ECOLOGICAL DYNAMICS OF *BACILLUS ANTHRACIS* IN WATER AND SOILS AT SELECTED SITES IN ETOSHA NATIONAL PARK, NAMIBIA

A THESIS SUBMITTED IN FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

OF

THE UNIVERSITY OF NAMIBIA

BY

Modesta Evard

201025698

SEPTEMBER 2021

Main supervisor: Prof. J. K. Mfune (University of Namibia)

Co-supervisors: Dr. Kyrre Kausrud (University of Oslo)

Ms Claudine Cloete (Etosha Ecological Institute; Ministry of Environment, Forestry and Tourism)

ABSTRACT

Bacillus anthracis, is an endospore-forming, soil borne bacterium, also found in water and vegetation, which causes the disease anthrax. The species most affected by anthrax in Etosha National Park are the plains zebra (*Equus quagga*), blue wildebeest (*Connochaetes taurinus*), springbok (*Antidorcas marsupialis*) and African elephant (*Loxodonta africana)* Anthrax occurs seasonally with a peak incidence during the late wet season in the plains zebra (*Equus quagga*), blue wildebeest (*Connochaetes taurinus*), springbok (*Antidorcas marsupialis*), and during the late dry and early wet seasons in the African elephant (*Loxodonta africana*). Anthrax is generally assumed to be transmitted through grazing.

An experimental study was conducted in the laboratory at the Etosha Ecological Institute to investigate the behaviour of an avirulent *B. anthracis* strain. Jars filled with water and soil were inoculated with blood containing avirulent anthrax spores and subjected to repeated settling and disturbance. One jar with no soil but only water served as a control. The aim of the experiment was to determine whether most of the anthrax spores remained in the water or attached to the soil particles. The study further investigated how *B. anthracis* behaves in the soil through an outdoor enclosure experiment in which thimbles were filled with three soil types, namely, ferralic arenosol, calcaric regosol and lithic leptosol. Soils were inoculated with blood containing virulent anthrax spores and water. The aim of the experiment was to determine deep transport of *B. anthracis* in the three soils. Sampling was done over four weeks, with the thimbles being tested for the presence of *B. anthracis* every centimeter (1-12cm). In both experiments, a selective media polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar was used to isolate *B. anthracis* from the water and soil samples in this study.

This study revealed that there was a significant difference in the colony counts of *B. anthracis* sampled from the different layers in the water jar ($\chi^2 = 54.244$, df =4, $p < 0.05$). Most of the anthrax spores were attached to the finer sediments of the soil and not in the suspension of the disturbed samples as hypothesized. Fewer *B. anthracis* spores were found at the surface of the water, which suggests that animals are unlikely to ingest a lethal dose of *B. anthracis* to cause an infection which may in turn lead to death. Furthermore, the outdoor experiment revealed that significantly higher numbers of *B. anthracis* spores were transported further down in the sandy ferralic arenosol throughout the study period compared to the other two soils. It is concluded that the likelihood of animals contracting anthrax from sandy soil is low.

Key words: *Bacillus anthracis,* anthrax, Etosha National Park, spore transport, water, soil type

TABLE OF CONTENTS

ABBREVIATIONS

ANOVA – Analysis of Variance

CFU - Colony forming unit

CM - Centimeters

EDTA - Ethylenediaminetetra-acetic acid

EEI - Etosha Ecological Institute

ENP - Etosha National Park

HRS - Hours

KNP - Kruger National Park

MINS - Minutes

PCR - Polymerase Chain Reaction

PLET - Polymyxin-lysozyme-EDTA-thallous acetate

PVC - Polvinyl chloride

rcf - relative centrifugal force

SEM - Standard error of the mean

SPSS - Statistical Package for the Social Sciences

USA - United States of America

WHO - World Health Organisation

LIST OF FIGURES

LIST OF APPENDICES

ACKNOWLEDGMENTS

First and foremost, I would like to thank God for being with me on this academic journey. It was a rough road but he was by my side from the beginning until the end. I would not have completed this study without the support and input of other people and institutions. With this said, I express my deep and sincere gratitude to my research supervisors, for giving me the opportunity to experience the world of anthrax with them. Professor John Mfune, thank you for your constant support, the motivation, for believing in me and for helping me to believe in myself during my studies. Dr. Kyrre Kausrud thank you for granting me the opportunity to be your student and for introducing me to the world of R and immense knowledge. Many, many thanks to Ms. Claudine Cloete for providing invaluable guidance throughout this research. Thank you for your patience and continuous support. I am also very grateful for your help when the anthrax spores were being so stubborn in the lab, for the late evenings in the lab and for the mini walks, just to make sure this research was not driving me crazy.

I greatly thank the Ministry of Environment and Tourism for granting me permission to conduct this study in Etosha National Park (ENP). I am also grateful for the assistance of the staff of the Etosha Ecological Institute in the field and for making me feel part of their team. I give thanks to the University of Namibia for assisting me with laboratory supplies just to make sure this project fully worked out.

My gratitude goes to Zoe Barandongo, Maggie Angula and Claudine Cloete for their help in the laboratory and out in the field under the scorching heat. I deeply appreciate you all, your time and help. I also thank the input of Prof Hipondoka for constant support and for helping me with GIS for the development of the maps in this thesis. I also thank Namene Nashandi for helping me with blood collection. My gratitude also goes out to Excel Dynamic Solutions for helping with data analysis, I really appreciate your help.

Finally, this research would not have been a success without my family and friends. My sincere gratitude goes out to my mother, Mrs Asteria Evard the woman who was and is still my mentor and coach. You went above and beyond to make sure this research was a success, always shared words of wisdom with me and, prayed for me and helped me reach my full potential. Many thanks also go out to my sister Angela and friend Erastus Ithana who always believed in me and supported me, you are highly appreciated. To all my friends, thank you for running this race with me and for lending a shoulder to cry on.

This research was funded by the University of Oslo, under the Climate Change and Zoonotic Epidemiology (ZEWS) project and my beloved mother Mrs Asteria Evard.

DECLARATION

I, Modesta L.N Evard, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

No part of this thesis may be reproduced, stored in any retrieval system, or transmitted in any form, or by any means (e.g. electronic, mechanical, photocopying, recording or otherwise) without the prior permission of the author, or The University of Namibia in that behalf.

I, Modesta L.N Evard, grant The University of Namibia the right to reproduce this thesis in whole or in part, in any manner or format, which The University of Namibia may deem fit, for any person or institution requiring it for study and research; providing that The University of Namibia shall waive this right if the whole thesis has been or is being published in a manner satisfactory to the University.

……………………… Date ……………………………

Modesta L.N Evard

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Anthrax, is a bacterial disease of mammals and birds (Bellan et al., 2013), which is caused by the endospore –forming bacterium, *Bacillus anthracis*, which is Gram positive and rod-shaped (World Health Organisation, 2008; Hellstrom, 2013). The bacterium is soil-borne and can persist in the environment for many years (Mullins et al., 2013). Anthrax occurs world-wide, with it being enzootic in most countries of Africa and Asia, and several European, North and South American states (WHO, 2008). The disease affects both animals and humans and is initiated by the invasion of endospores into the host body through skin lesions, ingestion of contaminated food or inhalation of contaminated air (Vilas-Boas et al., 2007; Hudson et al., 2008). Anthrax is a major cause of mortality amongst livestock and wildlife populations (Bellan et al., 2013). It is believed that grazing is the dominant transmission route of the disease (WHO, 2008; Hellstrom, 2013). Humans may contract anthrax from animals or animal products (WHO, 2008), either through contact with anthrax-infected animals or through handling of animal carcasses (Hellstrom, 2013). The incidence of the disease in humans is highest in Africa and in central and southern Asia (WHO, 2008). Possible reasons for this include the disease being poorly controlled and due to public ignorance of the disease (WHO, 2008; Hudson et al; 2008). The continuous sale and slaughter of affected livestock as a result of poor veterinary supervision could also lead to high anthrax incidences in humans in these parts of the world (WHO, 2008).

Anthrax is a neglected zoonotic disease, and although it occurs globally (Beyer et al., 2012; Hudson et al., 2008; WHO, 2008), more research still needs to done on it’s ecology. It continues to be a serious zoonosis in the developing countries and is enzootic in many national parks including the Etosha and Kruger National Parks (Hudson et al., 2008). Various environmental factors may also largely play a role in the incidence of anthrax, which may account for the observed differences in the incidence of human and animal anthrax cases in different parts of the world. Soil pH of the is one of the environmental parameters known to play a big role in the ecology of anthrax, with the alkaline (pH 9) being the most suitable pH for the survival of *B. anthracis* for longer periods (WHO, 2008). Soils in the western countries are characterized by a low soil pH which may have contributed to the low survival of the anthrax bacterium in soil which in turn leads to the absence of or lessens the disease in animals, resulting in low human anthrax cases in these areas (WHO, 2008). A study by Cloete (2013), revealed that soil types play significant roles on the persistence of *B. anthracis*, with sandy soils likely to have a greater effect on the persistence of the bacterium compared to the other study soil types. This could be attributed to distinct characteristics that the different soils possess, where the sandy soils had low soil moisture suitable for anthrax to thrive better. Another critical factor also known to affect the dynamics of an infectious disease such as anthrax is the variation in time and space in which it is transmitted (Turner et al., 2016). The disease shows seasonality in its occurrence in enzootic areas, with epidemics being observed mostly after longer periods of hot dry weather following some heavy rains (Hellstrom, 2013). These rains are believed to concentrate spores in low-lying areas where runoff collects (Dey et al., 2012; Fasanella et al., 2009). In the case of Etosha National Park in Namibia, Ebedes (1976), argues that gravel pits are the main reservoirs of anthrax. The gravel pits occur in most of the anthrax enzootic areas in Etosha National Park (ENP). These pits are low-lying areas that retain water for long periods, making it a focus for animal concentration. Ebedes also argued that these pits provided favorable conditions for the multiplication of *B. anthracis*.

The Adamax gravel pit in central ENP is a good example of a water source that retains water for about two months, where *B. anthracis* was found in the gravel pit and might have derived from carcasses of animals that died close to the water (Lindique and Turnbull, 1994). Animals drinking from this gravel pit may be infected, since the anthrax spores have a high buoyancy density and may contaminate water (Hellstrom, 2013). Another study by Turner et al., (2016) showed that seasonal water sources such as gravel pits hold significantly higher concentrations of *B. anthracis* in sediments than perennial sources. The study also found the concentrations of *B. anthracis* in gravel pits to be so low they were unlikely to be a major source of infection. Despite all these studies, not much is known about the ecology and behaviour of *B. anthracis* in environmental reservoirs such as water and soil to understand how it is able to contribute to completing the infectious cycle. Hence, the aim of this study was to investigate how the anthrax bacterium behaves in water and different soil types from ENP, in terms of deep transport as it may also play a big role on its persistence, as a determining factor of the transmission of anthrax. According to (WHO, 2008) most anthrax contaminated carcass sites in ENP are characterized by very dry dusty soils, in which anthrax spores still persist for many years despite strong seasonal winds that are proven to move some of the spores.

The dominant anthrax hosts in Etosha are the plains zebra (*Equus quagga*), accounting for >50% of recorded mortalities, followed by the blue wildebeest (*Connochaetes taurinus*), springbok (*Antidorcas marsupialis*) and African elephant (*Loxodonta africana*) (Turner et al., 2013). Anthrax cases in zebras, springboks and wildebeest peak during the late wet season (March and April), while in elephants it peaks in the late dry season or early wet season (October) (Beyer et al., 2012). This difference in the time when these species are affected by anthrax could also be attributed to exposure to the disease as a result of different behavioral and ecological traits and possibly host-specific differences in susceptibility (Hampson et al., 2011).

1.2 Statement of the problem

Anthrax outbreaks have been a cause for concern in Namibia for many years, affecting livestock, wildlife and humans (WHO, 2008). Very little is known about how *B. anthracis* endospores behave in the environment, despite such knowledge being crucial to understanding the transmission capabilities of anthrax. The routes of anthrax transmission are much debated, and the importance of the many possible transmission mechanisms is still poorly understood. Several studies have been carried out in ENP to find possible routes of anthrax transmission. A recent study suggests that grazing rather than drinking at waterholes is the dominant transmission route of *B. anthracis* in Northern Namibia (Turner et al., 2016), yet a possible role of water-borne anthrax cannot be ruled out. A study by Havarua et al. (2014), has shown that zebras have a higher bite rate and prefer short grasses in the wet season which could increase their chances of ingesting lethal doses of *B. anthracis* spores while foraging. Furthermore, Barandongo et al. (2018) showed that the dust bathing behaviour of zebras, wildebeest and elephants in ENP is less likely to cause anthrax by means of inhaling *B. anthracis* spores. *Bacillus anthracis* spores persist in the soil for years (Turner et al., 2016). A study by Cloete (2013) revealed that soil type has a great influence on the persistence and survival of *B. anthracis*. The sandy soils had the highest *B. anthracis* spore counts compared to the karstveld soils of central ENP, rendering them more conducive for anthrax spore persistence (Cloete, 2013). Although this study also revealed that the site in terms of soil moisture or rainfall did not have a significant influence on the persistence of *B. anthracis,* little is known regarding how the processes of water transportation may affect the distribution or movement of spores in the soil. These unknown mechanisms may be the root cause of anthrax outbreaks and how the disease responds to climate fluctuations. Hence, the purpose of the present study was to investigate the transport of anthrax spores in water and soil in order to contribute to knowledge regarding their distribution and transmission.

1.3 Objectives

The objectives of the study were:

a) To determine the influence of organic matter and soil moisture on the transport of *B. anthracis* among three soil types in ENP.

b) To determine how *B. anthracis* spores interact with water, soil particles and capillary forces in a natural soil environment.

c) To determine how *B. anthracis* spores interact with water and soil particles in a water environment, such as waterholes, that have been contaminated by anthrax and are regularly disturbed by animals.

1.4 Hypotheses

a) Significantly high amounts of *B. anthracis* spores will be leached deeper in the soil with low organic matter. Less organic matter would increase pore spaces of soil and allow more water to seep through the soil transporting more *B. anthracis* further down compared to those with more organic matter. The increase in pore spaces due to low organic matter may also increase the evaporation of water from the soil causing low soil moisture and affect the transport of *B. anthracis* in soil.

b) Significantly high amounts of *B. anthracis* spores remain at the surface of the soil rather than leaching deep into the soil. Transportation of *B. anthracis* spores deep into the soil would reduce the likelihood of spores becoming available to any passing host. Thus, spore behavior that limits deep transport would strengthen the hypothesis that the soil reservoir is important for *B. anthracis* transmission.

c) Significantly high amounts of *B. anthracis* spores are suspended in the turbid disturbed water sediment mixture and not in the underlying soil sediment. Significantly high amounts of *B. anthracis* spores in the water-sediment mixture makes them more readily available for drinking animals, thus strengthening the hypothesis that disturbed sediment-water mixture can be important for *B. anthracis* transmission.

1.5 Significance of the study

The mechanisms of how, where and when mammalian hosts contract *B. anthracis* in the environment has been poorly studied (Turner et al., 2014). Standing waters can be considered potential sites for the transmission of *B. anthracis* but no studies have been conducted to investigate the behaviour of the *B. anthracis* endospores in a water environment. This is important because it could determine the probability of their uptake by animals and in what amounts to cause anthrax. This study broadens understanding of the disease transmission and generates knowledge that will be important for the management of anthrax outbreaks. Knowledge on the transmission routes of the disease also helps in predicting the risk of infection which is important for wildlife conservation, livestock economies and public health (Hampson et al., 2011).

CHAPTER 2: LITERATURE REVIEW

2. Introduction

Anthrax is a disease of concern and has a worldwide distribution (Beyer et al., 2012; Hudson et al., 2008; WHO, 2008). The disease affects both animals and humans. The anthrax causative agent, *B. anthracis*, has long been known to form spores which can retain their virulence for many years in different media such as soil, water, vegetation and animal hides (Ebedes, 1976). Animals may contract anthrax from the soil while grazing, while in humans it occurs when they have contact with infected animals or exposure to contaminated animal products (WHO, 2008).

This literature review focuses on the history of anthrax, the bacterium *Bacillus anthracis*, the ecology of anthrax, possible routes of anthrax transmission, anthrax in Namibia and anthrax in humans and animals.

2.1 History of Anthrax

Anthrax is an ancient disease documented since biblical times, where it may have featured as the fifth and sixth plagues ravaging the livestock in Egypt (Hellstrom, 2013; Hudson et al., 2008; Fasanella et al., 2009). Anthrax occurs worldwide and is enzootic in most African and Asian countries, parts of Europe, the Americas, Australia and is believed to have originated in Sub-Saharan Africa (Saile, & Koehler, 2006). The disease is transmitted in various ways, with Louis Pasteur identifying carcass sites as key hotspots for anthrax transmission in the late 19th century (Ganz et al., 2014), and continues to be one of the major causes of mortality in livestock through recorded history (Hudson et al., 2008; Hugh-Jones & de Vos, 2002). Despite efforts put in place to reduce anthrax mortalities, anthrax continued to be one of the infectious diseases with major mortalities among domestic and wild animals between the late 19th and 20th century (Fasanella et al., 2009; WHO, 2008). The disease also caused deaths in humans throughout the 19th and 20th centuries via both, intentional and accidental means (Dragon & Rennie, 1995).

Although the *B. anthracis* pathogen is known for having played a crucial role in establishing modern medical microbiology, it was also used for unethical purposes such as terror attacks and bioweapons by different entities (Husdon et al., 2008). The microbe caused mortalities in humans, where for example, the British Army carried out the testing of anthrax bioweapons on an island in Scotland, while in a separate incident a Japanese Army unit experimentally infected prisoners with *B. anthracis* between 1932 and 1945 (Hudson et al., 2008). In 1979, in Sverdlovsk in the former Soviet Union (Russia), an accidental release of spores from a bioweapons research and production centre occurred resulting in the death of sixty-six people (Hudson et al., 2008; Fasanella et al.,2009).

Anthrax became a matter of global public interest after bioterrorist attacks in 2001in the United States of America (U.S.A) (Fasanella et al., 2009). The 2001 anthrax attacks were carried out in the form of letters which were distributed, containing very finely dispersed anthrax spores (Hudson et al., 2008). These attacks led to twenty- two confirmed anthrax cases of which five people died.

Anthrax is caused by the bacterium *Bacillus anthracis* and was named "anthracis" from the Greek word "*anthrax* (ἄνθραξ) meaning "coal" by Cohn in 1872 (de Vos & Turnbull, 2004; Stableforth & Galloway, 1959). It is the first known disease of humans and animals to be shown to be caused by a specific micro-organism (de Vos & Turnbull, 2004). In the year 1876, Robert Koch carried out intensive studies on anthrax and conclusively described the etiology of the disease (Hudson et al., 2008). He continued to do more work on rod-like microorganisms and was the first to demonstrate that anthrax spores developed under unfavorable conditions, and that the spores transformed into vegetative rod-like bacilli when exposed to nutrient-rich conditions (Hudson et al., 2008). Koch also discovered that the spores could survive for long periods of time in the soil after an anthrax outbreak (Hudson et al., 2008). The anthrax bacterium was only identified in the late 19th century by Robert Koch (Fasanella et al., 2009), who also linked the spore stage of anthrax with its ability to persist in the environment (Dipti, 2013). Anthrax was the cause of many mortalities in sheep, cattle and goats and humans until an effective vaccine was produced midway through the 20th century (Bengins & Frean, 2014).

In 1881 Pasteur introduced a new vaccine to help in reducing the number of anthrax mortalities in animals (Stableforth & Galloway, 1959; Hudson et al., 2008). It was one of the first vaccines to be developed. Prior to the introduction of the anthrax vaccine, Pasteur performed a public anthrax vaccine trial experiment on a group of sheep (Hudson et al., 2008), where half of the sheep were vaccinated with a culture of *B. anthracis* and the rest not. The sheep were later inoculated with virulent anthrax bacilli, where all the vaccinated sheep were alive and the unvaccinated sheep died. This experiment led to the introduction of the Pasteur's anthrax vaccine which was very effective and was used worldwide (Hudson et al., 2008). In the 1930s, the Sterne's vaccine was developed by Max Sterne after recalling an anthrax outbreak in which an estimated 30000-60000 animals died in South Africa in 1923 (Hudson et al., 2008). An estimated worldwide incidence of natural anthrax was put between 20000 to 100000 cases in 1958 by Glassman (Bhatnagar & Batra, 2001). The introduction of the Sterne vaccine drastically reduced the incidence of anthrax in most countries (Hudson et al., 2008), but its environmental persistence and high mortality causes it to remain a zoonotic pathogen of great concern for animal husbandry and biosecurity.

2.2 The bacterium *Bacillus anthracis*

Bacillus anthracis is a soil-borne, spore forming bacterium and the causative agent of anthrax in wildlife, livestock and humans worldwide (Saile & Koehler 2006; Mullins et al., 2013; Blackburn et al., 2014). It is zoonotic and is the only obligate pathogen which belongs to the family Bacillaceae (Fasanella et al., 2009: Dipti, 2013; Hellstrom, 2013. The bacterium is rod-shaped, Gram positive and about 1 to 9 micrometres in length (Dipti, 2013). It is facultatively anaerobic (Koehler, 2009), and forms spores which persist for years in the environment (Lindeque & Turnbull, 1994; Turner et al., 2016). The spores are highly resistant to a variety of environmental conditions such as heat, cold, ultraviolet and ionizing radiation and chemical agents (Vilas-Boas et al., 2007; Hellstrom, 2013). They are also known to have a high capacity of floating due to their low water content, which makes water an important aspect in the ecology of the bacterium (Fasanella et al., 2009; Hellstrom, 2013).

Inside a host, it is believed that the anthrax spores are transported by macrophages from the initial site of inoculation to the lymph nodes (Hudson et al., 2008). The spores then develop into vegetative bacilli which produce virulence factors: toxins and capsules, resulting in the infection extending to the successive nodes and entering the bloodstream where they multiply rapidly (Hudson et al., 2008). *B. anthracis's* unique capsule is considered to be the major contributor to its virulence (Misgie et al., 2015). The capsule enhances the pathogen's ability to evade host defenses (Misgie et al., 2015). The bacterium also has virulent strains which carry two large plasmids: pX01 and pX02 which contain the genes responsible for encoding the primary virulence factors: toxins and capsules (Hudson et al., 2008). Bacilli which are shed when an animal dies sporulates when in contact with air and are passed onto the next host (Hudson et al., 2008). Each bacterial cell produces one endospore, hence the spore of the bacterium is a dormant form of the vegetative bacteria (Hellstrom, 2013). In addition, the spores of the pathogen have an exosporium crucial for hydrophobicity. The exosporium aids in the adherence of spores to various surfaces and also protects the inside of the spore (Williams et al., 2012; Hudson et al., 2008; Hellstrom, 2013). This outer layer of the spore further helps the spore to communicate with its surroundings (Williams et al., 2012). This outer part of the bacterium plays a crucial role in soil attachment, which may contribute to its high levels of persistence and accumulation at restricted sites (Williams et al., 2012). The surface of the spores also has a paracrystalline basal layer and a hair-like outer layer, composed of proteins, carbohydrates and lipids (Hudson et al., 2008). *Bacillus anthracis* thrives well in ordinary medium, at temperatures between 12 ºC and 44 ºC, although optimal growth of the bacterium occurs around 37 ºC (Fasanella et al., 2009). The pathogen forms off-white colonies with irregular edges and a "round glass" appearance when incubated in conditions that are not suitable for capsule formation (Koehler, 2009; Hudson et al., 2008; Fasanella et al., 2009).

2.3 Ecology of anthrax

The ecological and evolutionary patterns of *B. anthracis* are highly dependent on its ability to form spores (Keim & Wegner, 2009). *Bacillus anthracis* has two life forms: vegetative cells and endospores, where the endospores are the form required to induce an oral infection (Turner et al., 2013). It occurs as vegetative cells within a host body, but forms endospores when exposed to conditions unfavourable for their vegetative growth after the host has died (WHO,2008; Hellstrom,2013; Figure 1). Metabolically dormant or environmentally persistent spores of *B. anthracis* spores are taken into a host via ingestion and inhalation, and are transported to the lymph nodes where they germinate (Bellan et al., 2013). Vegetative cells then reproduce in the blood and produce toxins which kill the host within 3 to 5 days (Bellan et al., 2013). Since anthrax lacks the ability of direct animal-to-animal transmission, it needs to kill its host in order to persist (Hellstrom, 2013). It is widely believed that the vegetative forms of *B. anthracis* sporulate when exposed to oxygen (Fasanella et al., 2009: Ebedes, 1976; Hudson et al., 2008). The bacterium normally rests in endospore form in the soil, and can survive for decades in this state (Dipti, 2013; Hudson et al; 2008).

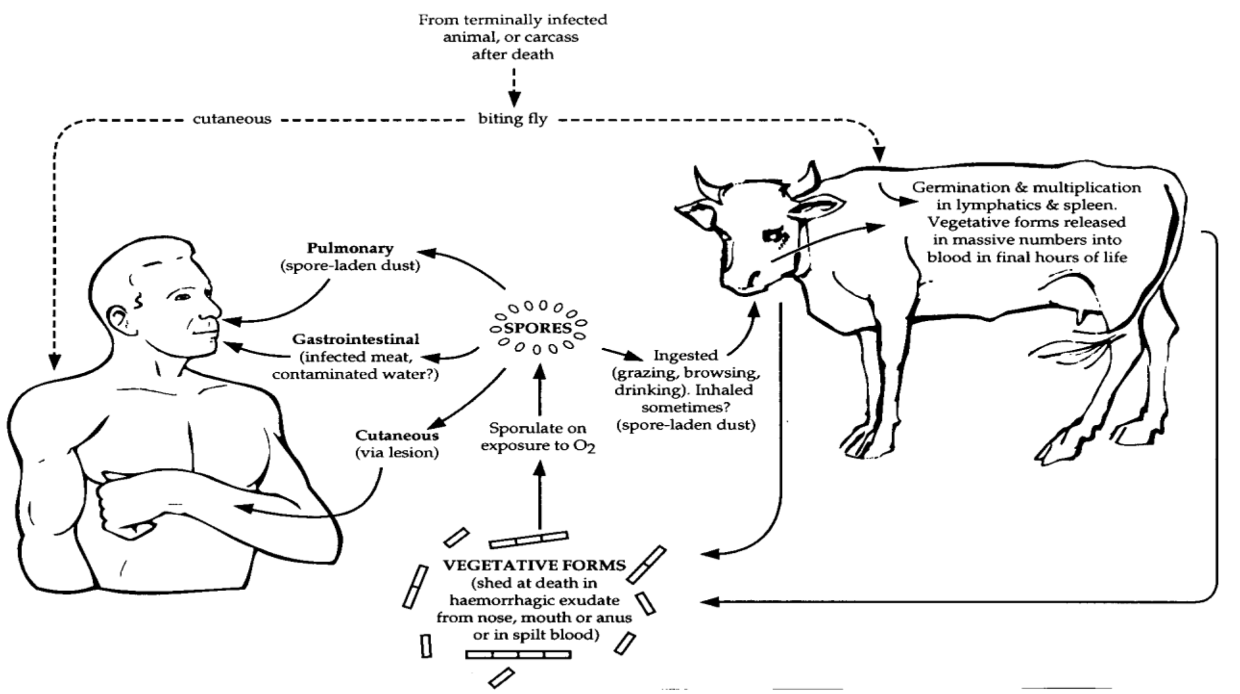


Figure 1: The life cycle of the infection of anthrax in humans and animals (Source: WHO, 2008).

A number of environmental factors affect long-term spore survival of the bacterium (Hampson et al., 2011), where its persistence has been associated with soil properties, weather and climate characteristics and thus increase the risk of anthrax (Turner et al., 2014; Hampson et al., 2011; Figure 2). The persistence of *B. anthracis* seems to favor soils that are high in calcium, rich in organic matter and have alkaline pH levels (Lindique & Turnbull, 1994; Hudson et al., 2008). In addition, Hugh-Jones (2002) found that anthrax endemic areas are associated with warmer temperatures and higher soil moisture content. Since the rate and extent of sporulation by vegetative cells released from infected animals is determined by the environmental conditions into which they fall, varying levels of anthrax outbreaks are expected in different regions (WHO, 2008). However, the ecology of the anthrax pathogen under natural conditions is still poorly understood due to varying climatic and environmental conditions, and the variable nature of anthrax outbreaks (Hampson et al., 2011). In particular, the interactions between anthrax and other microbiota in the soil and rhizosphere (Valseth et al., 2017) or in the host (Cizauskas et al., 2014) remain underexplored.

Outbreaks of anthrax occur mainly during the dry months that follow a prolonged period of rain (Fasanella et al., 2009). In the Kruger National Park, for example, a study carried out by De Vos (1990), has shown that the anthrax cycle is maintained by both biotic and abiotic factors. He carried out an investigation on the influence of rainfall on anthrax epidemics in the Fever Tree Depression area and reported that a high concentration of anthrax spores was found in the upper 3 centimeters of the soil but reduced overtime with an increase in rainfall. The observed that after heavy rains, the upper centimeters had little to no *B. anthracis*, whilst the deeper layers (10-15 cm) showed higher concentrations of *B. anthracis.* A reduction in the amounts of *B. anthracis* on the surface correlated with a drop in anthrax incidences in this area. Seasonal anthrax incidence patterns suggest that climatic factors such as ambient temperature and precipitation play a big role in the outbreak of the disease (Hampson et al., 2011).

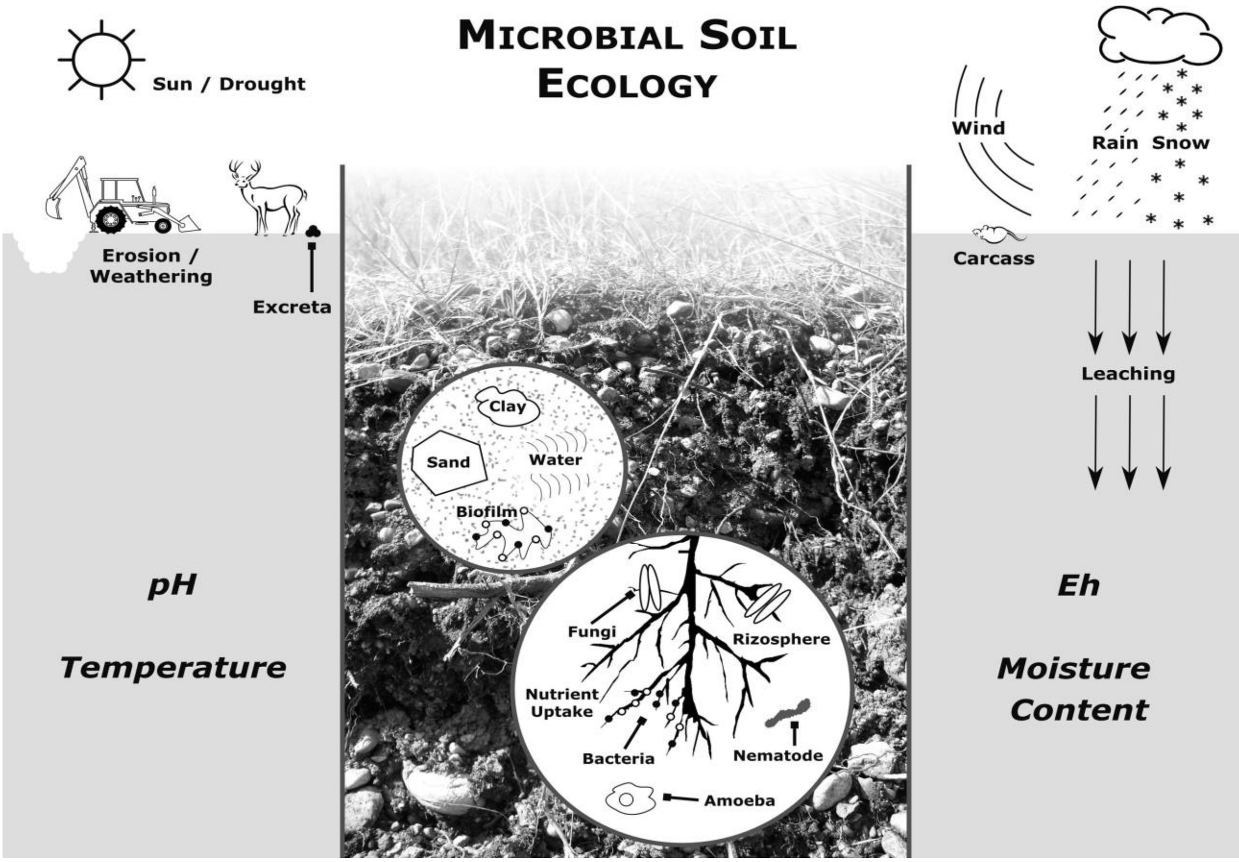


Figure 2: Pictorial representation of biotic and abiotic factors that determine the distribution and persistence of soil borne microorganisms such as *B. anthracis* (Source: Baumgardner, 2012).

2.4 Routes of transmission

Anthrax has been an issue for many years, yet much remains unknown about the disease, especially how and when it is transmitted. The transmission cycle of the disease is highly dependent on a host and is associated with soil as an environmental reservoir, in which spores persist for many years (Keim & Wegner, 2009). The transmission of anthrax requires the hosts' death and the release of bacterial endospores into the environment to be taken up by a susceptible host (Turner et al., 2016; Fasanella et al., 2009).

Although many questions were and are still being asked regarding the transmission of *B. anthracis*, there are several long-held beliefs considering the possible routes of anthrax (Hellstrom, 2013). It is long believed that animals take up anthrax spores while they are grazing or browsing, since *B. anthracis* is soil-dwelling (Hellstrom, 2013; WHO, 2008). A recent study suggests that grazing may be the dominant transmission route of the disease (Turner et al. 2016). The close grazing of animals on fresh shoots of grass after the rains is known to often lead to outbreaks of anthrax due to the ingestion of organisms picked from contaminated soils (Dipti, 2013). A study by Ruggiero (1992), on the seasonal utilization of forage by elephants showed that elephants had a higher forage intake on grass than browsing in the wet season compared to the dry season. However, he argued that this foraging behavior may reduce their exposure to *B. anthracis* during the wet season compared to the dry season, when grass is much taller and thus the elephants will not have close contact with the soil to ingest the pathogen. This foraging behavior may cause a variation in the transmission and incidence of the disease in the different seasons. In contrast, Lindique & Turnbull (1994), argue that feeding by cropping close to the soil is an unlikely means by which the Etosha National Park (ENP) ungulates can contract anthrax. However, a study by Havarua, Turner and Mfune (2014) demonstrated that it is not just foraging conditions that are relevant to anthrax transmission, but that the variation in foraging behaviour, in particular diet selection, may also alter seasonal exposure to soil-borne pathogens such as *B. anthracis*.

Flies also appear to play an important role in large anthrax outbreaks in endemic areas (WHO, 2008; Hudson et al., 2008). A study carried out by Blackburn et al., (2014) on Necrophagous flies has shown that *B. anthracis* can be carried away from carcasses into surrounding host plants by the flies. This was achieved through PCR analysis of leaf samples which tested positive for the bacterium. This may be a possible route of anthrax transmission when the anthrax infected plants are ingested by animals while foraging. A similar study by De Vos (1990) in the Kruger National Park showed that blowflies may play a significant role in the transmission of anthrax, where the blowflies deposit vomit droplets on vegetation after feeding on infected carcasses. These droplets were also found on leaves and twigs 1 to 3 metres above the ground, which is the preferred foraging height for kudus, the species most affected by anthrax in the KNP. The animal could then be exposed to anthrax while foraging as a result. Although, kudus are not one of the species affected by anthrax in ENP, the possibility that flies can play a role in anthrax transmission should not be ignored. Biting insects have also been argued by Russian scientists to be involved in spreading anthrax in reindeer herds in Siberia (Gainer, 2016).

Carcass sites are also known to be one of the main areas of anthrax transmission, whereby many factors such as the timing and frequency of carcass sites and animal's behavior at these sites may play a role in the transmission of the disease (Havarua et al., 2014). The avoidance or attraction of animals to carcass sites as one of the main sources of anthrax infection, may also have an influence on the transmission rates of the disease or bacterium to susceptible hosts (Turner et al., 2014). A study conducted by (Turner et al.,2014), revealed that nutrients from carcasses have positively altered the environment in ways that attract animals. In the same vein, a recent study by (Ganz et al.,2014) in Etosha has also shown that the presence of anthrax spores in the soil increases the germination rate of grass seeds. This further strengthens the view that anthrax has evolved to utilize grass as a transmission medium, as it may attract grazing hosts to infectious sites.

Etosha is also known to have a potential for drinking-based transmission of *B. anthracis* (Turner et al., 2016), as spores may accumulate in the sediments from carcasses in the water (Turner et al., 2016). Anthrax in Etosha has a peak in incidence during the late wet season (Lindeque & Turnbull, 1994; Havarua et al., 2014), where gravel pits known as "mini dams" form and retain water for long periods (Ebedes, 1976). These mini dams could be possible routes of anthrax transmission, as they have become favoured drinking places for zebras and wildebeests which are species commonly infected by anthrax (Ebedes, 1976). A study carried out by Lindique & Turnbull (1994) testing for the presence of *B. anthracis* in water and soil samples from different water bodies revealed that three out of ninety- two water samples positively contained *B. anthracis*, while seven out of two-hundred and thirty soil samples were *B. anthracis* positive. The presence of the anthrax pathogen in the different water bodies shows that water points or water holes are possible routes of the transmission of the disease. However, the lethal dose of *B. anthracis* which an animal needs to ingest in order to be infected with anthrax is largely unknown. It was not possible to draw many conclusions from the study of Lindique & Turnbull, on whether water points contribute to anthrax transmission because samples which were tested were only from one part of the water body, being either from the soil or the water and not both. Similarly, a study carried out by Berry (1993) revealed that elephants, as one of the species that are most infected by anthrax in Etosha, have a high mortality during the dry season. The study stated that the high anthrax mortality of elephants during this period could be due to their feeding behavior, whereby they prefer feeding on Acacia species. This would then possibly cause mouth lesions, which allows the transmission of anthrax from infected water bodies to the animal when drinking. Although anthrax is believed to be transmitted through water, not much is known on whether lethal amounts of anthrax can be transmitted through this medium to cause an infection. According to (Bellan et al., 2013), species vary in lethal doses and immunity against the anthrax disease, which also plays an important role in the transmission of the disease.

The disease may also occur through the cutaneous or pulmonary routes (Turner et al. 2013; Hellstrom, 2013), with the pulmonary form being considered the most dangerous (Vilas-Boas et al., 2007; Dey et al., 2012), yet there is no concrete evidence for this route. The cutaneous form is least deadly, it however accounts for over 90% of all human cases (Vilas-Boas et al., 2007; WHO, 2008). According to Hellstrom (2013), animals can contract anthrax from inhaling dust contaminated with anthrax spores though less frequently. In contrast, a recent study carried out by Barandongo et al. (2018), on the link between dust bathing activities and inhalational anthrax in ENP, showed that the dust bathing behaviour of zebras, wildebeest and elephants is less likely to cause anthrax by means of inhaling *B. anthracis* spores. Humans may also aid in the transmission of the disease through the transportation of contaminated animal products, such as meat, hides, hair and bones (Keim & Wegner, 2009). Anthrax is not contagious, implying that it cannot be transmitted from sick to healthy animals, but due to the intake of spores released into the environment (Fasanella et al.,2009). Although so many studies have been carried out to investigate and determine the possible transmission routes of anthrax in animals, much still remains to be elucidated concerning the transmission of the disease.

2.5 Anthrax in Namibia

Anthrax in Namibia has occurred since pre-colonial times (Ebedes, 1976). Several regions of Namibia, such as the Etosha National Park are well known to be areas endemic to anthrax (Turner et al., 2014; Beyer et al., 2012). Etosha National Park is one of the areas in the country enzootic to anthrax. In ENP, anthrax typically occurs as sporadic cases interspersed with small outbreaks (Lindique & Turnbull, 1994; Bellan, 2011). The earliest mortality records from the ENP are those of Ebedes (Beyer et al., 2012), with the disease occurring throughout the year with a peak in incidence occurring during the late wet season (Turner et al., 2013; Beyer et al., 2012). Anthrax outbreaks occur annually in the ENP and on private game and livestock farms (Beyer et al., 2012). Before any intensive studies were carried out on anthrax in Namibia from 1966, no anthrax mortalities in wildlife in Etosha national park were recorded (Berry, 1993). Anthrax mortalities among wildlife were not recorded because they did not play a big role in the economy of the country (Ebedes, 1976). However, it is known that in 1879, many Ovaherero people and their livestock died from an anthrax outbreak in the northern parts of Namibia which was then South West Africa (Ebedes, 1976).

Anthrax is an endemic disease in Namibia, and has a high number of cases of humans that died from anthrax (Beyer et al., 2012; Magwedere et al., 2012). An anthrax epidemic in Namibia killed thousands of wild animals between 1984 and 1989 (Edwards et al., 2006: Bhatnagar & Batra, 2001). Anthrax continues to kill many herbivorous species such as plains zebra, which are the main anthrax host species in Etosha, representing over 50% of all recorded anthrax mortalities (Turner et al., 2013). In the early 1970s the blue wildebeest was the most susceptible species to anthrax in ENP (Lindeque & Turnbull, 1994). Outbreaks in zebras, springbok and wildebeest are higher in the late wet season (March and April), while in elephants the disease peaks in the late dry/ early wet season (Beyer et al., 2012). Most elephant anthrax cases recorded in the park occurred during droughts in the 1980s (Lindeque, 1991). According to Ebedes (1976), contaminated waterholes might be the main source of infection in the Okaukuejo area where anthrax is enzootic, as the anthrax enzootic areas in the park are characterized by having lower-lying fountains, mini dams and being flat (Ebedes, 1976).

According to Shaanika (2013), in Omadhiya a small village in the Oshikoto region, dozens of cattle died of anthrax and two people died from eating the anthrax infected beef, while about 3000 were at the risk of being infected by the disease. Similarly, in a recent 2019 incident, an anthrax outbreak was also reported in the Kunene and Zambezi regions where 104 suspected cases of human anthrax were reported. The people were suspected of having eaten or had close contact with anthrax contaminated meat, resulting in lesions on their bodies (Shikongo, 2019). In another similar incident reported by Smith (2018), an anthrax outbreak occurred at the Sesfontein area in Kunene region, where 13 people were infected with anthrax after consuming anthrax-infected livestock meat. Forty –four (44) other people who were exposed to the anthrax infected meat were provided post-exposure prophylaxis. Another incident of a lion that died of anthrax in the Ugab area was also reported two years ago. The carcass was burned and buried as a precautionary measure to avoid further spread of the disease (Nakale, 2017). Furthermore, a case of anthrax in the Okavango region's Bwabwata National Park was reported in which the disease killed about 110 hippos and 20 buffalos (Kooper, 2017). Livestock deaths from anthrax were reported at Otjitanga village in the Kunene region on 25 September 2019, while deaths of hippos at the lake of Liambezi in the Zambezi region were reported on 29 August 2019 (Shikongo, 2019). Similarly, an anthrax case was reported in the Kavango region in 2017 where a total of 109 hippos died of anthrax (Kooper, 2017).

2.6 Anthrax in animals and humans

2.6.1 Clinical signs of anthrax in humans and animals

Anthrax infects both humans and animals. It develops as one of three forms, namely: cutaneous anthrax, intestinal anthrax and pulmonary anthrax (Hellstrom, 2013; Dipti, 2013; Turner et al, 2013). Cutaneous anthrax occurs when broken skin is contaminated with *B. anthracis* spores, while intestinal anthrax occurs when the spores of the anthrax pathogen are ingested, lastly the pulmonary anthrax which is the least common, occurs when *B. anthracis* spores are inhaled (Hellstrom, 2013). The cutaneous anthrax form accounts for more than 95% of anthrax cases (Misgie et al., 2015). Cutaneous anthrax in humans begins with a malignant pustule usually localized to the hands, legs, face, neck or arms (Fasanella et al., 2009;). This form of anthrax in humans also tends to form lesions on the skin, which are covered with a black eschar (Fasanella et al., 2009). Intestinal anthrax in humans occurs less frequently and is caused by the consumption of contaminated food (Fasanella et al., 2009; Misgie et al., 2015; WHO, 2008). This form of anthrax is associated with signs of nausea, vomiting, loss of appetite and fever followed by abdominal pain, severe diarrhoea, difficulty in swallowing and soreness of the throat (Fasanella et al., 2009). Untreated cases of intestinal anthrax have a case fatality rate of 25-75% (Misgie et al., 2015). Lastly, the pulmonary form of anthrax in humans is the most dangerous of the three and can be fatal if it goes untreated (Fasanella et al., 2009; Dey et al., 2012). This form of anthrax is very rare and is characterized by symptoms such as coughing, bloody sputum, fever, difficulty breathing, chest pain and muscle pains (Fasanella et al., 2009; Dipti, 2013; Misgie et al.,2015).

Figure 3: Cutaneous anthrax in acute and convalescent stages (Source: Bengis & Frean, 2014).

Anthrax can also affect animals, usually via ingestion of spores to cause cutaneous infection, (Dipti, 2013). Clinical forms of anthrax in animals are described as acute in which deaths occur suddenly from the onset of clinical signs (Dipti, 2013). Most of the animals are usually found dead without showing any signs of illness (Hugh-Jones & de Vos, 2002; Dipti, 2013). Thus, often the first and most obvious sign of anthrax in wildlife or domestic animals is finding dead animals (Hugh-Jones & de Vos, 2002). The common sign of anthrax in animals is a blood-stained fluid oozing from the anus, nostrils (Figure 4) and the mouth (Hugh-Jones & de Vos, 2002; Dipti, 2013). Another clinical sign of anthrax in animals is the oedematous swelling of the body (Hugh-Jones & de Vos, 2002). Herbivores such as goats, cattle and most wild ruminants manifest acute and peracute symptoms (Hugh-Jones & de Vos, 2002). In carnivores the disease usually shows subacute to chronic symptoms, which last for more than three days before the animal dies (Hugh-Jones & de Vos, 2002). The most common symptoms are oedematous swellings of the throat, face and or ventral parts of the body (Hugh-Jones & de Vos, 2002). Susceptible animals infected with anthrax can only walk short distances before succumbing to death (Keim & Wagner, 2009).

Figure 4: A zebra carcass with blood oozing from the nostrils as a sign of anthrax (Source: de Vos & Turnbull, 2004).

2.6.2 Treatment of anthrax in animals and humans

Cutaneous anthrax accounts for more than 95% anthrax cases in humans worldwide (WHO, 2008). It can be treated easily with antibiotics and should not be neglected as it may become fatal (Fasanella et al., 2009). Penicillin is the most favored antibiotic in treating anthrax in humans (WHO, 2008). Although various measures have been put in place to reduce the incidence of anthrax in livestock and wildlife, the disease still occurs worldwide in animals (Hugh-Jones & de Vos, 2002). It still retains a place in our natural ecosystems, because of practical difficulties experienced in vaccinating free living wild animals (Hugh-Jones & de Vos, 2002). In major game reserves and parks in Africa such as the Etosha National Park, most of the control measures similar to those used on livestock are very difficult and probably also impossible to use on free living wild animals (Hugh-Jones & de Vos, 2002), thus making it impossible to treat anthrax in wild animals but rather manage it. Anthrax management measures range from disease surveillance to disease regulatory actions. Surveillance is the gathering, summation and analysis of health data that allows an immediate dissemination of information to those who need to know, in order for appropriate action to be taken (WHO, 2008). Disease surveillance should include a proactive system put in place to monitor anthrax outbreaks and to maintain wildlife species and numbers (Hugh-Jones & de Vos, 2002; WHO, 2008). Effective surveillance is crucial in preventing and controlling anthrax (WHO, 2008). Measures that are put in place to regulate anthrax may involve treating and isolating sick animals, incinerating dead animals and when possible vaccinating susceptible stocks (Misgie et al., 2015). Domestic animals and livestock can mostly be vaccinated against anthrax since they can be approached and controlled to a much greater degree than wildlife. Vaccination plays a big role in controlling anthrax (Misgie at al., 2015). A well-known and used vaccine for animals is the

34F Sterne vaccine which is non-pathogenic, safe, cheap and provides effective protection of most livestock species (Hugh-Jones & de Vos, 2002; Dipti, 2013; Bengis & Frean, 2014). The vaccine, however, gives protection for only one year (Misgie et al., 2015). This need for repeated vaccinations to maintain immunity contributes to the difficulties in vaccinating wild animals and to the cost of keeping livestock vaccinated. To have better control and preventative measures of anthrax, the factors influencing its occurrence should be identified (Parthiban et al., 2015).

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

This chapter describes the study site, focusing on the size and location of the ENP, its mean annual precipitation and temperatures, as well as the types of vegetation it is composed of. Since one of the objectives of the study focused on the behavior of *B. anthracis* in the soil, a brief description of the major soil types found in the ENP is provided. This chapter also introduces the experimental techniques that were used in the study to meet the specified objectives. Lastly, the statistical methods that were used for data analysis are also outlined in this chapter.

3.2 Study area

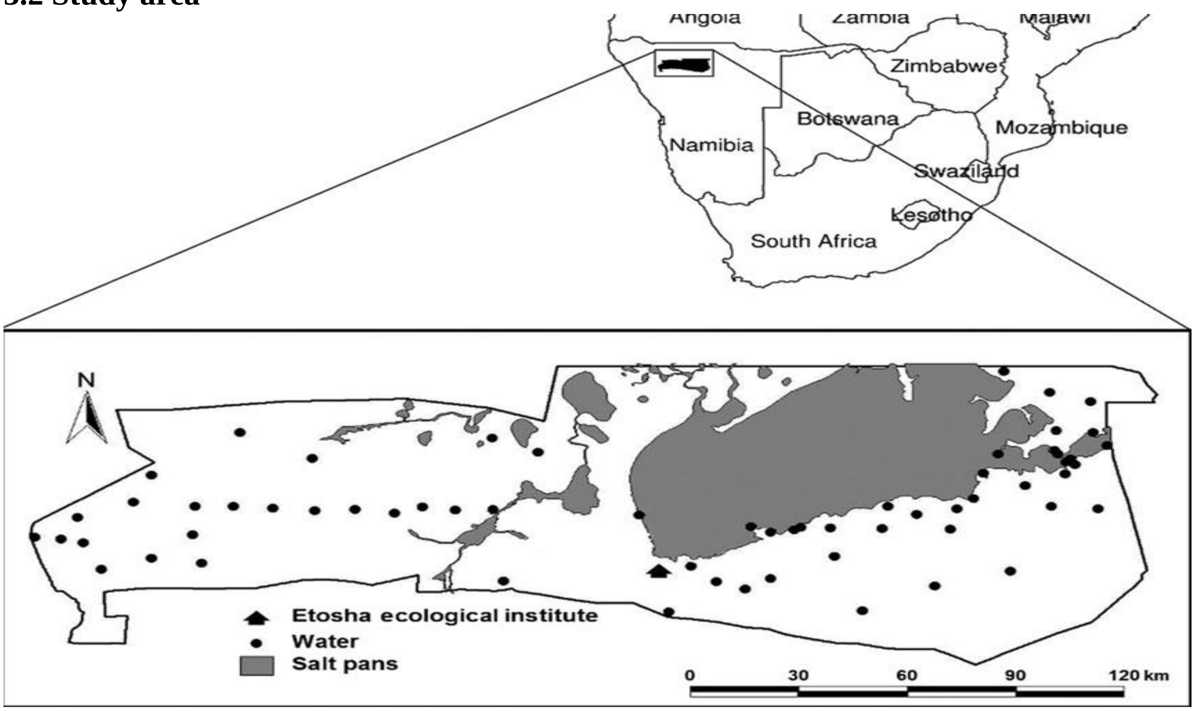


Figure 5: Map of Etosha National Park in northern Namibia (Source: Cizauskas et al., 2014).

This study was carried out in Etosha National Park which is in northern Namibia (18.85.56 ^{0}N; 16.32.93 ^{0}E) and covers an area of about 22,915 km². Three distinct climatic seasons are recognized in ENP, namely: the cool dry, hot dry and hot wet seasons (Ebedes, 1976; Turner et al., 2013). The rainfall pattern in the park varies greatly, decreasing from the east to the west of the park (Ebedes, 1977; Le Roux, et al., 1988). The annual rainfall in ENP ranges from 450 mm in the east to 300 mm in the west (Le Roux et al., 1988).

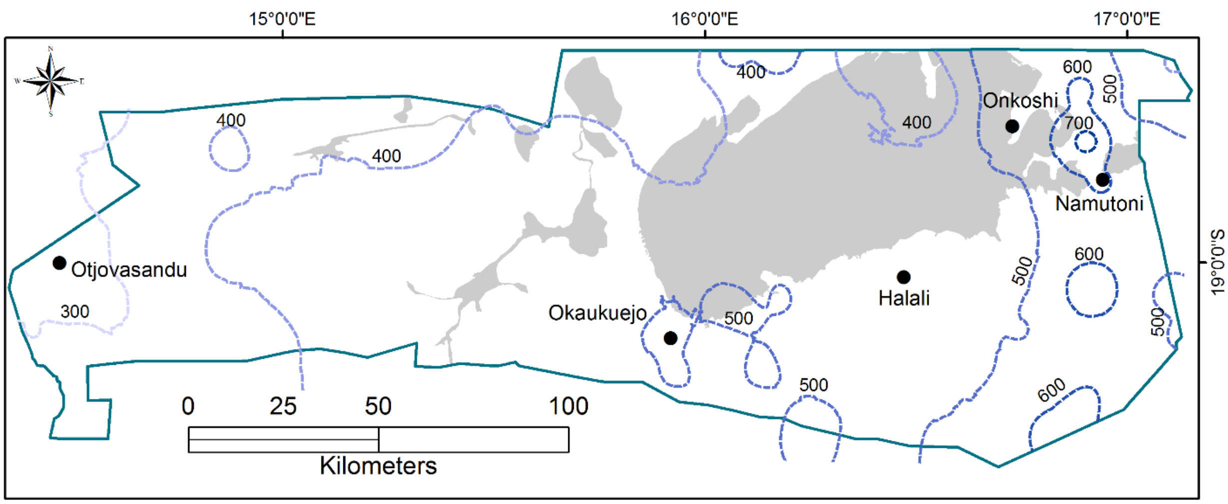


Figure 6: Rainfall map for ENP from June 2016 to May 2017 (Source: Hipondoka, 2017). This map indicates the variation in the amount of rainfall received throughout the park. Rainfall decreases from the east to the western part of the park. The isohyets in blue indicate the rainfall in each area. The Inverse Distance Weighted (IDW) method was used to produce this map.

Temperatures of the park range from an average minimum of 6.8 ^{o}C in winter and 17.4^{o}C in summer, to an average maximum of 28.4 ^{o}C in winter and 32.4 ^{o}C in summer (Ebedes, 1976). The vegetation of the park is classified in seven major zones (Lindeque, 1991) and is dominated by the mopane tree (*Colophospermum mopane*), which make up about 80% of all trees in the park. Other types of vegetation of the park vary from dwarf shrub savanna and grasslands (Le Roux et al., 1988). The soils of Etosha are categorised in five major groups (Beugler-Bell & Buch, 1997), the A: soil associations from deep sandy substrata, B: shallow to moderately deep sandy loamy to sandy soil associations, C: shallow to moderately deep sandy-loamy to loamy-clayey soil associations, D: soils from fluvial deposits and E: saline soils, respectively.

3.3 Behavior of *B. anthracis* in water and soil columns

3.3.1 Behavior of *B. anthracis* in water columns

A laboratory water experiment was carried out to investigate how *B. anthracis* endospores behave in a water environment. The experiment was conducted in the laboratory at the Etosha Ecological Institute (EEI). All benches and equipment were disinfected with 10% sodium hypochlorite at the start and end of the experiment. A plastic cover was laid out on a disinfected bench. Two clean 2liter plastic jars labelled A and B were placed on the bench to "kick start" the experiment. Into one jar (A) was added soil (600 g) and 700ml of distilled water, while the second jar (B) was only filled with distilled water (700ml) thus serving as a control. The soil used in the experiment was collected from the Tsumcor area 18.65.82 N^0; 016.89.51^0 E in Namutoni. The soil was collected from this area because it is characterized by sandy soils. Cloete (2013) showed that sandy soils are most conducive for anthrax spore persistence, since they had a higher spore count compared to other soil types in ENP that were used in her study. Similarly, Lindeque (1991) also found that maximum sporulation of *B. anthracis* occurred in sandy soils. An amount of 10 ml of sheep blood and 4 ml Sterne strain F2 of anthrax were introduced to each jar. The sheep blood was obtained from a farm 5 km outside Otjiwarongo. The blood and anthrax strain were added to the jars to simulate what happens in the natural world, when an animal dies near a waterhole or water point and becomes contaminated with anthrax as a result. Each jar was then stirred with a spoon to allow the blood and anthrax strain to mix well with the soil and water. The jars were then allowed to settle for 24 hours.

After 24 hours, nine samples (five from the soil jar and four from the control jar) were taken from the jars. The samples were drawn from different regions in the jars. The aim of sampling from different regions in the jar was to determine where most *B. anthracis* endospores ended up in a water environment. Using a 1 ml pipette two samples were drawn from the water regions termed: surface and middle (Figure 7). The remaining three samples were drawn from the soil regions termed: fine soil layer and the coarse soil, with the final sample being sampled after the jar was stirred (Figure 7). The jars were stirred for 1 minute before sampling to simulate an animal trampling in a waterhole. The sample was drawn immediately after stirring has ended. The remaining jar B only filled with distilled water had only four samples drawn from it: from the surface, middle, bottom and the last sample after stirring. The samples were put in 1.5 ml Eppendorf tubes and were cultured following the culture procedures in (section 3.4) below. Each jar was stirred with a separate spoon, simulating the effect of periodic animal movement. After the jars A and B were stirred and samples drawn from them, the jars were allowed to settle for 24 hours and the same procedure repeated the next day.

After the second day of repeating the sampling procedure, the jars were emptied, disinfected, rinsed well and allowed to air dry. The samples were drawn from the jars only after 24 and 48 hours before emptying. The jars were then again filled with the same amount of distilled water and soil and inoculated with the Sterne anthrax strain and blood. This procedure was repeated 20 times, resulting in ten sampling trials after 24hrs and ten sampling trials after 48hrs.

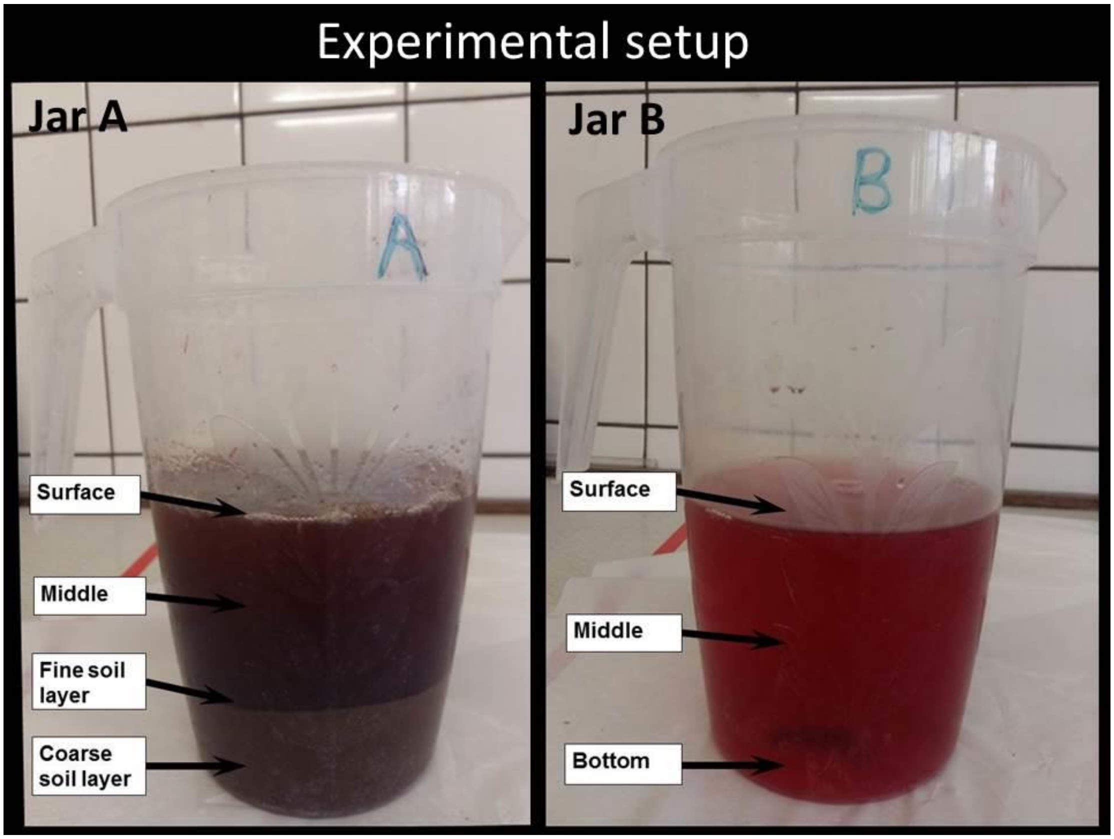


Figure 7: Experimental setup. Jar A was filled with soil and distilled water and inoculated with Sterne strainF2 of anthrax and blood. This jar served as the treatment of the experiment. The labelled regions are where the samples were drawn from. Jar B was only filled with distilled water and inoculated with Sterne strainF2 of anthrax and blood. This jar served as the control of the experiment. The labelled regions are where the samples were drawn from.

3.3.2 Behavior of *B. anthracis* in a soil column

Another experiment was carried out to determine the behavior of *B. anthracis* endospores in a soil environment. The study was carried out in a fenced area (26.5 m long × 15.5 m wide × 1.4 m high) in the savanna, 4.8 km north of Leeubron in central Etosha ($19.06.93^0$ N; 15.81530^0 E). This area was chosen in order to restrict access by grazing animals and scavengers. Three soil types were used in this experiment in order to meet the objective of how *B. anthracis* behaves in different soils in terms of deep transport of the pathogen. The three soils used in the experiment were collected from the Okaukuejo (S 19.17360^0; E 015.91828^0), Rietfontein (S 19.03640^0; E 016.34117^0) and Namutoni (S 18.65825^0; E 016.89515^0) areas respectively. The soils were named based on site of collection to ease follow up of soils and data collected in the field experiment

The three soils were later properly named based on their characteristics, using the map on soils in Northern Namibia by Beugler-Bell & Buch (1997). The soils were then named the calcaric regosols, lithic leptosols and the ferralic arenosols. The three soils used in the experiment were chosen because they are found in areas where most anthrax cases occur and where anthrax spores have been reported to persist in the soils as confirmed by the study by Cloete (2013).

3.3.2.1 Collection, transportation and analysis of soils

The soils were dug with a spade and transferred into properly labelled buckets. The soils were then taken to the soil laboratory at the EEI. At the laboratory three soil bags were filled with 500g of each soil type, sealed and sent to the Agricultural laboratory of the Ministry of Agriculture, Water and Rural Development and the Analytical Laboratory Services in Windhoek for further analysis and classification. The soils were analyzed for various parameters, including the texture of the soil (sand, silt or clay), organic matter content and pH of each soil using the FARM soil analysis (Personal communication, Ms Sipapo -Ministry of Agriculture, Water and Forestry). Thirty-six Whatman semi-permeable thimbles (43mm base x 123mm height) made from borosilicate glass were also filled with the three different soil types namely: calcaric regosols, lithic leptosols and the ferralic arenosols which served as treatments.

Figure 8: Whatman borosilicate glass semi-permeable thimble used in the soil experiment.

Twelve thimbles filled with the three soils served as controls of the experiment. The three soils were weighed out and each thimble filled with 250g of the soils (for both treatment and control). This was carried out in the soil laboratory at the EEI. The thimbles were randomly placed in an upright position in a basin and were supported with old newspapers and boxes to avoid them from falling and breaking during transport to the enclosure.

3.3.2.2 Setting up of experiment in the field

In the enclosure, holes were dug wide enough to avoid the breakage of the thimbles during their removal. The holes were dug in rows, with twelve holes in each row, placed 60cm apart. A total of 48 soil filled thimbles were placed into the holes and the surroundings filled with soil to keep them firm. Each of the 48 thimbles were identified by a labelled tag mounted into the ground next to them. The tags contained information on the soil type in the thimble and the sampling time point of the thimble. when they were going to be sampled from. The experiment was conducted over four time points $T_{0\ (24hrs)}$, $T_{1\ (1\ week)}$, $T_{2\ (2\ weeks)}$, and $T_{3\ (4\ weeks)}$. The experiment was conducted over 4 weeks due to limited resources. Three replicates of each soil type were made at each time point, bringing the number of thimbles to a total of nine at each time point. After all the thimbles were planted into the ground, they were each inoculated with 30 ml of sheep blood mixed with virulent anthrax spores. Two bottles of the anthrax- blood solution were prepared, where a volume of 60 ml of the virulent anthrax spores was diluted in 750 ml of sheep blood. The thimbles were watered with 130 ml of distilled water at the beginning of the experiment and during the collection of each set of thimbles at a particular time point, to mimic periodic rainfall. The thimbles were inoculated with blood, *B. anthracis* spores and water to simulate what happens in the natural world, when an anthrax infected animal dies and their blood seeps out contaminating the soil. The 12 thimbles which served as controls were only inoculated with 130 ml sterile distilled water. After 24 hrs (T_{0})

the first set of thimbles were collected with its set of 9 controls (3 for each soil type). The thimbles were placed in plastic Whirlpacks and properly sealed. The remaining 27 treatment thimbles and 27 controls were left out in the field. The T_1 thimbles with their controls were removed one week after inoculation, the $T_{2's}$ 2 weeks after inoculation and the $T_{3'S}$ 4 weeks after inoculation. After the removal of a set of thimbles at a particular time point (T_1-T_3), the remaining thimbles were watered with 130ml of distilled water until the last set (T_3) was removed. The thimbles were transported to the anthrax laboratory at the EEI.

Figure 9: Setup of the soil experiment in the Leeubron area of central Etosha.

3.4 Laboratory culture techniques

3.4.1 Water samples

Prior to the culturing of the samples, all equipment that were used in the culturing process including counter tops and biosafety cabinets were disinfected with 10% bleach (sodium hypochlorite). The polymyxin-lysozyme-EDTA-thallous acetate (PLET) plates were dried in the biosafety cabinet to remove condensation and were ready to be used for the culturing of samples.

The water samples (A1, A2, B1, B2, B3 and B4) drawn from the jars A and B (Figure 7) were cultured in the anthrax laboratory at the EEI, ″… using the following culture procedure. …" After the water samples from both jars were drawn, a volume of 1.5 ml of each sample was transferred into separate 1.5 ml Eppendorf tubes. The water sample tubes were then vortexed using a vortexer (Model: REAX 2000) for 5 minutes and centrifuged for 2 minutes at 0.3×1000 rcf (relative centrifugal force). The tubes containing the samples were properly placed in a disinfected tray and 1 ml supernatant of each sample transferred into new labelled tubes using a pipette. The Eppendorf tubes were labelled with letters in relation to the region of the jars from which samples were collected from. The tubes with the samples from Jar A (Figure 7) were labelled: A1, A2, A3, A4 and A5, while the tubes containing the samples from Jar B (Figure 8) were labelled B1, B2, B3 and B4. Tenfold dilutions of the 1 ml supernatant of each sample were made in a biosafety cabinet (ESCO class II, model: AC2-AE8). The samples were vortexed and 100 µl of each dilution placed onto polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar plates using a pipette. Two dilutions of 10^{-1} and 10^{-2} were made. The 100 µl dilution drop was evenly spread on the agar surface with a spreader. The culture plates were then incubated at 37 °C and checked for *B. anthracis* after 48-96 hours. The same procedure was used to culture the samples drawn after the jars were stirred.

3.4.2 Soil samples

The soil samples that were collected for both the soil (section 3.2.2) and water (section 3.2.1) experiments were cultured in the anthrax laboratory at the EEI using the following procedure. ″… using the following culture procedure. …"

3.4.2.1 Sampling and culture of soil (A4) (section 3.3, Figure 7)

After all the water samples (A1, A2 and A3) from Jar A (section 3.3.1, Figure 7) were sampled, the water in the jar was transferred to a separate clean Schott bottle, leaving only soil in the jar. Five grams of soil from the jar was weighed out and transferred into an empty falcon tube. The water in the Schott bottle was transferred back into the jar and stirred. The weighed out soil was then suspended in 45 ml of 0.1% sodium pyrophosphate, and the sample vortexed for 10 minutes to discharge and loosen spores from the soil particles. The samples were then centrifuged at 0.3x 1000 rcf for 2 minutes to separate the spores from the soil particles and to allow the soil to settle. The supernatant was transferred to a new clean 50ml falcon tube and centrifuged at 3.0 x 1000 rcf for 15 minutes to pellet the spores. The supernatant was discarded in a labelled waste bottle and the pellets re-suspended in 5ml sterile distilled water. The pellet/water mixtures were vortexed to get the spores in suspension and 1.0 ml of each sample transferred into 1.5ml Eppendorf tubes. Tenfold dilutions of the 1 ml suspension of each sample were made in a biosafety cabinet. The dilutions were made by transferring 100 µl of the suspension into another Eppendorf tube preloaded with 900 µl of sterile distilled water using sterile pipette tips. The samples were vortexed and 100 µl of each dilution placed onto polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar plates using a pipette. Two dilutions of 10^{-1} and 10^{-2} were made. The 100 µl dilution drop was evenly spread on the agar surface with a sterile spreader for each sample. The culture plates were incubated at 37 °C and checked for *B. anthracis* after 48-96 hours.

3.4.2.2 Slicing of thimbles and soil sample storage

Thimbles were collected from the soil experimental site (Figure 9) and brought to the anthrax laboratory at EEI. Thimbles were sliced to determine the depth of transport of *B. anthracis* endospores in these soils. They were sliced in 1 cm intervals from 1 to 11-12 cm depths, and marked off with a permanent marker at each centimeter. The soil samples for each depth in each soil were transferred into appropriately labelled WhirlPak bags using a clean plastic spoon. Each time this was done, a new clean spoon was used for each depth of each soil thimble to avoid contamination. The WhirlPak bags were labelled with the following information: Sampling time point, soil type, replicate number and the date of collection of the thimble or pipes. The soil samples from the different WhirlPak bags were weighed out using separate disinfected plastic spoons. The spoons were used to get soil from the bags into weighing boats during the culturing of the soils. Five (5) grams of each soil was weighed out and transferred into labelled 50 Falcon tubes and were cultured using the same procedure as described for the soils in the water experiment in (section 3.4.2.1). Three dilutions of 10^{-1}, 10^{-2} and 10^{-3} were made for the soil experiment. The remaining soil samples in the bags were stored in a walk-in fridge in the EEI for future usage.

3.5 Identification and counting of colonies

After the culture plates were incubated, the *B. anthracis* colonies on each plate of each dilution were counted after 48 hours and 96 hours of incubation and recorded on a data sheet. The number of colonies counted on each plate were also written on the base of the plates using a permanent marker pen. The counting of bacterial colonies after their growth on solid agar media is identified as a classical microbiological method that produces valuable data on the biological activity of samples (Almeida et al. 2008). *Bacillus anthracis* forms off-white colonies with irregular edges and a “round glass” appearance when incubated in conditions that are not suitable for capsule formation (Koehler, 2009). The plates with colonies that fell between 30 and 300 were considered for data analysis. The counts on the plates with <30 and >300 colonies were recorded on the data sheet although not considered for data analysis.

Whenever there was uncertainty on a microorganism that had grown on a plate, *B. anthracis* colonies were confirmed on blood agar using penicillin and gamma phage, because it is highly sensitive to them. The gamma phage was first recorded by Brown & Cherry (1995), as described in WHO, (2008). It was used in a study where it lysed all the *B. anthracis* strains, but none of the *B. cereus* strains (WHO, 2008), thus making it suitable for *B. anthracis* confirmation. In this study, a penicillin G disc and a 12.5 μL drop of diagnostic gamma phage were added to the bacterial streak of a suspected *B. anthracis* colony on blood agar. The blood agar plate was then incubated overnight at 35 °C and the colony confirmed as *B. anthracis* if it showed sensitivity to both the penicillin disc and the gamma phage (WHO, 2008).

3.6 Analysis of soil and water samples collected during the study

3.6.1 Soil samples

Three samples of each of the soil types collected during the study to carry out the experiments were sent to the Analytical Laboratory in Windhoek, Namibia for analyses. The parameters measured were soil texture (sand, silt and clay), soil pH (acidity or alkalinity of soil) and organic matter. Before any statistical analyses were done on the data, the *B. anthracis* colonies from the soil samples in both the soil and water experiments (Sections 3.3.1 and 3.3.2), were converted into colony forming units per gram of soil, using the following formulas (Maria Csuros & Csuba Csuros, 1999).

In order to obtain a measure of the concentration of colony forming units in the soil samples the moisture content of the soil is needed and was calculated by:

$$\textit{Moisture content} = \textit{Moist weight (g)} - \textit{dry weight (g) of soil} \div \textit{dry weight (g) of soil}$$

The wet weight of the soil samples was obtained by weighing out 10 g of the soil before drying it. The soil samples were then dried in an oven overnight at 100 ˚C to get the dry weight of the soil samples.

$$\textit{CFU/g} = \textit{(mean plate count) (dilution factor)} \div \textit{dry weight}$$

$$\textit{Dry weight soil} = 10 \times (1 - \%\ \textit{moisture} \div 100)$$

3.6.2 Water samples

Before any statistical analyses were done on the water experiment data, the *B. anthracis* colonies in the water samples were converted into colony forming units per milliliter, using the following formula (Maria Csuros & Csuba Csuros, 1999):

$$\textit{CFU/ml} = \textit{number of colonies} \times \textit{dilution factor} \div \textit{volume of plate culture } (0.1\,ml = 100\ \mu l)$$

3.7 Culture media preparations for *B. anthracis* isolation and spore production

3.7.1 PLET agar

The polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar media was used to grow the *B. anthracis* colonies in this particular study. PLET media is historically recognized to be the most successful selective media in isolating *B. anthracis* amongst others that were proposed (Knisely, 1966 as cited in WHO, 2008). "… the following procedure was used to prepare the media. …''

In a 2 L flask, 1.5 L distilled water, 78 g of brain heart infusion agar (BHI), 0.45 g EDTA and 1 ml thallous acetate were added. The thallous acetate solution was prepared by dissolving 0.6 g thallous acetate in 10 ml distilled water, and the lysozyme and polymyxin B sulphate by dissolving 0.279 g lysozyme and 0.143 g polymyxin B sulphate in 10 ml distilled water. The flask was then carefully sealed with foil and placed on a magnetic heat stirrer to dissolve the contents. The mixture was autoclaved with warming function for 45 minutes at 125 ˚C for 45. The warming function was set at 50 ˚C for 1 hour. After the removal of the flask from the autoclave, 400 µl of the lysozyme and polymyxin B sulphate solutions were added using sterile pipettes. The agar was added to the solution and gently mixed. Twenty milliliters of the agar were then pipetted into each petri dish placed on a disinfected working bench and left to set overnight. The plates were put into bags, labelled with the date of media preparation, and put in the fridge.

3.7.2 Sporulation agar

In a 2L flask, 1 L distilled water, 16 g BHI broth, 2 g Potassium Chloride (KCl), 0.5 g magnesium sulfate heptahydrate ($MgSO_{4.}7H_20$) and 16 g nutrient agar were added. Each of the solids used in the agar media were prepared by dissolving 1.98 g of Manganese (II) chloride tetrahydrate, 0.28g Iron (II) sulfate heptahydrate and 18g of glucose in 100 ml sterile distilled water.

The flask was then carefully sealed with foil and placed on a magnetic heat stirrer to dissolve the contents. The solution was autoclaved with warming function for 45 minutes at 125 ˚C. The warming function was set at 50 ˚C for 1 hour. After the removal of the flask from the autoclave, 1ml of 0.1 g Manganese (II) chloride tetrahydrate $MnCl_2.4H_20$, 100 µl of Iron (II) sulfate heptahydrate 10 Mm $FeSO_{4.}7H_20$ and 10ml of 10% glucose were added through sterelised filters. The agar was added to the solutions and gently mixed. Twenty milliliters of the agar were then pipetted into each petri dish placed on a disinfected working bench. After all the agar was poured in the petri plates, the plates were left to set overnight.

3.7.3 Blood agar

In this study the sensitivity of *B. anthracis* colonies was tested on blood agar using penicillin and gamma phage. In a 2 L flask, 20 g Bacto agar, 940 ml distilled water, and 8 g nutrient broth were added to make nutrient agar. The agar was autoclaved with the warming function for 40 minutes at 125 ˚C. The warming function was set at 50 ˚C for 1 hour. While the nutrient was in a cooling phase in the autoclave, 60 ml of horse blood was warmed in a water bath for an hour at 50 ˚C. The blood was then added to the cooled nutrient agar to achieve a blood concentration of 6%. The agar was gently mixed and poured into the petri plates. Twenty milliliters of the agar were then pipetted into each petri plate placed on a disinfected working bench. After all the agar was poured in the petri plates, the plates were left to set overnight. The plates were put into bags, labelled with the date of media preparation and put in the fridge.

3.7.4 Spore production

The virulent *B. anthracis* spores that were used in the soil experiment (section 3.3.2) were produced by culturing on sporulation agar (section 3.7.2) ″…using the following procedure. …".

A blood agar plate was streaked with a swab isolate that was collected from a springbok (carcass number 11-001). The plate was incubated overnight at 30˚.Twelve sporulation agar plates were also incubated overnight at 30˚ to check for sterility, by ensuring that no *B. anthracis* or any other microbes have grown on the plate. After checking the plate sterility, a loopful of *B. anthracis* colonies were harvested from the blood agar plate and suspended in a sterile Falcon tube containing 2 ml of sterile water. The falcon tube was vortexed and the sporulation agar plates inoculated with the cell suspension. The plates were inoculated with 100 µl of the cell suspension, which were spread on the plates with sterile spreaders and incubated for 48-72 hours. Plates were removed from the incubator and were left on a disinfected bench for 24 hours. The next day sporulation efficiency was checked under a microscope. Once the sporulation efficiency was close to 100%, the cell lawn on each plate was harvested with a sterile disposable cell scraper into a 50 ml falcon tube. The falcon tube containing the harvested cells was left on the bench for 72 hours. Thereafter, the tube was centrifuged at 3.000 g for 20 minutes to collect the cells. The cells were washed with 40 m sterile distilled water and centrifuged at 3.000 g for 3 minutes. The resulting pellet contained the spores and vegetative cells. Virulent anthrax spores were used in this experiment because it was carried out in the field away from people, to avoid lethal infection.

3.8 Data analyses

3.8.1 Soil experiment

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 22 statistical package. All data were tested for normality using the Shapiro-Wilk test, before further statistical analysis was done on them. The Shapiro Wilk test was used because it is one that is restricted for a sample size of less than 50 (Razali & Yap, 2011). The colony forming units per gram of soil (CFU/g/) data for this experiment of the study were tested for normality using the Shapiro Wilk test and was concluded that data did not follow a normal distribution. The pH ($W=0.893$, $df= 9$, $p >0.05$), clay ($W=0.943$, $df= 3$, $p >0.05$), silt ($W=0.832$, $df= 3$, $p >0.05$) and sand ($W=0.726$, $df= 3$, $p >0.05$) data were normally distributed. However, data for organic matter ($W=0.802$, $df= 9$, $p <0.05$) data were not normally distributed. A one-way analysis of variance (ANOVA) was used to compare the means of the soil properties (pH and texture (silt, sand, clay) of the three soil types (section 3.3.2.), while the means of the organic matter data of the three soils were compared using the Kruskal-Wallis test. These tests were carried out in order to determine the effect of soil type on the behavior of *B. anthracis* in water and soil reservoirs. The soil moisture at different time points and depths were also tested and data was not normally distributed. This test was done to determine if the soil moisture of the soils at different depths had an influence on the *B. anthracis* cell counts at every depth. The Chi-Square test of association was used to determine if there was an association between the *B. anthracis* colony counts and the depth, and the *B. anthracis* colony counts and the soil type.

3.8.2 Water experiment

Data were analyzed using the SPSS version 22 statistical package. A Shapiro-Wilk test was used to test all data for normality. The test revealed that the *B. anthracis* count data in jar A at both time periods (24 hrs and 48 hrs) were not normally distributed (24hrs: $W=0.632$, $df= 50$, $p < 0.05$; 48hrs: $W=0.496$, $df=50$, $p < 0.05$) and, therefore a Kruskal-Wallis test was used to compare the means of *B. anthracis* counts at the different layers in jar A, which served as the treatment. Similarly, the *B. anthracis* count data in jar B which served as a control did also not follow a normal distribution (24hrs: $W=0.573$, $df=40$, $p < 0.05$; 48hrs: $W=0.54$, $df=40$ $p < 0.05$) and thus a Kruskal-Wallis test was also used to compare the means of *B. anthracis* counts at the different layers in the jar.

CHAPTER 4: RESULTS

The study was conducted in order to investigate how *B. anthracis* spores interact with water, soil particles and capillary forces in a natural soil environment and in a water environment, such as waterholes, that have been infected by anthrax and are regularly disturbed by animals.

This chapter presents the results obtained from the laboratory and field experiments described in the previous chapter (sections 3.3.1 and 3.3.2) to address the objectives of the study. The results include tests done on the soil chemistry and soil moisture data, to validate the effect of soil type on deep transport of *B. anthracis* and the behavior of *B. anthracis* in a water environment.

4.1 The influence of soil chemistry, organic matter and soil moisture on the transport of *B. anthracis* in three different soil types

4.1.1 Soil chemistry and organic matter

Three soil types were used in this study to determine the effects of soil chemistry on the transport of *B. anthracis* in the different soils. These soils were collected from the Okaukuejo, Namutoni and Rietfontein areas of Etosha National Park, in June 2018. The soils were characterized and the means of the soil properties were calculated and compared and are presented in the figures below (Figures 10,11,12,13 and 14).

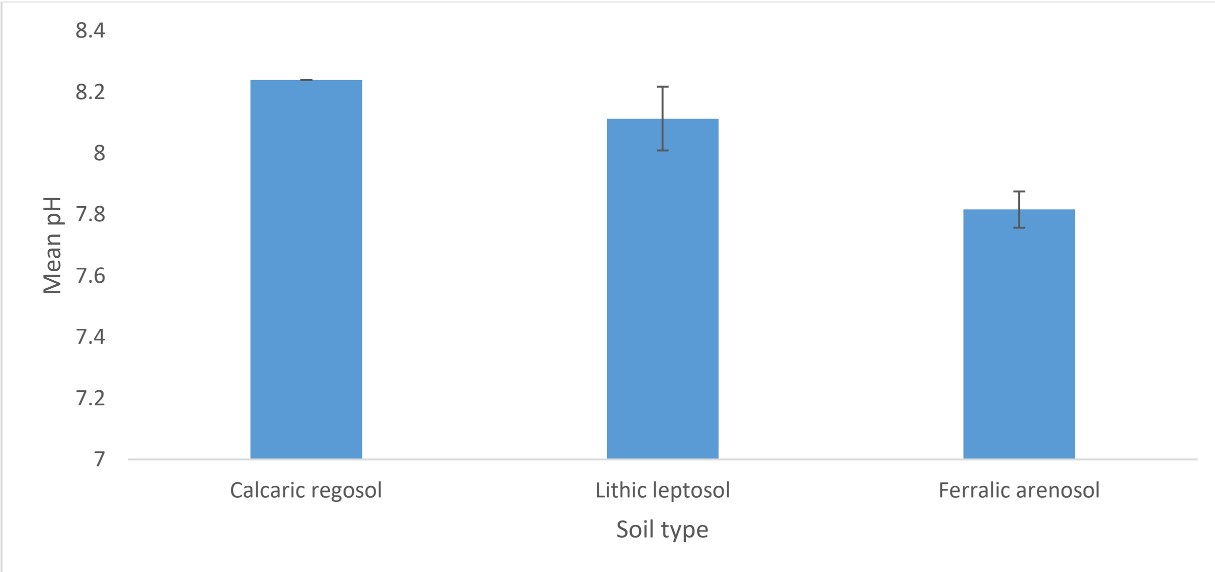


Figure 10: Means (±SEM) for the pH of the calcaric regosol, ferralic arenosol and lithic leptosol soil types collected from the Okaukuejo, Namutoni and Rietfontein areas in ENP, respectively. The bars indicate the standard error of the mean.

A one-way ANOVA revealed that the pH of the three soils differed significantly ($F=9.889061$, $df=2$, $p<0.05$). The calcaric regosol and the lithic leptosol soil types had a significantly higher pH amongst the three soils, while the ferralic arenosol soil type had the lowest pH amongst the three soils. A post hoc Tukey analysis test was also carried out to determine in which soils the pH were significantly different. The test revealed that there is a significant difference ($F=2.364$, $df=4$, $p<0.05$) in pH between the ferralic arenosol and calcaric regosol soil types. However, the pH for the lithic leptosol vs calcaric regosol ($F=1.301$, $df=4$, $p>0.05$) and the lithic leptosol vs ferralic arenosol ($F=1.371$, $df=4$, $p>0.05$) were not significantly different.

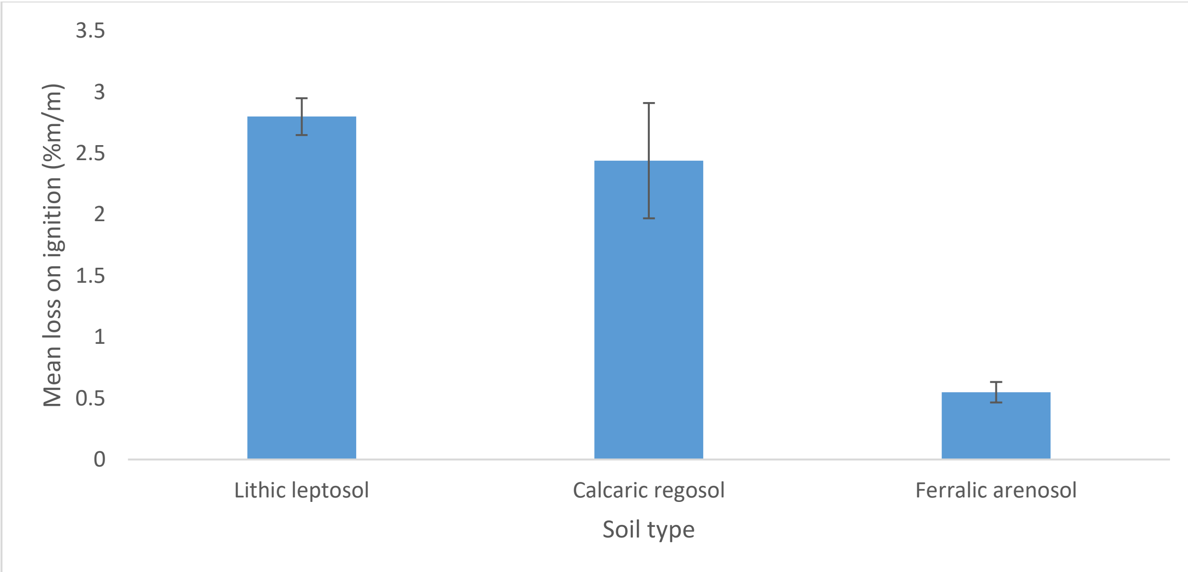


Figure 11: Means (± SEM) for the organic matter of the calcaric regosol, ferralic arenosol and lithic leptosol soil types collected from the Okaukuejo, Namutoni and Rietfontein areas in ENP. The bars indicate the standard error of the mean.

The Kruskal-Wallis analysis revealed that the organic matter of the three soils differed significantly ($H=5.422$, $df= 2$, $p<0.05$). The calcaric regosol and the lithic leptosol soil types had a significantly higher organic matter percentage. The ferralic arenosol soil type had a significantly lower organic matter percentage. A post hoc Tukey analysis test was also carried out to determine in which soils the organic matter was significantly different. The test revealed that there was a significant difference in organic matter between the ferralic arenosol and calcaric regosol ($F=3.994$, $df= 4$, $p<0.05$), as well as between the lithic leptosol and the ferralic arenosol ($F=13.06$, $df= 4$, $p<0.05$) soil types. The organic matter for the lithic leptosol and the calcaric regosol soil types did not differ significantly ($F=0.727$, $df= 4$, $p>0.05$).

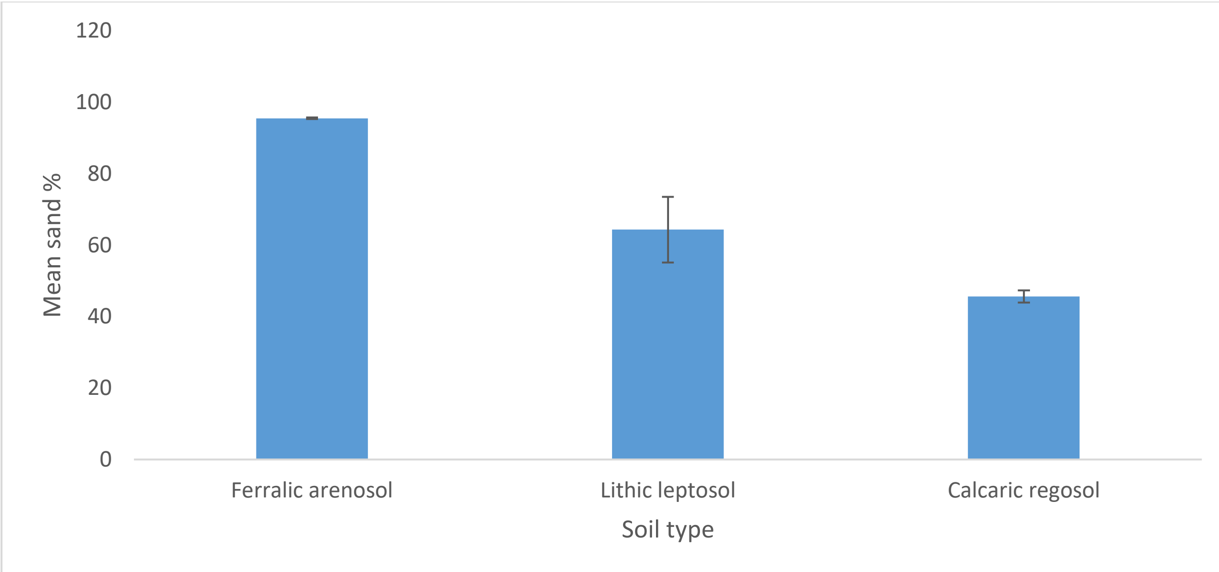


Figure 12: Means (± SEM) for the percentages of sand in the calcaric regosol, ferralic arenosol and lithic leptosol soil types collected from the Okaukuejo, Namutoni and Rietfontein areas in ENP. The bars indicate the standard error of the mean.

The one-way ANOVA analysis revealed that the percentage of sand in the three soils differed significantly ($F=21.85181$, $df= 2$, $p<0.05$). The ferralic arenosol had a significantly higher sand percentage amongst the three soil types. The calcaric regosol had the least sand percentage. A post hoc Tukey analysis test was also carried out to determine in which soils the sand percentage was significantly different. The test revealed that there was a significant difference in the percentage of sand between the ferralic arenosol and calcaric regosol ($F=29.42$, $df= 4$, $p<0.05$) soils, as well as between the lithic leptosol and the ferralic arenosol ($F=3.395$, $df= 4$, $p<0.05$) soil types. The percentage of sand for the lithic leptosol and the calcaric regosol soil types did not differ significantly ($F=2.002$, $df= 4$, $p>0.05$).

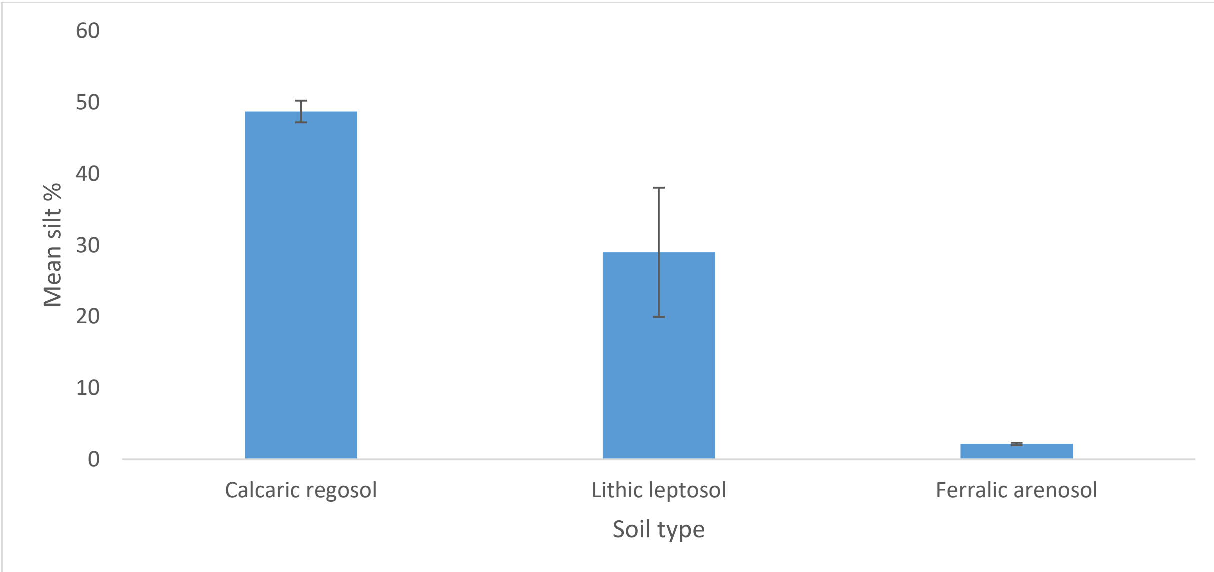


Figure 13: Means (± SEM) for the percentage of silt in the calcaric regosol, ferralic arenosol and lithic leptosol soil types collected from the Okaukuejo, Namutoni and Rietfontein areas in ENP. The bars indicate the standard error of the mean.

The one-way ANOVA analysis revealed that the percentage of silt in the three soils differed significantly ($F=19.45724$, $df= 2$, $p<0.05$). The calcaric regosol had a significantly higher percentage of silt amongst the three soil types. The ferralic arenosol had a significantly lower percentage of silt amongst the three soil types. A post hoc Tukey analysis test was also carried out to determine in which soils the percentage of silt was significantly different. The test revealed that there was a significant difference in the percentage of silt between the ferralic arenosol and calcaric regosol ($F=30.36$, $df= 4$, $p<0.05$) soils, as well as between the lithic leptosol and the ferralic arenosol ($F=2.966$, $df= 4$, $p<0.05$) soil types. The percentage of silt for the lithic leptosol and the calcaric regosol soil types did not differ significantly ($F=2.148$, $df= 4$, $p>0.05$).

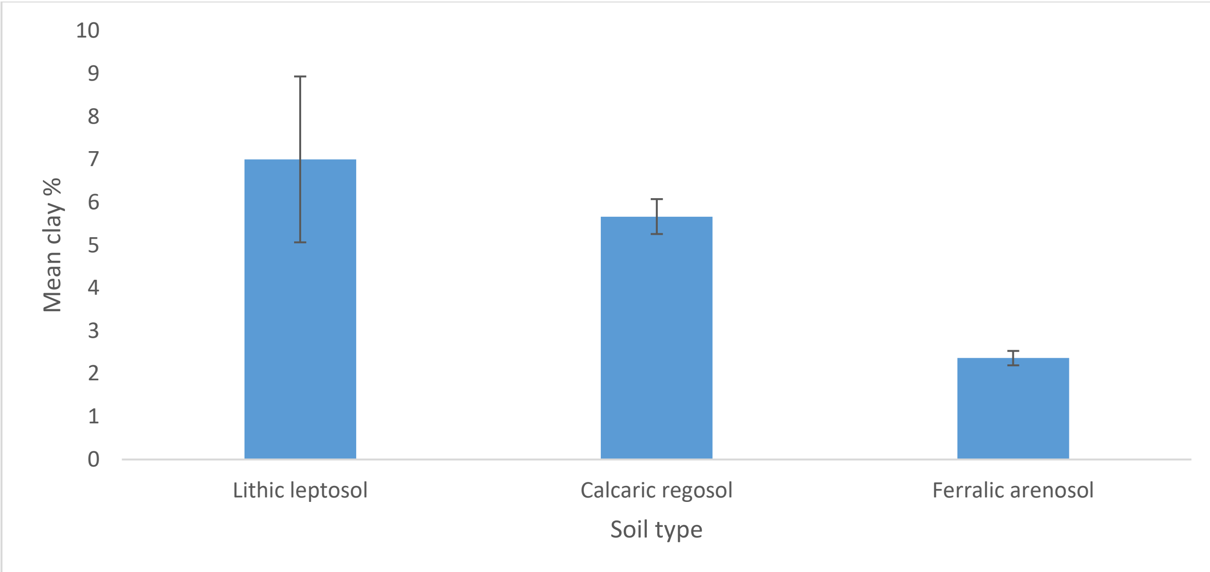


Figure 14: Means (± SEM) for the percentage of clay in the calcaric regosol, ferralic arenosol and lithic leptosol soil types collected from the Okaukuejo, Namutoni and Rietfontein areas in ENP. The bars indicate the standard error of the mean.

The one-way ANOVA analysis revealed that the percentage of clay in the three soils did not differ significantly ($F=4.336815$, $df= 2$, $p>0.05$). The lithic leptosol had a significantly higher percentage of clay amongst the three soil types. The ferralic arenosol had a significantly lower percentage of clay amongst the three soil types. A post hoc Tukey analysis test was also carried out to determine in which soils the percentage of clay was significantly different. The test revealed that there was no significant difference in the percentage of clay between the ferralic arenosol and calcaric regosol ($F=7.52$, $df= 4$, $p>0.05$) soils, as well as between the lithic leptosol and the ferralic arenosol ($F=2.38$, $df= 4$, $p>0.05$) soil types. The percentage of clay for the lithic leptosol and the calcaric regosol soil types did not differ significantly ($F=2.674$, $df= 4$, $p>0.05$).

4.1.2 Soil moisture

The mean proportions of soil moisture of the soil types used in the soil experiment were calculated and are presented in Figure 15. The proportion of soil moisture of the three study soils was not normally distributed. A Kruskal-Wallis test revealed that there was a significant difference in the proportion of soil moisture amongst the calcaric regosol, ferralic arenosol and the lithic leptosol soil types (H = 86.851, df = 10, p <0.05; Figure 15). The Chi-Square test of association revealed that there was marginal but significant association between the soil moisture and *B. anthracis* colony counts (χ^2 =17.21, df = 9, p =0.045) in the three soils used in this study.

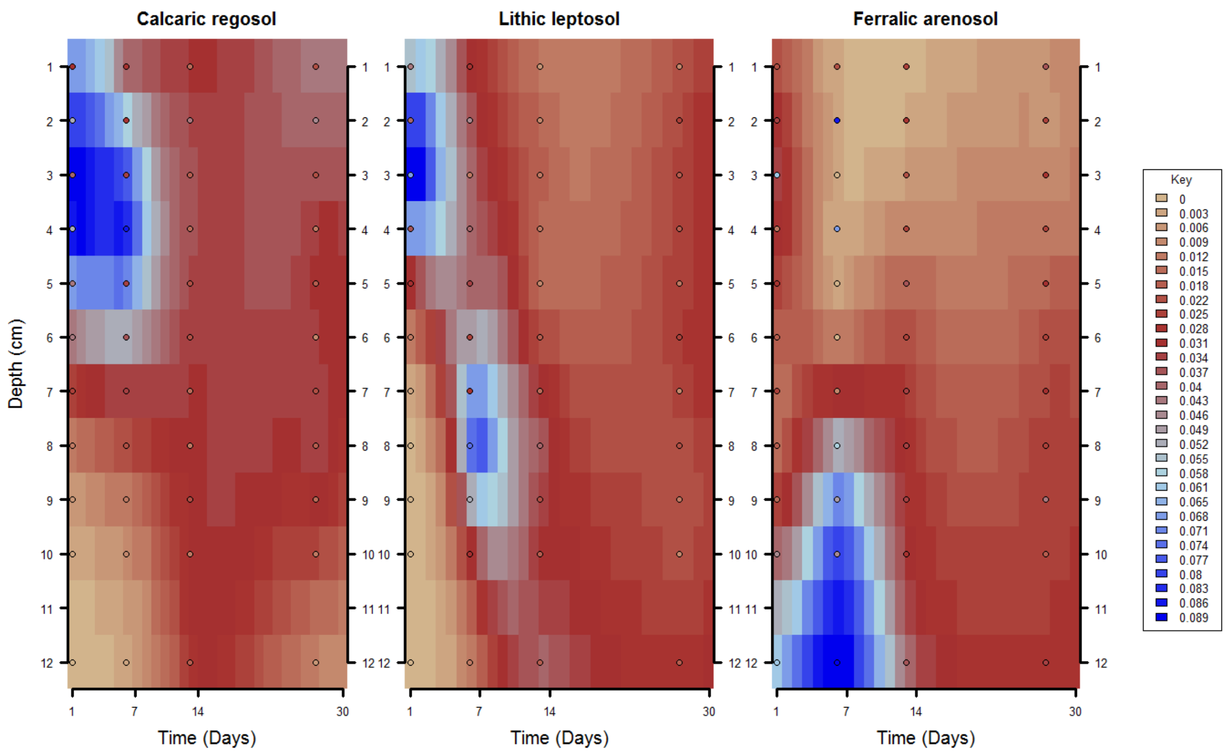


Figure 15: Proportion of soil moisture of the calcaric regosol, ferrallic arenosol and lithic leptosol soils at each depth (cm). The study was carried out over a month and soil moisture calculated from samples collected at four time points (1 day,7 days, 14 days and 30 days respectively).

Figure 15 shows the proportion of soil moisture between the calcaric regosol, lithic leptosol and ferralic arenosol study soil types at different depths and time points. The calcaric regosol retained more moisture in the upper layers (1-6 cm) in the first week (7 days) compared to the other two soils. The lithic leptosol retained more moisture in the upper layers of 1-6 cm in the first week. The water in the lithic leptosol soil gradually percolated further down, retaining more moisture at the depths of 6-12 cm after two weeks compared to the calcaric regosol soil type. The ferralic arenosol was the most different from the other two soils, as water was not retained in the upper layers but quickly seeped down to the bottom layers (8-12 cm), resulting in more moisture being retained at those depths in the first two weeks (14 days). The ferralic arenosol had significantly lower soil moisture on the soil surface compared to the other two soils throughout the study. The soil moisture content of the calcaric regosol and lithic leptosol soils decreased significantly with increasing depth (Figure 15).

4.1.3 Behaviour of *B. anthracis* in three soil types

The *B. anthracis* colony counts in the three soils were not normally distributed (A-Calcaric regosol ($W = 0.848$, $P<0.05$); B-Ferralic arenosol ($W=0.875$, $p<0.05$); C- Lithic leptosol ($W=0.8$, $p<0.05$). The results revealed that there was a significantly positive association between the depth and *B. anthracis* colony counts ($\chi^2 = 143.330$, df = 30, $p = .000$) and that there was a significantly positive association between the soil type and the *B. anthracis* colony counts ($\chi^2 = 24.77$, df = 6, $p = .000$).

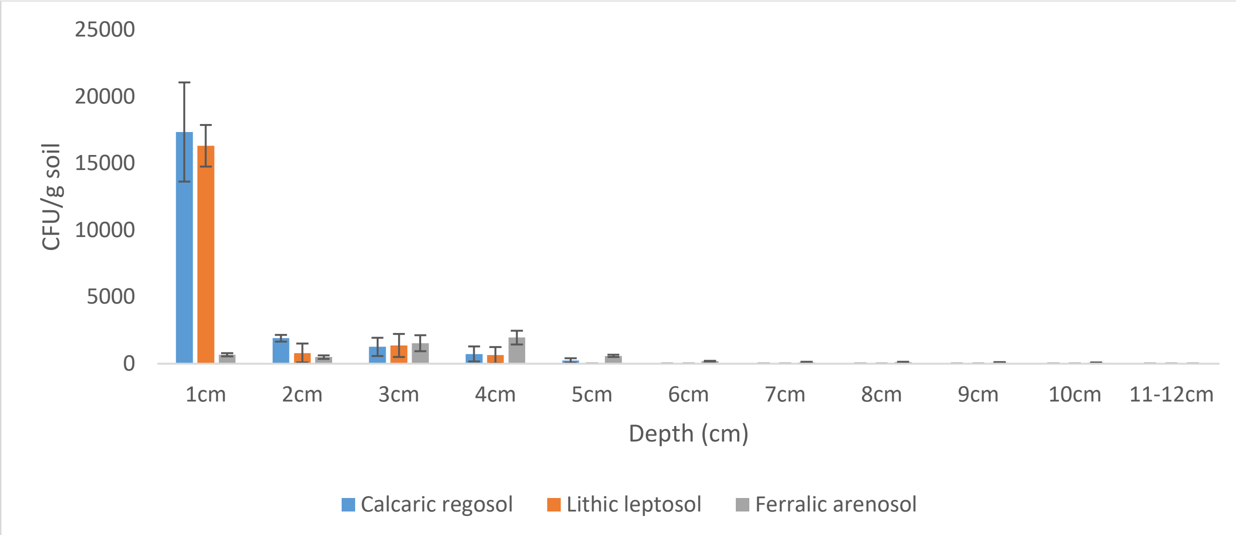


Figure 16: Mean number of *B. anthracis* counts per gram of soil (CFU/g) at sampling point T_0 (24 hrs), in each soil type at each depth. The key indicates the calcaric regosol, ferralic arenosol and lithic leptosol study soils. The bars indicate the standard error of the mean.

The *B. anthracis* cell counts decreased with increasing depth in the three soils calcaric regosol, ferralic arenosol and the lithic leptosol soil types after 24 hours (Figure 16). After 24hrs (T_0) the *B. anthracis* spores were leached further down to a depth of 10 cm in ferralic arenosol, while in the calcaric regosol they moved until the 5cm depth and until 4cm depth in the lithic leptosol soil type. The calcaric regosol and the lithic leptosols had a significantly higher number of *B. anthracis* counts at the 1-2 cm depth at T_0 (24hrs) compared to the ferralic arenosol (Figure 16). Calcaric regosol vs ferralic arenosol (1cm: $t=4.488$, $df= 4$, $p<0.05$; 2cm: $t=4.936$, $df= 4$, $p<0.05$;). Lithic leptosol vs ferralic arenosol (1cm: $t=10.028$, $df= 4$, $p<0.05$; 2cm: $t=0.418$, $df= 4$, $p<0.05$). However, there was no significant difference in the *B. anthracis* counts of the ferralic arenosol and the lithic leptosol and calcaric regosol soils (Figure 16; 3cm: calcaric regosol vs ferralic arenosol: $t=-0.29719$, $df= 4$, $p>0.05$; 3cm: lithic leptosol vs ferralic arenosol $t=-0.164$, $df= 4$, $p>0.05$)

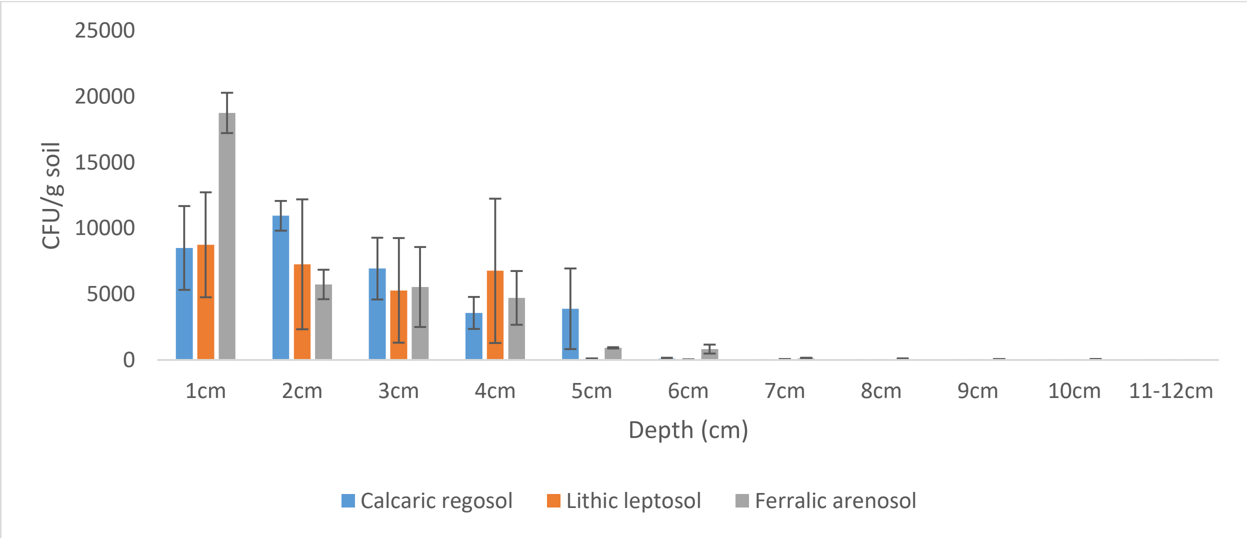


Figure 17: Mean number of *B. anthracis* counts per gram of soil (CFU/g) at sampling point T_1 (1 week), in each soil type at each depth. The key indicates the calcaric regosol, ferralic arenosol and lithic leptosol study soils. The bars indicate the standard error of the mean.

The *B. anthracis* cell counts decreased in the three soils, the calcaric regosol, ferralic arenosol and the lithic leptosol soil types with increasing depth after 1 week (Figure 17). After 1 week (T1) the *B. anthracis* spores were leached further down to a depth of 10 cm in the ferralic arenosol soil, while in the calcaric regosol they moved until the 6cm depth and until 7cm depth in the lithic leptosol soil type (Figure 17). The ferralic arenosol soil type had significantly higher *B. anthracis* counts at 1 cm depth compared to the calcaric regosol and the lithic leptosol and (Figure 17). The ferralic arenosol also had significantly higher counts of *B. anthracis* with increasing depths of 6-10cm. The calcaric regosols and lithic leptosol soils at this time pointT_1 had significantly higher counts of *B. anthracis* at the 2cm-4cm depths compared to the ferralic arenosol.

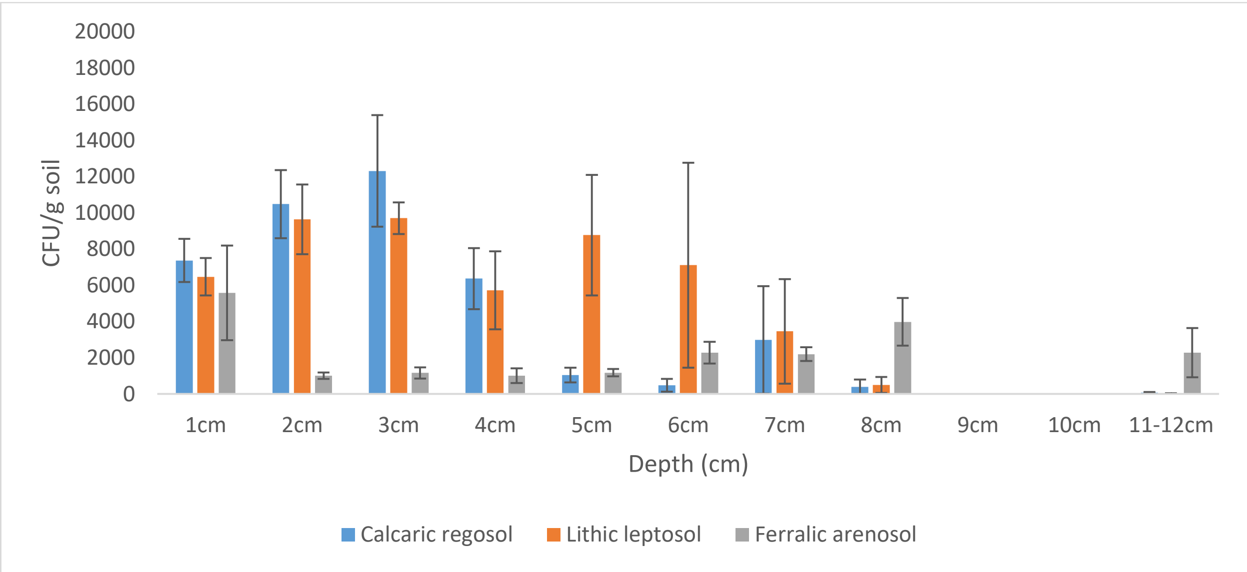


Figure 18: Mean number of *B. anthracis* counts per gram of soil (CFU/g) at sampling point T_2 (2 weeks), in each soil type at each depth. The key indicates the calcaric regosol, ferralic arenosol and lithic leptosol study soils. The bars indicate the standard error of the mean.

After 2 weeks (T_2) *B. anthracis* spores had leached to the 11-12cm depths in all three soils (Figure 18). There was no significant difference in the *B. anthracis* counts in the calcaric regosol and the lithic leptosol soils compared to the ferralic arenosol at the 1cm depth (calcaric regosol vs ferralic arenosol 1cm: $t=0.622$, $df=4$, $p>0.05$; lithic leptosol vs ferralic arenosol 1cm: $t=0.314$, $df=4$, $p>0.05$). The calcaric regosol and the lithic leptosol had significantly higher counts at 2-4cm depths compared to the ferralic arenosol (Figure 18), (calcaric regosol vs ferralic arenosol: 2cm: $t=5.023$, $df=4$, $p<0.05$; 3cm: $t=3.597$, $df=4$, $p< 0.05$; 4cm: $t= 3.091$, $df=4$, $p< 0.05$), lithic leptosol vs ferralic arenosol (2cm: $t=4.471$, $df=4$, $p<0.05$; 3cm: $t=9.215$, $df=4$, $p< 0.05$; 4cm: $t= 2.197$, $df=4$, $p< 0.05$). There was no significant difference in the counts of *Bacillus anthracis* in ferralic arenosol soil at the 11-12cm depths compared to the calcaric regosol and the lithic leptosol (ferrralic arenosol vs lithic leptosol 11-12cm: t=-1.657, df=4, p>0.05; ferrralic arenosol vs lithic leptosol 11-12cm: t=-1.622, df=4, p >0.05).

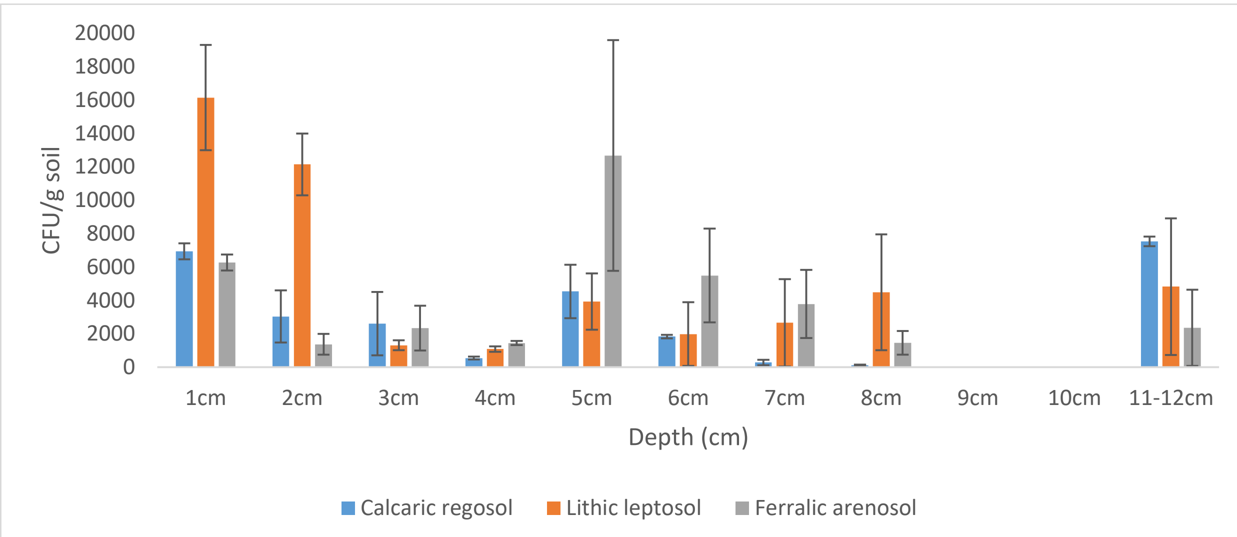


Figure 19: Mean number of *B. anthracis* counts per gram of soil (CFU/g) at sampling point T_3 (4 weeks), in each soil type at each depth. The key indicates the calcaric regosol, ferralic arenosol and the lithic leptosol study soils. The bars indicate the standard error of the mean.

After 4 weeks (T3) *B. anthracis* spores had leached to the 11-12cm depths in all three soils (Figure 19). There was no significant difference in the counts of *B. anthracis* counts in the calcaric regosol and lithic leptosol soils compared to the ferralic arenosol at the 1,2 and 11-12 cm depths compared to the ferralic arenosol soil. Calcaric regosol vs ferralic arenosol (1cm: t=0.982, df=4, p>0.05; 2cm: t=0.992, df=4, p> 0.05; 11-12cm: t= 2.257, df=4, p> 0.05), lithic leptosol vs ferralic arenosol (1cm: t=1.907, df=4, p>0.05; 2cm: t= 5.537, df=4, p> 0.05; 11-12cm: t= 0.528, df=4, p> 0.05). The lithic leptosol soil type had the highest number of *B. anthracis* counts on the surface (1-2cm) and 8cm depths compared to the calcaric regosol and the ferralic arenosol soil types (Figure 19). The ferralic arenosol soil type had significantly higher *B. anthracis* counts from 4cm-7cm depths compared to the calcaric regosol and the lithic leptosol. Calcaric regosol vs ferralic arenosol (4cm: $t=5.914$, $df=4$, $p>0.05$; 7cm: $t=-1.712$, $df=4$, $p> 0.05$;) and lithic leptosol vs ferralic arenosol (4cm: $t=1.714$, $df=4$, $p>0.05$; 7cm: $t= 0.339$, $df=4$, $p> 0.05$;)

4.2 Behaviour of *B. anthracis* in a water environment

4.2.1 Water-soil jar (JAR A)

The presence of anthrax in different layers of a water environment were quantified by estimating and comparing colony counts of *B. anthracis* and are presented in Figures 20-23 below. The Shapiro-Wilk test revealed that the mean colony count data of *B. anthracis* in this experiment were not normally distributed (24hrs: W= 0.632, df=50, $p<0.05$; 48hrs: W= 0.496, df=50, $p<0.05$). A Chi-square test revealed that there was a significant difference ($\chi^2 = 54.244$, df =4, $p < 0.05$) in the colony counts of *B. anthracis* sampled from the different layers in the jar (Figure 20).

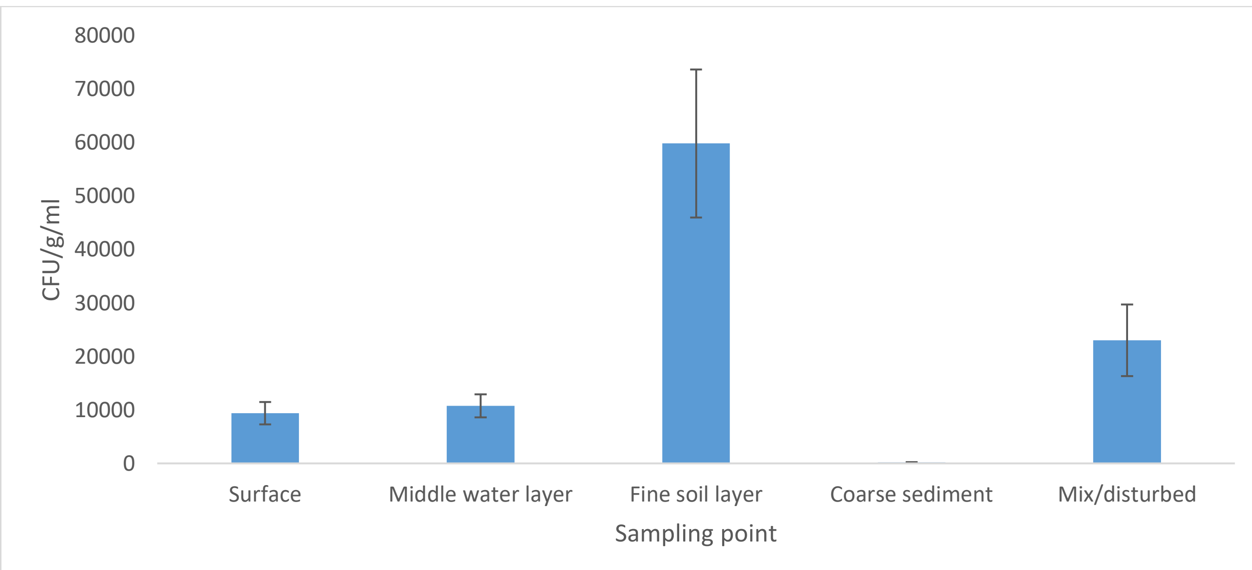


Figure 20: Mean of total counts of *B. anthracis* (CFU/g/ml) in different layers of a water environment (jar), irrespective of sampling time. The samples in the water jar from the surface of the water to the coarse soil later that settled at the bottom of the jar. The bar for 'mix' colony counts represents samples taken after mixing the water, soil, blood and inoculum of *B. anthracis* spores before allowing them to settle down. The bars indicate the standard error of the mean.

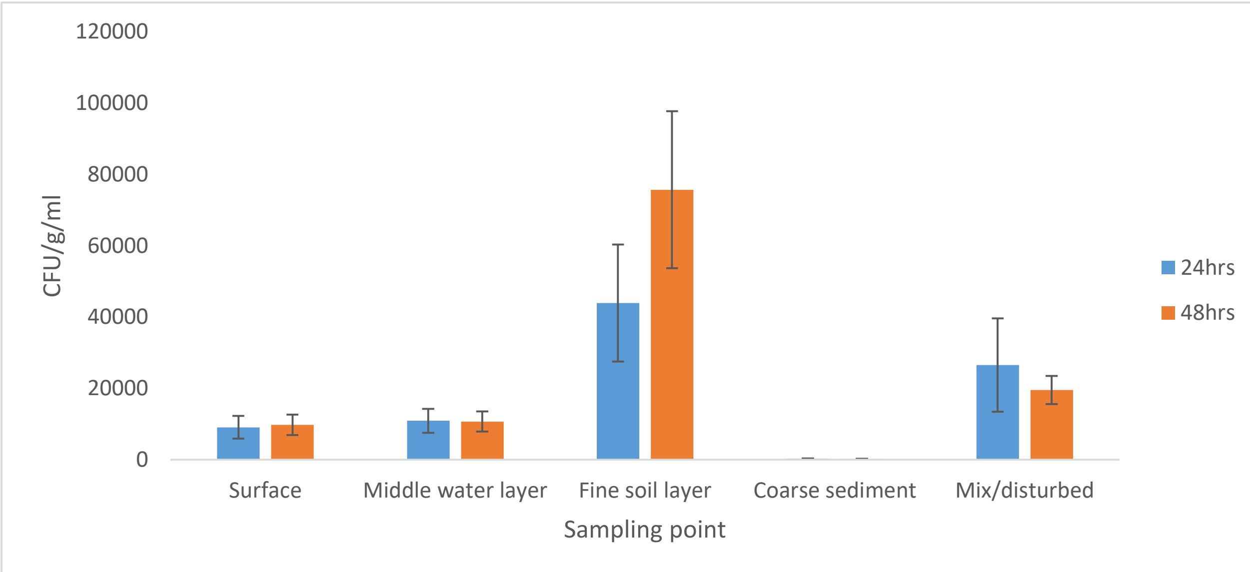


Figure 21: Mean of total counts of *B. anthracis* (CFU/g/ml) after 24 and 48 hours in different layers of a water environment (Jar A). Bars indicate the standard error of the mean.

A two sample t- Test revealed that there was no significant difference in *B. anthracis* counts in the different layers after 24 hours except the fine soil which had a significant difference in B. anthracis compared to the other sampling points. (Surface vs Middle (:t= 0.390, df=18, p>0.05); Surface vs Fine soil (t=-2.085, df=18, p<0.05); Surface vs coarse sediment (t= 2.763, df=18, p>0.05); Surface vs Mix (t= -1.294, df=18, p>0.05); Middle vs Fine soil (t= 1.971, df=18, p<0.05); Middle vs coarse sediment (t= 3.149, df=18, p>0.05); Middle vs Mix (t= 1.153, df=18, p>0.05); Fine soil vs Coarse sediment (t= 2.663, df=18, p<0.05); Fine soil vs Mix (t= 0.830, df=18, p<0.05); Coarse sediment vs mix (t= -2.008, df=18, p>0.05. The Tukey test also revealed that there was no significant difference in *B. anthracis* counts on the surface, in the middle and mix sampling points after 48 hours. However, there was a significant difference in the counts of *B. anthracis* in the fine soil and the coarse sediment compared to the other sampling points after 48 hrs. (Surface vs Middle (:t=-0.233, df=18, p>0.05); Surface vs Fine soil (t=-2.976, df=18, p<0.05); Surface vs coarse sediment (t= 3.332, df=18, p<0.05); Surface vs Mix (t= -2.018, df=18, p>0.05); Middle vs Fine soil (t=-2.934, df=18, p<0.05); Middle vs coarse sediment (t= 3.694, df=18, p<0.05); Middle vs Mix (t=-1.830, df=18, p>0.05); Fine soil vs Coarse sediment (t= 3.434, df=18, p<0.05); Fine soil vs Mix (t=2.514, df=18, p<0.05); Coarse sediment vs mix (t= -4.919, df=18, p<0.05).The coarse sediment had significantly low counts of *B. anthracis* compared to all the sampled layers in the jar after 24 and 48 hours (Figure 21).).

A series of t-tests of the Bonferroni correction post hoc test to compare the counts of *B. anthracis* after 24 and 48hrs revealed that there was no significant difference in the *B. anthracis* counts between the two sampling times (Surface: t= -0.154, df=17, p> 0.05; Middle: t= 0.0498, df=17, p> 0.05; Fine soil: t= 0.101, df=16, p> 0.05; Coarse sediment: t= -1.12, df=17, p> 0.05; Mix: t= 0.510, df=16, p> 0.05).

4.2.2 Water only jar (Control)

The mean colony count data of *B. anthracis* in this experimental jar were not normally distributed (24hrs: W= 0.573, df= 40, p<0.05; 48hrs: W= 0.54, df=40, p<0.05). A Chi-square test revealed that there was no significant difference (χ^2 = 7.723, df =3, p >0.05) in the *B. anthracis* counts in the different layers of the jar (Figure 22).

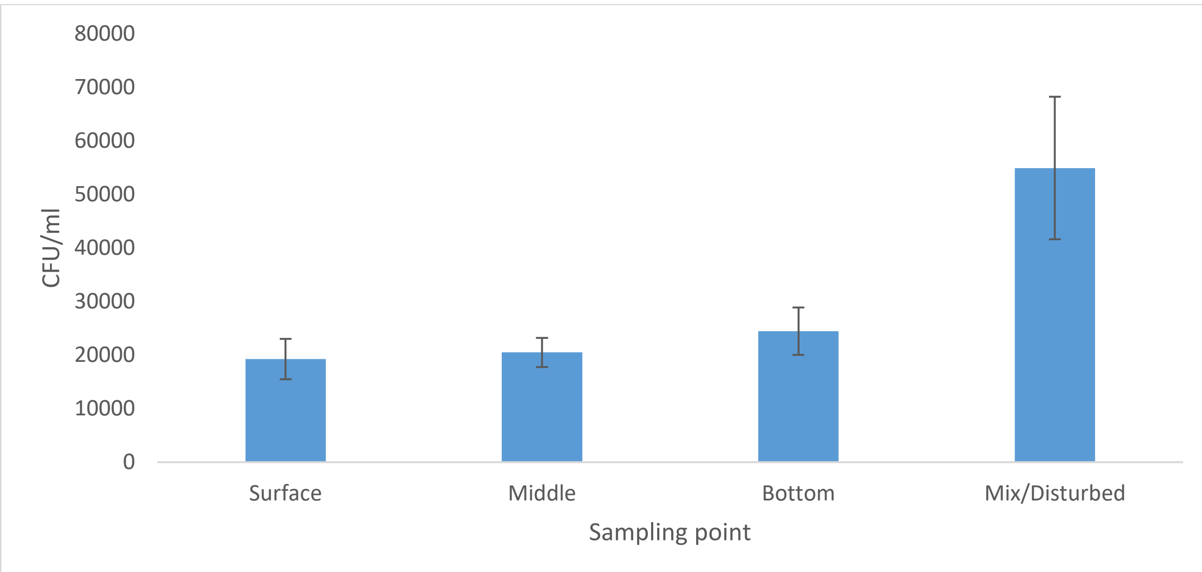


Figure 22: Mean of total counts of *B. anthracis* (CFU/ml) in different layers of a water environment, irrespective of sampling time. The bars indicate the standard error of the mean.

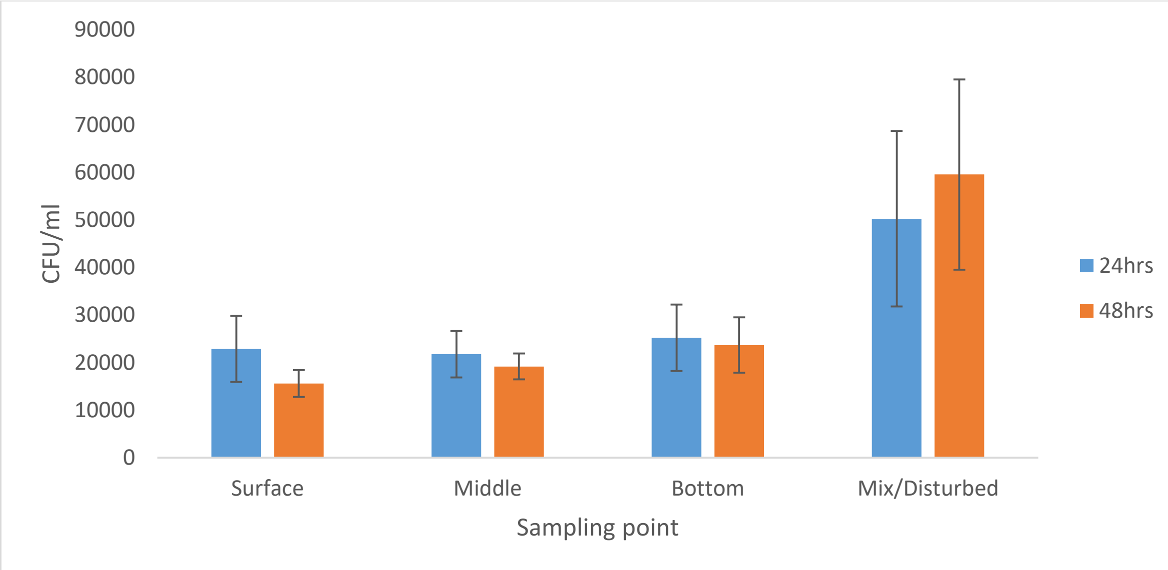


Figure 23: Mean of total counts of *B. anthracis* (CFU/ml) after 24 and 48 hours in different layers of a water environment (jar). The bars indicate the standard error of the mean.

A two sample t test revealed that there was no significant difference in *B. anthracis* counts in the different layers after 24 hours (Surface vs Middle layer ($t= 0.1316$, $df=18$, $p> 0.05$); Surface vs bottom layer ($t= -0.2367$, $df=18$, $p> 0.05$); Surface vs Mix ($t= -1.389$, $df=18$, $p> 0.05$); Middle vs bottom layer ($t= -0.406$, $df=18$, $p> 0.05$); Middle vs Mix ($t= -1.494$, $df=18$, $p> 0.05$); Bottom vs mix ($t= -1.270$, $df=18$, $p> 0.05$).

The two sample t test also revealed that there was no significant difference in *B. anthracis* counts in the different layers after 48 hours (Surface vs Middle layer ($t= -0.914$, $df=18$, $p> 0.05$); Surface vs bottom layer ($t= -1.253$, $df=18$, $p> 0.05$); Surface vs Mix ($t=-2.173$, $df=18$, $p> 0.05$); Middle vs bottom layer ($t=-0.700$, $df=18$, $p> 0.05$); Middle vs Mix ($t=-1.996$, $df=18$, $p> 0.05$); Bottom vs mix ($t=-1.719$, $df=18$, $p> 0.05$). A series of t-tests of the Bonferroni correction post hoc test to compare the counts of *B. anthracis* after 24 and 48hrs revealed that there was no significant difference in the *B. anthracis* counts between the two sampling times (Surface: $t= 0.97$, $df=11$, $p>$

0.05; Middle: $t= 0.464$, $df=14$, $p> 0.05$; Bottom: $t= 0.170$, $df=17$, $p> 0.05$; Mix: $t= -0.341$, $df=17$, $p> 0.05$).

CHAPTER 5: DISCUSSION

5.1 The influence of soil type on leaching or deep soil transport of *Bacillus anthracis*

This study investigated the dynamics of *Bacillus anthracis* in water and soils at selected sites in Etosha National Park. It was hypothesized that significantly high amounts of *B. anthracis* spores would remain at the surface of the sandy-loamy calcaric regosol and lithic leptosol soils instead of leaching deep into the soil compared to the sandy ferralic arenosol soil. This spore behavior would increase chances of animals contracting anthrax from the loamy sandy soils, and would render them as important for anthrax transmission. The results of the present study suggest that soil type has a great influence on the deep transport of *B. anthracis* in different soils. A Chi-Square test of association between the *B. anthracis* colony counts and the depth, and the *B. anthracis* colony counts and the soil type revealed that there was a significant positive association between the depth of the soil and *B. anthracis* colony counts (χ^2 =143.330, df = 30, p =.000) and that there was significant positive association between soil type and the *B. anthracis* colony counts (χ^2 =24.77, df = 6, p =.000) (Figure 16 & 17). The *B. anthracis* cell counts decreased in the three soils, the calcaric regosol, ferralic arenosol and the lithic leptosol soils with increasing depth (Figures 16 & 17). Although the ferralic arenosol soil also had a reduced number of *B. anthracis* counts with increasing depth, it had significantly lower *B. anthracis* counts at the depth of 1cm (Figure 16) and had *B. anthracis* leached to deeper depths of 10cm compared to the other two soils which had no *B. anthracis* present from 6-7 cm depths (Figure 16 & 17).

According to Bales et al. (2015), the transport of viruses and bacteria through the soil is influenced by various parameters such as soil characteristics and environmental physical properties. The organic matter is known to greatly influence persistence of spores, where it is believed that soils rich in organic matter are most suitable for anthrax survival (Dey et al., 2012; Parthiban et al., 2015; Mari et al., 2017). In this study it was hypothesized that significantly high amounts of *B. anthracis* spores would be leached deeper in the soil with low organic matter. Less organic matter would increase pore spaces of soil and allow more water to seep through the soil transporting more *B. anthracis* further down into the soil compared to those with higher organic matter. The present study revealed that there was a significant difference in the organic matter of the three soils. The calcaric regosol and lithic leptosol soils in this study had significantly higher organic matter content compared to the sandy ferralic arenosol soil (Figure 11). A study by William et al. (2012) found that the binding of spores to soils varied and depended on the characteristics of the soil such as organic matter content. In the study, spores bound strongly on the soils with the highest organic matter compared to those with lower organic matter, with a 25% reduction in binding. According to Pimentel & Burgess (2013), most soil organic matter is found on the surface of the soil, and could be a possible reason why there was a significantly higher number of *B. anthracis* spores on the surface of the calcaric regosol and lithic leptosol soils (1-4cm) (Figure 16 & Figure 17). Higher content of organic matter in the two soils could mean that the *B. anthracis* spores possibly bound to the organic matter which is mostly found on the surface of the soil, and were not leached deep into the soil as was the case for the ferralic arenosol with very little organic matter. Low organic matter content in the ferralic arenosol soil allows water to seep through faster, transporting the *B. anthracis* to deeper depths as a result. Fewer *B. anthracis* spores being leached in the lower depths of the three soils after 24hrs and after one week may be due to soil characteristics such as organic matter. A study by Havarua et al., (2014), has shown that zebras have a higher bite rate and prefer short grasses in the wet season which could increase their chances of ingesting lethal doses of *B. anthracis* spores while foraging. Zebras are likely to ingest lethal doses which can cause infection when foraging on the calcaric regosol and lithic leptsosol soils, due to more spores being attached on the surface of these soils compared to the ferralic arenosol in the present study. Although it is believed that soils rich in organic matter promote the survival and persistence of *B. anthracis* (Lindique & Turnbull, 1994; Hudson et al., 2008), a study by Cloete (2013) reported that *B. anthracis* persisted longer in the sandy ferralic arenosol soils with lower organic matter compared to the calcaric regosol and lithic leptosol soils. This may be due to the deep transport nature of *B. anthracis* in the ferralic arenosol soil.

A study by Kim & Boone (2009), revealed that pH, water flow and grain size have a positive relationship with bacterial transport through sand. In their study, an increase in sand grain size was associated with increase in spore movement by 82%. Similarly, increases of water velocity enhanced by the presence of bigger pore spaces in larger sand grains may have resulted in spore detachment and movement of the spores to increase (Kim & Boone, 2009). In the present study, the significantly higher percentage (95%) of sand in the ferralic arenosol soil compared to the calcaric regosol and the lithic leptosol (Figure 12), lends support to the possible role of soil texture in the transport of anthrax spores into deeper layers of the soil. High content of sand in the ferralic arenosol may have influenced in faster water movement due to larger pore spaces causing high numbers of the *B. anthracis* spores to be leached deeper compared to the calcaric regosols and the lithic leptosol which has a higher number of spores on the surface (Figures 16 & 17).

Furthermore, the increase in pH can facilitate the movement of microbes through soil due to more negative ions being produced in water further reducing surface interaction (Kim & Boone, 2009).

The study by Kim & Boone (2009) revealed that an increase in pH of sandy soils from 7.2 to 8.5 was associated with increase in the spore movement by 53%. Although the calcaric regosol and the lithic leptosol had a significantly higher pH compared to the ferralic arenosol soil type (Figure 10), *B. anthracis* spores leached deeper into the ferralic arenosol (Figure 16 & 17) in the present study. There was supporting evidence for the hypothesis that significantly higher amounts of *B. anthracis* spores remain at the surface of the soil rather than leaching deep into the soil for the calcaric regosol and lithic leptosols while the same hypothesis rejected for the ferralic arenosol. Transportation of more *B. anthracis* spores deep into the ferralic arenosol soil type would reduce the likelihood of spores becoming available to any passing host. In this study, the significantly higher pH values in the two loamy soils (calcaric regosol and lithic leptosol) compared to the sandy ferralic arenosol (Figure 10) suggest that the pH did not play a big role in the movement of spores deep into the soil.

Climatic factors such as precipitation are suggested to also play a big role in the outbreak of the anthrax disease (Hampson et al., 2011). In the Kruger National Park, an investigation on the influence of rainfall on anthrax epidemics in the Fever Tree Depression by De Vos (1990), showed that a high concentration of anthrax spores was found in the upper 3 centimeters of the soil but reduced overtime with an increase in rainfall. After the heavy rains it was revealed that the upper centimeters had little to no *B. anthracis,* whilst the deeper layers of 10-15 cm showed higher concentrations of *B. anthracis.* A reduction in the amounts of *B. anthracis* on the surface correlated with a drop in anthrax incidences in this area. This may imply that the calcaric regosol and the lithic leptosol soil types which had higher *B. anthracis* concentration on the surface (Figures 16 &17) may be favorable for anthrax transmission at the site of a fresh carcass which died from anthrax within a week. It however may not be the case after 2 and 4 weeks (Figures 18 & 19) because there was no significant difference in the *B. anthracis* counts in the three soils on the surface and the 11-12cm lower depths (Figures 18 & 19). In the present study the sandy ferralic arenosol with significantly lower organic matter coupled with an increase in water added would be expected to have higher counts of *B. anthracis* at the lower depths (11-12cm). It is difficult to compare the present study to the study of de Vos (1990) in terms of rainfall playing a big role in the transport of *B. anthracis* because de Vos (1990) study was exposed to real environmental conditions and not a simulation as the present study which was conducted over a short period of time. Although the *B. anthracis* spores were leached further down (11-12cm) in all soils after 2 and 4 weeks (Figures 18 & 19), it is evident that *B. anthracis* was leached faster compared to the lithic leptosol and the calacric regosol soils. A numbers of factors working together may have an influence on anthrax transmission.

In a study on the ecology of anthrax in a karstveld soil in ENP, Lindeque & Turnbull (1994), reported that the number of *B. anthracis* spores at anthrax carcass sites reduced over time from the superficial soil layers 0-2 cm. They suggested that this reduction might have been caused by dispersal due to wind and water. Similarly, the *B. anthracis* counts in the calcaric regosol, ferralic arenosol and lithic leptosol soils in the present study also declined at the surface 0-3cm (Figures 16,17 and 18), but the reduction in the spores is believed to be attributed more to the seepage of water draining the spores deep into the soil. The infiltration or seepage of spores deep into the soil may also play a big role. However, environmental factors such as wind can play a part and should not be ignored. Wind could possibly also have played a role in fewer spores being present with increasing depths, due to large numbers of spores being carried away by strong wind forces, reducing the number of spores which can pass through the soil. However, comparisons were hard to make between these two studies because the study by Lindeque & Turnbull (1994) was carried out over a longer period and did not focus on deep transport of the *B. anthracis* spores but more on the sporulation of the pathogen on the soil surface. The anthrax carcass sites in their study were also exposed to real rainfall, whereby the reduction in spores could possibly have been due to spores being washed away by water unlike the experiment in the present study which was exposed to simulated rainfall.

The soil moisture content of the soil may also play a role in anthrax transmission. There was a significant difference in the proportion of soil moisture amongst the calcaric regosol, ferralic arenosol and the lithic leptosol soil types (χ^2 = 86.851, df = 10, p <0.05; Figure15). The calcaric regosol and lithic leptosol soils retained more soil moisture compared to the ferralic arenosol in this study (Figure 15). The moisture content of soil depends on the soil type which may influence the persistence of anthrax spores (Parthiban et al., 2015; Mari et al., 2017). In the present study the soil texture may have greatly influenced the moisture content of the soils. This study revealed that there was a significant difference in the percentage of sand in the three soils (F=21.85181, df= 2, p<0.05), with the ferralic arenosol soil containing 95% of sand (Figure 12). A very high percentage of sand in the ferralic arenosol means that a lot of bigger pore spaces are available in the soil, which would result in a faster evaporation rate of water from the soil thus leading to a lower retention of water compared to the calcaric regosol and lithic leptosol soils (Figure 15). Similarly, the sandy soil in a study by Cloete (2013) had a lower soil moisture content and a higher persistence of *B. anthracis*. In the present study the soil moisture content declined with increasing depth and the *B. anthracis* counts increased at the lower depths of the ferralic arenosol (Figure 15). Besides the large pore space of the sandy ferralic arenosol playing a role in the increase of the anthrax spores further down in the soil, there is a possibility that *B. anthracis* prefers dryer zones for it to multiply and persist. According to WHO (2008), the survival of *B. anthracis* spores is enhanced by dryness. Although *B. anthracis* may persist longer in the sandy soil due to favourable drier soil, the fact that the spores are leached deep into the soil reduces the likelihood of a host ingesting large amounts of spores to cause anthrax. Hence, we can conclude that the sandy ferralic arenosol soil is not a possible source of anthrax infection.

5.2 Behavior of *Bacillus anthracis* in a water environment

In this study, it was hypothesized that significantly high amounts of *B. anthracis* spores are suspended in the turbid disturbed water sediment mixture and not in the underlying soil sediment. This spore behavior would increase chances of animals contracting anthrax rendering the water reservoir important for anthrax transmission. A laboratory experiment on the behavior of *B. anthracis* revealed that there was a significant difference (Figure 20 & 21) in the colony counts sampled from the different layers in the experimental jars. In this study the fine soil had a significantly higher number of *B. anthracis* spore counts and not in the turbid sediment as hypothesized (Figure 20 & 21). This could possibly be attributed to the spores having a strong adsorption to soil (Lindeque & Turnbull, 1994). This attachment of spores to the soil might have led to a reduced number of *B. anthracis* spores on the surface or in the water column. *B. anthracis* spores are believed to have a buoyant density allowing them to freely move on the surface of water (Dragon & Rennie, 1995), however it was not to be the case in this study. The amount of *B. anthracis* in the fine soil layer increased after 48 hours and reduced in the turbid sediment, hence there was no sufficient evidence to accept the hypothesis that most spores would end up in the turbid sediment. Furthermore, Dragon & Rennie (1995) suggest that clumps of organic matter which carry bigger numbers of spores may float. However, the soil used in the present study was a sandy ferralic arenosol which has very low organic matter. No clumps were formed in this soil and thus, *B. anthracis* spores did not float the surface.

A study was carried out by Lindique & Turnbull (1994), where *B. anthracis* was found in soil and water samples from different water bodies. Although it was known that *B. anthracis* was present in the soil and water samples, samples were only drawn either from only the soil or the water and not both samples from the same water body. In addition, the samples were natural samples which were exposed to natural environmental conditions and were not controlled, making comparisons between the two studies difficult. According to Ebedes (1976), spores are constantly ingested by animals when they drink water that may have anthrax yet do not get the disease. This has been attributed to the behavior of the spores that attached to the fine soil particles, with very few ending up in the water as revealed in the present study. This behavior of anthrax attaching to soil particles may reduce chance of animals being infected by anthrax in water bodies. Ebedes (1976) stated that although water sources have been found to contain *B. anthracis*, animals were unlikely to ingest a lethal dose of spores from the relatively low quantities of *B. anthracis* found in water samples (Lindique & Turnbull, 1994; Turner et al., 2016).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

A field based experiment was carried out to determine how deep transport of *Bacillus anthracis* may have an influence on the transmission dynamics of anthrax. The study revealed that soil type has an influence on deep transport of *B. anthracis* spores. The sandy ferralic arenosol soil used in this study had a lower number of spores on the surface compared to the calcaric regosol and lithic leptosol soils. It was hypothesized that the *B. anthracis* spores would remain on the surface in all three soils. It has been shown in the present study that the sandy soil has larger pore spaces and lower organic matter resulting in deeper leaching of *B. anthracis* spores into the soil. Deep leaching of *B. anthracis* spores into the soil reduces the chances of a passing host ingesting lethal doses to cause disease. Furthermore, this study also revealed that most *B. anthracis* spores were attached to the finer soil particles and were not suspended in the turbid sediment as hypothesized. In conclusion, sandy soils may not be an important reservoir in the transmission of anthrax. The attachment of most *B. anthracis* spores to soil does not strengthen the hypothesis that disturbed sediment-water mixture can be important for *B. anthracis* transmission. Sandy soils are less likely to support the spread of *B. anthracis* in both water and soil reservoirs.

6.2 Recommendations for future research

The experiments that were carried out in this study were done over a very short period and may not adequately explain how *B. anthracis* behaves in the environment in response to varying climatic and environmental conditions. Long term studies on *B. anthracis* dynamics should be carried out and with exposure to real environmental conditions such as rainfall and temperature. A study by Wolff & Stewart-Akers (2013) on endospores of *Bacillus* in sand revealed that large numbers of *Bacillus* were recoverable from depths as deep as 1 meter. This clearly shows that deep leaching of *B. anthracis* may also play a big role in the persistence of the bacterium and contribute to anthrax outbreaks. However, in this study the *B. anthracis* spores were limited to thimbles of short depth and were not exposed to water over a very long period. A similar study should be carried out in the natural environment where the *B. anthracis* spores are exposed to all environmental conditions and their influence on deep transport of the bacterium are being monitored over a period of years. Similarly, an experiment on the behavior of anthrax in a water environment should be carried out in the natural environment in larger water bodies and with exposure to sunlight. *B. anthracis* may act differently in larger water bodies as in the real world due to dilution effects of blood and spores from an infected animal. The exposure of *B. anthracis* spores to high levels of solar radiation might also cause spores on the surface of water bodies to be killed by high temperatures (Hugh-Jones & Blackburn, 2009). However, this experiment was carried out in the laboratory and was not exposed to solar radiation which can have an influence on spore dynamics. Different soil types with varying organic matter should also be used in this type of experiment since the organic matter may largely influence the attachment of spores to soils. A number of beetles were also seen feeding on the soil in the thimbles in the experimental enclosure during the collection of thimbles after 24 hrs and 7 days. It is recommended that controlled studies be carried out in the future to determine whether these ground-dwelling invertebrates can have a significant influence on the dynamics of anthrax, especially when in large numbers on fresh anthrax carcass sites. Future studies may also include vegetation, and must be done in a time frame that spans all the three seasons identified in the Etosha National Park. Understanding the dynamics of *B. anthracis* in different environmental reservoirs may add to knowledge already known about the disease and can also help in managing the disease where possible.

REFERENCES

1. Almeida, J.I., Harper, B. & Cole, K.D. (2008). *Bacillus anthracis* spore suspension: determination of stability and comparison of enumeration techniques. *Journal of Applied Microbiology*, 104:1-7
2. Bales, R.C., Li, S., Maguire, K.M., Yahya, M.T., Gerba, C.P. & Harvey, R.W. (2015). Virus and bacteria transport in a sandy aquifer. *Cape Cod MA Ground Water*, 33:653-661
3. Barandongo, Z.R, Mfune, J.K and Turner, W.C. (2018). Dust-bathing behaviors of African herbivores and the potential risk of inhalational anthrax. *Journal of Wildlife Diseases*, 54(1): 33-44
4. Baumgardner, D.J. (2012). Soil-Related Bacterial and Fungal Infections. *Clinical Review*, 25(5): 734-744
5. Bellan, S. (2011). Counting Wildlife Carcasses: Anthrax surveillance in Etosha National Park, Namibia. *SACEMA*, Pp 1-3
6. Bellan, S.E., Turnbull, P.C.B., Beyer, W., & Getz, W.M. (2013). Effects of Experimental Exclusion of Scavengers from Carcasses of Anthrax-Infected Herbivores on *Bacillus anthracis* Sporulation, Survival, and Distribution. *Applied and Environmental Microbiology*. *JournalsASM.org*,79 (12): 3759-3761
7. Bengis, R. G & Frean, J. (2014). Anthrax as an example of the One Health Concept. *Rev.sci.tech.Off.Int Epiz,* 33(2): 593-604
8. Berry, H.H. (1993). Surveillance and control of anthrax and rabies in wild herbivores and carnivores in Namibia. *Rev. sci.tech. Off. Int. Epiz*,12 (1):137-146
9. Beugler-Bell, H. & Buch, M.W. (1997). Solid and soil erosion in Etosha National Park, Northern Namibia. *MADOQUA*, *20* (1): 91 – 104.

10. Beyer, W., Bellan, S., Eberle, G., Ganz, H.H., Getz, W.M., Haumacher, R., Hilss, K.A., Kilian, W., Turner, W.C., & Turnbull P.C.B. (2012). Distribution and Molecular Evolution of *Bacillus anthracis* Genotypes in Namibia. *Neglected Tropical Diseases*, 6(3):1-12

11. Bhatnagar, R. and Batra, S. (2001). Anthrax toxin. *Critical Reviews in Microbiology*, 27(3): 1-35

12. Blackburn, J.K., Van Ert, M., Mullins, J.C, Hadfield, T.L., & Hugh-Jones, M.E. (2014). The Necrophagous fly Transmission Pathway: Empirical and Genetic Evidence from Wildlife Epizootics. *Vector-Borne and Zoonoic Diseases*, 14(8):1-9

13. Brown, E.R. & Cherry, W.B. (2015). Specific identification of *Bacillus anthracis* by means of a variant bacteriophage. *Journal of Infectious Diseases*, 96: 34-39

14. Cizauskas, C.A, Bellan, S.E, Turner, W.C, Vance, R.E and Getz, W.M. (2014). Frequent and seasonally variable sublethal anthrax infections are accompanied by short-lived immunity in an endemic system. *Journal of Animal Ecology*, 83:1078-1090

15. Cizauskas, C.A, Turner, W.C, Wagner, B., Kuster, M., Vance, R.E and Getz, W.M. (2014). Gastrointestinal helminths may affect host susceptibility to anthrax through seasonal immune trade-offs. *BMC Ecology*, 14(27): 1-1

16. Cloete, C. (2013). Influence of site and soil type on the distribution of anthrax infection sites and the contribution of anthrax to elephant mortality in Etosha National Park, Namibia. Unpublished Master's dissertation, University of Namibia, Windhoek.

17. Csuros, M. & Csuros, C. (1999). Microbiological examination of water and wastewater. *Lewis Publishers*,1-311

18. De Vos, V & Turnbull, P.C.B. (2004). Infectious Diseases of Livestock. *Bacterial diseases*, 3(3): 1-33

19. de Vos, V. (1990). The Ecology of Anthrax in the Kruger National Park, South Africa.

20. Dey, R., Hoffman, P.S., & Glomski, J.I. (2012). Germination and amplification of Anthrax Spores by Soil-Dwelling Amoebas. *Applied and Environmental Microbiology*, 78(22): 1-7

21. Dipti, M. (2013). Morphological and Immunological Characterization of Anthrax Vaccine in Cattle. Unpublished Masters dissertation, Bangladesh Agricultural University, Mymensingh.

22. Dragon, D.C., Rennie, R.P. (1995). The ecology of anthrax spores: Tough but not invincible. *Canadian Veterinary Journal*, 36: 295-301

23. Ebedes, H. (1976). Anthrax epizootics in Etosha National Park. *Madoqua,* 10(2): 99-118

24. Edwards, K.A., Clancy, H.A., & Baeumner, A.J. (2006). Bacillus anthracis: toxicology, epidemiology and current rapid-detection methods. *Review*, 384: 1-12

25. Fasanella, A., Galante, D., Garofolo, G., & Hugh-Jones, M. (2009). Anthrax undervalued zoonosis. *Veterinary Microbiology*, 140(3-4): 1-33

26. Gainer, R. (2016). Yamal and Anthrax. *The Canadian Veterinary Journal,* 57(9): 985-987

27. Ganz, H.H, Turner, W.C, Brodie, E.L, Kusters, M., Shi, Y., Sibanda, H., Tomk, T. & Getz, W.M. (2014). Interactions between *Bacillus anthracis* and Plants May Promote Anthrax Transmission. *Neglected Tropical Diseases*, PLOS:1-22

28. Hampson K, Lembo T, Bessel P et al. (2011). Predictability of anthrax infection in the Serengeti, Tanzania. *Journal of Applied Ecology* ,48(6): 1333-1344

29. Havarua, Z., Turner, W. and Mfune, J.K.E. (2014). Seasonal variation in foraging behaviour of plains zebra (*Equus quagga*) may alter contact with the anthrax bacterium (*Bacillus anthracis*). *Canadian Journal of Zoology,* 92: 331-337

30. Hellstrom, A.C. (2013). The Secret Life of *Bacillus anthracis*. 1-15

31. Hipondoka, M. (2017). Rainfall map for Etosha National Park. Windhoek.

32. Hudson, J.M., Beyer, W., Bohm, R., Fasanella, A., Garofolo, G., Golinski, R., Goossens, P.L., Hahn, U., Hallis, B., King, A., Mock, Michele., Cesare, M., Ozin, A., Tonello, F., Kaufmann. (2008). *Bacillus anthracis*: Balancing innocent research with dual-use potential. *International Journal of Medical Microbiolgy*, 298(2008):345-364.

33. Hugh-Jones, M. & Blackburn, J. (2009). The ecology of *Bacillus anthracis*. *Molecular Aspects of Medicine*, 30: 1-12

34. Hugh-Jones, M.E & de Vos, V. (2002). Anthrax and wildlife. *Rev Sci Tech. Off. Int. Epiz,* 21(2): 359-383. PubMed: 11974621

35. Keim, S.K., and Wagner, D.M. (2009). Humans and evolutionary and ecological forces shaped the phylogeography of recently emerged diseases. *Nature Reviews*, 8, 1-9

36. Kim, M. & Boone, S.A. (2009). Factors that influence the transport of *Bacillus cereus* spores through sand. *Water Air Soil Pollution*, 199: 151-157

37. Knisely, R.F. (1966). Selective medium for *Bacillus anthracis*. *Journal of Bacteriology, 92*: 784-786.

38. Koehler, T.M. (2009) *Bacillus anthracis* Physiology and Genetics. *Mol Aspects Med*, 30(6): 386-396

39. Kooper, L. (2017, September 10). 'Anthrax kills 109 hippos in Bwabwata'. *The Namibian*, p.1.

40. Le Roux C.J.G., Grunow J.O., Morris J.W, Bredenkamp G.J, and Scheepers, J.C. (1988). A classification of the vegetation of the Etosha National Park. *S.Afr. J. Bot*, 54(1):1-10

41. Lindeque, P.M. & Turnbull, P.C. (1994). Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. *Onderstepoort Journal of Veterinary Research, 61*(1), 71-83.

42. Lindique, P.M. (1991). Factors affecting the incidence of anthrax in the Etosha National Park, Namibia. Dissertation. Porton Down, Salisbury, UK.

43. Magwedere, M., Hemberger, M.Y., Hoffman, L.C., & Dziva, F. (2012). Zoonoses: a potential obstacle to the growing wildlife industry of Namibia. *Infection Ecology and Epidemiology*, 2: 1-16 in the text it must cited with et al

44. Mari, A.S., Kamboh, A.A., Bhutto, K., Sahito, I.A and Abro, S.H. (2017). Prevalence of *Bacillus anthracis* spores in soil of district Badin. *Journal of Animal Health and Production*, 5(2): 79-84

45. Misgie, F., Atnaf, A. & Surafel, K. (2015). A Review on Anthrax and its Public Health and Economic Importance. *Academic Journal of Animal Diseases*, 4(3): 196-204.

46. Mullins, J.C., Garofolo, G., Van Ert, M., Fasanella, A., Lukhnova, L., Hugh-Jones, M.E & Blackburn, J.K. (2013). Ecological Niche Modelling of *Bacillus anthracis* on Three Continents: Evidence for Genetic-Ecological divergence? *PLos ONE*, 8(8): 1-8

47. Nakale, A. (2017). 'Ugab lion died from anthrax'. *New Era*, p.1.

48. Parthiban, S., Malmarugan, S., Murugan, M.S, Rajeswar, J.J & Pothiappan, P. (2015). Anthrax: A re-emerging livestock disease. *International Journal of Nutrition and Food Sciences*,4(4): 7-12

49. Pimentel, D. & Burgess, M. (2013). Soil erosion threatens food production. *Agriculture,* 3: 443-463

50. Razali, N.M & Yap, B.W. (2011). Power Comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling Tests. Data Analytics. *Journal of Statistical, Modeling and Analytics*, 2(1): 21-33

51. Ruggiero, R.G. (1992). Seasonal forage utilization by elephants in central Africa. *Afr. J. Ecol*, 30:137-148

52. Saile, E. and Koehler, M.T. (2006). *Bacillus anthracis* Multiplication, Persistence, and Genetic Exchange in the Rhizosphere of grass plants. *Applied and Environmental Microbiology,* 72(5): 3168-3174

53. Shaanika, H. (2013,). Anthrax outbreak strikes Oshikoto. *New Era*, p.1.

54. Shikongo, A. (2019,). Anthrax fears in Kunene, Zambezi. *The Namibian*, p.3.

55. Smith, J. (2018, Anthrax outbreak in Sesfontein area. *Namibian Sun*, p.1.

56. Stableforth, A.W., and Galloway, I.A. (1959). Infectious diseases of animals: Diseases due to bacteria. *Sterne/ Anthrax*, 1-20

57. Turner, W.C, Kausrud, K.L, Beyer W et al. (2016). Lethal exposure: An integrated approach to pathogen transmission via environmental reservoirs. *Scientific Reports*, 1-13

58. Turner, W.C, Kausrud, K.L, Krishnapa S.Y et al. (2014) Fatal attraction: vegetation responses to nutrient inputs attract herbivores to infectious anthrax carcass sites. *Biological Sciences,* 1-9

59. Turner, WC, Imologhome P, Havarua Z et al. (2013) Soil ingestion, nutrition and seasonality of anthrax in herbivores of Etosha National Park. *Ecosphere, 4*(1): 1- 19.

60. Valseth, K., Nesbo, C.L., Easterday, W.R., Turner, W.C., Olsen, J.S., Stenseth, N.C, and Haverkamp, T.H.A. (2017). Temporal dynamics in microbial soil communities at anthrax carcass sites. *BMC Microbiology*, 17(206): 1-15

61. Vilas-Boas, G.T, Peruca, A.P.S. and Arantes, O.M.N. (2007). Biology and taxonomy of *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*. *Review /Synthese,* 53*:* 673-687

62. Williams, G., Linley, E., Nicholas, R. & Baillie, L. (2012). The role of the exosporium in the environmental distribution of anthrax. *Journal of Applied Microbiology*, 114: 396-403

63. Wolff, R.J & Stewart-Akers, A.M. (2013). Endospores of Bacillus in sand soils and the implications. *Military Applications of Medical Geology*,53(6): 1-2

64. World Health Organisation. (2008). Anthrax in humans and animals. Geneva, Switzerland. WHO Press.

APPENDICES

Appendix 1: Research permit issued by the Ministry of Environment and Tourism to carry out the study.

MINISTRY OF ENVIRONMENT AND TOURISM

RESEARCH/COLLECTING PERMIT

Permit Number 2276/2017
Valid from 1 April 2017 to 31 March 2018

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975) to:

Name: **Ms. C. Cloete**
Address: **Etosha Ecologial Institute**
Etosha National Park
P O Box 6
Okaukuejo via Outjo
Namibia
Coworkers: **W. Kilian, Dr. K. Kausrud, Dr. J. Mfune, M. Evard, Dr. R. Easterday, H. Schønhaug, Dr. W. Turner and Z. Barandongo**

Climatic fluctuations and the ecology of anthrax in Etosha National Park, subject to attached conditions.

IMPORTANT: This permit is not valid if altered in any way

13 MAR 2017

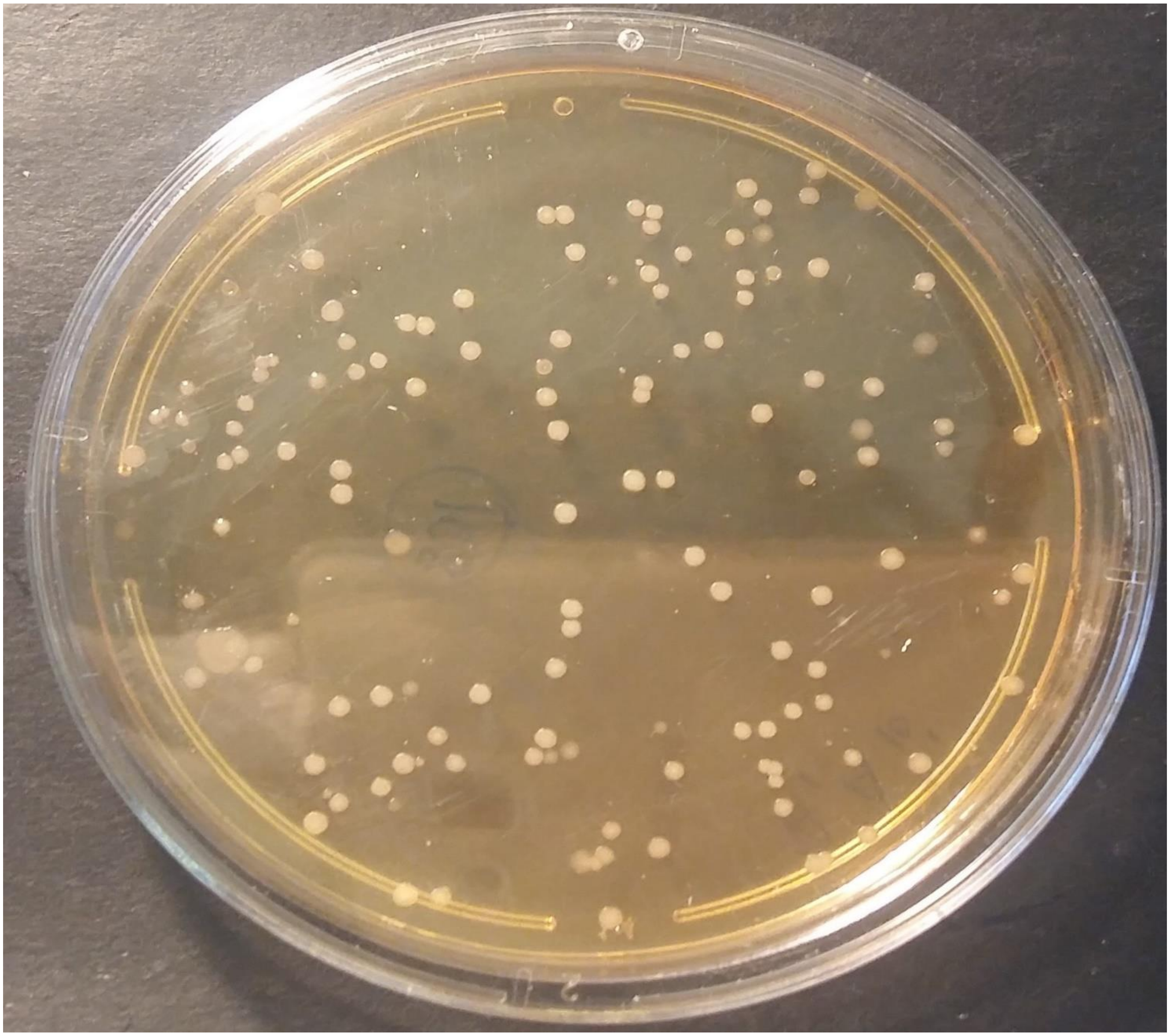
Private Bag 13306, Windhoek
Tel: 2842111 - Fax: 258861

Authorising Officer

IMPORTANT
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations.

Enquiries: Conservation Scientist, email: ita.mathaus@met.gov.na
Private Bag 13306, Windhoek, Namibia

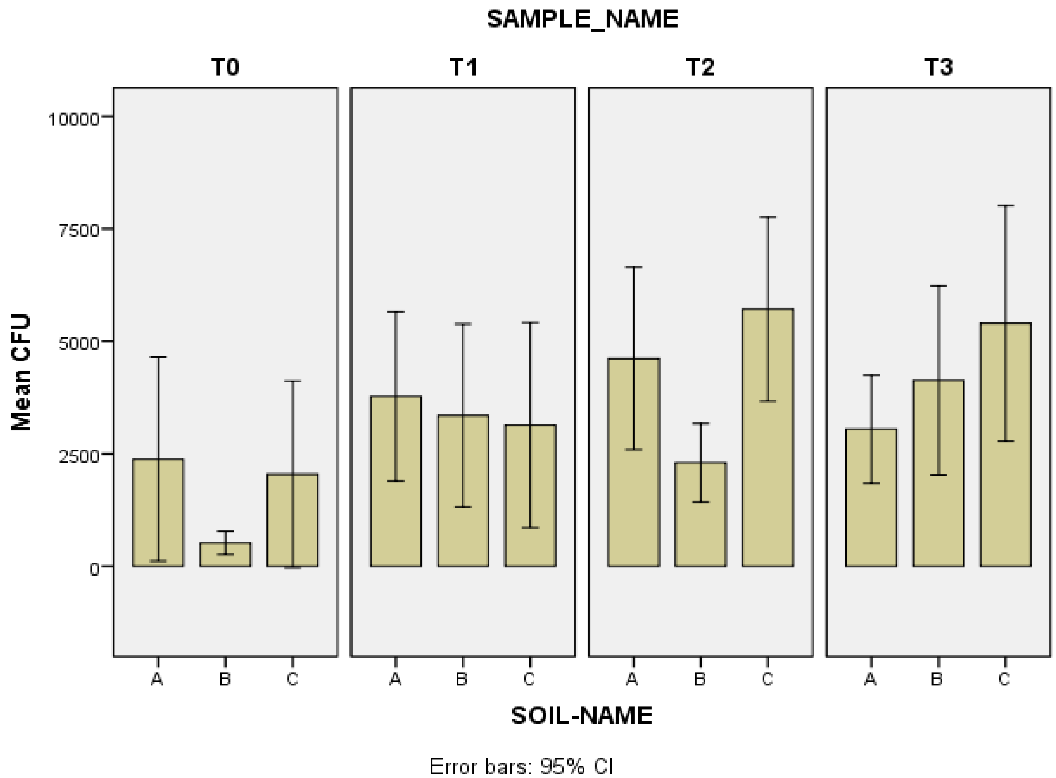
Appendix 2: Growth of *Bacillus anthracis* on PLET agar (Photo: Modesta Evard, 2018).



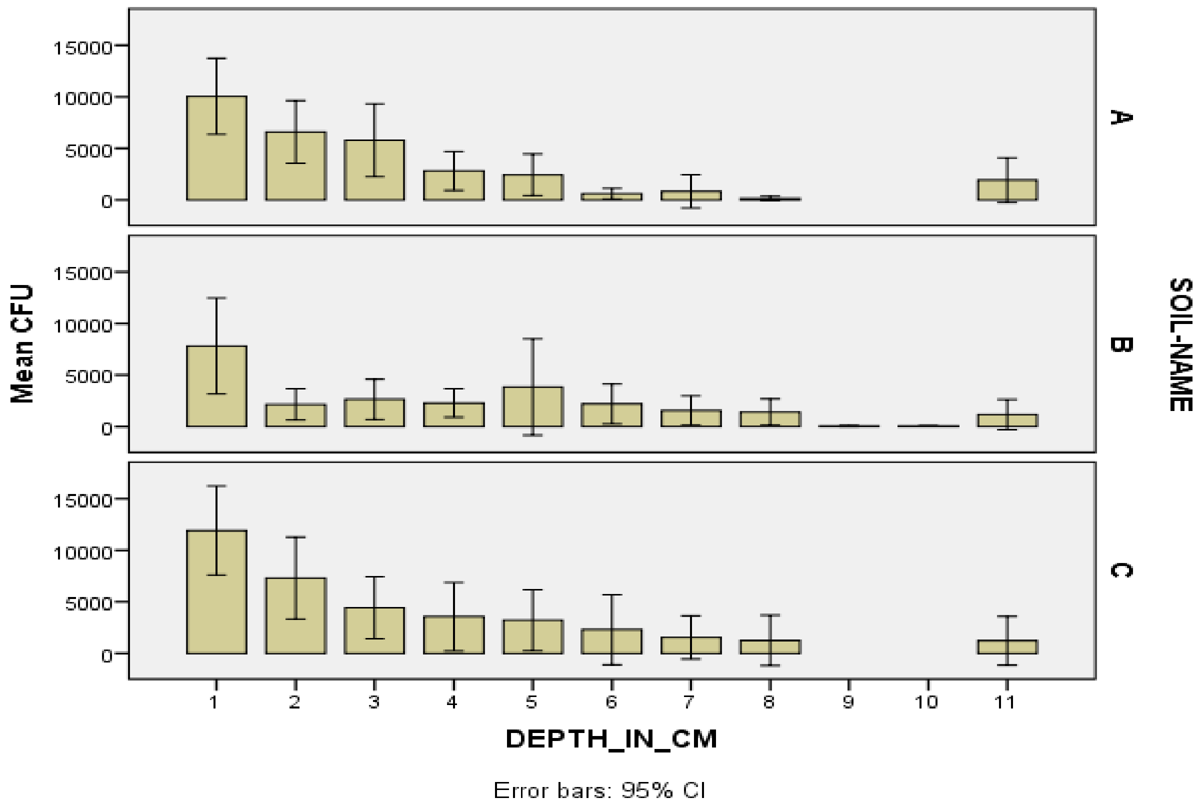
Appendix 3: Soil properties of the soils used in this study.

Soil identification	pHw	ECw uS/cm	OM %	Texture	Sand %	Silt %	Clay %
Okaukuejo	8.24		2.84	Sandy loam	48.6	46.4	5
Okaukuejo	8.24		2.98	Sandy loam	42.8	51.6	5.6
Okaukuejo	8.24		1.5	Sandy loam	45.5	48.2	6.4
Namutoni	7.86		0.56	Sandy	95.1	2.2	2.7
Namutoni	7.89		0.69	Sandy	96	1.8	2.2
Namutoni	7.7		0.4	Sandy	95.5	2.4	2.2
Rietfontein	7.94		2.97	Sandy loam	82.7	11.4	6.9
Rietfontein	8.1		2.93	Sandy loam	54.8	41.5	3.7
Rietfontein	8.3		2.5	Sandy loam	55.5	34.1	10.4

Appendix 4: Mean CFU/g of soil in the study soils at each time point irrespective of the depth (A-Calcaric regosol; B-Ferralic arenosol; C-Lithic leptosol).



Appendix 5: Mean CFU/g of soil in the study soils at each depth in each soil type irrespective of sampling point (A-Calcaric regosol; B-Ferralic arenosol; C-Lithic leptosol).



Appendix 6: *Bacillus anthracis* counts in different water layers of the treatment jar.

TRIAL NO	A1	A2	A3	A4	A5
	SURFACE	MIDDLE WATER LAYER	FINE SOIL LAYER	COARSE SEDIMENT	MIX/DISTURBED
1	6100	15300	60000	238	133000
2	3300	4100	147000	115	25600
3	1000	2800	6000	927	11600
4	200	1400	6000	20	700
5	2800	2400	28000	1210	18700
6	300	1000	43000	187	11000
7	1300	1700	21300	43	5800
8	200	1400	6000	24	700
9	10600	10500	15200	122	12400
10	800	600	10700	42	4000
11	13600	18800	141000	302	28400
12	100	300	3900	106	2400
13	22000	23000	108000	215	59000
14	15800	17000	71000	201	46000
15	24800	22300	76000	81	25300
16	19200	24600	60000	303	26500
17	13800	15500	3000	123	8300

18	4700	5000	23600	91	7000
19	25700	24600	154000	68	12000
20	22100	23700	213000	139	22500

Appendix 7*: Bacillus anthracis* counts in different water layers of the control jar.

TRIAL NO	B1 SURFACE	B2 MIDDLE	B3 BOTTOM	B4 MIX/DISTURBED
1	21000	11800	10400	11100
2	3200	2500	3300	3000
3	11500	28300	30000	75000
4	11000	12800	9400	13900
5	8100	12500	7700	16600
6	13200	13900	17500	19300
7	7700	10100	8700	14200
8	21300	22000	23300	24900
9	20300	19400	24900	27300
10	18300	24000	22100	25700
11	20800	24800	25600	80000
12	5300	11300	12500	191000
13	8300	20700	12100	137000

14	9400	13800	24000	13600
15	20700	17900	15500	196000
16	26700	24600	22900	28100
17	29000	26700	54000	29500
18	19700	20500	24200	64000
19	26300	28900	55000	16900
20	72000	55000	26100	57000