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MICROBIAL BIODIVERSITY AS AN INDICATOR OF HEALTH: A FOCUS ON SELECTED PEATLANDS OF THE SOUTHERN CAPE, SOUTH AFRICA

by

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DECLARATION

I, Shandre Sharnize Lavern Weels, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed S. Weels **Date** 31 July 2022

ABSTRACT

South Africa has around 497 peatlands, 308 of which are located in KwaZulu-Natal (1% of the global total). Compared to many peatlands found in the northern hemisphere, South African peatlands are generally not of sphagnum-origin but are rather composed of the decaying material of reeds, sedges, and grass. In the northern hemisphere extensive research has been performed on the microbial diversity of peatlands, including actinobacterial diversity, and how it is linked to the 'health' of the peatland. This study is the first of its kind in South Africa, focusing on the microbial biodiversity associated with selected peatlands and how this can be related to their health status.

In this study, we compared three different peatlands (with different health statuses) and looked at their physicochemical and molecular data in order to assess their 'health' status. Peat samples were collected from Vankervelsvlei (unimpacted and well-preserved peatlands), Goukou River (impacted by agricultural wastewater and has exposed deposits), and Springfield Farm (developing and impacted by agricultural wastewater). The physicochemical analysis performed on the samples included measuring the pH, humification, loss-on-ignition (LOI), and analysis of the major elements present in the samples. The molecular analysis included terminal restriction fragment length polymorphism (T-RFLP) to look at bacterial population similarity among the three sites, and next generation sequencing (NGS) to look at the actinobacterial, and fungal diversity of the selected sites. In addition, selective isolation techniques were applied in order to isolate and identify several actinobacterial strains from the Vankervelsvlei and Goukou River samples.

As expected, the physicochemical data indicated that there were many similarities and differences between the three selected sites, specifically Goukou River and the other two sites. This is because Goukou River, similar to Vankervelsvlei is a well-developed peatland, but also as is the case with Springfield Farm is affected by agricultural wastewater run-off which has resulted in exposed peat deposits. The only similarity between Goukou River and Vankervelsvlei was their pH levels, which were both acidic. While the pH of Springfield Farm is basic. Humification, organic matter (OM), and elements including carbon (C), and nitrogen (N), of Vankervelsvlei were high, while Goukou River and Vankervelsvlei had similar values. The T-RFLP data showed that the microbial communities present in the three sites were closely related, however different communities were found at different depths. A variety of actinobacterial and fungal orders were detected in the different sites using NGS. Actinobacteria such as the members of *Acidimicrobiales*, *Actinomycetales* and *Solirubrobacterales* were abundant in Goukou River and Springfield Farm. *Bifidobacteriales*

were abundant in Vankervelsvlei, whereas *Micrococalles*, *Euzebyales*, *Nitriliruptorales*, and *Rubrobacterales* were only detected in Springfield Farm. BEST analysis (linking microbial diversity patterns to environmental variables) showed that N, aluminium (Al), calcium (Ca), phosphorus (P), and titanium (Ti) were the most significant physicochemical drivers of actinobacterial community structure. Twenty-six actinobacterial strains were isolated and identified from Goukou River and Vankervelsvlei, which included *Nocardia africana*, *Kitasatospora albolonga*, and a variety of strains from the genus of *Streptomyces*. Fungi such as *Ascomycota* and *Basidiomycota* are abundant in all three sites. However, *Ascomycota* are abundant in Goukou River, *Basidiomycota* are abundant in Vankervelsvlei, while *Glomeromycota* are abundant in Springfield Farm. BEST analysis confirmed that sulphur (S), aluminium (Al), calcium (Ca), potassium (K), phosphor (P), titanium (Ti), silicon (Si), magnesium (Mg), iron (Fe), humification, and pH were the environmental parameters that affected the fungal communities the most. An overview of all the data obtained highlighted the fact that the Vankervelsvlei peatland represented a ‘healthy’ peatland, having all the ‘typical’ features of an unimpacted peatland. Both the impacted (Goukou River) and the developing (Springfield Farm) peatlands exhibited ‘typical’ features of ‘unhealthy’/impacted peatlands, which is highlighted in the conclusion section of this thesis.

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Notes:

All raw data pertaining to this study can be accessed via the CPUT eSango Research Data Platform via the following link: https://doi.org/10.25381/cput.17206676.v1. Raw sequence data obtained in this study have been deposited in the National Center for Biotechnology Information Database under BioProject PRJNA805212.

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GLOSSARY

Terms	Definition/Explanation
Biogeochemical processes	Biochemical process the are essential for the circulation of matter and denotes to the consideration of the biological, geological, and chemical aspects of each cycle.
Bogs	Bogs are poorly drained typically acidic areas that has an abundance of accumulated plant material, often surrounded by open water, with the characteristic vegetation of heaths, sedges, and sphagnum.
Fens	Fens are low areas of peaty, alkaline soil (sedges and reeds) that are covered solely or partially with water unless artificially drained.
Humification	When organic matter is transformed into humic substances such as humate, humin, humic acid, humus, and fulvic acid, by geo-microbiological mechanisms.
Hydrology	The existence, circulation, movement, and properties of the waters of earth and their connection with the environment within each phase of the hydrologic cycle.
Loss-on-ignition	Loss of ignition (LOI Loss on ignition) is when external moisture of raw material is lost after drying in a temperature range of 105-110 °C.
Mesotrophic	Having a moderate amount of dissolved nutrients.
Minerotrophic fens	Minerotrophic environments obtain nutrients through groundwater that flows through mineral-rich soils or rock, or surface water flowing over land.
Mires	Wet, springy soil (as of a bog or marsh)

Oligotrophic	An abundance of dissolved oxygen in the presence of a lack in plant nutrients.
Ombrotrophic bogs	Ombrotrophic bogs environments that obtain water and nutrients from rainfall.
Peat	Brown deposit that resembles soil, formed by the incomplete decomposition of plant matter in the wet acidic conditions of bogs and fens.
Peatlands	Peatlands are global wetland environments in which waterlogged conditions prevent the decomposition of plants.
Wetlands	Wetlands are environments in which water covers the soil or is present either at or near the surface of soil all year or for varying periods of time during the year, including during the growing season.
Zonality	Relating to, or being a soil or a major soil group marked by well-developed characteristics that are determined primarily by the action of climate and organisms (such as vegetation)

Abbreviations	Explanation
2D	Two dimensional
Al	Aluminium
ANOSIM	Analysis Of Similarities
BBPE	Bushveld Basin Peatland Eco-region
Bp	Base pair
C	Carbon
Ca	Calcium
$CaCO_3$	Calcium Carbonate
CAF	Central Analytical Facility
CFMPE	Cape Fold Mountains Peatland Eco-region
CH_4	Methane
CHPE	Central Highlands Peatlands Eco-region
CO_2	Carbon dioxide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DOC	Dissolved organic carbon
ECBPE	Eastern Coastal Belt Peatland Eco-region
EUPE	Eastern Uplands Peatlands Eco-region
FBIP	Foundational Biodiversity Initiative Programme
Fe	Iron
FISH	Fluorescent *in situ* hybridisation
GK	Goukou River
GRMPE	Great Escarpment Mountain Peatland Eco-region
H	Hydrogen
Ha	Hectare
HCO3	Bicarbonate
HPE	Highveld Peatland Eco-region
ISP	International Streptomyces Project

ITS	Internal Transcribed Spacer
K	Potassium
LOI	Loss-on-ignition
LPE	Lowveld Peatland Eco-region
LPPE	Limpopo Plain Peatland Eco-region
MCP	Mozambique coastal plain
MDS	metric multidimensional
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgCO_3$	Magnesium carbonate
MMO	methane monooxygenase enzyme
N	Nitrogen
NaOH	Sodium hydroxide
NCPPE	Natal Coastal Plain Peatland Eco-region
NGS	Next-generation sequencing
nMDS	Non-metric multidimensional scaling
O_2	Oxygen
OH^-	Hydroxide
OM	Organic matter
OTU	Operational taxonomic unit
P	Phosphor
PCA	Principal Component Analysis
PCR	polymerase chain reaction
RA	Relative abundance
rRNA	Ribosomal ribonucleic acid
S	Sulphur
SANBI	South African National Biodiversity Institute
SCBPE	Southern Coastal Belt Peatland Eco-region
SF	Springfield Farm
Si	Silicon
SOM	Soil organic matter
Spp.	Species

Ti	Titanium
T-RFLP	Terminal restriction fragment-length polymorphism
T-RFs	Terminal-restriction fragments
Tris-HCl	Tris hydrochloride
UK	United Kingdom
USA	United States of American
UV	Ultraviolet
VV	Vankervelsvlei
WRC	Water Research Commission
XRF	X-Ray Fluorescence

CHAPTER ONE: INTRODUCTION

South Africa is a country that contains a variety of landscapes ranging from tropical to desert-like terrains. The tropical landscapes are located on the eastern Indian Ocean shore, while desert-like landscapes can be found on the Atlantic Ocean side. The innermost part of South Africa consists of grasslands (the Highveld) and savannah woodlands (the Bushveld) (Grundling and Grobler, 2005:380). Table 1.1 summarises the different types of terrains found in South Africa, highlighting the fact that wetlands only make up 0.5% of all terrains.

Table 1.1: Different types of terrains found in South Africa (Fairbanks et al., 2000:73)

Surface types of South Africa	**%**
Shrubland and low fynbos	34.1
Grassland	21.3
Thicket and Bushveld	17.6
Cultivated lands	12.2
Forest and woodland	5.8
Degraded lands	4.9
Forest plantation	1.5
Urban/built-up lands	1.1
Wetlands (peatlands and mires)	0.5
Waterbodies	0.4
Forest	0.3
Barren rock	0.2
Herbland	0.2
Mines and quarries	0.14

South Africa receives an average rainfall of 497 mm per year, which is well below the world average of 860 mm. The total rainfall is more in the eastern parts of South Africa and systematically decreases westward, with some semi-desert areas along the western edge of South Africa. Therefore, it is not surprising that peat can be found along the eastern coastline, the subtropical interdune mires of the Mozambique coastal plain (MCP), and on the coastal plateau of South Africa. South African peatlands can also be found in the temperate Highveld area. Compared to the various types of peatlands worldwide, South African peatlands are classified as fens and originate from reeds, sedges, and grasses (Joosten and Clarke, 2002). Peatlands are vital ecosystems because of the biodiversity they harbour, and their limited size and distribution. There has also been an interest in peat-derived products, which includes peat harvesting and the use of acid extracted from peat for new technological pharmaceutical applications. This part of the peat industry has yet to be explored extensively. However, South Africa's peatlands are threatened mainly due to overpopulation and development (Grundling

and Grobler, 2005:379-390). The Working for Wetlands Programme of the South African National Biodiversity Institute (SANBI) focuses on restoring degraded wetland ecosystems, of which 40% of the current projects are associated with mires and peatlands (SANBI).

Over the years, a considerable amount of research that focused on aspects such as the depth, chemical properties, vegetation, age, etc., has been performed on peatlands worldwide. However, not much research has been conducted on South African peatlands, especially research focused on microbial diversity, including fungal and bacterial diversity. In addition, the chemistry of these peatlands is also not fully understood. Microorganisms, such as fungi and actinobacteria, play a crucial role in the formation of peat: they are responsible for the breakdown of the plant material that makes up the peatland. It is therefore proposed that organisms such as fungi and actinobacteria can be used as biomarkers for a peatland's health. For example, if a peatland is disturbed by human activities such as peat harvesting, agricultural waste, etc., or due to environmental events such as heavy rains, erosion, rivers, etc., this will impact the microbial communities present in the peatland. Understanding the microbial biodiversity and ecological integrity of both an impacted and unimpacted peatland could, therefore, be beneficial from an environmental health perspective. Ecological integrity can thus be defined as 'the ecosystem's structure, composition, and function are unaffected by human activity; natural ecological processes are intact and self-sustaining; the ecosystem evolves naturally and its capacity for self-renewal is maintained; and the ecosystem's biodiversity is ensured,' according to the BC Parks Legacy Panel in 1999.

Based on this background information, the main aim of this study was to identify and compare the biodiversity of fungi and actinobacteria in selected South African peatlands in order to assess the ecological integrity of the sampled sites.

In order to achieve this aim, the following objectives were identified:

- To collect peat samples from selected South African peatlands that represent, 1) a well-established, undisturbed peatland, 2) an impacted/disturbed peatland, and 3) a developing peatland;
- To extract metagenomic DNA from the peat samples;
- To perform fungal and bacterial community fingerprinting using terminal restriction fragmentation polymorphism analyses and next generation sequencing (NGS);
- To isolate actinobacteria from peat samples, and to identify the isolates by sequencing their 16S rRNA genes; and

- To characterise the peat samples for selected physicochemical parameters.

Thesis layout

The next chapter, Chapter two, is the Literature review. This chapter contains relevant material published, justifying the materials and methods used throughout this project. The experimental methodology is covered in Chapter three; it includes the methods used in the collection of the peat samples, the molecular analyses, isolation, and identification of actinobacterial strains, and the physicochemical analyses of all peat samples. Chapters four and five contains the results and discussions on the physicochemical properties and microbial diversity, respectively, while Chapter six contains a summary of the project, final thoughts, observations, and project recommendations.

CHAPTER TWO: LITERATURE REVIEW

2.1 Peatlands

2.1.1. What are peatlands?

There is often a confusion around the use of terminology such as wetlands, peatlands, and mires – mainly because these ecosystems are so closely related. According to the National Wetlands Work Group (1997), 'wetlands' are classified as land that has been saturated with water for a long enough time resulting in a wetland or aquatic processes that indicates poorly drained soils, hydrophytic vegetation, and various kinds of biological activity that has been adapted to a wet environment (National Wetlands Working Group, 1997; Bourbonniere, 2009:394). The term 'mire' is used for a terrain that is dominated by living peat-forming plants (Sjörs, 1950:241; Rydin and Jeghum, 2006), while 'peatlands' are wetland-like ecosystems that are characterised by the build-up of organic matter emanating from dead or decaying plant and animal material (to a lesser extent) under permanent waterlogged conditions (Grundling, 2001; Ellery et al., 2012:11-12; Figure 2.1).

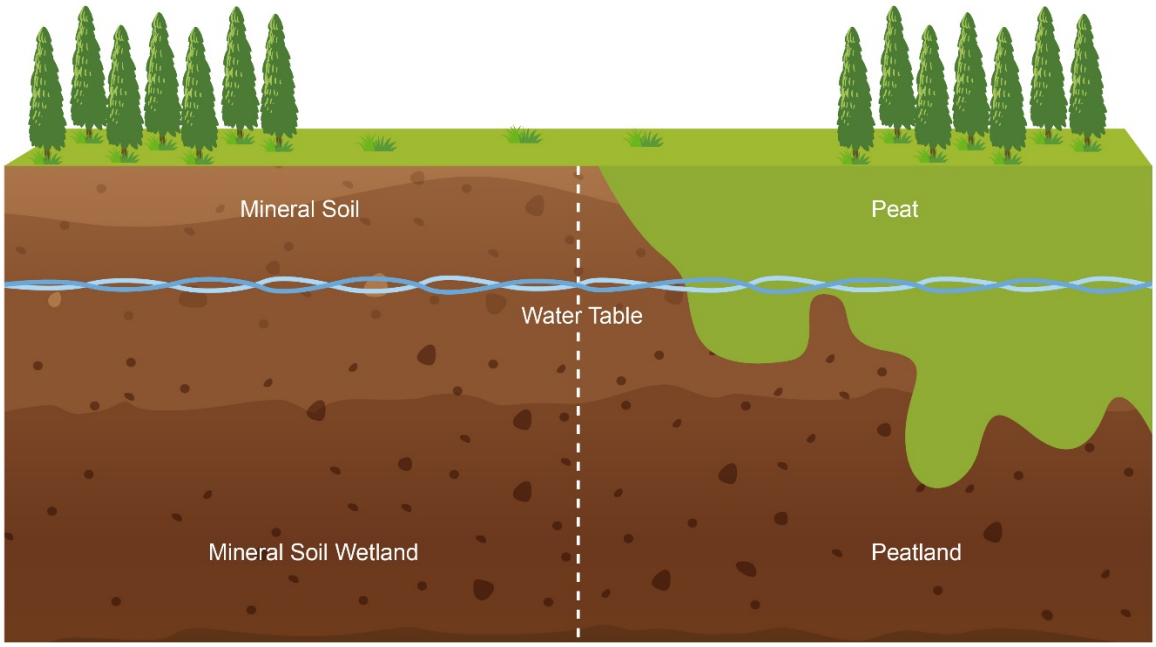

Figure 2.1: Diagram depicting the difference between wetlands and peatlands (adapted from Kolka et al., 2018:516)

Peat is formed over thousands of years in areas where the plant's growth rate is faster than the rate of decomposition. Peat formation requires low activity of aerobic and anaerobic microorganisms associated with particular environmental conditions, including recalcitrant organic material, low temperatures, low pH, low nutrient levels, and low oxygen availability

(Ellery et al., 2012:11-12). The decomposition process of the plant does not occur because these areas are constantly saturated with water. Soils saturated with water are often anaerobic or lack oxygen, and oxygen is vital for the growth of the soil microorganisms involved in the breakdown process of the plant material. Because of the delayed microbial activity, dead plants accumulate in the water. The soil's pH also contributes to the formation of peat, while the vegetation of the peatland controls the soil pH. The plants absorb cations (from rainwater) and release hydrogen ions (H^+) into the water, resulting in a decrease in the pH. The more acidic the soil water, the less favourable it becomes for the microorganisms to grow, and the plant remnants, therefore, accumulate at a faster rate (water-us.net; Figure 2.2).

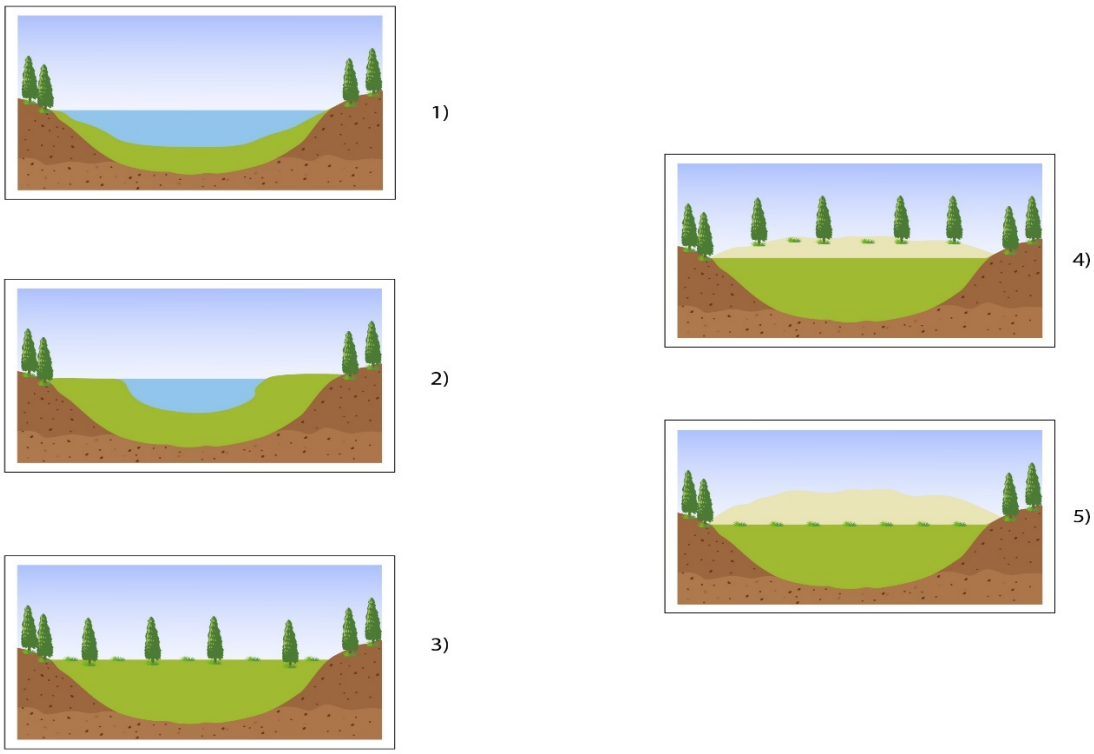

Figure 2.2: The peat formation process. [1-3] The accumulation of organic material forming a peatland. [4-5] Plant growth causing the formation of a raised bog (adapted from water-us.net)

Peatlands cover about 3% of the Earth's surface and are responsible for storing approximately 30% of global terrestrial carbon (Parish et al., 2008; Joosten et al., 2012; Grundling et al., 2017:1). Peatlands are areas of deep peat soils with an organic layer deeper than 40 cm. Even though the high organic content of peatlands is their primary defining characteristic, it is the water-retention and the hydrological properties of peatlands that allow their continued existence and produces their distinctive suite of habitats (Rydin and Jeghum, 2006).

Peatlands can further be divided into bogs or fens (Figure 2.3) based on the plant type they are composed of, water chemistry, and hydrology (Payette, 2001:39-40). Peatlands that are

nutrient-poor and fed only by rainwater are known as ombrotrophic bogs. Ombrotrophic bogs have wet, spongy, and poorly drained soil. These are acidic peatlands and have a surface pH ranging from 3.7 to 4.1. Ombrotrophic bogs are typically dominated by sphagnum moss and heaths such as *Chamaedaphne*. Bogs generally are surrounded by a body of open water (Andersen et al., 2013:1375-1376). Peatlands fed by rainwater and groundwater (minerals from rocks underground are pushed into the peatland via the groundwater) are known as minerotrophic fens (Lähteenoja et al., 2009:140; van Vuuren, 2010:17). Minerotrophic fens can further be divided into oligotrophic (poor) and mesotrophic (rich) fens with pH readings ranging between 3.8 to 6.5 for oligotrophic fens and 5.8 to 8.4 for mesotrophic fens (Sjörs, 1950:242-244). Mesotrophic forested fens, such as swamp forests, consist of ground layers containing sphagnum moss; these fens also contain layers with shrubs and sedges, including tree stands, grasses, grass-like plants, and reeds (Andersen et al., 2013:1375-1376). Fens and bogs regularly occur side by side. There is often no border that separates the two peatland types.

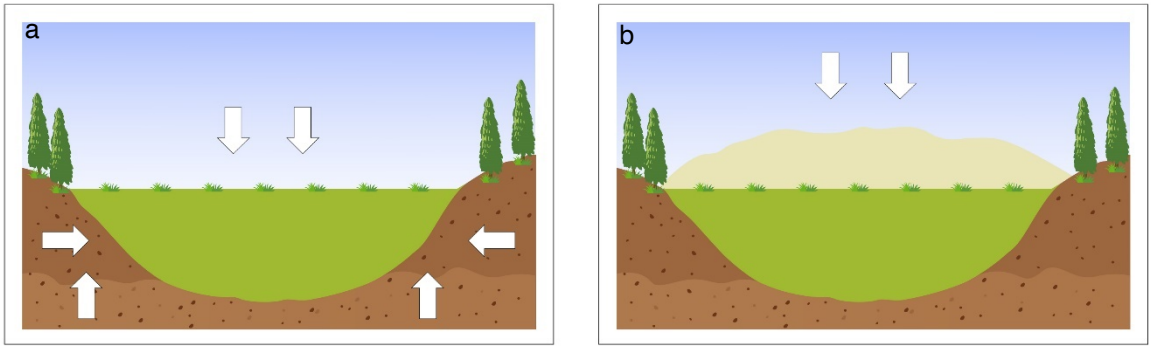

Figure 2.3: Schematic representation and the classical difference between a 'fen' (a) and a 'bog' (b); white arrow = water flow (adapted from Joosten and Couwenberg, 2008:16)

The waterlogged conditions make it possible for peatlands to store carbon and support the peat-forming plants that use the stored carbon. Healthy peatlands maintain the wet, acidic, and low oxygen conditions required to stop material from being fully decomposed and all of their carbon from being released back into the atmosphere. Damaged peatlands naturally release greenhouse gasses such as methane (CH_4) and carbon dioxide (CO_2). However, once the peatland dries out or is damaged, the rate at which the peatland releases these gasses increases, contributing to global warming [England's peatlands: carbon storage and greenhouse gases (NE257), 2010]. Peatlands can store large volumes of water, and around 20% of the world's fresh water is found in peatlands. They act as natural filters by removing sediment, pollutants, and pathogens from the water. The pollutant and pathogen removal abilities of peatlands are determined by the peat's chemical composition and a high cation

exchange capability (Painter, 1991:123-124). Compounds in peat vary from site to site, but commonly found compounds include N, organic compounds containing S, and phenolic compounds (Tfaily et al., 2012:447).

2.1.2 Peatland distribution

Peatlands are essential as they act as terrestrial carbon pools and play a crucial role in carbon soil-atmosphere exchange processes. They are found globally, with their greatest extent in boreal and temperate zones, particularly in North America and Eurasia. However, peatlands are also found in places such as Patagonia, Ethiopia, South Africa, Mongolia, and Iran (Renou-Wilson et al., 2011:157). The majority of the tropical peatlands are located at low altitudes; however, some occur at high altitudes and are located in mountainous areas (Bord na Mona, 1985:8; Lappalainen, 1996). Globally, peatlands are distributed unevenly; North America has the largest peatland area, contributing around 43.54% of the global peatland area. Asia and Europe contribute 28.08% and 24.02%, respectively. Table 2.1 summarises the different peat regions and area size.

Table 2.1: Global peatland distributions (Bord na Mona, 1985:8)

Global distribution	Area (Ha)
North America	209 640 000
Eastern Europe	151 957 750
Western Europe	25 986 200
Asia	24 886 500
South America	6 173 000
Africa	4 856 500
Central America	2 524 000
The Pacific	165 000

According to Liu & Liu (2008:145-150), peatland distribution demonstrates zonality. However, the distribution of Earth's land masses, the topography, geological conditions, etc., creates a degree of disturbance to regularity. Thus, each one of the continents has its own characteristic distribution of peatlands. The northern hemisphere contains a larger land area that is in the temperate climatic region in the middle and high latitudes. Temperatures range from cold and humid to mild and humid, which is ideal for the accumulation of peat. The northern hemisphere also has fewer mountains influencing peat distribution. There are no peatlands in Antarctica

because conditions (snow and ice) are not ideal for peat to accumulate (Liu and Liu, 2008:145-150).

It is clear from the information provided in Table 2.1 that most peatlands occur in the northern hemisphere, while only 1% occurs in the southern hemisphere. Peatlands found in the northern hemisphere are predominantly made up of sphagnum moss, grasses, and other plant-like materials, while peatlands in the southern hemisphere have a different vegetation composition (Renou-Wilson et al., 2011:157). The accumulation rate of peat depends on the matrix type; sphagnum-dominated peatlands accumulate around 1 mm to 2 mm annually, while non-sphagnum-dominated peatlands accumulate peat at a rate of 0.5 mm to 1 mm annually (van Vuuren, 2010:14-15). The peatlands present today were formed more than 10 000 years ago. The water, plants, and peat are very important to the survival of the peatland and, if disturbed, could lead to the destruction of that peatland (Marsden and Ebmeier, 2012).

South Africa is home to 467 peatlands, with 308 being found in Kwa-Zulu Natal (KZN). Eleven peatland eco-regions have been identified for South Africa, as shown in Figure 2.4, and as summarised in Table 2.2. These eco-regions all contain different vegetation types, resulting in the formation of the different types of peatlands (Grundling and Grobler, 2005:380). Peat originating from South Africa usually consists of degraded reeds, sedges, and grasses.

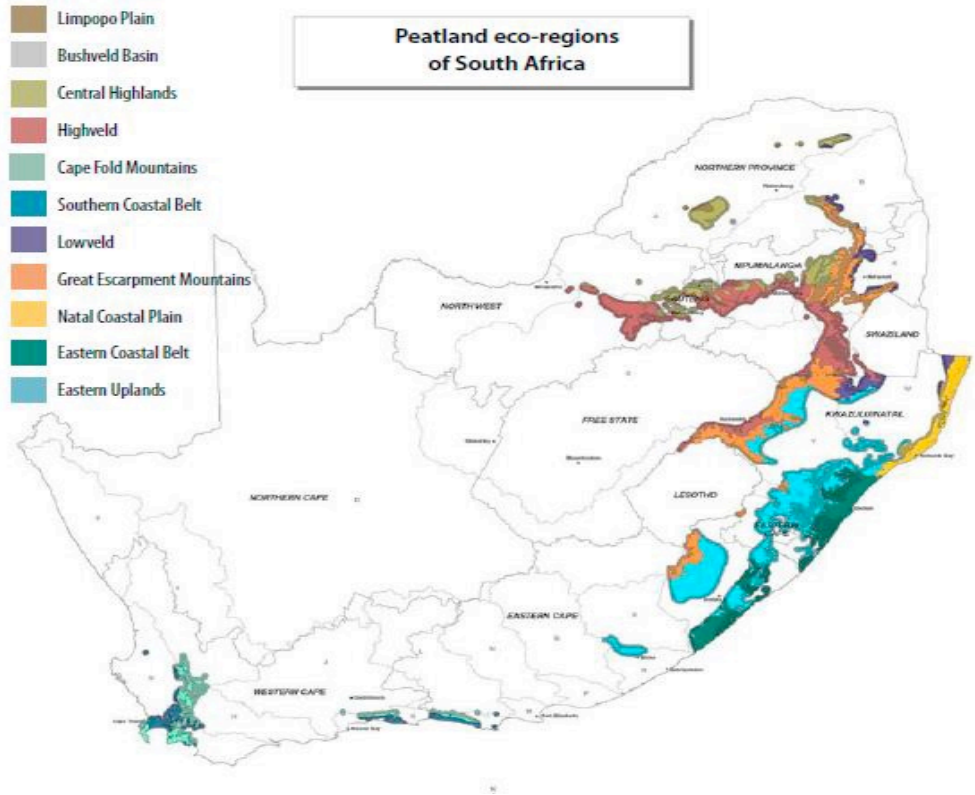

Figure 2.4: The different peatland eco-regions of South Africa (van Vuuren, 2010:17)

Table 2.2: Summary of the different peatland eco-regions in South Africa (Grundling and Grobler, 2005:380)

	Eco-region	Vegetation	Peat type	Uses and impacts
1	Limpopo Plain Peatland Eco-region (LPPE)	Valleybottom fens and grasses, and sedges are the dominant plants in this region.	Grass and sedge peatlands contain medium to fine-grained peat.	The mining of peat, abstraction of water, agriculture, grazing, afforestation, draining, headcut and donga erosion, roads, fences, and dams
2	Bushveld Basin Peatland Eco-region (BBPE)	Thorn and mixed bushveld types		Urban and about 45% of this area has been altered
3	Central Highlands Peatlands Eco-region (CHPE)	Valleybottom fens, Reeds, and *Carex* spp. (dominant), grasses and other sedges are not common in this region	Reed and sedge peatlands contain fibrous to medium-fine peat and grass, and sedge peatlands contain medium to fine-grained peat.	Abstraction of water, agriculture, draining, afforestation, headcut and donga erosion, peat fires, roads, grazing, fences, and dams (trout dams)
4	Highveld Peatland Eco-region (HPE)	Valleybottom fens, reeds (*Phragmites australis* and *Carex* spp.) are the region's dominant vegetation. Grasses and sedges occur less.	Reed and sedge peatlands contain more fibrous peat, while grass and sedge peatlands contain medium to finely grained peat.	Peat mining (most peat mining operations occur in the central and part of this region), grazing, abstraction of water, agriculture, afforestation, draining, headcut and donga erosion, urbanisation, fences, roads, and dams.
5	Lowveld Peatland Eco-region (LPE)	Low bushveld types, but in the north, mopane bushveld and shrubland occur.		Cultivation, about 14% of the area has been altered. Encroachment on peatlands is afforestation.
6	Great Escarpment Mountain Peatland Eco-region (GRMPE)	Valleybottom fens, grasses, and sedges, reeds (*Phragmites australis* and *Carex* spp.)	The largest of the peatlands are reeds, and sedges dominated and have developed the thickets peats. Reed and Sedge peatlands tend to contain peat that is fibrous to medium fine. And grass and sedge peatlands contain peat that is medium to finely grained.	Abstraction of water, fences, headcut and donga erosion, grazing, agriculture, afforestation, drainage, roads, dams, and the peatlands' siltation in this region.

	Eco-region	Vegetation	Peat type	Uses and impacts
7	Eastern Uplands Peatlands Eco-region (EUPE)	Valleybottom fens, bulrushes (*Typha capensis*) and sedges (dominant vegetation), grasses, and reeds (*Phragmites australis* and *Carex* spp.).	Reed/Sedge peatlands contain peat that is fibrous to medium fine. And Bulrush or grass peatlands contain medium to fine-grained peat.	Abstraction of water, afforestation, headcut and donga erosion, agriculture, grazing, grazing, draining, roads, and fences.
8	Eastern Coastal Belt Peatland Eco-region (ECBPE)	Valleybottom fens, swamp forests (dominant vegetation), reeds, and sedges.	Reeds and sedges peatlands contain medium to fine peat. Swamp forest peatlands are mostly fibrous to medium fine-grained peat.	Abstraction of water, agriculture, roads, urban development, grazing, afforestation, draining, fences, and dams
9	Natal Coastal Plain Peatland Eco-region (NCPPE)	Valleybottom fens, reeds and sedges (*Cyperus papyrus, Phragmites australis,* and *Carex* spp.), swamp forests and grasses and sedges (not common).	Reed/sedge and swamp peatlands contain peat that is either coarse fibrous or fine-grained.	Intensive *in situ* horticulture, water abstraction, agriculture, grazing, afforestation, and draining.
10	Southern Coastal Belt Peatland Eco-region (SCBPE)	Valleybottom Fens, reeds (*Phragmites australis* and *Carex* spp.) grasses, reeds, and palmiet.	Peatlands containing reeds and sedges have fibrous to medium-fine peat, while palmiet peat is sandy fibrous to fine. Vankervelsvlei is a sphagnum moss dominated peatland in this region	Abstraction of water, roads, agriculture, grazing, afforestation, draining, urban development, fences, and dams
11	Cape Fold Mountains Peatland Eco-region (CFMPE)	Valleybottom fens, palmiet (*Juncaceae* or rush family of plants), grass, and sedges.	Palmiet-based peat tends to be sandy, medium-fine to fibrous peat. Grass-sedge peat is mostly medium to fine-grained peat.	Abstraction of water, draining, fences and dams, agriculture, grazing, peat fires, headcut and donga erosion, roads, and alien invasive infestation

2.1.3 Threats to peatlands

Peatlands are being threatened by environmental degradation and harvesting. Currently, peat is still being harvested and used for various applications and processes, including its use as a growth medium, fertiliser, and fuel. Industrially, peat is used as a filter and purifier of municipal and domestic effluent and gas, for various medical applications, and as a colouring agent. When combined with bentonite, it can serve as a binding agent for iron (Grundling and Grobler, 2005:379-396). In South Africa, coco peat is used as a soil additive and growth medium for various crops, in commercial plant nurseries and mushroom industries. Sphagnum moss is preferred among gardeners as they mix the peat moss with the soil. The mushroom industries have started to import peat casting as South Africa does not have a lot of peat readily available. Peatlands are also used for subsistence agriculture by rural communities. Governmental conservation units are mandated to protect peatlands or at least ensure that the use of peat as a resource is controlled and sustainable. However, there is a need for improved policy aimed at protecting South African peatlands (van Vuuren, 2010:20).

2.1.3.1 Harvesting and commercial uses for peat

Globally, peat is used for a variety of applications such as a growth medium, fertiliser, fuel, water resource, filter and purifier of industrial, municipal, and domestic effluent, the treatment of radioactive waste and pesticides, as well as in oil production. It is used for lawn and garden soil amendments, potting soils, and turf maintenance on golf courses (Gruda, 2019:298). Because peat moss contains organic salts, it has medical properties and is used for applications such as therapeutic baths to heal ailments, including ulcers and cancer. Peat mosses are absorptive and possess antibacterial properties; thus, Native Americans use it as bandages, diapers, and menstrual pads. In folk medicine, peat was used to disinfect and treat skin alignments (Riegler, 1989:28-40; Saxena and Harinder, 2004:56-65; Gruda, 2019:298).

Countries such as Finland, Sweden, Ireland, Germany, Belarus, Canada, and Russia are the top producers of commercially used peat. Peat uses are very popular because of its low cost, exceptional chemical, biological and physical properties with low nutrient content, low pH, a combination of high water-holding capacity by air space and drainage characteristics; it is light weight and are free of bothers and ailments. Peat also has unique microporous properties and is resistant (slow) to degradation (Gruda, 2019:298). The extraction and harvesting of peat have negative impacts on the environment. Peat harvesting has limited the availability of peat and contributes disproportionally to greenhouse gas emissions. Peat harvesting might still be

widespread. However, research on alternative growth media such as soilless culture and growth media are currently being investigated (Grundling and Grobler, 2005:379-396; Gruda, 2019:298).

2.1.3.2 Environmental threats to peatlands and impact of peatland destruction

A warmer temperature in conjunction with summer drought increases the decomposition rate of peat, which in turn enhances erosion, promotes fires, and ultimately alters species composition towards non-peat forming graminoid plants and shrubs (Dise and Phoenix, 2011:309-311). This not only threatens the peatland integrity but also their ability to store carbon long-term. Reactive nitrogen, which originates from agricultural emissions and fossil fuel combustion, is high in deposition worldwide and is a very big worry for developing countries because of its contribution to global warming (Galloway et al., 2004:153-220; Bragazza et al., 2005a:106-107, 2005b:223-225; Dorrepaal et al., 2009:93-95). As a regulating nutrient, nitrogen supports the long-term establishment of vascular plants, enhancing peat decomposition. However, elevated levels of nitrogen in the decomposed plant material leads to nutrient imbalances and toxicity reactions on bryophytes (Bates, 2002:309-342). This leads to deformities in plants, causing them to involuntarily absorb pollutants and nutrients. These stressors not only possess the potential to shift impacted peatlands from carbon sinks to carbon sources but also negatively affect the microbial communities (Dorrepaal et al., 2003:93-95). The microbial community can thus be used to measure soil health as they respond to environmental changes quickly since 'the microorganism that is best adapted will be the one that flourishes' (Pankhurst et al., 19951015-1016). Due to their high surface-to-volume ratio, microorganisms can also provide an early indication of soil improvement or early warning of soil degradation, as they respond faster to environmental stressors compared to higher organisms (Pankhurst et al., 1995:1015-1016). Previously, peatlands have been drained, burned, or dug up without taking into consideration how precious this unique wetland-type is (Dise and Phoenix, 2011:309-311). The overuse of peatlands, either for agricultural or residential development, has slowly led to their destruction (van Vuuren, 2010:14-20).

Since its establishment in 2004, the Working for Wetlands Programme has invested around R 826.8 million in the rehabilitation of over 1,000 wetlands. Over the years, the budget for the programme has increased, and more employment opportunities have been created. Investment of public funds in wetland rehabilitation encouraged a range of supporting activities, which include the publication by the Water Research Commission (WRC) in 2008 of

an 11 volume series of reports, manuals, and guidelines for wetland rehabilitation and assessment, strengthening the technical and scientific foundation of the programme's work, combined with an investment in wetland classification and inventory, thus improving the scope to plan and undertake a systematic rehabilitation at catchment scale (www.environment.gov.za, n.d.).

2.1.4 Peatland ecosystem services

Ecosystem services can be defined as the benefits we as humans receive from studying and understanding ecosystems. A term first used in the 1980s, ecosystem services is a 'framework for studying the structural and synthesising biophysical understanding of ecosystem process in terms of human well-being', life would not be able to exist without these ecosystem services (Mooney and Ehrlich, 1997:11-19; Braumann et al., 2007:67-70). These services can be divided into four categories which include provisioning services (1) such as providing food (fruits, vegetables, trees, fish, and livestock ready for human consumption). These services also include drinking water, wood timber, natural gas, oil, plants, and other materials that can be used to make medicine and clothing. Regulating services (2) to control floods and diseases: these services include plants cleaning the air and filtering water, bacteria decomposing waste, flowers being pollinated by bees, and trees preventing erosion by keeping the soil intact. Therefore, regulation services are processes working together to provide clean, sustainable, functional, and resilient ecosystem services. Cultural services (3) provide educational, spiritual, and recreational benefits. It contributes to the development and cultural advancement of people, while support services (4) are needed for the construction of all other ecosystems. Some include the production of biomass and atmospheric oxygen, the formation of soil and retention, nutrient cycling, water cycling, and establishment of habitats (Sarukhán and Whyte, 2005:1-3). Peatlands are known to provide a variety of ecosystem services contributing to the well-being of humans: climate regulation, water purification, recreational and educational opportunities, and an increase in tourism (Kimmel and Mander, 2010:500). Another service provided by peatlands is regulating climate change through carbon sequestration and releasing fixed carbon into the biosphere (Sarukhán and Whyte, 2005:1-3). Peatlands are important natural ecosystems with a high value for biodiversity conservation, climate regulation and human welfare (see section 2.1.3). However, due to the lack of policies required to protect these valuable ecosystems, many peatlands have been drained, burned, and damaged.

2.1.5 Carbon sequestration and hydrology of peatlands

The procedure of capturing and storing atmospheric carbon dioxide (CO_2) is known as carbon sequestration. This method is used to reduce atmospheric CO_2, thereby reducing the climate change effect. This process can occur naturally or through anthropogenic activities. In nature, carbon is removed by sequestering it in naturally occurring sinks such as forests, grasslands, and soil [downtoearth.org/5-11]. Peatlands are also known as carbon sinks (Figure 2.5). They are one of the largest natural terrestrial carbon stores. The process of carbon sequestration occurs in peatlands as long as the formation of the new peat is faster than the decay losses of all the previously accumulated peat (Gorham et al., 2012:77-78). Plant remains, above and below the ground, decay faster in the acrotelm (i.e., the peatland's surface layer), which only gets water seasonally. Peat starts forming at the bottom of the acrotelm, which then becomes part of the catotelm (i.e., the lower layer), which is always saturated. The acrotelm thickness is dependent on the plant species distribution, litter production, and litter decay losses (Malmer, 1962:1-4; Clymo, 1984:605; Walle'n, 1987:73-74; Walle'n et al., 1988:70-72; Belyea, 1996:529; Belyea and Malmer, 2004:1043-1045). Therefore, the rate at which peat is formed is faster for intermediate microforms and slower for microforms with a large amount of water (Belyea and Clymo, 2001:1315-1320; Belyea and Malmer, 2004:1043-1045). The decomposition of peat occurs slowly in the catotelm, and even though recalcitrant materials accumulate in the catotelm, it does not affect the catotelm thickness. Thus, the sequestration of carbon depends on the composition of the peatland vegetation, acrotelm, and catotelm thickness (Clymo *et al*., 1998:368-380; Belyea and Malmer, 2004:1043-1045).

The surface structure of the peatland and topography are linked to the hydrology of the peatland. Near the vegetation surface, hydraulic activity is high because the litter is not tightly packed together. The hydraulic conductivity (the speed at which water passes through a substrate) decreases as peat is formed from the decayed plant material (Boelter, 1969:606-607; Hoag and Price, 1995:171; Belyea and Malmer, 2004:1043-1045). Therefore, the groundwater will be at its peak near or at the water table (Hoag and Price, 1995:172). For peatlands that have a thick acrotelm (i.e., thicker than zero), there is an increase in water loss when the water table goes down. Water is lost by overland flow when the water table rises above the surface layer. An increase in the thickness of the catotelm causes a change in the hydraulic activity gradient. Thus, the acrotelm and catotelm thickness work together in controlling water losses through seepage and overland flow (Hilbert et al., 2000:230-240; Wieder, 2001:327-340; Malmer and Walle'n, 2004:111-117; Belyea and Malmer, 2004:1043-1045).

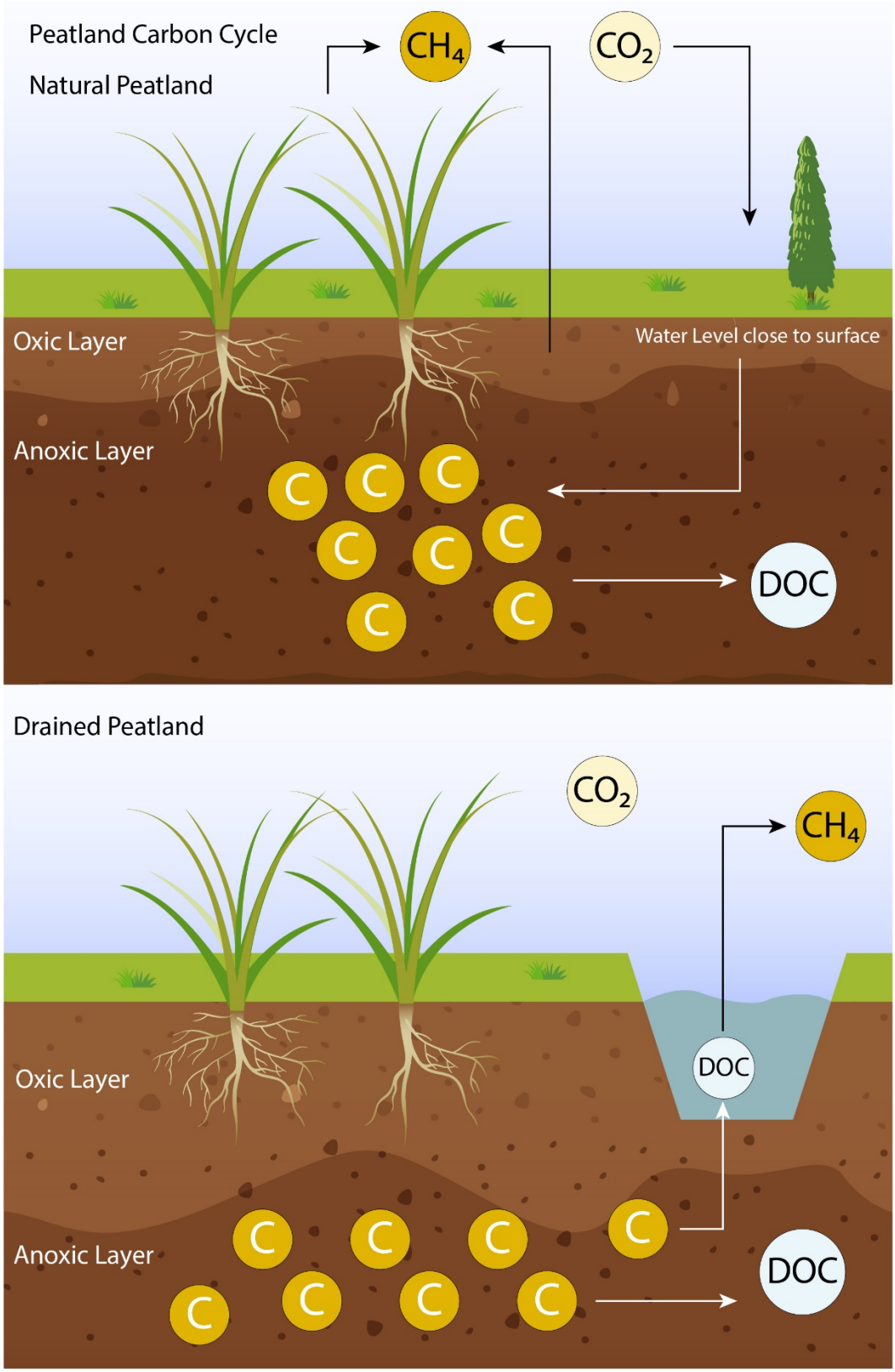

Figure 2.5: Schematic diagram of carbon cycling occurring in natural and drained peatlands (adapted from GRIDA, 2017). DOC = dissolved organic carbon

2.2 Macrophytes in peatlands

Macrophytes are very important as they are responsible for the organic material that makes up peatlands and or wetlands. They are aquatic plants such as flowering plants, bryophytes, lichens, and algae that are found in ponds, lakes, wetlands, and peatlands (Wilzbach and Cummins, 2019:594-596). Bryophytes are freshwater plants that consist of three non-vascular plants: liverworts, hornworts, and mosses (Figure 2.6). Aquatic mosses can be divided into *Sphagnales*, *Bryales* and *Andreales* (Hutchinson, 1975). The *Bryales* contain a wide variety of plants, such as *Fontinalis*, which is found at a depth of 120 m in Crater Lake and Fissidens. The *Sphagnales* and *Andreales* both contain aquatic vegetation of the genus *Sphagnum*. The total biomass of *Sphagnum* is greater than that of any of the other bryophytes and is often the dominant vegetation in shallow waters of peat bogs (Clymo and Hayward, 1982:229-250). Aquatic mosses are among the deepest living plants in lakes and have been found at 122 m in Lake Tahoe (Hutchinson, 1975).

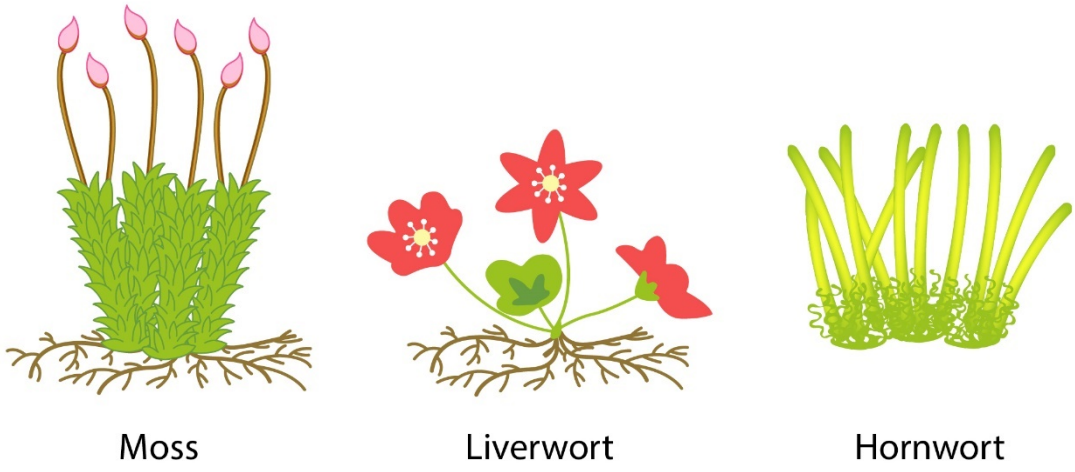

Figure 2.6: The different types of bryophytes found in nature and often in association with peatlands (adapted from https://tentativeplantscientist.wordpress.com/2013/04/02/plant-divisions-mosses-liverworts-and-hornworts/)

Macrophytes play an important role in maintaining the health of aquatic environments. They also harbour invertebrates that feed fish and wildlife, and provide organic carbon to bacteria (Bowden et al., 2017:243-245). Macrophytes are also responsible for reducing the turbidity in lakes by keeping down sediments; they remove excess nutrients and reduce phytoplankton (Timms and Moss, 1984:472-480; Dvořák, 1996:27-30; Rejmánková, 2011:333-340).

In 1975, Hutchinson created a detailed macrophyte classification method by combining various plants based on their water and rock relationship. This is a widely accepted system and is used to classify freshwater macrophytes. The four main categories differentiate between free-floating macrophytes and those that are attached to the various aquatic environments. The attached or rooted macrophytes can be divided into three categories: floating, submerged, and emergent macrophytes (Figure 2.7). Emerging and floating macrophytes remove contaminants using the plant's root, whereas submerged macrophytes absorb and accumulate metals (Rahman and Hasegawa, 2011:633-640; Nakbanpote *et al*., 2016:179-210).

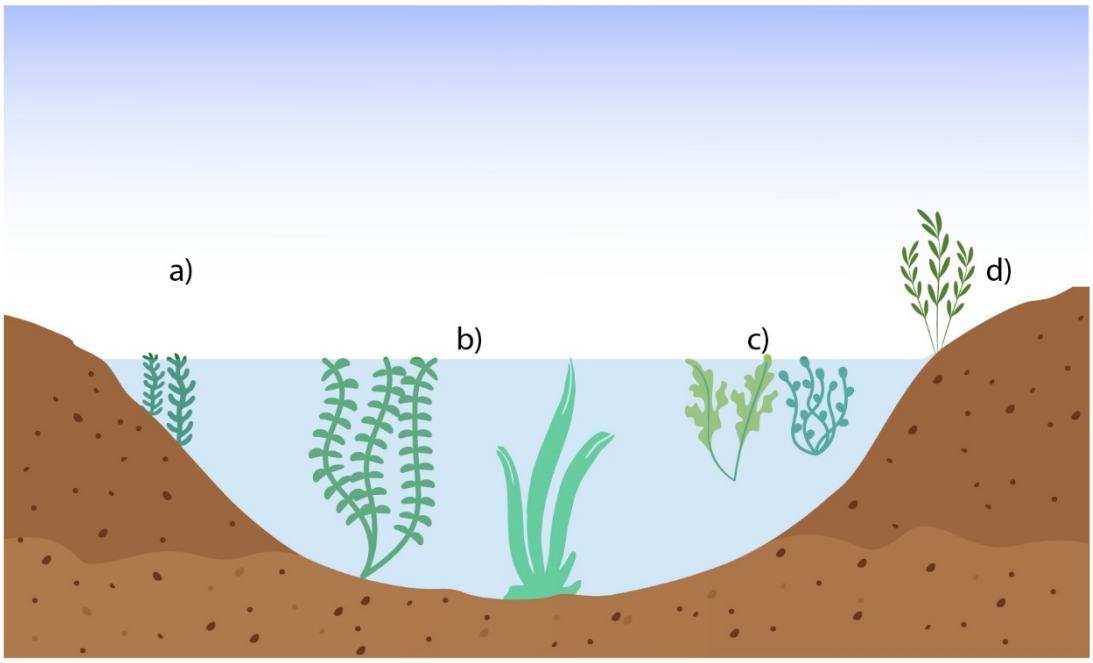

Figure 2.7: Different types of macrophytes according to their water and rock relationship, (a) submerged macrophytes, (b) floating macrophytes, (c) free-floating macrophytes, and (d) emergent macrophytes (adapted from Schoelynck et al., 2012: 760-766)

2.3 Physicochemical characteristics of peatlands

The peat properties tell a lot about the peat-forming environment, such as how the peat is formed and the type of plants that make up the peat-environment. These properties can also be used to classify and evaluate the quality of the peat. Peat's physical properties include the degree of decomposition or humification, water content, specific density, and bulk density (Nature and Management of Tropical Peat Soils,1988; Gao, 2009:1-39). Decomposition or humification is when organic matter is lost, resulting in the disappearance of the plant's physical structure and its chemical composition. Aerobic decomposition occurs and produces

CO_2 and water, which is caused by soil microflora, fungi, and bacteria (Huat et al., 2014). According to Biester et al. (2013:2691-2692), carbon and nitrogen levels are very low in the peat's surface layer but increases with depth. Aerobic decomposition is fast in the surface layer, but water presence lowers the oxygen levels, resulting in a reduction of aerobic microbial activity, promoting slower, anaerobic decomposition (Hashim and Islam, 2008:1-9). The degree of decomposition can be divided into low, moderate, and high decomposition according to the degree that the plant material decays (Nature and Management of Tropical Peat Soils,1988; Gao, 2009:1-39). In 1926, Lennart von Post created a scale (that is still used today) to measure peat's decomposition degree (Table 2.3). The scale contains various parameters used to measure the decomposition degree (Rokus, 2020).

Table 2.3: Summary of von Post's humification scale as applied to peat (Rokus, 2020)

Conditions of Peat before squeezing					**Conditions of peat when squeezing**		
Degree of humification	**Soil Colour**	**State of Decomposition**	**Plant structure**	**Content of amorphous material**	**Squeezed solution**	**Material extruded**	**Nature of Residue**
H1	White or yellow	None	Easily Identified	None	Clear, colourless water	No material	Not pasty
H2	Very pale brown	Insignificant	Easily Identified	None	Yellowish water/ pale brown-yellow	No material	Not pasty
H3	Pale brown	Very slight	Still identifiable	Slight	Brown, muddy water, not peat	No material	Not pasty
H4	Pale brown	Slight	Not easily identified	Some	Brown, muddy water, not peat	Some peat	Somewhat pasty
H5	Brown	Moderate	Recognised but vague	Considerable	Very dark brown muddy water	Some peat	Strongly pasty
H6	Brown	Moderately strong	Indistinct	Considerable	Very dark brown muddy water	About $\frac{1}{3}$ of peat squeezed out	Very strongly pasty
H7	Dark brown	Strong	Fairly recognisable	High	Very dark brown muddy water	About $\frac{1}{2}$ of peat squeezed out	Very strongly pasty
H8	Dark brown	Very strong	Very distinct	High	Very dark brown pasty water	About $\frac{2}{3}$ of peat squeezed out	Very strongly pasty
H9	Very dark brown	Nearly complete	Almost unrecognisable	Very high	Very dark brown paste	Nearly all the peat squeezed out as a uniform paste	Very strongly pasty
H10	Black	Complete	Not discernible	Whole	Very dark brown paste	All peat passes through the fingers	Very strongly pasty

The specific density of peat, which is closely related to the components of the plant residue found in peat, is relatively low and ranges between 1 to 1.6 kg/m^2; the bulk density depends on the ash content of the peat, decomposition degree and components of the plant residue is low and ranges between 0.1 and 0.5 Mg/m^3. In sphagnum peat, the humidity and saturated soil water content are high as these peatlands do not contain a lot of herbaceous-woody peat (Jinsheng, 2009:1-32).

The chemical properties of peat typically evaluated include element composition, organic components, and ash content. Carbon, hydrogen, oxygen, sulphur and nitrogen are the five basic elements of peat, with the elemental properties between wood and coal. The elemental composition of poorly decomposed peat is similar to that of wood, while the elemental properties of highly decomposed peat resemble that of lignite. The peat's organic material can be divided into four groups: bitumen, water-soluble, humus, and a mixture of lignin and other plant-like components (Gao, 2009:1-39). Soil with a 35% organic content is typically regarded as peat. However, various researchers have their own ideas as to what they regard as peat (Paul et al., 2018:692-700). Hobbs (1986:7-80) regards soil with an organic content of 27.5% as peat, while geotechnical engineers regard soil with an organic content of or greater than 75% as peat (Paul et al., 2018:692-700).

2.4 Microbial communities in peatlands

Peatlands act as a natural habitat for microorganisms such as fungi and bacteria. Aerobic bacteria, facultative anaerobes, and fungi (spore numbers decrease with depth) can be found in the peatland's top layer, while anaerobic microorganisms are located deeper within the peatland. The presence of these microorganisms is essential in peat as they aid in the breakdown of the plant material (Golovchenko et al., 2002:667-668; Ellery et al., 2012:11-13). It is believed that fungi are the principal aerobic decomposers in peatlands (Thormann, 2006b:101-110; Peltoniemi et al., 2009:1902-1903). Actinobacteria are also thought to be essential decomposers in peatlands. They can break down cellulose in a similar way to fungi, and several actinobacterial strains can metabolise lignin and other complex polymers (Pankratov et al., 2006:428-430).

2.4.1 Microbial community structures

The peatland microbial structure is highly affected by the capacity of the peatland to accumulate carbon as the photosynthesis input exceeds the carbon losses through decomposition. As organic material accumulates, the peatland microorganisms face an

increase in energy constraints with depth, which is caused by a combination of factors as well as oxygen (O_2) availability and other electron acceptors. This means that the deeper organic material region needs more specialised microorganisms that can breakdown the plant material. Aerobic decomposers such as saprotrophs dominate the top layer or acrotelm of the peatland. These microorganisms are thought to play a more important role during the early stages of the decaying processes (Newell et al., 1995:135-147*;* Kuehn et al., 2000:862-864*).* The interface between the peatland's oxic and anoxic layers is called the mesotelm (Clymo and Bryant, 2008:2048-2050). This is the layer in which water fluctuates. The presence of methane (CH_4) and O_2 makes this layer ideal for methanotrophs. Methanotrophic bacteria have a methane monooxygenase enzyme (MMO) that allows them to metabolise methane, producing both carbon dioxide and biomass (Hanson and Hanson, 1996:439-470). The methanotrophs found in this layer belong to the bacterial phyla *Proteobacteria* and *Verrucomicrobia.* Deeper in the peatland's anaerobic region, aerobic bacteria are not present because of the low hydraulic conductivity and small pore size. This, however, does not affect facultative anaerobes (Andersen et al., 2013:979-990).

The role of bacteria in anoxic environments is diverse (Westermann, 1993:215-230). Despite this, fungi are considered to be the main decomposers in oxic peat layers (Thormann et al., 2004a:710-720, 2004b:793-800; Thormann, 2006b:101-120). Most fungal decomposers in peatlands occur in the top layer of the peat (Latter et al., 1967:445-446; Nilsson and Rülcker, 1992:795-797). Following a water-level drawdown, fungal decomposers should become more abundant and active as the depth of the oxic layer rises (Jaatinen et al., 2007:492).

2.4.2 Microorganisms as indicators of soil health

Soil health focuses on the constant soil capacity to tolerate the growth of plants and maintaining its functions. Conservation and degradation processes strongly influence soil health and the soil biota, which in turn influence plant health, environmental health, food safety, and quality. Parameters that affect soil health include organic matter content, number or diversity of microorganisms, and microbial constituents (Singer and Ewing, 2000:271-298). Soil is dominated by a solid phase consisting of particles of various sizes in close contact with gas and water, the amount and composition of which noticeably change in space and time. Water usually is discontinuous, meaning that the pore space is filled with air and other gases, except when the soil is saturated with water (Stotzky, 1997:1-20). Physical, chemical, and biological processes control the constant interchange of molecules and ions between the solid, liquid, and gaseous phases. These processes represent a unique balance between the physical, chemical, and biological components, thus maintaining this balance is very important to soil health (Doran and Parkin, 1994:3-18). The microbial biomass, soil basal respiration,

enzyme activity, and nutrient transformations are important soil fertility characteristics that can be used to assess soil contamination, agricultural use, suitable management, and restoration efforts. Thus, we can use these cost-effective characteristics as biological indicators to monitor and evaluate environmental health and biogeographic changes in the environment (Niemeyer *et al*., 2012:103).

The microorganisms in the topsoil layer are essential in N, P, and S cycling and the degradation of organic matter, thus affecting carbon and nutrient cycling on a global scale. This means that the energy input into soil ecosystems is derived from the microbial degradation of dead plant and animal organic matter (Pankhurst et al., 1997:419-420). Microbial biomass is an important component of soil biological fertility, as it participates in the biogeochemical cycle of nutrients and other elements. Enzymes catalyse microbiologically mediated activities, which are crucial for soil functioning and provide the basis for C, N, P, and S cycling (Niemeyer et al., 2012:96-97). According to Torstensson et al. (1998:4-8), microorganisms can further be associated with the transformation and degradation of waste nutrients and synthetic organic compounds. By producing extracellular polysaccharides and other cellular compounds, the microbial community maintain soil structure by using these products as cementing agents to stabilise soil aggregates. However, this also affects the soil's water-holding capacity, infiltration and erosion rate, crushing, and susceptibility to compaction (Elliott et al., 1996:1-21). Soil health cannot be measured with physical or chemical applications. However, based on what is known about the role of microbial communities in soil and peat environments and the fact that microorganisms respond to environmental changes quickly, the microbial community structure may act as an indicator of change in soil health (Pankhurst et al., 1995:419-420; Kennedy and Papendick, 1995:243-247).

2.4.3 Actinobacteria

2.4.3.1 Introduction to actinobacteria

Actinobacteria are a group of Gram-positive to Gram-variable bacteria containing high guanine and cytosine content in their DNA that ranges from just under 50% to over 70%, which are recorded from 16S rRNA cataloguing and DNA-RNA pairing studies (Amin et al., 2020:1-2). Their morphology and physiology are very diverse and ranges from coccoid or rod-coccoid to fragmenting hyphal forms or permanent and highly differentiated branched mycelium (Figure 2.8 and Figure 2.9) (Atlas, 1997). Actinobacteria possess diverse physiological and metabolic properties. They are known to produce extracellular enzymes and various secondary metabolites, which makes these organisms biotechnologically valuable. Members of the

genus *Streptomyces* have been researched countless times and are used as antibiotic-producing organisms in the pharmaceutical industry. Actinobacteria possess many other uses, including being used as producers of enzyme inhibitors applied in the treatment of cancer, the production of immune modifiers, microbially transforming organic compounds, the production of antimicrobial drugs, and they even have the ability to degrade hydrocarbons, pesticides, aliphatic and aromatic compounds (Bérdy, 2005:1-26; Anandan et al., 2016). Actinobacteria are distributed everywhere: on land, aquatic, and marine areas. Soil actinobacteria are very important as they take part in the degradation of complex polymers (Gorden et al., 1999:881-890; Stach and Bull, 2005:3-4).

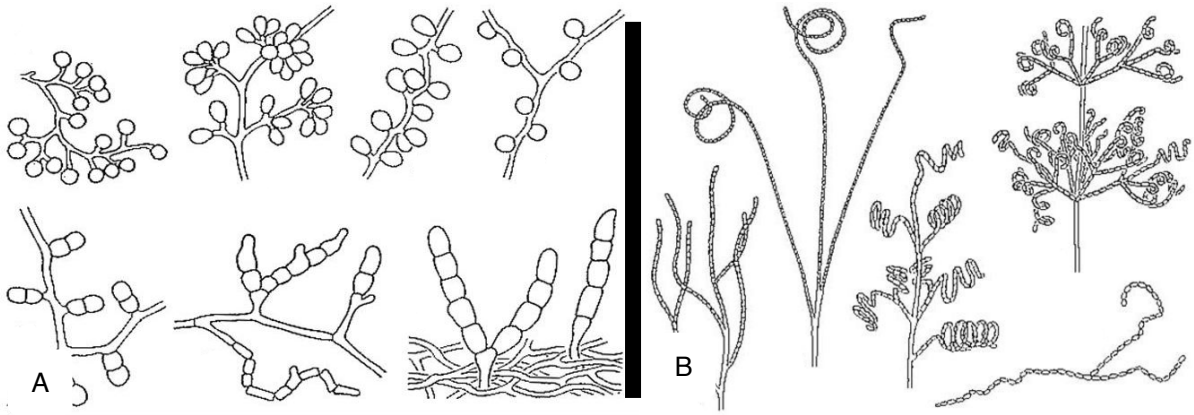

Figure 2.8: Examples of actinobacterial growth: [A] Single spore production and spores in short chains. [B] Spore production in long chains (Li et al., 2016:62)

2.4.3.2 Characteristics

Actinobacteria are unicellular and can be aerobic, anaerobic, or facultative anaerobic microorganisms. They reproduce via binary fission, sporulation, or conidia formation. On agar, actinobacteria may appear leathery with aerial mycelium or have a mat appearance, or even colonies with a shine. Filamentous actinobacteria often referred to as actinomycetes, contain aerial and substrate mycelium (Figure 2.9). The aerial mycelium is often thicker and can be used (i.e., under fixed conditions) to group isolates together. The substrate mycelium varies in size, shape, and thickness, and its colour ranges from being transparent to yellow, brown, pink, blue, grey, orange, green, or black (Anandan et al., 2016).

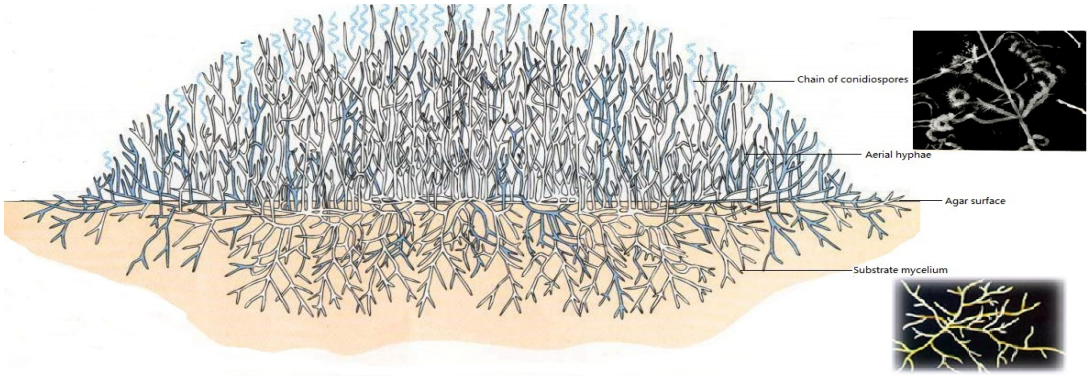

Figure 2.9: Diagram of an actinomycete colony growing on agar. The figure displays a clear depiction of the substrate mycelium and aerial mycelium with chains of conidiospores (Li et al., 2016:61)

Members of the Class Actinobacteria can further be grouped into 20 families (https://www.lpsn.dsmz.de/class/actinobacteria). As summarised in Table 2.4, there are many different types of actinobacteria, including thermophilic, acidophilic, halophilic, endophytic, symbiotic, endosymbiotic, and gut actinobacteria.

Table 2.4: Different types of actinobacteria: grouping based on environmental adaptation (Anandan et al., 2016)

Name	Description	Growth conditions	Examples
Thermophilic actinobacteria	These are actinobacteria that grow at extreme temperatures.	Mesophilic actinobacteria: 20°C – 42°C Moderately thermophilic actinobacteria: 45°C - 55°C Thermophilic actinobacteria: 37°C - 65°C	*Thermomonospora, Microbispora, Saccharopolyspora, Saccharomonospora,* and *Streptomyces*
Acidophilic actinobacteria	These are actinobacteria that grow at a very low pH.	pH 3.5 – 6.5 Optimum growth rate: pH 4.5 – 5.5	*Streptacidiphilus, Kitasatospora, Streptomyces*
Halophilic actinobacteria	These are salt-loving actinobacteria.	Halophiles: 1.5 – 4.0 M salt Extreme Halophiles: 2.5 – 5.2 M salt	*Dietzia, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium, Gordonia*
Endophytic actinobacteria	These are actinobacteria that inhibit the internal part of plants, causing no diseases in the plants.	Found in: *Palicourea longifolia, Calycophyllum acreanum, Monstera spruceana, Croton lechleri,* etc.	*Frankia, Streptomyces Streptoverticillium*, *Nocardia*, *Micromonospora, Kitasatospora, Pseudonocardia*
Symbiotic actinobacteria	These actinobacteria form a symbiotic relationship with plants called actinorhizal plants.	Ideal soil temperature for optimum growth is 25°C.	*Frankia*
Endosymbiotic actinobacteria	These actinobacteria are found living in the body or cells of another organism.	Sea sponges Animals	*Mycobacterium, Spheciospongia vagabunda, Hylobates hoolock*
Gut Actinobacteria	These actinobacteria are found in the gut of invertebrates and vertebrates.	They are able to adapt and grow at the pH and temperature level of the organisms they inhabit.	*Streptomyces*, *Nocardiopsis*, *Oerskovia*
Pathogenic actinobacteria	These actinobacteria are harmful to humans, animals, and plants.	They are able to adapt and grow at the pH and temperature level of the organisms they inhabit.	Including species from *Corynebacterium, Mycobacterium, Nocardia,* and *Rhodococcus*

2.4.3.3 Presence and function of actinobacteria in peatlands

Actinobacteria are found in various locations, especially aquatic, marine, and soil environments. They can be found in Egypt's sands or the deepest parts of the ocean and play various (and important) roles within these environments (Anandan et al., 2016). Some actinobacteria are leaf and root endophytes. A study by Opelt et al. (2007:38-50) reports that actinobacteria are very much present amongst the endophytes present in sphagnum (plant type that can be found all over in northern hemisphere peatlands). Table 2.5 summarises selected actinobacteria detected and identified in peat environments from around the world.

Table 2.5: Global microbial ecology of peatlands worldwide (bogs and fens): Actinobacteria

Actinobacterial Taxa	**Location**	**Reference**
Nocardiopsis	Algeria	Meklat et al., 2020
Agromyces, Arthrobacter, Isoptericola, Microbacterium, Micrococcus, Oerskovia, Rhodococcus, and *Streptomyces*	Korea	Yun et al., 2017
Gordonia, Leifsonia, Microbacterium, Micromonospora, Mycobacterium, Nocardia, Nocardioides, Sinomonas, Streptacidiphilus, Streptomyces, and *Terrabacter*	Malaysia	Lee et al., 2014
Microbacterium	Finland	Virpiranta et al., 2020
Streptomyces	Wisconsin	Mason et al., 2014
Microbacterium	Poland	Virpiranta et al., *2020*
Brevibacteriaceae, Corynebacteriaceae, Dermabacteraceae, Dietziaceae, Microbacteriaceae, Micrococcaceae, Nocardiaceae, and *Streptomycetaceae*	Chile	Undabarrena et al., 2016

In peatlands, these microorganisms play a major role in the breakdown of plant material, resulting in peat formation and releasing CO_2 and CH_4 (Myers et al., 2012:77-88). In ombrotrophic sphagnum-dominated peatlands, the breakdown of plant material (which include cellulose) occurs very slowly because of the acidic environment, the absence or presence of nitrogen – phosphorous agents and phenolics produced by the sphagnum mosses (Naplekova, 1974:250; Verhoeven and Toth, 1995:271-274; Mitchell et al., 2003:187-188). Pankratov et al. (2006:428-430) identified actinobacteria as one of the main decomposers of organic material (in peatlands) under acidic and oligotrophic conditions (in the absence of fungi). A later study revealed that these actinobacteria were mainly from the genus *Streptomyces* (Pankratov and Dedysh, 2009:227-230). Similar to fungi, actinobacteria can break down cellulose (even in its crystalline form) and metabolise lignin and other complex polymers. They are able to break down cellulose by secreting the enzyme cellulase (which is induced by cellulose, cellobiose, sophorose, and lactose) into their surroundings or has the enzyme located on their surface layer. The enzyme can break down the

insoluble macromolecules of cellulose into oligomers and is then taken up by the bacteria to be metabolised (Eriksson et al., 1990; Hasegawa et al., 2007:72-81). Actinobacteria are known to produce secondary metabolites, some even possessing antifungal properties (Strobel, 2003:535-536; Hasegawa et al., 2007:72-81; Opelt et al., 2007:38-50) and because of this could inhibit other microbes that take part in the decomposition possesses, thereby playing a role in microbial competition for carbon and nutrients. The microbial activity of *Frankiaceae* (a family within the class Actinobacteria) indicated that there are some microbes present in bogs and fens that form a symbiotic relationship with actinorhizal plants. These bacteria live in symbiosis with the plant providing nutrients to the plant; they are also known to produce carbohydrate degrading proteins, which aid with the breakdown of dead plant material (Peltoniemi et al., 2012:20-30). A study performed by Winsborough & Basiliko (2010:315-320) on the fungal and bacterial activities across three different peatlands in Canada showed that the bacteria present in the peatland were responsible for most of the microbial activity that was occurring in the peatland. Many different types of Actinobacteria can break down lignin, cellulose, and hemicellulose. Some types can decompose woody tissue, which contains all three of the components mentioned above (Hasegawa et al., 2007:72-81).

2.4.4 Fungi associated with peatlands

2.4.4.1 Introduction to fungi

Fungi are eukaryotic microorganisms that digest their food externally and take the resulting nutrients in through their cell wall. They reproduce asexually via spores, and their body is composed of hyphae (i.e., microscopic tubular cells) (Figure 2.10). Fungi are heterotrophs because they receive carbon from other organisms for their metabolism and nutrients (Little et al., 2012). The use of fungi has been applied in many industries, which makes them very important. Fungi are used in the recycling of dead and decaying organic material, as food, in medicine, as biocontrol agents to control pests, and are often associated with food spoilage (Webster and Webster, 2007).

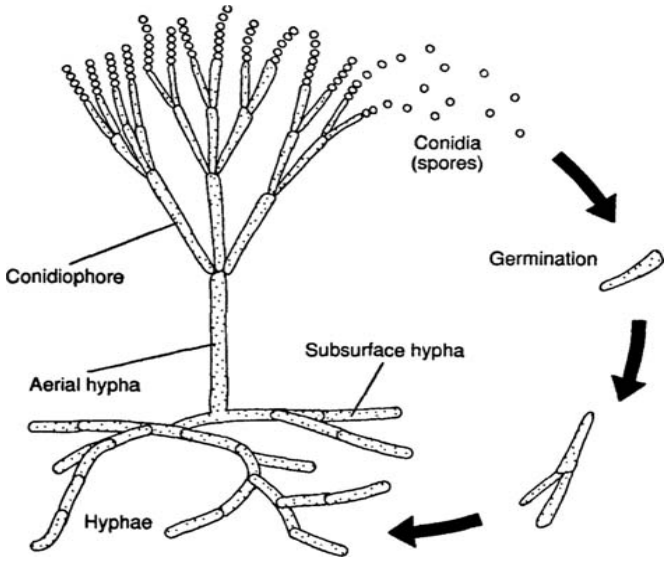

Figure 2.10: This image shows the microscopic structure of a septate fungus containing conidia produced asexually and shed off the fungus (CliffesNotes, 2021).

2.4.4.2 Characteristics

Fungi are achlorophyllous and can either be unicellular or multicellular. They reproduce through asexual and sexual spores. Most fungi are terrestrial. However, some occur in aquatic environments. Fungi are eukaryotes as they possess membrane-bound nuclei, which contain chromosomes and a range of membrane-bound cytoplasmic organelles such as mitochondria, vacuoles, endoplasmic reticulum, etc. (Whittaker, 1969:150-160; Deacon, 2013; Walker and White, 2017:1-35). Most fungi are filamentous as they are composed of microscopic filaments called hyphae, which exhibit apical growth and branching, forming a network of hyphae called mycelia (Harris, 2019:35-39). Fungi have nuclei that are typically haploid and hyphal compartments are often multinucleate. All fungi are achlorophyllous as they do not have chlorophyll pigments and are incapable of photosynthesis. They are chemoheterotrophic as they utilise pre-existing organic sources of carbon found in the environment and energy from the chemical reactions to synthesise the organic compounds required for growth and energy (Cole, 1996:73; Mehrotra and Aggarwal, 2013:24-28). Fungi possess a range of storage compounds such as trehalose, glycogen, sugar, alcohols, and lipids. They are organisms that can either occur free-living, as parasites or in a symbiotic relationship with other organisms (Lewis and Smith, 1967:143-144; Overstreet and Lotz, 2016:27-28).

Most fungal species can be challenging to isolate and culture, with some fungi being unculturable. Thus, molecular techniques can be used to identify or determine diversity. Genomic DNA or metagenomic DNA can be amplified using PCR (Polymerase chain reaction) techniques where specific primers [e.g., internal transcribed spacer (ITS) primers] can be used when targeting fungi. ITS primers target and amplify the ITS region in fungal DNA. These methods have been used to successfully uncover the fungal diversity and allows for community comparison of fungi in various substrates and areas (Tedersoo et al., 2015:936).

2.4.4.3 Presence and function of fungi in peatlands

Many studies suggest that in peatlands, the fungal communities dominate over the bacterial communities present in the peat, which indicates that fungi are responsible for the majority of the breakdown of plant material in many of these ecosystems (Kox, 1954:111; Latter et al., 1967:445-446; Williams and Crawford, 1983:201-202). This is because of their wide-ranging hyphal growth, fast growth rate and the ability to transport and relocate nutrients through their hyphal network. The fungal communities of various substrata worldwide have been examined, and more than 860 records were reported from peatlands that represent 656 different fungal species. These include 408 anamorphic species, 22 teleomorphic ascomycetes, 25 basidiomycetes, 67 zygomycetes, 28 chytridiomycetes, and 108 unidentifiable organisms (yeasts, etc.). Even though many fungal communities could be identified, there are still large amounts of unidentified fungal species present in peatlands (Thormann, 2006b:101-120). Table 2.6 summarises selected fungi detected and identified from peat environments around the world.

Table 2.6: Global microbial ecology of peatlands worldwide (bogs & fens): Fungi

	Fungal Taxa	Location	Reference

Ascomycota	*Acremonium* species	Canada, Italy, Russia, U.K., USA	Bisby et al.,1935; Stenton, 1953; Boswell, 1955; Thornton, 1956; Sewell, 1959 a,b; Christensen & Whittingham, 1965; Dickinson & Dooley, 1969; Dooley & Dickinson, 1971; Maciejowska-Pokacka, 1971; Dal Vesco, 1974, 1975; Cormier et al., 1988; Golovchenko et al., 2002; Thormann & Rice, 2007
	Aspergillus species	Argentina, Canada, Ireland, Russia, U.K.	Bisby et al., 1935; Stenton, 1953; Latter et al., 1967; Dickinson & Dooley, 1969; Maciejowska-Pokacka, 1971; Hurley, 1981; Croft et al., 2001; Golovchenko et al., 2002; Robson et al., 2004; Thormann & Rice, 2007
	Chrysosporium species	Canada, Russia	Christensen & Cook, 1970; Golovchenko et al., 2002
	Cladosporium species	Argentina, Canada, Ireland, Sweden, U.K., USA	Boswell, 1955; Christensen & Whittingham, 1965; Dickinson & Dooley, 1969; Christensen & Cook, 1970; Dickinson & Maggs, 1974; Cormier et al., 1988; Nilsson et al., 1992; Searles et al., 2001; Thormann & Rice, 2007
	Penicillium species	Argentina, Canada, Ireland, Italy, Russia, U.K., USA	Bisby et al., 1935; Stenton, 1953; Boswell, 1955; Thornton, 1956; Sewell, 1959a,b; Christensen & Whittingham, 1965; Latter et al., 1967; Dickinson & Dooley, 1969; Christensen & Cook, 1970; Gantimurova, 1970; Maciejowska-Pokacka, 1971; Dal Vesco, 1974, 1975; Hurley, 1981; Cormier et al., 1988; Croft et al., 2001; Searles et al., 2001; Golovchenko et al., 2002; Summerbell, 2005; Rice et al., 2006
Basidiomycota	*Amphinema* species	Canada, USA	Wurtzburger et al., 2004; Robertson et al., 2006
	Cortinarius species	Canada	Roberts et al., 2004
	Trichosporon species	Russia	Golovchenko et al., 2002
	Mycena sanguinolenta (Alb. & Schwein.) P. Kumm.	Canada, Norway, UK	Oudemans, 1919; Roberts et al., 2004
	Lepiota cepistipes var. flos-sulphuris (Schnizl.) Rick	France, India, Japan, The Netherlands	Oudemans, 1919
Chytridiomycota	*Rhizophydium* species	USA	Sparrow & Lange, 1977
	Rhizophydium keratinophilum Karling	Poland	Czeczuga, 1993
	Blastocladiopsis parva (Whiffen) Sparrow	USA	Czeczuga, 1993
Zygomycota	*Mortierella* species	Argentina, Austria, Canada, Ireland, Italy, Russia	Loub, 1960; Dickinson & Dooley, 1969; Dal Vesco, 1974, 1975; Hurley, 1981; Searles et al., 2001; Golovchenko et al., 2002

Penicillium, *Trichoderma*, *Aspergillus*, and many other microfungi (also known as prolific sporulators) have been isolated from peat and peatlands. These microorganisms are easily

culturable because they have a fast growth rate and can be cultured on standard media under standard growth conditions. The fact that these microfungi are easily culturable explains why they have been so dominant in studies, regardless of their location and substrate (Thormann, 2006a:281-290). Deacon (2013) made a list of five major "behaviour groupings" of saprobes. Group 1 (fungi, weak parasites, and pathogens) contain microbes that are able to endure mechanisms the host uses as a defence. These microbes are very active in dead organic matter, and examples include *Cladosporium*, *Aurebasidium, Botrytis,* and *Alternaria*. They use simple sugars and other plant exudates. The majority of group 2 microorganisms are zygomycetes which include *Mucor* and *Martierella*. In group 3, the polymer-degrading fungi are found and include the species of *Fusarium, Chaetomium, Trichoderma,* and *Penicillium.* The microorganisms responsible for the degradation of recalcitrant polymers, the basidiomycetes, and selected groups of ascomycetes, are found in group 4, while in group 5, secondary saprobes that are common during the entire process of the decomposition of organic material can be found (Thormann, 2006b:101-120).

Previous research shows that fungi from all the five "behavioural groups" have been isolated from peat and peatlands but that the fungi from group 4 are rarely reported. Research links these fungal groups found in group 4 to the breakdown of the most complex structural polymers, including lignin, tannins, and other polyphenolics, contributing up to 50% of the chemical composition of peat (Turestsky et al., 2000:379-380). According to Hutchinson (1990), there are two reasons why group 4 microorganisms are underrepresented in previous studies. They suggest that these fungi (basidiomycetes) are rare in peatlands. The rarity of these microorganisms explains why organic material is not entirely decomposed and accumulates in peatlands. Secondly, it could be that isolation protocols previously used may have been lacking (Thormann, 2006b:101-120).

The breakdown of sphagnum occurs in various ways, and this depends on the type of fungal communities present. This is thought to be as a result of them competing against other types of microorganisms living in their environment (Thormann, 2006b:101-120). The breakdown of the leaf or plant tissue occurs before the *hyphae* of fungus attacks the plants. This is happening because the microfungi release enzymes into nature; the plant first takes this up before the hyphae start growing (Thormann, 2006a:281-290). Some plant types (*Sphagnum fallax)* have the ability to form secondary cell walls when it experiences a fungal attack (Untiedt and Muller, 1985:757-758). However, in sphagnum plants, this defence mechanism does not work because the plants eventually succumb to the fungal attack (Thormann, 2006b:101-120). As mentioned

above, the different fungal communities of bryophytes work in different ways to attack, resulting in the plant's decomposition. These fungi attack the plant by producing unique enzymes that decompose the plant's organic material (Redhead, 1981:63-67; Untiedt and Muller 1985:757-758; Tsuneda et al., 2001a:1217-1218, 2001b:93-95; Thormann, 2006a:281-290). Ericoid mycorrhizal fungi such as *Oidiodendron maius* (produce enzymes that can break down tannic acid, cellulose, and starch) simultaneously causes the in vitro breakdown of all the components in the cell wall, causing the formation of holes (Thormann et al., 2002:204-210). However, *Acremonium* cf. *curvulum* possess the ability to cause the in vitro breakdown of starch and cellulose. This fungus starts the process of decomposition, releasing enzymes that fragments and removes the structure-less cell wall outer layer, then targets the inner and central cell walls. It then decomposes the cell wall's microfibrils leading to the formation of holes in the leaf tissue (Thormann et al., 2002:204-210; Thormann et al., 2004a:710-711, 2004b:793-794). The microfungus, *Scleroconidioma sphagnicola,* perforates the plant's chlorophyllous cell, causing the degradation of chloroplasts, which results in chlorosis and ultimately the death of the leaf tissue. The hyphae of *Scleroconidioma sphagnicola* then attack the surrounding leaf cells, which result in the formation of holes in the leaves (Tsuneda *et al*., 2001a:1217-1218, 2001b:93-95). *Ledum palustre* uses the same decomposition mode as *Scleroconidioma sphagnicola* (Redhead, 1981:63-67; Untiedt and Muller, 1985:757-758).

2.5 Studying peatland microbial communities: the use of molecular-based and culture-based methodologies

As previously indicated, little is known about the microbial communities of South African peatlands, and the majority of literature concerns studies on the microbial communities of northern hemisphere peatlands. Microbial diversity may be examined using a range of techniques, including molecular and culture-based methods. Because not all microorganisms can be grown, molecular techniques are preferred for determining overall diversity and/or providing a glimpse of variety at the sample location.

2.5.1 Polymerase chain reaction

Genotypic detection is a molecular DNA-based approach that amplifies target sequences using either direct cloning and sequencing of DNA fragments or polymerase chain reaction (Saiki et al., 1985:1350-1354). The polymerase chain reaction is a method for amplifying a nucleic acid target sequence; the amplified sequences might be gene-specific, repeating sections of the sequence,

or random sequences. However, in order for the PCR method to work, the amplified sequence of a specific section of DNA must be known (Olsen et al., 1995:1-10). Post-PCR detection methods include hybridisation analysis, gel electrophoresis, and the use of specific nucleic acid probes (Hakovirta, 2008).

The 16S rRNA gene that is present in bacteria is used to amplify a targeted sequence for identification and phylogenic purposes using universal or strain-specific primers (Weisburg et al., 1991:697-700). Databases such as the Ribosomal Data Base Project (http://rdp.cme.msu.edu/) and the National Centre for Biotechnology Information Blast Library (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) have been created to handle the ever-increasing number of data. Thus, by comparing the 16S rRNA gene sequences in the databases to those of the microbe recovered, these databases may be utilized to identify previously grown and uncultured microorganisms (Drancourt et al., 2000:3623-3625). For the simultaneous detection, quantification, and differentiation of live bacterial cells, many PCR applications have been developed. Multiplex PCR, which uses a variety of primers to target each bacterial strain, is one of these detection approaches (Touron et al., 2005:541-550; Yaron and Matthews 2002:633-638). Multiplex PCR techniques have been developed by Settanni & Corsetti (2007:1-22) to distinguish between numerous species belonging to a single genus as well as mixed microbial populations.

2.5.2 Non-restriction enzyme DNA fingerprinting techniques

2.5.2.1 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a fingerprinting technique used to separate PCR results. When ambient metagenomic DNA is amplified, it yields amplicons of diverse DNA sequences that represent many of the major microbial species occurring in that habitat. DGGE, as opposed to agarose gel electrophoresis, separates PCR amplicons based on their sequence, resulting in unique denaturing characteristics of the DNA (Muyzer and Smalla, 1998:127-128).

2.5.2.2 Fluorescent *in situ* hybridisation

Fluorescent *in situ* hybridisation (FISH) identifies both culturable and unculturable microorganisms and is very successful for identifying bacteria in complex environments such as soil, biofilms, activated sludge, and so on (Juretschko et al., 2002:84-85; Moter and Göbel, 2000:85-110). This approach identifies nucleic acid sequences by employing a fluorescently

tagged probe that preferentially binds to its complementary target sequence within the intact cell. FISH has limitations, such as permeability difficulties, and is only applicable to species with known DNA sequences (Moter and Göbel, 2000:85-110).

2.5.2.3 Restriction enzyme DNA fingerprinting techniques

Another form of genotypic detection method is based on restriction digests using bacterial enzymes (Towner and Cockayne, 1993). Different restriction enzymes break DNA in different locations, resulting in a restriction pattern of DNA fragments that is unique to the restriction enzyme used, sometimes known as a 'DNA fingerprint'. Restrictions enzymes can be used to break DNA extracted from pure microbial cultures or metagenomic DNA. Single-species strain discrimination for epidemiological reasons, comparison of microbial consortia in distinct environments, and evaluation of microbial consortia responses to physiochemical effects are all applications of fingerprinting. T-RFLP (which was employed in this experiment and is thus addressed in further detail in section 2.7.4), and amplified fragment length polymorphism (AFLP) are two fingerprinting approaches (Olive and Bean, 1999:1661-1667; Vos et al., 1995:4407-4412; Lin et al., 1996:3649-3650).

2.6 Analysis of the metagenomic DNA

To obtain high-quality data for interpretation, the sampling strategy and procedure must be carefully planned and followed according to established guidelines. However, the retrieved metagenomic DNA may not be an exact representation of the microbial community present in the sample location (Thomas et al., 2012:3). The sample's transportation time and storage conditions (at 4°C) are critical characteristics to consider for metagenomic DNA extraction (Paul and Clark, 1989). The preservation time should be kept as short as possible, and biological activities should take place as soon as possible after sampling. This decreases the potential of contamination and DNA loss, increasing the likelihood of reliable results (Felczykowska et al., 2015:151-159).

2.6.1 Terminal-Restriction Fragment Length Polymorphism analyses

T-RFLP (terminal-restriction fragment length polymorphism) analysis may be used to examine microbial community structures in various environmental materials, including peat (Mummey and Stahl, 2004:41-48). It combines PCR-amplified gene marker restriction fragment analysis with automated sequencing gel technology (Liesack and Dunfield, 2004:23-24). T-RFLP analysis may be used to efficiently evaluate the microbial community present in a given sample due to the

complexity of the microbial communities in soil. T-RFLP utilizes PCR amplification using fluorescently labeled primers (both the 5' and 3' primers are labeled with distinct dyes to detect higher variety and boost accuracy). It is then followed by restriction enzyme digestion and capillary electrophoresis to separate terminal restriction fragments (T-RFs) based on size utilizing automated sequencing methods (Marsh et al., 2000:3616). The intensity of the fluorescent signal reflects the relative abundance of the T-RFs. Analysis of the T-RFs and standardisation is performed using software programs such as Genemapper® or PeakScanner™. Data is exported to other software packages for alignment and noise reduction T-RFs (Liesack and Dunfield, 2004:23-24). Following that, more statistical studies are undertaken to understand the data. T-RFLP enables the comparison of the structure and diversity of microbial communities in various samples. This method does not rely on the culture of the microorganisms in the sample (Marsh, 2005:308-325). T-RFLP studies have the advantage of using degenerate primers, which expands the possible range of markers addressed by a single primer set. T-RFLP offers the potential for large throughput as well. Because of its low cost, the approach is often employed for ecological research that need large-scale sample collection. The technique's strength lies in its capacity to compare complicated microbial populations. The fact that T-RFLP is automated, resulting in extremely sensitive detection, is by far the most significant advantage (Marsh, 2005:308-325).

2.6.2 Next generation sequencing

The term ‘next generation sequencing’ (NGS) refers to the technique of high-throughput massively parallel DNA sequencing. NGS may be used to gather information for genome assembly or amplicon sequencing (Rizzo and Buck, 2012:887-890). The Illumina MiSeq platform is one example of a technology that might be utilized for amplicon sequencing (Figure 2.11), which involves the amplification of selected DNA with primers (including an overhang adaptor) (Illumina, 2016). In microbiology, NGS is used to replace traditional microbe characterisation by appearance, staining features, and metabolic factors with a genomic description of the bacterium. NGS may also be used to study diversity; a segment of the sample's 16S rRNA gene is PCR amplified, and the resulting amplicon is sequenced with a sequencer (Behjati and Tarpey, 2013:236-238).

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specific DNA with primers (including an overhang adaptor) (Illumina, 2016). NGS is used in microbiology to replace traditional microbe characterization by appearance, staining characteristics, and metabolic parameters with a genomic description of the bacteria. NGS may also be used to examine diversity by PCR amplifying a portion of the sample's 16S rRNA gene and then sequencing the resultant amplicon using a sequencer (Behjati and Tarpey, 2013:236-238).

Other molecular approaches that involve high-throughput sequencing, such as metabarcoding (a comprehensive, objective, and efficient way to assessing molecular biodiversity that may frequently exceed morphological surveys) (Garcés-Pastor et al., 2019:425-439) include Shotgun sequencing (allows researchers to sample all genes in all organisms present in a complex sample in one go), Long-read sequencing (an accurate method for sequencing genomes that are generally difficult to sequence, such as those with long sections of highly repetitive components), Single-Cell genomics (amplification of single-cell DNA), and whole genome sequencing (amplification of entire genomes) (Illumina, 2022).

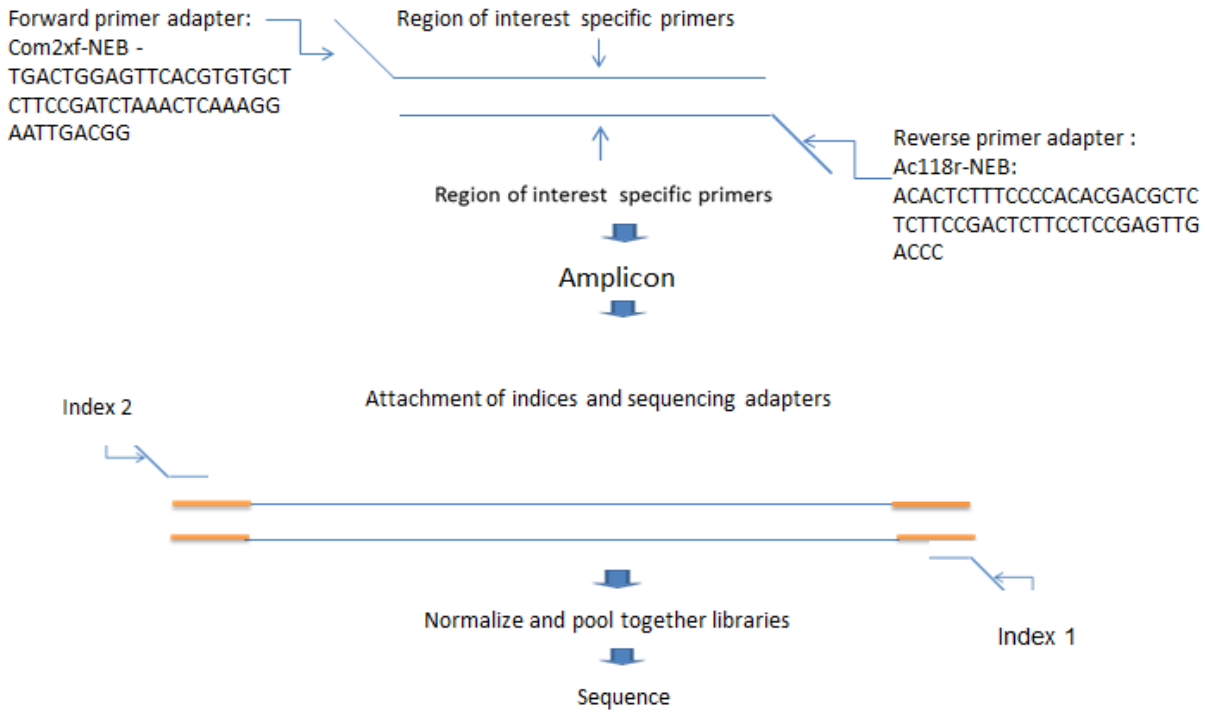


Figure 2.11: Flow-diagram of the process involved in next generation amplicon sequencing using Ac118r-NEB-Com2xf-NEB primers (adapted from Illumina, 2016)

2.7 Culture-based methodologies

Microorganisms can be cultured in artificial media using selective plating and enrichment procedures. Once the organisms have been isolated, various physiological tests can be performed to determine their ecological function. However, one of the disadvantages of this detection method is that the vast majority of microbes have not been or cannot be cultured under laboratory conditions as little is known about the specific growth requirements of most microorganisms, including their nutrient requirements, O_2 levels, co-factors and their interactions with other populations. Many microorganisms also possess very low metabolic activity, making them undetectable in the laboratory. In the environment, microbes also grow in consortia, each with their niche function. This cannot yet be replicated artificially (Wagner et al., 1993:1520-1521; Choi et al., 1994:1889-1893). In NGS-based studies, there are more chances to detect rare organisms than in culture-based studies.

Based on the background information provided, as well as reports in literature, specific methodologies were selected for this study. The following chapter, Chapter 3, will therefore outline the methodology followed in this study. The methodology that was selected was based on the overall aims and objectives of this study.

CHAPTER THREE: MATERIALS AND METHODOLOGY

All reagents used in this study were of analytical grade and were obtained from the suppliers as indicated in the main text.

3.1 Delimitation of the study

This study focused on three peatland sites located within the Western Cape Province of South Africa.

3.2 Sampling

Samples were obtained from three different wetland systems (in Spring) in the Western Cape: Vankervelsvlei near Sedgefield (-34º00'42.5", 22º54'12.0"), Gratitude Farm near Riversdale (-34º01'25.8", 21º18'12.1"), and Springfield Farm in the Agulhas plain (-34º44'16.4", 19º54'39.7") using a hand spade (Figure 3.1). Triplicate peat samples were collected from Gratitude Farm and Springfield Farm, and for each triplicate sample, the following layers were combined:

- Top Soil (0 to -5 cm), designated as 'top' in results and discussion
- Samples from -6 cm to -25 cm depth, designated as 'middle' in results and discussion
- Samples from -26 cm to -50 cm depth, designated as 'bottom' in results and discussion

This sampling approach was repeated at 30 m intervals. In total, nine samples were collected at Gratitude Farm and six samples were collected at Springfield Farm (as only two suitable sites within a 30 m interval were found) each sample further consisting of the three layers indicated. Using a measuring tape, the different depths were measured. In many instances, the top layer (1-2 cm) was discarded to obtain more 'pure' and less exposed peat samples. The samples were stored in sterile plastic bags in a refrigerated container for transport to the laboratory. All samples were collected with permission of the owners of Gratitude Farm and Springfield Farm.

Samples collected at Vankervelsvlei were kindly provided by Prof Torsten Haberzetl (University of Jena, Germany) and with permission from PG Bison (the company which owns the land on which the peatland is located). Samples were collected from three sites: Site 1 (at a depth of 3.8 m, 5.8 m, 7.8 m and 11.8 m); site 2 and 3 (at a depth of 4 m, 6 m, 8 m and 12 m). A total of 12 samples was received from the Vankervelsvlei sampling site. All samples were kept cool while

being transported to the laboratory (in an electrical refrigeration unit) and were processed within two days of collection.

Figure 3.1: Location of the sampling sites in context of the southern coastline of South Africa (Google Earth)

3.2.1 Historical and botanical site information

3.2.1.1 Vankervelsvlei

This undisturbed sampling site is located close to Sedgefield (-34º00'42.5", 22º54'12.0") (Figure 3.2) and is about 2 km north-east of Groenvlei. This wetland has no open water, and the peat found there is about 10 m thick; this wetland has been described as a floating bog by Irving & Meadows (1997:101-105). Vankervelsvlei is located 150 m above sea-level and spreads over a 0.5 km^2 area. It is an enclosed interdunal depression with no surface water inflow. The peatland's water is entirely concealed by a thick layer of matted sedge vegetation to a depth of approximately 2 m below the surface (Figure 3.2). Vankervelsvlei basal sediments have been dated as old as 40 000 years (Parsons, 2009:657-660).

Figure 3.2: [A] A photograph of Vankervelsvlei, clearly showing the elongated area covered by sedge vegetation. [B] Satellite image of the peatland, Vankervelsvlei (Google Earth)

3.2.1.2 Goukou River (Gratitude Farm)

This impacted peatland sampling site is close to Riversdale and is located on the upper reaches of the Goukou River in the Renosterveld biome area (-34º01'25.8", 21º18'12.1") (Figure 3.3). The site was originally dominated by palmiet (*Prionium serratum*). However, this site has been severely invaded by black wattle (*Acacia mearnsii*), and the wetland has subsequently lost its flood attenuation ability. Consequently, there is severe erosion during periods of high precipitation, and a deep channel has been formed. In some areas, over 5 m of peat deposits have been exposed (Figure 3.3). Anecdotal evidence suggests that the deeper deposits may be up to 12 000 years old.

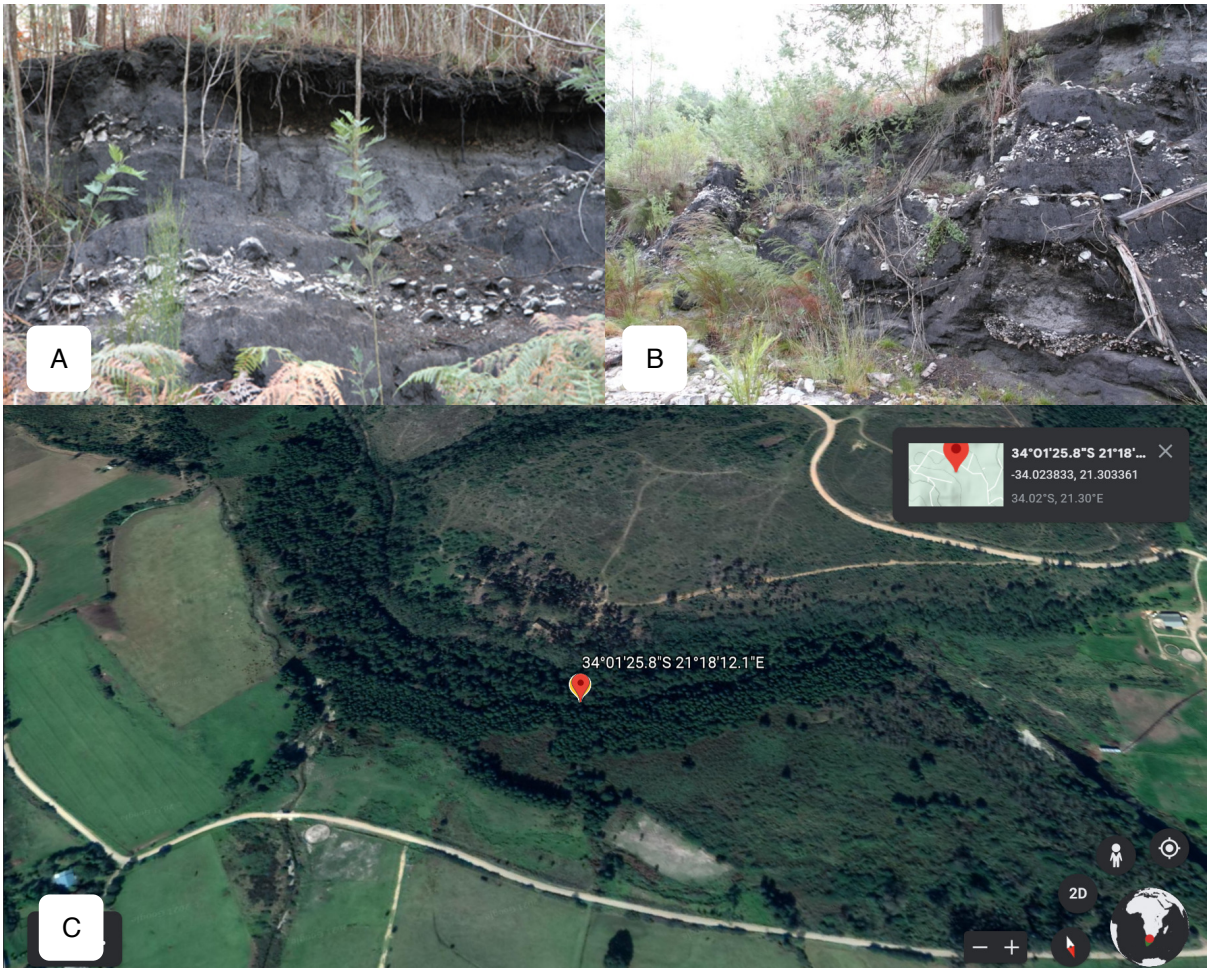


Figure 3.3: [A-B] Exposed peat deposits, Goukou River, Gratitude Farm, Riversdale. [C] Satellite image of the peatland, Goukou River, Gratitude Farm, Riversdale (Google Earth)

3.2.1.3 Springfield Farm

This sampling site forms part of the wetland system found on the Nuwejaars River's outskirts on the Agulhas plain (-34º44'16.4", 19º54'39.7") (Figure 3.4). This wetland contains newly formed peat. However, it has been severely impacted by agricultural run-off. This site is under constant flooding as it is fed by seepage and rainwater (Figure 3.4). This area consists of mineral deposits as well as peat. The original flora, which includes sedges, grasses and restios, can still be found on the plain; however alien vegetation such as Cluster pines (*Pinus pinaster*) and Port Jackson (*Acacia salinga*) have invaded the area. During 2012 the peat ignited and smouldered, destroying some of the invading vegetation and some of the peat.

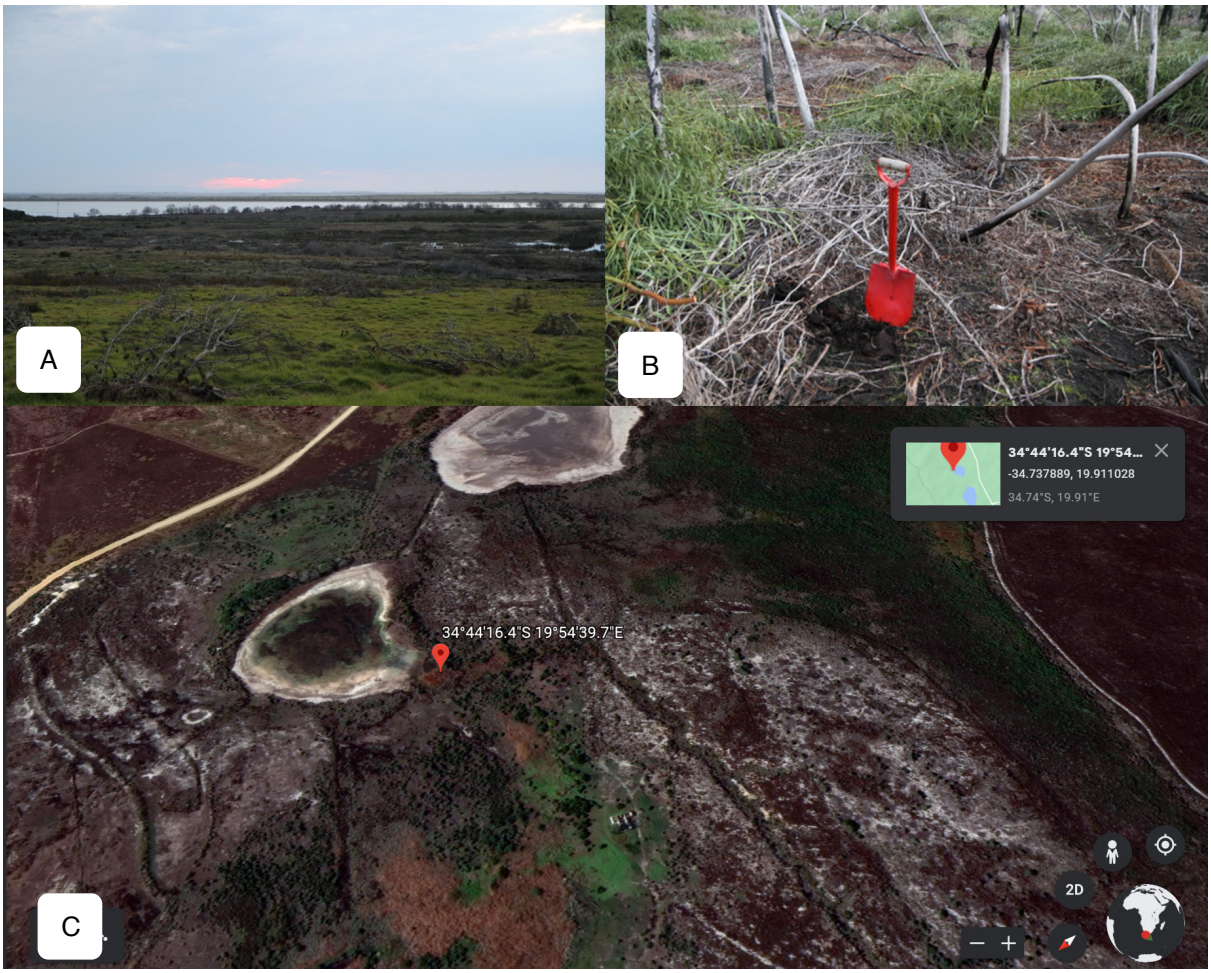


Figure 3.4: [A-B] Springfield Farm located on the Agulhas Plain. The dark colouring of the soil can be seen when plant debris has been removed. [C] Satellite image of the peatland, Springfield Farm (Google Earth)

3.3 Molecular analysis of actinobacterial, bacterial and fungal communities in peat samples

Figure 3.5 provides an overview of the three approaches taken in this study and is detailed below.

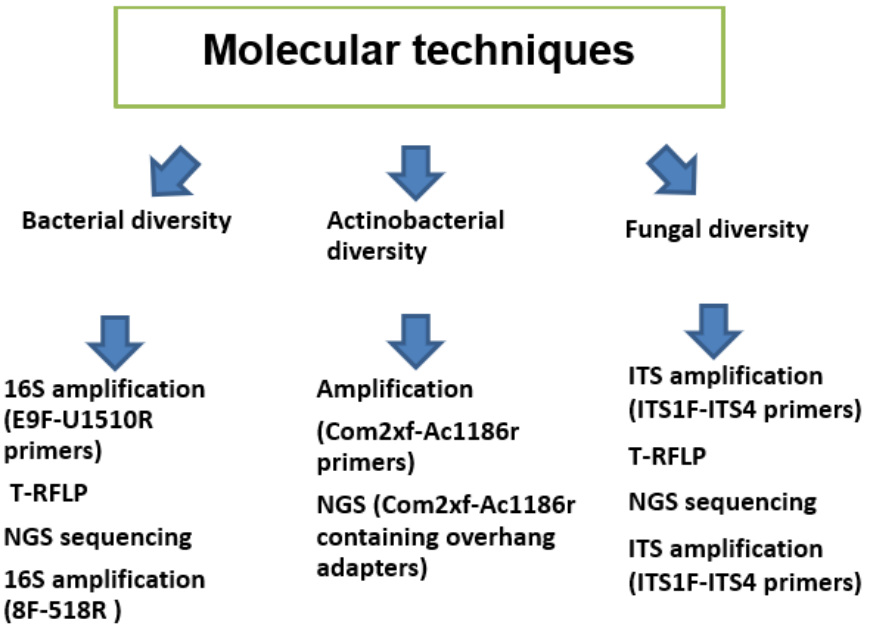


Figure 3.5: An overview of the molecular protocols followed in this study

3.3.1 Metagenomic DNA extraction

Metagenomic DNA extractions were performed on all the samples obtained from the three sites. The DNA was extracted using the PowerSoil® DNA extraction kit (MO BIO Laboratories, California, USA) with the following modification: 1 g (instead of 0.25 g) of the sample was used for the extraction. A control (no soil) was included during the processing to ensure that no contaminants were introduced during the isolation of metagenomic DNA. Using a nanodrop, the quality and concentration of the Metagenomic DNA was determined. After measuring the concentration, the DNA was stored at 0 - 4°C until further analysis.

3.3.1.1 Determination of similarities in bacterial community structure using terminal restriction fragment length polymorphism

The 16S rRNA gene amplification was performed using the forward and reverse primers, E9F (5'-GAGTTTGATCCTGGCTCAG-3') and U1510R (5'- GGTTACCTTGTTACGACTT-3'), respectively. The 5'- end of the E9F primer was FAM–labelled (labelled with fluorescein amidite dye). PCR was carried out in 50 µl reaction volumes, containing 1x DreamTaq PCR Mastermix (Thermo Fisher Scientific, Lithuania, Europe), 0.5 µM of each primer, 0.1% (w/v) molecular grade

bovine serum albumin, and between 1 and 10 ng of metagenomic DNA. Amplification was carried out using a Touchgene Gradient Thermal Cycler (Techne Incorporated, Princeton, USA), utilising the following PCR conditions: 4 minutes at 94˚C for denaturation; 30 cycles of: 30 s denaturation at 94˚C, 30 s annealing at 52˚C and 105 s elongation at 72˚C, and a final elongation step of 10 min at 72˚C. The PCR amplicons were visualised on a 1% (w/v) agarose gel containing 8μl of 10mg/ml ethidium bromide (visualisation at 254 nm). The KAPA Universal Ladder Kit was used to determine the amplicons' approximate size on the agarose gel. Amplicons were purified using the Nucleospin® gel and PCR clean-up kit (Macherey-Nagel, Durën, Germany).

The purified amplicons were digested, using the restriction enzymes *Hae*III and *Alu*I for 3 hours at 37˚C, following the manufacturer's instructions (Sigma–Aldrich, Darmstadt, Germany). Inactivation of the restriction enzyme was performed by heating at 80˚C for 10 minutes. The digested products were purified using the Nucleospin® gel and PCR clean-up kit. Restriction fragment lengths and quality was determined via capillary electrophoresis at the Central Analytical Facility (CAF), Stellenbosch University. The profiles were analysed using the freeware, PeakScanner™ (version 1.0) (Applied Biosystems, https://products.appliedbiosystems.com) and a data matrix was generated. The data table was exported in .csv format and converted to tab-delimited text format for the online software, T-REX (http://trex.biohpc.org/; Culman et al., 2009), and a label file with sample names was created. These two tab-delimited text files were used to create large data matrices through the use of the T-REX software. Peak height was used to characterise each unique terminal-restriction fragment (T-RF). Only peaks at positions between 30 and 500 bp were considered to avoid T-RFs caused by primer-dimers and to obtain peaks within the internal standard's linear range (Singh et al., 2006:7278-7280). The relative abundance of a T-RF in a T-RFLP profile was calculated by dividing the sample's peak height (a T-RF) by the total peak heights of all the samples (all T-RFs in the profile). T-RFs with intensities lower than 0.5%, which may have originated from background interference, were excluded from the matrices, thereby minimising the effect of the variations in the T-RFLP profiles caused by the starting quantities of DNA (Singh et al., 2006:7278-7280; Ding et al., 2013). Data matrices obtained from T-REX were exported to Microsoft Excel and analysed through the software, Primer 7 (Primer-E Ltd, UK). The community structures obtained from the T-RFLP data were used to determine the Bray-Curtis similarity coefficients (Bray and Curtis, 1957). The data generated was used to create similarity matrices and non-metric multidimensional scaling (NMDS) plots to determine the degree of similarity among the different microbial community profiles analysed in this study (Clarke and Warwick, 2001).

3.3.1.2 Amplification of fungal ITS region for NGS analysis

Primers ITS1F (5'-CTTGGTCTTTAGAGGAAGTAA-3') and ITS4Rv2 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the internal transcribed spacer (ITS) region of the fungal DNA present in the metagenomic DNA samples (Gardes and Bruns, 1993:113-118; White et al., 1990:315-320). PCR was carried out in a final reaction volume of 25 µl, containing 1x DreamTaq PCR Mastermix, 0.5 µM of each primer, and approximately 10-15 ng metagenomic DNA. Amplification was carried out using a Touchgene Gradient Thermal Cycler (Techne Incorporated, Princeton, USA), utilising the following PCR program: initial denaturation for 10 minutes at 95˚C, followed by 35 cycles of denaturation (94˚C for 30s), primer annealing (55˚C for 1 minute) and elongation (72˚C for 1 minute). The final extension was for 10 minutes at 72˚C. The PCR products were visualised, as previously described (Section 3.3.1.1). Amplicons were analysed on a 0.8% (w/v) agarose gel, excised as described above and submitted for sequencing by Inqaba Biotec (South Africa).

3.3.1.3 Amplification of bacterial 16S rRNA genes for NGS analysis

For the amplification of bacterial 16S rRNA genes from the metagenomic DNA samples, the 8F (5'-CCATCTCATCCCTGCGTGTCTCCGAC-3') and 518R (5'- ATTACCGCGGCTGCTGG-3') primer set, which covers the V2-V3 variable region of the bacterial 16S rRNA gene, was used (Turner et al., 1999:328-329; Muyzer et al., 1993:696-697). Once the PCR conditions were optimised, the PCR was repeated with primer sets containing 25 specific adapter sequences (as provided by the NGS service provider, Inqaba Biotech). The PCR amplification reactions were performed in a final volume of 25 µl, containing 0.25 µl KAPA HiFi DNA Polymerase, 0.2 µM of each primer, and approximately 10-15 ng metagenomic DNA. Amplification was performed using the following conditions: an initial denaturation step of 95˚C for 3 min and a final extension step at 72˚C for 15 min. Gradient amplification was used to determine the optimal annealing temperature. Amplification was carried out using Touchgene Gradient Thermal Cycler (Techne Incorporated, Princeton, USA) and the PCR comprised of 25 cycles. A negative control (which contains only the Mastermix, primers and nuclease-free water) and a positive control [containing 10-15 ng DNA that is known to amplify at the above conditions, and Mastermix (final volume of 25 µl, containing 0.25 µl KAPA HiFi DNA Polymerase, 0.2 µM of each primer, and nuclease-free water] were also used. Once the PCR conditions were optimised, the PCR was repeated with

primer sets containing specific adapter sequences [as provided by the NGS service provider (Inqaba Biotec)]. The quality of the sequence reads was confirmed using FastQC (Galaxy Version 0.72+galaxy1; https://www.usegalaxy.org) and analysed using Quantitative Insights Into Microbial Ecology (QIIME 2; Carporaso et al., 2010). OTUs were defined at a 97% sequence similarity, and OTUs called using the SILVA database (v132; Quast et al., 2013; Yilmaz et al., 2014). OTU output data was filtered to remove non-fungal and non-actinobacterial hits. The filtered data was applied in PRIMER 7 analyses.

3.3.1.4 Amplification of actinobacterial 16S rRNA genes for NGS analysis

Metagenomic DNA was also used for the amplification of actinobacteria-specific 16S rRNA genes. The amplification of the 16S rRNA gene was performed using the method described by Schäfer et al. (2010:104-110). The actinobacterial-specific 16S rRNA gene primer pair (Com2xf: 5'-AAACTCAAAGGAATTGACGG-3'; Ac1186r: 5'-CTTCCTCCGAGTTGACCC-3') was used in the following PCR reaction: 1x KAPA Taq Readymix (1.5 mM $MgCl_2$ and 0.2 mM of each dNTP), 0.2 µM of each primer, 1 µl of template (10 ng of metagenomic DNA) and water to a final volume of 25 µl. The amplification program of 25 cycles was started by an initial denaturation step at 95°C for 3 minutes, followed by denaturation at 94°C for 30 seconds, an annealing gradient with temperatures between 51.6°C – 60.2°C and extension step at 72°C for 30 seconds. A final extension step of 72°C for 15 minutes. Genomic DNA from *Streptomyces polyantibioticus* SPR^T was used as a positive control. Amplicons were analysed by electrophoresis on a 0.8% (w/v) agarose gel (containing 10 µg/ml ethidium bromide) and visualised under UV light. Amplicons were excised from the agarose gel and purified using the Machery-Nagel gel purification kit and submitted to the NGS facility at Inqaba Biotec (South Africa). All raw sequence reads have been submitted to GenBank and is available under the BioProject, PRJNA805212.

3.3.2 Statistical Analyses

Statistical analyses were carried out according to the makers' instructions using Primer 7® software (Quest Research Limited, Auckland, New Zealand) (Clarke and Gorley, 2016; Clarke et al., 2016). Spearman rank similarity was used in the analysis of similarity (ANOSIM). The actinobacterial and fungal NGS data, as well as the physicochemical data, were subjected to multivariate analysis using square root and fourth root transformed data. Non-metric multidimensional scaling (nMDS) plots and cluster plots (based on group average linkage) were

created for microbial communities using Bray-Curtis similarity matrices, while Principal Component Analysis (PCA) plots and cluster plots (based on group average linkage) were created for physicochemical data using similarity matrices based on Euclidean distances. The most important drivers were discovered utilizing BEST analysis to analyse the effect of physicochemical factors on microbial community architectures. The physicochemical dataset was whittled down to the maximum amount of parameters that could be used in BEST analyses: First, 4^{th} root transformed and normalised physicochemical data were checked for co-linearity. For S + N, C + N, LOI + N, and LOI + C, the (Spearman) correlation co-efficient was determined to be >0.95, allowing one parameter to be used as a proxy for the set of parameters. The highest R values were reported for N (0.420, p0.05) and S (R=0.295, p0.05) in a separate BEST analysis for actinobacteria and fungi, and they were used as proxy values for actinobacteria and fungi, respectively. Second, using the parameters established by X-Ray Fluorescence (XRF), independent BEST analyses were done for actinobacteria and fungi. Combinations of Al, Ca, P, and Ti (actinobacteria) and Al, Ca, K, P, and Ti (fungi) yielded the most significant (p0.05) global R values of 0.676 and 0.596, respectively. The parameters included in the final BEST analysis were balanced based on the highest single R values achieved. N (as a substitute for N, S, C, LOI), Al, Ca, P, Ti, K, Si, Fe, Mg, humification, and pH comprised the final physicochemical dataset for actinobacterial BEST analysis. S (as a substitute for N, S, C, LOI), Al, Ca, K, P, Ti, Si, Mg, Fe, humification, and pH comprised the final physicochemical dataset for fungal BEST analysis.

Constrained cluster analyses (LINKTREE) were done using Bray-Curtis similarity matrices (microbial) and the physicochemical datasets generated using BEST to discover the most relevant drivers (and their concentrations) for selection of the microbial community structures.

3.4 Culture-dependent isolation of actinobacteria from peat samples

Three processes were followed for the isolation of actinobacteria from the peat samples obtained from Vankervelsvlei and Goukou River:

1) Direct isolation from the peat;
2) Heat pre-treatment prior to isolation; and
3) Isolation from air-dried samples.

3.4.1 Direct isolation from peat

One gram of each peat sample was added to 5 ml of sterile distilled water, vortexed for 5 minutes and allowed to settle. The supernatant was serially diluted (10-fold dilutions) in sterile distilled water. The 10^0 to 10^{-4} dilution samples were plated onto various isolation media: 100 µl was inoculated onto the centre of the agar plate and spread-plated. Duplicate plates were incubated at 30˚C and room temperature (22 ± 3°C) until growth was observed.

3.4.2 Heat pre-treatment

One gram of each peat sample was placed on a glass slide and heated at 120°C for two hours. The samples were allowed to cool and were subjected to the same serial dilution and plating, as described in Section 3.4.1.

3.4.3 Air-drying as pre-treatment

One gram of each peat sample was placed in a sterile Petri dish, each containing one gram of calcium carbonate ($CaCO_3$) and left on the bench in the laboratory for 14 days prior to following the serial dilution and plating method as described in Section 3.4.1.

The following media were used for the isolation of actinobacteria (Appendix A):

- International *Streptomyces* Project (ISP) medium no.1; ISP medium no. 2 and ISP medium no. 4 (Shirling and Gottlieb, 1966:313-340)
- Czapek solution agar (Atlas, 2004); and
- Starch-casein-nitrate agar (Atlas, 2004).

The pH of the agar media was adjusted depending on the properties of the sampling site. Each agar medium was supplemented with 100 µg/ml cycloheximide and 5 ml of SV11 STAPH/STREP SELECTAVIAL™ (Mast Group – Merseyside, United Kingdom) to maximise isolation of actinobacterial strains.

3.5 16S rRNA gene sequence amplification and identification of actinobacterial isolates

Actinobacterial isolates were selected randomly and grown on ISP no. 2 agar, until pure isolates could be observed. The isolates were grown in ISP no. 2 (liquid media) and the DNA was extracted using the 'Bead-beating method'. Liquid media was inoculated with the isolates and

incubated at 30˚C, shaking at 160 rpm, until sufficient cell mass was produced. DNA extractions were performed as follows:

- 2 ml of the culture suspension was transferred to a 2 ml Eppendorf tube.
- The cultures were centrifuged for 60 s at 10 000 rpm, and the supernatant discarded.
- 1 ml 100 mM phosphate buffer (pH 8) was added to the pellet, vortexed and centrifuged for 10 minutes at 10 000 rpm. The supernatant was discarded.
- 300 µl 100mM phosphate buffer (pH 8), 10% (w/v) SDS and chloroform: isoamyl alcohol (24:1, v/v) was added to the pellet. The tubes were incubated at room temperature (22 ± 3°C) for 60 minutes, vortexed for 2 minutes and centrifuged for 5 minutes at 10 000 rpm and the supernatant (approx. 650 µl) transferred to a sterile Eppendorf tube.
- 360 µl 7M ammonium acetate was added to the tubes, vortexed and incubated at room temperature (22 ± 3°C) for 5 minutes.
- The tubes were centrifuged for 5 minutes at full speed and the supernatant (approx. 580 µl) transferred to a sterile 1.5 ml Eppendorf tube.
- 315 µl isopropanol was added to the tubes, vortexed and the tubes incubated at room temperature (22 ± 3°C) for 2 hours. The tubes were centrifuged for 15 minutes at 10 000 rpm.
- The supernatant was discarded and 500 µl 70% (v/v) ethanol was added to the tubes, vortexed and centrifuged for 10 minutes at 10 000 rpm.
- The liquid was discarded, and the tubes were left to air-dry for 1 to 2 hours.
- 70 µl 10 mM Tris-HCl (pH 8) or sterile distilled water was added to the pellet and the DNA stored at refrigeration temperature till further use.

Primer set F1-R5 (F1: 5'-AGAGTTTGATCITGGCTCAG-3'; R5: 5'-ACGGITACCTTGTTACGACTT-3') was used to amplify the 16S rRNA genes of actinobacterial isolates. PCR was carried out in 25 µl reactions, containing 0.1 µl KAPA Robust Taq, 0.4 µM of each primer (final concentration), 5 µl KAPA High-GC buffer, 0.5 µl 10 mM dNTPs, 2 µl metagenomic DNA and water to final volume of 25 µl. Amplification was carried out using a Touchgene Gradient Thermal Cycler (Teche Incorporated, Princeton, USA), at the following PCR conditions: 30 cycles of denaturation at 96˚C for 45 s, primer annealing at 56˚C for 30 s, elongation at 72˚C and a final extension at 72˚C for 2 min. The KAPA Universal Ladder Kit was used to determine the approximate size of the amplicons on the agarose gel. Amplicons were purified using the Machery-Nagel PCR and gel purification kit and sent to Inqaba Biotech for sequencing. Sequences were analysed using FinchTV (https://digitalworldbiology.com/FinchTV) and DNAman

(https://www.lynnon.com/dnaman.html) and the confirmed 16S rRNA sequences submitted to NCBI (https://www.ncbi.nlm.nih.gov/) and Eztaxon (https://www.ezbiocloud.net/) for identification of the closest phylogenetic neighbours. Accession numbers were assigned by NCBI for all 16S rRNA sequences submitted and have been indicated in the results section.

3.6 Physiochemical analyses of peat samples from peatlands

Physiochemical analyses performed on the peat samples obtained from the sampling sites include the pH analysis, total organic and inorganic carbon, total nitrogen, and the redox potential. Peat samples were submitted to Bemlab (Somerset West, South Africa) for carbon, hydrogen (H) and N analysis by elemental analyser and to the CAF at the Stellenbosch University for analysis of major elements by XRF. The XRF spectrometry was performed using an Almelo instrument fitted with an argon/methane mixture and an Rh tube. Initially, samples were ground into a fine powder by using a jaw crusher, followed by milling in a tungsten Zipmill. A fused glass disc was prepared with 1 g of sample, 10 g of trace element free and REE-free flux. In order to correct for sample matrix effects, raw intensity data were corrected with theoretical alpha factors and line overlap factors using SuperQ PANalytical software. BHVO-1 (basalt from the United States Geological Survey, Reston) was used as the control standard for calibration with NIM-G (Granite from the Council for Mineral Technology, South Africa).

3.6.1 pH analysis using $CaCl_2$ method (Sikora and Kissel, 2014)

In order to determine the pH of the samples, 10 ml of 0.01 M $CaCl_2$ was added to 10 g of soil. The soil-$CaCl_2$ was stirred and allowed to settle for 1 hour. The pH meter (pH700 meter and probe, Eutech Instruments, Singapore) was calibrated (according to the manufacturer's instructions), and the pH of the peat samples measured and recorded (in triplicate). For the samples, V5 and V10, litmus paper was used because there wasn't enough sample available to do the pH test with the pH probe.

3.6.2 Organic matter content

20 g of peat was weighed out and dried for 24 hours at 100˚C. After 24 hours, the crucibles were placed in a desiccator for 30 minutes allowing the samples to cool. The mass of the peat samples was recorded. The peat samples were placed in a furnace at 550˚C for 4 hours. After 4 hours,

the crucibles were placed in a desiccator, allowing the sample to cool down and the mass recorded (Chambers et al., 2011:1-10). Calculations were performed as follow:

Organic matter content % = [(dried weight at 100˚C – weight after ashing at 550˚C) / dried weight at 100˚C] × 100

3.6.3 Peat humification

Blackford & Chambers (1993:7-24) used a NaOH extraction procedure along with UV absorption tests to determine differences in the degree of peat humification based on the sample's colour. One gram of peat was dried at 50˚C for 2 hours or until the water was wholly evaporated. After drying the peat samples, the samples were ground separately using a pestle and mortar. The pestle and mortar were cleaned with ethanol and dried with a paper towel between each grind. 0.2 g of each sample was weighed out and transferred to a 150 ml beaker. Using a measuring cylinder, 100 ml of 8% (w/v) NaOH was added to each beaker containing 0.2 g of peat. The beakers were placed in a water-bath at a temperature of 80˚C to 95˚C and were allowed to simmer for one hour. The beakers were occasionally topped up with deionised water to prevent the solution from becoming too concentrated. The waterbath was turned off, and each beaker's contents were transferred into a separate correctly labelled 200 ml volumetric flask. The flasks were topped up with deionised water. Using Whatman no.1 grade filter paper, 50 ml of each 200 ml flask's contents was filtered into the corresponding labelled 50 ml volumetric flask. The 50 ml filtrates were transferred to 100 ml volumetric flasks. The flasks were topped up with deionised water. The spectrophotometer was blanked against a sample not containing peat, and the test samples' absorbances were read (in triplicate) at 540 nm.

CHAPTER FOUR: RESULTS AND DISCUSSION - PHYSICOHEMICAL DATA

In soil, microorganisms play a key role in biogeochemical processes. There is therefore a need to evaluate soil physicochemical properties and how these traits could be associated with differences in soil microbial profiles (Peixoto et al., 2010:403-410). Changes, such as pH, moisture or even exogeneous materials in soil can lead to a change in the distribution patterns of the microbial communities. For example, some actinobacteria, such as the members of *Streptomyces* and *Micromonospora* genera, prefer neutral to alkaline environments, whereas yeasts and filamentous fungus such as the members of *Basidiomycota*, *Ascomycota*, *Zygomycota*, and *Chytridiomycota* prefer acidic ones (Qin et al., 2017). Different bacterial species may also prefer various moisture conditions such as field capacity, maximum molecular capacity, and maximum adsorption capacity (such as *Streptomyces*, *Micromonospora*, *Actinomadura*, *Saccharopolyspora*, and *Microbispora*) or low moisture (such as *Thermomonospora* and *Kibdelosporangium*) contents in soils (Schäfer et al., 2010:103-110). Among the most important characteristics of soil ecosystems is soil health, which is linked to soil quality and fertility. Soil is assumed to be a living system, with different processes and properties interacting with each other to determine soil health. This strongly influences the activity of soil microbes. Various techniques are available to determine soil physical, chemical, and biological properties, but natural selection (i.e., a combination of these properties and the soil microbiome) is responsible for its adaptability to environmental changes (Frąc et al., 2018). This chapter provides the results and analyses of the physicochemical data and along with the next chapter, focus on discussions on how the physicochemical characteristics affected the microorganisms (both actinobacterial and fungal communities) in all three of the sampling sites, and critical analyses of the most dominant species found. The chapter also includes an analysis on how these factors are impacted by or have an impact on the 'health' of the study peatlands.

4.1 Physicochemical Results

4.1.1 Peatland pH

Generally, peatlands can be classified into bogs and fens. Researchers have categorised the two different types of peatlands based on their vegetation, hydrology, water chemistry, and pH (Davies and Anderson, 2001:1-3). In comparison with fens, bogs are generally more acidic with pH < 4.5, while fens can range from slightly alkaline to slightly acidic with pH ranging from 4.5 to 7.5 (Taskila et al., 2016:115-120; Szajdak et al., 2020:2587). Bog acidity results from the microbial decaying processes, cation exchange, and natural acid input from the atmosphere. During the process of

decay, bacteria and fungi break down dead animal and plant matter, releasing acid into the surrounding environment, which is then followed by cation exchange with plant matter, such as sphagnum moss. By adsorbing Ca and Mg from the soil water, it exchanges or releases H^+ in their place. Hence, as the plant type populates the peatland environment, the amount of H^+ in the ecosystem increases too, acidifying the bog (Johnson, 1985; Crum, 1988).

In fens (peat-forming system), pH values are influenced by the interactions between the substrate and water chemistry (Rydin et al., 1999:91-110). A fen can be classified as poor, intermediate, moderately rich, or extremely rich in terms of mineral abundance (Hogg and Potter, 2018:173-175). A poor fen is similar to a bog, with sphagnum mosses dominating and a few more vascular species, mostly grasses and sedges (*Carex* species). It can also be found in areas where silicaceous bedrock is a dominant component of the region, together with glacial till and glaciofluvial deposits as the overburden. Moderately rich fens can be found in areas with high supplies of non-acid terrestrial water or soils containing some Ca, and extremely rich fens can be found where limestone or calcareously-drifting sands are present. The plant life in rich fens can be quite different from that in poor fens. The dominant peat mosses in the bottom layer in poor mires is taken over by brown mosses and there is an increase of vascular species, for example *Carex* species (Rydin et al., 1999:91-110; Vitt, 2008:2656-2660). In this study, the samples from Vankervelsvlei and Goukou River were acidic, while those from Springfield Farm exhibited higher pH levels, ranging between 6 and 8 (Figure 4.1). Springfield Farm is a young, developing peatland that has been impacted by agricultural run-off and invasion by alien vegetation. This area is permanently flooded and is fed by groundwater and rainwater typical of a fen. Much of the area consists of large mineral deposits, mineral soil and material resembling peat. Apart from being less acidic, the Springfield Farm wetland also contains the typical vegetation of fens such as shrubs and sedges. Goukou River and Vankervelsvlei, however, are not young peatlands. Anecdotal evidence suggests that the deeper deposits in Goukou River may be up to 12 000 years old, while Vankervelsvlei contains 40 000-year-old sediments (Parsons, 2009). Samples from Vankervelsvlei used in this study were up to 8000 years old (Figure 4.2). Both these two peatlands are very old and are also very acidic. Similar to Springfield Farm, both these two peatlands are fed by rainwater and groundwater which also makes them fens.

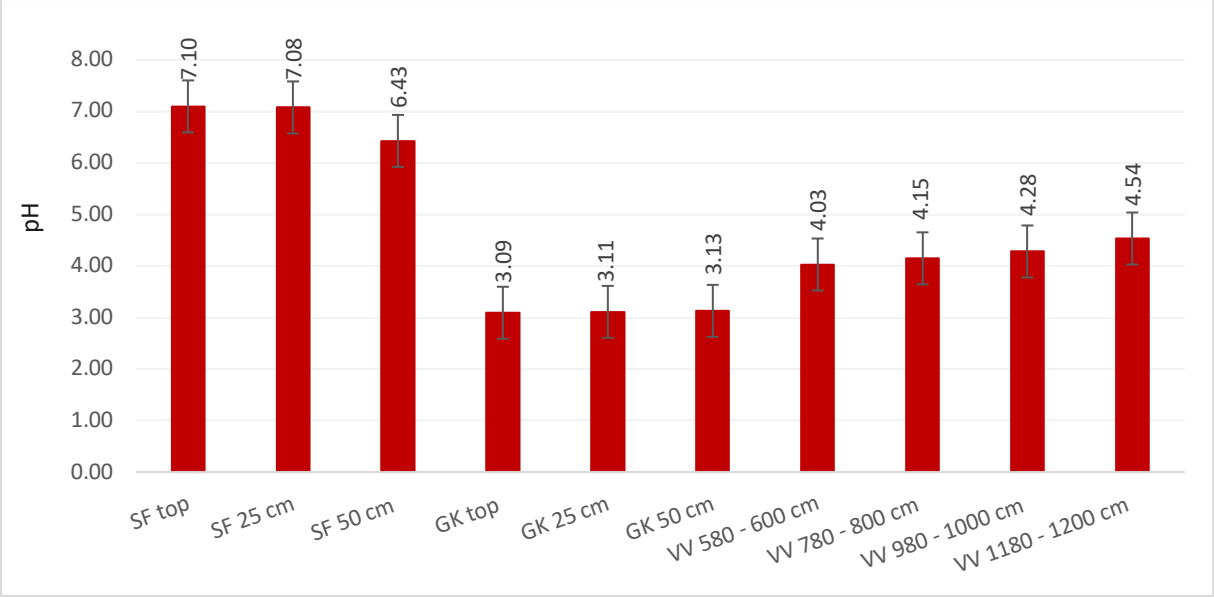


Figure 4.1: pH results of samples taken from the three study sites SF = Springfield; GK = Goukou River; VV = Vankervelsvlei. Error bars indicate the standard deviation of analysis, *n*=3

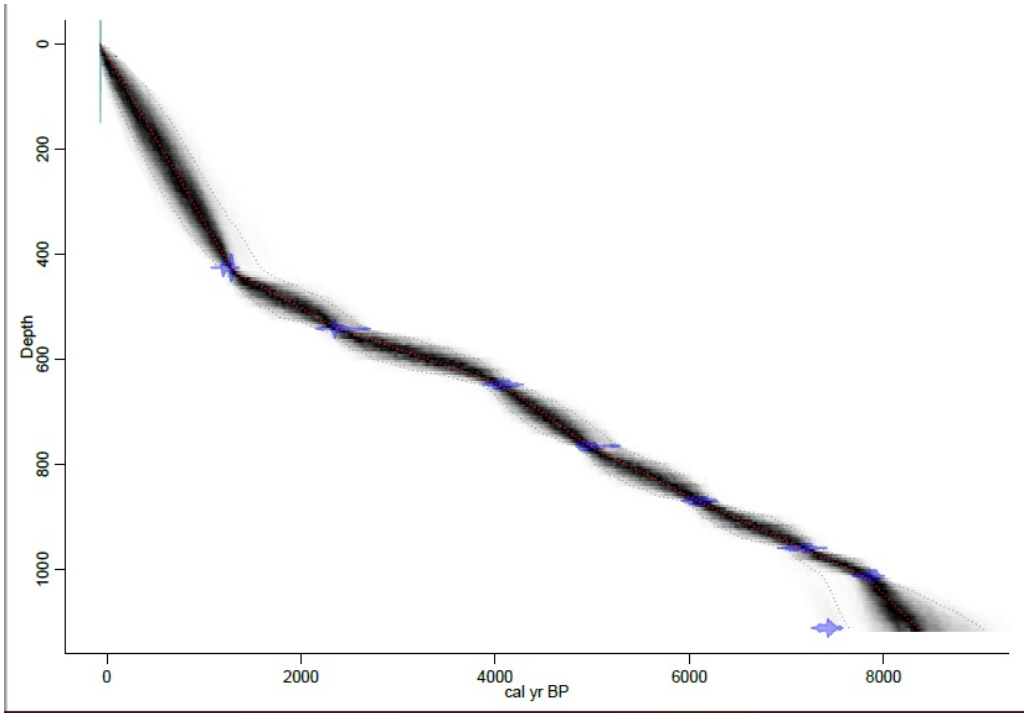


Figure 4.2: Age distribution of Vankervelsvlei samples using radiocarbon dating; samples used in this study is highlighted with purple arrows (results provided by Prof Torsten Haberzetl, University of Jena, Germany) (Strobel et al., 2019:206)

Interestingly, the pH levels of the Goukou River samples were lower than the pH levels of both Vankervelsvlei and Springfield samples. The divalent cation Ca^{2+} and Mg^{2+} concentrations were notably higher in the Vankervelsvlei samples (Figure 4.3). It was therefore speculated that higher concentrations of dissolved Ca and Mg minerals acted as buffers, causing the pH of Vankervelsvlei and Springfield to be higher than that of Goukou River (Gorham et al., 1984:77-80). Soil pH of 7 to about 8.5 is related to the solubility of $CaCO_3$ as the dissolution of soluble carbonates is a significant contributor to soil alkalinity. The dissolution of $CaCO_3$ in calcareous soils produces OH^- which leads to an increase in soil pH (Equation 4.1).

$$CaCO_3 + H_2O = Ca^{2+} + HCO_3 + OH^-$$

Equation 4.1

Thus, the pH range of calcareous soils is 7 to 8.3 as a result of hydrolysis of $CaCO_3$. The high levels of Mg and relatively high concentrations of Ca (Figure 4.3) would have contributed to the high pH in the Springfield samples. Magnesium carbonate ($MgCO_3$) increases the pH 1.2× more than $CaCO_3$ (Teir et al., 2006:3059-3066).

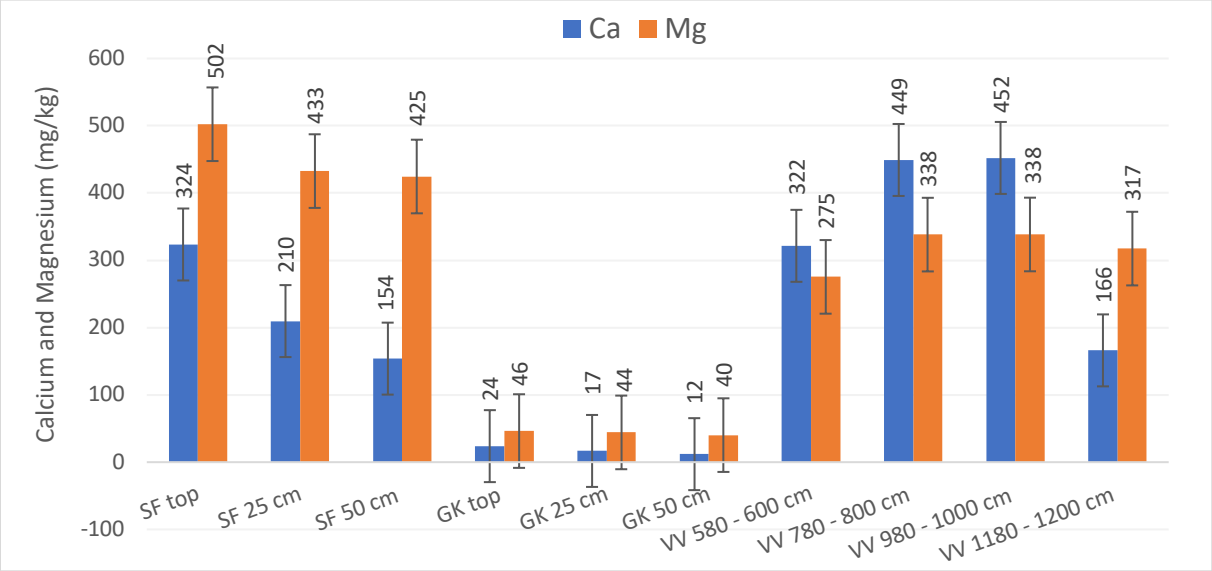


Figure 4.3: Calcium and Magnesium concentrations in samples taken from the study sites. SF = Springfield; GK = Goukou River; VV = Vankervelsvlei. Error bars indicate standard deviation from the mean, *n*=3

4.1.2 Organic matter and carbon content

It is commonly assumed that C content is a fixed proportion of either bulk density or organic matter density (estimated by loss on ignition, LOI). In peat, the total C can be quantified as the product of bulk density (g/cm^3) and total C content (gravimetric %) of the geobiological material. The C content in peat has been studied extensively and there has been an assumption made that organic C constitutes 50% of the total organic matter (OM) by mass. However, the C content of peat varies due to numerous factors such as the organic matter quality (cellulose, lignin), the composition of fossil plants (the various plants that make up the peatland), and OM chemistry changes that occur during decomposition. Well-preserved peatlands tend to have low OM densities because of the peat accumulating environment. As expected, the C content of the samples from Vankervelsvlei were notably higher than those from Goukou River and Springfield Farm (Figure 4.4). However, in this study, samples from all three sites displayed a decrease in the concentration of C with depth. The results are consistent with those of Benavides (2014:1-15), who observed that C accumulation was lower on drained peat sites, possibly because decomposition rates of the upper peat column were greater, or soil productivity was lower due to changes in plant communities.

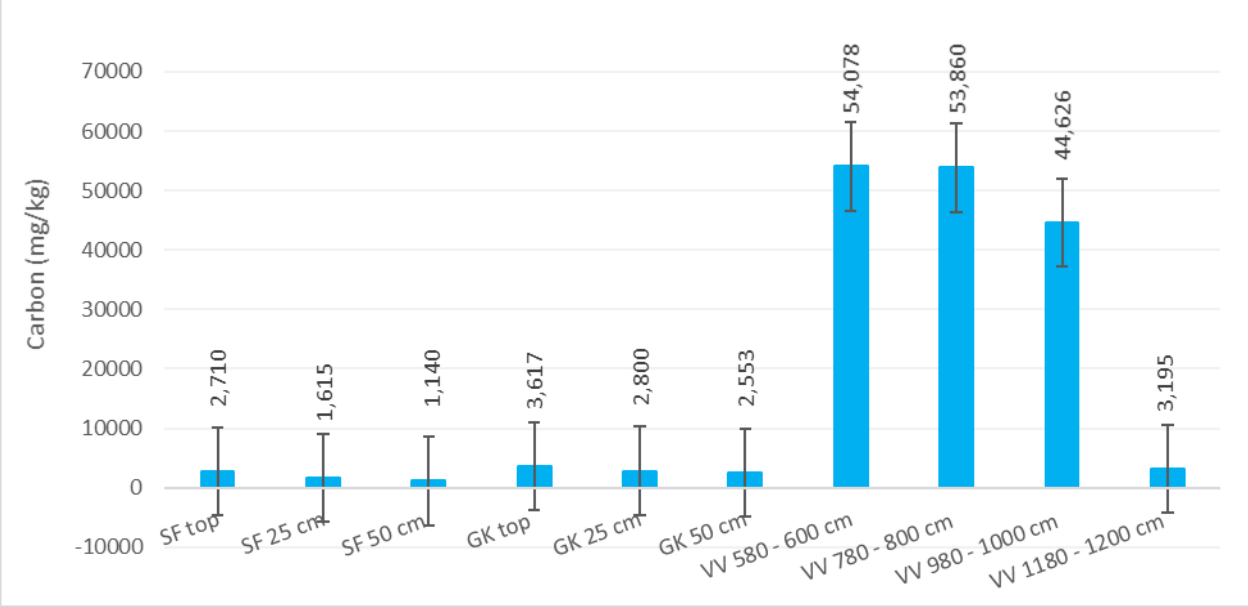


Figure 4.4: Carbon content of samples taken from the study sites. SF = Springfield; GK = Goukou River; and VV = Vankervelsvlei. Error bars indicate the standard deviation from the mean, *n*=3

Loss on ignition is a high-temperature analysis that is used to estimate the abundance of OM in peat (Heiri et al., 2001:101-103). After combustion, OM is calculated as the proportion of weight loss at 550°C. A high OM concentration occurs in peatlands that accumulate peat naturally, but the concentration can be negatively affected by non-organic inputs, such as exogenous windblown debris and waterborne particles, and/or *in-situ* carbonate precipitation in some peatland systems. In general, the OM density is more directly related to endogenous ecosystem processes. High levels of OM content were observed in Vankervelsvlei, ranging from 94.7% - 10.5%, while low levels we observed in Springfield Farm (14.4% - 5.6%) and Goukou River (12.8 % - 7.9%) (Figure 4.5). The peat found at Vankervelsvlei and Goukou River has been accumulating for a very long time (for about 10 000 years or more). However, the peat deposits at Goukou River are very exposed and damaged and not as tightly packed as the peat found at Vankervelsvlei, causing oxygen to penetrate the peat, which increases peat degradation and causes peat 'loss' (Leifeld et al., 2020:7634). In previous studies, Bauer et al. (2006:295-297) reported that highly organic peat contains an OM content of >70%, which is similar to the well-preserved and undisturbed Vankervelsvlei peatland as the OM content of the samples was > 90%. For Springfield Farm, the OM content in the samples was similar to the content in the samples from Goukou River. However, Springfield Farm is still a developing peatland, which could explain the low OM concentration. In addition, the effect of exogenous materials, and factors such as the presence of alien vegetation (Cluster pines (*Pinus pinaster*) and Port Jackson (*Acacia salinga*), fires (that destroyed much of the vegetation), and agricultural run-off may have inhibited peat accumulation (Figure 4.5 and Figure 4.6). An interesting observation is that all of the 1180-1200 cm depth samples taken from Vankervelsvlei produced OM readings that were similar to the OM content of Springfield Farm and Goukou River. The samples also had a clay-like consistency in contrast to the other Vankervelsvlei samples that had a peat-like appearance and texture. These results strongly suggested that the 'bottom' of the peatland had been reached with the sampling equipment in the study sampling areas focused on at Vankervelsvlei.

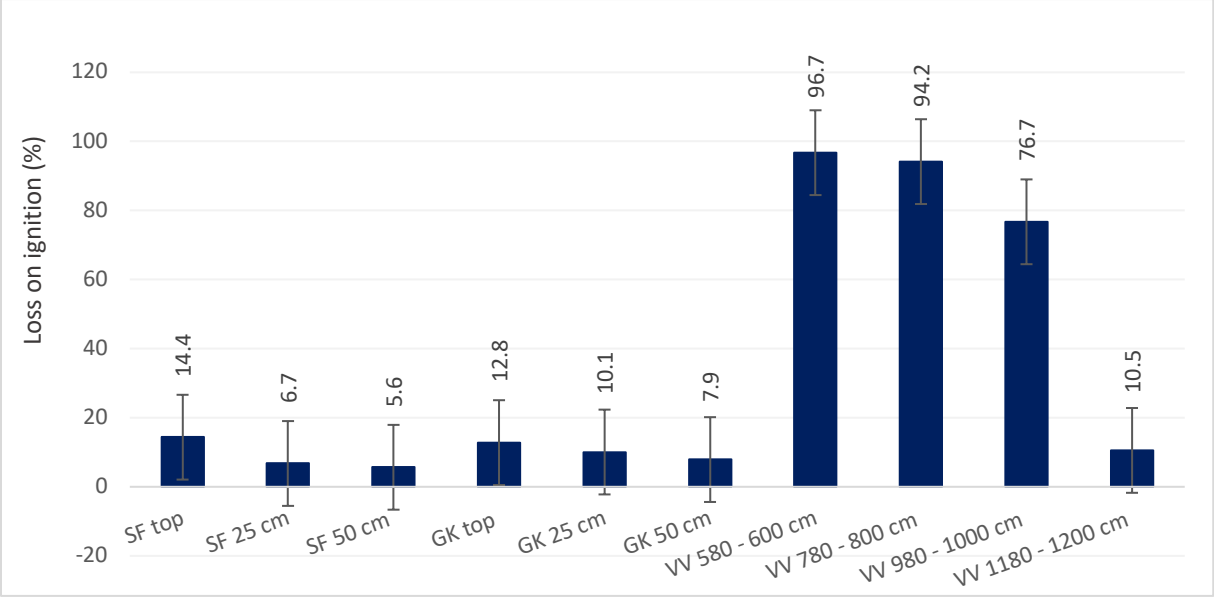


Figure 4.5: Loss on ignition results of samples taken from the three study sites. SF = Springfield; GK = Goukou River; and VV = Vankervelsvlei. Error bars indicate the standard deviation from the mean, *n*=3

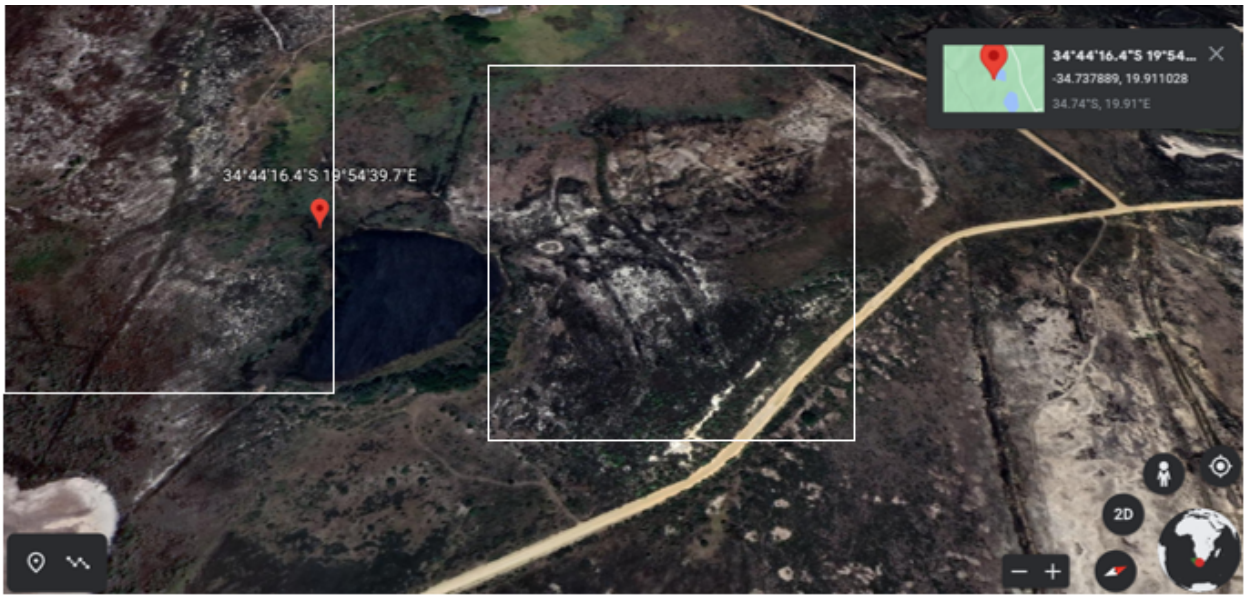
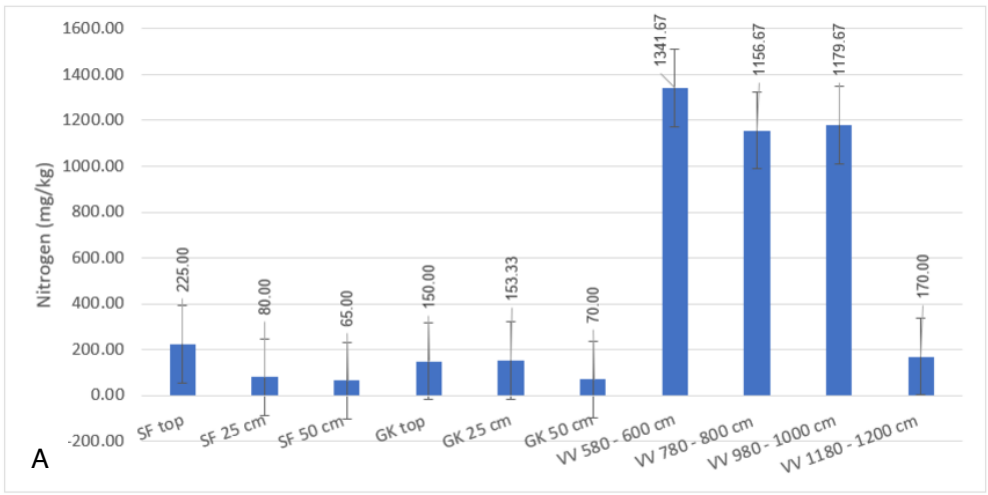
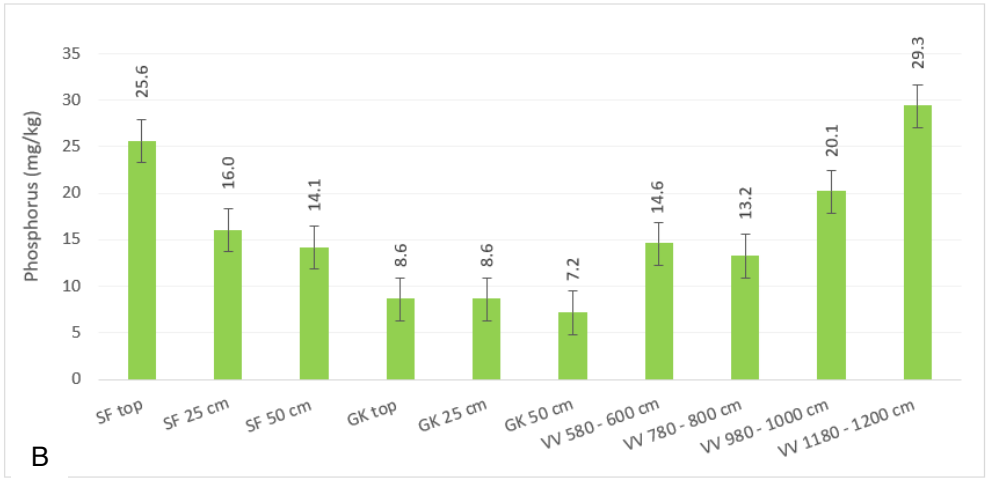


Figure 4.6: The effects of the fires and agricultural runoff on Springfield Farm can be seen in this satellite image, with burnt surfaces clearly visible in the highlighted areas (image obtained from Google maps)

Soil organic matter (SOM) is largely composed of C, H, oxygen (O), with contributions from N, P, and S (Figure 4.7). These elements can be used as semi-quantitative and qualitative indicators

of the SOM content. In soils with low levels of SOM, N, P, and S are higher than in soils containing higher levels of SOM. In this study high levels of N (143 mg/kg – 1568 mg/kg), P (11.14 mg/kg – 53 mg/kg), and S (36 mg/kg – 803mg/kg) were detected in the Vankervelsvlei samples compared to the other sites of interest, which substantiates the correlation with SOM (Tipping et al., 2016:117-118). Soil organic carbon and organic nutrients are co-sequestered in mineral soil and the net amount of N released from mineral soil inversely correlates with the C/N ratio (Springob and Kirchman, 2003:629-632; Kirkby et al., 2013). However, the SOM stoichiometry is not very well understood in organic soils formed in peatlands. Together with C, intact peat-accumulating areas also sequester N and other nutrients (Kuhry and Vitt, 1996:271-275).





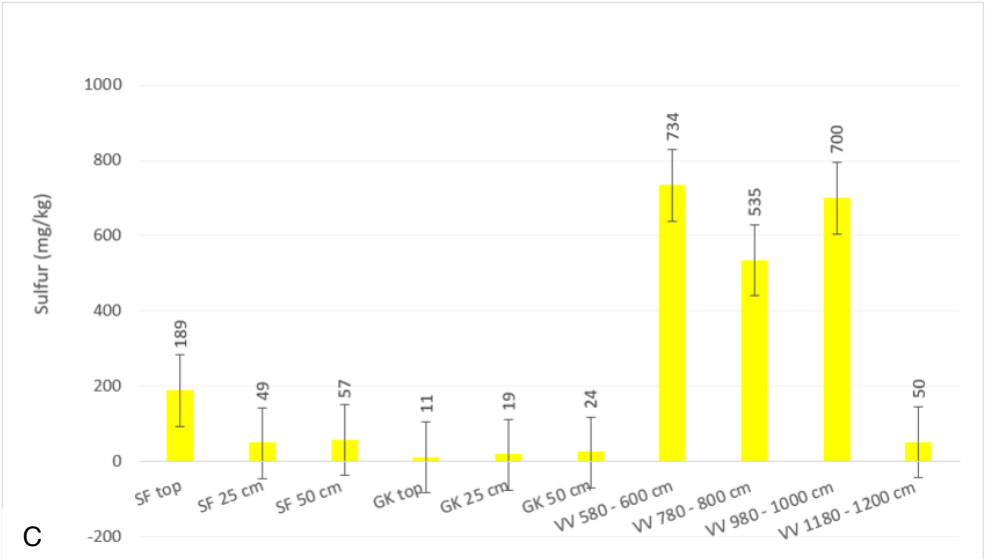


Figure 4.7: Nitrogen (A), phosphorous (B) and sulphur (C) content in samples taken from the three study sites. SF = Springfield; GK = Goukou River; and VV = Vankervelsvlei. Error bars indicate the standard deviation from the mean, *n*=3

4.1.3 C/N ratio of the sampling sites

Peat decomposition can also be assessed through the use of C/N ratios. As a result of the formation of SOM, significant residual enrichment of N over C with time can typically be observed. This is because the deeper the peat, and the older it is, the greater the extent of microbial decomposition of C. In addition, data from artificially drained and decomposed peat also exhibits low C/N ratios (Berglund et al., 2010:508-513). Therefore, the C/N ratio is expected to decrease with depth in peatlands (Leifeld et al., 2020:7634). However, C/N is also affected by differences in the vegetation, changing deposition rates of N, and/or environmental conditions such as dry weather and/or seasonal rains (Biester et al., 2013:2691-2692). In this study, the highest C/N ratios were found in the samples from Vankervelsvlei, but the C/N ratio was not well correlated with the depth of samples in any of the study sites (Figure 4.8). Due to the varying degrees of peat humification (Anderson et al., 1998:97-103), it is possible that fluctuating C/N ratios can be explained by changes in peat hydrology that altered N concentrations before being incorporated into the anaerobic catotelm. It can also be inferred that some of the differences in the C/N ratio may be attributed to the same palaeohydrological shifts responsible for changes in humification. Moreover, the increase and decrease of N in peat are highly dependent on rates of N mineralization at the surface (Anderson et al., 1998:97-103).

Decomposition rates and N mineralization both increase with increased peatland surface moisture (profiling of the oxygenated acrotelm), while N loss increases with increasing N mineralisation. Nitrate (NO_3^-) can leach across the acrotelm/catotelm interface or be volatilised as nitrous oxide (N_2O), nitrogen gas (N_2), or ammonia (NH_3). As N mineralisation exceeds the N demand of the biota on peat surfaces, especially when soils experience intermittent moisture (Patrick and Wyatt, 1964:647-648), then N will be lost in comparison to C. This results in a layer of peat in the catotelm with a relatively high C/N ratio. Consequently, it is probable that peat C/N ratios can provide information about past bog wetness changes, but different bog plants can have different C/N ratios, making the study of past bogs difficult (Anderson et al., 1998:97-103).

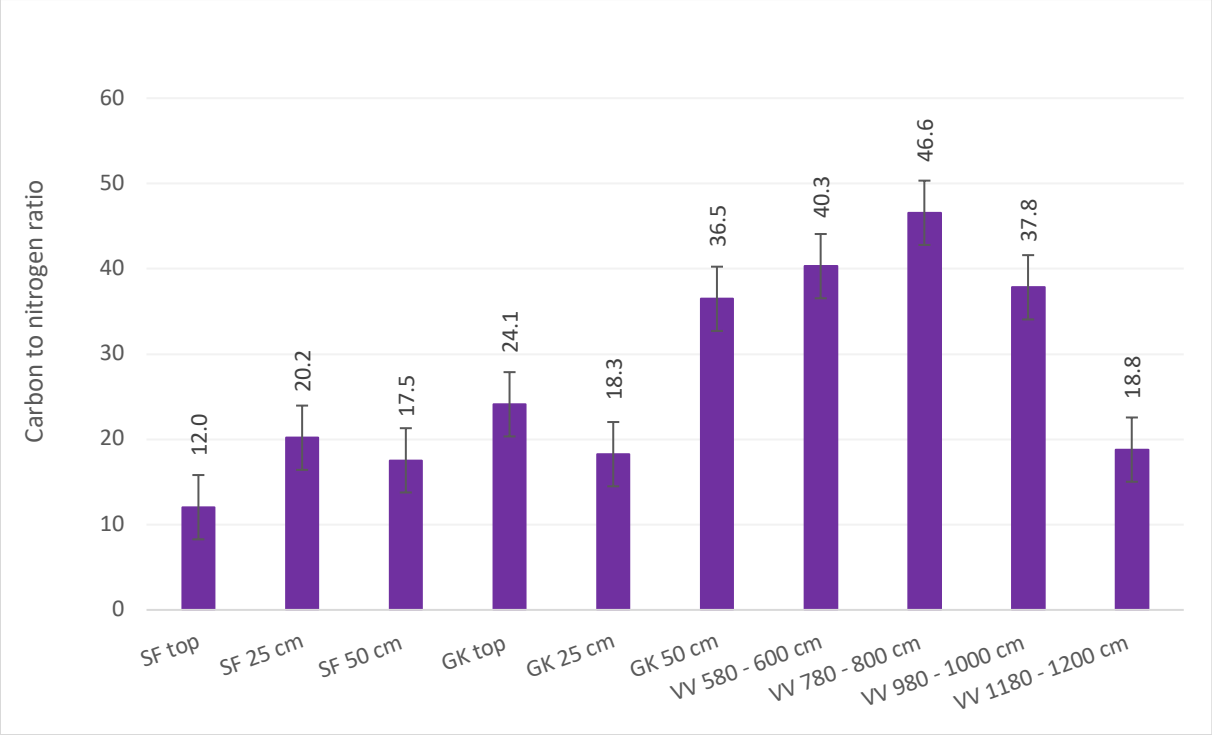


Figure 4.8: The carbon to nitrogen ratio in samples taken from the three sampling sites. SF = Springfield; GK = Goukou River; and VV = Vankervelsvlei. Error bars indicate the standard deviation from the mean, *n*=3

4.1.4 Humification results of the sampling sites

Peat humification can be described as a measure of the degree of peat breakdown or decomposition, most of which occurs when the plant material is in the upper part of the peatland (acrotelm). There are various ways to assess the process of peat humification. In the field, using a 10-point scale (von Post and Granlund, 1929) or a 5-point scale (Troels-Smith, 1959:1-73), a visual estimation of the humification of freshly extracted peat can be performed. Humification tests can also be performed in the laboratory, where the physical properties such as the fibrosity, chemical properties, and/or the chemical extraction of soluble materials are measured (Blackford and Chambers, 1993:7-24; Klavins et al., 2009:1-15). Using these techniques, peat texture and stratigraphy can be described. Bahnson (1968:55-63) proposed a colorimetry method for the determination of peat humification. Colorimetry is a method based on light-absorbance or light transmission measured with a colorimeter or a spectrophotometer at a fixed wavelength. It has been observed that when organic material decomposes, humic acids are produced which are dark brown in colour. The more humified the material, the darker the colour that is able to absorb

light at 540 nm. The humification test measures the absorbance of light by a solution of the OM (in this case peat) in NaOH at 540 nm (Aaby and Berglund, 1986:231-246).

The peat humification results (540 nm absorbance values in percentage) are shown in Figure 4.9. For Springfield Farm, the colour of the samples in NaOH solution was very light (Figure 4.10) and absorbance values were very low, which was expected because the peatland is still developing. The colour of the NaOH solutions of samples from Goukou River were generally medium brown in intensity, with absorbance values slightly higher than those for the Springfield Farm samples. In samples from Vankervelsvlei, very high absorbances were measured and the NaOH solutions were dark brown, with the exception of the samples taken from the bottom of the peatland, which is consistent with results described in Section 4.1.2.

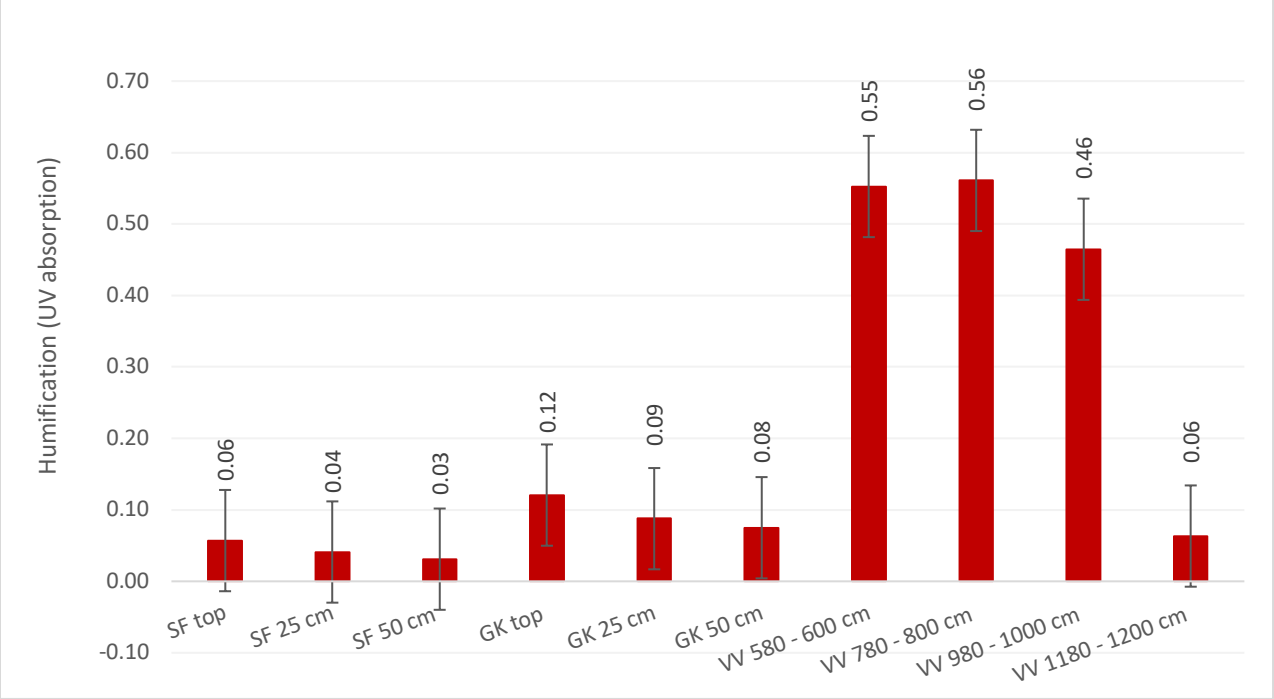


Figure 4.9: Humification results of samples taken from the three study sites. SF = Springfield; GK = Goukou River; and VV = Vankervelsvlei. Error bars indicate the standard deviation from the mean, *n*=3

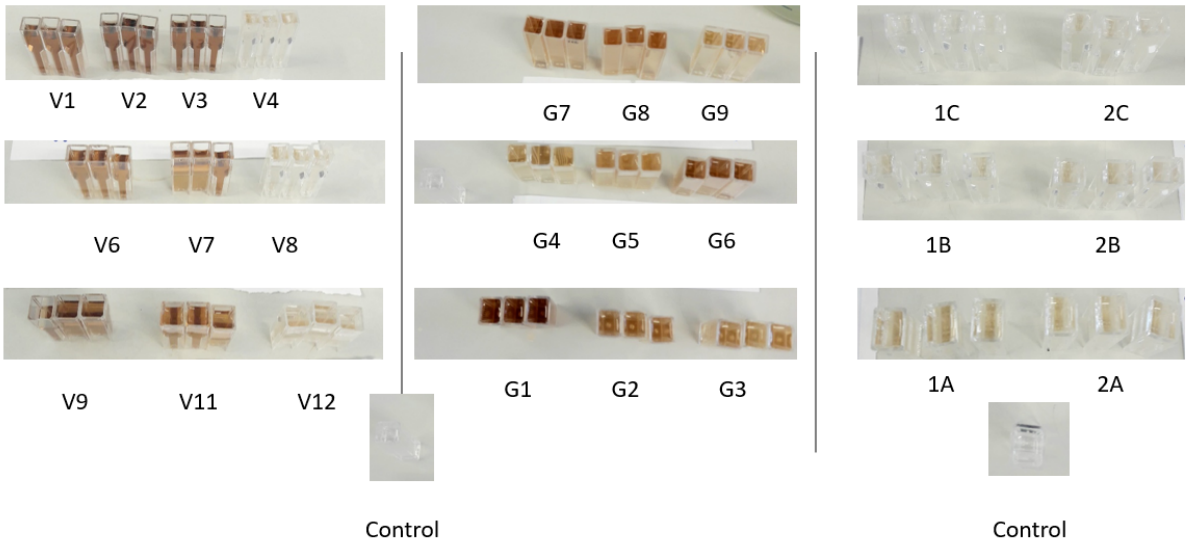


Figure 4.10: Cuvettes containing solutions of samples in NaOH for the colorimetric analysis to determine the degree of the humification VV = Vankervelsvlei (V1, V9: top; V2, V6: top – middle; V3, V7, V11: middle – bottom; V4, V8, V12: bottom) GK = Goukou River (G1, G4, G7: top; G2, G5, G8: middle; G3, G6, G9: bottom); and SF = Springfield (1a – 2a: top; 1b – 2b: middle; 1c – 2c: bottom)

4.2 Conclusion

As expected, the physicochemical data indicated that there were many similarities and differences between the three selected sites, specifically Goukou River and the other two sites. This is because Goukou River, similar to Vankervelsvlei is a well-developed peatland, but also as is the case with Springfield Farm is affected by agricultural wastewater run-off which has resulted in exposed peat deposits. The only similarity between Goukou River and Vankervelsvlei was their pH levels, which were both acidic. While the pH of Springfield Farm is basic. Humification, organic matter (OM), and elements including carbon (C), and nitrogen (N), of Vankervelsvlei were high, while Goukou River and Vankervelsvlei had similar values.

CHAPTER FIVE: RESULTS AND DISCUSSION - MOLECULAR DATA

5.1 Microbial Community composition

5.1.1 T-RFLP analyses: 16S rRNA gene

T-RFLP analysis involved comparing specific PCR fragments after digestion with a restriction enzyme. Using this technique, one can compare different communities quickly and economically; in addition to bacteria, it has been very useful for investigating microbial communities in general (Hoppe and Schnittler, 2015:216-218). Primers are designed to amplify and target a gene of interest using fluorescently labelled PCR primers. Following amplification, restriction enzymes are used to identify sequence polymorphisms, which results in fragments of varying lengths. A DNA sequencer is used to fractionate the fluorescently labelled fragments (Liu et al., 1997:4516-4518). In this study, the T-RFLP data that was generated was used to determine the degree of similarity between the different bacterial communities in the three peatlands using all of the sample replicates (Figure 5.1). There were significant differences (ANOSIM) between the bacterial communities in each peatland (Table 5.1). This can be seen visually on the nMDS plots as the data points representing the bacterial communities in the peatlands clustered together, albeit with some co-clustering of data points representing some of the samples from Springfield Farm and Vankervelsvlei. The 2D stress of the community is 0.16, and this could be an indication that the algorithm had difficulty visually representing the data distribution (of all three sites) in 2D.

Table 5.1: Unordered ANOSIM R of the T-RFLP amplicons (all samples)

	Springfield	**Gratitude**
Gratitude	0.751***	-
Vankervelsvlei	0.657**	0.168***

Level of significance: *0.05>p≥0.01 **0.01>p≥0.005 ***0.005<p

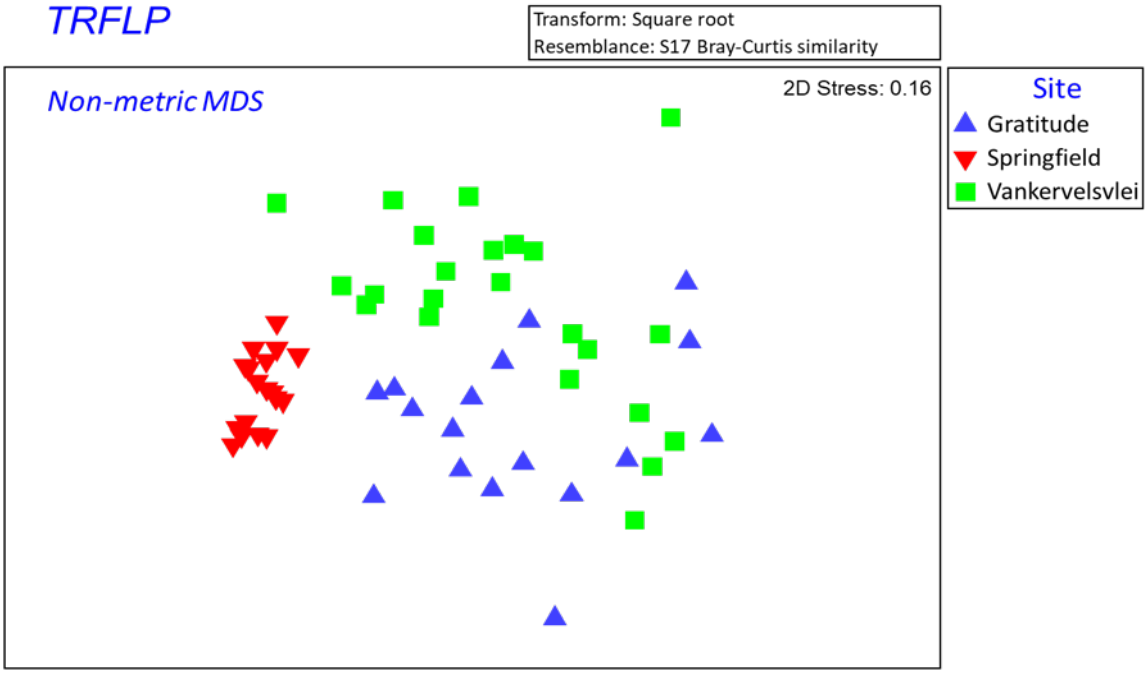


Figure 5.1: A Non-metric multidimensional scaling plot of T-RFLP data of the Bray-Curtis similarity of the bacterial communities from the three study sites

The data points denoting the bacterial community structures from the different sampling sites and depths at Springfield Farm clustered together in the nMDS plot (Figure 5.2), indicating high similarity with both factors 'site' and 'depth'. There were statistically significant differences (ANOSIM) between the bacterial communities in the upper (top) and middle and bottom layers, but not between the middle and bottom layers at each site (Table 5.2), indicating that the bacterial communities in the middle and bottom layers were highly similar at each site.

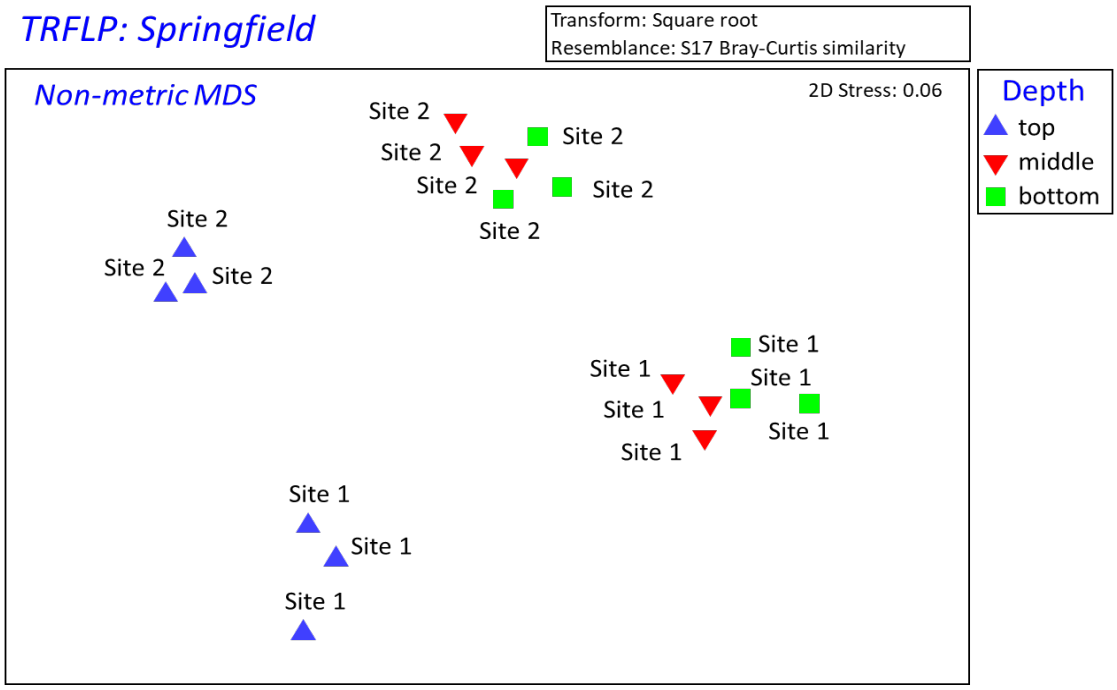


Figure 5.2: A Non-metric multidimensional scaling plot of T-RFLP data of the Bray-Curtis similarity of the actinobacterial communities from Springfield Farm

In samples taken from Goukou River, the difference between the bacterial communities of the different sites (Figure 5.3) increased with depth. There were no statistically significant differences (ANOSIM) between the bacterial communities in the top and middle layers (Table 5.2) and the data points representing the bacterial communities in the middle and top layers were randomly spaced on the nMDS plot. In contrast, there were significant differences between the bacterial communities in the samples in the the top/middle and bottom layers (Table 5.2), and the data points representing the bacterial communities in the bottom layers also clustered closely on the nMDS plot, indicating high levels of similarity.

Table 5.2 Unordered ANOSIM R of the T-RFLP amplicons for Springfield Farm and Goukou River

	Springfield		**Goukou River**	
	Top (0 to -5 cm)	Middle (-25 cm)	Top (0 to -5 cm)	Middle (-25 cm)
Middle (-25 cm)	0.800***	-	NS	-
Bottom (-50 cm)	0.913***	NS	0.331*	0.557***

Level of significance: *0.05>p≥0.01 **0.01>p≥0.005 ***0.005<p NS = not significant

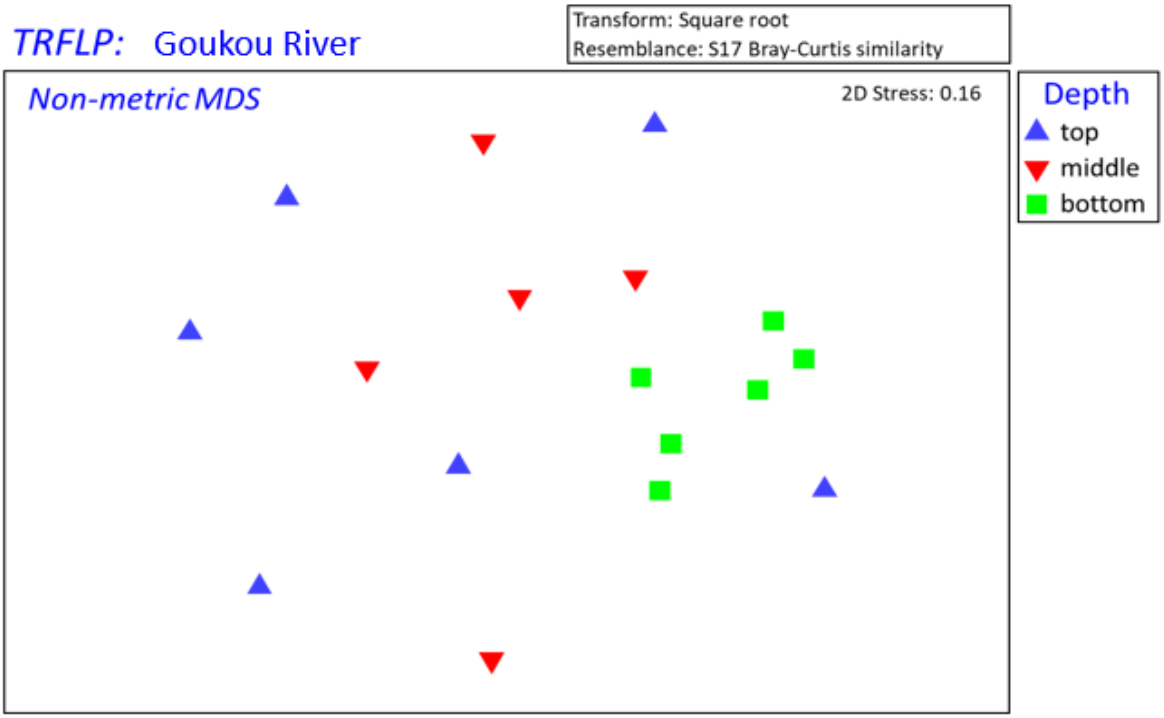


Figure 5.3: A Non-metric multidimensional scaling (MDS) plot of T-RFLP data of the similarity between the bacterial DNA communities of Goukou River

In the samples taken from Vankervelsvlei, although some clustering of data points representing bacterial communities in samples from 780-800 cm and 1180-2000 cm were noted, there were no significant differences between the communities at all depths from 580-1000 cm (Table 5.3). In line with the physicochemical differences at depth 1180-2000 cm, there were significant (ANOSIM) differences in the bacterial communities at 1180-2000 cm and the bacterial communities in all the upper layers (Table 5.3).

Table 5.3 Results of one-way unordered ANOSIM: Vankervelsvlei at different depths

	580-600 cm	**780-800 cm**	**980-1000 cm**
780-800 cm	0.111		
980-1000 cm	0.074	0.185	
1180-2000 cm	0.889*	0.704*	0.889*

Level of significance: *0.05>p≥0.01 **0.01>p≥0.005 ***0.005<p

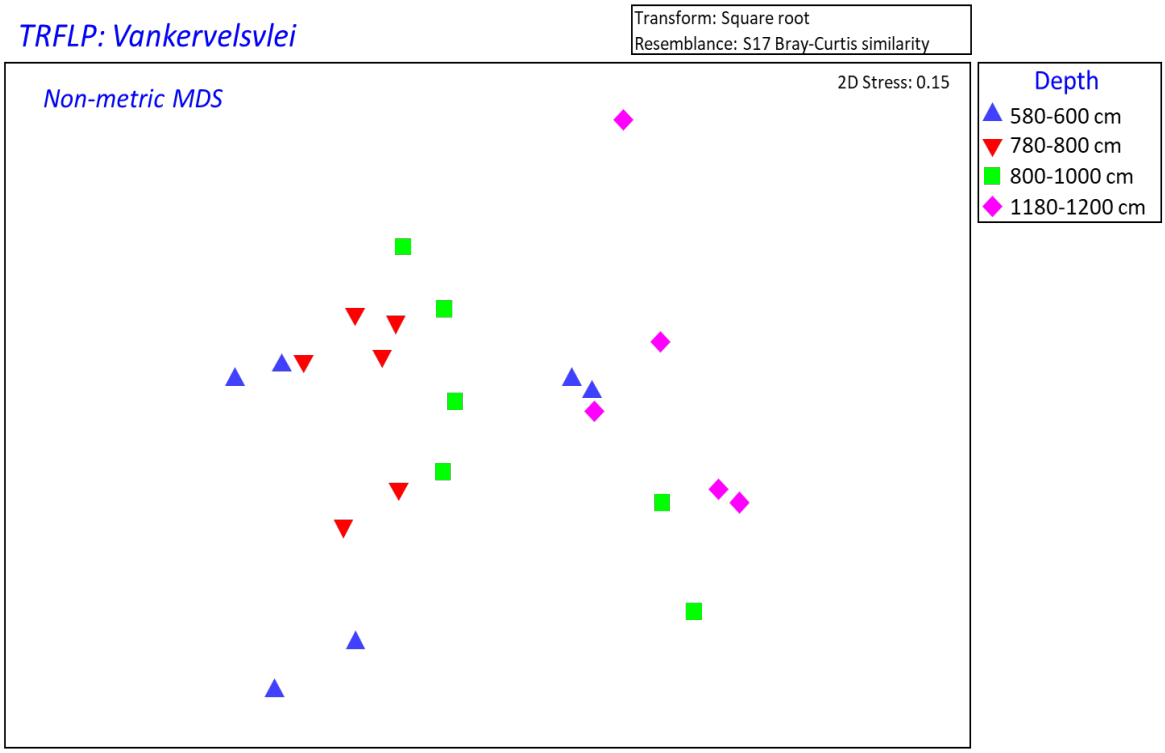


Figure 5.4: A Non-metric multidimensional scaling plot of T-RFLP data of the Bray-Curtis similarity of the bacterial communities from Vankervelsvlei

After analysing the T-RFLP data to determine the similarity of the communities, the identity of the bacteria and fungi were determined. The most abundant taxa are presented and discussed in Section 5.2.2.

5.1.2 16S rRNA-based microbial community analyses

5.1.2.1 Bacterial communities present in the selected peatlands

Bacterial and archaeal DNA was found in all of the samples. Common phyla included *Proteobacteria*, *Gemmatimonadetes*, *Planctomycetes*, *Bacteroidetes*, *Firmicutes*, *Cyanobacteria* and *Actinobacteria*. NGS was performed to establish the actinobacterial community structure in selected samples. The Actinobacterial orders *Acidimicrobiales* (Springfield Farm: 0.85-4.04%; Goukou River: 3.59-29.05%; Vankervelsvlei: 0.004-0.11%), *Actinomycetales* (Springfield Farm: 0.25-3.82%; Goukou River: 1.60-9.08%; Vankervelsvlei: 0.009-15.54%), 0319 – 7L14 (uncultured bacterium) (Springfield Farm: 0.054-1.97%; Goukou River: 0.027-0.26%; Vankervelsvlei: 0.009-0.22%), *Gaiellales* (Springfield Farm: 0.14-1.01%; Goukou River: 0.01-0.75%; Vankervelsvlei:

0.007-0.13%), and *Solirubrobacterales* (Springfield Farm: 0.70-2.16%; Goukou River: 0.94-9.76%; Vankervelsvlei: 0.005-0.52%) were present in the highest relative abundances (RA) in all three sampling sites from 0 to 50 cm (Figure 5.4). The RA of orders *Acidimicrobiales*, *Actinomycetales*, *Gaiellales* and *Solirubrobacterales* increased with sample depth (0-50 cm and 580-1200 cm) in mineral-rich environments as previously described by Khilyas et al. (2019). Their research suggests that the above-mentioned microorganisms were influenced by mineral porosity (Mg^{2+} decreasing with depth) and water circulation, which resulted in them having a positive correlation with depth. However, Khilyas et al. (2019) did not look at the other minerals that played a role in why these orders increased with depth. These actinobacterial orders were highly abundant in the Goukou River and Springfield Farm samples, and less abundant in the Vankervelsvlei samples, with the exception of the bottom layer where *Actinomycetales* was detected in high RA (2.86% to 15.54%). These results are very similar to that of Khilyas et al. (2019) and we observed an increase in the RA in the sampling sites with decreasing levels of Mg^{2+} (Figure 4.3) as can be seen for Springfield Farm, Goukou River, and the deep samples of Vankervelsvlei. However, the actinobacteria in the bottom layer were less abundant: *Bifidobacteriales* (≤ 0.33%), *Micrococalles* (0%), *Euzebyales* (0%), *Nitriliryptorales* (0%), and *Rubrobacterales* (0%), and only detected in the samples from Springfield Farm, while *Bifidobacteriales* was only detected in one of the bottom layer samples of Vankervelsvlei. Table 5.4 contains complete data of the RA of all the actinobacterial orders.

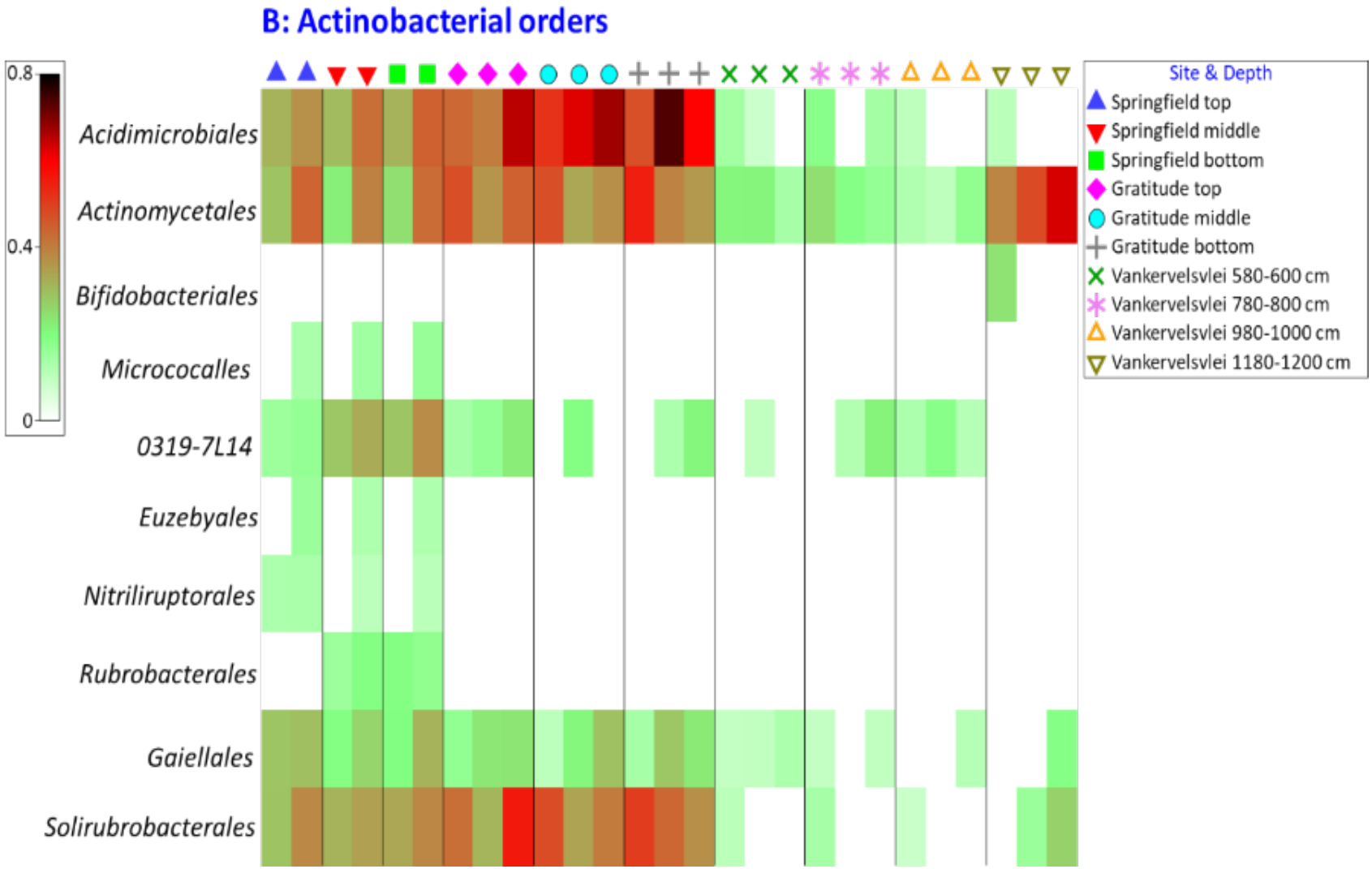


Figure 5.5: Shade plots of relative abundance of square root transformed relative abundance data of actinobacterial orders in the peat samples

Table 5.4: Relative abundance percentage of actinobacterial orders detected in the three sampling sites used in this study

Site & depth	Replicate	*Acidimicrobiales*	*Actinomycetales*	*Bifidobacteriales*	*Micrococcales*	0319-7L14	*Euzebyales*	*Nitriliruptorales*	*Rubrobacterales*	*Gaiellales*	*Solirubrobacterales*
Springfield farm Top	1A	0.973	0.703	0.000	0.000	0.054	0.000	0.027	0.000	0.676	0.703
Springfield farm Middle	1B	0.846	0.245	0.000	0.000	0.641	0.000	0.000	0.055	0.136	0.982
Springfield farm Bottom	1C	1.009	0.368	0.000	0.000	0.676	0.000	0.000	0.131	0.142	1.270
Springfield farm Top	2A	1.857	3.822	0.000	0.031	0.077	0.062	0.031	0.000	0.789	2.120
Springfield farm Middle	2B	3.287	2.383	0.000	0.047	1.162	0.023	0.012	0.129	0.470	1.409
Springfield farm Bottom	2C	4.036	3.378	0.000	0.062	1.974	0.025	0.012	0.087	1.006	2.161
Goukou River Top	G1	3.599	5.052	0.000	0.000	0.035	0.000	0.000	0.000	0.081	3.288
Goukou River Middle	G2	7.284	5.106	0.000	0.000	0.000	0.000	0.000	0.000	0.012	5.176
Goukou River Bottom	G3	4.930	9.083	0.000	0.000	0.000	0.000	0.000	0.000	0.035	6.485
Goukou River Top	G4	2.606	1.707	0.000	0.000	0.077	0.000	0.000	0.000	0.300	0.939
Goukou River Middle	G5	14.948	1.240	0.000	0.000	0.131	0.000	0.000	0.000	0.196	1.338
Goukou River Bottom	G6	29.048	2.347	0.000	0.000	0.027	0.000	0.000	0.000	0.667	3.708
Goukou River Top	G7	17.960	4.042	0.000	0.000	0.261	0.000	0.000	0.000	0.326	9.762
Goukou River Middle	G8	20.515	1.891	0.000	0.000	0.000	0.000	0.000	0.000	0.748	2.723
Goukou River Bottom	G9	12.453	1.604	0.000	0.000	0.189	0.000	0.000	0.000	0.283	1.887
Vankervelsvlei 580 cm – 600 cm	V1	0.041	0.197	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.012
Vankervelsvlei 780 cm -800 cm	V2	0.112	0.381	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.037
Vankervelsvlei 980 cm – 1000 cm	V3	0.011	0.021	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.005
Vankervelsvlei 1180 cm – 1200 cm	V4	0.000	15.537	0.000	0.000	0.000	0.000	0.000	0.000	0.126	0.522
Vankervelsvlei 580 cm – 600 cm	V5	0.005	0.206	0.000	0.000	0.009	0.000	0.000	0.000	0.009	0.000
Vankervelsvlei 780 cm -800 cm	V6	0.000	0.122	0.000	0.000	0.019	0.000	0.000	0.000	0.000	0.000
Vankervelsvlei 980 cm – 1000 cm	V7	0.000	0.010	0.000	0.000	0.113	0.000	0.000	0.000	0.000	0.000
Vankervelsvlei 1180 cm – 1200 cm	V8	0.000	5.429	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.061
Vankervelsvlei 580 cm – 600 cm	V9	0.000	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.000
Vankervelsvlei 780 cm -800 cm	V10	0.039	0.075	0.000	0.000	0.215	0.000	0.000	0.000	0.009	0.000
Vankervelsvlei 980 cm – 1000 cm	V11	0.000	0.085	0.000	0.000	0.016	0.000	0.000	0.000	0.016	0.000
Vankervelsvlei 1180 cm – 1200 cm	V12	0.012	2.286	0.334	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Acidimicrobiales was found in high RA, particularly in the Goukou River samples (RA = 2.61-29.05%). These microorganisms are very widespread and have been isolated from a copper leach dump, mine drainage water, a forested wetland that has been impacted by reject coal and from geothermal sites including Yellowstone National Park, Iceland and Milos, Greece while their DNA has been detected in river water samples, acid mine effluent, sulfidic mine waste and various hot springs (Johnson et al., 2003:60-67). The dominance of *Acidimicrobiales* could be as a result of the pH of the peatland because some of these microorganisms grow at an optimal pH of 2.0 to 3.0 (Clark and Norris, 1996:785-790), and the average pH of the Goukou River samples ranges between 2.93 to 3.31 which is very close to the optimal pH of members of the actinobacterial genera *Acidimicrobium* (pH 2.0), *Ferrmicrobium* (pH 2.0), and *Aciditerrimonas* (pH 3.0) (Clark and Norris, 1996:785-790; Johnson et al., 2003:60-67; Itoh et al., 2011:1281-1284). *Acidimicrobiales* was also detected in the Springfield Farm samples. However, the pH of the Goukou River samples was notably lower than the pH of the Springfield Farm samples (Figure 4.1). The pH results indicated that Goukou River is an acidic peatland, while Springfield Farm is basic. Members of the genus *Illumatobacter*, belonging to the order *Acidimicrobiales*, may have been preferentially selected at Springfield Farm (RA = 0.97-4.04%) as the optimal pH of *Illumatobacter* is 7 (Matsumoto et al., 2009:201-205). This peatland (with pH values ranging from 6.43 to 7.12) would theoretically be a more suitable site than the other study peatlands for growth of this genus in terms of pH.

The Goukou River samples show a clear decline in Fe^{3+} and an increase in RA of *Acidimicrobiales* with depth which is very characteristic of the environments in which some family members occur (Figure 5.6, Figure 5.7). The family members of the *Acidimicrobiales* order possess a versatile metabolism as they have the ability to be mixotrophs when grown in the presence of glucose and Fe, autotrophs in the presence of Fe or reduced S, and chemolitheterotrophs in the presence of Fe-yeast extract (Fe is used as the energy source and yeast extract is used as the carbon source) (Wood and Kelly, 1983:107-112; Bridge and Johnson, 1998:2181-2186). *Acidimicrobiales* are acidophilic and slightly alkaliphilic, as well as moderately thermophilic and mesophilic. Under aerobic conditions, the Fe-oxidizing members of this family oxidize Fe^{3+} and under anaerobic conditions Fe^{3+} is reduced (Bridge and Johnson, 1998:2181-2186). Similar trends of decreasing Fe concentration with depth were noted in the samples of Springfield Farm and Goukou River, albeit the concentrations were notably higher in the samples from Springfield Farm (Figure 5.7). Previous studies show that these microorganisms use the geomicrobial cycling of Fe and CO_2 in acidophilic ore-containing environments (González-Toril et al., 2003:4853-4854).

Members of the order *Acidimicrobiales* were less abundant or absent in samples from Vankervelsvlei (0-0.11%) than in samples from the other sites (Goukou River 0-14.95%; Springfield Farm 0-4%). Similar to Goukou River, Vankervelsvlei is also an acidic peatland, which makes this an ideal environment for these microorganisms in terms of pH. As mentioned in previous sections (Section 3.2.1.2, Section 3.2.1.3), both Goukou River and Springfield Farm have historically been impacted by agricultural run-off and farming activities, while Vankervelsvlei has not. *Acidimicrobiales* were more abundant in samples from Springfield Farm and Goukou River, compared to Vankervelsvlei and this could be as a result of the environment becoming less favourable for *Acidimicrobiales* also making it more difficult for them to compete with other microorganisms present in this peatland. However, some members, notably *Acidomicrobium capsulatum* (isolated from acid drainage sediments in Japan), *Acidomicrobium usitatus* and *Acidomicrobium versatili* (isolated from ryegrass grass/ clover pastures in Australia) have been found to be dominant in soils with high OM content because they are involved in the breakdown of lignocellulose and cellulose (Eichorst et al., 2018:1041-1042).

Organisms in the order *Actinomycetales* are Gram-positive to Gram variable, filamentous or fragmenting, branching bacteria, with a number of morphological features. They typically have a diameter of 1 µm, and they are pleomorphic. In addition to being non-motile, slow-growing organisms, they are mostly not acid tolerant. It is possible for them to be facultative, obligate, or microaerophilic (Li et al., 2016). Genera such as *Actinomyces*, *Propionibacterium*, *Bifidobacterium*, *Nocardia*, *Streptomyces*, and *Rhodococcus* are included in this group. These organisms were detected in all the samples, being most abundant in the Goukou River samples (1.60-9.08%) moderately abundant in the Springfield Farm samples (0.25-3.82%) and mostly less abundant in Vankervelsvlei samples (0.009-2.286%), with the exception of two samples which showed high RA (5.428% and 15.54%) (Table 5.4). According to Golovchenko et al. (2002:667-670), the abundance of Actinobacteria decreases with depth and is always the highest at the surface. This trend was noted in this study, with the exception of a few samples from the bottom of Vankervelsvlei. *Corynebacteriaceae* were observed in Vankervelsvlei samples, *Intrasporangiaceae* in Springfield Farm and Goukou River samples, *Microbacteriaceae*, *Propionibacteriaceae* and *Nocardiaceae* in both Goukou River and Vankervelsvlei, and both *Pseudonocardiaceae* and *Streptomycetaceae* in all of the sampling sites (Figure 5.6). According to the LINKTREE results (see Figure 5.14, Section 5.3.2.1), N was a significant driver for actinobacterial selection, and the majority of these microorganisms contain the *nifH* gene, which

encodes for the enzyme (nitrogenase) responsible for fixing atmospheric N_2 into NH_3 (Sellstedt and Richau, 2013:179-185). In this study, Al concentrations also correlated with actinobacterial selection (Table 5.9, Section 5.2.2). According to a study conducted by Silva et al. (2013:1-10), high levels of Al have no effect on actinobacterial community composition. However, in this study, Al played a significant role in actinobacterial community selection, a novel finding (discussed in section 5.2.2).

Members of the order *Gaiellales* were detected in all of three sampling sites. There is limited literature available on studies of this order. However, according to Kaiser et al. (2016:33696), *Gaiellales* abundance increases with an increase in pH. In our results *Gaiellales* was more abundant (Table 5.9) in samples from Goukou River (*p* value: 0.31) and Vankervelsvlei (*p* value: 0.42) compared to that of Springfield Farm (*p* value: -0.07). The PEARSON's correlation coefficient indicates that there is poor correlation between abundance and pH for Goukou River and Vankervelsvlei, while there is no correlation between the pH and abundance of *Gaiellales* in Springfield Farm. *Solirubrobacterales* were also highly abundant in the Goukou River and Springfield Farm samples. It was observed that the abundance of these microorganisms increased with depth. Previous research has indicated that *Solirubrobacterales* prefer alkaline environments (optimal pH 6.5 – 9.0), which falls well above the pH determined in the samples from Goukou River (pH 2.99 – pH 3.31). However, according to Shange et al. (2012), members of this order are very adaptive and have to ability to colonize different ecosystems, thus from our results we can speculate that the different actinobacterial species present in this study could tolerate different pH ranges.

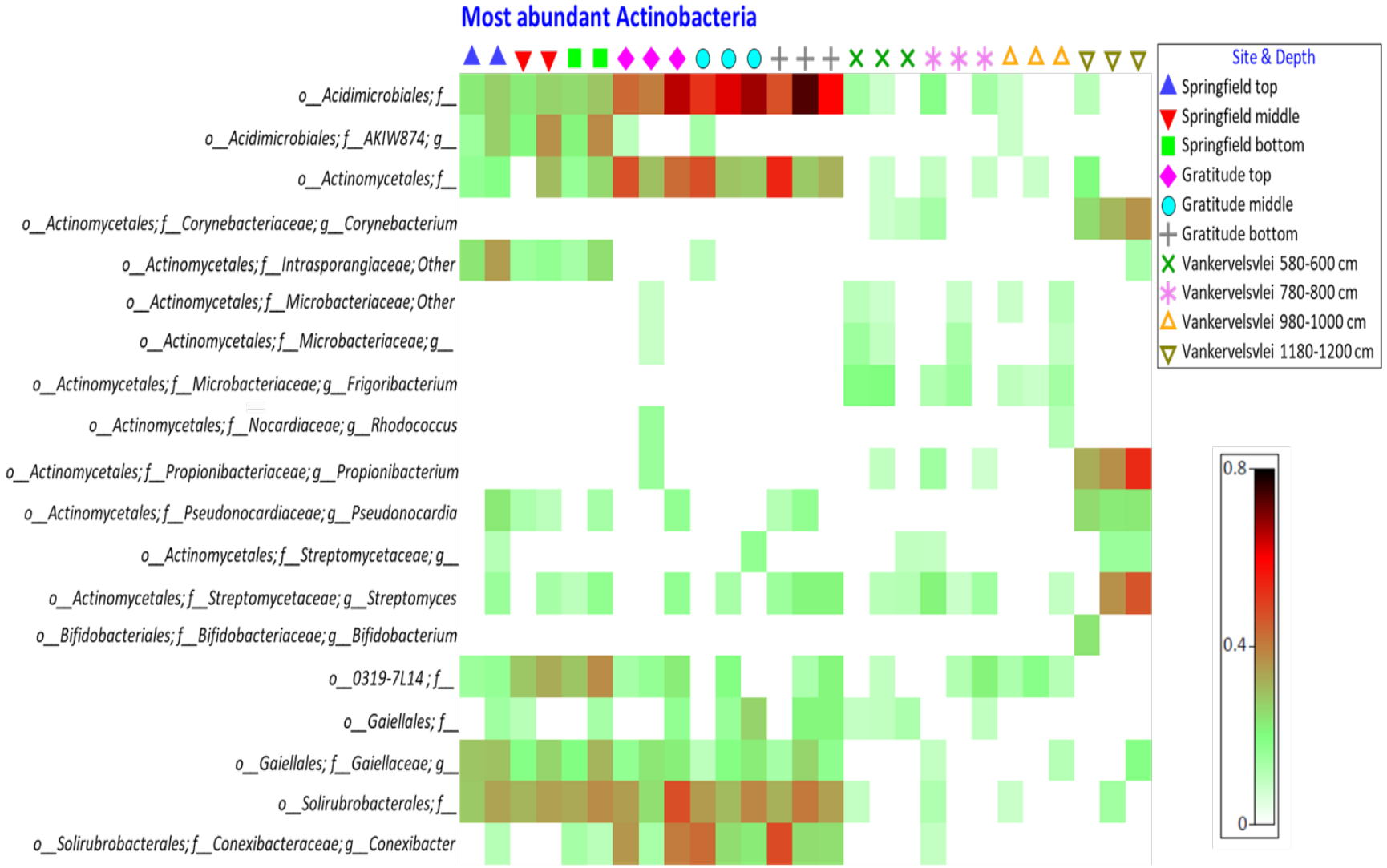


Figure 5.6: Shade plot of square root transformed relative abundance of the most abundant actinobacterial operational taxonomic units in the peat samples

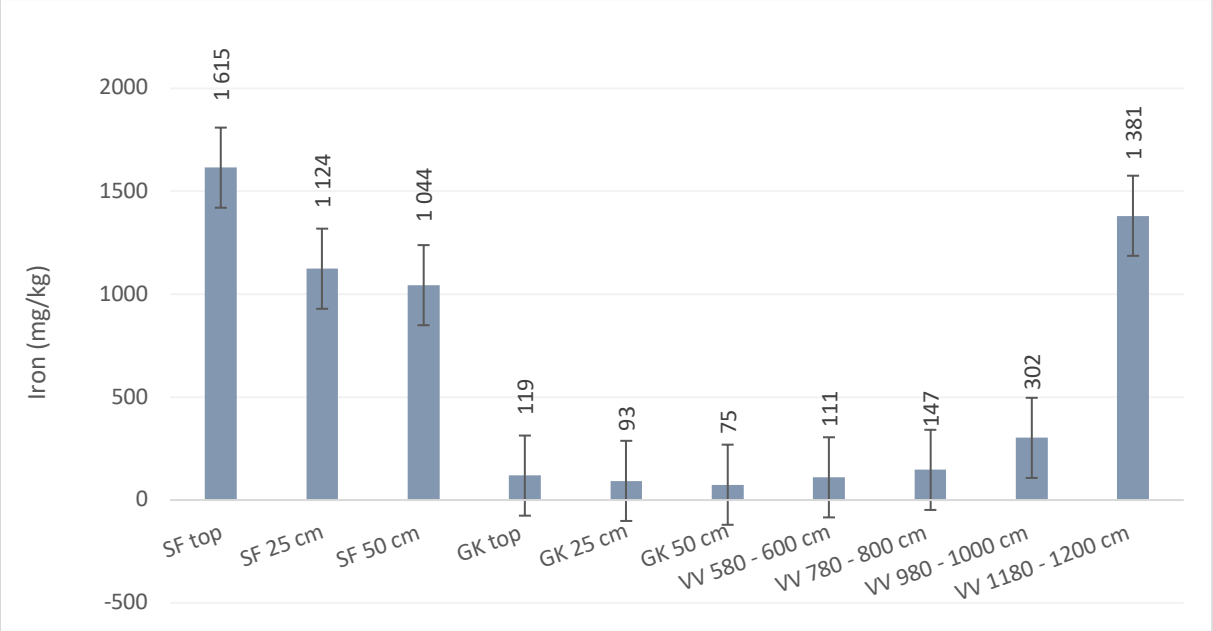


Figure 5.7: Iron content in samples taken from the study sites. SF = Springfield; GK = Goukou River; and VV = Vankervelsvlei. Error bars indicate the standard deviation of analysis, *n*=3

5.1.2.2 Fungal DNA barcoding for NGS analyses

Next generation sequencing of the DNA samples showed that fungal DNA was present in all of the samples. The common phyla for Goukou River, Springfield Farm and Vankervelsvlei includes *Ascomycota* (Springfield Farm: 22.48-84.26%; Goukou River: 3.25-16.41%; Vankervelsvlei: 0.38-12.04%), *Basidiomycota* (Springfield Farm: 0.44-5.09%; Goukou River: 0.16-57.73%; Vankervelsvlei: 20.07-94.06%), *Chytridiomycota* (Springfield Farm: 0.02-0.06%; Goukou River: 0.13-4.13%; Vankervelsvlei: 0-0.05%), *Glomeromycota* (Springfield Farm: 0.08-0.22%; Goukou River: 0-0.01%; Vankervelsvlei: 0.01-0.02%), and *Zygomycota* (Springfield Farm: 0-0.01%; Goukou River: 0.01-0.90%; Vankervelsvlei: 0%) (Figure 5.8). In this study, the fungal taxa primarily fell into two phyla: *Ascomycota* and *Basidiomycota*, in agreement with previous research on peat soils (Thormann, 2006b:101-120; Artz et al., 2007:508-520). In all three sampling sites *Ascomycota* abundance (Table 5.5) decreased with depth, while *Basidiomycota* abundance (Table 5.6) increased with depth only in samples from Vankervelsvlei. No particular trend in terms of *Basidiomycota* RA was noted in samples from Springfield Farm and Goukou River. Fungi, in general, prefer oxic and acidic environments, while bacteria prefer neutral and alkaline environments. Because of this, bacteria found in fens are more abundant and in bogs, fungi are dominant, however, their abundance decreases with depth as the oxygen level decreases (Golovchenko et al., 2002:667-668).

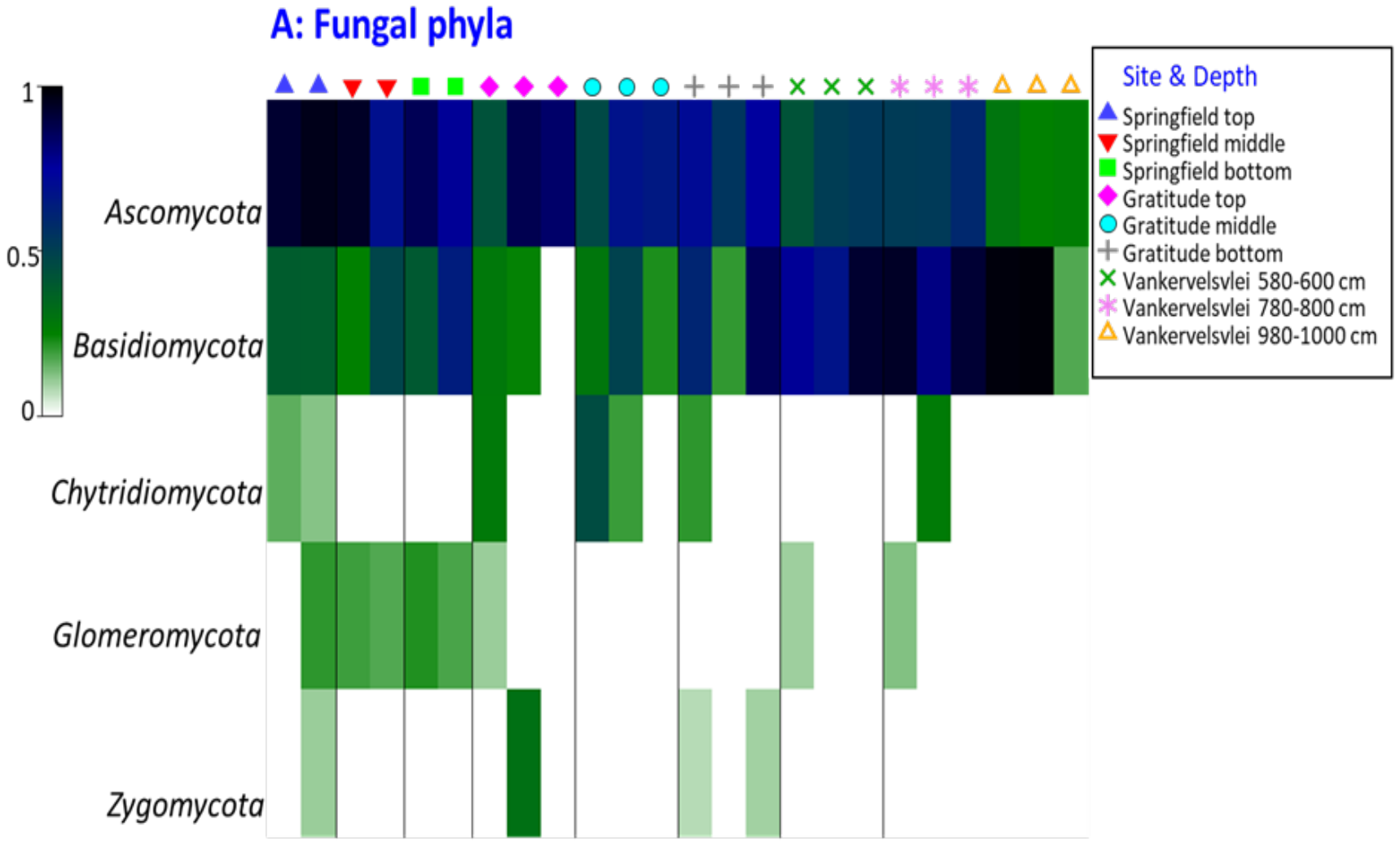


Figure 5.8: Shade plot of square root transformed relative abundance fungal communities detected in samples collected from Springfield Farm, Goukou River and Vankervelsvlei

Table 5.5: Relative abundance percentage of fungal phyla for all three sites targeted in this research study

Site & depth	Replicate	*Ascomycota*	*Basidiomycota*	*Chytridiomycota*	*Glomeromycota*	*Zygomycota*
Springfield farm Top	1A	72.26	1.06	0.06	0.00	0.00
Springfield farm Middle	1B	77.32	1.74	0.00	0.12	0.00
Springfield farm Bottom	1C	55.70	5.09	0.00	0.22	0.00
Springfield farm Top	2A	84.26	2.54	0.02	0.17	0.01
Springfield farm Middle	2B	22.48	0.44	0.00	0.08	0.00
Springfield farm Bottom	2C	33.44	2.53	0.00	0.10	0.00
Goukou River Top	G1	3.25	16.41	0.56	0.01	0.00
Goukou River Middle	G2	4.59	0.00	4.13	0.00	0.00
Goukou River Bottom	G3	24.77	0.23	0.17	0.00	0.00
Goukou River Top	G4	57.73	0.62	0.00	0.00	0.90
Goukou River Middle	G5	20.72	0.67	0.13	0.00	0.00
Goukou River Bottom	G6	8.40	13.14	0.00	0.00	0.00
Goukou River Top	G7	48.34	0.28	0.00	0.00	0.00
Goukou River Middle	G8	18.13	5.63	0.00	0.00	0.00
Goukou River Bottom	G9	31.76	0.16	0.00	0.00	0.01
Vankervelsvlei 580 cm – 600 cm	V1	3.21	59.49	0.00	0.01	0.00
Vankervelsvlei 780 cm -800 cm	V2	7.06	74.89	0.00	0.02	0.00
Vankervelsvlei 980 cm – 1000 cm	V3	0.69	59.83	0.00	0.00	0.00
Vankervelsvlei 1180 cm – 1200 cm	V4	6.69	28.74	0.00	0.00	0.00
Vankervelsvlei 580 cm – 600 cm	V5	7.29	74.06	0.50	0.00	0.00
Vankervelsvlei 780 cm -800 cm	V6	0.38	89.69	0.00	0.00	0.00
Vankervelsvlei 980 cm – 1000 cm	V7	7.70	20.07	0.00	0.00	0.00
Vankervelsvlei 1180 cm – 1200 cm	V8	12.04	49.92	0.00	0.00	0.00
Vankervelsvlei 580 cm – 600 cm	V9	0.44	94.06	0.00	0.00	0.00

Research has shown that fungi are the dominant decomposers in peatlands and play a more dominant role than bacteria in this process (Kox, 1954:111-112; Latter et al., 1967:445-446; Williams and Crawford, 1983:201-202; Andersen et al., 2006:1375-1376). In peatlands, most fungi are saprobes that decompose organic materials (including cellulose, lignin, and their derivatives) and this process is caused by extracellular enzymes which degrade simple leachates and complex structural plant polymers (Thormann and Rice, 2007:241-242). *Ascomycota* are fungi that have cells that are divided into septa by cellular walls. They produce spores in the form of ascospores, formed within sac-like structures called asci, as well as small asexual spores called conidia. There are some species of *Ascomycota* that do not reproduce sexually and do not construct asci or ascospores (McConnaughey, 2014). In peatlands, the *Ascomycota* such as *Bulgaria*, *Chaetomium*, and *Helotium* have been extensively researched because of their cellulolytic enzymes and wood (plant)- degrading abilities (Lynd et al., 2002:506-508). Previous research by Thormann and Rice (2007:241-242), have found that ascomycetes are the largest fungal group present in peatlands. This corresponds to the results of this study as high OTU numbers of *Ascomycota* were observed in all of the sampling sites. At lower taxonomic levels, the classes *Archaeorhizomycetes, Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Orbiliomycetes, Pezisomycetes, Saccharomycetes,* and *Sordatiomycetes* (Figure 5.9) dominated.

Dothideomycetes (Springfield Farm: 0.31-33.23%; Goukou River: 0.40-33.33%; Vankervelsvlei: 0.07-10.04%)*, Eurotiomycetes* (Springfield Farm: 0.26-10.69%; Goukou River: 0.07-23.45%; Vankervelsvlei: 0.02-0.14%)*, Lecanoromycetes* (Springfield Farm: 0.01-3.18%; Goukou River: 0.07-15.40%; Vankervelsvlei: 0.02-0.07%)*, Pezisomycetes* (Springfield Farm: 0.03-0.36%; Goukou River: 0.03-4.60%; Vankervelsvlei: 0-0.10%)*,* and *Sordatiomycetes* (Springfield Farm: 3.87-58.65%; Goukou River: 1.14-8.75%; Vankervelsvlei: 0.01-0.71%)*,* were detected in all of the sampling sites. *Archaeorhizomycetes* (0.09-1.57%) was detected in samples from Springfield Farm, *Saccharomycetes* (Vankervelsvlei: 0.02-5.47%; Springfield Farm: 0-0.02%; Goukou River: 0-0.13%) were detected in all the Vankervelsvlei samples (present in the top layer of Springfield Farm and Goukou River samples), and *Orbiliomycetes* in Vankervelsvlei (0.004-0.053%) and Goukou River (0-0.20%) samples (Table 5.6).

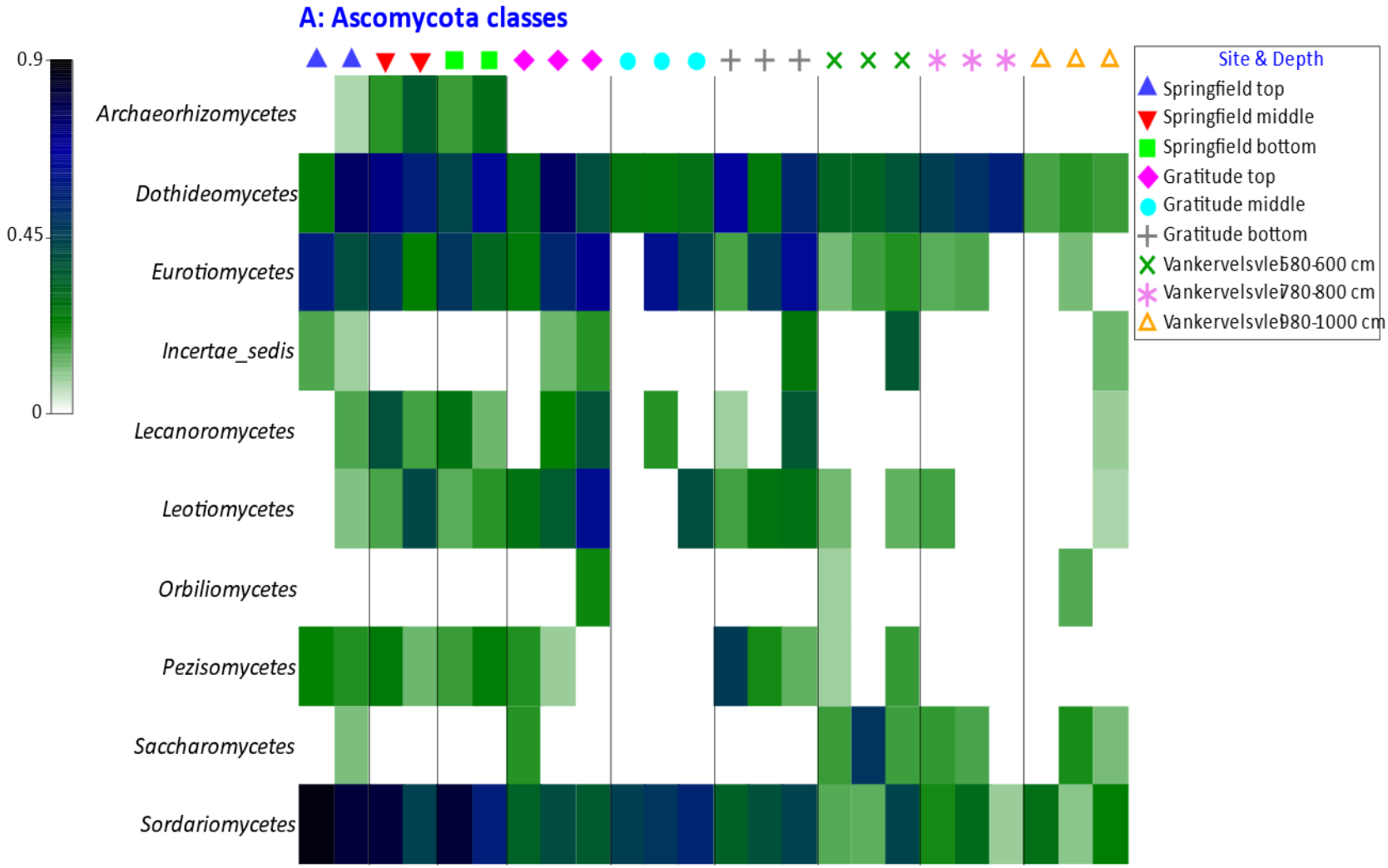


Figure 5.9: Shade plots of square root transformed relative abundance of fungal data of the class *Ascomycota* detected in the samples analysed in this study

Table 5.6: Relative abundance percentage of *Ascomycota* classes detected in the samples analysed in this study

Site & Depth	Site	*Archaeorhizomycetes*	*Dothideomycetes*	*Eurotiomycetes*	*Incertae_sedis*	*Lecanoromycetes*	*Orbiliomycetes*	*Pezizomycetes*	*Saccharomycetes*	*Sordariomycetes*	*Agaricomycetes*	*Agaricostilbomycetes*	*Exobasidiomycetes*	*Incertae_sedis*	*Microbotryomycetes*	*Tremellomycetes*	*Ustilaginomycetes*	*Chytridiomycetes*	*Glomeromycetes*	*Incertae*
SF Top	1A	0.00	0.31	10.69	0.06	0.00	0.00	0.23	0.00	58.65	0.37	0.00	0.00	0.00	0.00	0.23	0.34	0.00	0.00	0.00
SF Middle	1B	0.12	25.81	5.05	0.00	0.06	0.00	0.36	0.00	43.38	1.38	0.00	0.00	0.00	0.06	0.30	0.00	0.00	0.12	0.00
SF Bottom	1C	0.09	3.44	5.48	0.00	0.04	0.00	0.09	0.00	45.21	4.70	0.00	0.00	0.00	0.04	0.30	0.04	0.00	0.00	0.00
SF Top	2A	0.00	33.23	2.48	0.00	0.01	0.00	0.14	0.02	44.43	0.29	0.02	2.39	0.00	0.04	0.27	1.50	0.00	0.14	0.01
SF Middle	2B	1.57	9.83	0.26	0.00	3.18	0.00	0.03	0.00	3.87	0.03	0.00	0.00	0.05	0.00	0.26	0.03	0.00	0.08	0.00
SF Bottom	2C	0.69	16.66	0.86	0.00	0.12	0.00	0.29	0.00	10.33	2.21	0.00	0.00	0.00	0.00	0.25	0.02	0.00	0.00	0.00
GK Top	G1	0.00	0.56	0.38	0.00	0.54	0.00	0.14	0.13	1.14	16.24	0.00	0.00	0.00	0.14	0.00	0.00	0.56	0.01	0.00
GK Middle	G2	0.00	0.46	0.00	0.00	0.00	0.00	0.00	0.00	4.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.13	0.00	0.00
GK Bottom	G3	0.00	18.66	0.07	0.00	0.07	0.00	4.60	0.00	1.18	0.01	0.00	0.00	0.00	0.21	0.00	0.00	0.17	0.00	0.00
GK Top	G4	0.00	33.33	8.33	0.02	1.55	0.00	0.01	0.00	2.63	0.52	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.90
GK Middle	G5	0.00	0.40	14.44	0.00	0.00	0.00	0.00	0.00	5.48	0.40	0.00	0.00	0.00	0.13	0.13	0.00	0.13	0.00	0.00
GK Bottom	G6	0.00	0.41	4.50	0.00	0.47	0.00	0.18	0.00	2.25	13.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GK Top	G7	0.00	2.37	23.45	0.13	15.40	0.20	0.00	0.00	1.59	0.18	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00
GK Middle	G8	0.00	0.63	3.75	0.00	2.50	0.00	0.00	0.00	8.75	5.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GK Bottom	G9	0.00	8.47	16.22	0.43	0.55	0.00	0.03	0.00	3.74	0.13	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.01
580 cm – 600 cm	V1	0.00	1.01	0.02	0.00	0.02	0.00	0.00	0.09	0.05	0.26	0.00	0.00	0.00	29.22	20.67	4.16	0.00	0.01	0.00
780 cm – 800 cm	V2	0.00	4.25	0.04	0.00	0.07	0.00	0.00	0.11	0.17	0.09	0.00	0.39	0.00	26.62	45.16	0.00	0.00	0.02	0.00
980 cm – 1000 cm	V3	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.62	7.70	0.00	0.00	0.00	19.12	31.43	0.00	0.00	0.00	0.00
580 cm – 600 cm	V5	0.00	1.09	0.08	0.00	0.00	0.00	0.00	5.47	0.04	0.41	0.00	0.00	0.00	17.02	10.86	0.00	0.00	0.00	0.00
780 cm – 800 cm	V6	0.00	6.20	0.06	0.00	0.00	0.00	0.00	0.06	0.71	0.03	0.00	0.00	0.03	68.06	5.64	0.00	0.00	0.00	0.00
980 cm – 1000 cm	V7	0.00	0.13	0.02	0.00	0.00	0.05	0.00	0.16	0.01	0.03	0.00	0.00	0.01	67.15	22.41	0.00	0.00	0.00	0.00
580 cm – 600 cm	V9	0.00	2.01	0.14	1.68	0.04	0.00	0.10	0.08	3.55	7.70	0.00	0.00	0.01	3.10	9.18	0.01	0.00	0.00	0.00
780 cm – 800 cm	V10	0.00	10.04	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.08	21.79	17.00	0.00	0.00	0.00	0.00
980 cm – 1000 cm	V11	0.00	0.09	0.00	0.03	0.00	0.00	0.00	0.02	0.27	0.70	0.02	0.00	0.00	25.66	63.08	4.52	0.00	0.00	0.00

SF- Springfield Farm **GD – Goukou River** **V – Vankervelsvlei**

Archaeorhizomycetes, found in samples from Springfield Farm, are widespread worldwide and dominate in many soil habitats. The class contains filamentous species that form associations with plant roots and may play an important role in terrestrial ecosystems (Pinto-Figueroa et al., 2019). Previous research has shown that this class of fungi can dominate in peatlands that contain shrubs (Rosling et al., 2013:333-349; Wang et al., 2012:743-744). This could explain the absence of *Archaeorhizomycetes* in samples from Vankervelsvlei which was dominated by sphagnum moss. According to the results obtained by Pinto-Figueroa et al. (2019), there are various factors that affect the ecological distribution of *Archaeorhizomycetes*, including growth minimum temperature, and precipitation. Other researchers have also found that low pH, high P levels, aluminium oxide (Al_2O_3), H and phyllosilicates influence their presence (Carrino-Kyker et al., 2016; Pinto-Figueroa et al., 2019). Unlike the other two sampling sites, the Springfield Farm sampling sites are not acidic (Figure 4.1) and high concentrations of Al and Si (Figure 5.10) were observed. This could explain why it was only found in Springfield Farm samples, and not in Goukou River and Vankervelsvlei.

Yeasts are fungi with a predominantly unicellular thallus that reproduces by budding, fission, or both. Usually, ascospores are formed from a single somatic cell or from a zygote in a naked ascus. Acrotelm depth and peatland type influence yeast species richness and composition. These fungi are located in the acrotelm as most of them are obligate aerobes (Thormann and Rice, 2007:241-242). This corresponds with the results of this study, as *Saccharomycetes* were detected in the top layer of all the sampling sites. Thormann & Rice (2007:241-242) speculated that yeasts play a vital role during first 50 days of decomposition and are therefore typically located close to the surface. However, *Saccharomycetes* were also detected in Vankervelsvlei at depths close to 1 m. It was not clear whether *Saccharomycetes* DNA was from viable organisms or DNA that was preserved at those depths. *Orbiliomycetes* were detected in Goukou River and Vankervelsvlei, however, to date their role in peatlands have not been studied or recorded.

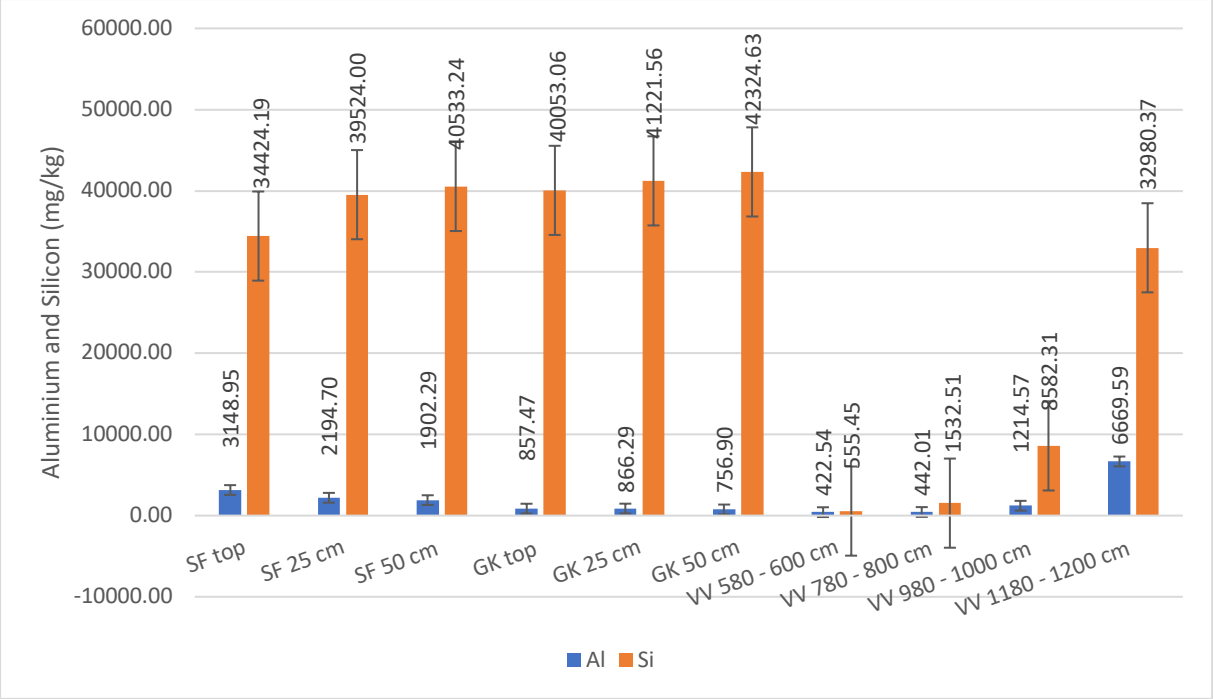


Figure 5.10: Aluminium and Silicon concentrations in samples taken from the study sites. SF = Springfield; GK = Goukou River; VV = Vankervelsvlei. Error bars indicate the standard deviation of analysis, *n*=3

Basidiomycota are fungi that consist of hyphae divided by cellular cross-walls called septa. *Basidiomycota* spores normally have external basidiospores when they reproduce sexually. A few *Basidiomycota* can also reproduce asexually (McConnaughey, 2014). In peatlands, *Basidiomycota* are the second largest fungal taxa present with *Galerina paludosa*, *Phaeogalera stagnina G. sphagnorum*, *Tephrocybe palustris*, and *Cryptococcus albidus* being the most reported. In contrast to the ascomycetes, which largely consist of anamorphic taxa, basidiomycetes are mostly teleomorphic (Thormann and Rice, 2007:241-242). The data from this study supports previous research as *Basidiomycota* were the second largest fungal taxa present in our samples. In contrast to the *Ascomycota*, there was an increase in *Basidiomycota* with depth (Table 5.7). The classes *Agaricomycetes*, *Microbotryomycetes*, and *Trellomycetes* were detected in samples from all three sites (Figure 5.11), while *Agricostilbomycetes* and *Ustilaginomycetes* were detected in samples from Springfield Farm and Vankervelsvlei, and *Exobasidiomycetes* was only detected in samples from Goukou River.

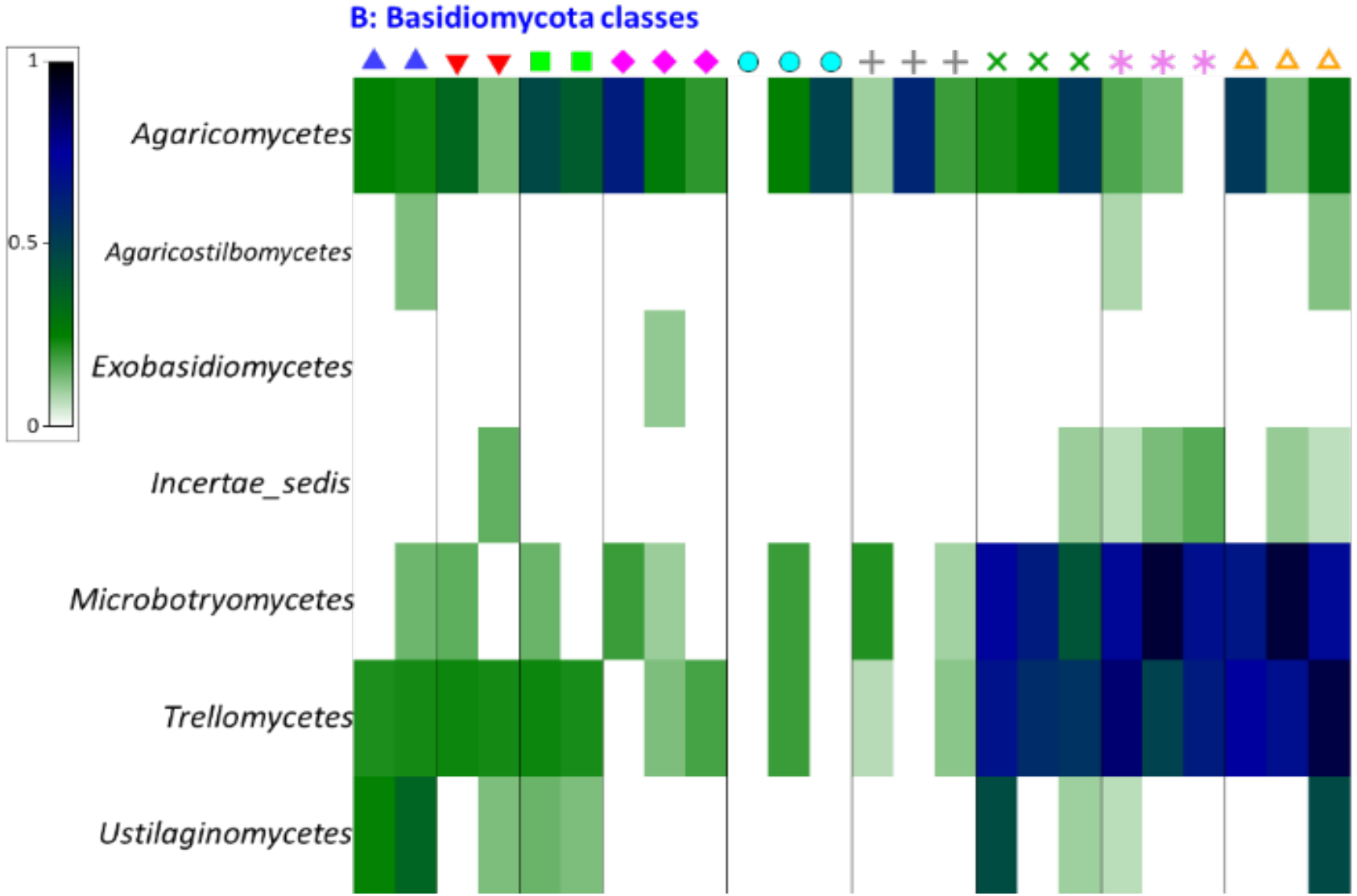


Figure 5.11: Shade plots of square root transformed relative abundance of fungal data of the class *Basidiomycota* detected in the samples analysed in this study

Table 5.7: Relative abundance percentage of *Basidiomycota* classes detected in the samples analysed in this study

Site & Depth	Site	*Agaricomycetes*	*Agaricostilbomycetes*	*Exobasidiomycetes*	*Incertae_sedis*	*Microbotryomycetes*	*Trellomycetes*	*Ustilaginomycetes*
SF Top	1A	0.37	0.00	0.00	0.00	0.00	0.23	0.34
SF Middle	1B	1.38	0.00	0.00	0.00	0.06	0.30	0.00
SF Bottom	1C	4.70	0.00	0.00	0.00	0.04	0.30	0.04
SF Top	2A	0.29	0.02	0.00	0.00	0.04	0.27	1.50
SF Middle	2B	0.03	0.00	0.00	0.05	0.00	0.26	0.03
SF Bottom	2C	2.21	0.00	0.00	0.00	0.00	0.25	0.02
GK Top	G1	16.24	0.00	0.00	0.00	0.14	0.00	0.00
GK Middle	G2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GK Bottom	G3	0.01	0.00	0.00	0.00	0.21	0.00	0.00
GK Top	G4	0.52	0.00	0.01	0.00	0.01	0.02	0.00
GK Middle	G5	0.40	0.00	0.00	0.00	0.13	0.13	0.00
GK Bottom	G6	13.14	0.00	0.00	0.00	0.00	0.00	0.00
GK Top	G7	0.18	0.00	0.00	0.00	0.00	0.11	0.00
GK Middle	G8	5.63	0.00	0.00	0.00	0.00	0.00	0.00
GK Bottom	G9	0.13	0.00	0.00	0.00	0.01	0.02	0.00
580 cm – 600 cm	V1	0.26	0.00	0.00	0.00	29.22	20.67	4.16
780 cm – 800 cm	V2	0.09	0.00	0.00	0.00	26.62	45.16	0.00
980 cm – 1000 cm	V3	7.70	0.00	0.00	0.00	19.12	31.43	0.00
580 cm – 600 cm	V5	0.41	0.00	0.00	0.00	17.02	10.86	0.00
780 cm – 800 cm	V6	0.03	0.00	0.00	0.03	68.06	5.64	0.00
980 cm – 1000 cm	V7	0.03	0.00	0.00	0.01	67.15	22.41	0.00
580 cm – 600 cm	V9	7.70	0.00	0.00	0.01	3.10	9.18	0.01
780 cm – 800 cm	V10	0.00	0.00	0.00	0.08	21.79	17.00	0.00
980 cm – 1000 cm	V11	0.70	0.02	0.00	0.00	25.66	63.08	4.52

SF- Springfield Farm GD – Goukou River V – Vankervelsvlei

Agaricomycetes, Microbotryomycetes, and *Trellomycetes* were detected in samples from all the sites. These fungal classes are saprotrophic and are involved in the breakdown of plant matter. As fungi are obligate aerobes, the increase in their abundance in this study was unexpected. However, Guan et al. (2021:145-160) also reported an increase in the RA of *Basidiomycota* with depth. They reported that an increase in pH (with an increase in depth), a decrease in C and N and the season played a large role in this phenomenon. In this study, pH, C and N were measured, but only at one time point, and this could have affected our results and is possibly one of the reasons why we observed an increase in *Basidiomycota* RA. However, Buscardo et al. (2021:55) reported a decrease in C and N occurred with an increase of *Basidiomycota* RA. They speculated that *Basidiomycota* utilized C and N and that's why there is a decrease. In this study we observed a similar trend and could also be why there is an increase in *Basidiomycota* RA.

Ustilaginomycetes, also known as 'smut' fungus, is a spore-forming plant pathogen that infects the stems and/or flowers and/or leaves and/or seeds of grasses and herbaceous plants (Webster and Weber, 2007). The presence of this fungus can therefore be used to provide an indication of the original wetland vegetation from which the peat was formed. In this study, a decrease in abundance was observed in samples from Springfield Farm (1.50-0.02%) and Vankervelsvlei (4.52-0.01%). The class *Agaricostilbomycetes,* was detected in the top layer of samples from Springfield Farm (0.024%) and in Vankervelsvlei at a depth of 780-800 cm (0.0039%) and at a depth of 980-1000 cm (0.0207%).

Concentrations of S, Al, Ca, K, P, Si, Mg, Fe, humification and pH were important drivers for structuring fungal distribution (LINKTREE, Figure 5.15), an aspect discussed more fully in Section 5.2.2.2. In samples from Vankervelsvlei, humification values were very high as this peatland was undisturbed (when compared to Goukou River and Springfield Farm). The fungal community composition in Springfield Farm and Goukou River appeared to be driven by Fe and S (Section 5.2.2). In microbial metabolism, Fe plays a vital role. Many enzymes require this metal as a cofactor (Howard, 1999:394-395) and it is essential during DNA synthesis (ribonucleotide synthetase) and cleavage (endonuclease III) (Matzanke, 1994:179-200). Furthermore, some wood decaying fungi need this metal to degrade lignocellulosic polysaccharides as well as lignin non-enzymatically by initiating the Fenton base reactions that generate oxygen-free radicals that cause wood degradation (Arantes et al., 2011:541-550). In many environments Fe is not readily bioavailable, but many fungi produce siderophores that can chelate Fe (Neilands, 1995:26723-26726; Philpott, 2006:636-640), while others over-excrete organic acids (Fomina et al., 2005:371-

380; Gadd, 2007:3-49) that assist in metal-chelating properties. As well as making various cellular metabolites and carrying out multiple metabolic processes, fungi use sulfur (S) to make proteogenic amino acids cysteine (Cys) and methionine (Met), iron metabolism (Fe–S clusters), ergothioneine (EGT)], oxidative stress defence [glutathione (GSH) methylation [S-adenosylmethionine (SAM)], and epipolythiodioxopiperazine (ETP) toxin biosynthesis (e.g., gliotoxin) (Traynor et al., 2019).

5.2 Culture-based analysis of actinobacterial communities

5.2.1 Identification of actinobacterial isolates

Typically, culture-based methods are used to detect and identify microorganisms that can be cultivated in the laboratory, whereas molecular approaches are used for non-cultivable microorganisms. There are a variety of molecular tools that are inexpensive, relatively rapid, practical, and appear to accurately represent microbial loads in nearly any environment. Using these two approaches together helps eliminate and make up their limitations. Where NGS shows all the microorganisms (viable or non-viable) present in the sites, culture-based methods are able to assess the culturable living microorganisms (recognizing the viable cells) present. It is also a simple way to quantitate cells and gives high sensitivity when specific (appropriate) media is used for the isolation process (Figdor and Gulabivala, 2008:62-70).

Using morphological and cultural characteristics, the actinobacterial isolates obtained in this study were identified using the methods described in the *International Streptomyces Project* (ISP; Shirling and Gottlieb, 1966:313-320). Using a slide culture technique described by Williams et al. (1989:2452-2492), we characterized spore-bearing hyphae, the structure and arrangement of the spore-chains with the substrate, and the aerial mycelia of the actinobacteria. A relevant colour chart was used to visually estimate the colour of the spore mass after growth in the culture (Pridham, 1965:43-61).

In this study, three different isolation methods were employed (Section 3.4) and many different isolates were obtained. Thirty-two actinobacterial isolates from the Goukou River and Vankervelsvlei samples were selected for identification through 16S rRNA gene sequencing. After receiving the sequences, they were submitted to NCBI BLAST as well as the EzBioCloud database for identification (Table 5.8). Nine different isolates were obtained by the direct isolation method and eight isolates were obtained using the heat pre-treatment method. Ten isolates (all from Vankervelsvlei samples) were obtained using the $CaCO_3$ pre-treatment method, which may be related to the fact that the Vankervelsvlei samples had the highest Ca levels (Figure 4.3).

Table 5.8: Actinobacterial isolates obtained from culture-based studies, with a focus on isolates obtained from Goukou River and Vankervelsvlei

Depth	Name	Isolation method	Closest relative	Strain	Accession	Similarity %	Diff/Total	Completeness
TL – 25 cm	GD9	Direct	*Streptomyces chattanoogensis*	NRRL ISP-5002	LGKG01000206	100	0/1102	100
TL – 25 cm	GD50	Direct	*Microbacterium oxydans*	DSM 20578	Y17227	99.85	2/1375	99.38
TL	GD53	Direct	*Streptomyces xanthocidicus*	NBRC 13469	AB184427	99.91	1/1088	99.24
TL – 25 cm	GD61	Direct	*Streptomyces luridiscabiei*	NRRL B-24455	LIQV01000394	100	0/1055	100
TL	GD64	Direct	*Streptomyces luridiscabiei*	NRRL B-24455	LIQV01000394	100	0/1100	100
25 cm	GD65	Direct	*Streptomyces pratensis*	ch24	JQ806215	95.36	57/1228	94.61
25-50 cm	GD77	Direct	*Nocardia africana*	NBRC 100379	BDAV01000043	97.90	28/1335	100
25-50 cm	GD84	Direct	*Streptomyces alfalfae*	XY25	KR080524	100	0/1083	100
50 cm	GD91	Direct	*Streptomyces albolongus*	NBRC 13465	AB184425	99.55	6/1326	99.93
TL	GH70	Heat	*Streptomyces avidinii*	NBRC 13429	AB184395	100	0/1099	99.93
8 m	V2	$CaCO_3$	*Streptomyces bacillaris*	NBRC 13487	AB184439	100	0/1093	99.93
12 m	V4	$CaCO_3$	*Streptomyces cavourensis*	NBRC 13026	AB184264	100	0/599	99.93
12 m	V5	$CaCO_3$	*Streptomyces luridiscabiei*	NRRL B-24455	LIQV01000394	100	0/1062	100
12 m	V7	Heat	*Streptomyces bacillaris*	NBRC 13487	AB184439	99.91	1/1096	99.93
8 m	V8	Heat	*Streptomyces bacillaris*	NBRC 13487	AB184439	99.91	1/1100	99.93

Depth	Name	Isolation method	Closest relative	Strain	Accession	Similarity %	Diff/Total	Completeness
8 m	V9	$CaCO_3$	*Streptomyces bacillaris*	NBRC 13487	AB184439	99.91	1/1100	99.93
8 m	V11	$CaCO_3$	*Streptomyces pratensis*	ch24	JQ806215	100	0/1245	94.61
6 m	V12	$CaCO_3$	*Streptomyces pratensis*	ch24	JQ806215	99.93	1/1363	94.61
4 m	V13	Heat	*Streptomyces pratensis*	ch24	JQ806215	99.64	4/1100	94.61
8 m	V14	Heat	*Streptomyces pratensis*	ch24	JQ806215	100	0/1319	94.61
4 m	V15	Heat	*Streptomyces bacillaris*	NBRC 13487	AB184439	99.91	1/1116	99.93
8 m	V17	$CaCO_3$	*Kitasatospora albolonga*	NBRC 13465	AB184425	100	0/1211	99.93
8 m	V18	$CaCO_3$	*Streptomyces bacillaris*	NBRC 13487	AB184439	100	0/1054	99.93
4 m	V20	$CaCO_3$	*Streptomyces albolongus*	NBRC 13465	AB184425	98.61	19/1370	99.93
8 m	V21	Heat	*Streptomyces bacillaris*	NBRC 13487	AB184439	99.91	1/1085	99.93
8 m	V24	Heat	*Streptomyces bacillaris*	NBRC 13487	AB184439	99.91	1/1111	99.93

GD – Goukou River **V – Vankervelsvlei** **TL – top layer**

The majority of the isolated actinobacteria belonged to the genus *Streptomyces*. However, looking at the community-based analysis results, *Streptomyces* was not as abundant as some of the other actinobacterial classes such as *Acidiomicrobiales* or *Gaiellales*. The isolated actinobacterial (and non-actinobacterial) strains gives us an indication of which microorganisms are viable while analysing the soil DNA indicates everything present in the sample; living and non-living. That could be why there is such a wide variety of actinobacterial results in the NGS data or it could be because these isolates could actually be cultured in the laboratory (media selected), while others could not. Another reason could be because it is difficult to extract DNA from spores using the PowerSoil® DNA extraction kit. Similar results have been observed in previous studies, in which various strains of streptomycetes were isolated from either peat samples or soil from the surrounding areas. However, in those studies they did not use NGS to look at the community members present in the sampling sites (Tahvonen, 1982:357-360; Pankratov and Dedysh,

2009:227-230; Tanasupawat et al., 2016a:290-295, 2016b:1950-1955; Lipun et al., 2020:435-440). We also isolated *Microbacterium* and *Nocardia* from Goukou River and *Kitasatospora* from Vankervelsvlei. In previous studies, *Microbacterium* have been isolated in soils that have been contaminated with heavy metals, and even groundwater from a radioactive waste deposit (Nedelkova et al., 2007:694-700; Heidari and Sanaeizada, 2020:901-910). Like many other Actinobacteria, *Microbacterium* members possess the ability to breakdown hydrocarbons and very complex polysaccharides. In soil they promote plant growth, via modulation of sulphur and nitrogen metabolism (Cordovez et al., 2018). *Nocardia* members are often found in organic material (decaying plant matter), water, plants, and soil, these organisms are saprophytes, ubiquitous in nature. Members of this species are known to cause Nocardiosis in immunocompromised people (Kachuei et al., 2012:474-478; de Farias et al., 2012:239). *Kitasatospora* was isolated from a sample that was 8 meters deep from Vankervelsvlei. However, the role of the members of this group in soil or in peatlands has not been researched or recorded before.

5.2.2 The correlation co-efficient analysis of the physicochemical parameters associated with the differences in peat microbial profiles

5.2.2.1 The correlation between physicochemical parameters of peat and actinobacterial populations

Bacteria from the phylum Actinobacteria are very diverse in their morphological and physiological characteristics, and their DNA contains a high percentage of Guanine and Cytosine, making them one of the major groups of bacteria within the domain Bacteria. Actinobacteria are composed of six orders: *Acidimicrobiales*, *Rubrobacterales*, *Coriobacterales*, *Bifidobacteriales*, *Actinomycetales*, and *Nitriliruptorales*. Among soil microorganisms, actinobacteria are dominant. Extracellular enzymes are produced by many species for degradation of macromolecules including lignin, cellulose, chitin, and mostly starch. Actinobacteria, therefore, tend to be found in materials where organic materials are being degraded (Schäfer et al., 2010:103-110). In particular, studies conducted in the indoor environment showed that they were present along with fungi in water-damaged materials and soils (Schäfer et al., 2010:103-110). Actinobacteria play a significant role in the breakdown of organic compounds in nature, and has been associated with soil organic matter production, as their black pigments, called melanins, are related to soil humic acids in some way (Coelho and Drozdowicz, 1978:459-470; Schäfer et al., 2010:103-110).

For the comparison between the soil physicochemistry of the sampling sites, a metric multidimensional scaling (MDS) method was applied using both Euclidian matrices and PCA to create similarity matrices. The Euclidean distance similarity displays how well the different sites cluster together (Figure 5.12), displaying high similarity between the physicochemical properties of the sites. While the PC analysis (Figure 5.13) visually displays a more detailed version of the sites and how they cluster together.

BEST analysis showed that N, Al, Ca, P, and Ti were the most significant physicochemical drivers of actinobacterial community structure (Table 5.9). The LINKTREE analysis, a binary divisive clustering plot (Figure 5.14) first separated the actinobacterial community into two groups dividing the actinobacterial communities into those that thrive at Al concentrations of <4690 mg/L or >6090 mg/L and Ti concentrations <of 335 mg/L or >448 mg/L (with an R value = 0.78). The high levels of Ti and Al drives the actinobacterial community composition in the deep samples from Vankervelsvlei (1180-1200 cm). Identification of the OTUs showed that these samples contained high RA of the orders *Actinomycetales* (15.54%), and low RA values of *Acidimicrobiales* (0.012%) in comparison to maximum RA ranges of the *Actinomycetales* (0.24-3.83% and 1.24-9.08%) and *Acidimicrobiales* (0.84-4.04% and 2.61-29.05%) in the other samples. *Bifidobacteriales* (0.33%) is the only actinobacterial group observed in the bottom layers of Vankervelsvlei. No other similar findings of this sort have been reported, which makes this phenomenon (high levels of Ti and Al increases the RA of selected actinobacterial taxa) a novel finding. On the next branch, N concentrations >943 mg/L are selective for the rest of the Vankervelsvlei samples (580-1000 cm) in comparison to samples from the other sites (N concentrations <250 mg/L). This split clearly separates the Vankervelsvlei actinobacterial communities from the Goukou River and Springfield Farm actinobacterial communities based on their N concentration. The RA of the OTUs showed that N concentration >943 mg/L were selective for *Acidimicrobiales* (0.005-0.11%), *Actinobacteriales* (0.01-0.20%), *Gaiellales* (0.008-0.03%), 0319-7l14 (0.01-0.22%), and *Solirubrobacterales* (0.005-0.04%) in comparison to maximum RA ranges of *Acidimicrobiales* (0.85-4.04% and 2.61-29.05%), *Actinobacteriales* (0.25-3.82% and 1.24-9.08%), *Gaiellales* (0.14-1.01% and 0.01-0.75%), 0319-7l14 (0.05-1.97% and 0.02-0.26%), and *Solirubrobacterales* (0.70-2.16% and 0.94-9.76%) respectively in Springfield Farm and Goukou River.

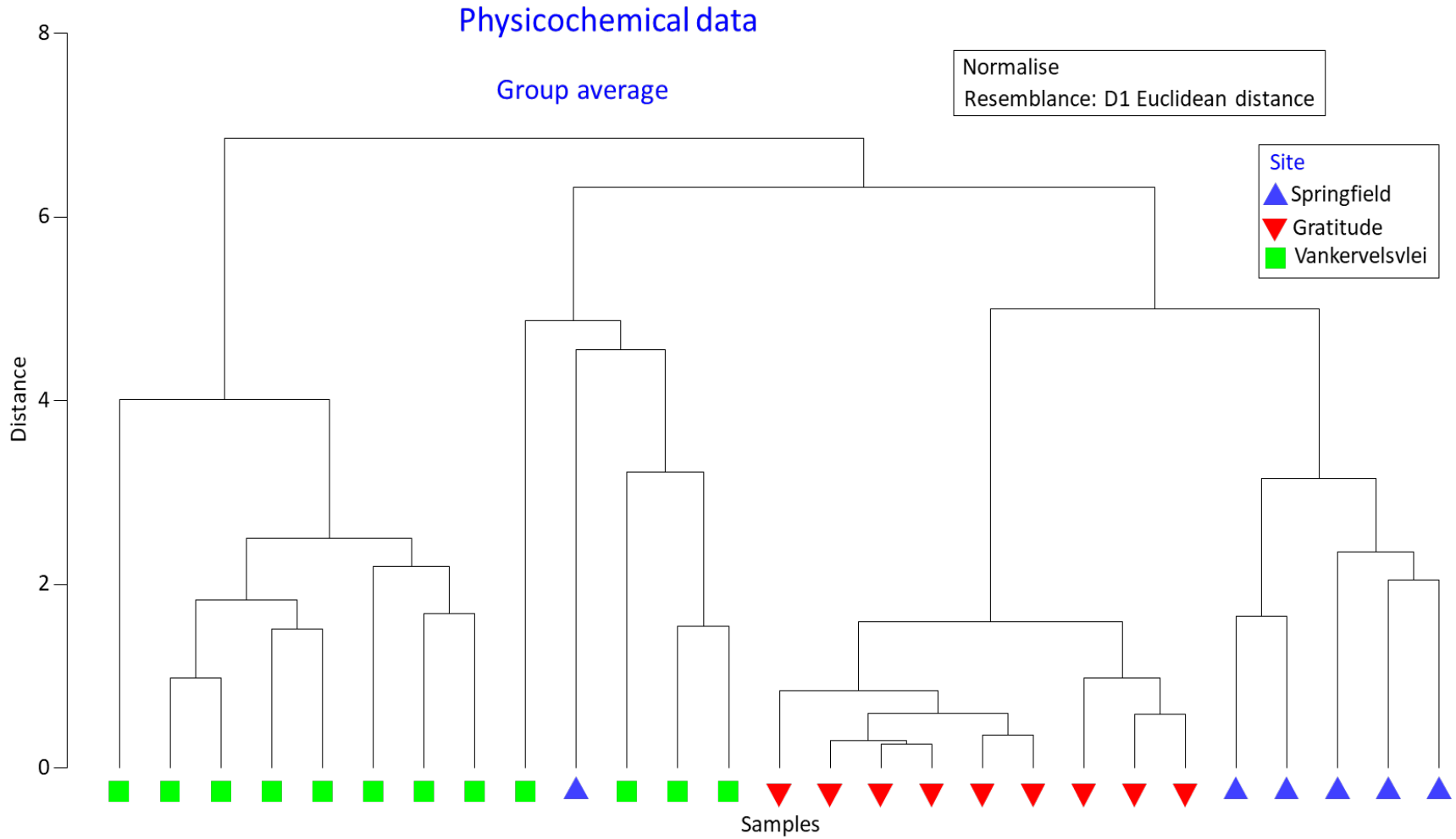


Figure 5.12: Cluster analysis (group average linkage) of normalised Euclidean distance similarity of the physicochemical data from the samples collected in this study

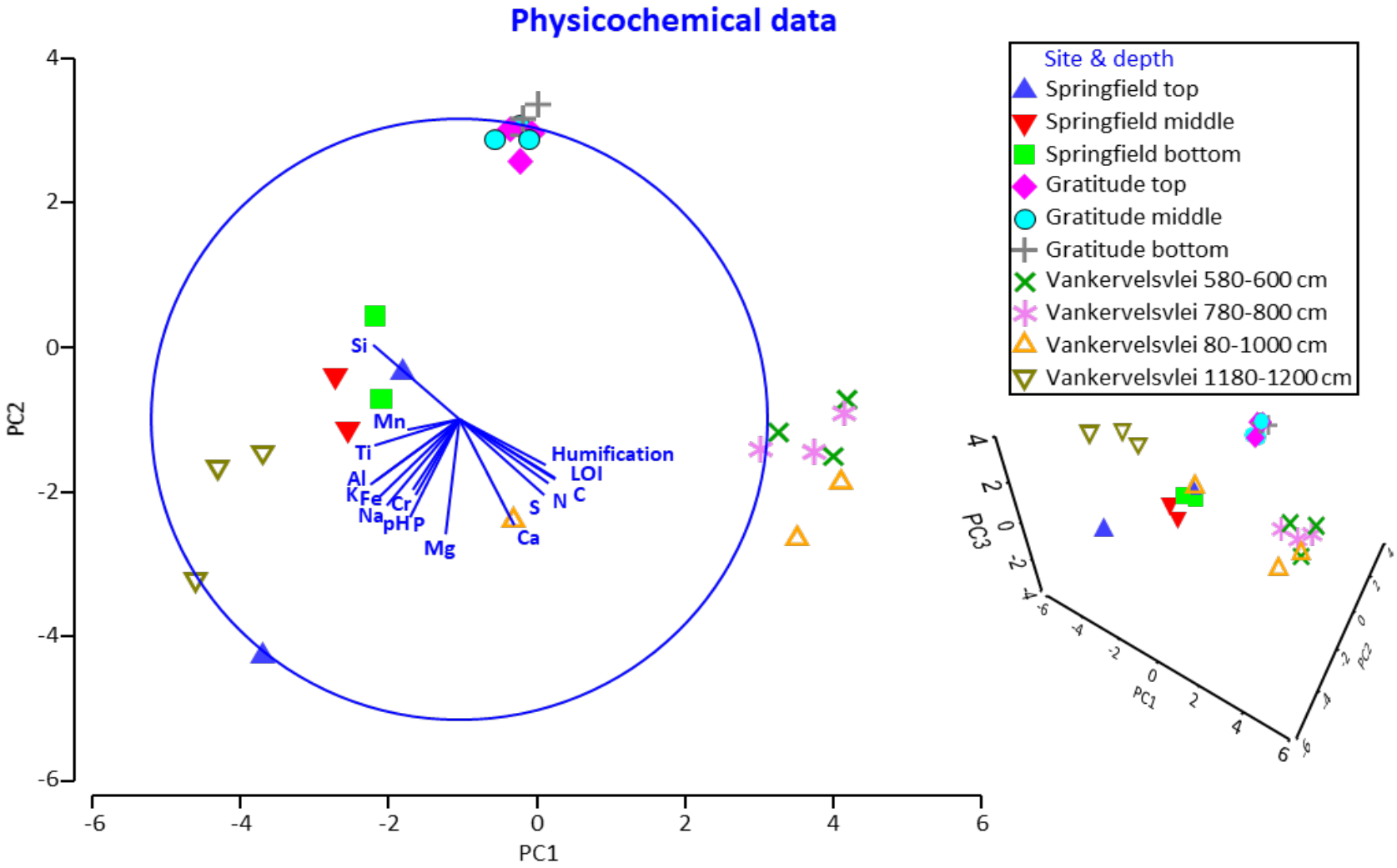
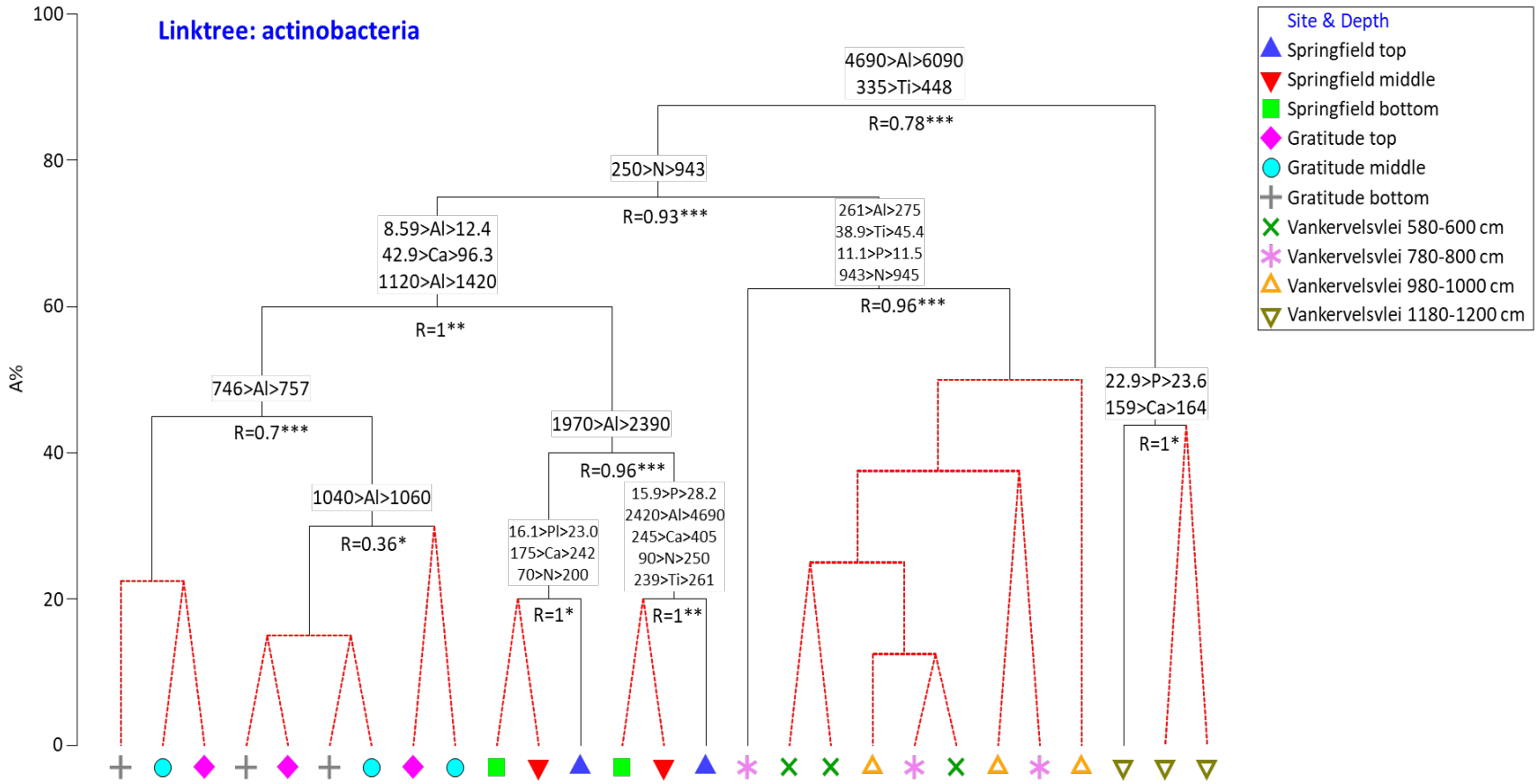


Figure 5.13: Two-dimensional and three-dimensional (insert bottom right) principal component analysis plots of normalised physicochemical data from the peat samples. 47.1%, 32.0% and 9.3 % (cumulative 88.4%) of the variations are explained by PC1, PC2 and PC3, respectively

As mentioned in Section 5.2.2.1, there are many actinobacterial members such as *Microbacterium*, *Corynebacterium*, and *Micromonospora* that possess the *nifH*-gene which causes them to convert atmospheric N into NH_4 and store it in the soil for plant utilization (Sellstedt and Richau, 2013:179-186). The samples from Springfield Farm and Goukou River are divided into two distinct clusters on the LINKTREE plot. The actinobacterial community composition in samples from the two sites are influenced by differences in P concentration (<8.59 mg/L or >12.4 mg/L), Ca concentration (<42.9 mg/L or >96.3 mg/L), and Al concentration (<1120 mg/L or >1420 mg/L), with the higher concentrations of these elements being selective for Springfield Farm actinobacterial communities. A larger variety of actinobacterial orders were observed in samples from Springfield Farm, including *Acidimicrobiales*, *Actinomycetales*, 0319-7L14, *Euzebyales* (0.02-0.06%), *Nitriliruptorales* (0.01-0.03%), *Rubrobacterales* (0.05-0.13%), *Micrococcales* (0.03-0.06%) *Gaiellales* and *Solirubrobacterales*. The orders of *Euzebyales*, *Nitriliruptorales*, *Rubrobacterales*, and *Micrococcales* were observed only in Springfield Farm.

Table 5.9: BEST analysis of actinobacterial diversity vs physicochemical parameters

Number of Variables	Correlation	Physicochemical selections
5	**0.702**	**C, Al, Ca, P, Ti**
4	**0.689**	**C, Al, P, Ti**
4	**0.688**	**C, Al, Ca, P**
3	**0.684**	**C, P, Ti**
4	**0.680**	**C, Ca, P, Ti**
4	**0.676**	**Al, Ca, P, Ti**
3	**0.674**	**Al, Ca, Ti**
4	**0.673**	**C, Al, Ca, Ti**
5	**0.672**	**Al, Ca, P, Si, Ti**
5	**0.672**	**C, Al, Mg, P, Ti**



Levels of significance: *0.05>p≥0.01 **0.01>p≥0.005 ***0.005<p

Figure 5.14: Linktree of showing concentrations of the most important environmental parameters identified using BEST analysis selecting the actinobacterial community structure in the samples. All concentrations are in mg/L. N is a proxy for C, S and LOI (loss on ignition)

5.2.2.2 The correlation co-efficient analysis between physicochemical parameters of soils and fungal populations

The BEST analysis and corresponding LINKTREE (Figure 5.15) display the environmental parameters that had a significant effect on the fungal communities in samples from the three sampling sites. For the fungal communities, BEST confirmed that S, Al, Ca, K, P, Ti, Si, Mg, Fe, humification, and pH were the environmental parameters that affected the fungal communities the most (Table 5.10). The first branch of LINKTREE analysis separates the communities of Vankervelsvlei with Goukou River and Springfield Farm on the basis of humification with a concentration <0.174 mg/L (Goukou River and Springfield Farm) to >0.31 mg/L (Vankervelsvlei). Humification plays a big role in Vankervelsvlei and this could be as a result of the age of Vankervelsvlei (Section 3.2.1.1) compared to Goukou River (Section 3.2.1.2) and Springfield Farm (Section 3.2.1.3). The fungal orders detected in Vankervelsvlei were *Ascomycota* (0.38-12.04%), *Basidiomycota* (20.07-74.89%), and *Glomeromycota* (0.01-0.02%) in comparison to the maximum RA ranges of *Ascomycota* (33.44-84.26% and 3.25-57.73%), Basidiomycota (0.44-5.09% and 0.16-16.41%), and *Glomeromycota* (0.08-0.22% and 0.00-0.01%) respectively to Springfield Farm and Goukou River.

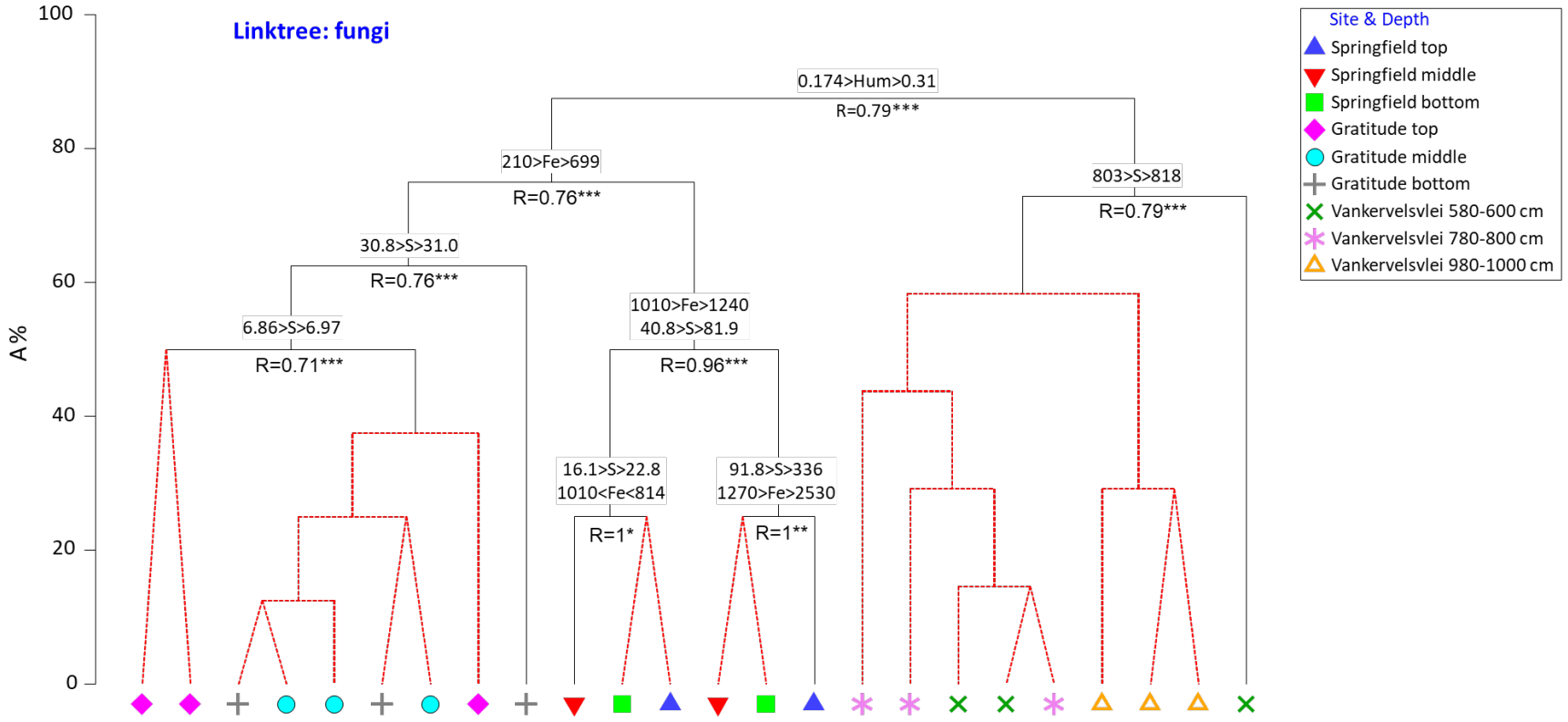
Fungi are one of the main groups of microorganisms involved in the degradation process of decaying plant material and many fungi from the *Ascomycota* and *Basidiomycota* phyla have the ability to incorporate S into various cellular metabolites and use S for multiple metabolic processes. The second split is observed between the fungal communities for Goukou River and Springfield Farm, which is caused by Fe concentrations <210 mg/L to Fe concentrations >699 mg/L. The Springfield Farm fungal communities are selected by Fe concentrations >699 mg/L, while the fungal communities in Goukou River are selected by Fe concentrations <210 mg/L. The orders *Ascomycota*, *Basidiomycota*, *Chytridiomycota* (0.02-0.06%), *Glomeromycota*, and *Zygomycota* (0.01%) were detected in Springfield Farm. The final split in the LINKTREE shows that the fungal communities of Goukou River are affected by S concentrations <30.8 mg/L or >31 mg/L. The fungal community detected in Goukou River are *Ascomycota*, *Basidiomycota*, *Chytridiomycota* (0.13-4.13%), and *Zygomycota* (0.01-0.90%). Looking at the RA (Figure 5.7) we observed that *Basidiomycota* and *Glomeromycota* are dominant in Springfield Farm samples, while only *Ascomycota* and *Basidiomycota* are dominant in Goukou River because of these parameters.

5.3 Conclusion

The T-RFLP data showed that the microbial communities present in the three sites were closely related, however different communities were found at different depths. A variety of actinobacterial and fungal orders were detected in the different sites using NGS. Actinobacteria such as the members of *Acidimicrobiales*, *Actinomycetales* and *Solirubrobacterales* were abundant in Goukou River and Springfield Farm. *Bifidobacteriales* were abundant in Vankervelsvlei, whereas *Micrococalles*, *Euzebyales*, *Nitriliruptorales*, and *Rubrobacterales* were only detected in Springfield Farm. BEST analysis (linking microbial diversity patterns to environmental variables) showed that N, aluminium (Al), calcium (Ca), phosphorus (P), and titanium (Ti) were the most significant physicochemical drivers of actinobacterial community structure. Twenty-six actinobacterial strains were isolated and identified from Goukou River and Vankervelsvlei, which included *Nocardia africana*, *Kitasatospora albolonga*, and a variety of strains from the genus of *Streptomyces*. Fungi such as *Ascomycota* and *Basidiomycota* are abundant in all three sites. However, *Ascomycota* are abundant in Goukou River, *Basidiomycota* are abundant in Vankervelsvlei, while *Glomeromycota* are abundant in Springfield Farm. BEST analysis confirmed that sulphur (S), aluminium (Al), calcium (Ca), potassium (K), phosphor (P), titanium (Ti), silicon (Si), magnesium (Mg), iron (Fe), humification, and pH were the environmental parameters that affected the fungal communities the most.

Table 5.10: BEST analysis of Fungal diversity vs physicochemical parameters

Number of variables	Correlation	Physicochemical Selections
5	**0.737**	**C, Ca, Fe, P, Humification**
5	**0.735**	**C, Al, Ca, P, Humification**
5	**0.735**	**C, Ca, K, P, Humification**
4	**0.731**	**C, Ca, P, Humification**
5	**0.730**	**C, Ca, Mn, P, Humification**
4	**0.728**	**C, Fe, P, Humification**
4	**0.726**	**C, Mg, P, Humification**
5	**0.723**	**C, Ca, Cr, P, Humification**
3	**0.721**	**C, P, Humification**
5	**0.715**	**C, Al, Mg, P, Humification**



Levels of significance: *0.05>p≥0.01 **0.01>p≥0.005 ***0.005<p

Figure 5.15: Linktree of showing concentrations of the most important environmental parameters identified using BEST analysis selecting the fungal community structure in the peat samples. All concentrations are in mg/L except humification (Hum). S is a proxy for N, C and LOI (loss on ignition)

CHAPTER SIX: CONCLUSION

The purpose of this research study was to compare three different sites of interest: Goukou River (impacted/disturbed peatland), Springfield Farm (a developing peatland), and Vankervelsvlei (a well-established peatland), and assessing the microbial biodiversity and physicochemical data to determine the health of the soil/peatland. This work was novel, as it was the first biodiversity study performed on these South African peatlands.

In terms of physicochemical and molecular data, all three sample locations gave extremely intriguing findings, as predicted. The physicochemical data revealed that Goukou River shared commonalities with both Vankervelsvlei and Springfield Farm, but Vankervelsvlei and Springfield Farm did not. The similarities between Goukou River and Vankervelsvlei may be due to the age of the two peatlands, whereas the similarities between Goukou River and Springfield Farm may be due to the exposed deposits present in Goukou River and the fact that both of these peatlands have been and continue to be heavily impacted by agricultural wastewater. Because Vankervelsvlei is undisturbed and unaffected by agricultural wastewater or human activity, no parallels between Vankervelsvlei and Springfield Farm were discovered. Both Vankervelsvlei and the Goukou River have acidic peatlands, although Springfield Farm has not. The same pH is owing to the fact that Vankervelsvlei and Goukou River are older than Springfield Farm, and the Ca and Mg levels at Springfield Farm are likewise greater than at the other locations, explaining why the pH range is more basic than acidic. Vankervelsvlei likewise had significant humification levels, although Goukou River and Springfield Farm did not. In terms of C content, the well-preserved peatland of Vankervelsvlei generated high amounts, but C levels were much lower in the damaged Goukou River and growing Springfield farm.

The findings of the metagenomic DNA and NGS analyses revealed a distinct microbial richness of the various locations. The T-RFLP findings revealed a degree of resemblance between the various bacterial groups. However, distinct bacterial populations were discovered at various depths. *Acidimicrobiales*, *Actinomycetales*, *Gaiellales*, and *Solirubacterales* were found in greater abundance in Goukou River and Springfield Farm than in Vankervelsvlei. *Bifidobacteriales*, on the other hand, were only found in Vankervelsvlei, while *Microccocalles*, *Euzebyales*, *Nitriliruptorales*, and *Rubrobacterales* were only found in Springfield Farm. Twenty-six actinobacteria were isolated and identified from the Goukou River and Vankervelsvlei, including *Nocardia africana*, *Kitasatospora albolonga*, and a number of *Streptomyces* species. Ascomycota

and Basidiomycota fungi were abundant in all three locations. Ascomycota dominated in the Goukou River (impacted), Glomeromycota in Springfield Farm (developing), and Basidiomycota in Vankervelsvlei (unimpacted). It should be noted that the presence of humic acids in dark soils or peat often make it difficult to isolate metagenomic DNA. Appendix C shows the rarefaction curves of the different sampling sites. A bend down towards the right side of the rarefaction graph of Vankervelsvlei can be observed, indicating that the majority of the bacterial and fungal communities were captured in the metagenomic sequencing data. However, the rarefaction curves for Goukou River and Springfield Farm indicate that the bacterial and fungal communities were not fully captured.

The findings of this study can be utilized to develop criteria for evaluating the health of peatlands (Table 6.1), based on physicochemical and molecular data. The presence of the actinobacteria and fungal communities detected in the studied peatlands can be used as bioindicators of whether a peatland is healthy or unhealthy. The findings from Vankervelsvlei were consistent with prior research on unimpacted peatlands (Kuhry et al., 1991:1070-1090; Pawlowski et al., 2014:269-288; Salvador et al., 2014:1-17). Acidic soil, high organic matter content, and the presence (high RA) of particular microorganisms such as Basidiomycota members are among the key features observed.

Table 6.1: Physicochemical (chemical indicator) and molecular (bioindicator) criteria can be to distinguish healthy peatlands from 'unhealthy' and developing peatlands

Parameters	**Vankervelsvlei (unimpacted; ideal/expected)**	**Goukou River (impacted)**	**Springfield Farm (developing)**
pH	Acidic	Acidic	Basic
Organic Matter content	High	Low	Low
C/N ratio	High	Low	Low
Humification	High	Low	Low
Actinobacteria detected	*Bifidobacteriales*	*Acidimicrobiales*, *Actinomycetales*, *Gaiellales*, and *Solirubacterales*	*Micrococalles*, *Euzebyales*, *Nitriliruptorales*, and *Rubrobacterales*
Fungi detected	*Basidiomycota*	*Ascomycota*	*Glomeromycota*

Work suggested for the future include similar studies on additional South African peatlands, which can be added to the criteria in Table 6.1 to make it more viable to be used to distinguish healthy peatlands from 'unhealthy'/impacted ones. Also analysing the agricultural wastewater that impacted the Goukou River and Springfield Farm will allow for a better understanding and comparison of physicochemical data of the effluent and the peat samples. Finally, an RNA-based analysis to detect viable microorganisms that are detected during community-based analyses but not during culture-based analyses, will provide further insight into the ‘true’ communities driving peatland health.

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APPENDIX A: MEDIA

Table A1: International *Streptomyces* Project Medium No.1

Ingredients	g/L
Casein enzymic hydrolysate	5
Yeast extract	3
Bacteriological agar	20
Final pH (at 25 °C)	7.0±0.2

Directions: Make up to 1 L deionised water. The pH was adjusted, and 15 g -20 g bacteriological agar was added. Media was sterilized by autoclaving at 121°C for 15-20 minutes. Cooled and poured plates.

Table A2: International *Streptomyces* Project Medium No.2
(Yeast extract-Malt extract Agar – YEME)

Ingredients	g/L
Yeast extract	4.0
Malt extract	10.0
Glucose	4.0
Bacteriological agar	20
Final pH (at 25°C)	7.3

Directions: Make up to 1 L deionised water. The pH was adjusted, and 15 g -20 g bacteriological agar was added. Media was sterilized by autoclaving at 121°C for 15-20 minutes. Cooled and poured plates.

Table A3: International *Streptomyces* Project Medium No.3 (Oatmeal Agar)

Ingredients	g/0.5L
Oat Meal	10
Trace salts	500µl
Bacteriological agar	9
Final pH (at 25°C)	7.3±0.2

Trace salts solution

g/100ml: 0.1 $FeSO_4.7H_2O$, 0.1 $MnCl_2.4H_2O$, 0.1 $ZnSO_4.7H_2O$, dH_2O to 100 ml. Filter sterilize.

Directions

1. Place 10g oatmeal in a 1litre bottle – add 500 ml distilled water and boil for 4-5 minutes.
2. Simmer for 20 minutes at 20% power – mix well.
3. Allow the suspension to cool and settle on the bench for 2 hrs or filter through cheesecloth.
4. Carefully pour off the sludge – make up the volume to 500 ml with distilled water.
5. Add 500µl trace salts solution and pH to 7.2.
6. Add 9.0 g agar and autoclave at 15 psi for 15-20 minutes.
7. Cool and pour plates

Table A4: Czapek solution agar (CZ)

Ingredients	g/L
Sucrose	30
K_2HPO_4	1
$NaNO_3$	2.0
KCl	0.5
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$	0.01
Bacteriological agar	15
Final pH	7.3 ± 0.2

Directions: Make up to 1 L deionised water. The pH was adjusted, and 15 g -20 g bacteriological agar was added. Media was sterilized by autoclaving at 121°C for 15-20 minutes. Cooled and poured plates.

Table A5: Starch-casein-nitrate agar (S-C-N)

Ingredients	**g/L**
Starch	**10**
Casein	**0.3**
KNO_3	**2**
$MgSO_4 \cdot 7H_2O$	**0.05**
$CaCO_3$	**0.3**
$FeSO_4.7H_2O$	**0.01**
Agar	**15**
Final pH	**7.0**

Directions: Make up to 1 L deionised water. The pH was adjusted, and 15-20 g bacteriological agar was added. Media was sterilized by autoclaving at 121°C for 15-20 minutes. Cooled and poured plates.

APPENDIX B: STATISTICAL ANALYSIS OF THE PHYSICOCHEMICAL DATA

Table B1: Nitrogen ANOVA Single Factor

SUMMARY						
Groups	Count	Sum	Average	Variance	**Samples**	
Column 1	2	450	225	1250	NR top	
Column 2	2	160	80	200	NR middle	
Column 3	2	130	65	450	NR bottom	
Column 4	3	450	150	9100	GK top	
Column 5	3	460	153.3333	2033.333	GK middle	
Column 6	3	210	70	300	GK bottom	
Column 7	3	4025	1341.667	4008.333	VV 580-600 cm	
Column 8	3	3470	1156.667	126960.3	VV 780-800 cm	
Column 9	3	3539	1179.667	66924.33	VV 980-1000 cm	
Column 10	3	510	170	787	VV 1180-1200 cm	
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7296274	9	810697.1	32.64861	5.42E-09	2.494291
Within Groups	422126.7	17	24830.98			
Total	7718401	26				

Table B2: Carbon ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**5420**	**2710**	**3200**	NR top	
Column 2	**2**	**3230**	**1615**	**36450**	NR middle	
Column 3	**2**	**2280**	**1140**	**168200**	NR bottom	
Column 4	**3**	**10850**	**3616.667**	**1690633**	GK top	
Column 5	**3**	**8400**	**2800**	**164800**	GK middle	
Column 6	**3**	**7660**	**2553.333**	**38233.33**	GK bottom	
Column 7	**3**	**162234**	**54078**	**1116193**	VV 580-600 cm	
Column 8	**3**	**161581**	**53860.33**	**1948596**	VV 780-800 cm	
Column 9	**3**	**133878**	**44626**	**2.32E+08**	VV 980-1000 cm	
Column 10	**3**	**9585**	**3195**	**544267**	VV 1180-1200 cm	
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**1.41E+10**	**9**	**1.57E+09**	**56.15019**	**7.07E-11**	**2.494291**
Within Groups	**4.76E+08**	**17**	**27972991**			
Total	**1.46E+10**	**26**				

Table B3: Sulphur: ANOVA: Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**377**	**188.5**	**43654.22**	NR top	
Column 2	**2**	**97.94**	**48.97**	**2167.453**	NR middle	
Column 3	**2**	**114.58**	**57.29**	**2384.642**	NR bottom	
Column 4	**3**	**32.58**	**10.86**	**97.5625**	GK top	
Column 5	**3**	**55.86**	**18.62**	**142.4325**	GK middle	
Column 6	**3**	**73.11**	**24.37**	**62.0575**	GK bottom	
Column 7	**3**	**2202**	**734**	**5917**	VV 580-600 cm	
Column 8	**3**	**1605**	**535**	**4548**	VV 780-800 cm	
Column 9	**3**	**2100**	**700**	**18877**	VV 980-1000 cm	
Column 10	**3**	**151**	**50.33333**	**264.3333**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**2320980**	**9**	**257886.6**	**40.58459**	**9.66E-10**	**2.494291**
Within Groups	**108023.1**	**17**	**6354.299**			
Total	**2429003**	**26**				

Table B4: Aluminium: ANOVA: Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**6297.894**	**3148.947**	**4737641**	NR top	
Column 2	**2**	**4389.406**	**2194.703**	**97652.08**	NR middle	
Column 3	**2**	**3804.577**	**1902.288**	**473196.8**	NR bottom	
Column 4	**3**	**2572.398**	**857.466**	**32022.12**	GK top	
Column 5	**3**	**2598.863**	**866.2877**	**138519.7**	GK middle	
Column 6	**3**	**2270.697**	**756.899**	**44825.36**	GK bottom	
Column 7	**3**	**1267.622**	**422.5405**	**57839.98**	VV 580-600 cm	
Column 8	**3**	**1326.025**	**442.0084**	**42978.32**	VV 780-800 cm	
Column 9	**3**	**3643.704**	**1214.568**	**2067474**	VV 980-1000 cm	
Column 10	**3**	**20008.77**	**6669.588**	**767137.1**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**95901508**	**9**	**10655723**	**15.60258**	**1.47E-06**	**2.494291**
Within Groups	**11610084**	**17**	**682946.1**			
Total	**1.08E+08**	**26**				

Table B5: Calcium ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**647.0354**	**323.5177**	**13213.84**	NR top	
Column 2	**2**	**419.3412**	**209.6706**	**2468.37**	NR middle	
Column 3	**2**	**307.7804**	**153.8902**	**6638.189**	NR bottom	
Column 4	**3**	**71.47**	**23.82333**	**272.4246**	GK top	
Column 5	**3**	**50.029**	**16.67633**	**17.02654**	GK middle	
Column 6	**3**	**35.735**	**11.91167**	**17.02654**	GK bottom	
Column 7	**3**	**964.6025**	**321.5342**	**5450.594**	VV 580-600 cm	
Column 8	**3**	**1347.451**	**449.1503**	**7495.801**	VV 780-800 cm	
Column 9	**3**	**1356.516**	**452.172**	**14548.96**	VV 980-1000 cm	
Column 10	**3**	**498.4203**	**166.1401**	**80.02747**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**756475**	**9**	**84052.78**	**18.29946**	**4.57E-07**	**2.494291**
Within Groups	**78084.12**	**17**	**4593.183**			
Total	**834559.1**	**26**				

Table B6: Chromium ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**5.616315**	**2.808157**	**15.7715**	NR top	
Column 2	**2**	**12.76049**	**6.380244**	**0.004294**	NR middle	
Column 3	**2**	**6.583566**	**3.291783**	**21.67167**	NR bottom	
Column 4	**3**	**0**	**0**	**0**	GK top	
Column 5	**3**	**0**	**0**	**0**	GK middle	
Column 6	**3**	**0**	**0**	**0**	GK bottom	
Column 7	**3**	**0**	**0**	**0**	VV 580-600 cm	
Column 8	**3**	**0**	**0**	**0**	VV 780-800 cm	
Column 9	**3**	**15.48891**	**5.16297**	**33.91691**	VV 980-1000 cm	
Column 10	**3**	**18.36388**	**6.121294**	**0.018339**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**183.127**	**9**	**20.34745**	**3.284403**	**0.016701**	**2.494291**
Within Groups	**105.318**	**17**	**6.195174**			
Total	**288.445**	**26**				

Table B7: Iron ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**3230.49**	**1615.245**	**1680215**	NR top	
Column 2	**2**	**2248.333**	**1124.166**	**25306.27**	NR middle	
Column 3	**2**	**2088.493**	**1044.246**	**105742.6**	NR bottom	
Column 4	**3**	**356.694**	**118.898**	**6310.169**	GK top	
Column 5	**3**	**279.76**	**93.25333**	**16.30535**	GK middle	
Column 6	**3**	**223.808**	**74.60267**	**16.30535**	GK bottom	
Column 7	**3**	**331.582**	**110.5273**	**1173.975**	VV 580-600 cm	
Column 8	**3**	**440.3972**	**146.7991**	**1540.534**	VV 780-800 cm	
Column 9	**3**	**906.737**	**302.2457**	**21584.55**	VV 980-1000 cm	
Column 10	**3**	**4144.425**	**1381.475**	**90412.79**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**8590811**	**9**	**954534.6**	**7.902649**	**0.00015**	**2.494291**
Within Groups	**2053373**	**17**	**120786.7**			
Total	**10644184**	**26**				

Table B8: Potassium ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**1307.569**	**653.7846**	**205581.4**	NR top	
Column 2	**2**	**974.4386**	**487.2193**	**6817.51**	NR middle	
Column 3	**2**	**840.8876**	**420.4438**	**27340.78**	NR bottom	
Column 4	**3**	**390.147**	**130.049**	**2090.167**	GK top	
Column 5	**3**	**406.749**	**135.583**	**4019.552**	GK middle	
Column 6	**3**	**406.749**	**135.583**	**160.7821**	GK bottom	
Column 7	**3**	**139.9929**	**46.66429**	**439.9788**	VV 580-600 cm	
Column 8	**3**	**150.2814**	**50.09379**	**257.4952**	VV 780-800 cm	
Column 9	**3**	**616.4634**	**205.4878**	**84145.04**	VV 980-1000 cm	
Column 10	**3**	**3104.169**	**1034.723**	**41990.55**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**2622349**	**9**	**291372.1**	**9.790211**	**3.77E-05**	**2.494291**
Within Groups	**505946.8**	**17**	**29761.58**			
Total	**3128296**	**26**				

Table B9: Magnesium ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**1004.483**	**502.2413**	**76579.2**	NR top	
Column 2	**2**	**865.2138**	**432.6069**	**4505.461**	NR middle	
Column 3	**2**	**849.1113**	**424.5557**	**19852.44**	NR bottom	
Column 4	**3**	**138.69**	**46.23**	**84.8421**	GK top	
Column 5	**3**	**132.66**	**44.22**	**48.4812**	GK middle	
Column 6	**3**	**120.6**	**40.2**	**48.4812**	GK bottom	
Column 7	**3**	**826.1411**	**275.3804**	**2557.891**	VV 580-600 cm	
Column 8	**3**	**1014.624**	**338.208**	**1819.107**	VV 780-800 cm	
Column 9	**3**	**1015.197**	**338.399**	**5720.985**	VV 980-1000 cm	
Column 10	**3**	**952.2878**	**317.4293**	**5513.75**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**699832.1**	**9**	**77759.12**	**9.974822**	**3.33E-05**	**2.494291**
Within Groups	**132524.2**	**17**	**7795.54**			
Total	**832356.3**	**26**				

Table B10: Manganese ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**13.26552**	**6.63276**	**0.151482**	NR top	
Column 2	**2**	**28.99411**	**14.49706**	**107.3759**	NR middle	
Column 3	**2**	**22.06737**	**11.03368**	**29.97287**	NR bottom	
Column 4	**3**	**7.745**	**2.581667**	**19.99501**	GK top	
Column 5	**3**	**15.49**	**5.163333**	**19.99501**	GK middle	
Column 6	**3**	**0**	**0**	**0**	GK bottom	
Column 7	**3**	**8.382809**	**2.79427**	**23.42383**	VV 580-600 cm	
Column 8	**3**	**0**	**0**	**0**	VV 780-800 cm	
Column 9	**3**	**4.542486**	**1.514162**	**6.878059**	VV 980-1000 cm	
Column 10	**3**	**13.97562**	**4.658541**	**16.28969**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**461.9332**	**9**	**51.32591**	**2.808636**	**0.031921**	**2.494291**
Within Groups	**310.6634**	**17**	**18.27432**			
Total	**772.5967**	**26**				

Table B11: Sodium ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**873.3929**	**436.6965**	**19688.87**	NR top	
Column 2	**2**	**358.9479**	**179.4739**	**5840.661**	NR middle	
Column 3	**2**	**403.4924**	**201.7462**	**6955.101**	NR bottom	
Column 4	**3**	**37.095**	**12.365**	**238.5134**	GK top	
Column 5	**3**	**0**	**0**	**0**	GK middle	
Column 6	**3**	**0**	**0**	**0**	GK bottom	
Column 7	**3**	**84.03352**	**28.01117**	**1082.329**	VV 580-600 cm	
Column 8	**3**	**119.2664**	**39.75546**	**2130.883**	VV 780-800 cm	
Column 9	**3**	**568.3729**	**189.4576**	**34764.73**	VV 980-1000 cm	
Column 10	**3**	**1380.903**	**460.3009**	**5460.43**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**736082.1**	**9**	**81786.9**	**11.6021**	**1.2E-05**	**2.494291**
Within Groups	**119838.4**	**17**	**7049.318**			
Total	**855920.5**	**26**				

Table B12: Phosphorus ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**51.16581**	**25.58291**	**13.61024**	NR top	
Column 2	**2**	**32.02618**	**16.01309**	**0.027049**	NR middle	
Column 3	**2**	**28.27293**	**14.13646**	**6.082559**	NR bottom	
Column 4	**3**	**25.758**	**8.586**	**0**	GK top	
Column 5	**3**	**25.758**	**8.586**	**0**	GK middle	
Column 6	**3**	**21.465**	**7.155**	**6.143283**	GK bottom	
Column 7	**3**	**43.70978**	**14.56993**	**12.07255**	VV 580-600 cm	
Column 8	**3**	**39.63435**	**13.21145**	**7.948807**	VV 780-800 cm	
Column 9	**3**	**60.43029**	**20.14343**	**119.1645**	VV 980-1000 cm	
Column 10	**3**	**88.01325**	**29.33775**	**111.7018**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**1360.564**	**9**	**151.1738**	**4.814616**	**0.00265**	**2.494291**
Within Groups	**533.7818**	**17**	**31.39893**			
Total	**1894.346**	**26**				

Table B13: Silica ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**68848.37**	**34424.19**	**33351835**	NR top	
Column 2	**2**	**79048.01**	**39524**	**893505.9**	NR middle	
Column 3	**2**	**81066.48**	**40533.24**	**5464078**	NR bottom	
Column 4	**3**	**120159.2**	**40053.06**	**5689739**	GK top	
Column 5	**3**	**123664.7**	**41221.56**	**1081179**	GK middle	
Column 6	**3**	**126973.9**	**42324.63**	**579152.1**	GK bottom	
Column 7	**3**	**1666.365**	**555.4549**	**290993.3**	VV 580-600 cm	
Column 8	**3**	**4597.52**	**1532.507**	**1191892**	VV 780-800 cm	
Column 9	**3**	**25746.93**	**8582.311**	**1.58E+08**	VV 980-1000 cm	
Column 10	**3**	**98941.12**	**32980.37**	**3168523**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**7.78E+09**	**9**	**8.65E+08**	**38.66016**	**1.42E-09**	**2.494291**
Within Groups	**3.8E+08**	**17**	**22363577**			
Total	**8.16E+09**	**26**				

Table B14: Titanium ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**453.3111**	**226.6555**	**2333.807**	NR top	
Column 2	**2**	**441.3579**	**220.679**	**645.3184**	NR middle	
Column 3	**2**	**377.7388**	**188.8694**	**1496.017**	NR bottom	
Column 4	**3**	**653.455**	**217.8183**	**2024.621**	GK top	
Column 5	**3**	**569.525**	**189.8417**	**443.2603**	GK middle	
Column 6	**3**	**449.625**	**149.875**	**1868.881**	GK bottom	
Column 7	**3**	**221.2959**	**73.76529**	**2198.854**	VV 580-600 cm	
Column 8	**3**	**301.0216**	**100.3405**	**5577.835**	VV 780-800 cm	
Column 9	**3**	**471.1636**	**157.0545**	**29429.64**	VV 980-1000 cm	
Column 10	**3**	**1554.443**	**518.1475**	**12812.21**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**397791.9**	**9**	**44199.1**	**6.63851**	**0.000436**	**2.494291**
Within Groups	**113185.7**	**17**	**6657.985**			
Total	**510977.6**	**26**				

Table B15: pH ANOVA Single Factor

SUMMARY					
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**
Column 1	**2**	**14.2**	**7.1**	**0.0008**	NR top
Column 2	**2**	**14.16**	**7.08**	**0**	NR middle

Column 3	**2**	**12.86**	**6.43**	**0**	NR bottom	
Column 4	**3**	**9.28**	**3.093333**	**0.031033**	GK top	
Column 5	**3**	**9.33**	**3.11**	**0.0304**	GK middle	
Column 6	**3**	**9.39**	**3.13**	**0.0156**	GK bottom	
Column 7	**3**	**12.55**	**4.183333**	**0.075433**	VV 580-600 cm	
Column 8	**3**	**12.78**	**4.26**	**0.0364**	VV 780-800 cm	
Column 9	**3**	**12.85**	**4.283333**	**0.004433**	VV 980-1000 cm	
Column 10	**3**	**13.71**	**4.57**	**0.0043**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**52.26927**	**9**	**5.807696**	**249.3203**	**2.99E-16**	**2.494291**
Within Groups	**0.396**	**17**	**0.023294**			
Total	**52.66527**	**26**				

Table B16: Humification ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**0.114**	**0.057**	**0.000002**	NR top	
Column 2	**2**	**0.082**	**0.041**	**8E-06**	NR middle	
Column 3	**2**	**0.177**	**0.0885**	**0.006613**	NR bottom	
Column 4	**3**	**0.362**	**0.120667**	**0.003814**	GK top	
Column 5	**3**	**0.263**	**0.087667**	**0.00054**	GK middle	
Column 6	**3**	**0.225**	**0.075**	**0.001591**	GK bottom	
Column 7	**3**	**1.306**	**0.435333**	**0.041526**	VV 580-600 cm	
Column 8	**3**	**1.323**	**0.441**	**0.099369**	VV 780-800 cm	
Column 9	**3**	**1.394**	**0.464667**	**0.03071**	VV 980-1000 cm	
Column 10	**3**	**0.19**	**0.063333**	**0.000169**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**0.826456**	**9**	**0.091828**	**4.311626**	**0.004672**	**2.494291**
Within Groups	**0.362064**	**17**	**0.021298**			
Total	**1.18852**	**26**				

Table B17: LOI ANOVA Single Factor

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**	**Samples**	
Column 1	**2**	**28721.49**	**14360.74**	**25253221**	NR top	
Column 2	**2**	**13497.69**	**6748.843**	**917291.6**	NR middle	
Column 3	**2**	**11298.77**	**5649.387**	**7010369**	NR bottom	
Column 4	**2**	**26129.43**	**13064.71**	**71921041**	GK top	
Column 5	**2**	**17709.55**	**8854.777**	**1913350**	GK middle	
Column 6	**2**	**17267.51**	**8633.753**	**1697081**	GK bottom	
Column 7	**2**	**194578.8**	**97289.41**	**16489.72**	VV 580-600 cm	
Column 8	**2**	**190533.6**	**95266.8**	**10985710**	VV 780-800 cm	
Column 9	**2**	**188829.7**	**94414.84**	**4430392**	VV 980-1000 cm	
Column 10	**2**	**20341.64**	**10170.82**	**7045517**	VV 1180-1200 cm	
ANOVA DATA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	**3.12E+10**	**9**	**3.47E+09**	**264.2834**	**1.09E-10**	**3.020383**
Within Groups	**1.31E+08**	**10**	**13119046**			
Total	**3.13E+10**	**19**				

APPENDIX C: RAREFACTION CURVES

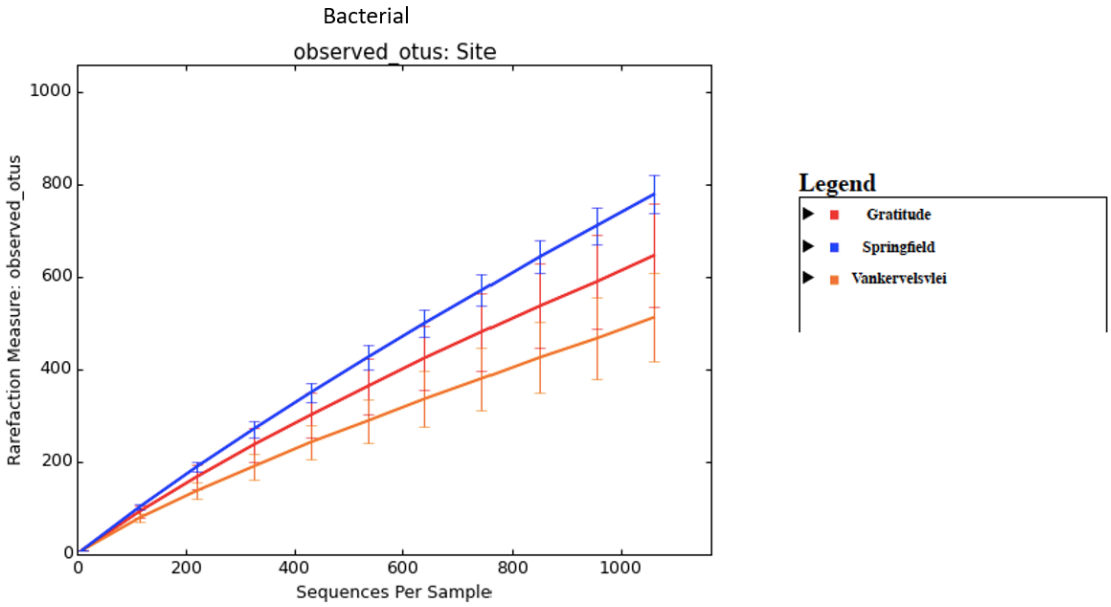

Figure C1: Bacterial rarefaction curve of all the sampling sites.

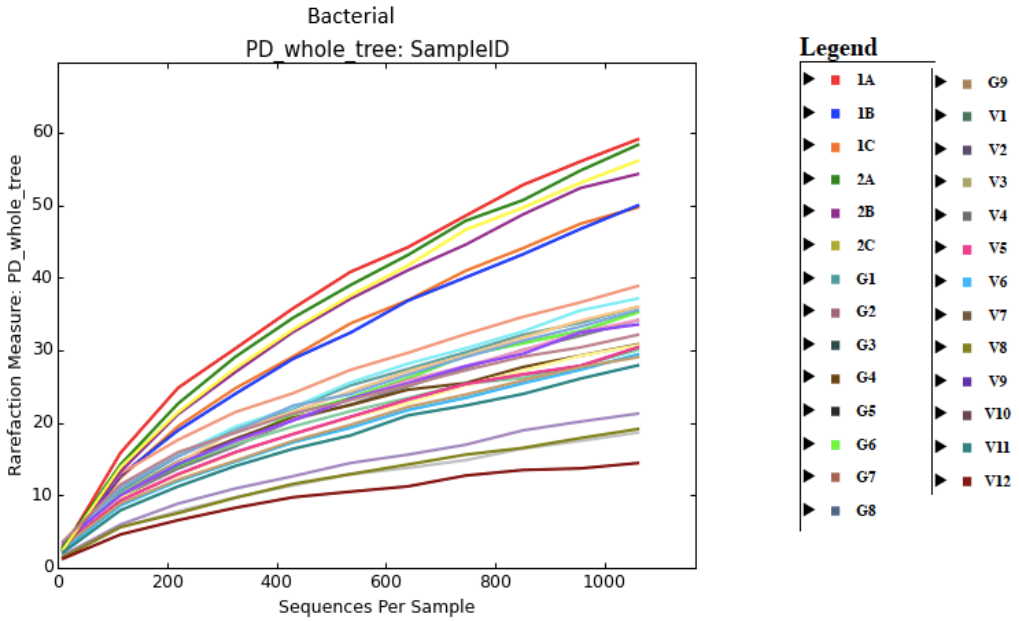

Figure C2: Bacterial rarefaction curve of the individual samples.

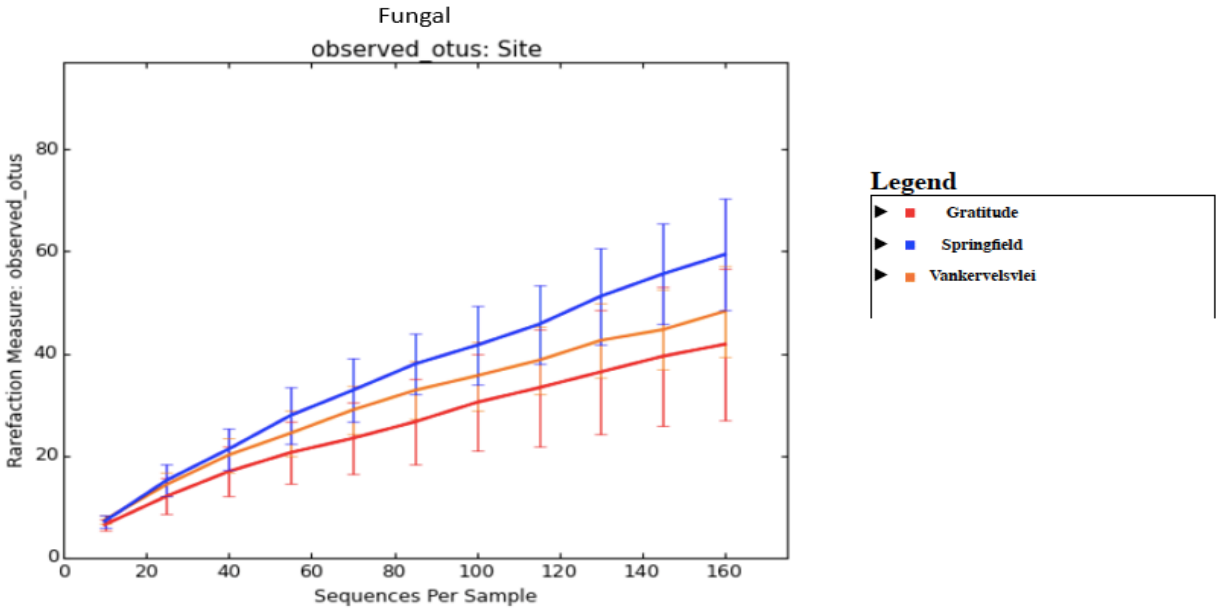

Figure C3: Fungal rarefaction curve of all the sites.

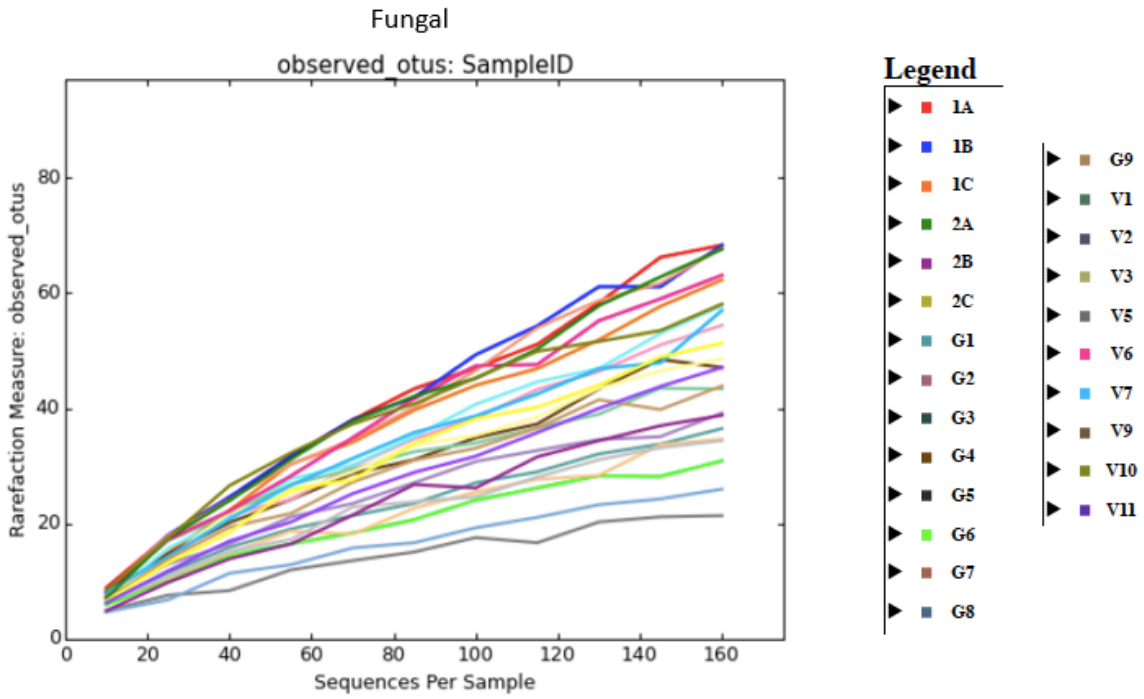

Figure C4: Fungal rarefaction curve of the individual samples.