# INFLUENCE OF SITE AND SOIL TYPE ON THE DISTRIBUTION OF ANTHRAX INFECTIOUS SITES AND THE CONTRIBUTION OF ANTHRAX TO ELEPHANT MORTALITY IN ETOSHA NATIONAL PARK, NAMIBIA

A THESIS SUBMITTED IN FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

OF

THE UNIVERSITY OF NAMIBIA

BY

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MARCH 2013

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## ABSTRACT

*Bacillus anthracis*, a soil-borne pathogen, causes the deadly zoonotic disease anthrax. Anthrax outbreaks occur frequently in Etosha National Park (ENP). ENP has nine major soil types and a distinct rainfall gradient decreasing from east to west. The sporulation success of *B. anthracis* is believed to be influenced by soil type, but the influence of soil type and site along a rainfall gradient on the persistence of *B. anthracis* in the soil is unknown.

The present study investigated the influence of site along the rainfall gradient and soil type on the persistence of *B. anthracis* in three soil types, ferralic arenosols, calcaric regosols, and haplic arenosols. To determine the effect of soil type and site on the persistence of *B. anthracis*, a reciprocal transplant experiment was conducted at three experimental sites across ENP following the rainfall gradient in which soil cores were spiked with a virulent *B. anthracis* strain. Sampling was done over 12 months to determine the persistence of the bacilli over time. Soil from elephant (*Loxodonta africana*) carcass sites was also collected and tested for the presence of *B. anthracis*. A selective media called polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar was used to isolate *B. anthracis* from the soil. This study revealed that site did not have a significant effect on *B. anthracis* cell counts. However, soil type had a significant effect on *B. anthracis* cell counts. The sandy ferralic and haplic arenosols of the east and west that had the lowest soil moisture content were found to have a significantly greater influence on the persistence of the bacterium compared to the calcaric regosols of central ENP. The sandy soils may, therefore, serve as endemic areas of anthrax infection in ENP. This study further revealed that anthrax did not contribute significantly to elephant mortality in ENP.

**Key words:** *Bacillus anthracis*, anthrax, Etosha National Park, anthrax infectious sites, *Loxodonta africana*, soil type, reciprocal transplant experiment.

All maps, graphs and tables presented in this thesis were produced by the author, unless otherwise stated.

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## ABBREVIATIONS

ANOVA – Analysis of Variance

$a_w$ – Water activity

BW – Biological warfare

BWC – Biological Weapons Convention

CDC – Centres for Disease Control and Prevention

CFU – Colony forming unit

CVL – Central Veterinary Laboratory

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetra-acetic acid

EEI – Etosha Ecological Institute

EF – Oedema factor

ENP – Etosha National Park

GIS – Geographic Information System

GPS – Global Positioning System

LF – Lethal factor

MET – Ministry of Environment and Tourism

NCC – Neurocysticerocosis

OIE – World Organisation for Animal Health

PA – Protective antigen

PCR – Polymerase Chain Reaction

PLET – Polymyxin-lysozyme-EDTA-thallous acetate

PVC – Polyvinyl chloride

rcf – relative centrifugal force

| | |
|---|---|
| RH | – Relative humidity |
| SEM | – Standard error of the mean |
| TE | – Tris/EDTA |
| Tris | – Trisaminomethane |
| USA | – United States of America |
| UV | – Ultra-violet |
| WHO | – World Health Organisation |
| WW I | – World War One |
| WW II | – World War Two |

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## ACKNOWLEDGMENTS

I could not have completed this study without the input of others. I would have been lost without the help of many people and I hope that I do not forget to thank anyone. Firstly, I would like to extend my appreciation to my three supervisors. My sincerest gratitude goes to Dr. John Mfune who has been a source of motivation since my undergraduate years. Thank you for your time, tireless effort and encouragement, and thank you for caring. I deeply appreciate the input of Dr. Holly Ganz. Holly, you have been invaluable to this study. Thank you for taking me as your student, and for introducing me to the incredible world of anthrax. I am also very grateful for everything you taught me and for your help in the lab, especially with the colony counts. I am also indebted to Dr. Percy Chimwamurombe for his insightfulness and contribution to the completion of this thesis.

I thank the Ministry of Environment and Tourism for granting me permission to do research in the magnificent Etosha National Park (ENP). I thank the Deputy Director of ENP, Mr. Boas Erkkie, and the Chief Control Warden of ENP, Rehabeam Erckie, for their assistance and encouragement. I appreciate the help of area wardens Mssrs. Shayne Kötting, Immanuel Kapofi, and Bonnie Simaata. I also appreciate the assistance and advice of the Chief Conservation Scientist, Mr. Werner Kilian, and the rest of the Etosha Ecological Institute staff. Thank you for allowing me the use of equipment, office, and laboratory space. A very special thank you goes to the members of the EEI tea club. That minimum one hour a day of your company kept me sane. I am also thankful to the Kötting and Nel families for making me feel at home in Okaukuejo.

I could not have done the collection and analyses of almost 400 soil samples without the help of Zepee Havarua, Martina Küsters, Sarah Bischoff, Christina Law, Kao Thao, Tiffany Chambers, Freedom Jatamunua, and Zoe Barandongo. I would also like to thank Dr. Wendy Turner for her sound advice and continued support. Wendy, your passion for science and Etosha is admirable. Thank you to Ms. Miriam Tsalyuk for her helpful ArcGIS tutorials that enabled me to create the maps in this thesis. I also appreciate the initial input of Dr. Martin Hipondoka for introducing me to the identification and classification of soils.

Finally, this thesis would not have been completed without the love and support of my family and friends. Freddy, I appreciate your patience and motivation. Thank you for believing in me. Sem, thank you for pushing me to reach my full potential. This is evidence of the Warrior within.

This research was funded by the University of California, Berkeley, and the Country Pilot Partnership (CPP) of Namibia under a Young Professional Research Associate (YPRA) Grant.

*“Infectious disease is one of the few genuine adventures left in the world.”*

HANS ZINSSER (1878–1940)

## DECLARATION

I, Claudine C. Cloete, 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.



…… Date ……

Claudine C. Cloete

# CHAPTER 1: INTRODUCTION

## 1.1 General Introduction

Anthrax is a deadly disease of global importance that mainly affects herbivorous wildlife and livestock, especially grazing mammals (Dragon & Rennie, 1995; World Health Organization [WHO], 2008). Grazing mammals are more at risk of contracting anthrax because their feeding habits make them come into contact with soil and dust (Nicholson, 2002). Humans may contract the disease through contact with infected animals or animal products, or through the deliberate release of *Bacillus anthracis* endospores (WHO, 2008). In humans, anthrax is manifested in three ways: pulmonary anthrax, gastrointestinal anthrax, and cutaneous anthrax. Of these, cutaneous anthrax is the most common but the least deadly, while pulmonary anthrax is the least common but the deadliest form of the disease (WHO, 2008).

Anthrax, bovine tuberculosis, rabies, zoonotic sleeping sickness, cysticercosis and neurocysticercosis (NCC), brucellosis, and hydatid disease are neglected zoonotic diseases (WHO, 2005). A zoonosis is a disease that can be transmitted from livestock and wildlife to humans (WHO, 2008). Neglected zoonotic diseases (also known as neglected infectious diseases) are zoonoses that are often overlooked in terms of the allocation of health resources and research (WHO, 2005). Therefore, not much is known about these diseases and they are often misdiagnosed (WHO, 2005). Neglected infectious diseases mostly affect impoverished communities that rely heavily on the livestock they keep for food and which, in some instances, is their only source of income (WHO, 2005). Anthrax is a neglected infectious disease because even though it has been causing disease and death in both animal and human populations for many years, not a lot is known about this disease and not much is

done to control the spread of the disease, especially in poor livestock-keeping communities that are most affected (WHO, 2005).

*Bacillus anthracis* (Cohn 1872) is the bacterial pathogen that causes anthrax. This bacterium is a soil-borne, spore-forming, Gram-positive microbe that is the only obligate pathogen of vertebrates in the genus *Bacillus* (Turnbull, 1996). *Bacillus anthracis* occurs as active vegetative cells within a host body, but forms endospores when exposed to air and conditions unfavourable for vegetative growth (Ebedes, 1976). Some microorganisms can protect themselves from harsh environmental conditions by entering into a dormant endospore state (Madigan, Martinko, Stahl & Clark, 2012). Endospores may germinate outside the host given the right conditions (Saile & Koehler, 2006). *Bacillus anthracis* endospores can survive for long periods of time and were found on animal bones dated approximately 200 years old in the Kruger National Park (KNP), South Africa (de Vos, 1990).

Soil and water sources become contaminated with *B. anthracis* through terminal haemorrhaging from an infected carcass when such a carcass is opened up by scavengers (Gates, Elkin & Dragon, 2001). *Bacillus anthracis* tends to be more concentrated in soils with high pH, soil moisture, and organic matter (Dragon & Rennie, 1995). As a result, the establishment and maintenance of anthrax contaminated sites (hereafter referred to as infectious sites) in nature depend in part on soil characteristics.

Soil types differ widely across Etosha National Park (ENP) and influence the distribution of vegetation in the park (Beugler-Bell & Buch, 1997; Le Roux, Grunow, Morris, Bredenkamp & Scheepers, 1988). Beugler-Bell and Buch (1997)

described the soils of ENP into nine major soil types according to the Food and Agriculture Organization (FAO) Soil Nomenclature. The major soil types in ENP are arenosols, calcisols, cambisols, fluvisols, leptosols, regosols, solonchaks, solonetzs, and vertisols (Beugler-Bell & Buch, 1997). The present study focused on the haplic arenosols, the ferralic arenosols, and the calcaric regosols, as described by Beugler-Bell and Buch (1997).

According to Coetzee (2004), arenosols cover approximately 225 000 $km^2$ of Namibia's surface area. In ENP, the ferralic arenosols are often found on sand plains, are brown in colour, have a sandy texture, are non-calcareous, are more than one metre deep, and are of aeolian origin (Kutuahupiro, Mouton & Coetzee, 2001; Beugler-Bell & Buch, 1997). The calcaric regosols cover the limestone surface and are relatively shallow, calcareous, and sandy-loamy to loamy-clay soils of aeolian origin (Beugler-Bell & Buch, 1997). The calcaric regosols are also known as the karstveld soils (Le Roux, Morris, Grunow, et al., 1988). The haplic arenosols cover a big part of western ENP and are less than one metre deep, are reddish brown in colour, are very low in organic matter, and have a fine sand texture (Beugler-Bell & Buch, 1997).

There is a distinct decreasing rainfall gradient across ENP; rainfall is highest in the east and lowest in the west (Engert, 1997). In ENP, anthrax outbreaks occur annually and mostly affect plains zebra (*Equus quagga*), springbok (*Antidorcas marsupialis*), blue wildebeest (*Connochaetes taurinus*), and the African elephant (*Loxodonta africana*; Lindeque & Turnbull, 1994). The peak in anthrax cases in plains zebra and elephant populations differs in space and time, and correlates with rainfall (Lindeque & Turnbull, 1994). The peak in anthrax mortality in plains zebra is

observed towards the end of the wet season, whilst the peak in anthrax mortality in elephants is observed towards the end of the dry season in ENP (Lindeque & Turnbull, 1994). This phenomenon sparked the interest to undertake extensive research on the occurrence of anthrax in ENP and led to the establishment of a large anthrax ecology research project in the park, which includes the present study. Studying the incidence of *B. anthracis* in the soil may help to identify endemic areas of infection which may be related to anthrax outbreaks, and thus contribute to a better understanding of the ecology of anthrax in ENP. It may also help facilitate the management and control of the disease if park managers deem the disease to be causing a decline in animal populations in ENP. Therefore, the aim of the present study was to determine the influence of site along the rainfall gradient and soil type on the distribution of anthrax infectious sites and the contribution of anthrax to elephant mortality in ENP. The distribution of anthrax infectious sites was identified by determining the effect of soil type and site on *B. anthracis* persistence in the soil.

### 1.2 Statement of the Problem

In ENP, anthrax outbreaks in elephants and plains zebra differ in space and time, and these outbreaks are associated with rainfall patterns (Lindeque, 1991). Anthrax outbreaks are highest in elephants during the dry season and mostly occur to the west of Okaukuejo (Lindeque & Turnbull, 1994). A peak in anthrax mortalities in plains zebra occurs during the wet season in the Okaukuejo area (Lindeque & Turnbull, 1994). Rainfall decreases from east to west in ENP while soil type varies throughout the park. The problem is that it is not known how the persistence of *B. anthracis* endospores is influenced by the different soil types and the rainfall gradient

across the park. Thus, the distribution of anthrax infectious sites in ENP is unknown. As a result, the present study investigated how these two variables influenced the persistence of *B. anthracis* endospores. This information would help to identify new areas of endemicity of anthrax in ENP. Together, the influence of soil type and change in rainfall patterns may influence seasonal anthrax outbreaks in ENP.

### 1.3 Objectives

The specific objectives of the present study were:

a. To determine and compare the effect of site along the rainfall gradient on the persistence of *B. anthracis* endospores in ENP.
b. To determine and compare the persistence of *B. anthracis* endospores among three soil types in ENP.
c. To determine the contribution of anthrax to elephant mortalities and the distribution of anthrax carcasses in ENP.

### 1.4 Hypotheses

a. The survival and persistence of *B. anthracis* endospores is enhanced by dryness. Therefore, the persistence of *B. anthracis* endospores is greater in sites that do not receive high annual rainfall.
b. Soils with higher pH and organic matter enhance *B. anthracis* endospore survival. Soil types vary in pH and organic matter. Thus, it is expected that soil type affects the persistence of *B. anthracis* endospores.

c. *Bacillus anthracis* spores can be detected at elephant carcass sites. Therefore, the contribution of anthrax to elephant mortalities and the distribution of anthrax carcasses in ENP can be determined.

## 1.5 Significance of the Study

This study characterizes the role of soil type on infectious zones of *B. anthracis* and identifies endemic areas of infection in ENP. Furthermore, this study provides a better understanding of the contribution of *B. anthracis* infections to elephant mortalities in the park. The findings of the present study may also provide park managers with insight into how *B. anthracis* endospore persistence is affected by changes in rainfall gradient. Knowledge of the influence of rainfall gradient on the dynamics of anthrax will help to predict how global climate change may affect future patterns of anthrax outbreaks, especially if it will lead to drying of some areas of ENP. Etosha National Park is one of the protected areas set aside for the conservation of Namibia's wildlife. The park is a huge tourist attraction area; more than 100 500 tourists – both local and foreign – visited ENP during 2011 (Ministry of Environment & Tourism [MET], 2012). African elephants make up one fifth of the Big Five, which comprises of the lion, leopard, buffalo, and rhinoceros, and are a huge attraction for tourists. The results of this study will contribute to the management of elephant and zebra populations, which both have touristic and conservation value. The results will also provide a better understanding of the natural anthrax transmission in herbivores and may pave a way for integrated sustainable land

management not only in ENP, but also the game and livestock farms surrounding the Park.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Introduction

*Bacillus anthracis* is a soil-borne, endospore-forming bacterium (Coker, 2002; Gates, Elkin & Dragon, 2001). *Bacillus anthracis* endospores are resistant to many environmental factors that normally limit or prevent bacterial growth, including ultra-violet (UV) radiation, desiccation, acidity (pH), and extreme temperature (WHO, 2008). The resistance of *B. anthracis* endospores to these environmental factors allows them to persist in the soil for a very long time (WHO, 2008). *Bacillus anthracis* causes the disease anthrax (WHO, 2008). In ENP, anthrax infects elephants and plains ungulates such as zebra and springbok at different times of the year (Lindeque, 1991). Since *B. anthracis* endospores are able to persist in the soil for so long, it is important to study the factors that influence sporulation and endospore survival, and relate these to anthrax mortalities.

This literature review focuses on the history of anthrax, the life cycle, pathogenesis and ecology of *B. anthracis*, anthrax in Namibia and ENP, and the use of anthrax in biological warfare.

## 2.2 History of Anthrax

The word anthrax, Latin for carbuncle, is derived from the Greek word for coal which refers to the black skin lesions characteristic of cutaneous anthrax (Schwartz, 2009; Turnbull & Shadomy, 2011). Anthrax is an ancient disease believed to have been the fifth and sixth plagues in the Bible in Exodus, chapters 7-9 (reviewed in Schwartz, 2009). Anthrax remained the cause of widespread mortality in livestock up to the 19$^{th}$ century (Dragon & Rennie, 1995). Robert Koch (1843-1910)

demonstrated that *Bacillus anthracis* is the causative agent of anthrax (Schwartz, 2009; Turnbull & Shadomy, 2011). This led Koch to formulate his well-known postulates which are a set of conditions to be met before a particular microorganism is identified as the cause of a particular disease (Madigan, Martinko, Stahl, et al., 2012). Although anthrax has a worldwide distribution, it is thought to have originated in sub-Saharan Africa (Smith, De Vos, Bryden, Hugh-Jones, Klevytska, et al., 1999). Anthrax is an ancient disease, yet there is still a lot that is unknown about the disease and the bacterium that causes it (Beyer & Turnbull, 2009).

## 2.3 *Bacillus anthracis* and Anthrax

### 2.3.1 Background on *B. anthracis*

*Bacillus anthracis* is the etiological agent of anthrax (WHO, 2008). This bacterium is classified in the family *Bacillaceae* which consists of heterotrophic bacteria that are able to produce endospores (Turnbull, 1996). The anthrax bacterium further belongs to the *B. cereus* group within the large and diverse genus *Bacillus* (Turnbull, 1996). Within the *B. cereus* group, *B. anthracis* is the only obligate pathogen of vertebrates and is probably one of the most well-known microorganisms (Turnbull, 1996; Koehler, 2009; Schwartz, 2009). *Bacillus anthracis* produces two anthrax toxins and the poly-γ-D-glutamic capsule that distinguishes this bacterium from other members in the closely related *B. cereus* group (Pilo & Frey, 2011; Koehler, 2009). *Bacillus anthracis* is a Gram-positive, rod-shaped, soil-borne, non-motile, aerobic bacterium that is also an endospore-former (WHO, 2008).

### 2.3.2 Anthrax, the Disease

#### 2.3.2.1 The Life Cycle of *B. anthracis*

*Bacillus anthracis* endospores enter the host by either ingestion, inhalation, or through skin lesions (Turnbull, 1996). In the environment, *B. anthracis* predominantly occurs in its spore form, but germinates into its vegetative form inside a host body due to favourable conditions that allow for the proliferation of the bacilli (WHO, 2008; Hugh-Jones & de Vos, 2002). The virulence of *B. anthracis* is distinguished by the presence of two plasmids, pXO1 and pXO2 (Koehler, 2009). The pXO1 plasmid is known as the toxin plasmid because it encodes for the lethal factor (LF) and the oedema factor (EF), two virulence factors that bind to the protective antigen (PA) and work together to kill the host cells (Pilo & Frey, 2011; WHO, 2008). The pXO2 plasmid encodes for the poly-γ-D-glutamic capsule which protects the bacterium from phagocytosis (Pilo & Frey, 2011; WHO, 2008). The host eventually dies of septicaemia (WHO, 2008). When the anthrax bacilli are released into the environment upon host death, the vegetative forms revert back to their spore form in the presence of oxygen (WHO, 2008; Hugh-Jones & de Vos, 2002; Lindeque & Turnbull, 1994). Endospores are able to withstand many environmental factors that inhibit bacterial growth (WHO, 2008). Endospores can therefore persist in the soil for decades, and in so doing, have the opportunity to infect more hosts (Hugh-Jones & de Vos, 2002). The cyclic sporulation and germination of *B. anthracis* is vital for this bacterium's survival (Koehler, 2009). A diagrammatic representation of the *B. anthracis* life cycle is provided in Figure 1.

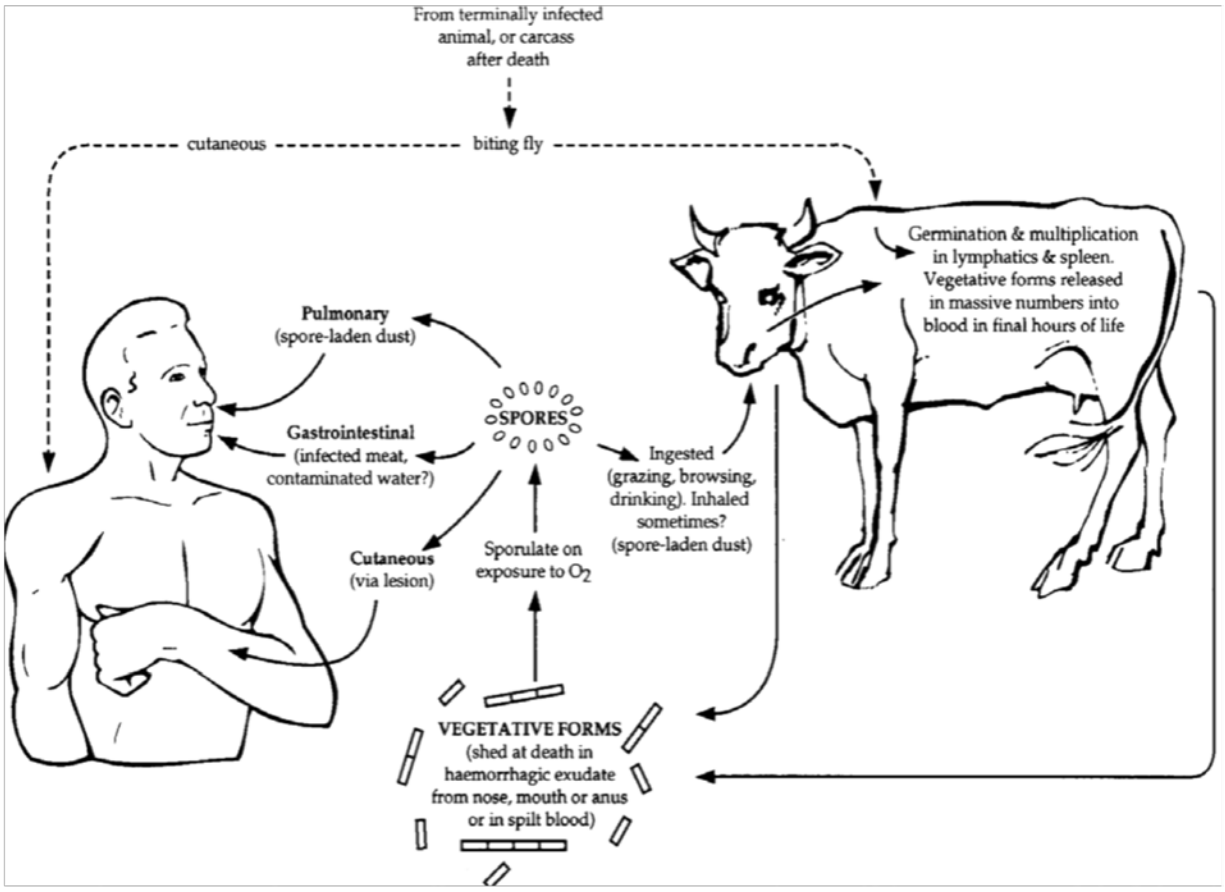


**Figure 1:** The cycle of anthrax infection (WHO, 2008).

### 2.3.2.2 Anthrax in Animals and Humans

Although anthrax is mainly manifested in herbivorous mammals, all mammals and some birds, such as ostrich, may become infected with the disease but the incidence in non-herbivores is less frequent (WHO, 2008). In experimental studies where laboratory animals were inoculated with virulent strains of *B. anthracis*, these animals (mice, guinea pigs, and rabbits) had an incubation period that ranged between 36 and 72 hours (Beyer & Turnbull, 2009), while cattle can have an incubation period of more than 14 days (WHO, 2008). In the natural environment the incubation period of anthrax is presumed to be between one and fourteen days (Hugh-Jones & de Vos, 2002). However, the World Organization for Animal Health (OIE) stipulates the incubation period of anthrax to be 20 days (World Organization

for Animal Health, 2011). In the natural situation, animals usually do not show any symptoms prior to their sudden death (WHO, 2008). Slow *rigor mortis* and terminal haemorrhaging from the nose, anus, and other openings are common signs that an animal died of anthrax (Hugh-Jones & de Vos, 2002). The fluid exuding during terminal haemorrhaging is usually dark in colour and fails to clot (Lindeque, 1991). Other signs of anthrax in animals include an enlarged spleen, swollen lymph nodes, rapid decomposition, and excessive oedema (Beyer & Turnbull, 2009; Hugh-Jones & de Vos, 2002). It was found that *B. anthracis* spores are able to persist at carcass sites for extended periods of time and is believed to be the cause of subsequent anthrax infection in herbivores (Lindeque, 1991). Lindeque (1991) further found that the *B. anthracis* spores declined over time at carcass sites where terminal bleeding occurred. However, Lindeque (1991) could not detect any viable *B. anthracis* spores at elephant carcass sites more than two months after the animal had died of anthrax.

In humans, anthrax infections occur in three forms namely inhalational anthrax, cutaneous anthrax, and gastrointestinal anthrax (Edwards, Clancy & Baeumner, 2006; Riedel, 2005). Humans can contract gastrointestinal anthrax by ingesting undercooked meat from an infected animal (Beatty, Ashford, Griffen, Tauxe & Sobel, 2003). This sometimes happens in areas where food is scarce and when people eat raw or inadequately cooked meat (Beatty, Ashford, Griffen, et al., 2003).

Of the three forms of anthrax infection in humans, pulmonary anthrax has the highest mortality, whilst cutaneous anthrax is the least deadly (WHO, 2008). Compared to gastrointestinal and cutaneous anthrax, the risk of contracting pulmonary anthrax is quite low (WHO, 2008). However, the risk of contracting pulmonary anthrax is increased significantly in the event of bioterrorist activities

when anthrax spores are released in high concentrations (WHO, 2008). Cutaneous anthrax is characterised by the formation of an eschar where the bacterium entered the host through a skin lesion (WHO, 2008). In the case of gastrointestinal anthrax, the bacterium can enter the bloodstream through a lesion in the gastrointestinal tract. This lesion generally forms an ulcer (WHO, 2008). Initial symptoms of this anthrax infection include nausea, vomiting, diarrhoea, and fever. These symptoms become more severe, and as the infection progresses without treatment, the host dies of septicaemia (WHO, 2008). The incubation period for both gastrointestinal and cutaneous anthrax is normally one week (WHO, 2008).

The initial symptoms of inhalational anthrax are similar to influenza. These symptoms include fever, fatigue, and a cough (Riedel, 2005). The incubation period for inhalational anthrax is approximately four to eleven days (WHO, 2008). Secondary infections like anthrax meningitis and anthrax sepsis may develop after exposure to any of the three primary anthrax infections (WHO, 2008; Riedel, 2005).

#### 2.3.2.3 Diagnosis and Treatment of Anthrax

*Bacillus anthracis* can be identified using a variety of techniques including bacterial culturing, staining and microscopy, serology, and genetics (WHO, 2008). Anthrax can be cured by starting early antibiotic treatment (WHO, 2008). Penicillin is still the antibiotic of choice, with ciprofloxacin and doxycycline as common alternatives (WHO, 2008).

## 2.4 Ecology of *Bacillus anthracis*

In order for the *B. anthracis* bacterium to survive as a species, it needs to maintain its sporulation-germination cycle (Koehler, 2009; WHO, 2008). There are various environmental factors affecting this cycle. The process of sporulation is influenced by temperature, pH, humidity, water activity ($a_w$), and available oxygen (WHO, 2008). In ENP it was found that the success of *B. anthracis* sporulation at anthrax carcass sites depended on soil type (Lindeque & Turnbull, 1994). The persistence of *B. anthracis* endospores in the environment is affected by $a_w$, temperature, pH, and the presence of nutrients and germinants (WHO, 2008). Davies (1960) emphasised that the persistence of anthrax spores in the soils of subtropical countries, such as Namibia, can be explained by high temperature and relative humidity (RH). Davies (1960) observed that maximum sporulation occurred within 12 hours at 37 °C and 100% RH.

The presence of germinants such as amino acids and ribonucleosides elicits germination in *B. anthracis* (Driks, 2009). In order for germination to commence, bacterial spores need temperature in the range of 8-45 °C, pH between 5 and 9, and 96% or higher relative humidity, in addition to germinants (WHO, 2008). In an experimental study, Davies (1960) found that maximum germination of *B. anthracis* endospores occurred within three hours at 39 °C and in the presence of germinants.

Van Ness (1971) hypothesised that anthrax spores germinate and proliferate in incubator areas that have favourably high pH and temperature above 15.5 °C. This is known as the “incubator area” hypothesis (Van Ness, 1971). However, Dragon and Rennie (1995) are of the opinion that *B. anthracis* spores do not germinate in the natural environment without artificial enrichment. Recent studies indicate that

germination may occur in association with other organisms, such as plants (Saile & Koehler, 2006), earthworms (Schuch & Fischetti, 2009), and soil amoeba (Dey, Hoffman & Glomski, 2012). Saile and Koehler (2006) observed that *B. anthracis* endospores germinate in the nutrient-rich rhizosphere of fescue (*Festuca arundinacea*) grasses. Horizontal gene transfer was also observed during that study (Saile & Koehler, 2006). Lysogenic bacteriophages enable *B. anthracis* to survive in its vegetative form in soil and in the gut of earthworms (Schuch & Fischetti, 2009). Dey, Hoffman and Glomski (2012) found that *B. anthracis* endospores germinate and proliferate inside *Acanthamoeba castellanii*, an amoeba commonly found in moist soils.

*Bacillus anthracis* endospores favour alkaline soils with high calcium and organic matter content (Hugh-Jones & Blackburn, 2009). Sporulation is a state of rest during which the organism is protected until conditions become conducive for growth (Nicholson, 2002). Anthrax spores are formed when conditions inside the dead host becomes unfavourable for cell growth and upon exposure to oxygen when bacilli are released into the environment during terminal haemorrhaging (Lindeque & Turnbull, 1994). The site where the bacilli were released has the potential to infect new hosts (Lindeque, 1991). The level of contamination by *B. anthracis* endospores at a carcass site decreases with time (Coker, 2002; Lindeque & Turnbull, 1994). This decrease in endospore numbers may be attributed to the effects of climatic conditions, such as wind and precipitation (Coker, 2002). Even though the number of anthrax spores decline over time at carcass sites, these spores are able to persist at infectious sites for long periods of time (Lindeque & Turnbull, 1994).

Certain soil factors like calcium levels may be responsible for the prolonged viability of spores in the soil and thus increase the likelihood of infecting new hosts (Dragon & Rennie, 1995). The relationship between sporulation and climatic factors such as temperature, $a_w$, and relative humidity greatly influences the occurrence of anthrax outbreaks (WHO, 2008). Dryness may also enhance the persistence of anthrax spores in the environment (WHO, 2008).

## 2.5 Anthrax in Namibia

Anthrax was documented in Namibia starting in pre-colonial times and sporadic outbreaks were recorded across the country since the 1870s (Ebedes, 1976). In Namibia, anthrax is considered an endemic disease with sporadic outbreaks occurring throughout the country in livestock and wildlife (Beyer, Bellan, Eberle, Ganz, Getz, et al., 2012). In the former South West Africa (SWA), 1541 anthrax cases were confirmed on White-owned farms from 1920 to 1971 (Ebedes, 1976). However, livestock were regularly vaccinated against the disease (Ebedes, 1976). No information is available on the incidence of anthrax in livestock in Owamboland in northern Namibia during colonial rule, but approximately 1413 people were treated for anthrax at mission hospitals and clinics during the period 1926-1941 (Ebedes, 1976). Of these, approximately 246 human fatalities due to anthrax were reported which indicated that the disease was prevalent in the northern regions of Namibia and resulted in intensive vaccination of livestock in the area (Ebedes, 1976).

More recently, anthrax has been recorded in both livestock and wildlife, mostly in communal farming communities. Mortality due to anthrax is a regular occurrence in domestic and wild animals in the Caprivi region. A major anthrax outbreak was

observed during 2004 in the Caprivi region where 152 buffalo, 42 cattle, 15 elephant, and two zebras dead of the disease (F. Chitate, pers. comm.).

During 2012, a commercial farm in the Omaheke region of eastern Namibia was placed under quarantine after it was suspected that three farm workers had died of anthrax after eating meat from a cow that died of unknown causes (Kisting, 2012). The quarantine was later lifted since no conclusive cause of death of the three deceased was found (Kisting, 2012). Farm Kroonster apparently suffered great financial losses due to the quarantine (Kisting, 2012). Currently, the socio-economic impact of anthrax in Namibia is unknown. However, it can be inferred that the quarantine of commercial livestock and game farms will have unfavourable financial implications on such businesses. Communal farmers may also be negatively affected should an outbreak occur in these areas. This is because poor livestock-keeping communities rely heavily on their livestock for their subsistence (WHO, 2005).

During January 2013 an anthrax outbreak was reported in the Oniipa Constituency in the Oshikoto region (Kisting, 2013). Three people died of anthrax during the outbreak and another 22 received treatment for the disease (Kisting, 2012). The Directorate of Veterinary Services of the Ministry of Agriculture, Water and Forestry started a mass livestock vaccination campaign in the area (Kleinhans, 2013). Communal farmers do not always vaccinate their livestock against anthrax and therefore outbreaks are not uncommon.

Anthrax is a soil-borne disease that causes sporadic outbreaks in southern Africa. Outbreaks are mostly detected in game reserves where it affects a variety of wildlife species. Examples of game reserves affected by the deadly disease in the southern Africa region are Etosha National Park, Namibia, Kruger National Park,

South Africa, Luangwa Valley, Zambia, and Malilangwe Wildlife Reserve, Zimbabwe (Clegg, Turnbull, Foggin and Lindeque, 2007).

### 2.6 Anthrax in Etosha National Park (ENP)

Even though anthrax was diagnosed in animals in the areas surrounding ENP, anthrax was first confirmed in the park in 1964 (Ebedes, 1976). Today, annual anthrax outbreaks are observed in ENP (Beyer, Bellan, Eberle, Ganz, Getz, et al., 2012). Anthrax may have become enzootic to the Okaukuejo, Namutoni, and Andoni areas due to the over-utilization of resources by animals (Ebedes, 1976). Ebedes (1976) further observed that anthrax outbreaks occurred in the Okaukuejo area since 1964. Lindeque (1991) found that the incidence of anthrax mortalities in the Okaukuejo area is proportional to monthly rainfall. A peak in anthrax mortalities of elephants was observed towards the end of the dry season whereas anthrax mortalities peak towards the end of the rainy season for the plains ungulates (Lindeque, 1991; Figure 2).

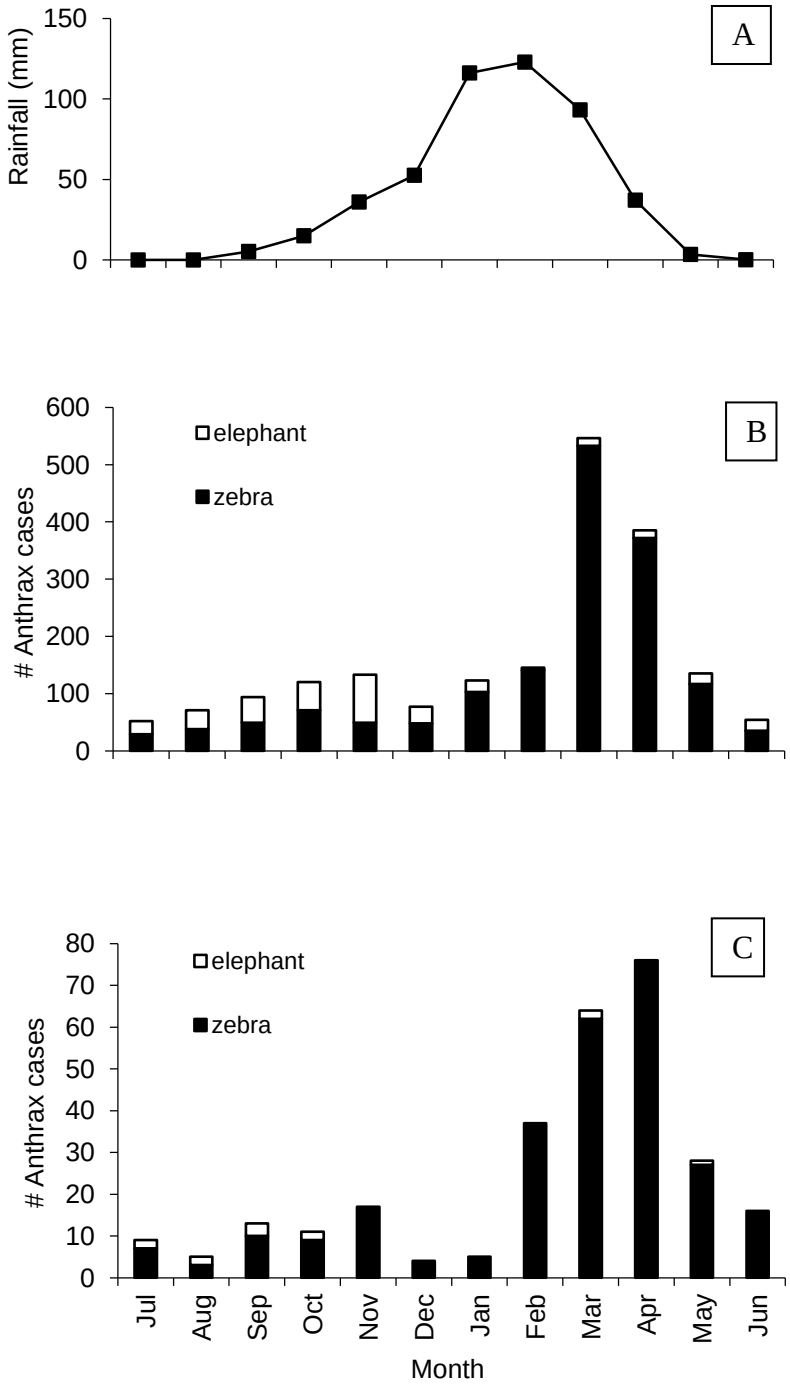


**Figure 2:** The seasonal incidence of anthrax mortalities in elephants and plains ungulates in relation to rainfall in Etosha National Park (Turner et al., 2013). (A) The mean monthly rainfall as recorded at Okaukuejo during 2005-2011, (B) number of anthrax mortalities in elephant and zebra during 1968-2011 throughout ENP, and (C) number of anthrax mortalities in elephant and zebra during 2005-2011 in central ENP (Okaukuejo area).

The incidence of anthrax in ENP differs in time and space. The majority of the plains ungulates, including the plains zebra, are found in the enzootic Okaukuejo area during the wet season (Ebedes, 1976). It is in this area where the highest number of anthrax mortalities in the plains zebra is observed (Lindeque, 1991; Figure 3).

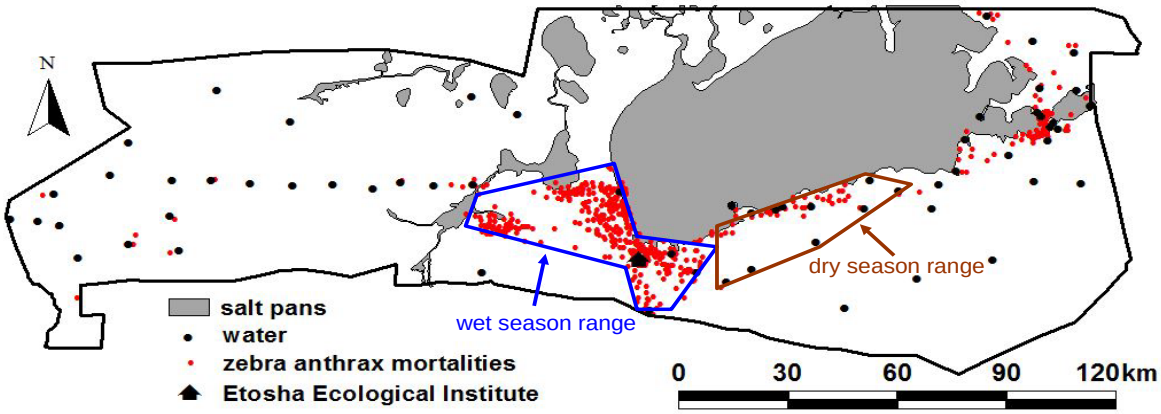


**Figure 3:** The seasonal distribution of plains zebra and the locality of anthrax mortalities in this species in Etosha National Park (Turner, unpublished data).

In contrast, anthrax mortalities in elephants were noted more during the dry season in the Okaukuejo area and towards the west of the park (Ebedes, 1976; Lindeque & Turnbull, 1994). Anthrax mortality among elephants is also observed during the dry season in the Luangwa Valley, Zambia (Turnbull, Bell, Saigawa, Munyenyembe, Mulenga & Makala, 1991) and in the Chobe National Park, Botswana (ProMed-Mail, 2000). Both male and female elephants are widely distributed throughout the park, especially during the wet season when anthrax outbreaks are more pronounced in plains zebra (Figures 4 & 5). It is their wide distribution that is presumed to be responsible for the increased incidence of anthrax in elephants in the western part of ENP (Lindeque, 1991). Unlike the ENP, the incidence of anthrax mortalities in plains ungulates peaks during the dry season in African game reserves such as the KNP in South Africa and Luangwa Valley in

Zambia (de Vos, 1990; Siamudaala, Bwalya, Munag'andu, Sinyangwe, Banda, et al., 2006). The situation is distinctly different in ENP where anthrax mortalities in the plains ungulates peak during the wet season (Lindeque & Turnbull, 1994).

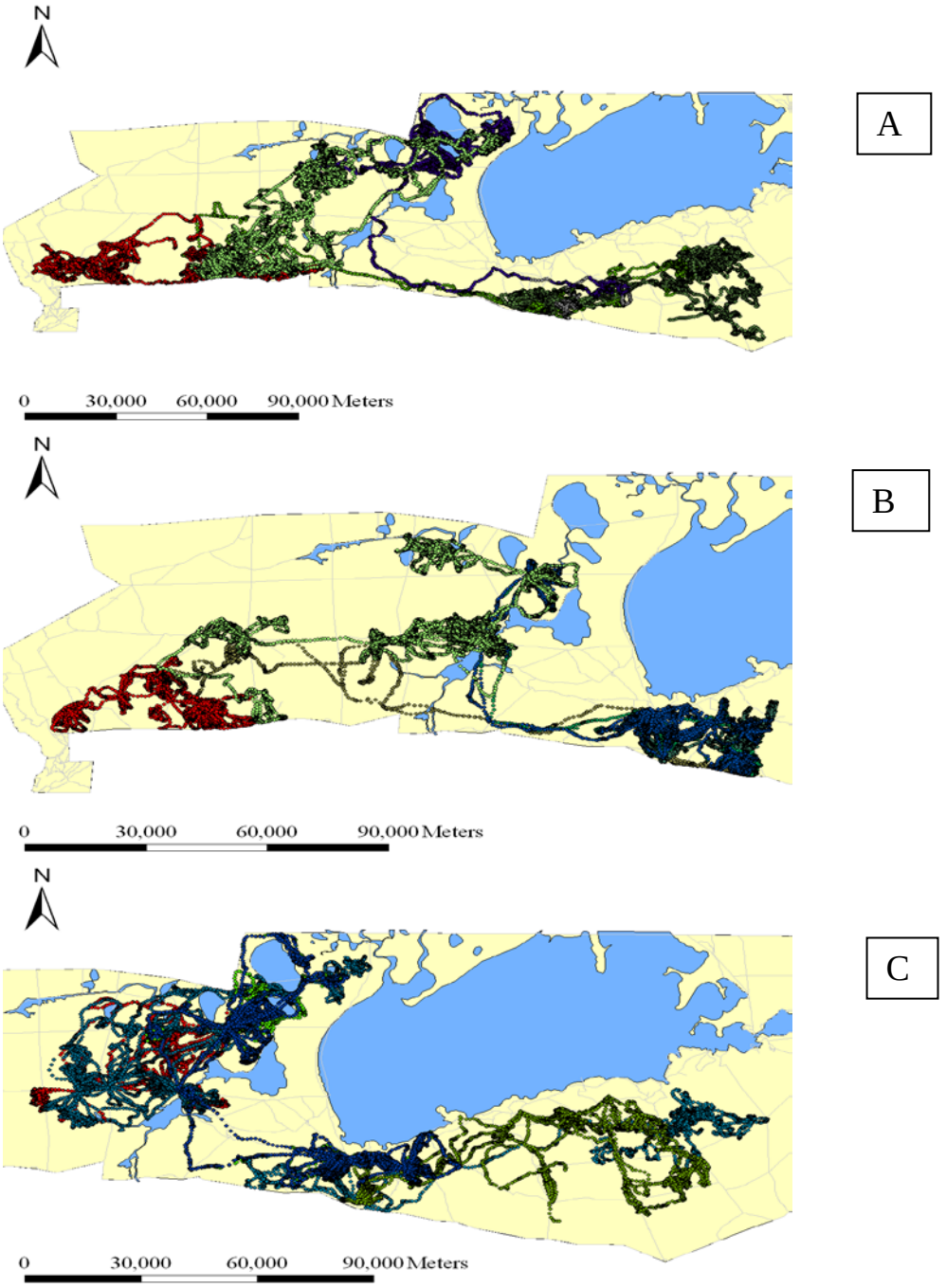


**Figure 4:** Global positioning system (GPS) locations of six female elephants. A – Data collected during January-April 2010. B – Data collected during May-August 2010. C – Data collected during September-December 2010 (Zidon, 2011). The blue polygons indicate the pans; the grey lines are gravel roads; and the coloured dots are GPS locations of six collared female elephants collected during the hot-wet season (A), cool-dry season (B), and the hot-dry season (C).

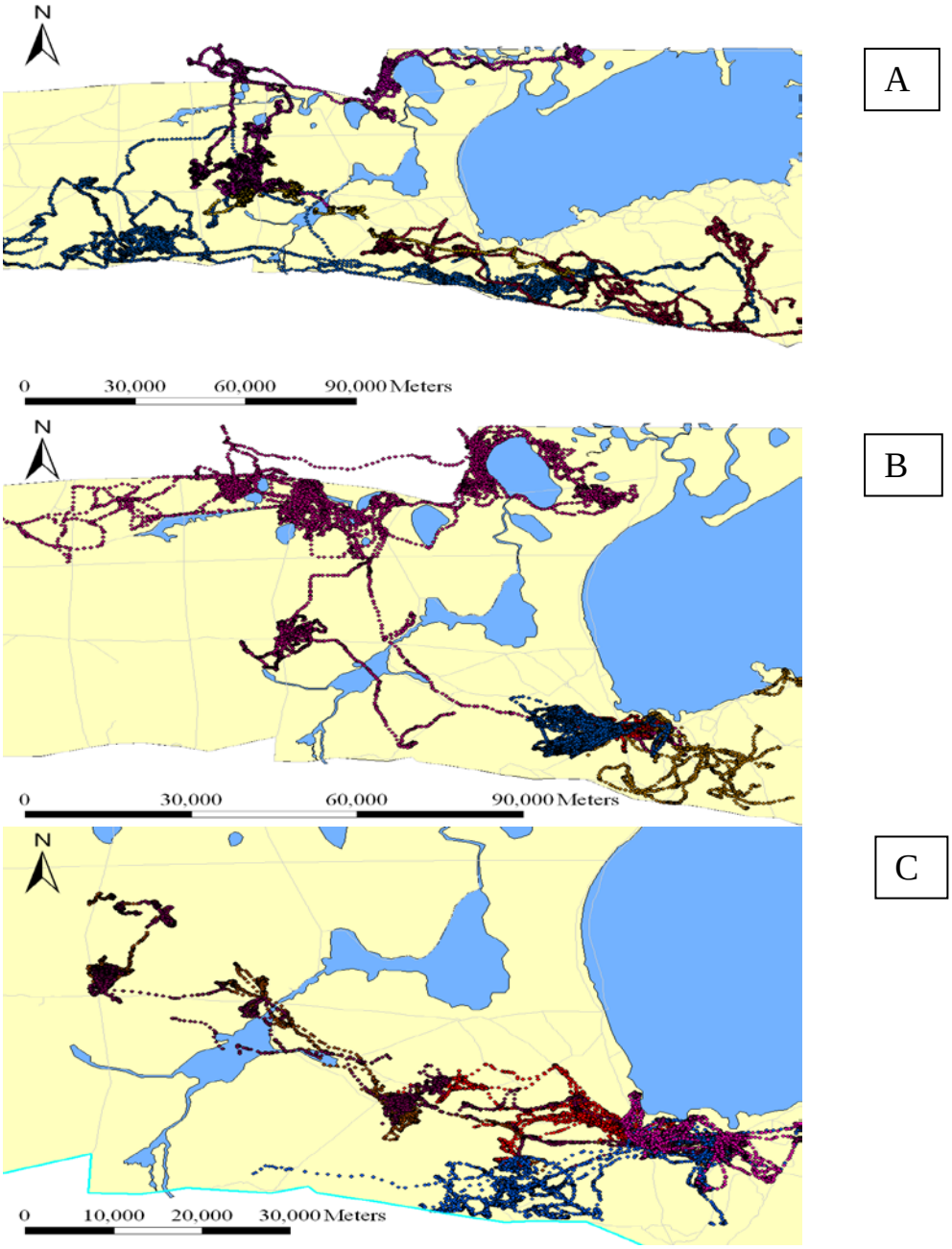


**Figure 5:** GPS locations of five male elephants. A – Data collected during January-April 2010. B – Data collected during May-August 2010. C – Data collected during September-December 2010 (Zidon, 2011). The blue polygons indicate the pans; the grey lines are gravel roads; and the coloured dots are GPS locations of five collared male elephants collected during the hot-wet season (A), cool-dry season (B), and the hot-dry season (C).

In ENP, gravel pits resulting from excavation of gravel for roads were thought to be related to the incidence of anthrax (Ebedes, 1976). This theory was in line with

the incubator area hypothesis of Van Ness (1971). However, further studies could not confirm this theory (Turnbull, Carman, Lindeque, Joubert, Hübschle, et al., 1989). In fact, only one out of 81 environmental samples that were not associated with anthrax carcass sites tested positive for *B. anthracis* (Turnbull, Carman, Lindeque, et al., 1989).

Lindeque (1991) investigated the effect of soil type on the distribution of anthrax mortalities in ENP. The three major soil mapping units used were described by Le Roux, Grunow, Morris, et al. (1988) as sandy soils, karstveld soils, and deep soils (Lindeque, 1991). There was no statistical difference in the pH values among the sandy, karstveld, and deep soils (Lindeque, 1991). Lindeque (1991) further found no relationship between the distribution of anthrax carcasses and soil type.

However, soil type was found to be an important factor in the sporulation and survival of *B. anthracis* in an experiment conducted in ENP (Lindeque & Turnbull, 1994). Sandy soils, with lower pH values and lower total spore counts, were better at promoting sporulation than the karstveld soils (Lindeque & Turnbull, 1994). On the other hand, carcass sites located on karstveld soils had a higher *B. anthracis* spore count than sites located on sandy soils (Lindeque & Turnbull, 1994). This relates to the notion that *B. anthracis* survives better in soils with high pH values (WHO, 2008). Lindeque and Turnbull (1994) further observed a decrease in *B. anthracis* endospore counts with time at anthrax carcass sites. This decline in endospore numbers was attributed to the effect of wind and water on surface soil (Lindeque & Turnbull, 1994).

## 2.7 Anthrax as an Agent of Biological Warfare

Bioterrorism is the intentional use of microorganisms and toxins produced by living organisms to cause death and disease in humans, animals, and plants (Centres for Disease Control and Prevention [CDC], n.d.). When microorganisms and their toxic by-products are used in bioterrorist attacks, they are called biological weapons (bioweapons) or biological warfare (BW) agents (Henneberry, 2001). The causative agent of anthrax, *B. anthracis*, is considered a bioweapon.

The first recorded use of anthrax as a bioweapon occurred during the First World War (WW I; 1914-1918) when Germany developed a biological warfare programme (Albarelli, 2001). The aim of this programme was to sabotage the attempts of neutral countries to export animals to the Allies by infecting the export animals with *B. anthracis* and *Pseudomonas pseudomallei* (Riedel, 2004). In 1925, after WW I, the Geneva Protocol for the Prohibition of the Use in War of Asphyxiation, Poisonous or Other Gases, and of Bacteriological Methods of Warfare was signed to prohibit the use of chemicals and microorganisms and their toxins as BW agents (Cole, 2011; Riedel, 2004; Christopher, Cieslak, Pavlin & Eitzen, 1997). However, the Geneva Protocol did not ban the research or possession of bioweapons (Riedel, 2004) and soon countries such as Japan, Great Britain, and the United States of America (USA) were developing their own bioweapons programmes (Cole, 2011; Riedel, 2005; Riedel, 2004; Christopher, Cieslak, Pavlin, et al., 1997). Even though many countries were conducting experiments on bioweapons and have accumulated stockpiles of BW agents including *B. anthracis*, no country was involved in biological warfare during the Second World War (WW II), with an exception to the Japanese experiments (Cole, 2011). The experimentation with bioweapons continued

in the years after WW II, and it became evident that the Geneva Protocol was ineffective and a new treaty had to be developed to prohibit the proliferation of biological weapons (Riedel, 2004). Thus, the "Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction" was established in 1972 (Riedel, 2004). This convention is also known as the Biological Weapons Convention (BWC; Cole, 2011). Not many incidents of bioterrorism with anthrax as the agent occurred after the establishment of the BWC, with an exception to the case of Sverdlovsk, Russia, in 1979 (Cole, 2011). The most recent anthrax scare occurred in 2001 in the USA where weaponised anthrax spores were sent in envelopes to journalists and politicians (Cole, 2011). These attacks had few fatalities but it demonstrated that such attacks can cause immense fear and panic, and can potentially be very destructive (Cole, 2011; CDC, n.d).

The natural occurrence of anthrax can be easily controlled, but its deliberate release in bioterrorist events cannot (WHO, 2008). The deliberate release of *B. anthracis* has immense repercussions because not only can it cause the deadly disease anthrax, but the spores of this bacterium can remain viable in the environment for decades without proper decontamination and still cause anthrax outbreaks years after the initial release. However, the impact of *B. anthracis* as a weapon may be reduced with adequate understanding of the bacterium, its survival in the environment, and the disease it causes.

## 2.8 Conclusion

This section highlighted the importance of anthrax as a zoonotic disease. Even though this bacterial disease occurs naturally in the soil in most parts of the world, it may be concentrated to deadly amounts and cause mass mortality to both humans and animals in bioterrorist activities. Sporadic anthrax outbreaks occur in Namibia. In ENP, where anthrax outbreaks occur yearly, the disease is an integral part of the ecosystem.

# CHAPTER 3: METHODOLOGY

## 3.1 Introduction

The present study investigated the influence of site and soil type on the distribution of anthrax infectious sites in ENP. The relative contribution of anthrax to elephant mortality in ENP was also investigated. The study was conducted under a research and collecting permit (permit number 1538/2011) granted by the Ministry of Environment and Tourism (MET). The investigation of the contribution of anthrax on elephant mortality was conducted from July 2010 to July 2012. The investigation of the influence of soil type and rainfall gradient on the distribution of anthrax infectious sites was conducted from February 2011 to February 2012. Samples were processed and analyzed at the Etosha Ecological Institute (EEI) at Okaukuejo, ENP.

## 3.2 Study Area

This study was carried out at Etosha National Park which is a 22 915 $km^2$ protected area in the northern part of Namibia between 18°30'-19°30'S and 14°15'-17°10'E. Three seasons are observed in ENP namely hot-wet (November-April), cool-dry (May-July), and hot-dry (August-October) (Lindeque, 1991). Rainfall occurs from November to April, with January and February being the wettest months (Engert, 1997). A rainfall gradient is observed from the east to the west of the park (Figure 6), with Namutoni camp in the east receiving an average annual rainfall of 452.1 mm, Okaukuejo, 356.4 mm, and Otjovasandu in the west 305.8 mm (Engert, 1997).

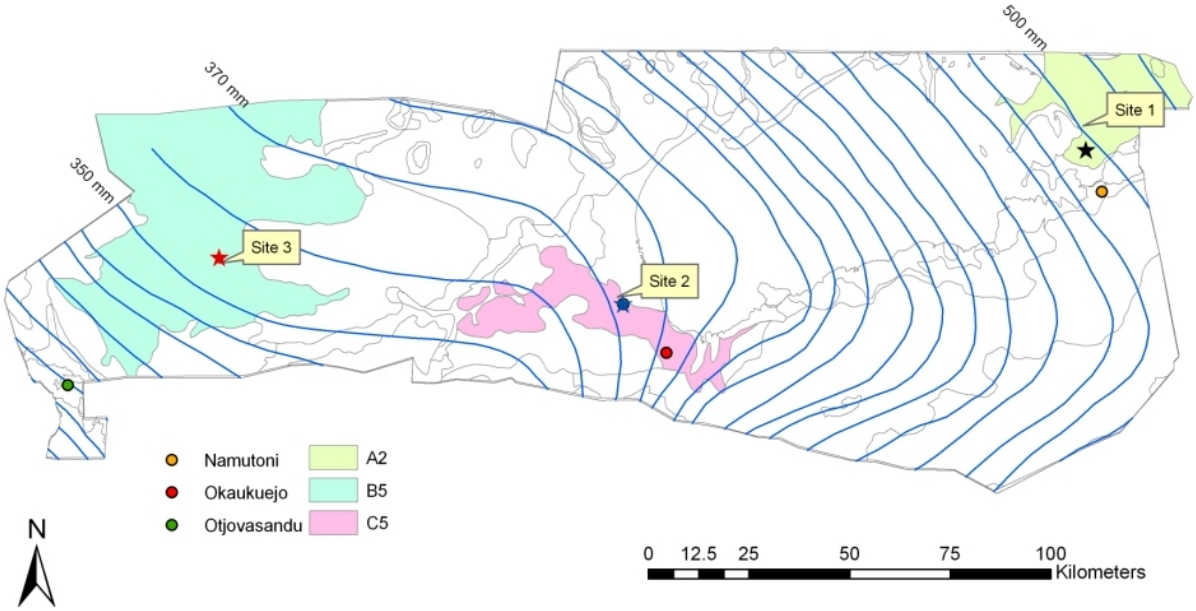


**Figure 6:** The rainfall gradient in relation to the three study sites and the weather stations in Etosha National Park (EEI Geographic Information System [GIS] database, 2012). The blue lines indicate the isohyets. Sites 1, 2, and 3 indicate the location of the three experimental sites in eastern, western, and central ENP. The three study soils are indicated by A2 (ferralic arenosols), B5 (haplic arenosols), and C5 (calcaric regosols). The seasonal rain gauges are indicated by the red star in the west at Olifantsrus, the blue star in the centre at Leeubron, and the black star in the east at Tsumcor. The weather stations are indicated by the coloured dots; the orange dot indicates Namutoni in the east, the red dot indicates Okaukuejo in the centre, and the green dot indicates Otjovasandu in the west.

The soils of ENP were classified into nine major soil units (Beugler-Bell & Buch, 1997; Appendix 1). The study on the distribution of anthrax infectious sites were located in the following soil types as described by Beugler-Bell and Buch (1997): ferralic arenosols (A2; eastern ENP), calcaric regosols (C5; central ENP), and haplic arenosols (B5; western ENP). Figure 7 indicates the soils of ENP as described by Beugler-Bell and Buch (1997).

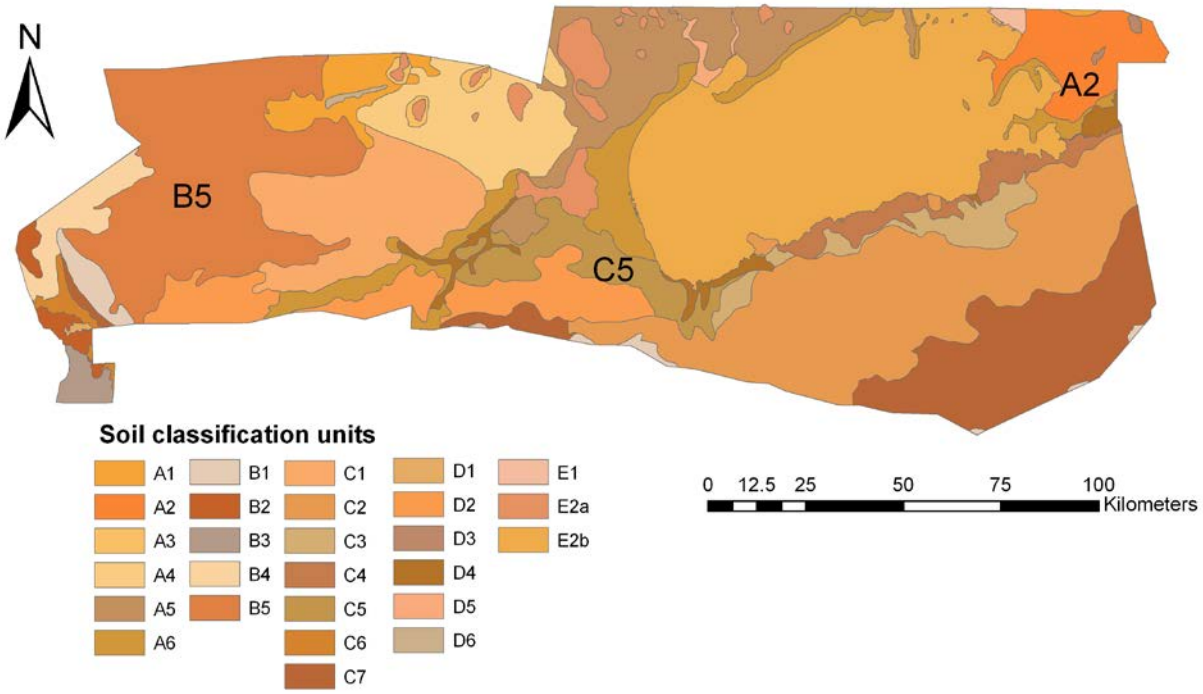


**Figure 7:** Soil classification of Etosha National Park (EEI GIS database, 2012; based on the surveys of Beugler-Bell & Buch, 1997). The ferralic arenosols, calcaric regosols, and haplic arenosols are indicated by A2, C5, and B5, respectively.

### 3.3 Influence of Site and Soil Type on the Distribution of Anthrax Infectious Sites

#### 3.3.1 Reciprocal Transplant Experiment

A reciprocal transplant experiment was conducted to determine and compare the effect of soil type and site along the rainfall gradient on the persistence of *B. anthracis* endospores in ENP. The study was carried out at three experimental sites in the wetter east, centre, and the drier west in ENP (Figure 6) from February 2011 to February 2012. The choice of these sites was based on the evident rainfall gradient from the east to the west in ENP (Figure 6). The eastern study site was located in the ferralic arenosols of mapping unit A2 at -18.65961°S and 16.89541°E, and 3 km north of Tsumcor waterhole. The closest seasonal rain gauge to the eastern study site was located at the Tsumcor waterhole pump at -18.71489 °S and 16.90328 °E. The

central study site was located in the calcaric regosols of mapping unit C5 at -19.05235°S and 15.80000°E, and 2.7 km north-west of Leeubron waterhole. The closest seasonal rain gauge to the central study site was located at -19.06861 °S and 15.81444 °E, and approximately 2 km south-west of the central study area. The western study site was located in the haplic arenosols of mapping unit B5 at -18.97846°S and 14.85937°E, and 1.45 km south of Olifantsrus waterhole. The closest seasonal rain gauge to the western study site was located at -18.96528 °S and 14.86056 °E, and approximately 1.5 km north of the western study area. Each study site was located approximately 20 m away from the road to enable easy access.

At each of the three experimental sites a 4 m × 4 m plot was fenced off for a reciprocal transplant experiment. During transplant experiments, also known as common garden experiments, individuals from the same species but different populations are removed from their original locations and planted into a new location where the environmental factors are kept constant to determine whether the observed phenotypic differences are due to underlying genetic variation (Molles, 2008). For the purpose of this study, a reciprocal transplant experiment was employed whereby three soil types were chosen and removed from their original locations and transplanted into three new locations which included the original location of each of the chosen soil types. This means that soil cores from the east, west, and centre of ENP were collected and transplanted into three sites in the east, west, and centre of ENP, i.e., each site contained soil cores from that site, plus soil cores from the two other sites. A fully factorial design for this experiment was set out and included two pathogen treatments (*B. anthracis* present and *B. anthracis* absent, i.e. control) at three experimental sites following a rainfall gradient (east, centre, and west) and

three soil types (ferralic arenosols, calcaric regosols, and haplic arenosols). This factorial experimental design yielded a total of eighteen treatments.

At each experimental site, the plot was cleared of all vegetation and ten random soil samples were collected from a depth of 10 cm. These soil samples were taken to the laboratory at the EEI and were cultured to confirm that the selected experimental sites were free of *B. anthracis* cells. For this experiment, *B. anthracis* endospores were grown in the laboratory and a known amount of endospores (80 million spores per millilitre) was inoculated into the soil and the persistence over time of these endospores was monitored. Therefore, it was important for the selected sites to be free of *B. anthracis* cells.

Each experimental plot was divided into two sections (*B. anthracis* present and absent) by placing sand bags in the middle of each plot (Appendix 2). The purpose of the sand bag barrier was to prevent *B. anthracis* endospores from being washed by rain water from the positive side and contaminating the negative side of the plot. Fifty-four 10 cm length polyvinyl chloride (PVC) pipes were hammered into the ground in each of the two sections (*B. anthracis* treatment and control) to give a total of 108 soil cores per soil type at each of the three experimental sites. The soil cores were carefully removed from the ground at each plot and placed in sterile WhirlPak® bags. The soil cores were collected in order to set up a reciprocal transplant experiment. This experimental set up yielded a combined total of 324 soil cores from the three soil types and the three study sites.

The reciprocal transplanting of the soil cores among the three sites was done over three days due to the distance between the three sites. The soil cores were stored at 4 °C during transit between the respective sites. At each site, the soil cores were

removed from the WhirlPak® bags and transplanted (Appendices 3 & 4) among the three plots so that each experimental plot had 36 soil cores of each soil type giving a total of 108 soil cores per experimental plot. The 108 soil cores were randomly placed into the ground at each plot, with 54 cores in the *B. anthracis* treatment section and 54 cores in the control section. The placement of the cores in each plot was assigned using a random number generator. The soil cores on the *B. anthracis* positive side of the experimental plots were spiked with 1 mL *B. anthracis* spore solution that was grown in the laboratory and then 1 mL sterile distilled water was added to allow drainage of spores below the soil surface to reduce the exposure to UV light that may have affected spore survival. The concentration of the spore solution was 80 million spores per millilitre. The soil cores on the control side (no *B. anthracis* inoculated) of the experimental plots were spiked with 2 mL sterile distilled water.

### 3.3.2 Spore Production and Purification

#### 3.3.2.1 Isolate Selection

For this spike experiment, pure *B. anthracis* endospores were grown from a known isolate in the laboratory at the EEI. The spores were grown from an isolate obtained from a swab collected from a springbok that died in February 2010 (EEI carcass number 10-007). This isolate was chosen because it belongs to genotype 6, which is the most common genotype in ENP (Beyer, Bellan, Eberle, Ganz, Getz, et al., 2012). This isolate was also genotyped by Hadfield and Blackburn (unpublished data) at the University of Florida, USA and was found to match genotype 40 in cluster A3a (Keim, Price, Klevytska, Smith, Schupp, et al., 2000; Figure 8).

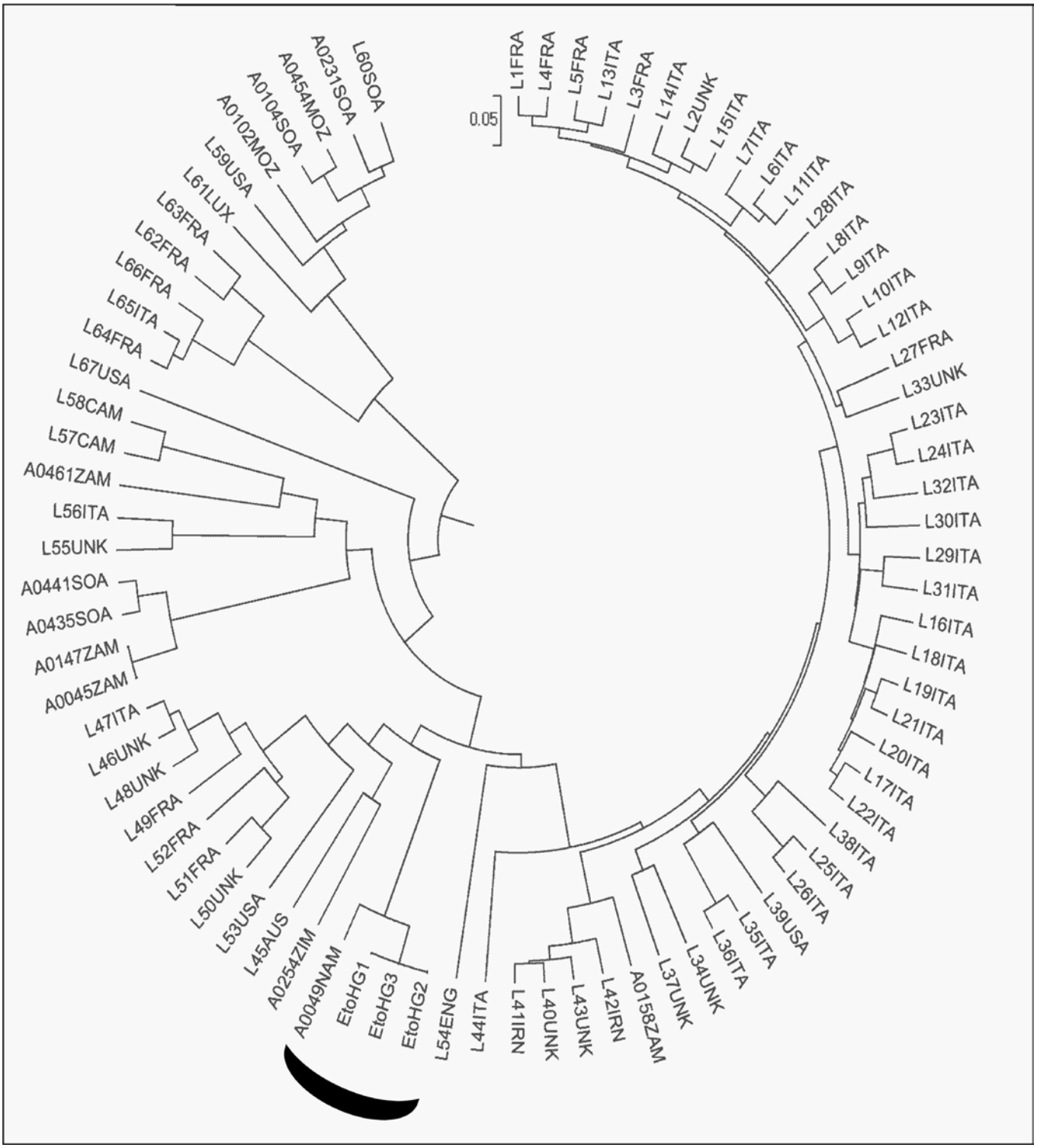


**Figure 8:** Tree with Lista genotypes and selected African strains (LSU study) and Etosha National Park genotypes (Hadfield & Blackburn, unpublished data). The isolate used in this study is indicated as EtoHG3.

### 3.3.2.2 Sporulation Agar

Sporulation agar was used to grow the *B. anthracis* spores that were used to inoculate the soil cores during the reciprocal transplant experiment. Leighton and Doi (1971) described 2x SG agar (modified Schaeffer's sporulation agar) as the best agar for promoting sporulation in the *Bacillus* species. This agar was made by adding 16 g nutrient agar (Difco), 2 g potassium chloride (KCl; SIGMA, P9541), 0.5 g magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$; SIGMA, M5921) and 32 g nutrient agar (Difco) to 1 L distilled water. The solution was mixed until all solvents dissolved. The solution was autoclaved for 15 minutes at 125 °C and 15 psi. Before the agar was poured into sterile Petri plates, the following filter sterilized components were added: 1 mL of 1M $Ca(NO_3)_2 \cdot 4H_2O$ (SIGMA, C4955), 1 mL of 0.1M $MnCl_2 \cdot 4H_2O$ (SIGMA, M8530), 100 µL of 10mM $FeSO_4 \cdot 7H_2O$ (SIGMA, F8263) and 10 mL of 10% (w/v) glucose (SIGMA, G5400). The solution was gently swirled to mix, poured into the Petri plates and allowed to set overnight.

### 3.3.2.3 Isolation and Concentration of *B. anthracis* Endospores

After the selection of the genotype that was used in this study, spores were grown to spike into the soil for the transplant experiment by streaking a blood agar plate with an isolate (EEI carcass number 10-007) that was collected from a springbok carcass swab collected on the Adamax-Okondeka road (-19.00353 °S; 15.73948 °E) in central ENP on 1 February 2010. The culture was incubated overnight at 30 °C. After the 2x SG plates solidified, twelve plates were incubated overnight at 30 °C. This was done to check for sterility of the agar plates. After all 12 plates were found to be sterile; a loopful of a pure *B. anthracis* colony obtained from

the blood agar culture was suspended in a sterile Falcon (BD) tube containing 2 mL sterile distilled water. Each of the twelve 2x SG agar plates was inoculated by spreading out 100 µL of the cell suspension. The plates were incubated overnight at 37 °C. Sporulation efficiency was confirmed microscopically.

The cell lawn was harvested into a sterile 50 mL centrifuge tube with a sterile disposable cell scraper. The cells were suspended by adding 30 mL of ice-cold sterile distilled water and vortexed vigorously. The cell suspensions were centrifuged for 20 minutes at 3 000g to collect the cells. The cells were washed three times with ice-cold sterile distilled water. The resulting pellet contained both *B. anthracis* spores and vegetative cells.

A step-density gradient was used to separate the spores from the vegetative cells and agar. For each washed pellet one 50 mL centrifuge tube containing 30 mL of 50% RenoCal-76 (Bracco Diagnostics) was prepared. This was done by diluting 19.7 mL of the original 76% concentration RenoCal-76 with 10.3 mL sterile water and mixing the layers well. A 3 mL cell/spore suspension in RenoCal-76 was prepared in a centrifuge tube. This was done to obtain a final RenoCal-76 concentration of 20%. A volume of 0.8 mL RenoCal-76 was added to the centrifuge tube. The cells were again washed three times with ice-cold sterile water. The pellet was then suspended in the remaining drops of wash water by gently tapping the centrifuge tube. An additional 1 mL of ice-cold sterile water was used to wash the 50 mL centrifuge tube. The wash was transferred to the centrifuge tube. This was repeated until 3 mL of the suspension was obtained in the centrifuge tube. The suspension was mixed by tapping.

The 20% RenoCal-76/cell/spore mixture was gently loaded onto the top layer of the 50% RenoCal-76 by slowly ejecting the sample to the side of the 50 mL centrifuge tube. The sample was centrifuged for 30 minutes at 3 000g and the supernatant was removed by aspiration. The pure spore pellet was suspended in 1 mL of ice-cold sterile distilled water and the suspension was transferred to a microcentrifuge tube. The suspension was centrifuged at 14 000g in a microcentrifuge (Labnet International) for one minute. The pellet was washed two more times with ice-cold sterile water. The pellet was then resuspended in sterile water to a concentration of 80 million spores per millilitre. The spore suspension was stored at 4 °C until taken to the experimental sites where 1 mL of the spore solution was applied to the soil cores on the *B. anthracis* treatment side of each experimental plot.

### 3.3.3 Sampling of Soil

In order to determine and compare the persistence of *B. anthracis* in the three soil types collected from the three different sites, soil samples at each experimental site were collected periodically after *B. anthracis* spores were inoculated into the soils. Soil samples were collected at six different time points: $T_0$ (start of the study, February 2011), $T_1$ (one month after inoculation, March 2011), $T_2$ (two months after inoculation, April 2011), $T_3$ (four months after inoculation, June 2011), $T_4$ (six months after inoculation, August 2011), and $T_5$ (one year after inoculation, February 2012). The following procedure was used to collect soil samples at each experimental site during each of the six sampling times. Three soil cores from each soil type were randomly selected and removed per treatment and control, giving a

total of nine soil samples from the *B. anthracis* treatment section and nine from the control section at each experimental site. This gave a total of 54 (18 from each site) samples collected at any given sampling time. Soil cores were gently dug out with a clean gardening spade, making sure not to disturb the soil core. The soil cores were placed in labelled, sterile WhirlPak® bags. The bags were placed upright in a cooler box with ice packs and transported to the EEI laboratory.

### 3.3.4 Isolation of *B. anthracis* from Soil Samples

#### 3.3.4.1 Culture Media

**PLET agar –** Polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar is a selective agar that is recommended for the isolation of *B. anthracis* from environmental samples (WHO, 2008). PLET agar was developed by Knisely (1966). It allows for the growth of *B. anthracis* but not common contaminants and closely-related spore formers such as *B. cereus*. Polymyxin B sulphate is an inhibitory agent allowing the growth of *B. anthracis* while inhibiting contaminants. Lysozyme specifically suppresses the growth of Gram negative contaminants. The combination of ethylenediaminetetra-acetic acid (EDTA) and thallous acetate inhibit most *B. cereus* strains but has no effect on *B. anthracis*.

In a 2 L flask, 1.5 L distilled water, 78 g brain heart infusion agar (BD BBL™, Becton, Dickinson & Company), 0.45 g EDTA, and 1 mL thallous acetate were added. The solution was heated and mixed well on a heating block until close to boiling point. The solution was autoclaved for 45 minutes at 125 °C and 15 psi with the warming function set to 50 °C for 1 hour. After removing the flask from the

autoclave, 400 µL lysozyme solution and 400 µL polymyxin B sulphate solution were added using a sterile micropipette tip for each solution. The agar was gently swirled to mix the solution. Twenty millilitres of the agar were aseptically pipetted out into each Petri plate. After all the agar was dispensed onto the Petri plates, the plates were allowed to set overnight after which they were put into bags and stored inverted at 4 °C in the refrigerator. The plates were dried in the biosafety cabinet to remove condensation from the lid before culturing of soil samples.

**Blood agar –** *Bacillus anthracis* is sensitive to gamma phage and penicillin (WHO, 2008; Appendix 5). In the present study this sensitivity was confirmed on blood agar. Blood agar was prepared by first mixing 20 g Bacto agar (BD), 8 g nutrient broth (BD) and 940 mL distilled water in a 2 L flask to make nutrient agar. The suspension was autoclaved for 40 min at 125 °C and 15 psi with the warming function set to 50 °C for one hour. While the nutrient agar was cooling in the autoclave, 60 mL donor horse blood was warmed to 50 °C in a water bath for one hour. After the one hour elapsed, the warmed blood was added to the agar to achieve a final blood concentration of 6%. The blood agar was gently swirled to ensure thorough mixing of the solution. Twenty millilitres of the agar were aseptically pipetted out into each Petri plate. The plates were allowed to set overnight after which they were put in bags and stored inverted at 4 °C in the refrigerator. The plates were dried in the biosafety cabinet to remove condensation from the lid before the colony confirmation tests were done.

### 3.3.4.2 Culture Techniques

Soil samples collected from each experimental site were cultured in the anthrax laboratory at the EEI to detect for the presence of *B. anthracis* endospores and vegetative cells. The procedures that were followed are described in this section.

All counters and the biosafety cabinet were disinfected with 10% bleach (sodium hypochlorite) before use. Spoons and weighing boats were cleaned by soaking in 10% bleach for 10 minutes. Each soil sample was thoroughly mixed with a clean plastic spoon in the WhirlPak® bags and 5 g was weighed out and transferred to a labelled 50 mL centrifuge tube. Forty five millilitres of 0.1% sodium pyrophosphate were added to the soil in the 50 mL centrifuge tubes. After adding sodium pyrophosphate to the soil, samples were vortexed to loosen spores from the soil particles by using a platform vortexer (Multi-Tube Vortexer, VWR Scientific Products) and shaking on maximum speed for 10 minutes. The 0.1% sodium pyrophosphate was used to discharge soil particles that become hydrophobic during the dry season (Katayama, Kai & Fujie, 1998).

Soil samples were briefly centrifuged at 0.3×1000 relative centrifugal force (rcf) for 2 minutes to allow the soil to settle. The resulting supernatant was transferred into a 50 ml centrifuge tube. The soil samples were centrifuged a second time at 3.0×1000 rcf for 15 minutes to pellet the spores. The supernatant was removed by decanting into a waste water container. The precipitate was suspended in 5 mL of 0.1% peptone water and vortexed until the pellet was completely dissolved. The 0.1% peptone water was used in order to provide a buffered environment for

microorganisms and to facilitate their growth on culture media. One millilitre (1 mL) of supernatant was transferred to a labelled 1.8 mL screw cap tube.

While the soil samples were vortexed and centrifuged, PLET agar plates were removed from the refrigerator and dried in the biosafety. Four sterile 1.5 mL Eppendorf tubes were labelled with successive factors of ten from $10^{-1}$ to $10^{-4}$ for each of the soil samples. The initial soil solution was diluted through serial dilutions in order to obtain a small number of colonies per plate. A volume of 100 µL of the supernatant from each soil solution was transferred to a 1.5 mL Eppendorf tube containing 900 µL 0.1% peptone water to obtain a $10^{-1}$ dilution. The tube was vortexed briefly to ensure even mixing. A volume of 100 µL from the first dilution tube was transferred to a 1.5 mL Eppendorf tube containing 900 µL 0.1% peptone water to obtain a $10^{-2}$ dilution. This was repeated for the remaining two tubes. The dilution procedure is diagrammatically depicted in Figure 10. After the last dilution was prepared, 100 µL of each diluted sample was dispensed onto a correspondingly labelled PLET agar plate and spread evenly on the agar surface. The dilution procedure is diagrammatically depicted in Figure 9.

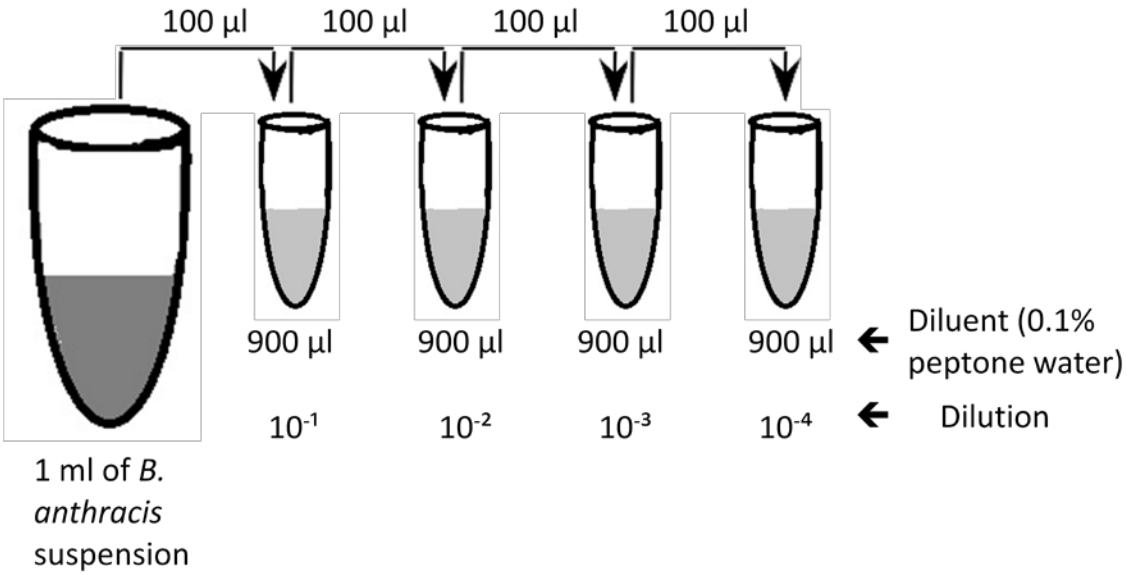


**Figure 9:** Preparation of a ten-fold dilution series of stock spore solution in sterile 0.1 % peptone water.

As a negative control, 100 µL of the dilution buffer (0.1% peptone water) was spread evenly on the agar surface of a clean PLET agar plate to detect any contamination in the buffer. Dilutions of stock solutions are made in order to accurately quantify the amount of bacteria in the culture (Madigan & Martinko, 2006). The plates were incubated at 37 °C and were read 48 and 96 hours after culturing. After incubation, the number of colonies per plate was counted by hand and recorded. All counters and the biosafety cabinet were bleached after use and all waste was autoclaved and incinerated.

### 3.3.5 Identification of *B. anthracis* colonies

The bacterial growth on the PLET plates was examined to identify *B. anthracis* colony forming units (CFUs). *B. anthracis* colonies typically have a cut-glass

appearance, are whitish, opaque, and do not have smooth edges (WHO, 2008; Appendix 6). The *B. anthracis* colonies were counted and recorded from each dilution plate per sample. Whenever the morphology of a colony was unfamiliar, the identity of such a colony was confirmed by sub-culturing on blood agar. A penicillin G disc and a 12.5 µL drop of diagnostic gamma phage were added to the bacterial streak. The blood agar plate was then incubated overnight at 35 °C. The colony was confirmed as *B. anthracis* if it showed susceptibility to both the penicillin disc and the gamma phage (WHO, 2008).

The total number of *B. anthracis* per plate per sample was obtained by taking the number of CFUs between 30 and 300 per plate per sample. This number of CFUs (30-300) was chosen because the number counted is high enough to have statistical accuracy, yet low enough to avoid nutrient competition among the developing colonies. The total number of *B. anthracis* cells per millilitre of the initial soil solution was calculated using the following formula:

$$CFU\ per\ ml\ of\ original\ spore\ solution = \frac{CFU}{(volume\ plated)\ (dilution\ factor)}$$

In order to quantify the total number of *B. anthracis* colony forming units in a soil sample, the proportion of soil moisture for each sample was calculated. Before drying, the mass of the soil samples was measured and recorded. The samples were then dried overnight at 100 °C to get the dry mass. The proportion of soil moisture in each sample was calculated by the following formula:

$$Proportion\ of\ soil\ moisture = \frac{Wet\ mass\ (g) of\ soil - Dry\ mass(g)\ of\ soil}{Dry\ mass\ (g) of\ soil}$$

The total number of *B. anthracis* per gram of soil was calculated using the formula:

$$CFU\ per\ g\ of\ soil = \frac{Total\ number\ of\ \frac{CFU}{ml}\ of\ original\ spore\ solution}{Proportion\ of\ soil\ moisture}$$

### 3.3.6 PCR amplification

After the number of colonies was recorded, six *B. anthracis* colonies were randomly selected from 15 plates (5 plates per experimental site) with a sterile toothpick. The selected colonies were placed in 20 µL Tris/EDTA (TE) buffer in a polymerase-chain reaction (PCR) tube and boiled for 10 minutes. Boiling causes deoxyribonucleic acid (DNA) to denature and thus render the bacterial cells non-pathogenic. The identity of the picked colonies was confirmed using PCR. The primers used to determine the presence or absence of *Bacillus anthracis* were:

BA177F 5’-TTG GAT CAG CGT TTC TGA ATT CAG C-3'

BA177R 5’-TCC CCA TAT CGC TCA ATT CCA TCT A-3'

These primers amplify the region DHP77.47 and are specific for *B. anthracis* (Radnedge, Agron, Hill, Jackson, Tichnor, et al., 2003).

### 3.4 Contribution of Anthrax to Elephant Mortality

A study was conducted from July 2010 to July 2012 in order to determine the proportion of elephant carcass sites that had *B. anthracis* endospores in the soil at these carcass sites in ENP. Elephant carcass sites were scouted for in the ENP during the study period. Once sited, surface soil samples were opportunistically collected in sterile WhirlPak® bags from elephant carcass sites using sterile spades for each carcass site. Soil samples were collected at the location of terminal haemorrhaging, e.g. the mouth, trunk, and anus. In the case of old, dry or fully decomposed carcasses, where it was unclear where terminal haemorrhaging occurred, random samples (six per carcass site) were collected in and around the area identified as the gut pile. The GPS locations of the elephant carcass sites were recorded and mapped (Figure 10). Soil samples were labelled and kept in a cooler box during transit to the laboratory. *B. anthracis* from soil samples collected from 52 elephant carcass sites was isolated and identified in the same manner as for the reciprocal transplant experiment (described in section 3.3.3).

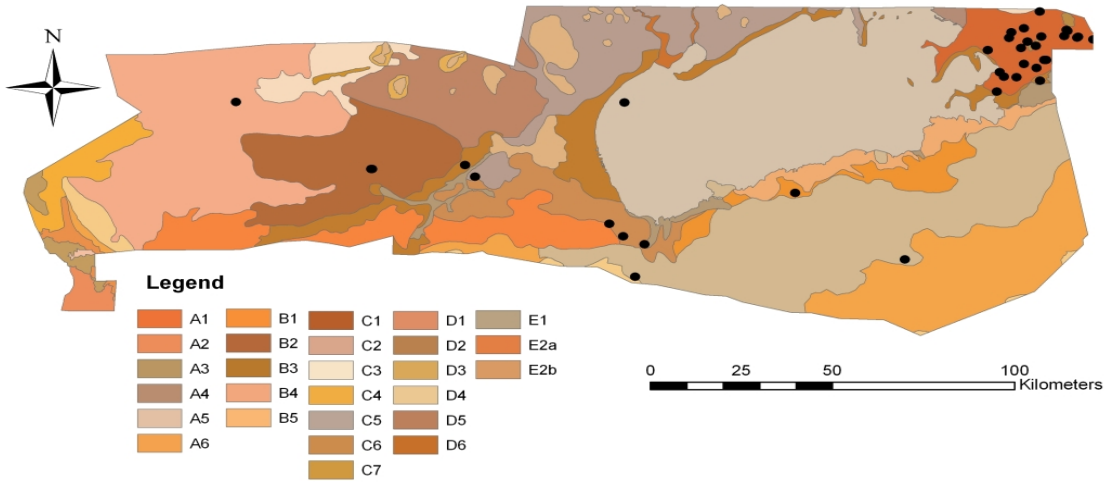


**Figure 10:** Location of soil samples collected from elephant carcasses during 2010 and 2011 in ENP (EEI GIS database, 2012).

**Swab collection and analysis –** When a fresh elephant carcass was found, its age, sex, and spatial location were recorded. Samples were collected of the soil surrounding the carcass where terminal bleeding occurred. Blood from the nasal cavity, or any other part of the body, was collected using a clean throat swab. In the case of old elephant carcasses, random soil samples were collected in the area where terminal haemorrhaging was presumed to have occurred. If the hide and bones were still present, a throat swab was used to collect a sample from the hide and bones by first wetting the swab with distilled water. The swabs were labelled with a laboratory number unique to each carcass. The swabs were sent to the Central Veterinary Laboratory (CVL) in Windhoek for selective culture.

Swabs were cultured at the CVL by suspending the swab in 2 mL sterile distilled water for two minutes. The suspension was then placed in a water bath and heated at 62-63 °C for 15 minutes. After heating for 15 minutes, ten-fold dilutions

were made to 1:100 in sterile distilled water. A volume of 250 µL of each dilution was dispensed and spread out on a sterile blood agar plate. The plates were incubated overnight and the presence of *B. anthracis* was determined within 24 hours. *Bacillus anthracis* colonies were confirmed with diagnostic gamma phage and penicillin discs.

### 3.5 Data analyses

**Analysis of soil collected during the transplant experiment –** After culturing the soil collected from the control side of all three sites at the first sampling time ($T_0$), three samples of each of the three soil types were sent to the Analytical Laboratory in Windhoek, Namibia for analyses. The parameters measured were soil acidity (pH), soil texture (sand, silt, and clay), organic carbon, organic matter, and iron. The loss on ignition method was used to determine the organic matter content of the soils (Heiri, Lotter & Lemcke, 2001).

**Statistical analyses –** Statistical analyses were done using JMP IN® (version 4; SAS, 2001) and R (version 2; R Development Core Team, 2008) statistical packages. All data were tested for normal distribution using the Shapiro-Wilk W test. A one-way analysis of variance (ANOVA) was used to compare the means for soil properties of the three study soils from site 1 (east), site 2 (centre) and site 3 (west) that had a normal distribution. These were soil pH, clay, iron, and organic matter. A Kruskal-Wallis test was employed for data that were not normally distributed. This was done for sand, silt, and carbon. These tests were done to validate the effect of soil type on *B. anthracis* cell counts. The total *B. anthracis* cell count data were not

normally distributed (W = 0.5318, $p$<0.0001) and, therefore, a linear mixed effects model was used to determine whether soil type and site had a significant effect on the total *B. anthracis* cell counts. A linear mixed effects model was also used to test for a significant effect of sampling date (month) and site on soil moisture. Linear mixed effects models are employed to analyse repeated measurements of nested data (Zuur, Ieno, Walker, Saveliev & Smith, 2009). A Chi-square test was used to determine whether there is an association between the age of elephant carcasses found and the presence of *B. anthracis* endospores in the soil at elephant carcass sites after the Shapiro-Wilk W test revealed that the data did not follow a normal distribution.

# CHAPTER 4: RESULTS

This chapter presents results of the influence of rainfall and soil type on the distribution of anthrax infectious sites in ENP. The findings include analyses of rainfall, soil moisture, and soil chemistry during the study period, as well as the effect of soil type and sampling time on *B. anthracis* cells. The occurrence of *B. anthracis* endospores at elephant carcass sites is also presented here.

## 4.1 Influence of Rainfall and Soil Type on the Distribution of Anthrax Infectious Sites in ENP

### 4.1.1 Rainfall

The seasonal rainfall data obtained for the three seasonal rain gauges closest to the three experimental sites were not normally distributed (W = 0.790401, df = 2; $p$ = 0.0918). There was no significant difference in the amount of rainfall amongst the three seasonal rain gauges ($\chi^2$ = 2.0000, df = 2, $p$ = 0.3679; Figure 11) during the study period.

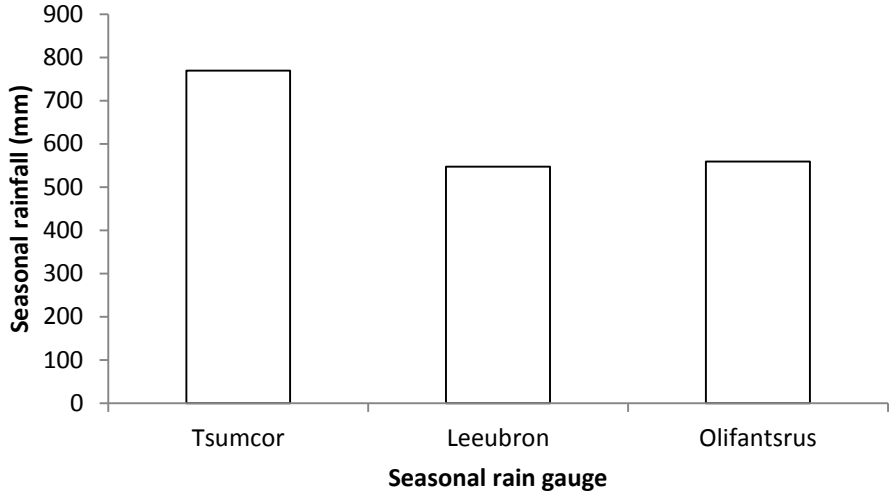


**Figure 11:** Seasonal rainfall (mm) at three seasonal rain gauges, Tsumcor (eastern ENP), Leeubron (central ENP), and Olifantsrus (western ENP) for the season.

Daily rainfall data collected from the MET weather stations at Namutoni (eastern ENP), Okaukuejo (central ENP), and Otjovazandu (western ENP) for the period February 2011 to February 2012, indicated that the eastern weather station (Namutoni; nearer site 1) had the lowest rainfall at the start of the study period and the western weather station (Otjovazandu; nearer site 3) had the highest rainfall at that time (Figure 12). However, at the end of the study period in February 2012, Namutoni had the highest recorded rainfall whilst the lowest rainfall was measured at the central weather station (Okaukuejo; nearer site 2; Figure 12). During the dry season months of May to August all three stations did not receive any rainfall (Figure 12).

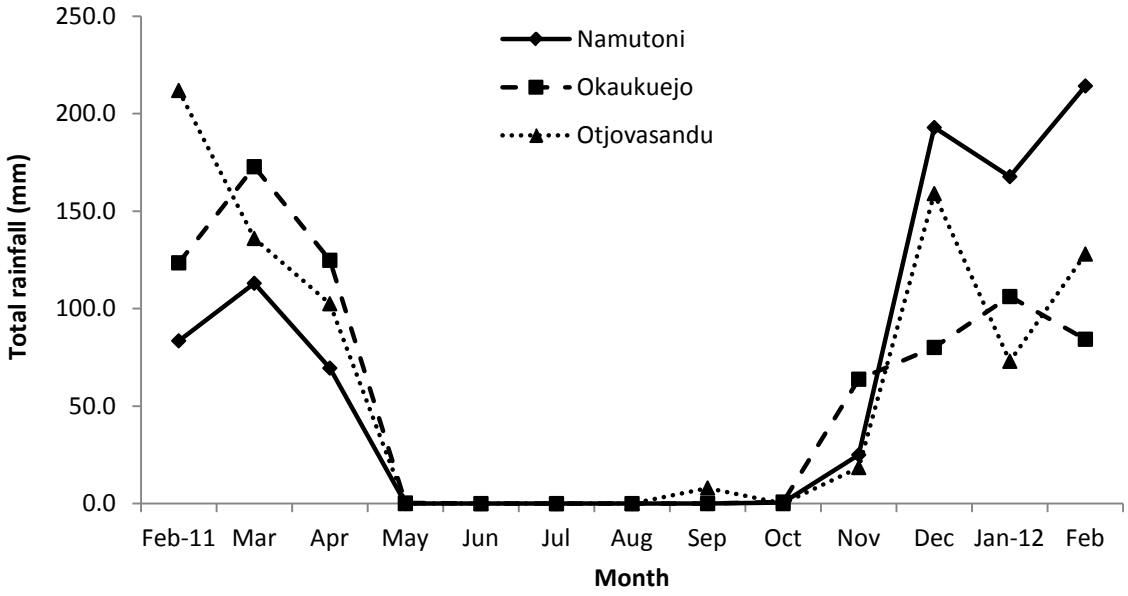


**Figure 12:** Monthly rainfall recorded at three weather stations (Namutoni, Okaukuejo, and Otjovazandu) in Etosha National Park for the period February 2011 to February 2012.

### 4.1.2 Soil Moisture

The mean proportions of soil moisture for all three study soils are presented in Figure 13 and were calculated irrespective of site and sampling time. The proportion of soil moisture for the three study soils was not normally distributed (W = 0.740855, $p$ <0.00001). The Kruskal-Wallis test revealed that there was a significant difference in the proportion of soil moisture amongst the ferralic arenosols, calcaric regosols, and the haplic arenosols ($\chi^2$ = 70.0948, df = 2, $p$ <0.0001; Figure 13).

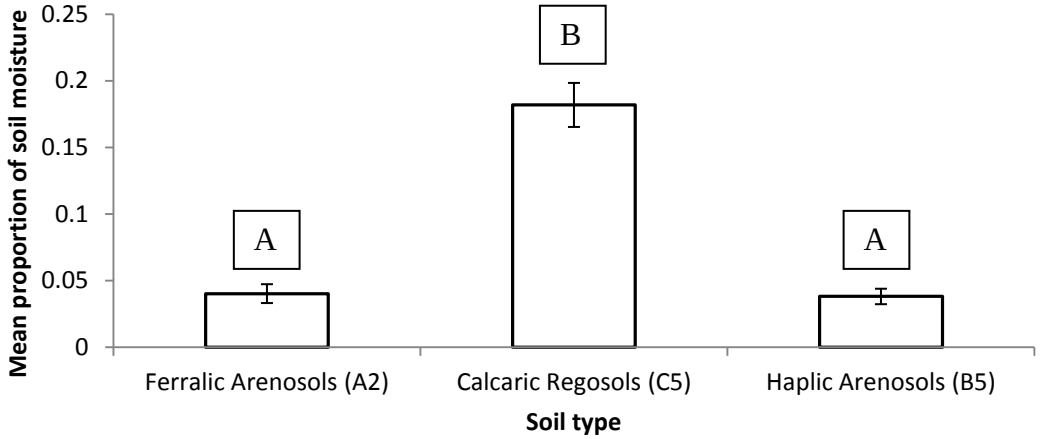


**Figure 13:** Mean (±SEM) proportion of soil moisture of ferralic arenosols (A2; east), calcaric regosols (C5; centre) and haplic arenosols (B5; west). The bars indicate the standard error of the mean (SEM). The letter B indicates that the calcaric regosols were significantly different from the ferralic arenosols and the haplic arenosols. There was no significant difference amongst the ferralic and haplic arenosols (indicated by the letter A).

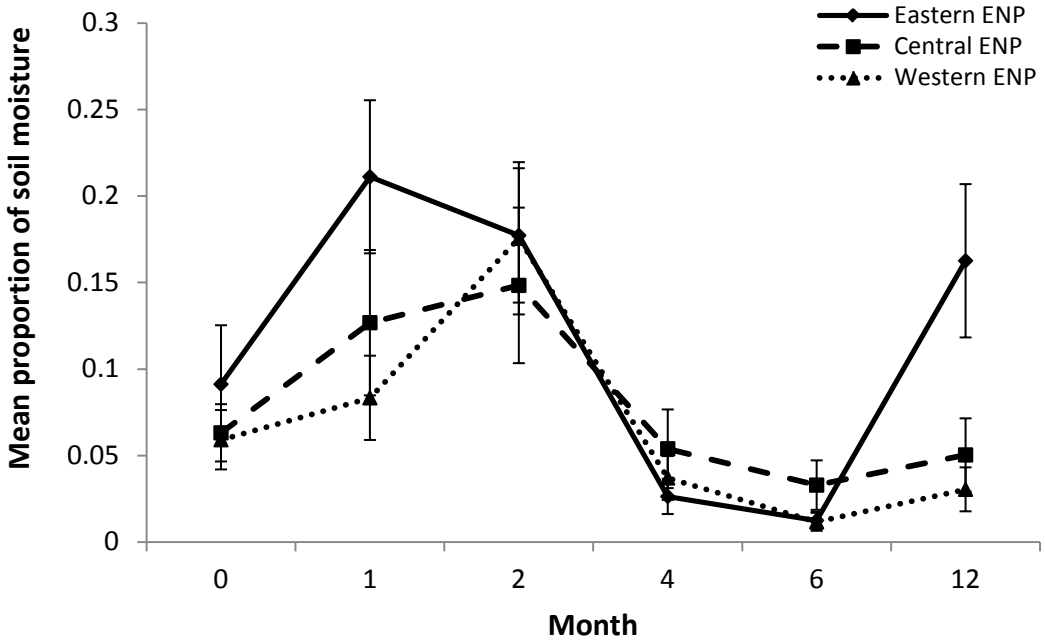


**Figure 14:** Mean (±SEM) proportion of soil moisture at three sites in eastern ENP, central ENP, and western ENP recorded at different times (month), coincident with time when soils were sampled for counting *B. anthracis*, from February 2011 to February 2012. Month 0 = time of inoculation of *B. anthracis* into soil, and month 12 = 12$^{th}$ month after inoculation. Bars indicate the standard error of the mean.

At all three study sites, the mean proportion of soil moisture changed throughout the duration of the study (Figure 14). This variation mirrored the variation in rainfall patterns (Figure 12). During sampling months four (June 2011) and six (August 2011) when no rainfall was recorded at the weather stations (Figure 12), the mean proportion of soil moisture declined considerably and was the lowest in the August 2011 sampling date (month 6; Figure 14).

There was also a significant variation in soil moisture throughout the duration of the study in all three soil types. Soil moisture decreased during the dry season and increased in the wet season (Figure 15). There was a significant difference in the proportion of soil moisture during different months after inoculation of *B. anthracis* spores (month: $t$ = -2.310, $p$ = 0.022). Although the three study sites did not differ significantly in total rainfall based on nearby weather stations (Figure 11), the sites differed significantly in proportion of soil moisture (study site: $t$ = -2.448, $p$ = 0.016). The calcaric regosols (soil 2) had the highest mean soil moisture at all three sites throughout the duration of this experimental study (Figure 15).

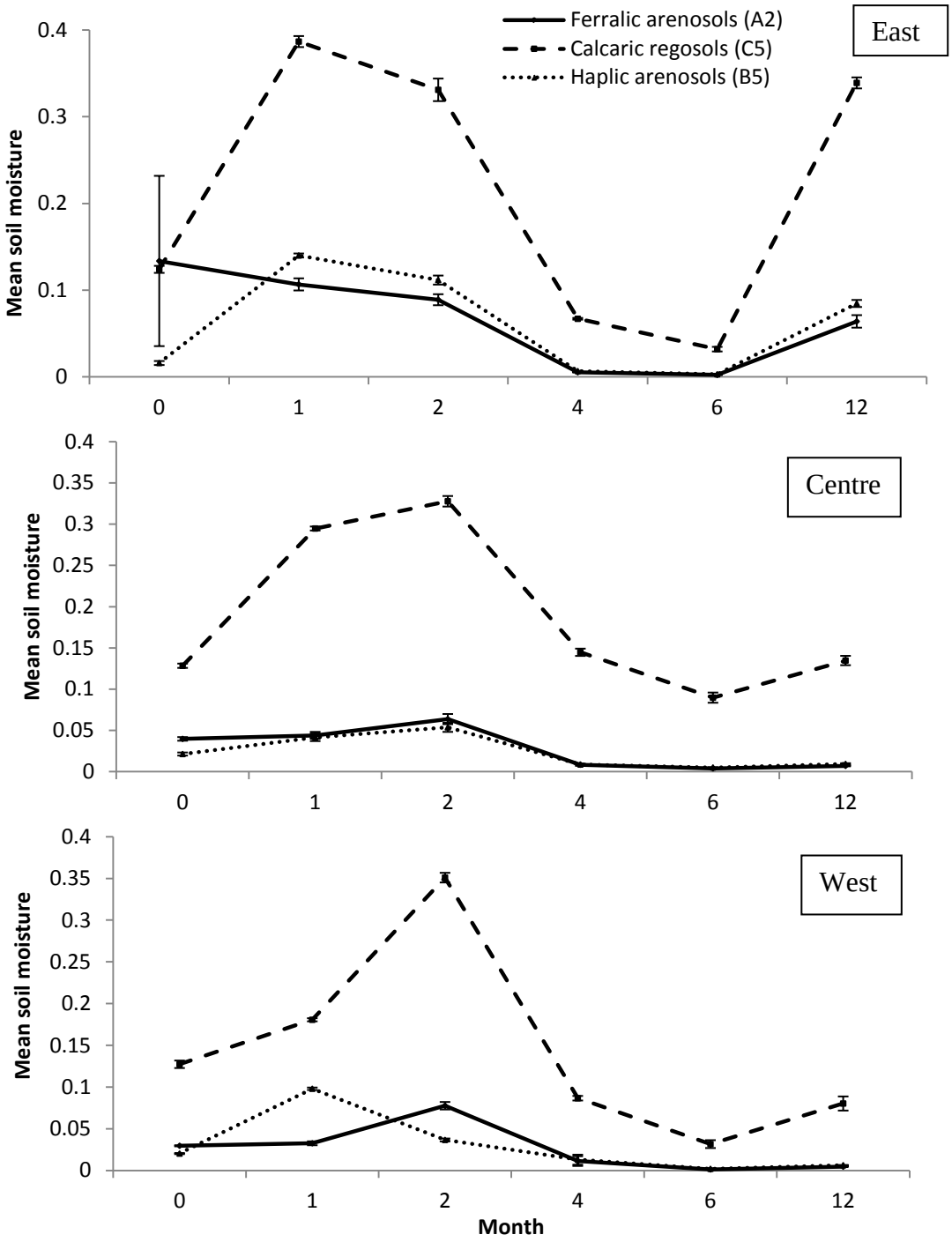


**Figure 15:** Mean proportion of soil moisture at the eastern, central, and western sites recorded at different times (month), coincident with the time when soils were sampled for counting of *B. anthracis*, from February 2011 to February 2012. Month 0 = time of inoculation of *B. anthracis* into soil, and month 12 = 12$^{th}$ month after inoculation. Months four (June) and six (August) are dry season months.

### 4.1.3 Soil Chemistry

The three soil types characterised in this study were used in the reciprocal transplant experiment to test for the effects of soil type and rainfall on the persistence of *B. anthracis* in ENP. The three soil types were collected at the start of the experiment during February 2011 (month 0). The soil samples were characterised and compared and these data are presented in Figures 16 and 17.

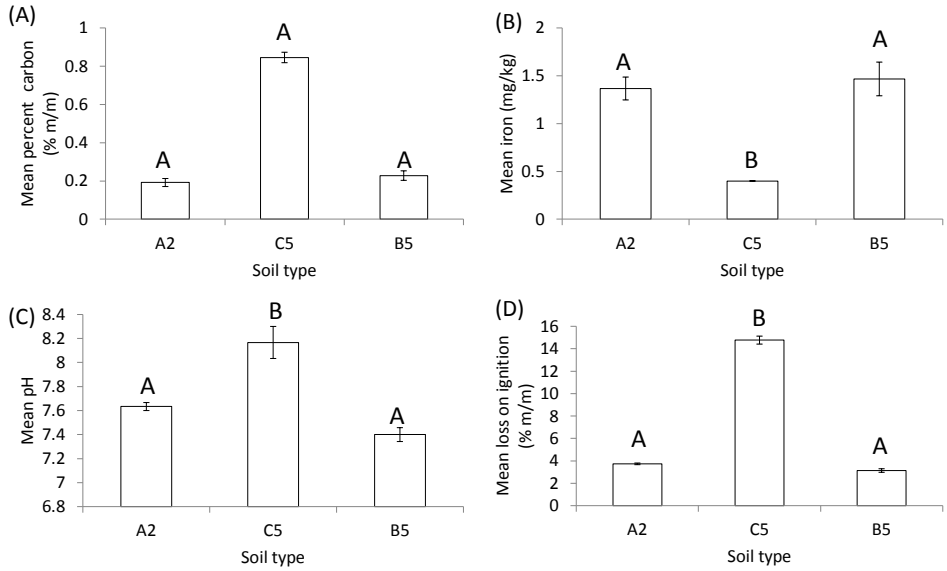


**Figure 16:** Mean (± SEM) for the concentration of (A) organic carbon, (B) iron, (C) soil pH, and (D) organic matter in ferralic arenosols (A2), calcaric regosols (C5), and haplic arenosols (B5) in ENP. The bars indicate the standard error of the mean.

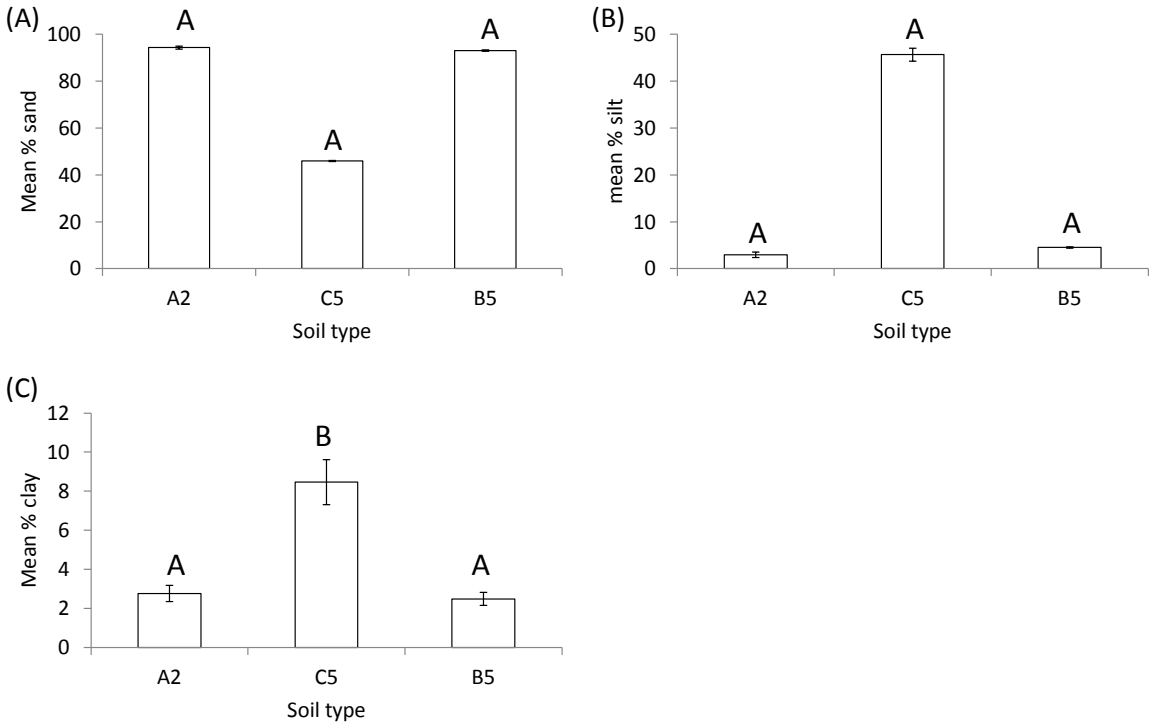


**Figure 17:** Mean (± SEM) percentage of (A) sand, (B) silt, and (C) clay in ferralic arenosols (A2), calcaric regosols (C5), and haplic arenosols (B5) in ENP. The bars indicate the standard error of the mean.

The calcaric regosols associated with site 2 in central ENP were characterised by higher soil moisture (Figure 13), higher pH, higher organic carbon content, higher organic matter content, higher clay content, and lower iron content compared to the ferralic and haplic arenosols associated with eastern and western ENP, respectively (Figures 16 & 17). The ANOVA and Kruskal-Wallis tests revealed that the calcaric regosols (C5) of central ENP differed significantly from the sandy soils of the east and west in terms of pH, iron, clay and organic matter content (Tables 1 & 2).

**Table 1**: ANOVA analysis of soil chemistry among the three study soils: ferralic arenosols (A2), calcaric regosols (C5), and haplic arenosols (B5) in ENP.

| Variable | df | *F* | *p* |
|---|---|---|---|
| pH (KCl) | 2 | 20.85 | **0.002** |
| Iron | 2 | 22.854 | **0.0016** |
| Clay | 2 | 22.345 | **0.0017** |

*Note*: Statistically significant *p*-values are indicated in bold; $\alpha = 0.05$.

**Table 2**: Kruskal-Wallis analysis of soil chemistry among the three study soils: ferralic arenosols (A2), calcaric regosols (C5), and haplic arenosols (B5) in ENP.

| Variable | df | $\chi^2$ | *p* |
|---|---|---|---|
| Organic Carbon | 2 | 5.8039 | 0.0549 |
| Organic matter | 2 | 7.322 | **0.0257** |
| Sand | 2 | 5.6 | 0.0608 |
| Silt | 2 | 5.6471 | 0.0594 |

*Note*: Statistically significant *p*-values are indicated in bold; $\alpha = 0.05$.

### 4.1.4 Total *B. anthracis* Cell Counts

The total counts of *B. anthracis* cells declined throughout the duration of the study (month: $t = -3.498$, $p = 0.0006$; Figure 18). The three study sites did not differ significantly in total cell counts (sites: $t = -0.814$, $p = 0.417$). Thus, there was no effect of the site treatment. However, within each study site, the three soil types differed significantly in total cell count (soil type nested within site: $t = 3.227$, $p = 0.0015$). At all three sites, cell counts of *B. anthracis* were consistently lower for the calcaric regosols that originated from site 2 than the other two soil types (Figure 18).

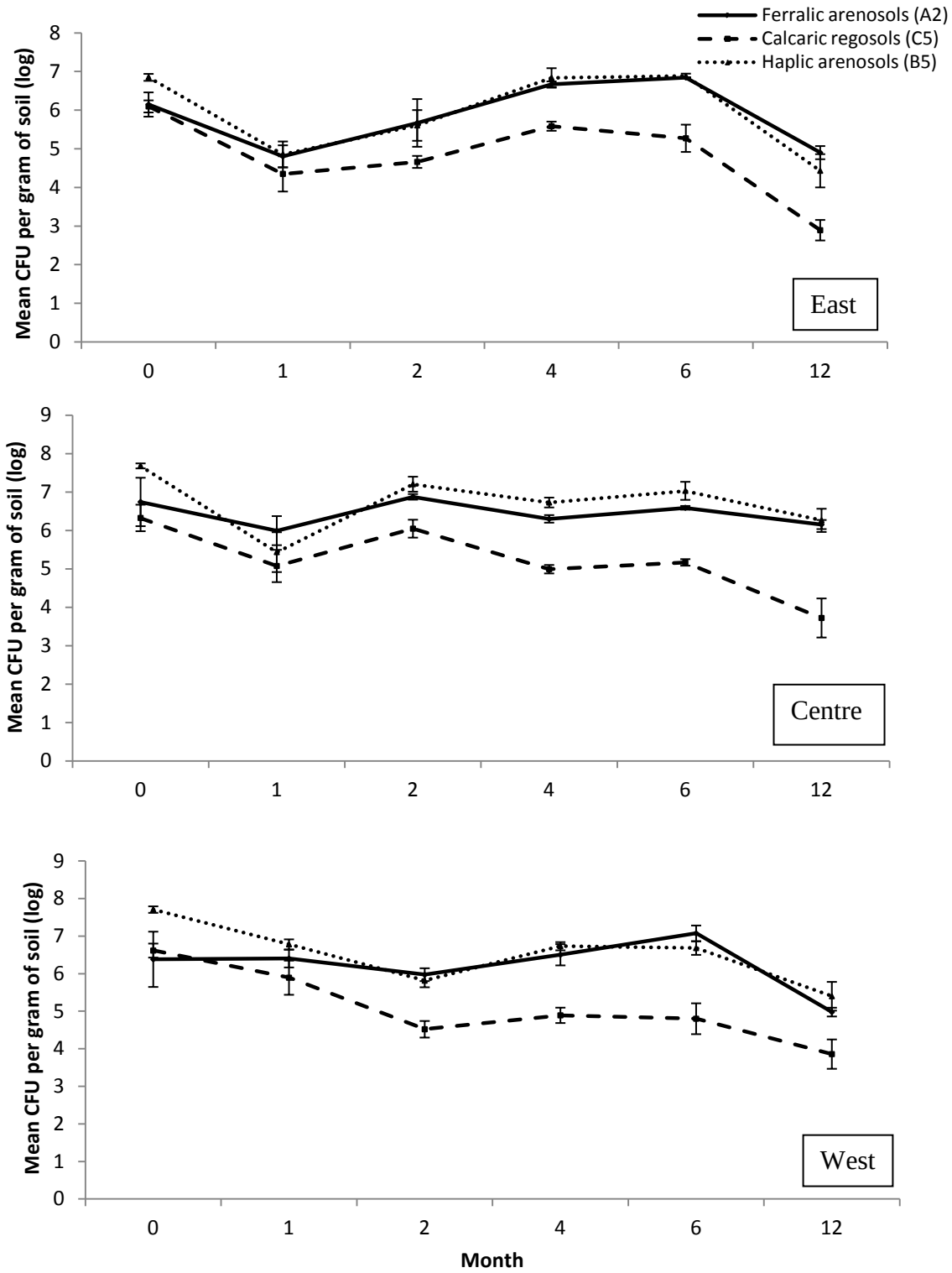


**Figure 18:** Mean number (± SEM) of *B. anthracis* cells per gram of soil from the point of inoculation (month 0) to the last sampling point (month 12) at the eastern, central and western study sites. Bars indicate the standard error of the mean. Months four (June) and six (August) are dry season months.

### 4.2 Contribution of Anthrax to Elephant Mortality

Of the 52 soil samples collected from elephant carcass sites, only 6% tested positive for *B. anthracis* (Figure 19). These data were not normally distributed (W = 0.2557, $p$ <0.00001). The Chi-square Goodness-of Fit test indicated that significantly more soil samples collected from fresh elephant carcasses were positive for *B. anthracis* than soil samples collected from old elephant carcasses ($\chi^2$ = 8.87, df = 1, $p$ = 0.003).

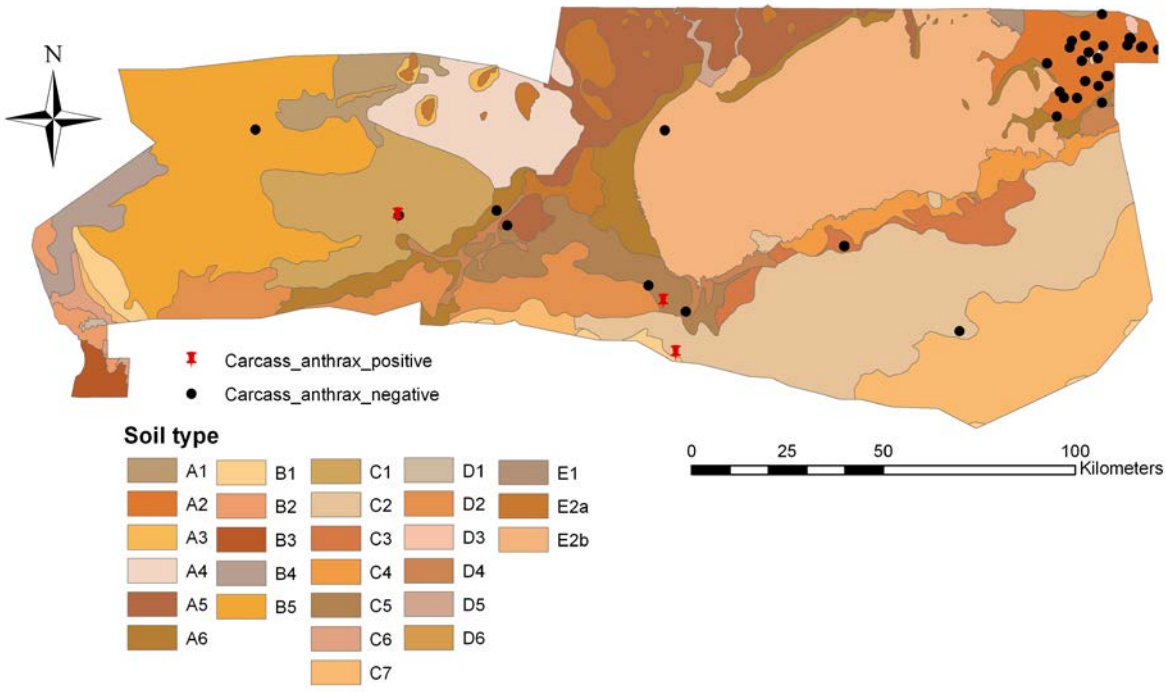


**Figure 19:** Location of soil samples collected from elephant carcasses during 2010 and 2011 in ENP (EEI GIS database, 2012; created by M. Tsalyuk). The black dots indicate anthrax negative soil and the red pins indicate soil samples that tested positive for *B. anthracis*.

During the study period, only eight of the 52 elephant carcasses found were tested for the presence of *B. anthracis* through both soil culture at the EEI and swab culture at the Central Veterinary Laboratory in Windhoek. Of the eight fresh elephant

carcasses found, 50% tested positive for *B. anthracis* through either soil and swab culture (Table 3).

**Table 3:** Detection of *B. anthracis* by soil and swab culture at eight elephant carcass sites located in ENP during 2010 and 2011.

| Elephant carcass swab number | Location of carcass | Swab culture | Soil culture |
|---|---|---|---|
| 09-147 | -18.97577 °S; 15.50385 °E | Negative | Negative |
| 10-213 | -19.18848 °S; 15.91694 °E | Positive | Positive |
| 10-222 | -19.30994 °S; 15.94879 °E | Positive | Positive |
| 10-228 | -18.98763 °S; 15.26144 °E | Negative | Negative |
| 10-229 | -18.98836 °S; 15.25981 °E | Negative | Positive |
| 10-232 | -19.01028 °S; 15.52975 °E | Positive | Negative |
| 10-250 | -19.15070 °S; 15.88015 °E | Negative | Negative |
| 11-092 | -18.78596 °S; 15.61829 °E | Negative | Negative |

# CHAPTER 5: DISCUSSION

## 5.1 Influence of Soil Type and Rainfall on *B. anthracis* Persistence

The aim of the present study was to determine the influence of site and soil type on the distribution of anthrax infectious sites in the Etosha National Park. This aim was achieved by conducting a reciprocal transplant experiment along the rainfall gradient that typically occurs in ENP.

It was hypothesized that the three study soils differ significantly in terms of soil chemistry, and that this difference would in turn enable the identification of the soil type most conducive for *B. anthracis* persistence. It was expected that the calcaric regosols of central ENP would have a higher pH value and higher organic matter content than the other two soils and, therefore, would have the highest *B. anthracis* cell count (Lindeque & Turnbull, 1994). It was further hypothesized that the experimental site with the lowest rainfall (western ENP) would have a greater persistence of *B. anthracis* cells in the soil than the sites in central and eastern ENP. This hypothesis was made on the assumption that the location and intensity of *B. anthracis* infectious sites are moisture-dependent and is enhanced by dryness (WHO, 2008).

The present study found that soil type significantly influenced the persistence of *B. anthracis* in the environment ($t = 3.227$, $p = 0.0015$; Figure 18). The sandy soils of the east (ferralic arenosols) and west (haplic arenosols) of ENP had the highest *B. anthracis* cell counts (Figure 18). The two sandy soils further had the lowest organic matter, organic carbon, pH (Figure 16) and soil moisture (Figure 13) compared to the calcaric regosols associated with the central study area. In a study on the distribution and persistence of anthrax in ENP, Lindeque (1991) found that the sandy soils of the

east and the west of ENP had the lowest pH values and the lowest *B. anthracis* spore counts compared to the karstveld soils of central ENP. The karstveld soils (calcaric regosols in the present study) had the highest pH values and the highest spore counts compared to the sandy soils (Lindeque, 1991). However, maximum sporulation of *B. anthracis* cells were achieved on the sandy soils (Lindeque, 1991). Unfortunately, no soil moisture data were available for Lindeque's study which made comparisons between her study and the present study difficult.

The present study further found that there was no significant difference in rainfall among the three seasonal rain gauges closest to the study sites ($\chi^2$ = 2.0000, df = 2, $p$ = 0.3679; Figure 11). Study site, therefore, did not have a significant influence on the persistence of *B. anthracis* in the soil (t = -0.814, $p$ = 0.417). It was expected that the rainfall gradient (decreasing rainfall from east to west) would be a prominent feature in ENP during the study period, but this was not the case. The annual rainfall received in ENP during 2011 was unusually high compared to previous years (Appendix 7) and no distinct rainfall gradient was observed during 2011 (Appendix 8). Even though there was no significant difference in rainfall among the three sites, soil moisture differed significantly among the three sites (t = -2.448, $p$ = 0.016; Figure 14) and the three soil types ($\chi^2$ = 70.0948, df = 2, $p$ <0.0001; Figure 13). The soil moisture of the calcaric regosols associated with central ENP was significantly higher than the ferralic and haplic arenosols (Figure 13). The hypothesis stating that *B. anthracis* spores survives and persists better in areas of low rainfall cannot be accepted because site did not have a significant effect on the persistence of *B. anthracis* spores in the soil. However, the sandy soils of the east (highest average rainfall) and west (lowest average rainfall) of ENP were found to be

the most conducive for anthrax spore persistence. It may therefore be said that drier soils, i.e. soils that do not retain water well, allow for greater persistence of *B. anthracis* endospores in the environment. Thus, it is not site, but rather soil type that enhances *B. anthracis* survival and persistence. However, other factors such as temperature and relative humidity that also may have contributed to the enhanced persistence of anthrax spores in the sandy soils were not accounted for in this study.

The ferralic and haplic arenosols originating from the east and west of ENP had the lowest recorded proportion of soil moisture. These two soil types also had the highest *B. anthracis* cell counts. The arenosols of the east and west of the park were described as deep, sandy soils (Beugler-Bell & Buch, 1997). The calcaric soils associated with the central study area not only had the highest pH value, it also had the highest soil moisture, carbon concentration and organic matter content of the three soils used in this experimental set up. Previous studies indicated that soils with high organic matter and soil moisture enhance *B. anthracis* survival (reviewed in Hugh-Jones & Blackburn, 2009). However, the present study found that *B. anthracis* persisted longer in sandy soils with characteristically lower pH values, carbon concentrations, organic matter, and soil moisture than the calcaric soils (Figure 18).

Sandy soils are very porous and do not retain water very well (Waugh, 2002). On the other hand, clay soils, like the soil associated with the calcaric regosols, are less porous and have a high organic matter content that captures water easily (Strahler & Strahler, 2006; Waugh, 2002). This allows for less evaporation to occur and hence the soil remains moist for a longer period of time (Strahler & Strahler, 2006).

In ENP, a flood-drought cycle is observed every two years. In the last four years (2009-2012) unusually wet and unusually dry conditions were observed, with flooding of the Etosha Pan in 2011 (Appendix 9). In 2011 which was an unusually high rainfall year, less anthrax positive carcasses were found compared to the previous year that was a low rainfall year (Appendix 10). A possible explanation for the higher persistence of *B. anthracis* in dry soils may be that the growth of the anthrax bacterium is adapted to the climatic conditions in ENP in that it can now survive better in sandy soils with low soil moisture. Anthrax has been in the park for many years, likely for longer than what has been recorded. This may have allowed the bacterium to mutate and adapt to the dry conditions in Namibia and the ENP, in particular. However, this is an assertion that requires further study.

The present study has shown that the sandy soils of eastern and western ENP with low soil moisture are more conducive for *B. anthracis* survival and persistence (Figure 18). The persistence of *B. anthracis* endospores is enhanced by dryness (WHO, 2008). Thus, the ferralic and haplic arenosols of eastern and western ENP were identified as endemic areas of anthrax infection. The persistence and survival of *B. anthracis* in the environment as a result of terminal haemorrhaging from anthrax carcasses may, therefore, also be greater in the ferralic and haplic arenosols. Infectious sites in these sandy soil types have the potential of infecting more animals than infectious sites in the calcaric regosols, because *B. anthracis* persists longer in the sandy soils than in the karstveld/clay soils.

## 5.2 Contribution of Anthrax to Elephant Mortality

In the present study, it was hypothesized that *B. anthracis* endospores are present at elephant carcass sites and, therefore, the contribution of anthrax to elephant mortality and the distribution of anthrax carcasses in ENP can be determined. Soil samples from elephant carcass sites were collected and cultured to determine whether anthrax was the cause of death. The results of the present study revealed that the proportion of soils that tested positive for *B. anthracis* spores at elephant carcass sites was small (17%; Figure 19). However, significantly more soil samples collected from fresh elephant carcass sites tested positive for *B. anthracis* than soil collected from old elephant carcass sites ($\chi^2 = 5.76$, df = 1, $p = 0.016$). This indicates that anthrax bacilli are more readily found at fresh carcass sites than at old carcass sites. The small proportion of anthrax positive soil at elephant carcass sites may be due to various factors. Not many fresh carcasses were found and this could have led to sampling incorrect soil since it was unknown where the terminal bleeding occurred or where the gut pile was located. Lindeque (1991) stated that *B. anthracis* endospores are formed upon release into the environment through terminal haemorrhaging, thus, the presence of *B. anthracis* can be detected at the site where the terminal bleeding occurred. The lack of locating fresh carcasses could be due to biased carcass surveillance that is more concentrated around the Okaukuejo area, but also due to the limitations of the road network. It would have been ideal to collect soil from only fresh elephant carcasses because it could then have been determined how long after death anthrax spores could be detected at said carcass sites. The degradation of *B. anthracis* cells and spores in the soil at old elephant carcass sites may also have contributed to the small proportion of anthrax positive soil samples. It

may also be that the soil samples were collected from carcass sites where the elephant did not die of anthrax. The low contribution of anthrax to elephant mortality has beneficial implications to the anthrax-elephant relationship because fewer elephants are succumbing to anthrax in ENP. However, the results of the present study are inconclusive and should be treated with caution, especially in terms of park management procedures and anthrax management in ENP.

Interestingly, the majority of elephant carcasses were found in the eastern sandveld area and all of these samples tested negative for the presence of *B. anthracis* (Figure 10). No published data could be found linking soil type to anthrax outbreaks in elephants. However, it was found that elephants die of anthrax during the dry season in game reserves such as Chobe National Park (ProMed-Mail, 2000) and Luangwa Valley (Turnbull, Bell, Saigawa, Munyenyembe, Mulenga & Makala, 1991). The timing of elephant mortality due to anthrax in these game reserves resembles the situation in ENP.

The present study identified the ferralic arenosols of the eastern sandveld and the haplic arenosols of the west as endemic soil types for *B. anthracis* since the bacterium had a greater persistence in these soils than in the calcaric regosols of the karstveld in central ENP (section 5.1). Again, the incidence of the anthrax bacterium in the soil may have been considerably reduced due to the age of the carcass which may have led to the sampling of soils that were not associated with the area where the blood and bacilli had spilled. Both Coker (2002) and Lindeque (1991) observed a decline in the number of *B. anthracis* endospores over time at carcass sites. This may be possible reason for the negative results obtained from soil samples collected at old carcass sites. Also, no swab results are available for most of the old carcasses that

were spotted during the aerial surveys. Therefore, it is unknown whether anthrax was the cause of death of these elephants. More intensive mortality surveillance is required in the east and west of ENP to obtain comparable data between these areas and the Okaukuejo area where the mortality surveillance effort currently is the highest. These data will also indicate whether the sandy soils with high *B. anthracis* persistence have higher anthrax mortality rates than the central area from which the calcaric soils originate.

### 5.3 Limitations of the study

The results of the present study were limited by a few factors. Firstly, the rainfall data collected during the present study were from seasonal rain gauges and weather stations that were located more than 20 km away from the sites used in the reciprocal transplant experiment. The reciprocal transplant experiment started out with a rain gauge in each of the three plots allocated for the study. However, there were instances where the rain gauges overflowed with rainwater. Long exposure to the sun caused the plastic gauges to become brittle and resulted in the gauges to leak. There was also an instance where one of the rain gauges was broken by elephants who tried to get to the water captured in the gauge during the dry season right after the initial rains started. All of this led to the rainfall measurements at the three sites to be disregarded because of the inaccuracy of the readings. The rainfall measurements at the three sites may actually have differed from each other and show that rainfall did have an effect on the *B. anthracis* cell count.

Another limitation to the present study was the collection of soil from old elephant carcasses where the location of terminal haemorrhaging and where the gut

pile spilled were unidentifiable. This may have led to the large number of negative carcass soils. Also, no swabs were taken from the bones or hides at any of these old carcasses. Thus, there is no way of telling whether these elephants died of anthrax or not, and comparisons between soil and swab culture is impossible.

# CHAPTER 6: CONCLUSION & RECOMMENDATIONS

## 6.1 Conclusion

Based on previous work by Lindeque and Turnbull (1994) and Lindeque (1991) in the Etosha National Park, it was expected that the persistence of *Bacillus anthracis* would be higher in the karstveld soils with high pH, organic matter content, and soil moisture than the sandy soils with lower pH, organic matter, and soil moisture. It was, however, not known what effect a combination of soil type and rainfall would have on *B. anthracis* persistence. Therefore, a reciprocal transplant experiment, a first in ENP, was conducted to determine and compare the effects of soil type and site on *B. anthracis* persistence in three soil types from the east, centre, and west of ENP. Also, the contribution of anthrax to elephant mortality in ENP was investigated.

The present study revealed that soil types that are sandy in texture and that do not retain water well are more conducive for *B. anthracis* persistence than soils with a finer texture and the ability to retain more water. This study also led to identifying the sandy soils of the east and west of ENP as anthrax endemic areas. Furthermore, the present study predicts that drying of ENP as a result of a change in the current rainfall gradient due possibly to the effects of global climate change will lead to the establishment of new areas of anthrax endemicity. In conclusion, soil type and soil moisture influence the distribution of anthrax infectious sites; and anthrax does not significantly contribute to elephant mortalities in ENP.

## 6.2 Recommendations for future work

Long-term investigations of *B. anthracis* in the soil are crucial in assessing the effect of climatic conditions (including temperature, humidity, and rainfall) on the survival of the bacterium in the environment. The interactions of *B. anthracis* with other microorganisms in the soil and during culture procedures should also be investigated to determine the possible inhibitory effects that other soil microbes may have on the anthrax bacterium. The persistence of *B. anthracis* spores at carcass sites, elephant carcass sites in particular, needs to be investigated and the role of factors like soil type and rainfall needs to be determined. The present study determined that soil type influences the distribution of anthrax infectious sites and that sandy soils are most conducive for anthrax spore persistence. Thus, knowledge of the persistence of *B. anthracis* at carcass sites and the role of soil type would strengthen the current understanding of anthrax infectious sites and how such sites could be responsible for future anthrax outbreaks. Intensive carcass surveillance and analyses throughout ENP is also important to determine the prevalence and survival of anthrax spores at elephant carcass sites. Continuing studies are also vital to determine how anthrax is transmitted within the Etosha ecosystem because it is still unclear how animals contract the disease. The sum of these investigations is fundamental for the better understanding of *B. anthracis* and the disease it causes, as well as the effective management thereof.

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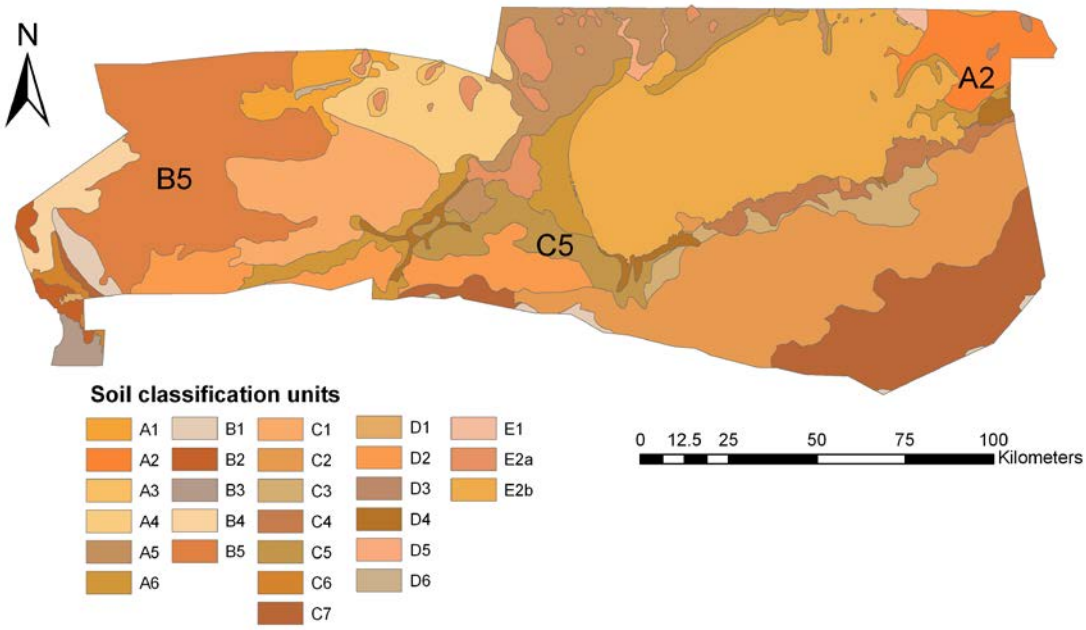
## Appendices

**Appendix 1:** Soils of Etosha National Park (adapted from Beugler-Bell & Buch, 1997). The description of each soil mapping unit below only lists the major soil types found in ENP, and does not describe each of the individual soil types.




**A: Soil associations from deep (>1 m) sandy substrata**

A1 – Arenosols
A2 – Arenosols
A3 – Arenosols; Cambisols
A4 – Arenosols; Cambisols
A5 – Arenosols; Cambisols
A6 – Arenosols; Cambisols

**B: Shallow to moderately deep (>1 m) sandy-loamy to sandy soil associations**

B1 – Leptosols; Cambisols, Arenosols
B2 – Leptosols
B3 – Cambisols; Fluvisols
B4 – Arenosols; Leptosols
B5 – Arenosols; Vertisols

**C: Shallow to moderately deep sandy-loamy to loamy-clayey soil associations**

C1 – Cambisols; Vertisols
C2 – Leptosols; Vertisols
C3 – Leptosols
C4 – Leptosols; Solonchaks; Regosols
C5 – Regosols; Leptosols
C6 – Cambisols; Leptosols
C7 – Leptosols; Cambisols; Fluvisols; Vertisols

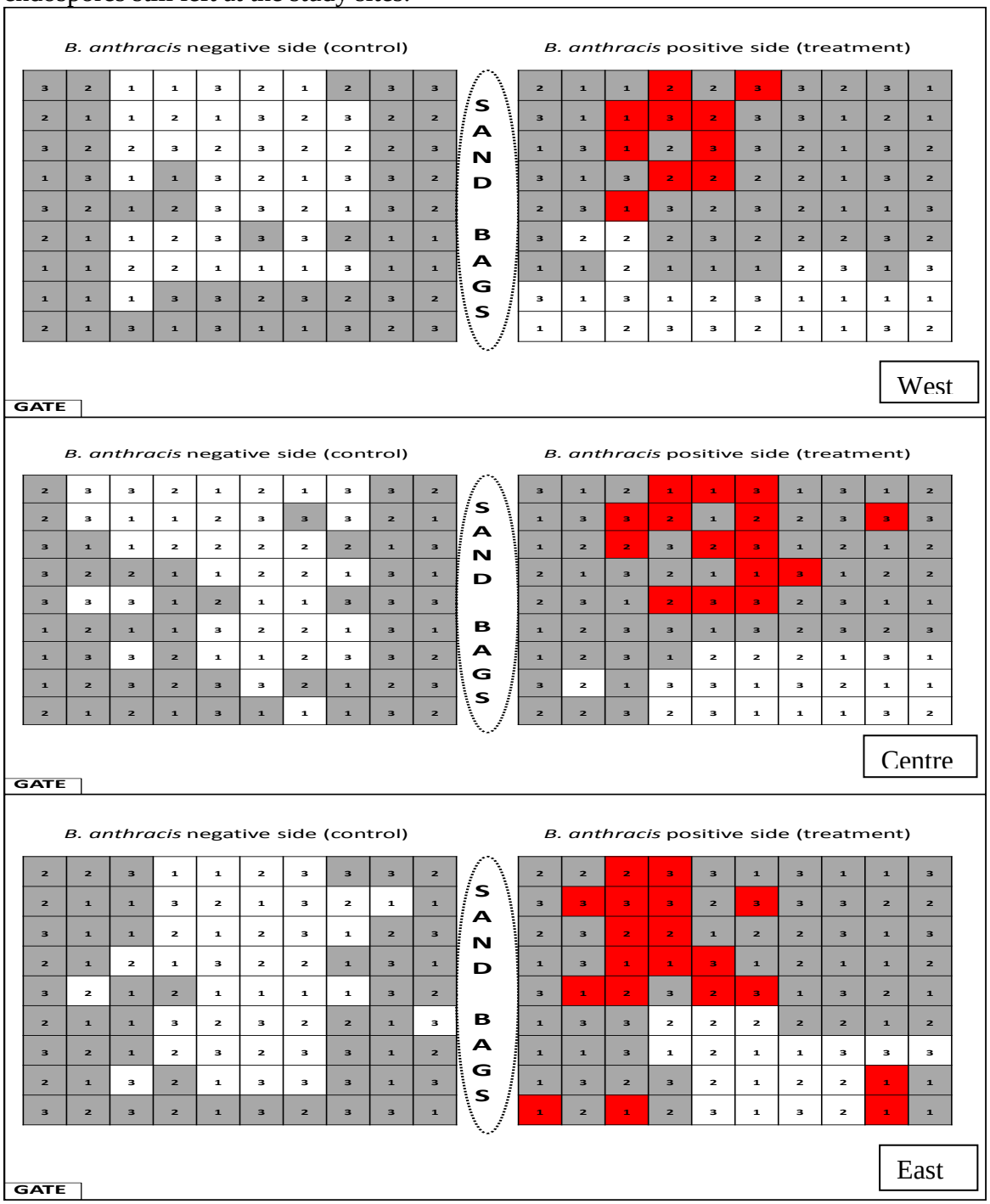
**D: Soils from fluvial sediments**

D1 – Fluvisols
D2 – Fluvisols; Regosols; Leptosols
D3 – Fluvisols; Regosols
D4 - Fluvisols
D5 - Fluvisols
D6 - Fluvisols

**E: Saline soils/sodium rich soils**

E1 – Solonetzs
E2 - Solonchaks

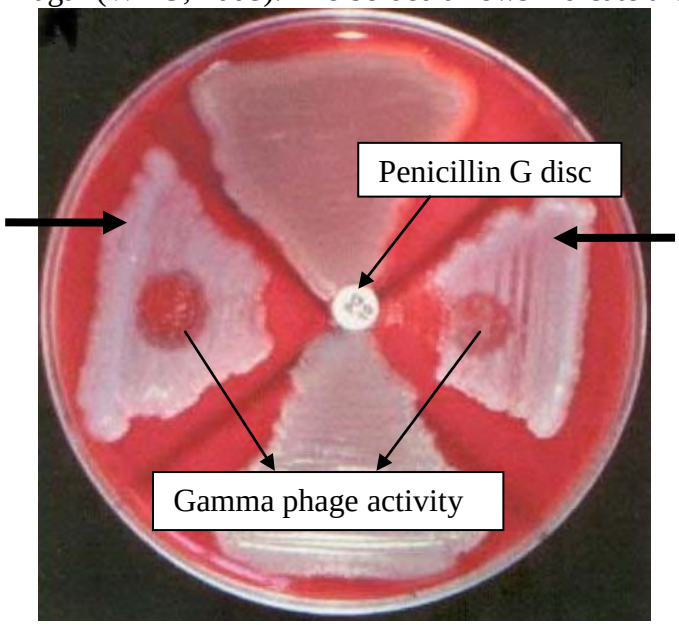
**Appendix 2:** Randomized layout of the ferralic arenosols (1), calcaric regosols (2), and haplic arenosols (3) at the three experimental sites – western ENP, central ENP, and eastern ENP. Gray indicates soil cores sampled; red indicates soil cores with *B.anthracis* endospores still left; white indicates soil cores without *B. anthracis* endospores still left at the study sites.



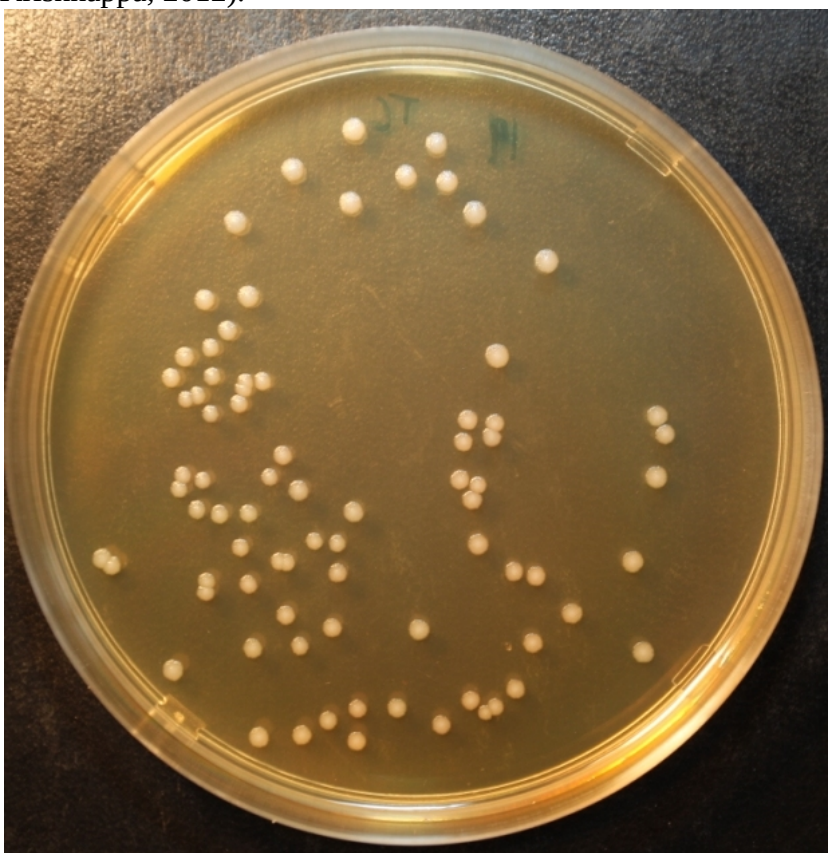
**Appendix 3:** Planting of soil cores (Photo: Z. Havarua, 2011).

**Appendix 4:** The random positioning of soil cores planted into the soil (Photo: S. Bischoff, 2011).

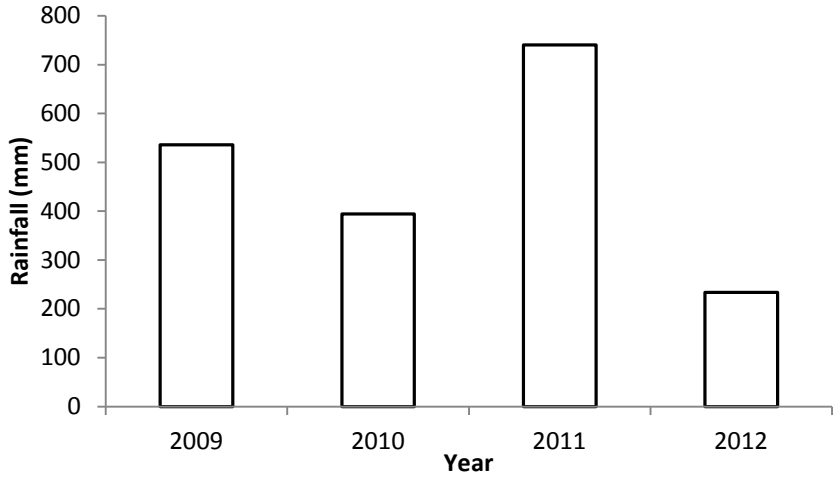
**Appendix 5:** Sensitivity of *B. anthracis* to gamma phage and penicillin on blood agar (WHO, 2008). The bolded arrows indicate the growth of *B. anthracis*.



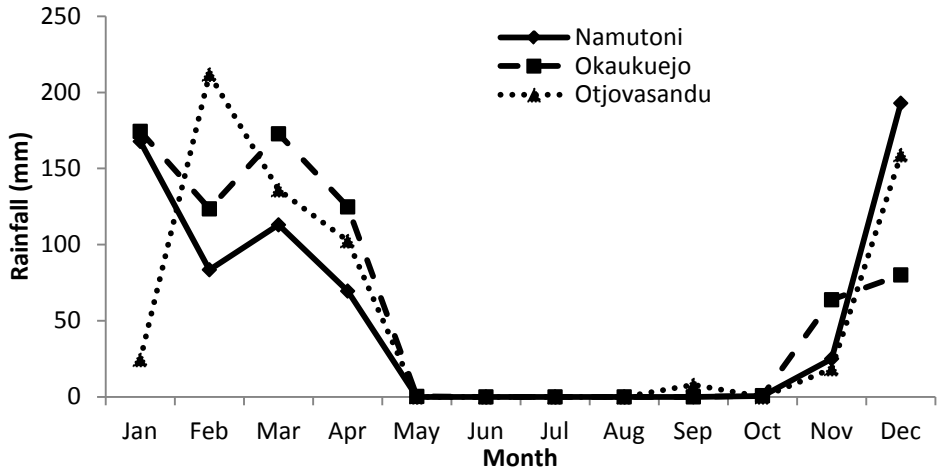

**Appendix 6:** *Bacillus anthracis* growth on PLET agar at 48 hours (Photo: Y. Krishnappa, 2012).



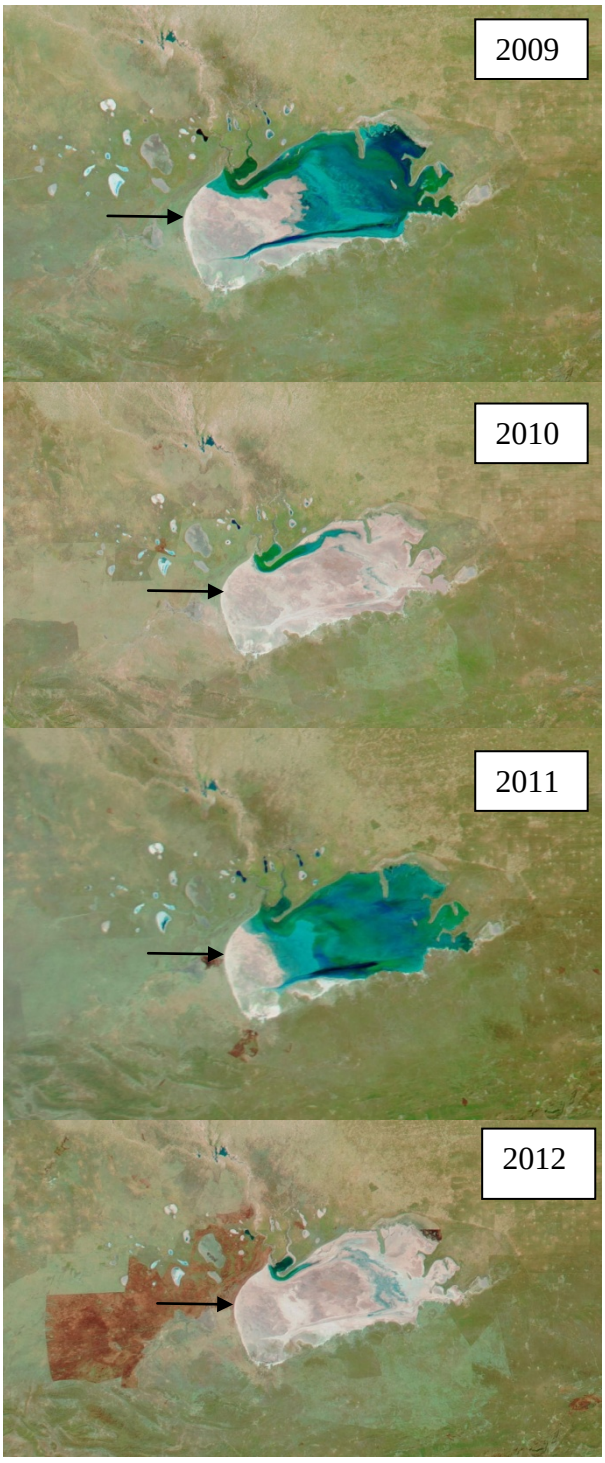
**Appendix 7:** Annual rainfall in ENP for the period July 2009 to June 2012.



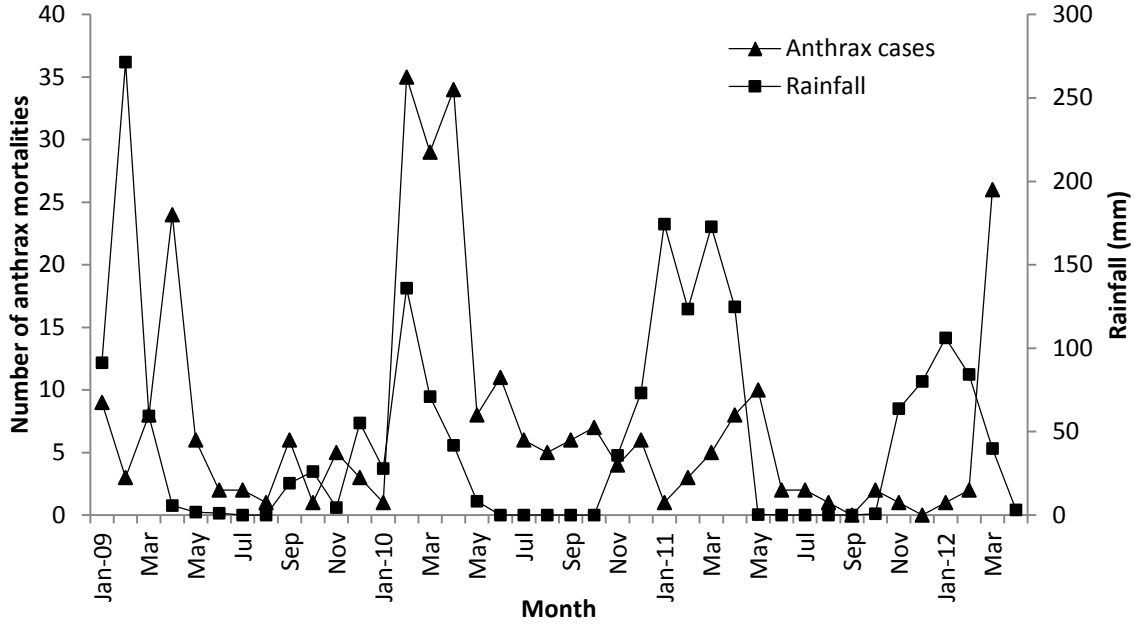

**Appendix 8:** Monthly rainfall at Namutoni, Okaukuejo, and Otjovazandu during 2011.



**Appendix 9:** Flood and drought in Etosha Pan for the years 2009, 2010, 2011, and 2012 (NASA Earth Observatory, 2012). All four pictures were taken during July. The arrow indicates the Pan.



**Appendix 10:** Monthly variation in anthrax in relation to rainfall in the Okaukuejo area, Etosha National Park for the period 2009-2012.




**Appendix 11:** Anthrax cases in herbivores in Etosha National Park during the period 1975-2011 (EEI mortality database, 2012).

| Species | Anthrax cases |
|---|---|
| Plains zebra | 967 |
| Elephant | 334 |
| Blue wildebeest | 297 |
| Springbok | 285 |
| Gemsbok | 20 |
| Greater kudu | 11 |
| Black rhinoceros | 9 |
| Giraffe | 7 |
| Red hartebeest | 3 |
| Eland | 2 |
| Impala | 1 |