REVIVAL AND CHARACTERIZATION OF AEROBIC ENDOSPORE-FORMING BACTERIA FROM AN ANCIENT SEDIMENT CORE OBTAINED FROM THE MFABENI PEATLAND, SOUTH AFRICA

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The research was financially supported by the National Research Foundation (NRF).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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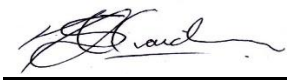
Signed: Dr. C.H. Hunter (Supervisor)

Date: June 2017

DECLARATION

I, Selisha Naidoo, declare that:

i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;
ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;
iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;
iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a) their words have been re-written but the general information attributed to them has been referenced;
    b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;
v) where I have used material for which publications followed, I have indicated in detail my role in the work;
vi) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.



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ABSTRACT

Peatlands contain an accumulation of organic material over extended periods of time which can provide biological and chemical proxies for determining palaeo-environments. The organic accumulation can be dated to provide a chronology of time that, integrated with various proxies, can help understand, interpret and infer past environmental conditions which prevailed at the time of deposition. One such proxy that has yet to be explored are aerobic endospore-forming bacteria (AEFB) which have the ability to form dormant endospores as a survival strategy to overcome unfavourable conditions. Dormant endospores may potentially serve as 'time capsules' which could provide an insight into the changes in microbial diversity of AEFB, from ecologically-sensitive environments, in relation to past physico-chemical conditions. This study sought to revive and determine the genetic and physiological diversity amongst dormant AEFB sampled from an ancient sediment core obtained from the Mfabeni Peatland, KwaZulu-Natal, South Africa. Samples were taken from radiocarbon-dated sections of the core at ca. 589, 1 964, 17 568, 33 328 and 37 906 cal years BP and subjected to a sequential extraction protocol prior to dilution series plating onto selected media. A total of 270 isolates were selected for genetic fingerprinting and screened using Repetitive extragenic palindromic-Polymerase Chain Reaction (Rep-PCR), targeting the BOX mosaic repetitive element to evaluate genetic diversity, and High Resolution Melt Analysis (HRMA), which was evaluated as a means to distinguish between fingerprint groupings and to validate the genotyping results. HRMA was carried out using primer sets targeting the *spo0A* gene and the V3 and V4 variable regions of the 16S rRNA gene. Taxonomic ranking and phylogeny of 83 representative genotypes were assessed through sequence analysis of amplified partial 16S rRNA gene fragments. As part of physiological testing, salt (0.5%–15% NaCl) and pH range (3–10) tolerances were determined using microtiter plate assays. Substrate utilization abilities of selected representative isolates were determined through physiological profiling using Biolog EcoPlates™ and statistically analyzed by means of Canonical Correspondence Analysis (CCA). Isolates were revived from all five depths and total colony-forming units (CFU) per gram were found to decrease with increasing depth and age of samples. The nutritionally-rich trypticase soy agar (TSA) medium produced lower counts of revived AEFB compared to the environmentally-based Marine Agar and nutritionally-limited Reasoner's 2A agar and 10% TSA media. Rep-PCR allowed for strain-level discrimination of the isolates. The V3 and V4 regions provided the best HRMA resolution and allowed for closely-related species to be

matched. Genotyping revealed that significant diversity was present amongst isolates from different sample depths. Ninety-four percent of operational taxonomic units (OTUs) distinguished were unique to their sampling depth. Phylogenetic analysis revealed the presence of several aerobic endospore-forming genera, namely, *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Domibacillus*, *Lysinibacillus*, *Solibacillus* and *Paenisporosarcina*. Strains of *Lysinibacillus* and *Brevibacillus* were unique to sample depths corresponding to ca. 589 and 1 964 cal years BP, whereas *Domibacillus* sp. isolates were revived from samples dated at ca. 17 568 and 33 328 cal years BP. Eighteen percent of isolates had 16S rRNA gene sequences displaying <97% similarity to reference species currently listed in the NCBI database, suggesting that some of these isolates may potentially represent novel species or strains. Substrate utilization profiling revealed that, collectively, isolates were able to utilize 27 of the 31 substrates tested; the most commonly utilized substrates being Tween 40, Tween 80 and pyruvic acid methyl ester. Canonical correspondence analysis revealed, with significance, that the age of the samples from which the isolates were obtained did have an effect on the variations observed in the patterns of substrate utilization. Tolerance ranges to salt and pH depicted less significant variations between isolates from different depths. The highest percentages of isolates favoured salt concentrations up to 2.5%. Thirty-seven percent of selected isolates were capable of growth at a 15% NaCl concentration. The majority of isolates tested from each depth preferred a pH range of 7.5–8.5, whilst a smaller percentage, namely, 5.7% and 34%, were capable of growth at pH values of 3 and 10, respectively. The results suggest that peatlands do serve as a reservoir for dormant AEFB, some of which are potentially uncharacterized. This study highlights the changes in bacterial genetic and physiological diversity which occur along an environmentally-variable sediment profile. Dormant endospores may serve as a proxy which can be used to determine palaeo-environments.

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LIST OF ABBREVIATIONS

AEFB	-	Aerobic endospore-forming bacteria
AMS	-	Accelerator mass spectrometry
ANOVA	-	Analysis of variance
ARDRA	-	Amplified ribosomal DNA restriction analysis
ATP	-	Adenosine triphosphate
CA	-	Correspondence analysis
$CaCl_2$	-	Calcium chloride
Cal years BP	-	Calibrated years before present (before 1950 AD)
CCA	-	Canonical correspondence analysis
CFU	-	Colony-forming units
CLPP	-	Community-level physiological profiling
DGGE/TGGE	-	Denaturing/temperate gradient gel electrophoresis
DNA	-	Deoxyribose nucleic acid
DPA	-	Dipicolinic acid
EDTA	-	Ethylenediaminetetraacetic acid
G+C	-	Guanine and cytosine
HRMA	-	High resolution melt analysis
LnAge	-	Natural logarithm of age
MST	-	Multilocus sequence typing
NaCl	-	Sodium chloride
OTU	-	Operational taxonomic unit
PCA	-	Principal component analysis
PCR	-	Polymerase chain reaction

R2A	-	Reasoner’s 2A agar
RAPD	-	Random amplified polymorphic DNA
RDA	-	Redundancy analysis
Rep-PCR	-	Repetitive extragenic palindromic-Polymerase chain reaction
RFU	-	Relative fluorescence units
RISA	-	Ribosomal intergenic spacer analysis
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
SASPs	-	Small acid soluble proteins
SSCP	-	Single strand conformation polymorphism
T-RFLP	-	Terminal restriction fragment length polymorphism
TSA	-	Trypticase soy agar
TSB	-	Trypticase soy broth
UV	-	Ultraviolet

CHAPTER ONE

INTRODUCTION

Wetlands are biologically diverse ecosystems that occupy approximately 6% of the total land surface on earth (Batzer and Sharitz, 2014). These systems can be sub-divided into areas of fresh-water, estuarine, fen, marsh or peatland. Of these, peatlands make up approximately 50% of wetlands in the world (Grundling *et al*., 2013a).

Within peatlands, the rate of biomass production exceeds that of decomposition (Whittle and Gallego-Sala, 2016). This causes an accumulation of organic material to occur in layers, over potentially thousands of years, in which biological organisms can become entrapped. Both anoxic and oxic conditions occur within these ecosystems (Bodelier and Dedysh, 2013). Aerobic endospore-forming bacteria (AEFB) are routinely found in the oxic zone of wetland environments and contribute towards the productivity and turnover within such environments (Ding *et al*., 2005; Nieder and Benbi, 2008; Kumar *et al*., 2012). When anoxic conditions dominate, AEFB have the ability to form dormant endospores for survival.

Endospore formation is a survival strategy which is triggered during adverse conditions and is employed by certain genera of Gram positive bacteria (Schlegel, 1993). The endospores are dormant, highly differentiated and exceedingly resilient structures which are formed within the bacterial cell and serve the function of providing resistance to the bacteria (Schlegel, 1993; Hilbert and Piggot, 2004). The structural composition of the endospore ensures DNA protection from the harmful effects of environmental conditions which may include exposure to chemicals, ultraviolet (UV) radiation or fluctuations in temperature, pH, salinity, moisture or oxygen levels (Hilbert and Piggot, 2004). This enables endospores to survive for extended time periods under unfavourable and potentially diverse conditions.

Dormant endospores may become deposited and preserved during the accumulation of peat material over time. As a result, they may remain trapped within layers over a significant time period in an unaltered form. The organic material forming the peatland can be dated to provide a chronology of time and age. In this way, dormant endospores may serve as ‘time capsules’, or environmental proxies, having the potential to provide information regarding changes in bacterial diversity and physiological activity arising from physico-chemical changes occurring within a given environment. By examining shifts in the presence, abundance and environmental tolerances of proxies trapped within the organic material, inferences can be made regarding

past environmental conditions, which is useful in the field of climate change (Meadows, 2014). By developing new proxies, a greater insight into palaeo-environments can be gained and could contribute to the list of existing proxies used, which include pollen, diatoms and macro-fossils, to allow for a more accurate and precise reconstruction of past environments (Meadows, 2014). Whilst dormant endospores have the potential to serve as palaeo-ecological indicators, their application in the examination of past environmental conditions has not been examined in great detail (Renberg and Nilsson, 1992). Therefore, by examining palaeo-environments, the physiological and taxonomic diversity, as well as the metabolic capabilities of AEFB, may be studied. Furthermore, the potential of AEFB to serve as proxies in palaeo-ecological research can be examined.

Within the iSimangaliso World Heritage Site, a site which houses approximately 3 280 km^2 of diverse ecosystems, lies St. Lucia, the largest estuarine-linked lake system in Africa (Grundling *et al*., 2013b). Approximately 66% of recorded peatlands in South Africa are contained within the greater St. Lucia Wetland Park, thereby making it one of the most significant peatland ecoregions in the country (Grundling *et al*., 2013a). Located within St. Lucia is the study site, the Mfabeni Peatland.

The Mfabeni Peatland is a system that is receiving research attention due to its age, dating back beyond the Holocene, which is exceptional in the region (Meadows, 2015). However, to date, this region has not been explored from a microbiological viewpoint and hence, there is a lack of information regarding the functional and genomic diversity and microbial communities which resided in this area. This is a region which is ideal for palaeo-environmental research (Finch and Hill, 2008) and due to the age of this peatland, would be suitable for examining the feasibility of reviving AEFB present in a sediment core extracted from the site.

The study was undertaken with the aim of reviving dormant AEFB from sections of an ancient sediment core obtained from the Mfabeni Peatland, KwaZulu-Natal and determining their genetic and physiological diversity. As such, the objectives of this study are as follows:

i) Isolation and revival of dormant AEFB from sections of an ancient sediment core that had been radiocarbon dated to ages ranging from ca. 589 to 37 906 cal years BP.

ii) Assessment of the genetic diversity of revived AEFB through utilization of two techniques, namely Repetitive extragenic palindromic-Polymerase Chain Reaction (Rep-PCR) and High Resolution Melt Analysis (HRMA).

iii) Determination of taxonomic ranking and phylogeny of representative genotypes through 16S rRNA gene amplification and sequence analysis.

iv) Assessment of the salt and pH tolerance ranges of selected representative isolates.

v) Determination of the metabolic diversity amongst isolates by examining substrate utilization capabilities through the use of Biolog EcoPlates™.

This study focussed on characterizing the diversity of AEFB at varying radiocarbon-dated ages down a sediment core, namely 589, 1 964, 17 568, 33 328 and 37 906 cal years BP. This will be the first report providing information regarding the bacterial diversity in an ecosystem within the Mfabeni Peatland. In addition, the changing climatic conditions of this peatland through time make it the ideal study site for examining the changes in bacterial genetic and physiological diversity which occur along an environmentally-variable sediment profile. This study fits into a multidisciplinary approach based on palaeo-environmental research for a fragile and important ecosystem in southern Africa and provides the potential to examine a new proxy which could contribute towards future studies of palaeo-environments.

CHAPTER TWO

LITERATURE REVIEW

2.1. INTRODUCTION

Wetlands are areas that are saturated with water on a temporary or permanent basis and are considered to be distinct ecosystems (Ramsar Convention Secretariat, 2013). These regions contain water which is either flowing or in a static state and support a diverse range of plant and animal life (Batzer and Sharitz, 2014). Wetlands play important roles in a number of ecological processes such as biogeochemical cycling, water purification, greenhouse gas emissions, shoreline stabilization and flood mitigation (Bodelier and Dedysh, 2013). These regions are extremely susceptible to climatic changes. Coastal wetlands may become flooded through sea level rise and are prone to erosion, runoff and depreciation in water quality (Jin *et al*., 2009). These changes have an impact on the biota and microorganisms which reside there, consequently affecting the diversity, abundance and distribution of species in these ecosystems (Jin *et al*., 2009). Microbial communities play a significant role in biogeochemical cycling and the overall maintenance of wetland processes; however, the study of microbial diversity in wetland environments is often overlooked (Bodelier and Dedysh, 2013).

Peatlands make up approximately half of all wetlands in the world (Grundling *et al*., 2013a). They are characterized by a build-up of organic material, consisting of partially-decomposed plant matter, which occurs over extended periods of time as a result of anaerobic conditions which predominate (Kroetsch *et al*., 2011). These regions are suitable for palaeo-environmental research, which is centred on the reconstruction of past environments (Gorham *et al*., 2001). Biological and chemical proxies contained within the organic material can be used for determining palaeo-environments (Gorham *et al*., 2001). These proxies generally exist in unaltered forms over long time periods and can be used to interpret and infer past environmental conditions which prevailed at the time of deposition. Proxies currently used include pollen, ichnofossils, diatoms and macro-fossils (Meadows, 2014). One such proxy that has yet to be explored are aerobic endospore-forming bacteria (AEFB) which have the ability to form dormant endospores, which are highly differentiated structures formed within the bacterial cell to provide protection against unfavourable conditions during metabolic dormancy (Hilbert and Piggot, 2004).

The occurrence of anoxic and oxic zones within peatlands support the presence of anaerobic and aerobic microbial communities (Bodelier and Dedysh, 2013). AEFB are heterotrophs that would be expected to be metabolically active within the aerobic zones. When anaerobic conditions prevail, AEFB form dormant endospores for survival. As peat layers are deposited over time, endospores would consequently remain trapped and preserved over long periods. Endospores are able to remain in sediment once deposited (Wunderlin *et al*., 2014) and viable endospores have been successfully isolated from ancient sediments dated to 13 000 years (Rothfuss *et al*., 1997), 9 000 years (Renberg and Nilsson, 1992) and 5 800 years (Bartholomew and Paik, 1966). Sediments may, therefore, serve as a suitable archival material which could provide stable conditions that allow for endospore survival. Since endospores are able to remain deposited and inactive, they could provide reflections of AEFB species diversity and abundance present at the time of deposition within ecologically-sensitive environments. In this way, dormant 'ancient bacteria', i.e. bacteria revived from ancient, radiocarbon-dated environmental samples, may serve as bioindicators of prevailing conditions for a given palaeo-ecological era.

The study of bacterial diversity across environmentally-variable profiles has been made possible through a wide range of molecular fingerprinting techniques which allow for microbial diversity to be differentiated at the species or strain-level (Mandic-Mulec and Prosser, 2011). Through the combination of sequencing technologies and phylogenetics, the taxonomic ranking and evolutionary relationships amongst isolated bacteria can be determined. In addition to genetic diversity, physiological dynamics may also provide insight into the changes in bacterial diversity occurring in palaeo-environments. By examining the physiological diversity of endospore-forming bacteria at different time scales, tolerance ranges to various environmental parameters can be ascertained (Jones *et al*., 1993). In addition, metabolic profiles can be established, thereby providing information on the potential functions specific bacteria may have played or the niches which they may have occupied within these ecosystems.

This review shall, therefore, address the following topics:

i) Wetland microbiology.
ii) Examination of AEFB as suitable candidates for palaeo-ecological studies.
iii) The diversity of AEFB.
iv) An evaluation of endospores, their structure, the mechanisms behind their resistance and their viability.
v) The revival of 'ancient' endospores and the associated challenges.

vi) Techniques used for the evaluation of bacterial genetic and physiological diversity.

2.2. WETLAND MICROBIOLOGY

2.2.1. What are wetlands and why are they important?

Several definitions exist which explain what wetlands are; however, from an ecological point of view, wetlands can be described as ecosystems which are characterized by land saturation with water (Bergh *et al*., 2004). The *Ramsar Convention on Wetlands* describes wetlands as areas of water, fen, marsh or peatland, which may be temporary or permanent, and contain water which is either flowing or in a static state (Ramsar Convention Secretariat, 2013). The water may either be saltwater, brackish or freshwater. In these areas, shallow water may cover the land or the water table may be present at the land surface. Due to the significant animal and plant life supported by wetlands, they are considered to be one of the most biologically diverse types of ecosystems.

Approximately 6% of the land surface on earth is occupied by wetlands (Batzer and Sharitz, 2014). Due to the significant effect that wetlands have on nutrient cycles, greenhouse gas emissions, hydrological processes, biogeochemical transformations and vegetation; wetland systems are considered to be of high ecological and economical value (Bodelier and Dedysh, 2013). Wetlands serve as buffers to run-off from terrestrial regions, thereby protecting inland and coastal waters from eutrophication. They also contribute to erosion control, stabilization of shorelines, water storage and the mitigation of floods (Ramsar Convention Secretariat, 2013). In addition, they play a pivotal role in water purification, nutrient retention, sequestration of carbon dioxide and groundwater control. Microbial communities are able to thrive in these systems due to the presence of oxic as well as anoxic conditions. The presence of these microbial communities boosts the productivity of wetland systems as they contribute significantly towards the biogeochemical cycling of nutrients and a global cycling of elements (Richardson, 1994; Bodelier and Dedysh, 2013). Ecologically, wetlands support a great range of aquatic and terrestrial biota and economically, they contribute towards fisheries, agriculture, food production, tourism and the assimilation of wastewater (Richardson, 1994).

2.2.2. Environmental characteristics of wetlands

Topography, geology and climate have an influence on the distribution and type of wetland. Wetlands are present in temperate lowlands, deserts, tropical, subtropical, alpine regions and

even the Arctic (Scott *et al*., 2014). The driving factor which dictates the characteristics, composition, productivity and biology of wetlands is hydrology (Mitsch and Gosselink, 1993). Wetlands experience saturation of water at the surface of the soil for extended periods of time annually. The hydrology includes many factors such as the level of groundwater, drainage characteristics, the degree of saturation of the soil and surface water outflow. The second major feature is the occurrence of hydric soil i.e. soil which is saturated with water (Mitsch and Gosselink, 1993). These soils generally have low oxygen contents. The third defining characteristic is the aquatic and plant life which is present (Mitsch and Gosselink, 1993). This generally includes plants such as hydrophytes which have adaptations to wet conditions.

2.2.3. The effects of climate changes on wetlands

The greatest effect that climate changes have on wetlands is the alteration of hydrological systems, since wetlands rely on the availability, supply and flow of water (Erwin, 2009). Inland wetlands may experience droughts when water inputs through groundwater recharge and surface runoff are outweighed by outputs through seepage and evaporation. Coastal wetlands can experience an increase in seawater due to the rise in sea levels (Jin *et al*., 2009). Extreme climate events can result in soil erosion, flooding, runoff and mudslides. These effects have a direct impact upon the recharging of floodplain aquifers, organic sediment oxidation and damage to local vegetation (Erwin, 2009).

Water quality is affected as a consequence since water temperatures in wetlands rise as a result of higher atmospheric temperatures. Certain plant species thrive with higher temperatures, resulting in algal blooms and a decrease of dissolved oxygen, which then promotes anaerobic decomposition (Jin *et al*., 2009). Additionally, the pH and salinity levels of wetlands may become significantly altered, thereby impacting upon the biota and microorganisms which reside there, consequently affecting the diversity and distribution of species in these regions (Jin *et al*., 2009). With the continued impact of climate changes, the functional capacity of wetlands can decline and the number of wetlands with high productivity may decrease (Erwin, 2009).

2.2.4. Microbial activity in wetland environments

Plant roots are largely responsible for the presence of anoxic and oxic conditions in wetlands, which generally occur in close proximity to each other (Bodelier and Dedysh, 2013). Due to the presence of these conditions, both anaerobic and aerobic microbial communities are active in these ecosystems. Nutrient recycling by these microbial communities contributes towards the productivity in wetlands (Bodelier and Dedysh, 2013). This occurs through the biogeochemical conversion of nutrients including carbon, nitrogen and sulfur, which are primarily derived from plant biomass (Bodelier and Dedysh, 2013).

Wetland microbiota comprise of microbial communities which belong to various functional guilds (Bodelier and Dedysh, 2013). Heterotrophic rhizosphere and phyllosphere microbial communities in wetland ecosystems may be involved in interactions with living plants and may contribute towards plant growth and productivity (Kumar *et al*., 2012). When plants die, heterotrophic aerobic and anaerobic microbial communities are responsible for the degradation of organic carbon through the production of extracellular enzymes. Carbon is one of the most predominant nutrients in wetlands and arises from plant breakdown into organic matter (Bodelier and Dedysh, 2013). The organic matter then gets broken down to release carbon dioxide (CO_2) and methane (CH_4). Methanogenic Archaea are responsible for methane production in these ecosystems, whereas aerobic methanotrophs play an important role in the oxidation of methane (Kolb and Horn, 2012). These communities thus contribute largely towards the atmospheric methane flux. Within the anoxic zones, denitrifiers are responsible for the conversion of nitrates to nitrous oxide (N_2O) and nitrogen gas (N_2) (Kolb and Horn, 2012). Nitrifiers are also present in the oxic zones and play a role in the conversion of ammonia (NH_3) to nitrate (NO_3^-), whilst diazotrophs carry out nitrogen fixation (Lovell and Davis, 2012). Peatlands are generally characterized by a low availability of nitrogen, thereby resulting in high C: N ratios, which contributes to the slow degradation process encountered under anaerobic conditions (Sheppard *et al*., 2013). Sulfate-reducing microorganisms also make up a part of wetland microbiota and are found to closely interact with microbes involved in carbon and nitrogen cycling (Pester *et al*., 2012).

Various species of AEFB have been isolated from wetland and peatland environments (Albert *et al*., 2005; Bae *et al*., 2010; Baik *et al*., 2011a; Baik *et al*., 2011b; Liu *et al*., 2015), thereby providing evidence for the occurrence of AEFB in these ecosystems. In addition to their role in degrading and metabolizing various forms of organic carbon as primary sources of carbon and energy, AEFB also contribute towards the solubilisation of phosphates, nitrogen fixation

and ammonification in sediment regions (Oren, 2002; Kumar *et al*., 2012). Endospore-formers are commonly associated with the plant rhizosphere and some species are responsible for the promotion of plant growth (Kumar *et al*., 2012). This generally occurs through the production of plant hormones and the solubilisation of elements such as phosphate and phosphorous (Han *et al*., 2006; Kumar *et al*., 2012). These elements are then released in forms which are available to surrounding plants. Endospore-forming bacteria may therefore also potentially contribute towards these functions within wetland environments.

2.3 USING AEFB IN CLIMATE CHANGE STUDIES

2.3.1. The effects of climate changes on the ecology of bacteria

Due to the roles that microbial communities play in biogeochemical processes and in maintaining ecosystem stability, it is important to examine the effects that climate changes may have on bacterial community structure and functionality. While many of the climate change effects might be indirect, such as impacts on sediment quality and on the abundance and types of plants present, the most common direct effects include fluctuations in moisture content and temperature (Singh *et al*., 2010). These factors can result in changes in microbial species richness and abundance.

The moisture content of peatlands impacts on the availability of oxygen and the rates of gas diffusion, thereby influencing the physiological activity of the extant microbial community. For instance, periodic reductions in sediment moisture may increase the availability of oxygen, thereby enhancing nutrient cycling, whereas an opposite effect is observed when moisture levels are high (Singh *et al*., 2010). The carbon: nitrogen ratio of a peatland environment can also be affected by climate changes, which can directly influence the rates of decomposition and microbial biomass in soils.

Temperature has an effect on the rate of growth, which has an impact on the rate and occurrence of microbial processes such as respiration (Singh *et al*., 2010). Temperature fluctuations within peatlands may thus potentially impact certain activities for some microbial species.

2.3.2. The use of endospore-forming bacteria in palaeo-ecological studies

Palaeo-ecology involves the reconstruction of previous environmental conditions, including the nature of past communities, using proxies (Gorham *et al*., 2001). The reconstruction of past

environments provides an insight into past climates as well as the effects of climate changes in specific regions and can be used to assess the impacts of future environmental changes (Meadows, 2014). A primary requirement for a biological indicator to serve as a proxy is that the organism, or associated structures, should exist in the preserving material over a significant period of time in an unaltered form (Gorham *et al*., 2001). Indicators which fit this requirement, and are commonly used, include siliceous microfossils and pollen grains. Past environmental conditions which prevailed at the time of deposition can be inferred using these proxies. It is necessary to explore and establish new proxies to add to the current list of proxies to aid in improving resolution and detail when reconstructing past environments (Meadows, 2014). Reconstructing past histories can enable various aspects to be studied, such as the effect of disturbances on ecosystem structure or the effect of climatic changes and anthropogenic activities on microbial diversity (Wunderlin *et al*., 2014). One such proxy which has not been examined in great detail is dormant bacterial endospores.

Wunderlin *et al*. (2014) used endospore-forming bacteria from a sedimentary record as an indicator to assess the impact of eutrophication on bacterial diversity and composition. Renberg and Nilsson (1992) concluded that whilst endospore-forming bacteria could serve as potentially useful contributors towards palaeo-ecological studies, their application is relatively unexplored. The isolation and identification of endospores, combined with molecular genetics and biochemical techniques could allow for past environmental conditions to be deduced (Renberg and Nilsson, 1992).

2.4. AEROBIC ENDOSPORE-FORMING BACTERIA (AEFB)

2.4.1. Taxonomic diversity of AEFB

The first genus of AEFB described, *Bacillus*, was proposed in 1872 by Ferdinand Cohn, a German biologist (Wipat and Hardwood, 1999). *Bacillus* is classified under the phylum *Firmicutes* and the family *Bacillaceae* (Wipat and Hardwood, 1999). This genus comprises of members which are facultatively anaerobic or aerobic, rod-shaped, Gram positive, capable of endospore-formation, with low guanine and cytosine (G+C) contents. Initially, variation in G+C content amongst different species ranged from 33–67 mol%; demonstrating higher levels of genomic heterogeneity within the *Bacillus* genus than expected (Priest, 1993). Subsequently, this genus underwent further taxonomic revision following 16S rRNA gene sequence analysis and phylogenetic evaluation (Ash *et al*., 1991). Since 1990, certain species which were

previously assigned to *Bacillus* were reclassified into 16 additional genera. Over 220 species are incorporated into these genera, with greater than 150 assigned to *Bacillus* (Logan and Halket, 2011). In addition to this, 38 new genera of AEFB have also been proposed since 1990. Collectively, there are currently 54 listed genera of AEFB (Logan and Halket, 2011). Together, these genera contain greater than 460 new species which have been proposed since the 1986 edition of *Bergey's Manual of Determinative Bacteriology* (Logan and Halket, 2011). *Paenibacillus* is the second largest genus of AEFB after *Bacillus*, containing 110 species (Logan and Halket, 2011).

2.4.2. Physiological diversity of AEFB

Physiological diversity amongst AEFB is extensive. Examples include: aerobic chemoheterotrophs such as *Bacillus*, *Sporolactobacillus* and *Paenibacillus*; sulfate reducers, such as *Desulfotomaculum* spp.; acidophiles, such as *Alicyclobacillus*; alkaliphiles, such as *Halalkalibacillus*; microaerophilic lactate fermenting bacteria, such as *Sporolactobacillus*; as well as coccoid representatives with morphological characteristics similar to actinomycetes e.g. *Thermoactinomycetes* and *Sporosarcina* (Nicholson *et al*., 2000; Slepecky and Hemphill, 2006; Ammann *et al*., 2011; Logan and Halket, 2011).

Endospore-formers have adapted to a wide range of environmental conditions and are capable of inhabiting diverse habitats (Nicholson *et al*., 2000). Endospore formation also provides bacteria with the ability to successfully colonize specific regions and out-compete other bacteria (Wipat and Hardwood, 1999). Representatives of the *Halobacillus*, *Thermobacillus* and *Psychrobacillus* genera have been isolated from halophilic, thermophilic and psychrophilic regions respectively (Ammann *et al*., 2011). AEFB have also been isolated from diverse environments including marine sediment, salt marshes, thermal acid waters, desert soils, volcanic soil, glaciers, subantarctic soil, marine sponges, wetlands, peat bogs and geothermal vents (Logan *et al*., 2000; Logan *et al*., 2004; Albert *et al*., 2005; Slepecky and Hemphill, 2006; Margesin and Miteva, 2011; Phelan *et al*., 2011; Cappa *et al*., 2014; Sonalkar *et al*., 2014; Aanniz *et al*., 2015; Liu *et al*., 2015). Many of these strains possess useful abilities, such as proteolytic, amylolytic and antimicrobial activities (Phelan *et al*., 2011; Aanniz *et al*., 2015).

Members of the *Bacillus* genus are capable of the production of various compounds including antibiotics, enzymes such as cellulases, proteases and amylases, and lipopeptides, which have

anti-fungal and anti-bacterial activities (Wipat and Hardwood, 1999). They may therefore be useful candidates in the biotechnological and medical sectors.

2.5. ENDOSPORES

2.5.1. Functions of endospores

Endospores are dormant, exceedingly resistant, highly differentiated structures which are formed within the bacterial cell (Hilbert and Piggot, 2004). These specialized structures serve the function of providing resistance to the cell during periods of unfavourable environmental conditions (Schlegel, 1993). The sporulation process has been described as a 'specialization of morphological characteristics' which is initiated in direct response to adverse conditions (Hilbert and Piggot, 2004). Unfavourable environmental conditions may include exposure to high temperatures, lack of moisture or nutrients and high concentrations of disinfectants. Protection needs to be present against factors which may result in direct damage to DNA, which could include UV or gamma radiation (Setlow, 1995). The resistance of endospores to heat is 10^5 times greater than that of vegetative cells and is a hundred-fold more resistant towards radiation (Roberts and Hitchins, 1969). Once favourable conditions arise, a vegetative state may once again resume. This may also occur through the relocation of the endospores to new environments through various agents of dispersal (section 2.5.5). Environmental samples are often abundant with bacterial endospores, which demonstrates the ability of endospore formation to serve as a successful means of dispersal and survival (Nicholson *et al*., 2000). Endospore formation is one of the primary reasons behind the effective establishment and colonization of endospore-forming bacteria in various niches, thereby allowing for vital roles in various ecosystem processes to be fulfilled.

2.5.2. Life cycle

2.5.2.1. Sporulation

Sporulation is described as the transitioning stage between a cell in the vegetative state and the completed endospore (Roszak and Colwell, 1987). Approximately 4% of the genome of *Bacillus subtilis* (ca. 4.2 Mbp) is linked to processes involved in endospore formation (Kunst *et al*., 1997). In the presence of abundant nutrients, there is a repression of the sporulation process (Trach *et al*., 1985). However, when conditions change, the sensing proteins SfrA and

NtrC are able to transmit a signal regarding the environmental conditions, in turn directly impacting upon the guanosine-5'-triphosphate levels within the endospore (Trach *et al*., 1985). Due to numerous similarities between sporulation and division in vegetative cells, it is believed that the process of endospore formation is a modified form of cell division found within prokaryotes (Slepecky and Hemphill, 2006).

A range of environmental and internal signals act as triggers for systems which are involved in regulation. The most important transcriptional regulator involved in these systems is known as *Spo0A*, which is responsible for the control of greater than 10% of the genes present in the *Bacillus* genome (Errington, 2003). Approximately 200 genes are involved in the entire sporulation process and the first stage in the development of endospores is referred to as endosporulation (Slepecky and Hemphill, 2006). This process takes approximately eight hours to reach completion. This step commences when *Spo0A* undergoes phosphorylation through the action of numerous kinases, each of which responds to a range of stimuli from the environment (Errington, 2003; Piggot and Hilbert, 2004). Once *Spo0A* has been phosphorylated, it serves to activate the transcription of genes such as *spoIIE* and *spoIIA*, which have key roles in the sporulation process. Remaining genes which are vital in the sporulation process are transcribed by the RNA polymerase enzyme, which is regulated by a sigma factor known as σ^H (Errington, 2003). Initially, the cell undergoes DNA replication. A protein known as FtsZ forms two distinct rings, one at each pole. The cell then divides through the formation of a membrane referred to as a septum, which occurs as a result of one of the Z-rings (Errington, 2003). This results in the production of a distinct pre-spore and a mother cell and is termed septation, also known as an asymmetric sporulation division (Piggot and Hilbert, 2004). The pre-spore and the mother cell follow independent developmental pathways (Figure 2.1).

Approximately one-third of the chromatin material is captured in the pre-spore during septum formation. The remainder of the chromatin material is translocated by means of a protein known as SpoIIIE (Errington, 2003). Cell-specific gene expression is co-ordinated by means of intercellular communication systems through the activation of specialized sigma factors (Piggot and Hilbert, 2004). These sigma factors are responsible for the initiation of gene expression by directing RNA polymerase to the required DNA sites. σ^E is the sigma factor present in the mother cell, whereas σ^F is active in the pre-spore. The mother cell then produces proteins which result in the degradation of the septum, with one of these proteins being SpoIIB, which is responsible for catalysis of the process. Once the developing spore obtains a double membrane, it is also sometimes referred to as a forespore (Piggot and Hilbert, 2004). The

double membrane occurs as a result of the cell plasma membrane surrounding the septum and pinching off. This process is termed engulfment and follows the degradation of peptidoglycan in the septum (Roszak and Colwell, 1987). At the completion of engulfment, different sigma factors become activated, namely σ^G in the forespore and σ^K in the mother cell, respectively. These sigma factors are responsible for the activation of gene transcription, which function towards building the endospore structural components (Piggot and Hilbert, 2004). The forespore is protected by the presence of a combination of calcium and dipicolinic acid (DPA), also referred to as calcium dipicolinate (Piggot and Hilbert, 2004). At this stage, the forespore is still present in the cytoplasm of the mother cell as a protoplast. The forespore consequently becomes the endospore core and the outer endospore layers are produced by the mother cell. The cortex, which consists primarily of peptidoglycan, is then added between the two membranes (Roszak and Colwell, 1987). An endospore coat then develops around the entire structure, after which an optional exosporium may be laid down in certain bacteria. One of the concluding stages involves the dehydration of the endospore, a stage which is vital for its resistance. The endospore now also contains pyrimidine nucleotides and nucleoside triphosphates in low concentrations (Roszak and Colwell, 1987). Upon maturation of the endospore, lysis of the mother cell occurs (Errington, 2003). This transpires through a process known as apoptosis, after which the release of the endospore into the surrounding environment takes place. The differences in resistance between species may be attributed to the variation in the structure of the polypeptides incorporated into the coat. The average time for sporulation to occur in *Bacillus subtilis* is 6–8 h (Errington, 2003).

Endospores may be produced in different regions of the cell, which may also aid in distinguishing bacterial species. The three locations include the end of the cells, referred to as terminal endospores, the middle of the cell, known as central endospores and lastly, the region between these two locations which is referred to as the sub-terminal endospores (Errington, 2003).

2.5.2.2. Germination

Once favourable environmental conditions arise, the endospore then undergoes germination. This arises through the activation of specific receptors present within the inner membrane of the endospore (Hudson *et al*., 2001). These are known as germinant or nutrient receptors and are protein in nature. They act as sensors and are therefore able to detect the occurrence of

changes in the surrounding environment, for instance, the presence of nutrients or germinants such as amino acids (Hudson *et al.,* 2001). Germinant receptors such as gerA only identify one type of germinant, whereas other receptors such as gerK and gerB are able to distinguish multiple types of germinants (Moir *et al*., 2002). Other studies have found evidence for the formation of complexes through the interaction of various germinant receptors (Igarashi and Setlow, 2005). The conditions under which activation occurs determines the rate at which the germination process takes place. The transmission of a signal occurs when the germinant binds to the specific receptor (Setlow, 2003). Following this, a partial loss of resistance occurs as the core becomes hydrated. The fluidity of the endospore membrane increases due to conformational changes in receptor proteins located within the inner membrane (Foster and Johnstone, 1989). The amounts of zinc ions and DPA within the endospore core decrease due to the release of the DPA and cations. It is also believed that a proton gradient may be established due to the formation of a proton motive force (Setlow, 2003). The removal of the endospore coat also occurs. The subsequent stage entails the hydrolysis of the endospore cortex region which allows for a greater degree of hydration and expansion to take place within the core (Setlow, 2003). The endospore now loses a greater degree of dormancy and becomes less resistant to factors such as radiation, chemicals and heat. The succeeding stage in the germination process involves the commencement of metabolic activities, through utilization of high-energy reserves present in the endospore. Since amino acids are not synthesized in the early stages of germination, the degradation of small acid soluble proteins (SASPs) takes place through utilization of specific enzymes which break down these proteins (Setlow, 2003). These are then used for the biosynthesis of macromolecular compounds and new proteins. GPR, an endoprotease which is sequence-specific, is responsible for the cleavage which initiates the degradation of the SASPs (Setlow, 2003). The two major genes involved in this process are the *ger* and *spo* genes, for germination and sporulation respectively (Moir *et al.*, 2002).

DNA replication and division occurs during the outgrowth stage, which is controlled by the *out* genes. Outgrowth is described as the phase in which the endospore transitions into the characteristics of a vegetative cell. If conditions remain favourable, biochemical and morphological transformations occur, resulting in symmetric cell division (Slepecky and Hemphill, 2006). A variety of proteases is also present, such as esterase A and B (Moir *et al*., 2002). These enzymes perform numerous vital functions such as the degradation of intracellular proteins and the inactivation of sporulation-specific enzymes (Slepecky and Hemphill, 2006). ATP synthesis also commences during the primary period of germination,

prior to the production of macromolecules (Roszak and Colwell, 1987). The primary molecules involved in ATP synthesis are basic proteins and phosphoglycerate (Roszak and Colwell, 1987).

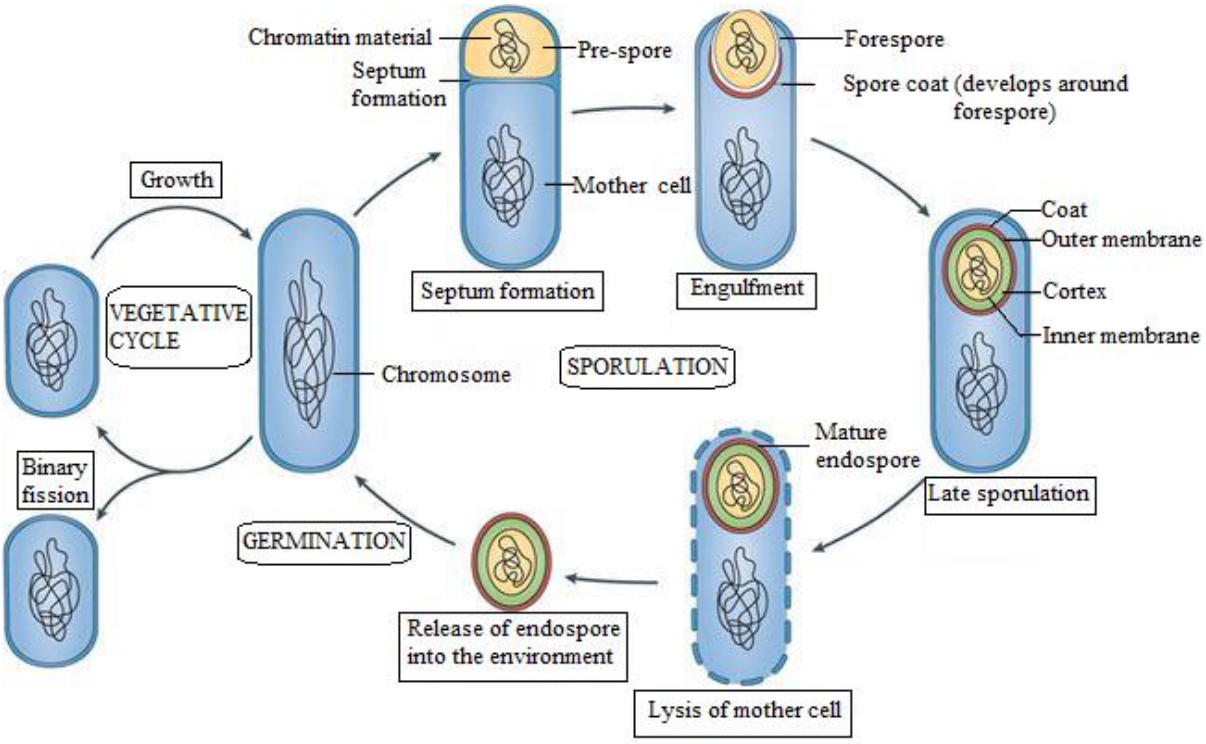


Figure 2.1. Cycle of sporulation and germination in AEFB (Adapted from McKenney *et al*., 2013)

2.5.3. Structure of endospores

The endospore is a multi-layered structure which comprises of six basic components. The innermost region of an endospore is referred to as the core (Setlow, 1988). The core consists of the cytoplasm as well as various components including RNA, DNA, ribosomes, enzymes and proteins. The cytoplasm of the core contains proteins in an immobile form and has reduced quantities of high-energy yielding compounds which are present in the vegetative cell cytoplasm. One of the significant differences between the composition of the core and that of the vegetative cell is the total water content (Potts, 1994). The cytoplasm of the vegetative cell is largely hydrated, with a total water composition of approximately 70–88% (Potts, 1994). However, the water content of the endospore core is a much reduced 28–57% (Leggett *et al*., 2012). This change plays a substantial role in the survival of the endospore. Durability, as well

as resistance, are factors which are considerably increased due to the dehydrated nature. Wet heat resistance is also increased with reduced hydration (Setlow, 2006). This resistance is brought about through the high quantity of DPA complexed with divalent cations, such as calcium, which are in abundance in the endospore core. Approximately 5–15% of the total weight of the endospore is made up by DPA (Murrell and Warth, 1965). Excretion of the calcium cations and DPA occurs at the onset of germination. Another aspect which differs between the vegetative cell state and the core is pH, which is lower in the core, usually ca. 6.3–6.5 (Setlow and Setlow, 1980). A large amount of SASPs is also present within the core (Setlow, 1988). These proteins contribute towards the protection of DNA, most especially by DNA-degrading chemicals and UV light, by complexing with the DNA, thus decreasing the surface area of exposure (Douki *et al*., 2005).

The next layer which surrounds the core is referred to as the inner membrane. Within the endospore, this layer occurs in a compressed form compared to the vegetative cell and, thus, swells when activation and germination commence (Leggett *et al*., 2012). The inner membrane is considered to play a very significant role as a permeability barrier (Leggett *et al*., 2012). In the dormant state, lipids present in the membrane are highly immobile compared to the fluid nature after germination; this is attributed to membrane compression and reduced water content (Cowan *et al*., 2004; Leggett *et al*., 2012). This membrane exhibits very low permeability as demonstrated by studies which reveal that water and lipophilic, uncharged molecules are only taken up at an extremely slow rate (Setlow and Setlow, 1980). This membrane also plays an important role in germination due to the presence of germination receptor proteins within this layer. The composition of the membrane proteins also differs to that of the vegetative cell with additional proteins such as SpoVA, which mediate DPA uptake, being present (Leggett *et al*., 2012). Furthermore, this layer also serves as a protective barrier towards unfavourable chemicals and UV light.

The layer surrounding the inner membrane is known as the cortex and comprises of a modified peptidoglycan layer (Warth and Strominger, 1972). The cross-linking of the peptidoglycan polymer varies at different areas of the cortex and this directly affects the degree of pressure exerted on the core (Popham, 2002). Approximately fifty percent of N-acetylmuramic acid (NAM) molecules in the cortex peptidoglycan form cyclic muramic-δ-lactam residues due to the absence of peptide side chains (Leggett *et al*., 2012). This differs to vegetative cells, in which NAM molecules form cross-links form with other glycan strands. The cortex is thought to promote core dehydration. For this reason, the cortex is considered to be largely responsible

for the temperature resistance of the endospore (Gould, 1983). Lytic enzymes are responsible for the degradation of peptidoglycan upon germination. The germ cell wall is positioned in the inner region of the cortex and has a differing peptidoglycan structure to the remainder of the cortex, thus shielding it from selective degradation upon germination. This structure therefore remains and serves to function as the cell wall in newly vegetative cells (Popham, 2002).

The layer surrounding the cortex is referred to as the outer membrane and possesses a polarity which is opposite to that of the inner membrane (Crafts-Lighty and Ellar, 1980). This layer has its underlying role in permeability by preventing larger ions from diffusing across the membrane (Crafts-Lighty and Ellar, 1980; Kozuka and Tochikubo, 1991). Germinants, however, are able to diffuse through the outer membrane and reach the receptors located within the inner membrane. It has been reported that this layer does not contribute towards a high degree resistance in the endospore (Nicholson *et al*., 2000). Proteins associated with the electron transport chain and cytochrome systems have been identified in the outer membrane structure (Crafts-Lighty and Ellar, 1980).

The endospore coat, which covers the outer membrane, is mostly protein in nature, comprising of approximately 50–80% of the total protein content of the endospore (Leggett *et al*., 2012). Two fractions make up the protein content, namely the insoluble and soluble fractions. Coat proteins involved in the germination process, such as CotD, are present in the soluble fraction. The insoluble fraction of the endospore coat contains a high concentration of cysteine (Behravan *et al*., 2000). Consequently, a high percentage of sulfur cross-linkages occurs in this layer; this results in endospores having approximately five times greater sulfur content than that of vegetative cells (Behravan *et al*., 2000). Cross-linking is thought to contribute towards chemical and mechanical resistance. When these cross-linkages are reduced, the tertiary structure of the coat proteins changes, thereby altering the protein conformation. This, in turn, exposes enzyme sites which are essential for activation for endospore germination to occur. If these cross-linkages are disrupted, the endospore becomes susceptible to agents such as hydrogen peroxide and lysozyme. The coat protects the endospore from heat, UV radiation and oxidizing agents (Riesenman and Nicholson, 2000). Lytic enzymes present in the coat aid in the degradation of the cortex when germination is initiated. Proteins present in the coat also play a role in facilitating the movement of germinants through the coat to the inner membrane.

The outermost layer of the endospore is known as the exosporium and is only found in certain bacterial species. Proteins make up the most significant portion of the exosporium, which also

comprises carbohydrates, such as glucose and glucosamine, and lipids which include neutral lipids and fatty acids (Leggett *et al*., 2012). Due to the homology of proteins associated with the coat and the exosporium, it is believed that the exosporium may be a specialized extension of the preceding coat layer (Redmond *et al*., 2004). The exosporium exhibits hydrophobic properties and plays an important role adhering endospores to various surfaces (Faille *et al*., 2002). Superoxide dismutase has been found in association with the exosporium and is thought to play a role in detoxifying chemicals at the surface of the endospore before entry (Nicholson *et al*., 2000).

2.5.4. Molecular mechanisms behind endospore resistance

Dipicolinic acid (DPA), otherwise known as Pyridine-2,6-dicarboxylic acid, is present in large amounts and constitutes between 10–15% of the total dry weight of the endospore (Schlegel, 1993). DPA is present in the core together with divalent calcium cations in a 1:1 ratio (Setlow, 2007). This Ca-DPA chelate is unique to endospores and is responsible for the reduction of the water content of the core to low levels. There are several mechanisms by which a decreased water content aids in protection against DNA damage. Firstly, the rate at which depurination of DNA occurs is decreased, since this is a process which occurs in conjunction with the catalysis of water (Lindahl and Nyberg, 1972). Secondly, the frequency of thymine-thymine dimers is reduced 2–3 fold when partially hydrated DNA is exposed to UV (Nicholson *et al*., 1991). Thirdly, the interaction present between DNA and SASPs is enhanced by lowering the water content within the core. The Ca-DPA chelate also aids in providing resistance against oxidizing chemicals, desiccation and dry and wet heat. (Schlegel, 1993; Setlow *et al*., 2006).

Damage to DNA is reduced via two mechanisms, namely, DNA repair, which can occur at the commencement of germination, and protection arizing during dormant periods (Nicholson *et al*., 2002; Setlow, 2007). The repair process does, however, have two problems. Firstly, the repair process is delayed since it only occurs at the completion of endospore germination and outgrowth. Secondly, DNA repair may be susceptible to mutations.

Another form of DNA protection is brought about by the presence of α/β-type SASPs, which are produced in the late stages of sporulation. These proteins have been shown to protect against desiccation, toxic chemicals, as well as dry and wet heat (Yang *et al*., 2011). These proteins are coded by *ssp*, which are monocistronic genes belonging to a multigene family present in endospore-forming bacteria (Setlow, 2007) and comprise 5–10% of the total protein content of

the core (Driks, 2002). These proteins bind to DNA to afford protection. This binding causes the UV photochemistry of the DNA to be altered, thereby bringing about a greater degree of resistance against UV radiation (Nicholson *et al*., 2005). Another type of SASP present in aerobic endospore-formers is the γ-type SASP. These proteins play a role during outgrowth of the endospore by providing amino acids for biosynthesis of proteins upon degradation (Setlow, 1995).

Two repair systems are present to remove spore photoproduct (SP), a thymine dimer generated as a result of exposure to UV radiation, at the onset of germination (Xue and Nicholson, 1996). The first system involves a nucleotide excision repair pathway, which works by excising the SP from DNA and thereafter filling the single-stranded gap which results after excision (Moeller *et al*., 2007). The second system involves endospore photoproduct lyase which functions by reversing the endospore photoproduct (Yang *et al*., 2011).

Unfavourable conditions or specific treatments may also adversely affect proteins. For this reason, repair of proteins such as heat shock proteins and aspartate-O-methyltransferase may also be activated upon germination (Nicholson *et al*., 2000).

A lack of metabolic activity leads to a state of dormancy which allows endospores to survive over long periods of time (Setlow, 1994). Under dormant conditions, no ATP is produced within the endospore and enzymatic systems remain inactive. However, once germination is initiated, mechanisms behind dormancy and resistance are removed.

2.5.5. Endospore dispersal

An important aspect of the ecology of endospore formers is their dispersal over large distances across differing geographical locations (Yamaguchi *et al*., 2012). The proliferation of endospore-forming bacteria in a new habitat is dependant on the prevailing environmental conditions. Mechanisms which influence the dispersal of endospores include water, wind, dust particles and animal hosts (Nicholson *et al*., 2000; Yamaguchi *et al*., 2012). Endospores have also been isolated from frozen environments; dispersal of endospores in these regions has been attributed to snowflakes and wave action (Christner *et al*., 2000). As a result of their widespread dispersal, endospores have been isolated from most types of environmental samples from diverse locations (Nicholson *et al*., 2000).

2.5.6. Mechanisms of destruction of endospores

One of the first methods developed to eliminate endospores is Tyndallization which was developed in the 19th century (Parija, 2009). This method was used before the advent of autoclaves. It is an indirect procedure which involves providing favourable conditions that favour endospore germination. Subsequently, vegetative cells are destroyed through the use of free-flowing steam; the process is then repeated to ensure that endospores undergo germination (Parija, 2009).

Boiling at 100°C with the addition of 2% sodium carbonate during the boiling process has been shown to have a sporicidal effect (Parija, 2009). Currently, autoclaves (121°C at 105 kPa) are the preferred method for the inactivation of endospores. Autoclaves create steam through the heating of water within a high-pressure environment (Parija, 2009). The use of pressure allows for a greater temperature to be achieved with a lower degree of energy and provides a superior sterilizing ability over free-flowing steam. A holding time of 15–20 minutes at 121°C is sufficient to kill all known organisms (Parija, 2009). Endospores are destroyed as a result of protein denaturation and coagulation, which occurs due to the high penetration ability of moist heat.

Alkylating agents, such as ethylene oxide, are more effective in destroying endospores than detergents. Sterilants containing sodium hypochlorite, e.g. bleach, exhibit sporicidal activity (Parija, 2009). Exposure to a 1:10 dilution of bleach for a period of five minutes is sufficient to kill endospores arising from *Bacillus anthracis* (Heninger *et al*., 2009). The duration of exposure, temperature and concentration of the sterilizing agent are important parameters to take into consideration (Parija, 2009). Alkylating agents typically cause oxidation and hydrolysis of proteins within the endospore, thereby leading to its destruction. They may also negatively affect the permeability of the inner membrane, thereby influencing its ability to function as an effective barrier (Parija, 2009). This results in an inability to germinate and outgrow when favourable conditions arise. The permeability of the inner membrane of the endospore may also be increased through the use of oxidizing agents such as hydrogen peroxide and chlorine dioxide, which leads to a loss of DPA and simultaneously allows for the entry of potentially damaging chemicals (Cortezzo *et al*., 2004; Leggett *et al*., 2012). Rogers *et al*. (2007) had found that formaldehyde exposure over a ten hour period maintained at 1 100 ppm was able to significantly reduce the number of spores of *Bacillus anthracis*, *B. subtilis* and *Geobacillus stearothermophilus.* Methyl bromide is capable of disinfecting Anthrax endospores due to its ability to react with carboxyl, hydroxyl and protein groups present within

the endospore (Hugo, 2012). Nitrous acid can be used to destroy endospores by damaging DNA through mutagenesis (Tennen *et al*., 2000). Glutaraldehyde is commonly used as a sporicidal agent since it functions by inactivating endospore germination. Iodine-based treatments may also be effective by inactivating enzymes present in the endospore cortex (Tennen *et al*., 2000).

Radiation may also be used to destroy endospores (Parija, 2009). The most common forms of radiation used include gamma rays and X-rays. Gamma rays exert their sporicidal action through damage of the endospore DNA. Non-ionizing forms of radiation are less effective due to their reduced penetration abilities (Parija, 2009).

In hospitals, the concentrated vapour of hydrogen peroxide at low temperatures is used to achieve the destruction of endospores on medical instruments (Linley *et al*., 2012). This functions as a non-toxic and non-corrosive treatment.

2.5.7. Viability of endospore-forming bacteria

Viability can be described as the ability of bacterial populations to multiply when presented with conditions which are suitable for growth (Roszak and Colwell, 1987). Linked to viability is the concept of survival, which relates to an organism's ability to persist and remain viable even under stressful conditions. One of the main attributes of endospore-forming bacteria which allows them to remain viable under adverse circumstances is the ability to decrease the endogenous rate of metabolism of the endospore. Some of the methods utilized for the detection of viability include fluorescence microscopy (Laflamme *et al*., 2004) and hyperspectral reflectance microscopy (Anderson *et al*., 2008).

There is growing interest in establishing how long endospore formers may remain viable. Determination of viability and longevity in bacteria is essential in studies involving pathogenicity, evolution, ecology and physiology (Renberg and Nilsson, 1992). Currently, the oldest endospores that have been claimed to have been successfully revived were isolated from 250 million year old salt crystals (Vreeland *et al*., 2000). Due to their resistant nature, endospores have also become the focus of studies investigating the response of microorganisms to extra-terrestrial environments (Nicholson *et al*., 2000). Bucker *et al*. (1974) analyzed the viability of *B. subtilis* endospores aboard Apollo 16 in response to space environments and found that endospores were able to survive under space vacuum conditions. These results were confirmed by experiments conducted by Horneck *et al*. (1994) when *B. subtilis* endospores

were exposed to conditions mimicking those found in space, specifically vacuum pressure, solar electromagnetic radiation and cosmic radiation over a six-year study period and discovered that even in unprotected samples, a significant number of endospores had survived. The possibility of endospore survival in space has been proposed and examined by Nicholson *et al*. (2000).

2.6. REVIVAL OF BACTERIAL ENDOSPORES TO A VEGETATIVE STATE

2.6.1. Isolation and revival of endospores from diverse locations

Ancient endospores have been revived from various sample types including fossilized amber, salt crystals, ice and sediment. Currently, the oldest bacteria to have been revived were isolated from salt crystals that are thought to be 250 million years old (Vreeland *et al*., 2000). These bacteria represent a formerly unidentified endospore-forming *Bacillus* sp., designated strain 2-9-3, and were found to be closely related to *Virgibacillus pantothenticus* and *Bacillus marismortui*.

In another study, endospores were isolated from the abdominal tissue of a bee trapped in fossilized Dominican amber that was dated 25 to 40 million years old (Cano and Borucki, 1995). Morphological and biochemical characterization techniques were used to identify the isolate as a strain of *Bacillus sphaericus.*

Dormant endospores have been isolated from a range of diverse locations including deep subsurface sediment cores extracted from brine lakes located in the Mediterranean Sea (Sass *et al*., 2008), from Atlantic coastal plains (Fredrickson *et al*., 1991), from 40 million year old biosphere sediments below the sea floor in the Peru Margin and Equatorial Pacific Ocean (Batzke *et al*., 2007) and from 35 000 year old crystallized halite (Schubert *et al*., 2009). Endospores have also been isolated from lake sediments which have accumulated over years and have been dated to 5 800 years old (Bartholomew and Paik, 1966), 9 000 years old (Renberg and Nilsson, 1992) and 13 000 years old (Rothfuss *et al*., 1997).

Studies have also been conducted whereby endospores were isolated from ice deposits (Shivaji *et al*., 2011; Antony *et al*., 2012). Ice aids in preserving trapped microbial cells (Christner *et al*., 2000; Antony *et al*., 2012). Using advanced drilling equipment, ice cores can now be obtained with minimum risk of contamination as opposed to traditional drilling and handling procedures (Christner *et al*., 2000).

The entrapment of ancient endospores in ice has lead scientists to investigate the possibility of microorganisms existing on Mars. This is due to the postulation that atmospheric conditions on Mars were similar to that of Earth ca. four billion years ago; roughly the same time when life began to develop on Earth (Christner *et al*., 2000). The possibility of interplanetary transfer has been reviewed by Horneck (1993), Nicholson *et al*. (2000) and Horneck *et al*. (2002).

2.6.2. Applications of endospore revival

The study of ancient endospores has potential in several applications. Revival of dormant endospores from ancient environmental samples may lead to the isolation of novel endospore-forming bacterial species or strains. Molecular fingerprinting, combined with bioinformatics and genetics, could allow for the evolutionary history and phylogenetic relationships between ancient bacteria to be established. The study of ancient bacteria could provide an insight into evolutionary processes and the change in genomic bacterial DNA over generations. The mapping of mutation rates can also be conducted using ancient bacteria (Christner *et al*., 2000). Studies evaluating bacterial resistance can also benefit from information derived from ancient bacteria. For example, D'Costa *et al*. (2011) isolated bacterial DNA from ancient frozen sediment samples and detected the presence of antibiotic resistance genes, thereby establishing that resistance to antibiotics occurs naturally in environments and predates the antibiotic era.

Aerobic endospore formers have contributed significantly towards the pharmaceutical and medical sectors through the production of extracellular enzymes and antimicrobial compounds (Raaijmakers *et al*., 2010). Lipopeptides produced by AEFB have been applied in agriculture and biotechnology as pest control agents for plant protection and surfactant-enhanced bioremediation (SEB) due to their metal-chelating and aromatic compound-degrading capabilities (Raaijmakers *et al*., 2010). Biomining could be used to examine if ancient bacteria may be a potential source of novel compounds or enzymes.

Bacteria from polar regions possess modified protein and lipid structures which allow for adaptation to cold environments. Psychrophilic microorganisms display substantial functional diversity and the commercial potential of proteins and bioactive compounds produced by these organisms is being examined (de Pascale *et al*., 2012). The application of endospore-forming bacteria from ancient ice samples for bioprospecting may provide a novel and untapped source of useful compounds.

Dormant endospores may also have an application in palaeo-ecological research, which is centered on the use of proxies, from prehistoric palaeo-environmental samples, for the reconstruction of past environmental conditions (Wunderlin *et al*., 2014). Due to the lack of focus placed on using ancient bacteria in palaeo-ecological research, other indicators are used, however, the dormant nature of endospores could make them suitable for these studies. The revival of ancient bacteria along a geographical distribution could allow for microbial diversity changes and variations in community structures over time to be mapped out (Wunderlin *et al*., 2014). This could be used as a bioindication of the effects of climatic changes and anthropogenic activities on microbial diversity.

The exploration of diverse ancient environments may therefore provide reservoirs of endospore-forming bacteria that may contribute towards various fields of research.

2.6.3. Challenges behind the analysis of ancient bacteria

Appropriate environmental and nutrient conditions need to be established to successfully revive endospores which have been dormant for hundreds or thousands of years. The sampling and screening procedures have to be conducted aseptically with the utmost caution in order to avoid modern contamination. Precautions include extensive decontamination of all equipment and reagents. Controls for PCR and DNA extraction should always be included for detection of possible contamination, however, independent validation provides the greatest authentication (Willerslev and Cooper, 2005). In some cases, ancient endospore DNA may be partially degraded or fragmented due to possible damage over time (Rawlence *et al*., 2014). The sampling site itself should be a factor of consideration before initiation of the study. Stable, low-energy ecosystems in which DNA remains in an intracellular form is preferred (Rawlence *et al*., 2014).

2.7. TECHNIQUES FOR DETERMINING THE AGE OF ENVIRONMENTAL MATERIALS

2.7.1. Radiocarbon dating

Radiocarbon dating is one of the most popular radiometric techniques which is used to determine the ages of materials and objects. Carbon comprises of three isotopes namely C^{12}, C^{13} and C^{14}, the third of which is known as radiocarbon (Walker, 2005). Radiocarbon is not a

stable isotope and decays over time, resulting in radioactivity. Plants obtain C^{14} in the form of $^{14}CO_2$ from the surrounding atmosphere. Animals subsequently obtain this carbon through the consumption of plants. The constant decay of C^{14} is balanced by replenishment, allowing for an equilibrium to be established. However, when living organisms die, their radiocarbon levels decrease and hence, the ratio of radiocarbon to stable carbon declines due to decay of the radiocarbon. An age can then be determined by comparing the residual C^{14} content to that of modern radiocarbon material from a standard. Radioactive decay occurs at a rate of 1% every 83 years (Walker, 2005). The spectrum of materials which can be dated using this technique includes lake sediments, peat, plant remains and wood, amongst others. Radiocarbon dating is most advantageous for providing the ages of material from the last 50 000 years with relative accuracy (Blaauw, 2010). This technique can be performed using one of two techniques, namely Beta Counting, which is further divided into Liquid Scintillation Counting and Gas Proportional Counting, or Accelerator Mass Spectrometry dating (Walker, 2005).

Accelerator mass spectrometry dating (AMS) is one of the more extensively used techniques and works by using particle accelerators to discriminate between C^{14} and other isotopes present (Walker, 2005). The ratio between C^{14} and other carbon isotopes such as C^{12} and C^{13} is then determined and serves as the basis for determining the age of the sample by comparison of this ratio with a known standard (Walker, 2005). AMS is considered superior to Beta Counting, which works by detecting and counting β emissions which are released by C^{14} atoms over a set time period. AMS is a faster and more effective process with a higher throughput and has a greater degree of convenience due to the smaller sample size requirements for measurements to occur. The precision of AMS in recent years is approximately on the same level as that achieved by beta counting (Walker, 2005).

2.7.2. Age-depth modelling

Cores which have been processed using radiocarbon dating can be used for the generation of age-depth models. These models rely on depths which have been dated and work to estimate the deposition between those depths (Blaauw, 2010). However, radiocarbon dates cannot be plotted directly onto a calendar year timescale and have to undergo calibration first. This is essential since calibration accounts for past variations in the radiocarbon. Modelling is necessary in order to produce these calendar years for the depths of a core (Blaauw, 2010). Age-depth models usually incorporate either polynomial regression, spline regression or linear

interpolation, each carrying their own advantages and drawbacks (Blaauw, 2010). Linear interpolation is the most utilized technique and works on the basis of plotting radiocarbon ages against the depth of a sample (Bennet, 1994). Gradients between adjacent points are used for the estimation of deposition time and the interpolated ages are calculated for intermediate depths (Bennet, 1994). Age modelling software can be used to generate age-depth models and takes into account age variation, extrapolation and outliers (Blaauw, 2010). These software allow for exponential, polynomial or straight curves to be constructed using the age-depth data. *Clam* is an example of software used for the construction of classical age-depth models by calibrating the calendar C^{14} dates (Blaauw, 2010). *Clam* has the advantage of providing outputs which are simple, organized and reliable.

2.8. ANALYSIS OF BACTERIAL DIVERSITY

2.8.1. The study of bacterial diversity from the environment

It is important to study the physiological and taxonomic diversity of bacteria present in sediment due to the essential roles they play in mediating various processes and functions within these systems (Mandic-Mulec and Prosser, 2011). These processes include mineral solubilisation, the production of plant growth promoters and the decomposition of organic and inorganic matter such as humic material and cellulose (Chaudhry *et al*., 2005). AEFB contribute towards various elemental cycles namely the carbon, nitrogen and sulfur cycles. Within the nitrogen cycle, AEFB play important roles in the degradation of organic nitrogen, denitrification and nitrogen fixation (Chaudhry *et al*., 2005). Several AEFB species also participate in sulfur oxidation, a process vital to the success of soil environments (Chaudhry *et al*., 2005). Some species of thermophiles are able to break down BTEX (benzene, toluene, ethylbenzene and xylene) and hence, show potential for bioremediation applications (Pichinoty and Asselineau, 1984).

The ability of AEFB to form extremely resistant endospores, in addition to their synthesis of structurally-diverse antibiotics and enzymes, allows these bacteria to establish communities and thrive successfully in various habitats (Wipat and Harwood, 1999). *Bacillus* strains in soil ecosystems generally display a large degree of phenotypic and genotypic variation. Plants benefit considerably from the presence of *Bacillus* for several reasons. For instance, *B. thuringiensis* is used as an insecticide in agriculture (Betz *et al*., 2000). Furthermore, certain *Bacillus* species are able to stimulate the uptake of nutrients in plants either through

solubilization of minerals or through the promotion of symbiotic relationships between plants and mycorrhiza (Gardener, 2004). Also beneficial to plants are those *Bacillus* species which produce surfactants exhibiting a range of antibacterial and antifungal activities, thereby aiding in suppressing plant pathogens (Ali *et al*., 2010).

The general methods of isolating AEFB from soil/sediment involve the collection of a sample, followed by a pasteurization step (80^{o}C for 10–30 min) to destroy all vegetative cells (Slepecky and Hemphill, 2006). A dilution series may be carried out, followed by plating onto a favourable medium to obtain distinct colonies. Analysis of 16S rRNA sequences has greatly contributed towards studies of microbial species diversity in environmental samples (Muyzer *et al.*, 1993). Through utilization of universal primers, gene clone libraries may be constructed. Use of these libraries allows for AEFB sequences to be tentatively identified, thereby allowing for the diversity of endospore-formers to be established (Garbeva *et al*., 2003). Bacterial diversity has been studied alongside factors relating to speciation, evolution, environmental changes and biogeography. In this regard, diversity-driving mechanisms may be established.

Expansion of the current knowledge base regarding bacterial diversity is essential since bacteria represent an enormous untapped resource of metabolic and physiological diversity (Horner-Devine *et al*., 2004). There are various factors which have influential effects upon bacterial diversity in environmental samples and these include, amongst others, nutrient availability, moisture and oxygen levels, salinity, plant species present and habitat heterogeneity (Horner-Devine *et al*., 2004; Fierer and Jackson, 2006). Fierer and Jackson (2006) investigated variables that influence bacterial diversity in samples obtained from different geographic locations and found that pH was a major factor influencing species richness and diversity within these habitats. This may be attributed to pH being a strong diversity driver due to its association with factors such as C: N ratio and organic carbon content. The influence of pH was also studied by Matthies *et al*. (1997), who found that pH affected the type of microorganisms present in soils. Microbial turnover and activity are influenced by temperature. Microbial community structure may even shift towards representatives of the community better adapted to the temperatures present in a particular region (Castro *et al*., 2010). By studying the changes in bacterial diversity alongside physico-chemical changes of the surrounding environment, the effects of these factors on the richness and abundance of bacterial species can be established.

2.8.2. Molecular fingerprinting techniques for the analysis of bacterial genetic diversity

A major challenge faced when studying environmental microbial diversity is that generally a significant proportion of the population is non-culturable (Horner-Devine *et al*., 2004); consequently, there is an important requirement for culture-independent techniques to be employed.

Molecular fingerprinting encompasses numerous nucleic acid-based techniques which are used to describe and characterize microbial genetic diversity (Rastogi and Sani, 2011). These techniques make it possible to obtain information about organisms which cannot be cultivated and thereby eliminate the need for phenotypic tests, which can often become quite tedious (Mandic-Mulec and Prosser, 2011). Diversity amongst AEFB species may be examined using specific markers or housekeeping genes (Garbeva *et al.*, 2003). Comparison between various strains is achieved through use of techniques including Random Amplified Polymorphic DNA (RAPD), Repetitive extragenic palindromic-polymerase chain reaction (Rep-PCR), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Terminal-Restriction Fragment Length Polymorphism (T-RFLP), Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TGGE), Single Strand Conformation Polymorphism (SSCP), Ribosomal Intergenic Spacer Analysis (RISA) and Multilocus Sequence Typing (MST) (Hadrys *et al*., 1992; Smit *et al*., 1997; Fisher and Triplett, 1999; Kirk *et al*., 2004; Martens *et al*., 2008; Ishii and Sadowsky, 2009; Rastogi and Sani, 2011), all of which will be discussed below.

RAPD involves the amplification of random DNA fragments through PCR with the use of an ca. 10 bp synthetic sequence primer (Hadrys *et al*., 1992). This technique is advantageous due to its ease of use and the fact that no prior knowledge of the sequence is required. RAPD does, however, have its limitations; for instance, an absence in banding patterns cannot be differentiated as being a lack of amplification due to poor quality DNA or through the absence of a target sequence (Kumari and Thakur, 2014). This technique, therefore, has a lower reproducibility than competing fingerprinting techniques.

In Rep-PCR, DNA fingerprints are generated through the PCR amplification of repetitive sequence elements which are present in multiple regions of bacterial genomes (Ishii and Sadowsky, 2009). Rep-PCR allows for identification and distinction of microorganisms at a subspecies or strain-level (Versalovic *et al*., 1998). These repetitive sequences are classified as REP if they are palindromic, make up greater than 0.5% of the total extragenic space of the genome and are between 21 to 65 bases in length (Tobes and Ramos, 2005). These elements

may play a role in gene expression and the stabilization of mRNA (Khemici and Carpousis, 2004; Tobes and Pareja, 2006). Furthermore, the Intergration Host Factor (IHF), which is necessary for DNA transcription and replication, uses REP sequences as a binding site (Tobes and Ramos, 2005). REP sequences also provide a site for recombination and provide protection against exonuclease activity (Tobes and Ramos, 2005). Several studies have found that this technique has a higher discriminatory power compared to alternative methods such as multiplex-PCR and plasmid profiling (Hahm *et al*., 2003; Foley *et al*., 2006).

ARDRA is a technique which involves the PCR amplification and restriction digestion of regions in the 16S rRNA gene (Smit *et al*., 1997). Variations in sequences can be used to compare diversity or to detect changes in the structure of microbial communities. A limitation of this procedure is that complex banding patterns do not resolve well on gels (Smit *et al*., 1997).

T-RFLP works on a similar basis to that of ARDRA, except that fluorescently-labelled terminal restriction fragments are generated through the use of a 5' labelled primer (Rastogi and Sani, 2011). This procedure has high reproducibility and banding patterns are more simplified. However, restriction enzymes have an impact on the community fingerprint and the same terminal restriction fragment length may be shared between differing bacterial species (Rastogi and Sani, 2011).

DGGE and TGGE are techniques which utilize gradients on acrylamide gels for the separation of DNA fragments. The gradients used for DGGE and TGGE are chemical and temperature-based respectively. Both techniques are reproducible and reliable, however, they have limitations. If co-migration occurs, then multiple species may be represented by one band (Kirk *et al*., 2004). A single bacterium which displays sequence heterogeneity amongst several rRNA operons would increase the number of bands present, thereby resulting in an overestimation of the diversity present (Rastogi and Sani, 2011).

SSCP is a technique which involves PCR amplification, followed by the separation of DNA into single-strands (Schwieger and Tebbe, 1998). Differences in the single-stranded DNA causes conformational differences after folding, resulting in differences in migration, which can be visualized using a non-denaturing gel. This technique is however restricted to small fragment sizes and limited by a high rate of DNA re-annealing. On the other hand, SSCP is less complex compared to DGGE in that no gradients or GC clamps are required (Rastogi and Sani, 2011).

The Intergenic Spacer Region (ISR) located between the 16S and 23S ribosomal subunits are amplified as the basis for RISA (Fisher and Triplett, 1999). ARISA, the automated version, employs a forward primer, which is fluorescently labelled, followed by the automatic detection of ISR fragments using laser detectors (Rastogi and Sani, 2011). These techniques are sensitive and highly reproducible. Limitations include the large quantity of DNA required and the overestimation of microbial diversity if multiple samples are simultaneously analyzed (Fisher and Triplett, 1999).

Another useful technique is the employment of MST. This technique is capable of characterizing isolates of various species through measurement of variations in DNA sequences of numerous housekeeping genes (Martens *et al*., 2008). MST serves as a reliable and practical alternative to DNA-DNA hybridization and 16S rRNA gene sequence analysis. Sequence data from housekeeping genes can be applied to intraspecies-level discrimination and evaluation of genetic relatedness (Martens *et al*., 2008).

Information may be obtained with molecular techniques even using preserved samples, thereby eliminating the necessity for cultivating organisms (Dolfing *et al*., 2004). This could have numerous applications such as examining the effects of environmental and climatic changes on microbial diversity and studying changes in diversity over time (Egert *et al*., 2004).

2.8.3. Use of High Resolution Melt Analysis (HRMA) for genotyping

HRMA is a fast and simple post-PCR technique that is used for the detection of sequence variation through the generation of melting curve profiles of PCR products (Reed *et al*., 2007). This technique makes use of a fluorescent dye which binds to double-stranded DNA (Borun *et al*., 2015). The PCR product is exposed to increasing temperature, resulting in dissociation of the double-stranded DNA into single DNA strands, which release the dye (Borun *et al*., 2015). Fluorescence emitted by the dye is correlated to the amount of double-stranded DNA present. The emission of fluorescence results in an amplicon-specific melting curve profile being produced. The length, sequence and G+C content of the DNA fragment have an effect on the characteristics of the resulting curve (Reed *et al*., 2007).

The HRM technique is sensitive to factors such as the quality of DNA, amplicon length and concentration of the dye used. Optimization of factors such as the annealing temperature, template DNA, primers and magnesium chloride concentrations are vital for successful HRMA (Reed *et al*., 2007).

HRMA has various applications including allele discrimination, mutation and single nucleotide polymorphism (SNP) detection, heterozygote identification and genotyping (Reed *et al*., 2007; Smith *et al*., 2009). This technique is advantageous due to its accuracy, high throughput, speed and simplicity. It is also a more cost-effective approach in relation to alternative methods commonly employed in studying genetic diversity (Reed *et al*., 2007).

2.8.4. Characterization of microbial metabolic diversity

2.8.4.1. Community-level physiological profiling (CLPP)

Through assessment of functional and metabolic diversity, factors influencing the distribution of microbial communities and the specific roles which these communities play in their environments can be determined (Calbrix *et al*., 2005). The carbon sources present in an environment have an effect on the metabolic diversity amongst microbial communities. Therefore, by studying substrate utilization profiles, differences in microbial community diversity can be established. CLPP is a technique which allows for the characterization of microbial communities based on substrate utilization testing. The procedure involves inoculation of environmental samples, usually suspended in saline or distilled water, into 96-well microtiter Biolog EcoPlates™ containing 31 environmentally-relevant substrates (Garland, 1997). Differences in bacterial communities can be distinguished based on the variations in the carbon source utilization profiles (Garland, 1997). Tetrazolium violet, a colourless, redox sensitive dye, is present in each well (Garland, 1997). This compound acts as an electron acceptor when substrate oxidation occurs and is reduced to produce formazan, a purple coloured product. This colour change is quantifiable using spectrophotometry and can be used to determine the diversity and relative rates of substrate utilization (Garland, 1997).

An important consideration for CLPP is that the inoculum requires standardization, since this factor will impact the rate of colour development (Hill *et al*., 2000). Another significant factor is that certain strains may predominate, thereby resulting in a more efficient utilization of substrates by the faster-growing bacteria. A third consideration is that the substrates included in the Biolog EcoPlates™ are not necessarily representative of all the potential substrates present in the targeted environment (Hill *et al*., 2000). Hence, only the organisms which have the capability of utilizing the available substrates would be represented.

Advantages of CLPP include its high reproducibility, ease of use and cost effectiveness (Kirk *et al*., 2004). Significant amounts of valuable data can be generated readily for a large number

of samples to allow for differentiation of microbial communities. Information relating to overall community functions and adaptation to environmental changes can be established (Weber and Legge, 2009). Substrate patterns from soil samples obtained from different locations or timeframes could allow for a comparison of physiological functions and a determination of the factors driving these variations (Hill *et al*., 2000). When this technique is used in conjunction with molecular, culture-independent based approaches, a powerful combination is possible.

Biolog EcoPlates™ have been used for a range of applications including studying metabolic diversity in contaminated sites (Konopka *et al*., 1998), assessing functional diversity in Arctic soils (Derry *et al*., 1999; Tam *et al*., 2001), determining physiological differences in microbial communities in plant rhizospheres (Garland, 1996), assessing the effects of pollution on community structure (Röling *et al*., 2000; Dobler *et al*., 2001) and determining the effects of organic amendments on microbial activity in soils (Frąc *et al*., 2012).

2.8.4.2. Statistical analysis of CLPP data

Average well colour development (AWCD) can be used for the analysis of CLPP data (Garland, 1997). AWCD is calculated as the mean value of the absorbance readings from the Biolog Ecoplate™ (Frąc *et al*., 2012). The formula is as follows: $AWCD = \sum(C - R)/n,$ in which C and R represent optical densities in each experimental (C) and control well (R) respectively and n represents the total number of substrates (Choi and Dobbs, 1999). AWCD serves as a way to remove variation arising from differing initial cell densities.

Colour development arising from substrate utilization generally follows a sigmoidal graph which can be used for kinetic analysis (Stefanowicz, 2006). Kinetic analysis is based on the Gompertz equation: $y = Aexp\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\}$, where *y* and t represent absorbance and time respectively and λ, , μ_m and *A* represent the lag time, slope and asymptote of the curve respectively (Stefanowicz, 2006).

To obtain insight into substrate richness (amount of substrate utilization) and substrate evenness (extent of substrate utilization), the Shannon-Weaver index (H) may also be used (Stefanowicz, 2006). This index is calculated using the formula: $H = -\sum piInpi$ (Frąc *et al*., 2012). In this equation, p_i represents the ratio of utilization of individual substrates to the sum

of utilization of all substrates. This index helps to provide insight into the physiological diversity of microbial communities.

For a comprehensive assessment of CLPP data, multivariate statistical techniques should be applied (Garland, 1996). A commonly used technique is Principal Component Analysis (PCA) (Stefanowicz, 2006). PCA is employed to decrease the total number of variables obtained (i.e. absorbances readings for all substrates) to a smaller group of variables, termed principal components (Stefanowicz, 2006). These principal components are used to account for the variability which is present in the data.

Canonical Correspondence Analysis (CCA) is a multivariate statistical method that has been used to determine the effect that environmental variables have on substrate utilization patterns (Bossio and Scow, 1995). It is typically paired with the Monte Carlo permutation test to evaluate the statistical validity of the relationship between substrate data and environmental parameters.

Another multivariate technique used to interpret CLPP data is cluster analysis (Stefanowicz, 2006). This technique classifies units (e.g., substrates) into different groups, such that units with similarity are grouped together (Preston-Mafham *et al*., 2002). The functional diversity for each grouping can then be determined.

Analysis of variance (ANOVA) is another statistical method which can be used to calculate differences in functional diversity between microbial communities (Frąc *et al*., 2012). ANOVA allows for differences between group means to be compared and analyzed, thereby providing a means for comparing factors such as functional diversity, richness, AWCD and the Shannon-Weaver index (Frąc *et al*., 2012).

2.9. PHYLOGENETIC EVALUATION OF ENDOSPORE-FORMING BACTERIA

2.9.1. 16S rRNA gene sequencing and applications

Insight into the microbial diversity within environmental samples can be gained through phylogenetic and taxonomic classification of the species present (Poretsky *et al*., 2014). The 16S rRNA gene is considered to be one of the most useful genetic markers for bacterial taxonomy and phylogeny studies (Janda and Abbott, 2007). Primers chosen are complementary to regions of the sequence which are conserved, whilst the variable sequence regions which

occur in-between are used for taxonomic comparisons (Clarridge, 2004). The identification criteria for percentage identity scores which is currently accepted by most taxonomists is ≥98.7% classification when sequence comparisons are being made (Stackebrandt and Ebers, 2006).

In addition to identification and classification, the 16S rRNA gene aids in determining the relatedness between organisms, the rate of species divergence as well as evolutionary distances (Clarridge, 2004). It has broadened understanding in the studies of microbial taxa diversity and allowed for novel and clinically important pathogenic bacteria, which cannot be cultivated, to be identified (Petti, 2007). This gene displays broad applicability across all taxonomic groups (Clarridge, 2004). 16S rRNA gene sequencing has gained popularity due to it being a cost-effective, rapid and non-labour intensive procedure (Janda and Abbott, 2007).

One of the restrictions of 16S rRNA gene sequencing is its limited taxonomic resolution for closely-related species. For example, *Bacillus psychrophilus* and *B. globisporus* share >99.5% 16S rRNA gene sequence similarity but only exhibit 23–50% relatedness when DNA-DNA hybridization is performed (Janda and Abbott, 2007). To overcome this problem, alternative gene targets can be used to separate species which are closely related. Examples of genes which achieve this function are the *rpoB* gene, which codes for the β-subunit in RNA polymerase, or *gyr A* and *gyr B*, for gyrase A and B, respectively (Petti, 2007). 16S rRNA-based analysis cannot provide information pertaining to function, as compared to metagenomic data which is superior (Poretsky *et al*., 2014).

One of the major applications of 16S rRNA gene sequence analysis is the determination of phylogenetic relationships amongst bacteria. It is a popular and reliable method since phylogenetic trees produced using the 16S rRNA gene and trees produced through whole-genome analysis were shown to be similar (Clarridge, 2004).

2.9.2. Gene mapping for the comparison of ancient and modern bacterial genes

Studies involving the isolation and revival of endospore-formers from ancient sources generally require some form of substantiation to address scepticism about the true ages of the bacteria found. To determine if the ages of two isolates are the same, a comparison of evolutionary rates using 16S rRNA sequence data and protein-coding genes can be conducted by performing the relative rate test (Maughan *et al*., 2002). This test is used for the comparison of evolutionary and mutation rates by using closely-related species as well as a reference

species. Ochman *et al*. (1999) proposed that conserved gene sequences amongst pairs of bacterial species which are closely related can be used for estimation of molecular evolution rates. In a study conducted by Maughan *et al*. (2002), the *splB* gene, a gene unique to Gram positive endospore-forming bacteria which codes for spore-photoproduct lyase, and the *recA* gene, a gene involved in DNA repair and homologous recombination, were able to serve this purpose.

To calculate the evolutionary history of bacteria, a molecular clock technique which bases its calculations on homologous gene substitution rates may be applied. This technique is used for the estimation of bacterial divergence times through the assumption that amino acid and nucleotide substitution accumulation occurs at a constant rate (Kuo and Ochman, 2009). The rate of mutation accumulation may also be used as an additional technique for the estimation of bacterial lineage ages (Kuo and Ochman, 2009).

2.10. CONCLUSION

The bacterial endospore contains numerous mechanisms of resistance which allow it to survive harsh conditions and remain viable over extended periods of time. The ability of endospore-forming bacteria to maintain a dormant, unaltered state for survival may allow them to be promising candidates as proxies in palaeo-ecological studies. The preservation of dormant endospores in palaeo-environments could be used to determine the changes in AEFB diversity across time. Studies of microbial diversity in wetland environments and the application of dormant endospores in palaeo-ecological studies are relatively unexplored. The use of molecular fingerprinting and phylogenetics could be used to examine changes in genetic diversity, whilst physiological testing could provide information regarding the metabolic and functional diversity of AEFB over time. Therefore, the revival and characterization of dormant AEFB from palaeo-environments may allow for the opportunity to explore diversity changes over time as well as examine their potential as indicators for changing environmental conditions.

CHAPTER THREE

ISOLATION AND ASSESSMENT OF THE PHYLOGENETIC DIVERSITY OF AEROBIC ENDOSPORE-FORMING BACTERIA REVIVED FROM AN ANCIENT PEATLAND SEDIMENT CORE

3.1. INTRODUCTION

AEFB form dormant endospores when unfavourable conditions (e.g. anoxic conditions) arise within peatlands. Endospores are frequently recovered from sediment layers (Wunderlin *et al*., 2014) and it is therefore feasible that dormant endospores become trapped and 'preserved' within the successive layers of organic material deposits associated with peatlands. In this way, endospores may be able to provide insight into the diversity of AEFB from ecologically-sensitive environments such as peatlands and allow their potential to serve as palaeo-proxies to be investigated.

To examine the changes in AEFB diversity over time across varied environmental profiles, successful isolation of dormant endospores must first be carried out. Isolation and revival of endospores from environmental sources requires the development and utilization of selective cultivation techniques combined with suitable media and culture conditions for growth promotion (Gontang *et al*., 2007).

Following isolation, molecular fingerprinting techniques which can differentiate isolates at the species and/or strain-level may be employed (Rastogi and Sani, 2011). Repetitive extragenic palindromic-Polymerase Chain Reaction (Rep-PCR) is a DNA fingerprinting method which targets repetitive, palindromic sequences present in microbial genomes to create unique DNA fingerprints for microbial strains (Tobes and Pareja, 2006; Ishii and Sadowsky, 2009). Rep-PCR has gained favour as a fingerprinting technique due to its simplicity, speed, reproducibility and resolution ability (Versalovic *et al*., 1998). A greater degree of variability is present between repetitive regions, in comparison to other genomic regions, thereby enabling analysis of strain-level genetic relationships to be possible (Cherif *et al*., 2003). Rep-PCR, utilizing the BOX-A1R primer which targets the BOX mosaic repetitive element, has been successfully applied for strain-level differentiation of endospore-forming bacteria (Cherif *et al*., 2002; Cherif *et al*., 2003; Ishii and Sadowsky, 2009). For these reasons, Rep-PCR was selected to provide strain-level discrimination amongst the aerobic endospore-forming bacterial isolates in the current study.

To facilitate identification and characterization of unknown bacterial isolates, sequence analysis of the 16S rRNA gene is generally conducted (Janda and Abbott, 2007). This gene is widely used for taxonomy and phylogeny studies due to its universal distribution amongst bacteria, its core function which has not changed over time and its convenient length (1 500 bp), which incorporates both conserved and variable sequence regions, that allows taxonomic and phylogenetic relationships to be inferred (Janda and Abbott, 2007). This technique was therefore selected for identification of the revived AEFB.

The Mfabeni Peatland, located within the St. Lucia Wetland Park, is one of the most significant peatland ecoregions in South Africa and is considered ideal for palaeo-environmental research due to its age and pristine nature (Finch and Hill, 2008; Grundling *et al*., 2013a). The present study was undertaken to assess the feasibility of isolating and reviving AEFB from 'ancient' sections of a sediment core dating back to ca. 44 000 cal years BP. Several extraction methods were first assessed to determine endospore extraction efficiencies. The abundance of viable AEFB at selected points along the core was determined. The points chosen correlated to radiocarbon ages ranging from ca. 589 to 37 906 cal years BP. Genomic diversity amongst revived AEFB isolates was established using a Rep-PCR fingerprinting approach. The taxonomic ranking and phylogeny of representative genotypes were then established by 16S rRNA gene sequence analysis.

3.2. MATERIALS AND METHODS

3.2.1. Site description

3.2.1.1. Introduction to the Maputaland region

The Maputaland coastal plain is located on the eastern seaboard of South Africa and runs parallel to the north coast of the KwaZulu-Natal province, stretching into Mozambique (Finch and Hill, 2008). This coastal plain covers an area approximately 943 000 ha in size and is boarded by the Indian Ocean in the east and the Lebombo Mountain range along its western boundary (Grundling *et al*., 2013b). Two types of coastal lake systems occur in this region, namely freshwater lakes and estuarine-linked lakes. Lake St. Lucia forms part of the largest estuarine-linked lake system in Africa and is located within the Greater St. Lucia Wetland Park (Grundling *et al*., 2013b). Approximately 66% of recorded peatlands in South Africa are found here, making it one of the most significant peatland ecoregions in the country (Grundling *et*

al., 2013a). On the eastern shore of St. Lucia lies the Mfabeni Peatland, which is amongst the largest and deepest peatlands in Africa (Grundling *et al*., 1998).

3.2.1.2. Location, climate and hydrology of the Mfabeni Peatland

The Mfabeni Peatland (28°8.92'S, 32°31.12'E) is situated on the eastern shore of St. Lucia in KwaZulu-Natal (Grundling *et al*., 2013a) (Figure 3.1). The peatland occurs 11 m above sea level, with an approximate length of 10 km, extending to a width of 3 km (Grundling *et al*., 2000; Grundling *et al*., 2013a). The Indian Ocean is located to the east of this peatland (Grundling *et al*., 2013a). Beach ridges separate the Mfabeni Peatland from Lake Bhangazi and Lake St. Lucia in the north and south respectively (Grundling *et al*., 2013a). Sedge/reed vegetation dominates the east and north regions, whilst a swamp forest is present in the west and south regions of the peatland (Grundling *et al*., 2013a). The peatland is surrounded by The Eastern Shores Nature Reserve, which comprises of a wide variety of habitats including grasslands, a dune forest and a swamp forest (Finch and Hill, 2008). The region is characterized by subtropical climatic conditions with 60% of the mean annual precipitation falling between the months of November and March (Grundling *et al*., 2013a). Climatic conditions in this region are characterized by wet and hot summers with dry, mild winters (Finch and Hill, 2008). The mean annual temperature recorded for this area ranges between ca. 21^{o}C and 23^{o}C (Turner and Plater, 2004). The Mfabeni Peatland is positioned in a low-lying region and obtains an input of water primarily from groundwater, precipitation and surface inflow (Grundling *et al*., 2013a). Perched aquifers from sand dunes surrounding the peatland are the primary sources of groundwater that flow into the peatland (Grundling *et al*., 2000). Detailed hydrological characteristics have been reported by Grundling *et al*. (2015).

3.2.1.3. Age of the Mfabeni Peatland

The Mfabeni Peatland is one of the oldest and deepest (9.9 m) known active peatlands in Africa and has a base age of ca. 44 000 cal years BP (Grundling *et al*., 1998; Grundling *et al*., 2013a). This timeframe covers the Late Pleistocene and Holocene periods (Finch and Hill, 2008).

3.2.2. Sample collection

A continuous sediment core sample was obtained from the Mfabeni Peatland using a vibracorer for a palaeo-ecological study (Figure 3.2) (Personal communication: Prof. Trevor Hill, hillt@ukzn.ac.za, Discipline of Geography, School of Agriculture, Earth and Environmental Sciences, University of KwaZulu-Natal, Private bag X01, Scotsville 3209, South Africa). The determination of a suitable coring location was conducted using age-depth transects. Aluminum irrigation tubes were used for enclosure of the sediment core. The core was transported back to the laboratory and heavy-duty aluminum foil was used to wrap the core, after which it was stored at 2-5^{o}C. Due to compaction, the sediment percentage recovery was 79.5%. A saw and moulded jig was utilized to split the core longitudinally.

3.2.3. Radiocarbon dating

Accelerator mass spectrometry (AMS) dating was used to radiocarbon date subsamples of the core (Personal communication: Dr. Jemma Finch, jemma.finch@gmail.com, Discipline of Geography, School of Agriculture, Earth and Environmental Sciences, University of KwaZulu-Natal, Private bag X01, Scotsville 3209, South Africa). These radiocarbon dates were calibrated using classical age-modelling software (CLAM, v 2.2) and a weighted mean for each of the sample's calendar ages was then calculated (Blaauw, 2010) (Figure 3.3). Based on this data, subsamples that represented time periods of varying environmental conditions were selected for analysis in this study (Figure 3.3). The interpolated radiocarbon data for each of these samples is presented in Table 3.1.

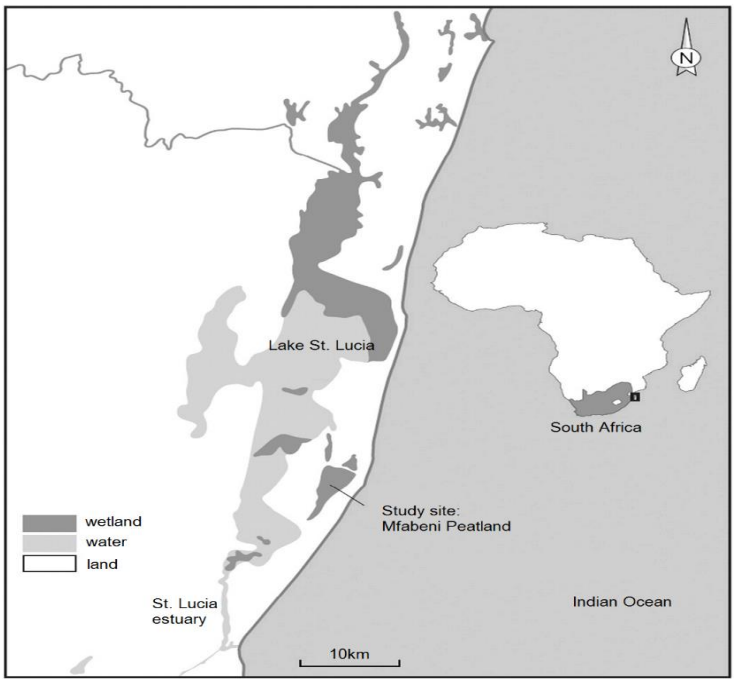


Figure 3.1. Location of the Mfabeni Peatland, the site used in the current study, on the eastern shore of Lake St. Lucia, northern KwaZulu-Natal, South Africa (adapted from Grundling *et al*. (2013a))

Figure 3.2. Core extraction using a vibracorer, Mfabeni Peatland

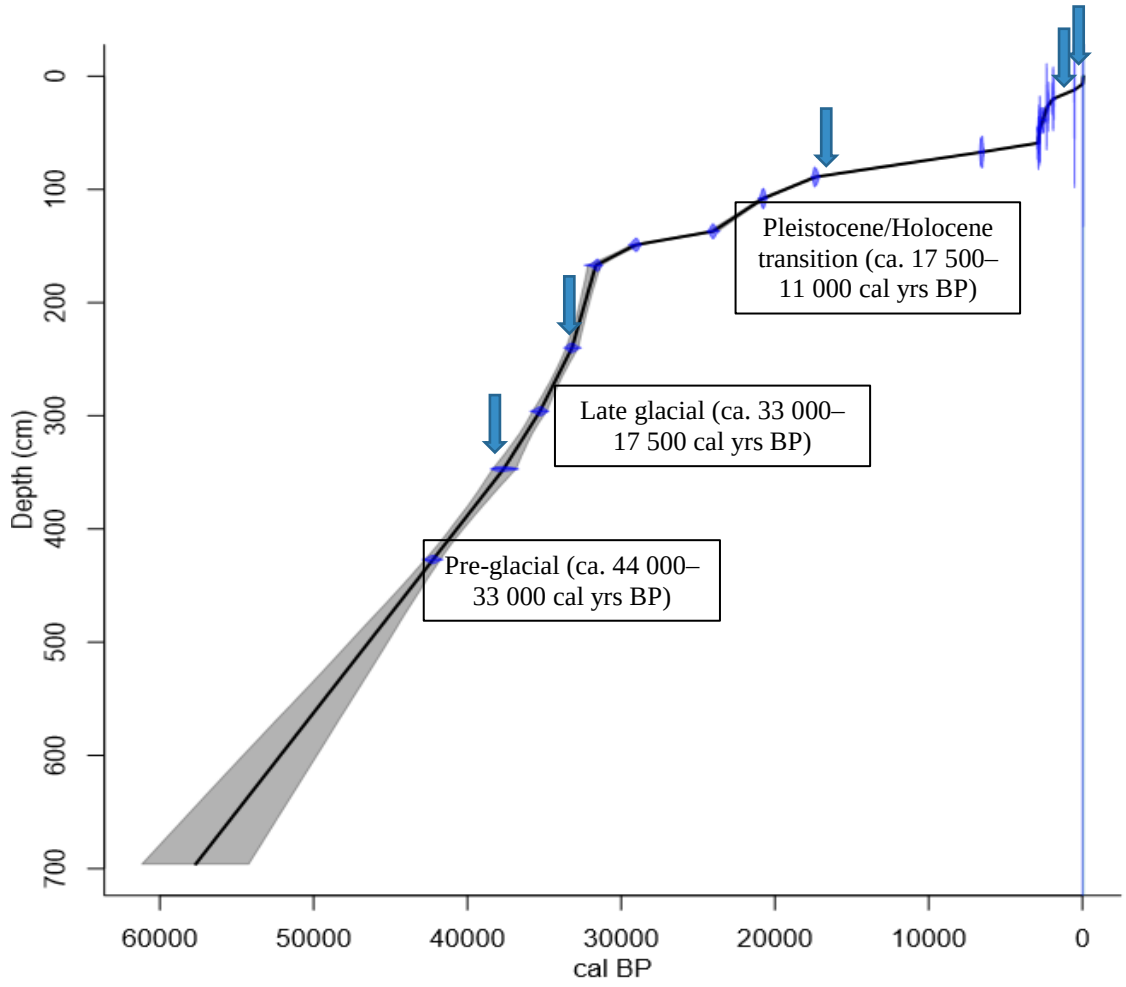


Figure 3.3. Age depth model based on linear interpolation depicting the calibrated ^{14}C dates for the Mfabeni Peatland region generated using the classical age-modelling software, CLAM (Finch and Hill, 2008). Arrows indicate where subsampling was conducted for the current study.

Table 3.1. Interpolated ages for each of the five sub-samples taken from an Mfabeni Peatland sediment core

SAMPLE	DEPTH (cm)	MIN AGE (cal yrs *BP)	MAX AGE (cal yrs BP)	MEDIAN (cal yrs BP)	WMEAN (cal yrs BP)
A	12	482.9	671.5	589.2	570.9
B	21	1 849.6	2 061.8	1 963.7	1 965.9
C	89	17 280.4	17 941.5	17 567.9	17 579.4
D	237	32 604.6	33 867.2	33 328.2	33 311.9
E	344	37 079.5	38 622.4	37 905.7	37 901.7

*BP= before 1950 AD

Physico-chemical characteristics of the sediment core, namely, average particle size, the size fraction of sand, silt and clay, total nitrogen, total organic carbon and pH, are provided in Appendix A (Personal communication: Dr. Jemma Finch, jemma.finch@gmail.com, Discipline of Geography, School of Agriculture, Earth and Environmental Sciences, University of KwaZulu-Natal, Private bag X01, Scotsville 3209, South Africa).

3.2.4. Sub-sampling from the sediment core

Five points along the core which represented time periods of varying environmental conditions were chosen for sampling for the current study. The depths and radiocarbon dates for these samples are provided in Table 3.1. During subsampling, the surface layer was scraped away using sterile scalpels before the samples were extracted. Duplicate samples (ca. 2 g) were aseptically dissected out from the interior of the sediment core using sterile scalpels and tweezers. Samples were transferred to sterile Falcon tubes and kept on ice until they could be stored at -20^{o}C.

3.2.5. Evaluation of extraction methods to recover endospores from peat sediment

Three extraction procedures were tested to determine which method was the most efficient for recovering endospores from the peat sediment. All experiments were carried out in a laminar flow hood with all surfaces disinfected (10% $^{v}/_{v}$ bleach solution) and all materials and equipment double autoclaved (20 min holding time, 121^{o}C, 100 kPa). Agar plates (Trypticase Soy Agar (TSA), 10% TSA, Marine Agar and Reasoner's 2A agar) were left open for the duration of all experiments to validate the absence of contamination from within the working environment (Cano and Borucki, 1995).

The first extraction procedure involved a mechanical treatment incorporating a vortexing agitation step. The second treatment tested the use of calcium chloride ($CaCl_2$) to disperse particulate matter and stabilize peat material in solution (Parent and Tremblay, 2003). The third extraction method involved a TE buffer-mediated extraction, accompanied by bead beating. This method was modified from a procedure used for indirect DNA extraction from sediment (de Bruijn, 2011). Two random samples (ca. 2 g) were taken from the sediment core at the 110 cm and 173 cm for the initial evaluation of each extraction procedure.

3.2.5.1. Mechanical treatment via agitation

One hundred milligrams of sediment from the two sample depths were added in duplicate to 900 µl sterile saline solution (0.85% $^{w}/_{v}$) in 1.5 ml microfuge tubes (Whitehead Scientific (Pty) Ltd., RSA). Each suspended sample was heated at 80^{o}C for 15 min to kill all vegetative cells (Scheldeman *et al*., 2006). To dislodge sediment-bound endospores, samples were vigorously agitated using a vortex (Maxi-Mix 1, Thermolyne Corporation, USA) for 30 min at room temperature. Samples were centrifuged at 850 rpm for 5 min (Neofuge 13, Heal Force, China) to remove debris and particulate matter. A dilution series was conducted by sequentially transferring 100 µl of supernatant from each tube to 900 µl of saline until a 10^{-3} dilution factor was achieved. One hundred microlitres of aqueous sample from each dilution was spread-plated onto four media types to assess endospore recovery efficiencies. Spread plating was carried out using separate sterile glass hockey sticks to minimize chances of cross-contamination. The four media types used were TSA (Biolab, Merck, RSA), 10% TSA (supplemented with 11.7 g/l bacteriological agar), Marine Agar (19.4 g/l NaCl, 8.8 g/l magnesium chloride, 5 g/l bacteriological peptone, 1.8 g/l calcium chloride, 1 g/l yeast extract, 0.16 g/l sodium bicarbonate, 3.24 g/l sodium sulphate, 0.1 g/l ferric citrate, 0.55 g/l potassium chloride, 0.08 g/l potassium bromide, 0.022 g/l boric acid, 0.004 g/l sodium silicate, 0.0024 g/l sodium fluorate, 0.0016 g/l ammonium nitrate, 0.016 g/l disodium phosphate, 15 g/l bacteriological agar) (Zobell, 1941) and Reasoner's 2A agar (0.5 g/l yeast extract, 0.5 g/l protease peptone, 0.5 g/l enzymatic digest of casein, 0.5 g/l glucose, 0.5 g/l soluble starch, 0.3 g/l sodium pyruvate, 0.3 g/l dipotassium phosphate, 0.05 g/l magnesium sulfate heptahydrate, 15 g/l bacteriological agar) (Reasoner and Geldreich, 1985). All media were made up in distilled water and autoclaved at 121^{o}C for 15 min prior to use. Once spread-plated, all media plates were incubated at 30^{o}C for a minimum of six days and viewed daily for evidence of microbial growth. For control purposes, an identical dilution series was conducted using 100 µl of saline solution instead of sediment sample and plated onto each agar medium evaluated. The use of a control dilution series was used to verify the absence of contamination from reagents, equipment or aerosols.

3.2.5.2. Calcium chloride treatment

The second treatment involved the use of calcium chloride ($CaCl_2$) as a dispersant, tested at two different concentrations, to release endospores bound to sediment particles. One hundred milligrams of sediment from two selected depths was added in duplicate to microfuge tubes

(Whitehead Scientific (Pty) Ltd.) containing either 900 µl 0.5 M or 1 M $CaCl_2$. Each sample was then heated at 80ºC for 15 min to kill all vegetative cells. Samples were vigorously agitated for 15 min using a vortex (Maxi-Mix 1) before being centrifuged at 850 rpm for 5 min to remove particulate matter and debris. A dilution series for experimental and control samples was conducted by sequentially transferring 100 µl of supernatant from each tube to 900 µl of each $CaCl_2$ solution until a 10^{-3} dilution factor was achieved. Control dilution series were conducted using 100 µl of each $CaCl_2$ solution instead of sediment sample and plated onto each agar medium evaluated. One hundred microlitres of aqueous sample from each dilution was spread-plated onto the four media types described previously (section 3.2.5.1). All media plates were incubated at 30ºC for a minimum of six days.

3.2.5.3. Buffer-mediated extraction accompanied by bead-beating

The third procedure, which was modified from de Bruijn (2011), combined a mechanical approach, namely bead-beating, with a chemical approach using TE (Tris-EDTA) buffer to aid in extraction. One gram of sediment sample was added in duplicate to 1.5 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). A control tube which lacked sediment sample was processed in the same manner as the experimental samples. Experimental and control tubes were subjected to bead beating (Minibeadbeater, Biospec Products, Inc., USA) for 35 s using six tanzanite beads (2 mm diameter) per tube (Biospec Products, Inc.). The tubes were incubated at 4ºC for 10 min, followed by centrifugation at 800 rpm for 10 min to remove debris and particulate matter. The supernatant was pooled for later use and the pellet was resuspended in 1 ml of TE buffer and vortexed (10 s). Samples were incubated at 37ºC for 5 min before the extraction process was repeated. The supernatant fractions were pooled into new microfuge tubes and centrifuged at 1 000 rpm for 10 min to remove the residual sediment particles. Samples were then incubated at 4ºC for 2 min; this was followed by centrifugation of the supernatant (10 000 rpm for 8 min) for final recovery of the bacterial endospores. Pellets were resuspended in 1 ml of sterile 0.85% saline solution and heated in a water bath at 80ºC for 15 min before performing dilution series plating (10^{-3}) onto selected media. One hundred microlitres of sample was spread-plated onto the four different media types as described (section 3.2.5.1). All media plates were incubated at 30ºC for a minimum of six days.

Dilution series were performed in duplicate and the results from each extraction procedure were compared in terms of average colony forming units (CFU) per gram of sediment (wet

weight). The procedure which exhibited the greatest efficacy in extraction of viable AEFB was chosen to evaluate each of the five selected sampling depths. Sample depths were processed from the oldest (i.e., the sample radiocarbon-dated to 37 906 cal years BP) to the youngest sample (i.e., the sample radiocarbon-dated to 589 cal years BP). This was done to avoid the possibility of sample carryover and cross-contamination of bacteria from 'younger' to 'older' samples. Surface sterilization was carried out between sample processing. Materials used for each sample extraction were prepared independently to minimize the risk of cross-contamination and sample carryover.

Endospore extraction and dilution series plating were undertaken for duplicate samples from five depths, viz., 12 cm, 21 cm, 89 cm, 237 cm, and 344 cm, which correlated to median radiocarbon dates of 589, 1 964, 17 568, 33 328, 37 906 cal years BP, respectively. For each sample set, a maximum of 30 morphologically distinct colonies were selected from each medium type for further characterization. Colonies were picked off from media plates at the highest dilutions from which single colonies were readily distinguished. In cases where low numbers of colonies were obtained, all colonies from each media type at the highest dilution were taken. Colonies were inoculated into 800 µl of 10% TSB in microfuge tubes (Whitehead Scientific (Pty) Ltd.). Tubes were incubated at 30^{o}C for 48 h after which 200 µl of sterile glycerol was added. These served as the master stock cultures. All master stocks were stored at -20^{o}C.

3.2.6. Genomic fingerprinting and phylogenetic analysis of revived AEFB

To confirm the suitability of obtaining template DNA for Rep-PCR directly from a colony 'pick-off' procedure, this approach was compared to a Rep-PCR protocol that used template DNA derived from a commercial DNA extraction kit method. Eight AEFB isolates were randomly selected from cultures obtained from the plating out of sediment core samples. *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7 and *Bacillus subtilis* subsp. *spizizenii* DSM 347 (ATCC 6633) were included as positive controls.

Isolates obtained from dilution series plating (total: 270) were then subjected to Rep-PCR. Fingerprint patterns were compared by measurement and visual comparison of band profiles between isolates. Isolates were assigned to operational taxonomic units (OTUs) on the basis of fingerprint profiles. Representatives of each OTU were then selected for 16S rRNA gene sequencing and phylogenetic analysis.

All molecular work was conducted in an enclosed PCR workstation (PCR cabinet, Esco, RSA) equipped with a HEPA air-filter, to minimize potential sources of contamination, and an ultraviolet (UV) light for surface sterilization.

3.2.6.1. DNA extraction using a kit protocol

Isolates were cultured overnight in 20 ml Luria Bertani broth (10 g/l Tryptone, 5 g/l yeast extract, 10 g/l NaCl) at 30^{o}C at 120 rpm in a rotary shaker (Orbital shaker incubator, MRC Laboratory Equipment, Israel). Cells were pelleted by centrifugation at 5 000 rpm for 10 min (Neofuge 13) in 1.5 ml microfuge tubes. DNA extraction of selected isolates was conducted using a GeneJET Genomic DNA kit (ThermoScientific, USA), according to the manufacturer's protocol for extracting genomic DNA from Gram positive bacteria. To confirm the success of extraction, DNA products were electrophoresed using a 1.5% $^{w}/_{v}$ agarose gel. The gel was run in 1 x Tris-Borate-Ethylenediaminetetraacetic acid (TBE) buffer (89 mM Tris base, 89 mM Boric acid and 2 mM EDTA, pH 8.3) at 80 V for 65 min. A 1 kbp DNA ladder (Fermentas, USA) was included as a reference standard for estimating fragment sizes. Five microlitres of DNA were mixed with 3 µl of 6 x loading dye (Promega) prior to loading into wells. Gels were stained with (1 x) SYBR™ Safe dye (Invitrogen, USA) and visualized under UV light. Images were taken using GeneSnap™ software (Syngene v 7.09, England). The purity (A_{260}:A_{280}) and concentrations (ng/µl) of the extracted DNA were analyzed using a NanoDrop^{TM} spectrophotometer (ThermoScientific). All DNA products were stored at -20^{o}C.

3.2.6.2. DNA extraction using a colony 'pick-off' approach

A colony 'pick-off' procedure was used to obtain genomic DNA for PCR purposes according to a procedure adapted from Nilsson *et al.* (1998). Selected isolates were sub-cultured onto 10% TSA plates and incubated for 72 h at 30^{o}C. A single distinct colony from each isolate was aspetically transferred to 50 µl of TE buffer using a sterile 200 µl pipette tip. The tubes were vortexed for 5 s and heated at 95^{o}C for 15 min in a dry heating block (Accublock$^{™}$, Labnet International, Inc., USA). All tubes were then centrifuged at 10 000 rpm for 1 min (Neofuge 13). The supernatant was stored at -20^{o}C for future use.

3.2.6.3. Repetitive extragenic palindromic-Polymerase Chain Reaction (Rep-PCR)

Genomic fingerprinting of selected isolates was undertaken following a Rep-PCR method described by Urzi *et al*. (2001), using a Box-A1R primer (Versalovic *et al*., 1994) (Table 3.2). Reactions were carried out in 200 µl sterile thin-walled PCR tubes (Whitehead Scientific (Pty) Ltd.) and consisted of 1 x GoTaq® Flexi Buffer (Promega), 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 µM of primer and 0.07 U GoTaq® DNA polymerase (Promega). Each tube contained 1 µl of template DNA (from either the kit extraction or the colony 'pick-off' method) and the final reaction volume was made up to 25 µl using nuclease-free water (Promega). Reaction mixtures which lacked template DNA and contained 1 µl nuclease-free water (Promega) were included as controls. The amplification reactions were carried out in a Bioer thermal cycler (XP Cycler Model TC-XP-G, Bioer Technology Co. Ltd., China). The temperature profile consisted of an initial denaturation at 95ºC for 5 min, followed by 35 cycles comprising of denaturation of 95ºC for 1 min, annealing at 45ºC for 1 min and extension at 72ºC for 2 min. A final extension of 72ºC for 10 min was used. All samples were stored at 4ºC prior to electrophoresis. PCR products were analyzed by electrophoresis using 1.5% $^{w}/_{v}$ agarose gels as described in section 3.2.6.1.

Table 3.2. Nucleotide sequences for primers used for Rep-PCR and 16S rRNA gene sequence amplification

*Primer	Sequence (5'–3')	Length (bp)	Tm (ºC) (min/max)	Reference
Rep-PCR				
Box-A1R	CTACGGCAAGGCGACGCTGACG	22	70.13/70.13	Versalovic *et al*. (1994)
16S rRNA-PCR				
16S rRNA-F	AGAGTTTGATCCTGGCTC	18	57.62/57.62	Ström *et al*. (2002)
16S rRNA-R	CGGGAACGTATTCACCG	17	59.61/59.61	

*All primers obtained from Inqaba BioTech™ (Hatfield, Pretoria, South Africa)

3.2.6.4. 16S rRNA gene amplification

The amplification of partial 16S rRNA gene fragments from selected isolates was carried out according to the method described by Tzuc *et al*. (2014) and Ström *et al*. (2002). PCR reactions were performed in a Bioer thermal cycler (XP Cycler Model TC-XP-G) using 25 µl reaction volumes consisting of 1 x GoTaq® Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM each of 16S rRNA forward and reverse primer (Table 3.2) and 0.04 U GoTaq® DNA polymerase (Promega). Two microlitres of template DNA were added to each reaction and the final volume was made up to 25 µl with nuclease-free water (Promega). Template DNA from *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7 was included as a positive control. Reaction mixtures which lacked template DNA were also included as controls. The cycling conditions used involved an initial denaturation at 94^{o}C for 5 min; this was followed by 30 cycles which comprised of a denaturation step at 94^{o}C for 30 s, annealing at 54^{o}C for 30 s and an extension step at 72^{o}C for 80 s. The final extension was carried out at 72^{o}C for 5 min. Confirmation of the PCR amplification of the targeted gene fragment (~1400 bp) was performed using agarose gel electrophoresis (section 3.2.6.1).

3.2.6.5. 16S rRNA gene sequencing and phylogenetic analysis

Amplified 16S rRNA gene fragments were sequenced at Inqaba BioTech™ (Hatfield, Pretoria, South Africa). Amplicons were first purified using Wizard PCR Prep Kits (Promega), before sequencing using a ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). Both 16S rRNA forward and reverse primers were used. Analysis of reaction sequences were conducted with an ABI 3130*XL* sequence analyzer (Applied Biosystems).

Editing of sequence chromatograms for all the isolates was performed with Chromas LITE© software (v 2.01, Technelysium (Pty) Ltd., Australia). BioEdit software (v 7.0.9.0) (Hall, 1999) was used to align the sequence data and generate contiguous sequences. Consensus sequences were then compared to 16S rRNA gene sequences deposited in Genbank (http://www.ncbi.nlm.nih.gov) using the Megablast algorithm. The Genbank search was limited to sequences from type and reference material within the 16S rRNA gene sequence database.

Evolutionary relationships were represented by creating phylogenetic trees using the 16S rRNA gene sequence data. Phylogenetic trees were constructed through use of the Neighbour-Joining (Saitou and Nei, 1987) and Maximum-Likelihood (Fisher, 1922; Huelsenbeck and Crandall,

1997) methods. All phylogenetic analyses were conducted using Mega software (v 6.06) (Tamura *et al*., 2013). For the Neighbour-Joining analysis, genetic distances were computed using the Jukes-cantor model (Tamura *et al*., 2007) by means of bootstrap values based on 1 000 replicates (Felsenstein, 1985). For the Maximum-Likelihood analysis, evolutionary distances were estimated using the Tamura-Nei substitution model by means of bootstrap values based on 1 000 replicates.

All sequences were aligned with Clustal and trimmed to the same base pair length (1 254 bp). Manual gap evaluation was carried out on the sequences and all gaps were removed for pairwise sequence comparisons. 16S rRNA gene sequences of phylogenetically-related taxa were included for comparison. Trees were rooted using the 16S rRNA gene sequence of *Clostridia beijerinckii* JCM 1390 as an outgroup.

3.3. RESULTS

3.3.1. Evaluation of extraction methods to recover endospores from peat sediment

The efficiency of recovering viable endospore-forming bacteria from sediment core samples varied between the three extraction methods (Figure 3.4). The results obtained exhibited a similar trend for all media types tested. The results obtained using 10% TSA are shown (Figure 3.4).

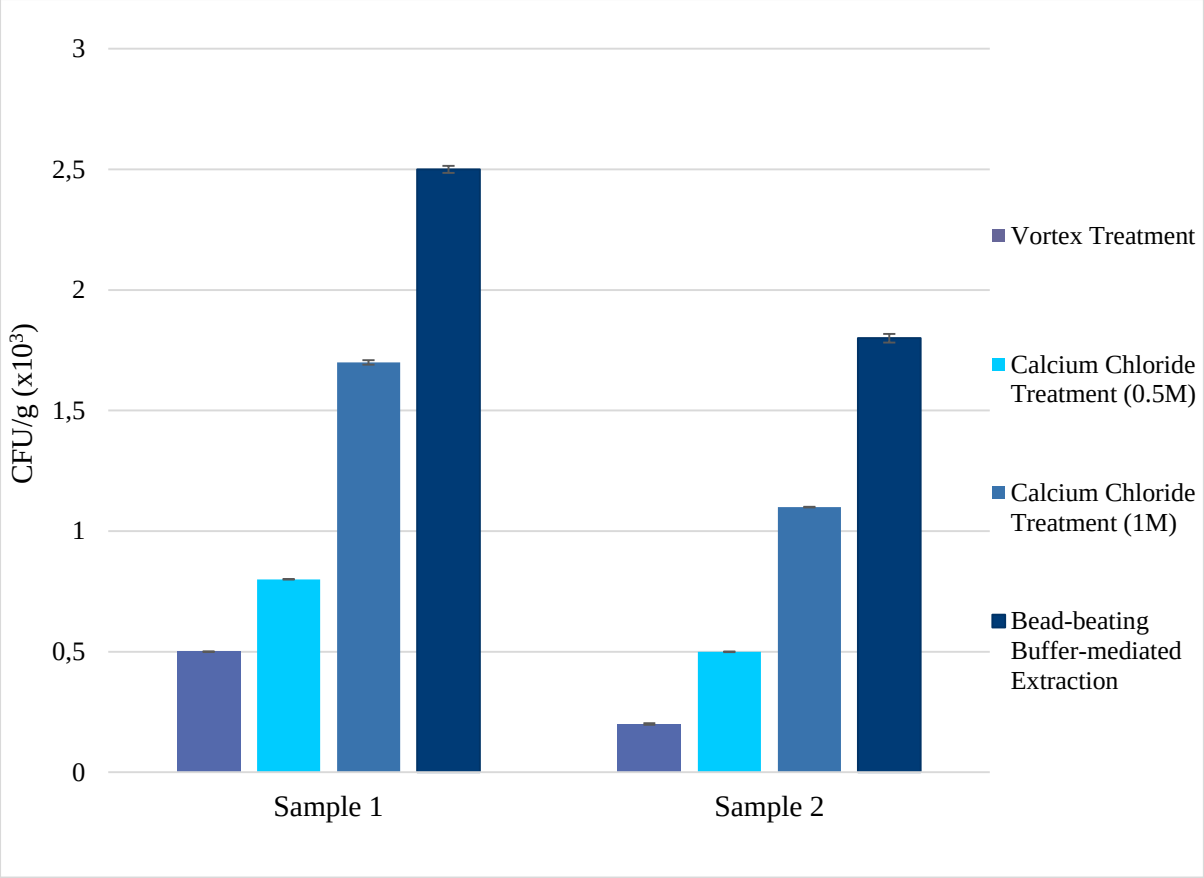


Figure 3.4. The recovery of viable endospore-forming bacteria from a peatland sediment core using three different extraction procedures. Error bars indicate the standard deviation between replicates (n=2).

A similar trend was observed for both samples that were evaluated. The vortex treatment yielded the lowest CFU/g values in both instances. The calcium chloride ($CaCl_2$) extraction method proved to be more effective than the mechanical (vortex) treatment. In both instances, the 1 M concentration of $CaCl_2$ proved to be twice as effective as compared to the lower concentration (0.5 M) of $CaCl_2$ used. These trends were observed across the four media tested. The bead-beating buffer-mediated extraction procedure yielded CFU/g values which were 5 x and 9 x greater than that of the vortex treatment for sample 1 and 2, respectively. This procedure was also more effective than the 1 M $CaCl_2$ treatment (1.5 x and 1.6 x more effective for sample 1 and 2, respectively). The bead-beating buffer-mediated extraction procedure was therefore chosen for isolating endospore-forming bacteria from sediment samples from the five depths under examination.

3.3.2. Endospore revival from sediment core samples

Colony counts (CFU/g) for AEFB revived from sections of a sediment core from the Mfabeni Peatland are presented in Table 3.3 and Figure 3.5.

Table 3.3. Colony-forming units per gram of sediment CFU/g (x10^3) showing revival efficiency across depths on four media types

Sample	Depth (cm)	CFU/g x 10^3			
		10% Trypticase Soy Agar	Trypticase Soy Agar	Marine Agar	Reasoner's 2A Agar
A	12	4.780 ± SD 0.049	0.930 ± SD 0.057	8.143 ± SD 0.424	6.108 ± SD 0.247
B	21	0.280 ± SD 0.028	0.055 ± SD 0.007	0.028 ± SD 0.014	0.533 ± SD 0.037
C	89	0.240 ± SD 0.018	0.003 ± SD 0.004	0.033 ± SD 0.021	0.083 ± SD 0.011
D	237	1.825 ± SD 0.071	0.083 ± SD 0.011	0.145 ± SD 0.035	0.043 ± SD 0.010
E	344	0.003 ± SD 0.004	0	0	0.025 ± SD 0.007

AEFB isolates were obtained from each of the sediment core samples assayed. The highest numbers of endospores revived were from sample A. A significant decrease in CFU counts was evident for samples taken from deeper sections of the core. For sample A, the AEFB revival efficiency ranged from 0.93 x 10^3 CFU/g to 8.143 x 10^3 CFU/g, whereas for sample B, revival ranged from 0.28 x 10^2 CFU/g to 0.533 x 10^3 CFU/g, representing an 11–300 fold decrease in overall AEFB numbers depending on medium type. The lowest total count (3.0 CFU/g) was observed for sample E (344 cm) on 10% TSA. With the exception of sample E, AEFB isolates were revived from all samples on each medium type evaluated. For sample E, AEFB were only revived on 10% TSA and R2A media. The proportions of isolates revived varied between the media. Using sample A as an example, the highest proportion of isolates were revived on Marine Agar, followed by R2A, 10% TSA and lastly, TSA. However, the abundance of isolates

cultured on each media type differed across sample depths. For example, the 10% TSA medium accounted for 23.9%, 31.3%, 67.6%, 87.1% and 9.09% of the total CFU/g at sample A, B, C, D and E, respectively. For samples A and B, the highest number of AEFB were revived on Marine Agar (40.8%) and R2A (59.5%), respectively, whereas, for samples C and D, the highest CFU counts were obtained using the 10% TSA medium (Figure 3.5).

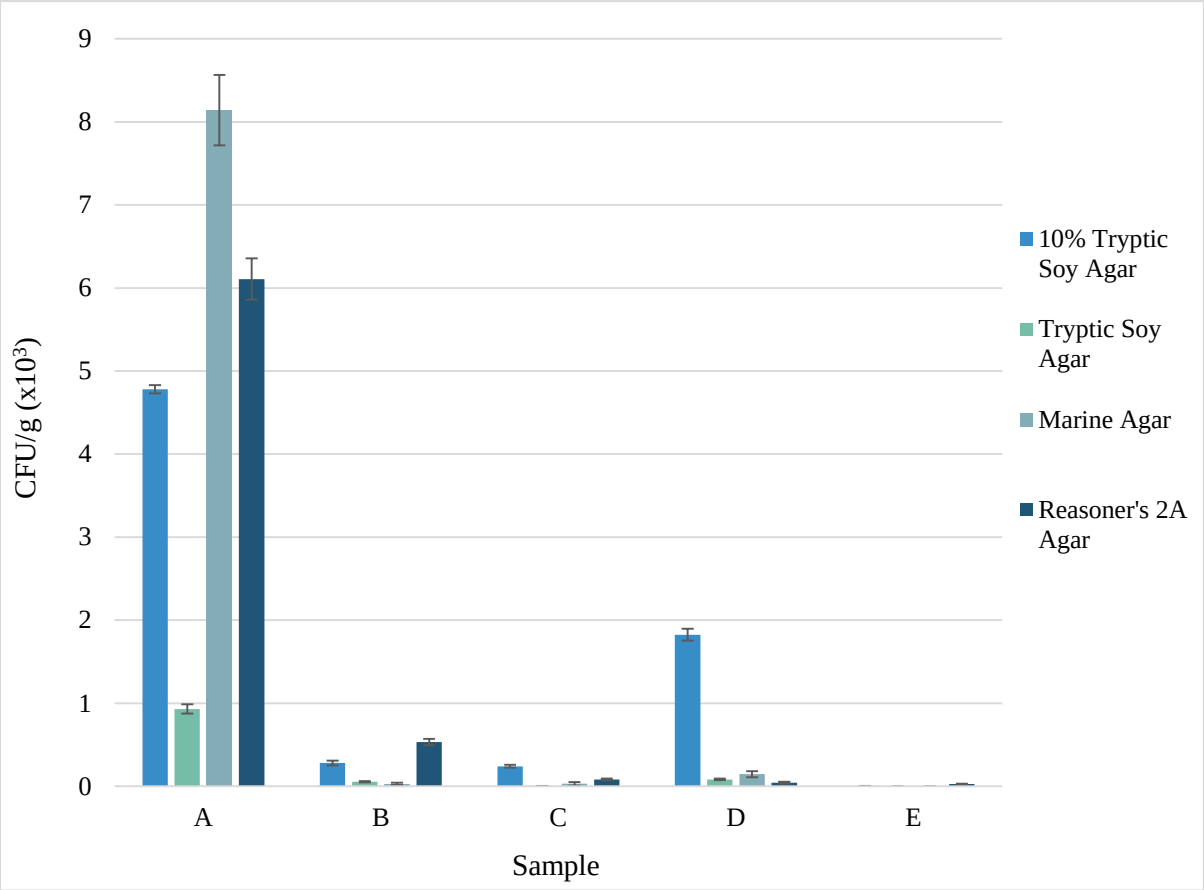


Figure 3.5. Total AEFB colony-forming units (CFU) per gram of sediment for each media type across five sampling depths examined along a sediment core from the Mfabeni Peatland. Error bars are used to indicate the standard deviation between replicates (n=2). Samples A, B, C, D amd E correspond to depths of 12 cm, 21 cm, 89 cm, 237 cm and 344 cm respectively.

A total of 270 isolates were selected for genomic fingerprinting using Rep-PCR. For sample A, 30 morphologically distinct isolates from the highest dilution of each medium type were selected, giving a total of 120 isolates. For the remaining sample depths, due to the low CFU/g

counts obtained, all isolates at the highest dilution from each medium type were selected for Rep-PCR fingerprinting.

3.3.3. Rep-PCR fingerprinting

A preliminary experiment was undertaken to determine the suitability of using template DNA derived from colony 'pick-off' for Rep-PCR. Eight isolates were randomly chosen from the endospore-forming bacteria collected and their Rep-PCR fingerprints, derived from kit-extracted DNA and colony 'pick-off', were compared (Figure 3.6). *Bacillus subtilis* subsp. *spizizenii* DSM 347 (ATCC 6633) and *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7 were included as positive controls.

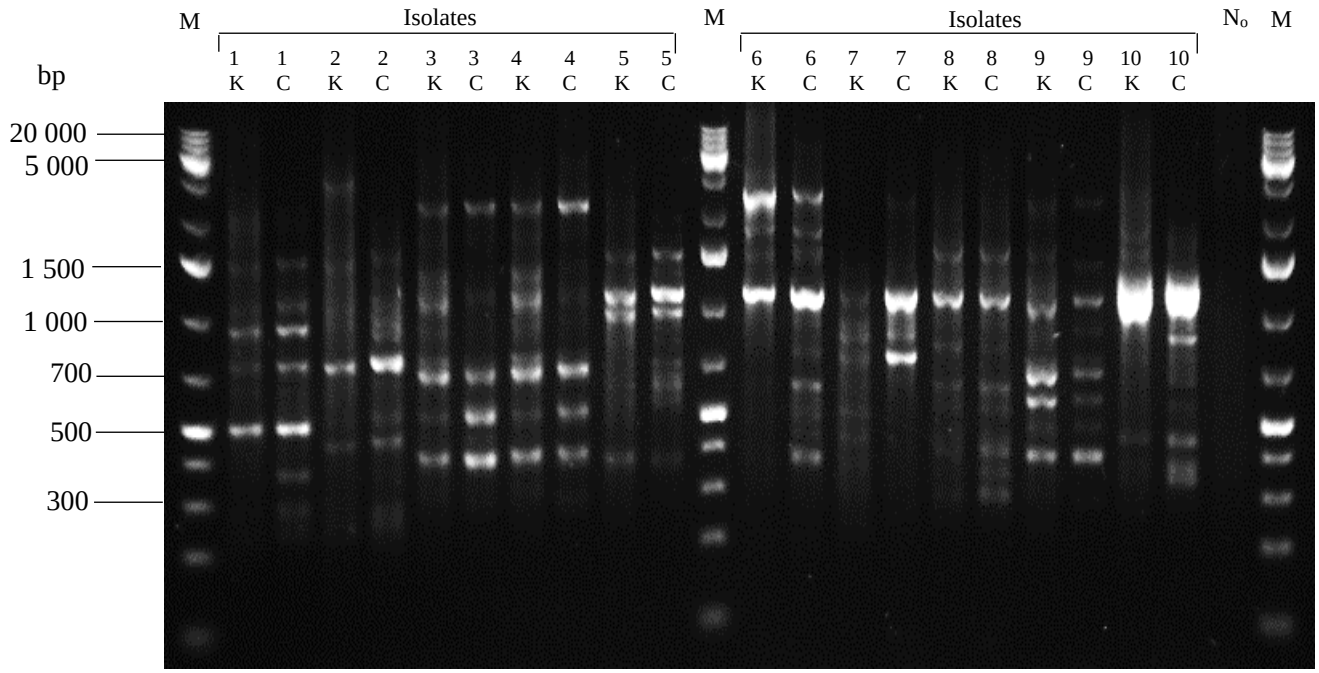


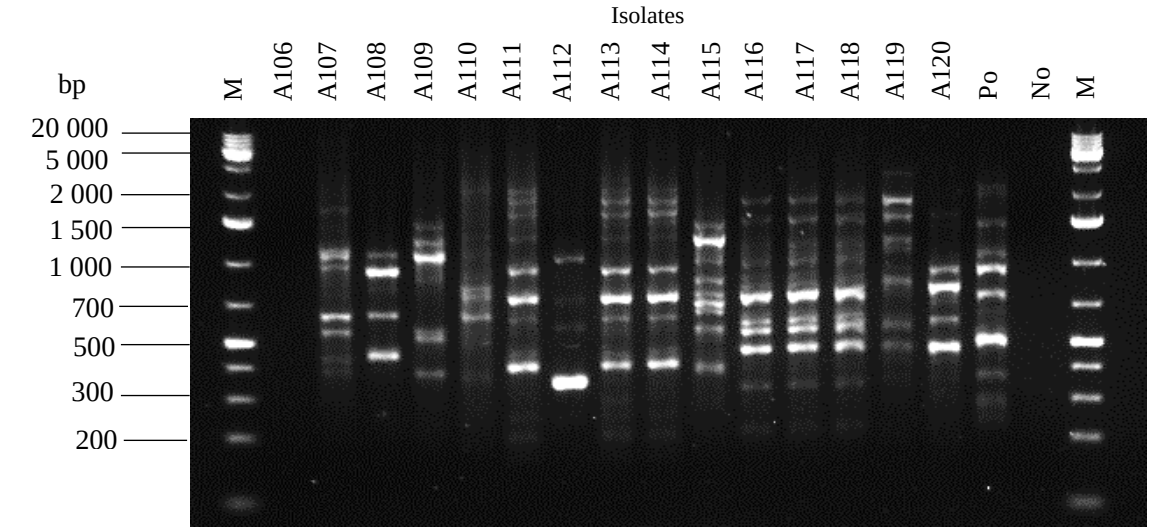
Figure 3.6. Agarose gel electrophoresis comparing Rep-PCR fingerprints of selected isolates using template DNA from GeneJET Genomic DNA extraction kits (K) and colony pick-offs (C). Lane: M, 1 kbp DNA ladder; isolate 1: *B. subtilis* subsp. *spizizenii* DSM 347; isolate 2, *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7; isolates 3–10, AEFB obtained from sediment core samples; N_o, negative control.

The concentration of template DNA obtained using a kit extraction ranged from 1.604 ng to 6.952 ng in 25 µl. DNA purity (A_{260}:A_{280}) of kit extracted template DNA ranged from 1.78–2.09.

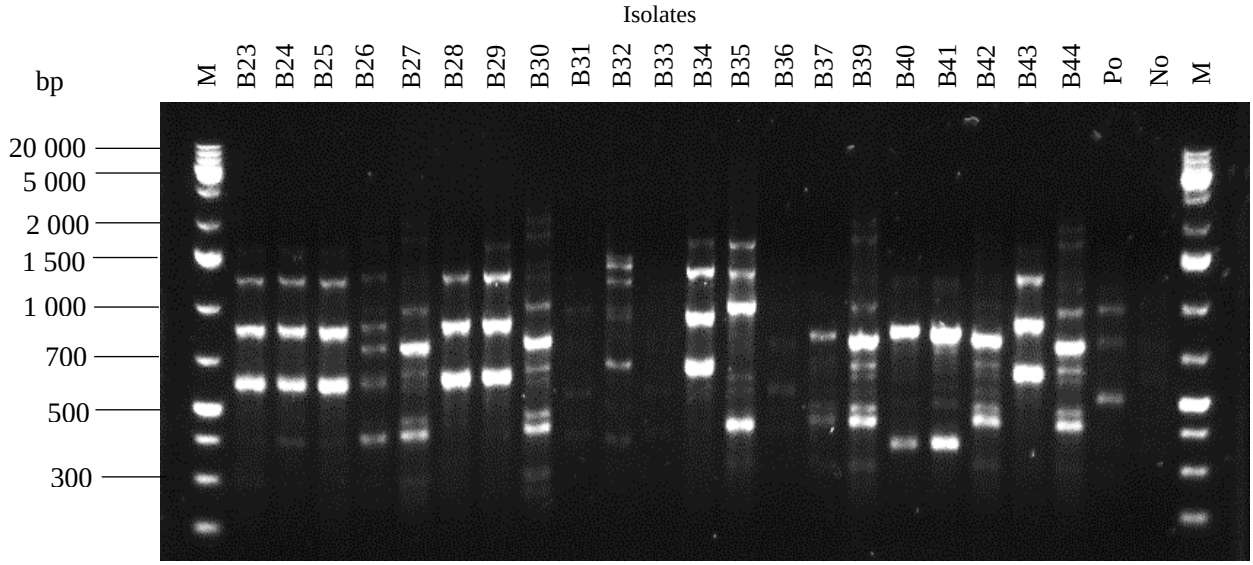
Rep-PCR fingerprint profiles for each template DNA source produced comparable banding patterns. In some instances, the intensity of the bands demonstrated some variability however, this was attributed to differences in template DNA concentration. Overall, colony-pick off was confirmed to produce distinct, comparable and reproducible banding patterns consistent with kit-extracted DNA in all cases. This procedure was simple, rapid and cost-effective. On the basis of these findings, this method of DNA extraction was used to obtain template DNA for subsequent Rep-PCR fingerprinting studies.

A total of 270 isolates, which had been sub-cultured on 10% TSA, were screened using Rep-PCR. Profiling was done for each depth separately. Banding patterns between all isolates were compared visually. On the basis of the visual comparisons of isolates, within and across samples, OTUs were assigned (Table 3.4). Examples of the fingerprint patterns obtained for selected isolates from each depth are shown in Figure 3.7 (A–E).

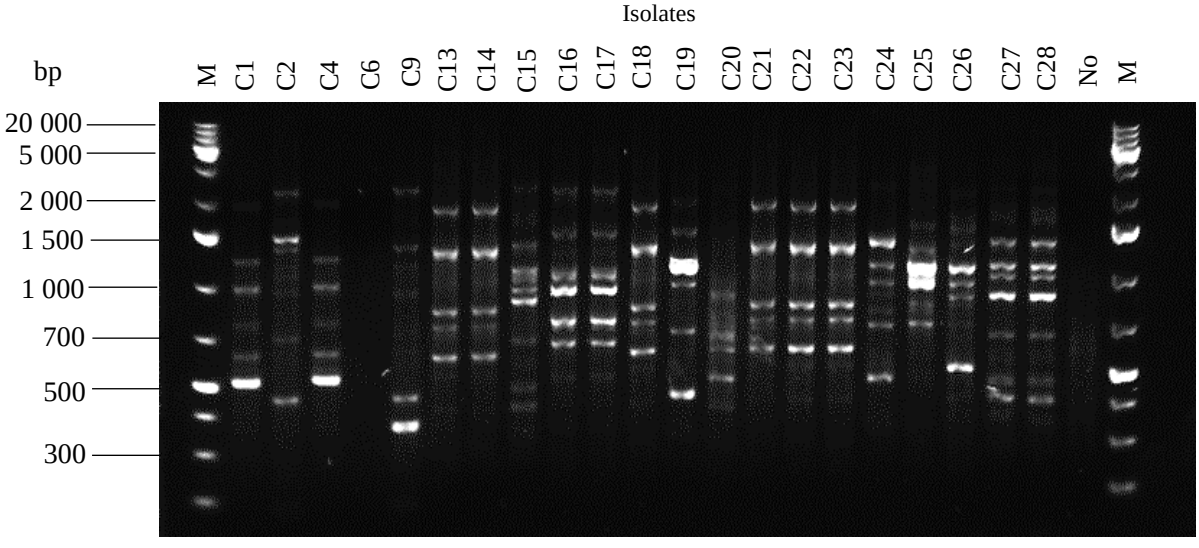
Sample A (12 cm ca. 589 cal years BP)



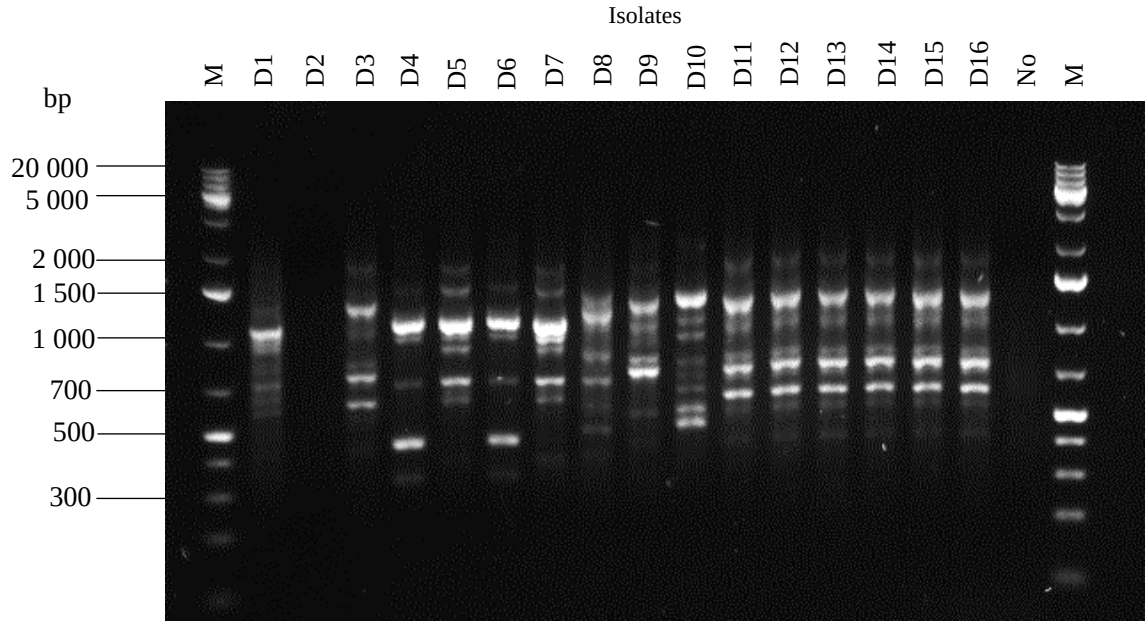
Sample B (21 cm ca. 1 964 cal years BP)



Sample C (89 cm ca. 17 568 cal years BP)



Sample D (237 cm ca. 33 328 cal years BP)



Sample E (344 cm ca. 37 906 cal years BP)

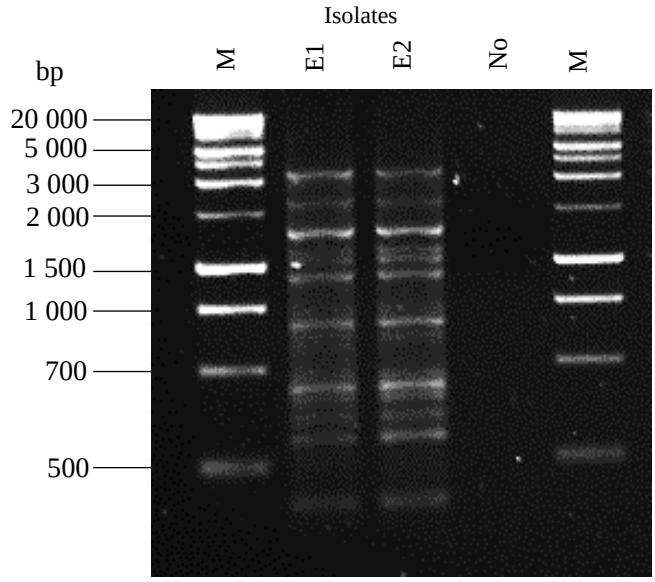


Figure 3.7. Examples of Rep-PCR fingerprint profiles of selected AEFB isolates from each sediment core sample visualized using 1.5% agarose gel electrophoresis. Lane: M, 1 kbp DNA ladder; Po, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7; No, negative control.

The number of bands in the fingerprinting patterns for all isolates screened with Rep-PCR (inclusive of those not included in the example figures above) ranged from 1 to 15. Approximate molecular weights of bands varied from 200 bp up to 3 000 bp in total. The Rep-PCR fingerprinting was repeated for selected isolates and in all cases, banding profiles were found to be reproducible.

Rep-PCR is a fingerprinting technique which is able to distinguish isolates at a strain level (Cherif *et al*., 2002). Banding profiles for each isolate were compared visually across all sample sets. Isolates exhibiting identical banding profiles were regarded as being the same strain and were grouped together as the same OTU. Each isolate which displayed a distinct banding profile was considered to be a unique strain and was assigned a separate OTU designation. On this basis, all of the isolates from each depth were assigned to an OTU (Table 3.4).

Table 3.4. Assignment of Operational Taxonomic Units (OTUs) to AEFB isolates from sections of a sediment core based on Rep-PCR fingerprinting profiles and their distribution across sample depths and cultivation media

DEPTH (cm)	TYPES OF MEDIA							
	Marine Agar	*RA	R2A agar	RA	TSA	RA	10% TSA	RA
12	OTU 1	1/30	OTU 4	1/30	OTU 15	11/30	OTU 15	10/30
	OTU 2	4/30	OTU 13	2/30	OTU 20	2/30	OTU 22	2/30
	OTU 3	3/30	OTU 14	1/30	OTU 21	2/30	OTU 25	3/30
	OTU 4	4/30	OTU 15	17/30	OTU 22	3/30	OTU 29	1/30
	OTU 5	3/30	OTU 16	1/30	OTU 23	1/30	OTU 30	2/30
	OTU 6	2/30	OTU 17	1/30	OTU 24	5/30	OTU 31	3/30
	OTU 7	1/30	OTU 18	1/30	OTU 25	1/30	OTU 32	4/30
	OTU 8	1/30	OTU 19	2/30	OTU 26	1/30	OTU 33	5/30
	OTU 9	4/30	OTU 20	2/30	OTU 27	3/30		
	OTU 10	3/30	OTU 21	1/30	OTU 28	1/30		
	OTU 11	2/30	OTU 22	1/30				
	OTU 12	2/30						
21	OTU 28	1/9	OTU 7	1/29	OTU 34	1/5	OTU 19	2/21
	OTU 34	2/9	OTU 15	16/29	OTU 35	1/5	OTU 40	7/21
	OTU 35	1/9	OTU 40	1/29	OTU 37	1/5	OTU 47	3/21
	OTU 36	2/9	OTU 41	5/29	OTU 45	1/5	OTU 48	2/21
	OTU 37	1/9	OTU 42	2/29	OTU 46	1/5	OTU 49	4/21
	OTU 38	1/9	OTU 43	2/29			OTU 50	3/21
	OTU 39	1/9	OTU 44	2/29				
89	OTU 51	1/3	OTU 51	2/6	OTU 51	1/1	OTU 51	5/18
	OTU 52	1/3	OTU 54	1/6			OTU 58	2/18
	OTU 53	1/3	OTU 55	1/6			OTU 59	1/18
			OTU 56	1/6			OTU 60	1/18
			OTU 57	1/6			OTU 61	3/18
							OTU 62	4/18
							OTU 63	2/18
237	OTU 53	1/13	OTU 64	3/8	OTU 64	1/5	OTU 73	7/30
	OTU 64	2/13	OTU 67	2/8	OTU 70	1/5	OTU 74	6/30
	OTU 65	3/13	OTU 68	2/8	OTU 71	1/5	OTU 75	3/30
	OTU 66	7/13	OTU 69	1/8	OTU 72	2/5	OTU 76	2/30
							OTU 77	4/30
							OTU 78	4/30
							OTU 79	3/30
							OTU 80	1/30
344			OTU 81	1/1			OTU 81	1/1

*Relative abundance expressed as the number of isolates belonging to the respective OTU over the total number of isolates selected from the respective media at the specific depth

Of the 270 isolates screened, 81 OTUs were distinguished. At each depth, with the exception of the 344 cm sample, isolates were recovered from each of the media evaluated. The richness and relative abundance of OTUs associated with each medium type varied between sampling depths (Table 3.5).

Table 3.5. OTU richness and relative abundance for the four media at each depth

Depth (cm)	Medium							
	MA		R2A		TSA		10% TSA	
	*Richness	¥Abundance	*Richness	¥Abundance	*Richness	¥Abundance	*Richness	¥Abundance
12	12	0.40	11	0.367	10	0.333	8	0.267
21	7	0.778	7	0.241	5	1	6	0.286
89	3	1	5	0.833	1	1	7	0.388
237	4	0.308	4	0.50	4	0.80	8	0.267
344	0	0	1	1	0	0	1	1
TOTAL	**26**	**2.486**	**28**	**2.941**	**20**	**3.133**	**30**	**2.208**

*Richness reflected as number of OTUs present on the respective media at each depth. ¥Abundance reflected as the number of OTUs divided by total number of isolates selected from the respective media type at the specific depth. This proportion was used to account for the differences in the number of isolates selected from each media type at each depth.

Based on OTU numbers across all depths, the 10% TSA medium displayed the highest level of OTU richness, closely followed by R2A and Marine Agar medium. Collectively, the lowest OTU richness was associated with TSA. OTU richness and abundance varied between depths. For example, at the 12 cm depth (sample A), a total of 33 OTUs were distinguished, with the highest number of OTUs being isolated on Marine Agar. For sample B (21 cm), the highest numbers of OTUs were associated with Marine Agar and R2A media. At the 89 cm and 237 cm sample depths, 10% TSA displayed the highest levels of OTU richness. The high relative abundances reflected for TSA needs to be viewed with caution due to the low CFU/g values obtained at certain depths.

The majority of the OTUs distinguished were specific to one medium type. Of the 81 OTUs differentiated, only 19.8% were isolated from more than one type of medium (Figure 3.8).

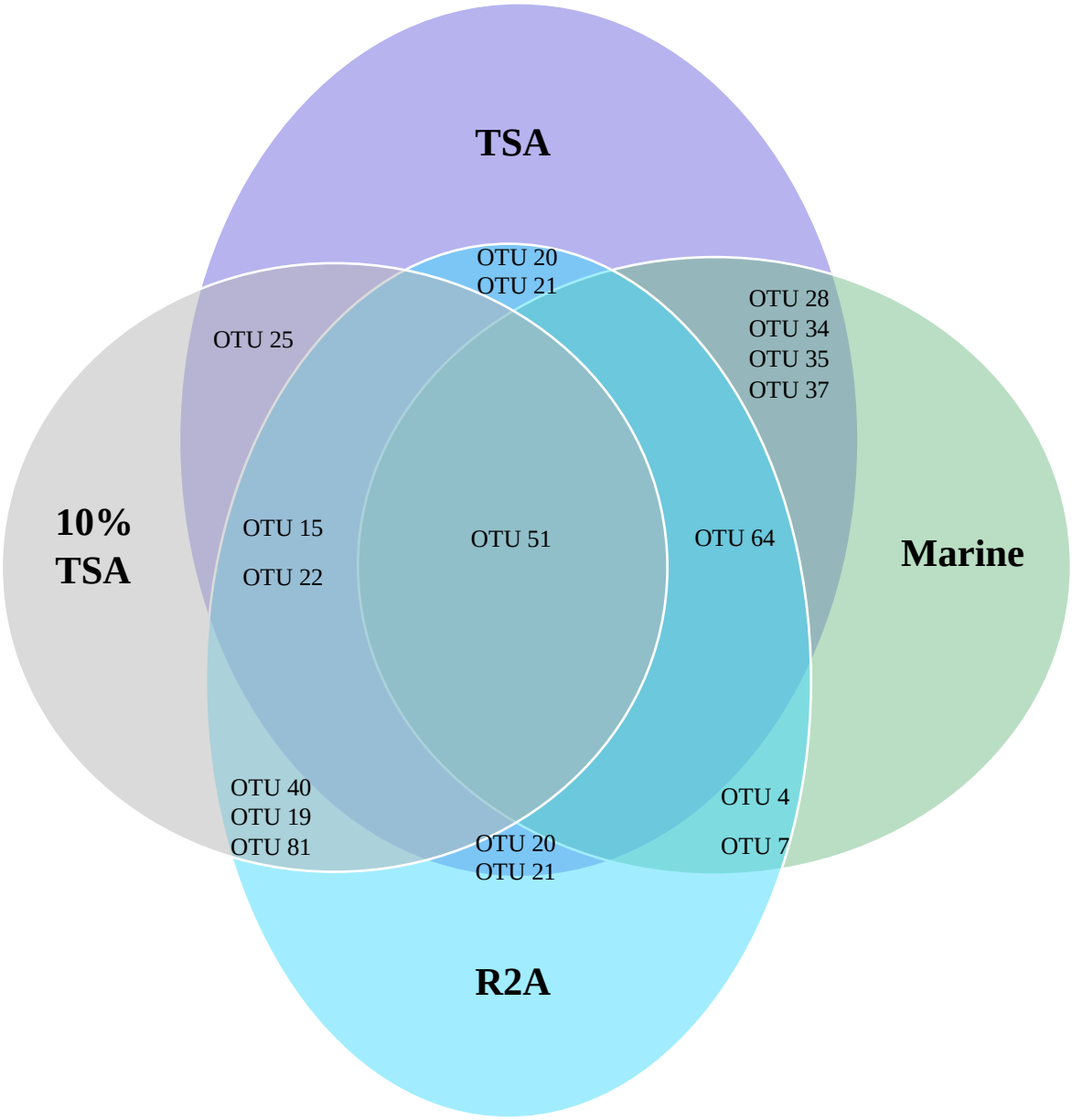


Figure 3.8. Venn diagram illustrating the distribution of Operational Taxonomic Units representing AEFB isolates revived on different agar media

OTU 51, which was unique to sample C (89 cm), was the only OTU which comprised of isolates which grew on each medium type evaluated. Isolates assigned to OTU 15 were the most abundant representatives in the core sample with examples cultured from both the 12 cm

and 21 cm depths. The high number of isolates which made up this OTU were isolated from three of the four media types tested, namely R2A, TSA and 10% TSA. OTU 22, comprising of isolates exclusively from the 12 cm depth, were also cultured on the same three media types. OTU 64, which was unique to the 237 cm depth, was the only other OTU which occurred across three media types, namely TSA, Marine Agar and R2A agar.

Of the 81 OTUs differentiated, 93.8% were found at only one depth. Only 5 OTUs were isolated from more than one depth (Figure 3.9).

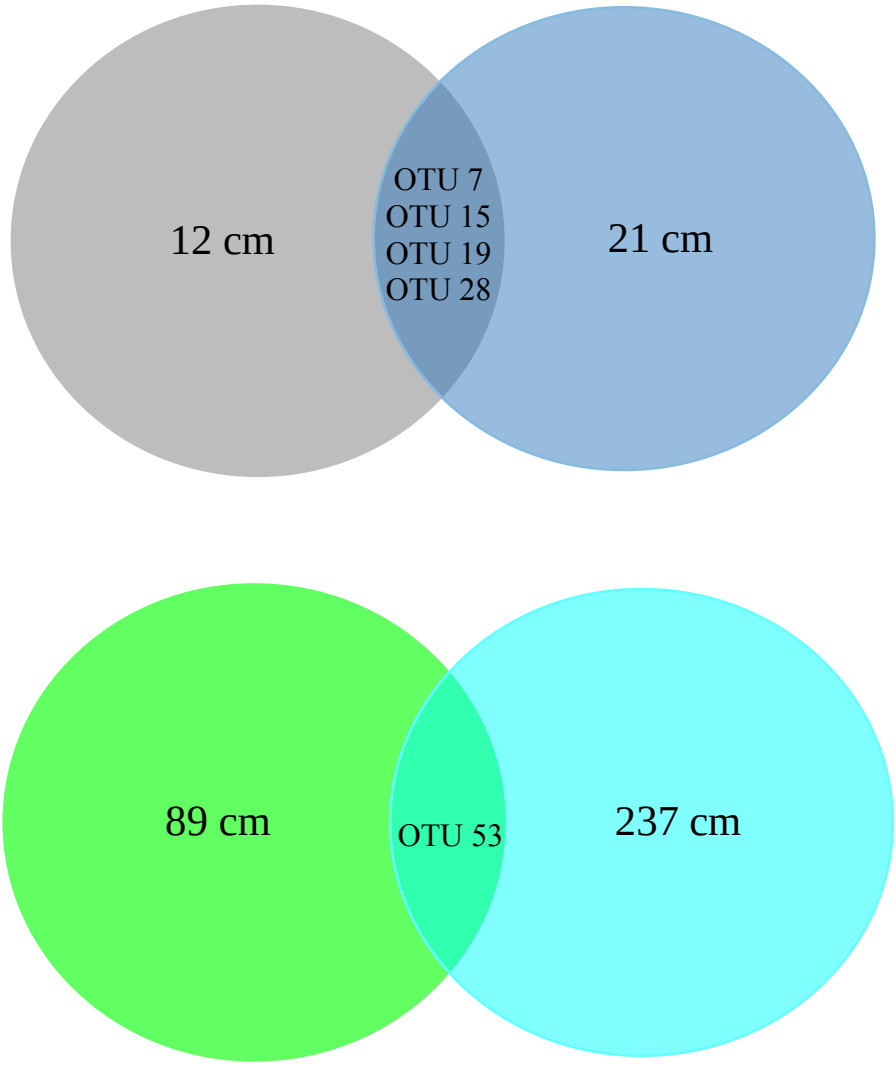


Figure 3.9. Venn diagram illustrating the distribution of Operational Taxonomic Units which were present at more than one sample depth examined

3.3.4. 16S rRNA gene sequencing and phylogenetic analysis

A representative isolate of each distinct OTU was selected for 16S rRNA gene sequence analysis. In addition, both isolates belonging to OTU 43 which possessed identical banding patterns were chosen for sequencing to validate the accuracy of the Rep-PCR technique. Both isolates belonging to OTU 53 (the OTU present at sample depths C and D) were also sequenced. In total, 83 isolates were selected for sequencing. PCR amplicons of approximately 1 400 bp in length were generated from the amplification of partial 16S rRNA gene fragments, which was consistent with results obtained by Ström *et al*. (2002) (Figure 3.10).

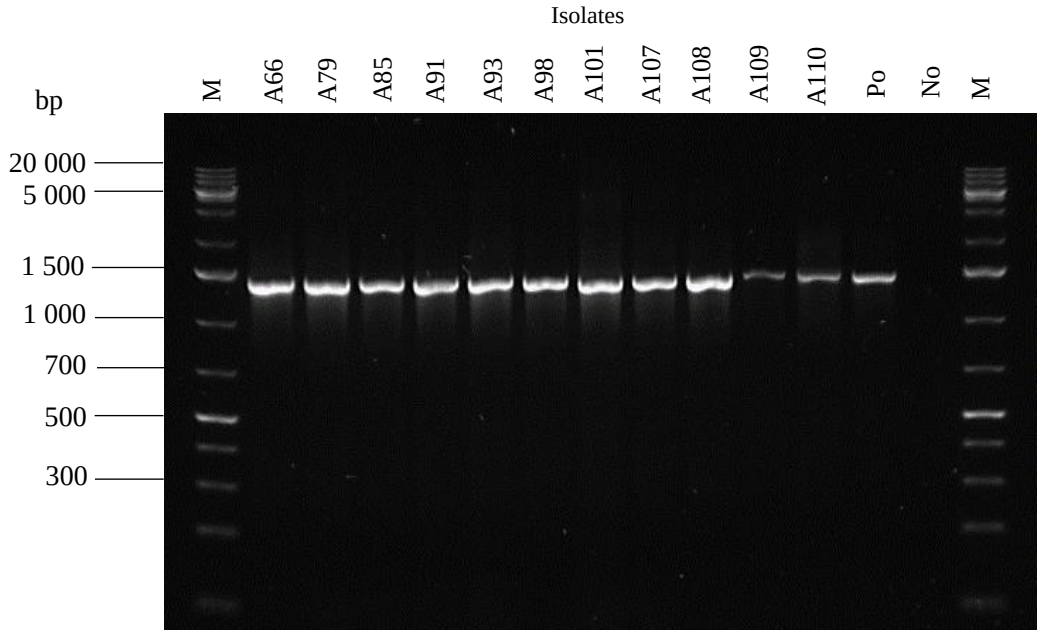


Figure 3.10. Agarose gel (1.5%) electrophoresis image displaying 16S PCR amplification products of selected AEFB isolates using 16S rRNA forward and reverse primers. Lane: M, 1 kbp DNA ladder; lanes 2-12: isolates selected from sample A (12 cm); Po, positive control (*Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7); N_o, negative control.

Phylogenetic trees constructed using the Neighbour-Joining and Maximum-Likelihood methods both displayed similar topologies. Only the Maximum-Likelihood tree is displayed to show the genera present (Figure 3.11). Selected sub-clades of the phylogenetic tree are provided in Figure 3.12 for focus on specific genera and ease of visualization. A total of 81.7% of the isolate sequences matched currently-listed sequences from type and reference material in the Genbank database with a percentage similarity of ≥97%. The remaining isolates (18.3%) aligned with lower percentage similarities which ranged from 84% to 96%, which is below the accepted threshold of 98.7% for species identification (Stackebrandt and Ebers, 2006). The top sequence similarity matches and OTU designations for isolates are provided in Tables A1–A5 (Appendix A).

In addition to 16S rRNA gene sequencing of representative OTUs, two isolates with identical banding profiles (namely B23 and B24, Figure 3.7) were subjected to 16S rRNA gene sequencing to confirm their taxonomic/phylogenetic relatedness. This was done to validate the accuracy of Rep-PCR for isolates exhibiting identical fingerprint profiles. 16S rRNA gene sequencing revealed that both isolates had near-identical sequences (>99.5% similarity) and identical hits on a BLAST database search for sequence similarity matches (Table A2, Appendix A).

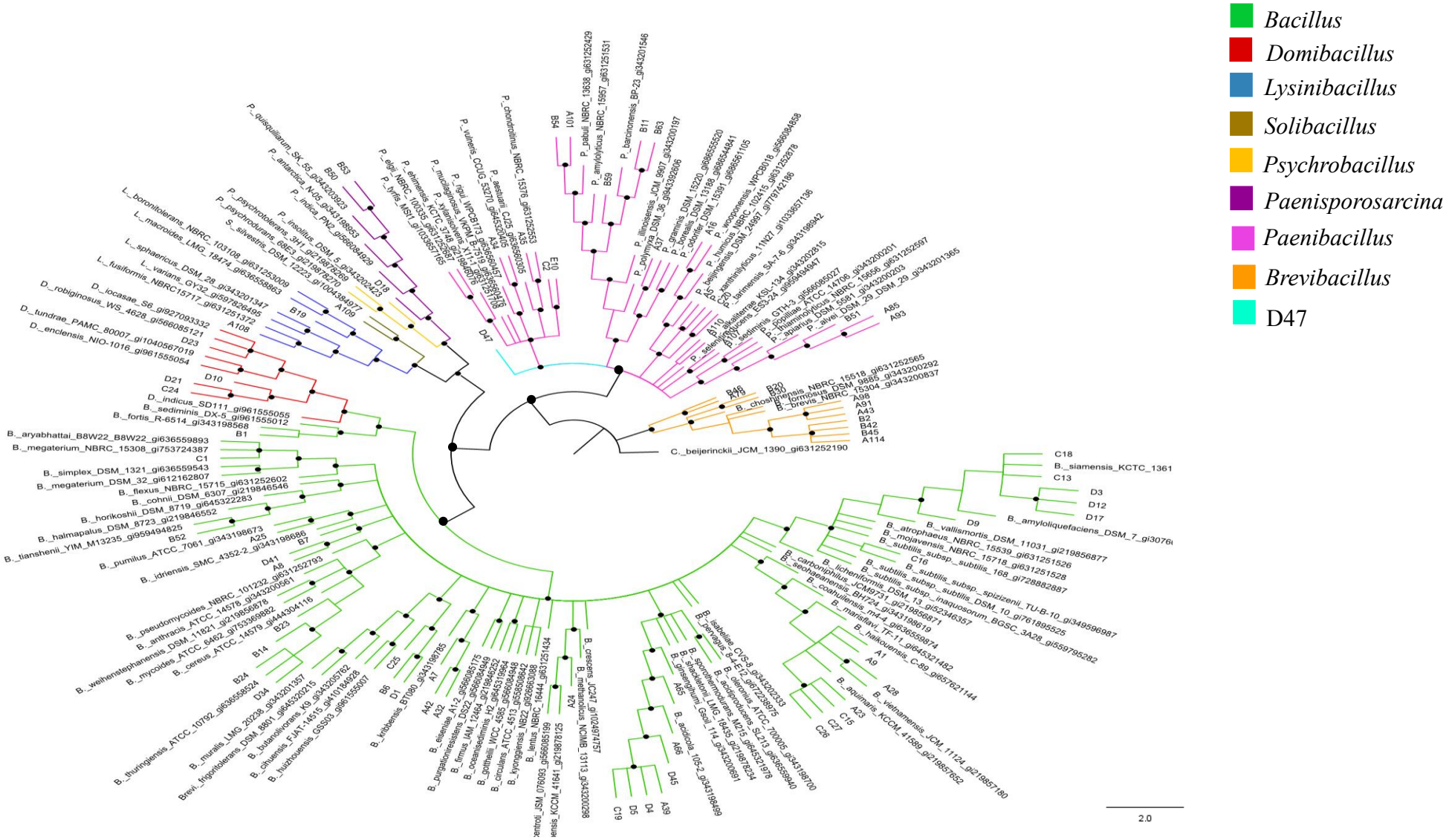
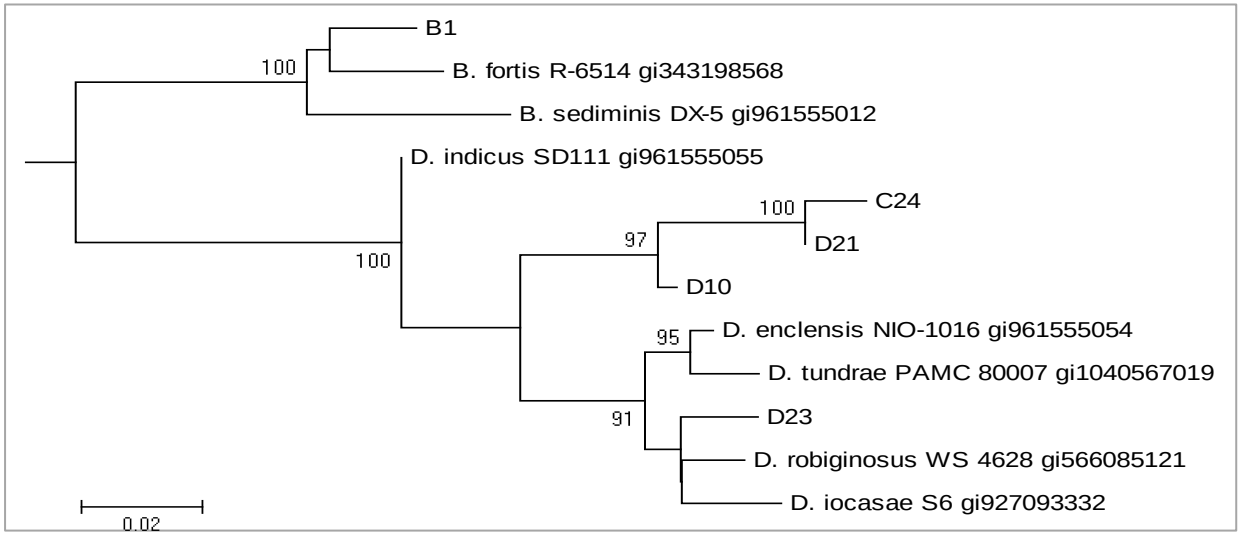


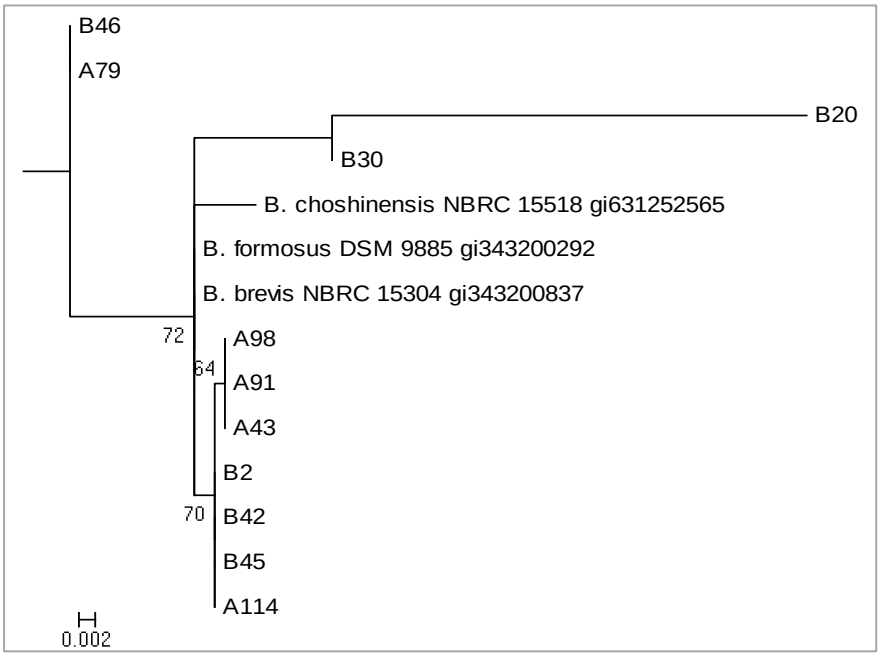
Figure 3.11. Maximum-Likelihood phylogenetic tree based on partial 16S rRNA gene sequences inferring the evolutionary relationships between selected AEFB isolates and reference sequences. The tree was constructed using the Tamura-Nei substitution model by means of bootstrap values based on 1 000 replications. The tree was rooted using *Clostridia beijerinckii* JCM 1390. The scale bar represents 2.0 substitutions per nucleotide position. Black dots represent bootstrap values ≥50%. The key represents the aerobic endospore-forming genera.

Several aerobic endospore-forming genera were distinguished amongst the isolates characterized, namely *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Domibacillus*, *Lysinibacillus*, *Paenisporosarcina* and *Solibacillus*. The majority of isolates were matched to the genus *Bacillus* and representatives of this genus were found at all depths sampled, with the exception of sample E (344 cm). Isolates matched to the *Paenibacillus* genus had representatives from each of the sample depths examined (Figure 3.12D). Isolates matched to the *Brevibacillus* and *Lysinibacillus* genera were only found at the two shallowest depths examined, namely samples A (12 cm) and B (21 cm) (Figures 3.12B and 3.12C). Conversely, isolates matched to the *Domibacillus* genus were only found in association with samples C (89 cm) and D (237 cm) (Figure 3.12A). Isolate D47 clustered as an outgroup of the *Paenibacillus* clade and was unusual in that it shared ≤93% sequence similarity to reference or type sequences currently listed in the GenBank database (Figure 3.12D). Isolates matched to the *Paenisporosarcina* genus were found at samples B (21 cm) and D (237 cm). The sole isolate matched to the *Solibacillus* genus was found in association with sample A (12 cm) (Figure 3.12C).

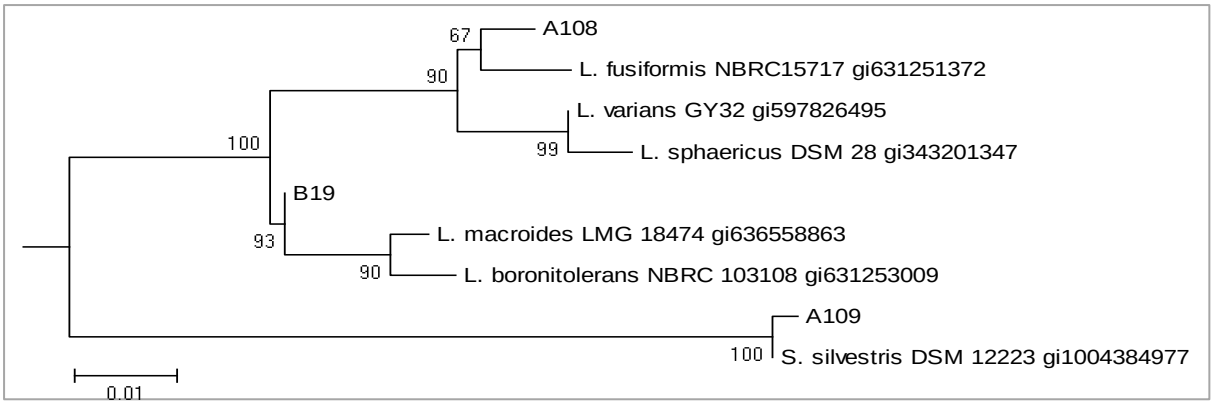
A


B1
100
B. fortis R-6514 gi343198568
B. sediminis DX-5 gi961555012
D. indicus SD111 gi961555055
100
C24
97
D21
100
D10
95
D. enclensis NIO-1016 gi961555054
D. tundrae PAMC 80007 gi1040567019
91
D23
D. robiginosus WS 4628 gi566085121
D. iocasae S6 gi927093332
0.02

B


B46
A79
B20
B30
B. choshinensis NBRC 15518 gi631252565
B. formosus DSM 9885 gi343200292
B. brevis NBRC 15304 gi343200837
72
A98
64
A91
A43
B2
70
B42
B45
A114
0.002

C


67
A108
90
L. fusiformis NBRC15717 gi631251372
L. varians GY32 gi597826495
100
99
L. sphaericus DSM 28 gi343201347
B19
93
L. macroides LMG 18474 gi636558863
90
L. boronitolerans NBRC 103108 gi631253009
A109
100
S. silvestris DSM 12223 gi1004384977
0.01

D

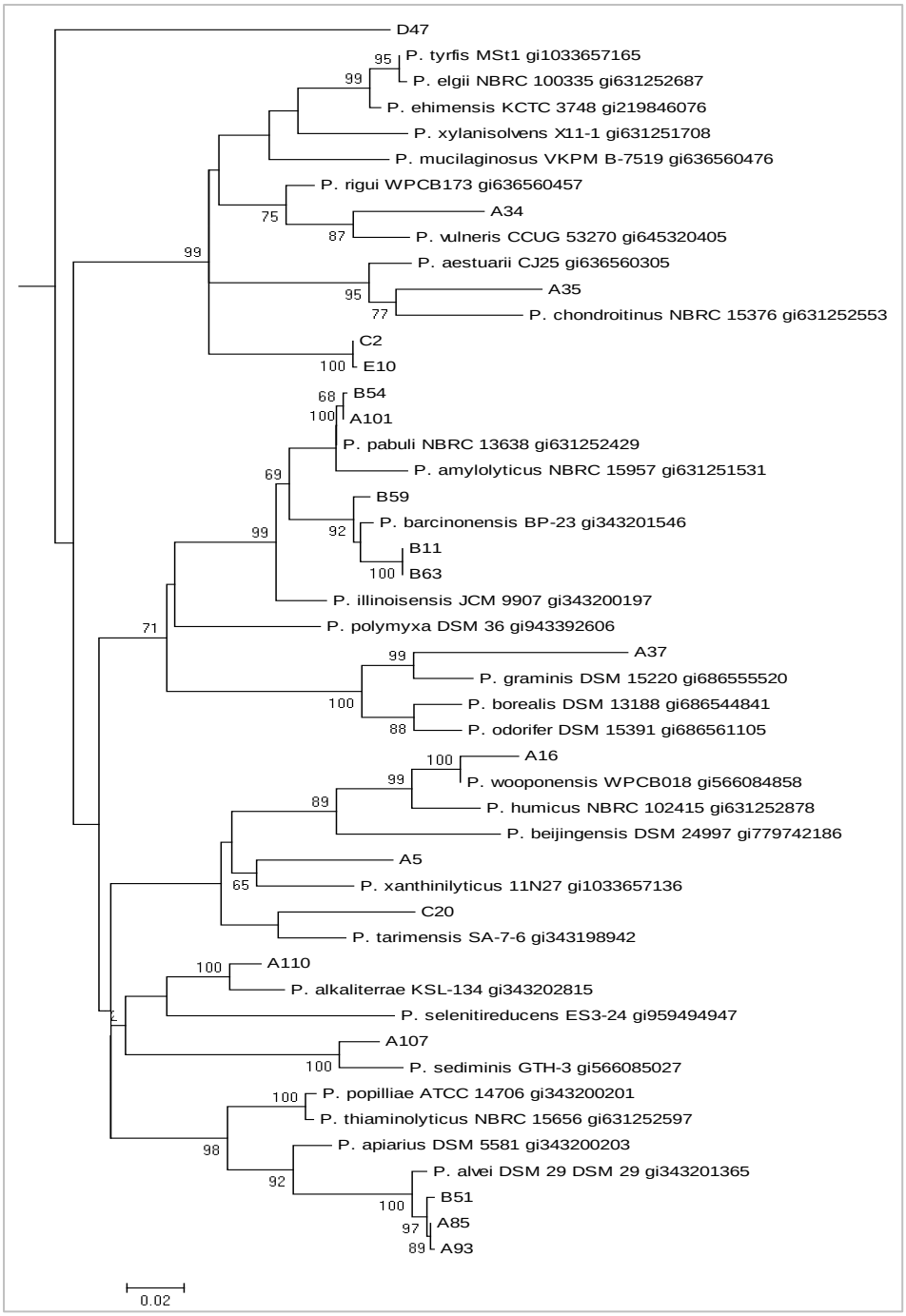


Figure 3.12. Sub-clades of the Maximum-Likelihood phylogenetic tree (Figure 3.11) illustrating the evolutionary relationships of revived AEFB isolates matched to reference strains of *Domibacillus* spp. (A) *Brevibacillus* spp. (B) *Lysinibacillus* spp. (C) and *Paenibacillus* spp. (D)

3.3.5. Strain comparison of taxonomically-related isolates

From the phylogenetic tree (Figure 3.11), it was evident that certain groups of isolates obtained from different depths clustered closely together, suggesting they were of the same species or closely related. To ascertain whether strain variation existed amongst these isolates, Rep-PCR fingerprinting profiles for each group of isolates were compared.

Isolates A65, A66, C19 and D5 were all matched to strains of *Bacillus acidicola* and *B. shackletonii*, with 98–99% similarity (Genbank, Megablast). Although each isolate shared a common fragment (ca. 1 550 bp), the overall differences in fingerprint banding profiles suggest that each isolate was a distinct strain (Figure 3.13). Interestingly, A65 and A66 displayed distinct banding patterns, indicating the occurrence of two different strains within the same sample depth.

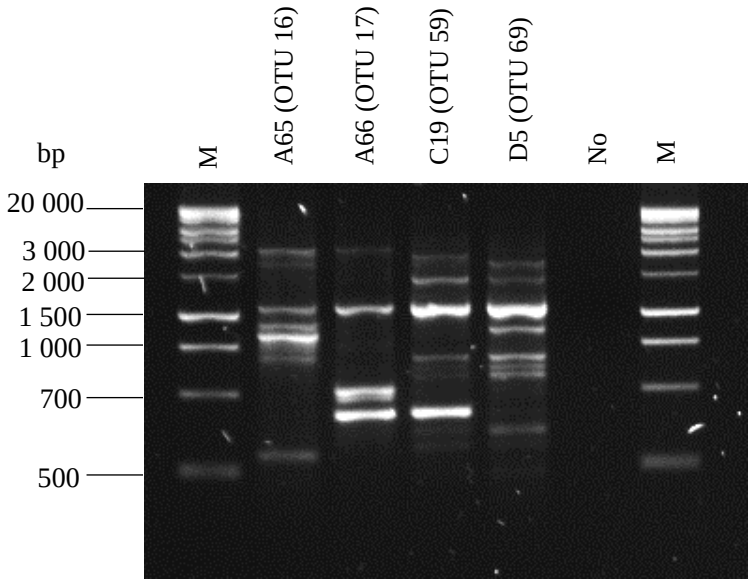


Figure 3.13. Rep-PCR comparison of sediment core AEFB isolates exhibiting 16S rRNA gene sequence similarity (98–99%) to strains of *Bacillus acidicola* and *B. shackletonii*.

Isolates C2, E1 and E2 displayed high levels of sequence similarity and were matched to strains of *Paenibacillus elgii* and *P. ehimensis* (97% identity). Based on the Rep-PCR banding patterns, isolates E1 and E2 from the 344 cm sample depth were found to be identical to each other and could be distinguished from isolate C2 (89 cm sample) on the basis of presence/absence of two fragments, namely a ca. 550 bp fragment which is present for the E isolates and a ca. 1 900 bp fragment present in isolate C2 (Figure 3.14).

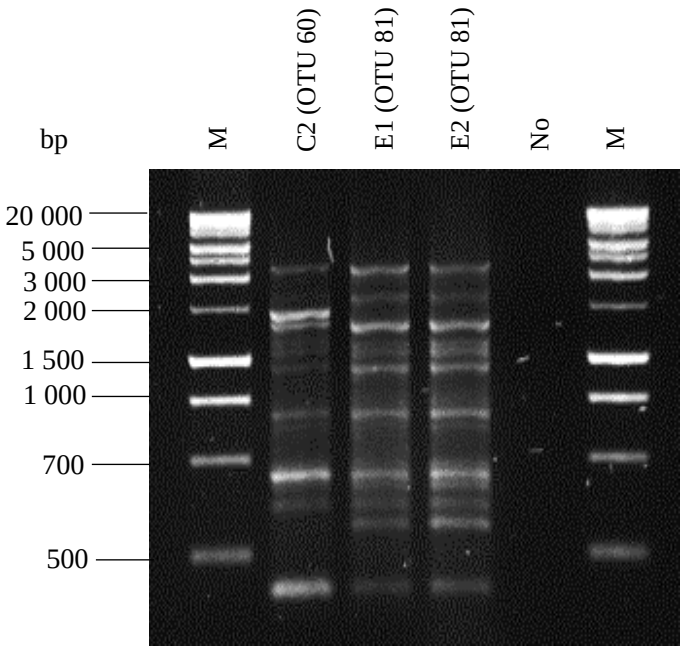


Figure 3.14. Rep-PCR comparison of fingerprint banding profiles of isolates C2, E1 and E2 which displayed 16S rRNA gene sequence similarity (97%) to strains of *Paenibacillus elgii* and *P. ehimensis*

Similarly, a Rep-PCR comparison of isolates A1 (12cm) and C26 (89 cm), which both produced BLAST sequence similarities which matched closely to *Bacillus aquimaris* and *B. vietnamensis* strains (99%), revealed that these isolates were distinct strains (Figure 3.15).

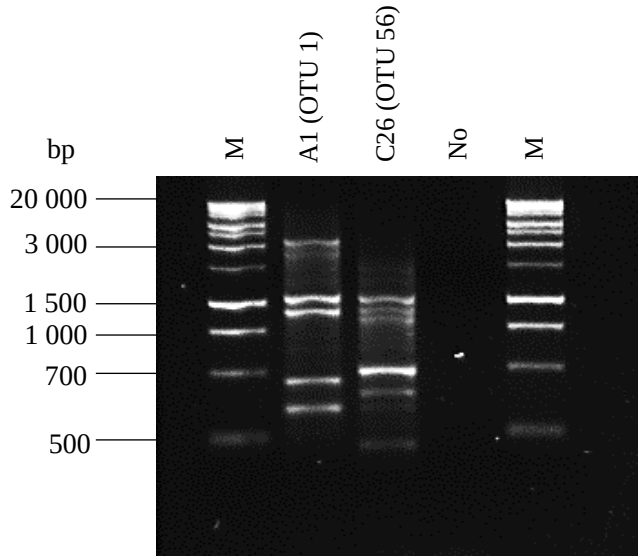


Figure 3.15. Rep-PCR comparison of fingerprint banding profiles of isolates A1 and C26 which displayed 16S rRNA gene sequence similarity (99%) to strains of *Bacillus aquimaris* and *B. vietnamensis*

Rep-PCR profiles for isolates C24, D23, D10 and D21, which were matched to strains of *Domibacillus robiginosus* (94–99% similarity), are shown in Figure 3.16. Isolates C24 (89 cm) and D21 (237 cm), which displayed similarities of 94% and 95% respectively, had identical fingerprint profiles, indicating that both isolates belong to the same strain. Isolate D23 could be clearly distinguished from the other isolates whereas isolate D10 showed some similarity to the banding profiles of C24 and D21 but could be differentiated on the basis of additional fragments (ca. 850 bp and ca. 750 bp) being distinguishable.

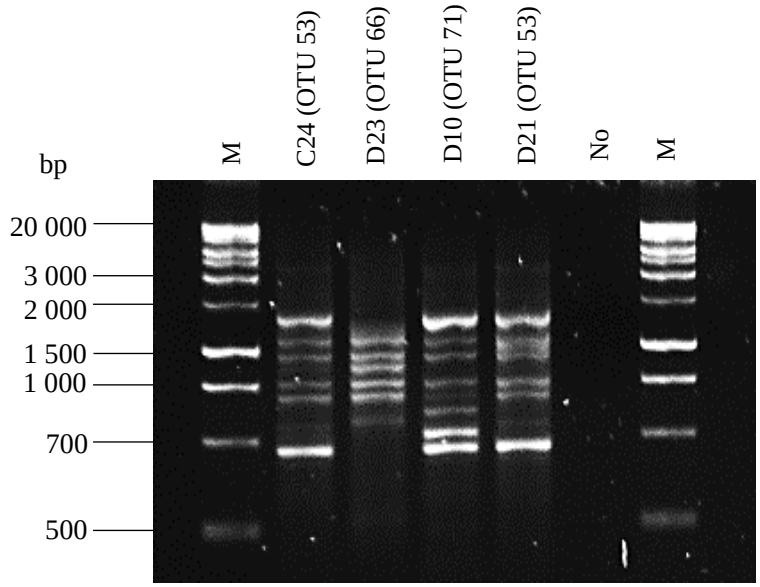


Figure 3.16. Rep-PCR comparison of fingerprint banding profiles of isolates C24, D23, D10 and D21 which displayed 16S rRNA gene sequence similarity (94–99%) to strains of *Domibacillus robiginosus*

Lastly, isolates B53 (21 cm) and D18 (237 cm) which displayed high levels of 16S rRNA gene sequence similarity (99%) to strains of *Paenisporosarcina quisquiliarum* and *P. indica* both displayed very distinct fingerprint profiles indicating that they belong to different strains (Figure 3.17).

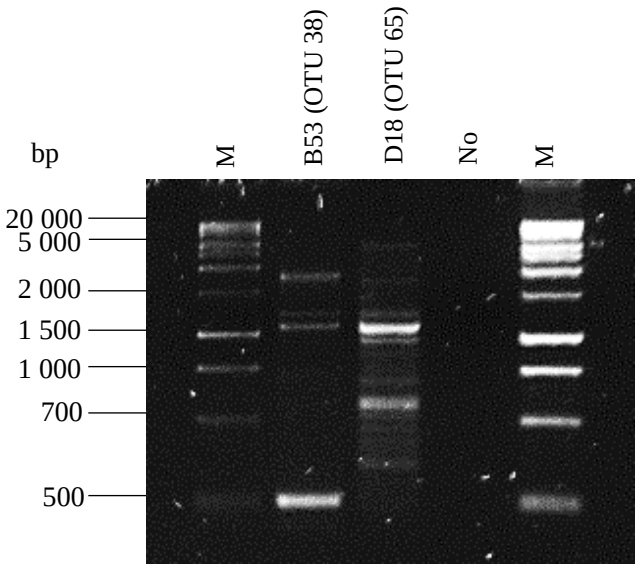


Figure 3.17. Rep-PCR comparison of fingerprint banding profiles of isolates B53 and D18 which displayed 16S rRNA gene sequence similarity (99%) to strains of *Paenisporosarcina quisquiliarum* and *P. indica*

3.4. DISCUSSION

Endospore-formation is a survival strategy which enables AEFB to survive unfavourable conditions and remain dormant for extended periods of time (Nicholson *et al*., 2000). It has been proposed that dormant, but viable, endospores show potential as proxies for palaeo-ecological studies aimed at reconstructing past environments (Wunderlin *et al*., 2014). However, there is little evidence in the literature suggesting that this hypothesis has been explored in detail. On this basis, a study was undertaken to attempt to isolate and revive dormant AEFB from sections of an ancient sediment core, extracted from the Mfabeni Peatland, to determine the genetic diversity amongst AEFB isolates and explore their potential as palaeo-ecological proxies.

Bacterial endospores adhere strongly to organic material, which influences their dispersal and distribution within the environment, as well as their recovery and isolation from environmental samples (Nicholson and Law, 1999). To overcome this adherence, vigorous extraction and purification procedures are often required. In the current study, three extraction procedures were evaluated for their suitability and efficiency in recovering viable endospores from Mfabeni peat samples. A mechanical agitation method employing simple vortexing was found to be the least efficient in extracting endospores (Figure 3.4). The inclusion of calcium chloride as a dispersing agent increased the endospore yield, however, the best yields were obtained

when a buffer-mediated/bead-beating extraction method was employed. This method was adapted from an indirect DNA extraction procedure designed to maximize detachment of bacterial cells and endospores from environmental material to allow for indirect DNA extraction (de Bruijn, 2011). The incorporation of TE buffer in the extraction method has been shown to improve bacterial cell extraction and may be attributed to the chelating effect of EDTA, which binds to ions in solution (de Bruijn, 2011). The adoption of sequential extraction/centrifugation steps also increases the likelihood of maximizing endospore displacement and recovery. Using this approach the recovery of viable endospores from sections of the sediment core ranged from 3.0 CFU/g to 8.1 x 10^3 CFU/g (Table 3.3). Whilst this procedure proved to be more time-consuming and intensive compared to the other extraction methods evaluated, the higher endospore yields obtained outweigh these disadvantages.

The CFU counts of revived endospores were highest at the shallowest depth sampled (youngest age) and generally, were found to decrease significantly by one to two orders of magnitude at deeper, older sampling points (Figure 3.5). These findings were not unexpected since sample A (12 cm) correlated to the youngest radiocarbon-dated sample and may have been influenced by a close proximity to oxic regions within the peatland which might support active populations of AEFB. Variables such as root penetration, air infiltration and fluctuations in water levels could also influence overall AEFB CFU counts in this region. The ability of endospores to survive extended periods of dormancy would depend upon a stable environment in which environmental stresses are minimized. Peatlands are characterized by acidic, anoxic environments which support the accumulation of organic material (Kroetsch *et al*., 2011). The preservation potential of organic material under anoxic conditions, combined with binding to organic particle matrixes, aids in reducing endospore and DNA damage (Torti *et al*., 2015), which could account for endospore survival at the 33 328 and 37 906 cal year BP samples. However, it is generally accepted that endospores lose viability over time, primarily caused through excessive damage during dormancy, resulting in fewer endospores surviving extended time periods (Logan and De Vos, 2011). The findings of this study support this assertion. They are also consistent with several studies that have shown that the numbers of culturable bacteria decrease with increasing depth and age along sediment and ice cores (Shivaji *et al*., 2011; Antony *et al*., 2012).

The revival of endospores requires the availability of suitable nutrients and/or growth conditions which favour germination and a return to an active metabolic state (Mandic-Mulec

and Prosser, 2011). Media selection, therefore, plays a critical role in the revival and isolation of endospores. In this study, four types of agar media were utilized to maximize isolation and determine their effect on the diversity of revived endospores. A significant degree of variation was observed in the CFU/g values between the various media types tested (Table 3.3), indicating that differences in the selective properties of the media types had an effect on the abundance of AEFB isolated. Of the 81 OTUs distinguished based on Rep-PCR fingerprinting data, 80.2% of isolates were revived from a single media type. This indicated that the isolated AEFB had varying physiological capabilities which resulted in differences in nutritional requirements, thereby illustrating the need for multiple media types to be used.

Marine Agar is a medium designed to support the growth of halophilic or halotolerant heterotrophs favouring marine environments (Zobell, 1941). Characteristically, this medium contains a relatively high salt concentration and low concentrations of organic nutrients. Approximately 32% of the OTUs distinguished in the study were isolated from this medium (Table 3.4), indicating that these AEFB were moderately halophilic or halotolerant.

Similarly, R2A medium also supported the growth of a high level of the OTU diversity (ca. 35%) distinguished in the study. R2A is a medium that was initially designed to maximize isolation of oligotrophic heterotrophic bacteria present in treated water (Reasoner and Geldreich, 1985). As such, R2A contains relatively low concentrations of complex carbon and energy sources to promote oligotrophic bacterial growth. The relatively high levels of OTU diversity obtained using this medium indicates its suitability for isolating AEFB from environmental samples.

TSA medium was found to support the lowest CFU recovery of AEFB isolates (Table 3.3, Figure 3.5) as well as the lowest level of OTU richness (Table 3.5). TSA is considered a general medium which supports a wide range of non-fastidious, copiotrophic heterotrophic bacteria. Interestingly, when a 10% dilution of TSA was used, a significantly higher number of CFU counts were obtained. This medium also supported the highest level of OTU diversity (ca. 37%) isolated from the sediment samples. These findings suggest that a greater proportion of the revived isolates preferred a lower concentration of nutrients as opposed to a nutrient-rich medium. This is not surprising since many environmental organisms are adapted to nutrient-limited conditions which are prevalent in terrestrial and aquatic environments. Similar trends have been reported by Christner *et al*. (2000) and Antony *et al*. (2012) who observed that

nutritionally-limited media such as R2A or 10 to 100-fold dilutions of tryptone soy broth (TSB) yielded higher CFU counts compared to nutritionally-rich alternatives.

Alternatively, some germinating endospores may experience a metabolic "shock" when exposed to nutritionally-rich media, which can interfere with cellular mechanisms aimed at repairing damaged DNA (Dodd *et al*., 1997). Slower-growing bacteria may also prefer nutritionally-limited media since they are afforded an opportunity to grow without being suppressed by faster-growing isolates, as is sometimes observed on nutritionally-rich media (Eevers *et al*., 2015).

Environmental organisms have diverse nutrient requirements due to their varied physiological capabilities. In many instances, a single medium type has been used to isolate and enumerate bacteria from soil and sediment samples (Fredrickson *et al*., 1991; Shivaji *et al*., 2011). This study has shown that media selection has a profound impact on the abundance and diversity of AEFB recovered from peatland sediment core samples. In order to maximize the recovery of diverse populations of AEFB from environmental samples, the inclusion of a range of environmentally-relevant selective or enrichment media is recommended.

To assess the diversity of selected revived bacterial isolates, Rep-PCR genotypic fingerprinting was employed. This method can differentiate isolates at the strain level and has been employed as an effective way to screen AEFB isolates; including closely-related members of the *Bacillus cereus* group (Cherif *et al*., 2002; Kim *et al*., 2002; Cherif *et al*., 2003). The location of repetitive sequences of nucleotides occurs in different regions of the genome for individual bacterial strains (Ishii and Sadowsky, 2009). By targeting the BOX-mosaic repetitive element, the generation of amplicons of varying sizes resulted in the production of unique fingerprinting profiles for each isolate (Figure 3.7) which allowed for the assignment of OTUs based on strain differences (Table 3.4).

Approximately 94% of OTUs distinguished were unique to their depths, i.e., they were isolated from a single sample point. This contributed to the notion that endospores are able to remain immobile within the sediment layers, supporting the assertion that the endospores were of equivalent age to the radiocarbon-dated sample material. This further suggested that the endospores were not transferred along the sediment core during the sampling process. For samples A (12 cm) and B (21 cm), only four OTUs (4.9%) of the total OTUs distinguished were common between these depths. This similarity may be feasible with only a 9 cm difference between sampling points. This overlap could have occurred during compaction of

the core or potentially as a result of natural disruptions or disturbances within the sediment layers. The only other significant OTU overlap between depths occurred with isolates C24 and D21, sampled at 89 cm and 237 cm, respectively. The identical Rep-PCR banding profiles revealed that these isolates belonged to the same strain (Figure 3.16). This suggests that there may have been a possible transfer of this strain between the C and D samples. Phylogenetic analysis revealed other instances where isolates from younger and older radiocarbon-dated samples were taxonomically-related (Figures 3.13–3.17). In all of these cases, it was found that different strains were present across the depths, indicating that they were unique to a sample. The high abundance of certain OTUs (e.g. OTUs 15 and 51, Table 3.4) at some depths suggests the possibility of these AEFB residing in the region, as opposed to being transported there.

The Rep-PCR technique proved to be one which was relatively fast, simple, reproducible and cost-effective to perform. The suitability of using template DNA obtained directly from a colony-pick off for Rep-PCR was also confirmed, thereby removing the cost and time implications associated with using a DNA extraction kit. Furthermore, Rep-PCR served as an effective way of differentiating and grouping related isolates which allowed for 'pre-screening' to rationalize the numbers of isolates needed for sequencing and to reduce the time and cost associated thereof. As an alternative, High Resolution Melt Analysis (HRMA) has shown promise as an emerging technique for genotyping (Dhakal *et al*., 2013). As such, assessment of HRMA as an alternative to Rep-PCR for the screening of AEFB shall be addressed in the subsequent chapter.

To further determine the differences between taxonomically-related isolates from different time periods, molecular clocking could be carried out (Maughan *et al*., 2002). Isolates occurring at depths of different ages which display taxonomic similarity (e.g. isolates A65/A65, C19 and D5, Figure 3.13) could be selected for this purpose. The common band present at ca. 1 550 bp could be excised and cloned to determine if differences in genetic make-up between 'ancient' and 'current-day' bacteria are present.

16S rRNA gene sequencing is widely considered the benchmark technique for identifying unknown bacterial isolates (Woo *et al*., 2008). It is relatively simple to perform and due to the size of the gene and the occurrence of interspecific polymorphisms, phylogenetic analyses based on valid bacterial identifications can be made (Clarridge, 2004).

Phylogenetic analysis revealed the presence of several aerobic endospore-forming genera (Figure 3.11). In some instances, representatives of these genera were specific to distinct

depths. For example, many groups of taxonomically-related isolates were found to be specific to the 12 cm and 21 cm depths, which date to 589 and 1 964 cal years BP, respectively. This included isolates matched to the *Lysinibacillus* and *Brevibacillus* genera, which were unique to the younger depths, whereas isolates matched to the *Domibacillus* genus were isolated exclusively from the older sections of the core, namely, those dated to 17 568 and 33 328 cal years BP (Figure 3.12). This indicates the presence of certain species which may have only been present at specific time periods within the region. In other cases, taxonomically-related isolates which could be differentiated at the strain level, were found at more than one sample depth which could indicate the occurrence of conditions which supported similar populations of AEFB. Representatives of the *Paenibacillus* genus were present at all five depths and was the only genus represented at the oldest depth, i.e., at 37 906 cal years BP. Furthermore, the OTU fingerprints of the isolates revived from this sample (sample E) were not found anywhere else along the core. This provides further evidence, albeit circumstantial, that these isolates were 'ancient' in origin. The changes in AEFB diversity between depths further support that the endospores were unique to the depths from which they were isolated and discounts against the possibility of carry-through or endospore migration along the sediment core.

Phylogenetic analysis revealed that a significant degree of diversity was present amongst the AEFB isolates. A number of isolates from the current study were closely matched to species which have been previously isolated from wetland and sediment environments; for example, *Brevibacillus laterosporus* has been isolated from suburban wetlands (Liu *et al*., 2015) and *Domibacillus enclensis*, from marine sediment (Sonalkar *et al*., 2014). Isolates obtained from three sample depths were closely matched to strains of *Bacillus acidicola*, an acidophilic mesophile which was first isolated from an acidic peat bog (Albert *et al*., 2005). A significant proportion of isolates were found to belong to the genus *Paenibacillus*. Isolates matched to several *Paenibacillus* species including *P. aestuarii*, which is common to wetland regions (Bae *et al*., 2010) and *P. wooponensis* and *P. rigui*, both of which were isolated from freshwater wetlands (Baik *et al*., 2011a; Baik *et al*., 2011b).

Additional species matches included a xylan-degrading strain of *P. sediminis* (Wang *et al*., 2012), *P. selenitireducens*, a bacterium capable of selenite reduction (Yao *et al*., 2014) and *P. polymyxa*, a species associated with biotechnological applications including antibiotic, enzyme and exopolysaccharide production (Lal and Tabacchioni, 2009). AEFB isolates from the sediment core were also matched to species that have been isolated from diverse environments including volcanic soil (Logan *et al*., 2004), marine sediment (Zhang *et al*., 2010), mural

paintings (Heyrman *et al*., 2005), glaciers (Reddy *et al*., 2013) and the Antarctic (Yu *et al*., 2008).

The threshold for the identification of same species is a sequence similarity of ≥98.7% (Stackebrandt and Ebers, 2006). Approximately 17% of isolates had sequence similarity matches which were 97% and below. A proportion of these isolates could potentially represent novel species or strains, for example, isolate D47 which appeared as a distinct outgroup from the *Paenibacillus* genus (Figure 3.12). These isolates require further characterization through the employment of alternate techniques such as DNA-DNA hybridization, a technique which is considered the 'gold standard' when dealing with a potentially new species (Janda and Abbott, 2007). This indicates that peatland regions may potentially harbour novel bacteria.

The genotyping and phylogenetic results provide evidence which strongly supports the notion that endospores were revived from the sample stated; this points towards the possibility of these endospores being of an age consistent with the age of the sample from which they were isolated. These findings also indicate that potential sample carryover or cross-contamination resulting from the sampling approach used did not appear to be a major contributing factor. These assertions are made based on the premise that the endospores readily adhere to organic material and are generally able to remain in a static state after deposition, preventing migration between depths (Wunderlin *et al*., 2014). However, environmental disturbances such as erosion or run-off cannot be ruled out. Although, if large-scale infiltration between depths had occurred, it is likely that a higher incidence of common species and strains would have been detected between depths along the sediment core.

The oldest sample examined was obtained from material radiocarbon-dated to 37 906 cal years BP. This falls within a pre-glacial time period which was dominated by the occurrence of wet and cool conditions (Finch and Hill, 2008). At 33 328 cal years BP (the time at which the 237 cm sediment deposits were formed), cool and wet conditions were prevalent (Finch and Hill, 2008). The 89 cm depth, radiocarbon dated to ca. 17 568 cal years BP, correlated to a significant climatic period as the Pleistocene/Holocene transition began ca. 17 500 cal years BP. Adverse and dry conditions were present during this time as a result of the Last Glacial Maximum, which was in effect from ca. 21 000 cal years BP until ca. 11 570 years BP (Grundling *et al*., 2013a). During the early Holocene (ca. 11 000–5 000 cal years BP), a shift in conditions towards a cooler and moist climate was detected (Finch and Hill, 2008; Grundling *et al*., 2013a). At ca. 2 500 cal years BP (late Holocene period), dry conditions and lower temperatures

prevailed (Finch and Hill, 2008). The impact of climatic changes and their subsequent effects on factors such as vegetation and peat composition may be contributing factors towards the changes in genetic diversity of AEFB over time. Changes in the diversity and abundance of a selected proxy, which is able to remain dormant and static within environmental material, can be examined in relation to the surrounding environment. This could potentially enable the effects of climate changes on the selected proxy to be established (Wunderlin *et al*., 2014). The proxy can be used for the inference of past environmental conditions, which can then be extrapolated to climate change.

Overall, AEFB were revived from ancient sediment samples from one of the oldest peatland regions in Africa. Media selection is critical to obtaining an accurate reflection of the diversity present. Genotyping revealed that the diversity of AEFB changed with depth. Almost all isolates were unique to their depths and phylogenetic analysis revealed the presence of several aerobic endospore-forming genera, members of which were isolated from distinct depths of the sediment core. This supports the notion that the endospores were revived from the respective samples at their ages and discounts against carryover between samples. This also satisfies the primary requirement that a biological indicator needs to remain static in preserved material in an unaltered state over a significant period of time to serve as a suitable proxy (Gorham *et al*., 2001). The proportion of isolates which had low sequence similarity matches to currently-listed species in the Genbank database suggests the possibility of new species or strains. This suggests that peatland environments potentially harbour novel microbial diversity. Regions such as the Mfabeni Peatland, which have the ability to remain stable over long periods of time and which contain records of periods in the Earth's history, may aid in preserving ancient endospore-forming bacteria, thereby allowing for their recovery and examination.

CHAPTER FOUR

ASSESSMENT OF HIGH RESOLUTION MELT ANALYSIS (HRMA) AS A GENOMIC FINGERPRINTING METHOD FOR THE SCREENING AND GROUPING OF AEROBIC ENDOSPORE-FORMING BACTERIA

4.1. INTRODUCTION

To analyze microbial diversity, a range of molecular fingerprinting techniques, such as RAPD, Rep-PCR and DGGE, have been developed (Rastogi and Sani, 2011). The choice of the fingerprinting technique predominantly depends on factors such as ease of use and the degree of taxonomic resolution required. When dealing with closely-related species, methods that have a greater resolution ability are necessary. Furthermore, many fingerprinting techniques require some form of post-PCR processing (e.g. gel electrophoresis), which is time-consuming and resource-intensive. For these reasons, it is useful to assess and/or develop alternative procedures to existing fingerprinting methods, which may be quicker, simpler, show automation potential, exhibit high resolution and reproducibility and do not require the need for further downstream processing.

HRMA has gained popularity in recent years as a fast and simple post-PCR technique that is used for the generation of melting curve profiles of PCR products for the detection of sequence variation (Reed *et al*., 2007). The first step of HRMA involves PCR to amplify a gene fragment of interest. The reaction mixture contains a fluorescent dye (e.g. SYBR® Green) which binds to double-stranded DNA. When the PCR amplicon is subjected to a temperature melt profile, the fluorescent dye is released as the double-stranded DNA fragments denature (Reed *et al*., 2007). Measurement of the resulting fluorescence, over a temperature range, is used to generate a melt curve for a target amplicon. Melt curves generally follow a characteristic pattern based on sequence composition. At the onset of the melt, fluorescence is at its highest due to the double-stranded conformation of the amplicon at this stage (Reed *et al*., 2007). As the temperature is slowly increased, dissociation of the double-stranded DNA occurs, yielding single DNA strands, which results in a decrease in the fluorescence signal. This results in a characteristic melt curve profile being produced for each amplicon. Factors such as DNA strand length, guanine and cytosine (G+C) content and sequence all have effects on the characteristics of the resulting curve (Reed *et al*., 2007). Distinctions between amplicons can be made on two

bases namely, the profiles of the melt curves and the differences in melting temperature (T_m) (Chauhan *et al*., 2013).

The advantages of this technique include its accuracy, reproducibility, high throughput, speed and simplicity. The range of HRMA applications includes mutation and single nucleotide polymorphism (SNP) detection, heterozygote identification, genotyping, allele quantification, clone screening and the comparison and matching of sequences between isolates (Reed *et al*., 2007; Vossen *et al*., 2009). HRMA has been used to establish compositional differences between bacterial communities from different soil ecosystems (Hjelmsø *et al*., 2014). It has also been applied to the identification of foodborne endospore-forming bacteria (Chauhan *et al*., 2013) and the discrimination of repetitive markers such as CRISPR (Clustered Regularly Interspaced Short-Palindromic-Repeats) amongst *Campylobacter jejuni* (Price *et al*., 2007).

The current study was undertaken with the aim of determining whether HRMA can serve as an alternative to Rep-PCR fingerprinting for analyzing diversity amongst aerobic endospore-forming bacterial isolates. Three gene targets, namely, the V3 variable region, the V3+V4 variable regions and the *spo0A* gene, were evaluated to determine their suitability for HRMA. Variable regions of the 16S rRNA gene have been shown to be suitable targets for differentiating between species (Chakravorty *et al*., 2007). Vasileiadis *et al*. (2012) tested the suitability of different variable regions in the 16S rRNA gene in comparison to the whole gene and found that the V3 region (182 bp) was suitable for assessing soil bacterial diversity and community structure. This region was therefore selected as the first target for HRMA. A primer set spanning the V3 and V4 regions (464 bp) of the 16S rRNA gene was also assessed to determine whether or not this larger fragment yielded greater discriminatory information.

Spo0A is a housekeeping gene that encodes an essential regulator which is responsible for activating sporulation genes at the onset of unfavourable environmental conditions (Hoch, 2000; Errington, 2003). The *spo0A* gene is highly conserved and present in almost all members of endospore-forming *Firmicutes* (Bueche *et al*., 2013); for this reason, this gene was selected as the third target for HRMA.

4.2. METHODS AND MATERIALS

4.2.1. High Resolution Melt Analysis (HRMA)

Forty-two isolates from the pool of AEFB isolated from the Mfabeni Peatland sediment core were selected randomly as part of a blind study without knowledge of prior Rep-PCR or phylogenetic affinities. They were then screened using HRMA to assess the accuracy of the procedure. Amplification of each target fragment (i.e., V3 region of 16S rRNA, V3+V4 regions of 16S rRNA and the *spo0A* gene) was carried out for all isolates. Amplicons were then subjected to melting in 0.1^{o}C increments. All reactions were carried out using a Rotor-Gene™ 6000 (Corbett Research Pty Ltd., Australia). The resultant melting curves were then normalized to relative fluorescence units (RFU) of the melt region specific to each amplicon. Normalized melt curves were converted into difference plots which provide an alternative graphical representation of the melt data. This allowed for a visual comparison of the melt curve profiles between isolates to be made. Acquisition and analysis of all HRM data were conducted using Rotor-Gene™ 6000 Series Software (v 1.7.28). For further analysis of the melt curves, a student *t-test* analysis (Microsoft Excel® 2013 v 15.0.4823.1000) was undertaken. The *t-test* was conducted using the RFU values for each 0.1^{o}C temperature increment across the normalized melt phase for comparisons between isolates. The comparisons of melt curve data were then used to group the isolates. A significance level of $P < 0.05$ was used to differentiate unrelated isolates, whereas $P \geq 0.05$ was considered to represent isolates which were not significantly different from each other and could be grouped together. Groupings of isolates based on each primer set were compared to each other. These groupings were also compared alongside the Rep-PCR fingerprinting data (section 3.3.3) and 16S rRNA gene sequence similarities (section 4.2.2) in order to evaluate HRMA accuracy.

For preliminary testing, HRMA was carried out on test isolates using template DNA obtained through the colony-pick off procedure (section 3.2.6.2). Curves carried out in independent runs using the same template DNA were compared to determine if HRMA using this DNA was reproducible.

4.2.1.1. HRMA of the V3 variable region of 16S rRNA

HRMA was carried out using 338f and 520r primers targeting the V3 variable region (182 bp) of 16S rRNA (Bueche *et al*., 2013) (Table 4.1). Twenty microlitre reactions consisting of 1 x KAPA SYBR® FAST qPCR universal master mix (Kapa Biosystems, RSA), 0.2 µM of forward and reverse primer and 1 µl of template DNA were used. Final volumes were made up to 20 µl using nuclease-free water (Promega, USA). A reaction mixture lacking template DNA was included as a control. The cycling conditions used were 95^{o}C for 10 min, followed by 30 cycles of 95^{o}C for 10 s, 58^{o}C for 15 s and 72^{o}C for 20 s. HRM was conducted using a gradual increase of temperature from 72^{o}C to 95^{o}C in temperature increments of 0.1^{o}C.

4.2.1.2. HRMA of the V3 and V4 variable regions of 16S rRNA

For comparative purposes, a 16S rRNA gene fragment (464 bp) spanning the V3 and V4 variable regions was evaluated for HRMA using the primer set S-D-Bact-0341-b-S-17/ S-D-Bact-0785-a-A-21 (Klindworth *et al*., 2013) (Table 4.1). Each 20 µl reaction consisted of 1 x KAPA SYBR® FAST qPCR universal master mix (Kapa Biosystems), 0.2 µM of forward and reverse primer and 1 µl of template DNA. Final volumes were made up to 20 µl using nuclease-free water (Promega). A reaction lacking template DNA was included as a control. Cycling conditions consisted of an initial denaturation of 95^{o}C for 5 min, followed by 25 cycles consisting of 95^{o}C for 40 s, 55^{o}C for 2 min and 72^{o}C for 1 min. Following the amplification, a melt analysis consisting of a temperature increase from 72^{o}C to 90^{o}C in 0.1^{o}C increments was used.

4.2.1.3. HRMA of the spo0A gene

The *spo0A* gene (179 bp) was amplified and subjected to analysis by HRM using the *spo0A* 655f and *spo0A* 834r primer set (Bueche *et al*., 2013) (Table 4.1). Reactions consisted of 1 x KAPA SYBR® FAST qPCR universal master mix (Kapa Biosystems, RSA), 1 µM of *spo0A* 655f forward primer, 0.45 µM of *spo0A* 834r reverse primer and 1 µl of template DNA. Final volumes were made up to 20 µl using nuclease-free water (Promega). A reaction lacking template DNA was included as a control. Cycling conditions consisted of an initial denaturation of 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 s, 48^{o}C for 15 s and 68^{o}C for 10 s. This was followed by the melt analysis which involved a temperature increase from 68^{o}C to 95^{o}C in 0.1^{o}C increments.

4.2.2. Generation of sequence matrixes for AEFB isolates based on partial amplified 16S rRNA gene fragments

A sequence similarity matrix was constructed for the selected isolates. The matrix was based on the partial 16S rRNA gene sequences (1 254 bp) which were amplified, sequenced and edited (sections 3.2.6.4 and 3.2.6.5). The sequence similarity matrix was constructed using BioEdit software (v 7.0.9.0) (Hall, 1999). A pairwise distance matrix was also constructed using Mega software (v 6.06) for confirmation and comparison. The Jukes-Cantor model was employed (Tamura *et al*., 2007) with uniform rates among sites and complete deletion of gaps. The sequence similarity matrix is provided in Appendix B.

Table 4.1. Nucleotide sequences for primers used for HRMA

*Primer	Sequence (5'–3')	Length (bp)	Tm (°C) (min/max)	Reference
V3 region				
338f	ACTCCTACGGGAGGCAGCAG	20	66.55/66.55	Bueche *et al*.
520r	ATTACCGCGGCTGCTGG	17	62.02/62.02	(2013)
V3 & V4 region				
S-D-Bact-0341-b-S-17	CCTACGGGNGGCWGCAG	17	64.43/66.84	Klindworth
S-D-Bact-0785-a-A-21	GACTACHVGGGTATCTAATCC	21	58.66/62.57	*et al*. (2013)
Spo0A				
Spo0A 655r	GGHGTDCCNGCNCATATHAA	20	56.30/66.55	Bueche *et al*.
Spo0A 834r	CCAHGCNACTTCWATNGCRT	20	56.30/64.50	(2013)

*All primers obtained from Inqaba BioTech™ (Hatfield, Pretoria, South Africa)

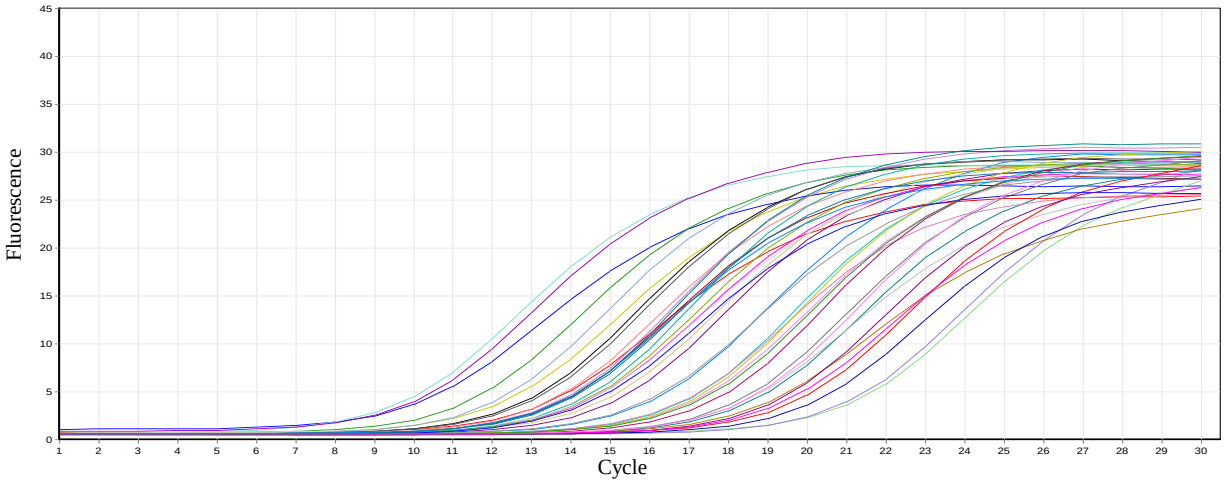
4.3. RESULTS

Results from preliminary testing revealed that HRM using DNA obtained through colony-pick off produced comparable and reproducible melt curve profiles in independent runs. Based on this, HRMA was then carried out on the 42 isolates selected from the blind study and evaluated with the three primer sets. The first step involved amplification of the selected target regions.

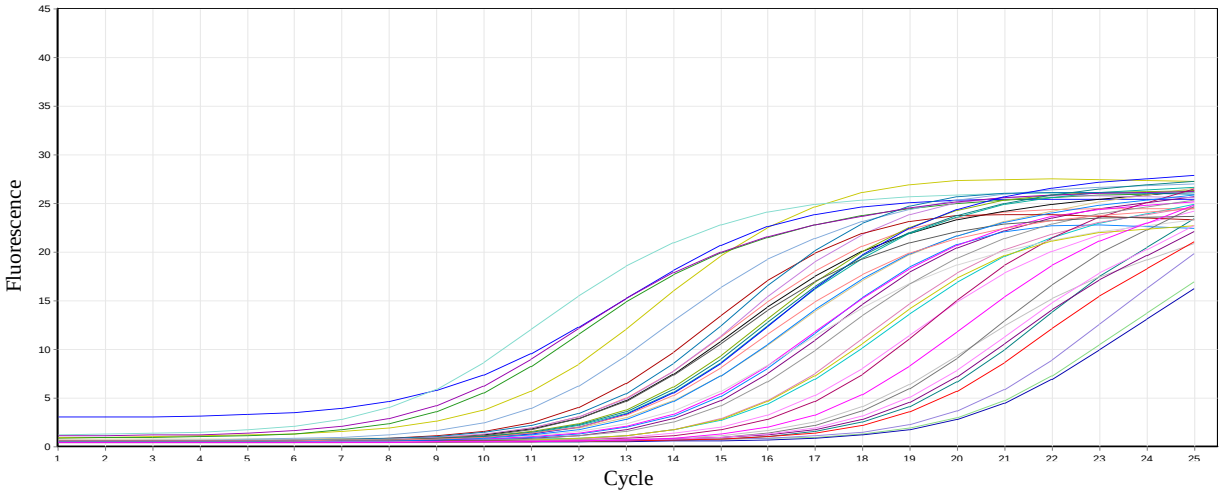
4.3.1. PCR amplification using the three primer sets evaluated

The first part aimed at assessing each of the three primer sets to determine if successful amplification of the target region was achieved. Amplification plots should follow a characteristic shape, in which a plateau is reached, indicating complete amplification of the target region. At the plateau phase, reactions are amplified to a similar extent. The amplification curves for each primer set are presented in Figure 4.1.

A



B



C

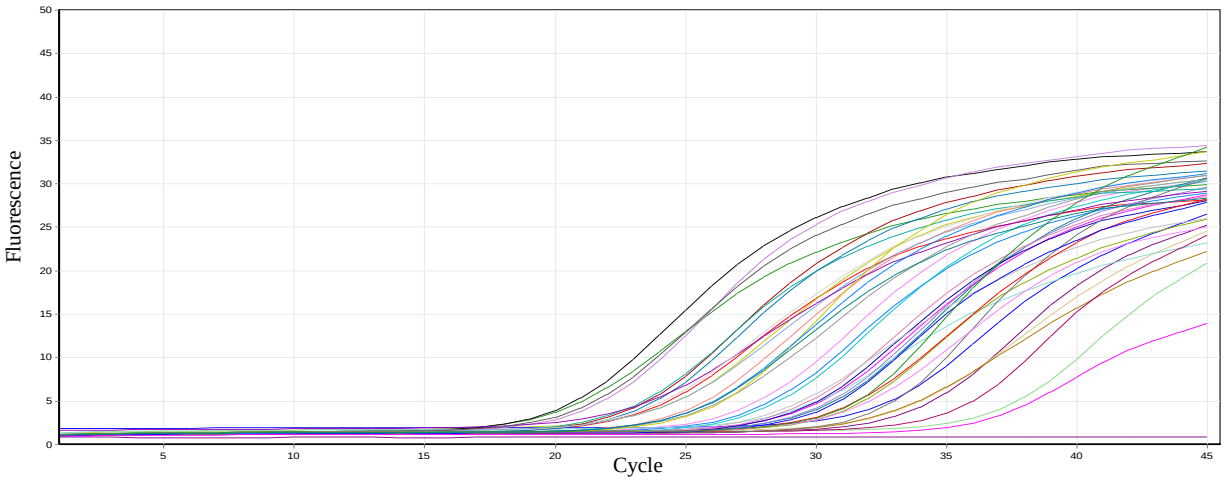


Figure 4.1. Amplification plots displaying RT-PCR amplification represented by increases in fluorescence for 42 AEFB isolates (A) the V3 region of 16S rRNA (B) the V3 and V4 regions of 16S rRNA and (C) *spo0A*

A steep log-linear phase was present with the amplification of the V3 region (Figure 4.1). Thirty cycles were sufficient as all reactions reached a plateau phase. This indicates that all reactions amplified to a comparable extent. For amplification of the V3+V4 region, the number of cycles may need to be increased as some of the reactions did not amplify to a plateau phase. Late amplification was detected with the *spo0A* gene, with a longer log-linear phase as compared to the amplification of the variable regions. After 45 cycles, many reactions do not reach a plateau, indicating incomplete amplification. The fluorescence signals of some reactions are much lower compared to others. Late amplification can occur as a result of low DNA template concentration or DNA degradation. However, the same concentration of template DNA was used for the variable regions therefore, this may not be the cause of the late amplification. Primer specificity or the lower concentration of the reverse primer used could have been contributing factors to the poor amplification.

4.3.2. HRMA of PCR amplicons

Following amplification of the targeted regions, the PCR products were subjected to a melt analysis consisting of 0.1^{o}C temperature increments, thereby generating melt curves for the individual isolates (Figures 4.2–4.3).

4.3.2.1. V3 variable region of 16S rRNA

The HRM generated for the V3 region of 16S rRNA for the isolates is shown in Figure 4.2.

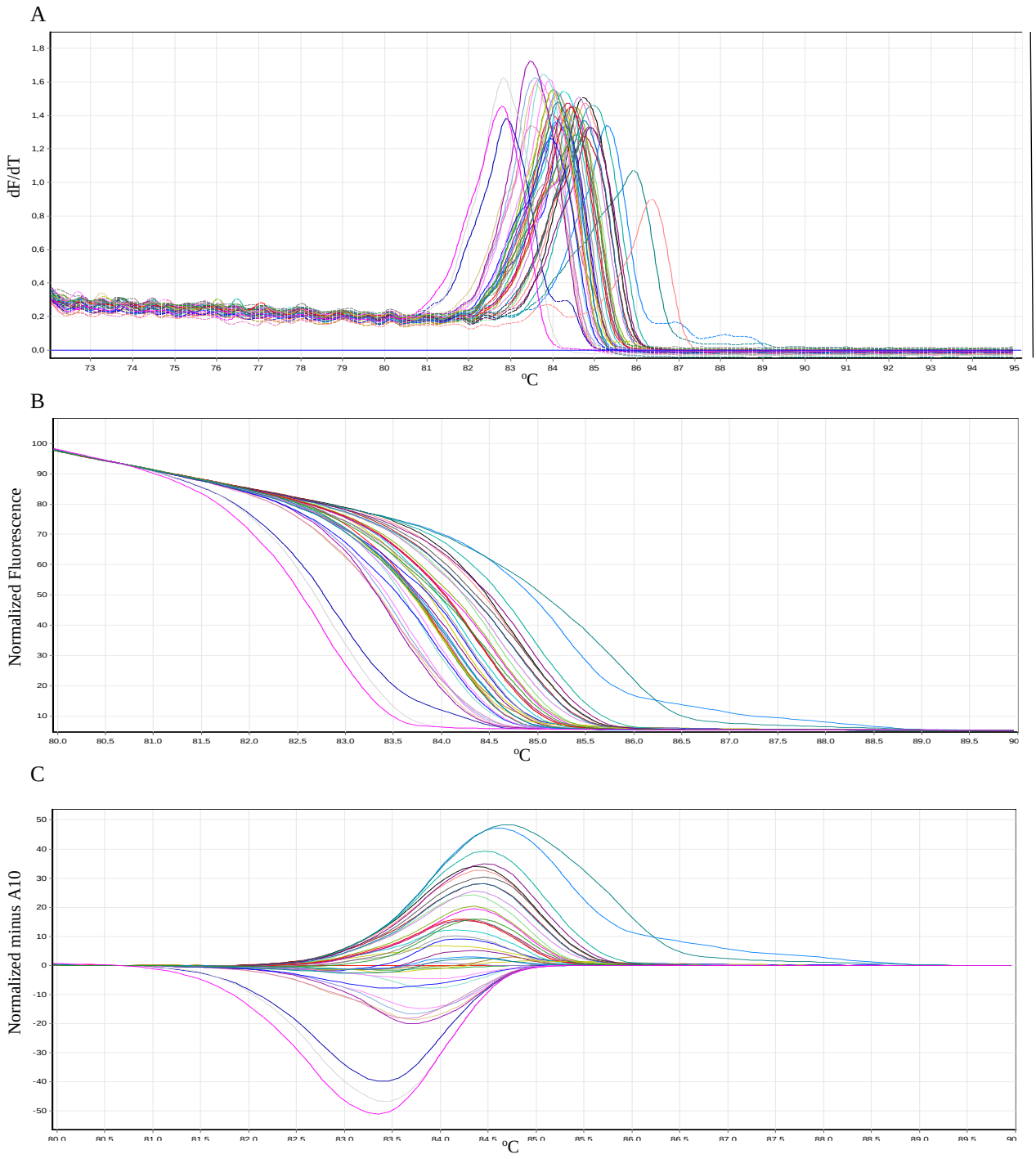


Figure 4.2. Melt curves displaying the HRMA of the V3 region amplicons for selected AEFB isolates (A) melt curves depicting fluorescence data as derivative plots of fluorescence against temperature (B) normalized melting curves (79.96ºC to 90.05ºC) displaying the melt phase for each isolate (C) difference plots of normalized fluorescence data in which a selected isolate (i.e., A10) was used as the baseline (pink line) from which to compare and match melt curves from different isolates.

4.3.2.2. V3 and V4 variable regions of 16S rRNA

The HRM for the V3 and V4 regions of 16S rRNA for the isolates is shown in Figure 4.3.

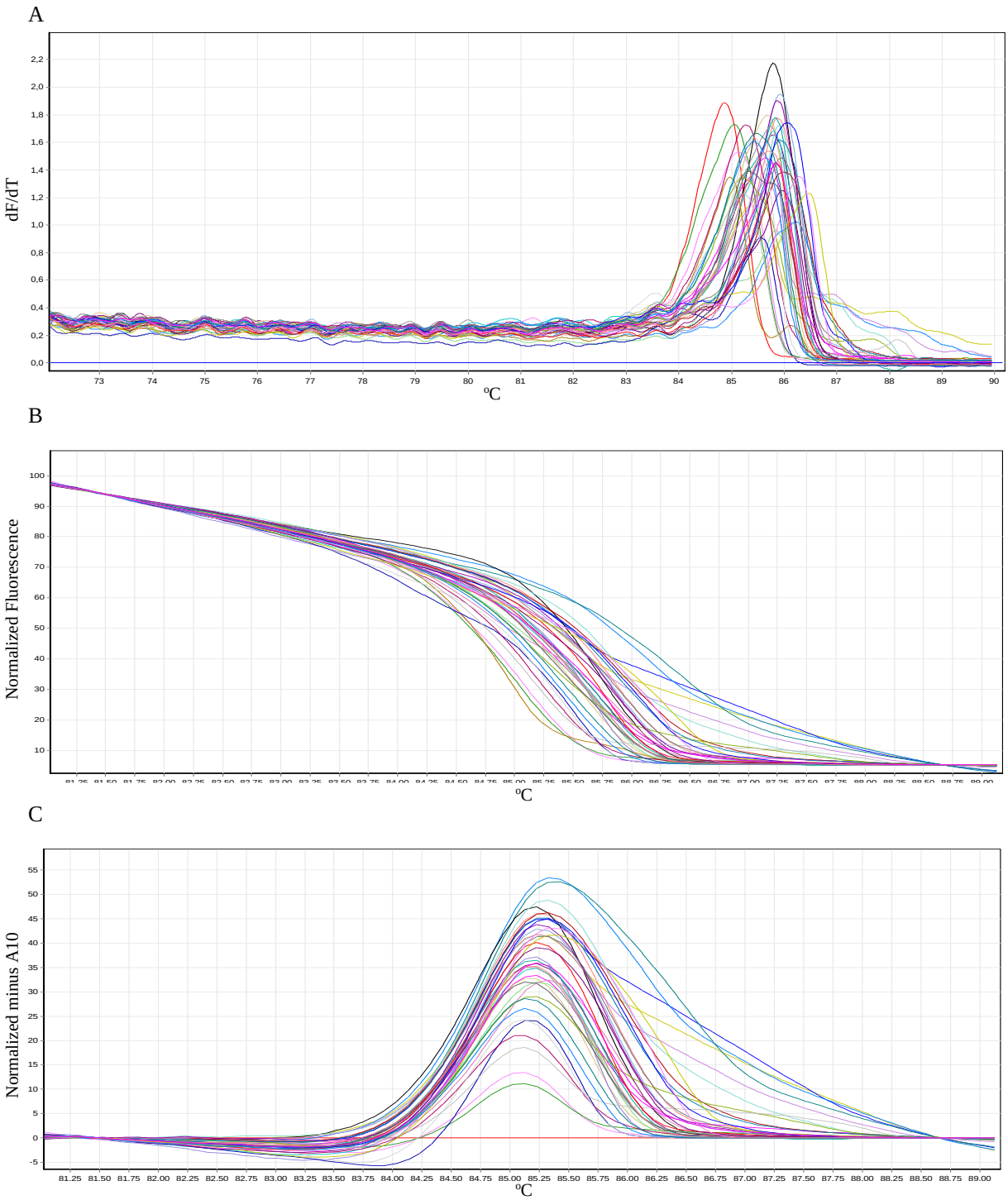


Figure 4.3. Melt curves displaying the HRMA of V3+V4 region amplicons for selected AEFB isolates (A) melt curves depicting fluorescence data as derivative plots of fluorescence against temperature (B) normalized melting curves (81.03ºC to 89.20ºC) displaying the melt phase for each isolate (C) difference plots of normalized fluorescence data in which a selected isolate (i.e., A10) was used as the baseline (red line) from which to compare and match melt curves from different isolates.

Variation in melting characteristics generates the differences in melting curves (panel A of Figures 4.2 and 4.3). In these plots, it can be seen that each melt curve generates a single peak which represents a single PCR product. Each plot has a similar profile but distinctions can be made between isolates based on the melting temperature (T_m) differences, which occur as a result of sequence variation. As DNA melts from a double-stranded to a single-stranded conformation, the amount of fluorescence decreases. This pattern can be observed in panel B, in which the start and end points of the curves represent 100% and 0% fluorescence, respectively. The highest fluorescence is detected at the start of the pre-melt phase and decreases through the melt phase to reach the basal level at the end of the post-melt phase. The rate of change of fluorescence is highest at the melt phase midpoint and this represents the T_m. Fluorescence normalization was performed in order to eliminate the impact of background fluorescence and to minimize variability from the run (panel B of Figures 4.2 and 4.3). This allowed for a more effective alignment of the data and for subtle differences in melt profiles to be detected. To provide an alternate visualization of the melt curve differences, difference plots were generated (panel C of Figures 4.2 and 4.3). These plots display the difference in fluorescence for each sample in relation to a randomly selected baseline isolate, which in this case was isolate A10.

The melt curve profiles and T_m values are characteristic of the specific amplicon. In this regard, detection of differences in the PCR product and hence, differentiation between the isolates, was possible.

4.3.2.3. Spo0A gene

An analysis of the melt curves of the fluorescence data from the *spo0A* gene amplification revealed that clearly defined peaks for most of the isolates were not produced. For some isolates, double-peaks and peaks with shoulders resulted. This generated melt curves with widespread peaks after normalization. This could have been as a result of the poor amplification. Reactions which amplify late produce inconsistent HRM data due to PCR artefacts or non-specific products. The amplification results were not optimal and may be attributed to the annealing temperature, primer specificity or primer concentration. As such, further HRMA characterization of the *spo0A* gene was not pursued. Therefore, for the purposes of further assessment and testing of HRMA, the V3 region and the V3+V4 regions were used.

Based on the HRM data, the selected AEFB isolates were compared to each other to determine melt profile similarities and differences between the isolates. Isolates were assigned to groupings based on visual comparisons of difference plots (since the difference plots allow for a greater accentuation of differences between isolates which have similar melting curve profiles as compared to the normalized curves) and on the results of the *t-tests* which were carried out using RFU values.

Examples of isolates which were grouped and separated on this basis are shown in Figures 4.4 to 4.7.

4.3.3. The ability of HRMA to identify similar 'unknown' isolates

The first step in determining whether HRMA would be a suitable alternative to Rep-PCR fingerprinting was to analyze the ability to generate similar melt profiles for isolates of the same species, thereby effectively allowing them to be grouped together.

Two examples of isolates which were grouped together are provided (Figures 4.4–4.6).

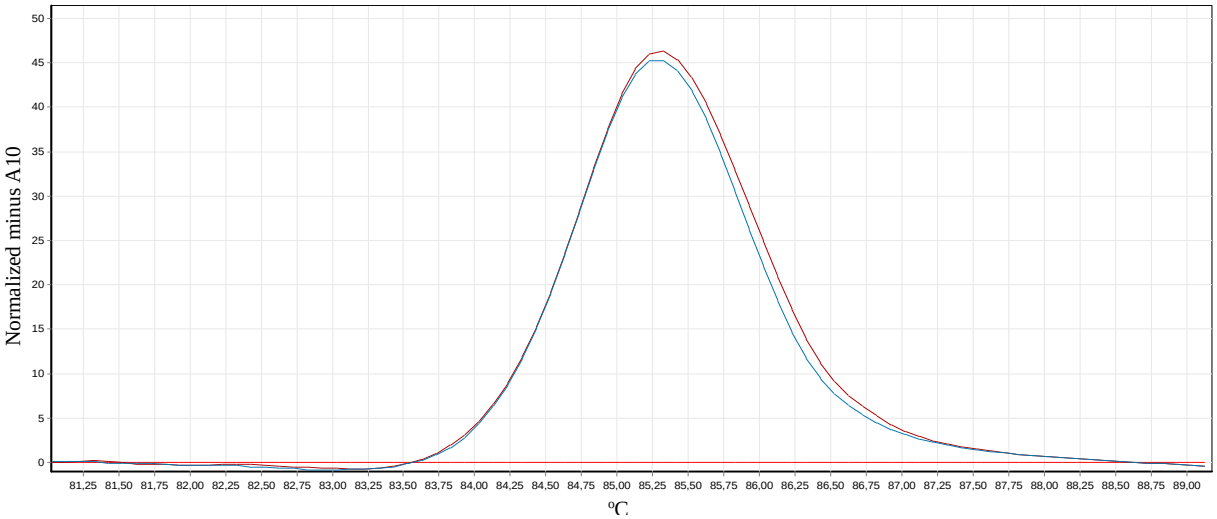


Figure 4.4. Difference plots displaying the melt curves of Isolates B11 (blue curve) and B63 (dark red curve) of amplified variable regions V3 and V4 against the selected baseline isolate A10 (red line)

The plot (Figure 4.4) displays the melt profile similarity between isolates B11 and B63. To further confirm the visual results produced by the melt curves, the RFU values of each 0.1°C temperature increment across the normalized range for both isolates were subjected to a *t-test* to determine if the RFUs making up the normalized melt phase for both isolates were

significantly different from each other. The results of the *t-test* indicated that the change in fluorescence over the temperature range of the melt phase was not significantly different from each other (P= 0.323). The sequence similarity based on partial 16S rRNA gene fragments revealed a 100% similarity between the two isolates (see Appendix B for sequence similarity matrix).

This result was further confirmed when the V3 variable region was tested alone (Figure 4.5).

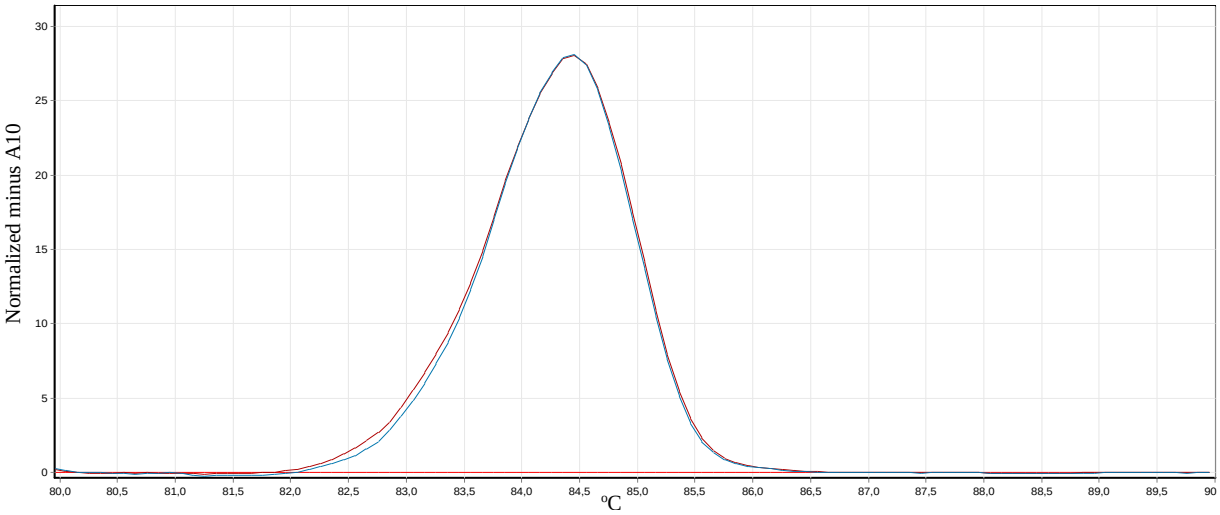


Figure 4.5. Difference plots displaying the melt curves of Isolates B11 (blue curve) and B63 (dark red curve) of the amplified variable V3 region against the selected baseline isolate A10 (red line)

The results of the *t-test* conducted on the RFU values across the normalized melt phase for the V3 region revealed that the isolates were not significantly different from each other (P= 0.789).

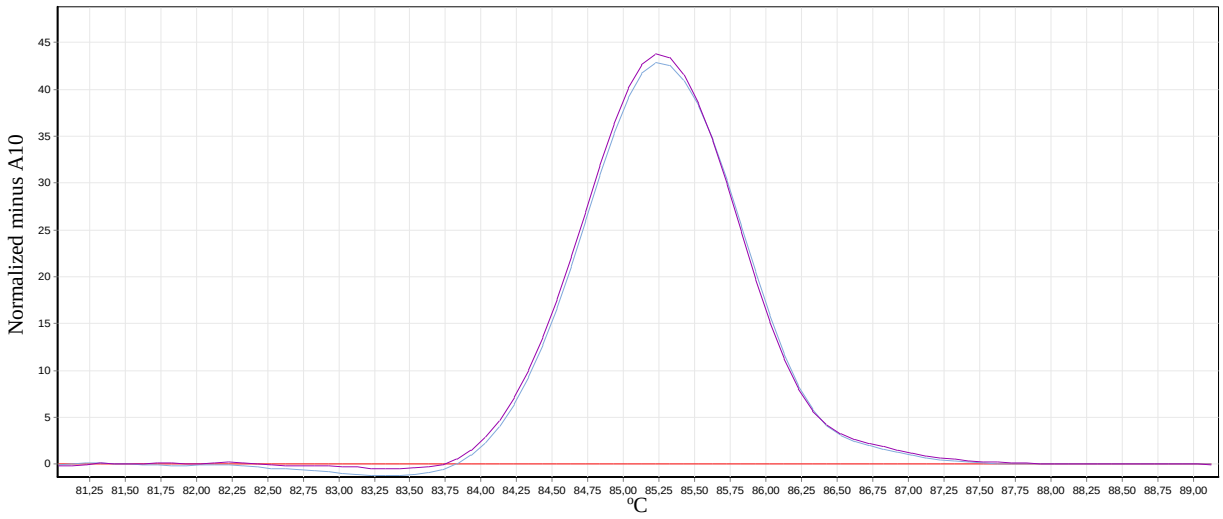


Figure 4.6. Difference plots displaying the melt curves of Isolates A98 (blue curve) and B45 (purple curve) of amplified variable regions V3 and V4 against the selected baseline isolate A10 (red line)

The plot (Figure 4.6) displays the close alignment of the melt curves for isolates A98 and B45. The result of the *t-test* confirmed that the isolates were not significantly different from each other (P= 0.891). The sequence similarity based on partial 16S rRNA gene fragments revealed a 99.9% similarity between the two isolates (Appendix B).

4.3.4. Determination of the ability of HRMA to distinguish between isolates

HRMA was used to establish whether isolates could be distinguished from each other based on melt profiles. Figure 4.7 illustrates the melt curve differences for two distinct isolates based on the V3+V4 amplified regions.

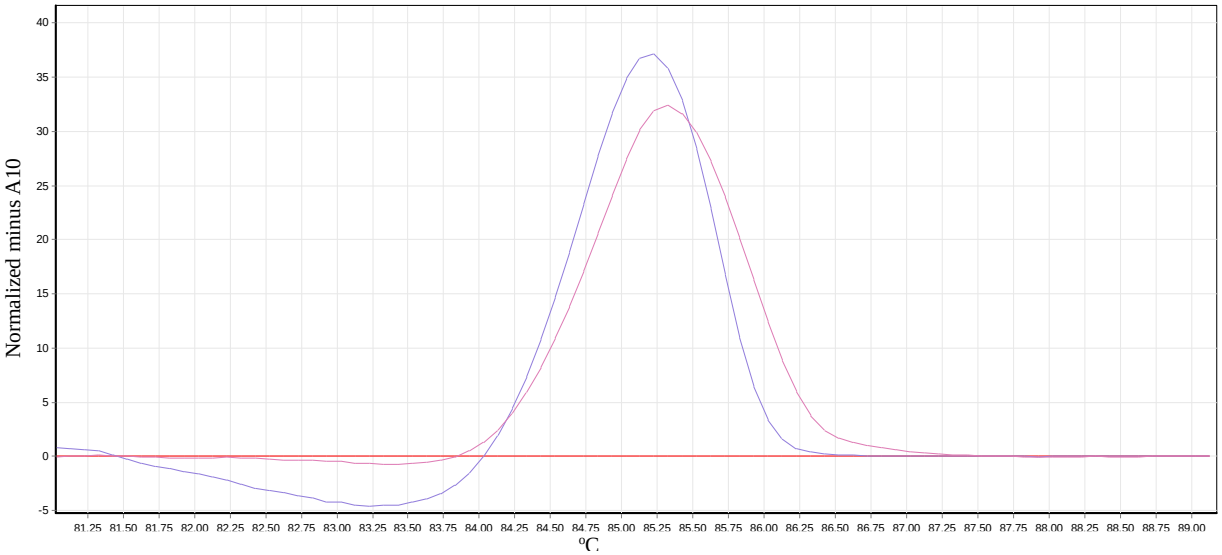


Figure 4.7. Difference plots displaying the melt curves of Isolates A25 (blue curve) and B30 (purple curve) of amplified variable regions V3 and V4 against the selected baseline isolate A10 (red line)

Distinct melt curve profiles were produced for Isolates A25 and B30, indicating variation in the amplified V3 and V4 region. This can be visualized by the shift in the melt curves along the x-axis, which is indicative of differences in the melting temperature. The *t-test* conducted on the RFU values across the normalized melt phase for these isolates indicate that the change in fluorescence over the temperature range of the melt phase was significantly different from each other (P= 0.007). This difference was further confirmed by the sequence similarity matrix which demonstrated an 86.3% similarity in partial 16S rRNA gene sequences between the isolates (Appendix B).

4.3.5. Comparison of V3 and V3+V4 variable regions as gene targets for HRMA

The discriminatory power of the V3 region (182 bp) was compared to that of the larger V3+V4 fragment size.

In most cases, groupings of isolates based on the V3 primer set were similar to those obtained using the V3+V4 primer set (Figures 4.4 and 4.5 as examples). However, in some cases, the V3+V4 melt curves showed a greater degree of differentiation compared to the V3 region alone (Figure 4.8).

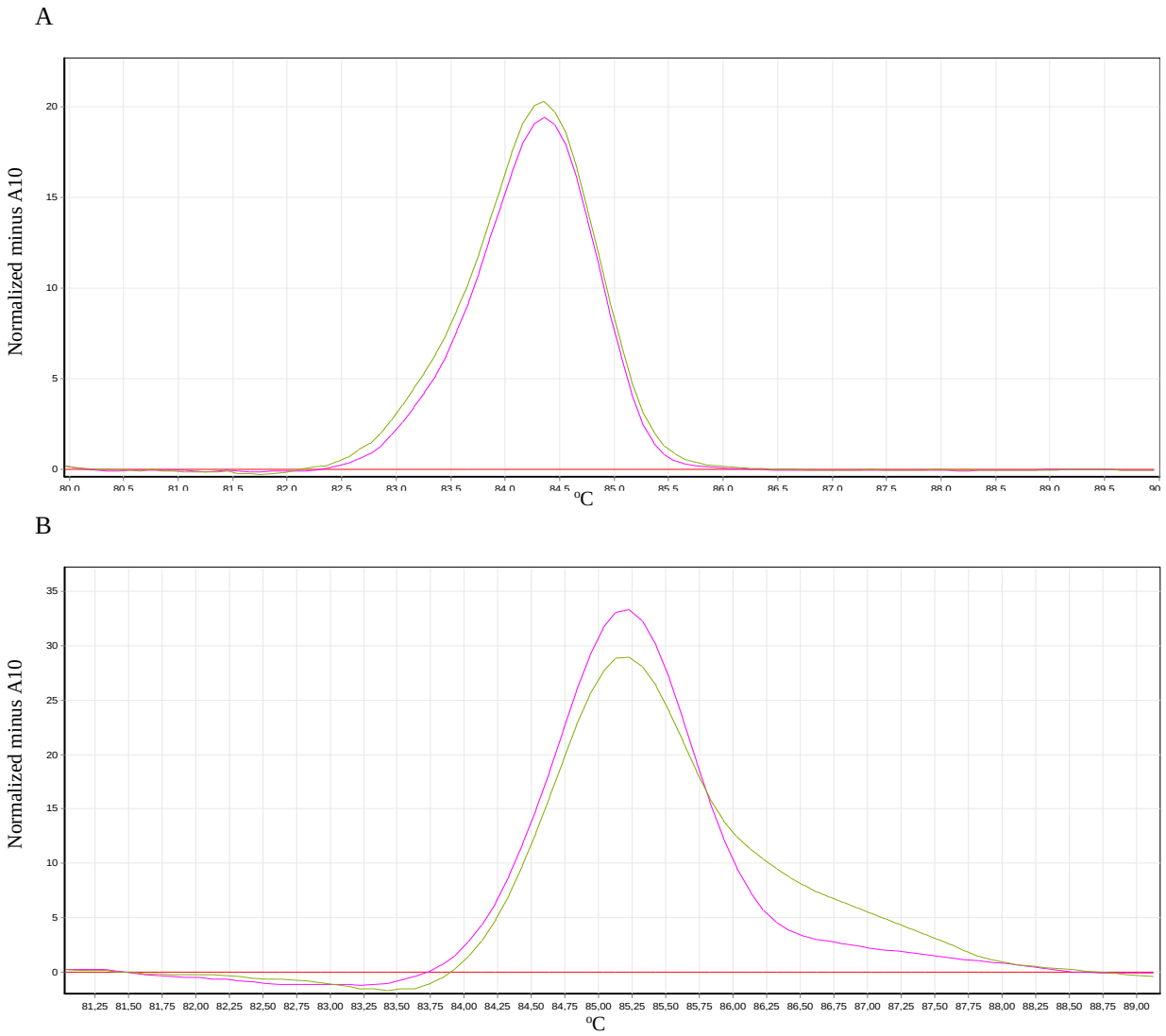


Figure 4.8. Difference plots displaying HRMA melt curves for Isolates A93 (pink curve) and B51 (green curve) using the V3 (A) and V3+V4 (B) amplicons compared to the baseline isolate A10 (red line)

Visually, melt curves of the V3 region for each isolate was virtually identical, whereas for the V3+V4 regions, differences in the melt profile were more pronounced. The student *t-test* analysis conducted using the RFU values for each 0.1ºC temperature increment across the normalized melt phase revealed that in both cases, A93 and B51 were not significantly different from each other (P= 0.902 and P= 0.496 for V3 and V3+V4 respectively), however variation was higher for the V3+V4 region than the V3 region alone.

4.3.6. Grouping of AEFB isolates based on HRMA

For the HRMA data, melt curve profiles and *t-test* results were used to group isolates as shown in the example Figures 4.4–4.7. *T-test* results of isolates with normalized melt phase RFU values which were not significantly different to each other were assigned to the same group, whilst those with unique melt curve profiles were assigned to their own groups. This was conducted for the V3 and V3+V4 regions. All 42 isolates were screened and evaluated in the same manner. Isolates were then grouped according to the 16S rRNA gene sequence data (based on the sequence similarity matrix, Appendix B). Isolates which had 16S rRNA gene sequences with ≥98.7% similarity were assigned to the same group, as this is the threshold sequence similarity value used for the identification of same species (Stackebrandt and Ebers, 2006). Isolates were also grouped according to the Rep-PCR fingerprinting data (based on OTUs provided in Tables A1-A5, Appendix A).

The groupings of AEFB isolates distinguished using HRMA were then compared to those obtained using Rep-PCR fingerprinting and 16S rRNA gene sequence analysis to assess HRMA accuracy (Table 4.2).

Table 4.2. Groupings of isolates based on the target variable regions for HRMA, Rep-PCR fingerprinting data and partial 16S rRNA gene sequence similarities

16S rRNA GROUP (≥98.7% SIMILARITY)	Rep-PCR	HRMA	
		V3	V3+V4
A98, B45, A79, B30	A98 (OTU 23)	A98, B45	A98, B45
	B45 (OTU 49)	-	-
	A79 (OTU 15)	A79	A79
	B30 (OTU 47)	B30	B30
B11, B63	B11 (OTU 45)	B11, B63	B11, B63
	B63 (OTU 46)		
B6, C25, D1	B6 (OTU 39)	B6, C25, D1	B6
	C25 (OTU 57)		C25, D1
	D1 (OTU 67)		
A39, D5, D45	A39 (OTU 32)	A39, A65, D5, D45	A39, A65, D5, D45
	D5 (OTU 69)		
	D45 (OTU 74)		
A93, B51	A93 (OTU 14)	A93, B51	A93, B51
	B51 (OTU 35)		
C2, E1	C2 (OTU 60)	C2, E1	C2, E1
	E1 (OTU 81)		
C13, D12	C13 (OTU 51)	C13, D12	C13
	D12 (OTU 76)		D12
B24, D34	B24 (OTU 43)	B24, D34	B24
	D34 (OTU 80)		D34
A23, A28, C15	A23 (OTU 9)	A23	A23
	A28 (OTU 11)	A28	A28
	C15 (OTU 52)	C15	C15
A9	A9 (OTU 5)	A9	A9
A10	A10 (OTU 7)	A10	A10
A16	A16 (OTU 6)	A16	A16
A24	A24 (OTU 8)	A24	A24
A25	A25 (OTU 10)	A25	A25
A34	A34 (OTU 29)	A34	A34
A35	A35 (OTU 30)	A35	A35
A37	A37 (OTU 31)	A37	A37
A42	A42 (OTU 33)	A42	A42
A65	A65 (OTU 16)	-	-
A101	A101 (OTU 25)	A101	A101
A110	A110 (OTU 24)	A110	A110
B19	B19 (OTU 44)	B19	B19
B52	B52 (OTU 36)	B52	B52
B53	B53 (OTU 38)	B53, D18	B53
D18	D18 (OTU 65)	-	D18
D21	D21 (OTU 53)	D21	D21
D23	D23 (OTU 66)	D23	D23
D47	D47 (OTU 75)	D47	D47

Rep-PCR was able to group isolates with a greater resolution and as a result, isolates were seperated into a larger number of groups compared to the variable regions for HRMA (Table 4.2). The V3+V4 regions were able to differentiate certain distinct isolates to a greater extent compared to the V3 region alone. For example, isolates C13 and D12 were found to be significantly different from each other (P= 0.043) when the V3+V4 regions were tested but not significantly different (P= 0.796) when the V3 region was used. The separation of these isolates into distinct groups with the V3+V4 regions matched the result obtained by Rep-PCR. Isolates grouped together using the V3 and V3+V4 regions were all found to belong to be same species (Tables A1–A5, Appendix A) however, according to Rep-PCR, were unique strains. This indicates that HRMA using the selected variable regions was able to differentiate groups of isolates to a species-level but not a strain-level.

An examination of melt curve profiles for taxonomically-related isolates from different depths revealed that, in some cases, the isolates from different depths which displayed phylogenetic relatedness in terms of their partial 16S rRNA gene sequences, were grouped together with HRMA; however, Rep-PCR was able to show that different strains of isolates were present between the depths. An example of this can be seen with isolates A39 and D45, which were grouped together when testing the V3+V4 region (Figure 4.9).

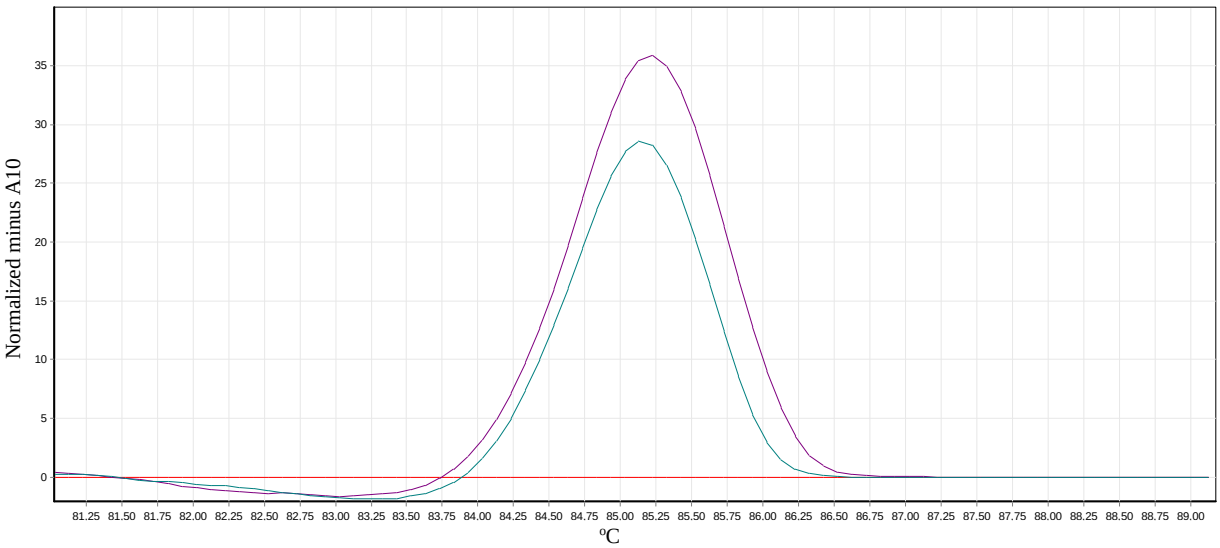


Figure 4.9. Difference plots displaying the melt curves of Isolates A39 (purple curve) and D45 (green curve) of amplified variable regions V3 and V4 against the selected baseline isolate A10 (red line)

Isolate A39 displayed a 99.1% sequence similarity to Isolate D45 (Appendix B). The *t-test* conducted on the RFU values across the normalized melt phase revealed that A39 was not significantly different from D45 (P= 0.319). However, both of these isolates belonged to distinct OTUs and hence, were different strains. Therefore, Rep-PCR was able to provide a more accurate representation of strain diversity occurring between the depths.

4.4. DISCUSSION

HRMA is a technique based on the melting of PCR amplicons and the subsequent generation of melt curves (Reed *et al*., 2007). It allows for nucleic acid sequences to be screened to determine the presence of genetic variation based on differences in DNA melting. In the current study, HRMA was used to screen selected revived AEFB in order to determine whether or not it could serve as an alternative to the Rep-PCR fingerprinting technique for the assessment of diversity amongst the isolates. To this end, three regions were selected as targets for HRMA.

One of the limitations of HRMA is that smaller amplicon fragments are generally recommended since better results are obtained with shorter gene targets (Vossen *et al*., 2009). Vasileiadis *et al*. (2012) tested individual variable regions associated with the 16S rRNA gene to determine the efficacy of each region in discriminating between isolates. The V3 region was found to contain a greater degree of per base variability in comparison to the other variable regions. The high resolution ability provided by the V3 region was also highlighted by Chauhan *et al*. (2013), in a study aimed at the differentiation of foodborne endospore-forming bacteria.

In most cases, isolates which displayed diverse fingerprinting profiles using Rep-PCR also displayed unique melt curve profiles for the V3 region (Table 4.2). However, in certain cases, closely-related isolates which displayed strain differences based on Rep-PCR did not display significantly different melt curve profiles and were grouped together. This resulted in some isolates of distinct OTUs being represented in the same groupings, resulting in AEFB diversity being under-represented with the V3 region as compared to using Rep-PCR.

The larger V3+V4 region was also assessed as a target for HRMA. In most cases, this combined region displayed a similar resolving power to the V3 region for grouping and differentiating isolates (Table 4.2). However, in some instances, HRMA of the V3+V4 regions resulted in a greater level of differentiation between the melt curves compared to the V3 region alone. This indicates that by increasing coverage to a second variable region, a higher degree of variability

may be detected between sequences. HRMA is a technique which is sensitive to amplicon length (Vossen *et al*., 2009). However, despite the sequence length of the amplicon being increased by using two variable regions, HRMA was still able to provide sufficient resolution for the 464 bp amplicon. This suggests that longer length sequences may be used for HRMA. Some studies have explored the use of amplicon lengths greater than 600 bp with successful results (Vossen *et al*., 2009).

When examining the V4 region, Vasileiadis *et al*. (2012) found that this region displayed a high performance in relation to the full 16S rRNA gene sequence, however if used alone, diversity may be under-represented due to a conservation loss between flanking sequence sites. However, when the V3 region was tested in combination with the V4 region, Vasileiadis *et al*. (2012) discovered a high resemblance to results obtained from that of the full length of the 16S rRNA gene sequence. Vasileiadis *et al*. (2012) also found that the V3 and V4 pair was able to reflect the results of the complete 16S rRNA gene more accurately than the V5 and V6 pair and concluded that a greater representation was provided by the V3 and V4 region pair. Klindworth *et al*. (2013) found that the S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 primer set used in the current study to amplify the V3+V4 regions was deemed to be optimal for bacterial diversity analysis.

The *spo0A* gene, which is a functional gene conserved and present in almost all members of endospore-forming *Firmicutes* (Bueche *et al*., 2013), was chosen as the third target for screening of the selected AEFB isolates through HRMA. Housekeeping genes are suitable alternatives which can, in some cases, provide greater resolution than variable regions (Chauhan *et al*., 2013). In the current study, it was found that successful amplification of the *spo0A* gene was not achieved. This could potentially be attributed to reduced primer specificity or a low primer concentration. In some instances, double peaks were produced. This could have occurred due to the presence of PCR artefacts. Double peaks for a single amplicon may also occur if certain regions of the amplicon possess high stability (Montgomery *et al*., 2007). A maintenance of the double-stranded configuration can occur in these regions, resulting in two melting phases. Secondary structures or amplicon misalignment could also be possible causes of the melt curves produced. However, to fully determine the cause, further retesting and examination of the suitability of this gene for HRMA is required.

HRMA proved to be able to demonstrate differences between isolates either through variation in melting temperatures or through distinctions in shapes of the individual melt curve profiles.

The comparison of RFU values was found to be a useful aid for determining whether changes in fluorescence across the temperature increments of the normalized melt phase were significantly different from each other. The ability of HRMA to distinguish between isolates with sequence variation provides a potential beneficial applicability in bacterial diversity studies. However, for the purposes of the current study, a greater strain-level resolution was required to determine if genetic differences were present between isolates revived from different time periods. The resolution provided by HRMA depends on the region chosen for amplification and melt analysis. Regions selected for this purpose should display significant intra-species sequence divergence and intra-strain conservation amongst strains of the same species (Chauhan *et al*., 2013). In this way, isolates of different species can be distinguished from each other and similar strains can be grouped together. Due to the presence of closely-related AEFB isolates, a region which provides greater resolution may need to be chosen to be able to produce analyses of the changes occurring in the diversity of AEFB from different depths. The use of the *spo0A* gene or alternative housekeeping genes could be further examined.

It was confirmed that HRMA worked using amplified DNA product obtained through the colony-pick off approach. This eliminated the additional cost and time associated with using a kit for DNA extraction. HRMA has an advantage over Rep-PCR in that the need for agarose gel electrophoresis for confirmation of PCR products is avoided, which makes the procedure more time efficient. Similar to information provided by electrophoresis gels, HRMA can be used to determine product purity by checking for the presence of multiple peaks and/or distortions, whilst the amount of signal given off and the melt profile provide information about yield and fragment identity respectively (Vossen *et al*., 2009). Another factor which makes this technique advantageous is its non-destructive nature hence, PCR products can still be sequenced post-HRMA (Vossen *et al*., 2009).

Both Rep-PCR and HRMA proved to be effective for pre-sequencing screening. As outlined in Chapter three, effective strain-level resolution of isolates was possible with Rep-PCR. In this way, representatives for each strain could be selected, which consequently allows for a reduction of the number of isolates which need to be chosen for sequencing and identification. HRMA can allow for isolates of the same genotype to be matched and compared to each other based on melt curve profiles and melting temperatures. This could have an application when isolating bacteria from environmental sources. Isolation of bacteria from environmental materials often leads to high CFU values being obtained. This can result in many isolates which

need to be sequenced for identification. However, if the isolates are first pre-screened using HRMA, isolates of the same genotype can be grouped together based on melt curves. By sequencing representatives of these groups, a reduction in sequencing costs can be achieved. If a database of melt curves of isolated environmental bacteria of interest is generated, then this could be used as a basis against which to compare new unknown isolated bacteria, thereby serving as a potential preliminary first step in identification. Isolated bacteria could be used to build up this melt curve database, against which to match new isolates. This may allow the new isolates to stand out, thereby making the selection process for those bacteria which require further sequencing and characterization easier.

Overall, HRMA was found to be useful and convenient due to the ability to visualize and analyze differences between isolates based on the amplified products through software which is user-friendly. Due to the sensitivity of HRMA, which is based on factors such as sequence length and G+C content; variations in the shape of the melt curve and melting temperature can be easily ascertained (Chauhan *et al.*, 2013). In addition, the application of statistical analysis on the actual fluorescence values can allow for a further validation of the melt curve results. High resolution is required when studying closely-related species, such as those belonging to the genus *Bacillus*. Due to insufficient 16S rRNA divergence, there is a loss of differentiation between members of this genus, thereby making the selection of a procedure which can provide effective strain-level resolution difficult (Maughan and van der Awera, 2011). However, to achieve this with HRMA, a suitable gene fragment which would provide the required level of discrimination must be chosen.

In conclusion, HRMA can be used to group closely-related isolates whilst also demonstrating the ability to differentiate genomically-distinct isolates. This technique may possibly serve as an alternative to gel-based fingerprinting procedures. However, the choice of primers and resulting amplicons must be taken into consideration. Whilst the results indicate the suitability of the selected 16S rRNA variable regions for a species-level identification, they highlight the potential for further methodological development and optimization in order to improve resolution using alternative gene targets. Factors pertaining to the length of the amplicon and concentration of master mix components such as magnesium chloride must be considered. Amplification can be influenced by template DNA and primer concentration. When applied and optimized correctly, this technique displays potential as a good alternative for pre-screening to reduce the number of isolates which may need to be sequenced for identification. It is a technique which was found to be relatively quick, simple to perform, with automated

data analysis and no requirement for gel electrophoresis. Due to the resolution of HRMA and its ability to detect single nucleotide differences, with a suitable gene target, this technique may potentially be applied to investigate changes in selected genes between related endospore-forming bacteria. HRMA could possibly serve as a useful technique for studies centred around the determination of differences in bacterial diversity across varying environmental locations, conditions, treatments or timescales.

CHAPTER FIVE

DETERMINATION OF PHYSIOLOGICAL DIVERSITY OF SELECTED REVIVED AEROBIC ENDOSPORE-FORMING BACTERIAL ISOLATES

5.1. INTRODUCTION

Within peatland ecosystems, microorganisms play an important role in decomposition, biogeochemical cycling, the release of nutrients and element turnover (Calbrix *et al*., 2005). Changes in abiotic factors, such as pH, salinity and organic matter composition, influence microbial metabolic activity within ecosystems (Stefanowicz, 2006). Since ecosystem stability is strongly influenced by the activities of microorganisms, the concept of physiological profiling has gained attention as a means of assessing functional and metabolic diversity (Insam and Goberna, 2004).

By assessing functional and metabolic diversity, environmental factors which influence the distribution of microbial species and the roles they play in the environment can be determined (Calbrix *et al*., 2005). Physiological characterization is also key to understanding the effects of anthropogenic impacts and environmental changes on the diversity, distribution and structure of microbial communities. A major factor which influences functional diversity in environmental samples is the type of carbon source present (Insam and Goberna, 2004). Information regarding the functional diversity of microbes present in an environmental sample can be gained by studying substrate utilization profiles.

Community-level physiological profiling (CLPP) was developed to determine the substrate utilization profiles and metabolic capabilities of microbial communities present in environmental samples using Biolog EcoPlate™ assays comprising microplates containing triplicate sets of 31 environmentally-relevant carbon substrates (Stefanowicz, 2006). Each experimental well contains a substrate plus tetrazolium dye which is reduced to formazan when substrate oxidation occurs (Stefanowicz, 2006). This results in a colour change from colourless to violet, which can be measured spectrophotometrically. An analysis of the substrate utilization patterns is used to determine a physiological profile or metabolic fingerprint for a particular environmental sample. For community analysis, direct inoculation of environmental samples into the EcoPlates™ is carried out (Garland, 1997). In the case of sediment, the sample material is usually first suspended in distilled water or saline solution. An isolate-based analysis

is also possible and would involve inoculation of a pure isolate into the microplate to obtain an individual physiological profile (Garland, 1997).

The Biolog™ procedure is user-friendly, reproducible and relatively low cost (Kirk *et al*., 2004). The triplicate tests on the EcoPlates™ serve as a means of increasing the validity of the results (Stefanowicz, 2006). However, the procedure has limitations which must be taken into consideration to increase the value and strength of the data obtained. For example, the procedure is sensitive to the initial density of inoculum used since this can influence the rate of colour development. For this reason, the initial inoculum preparation requires standardization (Kirk *et al*., 2004).

In addition to carbon utilization profiles, physiological profiling can also involve an assessment of environmental factors, such as pH and salinity, which influence microbial species diversity. For instance, several studies have shown that the pH of sediment is a significant determinant of the richness and abundance of microbial species present in ecosystems (Matthies *et al*., 1997; Fierer and Jackson, 2006). The impact of these environmental factors on microbial activity will ultimately affect the roles these organisms play in their ecosystems (Rietz and Haynes, 2003). Therefore, it is important to assess the tolerance ranges of bacteria to these conditions. This could be applied to bacteria which have been revived from ancient samples as this may aid in providing clues regarding environmental conditions present at the time the bacteria were deposited (Jones *et al*., 1993).

Microbial physiological diversity is relatively unexplored in peatland environments. AEFB within these environments form dormant endospores for survival when anaerobic conditions prevail. Endospores trapped within the accumulation of peat over extended periods of time may provide the opportunity to examine the changes in physiological diversity of the aerobic population of endospore-forming bacteria along a vertical distribution over time.

The aim of the present study was to assess the physiological characteristics of selected representative AEFB isolates from an Mfabeni peatland sediment core. Microtiter plate assays were used to determine salinity and pH tolerance levels. Physiological profiling of the isolates using Biolog EcoPlates™ was also undertaken to determine their substrate utilization capabilities.

5.2. METHODS AND MATERIALS

5.2.1. Determination of salinity and pH tolerance of selected AEFB isolates

Thirty-five isolates, which had been isolated from different depths along the sediment core, were selected for salinity and pH tolerance testing. The chosen isolates represented distinct taxonomic units which were distinguished at the species level using 16S rRNA gene sequence analysis. The isolates evaluated were A5, A7, A16, A24, A25, A34, A35, A37, A79, A107, A108, A109, A110, B1, B2, B7, B10, B11, B14, B19, B35, B41, B50, B51, B52, B54, C1, C18, C20, C25, C26, D4, D21, D47 and E1 (Taxonomic and Rep-PCR OTU groupings for the isolates are provided in Tables A1-A5, Appendix A). These isolates were purified and grown on 10% TSA plates prior to testing. Isolates were tested in 96-well F-bottom microtiter plates (Cellstar, Greiner bio-one, Austria) containing 10% Trypticase Soy Broth (TSB). The 10% TSB medium was amended with salt (NaCl) at concentrations ranging from 0.5% to 15.0% for salinity testing and amended to pH values ranging from 3.0 to 10.0 for pH testing.

5.2.1.1. Salinity testing using microtiter plate assays

Each isolate was cultured in 20 ml 10% TSB (Biolab, Merck, RSA), at 30^{o}C and 120 rpm for 16 h. Thereafter, the optical density of each culture was determined (OD_{595}). Cells were harvested by centrifugation at 8 500 rpm for 7 min (Neofuge 13, Heal Force, China). The supernatant was discarded and pelleted cells were resuspended and washed in 0.85% $^{w}/_{v}$ sterile saline solution. This step was conducted twice more using centrifugation at 8 500 rpm for 5 min to pellet cells between each wash. Cell pellets were re-suspended in saline solution and adjusted to a final OD_{595} of 0.12–0.14 using saline. Thirty microlitres of inoculum was inoculated into 150 µl of 10% TSB in each well. Six NaCl concentrations were assessed, viz., 0.5%, 2.5%, 5%, 7.5%, 10% and 15%. Controls included wells containing 180 µl uninoculated broth and wells containing 150 µl saline solution (0.85% $^{w}/_{v}$) with 30 µl of inoculum. Uninoculated control wells were used to verify the absence of contamination. Control wells with cells inoculated into saline solution were used to confirm the absence of media carryover with the cells used for inoculation. All experimental treatments and controls were performed in duplicate (see Appendix C for plate layout). Microtiter plates were incubated at 30^{o}C and 80 rpm for 24 h, after which readings were taken at 595 nm using a microtiter plate reader (FLUOstar Optima, BMG Labtech, Germany). A replicate control plate was made and read at time 0 as a basis of comparison for growth over 24 h.

5.2.1.2. pH testing using microtiter plate assays

Selected isolates were tested using 10% TSB poised at different pH values to determine pH tolerance ranges. Inoculum was prepared as described in section 5.2.1.1. Thirty microlitres of inoculum was pipetted into each well containing 150 µl of 10% TSB at six pH values viz. 3.0, 4.5, 5.5, 7.5, 8.5 and 10.0. The pH of each medium was maintained using buffers appropriate to the pH being evaluated. Media poised at pH 3.0, 4.5 and 5.5 were maintained with the addition of a 0.1 M citrate buffer. The pH 7.5 broth was maintained using a 0.1 M phosphate buffer whereas the pH 8.5 and pH 10.0 media were maintained using a 0.05 M Tris buffer and a 0.1 M bicarbonate buffer respectively (see Appendix C for buffer compositions). Controls included wells which contained 180 µl uninoculated broth and wells containing 150 µl saline solution (0.85% $^{w}/_{v}$) with 30 µl of inoculum. All experimental and control wells were conducted in duplicate (see Appendix C for plate layout). Microtiter plates were incubated at 30^{o}C and 80 rpm for 24 h, after which readings were taken at 595 nm using a microtiter plate reader (FLUOstar Optima). A replicate control plate was made and read at time 0 as a basis of comparison for growth over 24 h.

5.2.1.3. Multivariate analysis of salinity and pH results

Final absorbance data for each isolate was obtained by first subtracting the optical density values obtained at the time 0 reading from that of the optical density values obtained after the 24 h incubation period. This control aided in removing absorbance resulting from the media, the polystyrene wells and any turbidity from the starting inoculum. Following this, final values were obtained by averaging the replicate wells (n=2) for each isolate.

Redundancy analysis (RDA), a canonical form of Principal Component Analysis (PCA), was employed to detect whether the pattern of responses to varying levels of salinity and pH were influenced by the age of the sample from which the isolate was obtained. This was performed with CANOCO v 5.0 (Microcomputer Power Inc., Ithaca, NY) (ter Braak and Šmilauer, 2012). RDA uses matrices which comprise of independent (age of the samples) and dependant variables (response to salinity and pH) and is used to examine the degree to which variation in one variable set is able to account for the variation in the other variable set (Israëls, 1992). Age data for the radiocarbon-dated samples was transformed so that the natural logarithm of the ages were used (LnAge). This was performed to account for variation within the age scale. A Monte Carlo permutation test, which employed n (number of permutations) = 999, was used

for the determination of significance (Anderson and ter Braak, 2003). In permutation tests, values for all possible rearrangements of the test statistic on the observations are used to calculate the reference distribution for that statistic.

5.2.2. Physiological profiling for the determination of metabolic diversity of isolates

Biolog EcoPlates™ (Biolog Inc., USA) were used to determine the environmental substrate utilization patterns of selected AEFB isolates. Layout of the substrates present in each well of the EcoPlate™ is provided in Appendix C. Isolates which were selected for salinity and pH tolerance testing were used for substrate utilization profiling.

5.2.2.1. Determination of substrate utilization abilities of selected AEFB isolates using Biolog EcoPlates™

Isolates were cultured in 50 ml 10% TSB, in duplicate, at 30ºC and 150 rpm for 14 h. Thereafter, the optical density of each culture was determined (OD_{595}). Centrifugation at 6 000 x g for 5 min (Avanti Centrifuge, Beckman Coulter, USA) using sterile 50 ml centrifuge tubes (Beckman Coulter) was used to obtain pelleted cells. The supernatant was discarded and cell pellets for each isolate were pooled. Cells were then washed twice in 0.85% $^{w}/_{v}$ saline solution with a centrifugation step (6 000 x g for 3 min) being used after each wash. Cells were resuspended in 15 ml of saline solution and the optical density determined at 595 nm before being adjusted to OD_{595} 0.235–0.240 by dilution. Thirty microlitres of 5 mM thioglycolic acid was added to each inoculum to inhibit the production of capsules (Franco-Buff *et al*., 1998). One-hundred and fifty microlitres of inoculum was added to each well of the Biolog EcoPlate™ using a multichannel pipette. Microtiter plates were incubated at 30ºC, after which optical density readings were taken at 590 nm (Versa max microplate reader, Molecular Devices, USA and SoftMax® Pro 7 v 7.0, Molecular devices) to measure formazan colour development after days 1, 3, 5 and 7.

5.2.2.2. Analysis of Biolog EcoPlate™ data

Readings from day 7 were selected for data analysis. Colour development for each isolate tested was evaluated by averaging the triplicate readings for each substrate. A net optical density value for each substrate was obtained by subtracting the average optical density value derived

from control wells lacking substrate from the average optical density value of substrate-containing wells (Bossio and Scow, 1995; Kelly and Tate, 1998; Stefanowicz, 2006; Muñiz *et al*., 2014). In instances where a net negative optical density value was obtained, a value of zero was inferred (Bossio and Scow, 1995; Hu *et al*., 2007; Weber *et al*., 2007).

A student *t-test* analysis (Microsoft Excel® 2013 v 15.0.4823.1000) was undertaken to determine if the triplicate readings for each isolate in response to each substrate was significantly different to the control. Only *t-test* values which were significantly different ($P < 0.05$) to the controls were considered to be a positive indicator of substrate utilization. The transformed data was then carried forward for further statistical analysis.

5.2.2.3. Multivariate analysis of physiological profiling data

CANOCO v 5.0 (Microcomputer Power Inc.) statistical software was used to assess the data and determine the appropriate statistical model to use. The data was compositional with a gradient 3.2 standard deviation units long, indicating a unimodal ordination model was suitable. Correspondence analysis (CA) (CANOCO v 5.0) was used to summarize the main patterns of variation present in the substrate utilization profiles amongst the isolates (ter Braak and Šmilauer, 2012). CA is a method of dimension reduction which depicts results graphically (Greenacre, 2010). CA is used to analyze data tables which contain columns and rows. Each column and row is represented as a point. Distances between the points are based on the variances in each of the columns and rows, which have a proportional relationship to the mean (Greenacre, 2010). In this way, tabulated data can be transformed into graphical displays. CA allowed for isolates with similar metabolic profiles to be grouped closely together, whereas isolates with varying profiles were more distantly separated. A logarithmic (X+1) transformation was used for normalization of the data to reduce the weight of large values and allow for the patterns of less consumed substrates to be represented (Weber *et al*., 2007).

Canonical Correspondence Analysis (CCA), a linear variant of CA, can be used to determine relationships between biological variables, i.e. species, and corresponding environmental variables (ter Braak and Verdonschot, 1995; Greenacre, 2010). With CCA, linear combinations of the specific environmental variables dictate the ordination axes (Bossio and Scow, 1995). In this way, the direct effect of the environmental variable of interest (i.e. age) on the changes in substrate utilization patterns can be evaluated. CCA was therefore used to determine if a sample age trend was evident within the data. The radiocarbon-dated age of each sample was

transformed to a natural logarithm scale (LnAge) to account for any variation within the age scale. Significance was determined using a Monte Carlo permutation test, with n (number of permutations) = 999 (Anderson and ter Braak, 2003). Substrates which displayed greater than 10% of their variation explained by age were selected for further analysis by CCA.

5.3. RESULTS

5.3.1. Screening of AEFB isolates for salinity and pH tolerance

5.3.1.1. Salinity tolerance testing

The tolerance ranges of isolates to salt (NaCl) concentrations ranging from 0.5–15% are shown in Table 5.1. A summary of the statistical parameters associated with this data is presented in Table 5.2. Data was transformed by subtracting controls from experimental values, followed by the averaging of replicates (n=2), before being statistically analyzed.

Table 5.1. Tolerance of AEFB isolates to varying salt (NaCl) concentrations using microtiter plate assays (OD_{595})

Isolate	Percentage (%) concentration of NaCl					
	0.5	2.5	5.0	7.5	10.0	15.0
	Optical density rating*					
A5	±	-	-	-	-	-
A7	+	-	-	-	±	±
A16	++	+	+++	++	++	+
A24	-	-	-	-	-	-
A25	+	+	++	+	±	-
A34	+	++	++	+	++	++
A35	±	±	±	+	-	-
A37	+	+	+	+	+	+
A79	-	-	-	-	-	-
A107	+++	+++	+++	++	++	+
A108	+	+	+	+	+	+
A109	++++	+++	+	+	±	±
A110	+	+	±	±	-	-
B1	-	-	-	-	±	±
B2	+	+	+	+	-	-
B7	-	-	-	-	-	-
B10	-	-	-	-	-	-
B11	+	-	-	-	-	-
B14	-	-	±	±	±	-
B19	++	+	++	+	+	+
B35	+	±	±	++	-	-
B41	±	-	-	-	-	-
B50	±	±	±	-	-	-
B51	-	-	-	-	-	-
B52	-	-	±	+	+	+
B54	+	±	±	-	-	-

Table 5.1. continued

Isolate	Percentage (%) concentration of NaCl					
	0.5	2.5	5.0	7.5	10.0	15.0
	Optical density rating *					
C1	+	±	±	+	-	-
C18	+	±	-	-	-	-
C20	+	+	+	+	+	±
C25	+++	++	++	++	-	-
C26	+	+	+	++++	-	-
D4	+	+	±	-	-	-
D21	+	+	+	+	+	±
D47	-	-	-	-	-	-
E1	++	++	+	+	+	+

*± intermittent growth between transformed OD_{595} 0.05-0.099 + positive for growth between transformed OD_{595} 0.1–0.199 ++ positive for growth between transformed OD_{595} 0.2–0.299 +++ positive for growth between transformed OD_{595} 0.3–0.399 ++++ positive for growth at transformed $OD_{595} \geq 0.4$ - negative for growth (transformed $OD_{595}<0.05$)

Six isolates, namely A24, A79, B7, B10, B51 and D47, displayed no growth during the course of the experiment. These isolates were judged to be slow growing on 10% TSB medium which could account for their negative results obtained and may indicate the requirement for a longer incubation period. Isolates A5, B11 and B41 displayed growth only at a 0.5% NaCl concentration whereas isolate C18 exhibited growth restricted to 0.5–2.5% NaCl, indicating the low salt tolerance levels of these isolates. Only one isolate, namely C26, depicted an elevated level of growth at 7.5% NaCl. Greater than 28% of isolates were capable of growth at all six NaCl concentrations tested.

Table 5.2. Summary of statistical parameters calculated for AEFB isolate responses at varying concentrations of NaCl

% NaCl	¥MEAN (OD_{595})	ǂMAX (OD_{595})	§FREQUENCY	*% FREQUENCY	ᶍMEAN OD_{595} OF POSITIVE RESPONSES
0.5	0.121	0.421	26	74.29	0.163
2.5	0.092	0.346	22	62.86	0.146
5.0	0.091	0.323	23	65.71	0.138
7.5	0.096	0.485	20	57.14	0.167
10.0	0.057	0.247	15	42.86	0.133
15.0	0.046	0.261	13	37.14	0.125

¥average of transformed absorbance values for isolates in response to the 6 NaCl concentrations ǂhighest transformed absorbance response value for each NaCl concentration §total number of isolates which displayed growth for each NaCl concentration *(total number of isolates which displayed growth ÷35) x100 ᶍaverage, with the exclusion of isolates which did not display a positive response

A majority of isolates were able to grow on a baseline 0.5% NaCl concentration (Table 5.2). The frequency of isolates exhibiting growth displayed a downward trend with increasing NaCl concentration; with the exception of the NaCl concentration increase from 2.5% to 5%, in which the frequency increased. The 'mean of positive responses' reflects a more accurate average of microbial growth as this excludes the 6 isolates which displayed no growth, thereby preventing a skewed mean. In this case, the highest average optical density was observed for the 7.5% NaCl concentration. At this concentration, the greatest maximum optical density value was observed. Whilst fewer isolates were able to grow at this NaCl concentration compared to the 0.5% concentration, the isolates which did grow displayed high OD values indicating a significant growth response.

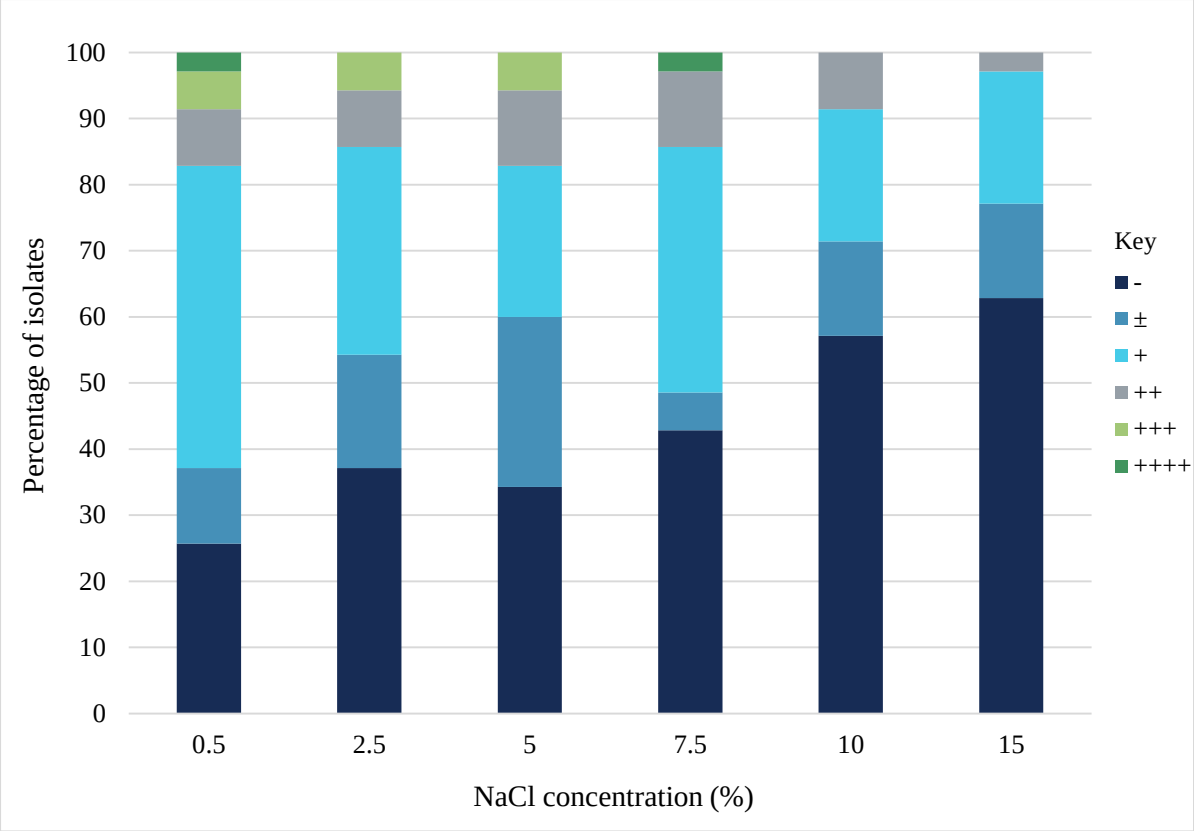


Figure 5.1. Distribution of relative growth intensity of revived AEFB isolates cultured at NaCl concentraions ranging from 0.5–15%. (Key: - no growth OD_{595} < 0.05; ± intermittent growth OD_{595} 0.05–0.099; + positive growth OD_{595} 0.1–0.199; ++ positive growth OD_{595} 0.2–0.299; +++ positive growth OD_{595} 0.3–0.399; ++++ positive growth $OD_{595} \geq 0.4$).

A total of 8.6% of isolates displayed a salt tolerance range of 0.5–5%, whilst 20% demonstrated growth from 0.5–7.5%. Approximately 43% of isolates were positive for growth at a 10% NaCl concentration. This percentage dropped to 37.1% for isolates which demonstrated an ability to grow at 15% NaCl. With increasing NaCl concentration, the percentage of isolates able to grow decreased. None of the isolates displayed optimal growth at the 10% and 15% NaCl concentrations.

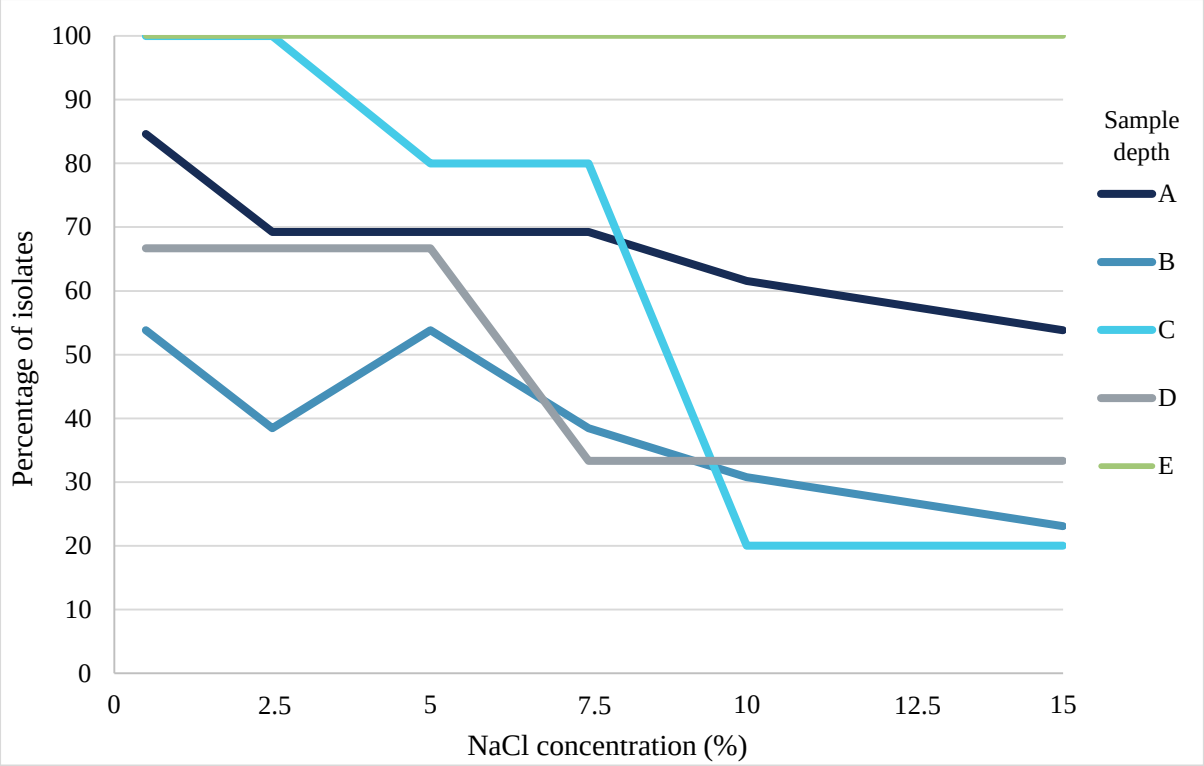


Figure 5.2. The effect of increasing salt concentration on AEFB isolate growth. Samples A, B, C, D and E correspond to depths radiocarbon-dated to 589, 1 964, 17 568, 33 328 and 37 906 cal years BP respectively.

Figure 5.2. illustrates the effect of increasing salt concentration on the proportion of isolates across different sampling depths. It was observed that at least one isolate from each depth was able to grow at each of the salt concentrations tested. For isolates from sample depths A, C and D, the increase in salt concentration resulted in a decrease in the percentage of isolates which were able to grow on the media. For sample depth B, a higher proportion of isolates were able to grow on a 5% NaCl adjusted medium, compared to the 2.5% concentration. The sole isolate (E1) tested from sample depth E was found to grow at all NaCl concentrations tested, with the greatest levels of growth associated with the 0.5–2.5% NaCl concentration range.

5.3.1.2. pH tolerance testing

The tolerance ranges of isolates to each pH value tested in the range from 3.0–10.0 is shown in Table 5.3. A summary of the statistical parameters associated with this data is presented in Table 5.4. Data was transformed by subtracting controls from experimental values, followed by the averaging of replicates (n=2), before being statistically analyzed.

Table 5.3. Tolerance of AEFB isolates to pH variation tested using microtiter plate assays (OD_{595})

Isolate	pH value					
	3.0	4.5	5.5	7.5	8.5	10.0
	Optical density rating*					
A5	-	-	-	++	+	-
A7	-	-	-	±	-	-
A16	±	±	±	++	+	±
A24	-	-	+	+	+	++
A25	-	-	-	++	+	+
A34	-	-	-	±	±	±
A35	-	-	-	++	+	-
A37	-	-	+	+	+	+
A79	-	-	-	+	+	-
A107	-	-	±	±	±	-
A108	-	-	-	++	+	-
A109	-	-	-	±	+	±
A110	-	-	-	±	+	-
B1	-	-	-	++	±	-
B2	-	-	-	+	±	-
B7	-	-	-	±	±	-
B10	-	-	++	++	++	+
B11	-	-	-	±	±	-
B14	-	-	-	±	-	-

Table 5.3. continued

Isolate	pH value					
	3.0	4.5	5.5	7.5	8.5	10.0
	Optical density rating*					
B19	-	-	-	+	+	±
B35	-	-	-	+	+	-
B41	-	-	+	+	+	+
B50	-	-	-	+	+	-
B51	-	-	+	+	+	-
B52	-	-	-	++	+	-
B54	-	-	-	+	+	±
C1	-	-	-	++	+	+
C18	-	-	-	+	+	+
C20	-	-	-	++	+	-
C25	-	-	-	+	-	-
C26	±	-	-	-	+	-
D4	-	+	+	+	-	-
D21	-	-	-	+	±	-
D47	-	-	-	+	+	-
E1	-	-	-	+	+	-

*± intermittent growth between transformed OD_{595} 0.05–0.099 + positive for growth between transformed OD_{595} 0.1–0.199 ++ positive for growth between transformed OD_{595} 0.2–0.299 +++ positive for growth between transformed OD_{595} 0.3–0.399 ++++ positive for growth at transformed $OD_{595} \geq 0.4$ - negative for growth (transformed $OD_{595}<0.05$)

All isolates displayed growth for at least one pH value tested. Isolates A16 and C26 were the only isolates which displayed growth at pH 3 (Table 5.3). Furthermore, isolate A16 was also the only one which displayed growth across the full pH range tested, with optimal growth at a pH of 7.5 being determined. Only one isolate (C26) did not display growth at a pH of 7.5, whilst four isolates (A7, B14, C25 and D4) were unable to grow at a pH of 8.5. The results indicate that the neutral to slightly alkaline pH range was more favourable for the majority of isolates, which was consistent with the initial selection criteria.

Table 5.4. Summary of statistical values calculated for AEFB isolate responses to varying pH values

pH	¥MEAN	ǂMAX	§FREQUENCY	*% FREQUENCY	ᶻMEAN OF POSITIVE RESPONSES
3.0	0.005	0.065	2	5.714	0.060
4.5	0.005	0.126	2	5.714	0.092
5.5	0.027	0.290	8	22.86	0.137
7.5	0.144	0.297	34	97.14	0.153
8.5	0.114	0.248	31	88.57	0.129
10.0	0.041	0.243	12	34.29	0.131

¥average of transformed absorbance values for isolates in response to each pH value ǂhighest transformed absorbance response result for each pH value §total number of isolates which displayed growth for each pH value *(total number of isolates which displayed growth ÷35) x100 ᶻaverage, with the exclusion of isolates which did not display a positive response

Only two isolates (5.7%) were capable of growth at pH values of 3 and 4.5, whilst a higher percentage, namely 22.9%, were able to grow at a pH of 5.5 (Table 5.4). The largest proportion of isolates (97%) grew at a pH of 7.5. A high proportion (88.6%) of isolates also showed growth at pH 8.5. A significant proportion of isolates (65.7%) were unable to grow at a basic pH of 10. The high average optical density values observed at a pH of 5.5 and 10 indicate that the isolates which were capable of growth at these pH values displayed significant growth responses.

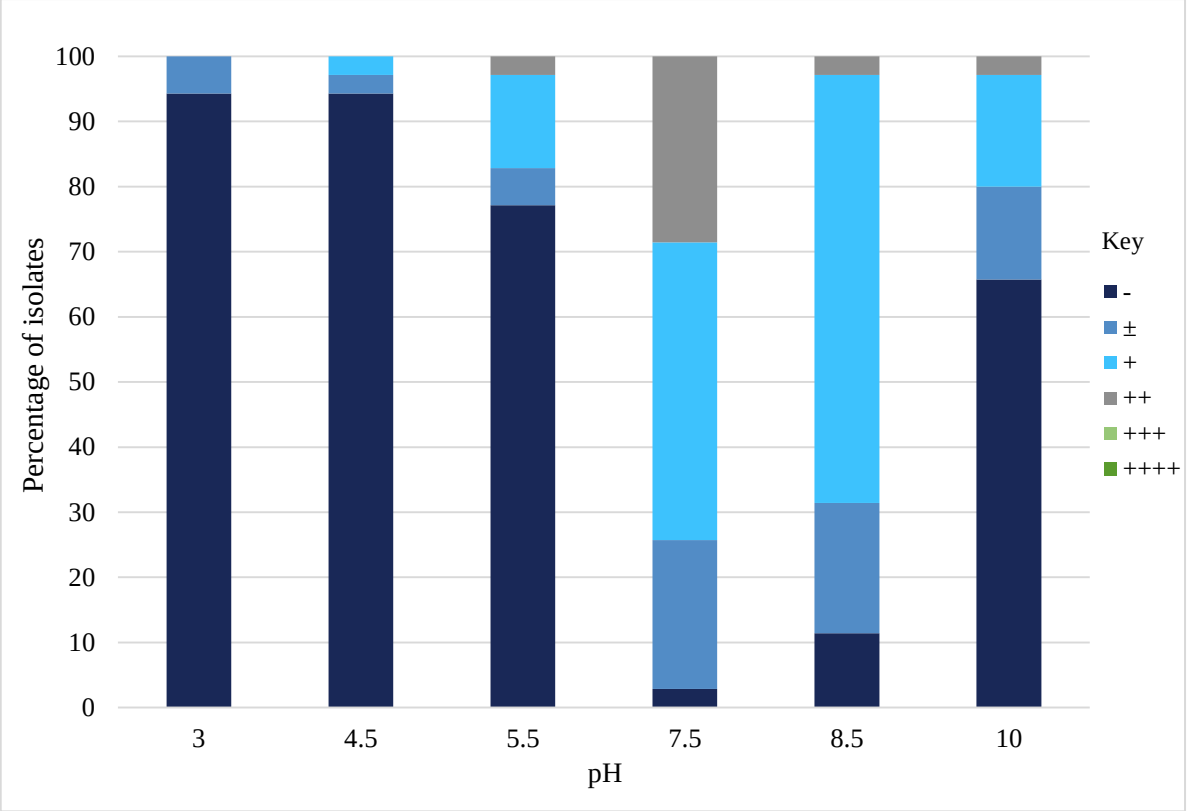


Figure 5.3. Distribution of relative growth intensity of revived AEFB isolates cultured at six pH values ranging from 3–10. (Key: - no growth OD_{595} < 0.05; ± intermittent growth OD_{595} 0.05–0.099; + positive growth OD_{595} 0.1–0.199; ++ positive growth OD_{595} 0.2–0.299; +++ positive growth OD_{595} 0.3–0.399; ++++ positive growth $OD_{595} \geq 0.4$).

Approximately 74% of isolates tested did not grow in acidic media (pH 3–5.5). The isolates which grew at pH 3 and 4.5 displayed low OD_{595} values. Twelve isolates (34.3%) grew across the basic pH range tested (pH 7.5–10). Forty-six percent of isolates demonstrated growth only within the pH range of 7.5–8.5. The highest optical density values were observed at a pH of 7.5. The majority of isolates favoured a near-neutral to alkaline pH (7.5–8.5).

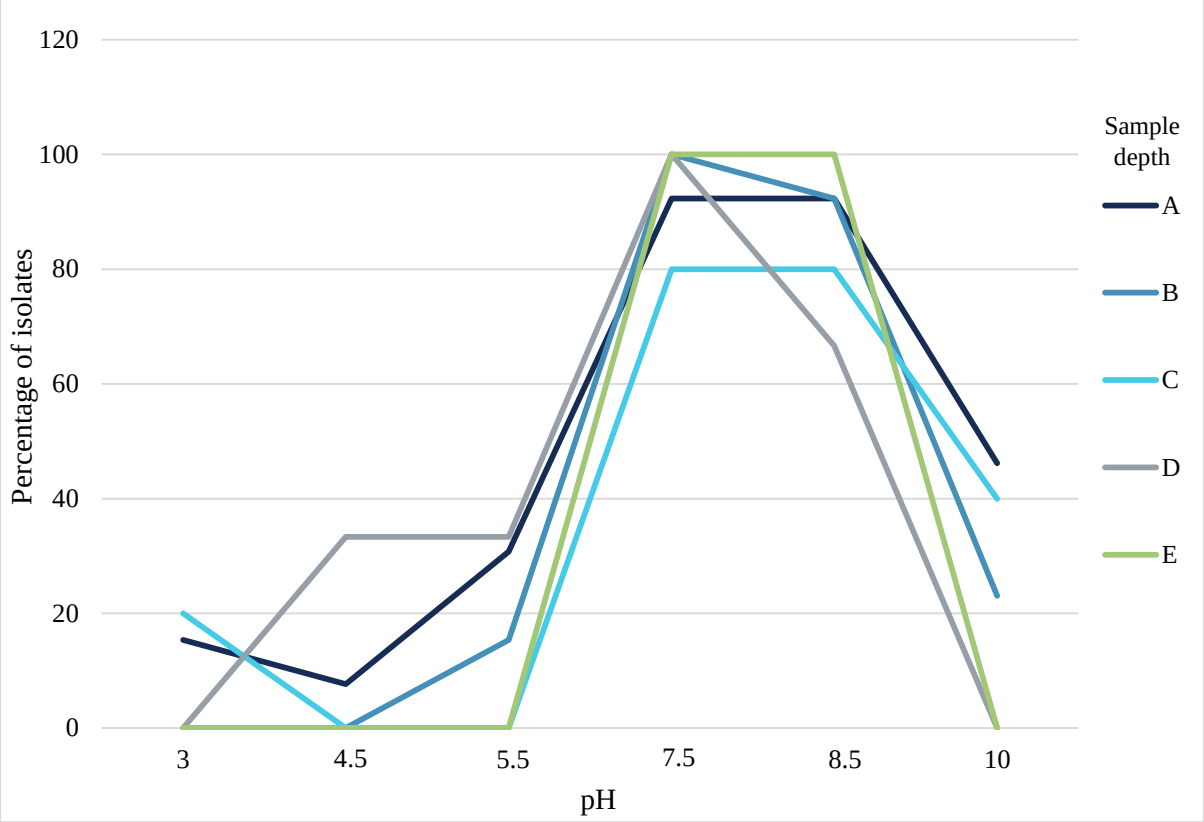


Figure 5.4. The influence of pH on AEFB isolate growth. Samples A, B, C, D and E correspond to depths radiocarbon-dated to 589, 1 964, 17 568, 33 328 and 37 906 cal years BP, respectively.

At pH 3, only isolates from sample depths A and C showed evidence of growth, whilst at pH 4.5, only isolates from sample depths A and D displayed growth. Isolates from sample depths A, B and D were able to grow at pH 5.5. The sole isolate (E1) tested from sample depth E was found to grow at a pH of 7.5 and 8.5 only. The highest numbers of isolates from all depths grew at pH 7.5 and 8.5. At pH 10, only isolates from sample depths A, B and C were able to grow.

RDA of LnAge (natural log of sample depth age) conducted on salinity response data revealed that the age of the samples from which isolates were obtained did not have an effect on the responses of the isolates to the various NaCl concentrations tested ($F= 0.5$, $P= 0.633$).

Permutation test results for RDA of LnAge on pH response revealed that sample depth age did not have an effect on the isolate responses to the range of pH values tested ($F= 0.9$, $P= 0.409$).

The results of these tests suggest that the responses are random, with no definitive pattern evident for salinity and pH responses in relation to sample age.

5.3.2. Physiological profiling of AEFB isolates to determine metabolic diversity

Substrate utilization profiles of the selected isolates were examined using Biolog EcoPlates™. Substrate utilization was determined by assessing a colour change in the microtiter plate well, with a violet colour production indicating a positive response (Figures 5.5–5.6). For each isolate, the intensity of the violet colour differed between substrates, indicating varying degrees of utilization of the compounds.

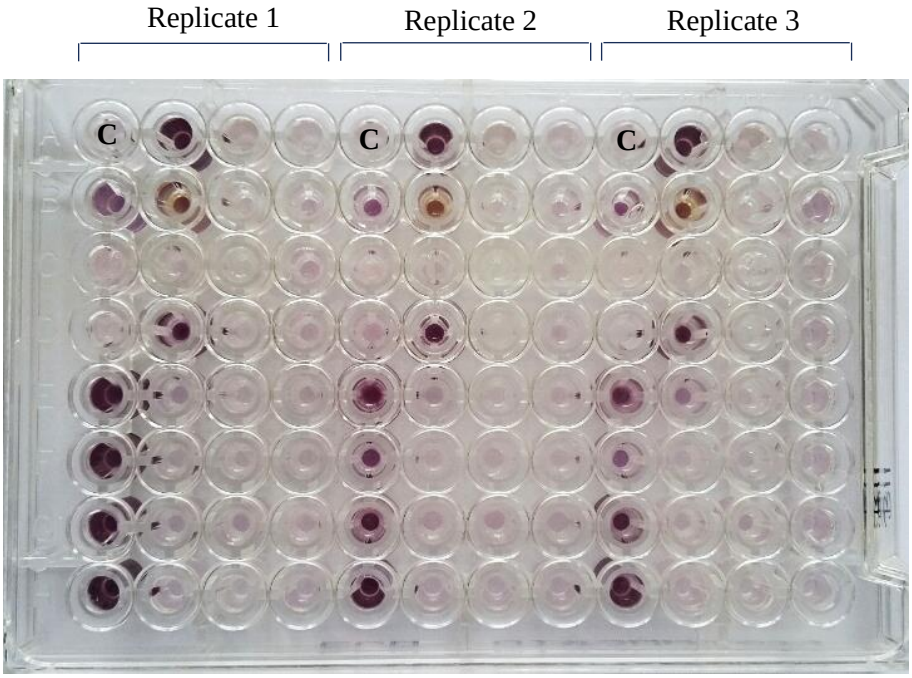


Figure 5.5. Substrate utilization profile determined for isolate B11 using a Biolog EcoPlate™ after 7 days incubation at 30°C. The violet colouration is interpreted as a positive reaction resulting from tetrazolium dye reduction. C is used to indicate the control wells which lack substrate.

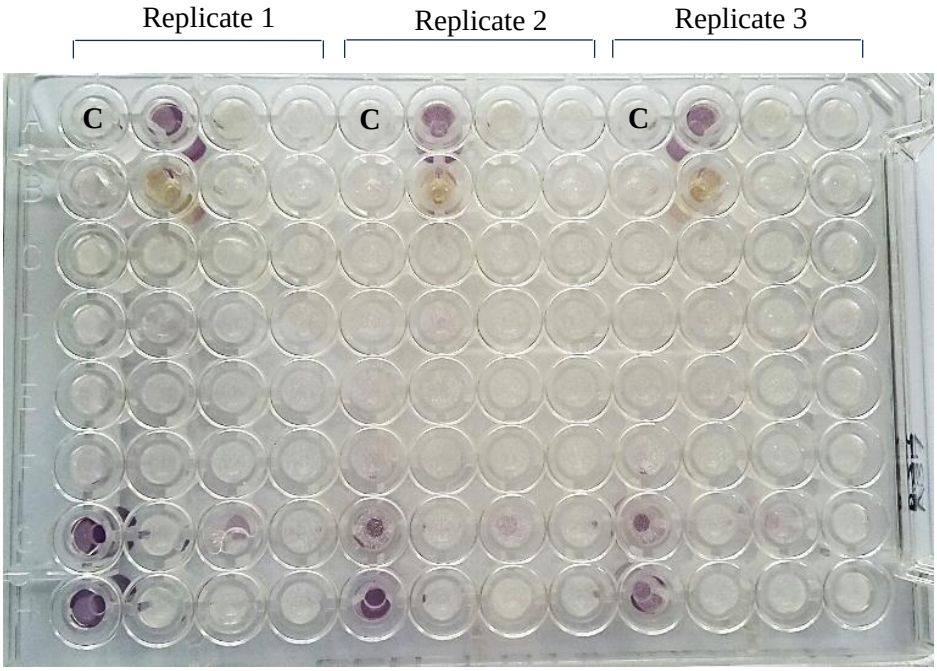


Figure 5.6. Substrate utilization profile determined for isolate A37 using a Biolog EcoPlate™ after 7 days incubation at 30°C. C is used to indicate the control wells which lack substrate.

Colour production in triplicate wells of each substrate evaluated confirmed the substrate utilization profiles and, hence, the result obtained. Figures 5.5 and 5.6 are examples of substrate utilization profiles for two isolates. Colour production of different intensities were determined for different substrates, with stronger colour development responses being interpreted as higher degrees of utilization. Variation in colour intensity resulted in differences in the transformed OD_{590} values obtained for each isolate in response to each substrate, thereby making it possible to determine which substrates were favoured more by each isolate (Table 5.5).

Based on the transformed OD_{590} data, the responses of all isolates to each of the 31 substrates were analyzed statistically to determine substrate utilization frequencies (Table 5.5).

Table 5.5. Summary of Biolog EcoPlate™ substrate utilization frequency parameters of isolate responses to each of the 31 substrates

SUBSTRATE	¥MEAN (OD_{590})	ǂMAX (OD_{590})	§FREQUENCY	*% FREQUENCY	ᵡMEAN OF POSITIVE RESPONSES
Pyruvic acid methyl ester	0.123	0.892	24	68.60	0.179
Tween 40	0.491	1.149	29	82.90	0.592
Tween 80	0.609	1.306	25	71.40	0.852
α-Cyclodextrin	0.080	1.654	4	11.40	0.698
Glycogen	0.081	0.979	7	20.00	0.405
D-Cellobiose	0.107	1.538	6	17.10	0.623
α-D-Lactose	0.095	1.768	4	11.40	0.830
β-Methyl-D-Glucoside	0.089	1.647	6	17.10	0.517
D-Xylose	0.123	0.934	16	45.70	0.270
i-Erythritol	0	0	0	0.00	-
D-Mannitol	0.056	1.667	5	14.30	0.391
N-Acetyl-D-Glucosamine	0.090	0.967	5	14.30	0.628
D-Glucosaminic acid	0	0	0	0.00	-
Glucose-1-Phosphate	0.037	0.705	3	8.60	0.426
D,L-α-Glycerol Phosphate	0.041	0.695	5	14.30	0.284
D-Galactonic Acid γ-Lactone	0.024	0.104	15	42.90	0.057
D-Galacturonic Acid	0	0	0	0.00	-
2-Hydroxy Benzoic Acid	0.011	0.100	6	17.10	0.064

Table 5.5. continued

SUBSTRATE	¥MEAN	ǂMAX	§FREQUENCY	*% FREQUENCY	ᵡMEAN OF POSITIVE RESPONSES
4-Hydroxy Benzoic Acid	0.002	0.051	2	5.70	0.043
γ-Hydroxybutyric Acid	0.001	0.044	1	2.90	0.044
Itaconic Acid	0.002	0.064	1	2.90	0.064
α-Ketobutyric Acid	0.051	0.852	6	17.10	0.299
D-Malic Acid	0.002	0.062	1	2.90	0.062
L-Arginine	0.024	0.421	2	5.70	0.421
L-Asparagine	0.060	0.592	6	17.10	0.353
L-Phenylalanine	0	0	0	0.00	-
L-Serine	0.084	0.921	5	14.30	0.591
L-Threonine	0.039	0.670	3	8.60	0.450
Glycyl-L-Glutamic Acid	0.050	0.727	3	8.60	0.578
Phenylethylamine	0.002	0.060	1	2.90	0.060
Putrescine	0.014	0.312	5	14.30	0.101

¥average of transformed absorbance values for isolates in response to the 31 substrates ǂhighest transformed absorbance response value for each substrate §total number of isolates which utilized each substrate *(total number of isolates which utilized each substrate ÷35) x100 ᵡaverage, with the exclusion of isolates which did not display a response to the substrate

Four substrates, namely i-erythritol, D-glucosaminic acid, D-galacturonic Acid and L-phenylalanine, were not utilized by any of the isolates evaluated. Four carbon sources (12.9%), namely γ-hydroxybutyric acid, itaconic acid, D-malic acid and phenylethylamine, were utilized by only one isolate (i.e. a frequency of 1). Isolates utilizing these substrates were only found at sample depths A and B. Isolates from sample depth A were able to utilize 22 of the 31 substrates (62.9%), whilst isolates from sample depth B were able to utilize 23 substrates (74.2%). Tween 40 and 80 were utilized at the highest frequencies, with 82.9% and 71.4% of isolates utilizing

each of these compounds as carbon sources respectively. For certain compounds, namely α-cyclodextrin, α-D-lactose and β-methyl-D-glucoside, it is evident that although they were utilized at a low frequency, the degree of utilization by isolates was high (i.e. high maximum optical density value). This indicates that whilst only a few isolates could utilize these substrates, they were able to do so efficiently. Conversely, D-galactonic acid γ-lactone was utilized at a relatively high frequency (42.9%), however, the relative utilization of this substrate was low (mean positive response OD_{590}= 0.057; maximum OD_{590}=0.104). This suggests that although a significant proportion of isolates were able to use this substrate as a carbon source; it was not utilized to a high degree, relative to other substrates with high frequencies. The 'mean of positive responses' was found to afford a more accurate representation of the data than the standard 'mean'. This is due to the nil responses of many isolates to certain substrates, which resulted in the mean absorbance being under represented. Tween 80 showed the highest mean absorbance illustrating that a high proportion of isolates were able to utilize this carbon source efficiently. Conversely, excluding substrates that were not utilized, 4-hydroxy benzoic acid displayed the lowest mean absorbance value, with only two isolates displaying an ability to utilize this substrate and to a significantly low degree.

Pyruvic acid methyl ester (carboxylic ester) and tween 40 (polymer) were the substrates that were utilized by the highest proportion of isolates from sample depth A. For sample depth B, tween 40 was the most commonly utilized substrate amongst isolates. For sample C, all isolates tested were capable of utilizing pyruvic acid methyl ester, tween 40 and tween 80. Both tween compounds were also utilized by all isolates from sample depth D. Isolate E1, the sole isolate tested from sample depth E, was able to utilize tween 40, tween 80, D-galactonic acid γ-lactone and 2-hydroxy benzoic acid.

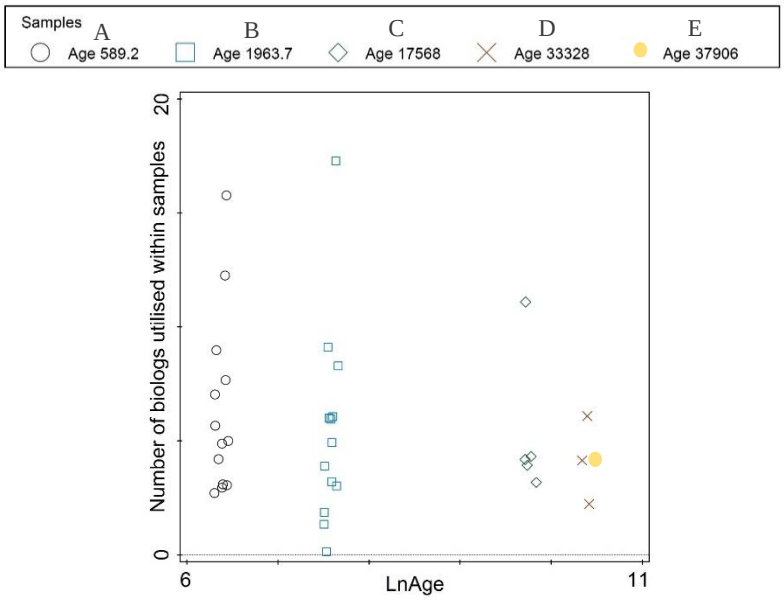


Figure 5.7. The distribution of Biolog EcoPlate™ substrate utilization by AEFB isolates from each depth across a LnAge scale

Figure 5.7 provides a comparison of the number of substrates utilized by isolates from each sample depth. Most of the isolates from sample depth C (17 568 cal year BP) were shown to utilize a similar number of substrates. The three isolates from sample depth D (33 328 cal year BP) also appeared in the lower spectrum indicating that these isolates only utilized a limited number of substrates. Isolates from sample depths A and B (589 and 1 964 cal year BP samples respectively) displayed variation in the number of substrates utilized. The maximum number of substrates utilized by an isolate (B11) was 17.

To determine the level of variation existing amongst isolates in terms of substrate utilization, a correspondence analysis (CA) plot was constructed using the log (X+1) transformed data (Figure 5.8). Eigenvalues (the amount of variability captured on the axes) was 0.668 and 0.343 for CA axis 1 and CA axis 2 respectively. The cumulative explained variation of CA axis 1 and 2 were 30.95% and 46.87% respectively. The pseudo-canonical correlation (correlation of the axes to the variable of interest i.e. age) for CA axis 1 and 2 were 0.331 and 0.056 respectively.

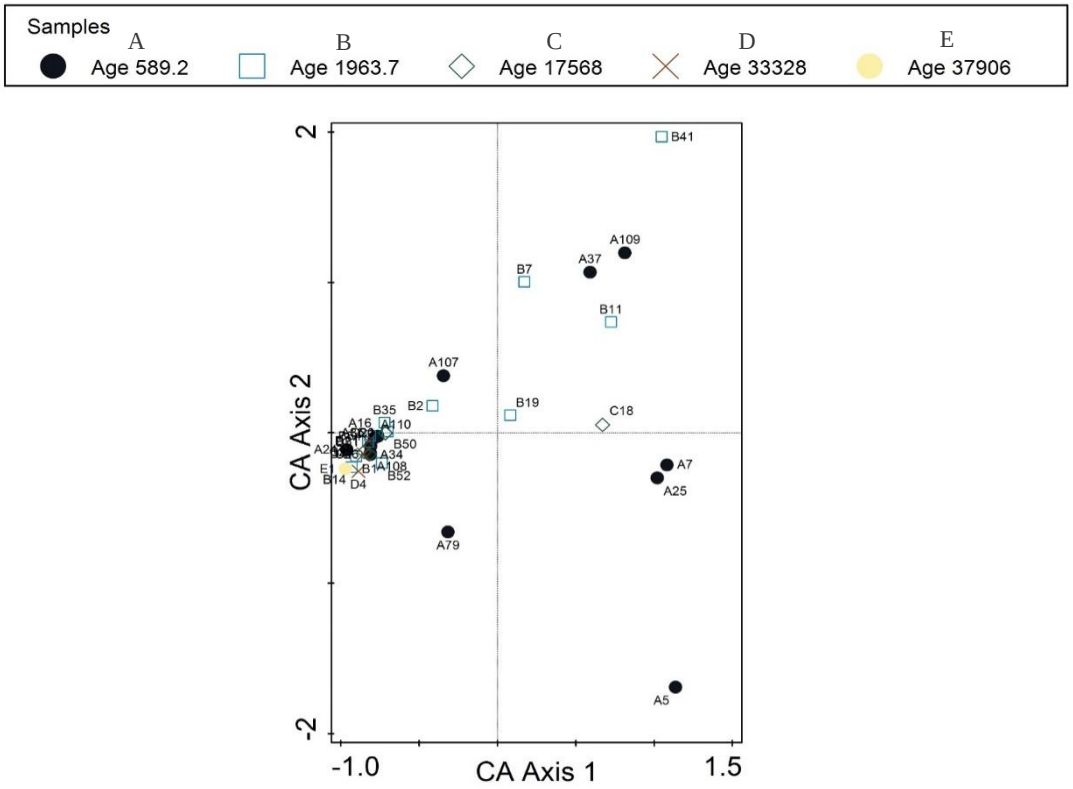


Figure 5.8. A CA plot depicting the level of variation amongst AEFB isolates (log (X+1) transformed values) based on their substrate utilization profiles. Points on the plot represent the each of the isolates.

Isolates which are close together, such as A7 and A25, represent those which have similar metabolic profiles, whilst those further apart, for example A5 and A25, are indicative of isolates which have higher degrees of variation in their substrate utilization profiles (Figure 5.8). The CA plot reveals that the A and B isolates (from the respective 589 and 1 964 cal year BP age depths) displayed a greater degree of variation in the utilization of substrates from the EcoPlate™ than the isolates from the other sample depths. This can be seen from the wide distribution amongst the A and B isolates throughout the plot. This was expected since a greater number of isolates were obtained at these depths. With the exception of isolate C18, all isolates from the 17 568 cal year BP sample and older cluster closely together, indicating a greater similarity in substrate utilization profiles amongst isolates from the older depths. Overlap is also evident between many A and B isolates and isolates from sample depths C, D and E.

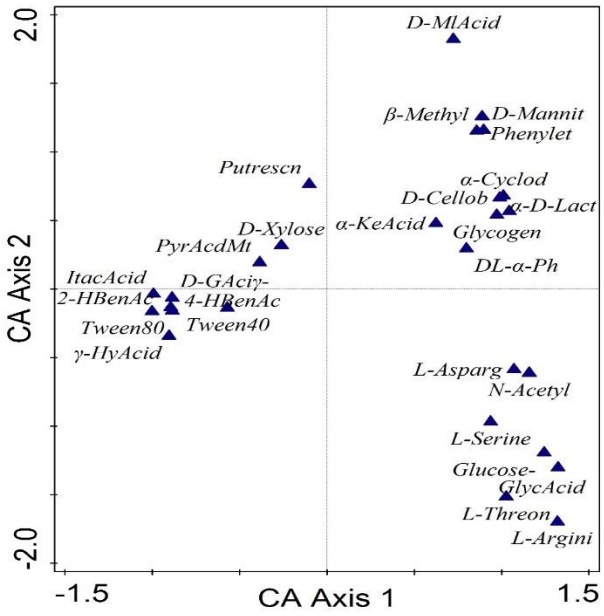


Figure 5.9. CA plot depicting the centroid locations of the utilized substrates to determine the pattern of utilization amongst the AEFB isolates

Centroids for the substrates are represented independently of the CA plot (Figure 5.8) and are shown in Figure 5.9 for ease of visualization. Positions of substrates in Figure 5.9 correlate to utilization by isolates displayed in Figure 5.8. i-Erythritol, D-Glucosaminic acid, D-Galacturonic Acid and L-Phenylalanine are not included since none of these substrates were utilized by the isolates tested.

To determine if an age trend was present, i.e. to test the effect of sample age on the substrate utilization profiles, canonical correspondence analysis (CCA) was carried out using the natural logarithm of the age data (Figure 5.10).

Eigenvalues for CCA axis 1 and 2 were 0.124 and 0.580 respectively. CCA axis 1 accounted for 5.72% and 100% of the cumulative explained variation and the fitted variation respectively, whilst CCA axis 2 accounted for 32.60% of the cumulative explained variation. The pseudo-canonical correlation (correlation between the axes representing the isolates and the tested environmental variable of age) of CCA axis 1 was 0.480. The Monte Carlo permutation test results (n= 999) on all axes revealed that age did have an effect on the substrate utilization patterns observed (F= 1.9, P= 0.036).

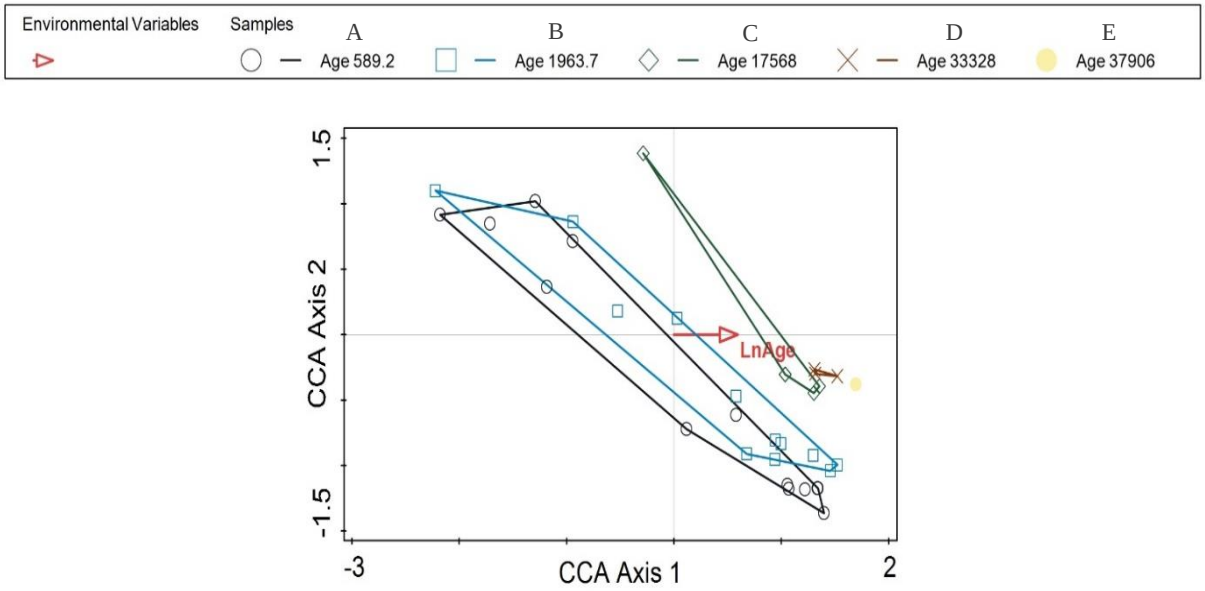


Figure 5.10. CCA plot of AEFB isolates along a LnAge (natural logarithm of age) gradient. CCA axis 1 is used to show the variability as a result of the age effect. Lines are used to group together isolates from the same ages for ease of visualization.

Substrate utilization patterns between the isolates from sample depth D are very similar, as they cluster together closely in the CCA plot. With the exception of one isolate from sample depth C, all other isolates from the older depths, i.e. sample depths C, D and E, displayed very similar utilization patterns to each other. It is evident that the isolates from the more recent ages, i.e. from sample depths A and B, have a greater degree of diversity and variability within their substrate utilization profiles. Significant overlap is evident between the A and B isolates, even with the high degree of variability amongst profiles. Substrate utilization profiles of A and B isolates are distinct from the isolates from older samples.

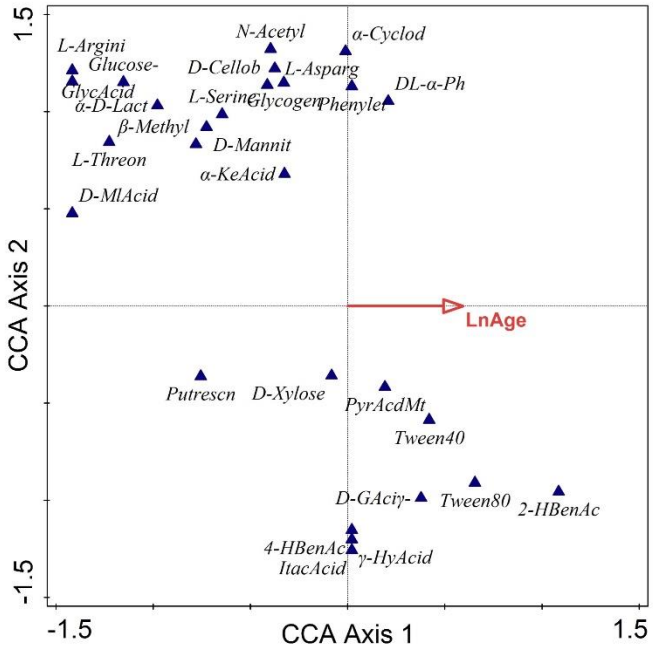


Figure 5.11. The centroid positions for Biolog EcoPlate™ substrates along an age gradient according to the CCA plot (Figure 5.10)

Each substrate was analyzed using CCA to determine the degree to which the variation in substrate utilization occurred as a result of age (Table 5.6). In this way, the substrates with the highest variation which could be explained due to changes over time were distinguishable.

Table 5.6. Variation in substrate utilization as a function of age for Biolog EcoPlate™ substrates

SUBSTRATE	VARIATION EXPLAINED BY AGE (%)
Glycyl-L-Glutamic acid	17.04
Tween 80	16.28
α-D-lactose	13.80
Tween 40	12.93
L-arginine	11.48
Glucose-1-phosphate	11.00
L-threonine	10.88
2-Hydroxy benzoic acid	8.06
L-serine	4.66

Table 5.6. continued

SUBSTRATE	VARIATION EXPLAINED BY AGE (%)
β-Methyl-D-Glucoside	4.23
D-mannitol	4.16
D-Malic acid	3.87
D-cellobiose	3.51
Putrescine	3.34
N-Acetyl-D-Glucosamine	2.76
D-Galactonic Acid γ-Lactone	2.71
L-Asparagine	2.46
Glycogen	2.19
Pyruvic acid methyl ester	1.93
α-Ketobutyric Acid	1.69
D,L-α-Glycerol Phosphate	0.52
D-Xylose	0.24
α-Cyclodextrin	0
4-Hydroxy Benzoic Acid	0
γ-Hydroxybutyric Acid	0
Itaconic Acid	0
Phenylethylamine	0

Substrates with low percentage values, i.e. <5%, denote compounds whose variation in utilization was less sensitive to age. These substrates generally had extremely low frequencies of utilization as they were used by only a few isolates therefore, an accurate age comparison cannot be made. For the first seven compounds, i.e. glycyl-L-glutamic acid, Tween 80, α-D-lactose, Tween 40, L-arginine, glucose-1-phosphate and L-threonine, greater than 10% of their variation was explained as a factor of age (Table 5.6).

Further analysis of these seven compounds using CCA was then conducted (Figures 5.12–5.15).

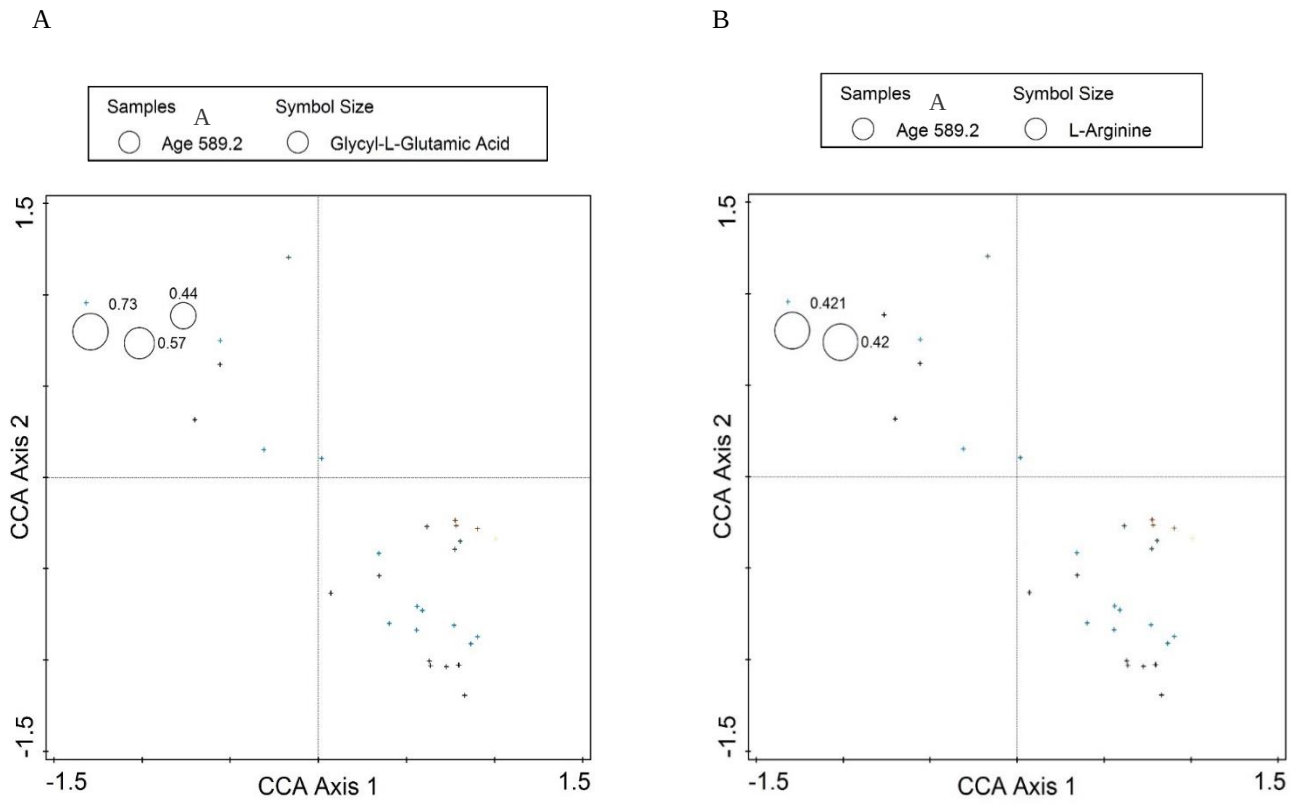


Figure 5.12. CCA plots of substrates which were utilized by AEFB isolates exclusively from sample depth A (A) Glycyl-L-glutamic acid and (B) L-arginine. The size of the symbols used to depict sample depth age and a specific substrate correlates to the degree of utilization. Numerical values on the graph represent the transformed OD_{590} values for isolates which were able to utilize the respective substrate.

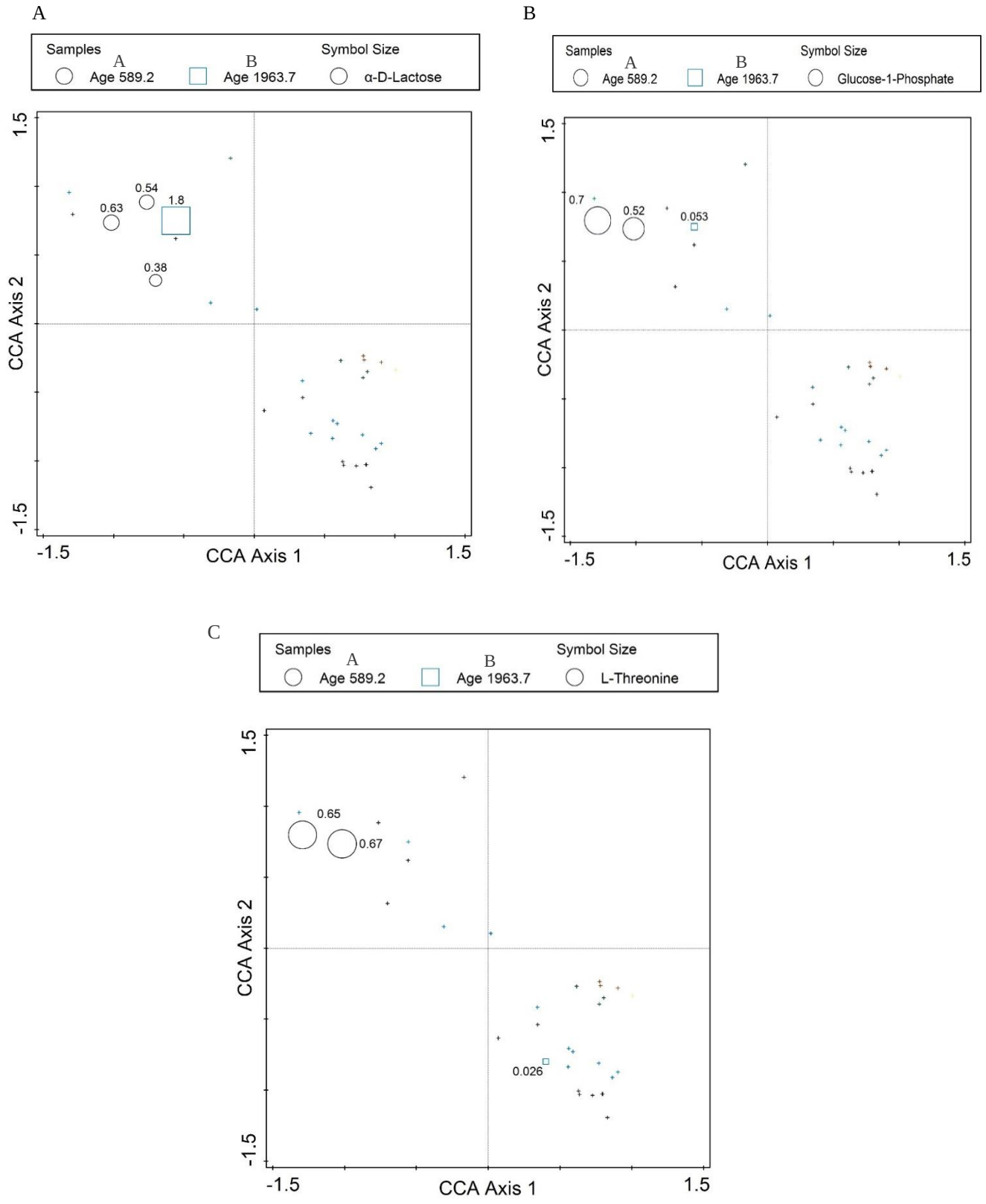


Figure 5.13. CCA plots of substrates utilized by AEFB isolates exclusively from sample depths A and B for (A) α-D-lactose, (B) Glucose-1-phosphate and (C) L-threonine

Glycyl-L-Glutamic acid and L-arginine were exclusively utilized by isolates from the 589 cal year BP sample, whilst α-D-lactose, glucose-1-phosphate and L-threonine were utilized solely by isolates from the two younger age depths, namely from the 589 and 1 964 cal year BP samples (Figures 5.12–5.13).

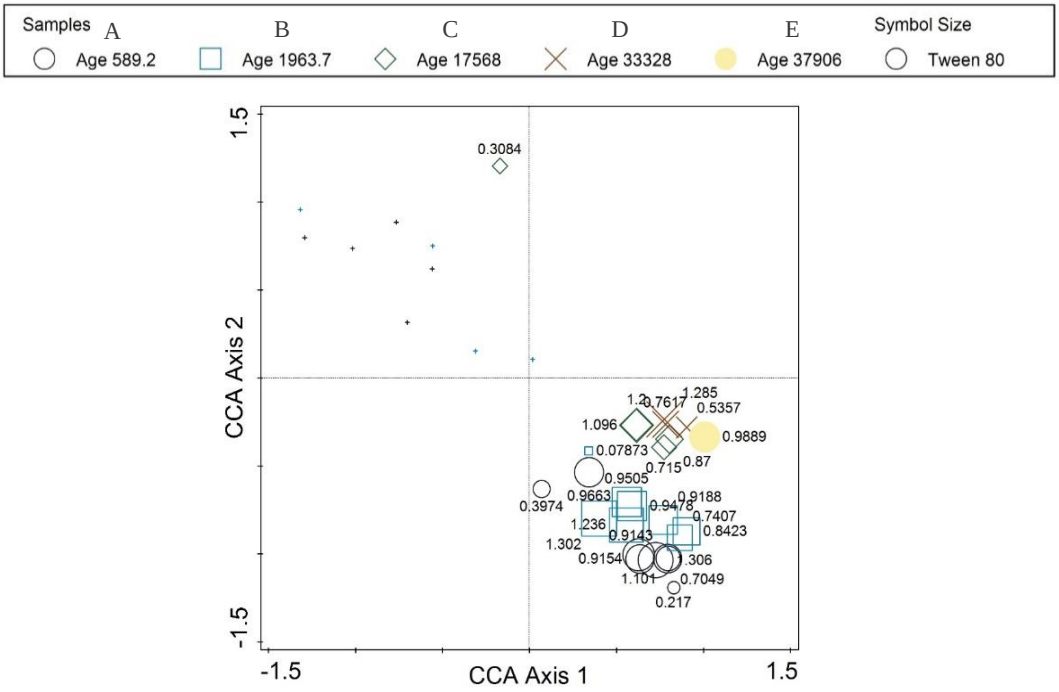


Figure 5.14. CCA plot displaying utilization of Tween 80 across sampling age

It was established that Tween 80 was a highly utilized substrate amongst the isolates from each of the different sample depths (Figure 5.14). This was confirmed by the high percentage frequency of 71.40% (Table 5.5). Isolates from all depths were able to use Tween 80 as a carbon source. All isolates tested from sample depths C, D and E used this substrate, whereas only 38.50% of isolates from both the A and B sample depths were unable to utilize Tween 80.

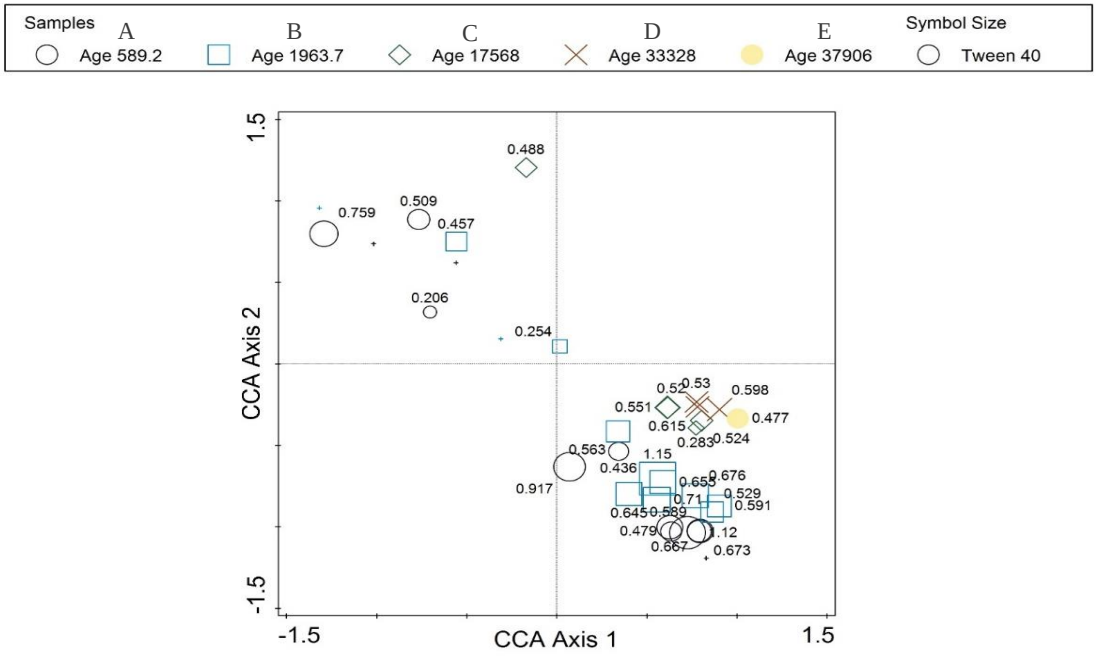


Figure 5.15. CCA plot displaying utilization of Tween 40 across sampling age

Similarly, tween 40 was highly utilized amongst isolates from each of the different sample depths (Figure 5.15). This substrate was utilized at a high frequency, with 82.90% of isolates exhibiting the ability to use this compound as a carbon source (Table 5.5). Whilst all isolates from sample depths C, D and E used this substrate, 23.08% of isolates from both the A and B sample depths were unable to do so. The degree of utilization for this substrate was high, with the transformed OD_{590} values for this compound having an average of 0.59, indicating a significant level of usage by most isolates.

5.4 DISCUSSION

The physiological characteristics of AEFB associated with peatlands are relatively unknown. Determination of these characteristics is of interest when dealing with revived bacteria from ancient samples, since their physiological capabilities may differ significantly from active bacterial populations, thereby providing a possible insight into past conditions. For the current study, physiological profiling was undertaken to establish substrate utilization capabilities, salinity tolerances and pH range tolerances of isolates representative of the taxonomic diversity from varying depths along the sediment core. This served to provide a representation of the physiological profiles of AEFB species present in the region.

At each sample depth evaluated, isolates displayed a range of tolerances to the salt concentrations tested (Table 5.1, Figure 5.1). Bacteria may be classified as non-halophiles (grow at 0-2% NaCl concentration), slight halophiles (2-5%), moderate halophiles (5-10%), extreme halophiles (≥10%) and halotolerant (capable of growth in saline conditions but can survive without salt) (Ollivier *et al*., 1994; Agwu and Oluwagunke, 2014). Approximately 11.4% of isolates were non-halophilic and were present at the 589, 1 964 and 33 328 cal year BP samples. Forty percent of isolates tolerated a 10% salt-based medium, indicating the presence of halotolerant bacteria. Isolate B1, from the 1 964 cal year BP sample, was the only strictly halophilic isolate present. The presence of these bacteria was expected as the use of Marine Agar during isolation selected for salt-tolerant bacteria. It is not uncommon for the Mfabeni Peatland to experience changes in salinity, which is caused through fluctuations in water levels (MacKay *et al*., 2010). The history of this region reveals occurrences of saline intrusion at these time periods. Water levels in the region have been influenced by climate changes and anthropogenic activities (Finch and Hill, 2008; Clulow *et al*., 2013; Grundling *et al*., 2013a). Rises in salinity levels could thus support the presence of halotolerant and halophilic bacteria.

pH tolerance testing revealed that majority of isolates from all depths were found to prefer neutral to slightly basic pH (Figure 5.3–5.4). Only 26% of isolates displayed growth in the acidic pH range tested (3–5.5). This was not surprising since AEFB were selectively isolated on media with a neutral baseline pH. Acidotolerant bacteria were isolated from all sample depths except the 37 906 cal year BP sample. Approximately 34% of isolates were able to grow at a pH of 8.5–10. A potential reason for the occurrence of alkalitolerant bacteria in an acidic environment is the presence of temporary alkalinity that can occur within microhabitats as a result of biological activities such as sulfate reduction and ammonification (Grant *et al*., 1990).

In addition, the accumulation of organic matter in these regions and the presence of biofilms may contribute to a pH-protected environment. However, the types of alkalitolerant bacteria which are able to survive within such environments are often restricted to more resistant organisms, such as endospore-forming bacteria, which are able to persist during adverse growth conditions (Grant *et al*., 1990).

Redundancy analysis revealed that isolate responses to varying salt concentrations and pH across all sample depths were not significantly different to each other. This suggests that salinity and pH conditions may not have been primary environmental factors affecting physiological diversity between the isolates. This could suggest that salinity and pH conditions in the region may not have changed significantly over time.

The use of microtiter plates served as an efficient means of screening multiple isolates across several NaCl concentrations and pH values, in duplicate, with the inclusion of controls. This approach served as an improved alternative to using multiple agar plates or test tubes as it is quicker and less labour-intensive. This method requires fewer materials and allowed for multiple optical density readings to be taken simultaneously. In the current study, inoculum densities were standardized and although this prolonged the procedure, it provided uniformity thereby minimizing differences arising from variation in inoculum density. In the study, optical density was measured to determine growth. As an alternative, redox indicator dyes such as 2,3,5-triphenyltetrazolium chloride (TTC) can be used (Beloti *et al*., 1999). By incorporating redox indicators into the media, colour changes can be detected, which would be used to indicate a positive result. However, utilization of such dyes requires careful selection since dyes such as TTC are pH sensitive and generally are not suitable for pH-based assays (Beloti *et al*., 1999). The application of redox indicator dyes to the microtiter plate assays is an area that could be explored in future studies. Further examination could provide information into whether these dyes have the potential to be more sensitive and provide measures of growth changes over time.

Heterotrophic bacteria residing in peatlands generally utilize organic matter derived from plant material as a primary source of carbon and energy (Reddy and DeLaune, 2008). The decomposition of plant material in peatlands results in the release of many plant-based compounds into the environment. These may include substrates produced by plant cells, such as carbohydrates, and various constituents of plant cells including amino acids, fatty acids and polyamines. Myers *et al*. (2001) found that substrate utilization and the composition of

microbial communities varied between regions with differing dominant plant species. It was concluded that the availability and composition of organic compounds from plant material will affect the diversity and composition of microbial species present. There is evidence suggesting that the Mfabeni Peatland has experienced major changes in plant species over the course of its history (Finch and Hill, 2008). Consequently, any changes in plant communities over time could be expected to have a significant impact on the types and quantities of organic compounds being released into the surrounding environment. This, in turn, could be expected to influence microbial diversity and substrate utilization responses.

The metabolic capabilities of selected AEFB were evaluated using Biolog Ecoplate™ assays. Twenty-seven of the thirty-one substrates tested were utilized, to varying degrees, by representatives of the AEFB screened. Tween 40 and Tween 80 were the two most widely utlized substrates with approximately 83% and 71% of the isolates tested being able to use these compounds respectively (Table 5.5). These substrates were also utilized readily, i.e. they had good utilization responses, as indicated by the high average OD_{595} readings, indicating that these substrates were favoured. Tween 40 and Tween 80 are polysorbates (non-ionic surfactants) derived from ethoxylated sorbitan which is esterified with fatty acids (Fredrickson *et al*., 1991; Chou *et al*., 2005). The fatty acids present in Tween 40 and Tween 80 are palmitic acid and oleic acid respectively (Lojda *et al*., 2012). Palmitic acid is the most common saturated fatty acid associated with microorganisms, plants and animals (Gunstone *et al*., 2007). Oleic acid is a naturally occurring fatty acid present in animals and plants (Rustan and Drevon, 2005). These substrates were utilized by representative AEFB from all five sample depths evaluated. This finding suggests that AEFB populations within the peatland environment may play an important role in fatty acid metabolism.

Heterotrophic bacteria isolated from sediment and water samples from a Pleistocene sand aquifer (Kolbel-Boelke *et al*., 1988), as well as Atlantic coastal plain sediments (Fredrickson *et al*., 1991), have also shown a strong signature for Tween 40 utilization. Similarly, Tween 80 has also been found to be commonly used by subsurface bacteria from Atlantic coastal plain sediments (Fredrickson *et al*., 1991).

The polymers α-cyclodextrin and glycogen were utilized to a relatively low degree and only by isolates from the first three sample depths i.e. from ages of 589 to 17 568 cal years BP. Glycogen is a glucose polysaccharide which is used for energy storage in fungi, animals and in bacteria when excess carbon is present and other nutrients may be limiting (Bourassa and

Camilli, 2009). Cyclodextrin is produced during the bacterial degradation of cellulose, which occurs predominantly in plants and in the intestinal tracts of herbivores (Lengeler *et al*., 2009). This could possibly represent AEFB introduced into the environment via animal matter. Cyclodextrin can occur in one of three chemical configurations, namely alpha, beta and gamma, and bacterial utilization of this compound is influenced by the form which is present (Oros *et al*., 1990). α-Cyclodextrin has the highest degree of rigidity of the three forms, with γ-cyclodextrin being more readily utilized by bacteria due to its less rigid, more flexible structure (Oros *et al*., 1990). Therefore, the degree of consumption would have varied based on the type of cyclodextrin tested and this may account for the low level of utilization of this polymer.

The most commonly utilized carbohydrate (ca. 46% frequency) was D-xylose, with representative isolates from four of the five depths using this substrate. D-xylose is a pentose sugar which is present in hemicellulose and occurs in wood and plant biomass and would thus be expected to be present or released during plant biomass decomposition (Chen, 2014). D-xylose is converted into D-xylulose within bacterial cells, the phosphorylated form of which is utilized in the glycolytic and pentose phosphate pathways for energy derivation (Glazer and Nikaido, 2007).

N-acetyl-D-glucosamine, a glucose derivative, was utilized by 14% of the isolates tested. This compound is a monomeric unit that makes up the polymer chitin which is present in fungi, insects and crustaceans (Fraser-Reid *et al*., 2012). This compound is also found in bacteria as part of the peptidoglycan component of the cell wall (Fraser-Reid *et al*., 2012). The degradation of this compound results in nitrogen and carbon release, thereby making it a useful energy source (Fraser-Reid *et al*., 2012).

D-mannintol (present in plants), β-methyl-D-glucoside (monosaccharide produced from glucose), D-cellobiose (plant derived glucose disaccharide) and D,L-α-glycerol phosphate (phosphoric glycerol ester) were not widely utilized by AEFB, with the latter two being utilized solely by a small proportion of bacteria from the 589 and 1 964 cal year BP samples only. It was also determined that α-D-lactose was only utilized by four isolates from the 589 and 1 964 cal year BP sample depths. This compound is not commonly associated with aquatic or terrestrial environments and is usually associated with milk and lactating animals (Robinson, 2005). It is possible that the AEFB that utilize this substrate could represent bacterial

populations which were introduced into the peatland from exogenous sources (e.g. via faecal matter).

Putrescine, a polyamine, was used exclusively by isolates from the 589 and 1 964 cal year BP samples. Putrescine is one of the more common polyamines present in plants (Tiburcio *et al*., 1993) and may, therefore, become available in the environment as a result of plant decay and decomposition. This compound plays a role in replication, cell growth and siderophore biosynthesis (Shah and Swiatlo, 2008). It is also responsible for protein synthesis stimulation and the induction of 30S ribosomal subunit assembly (Yoshida *et al*., 2008). Putrescine, being a nitrogenous compound, also serves as a source of nitrogen, in addition to carbon, for bacterial cells (Bachrach and Heimer, 1989). The other amine, phenylethylamine, was poorly utilized by a single isolate, suggesting that it is not a favoured or readily available carbon source.

Amino acids are used for the synthesis of proteins and are present in plant and animal cells (Hildebrandt *et al*., 2015). The amino acid substrates were utilized to a low degree, indicating that they may not be abundantly available or favoured as an energy source by the isolates tested. The highest utilization occurred by isolates from the 589 cal year BP depth. Of these, L-asparagine was the most frequently utilized. L-asparagine undergoes degradation in bacterial cells to yield oxaloacetate, a key component of the citric acid cycle (Barton, 2005). The second most utilized amino acid was L-serine, which is used for pyrimidine and purine biosynthesis and serves as a precursor to amino acids such as tryptophan and glycine (Litwack, 2008). With the exclusion of L-phenylalanine, which was not used by any isolates, L-arginine, an amino acid used in the biosynthesis of polyamines such as putrescine (Bachrach and Heimer, 1989), was the least utilized (frequency 5.7%). In contrast to the carboxylic acids and carbohydrates, amino acids are rich in nitrogen (Madigan *et al*., 1997). These results suggest that the larger molecules with higher carbon contents (carboxylic acids and carbohydrates) were preferred over the nitrogen sources with lower carbon contents (amino acids).

Pyruvic acid methyl ester (ester produced from carboxylic acid) was the third most utilized substrate, with isolates from depths of ages 589 to 33 328 cal year BP using it as a carbon source. Methyl esters make up animal fats and plant-based oil compounds and may, therefore, be present in organic matter (Fugmann *et al*., 2014). Dissociation of the hydrogen atom in pyruvic acid yields pyruvate, a necessary component of the citric acid cycle required for energy production (Barton, 2005). Conversion of pyruvic acid back to glucose through gluconeogenesis aids cells during glucose deficiency. In addition, pyruvic acid is used to

produce alanine within cells (Schaechter, 2009). The high utilization of this compound by most of the isolates tested was not unexpected due to its necessary functions within aerobically respiring organisms. Carboxylic acids accumulate abundantly in animal and plant cells (Baxter *et al*., 1998). From the substrates belonging to the carboxylic acids group, D-galactonic acid γ-lactone was the most utilized substrate, with representative isolates from all depths being able to use this substrate as a carbon source. Two-hydroxy benzoic acid and α-ketobutyric acid were both moderately used. The remaining carboxylic acids, namely γ-hydroxybutyric acid and D-malic acid, were utilized poorly, suggesting that these were not favoured as carbon sources. Compared to the other substrate classes, the carboxylic acids display the greatest diversity as a result of the various chemical configurations and molecular weights (Christian and Lind, 2007). This could account for the variation between the high utilization of some carboxylic acids such as D-galactonic acid γ-lactone and low utilization of others.

Multivariate analysis was performed to determine of patterns of utilization amongst the isolates. It was revealed that the age of the sample from which the isolates were obtained had an influence on the differences in substrate utilization patterns. This could be seen with glycyl-L-glutamic acid and L-arginine which were only utilized by isolates from the 589 cal year BP sample (Figure 5.12) and substrates such as α-D-lactose, glucose-1-phosphate and L-threonine, which were solely utilized by isolates from the upper two depths of the sediment core (Figure 5.13). Isolates from these two depths had more diversity and variability in their substrate utilization capabilities (Figure 5.10), which allowed for utilization of a wider range of carbon sources.

A limitation of the approach used in the current study is that only a proportion of the total revived bacteria were screened for physiological profiling. However, representative isolates differentiated at the species-level were selected, enabling differences in metabolic profiles between the different species revived to be established. Fredrickson *et al*. (1991) found that even when a select proportion of isolates from core samples were chosen for physiological screening, it was sufficient to allow descriptions regarding diversity to be made. This was also observed by Bianchi and Bianchi (1982), who found that diversity could be established with numbers of isolates ranging from 20–30.

Whilst the use of Biolog Ecoplates™ for physiological profiling has proven to be a reproducible and effective method for the determination of microbial metabolic and functional diversity, there are several considerations which must be taken into account if the technique is to be

employed (Hill *et al*., 2000). The rate of colour development within the wells is dependent upon the initial inoculum density used. One way to avoid the effect of variation in initial densities, and the one which was applied in the current study, is through a direct adjustment of the concentration of the inoculum (Zak *et al*., 1994; Garland, 1997; Hill *et al*., 2000; Preston-Mafham *et al*., 2002). Whilst this may lengthen the procedure, it is not difficult to achieve when pure cultures are being used and allows for an increased accuracy of the results. This was selected as an alternative to the application of the transformed average well colour development (AWCD) formula. This transformation allows for a comparison of standardized patterns, which is recommended when environmental samples are used as the inoculum (Garland and Mills, 1991). In the current study, the aim was to determine metabolic profiles of individual isolates and not to compare communities from raw environmental samples. Furthermore, the validity behind the AWCD transformation is one which is still debated (Bossio and Scow, 1995; Howard, 1997) and has been shown to sometimes result in a skewing of data (Ellis *et al*., 2001). Another factor for consideration is the incubation period for colour development. Haack *et al*. (1995) state that the total cell count should reach an approximate value of 10^8 cells/ml for colour development to occur. To account for the lag phase, microtiter plate readings should not be taken too soon as this could lead to resulting false negatives (Hill *et al*., 2000). This was avoided in the current study by monitoring colour development over a number of days, after which final readings at day seven were used for statistical analysis, as this was determined as the time by which colour development had sufficiently occurred. In the current study, the Biolog EcoPlate™ assay was adapted to obtain physiological profiles for individual isolates as opposed to entire communities. This avoided the issue which arises when dominant, fast-growing species within an environmental sample mask strains which are incapable of using certain substrates (Garland and Mills, 1991; Bossio and Scow, 1995). In this way, an unbiased profile for each of the selected isolates was determined. Furthermore, reproducibility of response patterns to substrates when using pure cultures is extremely high with this technique (Haack *et al*., 1995).

It can be concluded that responses to variation in salinity and pH were similar between the groups of isolates from the different depths, suggesting that these two environmental factors did not influence diversity across time periods. It was evident that sample age did have an impact on the substrate utilization profiles for some of the carbon sources tested, thereby indicating that over time, the utilization of certain substrates changed. This could potentially be due to a change in the availability of these substrates in the region over time or due to the

change in AEFB species at each depth, with different species having the ability to utilize varying substrates. Biolog EcoPlates™ are effective tools for gaining an insight into the metabolic capabilities of bacterial isolates and statistical analysis of the subsequent data is necessary to allow these substrate utilization profiles to be correlated to environmental variables, thereby enabling meaningful conclusions to be drawn.

Physiological profiling is necessary to determine the metabolic capabilities of isolates, in particular, from environmental regions such as peatlands, which have not been explored in great detail. This is necessary in freshwater ecosystems, in which heterotrophic bacterial utilization of various organic carbon sources has an impact on the internal cycling of nutrients (Christian and Lind, 2007). Physiological profiling can provide information regarding the versatility of bacteria in utilizing different compounds and how utilization of these compounds may change over time. It can allow inferences to be made regarding the types of carbon sources occurring in the regions from which the bacteria were isolated and aid in understanding which substrates present in organic matter are consumed. Physiological capabilities may potentially provide information in relation to past physico-chemical conditions of the region of isolation (Jones *et al*., 1993). Furthermore, the discovery of the utilization and degradation of unique compounds could have applications in bioremediation and biotechnology.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSIONS

6.1. RESEARCH OVERVIEW

Palaeo-ecological reconstructions are able to provide an insight into previous environmental conditions, including the nature of past communities (Gorham *et al*., 2001). These studies are, therefore, able to afford an insight into the past which can then be extrapolated to a range of applications, including an assessment of the impacts of climate changes on ecosystems, the effects of anthropogenic influences on particular regions and ecosystem recovery in response to these impacts.

The strength and accuracy of such reconstructions are largely governed by the selected palaeo-ecological indicator, or proxy, which is used (Meadows, 2014). It is important to screen for new proxies since they may potentially provide further information regarding past conditions and, when used in combination with existing proxies, have the potential to improve the confidence in the assessments made and conclusions drawn in palaeo-ecological studies (Meadows, 2014). Endospore-forming bacteria have the ability to form dormant structures which could serve as potential candidates for these studies.

Dormant endospores, formed by AEFB at the onset of unfavourable anaerobic conditions, may become trapped within layers of accumulating organic material within peatlands. AEFB which are isolated from ancient deposits that have remained under anaerobic conditions would not be a part of a metabolically-active population. Due to the ability of endospores to adhere to organic matter, and the ability of sediment to serve as an archival material (Wunderlin *et al*., 2014), it was hypothesized that endospores would become preserved within sediment layers and would remain in a static, unaltered state. These factors would also prevent the dispersal of endospores over a wide range and allow for the inference that the AEFB are likely to have an age commensurate to the radiocarbon date determined for the sample from which they were isolated. In this way, the survival of dormant endospores over extended periods within ecosystems may provide insight into the diversity and physiological activities of AEFB over time, in relation to the changing physico-chemical conditions of the surrounding environment.

This study was undertaken with the aim of reviving and determining the genetic and physiological diversity amongst dormant aerobic endospore-forming bacteria (AEFB) present in sections of an ancient sediment core extracted from the Mfabeni Peatland. Furthermore, the

study served as a screening tool to establish if dormant endospores fulfil the primary requirements of a palaeo-ecological proxy.

The Mfabeni Peatland in KwaZulu-Natal, South Africa is suitable for palaeo-environmental research (Finch and Hill, 2008) and was therefore selected as the study site. This peatland is one of the oldest known active peatland regions in Africa, in which peat accumulation began approximately 44 000 cal years BP (Finch and Hill, 2008). This site is a part of one of the most significant peatland ecoregions in South Africa and contains a vast biological diversity (Grundling *et al*., 2013a). However, to date, this region has not been explored from a microbiological perspective and there is a paucity of research regarding the microbial diversity in this area. The age and stability of the region made it a suitable study site for the examination of changing AEFB diversity over time and afforded an opportunity to examine a potentially new proxy which could be applied in palaeo-environmental studies.

6.2. SUMMARY OF FINDINGS

The first part of the study involved revival and isolation of AEFB from five depths along a sediment core. Three procedures were used to extract endospores from sediment particle matrixes to determine which yielded the highest counts of AEFB. The optimal procedure was then applied to screen samples from the five depths, followed by plating onto four different media types to achieve growth and maximize isolation. The study explored two means of diversity analysis of AEFB isolates, namely, Rep-PCR and HRMA. Rep-PCR provided a strain-level resolution of the isolates and was used for classification of isolates into OTUs. Phylogenetic analysis was employed to determine the evolutionary relationships present between representatives of each OTU. As part of physiological profiling, a protocol involving microtiter plate assays was developed to test salinity and pH tolerance ranges of selected AEFB isolates. Lastly, metabolic diversity was evaluated by testing the substrate utilization capabilities of selected isolates using Biolog EcoPlates™.

Based on the findings of this study, the following was established:

♦ AEFB were present at all five sample depths along the Mfabeni Peatland sediment core, ranging in age from 589 to 37 906 cal years BP. This indicates that ancient sedimentary deposits do serve as a reservoir for dormant endospores. It was found that a bead-beating, buffer-mediated technique based on a modified indirect DNA extraction protocol yielded the highest CFU/g values based on preliminary testing. Extraction of endospores from different types of

sediment could be carried out using this technique to determine if it could be applied across a wider spectrum of archival material.

♦ The decreasing trend observed in CFU/g with increasing sample age indicates that rates of survival of endospores decrease as they lose viability over time. The higher numbers of AEFB observed at the shallowest depth may indicate that AEFB populations at this depth are influenced by a close proximity to oxic conditions. The survival of AEFB at a depth of 344 cm, albeit at very low CFU/g, suggests that the accumulating layers of organic material within peatlands do serve to trap and preserve dormant endospores over extended periods of time.

♦ Most OTUs were unique to the media types upon which they were isolated, demonstrating the diverse nutritional requirements amongst the AEFB isolates. A large proportion of OTUs would not have been isolated if only a single type of media had been selected. This was an important finding since several studies use only a single media type for isolation (Fredrickson *et al*., 1991; Shivaji *et al*., 2011). It was also found that the nutritionally-limited 10% TSA and R2A media and environmentally-based Marine Agar were able to support a higher level of OTU diversity. This was not unexpected since many environmental organisms are adapted to the nutrient-limiting conditions which are often prevalent in natural environments.

♦ Rep-PCR was found to be an effective tool for providing strain-level resolution of isolates. Fingerprinting profiles could be easily compared allowing for the differentiation of isolates into OTUs. HRMA was found to be suitable for comparing and grouping closely-related isolates and was able to distinguish isolates at a species level. The high throughput and elimination of gel electrophoresis made this a fast and simple technique to conduct. Rotor-Gene™ Software aided in the analysis of the melt curves and provided raw fluorescence data which could then be used for statistical analysis. Both techniques were found to be suitable for the analysis of diversity amongst AEFB in terms of the initial cost and throughput demands. However, for the degree of resolution required for the current study, Rep-PCR was able to provide a higher level of discrimination and differentiation amongst AEFB isolates, allowing for a greater degree of genetic diversity to be distinguished.

♦ Genotyping revealed distinctions in diversity amongst isolates obtained from sample depths of different ages. Based on the results of Rep-PCR, 94% of OTUs were found to be unique to their depths. This supported the notion that endospores are able to remain static and immobile once deposited. This further supports the inference that the endospores revived were of an age equivalent to that of the radiocarbon-dated sample material from which they were isolated. The

high numbers of certain OTUs at some of the depths suggest that these bacteria may have been residing in the ecosystem, as opposed to being transported there.

♦ Several aerobic endospore-forming genera were present along the sediment core. Members of some of the genera were found to be unique to specific sample depths, providing further evidence which discounts endospore migration through the core. For instance, members of the *Lysinibacillus* and *Brevibacillus* genera were only present in the younger sampling depths, whereas members of the *Domibacillus* genus were unique to the older depths. On the other hand, members of the *Bacillus* and *Paenibacillus* genera were present at multiple depths along the sediment core, indicating that they may be abundant in the region. Many isolates also matched species which have been previously isolated from peatlands. Some isolates from the younger sampling depths were taxonomically-similar to isolates from older depths. However, it was found that no common strains were present across the younger and older depths.

♦ The changes in diversity across different sampling depths refute the possibility of carry-through or contamination between the samples. The results support the notion that the bacteria isolated were ancient in origin.

♦ 16S rRNA gene sequencing results also revealed a proportion of isolates with low sequence similarity matches to currently-listed species. This suggests the possibility of some of these isolates being novel species or strains which require further characterization. These results suggest that by examining ecosystems which are able to remain stable over time, it is possible to isolate potentially uncharacterized bacterial species or strains.

♦ Responses of selected AEFB isolates to salinity testing revealed that a high percentage of isolates preferred lower salt concentrations. Halotolerant bacteria were also present throughout the core. Water fluctuations within peatlands have direct impacts on salinity levels, which may have an influence on the residing AEFB.

♦ pH testing revealed that a high percentage of isolates favoured near-neutral to basic pH. This could be attributed to the initial cultivation media used, which were neutral-based, thereby favouring the selection of neutrophiles. In addition, the presence of alkaline microenvironments within the peat may support the growth of alkaliphiles. A higher proportion of acidophiles may be isolated by enriching for these bacteria with the initial cultivation media used. This could allow for the bacteria, which are able to thrive in the acidic peat, to be isolated.

♦ Overall, it was determined that salinity and pH were not major factors driving the changes in diversity between isolates from different depths. This could suggest the possibility of

relatively stable salinity and pH conditions over time within the region. For future studies, temperature tolerance ranges can be tested to determine the effect of this environmental parameter on AEFB diversity.

♦ Biolog EcoPlates™ were found to be a useful tool for determining the substrate utilization capabilities of the isolates against environmentally-relevant substrates. It provided an efficient means of producing results relating to metabolic diversity, which could then be statistically analyzed for comparison between isolates of different depths. The utilization of a diverse range of substrates by each isolate demonstrated the varied metabolic capabilities present amongst the AEFB. The most commonly utilized substrates were found to be Tween 40 and Tween 80. The high degree of utilization of these compounds suggests that fatty acid metabolism may be prevalent amongst AEFB populations associated with the peatland environment. Substrates such as D-xylose and pyruvic acid methyl ester were also utilized to a high degree. These are substrates which are associated with decomposing plant material and thus, would be expected to be present in peat.

♦ Distinct patterns of substrate utilization were discerned between isolates from the younger and older depths. Multivariate analysis revealed, with significance, that the age of the sample from which the isolates were obtained did have an effect on the variation observed in substrate utilization patterns amongst isolates. This finding is promising since it supports the notion that past climate changes, which impacted on vegetation and conditions within the peatland, could have influenced the genetic and physiological diversity of the AEFB isolates recovered from different sections of the sediment core.

6.3. DO DORMANT AEFB MEET THE REQUIREMENTS OF A PALAEO-ECOLOGICAL PROXY?

The findings of the current study indicate that dormant AEFB appeared to be well preserved and refutes the possibility or probability of endospore migration along the sediment core through natural means or during the coring process.

AEFB endospores have the potential to enhance the accuracy of palaeo-records since diversity changes can be determined at the strain-level, a level of resolution that is not possible for most proxies (Gorham *et al*., 2001). Furthermore, the use of 'living' proxies also allows for the metabolic and physiological capabilities of AEFB to be determined, thereby providing clues as to the prevailing conditions within the peatland at the time of deposition.

The limitation to using dormant AEFB as a palaeo-ecological proxy is the decrease in abundance with increasing depth. However, it was found that even with declining CFU/g values, patterns of similarities and differences between isolates from different depths could still be established. For example, despite the lower numbers of isolates screened from the older depths for physiological profiling, commonalities in the utilization of the Tween compounds could still be identified.

6.4 ADDITIONAL APPLICATIONS OF ANCIENT AEFB

Revived endospore-forming bacteria could be screened to determine if these organisms possess useful or unique metabolic capabilities. Utilization of compounds such as polymers and hydrocarbons may prove to be useful for biotechnological or bioremediation applications. Furthermore, ancient bacteria may serve as a potential source of novel bioactive compounds including lipopeptides, enzymes or antimicrobial compounds. Therefore, biomining could be used to obtain potentially-novel metabolites which could be beneficial in areas such as pharmacy and agriculture.

Ancient bacteria could have an application in the molecular clocking of genes. Sequence divergence between various housekeeping or protein-coding genes could be used for the comparison of evolutionary rates amongst bacteria (Maughan *et al*., 2002). This may enable differences and mutations in these genes to be detected.

6.5. CONCLUSION

Due to the impact of rapid climate changes on ecosystems, there is a need to find additional proxies to allow for more accurate and reliable environmental reconstructions to be made. Palaeo-ecology is seen as the key to predicting and facing climate changes in the future (Meadows, 2014). The current study demonstrates that dormant AEFB can serve as 'time capsules', which can provide records of the changing microbial diversity across time. Examination of the vicissitudes in aerobic endospore-forming bacterial species abundance and composition over time has the potential to contribute towards the determination of past environments, as well as establish the effects which climate changes have on AEFB populations. Dormant AEFB thus have the potential to be applied in palaeo-ecological studies and can be a powerful tool if used in combination with existing proxies. Ecosystems such as the Mfabeni Peatland, which provide a long-term environmental archive of periods in the

Earth's history, may thus aid in preserving this diversity, thereby allowing for its examination. This provides the potential for ancient bacteria to contribute towards new knowledge.

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APPENDIX A

Physico-chemical characteristics of the sediment core

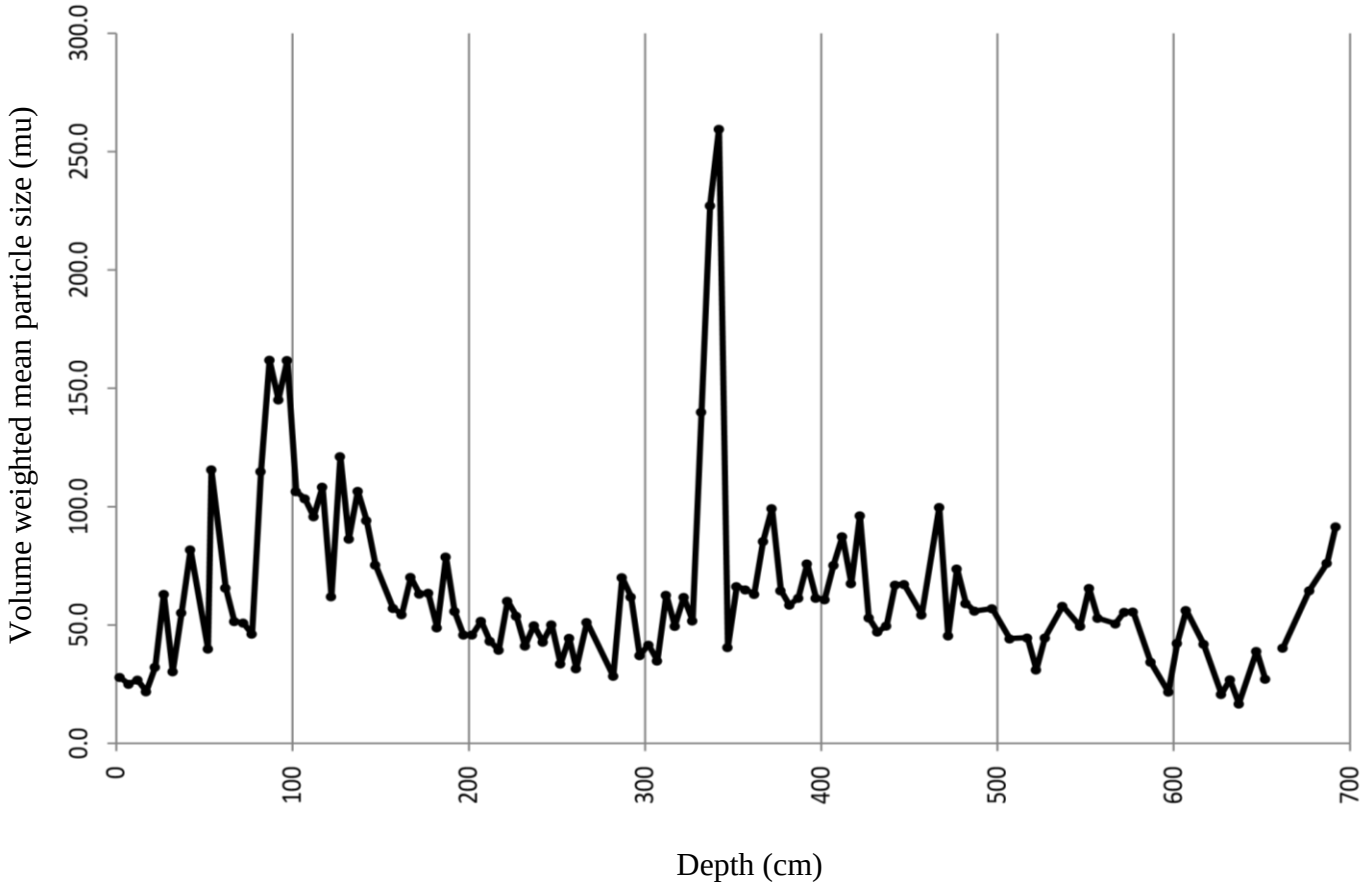

Figure A1. Volume weighted average particle size at each depth along the Mfabeni Peatland sediment core

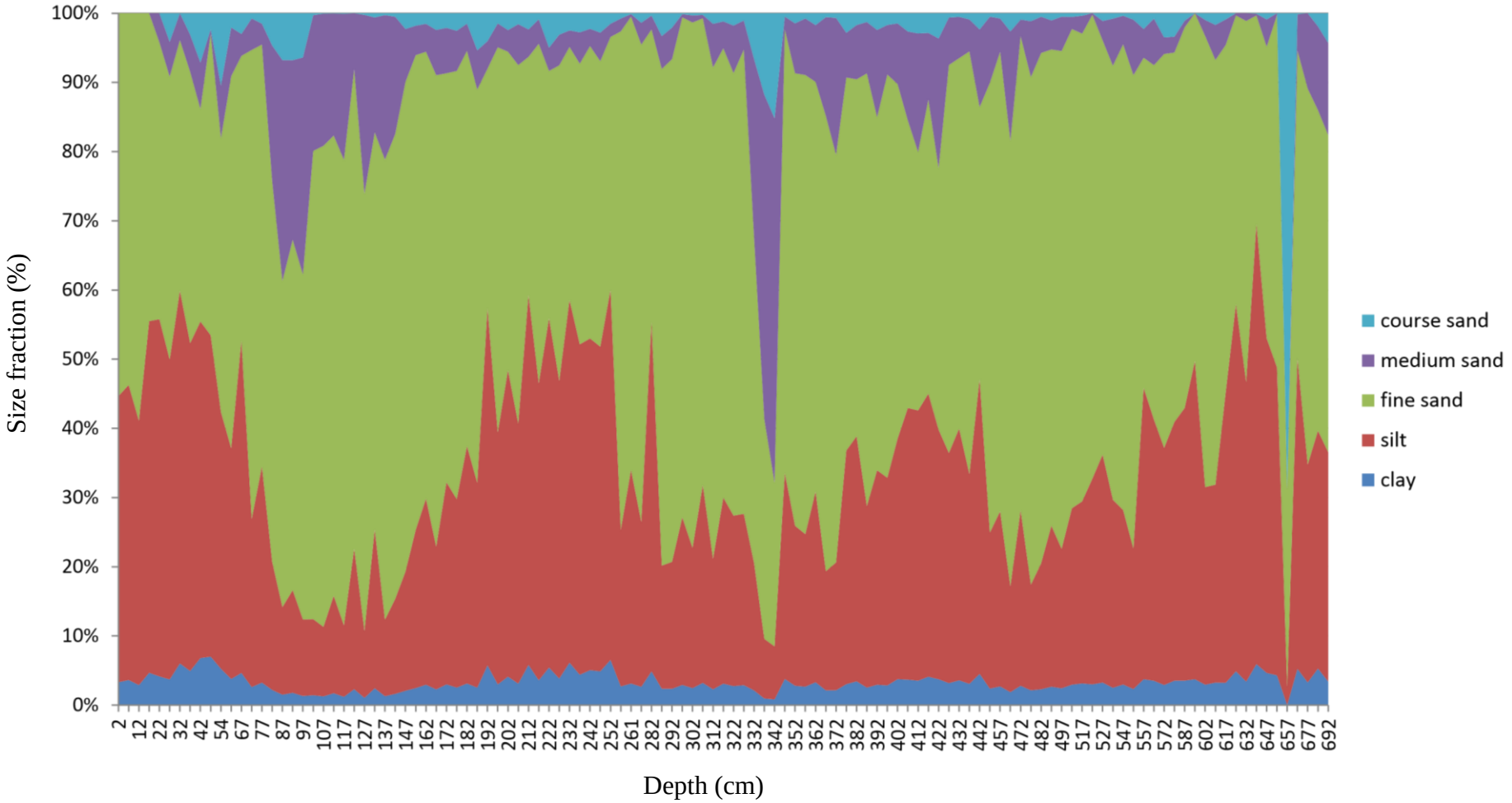

Figure A2. Percentage size fraction of sand, silt and clay along the sediment core

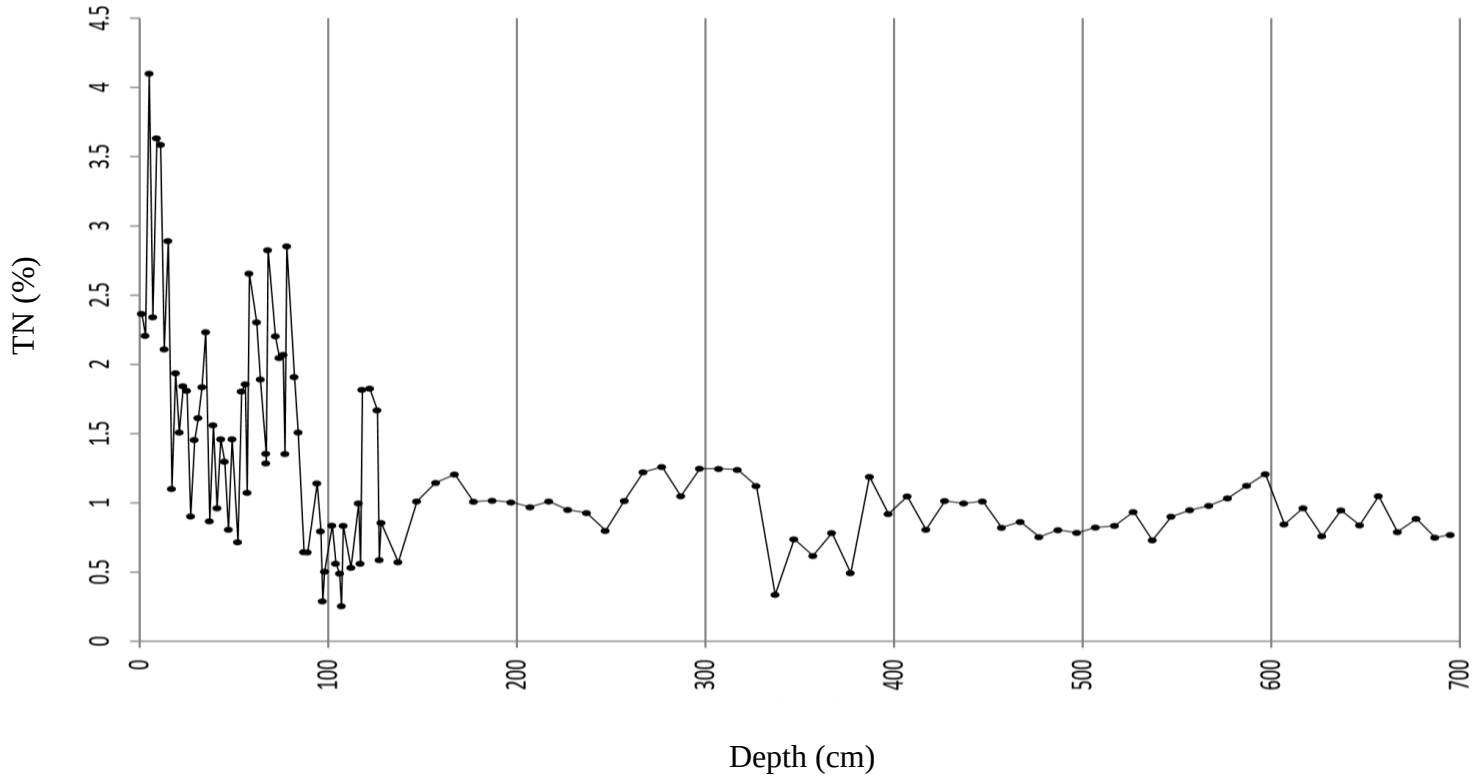

Figure A3. Percentage of total nitrogen at each depth along the sediment core

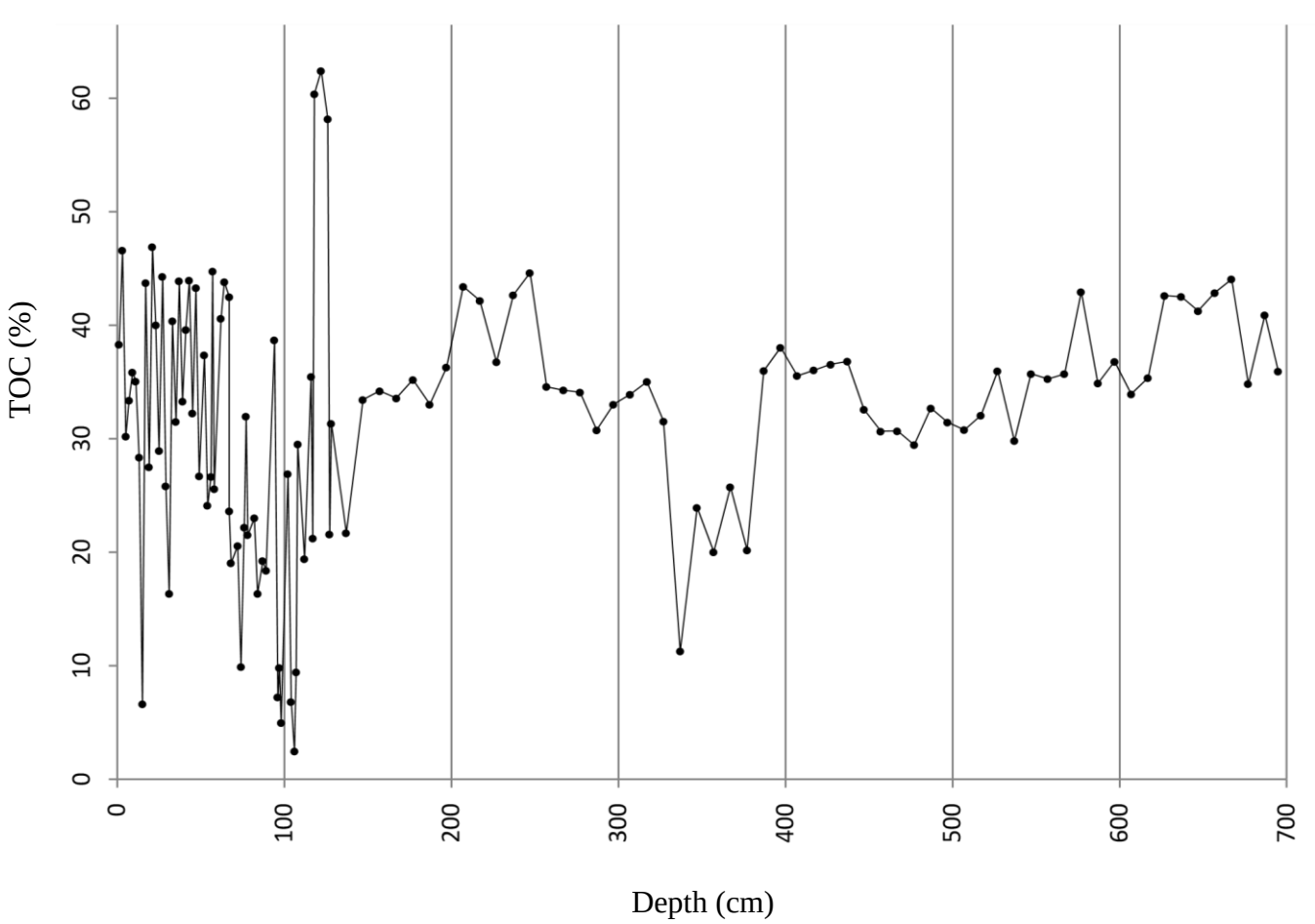

Figure A4. Percentage of total organic carbon at each depth along the sediment core

SEDIMENT pH TESTING

pH tests were conducted for sediment samples for the five depths under examination. Two grams of sediment were added to 5 ml 0.01 M calcium chloride ($CaCl_2$) solution in flasks to create a 1: 2.5 ratio of sediment: $CaCl_2$ solution (Ryti, 1965). Flasks were shaken at 120 rpm for 30 min. The pH was then measured at room temperature using a calibrated pH meter (Basic 20, Crison, Spain) while stirring.

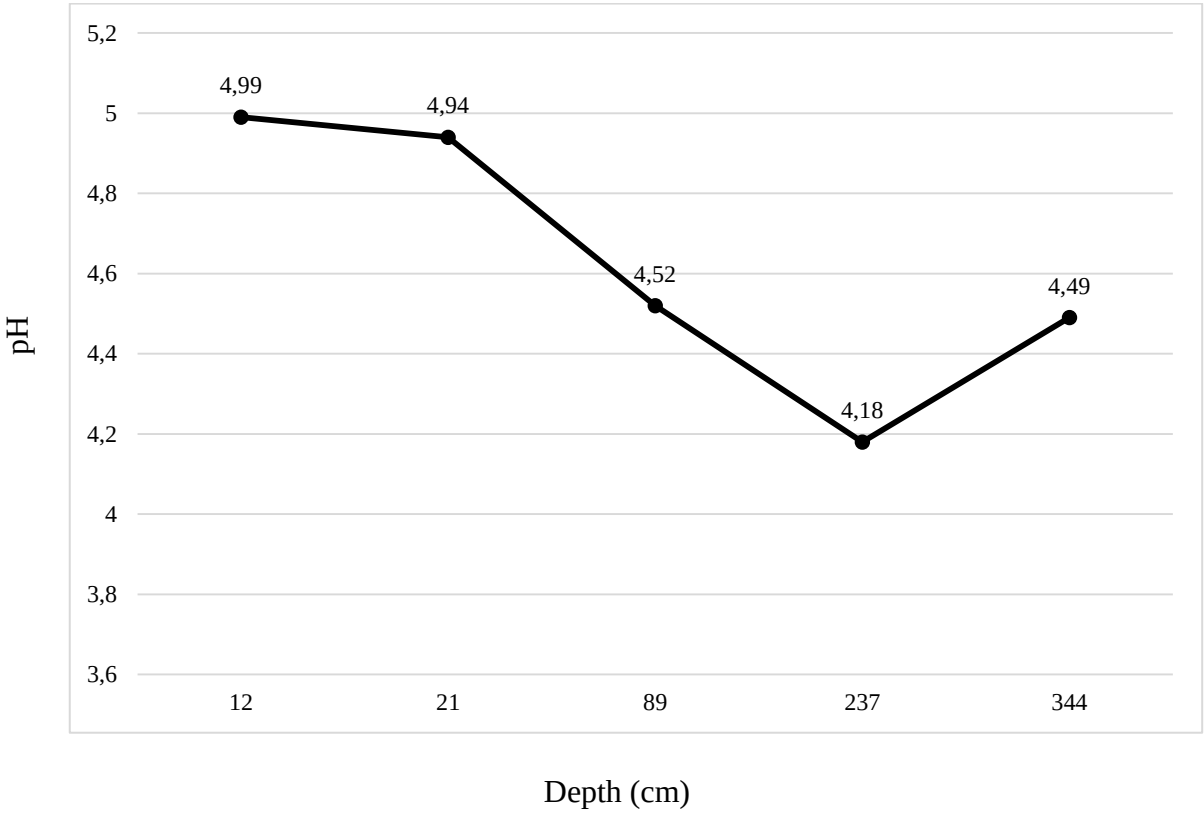

Figure A5. pH values of each depth selected for examination in the current study

APPENDIX A continued

Identity matches of selected isolates to currently-listed sequences in the Genbank database as obtained through a nucleotide BLAST search are provided in Tables A1–A5.

Table A1. BLAST similarity matches and percentage identity scores of isolates from sample depth A (12 cm)

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
Marine Agar	A1	1	*Bacillus aquimaris* strain TF-12 *Bacillus vietnamensis* strain 15-1	99 99	0.0 0.0	NR 025241.1 NR 024808.1
	A5	2	*Paenibacillus* sp. SCSIO N0306	98	0.0	KC978082.1
	A7, A32	7 12	*Bacillus kribbensis* strain BT080	99 93	0.0	NR 043682.1
	A8	4	*Bacillus idriensis* strain SMC 4352-2 *Bacillus kyonggiensis* strain NB22 *Bacillus kyonggiensis* strain NB22	97 97 97	0.0 0.0 0.0	NR 043268.1 NR 132682.1 JF896450.1
	A9	5	*Bacillus aquimaris* strain TF-12 *Bacillus marisflavi* strain TF-11 *Bacillus marisflavi* strain TF-11	96 96 96	0.0 0.0 0.0	NR 025241.1 NR 118437.1 NR 025240.1
	A16	6	*Paenibacillus wooponensis* strain WPCB018	96	0.0	NR 108291.1
	A21	3	Poor sequence (BLAST result omitted)			
	A23	9	*Bacillus aquimaris* strain TF-12 *Bacillus vietnamensis* strain 15-1 *Bacillus vietnamensis* strain NBRC 101237	99 99 99	0.0 0.0 0.0	NR 025241.1 NR 024808.1 NR 113995.1
	A24	8	*Bacillus hwajinpoensis* strain SW-72	99	0.0	NR 025264.1
	A25	10	*Bacillus pumilus* strain ATCC 7061, *Bacillus pumilus* strain NBRC 12092, *Bacillus safensis strain* NBRC 100820,	99 99 99	0.0 0.0 0.0	NR 043242.1 NR 112637.1 NR 113945.1
	A28	11	*Paenibacillus alkaliterrae* strain KSL-134 *Paenibacillus harenae* strain B519	98 98	0.0 0.0	NR 043293.1 NR 043220.1
10% TSA	A34	29	*Paenibacillus vulneris* strain CCUG 53270	95	0.0	NR 117618.1
	A35	30	*Paenibacillus aestuarii* strain CJ25 *Paenibacillus* sp. R55 16S	96 96	0.0 0.0	NR 116365.1 KP056549.1
	A37	31	*Paenibacillus graminis* strain DSM 15220 *Paenibacillus jilunlii* strain Be17 *Paenibacillus sonchi* strain X19-5	91 91 91	0.0 0.0 0.0	CP009287.1 NR 108639.1 NR 115751.1

Table A1 continued

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
10% TSA	A39	32	*Bacillus acidicola* strain 105-2 *Bacillus shackletonii* strain LMG 18435	99 99	0.0 0.0	NR 041942.1 NR 025373.1
	A42	33	*Bacillus kribbensis* strain BT080	92	0.0	NR 043682.1
R2A	A43, A91, A115, A117	18 19 20 21	*Brevibacillus brevis* strain NBRC 15304, *Brevibacillus choshinensis* strain NBRC 15518, *Brevibacillus formosus* strain DSM 9885	99 99 99	0.0 0.0 0.0	NR 113763.1 NR 040979.1 NR 041524.1
	A65	16	*Bacillus acidicola* strain 105-2 *Bacillus shackletonii* strain LMG 18435	98 98	0.0 0.0	NR 041942.1 NR 025373.1
	A66	17	*Bacillus acidicola* strain 105-2 *Bacillus shackletonii* strain LMG 18435	99 99	0.0 0.0	NR 041942.1 NR 025373.1
	A79	15	*Brevibacillus formosus* strain NBRC 15716 *Brevibacillus formosus* strain DSM 9885 *Brevibacillus brevis* strain NBRC 15304	99 99 99	0.0 0.0 0.0	NR 113801.1 NR 040979.1 NR 041524.1
	A85, A93	13 14	*Paenibacillus alvei* strain NBRC 3343 *Paenibacillus alvei* strain DSM 29	99 99	0.0 0.0	NR 113577.1 NR 042091.1
	A116	22	*Brevibacillus formosus strain NBRC 15716* *Brevibacillus brevis* strain NBRC 15304, *Brevibacillus formosus* strain DSM 9885	91 91 91	0.0 0.0 0.0	NR 113801.1 NR 113763.1 NR 041524.1
TSA	A98	23	*Brevibacillus brevis* strain NBRC 15304, *Brevibacillus choshinensis* strain NBRC 15518, *Brevibacillus formosus* strain DSM 9885	99 99 99	0.0 0.0 0.0	NR 113763.1 NR 040979.1 NR 041524.1
	A101	25	*Paenibacillus taichungensis* strain BCRC 17757 *Paenibacillus pabuli* strain NBRC 13638 *Paenibacillus pabuli* strain HSCC 492	99 99 99	0.0 0.0 0.0	NR 044428.1 NR 113627.1 NR 040853.1
	A107	26	*Paenibacillus sediminis* strain GTH-3	98	0.0	NR 108601.1
	A108	27	*Lysinibacillus parviboronicapiens* strain NBRC 103144 *Lysinibacillus mangiferihumi* strain M-GX18 *Lysinibacillus parviboronicapiens* strain BAM 582	99 99 99	0.0 0.0 0.0	NR 114213.1 NR 118146.1 NR 041589.1
	A109	28	*Bacillus isronensis* strain B3W22 *Solibacillus silvestris* strain HR3-23 *Bacillus isronensis* strain B3W22	99 99 99	0.0 0.0 0.0	NR 115952.1 NR 028865.1 NR 118049.1
	A110	24	*Paenibacillus alkaliterrae* strain KSL-134 *Paenibacillus harenae* strain B519	98 98	0.0 0.0	NR 043293.1 NR 043220.1

Table A2. BLAST similarity matches and percentage identity scores of isolates from sample depth B (21 cm)

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
Marine Agar	B50	34	*Paenisporosarcina quisquiliarum* strain SK 55	99	0.0	NR 043720.1
			Paenisporosarcina indica strain PN2	99	0.0	NR 108473.1
			Sporosarcina antarctica strain N-05	99	0.0	NR 044122.1
	B51	35	*Paenibacillus alvei* strain NBRC 3343	99	0.0	NR 113577.1
			Paenibacillus alvei strain DSM 29	99	0.0	NR 042091.1
	B52	36	*Bacillus halmapalus* strain DSM 8723	99	0.0	NR 026144.1
			Bacillus tianshenii strain YIM M13235	99	0.0	NR 133704.1
			Bacillus tianshenii strain YIM M13235	99	0.0	KF811034.1
	B53	38	*Paenisporosarcina quisquiliarum* strain SK 55	99	0.0	NR 043720.1
			Paenisporosarcina indica strain PN2	99	0.0	NR 108473.1
	B54	37	*Paenibacillus taichungensis* strain BCRC 17757	99	0.0	NR 044428.1
			Paenibacillus pabuli strain NBRC 13638	99	0.0	NR 113627.1
			Paenibacillus pabuli strain HSCC 492	99	0.0	NR 040853.1
	B6	39	*Bacillus muralis* strain LMG 20238	98	0.0	NR 042083.1
			Bacillus simplex strain LMG 11160	98	0.0	NR 114919.1
			Bacillus simplex strain NBRC 15720	98	0.0	NR 112726.1
10% TSA	B30	47	*Brevibacillus brevis* strain NBRC 15304,	98	0.0	NR 113763.1
			Brevibacillus choshinensis strain NBRC 15518,	98	0.0	NR 040979.1
			Brevibacillus formosus strain DSM 9885	98	0.0	NR 041524.1
	B35	48	*Paenibacillus rigui* strain WPCB173	90	0.0	NR 116517.1
	B45	49	*Brevibacillus brevis* strain NBRC 15304,	99	0.0	NR 113763.1
			Brevibacillus choshinensis strain NBRC 15518,	99	0.0	NR 040979.1
			Brevibacillus formosus strain DSM 9885	99	0.0	NR 041524.1
	B46	50	*Brevibacillus formosus* strain DSM 9885	99	0.0	NR 040979.1
			Brevibacillus formosus strain NBRC 15716	99	0.0	NR 113801.1
			Brevibacillus brevis strain NBRC 15304	99	0.0	NR 041524.1
R2A	B1	40	*Bacillus fortis* strain R-6514	98	0.0	NR 042905.1
			Bacillus fordii strain R-7190	98	0.0	NR 025786.1

Table A2 continued

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
R2A	B7	41	*Bacillus idriensis* strain SMC 4352-2	96	0.0	NR 043268.1
			Bacillus kyonggiensis strain NB22	96	0.0	NR 132682.1
			Bacillus indicus strain Sd/3	96	0.0	NR 029022.1
	B10	42	*Paenibacillus guangzhouensis* strain GSS02	84	0.0	NR 134114.1
			Paenibacillus guangzhouensis strain GSS02	84	0.0	KJ000691.1
	B23, B24	43	*Bacillus toyonensis* BCT-7112	99	0.0	CP006863.1
			Bacillus thuringiensis strain NBRC 101235	99	0.0	NR 112780.1
			Bacillus thuringiensis strain ATCC 10792	99	0.0	NR 114581.1
	B19	44	*Lysinibacillus pakistanensis* strain NCCP-54	99	0.0	NR 113166.1
			Lysinibacillus macroides strain LMG 18474	99	0.0	NR 113166.1
			Lysinibacillus boronitolerans strain NBRC 103108	99	0.0	NR 114207.1
TSA	B11, B63	45 46	*Paenibacillus barcinonensis* strain BP-23	99	0.0	NR 042272.1

Table A3. BLAST similarity matches and percentage identity scores of isolates from sample depth C (89 cm)

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
Marine Agar	C15	52	*Bacillus aquimaris* strain TF-12 *Bacillus vietnamensis* strain 15-1 *Bacillus vietnamensis* strain NBRC 101237	99 99 99	0.0 0.0 0.0	NR 025241.1 NR 024808.1 NR 113995.1
	C24	53	*Domibacillus robiginosus* strain WS 4628	94	0.0	NR 108861.1
10% TSA	C1, C27	61 63	*Bacillus aryabhattai* strain B8W22 *Bacillus megaterium* strain ATCC 14581 *Bacillus megaterium* strain NBRC 15308	99 99 99	0.0 0.0 0.0	NR 115953.1 NR 116873.1 NR 112636.1
	C2	60	*Paenibacillus ehimensis* strain KCTC 3748 *Paenibacillus ehimensis* strain IFO 15659 *Paenibacillus elgii* strain SD17	97 97 97	0.0 0.0 0.0	NR 025666.1 NR 112054.1 NR 115140.1
	C19	59	*Bacillus acidicola* strain 105-2 *Bacillus shackletonii* strain LMG 18435	99 99	0.0 0.0	NR 041942.1 NR 025373.1
	C20	58	*Paenibacillus tarimensis* strain SA-7-6	96	0.0	NR 044102.1
	C27	62	*Bacillus aquimaris* strain TF-12 *Bacillus vietnamensis* strain 15-1 *Bacillus vietnamensis* strain NBRC 101237	99 99 99	0.0 0.0 0.0	NR 025241.1 NR 024808.1 NR 113995.1
R2A	C16	54	*Bacillus subtilis subsp. spizizenii* TU-B-10 *Bacillus subtilis* strain DSM 10 *Bacillus subtilis subsp. inaquosorum* strain BGSC 3A28	99 99 99	0.0 0.0 0.0	CP002905.1 NR 027552.1 KR818221.1
	C18	55	*Bacillus siamensis* KCTC 13613	100	0.0	KT781674.1
	C25	57	*Bacillus muralis* strain LMG 20238 *Bacillus simplex* strain LMG 11160 *Bacillus butanolivorans* strain K9	98 98 98	0.0 0.0 0.0	NR 042083.1 NR 114919.1 NR 044170.1
	C26	56	*Bacillus aquimaris* strain TF-12 *Bacillus vietnamensis* strain 15-1 *Bacillus vietnamensis* strain NBRC 101237	99 99 99	0.0 0.0 0.0	NR 025241.1 NR 024808.1 NR 113995.1
TSA	C13	51	*Bacillus siamensis* KCTC 13613	100	0.0	KT781674.1

Table A4. BLAST similarity matches and percentage identity scores of isolates from sample depth D (237 cm)

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
Marine Agar	D18	65	*Paenisporosarcina indica* strain PN2	99	0.0	NR 108473.1
			Paenisporosarcina quisquiliarum strain SK 55	99	0.0	NR 043720.1
	D23, D21	66 53	*Domibacillus robiginosus* strain WS 4628	99 95	0.0	NR 108861.1
10% TSA	D34	80	*Bacillus toyonensis* strain BCT-7112	100	0.0	NR 121761.1
			Bacillus thuringiensis strain ATCC 10792	100	0.0	NR 114581.1
			Bacillus thuringiensis strain IAM 12077	100	0.0	NR 043403.1
	D41, D48	73 77	*Bacillus idriensis* strain SMC 4352-2	97	0.0	NR 043268.1
			Bacillus kyonggiensis strain NB22	97	0.0	NR 132682.1
	D45, D49	74 78	*Bacillus acidicola* strain 105-2	98	0.0	NR 041942.1
			Bacillus shackletonii strain LMG 18435	98	0.0	NR 025373.1
	D47	75	*Paenibacillus filicis* strain S4	93	0.0	NR 117365.1
			Paenibacillus alkaliterrae strain KSL-134	93	0.0	NR 043293.1
			Paenibacillus mendelii strain C/2	93	0.0	NR 041929.1
	D12, D17	76 79	*Bacillus siamensis KCTC 13613*	99	0.0	KT781674.1
			Bacillus velezensis strain CR-502	99	0.0	AY603658.1
			Bacillus amyloliquefaciens DSM 7	99	0.0	FN597644.1
R2A	D1	67	*Bacillus muralis* strain LMG 20238	98	0.0	NR 042083.1
			Bacillus simplex strain LMG 11160	98	0.0	NR 114919.1
			Bacillus butanolivorans strain K9	98	0.0	NR 044170.1
	D3	64	*Bacillus siamensis KCTC 13613*	99	0.0	KT781674.1
			Bacillus velezensis strain *CR-502*	99	0.0	AY603658.1
			Bacillus amyloliquefaciens DSM7	99	0.0	FN597644.1
	D4, D5	68 69	*Bacillus acidicola* strain 105-2	99	0.0	NR 041942.1
			Bacillus shackletonii strain LMG 18435	99	0.0	NR 025373.1

Table A4 continued

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
TSA	D9	70	*Bacillus subtilis subsp. subtilis* str. 168	99	0.0	CP010052.1
			Bacillus vallismortis strain DSM 11031	99	0.0	NR 024696.1
			Bacillus amyloliquefaciens DSM 7	99	0.0	FN597644.1
	D10, D50	71 72	*Domibacillus robiginosus* strain WS 4628	97	0.0	NR 108861.1

Table A5. BLAST similarity matches and percentage identity scores of isolates from sample depth E (344 cm)

MEDIA	ISOLATE NAME	OTU	NCBI BLAST Nucleotide Database Search Results			
			BLAST Sequence matches	Percentage Identity (%)	E-value	Accession Number
R2A	E1	81	*Paenibacillus elgii* strain SD17	97	0.0	NR 115140.1
			Paenibacillus ehimensis strain KCTC 3748	97	0.0	NR 025666.1
			Paenibacillus ehimensis strain IFO 15659	97	0.0	NR 112054.1

APPENDIX B

Table B1. Sequence similarity matrix based on partial 16S rRNA gene sequences for selected isolates generated through BioEdit software (v 7.0.9.0)

Seq->	B24	D34	A9	A23	C15	A28	C13	D12	A65	D45	A39	D5	A24	D1	C25	B6	A42	A25	B52	D21	D23
B24	ID	1	0,935	0,946	0,946	0,949	0,936	0,935	0,945	0,941	0,947	0,947	0,928	0,944	0,941	0,944	0,896	0,933	0,951	0,919	0,923
D34	1	ID	0,935	0,946	0,946	0,949	0,936	0,935	0,945	0,941	0,947	0,947	0,928	0,944	0,941	0,944	0,896	0,933	0,951	0,919	0,923
A9	0,935	0,935	ID	0,977	0,977	0,976	0,936	0,936	0,945	0,957	0,953	0,953	0,924	0,941	0,939	0,941	0,89	0,926	0,936	0,914	0,908
A23	0,946	0,946	0,977	ID	1	0,992	0,953	0,953	0,961	0,961	0,965	0,965	0,942	0,954	0,952	0,954	0,899	0,94	0,946	0,919	0,921
C15	0,946	0,946	0,977	1	ID	0,992	0,953	0,953	0,961	0,961	0,965	0,965	0,942	0,954	0,952	0,954	0,899	0,94	0,946	0,919	0,921
A28	0,949	0,949	0,976	0,992	0,992	ID	0,956	0,955	0,961	0,961	0,965	0,965	0,936	0,957	0,954	0,957	0,899	0,941	0,949	0,917	0,919
C13	0,936	0,936	0,936	0,953	0,953	0,956	ID	0,999	0,949	0,944	0,951	0,951	0,932	0,932	0,929	0,932	0,89	0,959	0,937	0,915	0,922
D12	0,935	0,935	0,936	0,953	0,953	0,955	0,999	ID	0,949	0,943	0,95	0,95	0,932	0,931	0,928	0,931	0,889	0,958	0,937	0,915	0,922
A65	0,945	0,945	0,945	0,961	0,961	0,961	0,949	0,949	ID	0,976	0,984	0,984	0,933	0,948	0,945	0,948	0,902	0,938	0,935	0,929	0,928
D45	0,941	0,941	0,957	0,961	0,961	0,961	0,944	0,943	0,976	ID	0,991	0,991	0,931	0,938	0,936	0,938	0,903	0,93	0,93	0,926	0,923
A39	0,947	0,947	0,953	0,965	0,965	0,965	0,951	0,95	0,984	0,991	ID	1	0,937	0,945	0,942	0,945	0,909	0,937	0,934	0,928	0,93
D5	0,947	0,947	0,953	0,965	0,965	0,965	0,951	0,95	0,984	0,991	1	ID	0,937	0,945	0,942	0,945	0,909	0,937	0,934	0,928	0,93
A24	0,928	0,928	0,924	0,942	0,942	0,936	0,932	0,932	0,933	0,931	0,937	0,937	ID	0,926	0,924	0,926	0,893	0,916	0,931	0,905	0,927
D1	0,944	0,944	0,941	0,954	0,954	0,957	0,932	0,931	0,948	0,938	0,945	0,945	0,926	ID	0,997	1	0,923	0,943	0,961	0,918	0,92
C25	0,941	0,941	0,939	0,952	0,952	0,954	0,929	0,928	0,945	0,936	0,942	0,942	0,924	0,997	ID	0,997	0,92	0,94	0,958	0,915	0,918
B6	0,944	0,944	0,941	0,954	0,954	0,957	0,932	0,931	0,948	0,938	0,945	0,945	0,926	1	0,997	ID	0,923	0,943	0,961	0,918	0,92
A42	0,896	0,896	0,89	0,899	0,899	0,899	0,89	0,889	0,902	0,903	0,909	0,909	0,893	0,923	0,92	0,923	ID	0,899	0,903	0,889	0,899
A25	0,933	0,933	0,926	0,94	0,94	0,941	0,959	0,958	0,938	0,93	0,937	0,937	0,916	0,943	0,94	0,943	0,899	ID	0,944	0,925	0,924
B52	0,951	0,951	0,936	0,946	0,946	0,949	0,937	0,937	0,935	0,93	0,934	0,934	0,931	0,961	0,958	0,961	0,903	0,944	ID	0,914	0,926
D21	0,919	0,919	0,914	0,919	0,919	0,917	0,915	0,915	0,929	0,926	0,928	0,928	0,905	0,918	0,915	0,918	0,889	0,925	0,914	ID	0,961
D23	0,923	0,923	0,908	0,921	0,921	0,919	0,922	0,922	0,928	0,923	0,93	0,93	0,927	0,92	0,918	0,92	0,899	0,924	0,926	0,961	ID
B19	0,912	0,912	0,907	0,924	0,924	0,922	0,905	0,904	0,909	0,912	0,916	0,916	0,904	0,918	0,916	0,918	0,881	0,905	0,919	0,91	0,92
D18	0,912	0,912	0,904	0,921	0,921	0,919	0,907	0,906	0,908	0,91	0,914	0,914	0,911	0,92	0,917	0,92	0,887	0,912	0,92	0,925	0,932
B53	0,914	0,914	0,906	0,924	0,924	0,92	0,904	0,903	0,908	0,91	0,914	0,914	0,909	0,924	0,921	0,924	0,887	0,912	0,923	0,924	0,928
A79	0,876	0,876	0,87	0,886	0,886	0,884	0,882	0,882	0,882	0,882	0,886	0,886	0,877	0,878	0,875	0,878	0,846	0,871	0,874	0,88	0,889
B45	0,871	0,871	0,868	0,883	0,883	0,882	0,877	0,876	0,878	0,878	0,882	0,882	0,875	0,875	0,873	0,875	0,842	0,868	0,87	0,877	0,886
A98	0,87	0,87	0,867	0,882	0,882	0,881	0,876	0,875	0,877	0,877	0,881	0,881	0,874	0,874	0,872	0,874	0,843	0,867	0,869	0,876	0,886
B30	0,865	0,865	0,864	0,878	0,878	0,876	0,873	0,872	0,871	0,873	0,875	0,875	0,868	0,87	0,868	0,87	0,839	0,863	0,865	0,873	0,881
A34	0,852	0,852	0,844	0,854	0,854	0,855	0,848	0,847	0,844	0,847	0,848	0,848	0,847	0,862	0,86	0,862	0,831	0,857	0,858	0,85	0,85
A35	0,846	0,846	0,842	0,855	0,855	0,857	0,845	0,844	0,842	0,844	0,843	0,843	0,841	0,862	0,86	0,862	0,822	0,853	0,853	0,838	0,841
C2	0,868	0,868	0,861	0,876	0,876	0,876	0,864	0,863	0,865	0,865	0,868	0,868	0,864	0,881	0,878	0,881	0,848	0,869	0,87	0,861	0,865
E10	0,867	0,867	0,86	0,875	0,875	0,875	0,863	0,863	0,864	0,864	0,867	0,867	0,863	0,88	0,878	0,88	0,847	0,869	0,869	0,86	0,864
A16	0,848	0,848	0,83	0,845	0,845	0,848	0,841	0,84	0,843	0,84	0,843	0,843	0,845	0,859	0,857	0,859	0,829	0,847	0,858	0,837	0,847
A110	0,867	0,867	0,854	0,869	0,869	0,872	0,866	0,866	0,861	0,857	0,86	0,86	0,853	0,877	0,875	0,877	0,836	0,874	0,872	0,867	0,863
D47	0,851	0,851	0,847	0,864	0,864	0,863	0,858	0,858	0,856	0,854	0,855	0,855	0,852	0,866	0,864	0,866	0,827	0,868	0,866	0,864	0,862
B51	0,865	0,865	0,854	0,871	0,871	0,869	0,86	0,859	0,865	0,863	0,866	0,866	0,859	0,871	0,869	0,871	0,841	0,865	0,863	0,86	0,866
A93	0,865	0,865	0,85	0,867	0,867	0,866	0,862	0,861	0,865	0,861	0,864	0,864	0,858	0,871	0,869	0,871	0,841	0,866	0,865	0,861	0,866
A37	0,827	0,827	0,824	0,83	0,83	0,831	0,828	0,827	0,828	0,823	0,825	0,825	0,823	0,838	0,836	0,838	0,803	0,83	0,836	0,833	0,827
A101	0,869	0,869	0,851	0,867	0,867	0,87	0,865	0,864	0,863	0,86	0,864	0,864	0,865	0,877	0,875	0,877	0,854	0,876	0,875	0,868	0,872
B11	0,857	0,857	0,844	0,86	0,86	0,863	0,858	0,857	0,856	0,854	0,858	0,858	0,858	0,871	0,868	0,871	0,846	0,863	0,87	0,849	0,861
B63	0,857	0,857	0,844	0,86	0,86	0,863	0,858	0,857	0,856	0,854	0,858	0,858	0,858	0,871	0,868	0,871	0,846	0,863	0,87	0,849	0,861

Table B1 continued

Seq->	B19	D18	B53	A79	B45	A98	B30	A34	A35	C2	E10	A16	A110	D47	B51	A93	A37	A101	B11	B63
B24	0,912	0,912	0,914	0,876	0,871	0,87	0,865	0,852	0,846	0,868	0,867	0,848	0,867	0,851	0,865	0,865	0,827	0,869	0,857	0,857
D34	0,912	0,912	0,914	0,876	0,871	0,87	0,865	0,852	0,846	0,868	0,867	0,848	0,867	0,851	0,865	0,865	0,827	0,869	0,857	0,857
A9	0,907	0,904	0,906	0,87	0,868	0,867	0,864	0,844	0,842	0,861	0,86	0,83	0,854	0,847	0,854	0,85	0,824	0,851	0,844	0,844
A23	0,924	0,921	0,924	0,886	0,883	0,882	0,878	0,854	0,855	0,876	0,875	0,845	0,869	0,864	0,871	0,867	0,83	0,867	0,86	0,86
C15	0,924	0,921	0,924	0,886	0,883	0,882	0,878	0,854	0,855	0,876	0,875	0,845	0,869	0,864	0,871	0,867	0,83	0,867	0,86	0,86
A28	0,922	0,919	0,92	0,884	0,882	0,881	0,876	0,855	0,857	0,876	0,875	0,848	0,872	0,863	0,869	0,866	0,831	0,87	0,863	0,863
C13	0,905	0,907	0,904	0,882	0,877	0,876	0,873	0,848	0,845	0,864	0,863	0,841	0,866	0,858	0,86	0,862	0,828	0,865	0,858	0,858
D12	0,904	0,906	0,903	0,882	0,876	0,875	0,872	0,847	0,844	0,863	0,863	0,84	0,866	0,858	0,859	0,861	0,827	0,864	0,857	0,857
A65	0,909	0,908	0,908	0,882	0,878	0,877	0,871	0,844	0,842	0,865	0,864	0,843	0,861	0,856	0,865	0,865	0,828	0,863	0,856	0,856
D45	0,912	0,91	0,91	0,882	0,878	0,877	0,873	0,847	0,844	0,865	0,864	0,84	0,857	0,854	0,863	0,861	0,823	0,86	0,854	0,854
A39	0,916	0,914	0,914	0,886	0,882	0,881	0,875	0,848	0,843	0,868	0,867	0,843	0,86	0,855	0,866	0,864	0,825	0,864	0,858	0,858
D5	0,916	0,914	0,914	0,886	0,882	0,881	0,875	0,848	0,843	0,868	0,867	0,843	0,86	0,855	0,866	0,864	0,825	0,864	0,858	0,858
A24	0,904	0,911	0,909	0,877	0,875	0,874	0,868	0,847	0,841	0,864	0,863	0,845	0,853	0,852	0,859	0,858	0,823	0,865	0,858	0,858
D1	0,918	0,92	0,924	0,878	0,875	0,874	0,87	0,862	0,862	0,881	0,88	0,859	0,877	0,866	0,871	0,871	0,838	0,877	0,871	0,871
C25	0,916	0,917	0,921	0,875	0,873	0,872	0,868	0,86	0,86	0,878	0,878	0,857	0,875	0,864	0,869	0,869	0,836	0,875	0,868	0,868
B6	0,918	0,92	0,924	0,878	0,875	0,874	0,87	0,862	0,862	0,881	0,88	0,859	0,877	0,866	0,871	0,871	0,838	0,877	0,871	0,871
A42	0,881	0,887	0,887	0,846	0,842	0,843	0,839	0,831	0,822	0,848	0,847	0,829	0,836	0,827	0,841	0,841	0,803	0,854	0,846	0,846
A25	0,905	0,912	0,912	0,871	0,868	0,867	0,863	0,857	0,853	0,869	0,869	0,847	0,874	0,868	0,865	0,866	0,83	0,876	0,863	0,863
B52	0,919	0,92	0,923	0,874	0,87	0,869	0,865	0,858	0,853	0,87	0,869	0,858	0,872	0,866	0,863	0,865	0,836	0,875	0,87	0,87
D21	0,91	0,925	0,924	0,88	0,877	0,876	0,873	0,85	0,838	0,861	0,86	0,837	0,867	0,864	0,86	0,861	0,833	0,868	0,849	0,849
D23	0,92	0,932	0,928	0,889	0,886	0,886	0,881	0,85	0,841	0,865	0,864	0,847	0,863	0,862	0,866	0,866	0,827	0,872	0,861	0,861
B19	ID	0,952	0,954	0,872	0,871	0,87	0,867	0,852	0,847	0,87	0,869	0,845	0,863	0,855	0,858	0,859	0,83	0,874	0,86	0,86
D18	0,952	ID	0,982	0,879	0,876	0,875	0,871	0,855	0,858	0,87	0,87	0,846	0,876	0,872	0,87	0,87	0,836	0,88	0,868	0,868
B53	0,954	0,982	ID	0,877	0,875	0,874	0,87	0,854	0,855	0,87	0,87	0,845	0,876	0,872	0,869	0,869	0,837	0,88	0,864	0,864
A79	0,872	0,879	0,877	ID	0,988	0,987	0,978	0,864	0,857	0,868	0,867	0,858	0,871	0,868	0,876	0,875	0,83	0,863	0,857	0,857
B45	0,871	0,876	0,875	0,988	ID	0,999	0,989	0,859	0,852	0,861	0,86	0,852	0,871	0,864	0,87	0,868	0,829	0,861	0,853	0,853
A98	0,87	0,875	0,874	0,987	0,999	ID	0,99	0,86	0,853	0,862	0,861	0,853	0,872	0,863	0,869	0,867	0,829	0,86	0,852	0,852
B30	0,867	0,871	0,87	0,978	0,989	0,99	ID	0,857	0,849	0,857	0,856	0,849	0,868	0,858	0,864	0,863	0,828	0,856	0,848	0,848
A34	0,852	0,855	0,854	0,864	0,859	0,86	0,857	ID	0,932	0,927	0,926	0,893	0,909	0,885	0,902	0,905	0,869	0,914	0,902	0,902
A35	0,847	0,858	0,855	0,857	0,852	0,853	0,849	0,932	ID	0,938	0,937	0,897	0,914	0,907	0,898	0,9	0,869	0,906	0,9	0,9
C2	0,87	0,87	0,87	0,868	0,861	0,862	0,857	0,927	0,938	ID	0,999	0,912	0,932	0,908	0,916	0,918	0,876	0,921	0,91	0,91
E10	0,869	0,87	0,87	0,867	0,86	0,861	0,856	0,926	0,937	0,999	ID	0,911	0,931	0,907	0,915	0,917	0,876	0,92	0,909	0,909
A16	0,845	0,846	0,845	0,858	0,852	0,853	0,849	0,893	0,897	0,912	0,911	ID	0,923	0,893	0,898	0,9	0,863	0,906	0,906	0,906
A110	0,863	0,876	0,876	0,871	0,871	0,872	0,868	0,909	0,914	0,932	0,931	0,923	ID	0,925	0,93	0,928	0,881	0,935	0,923	0,923
D47	0,855	0,872	0,872	0,868	0,864	0,863	0,858	0,885	0,907	0,908	0,907	0,893	0,925	ID	0,914	0,914	0,857	0,911	0,907	0,907
B51	0,858	0,87	0,869	0,876	0,87	0,869	0,864	0,902	0,898	0,916	0,915	0,898	0,93	0,914	ID	0,995	0,886	0,916	0,916	0,916
A93	0,859	0,87	0,869	0,875	0,868	0,867	0,863	0,905	0,9	0,918	0,917	0,9	0,928	0,914	0,995	ID	0,887	0,914	0,915	0,915
A37	0,83	0,836	0,837	0,83	0,829	0,829	0,828	0,869	0,869	0,876	0,876	0,863	0,881	0,857	0,886	0,887	ID	0,891	0,901	0,901
A101	0,874	0,88	0,88	0,863	0,861	0,86	0,856	0,914	0,906	0,921	0,92	0,906	0,935	0,911	0,916	0,914	0,891	ID	0,969	0,969
B11	0,86	0,868	0,864	0,857	0,853	0,852	0,848	0,902	0,9	0,91	0,909	0,906	0,923	0,907	0,916	0,915	0,901	0,969	ID	1
B63	0,86	0,868	0,864	0,857	0,853	0,852	0,848	0,902	0,9	0,91	0,909	0,906	0,923	0,907	0,916	0,915	0,901	0,969	1	ID

APPENDIX C

Table C1. The layout of the Biolog EcoPlate™ containing 31 carbon sources in triplicate sets (Biolog, 2007)

A1 Water	**A2 β-Methyl-D-Glucoside**	**A3 D-Galactonic Acid γ-Lactone**	**A4 L-Arginine**	**A1 Water**	**A2 β-Methyl-D-Glucoside**	**A3 D-Galactonic Acid γ-Lactone**	**A4 L-Arginine**	**A1 Water**	**A2 β-Methyl-D-Glucoside**	**A3 D-Galactonic Acid γ-Lactone**	**A4 L-Arginine**
B1 Pyruvic Acid Methyl Ester	**B2 D-Xylose**	**B3 D-Galacturonic Acid**	**B4 L-Asparagine**	**B1 Pyruvic Acid Methyl Ester**	**B2 D-Xylose**	**B3 D-Galacturonic Acid**	**B4 L-Asparagine**	**B1 Pyruvic Acid Methyl Ester**	**B2 D-Xylose**	**B3 D-Galacturonic Acid**	**B4 L-Asparagine**
C1 Tween 40	**C2 i-Erythritol**	**C3 2-Hydroxy Benzoic Acid**	**C4 L-Phenylalanine**	**C1 Tween 40**	**C2 i-Erythritol**	**C3 2-Hydroxy Benzoic Acid**	**C4 L-Phenylalanine**	**C1 Tween 40**	**C2 i-Erythritol**	**C3 2-Hydroxy Benzoic Acid**	**C4 L-Phenylalanine**
D1 Tween 80	**D2 D-Mannitol**	**D3 4-Hydroxy Benzoic Acid**	**D4 L-Serine**	**D1 Tween 80**	**D2 D-Mannitol**	**D3 4-Hydroxy Benzoic Acid**	**D4 L-Serine**	**D1 Tween 80**	**D2 D-Mannitol**	**D3 4-Hydroxy Benzoic Acid**	**D4 L-Serine**
E1 α-Cyclodextrin	**E2 N-Acetyl-D-Glucosamine**	**E3 γ-Hydroxybutyric Acid**	**E4 L-Threonine**	**E1 α-Cyclodextrin**	**E2 N-Acetyl-D-Glucosamine**	**E3 γ-Hydroxybutyric Acid**	**E4 L-Threonine**	**E1 α-Cyclodextrin**	**E2 N-Acetyl-D-Glucosamine**	**E3 γ-Hydroxybutyric Acid**	**E4 L-Threonine**
F1 Glycogen	**F2 D-Glucosaminic Acid**	**F3 Itaconic Acid**	**F4 Glycyl-L-Glutamic Acid**	**F1 Glycogen**	**F2 D-Glucosaminic Acid**	**F3 Itaconic Acid**	**F4 Glycyl-L-Glutamic Acid**	**F1 Glycogen**	**F2 D-Glucosaminic Acid**	**F3 Itaconic Acid**	**F4 Glycyl-L-Glutamic Acid**
G1 D-Cellobiose	**G2 Glucose-1-Phosphate**	**G3 α-Ketobutyric Acid**	**G4 Phenylethylamine**	**G1 D-Cellobiose**	**G2 Glucose-1-Phosphate**	**G3 α-Ketobutyric Acid**	**G4 Phenylethylamine**	**G1 D-Cellobiose**	**G2 Glucose-1-Phosphate**	**G3 α-Ketobutyric Acid**	**G4 Phenylethylamine**
H1 α-D-Lactose	**H2 D,L-α-Glycerol Phosphate**	**H3 D-Malic Acid**	**H4 Putrescine**	**H1 α-D-Lactose**	**H2 D,L-α-Glycerol Phosphate**	**H3 D-Malic Acid**	**H4 Putrescine**	**H1 α-D-Lactose**	**H2 D,L-α-Glycerol Phosphate**	**H3 D-Malic Acid**	**H4 Putrescine**

APPENDIX C continued

Table C2. The layout of the microtiter plate used for salinity testing displaying the varying concentrations of NaCl (%) in duplicate allowing for the inoculation of six isolates per plate

0.5	10	0.5	10	0.5	10	0.5	10	0.5	10	0.5	10
0.5	10	0.5	10	0.5	10	0.5	10	0.5	10	0.5	10
2.5	15	2.5	15	2.5	15	2.5	15	2.5	15	2.5	15
2.5	15	2.5	15	2.5	15	2.5	15	2.5	15	2.5	15
5	*30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline
5	*30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline	5	30 µl inoculum + 150 µl saline
7.5	*180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth
7.5	*180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth

*control wells

APPENDIX C continued

Table C3. The layout of the microtiter plate used for pH testing displaying the varying pH values in duplicate allowing for the inoculation of six isolates per plate

3	8.5	3	8.5	3	8.5	3	8.5	3	8.5	3	8.5
3	8.5	3	8.5	3	8.5	3	8.5	3	8.5	3	8.5
4.5	10	4.5	10	4.5	10	4.5	10	4.5	10	4.5	10
4.5	10	4.5	10	4.5	10	4.5	10	4.5	10	4.5	10
5.5	*30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline
5.5	*30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline	5.5	30 µl inoculum + 150 µl saline
7.5	*180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth
7.5	*180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth	7.5	180 µl uninoculated broth

*control wells

APPENDIX C continued

The composition of the various buffers used for pH tolerance testing of selected AEFB isolates (section 5.2.1.2) are presented below.

CITRATE BUFFER COMPONENTS

pH	Solution A (0.1 M citric acid monohydrate*)	Solution B (0.1 M trisodium citrate, dihydrate¥)
3.0	82.0 ml	18.0 ml
4.4	49.5 ml	50.5 ml
5.4	25.5 ml	74.5 ml

* citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) ¥ trisodium citrate, dihydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$)

PHOSPHATE BUFFER COMPONENTS

pH	Solution A (0.2 M mono-potassium phosphate*)	Solution B (0.2 M di-potassium phosphate¥)	Solution C (distilled water)
7.5	16.0 ml	84 ml	100 ml

*mono-potassium phosphate (KH_2PO_4) ¥di-potassium phosphate (K_2HPO_4)

TRIS BUFFER COMPONENTS

pH	Solution A (0.1 M Tris base*)	Solution B (0.1 M hydrochloric acid¥)	Solution C (distilled water)
8.5	50.0 ml	14.7 ml	35.3 ml

*tris base ($C_4H_{11}NO_3$) ¥hydrochloric acid (HCl)

BICARBONATE BUFFER COMPONENTS

pH	Solution A (0.1 M sodium bicarbonate*)	Solution B (0.1 M sodium carbonate anhydrous¥)
10.1	40 ml	60 ml

*sodium bicarbonate ($NaHCO_3$) ¥sodium carbonate anhydrous (Na_2CO_3)

CONFERENCES

This research has been presented at the following conferences:

1. POSTER PRESENTATION: South African Society for Microbiology (SASM) 19th Biennial Congress, 17–20th January 2016.

2. ORAL PRESENTATION (1st place): University of KwaZulu-Natal, College of Agriculture, Engineering and Science Annual Postgraduate Research Day, 29th November 2016.