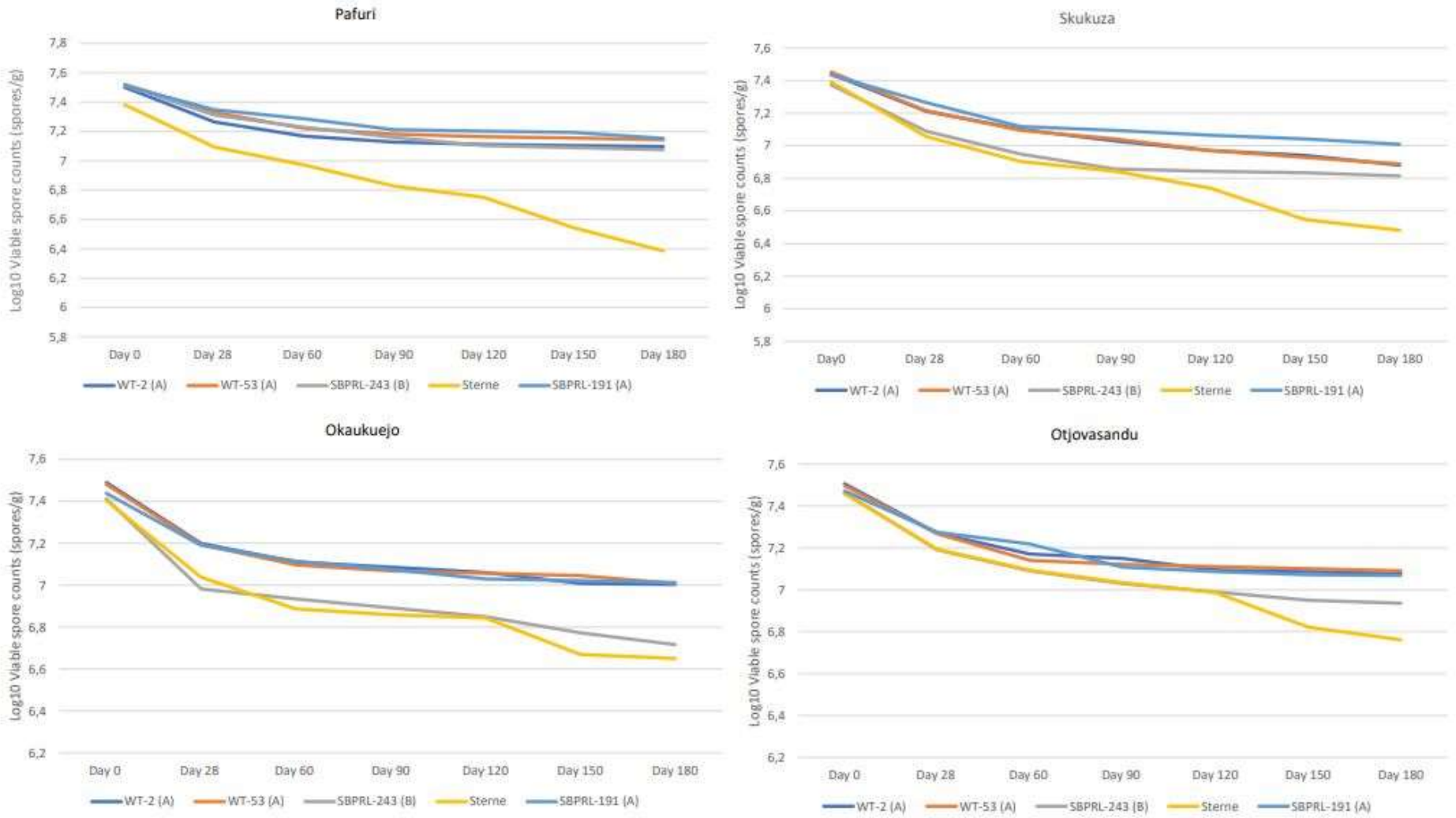


Figure 18: *Bacillus anthracis* spore survival trends over six months (Day 0 – 180) across the four different soil types from the anthrax high and low incidence regions of Kruger (Pafuri and Skukuza) and Etosha (Okaukuejo and Otjovasandu) National Parks. This image also shows the drop in the viable spore counts for the Sterne strain between day 120 -150. SBPRL191 and SBPRL 243 are South African A- and B-subclade strains, respectively. WT2 and WT53 are Nambian A-strains.

4.2.2. Soil analyses of soils from collected from Pafuri and Skukuza in Kruger National Park and Okaukuejo and Otjovasandu in Etosha National Park

The exchangeable cations (Sodium, Na; Calcium, Ca; Magnesium, Mg; Potassium, K); Manganese (Mn), the soil organic matter (SOM), soil density, and the pH of the soils from Pafuri and Skukuza (KNP) and Okaukuejo and Otjovasandu (ENP) are shown in Table 8.

Table 8: Soil properties such as exchangeable cations (sodium, calcium, magnesium and potassium), manganese, soil organic matter, soil density and pH of the soils from Pafuri and Skukuza (Kruger National Park) and Okaukuejo and Otjovasandu (Etosha National Park)

	Exchangeable cations (mg/kg)							
Region	Sodium (Na)	Calcium (Ca)	Magnesium (Mg)	Potassium (K)	**Manganese (Mn, mg/kg)**	**Soil organic matter (SOM, %)**	**Soil density (g/mL)**	**pH**
Pafuri	434	2623	1182	289	45,89	1,18	1,31	6,8
Skukuza	24	3428	559	303	79,54	3,28	1,12	6,7
Okaukuejo	3186	6871	698	513	37,81	0,87	1,22	7,8
Otjovasandu	17	1381	493	95	90,55	0,25	1,61	7,4

There were clear differences between all four soil types. Pafuri had moderate levels of Ca, Mn, soil organic matter and the highest Mg of all the soils (Table 8). Skukuza had a high level of SOM compared to other soils and had moderate levels of all other soil characteristics presented in Table 8. Okaukuejo had the highest levels of Na and Ca but the lowest level of Mn (Table 8). Otjovasandu had the highest levels of Mn and the highest soil density but the lowest levels of soil organic matter and Ca (Table 8). The soils in ENP are slightly alkaline whereas the soils in KNP are more neutral according to the classification of soil pH ranges. Okaukuejo had a soil moisture content of 20,2% and had a sandy, loam texture with 58,7% sand, 34,9% silt and 6,47 clay. Otjovasandu had a soil moisture content of 0,202% and had a sandy texture with 90,4% sand, 6,6% silt and 3,0% clay.

4.2.3 Data analysis

The different soil types treated with the different strains of *B. anthracis* spores showed variation in their spore concentration across the different time points (Table 9 and Figure 19). Overall, Pafuri had the highest spore counts, followed by Otjovasandu, Okaukuejo and Skukuza, although Sterne showed relatively lower spore counts in Pafuri than Otjovasandu (Figure 19). The strain type was a significant determinant of spore survival over time (Table 9). SBPRL191 had the highest spore count at the final month, although a similar trend of decline was observed across the three A-strains SBPRL191, WT53 and WT2 for the different soil types (Figure 19).

Time had a significant effect on spore survival as there was a significant decline over time with reference to the first month ($p<0.0001$). The average day temperature over time had a significant effect on spore survival across the different soil types treated with the various strains (Table 9). A unit increase in day temperature led to an increase of 2.3×10^6 in spore concentration ($p<0.0001$). Also, the night average temperature and average relative humidity significantly affected the spore survival over time across all the soil types ($p<0.0001$; Table 9). Also, for every 1 % unit increase in the relative humidity, the spore concentration reduced by 3.4×10^6 ($p<0.0001$). The results are given as an ANOVA table (Table 9) containing the significance of main effects and interactions between soil type and strains (Figure 20) (degrees of freedom: 200 total; 147 residual). The temporal autocorrelation in residuals was moderately high (0.57, likelihood ratio test, $P < 0.001$).

Table 9: Effect of environment and pathogen factors on *Bacillus anthracis* spore count from a Generalised Least Squares regression fit with REML

	numDF	F-value	p-value
(Intercept)	1	16269.42	<.0001
Soil_type	3	64.283	<.0001
Strains	4	73.917	<.0001
Month	6	926.742	<.0001
Average_Day_temp	1	22.371	<.0001
Average_Night_temp	1	379.97	<.0001
Average_Humidity	1	1026.676	<.0001
Soil_type:Strains	12	7.886	<.0001
Strains:Month	24	2.624	<.0001

Following a pairwise comparison for the different strains across the four soil types within KNP and ENP over time, for Sterne, there was a significant difference between Pafuri and Skukuza and between Okaukuejo and Otjovasandu ($p<0.0001$). There was also a significant difference between Pafuri and Skukuza and between Okaukuejo and Otjovasandu ($p<0.0001$) for the South African B-subclade strain (SBPRL-243). For the South African A-subclade strain (SBPRL-191), there was a significant difference between Pafuri and Skukuza ($p<0.0001$) but no significant difference between Okaukuejo and Otjovasandu ($p=0.2599$). Also, for the Namibian A-subclade strains, (WT-2 and WT-53) there was a significant difference between Pafuri and Skukuza ($p<0.0001$), however, there was no significant difference between Okaukuejo and Otjovasandu ($p=0.6118$ and $p=0.3428$ respectively).

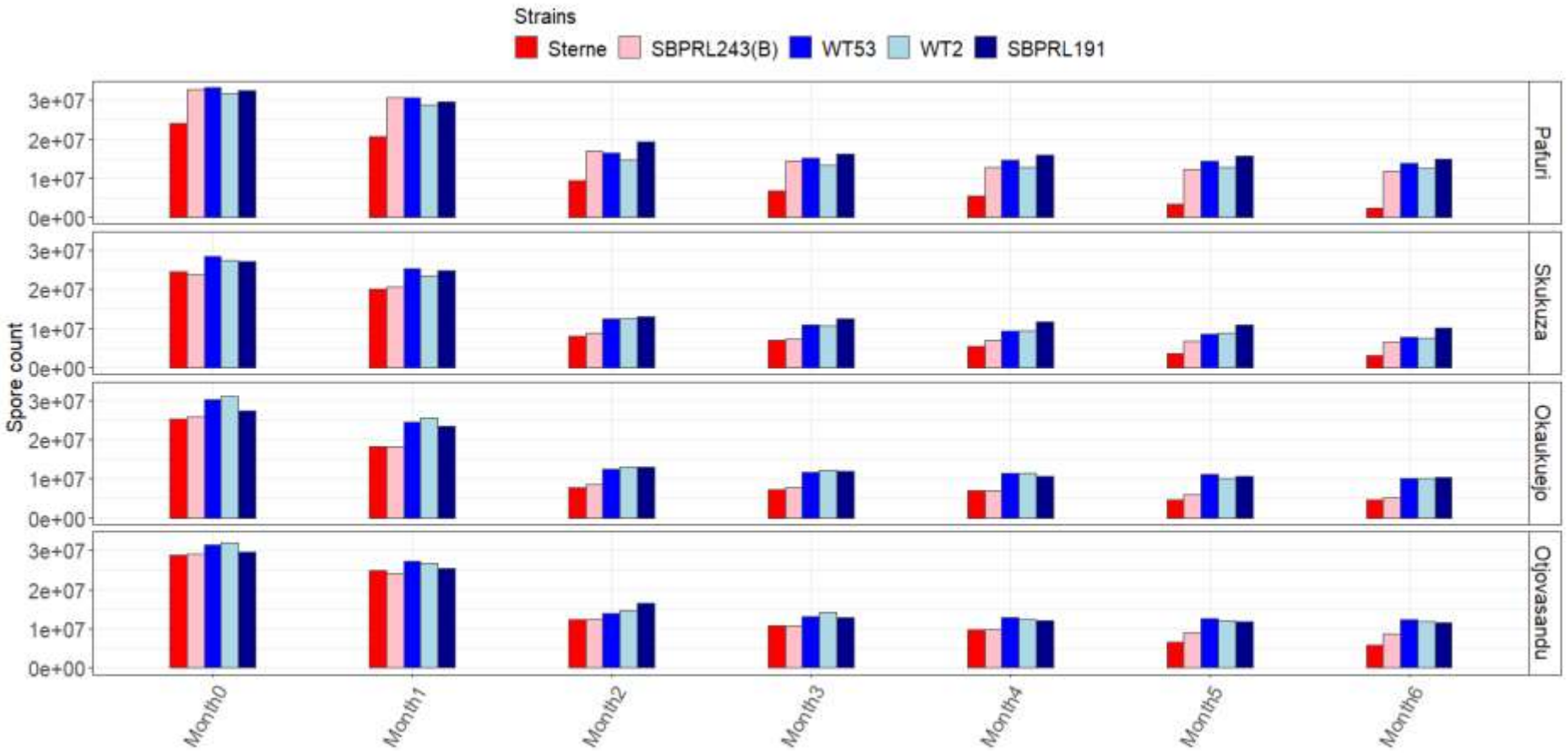
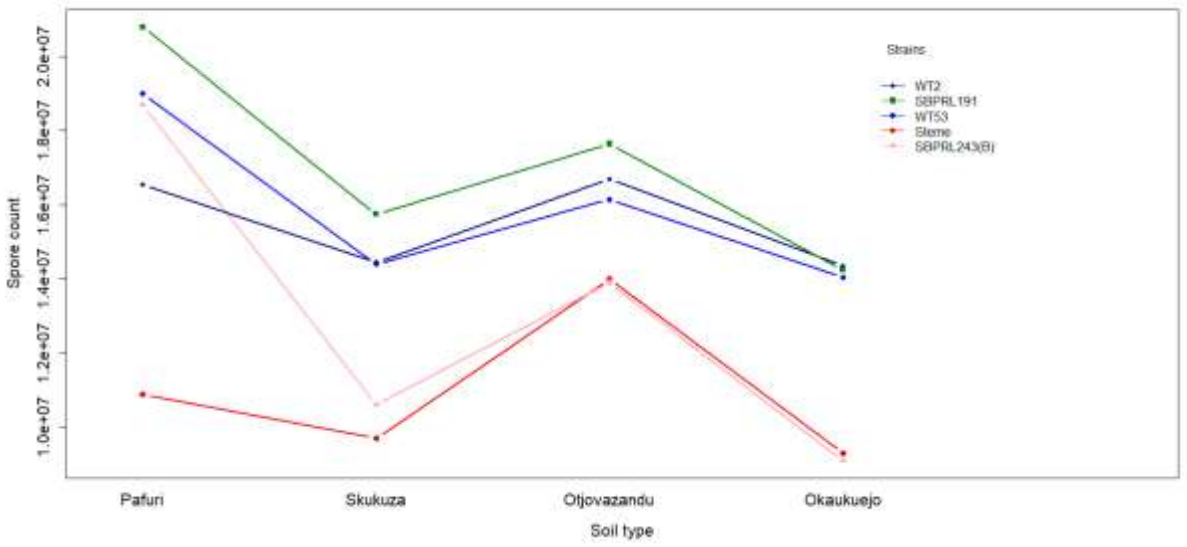
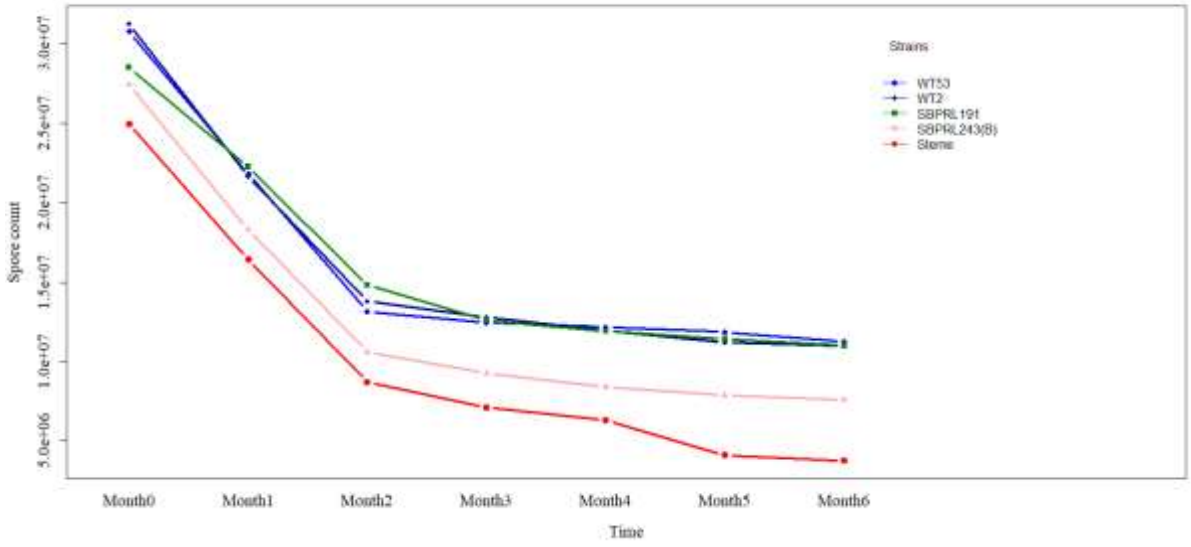


Figure 19: Bar plots representing spore counts obtained from the four soil types included in this study: Pafuri and Skukuza in Kruger National Park (KNP; South Africa) and Okaukuejo and Otjovazandu in Etosha National Park (ENP; Namibia) treated with five strains of *Bacillus anthracis* (Sterne, SBPRL243(B-subclade), WT53, WT2, SBPRL191). WT-2 and WT-53 are Namibian A-subclade strains. SBPRL191 and SBPRL243 are South Africa A- and B-subclades, respectively. Spore counts were performed monthly for six months and reported as spores/g.



A



B

Figure 20: A: Interaction plot showing points of interaction between soil types and strains over time. B: Interaction plot showing points of interaction between time and strains across the different soil types.

The interaction effect between soil type and strains was significant as we see all lines intersecting (Figure 20A; $p<0.0001$) across all the soil types. The spore counts represent the mean spore counts over time. This shows that the spore survival is influenced by the different soil types and the different strains. The interaction between the A-strains and month was significant across all the months (Figure 20B, $p<0.0500$) as all three lines intersect. This shows that their decline was similar over time in comparison to the Kruger-B and Sterne strains.

4.2.4 Molecular results

4.2.4.1. Molecular detection of *Bacillus anthracis* from soil

The DNA extracted from the negative control soil samples from the study were tested with the *B. anthracis* qPCR which amplified the *pag* gene. This was done to monitor the possibility of contamination. Microbiological and molecular tests were done throughout the study and confirmed that *B. anthracis* could not be detected in those soil. This also helped to confirm the validity of the rest of the results obtained from the study.

4.2.4.2. Variable number tandem repeat (VNTR) analysis of selected *Bacillus strains* with focus on the bams13 and bams30 loci

The VNTR analysis was focused on two loci – bams13 and bams30 which are two of the most polymorphic tandem repeats in *B. anthracis*. Based on these loci, we determined the unit copy number (Table 10) for each strain based on the approximate fragment sizes which we obtained from the gels (Figure 21). We analysed the spores that were stored in sporulation agar, and the spores that were isolated from the soil experiment before and after the conditions of the tank were changed to investigate if any differences occurred.

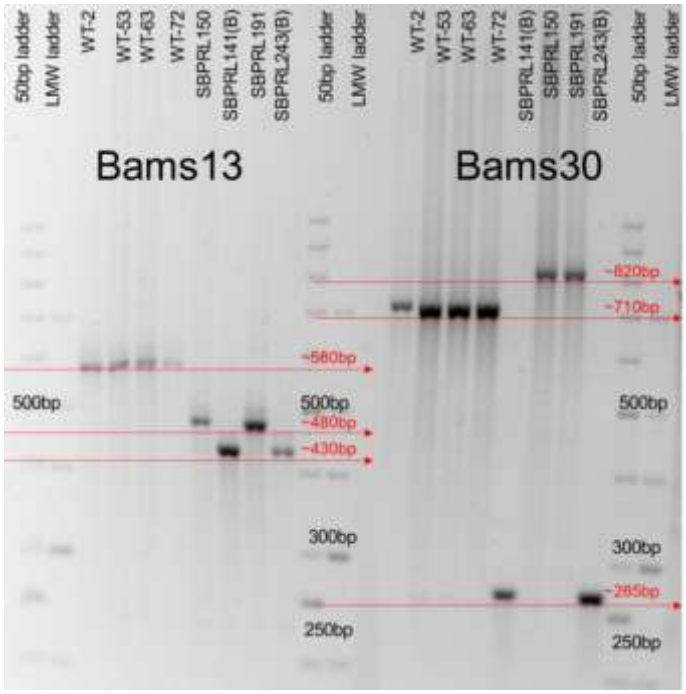


Figure 21: Variable number tandem repeat analysis of the bams13 and bams30 loci for selected *Bacillus anthracis* strains that were stored in sporulation agar. WT-2, WT-53, SBPRL191 and SBPRL243 were focused on in this study. Approximate band sizes are indicated in red.

Table 10: The fragment size and unit copy number for bam13 and bams30 loci of selected *Bacillus anthracis* strains from Namibia (WT-2 and WT-53, both A-subclade) and South Africa (SBPRL-191 and SBPRL-243, A- and B-subclade, respectively)

		Bams13		Bams30	
Country/subclade	**Strain**	**Fragment size (bp)**	**Unit copy number**	**Fragment size (bp)**	**Unit copy number**
Namibia, A	WT-2	580	44u	710	58u
	WT-53	580	44u	710	58u
South Africa, A	SBPRL-191	480	33u	820	71u
South Africa, B	SBPRL-243	429	27u	265	8u

We did not observe any changes on bams13 on a genetic level for the single spores that were the isolated from the soil before and after the conditions were changed in the tank (Figures 22 and 23) and also obtained the same size fragments that we saw with the spores that we stored in sporulation agar. Figure 22I shows a clearer picture of the situation where the same strain was isolated at different time points from the soils (indicated by 1. Pafuri-WT2 and 2. Pafuri-WT2) and was run next to each other. We did not observe any changes in the fragment size of bams13 in any of those isolates (Figure 22I). Since there were no changes to bams13, bams30 was not analysed for any of the isolates from soil. In addition, selecting random colonies from the plates also served as a method to monitor for cross contamination since all isolates that were grouped together had the same bams13 fragment size as can be seen in Figures 24 and 25 and all are therefore the same strains. This is especially clear with SBPRL-191, SBPRL-243 and the Sterne strain which all have different fragment sizes from each other in comparison to WT-2 and WT-53.

Lastly, the differences observed in the bams13 locus as shown in Figure 21 and Table 10 indicates that the lengths of the filaments in the nap layer of the exosporium between the Namibian A-strains (WT-2 and WT-53) and South African A- (SBPRL-191) and B- (SBPRL-243) strains are expected to differ such that the Namibian strains should have longer filaments in the nap layer when compared to the South African A- and B-strains as the Namibian strains have a higher unit copy number for bams13 (Table 10) than both the South African A- and B-strains.

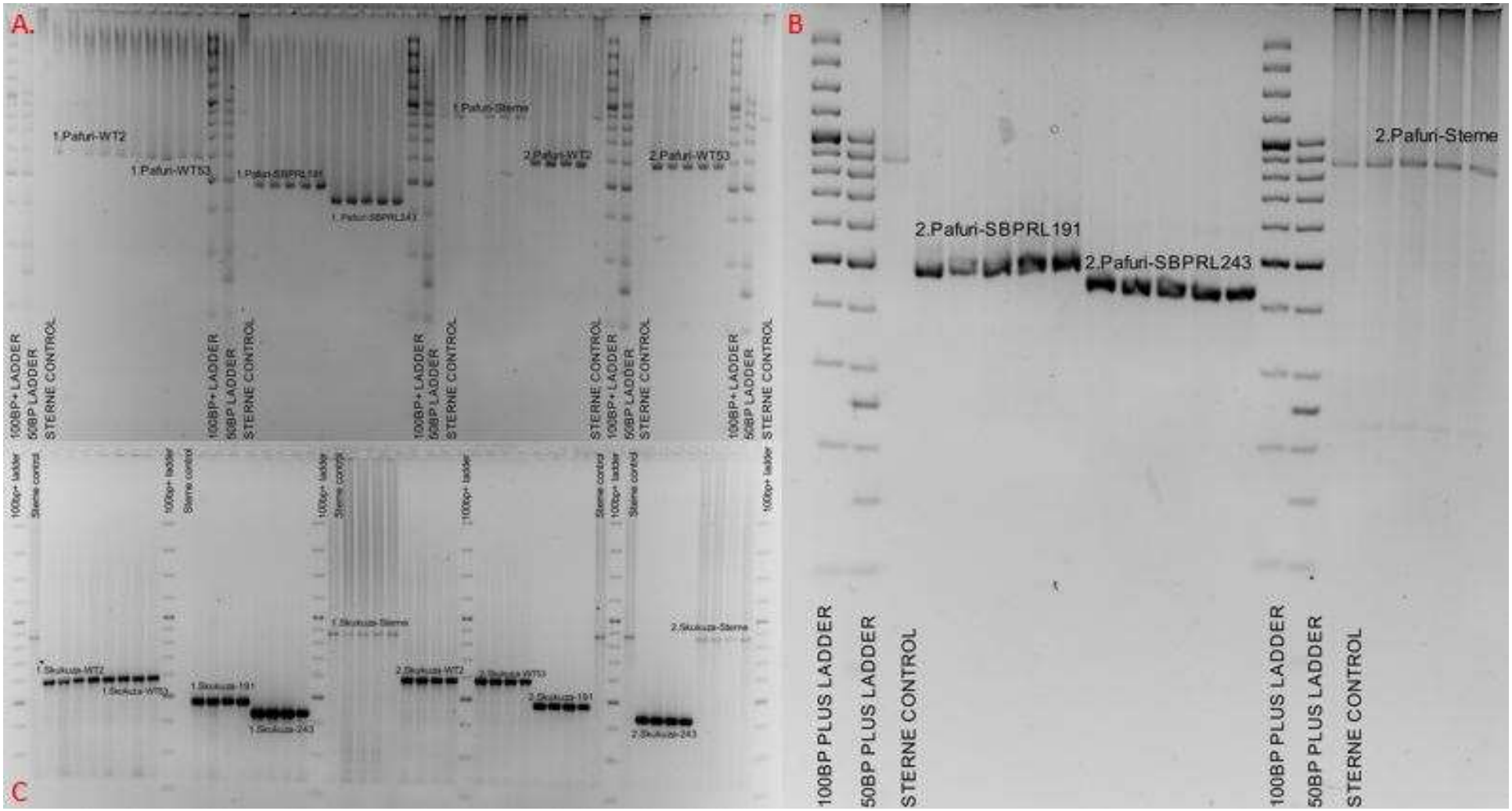


Figure 22A-C: Gel images of the bams13 locus of the single *Bacillus anthracis* colonies which were isolated from soil to investigate if differences occurred before and after the conditions in the tank were changed. This image shows the *Bacillus anthracis* isolates from Pafuri (A and B) and Skukuza (C). "1" indicates isolates that were extracted before the changes occurred and "2" indicates isolated that were extracted after the changes occurred. A 100bp plus ladder was used and the Sterne strain served as the PCR positive control.

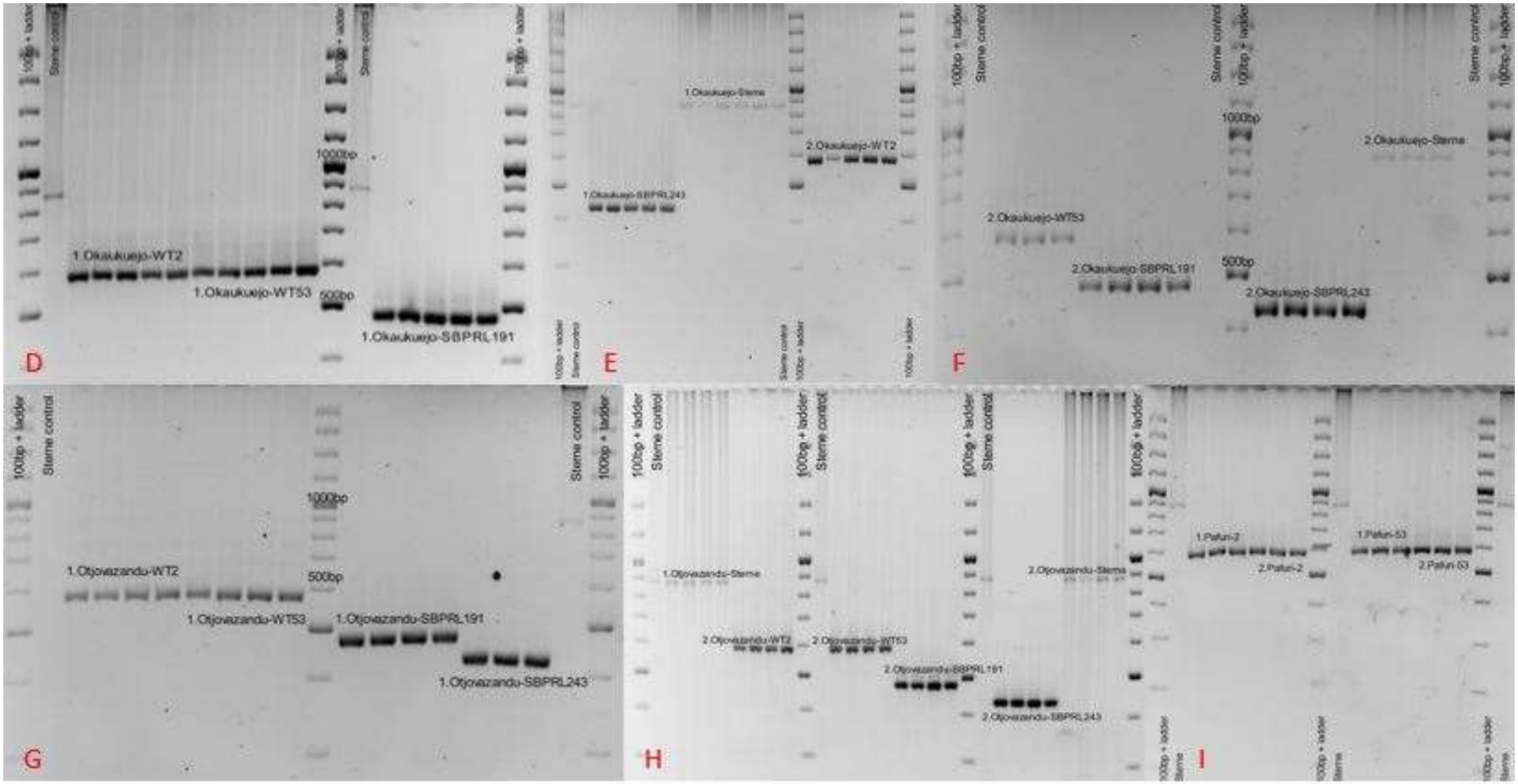


Figure 22D-I: Gel images of the bams13 locus of the singe *Bacillus anthracis* colonies which were isolated from soil to investigate if differences occurred before and after the conditions in the tank were changed. This image shows the *Bacillus anthracis* isolates from Okaukuejo (D-F) and Otjovasandu (G-H). Image I compares the same strain that was isolated at different time points from the soils and was run next to each other to show that differences did not occur in the bams13 loci during this time. "1" indicates isolates that were extracted before the changes occurred and "2" indicates isolated that were extracted after the changes occurred. A 100bp plus ladder was used and the Sterne strain served as the PCR positive control.

4.2.5 Transmission Electron Micrographs of *Bacillus anthracis* spores using negative staining, conventional resin-embedding and sectioning

The images of the selected strains are shown in Figures 23-27. As a reminder, WT-2 and WT-53 are the Namibian A-subclade strains; SBPRL-191 and SBPRL-243 are the South African A- and B-subclade strains. Quantitative analyses were not done, as an insufficient number of samples were fully processed and imaged (due to time constraints and technical difficulties with reagents and equipment). We first analysed spores that were stored in sporulation agar (Figure 23A, B, D). From the negative staining of whole cells, it was rather challenging to clearly visualise the nap layer (hairy outer layer of the spores) of the exosporium in all isolates, with the exception of WT-2, as the nap layer appeared to be absent or composed of poorly defined filaments that were difficult to distinguish against the underlying spore layers. The nap filaments also did not take the stain up well, irrespective of the heavy metal salt used (we tried phosphotungstic acid, uranyl acetate and ammonium molybdate) which made it difficult to make any useful comparative measurements for statistical analyses. However, an interesting finding was the presence of endospore appendages, 7.5 nm in diameter, in isolates from both Namibia and South Africa (Figure 24). Such appendages haves only been described previously in pathogenic *B. cereus* strains by Pradhan, 2021 (Pradhan et al., 2021) in which they emerged from the spore in bundles or individually, as seen in Figure 25 (shown for comparison).

While processing the spores for ultramicrotomy, technical difficulties were encountered during the resin embedding process which resulted in a lot of background on the electron micrographs (Figure 26) and some samples had to be discarded. Some of the ultrastructural details were discernible, including a faint nap layer, exosporium, spore coat layers, spore membrane around the core, and core contents. Given the pleomorphic nature of the exosporium, together with the many varied planes of section, it proved impossible to accurately measure the length of nap layer filaments for comparative purposes, as it was extremely rare to find an endospore in perfect longitudinal or cross section.

Representative images of the negatively stained spores isolated from the soil experiment are shown in Figure 23D and 23E. All strains were isolated from the different soil types and fully processed. However, due to time constraints and unforeseen circumstances with the electron microscope, we only focused on spores that were isolated from the Skukuza soil as this soil type had the poorest spore survival rates. The rest of the samples will be viewed at a later stage. We looked at the South African A- and B-subclades and clear differences can be seen in the abundance of the filaments around the spores in addition to the differences in the length. There is a well-defined layer of filaments which

surrounds the exosporium and forms the nap layer around SBPRL-191 (Figure 23D) but is not as abundant around SBPRL-243 (Figure 23E).

We also repeated the ultramicrotomy and resin embedding with a new batch of resin and obtained better quality images, as there is less adherent dirt, and the nap layer is slightly more well defined (Figure 27). Figure 26 shows the first attempt which was unsuccessful but obtained better images during the second attempt as seen in Figure 27. All samples have been processed, but sectioning and viewing will be completed at a later stage to provide more comprehensive data.

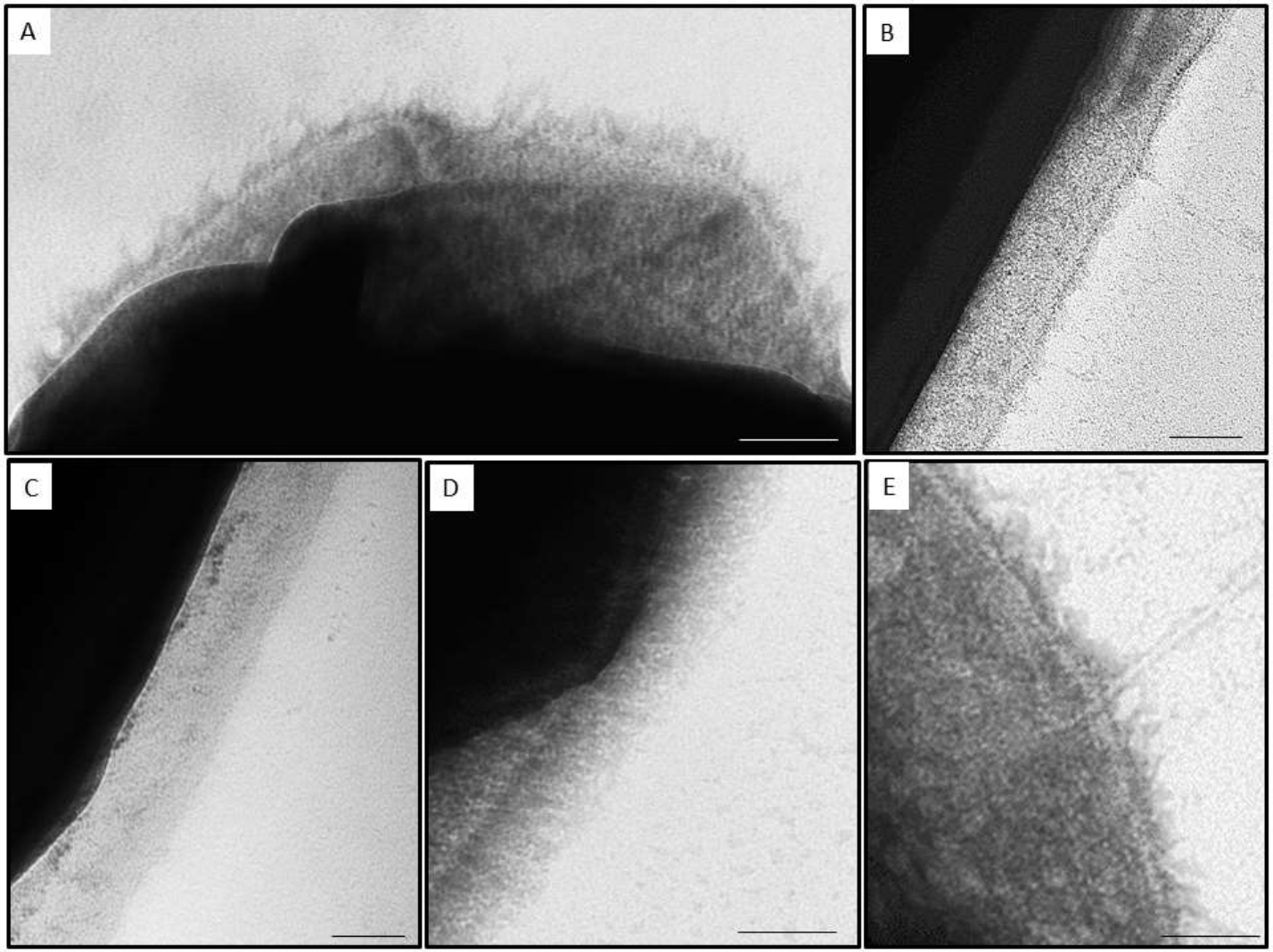


Figure 23: Negatively stained *Bacillus anthracis* spores for nap layer visualisation, from sporulation agar (A, B, C) and soil (D, E). A – WT2, B and D = SBPRL191, C and E = SBPRL 243. Scale bars: A, D, E -50 nm; B and C = 100 nm

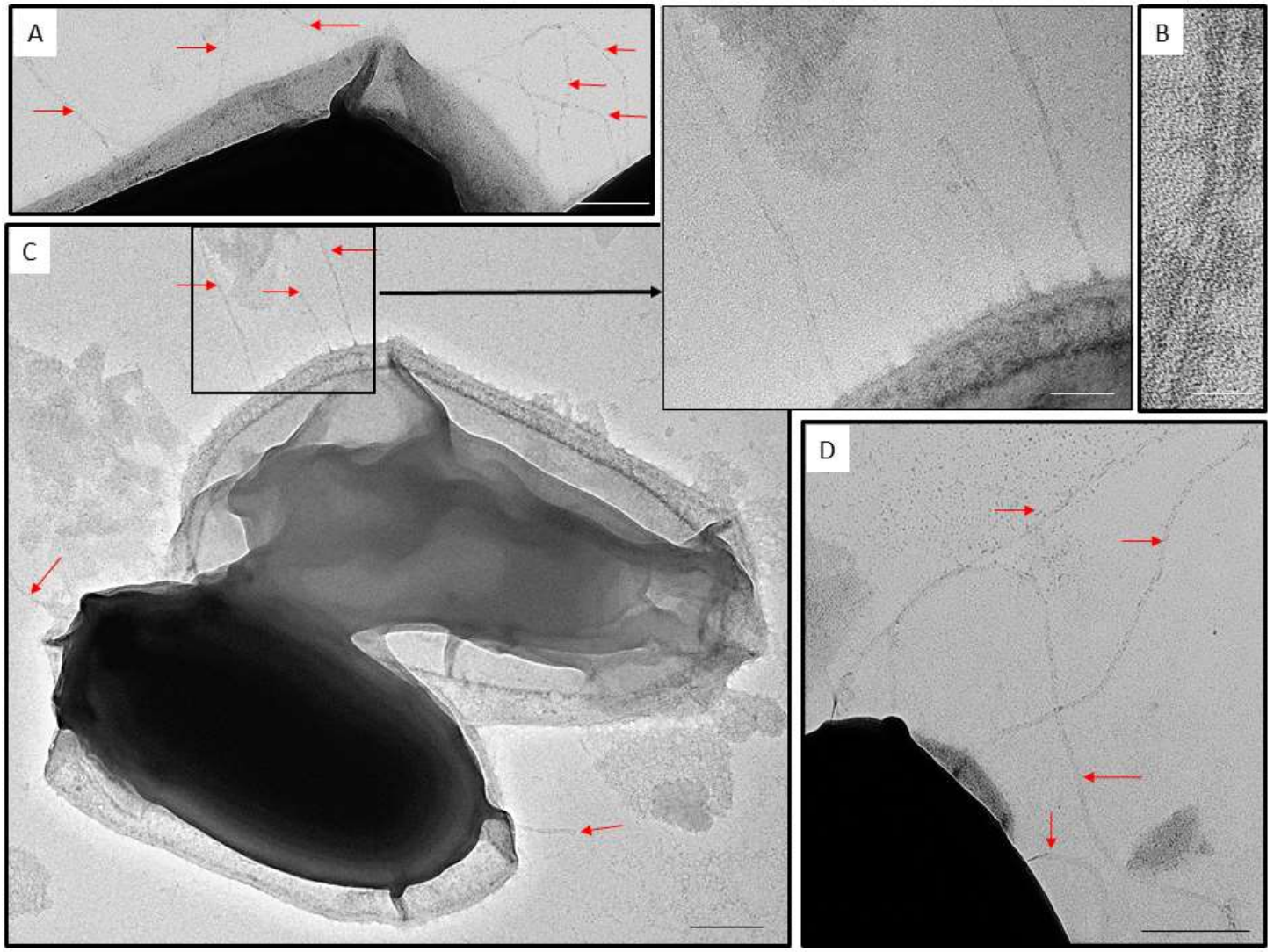


Figure 24: *Bacillus anthracis* endospore appendages apparent after negative staining. The red arrows indicate the appendages. A and D = SBPRL191; B and C =SBPRL243. Scale bars: A=200 nm; B=22 nm; C=270 nm (inset = 80 nm); D=200 nm.

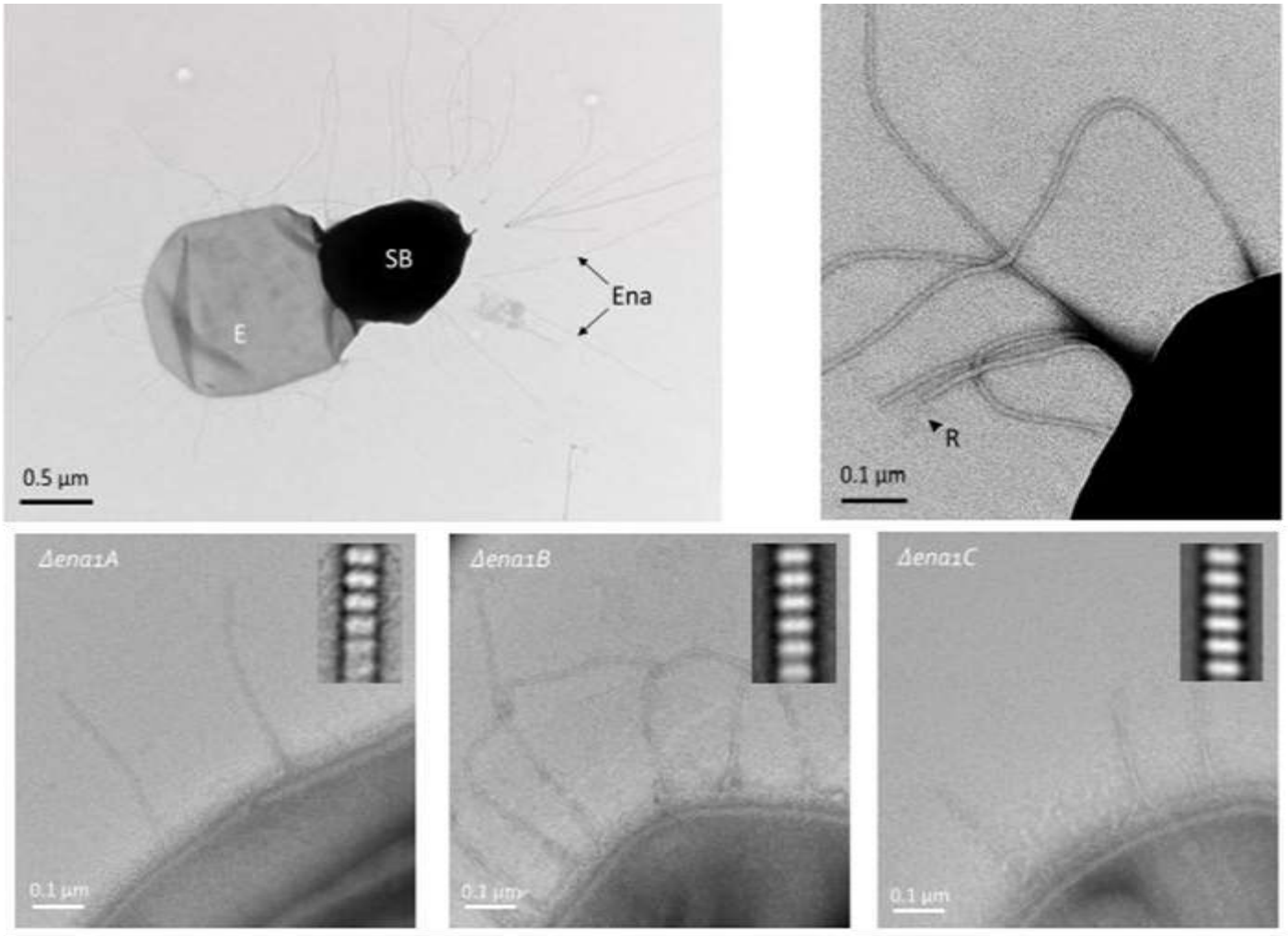


Figure 25: The *Bacillus cereus* endospore appendages (Ena) described by Pradhan et al (2021). The appendages may be seen individually or in bundles. Images from https://doi.org/10.15252/embj.2020106887.

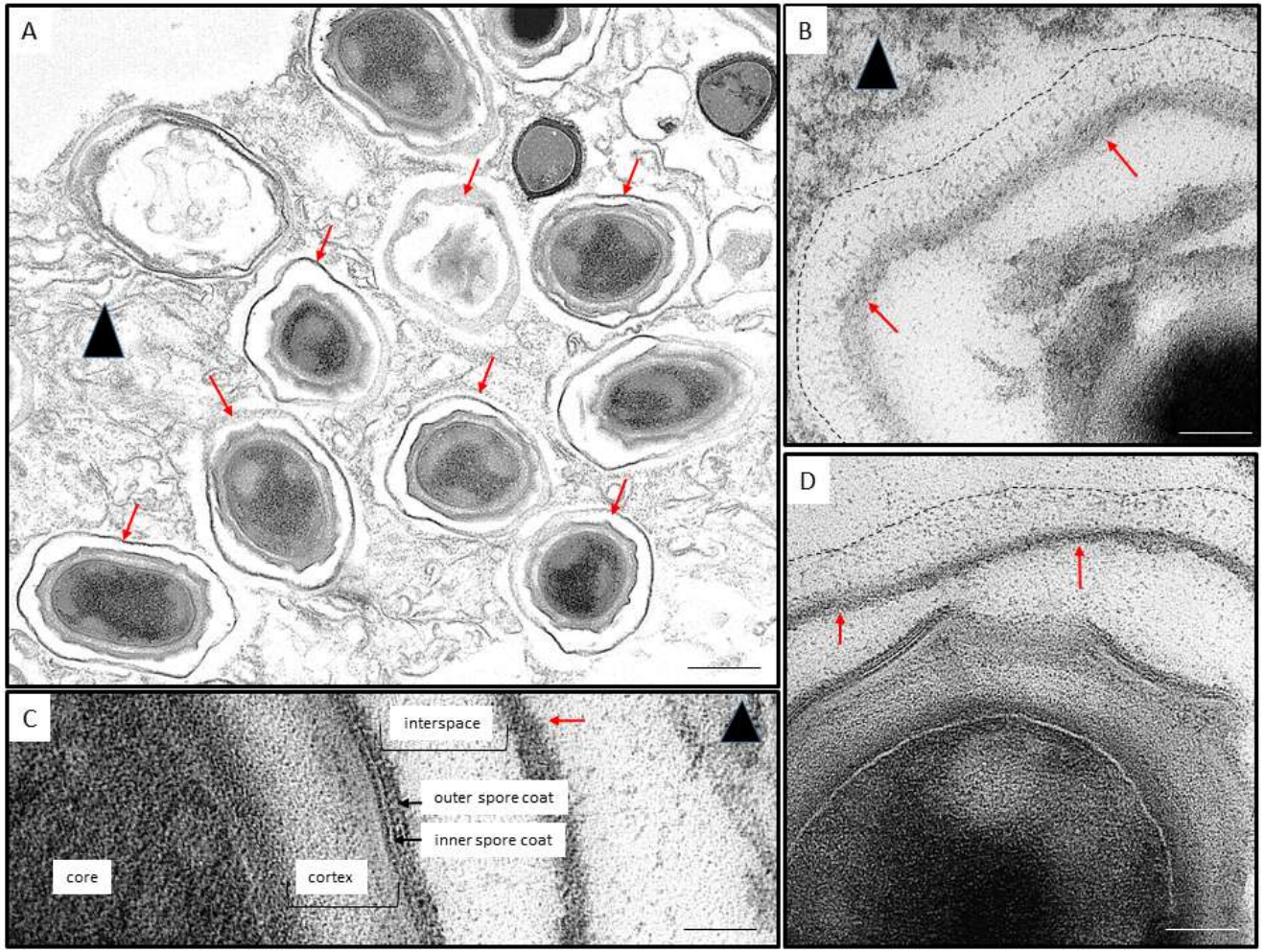


Figure 26: Unsuccessful first resin embedding run. The red arrows indicate the *Bacillus anthracis* exosporium, black triangles mark vegetative and spore debris. (A) pelleted spores randomly positioned in vegetative cell remnants; (B) dotted line demarcates the nap layer filaments along the exopsorium; (C) the crystalline S-layer of the spore outer coat. Note the complete absence of any nap filaments; (D) faint nap layer beneath the dotted line, other spore components were well preserved, but the section is speckled with a background contaminant as it is in (B) above. Scale bars: A=0,5 µm; B=60 nm; C =35 nm; D=100 nm.

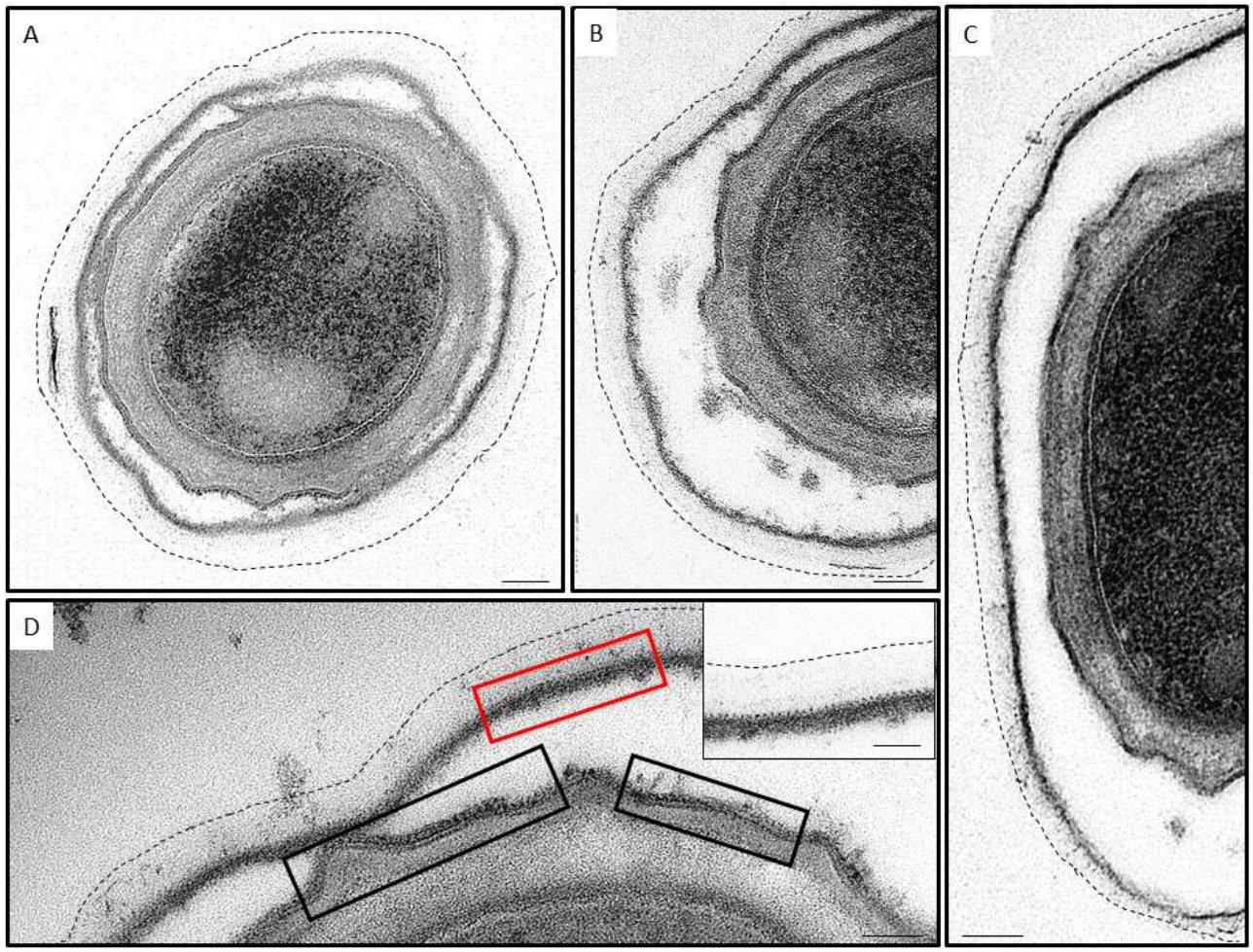


Figure 27:Second resin-embedding run with slightly more consistent *Bacillus anthracis* nap layers present (demarcated by the dotted lines), but still lacking in contrast despite the use of Ruthenium red and L-lysine acetate. In (D), note the repeating units of the spore's outer coat crystalline layer (black rectangles), as well as those of the exosporium's basal layer (red rectangle). Inset of D shows basal crystalline layer and faint nap layer in more detail. Scale bars: A, B and C=100 nm; D=50 nm, D inset = 30 nm.

5. CHAPTER FIVE: DISCUSSION

This study revealed differences in *B. anthracis* spore survival for the A- and B-subclades and the Sterne strains in soils from the anthrax high and low incidence regions of both KNP (Pafuri and Skukuza) and ENP (Okaukuejo and Otjovasandu). Our findings showed that the spore counts were becoming relatively stable by six months and that the highest decline was observed in the first four weeks after inoculation into the soil for all strains included in this study. The A-strains survived better than the B-strain, and the virulent strains survived better than the Sterne vaccine strain. The Pafuri soil showed the best spore survival for all strains, except Sterne. The second best soil type was from Otjovasandu where all the strains had high spore counts at the end of the project, including Sterne (Table 6).

The soil from Skukuza did not support the spore survival as well as the other soil types did. We observed a noticeable decline for all strains between the last sampling time point at six months and the termination of the project (~17 months) as seen in Table 6. Based on the anthrax suitability models designed for KNP, three regions in the park, namely Pafuri; Shingwedzi; and Letaba were favourable for spore survival and persistence (Steenkamp et al., 2018). Our results also showed that the Skukuza soil is not suitable for spore persistence as the spore counts continued to decline from the last sampling time point at six months until the termination of the project (Table 6). However, Pafuri has been previously described as the high incidence anthrax region (northern KNP) and is comprised of alluvial, lithosol and smectic clay soils. Skukuza (southern KNP), the low incidence region anthrax (southern KNP) is comprised of mostly weakly developed shallow and solonetzic duplex soil (Venter, 1986). The distribution of *B. anthracis* strains isolated in the park also differs over time and across KNP itself where the A-subclade isolates were found throughout the park, while the B-subclade was restricted to the north (Smith et al., 2000). Our experimental findings support this pattern, with the B-strains surviving better in the Pafuri soil (Table 6), where the strain was historically prevalent as opposed to the other three soil types.

The differences in the spore survival of the A- and B-subclade *B. anthracis* strains also speaks to the hypothesis of the adaptability of the strains to different soil types with different soil chemistries (Smith et al., 2000). The A-subclade strains appear to be more adaptable and have wider geographic distribution globally than the B-subclade strains (Sahl et al., 2016, Van Ert et al., 2007, Vergnaud et al., 2016). It has also been hypothesised that the geographic restriction of the B- and C-subclade strains may be attributed to a reduced capacity for spore survival (Smith et al., 1999, Smith et al., 2000) which we have also seen in our results with the South African B-subclade strain (SBPRL-243). This B-strain survived best in the Pafuri soil, but did not survive as well as the A-strains in the other soil types included in this study as seen in Table 6. In addition, Carerra et al (2007) also suggested that the differences in spore

size and length may be associated with the phylogenetic variation found in the polymorphic chromosomal regions of *B. anthracis* isolates (Carrera et al., 2007). This essentially means that the spore characteristics do vary across the clades and this may potentially affect the spore survival.

The Sterne strain continued to decline throughout this and showed the highest overall decline at the end of the project followed by the South African B-subclade strain (SBPRL-243) as seen in Figure 18 and 19. The Sterne strain and the South African B-subclade strain showed significant differences in spore survival over time between between Pafuri and Skukuza and between Okaukuejo and Otjovasandu. The A-subclade strains from South Africa (SBPRL-191) and Namibia (WT-2 and WT-53) showed similar survival trends across all four soil types (Figure 18) however, there was a significant difference in the spore survival of SBPRL-191 between Pafuri and Skukuza but no significant difference between Okaukuejo and Otjovasandu. The spore survival of the Namibian A-subclade strains, (WT-2 and WT-53) showed a significant difference between Pafuri and Skukuza, but no significant difference between Okaukuejo and Otjovasandu. This is summarsied well in Figure 20 which shows the interaction effect between soil type and strains. The interaction effect between soil type and strains was significant as we see all lines intersecting across all the soil types (Figure 20A). This means the effect of soil type on spore survival varies according to the strain. The interaction between the A-strains and month was significant across all the months (Figure 20B) as all three lines intersect. This shows that their decline was similar over time in comparison to the Kruger-B and Sterne strains.

The exchangeable cations (Sodium, Na; Calcium, Ca; Magnesium, Mg; Potassium, K) are also soil macronutrients and describes the amount of available cations that are able to supply nutrients to the soil and to plants; Manganese (Mn) is a soil micronutrient which sustains the metabolic roles in plants but is also an essential ion for endospore formation in *Bacillus* spp. (Charney et al., 1951); the soil organic matter (SOM) is composed of soil organisms, decomposing organic matter and stable organic matter; soil density which shows the relationship between the mass and the volume of dry soil. All these factors are hypothesised to play a role in *Bacillus* spp. spore survival and viability as they are directly affected by the soil chemistry (Carlson et al., 2018, Pepper and Gentry, 2002, West et al., 1985).

Notable differences in the soils from the low incidence anthrax regions from both KNP and ENP were observed. As per the soil chemistry results described in Table 7, Skukuza had a very similar soil profile to soils that *B. anthracis* spores are known to persist in which is alkaline, calcium rich soils with high levels of organic matter (Van Ness, 1971). Despite the high levels of Ca, SOM and Mn, the spores still had the poorest survival in the Skukuza soil. In contrast, the Otjovasandu soil showed the best spore survival after Pafuri, even though it did not have typical soil characteristics described by Van Ness (1971) which supports the survival of *B. anthracis* spores. The Okaukuejo soil had the lowest Mn and

very low SOM and despite the soil being slightly alkaline with high Ca, the spores still did not survive as well as they did in Otjovasandu. Pafuri had the typical characteristics (apart from being neutral instead of alkaline) of soils that support the survival of *B. anthracis* spores and showed the best spore survival from all other soil types.

In a study conducted by Mari (2017), it was found that the highest prevalence of *B. anthracis* spores were detected in clay loam type soils which had high levels of organic matter and Ca (Mari et al., 2017) which also agrees with Van Ness (1971), yet all strains, including Sterne, which had very low spore counts in other soils, survived very well in the Otjovasandu soil type despite the fact that its soil properties contrasts the soil properties described by the literature for optimal spore survival and persistence. The Otjovasandu soil is composed of 90,4% sandy soil, had the lowest Ca (1381 mg/kg) and soil organic matter (0,25%) levels of the studied soils, but had the highest Mn level (90,55 mg/kg). Charney (1951) showed that Mn is an essential element for endospore formation in *Bacillus* spp. and is hypothesised to sustain and maintain spores (Charney et al., 1951) which may have contributed to why the spores survived so well in this soil type. Cloete (2013) showed that *B. anthracis* spores had a better survival in sandy soils (Cloete, 2013), in addition we also oberserved that this soil had a very poor soil microbial activity with mostly *B. subtilis* being identified in the negative soil and no bacteriophage activity and this may in part be explained by the low soil organic matter content mentioned above.

Cloete (2013) also showed that the spores survived equally well in both Okaukuejo and Otjovasandu which means that both the previously described high and low incidence regions support the survival of the spores at a similar rate (Cloete, 2013). Ochai et al. (2022) found that the PA antibody titres of animals from both these areas were comparable and suggested that the whole park may be endemic for the disease (Ochai et al., 2022). The Namibian strains (WT-2 and WT-53) did not show any significant differences in spore survival in the Okaukuejo and Otjovasandu soils but they did significantly differ between Pafuri and Skukuza. These strains survived equally well in both ENP soil types which further agrees with the findings of Cloete (2013) and Ochai (2022).

This also leads us to consider the clear differences in the landscapes and ecology of the parks and the snowball effect this may have on *B. anthracis* spore survival. ENP is at the bottom of a shallow, inland basin, and as such is largely flat and consists of a large salt pan with grasslands and woodlands surrounding the pan (Beugler-Bell and Buch, 1997, Ebedes, 1977). KNP has various land elevations (Venter, 1986) and river systems (Muller and Villet, 2004), which already creates many topographical and habitat differences within KNP. Along with these differences that naturally occur, these parks also have varying levels of rainfall and differ in their wet and dry cycles (De Vos et al., 1990, Le Roux et

al., 1988, Turner et al., 2022). The combined effect of the natural characteristics of each park in addition to the temperature and humidity may affect *B. anthracis* spore survival. Our findings suggested that temperature ($p<0.0001$) and relative humidity ($p<0.0001$) are significant determinants of spore survival whereby an increase in temperature increased spore concentrations while an increase in relative humidity decreased the spore concentrations which agrees with the findings of Howie (1949) and Davies (1960). Temperature and humidity are factors that are always changing, and may differ between KNP and ENP, especially over time. Thus, anthrax predictive modelling systems are not "one size fits all".

Moreover, we also identified differences in the *Bacillus* spp. isolated from the negative soils and their bacteriophage activity as seen in Table 7, which suggests that the soil microbiome differs across all the soil types included in this study and these biotic components of soils could have an impact on spore survival. The effects that this has on the spore survival in these soil types is unknown currently, but other studies suggest that there may be high levels of competition in the soil between the natural soil microbiota and human bacterial pathogens for nutrients in addition to the production of antimicrobials and other metabolites by the pathogenic bacteria (Mendes et al., 2013). Biofilm formation is also an important factor to consider in the soil microbial community as it has been shown that *B. subtilis* is able to enhance its survivability by increasing the number of matrix-producing cells in response to the antimicrobials produced by other soil bacteria (Shank et al., 2011). In contrast, Hassim et al. (2020) isolated a lytic bacteriophage from a suspected anthrax carcass site but the isolation of *B. anthracis* from the soil proved challenging as the phage hampered the growth of *B. anthracis* using typical culture methods. This highlighted the possibility of the presence of certain bacteriophages in the environment which may interrupt the natural life cycle of *B. anthracis* in the soil (Hassim et al., 2020). Interestingly, this bacteriophage was isolated in Pafuri, the anthrax high incidence region in KNP. In comparison, Pafuri and Otjovasandu showed the best spore survival, yet their soils differed drastically, especially in terms of the microbial diversity as seen in Table 7 where Pafuri had a greater diversity of *Bacillus* spp identified and a higher bacteriophage activity compared to Otjovasandu. This highlights the importance of considering the soil microbiome and how its interactions impact the spore dynamics and the epidemiology of anthrax.

Our findings also drew attention to the poor environmental survival of the Sterne strain. We not only saw that the Sterne strain performed poorly during the main study, but also during the pilot study where huge fluctuations in temperature and rainfall was observed in Pafuri over a short period of time (Appendix 2). The conditions from day 0 to day 15 and from day 16 to day 28 during the pilot study differed considerably. Pafuri experienced high levels of rainfall and relatively low temperatures and then no rainfall at all with very high temperatures. The effect of such extreme weather conditions over

a relatively short period of time may have "shocked" the spores and the attenuated nature of the Sterne strain may make it less hardly than the virulent strains, where the Sterne spores could not respond or adapt to the changes as virulent *B. anthracis* spores would. It is known that temperature and humidity affect many aspects of sporulation such as formation and persistence (Davies, 1960, Minett, 1950). In contrast, during the main study, we did not observe this phenomenon with the Sterne strain. Instead, we observed a gradual decline in the spore counts over time rather than a rapid decline over time as we saw in the pilot study. The conditions in the tank were more stable during the period of the main study as it started during the winter months in South Africa, which has moderate temperatures and little to no rainfall. In addition, the conditions of the tank were also changed during the course of the main study to repeat to conditions of the pilot study, and we once again saw a drop in the viable spore counts of the Sterne strain as seen in Figure 18 while the virulent strains remained relatively stable during the change.

Jiranantasak et al. (2022) suggested that sporulation rates differ between wild type, laboratory designed (strains which have been repeatedly cultured and have adapted to laboratory conditions) and attenuated strains. It was found that the Sterne strain, which is attenuated as it lacks the pXO2 plasmid, sporulates faster but does not survive in the environment for as long as wild type strains do (Jiranantasak et al., 2022). It was suggested that the pXO2 plasmid, which is responsible for producing the poly-D-glutamic acid capsule, is essential for *B. anthracis* spore persistence in the environment (von Terzi et al., 2014) however, the causes as to why Sterne responds so differently to the natural environment in comparison to virulent *B. anthracis* strains is still poorly understood therefore, further investigation is required on this topic (Yang et al., 2020). In addition, it was also found that the wild-type strains appeared to be more stable in the environment when compared to the attenuated and laboratory designed strains as the wild type strains could utilise nutrients more efficiently and also sporulate faster. Our results highlighted the stability of the virulent strains in comparison to the Sterne strain as shown in Table 6 and Figure 18.

Highlights from the analysis of the transmission electron micrographs include the noticeable impact of storage conditions on the nap layer of the exosporium as it appears that the nap layer is more well-defined when isolated from soil (Figure 23) rather than when stored in sporulation agar as a response to the difference in the environments even though differences were not observed in bams13. We noticed a well-defined layer of filaments which surrounded the exosporium and forms the nap layer around the A-subclade stain (SBPRL191) and shown in Figure 23D, but it is not as abundant around the B-subclade strain (SBPRL243) as shown in Figure 23E. However, more measurements of the nap layer would be ideal to statistically confirm this finding. The identification of possible endospore appendages on *B. anthracis* spores (Figure 25) was also an interesting finding. Our preliminary results thus far, do suggest

that differences may occur with regards to the filament lengths of the nap layer according to the A- and B- subclades which also differ in size (bp) of the bams13 loci which is associated with the BclA protein (Sylvestre et al., 2003).

There is a great importance in looking for molecular or physiological differences across different *B. anthracis* strains since this a monophyletic bacterial species (Keim et al., 2000, Read et al., 2003). The differences in spore properties may prove to be a helpful tool in identifying spore adaptations or responses to different environments. From our results, we observed differences in the nap layers of the spores which were stored in sporulation agar and the spores that were isolated from the soil, whereby the spores isolated from the soil had a more well defined nap layer. Since all the strains were not analysed from the different soil types, it would be interesting to see if there are differences in the exosporium as a response to the different soil types. Futhermore, the molecular and physiological differences can be used to easily distinguish and differentiate between strains. The variation found in the collagen-like repeats of the BclA protein as by Sylvestre (2003) where the differences observed in the bams13 loci can be seen phenotypically in the variation of the filament length of the nap layer of the exosporium as suggested by our preliminary results. Morever, the *Bcl* genes also prove to be a useful aid for *B. anthracis* fingerprinting and identification as described by Leski (2009). The spore coat itself may also differ between strains and this is also another phenotypic difference that can be used to differentiate between strains (Mallozzi et al., 2008).

Conclusion and recommendations:

These results highlight the importance of considering the variation in the soil characteristics which support spore survival, for example, in the case of Otjovasandu and how some soils may seem like spore survival could be well supported but is not as can be seen in the Skukuza soil. It is also important to consider that these variations may stem from more than just the soil chemistry, but also from soil textures which affects its ability to hold water and other nutrients along with natural microbiome of the soil, the vegetation type and animal selection for these habitats. Furthermore, the adaptability of the strains from the different subclades also needs to be considered as there are notable differences with regards to survivability and persistence as described in this study and across the literature. The A- strains appear to be more adaptable and showed a better survival across all soil types when compared to the B-subclade strain and the Sterne strain. We also noted that the filament lengths in the nap layer of the Namibian A-subclade strains are longer than both the South African A- and B-subclade strains, however it has not been determined what effect this finding has on spore survival, therefore, more work is required on this aspect of the study.

The complete effects of the soil chemistry, texture and the soil microbiome on *B. anthracis* spore survival are still unknown in its entirety but these results provide a clearer picture of the soil chemistry in the high- and low-incidence anthrax regions of KNP and ENP. This study also indicates the importance of considering climate in conjunction with the soil characteristics and spore properties when talking about anthrax. Our findings also suggested that temperature and humidity may significantly affect spore survival. These are factors which are directly affected by our ever-changing climate, and we need to improve our predictive modelling systems for anthrax outbreaks to consider and account for these biotic as well as abiotic factors, and to consider soil types that do not fit the typical expectation for supporting anthrax endemicity.

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Appendix 1: Risk assessment

This risk assessment was conducted to highlight the recommended safety procedures for working with virulent *Bacillus anthracis* strains in addition to safely working in the terra-simulator and the mitigation strategies thereof in the BSL-3 facility at the Special Bacterial Pathogens Reference Laboratory (SBPRL) at the National Institute for Communicable Diseases (NICD). All standard operating procedures mentioned below are the intellectual property of the SBPRL and the National Health Laboratory Service (NHLS).

Bacillus anthracis risk assessment

Risk	Existing controls	Likelihood	Consequence	Mitigation strategy
Exposure to *B. anthracis* spores or inhalation of aerosols containing spores	All required PPE as per POLS0004 will be followed: Boot covers, folio gown, N95 mask, safety goggles and double gloves. Working in a class two biosafety cabinet.	Moderate	High	Check PPE thoroughly before donning to ensure that it is not compromised. Discard contaminated PPE as working. Specimens are only manipulated in the biosafety cabinets.
Failure in any of the engineering controls systems may increase the chances of exposure to spores.	Maintenance is conducted on all systems a regular basis. Biosafety class two cabinet Inward air flow and controlled ventilation system. All work should be suspended if a failure in the air pressure system has been detected. Double glaze windows, seals on doors and windows	Low	High	Smoke tests are done weekly, and the air pressure is checked daily on the biosafety cabinets. HEPA filtration of exhausted laboratory air. Any system not functioning optimally will be repaired or replaced. The seals and double-glazed windows provide an additional barrier to the external environment
Contamination of surroundings with aerosolised *B. anthracis* spores	All surfaces and equipment will be cleaned with 10% F10 before and after working with a contact time of 30 minutes and wiped with 70% ethanol thereafter. Wipe any plates or containers with 10% F10 before removing it from the biosafety cabinet.	Moderate	High	1% bleach may be used instead of 10% F10. Always remove contaminated PPE and don fresh PPE. Chemical and Biological spill kits are available for major spills in the lab. The lab manager and health and safety representative need to be informed. Prophylactic treatment may be necessary depending on the severity of the situation.

	Remove outer pair of gloves inside biosafety cabinet. Major spills (chemical or biological) need to be reported. Ventilation system and inward air flow.			Exhausted air is not recirculated and is HEPA filtered.
Contamination of water system with *B. anthracis* spores	All water waste from the BSL-3 is stored in an effluent waste tank.	Low	High	Once the tank is full, it is decontaminated overnight with bleach and then released into the municipal drainage system.
Incorrect disposal of waste that contains *B. anthracis* spores	All culture plates are to be sprayed with 10% F10, taped and double bagged before being discarded into biohazard boxes. All waste generated in the BSL-3 is autoclaved out. It is then sealed in biohazard boxes and sent for incineration.	Moderate	High	Plates are taped to ensure that its contents are contained and then double bagged to prevent any leakages. A biological indicator is included in every autoclave run for the waste to ensure that all spores have been destroyed.

Terra-simulator risk assessment

All safety measures for working with *B. anthracis* should also adhered to when working in the terra-simulator.

Safety measure	Potential hazard & implication?	Existing controls	Likelihood	Consequence	Mitigation strategy
Access control to the BSL-3 and the terra-simulator system	The terra-simulator will contain virulent *B. anthracis* spores.	Only authorised personnel are allowed into the lab.	Low	High	The terra-simulator also has combination locks securing the lid for additional safety. Codes of the lock will only be available to researchers already authorised to work on this project.
Design of the terra-simulator	Aerosolisation of *B. anthracis* spores during sample collection or spillages of soil	The terra-simulator was made from double-glazed (vacuum in between the glass) E glass (for the sides) and shatterproof plexiglass (for the front and base) as well as protected silicone corners for added structural integrity. All materials in the terra-simulator are also non-porous which ensures that it will be sufficiently sterilised before incineration. The soil samples will be placed in re-sealable, plastic containers which ensures that the *B. anthracis* spores are self-contained.	Low	High	If aerosolisation of spores or spillages of the soil samples do occur, the terra-simulator will be decontaminated with F10 foggers and wiped with 1% bleach solution since everything in the system can be sealed and will be self-contained. This is advantageous as it does not expose the entire BSL-3 to *B. anthracis* spores. The entire system will be swabbed and cultured to check for the growth of *B. anthracis*. Should growth still occur, the entire system will then be decontaminated with 10% formalin or peracetic acid.

Collecting samples from the terra-simulator	Exposure to *B. anthracis* spores	When collecting samples from the terra-simulator, all plastic containers will be sealed. A total of 1 g of each sample will be collected using a sterile spoon and transferred to sterile falcon tubes. There are membranous seals on both glove access panels, long gloves will be used and disposed after each use and hands are disinfected with 10% F10.	Moderate	High	Soil samples collected from the terra-simulator will only be manipulated in a Biosafety Class 2 cabinet. All further processing of the samples will follow the workflow outlined in Appendix 6 of NIC0881. All waste generated will follow the disposal methods highlighted in NIC0881.
Disposal of the soil samples when the study is completed.	Incorrect handling of the inoculated soil samples and insufficient contact time for decontamination before being sterilised	Concentrated F10 solution was used to cover the soils and allow for a sufficient contact time to decontaminate the soils before being autoclaved out of the BSL-3. This includes the soil and light sensors as well.	Moderate	High	The decontaminated soil samples will be cultured onto blood agar plates to check for the growth of *B. anthracis.* If confirmed negative, all samples will be double bagged in autoclave bags and sealed in preparation for being autoclaved out of the BSL-3 and following the subsequent procedures outlined in NIC0881. Should the soil samples still be positive upon culturing on blood agar, peracetic acid will be used to disinfect the soils.
Removal of the terra-simulator from the BSL-3 upon completion of the study	Proper decontamination and sterilisation of the system before removing it from the BSL-3	After the lab has been fully decontaminated with F10 foggers, all removable components will be wiped with 10% formalin. It will also be wiped with 10% F10 solution and 70% ethanol to ensure that no residuals remain. The system will also be swabbed and cultured again to ensure that there is no growth before being removed.	Low	High	Should the cultures be positive, peracetic acid will be used and also wiped with 10% F10 solution and 70% ethanol to ensure that no residuals remain.

Overall risk

The purpose of the terra-simulator was to ensure that the spores were contained within the system and to limit its exposure to the laboratory environment. The use of the terra-simulator does not eliminate all the risks associated with working on *B. anthracis* but it was designed to provide an efficient containment pod during the incubation period in addition to regulating the inside environment for the spores and soil. Due to the nature of this bacterium and the subsequent tests that will be conducted, all the risks mentioned above still need to be considered and all safety measures should be adhered to. In conclusion, the overall risk for working with *B. anthracis* when following the above-mentioned safety measures has a moderate likelihood with high consequences.

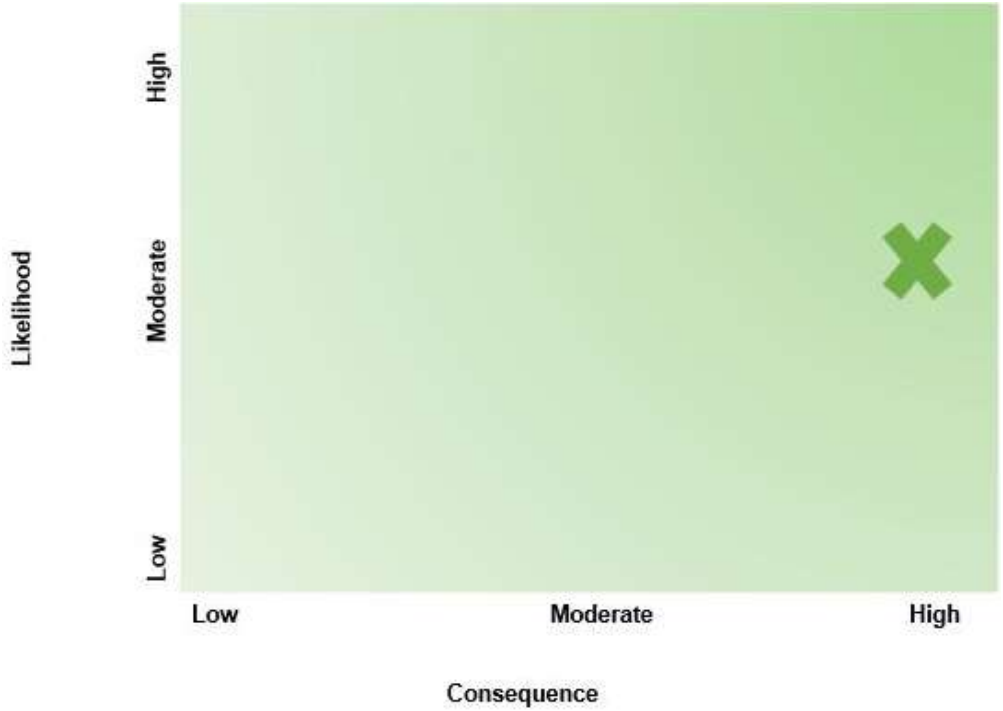

Figure 28:Overall risk of working with *Bacillus anthracis*. The likelihood of exposure is moderate but with high consequences should an exposure occur.

Appendix 2: Conditions in the Terra-simulator during the pilot study

The terra-simulator was programmed to mimic the weather conditions in Pafuri and Figure 29 depicts the temperature and humidity fluctuations over 28 days. When high temperatures are experienced, a lower relative humidity occurs which results in the air becoming drier. The humidity levels were managed by adding PBS to the soils to mimic rainfall on days when it did occur, and PBS was placed in a container to stimulate humidity in the entire terra-simulator.

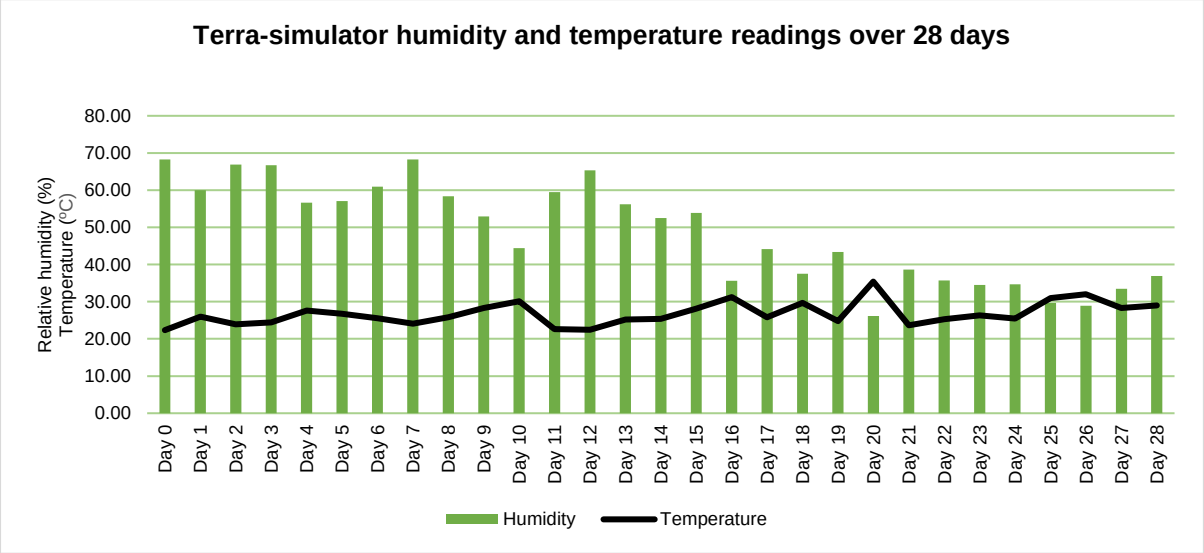

Figure 29: Average humidity (green bars) and temperature (black line) during October 2020 in Pafuri.

This experiment took place in October 2020 which is typically the beginning of the summer months where high temperatures and high levels of precipitation are observed. The first 15 days of this experiment had relative humidity's between 50% – 70% and the temperatures ranged from 20 ºC to 25ºC, with the exception of day 10. It was the beginning of the rainy season and almost 13mm of rainfall was recorded (https://www.sanparks.org/parks/kruger/tourism/climate.php) for this period. In addition, higher temperatures are usually observed during these heavy rains, however, the temperatures were considerably lower than past years which also resulted in a higher relative humidity and more water particles being present in the air. After day 15, the temperatures increased, and the humidity levels also decreased by a significant amount. No rainfall occurred for the rest of the month, so the conditions became very dry and humid in Pafuri.