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UNIVERSITY OF THE
WITWATERSRAND,
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Identifying the core microbiome of two South African lichens

By

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Dissertation

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Master of Science

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Co-supervisor: Prof Pieter De Maayer

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Declaration

I, Pascale Naude, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

15th day of May 2023 at the University of the Witwatersrand, Johannesburg.

Abstract

Lichens are a symbiotic relationship formed between fungi (the mycobiont) and algae and/or cyanobacteria (the photobiont). The microbiome (i.e. fungi and bacteria) associated South African lichens have not been investigated. Given the unique South African climate, and the many different lichen taxa found in South Africa, lichens could represent a rich source of novel microorganisms that produce biotechnologically relevant molecules and enzymes and could serve as a tool for environmental and climate change monitoring. The aim of this study was to investigate the microbiomes of two lichens, *Parmotrema* and *Flavopunctelia* in three locations in Gauteng, across both the winter and summer seasons, to determine the influence of biotic and abiotic factors on the microbial community structure of these lichens. The total genomic DNA was extracted from the lichens and subjected to 16S ribosomal RNA (16S rRNA) / Internal Transcribed Spacer (ITS) based tag-encoded FLX amplicon pyrosequencing (bTEFAP) at MR DNA (Texas, USA) to identify the bacterial and fungal components of the lichen microbiome, respectively. The core microbiome was identified, and the alpha and beta diversities were calculated to determine the composition of the microbiome and the influence of the lichen mycobiont, lichen sampling location, and seasonal changes on its structure. The core bacteriome was found to be mainly denominated by the phyla Proteobacteria, Acidobacteria and Bacteroidetes. The core mycobiome was largely made up of black meristematic fungi which provide lichens with nutrient sources and protection in nutrient poor environments and was dominated by the phylum Ascomycota. The lichen mycobiont was observed to have little to no influence on the composition and diversity of the lichen microbiome. This finding may be attributed to that fact that both sampled lichens are members of the same family. In contrast, significant differences in the microbial composition and diversity were observed between the sample locations and the seasons, which are characterised by differences in temperature and rainfall. Additionally, the microbial diversity was found to increase with an increase in rainfall. All of this shows that the lichen microbiome may vary with different environmental conditions. This study provides the first look at the South African lichen microbiome and paves the way for more studies to investigate a broader range of lichen taxa over a wider geographical area and over a longer study period.

Dedicated to my family,

And my friends,

And Tiffany,

Thank you for all your support.

Presentations arising from this research project

Conference output - Gradflash

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List of abbreviations

16S ribosomal RNA (16S rRNA)

16S rRNA/ITS based tagged amplicon pyrosequencing (bTEFAP)

Algal binding protein (ABP)

Amplicon sequence variants (ASVs)

Analysis of variance (ANOVA)

Cyanobacterium-binding protein (CBP)

Degrees of freedom (Df)

Genomic DNA (gDNA)

Indole-3-acetic acid (IAA)

Internal Transcribed Spacer (ITS)

Multidimensional scaling (MDS)

Multivariate analysis of variance (MANOVA)

National Centre for Biotechnology Information (NCBI)

Operational taxonomic units (OTUs)

Permutational multivariate analysis of variance (PERMANOVA)

Polymerase chain reaction (PCR)

Zero-radius Operational Taxonomic Units (zOTUs)

Chapter 1: Literature review

1.1. Lichens

Lichens are a symbiotic relationship formed between fungi (the mycobiont) and algae and/or cyanobacteria (the photobiont) (Nash, 2008). Within the lichen association, the mycobiont provides structural support and allows the lichen thallus to undergo atmospheric exchange whereby water is absorbed from the atmosphere, and in the process nutrients, minerals and other molecules are unbiasedly absorbed (Turetsky, 2003). The atmospheric exchange by the mycobiont allows the photobiont to utilise water for photosynthesis which in turn provides the mycobiont with a carbohydrate source (Stolte *et al*., 1993). The mycobiont component is mostly made up of fungi from the phyla Ascomycetes and Basidiomycetes (Gams, *et al.*, 2004), while the lichen can also be distinguished based on the primary photobiont type: chlorolichens due to the colonisation of green algae, and cyanolichens due to the colonisation of cyanobacteria (Lange and Wagenitz, 2003). It has been postulated that a tripartite relationship exists between the mycobiont and photobiont in chlorolichens, where cyanobacteria serve as additional photobionts (Armaleo and Clerc, 1991; Goffinet and Bayer, 1997). Cyanolichens have also been observed to be colonised by cyanobacteria as well as green algae which reside in the photosynthetic layer of the lichen thallus and both contribute to photosynthesis (Henskens *et al.,* 2012).

Despite all lichens being a symbiosis between the mycobiont and the photobiont, there are still variations in the morphology of lichens (Büdel and Scheidegger, 2008). They can be categorised into four main types of growth forms: crustose, foliose, fruticose and squamulose. Crustose lichens present like a crust and are classified by having small lobes that are either firmly or loosely attached to the substrate via the entirety of its lower surface. Foliose lichens have leaf-like lobes which are partly attached to the substrate. Squamulose lichens consist of small scales. Finally, fruticose lichens are attached to the substrate by one point and appear as a shrubby or beard-like growth form (Büdel and Scheidegger, 2008).

While lichen growth forms do vary in structure they are all characterised by the base components, namely the cortex (sometimes both upper and lower), algal zone, medulla and rhizines (Ranković and Kosanić, 2015) (**Fig 1.1**). The upper cortex and lower cortex contain a thick layer of compactly structured fungal hyphae which serve a protective function in the lichen (Jóźwiak, 2013; Morillas *et al*., 2022), while the medulla contains loosely packed hyphae (Morillas *et al*., 2022). The algal zone contains hyphae derived from the medulla that wind around algal or cyanobacterial cells (Frey and Scheidegger, 2002). This zone is protected by the upper cortex (Jóźwiak, 2013). The rhizines are the structures that attach the lichen thallus

to the substrate (Morillas *et al*., 2022). Other structures present on the lichen thallus include structures formed for reproduction, these being isidia and soredia.

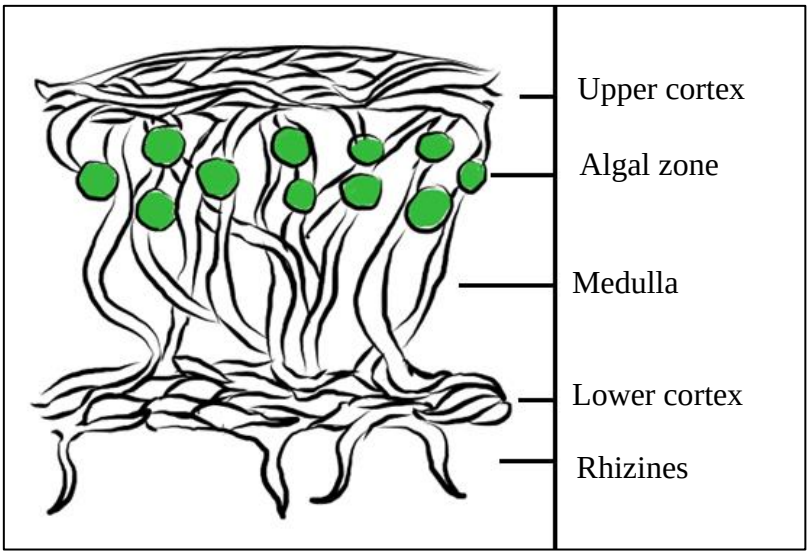


Figure 1.1: The cross-section of the lichen thallus. The algal or cyanobacterial cells are represented by the green circles. The white lines represent the fungal hyphae.

Lichen reproduction is complex, as lichens can undergo both sexual and asexual reproduction (Honegger, 1998). Sexual reproduction occurs through the production of spores by the lichen mycobiont (Büdel and Scheidegger, 2008). Ascomycetes are the type of lichen mycobiont that most commonly reproduce sexually through ascospores (Nash, 2008). These germinating spores are efficient at reproduction across a long distance (Ronnås *et al*., 2017), but will need to associate with a corresponding photobiont to form a lichen structure (Honegger, 1996; Seymour *et al.*, 2005). To undergo asexual reproduction, lichens can produce vegetative structures like isidia or soredia (Büdel and Scheidegger, 2008), which contain both the mycobiont and the photobiont (Fahselt, 2008). These propagules break off from the lichen, are dispersed throughout the environment and colonise a new substrate (Fahselt, 2008).

1.2. The relationship between the mycobiont and photobiont

A clear relationship between the mycobiont and the photobiont within a lichen is observed whereby the lichen mycobiont may either select for a single photobiont or multiple photobiont types (Yahr *et al.*, 2004). The mycobiont may choose to associate with multiple photobionts (if it is in the mycobiont's capability) to increase the mycobiont's chance for survival during shifts in climate (Casano *et al*., 2011). This ability for the mycobiont to choose one or several

photobionts is based on the selectivity of the mycobiont's and photobiont's respective lineages, their geographical distribution and surrounding ecological conditions (Muggia *et al*., 2014). The range of specificity for the selection of the photobionts by the mycobiont can be split into three main categories: photobiont specialists, intermediates and generalists (Miadlikowska *et al*., 2006). Specialists associate with single algal lineages and generalists have a large range of photobionts they can associate with depending on environmental factors. Lichens that have low selectivity for photobionts have a wider range of distribution (Muggia *et al*., 2014), which contributes to the lichen's survival.

Determining how the mycobiont and photobiont interact gives greater insights into the selectivity of the mycobiont for the photobiont. It was hypothesised by Lutzoni and colleagues (2009) that the mycobiont may communicate with its respective photobiont through chemical signalling (Lutzoni and Miadlikowska, 2009), such as through the release of phytohormones (Pichler *et al*., 2020). This communication between the mycobiont and the photobiont was demonstrated when the phytohormone indole-3-acetic acid released by three mycobionts (*Cladonia grayi*, *Tephromela atra*, and *Xanthoria parietina*) was tested against their compatible photobionts and it was found that the photobionts increased their water retention by 4.4% (Pichler *et al*., 2020). Chemical signalling has also been seen to play a role in the initiation of the symbiosis between the mycobiont and photobiont with several mycobiont-produced molecules proposed to be involved in this process. Some of these molecules include the algal binding protein (ABP) produced by *X. pareitina*, which plays a role in the recognition of the corresponding photobiont ligand (Molina *et al*., 1993; Molina and Vicente, 2000), the cyanobacterium-binding protein (CBP) produced by *Peltigera canina* that plays a role in the recognition of the corresponding cyanobacterium (Díaz *et al*., 2009), and agglutinin produced by *Nephroma laevigatum*, which is involved in the specificity of the symbiosis at the beginning of the symbiotic relationship (Kardish *et al*., 1991). The signalling molecules involved in the initiation of symbiosis between the mycobiont and photobiont appears to be unique to the specific mycobionts in which they were identified, while common molecules for the initiation of symbiosis between multiple lichens have not been observed (Nazem-Bokaee *et al*., 2021). This may be because lichens have evolved into different fungal lineages (Gueidan *et al*., 2008; Schoch *et al*., 2009). The specificity in communication that occurs between the mycobiont and photobiont highlights the selective nature of the mycobiont when associating with a photobiont (Pichler *et al*., 2020) .

Despite overwhelming evidence supporting the specificity of the mycobiont for the photobiont, Wirtz and colleagues (2003) found that the selection of the photobionts of lichens sampled from the Antarctic was not determined by the mycobiont (Wirtz *et al*., 2003). Five different lichen species were collected that had cyanobacteria as either the primary or secondary photobiont, and free-living cyanobacteria (*Nostoc* strains) were collected from the sampling sites. This was to determine the specificity of the cyanobiont as well to determine the effect of harsh environmental conditions on the distribution of the *Nostoc* strains. The cyanobiont was found to not be specific to individual lichens, theorised to be due to selection pressure exerted by the harsh Antarctic environment (Wirtz *et al*., 2003).

The photobiont also contributes to the distribution of lichens as photobionts have been observed to have clear preferences for certain environmental factors (Peksa and Škaloud, 2011). With this in mind, the mycobiont most probably selects for a photobiont which is well adapted to its environment (Peksa and Škaloud, 2011). In turn, this then shows how the photobiont can influence the distribution of the lichen. Expanding on the selection for well adapted photobionts in specific environments by mycobionts, photobiont switches have been observed to allow mycobionts to colonise an expanded range of habitats, such as *Cetraria aculeata* which changed from colonising temperate regions to both temperate and polar regions (Fernández-Mendoza *et al*., 2011).

1.3. Lichens and their role in the environment

Lichens play key roles in the biological functioning and maintenance of healthy ecosystems (Nash, 2008). These roles include mineral cycling (Nieboer *et al.*, 1978), nitrogen fixation (Seneviratne and Indrasena, 2006), and assisting in maintaining soil integrity by retaining soil moisture, decreasing soil erosion, and fixing minerals which can be utilised by plants and generally influencing soil properties (Lázaro *et al*., 2008). Their localisation in the environment offers food sources to various animals, and provides microhabitats for small animals and insects such as snails which will provide another nitrogen source to soil through their excrement (Jones and Shachak, 1990; Yang *et al*., 2021).

Anthropogenic uses of lichens include their consumption as a food source and the production of traditional medicines and natural dyes (Crawford, 2019; Shukla *et al*., 2014). *Cetraria islandica* is commonly eaten in soups and porridges by northern Europeans (Perez-Llano, 1944) and *Rocella spp.* are made into a purple dye (most commonly known as “royal purple”)

through an ammonia fermentation method (Shukla *et al*., 2014). Lichens are currently extensively used by scientists to study the environment due to their unique qualities. Most notably, lichens are used as bioindicators and biomonitors (Loppi, 1996; Shukla *et al.*, 2012). Bioindicators are organisms that give a reflection of the pollution levels in the environment they reside in as they are sensitive to environmental changes, while biomonitors are organisms that bioaccumulate pollutants which is used to proportionally calculate the levels of pollutants in an environment (Bosch-Roig *et al*., 2013). Lichens are efficient bioindicators and biomonitors due to several properties. Firstly, lichens are ubiquitous in nature as they are able to inhabit many different substrates and some can withstand harsh environmental conditions like extreme weather and high pollution levels (Garvie *et al*., 2008; Hill, 1994; Shimizu, 2004). The resilience of some lichens to harsh environmental conditions may be because these lichens are more efficient at switching from an active to a dormant state through the rapid dehydration of the lichen thallus (Nash, 2008). Factors like the microclimate, the pH of the substrate and topography also affects the ability of some lichens to colonise certain environments. Secondly, some lichens are sensitive to harsh environmental conditions which gives an indication of high pollution levels (Bates and Farmer, 1992; Nash and Gries, 2002). Thirdly, lichens undergo atmospheric exchange which renders them susceptible to environmental changes (Van der Wat and Forbes, 2015) and allows them to bioaccumulate pollutants in their thalli (Brunialti and Frati, 2014; Garty, 2001). Finally, lichens have a slow growth rate and stay morphologically consistent over time which also makes them ideal for studying the surrounding environment (Aprile *et al*., 2011). This relationship between lichens and the environment makes them great research tools to study the environment. However, understanding all the mechanisms involved in the lichen's functioning and the lichen's protective response that allows some lichens to be more resilient than others to harsh environmental conditions needs to be explored (Gasulla *et al*., 2021).

1.4. The lichen microbiome

There are associations beyond the mycobiont and photobiont within the lichen which contribute to the lichen's functioning in the environment, and these constitute the greater lichen microbiome. A microbiome is classified as the collection of genomes of the residing microorganisms within a specific environment (Cavicchioli *et al*., 2019). Terrestrial microbiomes are essential for the environmental processes within the ecosystems they reside (Cavicchioli *et al*., 2019). Such processes include influencing the composition of plant

communities (van der Heijden *et al.*, 2008), partaking in nutrient cycling and contributing to the state of soil and water quality (Battin *et al*., 2016). Microbial communities are also important in the reaction of terrestrial systems to climate and environmental changes (Fierer, 2017). The composition of such microbial communities can be structured according to biotic factors such as the host (Shapira, 2016), inter-microbial interactions (Hacquard *et al*., 2015), as well as abiotic factors such as temperature, pH (Thomas *et al*., 2016), and the availability of nutrients (Shapira, 2016). Since it has been found that the composition of microbial communities within given environments influence ecosystem processes, it is essential to further study the dynamics between microbiomes and the environment (Dodds *et al*., 2020).

The lichen microbiome forms a major component of the lichen symbiosis, being described as the third player in the lichen association (Hawksworth and Grube, 2020). The lichen microbiome has been said to consist of two components: (1) the core microbiome, which is stable throughout different environmental pressures, and (2) the accessory microbiome, which is shaped by the surrounding environment or when parasitic invasion occurs (Grimm *et al*., 2021; Wedin *et al*., 2016). This microbiome contributes to the functioning of the lichen, being involved in processes like the decay of older lichens, the production of biomolecules, detoxification and assistance in the protective response, nutrient cycling and acquisition and growth of the lichen (Eymann *et al*., 2017; Grimm *et al*., 2021; Grube *et al*., 2015), which will be expanded upon in subsequent sections.

As lichens can colonise an array of substrates (Nash, 2008), and can withstand harsh environmental conditions (Garvie *et al*., 2008; Shimizu, 2004), they are a vessel for a diverse set of niche microorganisms (Sierra *et al.*, 2020) that could potentially produce a wide range of secondary molecules that could be used as antimicrobials or other therapeutics such as anticancer or Alzheimer's disease drugs (González *et al.*, 2005; Wang *et al.*, 2012; Dou *et al.*, 2014; Zhang *et al.*, 2014; Zheng *et al.*, 2014; Zhao *et al.*, 2015; Sierra *et al.*, 2020). Studying the lichen microbiome will not only help identify the core microbiome and understand the effect of biotic and abiotic stresses on the composition and diversity of the lichen microbiome, but also the functions that the microbiome plays within the lichen. The lichen microbiome can be used further to assist in monitoring the health of the environment, potentially identifying novel microorganisms that can be used for bioremediation and to help tackle antimicrobial resistance (Suzuki *et al.*, 2016; Sierra *et al.*, 2020).

1.5. The lichen bacteriome

The bacteriome represents the bacterial counterpart inhabiting a specific environment (Shay *et al*., 2020). A single lichen can harbour over 800 bacteria (Grimm *et al*., 2021) which will mainly consist of bacteria from the phyla Acidobacteria, Actinobacteria, Bacteroidota, Proteobacteria, and Verrucomicrobia (Grube and Berg, 2009; Bates *et al.*, 2011; Sierra *et al.*, 2020). Growing evidence suggests that the lichen bacteriome is a crucial component of the lichen symbiosis (Aschenbrenner *et al*., 2016). The bacteria isolated from the lichen microbiome are known to play roles in environmental processes such as nitrogen fixation (Bates *et al.*, 2011) and phosphorous uptake (Liba *et al.*, 2006; Grube and Berg, 2009; Bates *et al.*, 2011). Grube and colleagues (2015) found that lichen associated bacteria also contribute to the functioning of the lichen through the synthesis of vitamins and hormones, detoxification, lichen degradation and protection against biotic and abiotic stresses (Grube *et al*., 2015). Lichen -associated bacteria were found to produce vitamins like Vitamin B12 which supports photosynthesis within lichens; and to produce hormones like auxin which may promote the growth of lichen associated fungi (Grube *et al*., 2015), as auxin has been found to promote fungal cell lengthening (Yanagishima, 1963). Lichen-associated bacteria were also found to contribute to detoxification within lichens by being able to degrade and metabolise xenobiotics as well as the degradation of phenolic compounds (Grube *et al*., 2015). The production of lytic enzymes by bacteria degrades older pieces of lichen which in turn provides nutrients to the younger lichen components(Grube *et al*., 2015). Finally, they were found to protect the lichen against biotic stresses, through the acquisition of virulence factors such as genes coding for efflux pumps, as well as the production of antimicrobials, and also protect against abiotic stresses by producing metabolites that counteract oxidative stress and heavy metals (Grube *et al*., 2015). All of this shows that the lichen bacteriome is important for the functioning of lichens but more work needs to be done to identify the exact roles of the lichen bacteriome within the lichen symbiosis (Bates *et al.*, 2011).

1.6. The lichen mycobiome

A mycobiome represents the fungal counterpart inhabiting a specific environment (Shay *et al*., 2020). The lichen mycobiome is comprised of symptomatic lichenicolous fungi (parasitic fungi living on the lichen) (Hawksworth, 1979; Lawrey and Diederich, 2003) and endolichenicolous fungi which are present throughout the lichen thalli (Petrini *et al.*, 1990; Girlanda *et al.*, 1997; Muggia and Grube, 2018). The mycobiome of lichens has been found to mainly consist of

Ascomycota and Basidiomycota, and at lesser abundances Chytridiomycota, Glomeromycota, Zygomycota and Kickxellomycota (Zhang *et al.*, 2015; Smith *et al.*, 2020). Most studies investigating endolichenicolous fungi have focused on the lichen-specific secondary metabolites they produce and their functional roles (Elix and Stocker-Wörgötter, 2008; Kellogg and Raja, 2017; Zhang *et al*., 2015). A great portion of the secondary metabolites produced by endolichenicolous fungi are antimicrobials (Kellogg and Raja, 2017), that protect the lichen and shape the bacterial composition within the lichen (Kosanić and Ranković, 2019). Other metabolites produced play roles in lichen decomposition and the formation of fungal galls (Muggia *et al.*, 2017). Lichen decomposition occurs because some lichen-associated fungi prefer the decayed components of lichens (Aptroot and Alstrup, 1999), while the formation of fungal galls allows for the habitation of other fungi in the lichen symbiosis (Millanes *et al*., 2016). The chemical composition and bioactivity of secondary metabolites produced by endolichenicolous fungi needs to be further investigated as there have been multiple studies identifying endolichenicolous fungi but few studying the metabolites produced by them (Gao *et al*., 2016; Wethalawe *et al*., 2021). The lack of studies investigating endolichenicolous fungal secondary metabolites demonstrates that little effort has been made to identify the direct links between the identified fungi and their functional roles in lichens (Zhang *et al*., 2015). Thus, there is an opportunity for more studies to be conducted to further characterise the lichen mycobiome and its functional roles within lichens and the environment, possibly through metagenome and metatranscriptome analysis. There is also the possibility to identify fungal species that produce novel antimicrobials and other secondary metabolites (Shrestha and St. Clair, 2013).

1.7. Lichen species and the lichen microbiome

It has been demonstrated that the bacteria that reside in lichens are largely shaped by the main mycobiont (Grube and Berg, 2009; Grube *et al.*, 2009; Bates *et al.*, 2011). The role of the mycobiont is so strong that variations in diversity (Grube *et al.*, 2009; Aschenbrenner *et al.*, 2014) and abundance of bacterial phyla have been observed between lichen species within a single genus (Grube *et al.*, 2009; Hodkinson and Lutzoni, 2009). Some studies have found that despite a predominance of Alphaproteobacteria across lichens, there was still differences in the bacterial communities between different lichen species (Bates *et al.*, 2011; Hodkinson *et al.*, 2012). The lichen species is further shown to play a role in the composition of the lichen bacteriome because when the mycobiont is destroyed by a parasitic fungus, a shift in the

bacterial composition occurs (Wedin *et al*., 2016), which highlights the importance of the mycobiont for the distribution of the bacteriome. This influence on the bacterial composition within lichens by the mycobiont may be because the lichen itself produces molecules such as depsides and depsidones which harbour antimicrobial properties which may shape the bacteriome through the selection of bacteria that can withstand these molecules (Hodkinson *et al*., 2012).

Some studies have also found that the mycobiome differs depending on the mycobiont, with a diverse set of mycobiomes being present in different lichens (Grube and Berg, 2009; Grube *et al.*, 2009; Bates *et al.*, 2011). In one study investigating the lichen mycobiomes from five lichen types, it was found that closely related lichens harboured more similar mycobiomes despite ecological differences, showing the specificity of the mycobiont for the habitation of different fungal communities (Smith *et al.*, 2020). In contrast, some studies have found that the formation of the lichen mycobiome is largely independent of the host lichen, with environmental factors, such as altitude and geography, having a greater influence (Wang *et al.*, 2016; Smith *et al.*, 2020).

Even though the photobiont does not dictate the species of the lichen (Grimm *et al*., 2021), it is still important to determine whether the photobiont holds any influence on the lichen microbiome structure. Bacterial communities have been found to differ in relation to the associated photobiont (Hodkinson *et al*., 2012) which can consist of a collection of algae and/or cyanobacteria (Casano *et al*., 2011). Hodkinson and colleagues (2012) found significant differences in the bacterial composition within lichens between cyanolichens and chlorolichens. This difference may be due to the availability of fixed nitrogen and carbon produced by different photobionts for use by the inhabiting lichen bacterial communities (Hodkinson *et al*., 2012). This difference in fixed nitrogen may be because cyanobacteria within cyanolichens more readily fix atmospheric nitrogen (Belnap, 2001; Rai, 2003). The difference in available carbon may be because green algae release carbon in the form of sugar alcohols while cyanobacteria release carbon in the form of glucose (Adams *et al*., 2013; Palmqvist, 2003). Despite several studies conducted to investigate the relationship between the photobiont and the lichen bacteriome, not much has been done to investigate the relationship between the photobiont and the fungal communities within lichens. It is possible that the photobiont type could influence the lichen mycobiome as lichen associated fungi also rely on the photobiont for a carbohydrate source (Xu *et al*., 2022). Thus, future studies are required to investigate the influence of the lichen photobiont on the lichen mycobiome (Xu *et al*., 2022).

1.8. Lichen location and the lichen microbiome

Several studies have demonstrated that the location of the lichen influences the composition and diversity of the lichen microbiome (Aschenbrenner *et al*., 2014; Hodkinson *et al*., 2012; Printzen *et al*., 2012). Bacterial classes such as the Alphaproteobacteria have been found to vary in abundance between individual lichens within the same genus, showing the influence of environmental factors such as altitude, substrate and sampling location (Cardinale *et al*., 2012; Coleine *et al*., 2019; Fernández-Brime *et al*., 2019; Hodkinson *et al*., 2012; Printzen *et al*., 2012). In a study of the lichen *Cetraria aculeata* across four geographically distant locations, Printzen and colleagues (2012) found that lichens sampled from polar regions harboured a less diverse bacteriome than lichens sampled from temperature regions (Printzen *et al*., 2012). This shows the influence of the surrounding environment on the diversity of the bacteriome as well as differences in the bacterial composition between geographically distant locations. Interestingly, earlier studies found no influence of geographical distribution on the lichen bacteriome (Grube *et al.*, 2009; Bates *et al.*, 2011), however these studies did not test trends across a wider geographical distribution or across different climates (Hodkinson *et al*., 2012).

The lichen mycobiome has been found to be shaped to some extent by the environmental conditions (Muggia and Grube, 2018). Lichens that reside in humid, mild weathered and northern environments harbour Sordariomycetes and Leotiomycetes (Arnold *et al*., 2009), while rock colonising lichens are dominated by melanised fungi (Harutyunyan *et al.*, 2008; Selbmann *et al.*, 2013). The influence of location and altitude on the fungal composition within *Hypogymnia hypotrypa* was studied and found to have a significant effect on the mycobiome structure (Wang *et al*., 2016). Altitude also played a role in the mycobiome structure in lichens sampled from the Antarctic, but Coleine and colleagues (2019) theorised that factors such as water availability, the temperature of rocks and sunlight may play greater roles in shaping the fungal community within lichens (Coleine *et al*., 2019). Altitude levels most probably hold such a great influence on the microbiome of lichens because high altitude levels are associated with low levels of pressure, oxygen and temperature and high levels of sunlight (Li *et al*., 2019), which can all put pressure on different microorganisms, allowing some to become more abundant than others. Some studies however, have found that the formation of the lichen mycobiome is largely independent of environmental influences (Fernández-Mendoza *et al*., 2017; Fleischhacker *et al*., 2015; Yu *et al*., 2018). With the impact of location yielding contrasting results, it is important to further investigate this factor to determine whether there is an influence on the lichen microbiome.

1.9. Climate and the lichen microbiome

Changes of the seasons bring about variation in the microbiome within various systems, due to temperature changes and moisture changes (Núñez *et al*., 2021). Environmental stresses such as drought may influence the composition of the lichen microbiome as drought has been found to structure the microbiome of sorghum, with the bacteriome protecting the plant against drought stress (Xu *et al*., 2018). Since lichens are able to withstand harsh environmental conditions, it is plausible that the lichen microbiome may assist with the lichen's adaptability and should be further studied (Cernava *et al*., 2019). Cernava and colleagues (2019) found that during dehydration, the bacteriome of the lung lichen, *Lobaria pulmonaria*, exhibits an increase in stress-reducing mechanisms, and sugar polymer and ketone metabolism (Cernava *et al*., 2019). The switch to the breakdown of ketones indicates a shift to lipid-based metabolism within the bacteriome, which allows for the synthesis of osmotically active substances that can assist with water retention (Cernava *et al*., 2019). During rehydration of the lichen there is an increase in the diversity of functional activities of microorganisms, including increased expression levels of bacterial porins for the prevention of cell death, increased levels in various transport proteins, increased transcripts associated with carbohydrate degradation, vitamin synthesis and respiration (Cernava *et al*., 2019).

Lichens have been found to reside in harsh environments, including volcanoes (Wang and Pecoraro, 2021) and the Antarctic continent (Selbmann *et al*., 2013), and have been found to harbour endolichenicolous fungi that are well suited to these harsh environmental conditions and changing environments (Selbmann *et al*., 2005, 2013). Black meristematic fungi have been isolated from lichens in Antarctica that are well adapted to fluctuating environments (Harutyunyan *et al.*, 2008; Selbmann *et al.*, 2013). Lichens have been found to harbour melanised fungi and other fungi that are well adapted to fluctuating environments (Harutyunyan *et al*., 2008; Selbmann *et al.*, 2013). Melanised fungi can reside in nutrient lacking environments as melanins are known to be protective against abiotic stresses (Gostincar *et al*., 2010), keep the lichen hydrated and protect the photobiont from excessive sunlight (Selbmann *et al*., 2005). Investigating the effect of climate on the lichen microbiome may help identify the key players in the survival strategy of lichens. Due to the projected increase in harsh weather conditions, like drought and extreme rainfall, exploiting the protective response of microbiomes within host organisms such as lichens will become pivotal (Schwalm *et al*., 2017).

1.10. The lichen microbiome and its value in industry

Environmental microbiomes are being studied to identify new sources of therapeutics, which are becoming more of a necessity due to the increase in antibiotic resistance across the world (World Health Organization, 2014). A few studies have found lichens harbour bacteria that produce therapeutically relevant molecules (González *et al.*, 2005; Sierra *et al.*, 2020). In one study, it was found that a total of thirty-five bacterial and fungal isolates from lichens exhibited antimicrobial properties (Sierra *et al.*, 2020). In addition, two of the bacterial taxa, belonging to the genera *Psoroma* and *Yoshimuriella*, respectively, were found to inhibit the growth of a multi-drug resistant *Klebsiella pneumoniae* strain (Sierra *et al.*, 2020). This shows that lichens can be important sources of novel antimicrobials. González and colleagues (2005) found that lichen isolates from the Actinomycete family *Pseudonocardiaceae* exhibited antimicrobial activity when tested against *Candida albicans, Escherichia coli* and *Staphylococcus aureus* (González *et al.*, 2005). It is assumed that these antimicrobial producing microbes may be important for the protection of the lichen thallus against pathogens (Sierra *et al.*, 2020), such as the fungal pathogen *Rhagadostoma lichenicola* (Grube *et al*., 2012) and lichenicolous fungi such as *Athelia arachnoidea* (Grimm *et al*., 2021).

Endolichenicolous fungi has been found to be a rich source of novel secondary metabolites with therapeutic and industrial relevance (Kellogg and Raja, 2017). Some of the secondary metabolites produced by endolichenicolous fungi include alkaloids (Zheng *et al*., 2014), quinones (Dou *et al*., 2014) and oxygen heterocycles (Wang *et al*., 2012; Zhang *et al*., 2014), which have been evaluated for their anticancer effects (Dou *et al*., 2014; Zhang *et al*., 2014; Zheng *et al*., 2014) or have been found to have antimicrobial properties (Wang *et al*., 2012). As lichens harbour microorganisms that are often rich sources of novel secondary metabolites, it is important to investigate lichens from varying environments for the potential to discover other novel secondary metabolites (Ranković and Kosanić, 2015).

1.11. South African lichens and their microbiome

The biodiversity of South African lichens is still being fully characterised. Some lichen species that occur in many parts of the world and are also found in South Africa include *Candelaria concolor*, *Lobaria pulmonaria*, *Peltigera canina* and *Xanthoria parietina*. Lichen species endemic to South African include *Combea mollusca*, *Rocella hypomecha*, *Teloschistes capensis* and *Xanthomaculina hottentotta* (Almborn, 1987). The first South

African lichen checklist was published in 1950 (Doidge, 1950). Since the first South African lichen checklist has become outdated, the official, "Lichens of South Africa," list by Fryday (2015) was created and currently consists of 1,750 lichen taxa in 250 genera (Fryday, 2015). It is estimated that the South African lichen consortium will amount to 2,500 to 3,000 species once fully investigated (Fryday, 2015). A fault of this list is that it was constructed off pre-existing literature and there was no attempt at confirming these reports in the field (Fryday, 2015). Since then, there have been updates to the list, making corrections, additions and deletions of lichen taxa (Ahti *et al*., 2016). Some of these corrections include the addition of twelve species from the genus *Cladonia*, thirteen species from the genus *Rinodina* and twelve species from the genus *Usnea*. Due to the list being based off previous literature which dates back to the 19th century, more in depth lichen research needs to be undergone through collecting, examining and identifying lichens found in South Africa (Ahti *et al*., 2016).

South African lichen microbiomes have to our knowledge not been explored. Most South African lichen studies have focused on studying the biodiversity of lichens. One study recorded thirty-five terricolous lichen taxa, of which 52% of the lichens were determined to be endemic to Namaqualand (Zedda and Rambold, 2009). The response of lichens to air pollution levels has also been characterised in South Africa. One study of the bioaccumulation of thirty-three trace and other toxic elements found South African lichens to be efficient at bioaccumulating halogens (Ndlovu *et al*., 2019). Another study focused specifically on the bioaccumulation of mercury in *Parmelia caperata* (Panichev *et al*., 2019). The antimicrobial properties of South African lichens including *Flavoparmelia sp.* and *Usnea sp.* has been investigated (Seaman *et al*., 2007). It was found that many of the molecules produced that exhibited antimicrobial activity contained usnic acid which had antibacterial and antifungal properties and exhibited antimalarial activity (Seaman *et al*., 2007). These studies demonstrate the importance of South African lichens for the biomonitoring of air pollutants and the acquisition of therapeutic molecules.

1.12. Aims and Objectives

The main aims of this project were to (1) conduct the first study of the bacteriome and mycobiome associated with South African lichens and (2) to study the effect the lichen species, sampling location and climate on the microbiome of these lichens.

These aims were approached through the following objectives:

1) Collection of two lichen species, from three locations within Gauteng, across two seasons, summer and winter.
2) Identification of the lichen species by examining morphological characteristics.
3) Extraction of the total genomic DNA from the lichen samples.
4) Metataxonomic sequencing of the lichen microbiome with 16S ribosomal RNA/Internal Transcribed Spacer (ITS) based tagged amplicon pyrosequencing (bTEFAP), targeting the V1-V3 of the 16S rRNA gene and the ITS2 hypervariable region of the ITS gene sequence to identify bacteria and fungi, respectively.
5) Conducting statistical analysis to identify the core lichen microbiome, analyse alpha and beta diversity metrices and determine the influence of the lichen species, sampling location, and season on the microbiome distribution.

Chapter 2: Methodology

2.1. Lichen sampling and identification

Two distinct lichens (**Fig. 2.1**) were collected from three areas; the Melville Koppies (Permit Appendix 2), and within residential areas in Bryanston and Pretoria (**Fig. 2.2**) during summer and winter. A biodiversity permit was obtained to collect lichen samples (BABS/001521N; Appendix 1). The winter samples were collected from the Melville Koppies and Bryanston in August 2021, while the Pretoria samples were collected in August 2022. The summer samples were collected in February 2022. Lichen samples were aseptically collected from trees using an autoclaved stainless-steel scalpel that was dipped in 70% (v/v) ethanol prior to each collection. The samples were stored in autoclaved glass jars. The samples were kept in a cooler bag with ice packs and transported to the University of the Witwatersrand. All downstream processing was completed on the same day as collection.

These lichen species were selected and identified based on morphological characteristics which included the location of the soredia, the type of rootlets, the appearance of the thallus, the surface pattern, the colour of the upper and lower surfaces, the presence or absence of fruiting bodies, isidia and cilia and the appearance of the cross-section of the lichen (*ISpot Keys | Foliose Lichens on Trees (v. 1.0)*, n.d.). These characteristics were put into the online interactive lichen identification guide: Foliose lichens grown on trees (v.1.0.) (*ISpot Keys | Foliose Lichens on Trees (v. 1.0)*, n.d.) to identify the lichen type down to the species level, and then cross-checked with Fryday's (2015) official South African lichen list to confirm that the identified lichen is found in South Africa (Fryday, 2015). The lichen species were identified as *Punctelia subrudecta* (**Fig. 2.1A**) and *Parmotrema perlatum* (**Fig. 2.1B**), respectively.

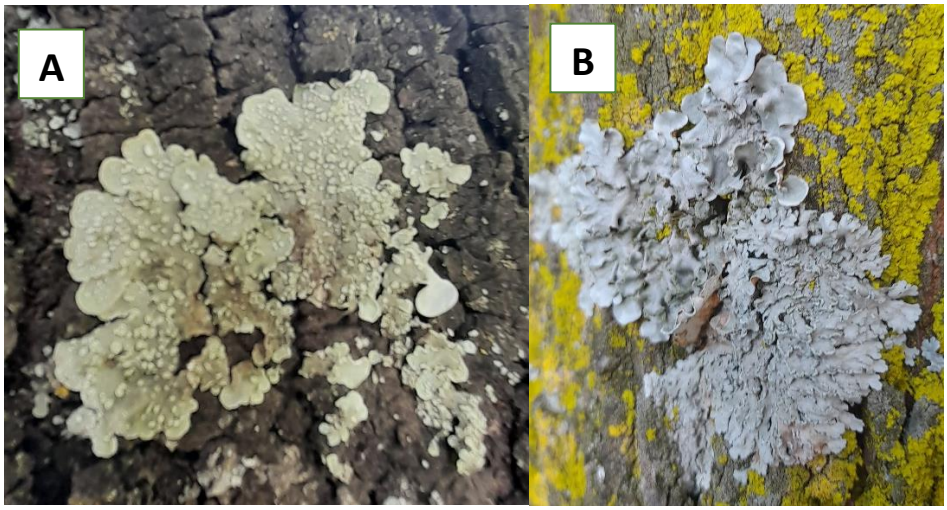


Figure 2. 1. Lichen samples. A- *Punctelia subrudecta* and **B**- *Parmotrema perlatum*. (Photos taken by Pascale Naude using Samsung A12 camera)

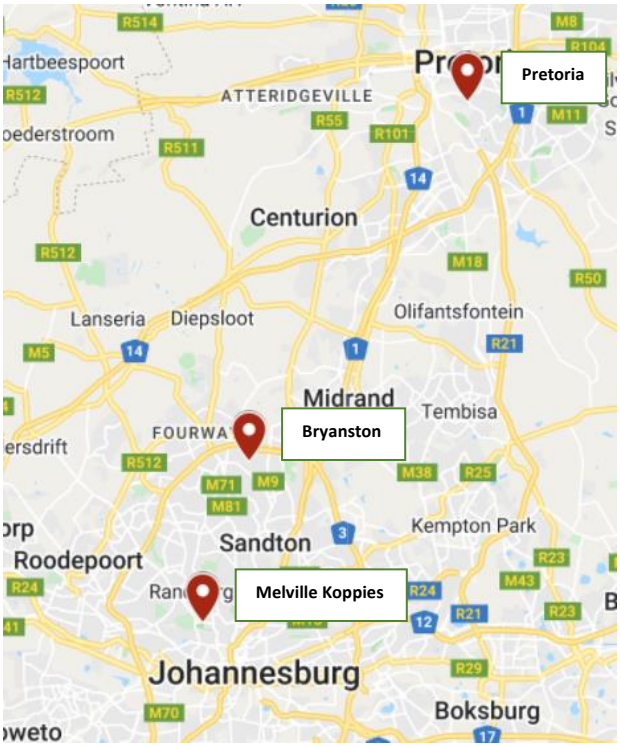


Figure 2. 2. Map of lichen sampling locations. Red pins indicate sampling areas. Melville Koppies (-26.169016, 28.005829); Bryanston (-26.046810, 28.043731) and Pretoria (-25.778829, 28.225244). All areas fall within the Gauteng Province of South Africa.

2.2. Lichen homogenisation

The lichen samples were aseptically cleaned of non-lichen material including bark, insects, dirt, and other debris using an autoclaved pair of tweezers. Lichen samples were sheared with autoclaved stainless-steel scissors and crushed with a mortar and pestle that was rinsed with 70% (v/v) ethanol. Tools were autoclaved prior to use to limit the introduction of microorganisms to the lichen samples.

2.3. Genomic DNA extraction

Total genomic DNA (gDNA) was extracted using the Zymobiomics DNA miniprep kit (ZymoBIOMICS, USA) according to the manufacturer's instructions with minor alterations. Briefly, a total of 0.1g of lichen was used as the starting weight, using three replicates per sample. Samples were subject to bead beating on the disruptor genie for eight minutes and then centrifuged at 14000xg for two minutes in the Heraeus Multifuge X1R (ThermoFisher Scientific) at 4°C. Subsequently, the extracted gDNA was eluted in 50µl of DNase/RNase free water rather than the recommended 100µl. The extracted gDNA was visualized on 0.8% (w/v)

agarose gel supplemented with 10µg/µl ethidium bromide under UV light to evaluate the quality of the gDNA (Lee *et al*., 2012; Sharp *et al*., 1973). The concentration and purity of the gDNA was determined using the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA).

The Internal Transcribed Spacer (ITS) (for the identification of fungi) and 16S ribosomal RNA (rRNA) (for the identification of bacteria) regions were amplified using polymerase chain reaction (PCR) on the Bio-Rad T100 Thermocycler with the ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS2R (5'-TGTGTTCTTCATCGATG-3') (Andreev *et al*., 1998), and 341F (5'-CCTACGGGN GGCWGCAG-3') (Klindworth *et al*., 2013) and 908R (5'-CCTACGGGNGGCWGC AG-3') (Lane *et al*., 1985) primers, respectively. The PCR amplification was undertaken using Biolabs Taq 5X Master mix (New England Biolabs) to confirm the presence of fungal and bacterial gDNA. The ITS PCR conditions were as follows: (1) An initial denaturation of 30 seconds at 95°C. (2) Denaturation of 95°C for 30 seconds. (3) Annealing at 55°C for 30 seconds. (4) Elongation at 68°C for 1 minute. (5) Steps (2) – (4) were repeated 32 times. (6) A final elongation of 68°C of 5 minutes. The 16S PCR conditions were similar to the ITS PCR conditions except that steps (2) – (4) were ran 30 times instead of 32 times. The amplicons were visualized on 0.8% (w/v) agarose gel supplemented with 10µg/µl ethidium bromide under UV light.

2.4. 16S rRNA and ITS based tagged amplicon pyrosequencing

The lichen gDNA extracts were submitted to MR DNA (Texas, USA) for 16S rRNA/ITS based tagged FLX amplicon pyrosequencing (bTEFAP) using Illumina Miseq per the manufacturers' instructions. This sequencing process has been commonly used for characterising microbiomes, both in the environment as well as health related microbiomes (Dowd *et al.*, 2008a; Dowd *et al.*, 2008b; Mohammadi-Aragh *et al.*, 2022). The 27F (5'-AGRGTTTGATCMTGGCTCAG-3' where R is A or G, M is A or C) (Frank *et al*., 2008) and 519R (5'-GTNTTACNGCGGCKGCTG-3' where N is any nucleotide and K is G or T) (Myer *et al*., 2016) primers were used to amplify the hypervariable regions V1-V3 of the 16S rRNA gene, and the ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990) primers amplified the ITS2 hypervariable region of the ITS gene sequences. Following sequencing, the

raw sequencing data was processed through the MR DNA analysis pipeline (*Sequencing Services*, n.d.). The MR DNA analysis pipeline started with joining the sequences, removing sequences with less than 150 base pairs and sequences that have unknown base pairs. Sequences were then quality filtered at a maximum expected threshold of 1.0 and dereplicated. Following dereplication, the sequences were denoised, chimeras were removed, sequences were then cleaned and removed. Finally, zero-radius Operational Taxonomic Units (zOTUs) were identified at different taxonomic levels by running the denoised sequences against a databased created on BLASTn obtained from National Centre for Biotechnology Information (NCBI). Zero-radius OTUs , otherwise known as amplicon sequence variants (ASVs), were determined instead of traditional OTUs as zOTUs cluster closely matching sequences and find single nucleotide differences (Callahan *et al.*, 2017).

2.5. 16S rRNA and ITS data processing

Reads belonging to the primary mycobiont (i.e., *Flavopunctelia* and *Parmotrema*) and the photobiont *Trebouxia aggregata* were first removed as these dominated the read datasets. Removal of these ensured that only the non-mycobiont and non-photobiont mycobiome and bacteriome, respectively, were analysed. Subsequently the read data was rarefied. Rarefaction is the process whereby reads from large samples are randomly discarded until the total number of reads is equal to the smallest number of reads (Sanders, 1968), this is done to aid in the analysis of diversities (Willis, 2019). Bar graphs were constructed on the rarefied read datasets for both the bacteriome and mycobiome at the different taxonomic ranks.

Operational Taxonomic Unit, metadata, and taxonomic data tables were created for both the 16S rRNA and ITS data, with the main photobiont and lichen species reads being removed for the 16S and ITS data, respectively. These tables were then uploaded into Rstudio (v.4.1.3) (R Core Team, 2022). Once in R, the OTU and taxonomy tables were combined via the OTU ID column using the “dplyr” package (Wickham, François, *et al*., 2023). The combined data was then converted into a taxmap format. Low abundance counts were removed, the data was rarefied, and a rarefaction curve was generated. From there, the subsequent analyses were conducted on the processed and rarefied data to determine the breakdown of the microbiome, the alpha and beta diversities and the effects of different factors (the lichen genus, sampling location and season) on the composition and diversity on the microbiome.

2.6. The lichen microbiome in terms of weather factors

Weather data (total daily rainfall (mm), and the daily maximum and minimum temperatures (°C)) was obtained from the South African Weather Service for the period between February 2021 to August 2022 (Disclosure statement; Appendix 3). The weather stations from which the weather data was collected were the Johannesburg Botanical Gardens (-26.156000, 27.999000 1624m), to account for the samples collected from the Melville Koppies and Bryanston, and the Pretoria University Proefplaas (-25.752000, 28.258000 1380m) to account for the samples collected from Pretoria. For each collection period, the average maximum, and minimum temperatures (°C) and total rainfall (mm) was calculated for six months prior to the collection. This was to account for differences in the maximum and minimum temperatures, and total rainfall between the collection locations and collection times. These values were used in downstream statistical analyses which will be discussed in the subsequent sections.

2.7. The core microbiome

The core microbiome is defined as the microorganisms that are present in an environment which are characteristic of that specific environment (Turnbaugh *et al.*, 2007; Risely, 2020; Neu *et al*., 2021). One method to determine the core microbiome is by calculating the relative abundance of taxa across all samples which is a measure of the percentage that a microorganism occupies the microbiome (https://biomcare.com/services/absolute-bacterial-abundance). By calculating the relative abundance, the core microbiome is identified by determining the most abundant species that are the most prevalent across all samples (Shetty *et al*., 2017). The relative abundances for both the 16S and ITS OTU data were calculated in RStudio (v.4.1.3) (R Core Team, 2022) to determine the core microbiome. Firstly an object was created from the rarefied data and metadata tables using the function "as_phyloseq" from the "phyloseq" package (McMurdie and Holmes, 2013). The "microbiome::transform" function from the "microbiome" package (Lahti and Shetty, 2023) was then used to transform the data into compositional data. The relative population frequencies at a 1% compositional threshold were calculated with the "prevalence" function from the microbiome package (Lahti and Shetty, 2023). The core taxa were calculated at a 1% detection threshold and at 50% prevalence using the "core" function from the "microbiome" package (Lahti and Shetty, 2023) on the relative population frequencies. A heatmap was then plotted with the relative population frequencies using the "plot_core" function from the "microbiome" package (Lahti and Shetty, 2023) and the "RColorBrewer" (Neuwirth, 2022) and "reshape" (Wickham, 2022) packages.

2.8. The taxonomic breakdown of the lichen microbiome

Bar graphs of the bacteriome and mycobiome at the phylum level were constructed to illustrate the differences between samples. To compare the breakdown of the genera of the microbiome across lichen samples to one another and to the members identified as the core microbiome, heatmaps comparing the different lichen genera, sampling locations and seasons were generated using the rarefied genera counts for the 16S rRNA and ITS data. Tables comprising the read counts for the twenty most abundant genera (for both the mycobiome and bacteriome samples) were uploaded in Rstudio (v.4.1.3.) (R Core Team, 2022) to construct the heatmaps with the libraries "gPlots" (Warnes *et al*., 2022), "Heatplus" (Ploner, 2023), "vegan" (Oksanen *et al*., 2022) and "RcolorBrewer" (Neuwirth, 2022). The data was proportionalised and a dendogram was constructed using the Bray Curtis method (Bray and Curtis, 1957).

2.9. 16S rRNA and ITS alpha diversity

Alpha diversity is a measure of the richness (the number of taxonomic groups) and/or evenness (the dispersal of abundances) within a single community (Willis, 2019). This measure is commonly used to demonstrate the impact of environmental factors on community structures (Willis, 2019). The alpha diversities were calculated on Rstudio (v.4.1.3) (R Core Team, 2022) to determine the diversities within the individual samples (Andermann *et al*., 2022) and to determine the influence of the lichen species, sampling location and season on alpha diversity. To calculate the alpha diversities, the object created previously to identify the core microbiome using the "phyloseq" package (McMurdie and Holmes, 2013) and the "diversity" function from the "vegan" package (Oksanen *et al*., 2022) were used to calculate the alpha diversity indices. It was then determined whether the data followed a normal distribution using the Shapiro-Wilk test for normality (King and Eckersley, 2019) for the Observed OTU richness (number of taxons per sample) (Stegen *et al*., 2016) and the Inverse Simpson index (the Simpson index which indicates richness with a constant evenness) (Simpson, 1949). For the indices found to follow a normal distribution, sample replicates were grouped, and Analysis of variance (ANOVA) tests were calculated to determine any significant differences in the alpha diversities between the lichen samples. ANOVA is used to determine for significant differences in the mean between samples (Chambers and Hastie, 2017). ANOVA analyses were conducted using the "aov" function from the "dplyr" package (Wickham, François, *et al*., 2023), which calculates a one way ANOVA, and the Tukey "HSD.test" function from the "agricolae" package (Mendiburu, 2021), which makes multiple pairwise comparisons between groups. A

plot object using the "ggplot" function from the "ggplot2" package (Wickham, Chang, *et al*., 2023), was used to visualise the ANOVAs. For those samples that were found to not follow a normal distribution after calculating the Shapiro-Wilk test, the alpha diversities were evaluated using the Kruskal-Wallis rank sum test (Hollander *et al.*, 2014). The Kruskal-Wallis test is a non-parametric equivalent to the ANOVA which determines whether different groups have the same mean (Xia, 2020). The Kruskal-Wallis test was conducted by using the "kruskal.test" function from the "stats" package (R Core Team, 2022) to determine if there were differences between climate, location and species.

2.9. 16S rRNA and ITS beta diversity

Beta diversity is the ratio of diversity between the gamma (total diversity within a region) and individual alpha diversity (previously described) (Whittaker, 1960, 1972). It is important to calculate the beta diversity when working with microbial data because the taxonomic or functional diversity can then be linked to environmental factors (Knight *et al*., 2018). The beta diversity of the data was generated using the objects that were created previously to calculate the alpha diversity. The vegan function "vegdist" (Oksanen *et al*., 2022) was used to calculate the pairwise beta diversity indices for the samples using the rarefied OTU table combined with the metadata. Then the "metaMDS" function from the "vegan" package (Oksanen *et al*., 2022) was used to create a Multidimensional scaling (MDS) plot (illustrates the dissimilarity between samples across two dimensions) (Faith *et al.*, 1987), using the Bray Curtis index which measures beta diversity by taking into account both the abundance per sample and the abundance of each taxon within each sample (Bray and Curtis, 1957). The MDS plots were built using ggplots.

Beta dispersion was calculated using the "betadisper" and "permutest" functions from the "vegan" package to determine the deviation from the mean community structure between sample groups under different conditions (Oksanen *et al*., 2022). A Permutational multivariate analysis of variance using distance matrices (PERMANOVA) (McArdle and Anderson, 2001) was also calculated with the "adonis2" function from the "vegan" package (Oksanen *et al*., 2022), with the Bray-Curtis indices. The "adonis2" function was developed based off the work from McArdle and Anderson (2001) (McArdle and Anderson, 2001) and is very similar to a Multivariate analysis of variance (MANOVA) (Anderson, 2001; McArdle and Anderson, 2001).

The average maximum and minimum temperatures and total rainfall that was calculated earlier were factored into the statistical analyses conducted on RStudio (v 4.1.3) by including them into the metadata tables. These values were then compared using the beta dispersion and PERMANOVAs as described previously to determine whether the weather conditions had any influence on the microbiome.

Chapter 3: Results

3.1. Lichen identification

Two green and grey foliose lichen types were found to be most abundant on trees in the sampled locations during both summer and winter sampling periods (**Fig. 2.2**). By comparing their morphological characteristics (colour, presence or absence of cilia, soredia) to online lichen identification resources (Fryday, 2015; *ISpot Keys | Foliose Lichens on Trees (v. 1.0)*, n.d.), they were identified as belonging to the genera *Parmotrema* and *Punctelia*. Lichens identified as *Parmotrema* had a grey upper cortex colour, the lower cortex was black, there was the presence of cilia, the thallus was absent of reproductive structures like isidia and soredia while margins were present on the edges of the lobes (**Fig. 2.1A**). *Punctelia* displayed an upper cortex that was green in colour, the lower cortex was black, cilia were absent, while soredia were present on the thallus surface (**Fig. 2.1B**). These two lichen types were collected, their gDNA was extracted and fungal and bacterial diversity associated with the lichens were analysed through 16S rRNA/ITS based tagged amplicon pyrosequencing.

3.2. Lichen microbiome

After quality filtering, a total of 1,101,434 (averages reads/sample: 30,595; {average across 36 samples = 2 lichen species x 3 sample locations x 2 seasons x 3 replicates}) and 1,887,478 (average reads/sample: 52,430) reads were obtained from 16S rRNA (bacterial) and ITS (fungal) amplicon sequencing, respectively. OTU clustering (>97% sequence identity) resulted in 719 (average OTUs/sample: 20) and 474 (average OTUs/sample: 13) distinct OTUs for the bacterial and fungal counterpart, respectively. This suggests that overall, there is greater bacterial OTU diversity than fungal OTU diversity associated with the sampled lichens. On average, there was a consistent number of bacterial reads per winter sample (average reads: 30,529; average OTUs: 29; {averages across 18 samples = 2 lichen species x 3 sample locations x 3 replicates}) as the summer sample (average reads: 30,662; average OTUs: 25). In contrast, the fungal reads from the summer lichen samples (average reads: 63,043; average OTUs: 17) were higher than the reads from winter (average reads: 41,816; average OTUs: 18). The bacterial reads were similar in number between Bryanston (average reads: 30,947; average OTUs: 45 {average across 12 samples = 2 lichen species x 2 seasons x 3 replicates}), Melville (average reads: 31,117; average OTUs: 41) and Pretoria (average reads: 29,722; average OTUs: 42). However, the fungal reads were not as similar across the sampling locations, where the most reads were observed for lichen samples from Pretoria, (average reads: 58,785; average

OTUs: 28), followed by Melville (average reads: 52,374; average OTUs: 34) and Bryanston (average reads: 46,131; average OTUs: 32).

The total bacterial reads were found to be similar between the two lichen genera *Parmotrema* (average reads: 30,719; average OTUs: 39 {average across 18 samples = 2 seasons x 3 sampling locations x 3 replicates}) and *Flavopunctelia* (average reads: 30,472; average OTUs: 39). In contrast there were fewer fungal reads in the *Parmotrema* (average reads: 48,526; average OTUs: 26) samples compared to the *Flavopunctelia* (average reads: 56334; average OTUs: 25) samples. This increased abundance in the *Flavopunctelia* samples compared to the *Parmotrema* samples in the fungal reads is attributed to the higher abundance of reads of the mycobiont *Flavopunctelia* compared to *Parmotrema*. The main mycobionts (thus the lichen species) made up 357,921 (19%; average reads/sample: 9,942) and 782,405 (42%; average reads/sample: 21,733) of the total reads from the *Parmotrema* and *Flavopunctelia* fungal ITS read sets. When the *Flavopunctelia* and *Parmotrema* reads are removed from the ITS data, the *Parmotrema* samples (average reads: 27,994) have a higher abundance than the *Flavopunctelia* (average reads: 13,515) samples. The main photobiont of both sampled lichen types was found to be the green alga *Trebouxia aggregata*. This photobiont contributed a total of 661,199 (~60% of the 16S rRNA reads; average reads/sample: 18,367) reads.

3.3. Weather data

To account for seasonal differences and differences between lichen sampling locations, weather data was obtained from the South African Weather Service for two stations, the Johannesburg Botanical Gardens; to account for the Melville Koppies and Bryanston lichen samples, and Pretoria University Proefplaas to account for the Pretoria lichen samples. Lichen samples were collected towards the end (February) of summer (warm and high levels of precipitation) and the end (August) of winter (cooler and drier). To determine the difference between the locations and the season, the average temperatures and total rainfall for six months prior to the sample collection (winter: February 2021 to August 2021 and February 2022 to February 2022; summer: August 2021 to February 2022) were calculated (**Table 3.1**). From the data, it could be observed that, on average, Pretoria experienced slightly warmer temperatures than Johannesburg, with average maximum temperatures for summer and winter being 1.9 °C and 1.5 °C higher, respectively, and average minimum temperatures for summer and winter being 2.0 °C and 2.1 °C higher for Pretoria, respectively. The coldest day within

the winter sampling period (within the six months prior to collection) had an average temperature of 6.45°C in Johannesburg and 10.6°C in Pretoria. The hottest day within the summer sampling period (within the six months prior to collection) in Johannesburg had an average temperature of 23.5°C, while the hottest day within Pretoria during the summer sampling period had an average temperature of 25.05°C.

Only marginal differences could be observed between the Pretoria and Johannesburg weather stations for the summer period. However, of note for the current study, is that more than double the amount of rainfall was observed for the winter period in Pretoria compared to Johannesburg. The most rainfall that occurred in a day in Johannesburg during the winter sampling period was 19.6 mm, while the most rainfall that occurred in a day in Pretoria during the winter sampling period was 82.4mm.

The average daily minimum and maximum temperatures and total rainfall were factored into the statistical analyses performed by including them as parameters in the meta data to determine their significance on the composition and distribution of the lichen microbiome between locations and seasons. Since the Johannesburg station was made to account for both the Melville Koppies and Bryanston locations, differences between these locations could not be determined.

Table 3. 1. Collated weather data over six months prior to lichen collections.

Location	Collection Date	Average daily Maximum Temperature (°C)	Average daily Minimum Temperature (°C)	Total rainfall (mm)
JHB Botanical Gardens	Winter (February 2021-August 2021)	22.3	7.8	153.4
JHB Botanical Gardens	Summer (August 2021-February 2022)	25.2	11.5	850.3
Pretoria University Proefplaas	Summer (August 2021-February 2022)	27.3	13.5	844.6
Pretoria University Proefplaas	Winter (February 2022-August 2022)	23.8	9.9	391

3.4. The core microbiome

To identify what members make up the core lichen microbiome (Turnbaugh *et al.*, 2007; Risely, 2020; Neu *et al.*, 2021), the relative abundance was calculated at a 1% detection threshold. The core bacterial microbiome was found to be comprised of fourteen species belonging to nine genera (**Fig. 3.1**), namely *Methylosinus*, *Terriglobus*, *Granulicella*, *Methylobacterium*, *Sphingomonas*, *Acidisphaera*, *Beijerinckia*, *Rhodopila* and *Candidatus solibacter*. Of these core bacterial components, two bacterial genera, *Methylosinus* (100% prevalence) and *Terriglobus* (100% prevalence), were present in all thirty-six samples.

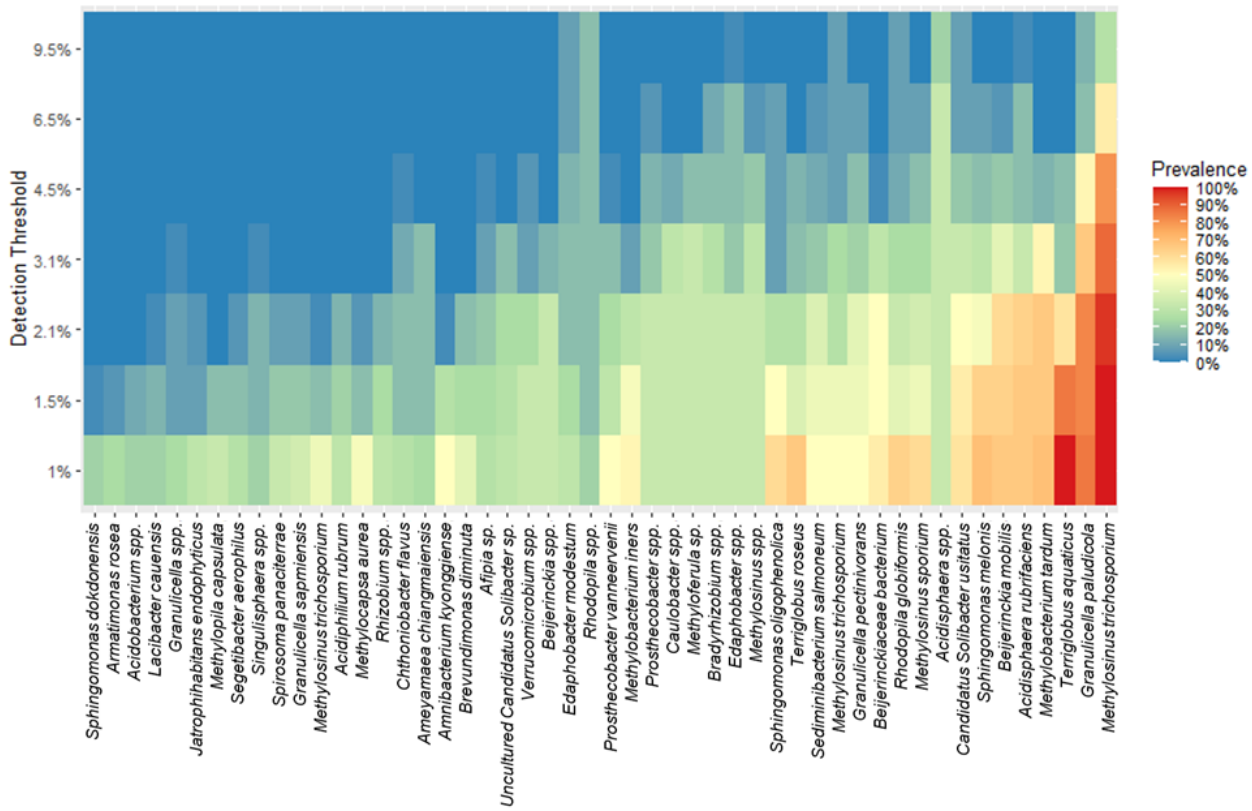


Figure 3. 1. Prevalence of core bacterial taxa in lichen microbiome. The heatmap shows the most prevalent Operational Taxonomic Units across all lichen samples. The core bacterial taxa were determined based off a prevalence above 50% at a 1% detection threshold.

The core lichen mycobiome was comprised of seven species from seven distinct genera (**Fig 3.2**), namely *Curreya*, *Microcera*, *Cladophialophora*, *Austroafricana*, *Knufia*, *Physconia* and *Penidiella*. None of the identified core members were present throughout all the lichen samples, although *Curreya* was found to be the most prevalent, being present in 81% of samples. *Cladophialophora* and *Microcera* were also highly prevalent, being present in 75% of samples.

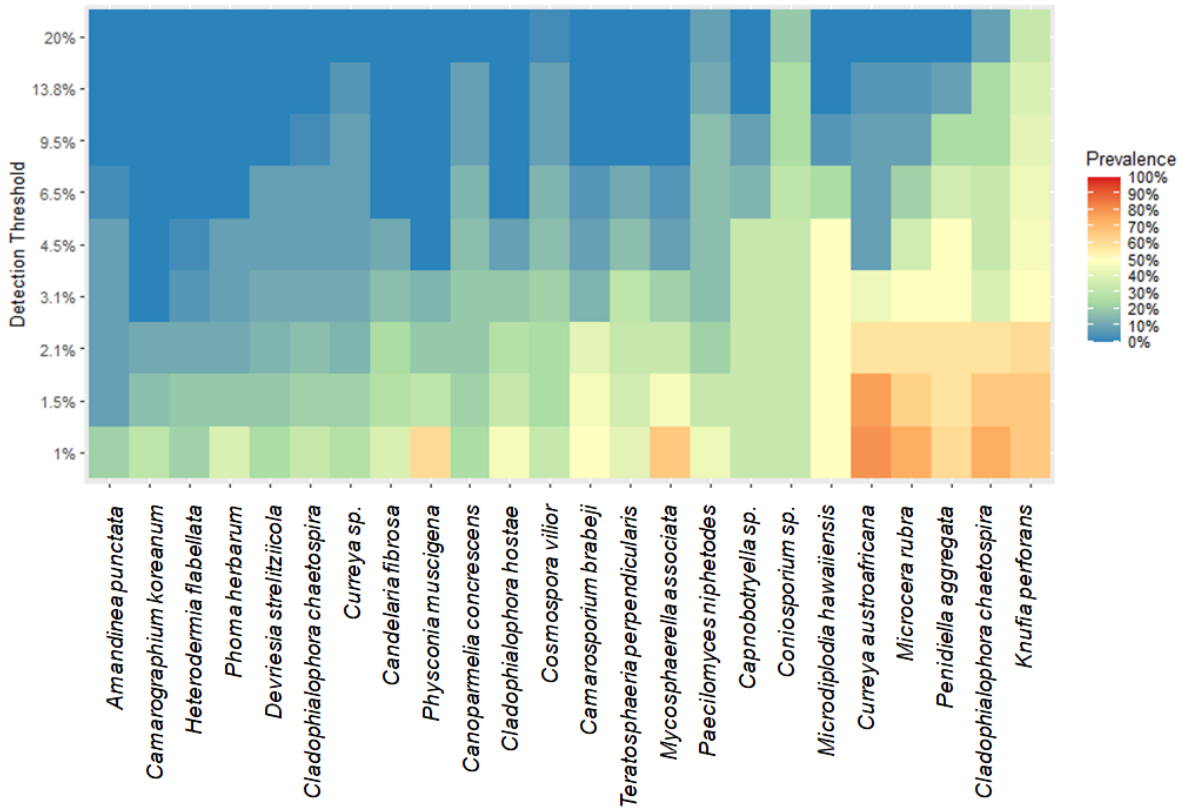


Figure 3. 2. Prevalence of core fungal taxa in lichen microbiome. Along the x-axis is the most prevalent Operational Taxonomic Units across all lichen samples. The core fungal taxa were determined based off a prevalence above 50% at a 1% detection threshold.

3.5. Taxonomic composition of lichen microbiome

To gain a perspective of the broader taxonomic composition of the lichen microbiome, the bacterial and fungal data was analysed at the phylum level. The bacterial counterpart is predominated by the phylum Proteobacteria (60% of total bacteria across all samples) followed by Acidobacteria (18% of total bacteria) (**Fig. 3.3**). Bacteroidetes was the third most abundant phylum (8% of total bacteria).

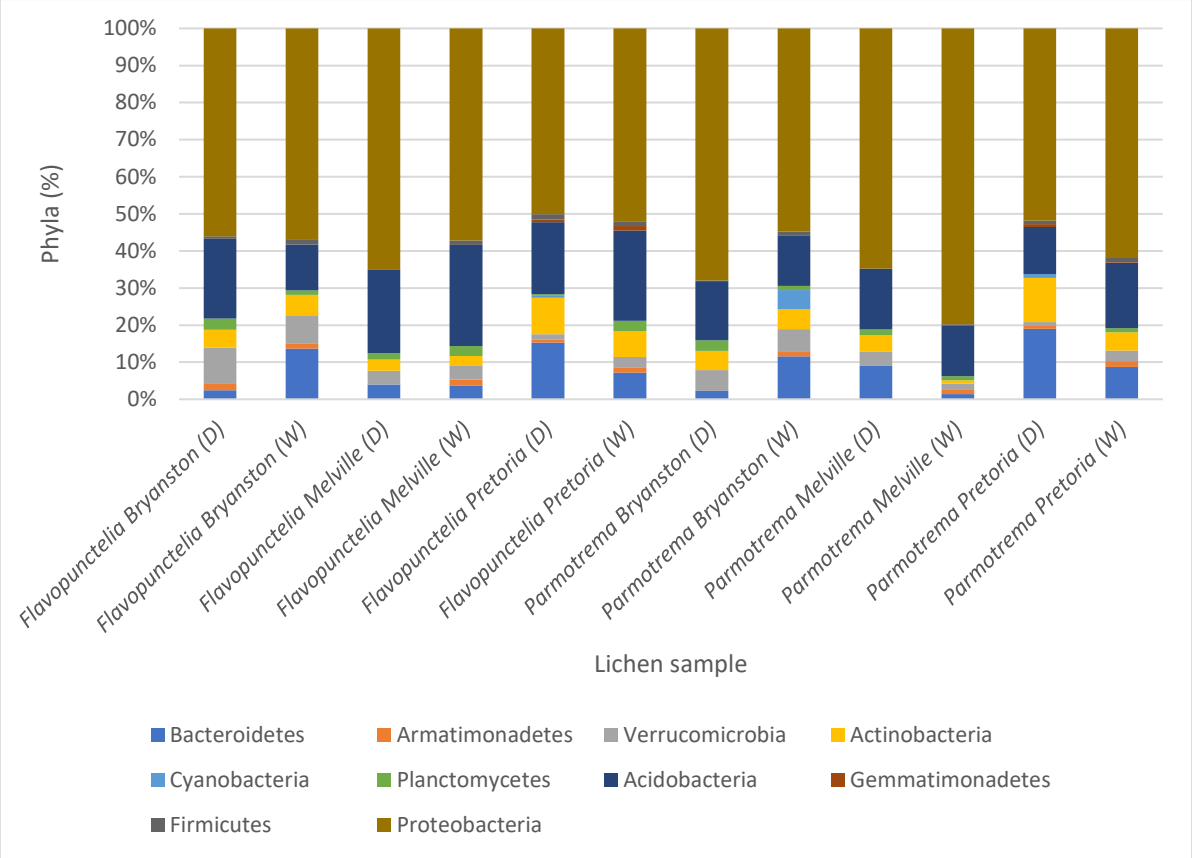


Figure 3. 3. Taxonomic breakdown at the phylum level of the lichen bacteriome in terms of abundance. D indicates winter (the dry season) while W indicates summer (the wet season). Sample replicates were combined and averaged (n=3).

The fungal counterpart of the lichen microbiome is predominated by members of the phylum Ascomycota across all samples (99% of total fungi) and, at a much lower abundance, Basidiomycota (0.75%) (**Fig. 3.4**).

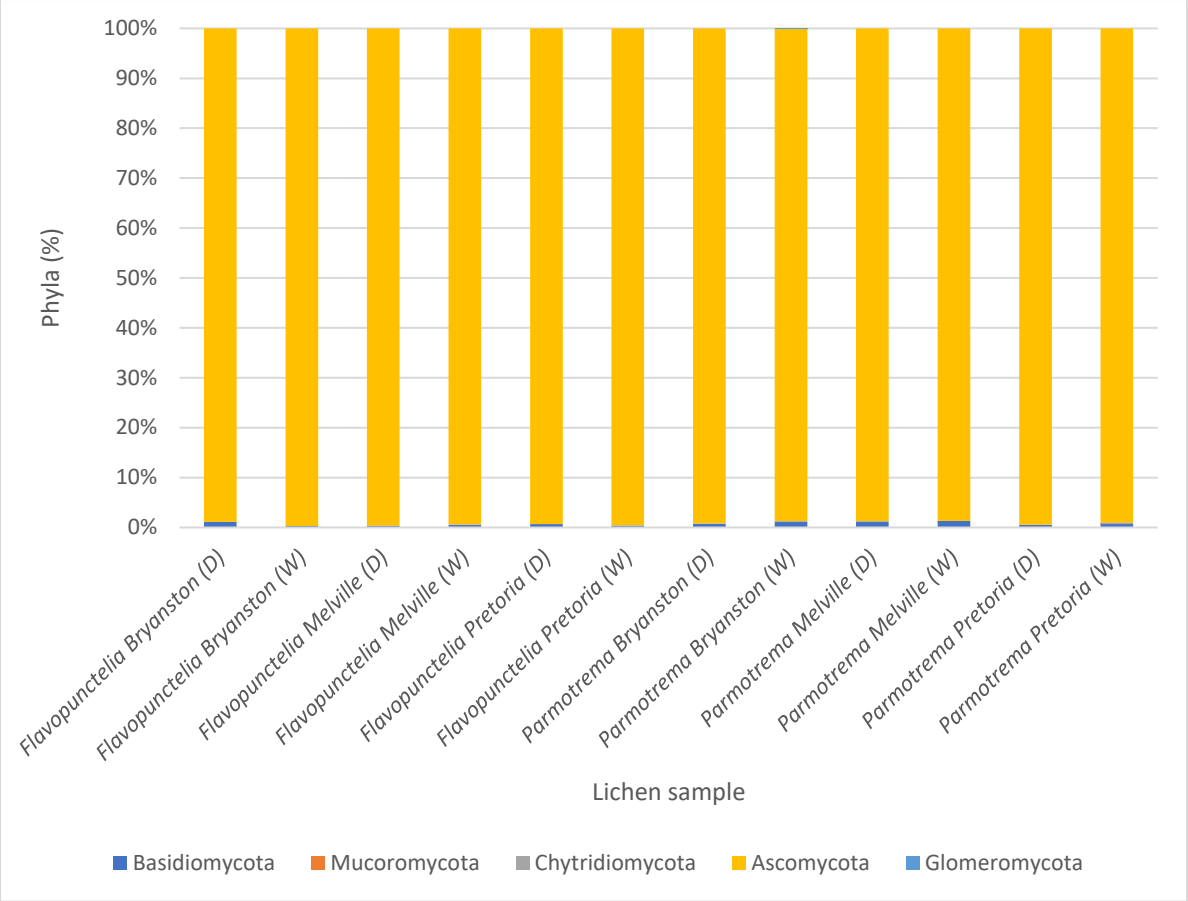


Figure 3. 4. Taxonomic breakdown at the phylum level of the lichen mycobiome in terms of abundance. D indicates winter (the dry season) while W indicates summer (the wet season). Sample replicates were combined and averaged (n=3).

Heatmaps were generated of the twenty most abundant bacterial (**Fig. 3.5**) and fungal (**Fig. 3.6**) genera. The bacterial genera identified as the core members are from the phyla Proteobacteria (*Methylosinus*, *Sphingomonas*, *Acidisphaera* and *Methylobacterium*) and Acidobacteria (*Granulicella* and *Terriglobus*) and these genera were observed to be abundant across all lichen samples. *Methylosinus* is the most abundant genus, contributing 14% of the entire bacteriome across all thirty-six samples (average reads: 536; {average across 36 samples = 2 lichen species x 3 sample locations x 2 seasons x 3 replicates), but also occurs at an exceptionally high abundance in the *Parmotrema* sample collected from Melville during summer (1,863 reads per sample; {3 x replicates}). *Sphingomonas* (10%) was also abundant across all lichens. *Granulicella* (7%), *Acidisphaera* (5%) and *Methylobacterium* (6%) occurred at high abundances within the lichen bacteriome but were not as evenly distributed across all lichen samples. *Granulicella* was found to be more abundant in the samples collected in

summer (average reads: 413; {average across 18 samples = 2 lichen species x 3 sample locations x 3 replicates) compared to winter (average reads: 311), while *Acidisphaera* occurred at higher proportions in the winter samples (average reads: 306) compared to the summer samples (average reads: 95). *Methylobacterium* contributed 6% of the total bacterial reads but was influenced by location as it was least abundant in the samples collected from Melville (average reads: 51; {average across 12 samples = 2 lichen species x 2 seasons x 3 replicates) but was highly abundant in the samples collected from Pretoria samples (average reads: 354), while also having a high number of reads in Bryanston (average reads: 234). *Terriglobus* contributes 4% of the total bacterial reads.

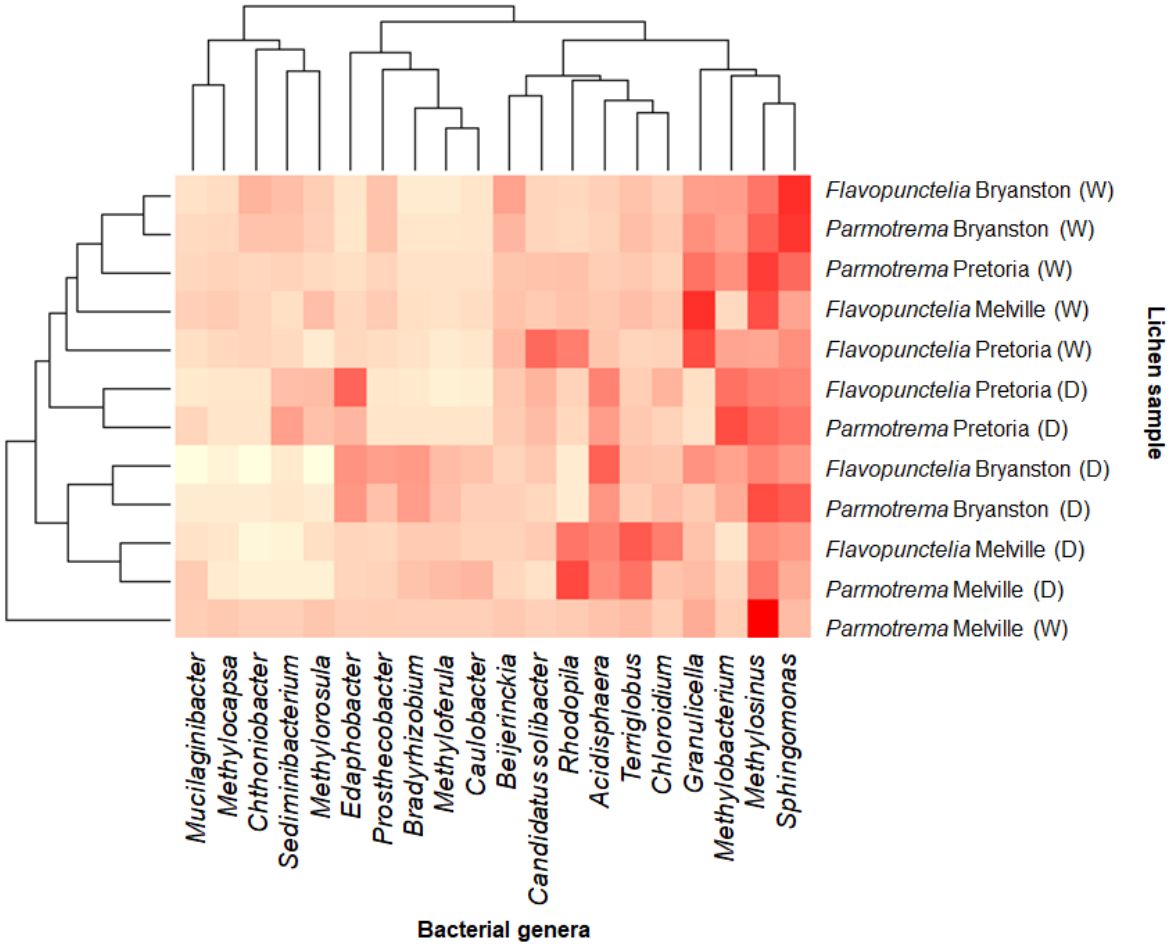


Figure 3. 5. Heatmap for twenty most abundant bacterial genera present across all lichen samples. The range from least abundant to most abundant is indicated by the colour range from white/yellow to red. W indicates summer (the wet season) from January to March where lichens were collected in February while D indicates winter (the dry season) collected in August. Samples replicates combined and averaged (n=3).

When considering the fungi, all core fungal genera are from the phylum Ascomycota. *Curreya* contributed 5% of the total mycobiome (average reads per sample: 224 {average across 36 samples = 2 lichen species x 3 sample locations x 2 seasons x 3 replicates) but more reads were observed in the *Parmotrema* sample collected in summer from Pretoria (average reads per sample: 657 {3 x replicates}) and *Flavopunctelia* from Melville in winter (average reads: 630 {3 x replicates}). *Cladophialophora* (10% of total mycobiome; average reads per sample: 476; 36 samples) was highly abundant in the samples collected in winter (average reads per sample: 615 {average across 18 samples = 2 lichen species x 3 sample locations x 3 replicates) compared to the samples collected in summer (average reads: 336) but was also highly abundant in the Pretoria samples collected in the summer (average reads: 1,002 {3 x replicates}). In contrast, *Knufia* (13% of total mycobiome; average reads: 618) was only observed in samples collected during summer (average reads: 934; 18 samples) across all three locations and in winter in lichens collected from Pretoria (average reads: 903 {average across 6 samples = 2 lichen species x 3 replicates). *Microcera* (4% of total mycobiome; average reads: 180; 36 samples) was also observed to be more abundant in the lichens collected during winter (average reads: 320; 18 samples) compared to lichens collected during summer (average reads: 41).

Outside of the genera identified as members of the core microbiome, other fungal genera were highly abundant throughout some lichen samples. *Coniosporium* (6% total mycobiome; average reads: 293; 36 samples) is highly abundant in the samples collected in Bryanston and Melville in winter (average reads: 876 {average across 12 samples = 2 locations x 2 lichen genera x 3 replicates) but is not present in any summer samples. *Paecilomyces* (average reads: 293 {average across 36 samples = 2 lichen species x 3 sample locations x 2 seasons x 3 replicates}) is seen to be highly abundant in only one lichen sample, *Parmotrema* collected from Melville in summer (average reads: 2626 {3 x replicates}).

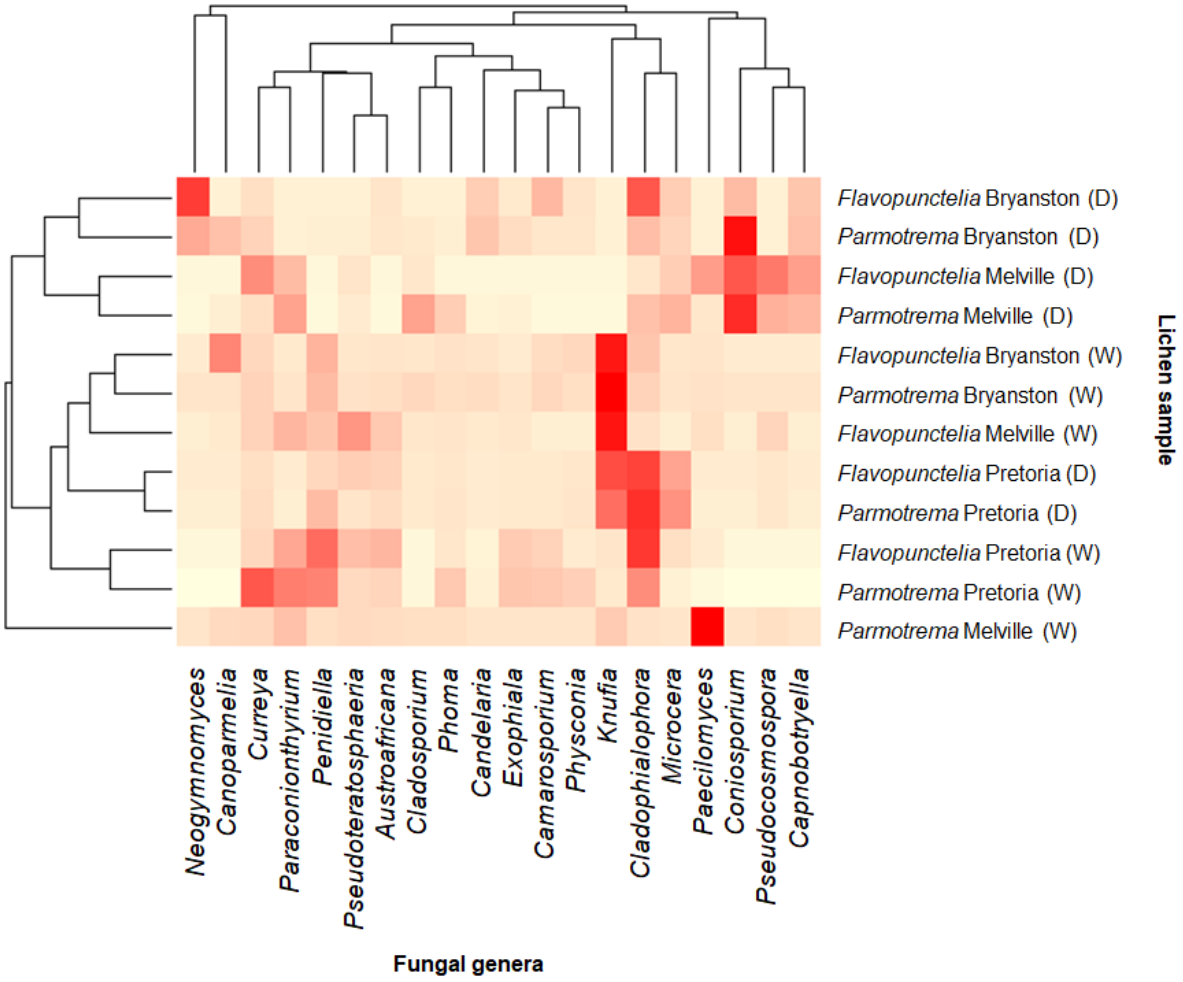


Figure 3. 6. Heatmap for twenty most abundant fungal genera present across all lichen samples. The range from least abundant to most abundant is indicated by the colour range from white/yellow to red. W indicates the wet season from January to March where lichens were collected in February while D indicates the dry season collected in August. Samples replicates combined and averaged (n=3).

3.6. Bacterial and fungal alpha diversity in lichens

The Shapiro-Wilk test for normality was used to determine which alpha diversity indices followed a normal distribution (King and Eckersley, 2019). Data is found to follow a normal distribution when the p-value achieved from the Shapiro-Wilk test is > 0.05 (King and Eckersley, 2019). Bacterial alpha-diversity was found to follow a normal distribution for the Observed OTU richness ($p = 0.5883$), Chao 1 ($p = 0.5883$), ACE ($p = 0.3315$) and Fisher (0.5946) indices ($p > 0.05$), while the fungal diversity was found to follow a normal distribution for the Inverse Simpson ($p = 0.6078$) and ACE indices ($p = 0.7074$) ($p > 0.05$). Since the Observed OTU richness followed a normal distribution for the bacterial data, and the Inverse Simpson followed a normal distribution for the fungal data, the observed OTU richness was

used to calculate Analysis of variance (ANOVA) (Chambers and Hastie, 2017) for the bacterial alpha diversity (**Fig. 3.7**) and the Inverse Simpson was used to calculate ANOVA for the fungal alpha diversity (**Fig. 3.8**). The ANOVAs were calculated to determine if there were statistical differences in the alpha diversities between the lichen samples.

The observed OTU richness refers to the number of taxa in a sample (Stegen *et al*., 2016), thus the greater the Observed OTU richness, the more taxa present, and the greater the richness. The bacterial alpha diversity in the summer lichen samples was observed to be higher than the winter samples, except for the *Flavopunctelia* samples from Pretoria, where the winter sample had a higher diversity (**Fig. 3.7**). This suggests that the warmer, wetter summer season may serve as a driver for bacterial species accrual in the lichen microbiome.

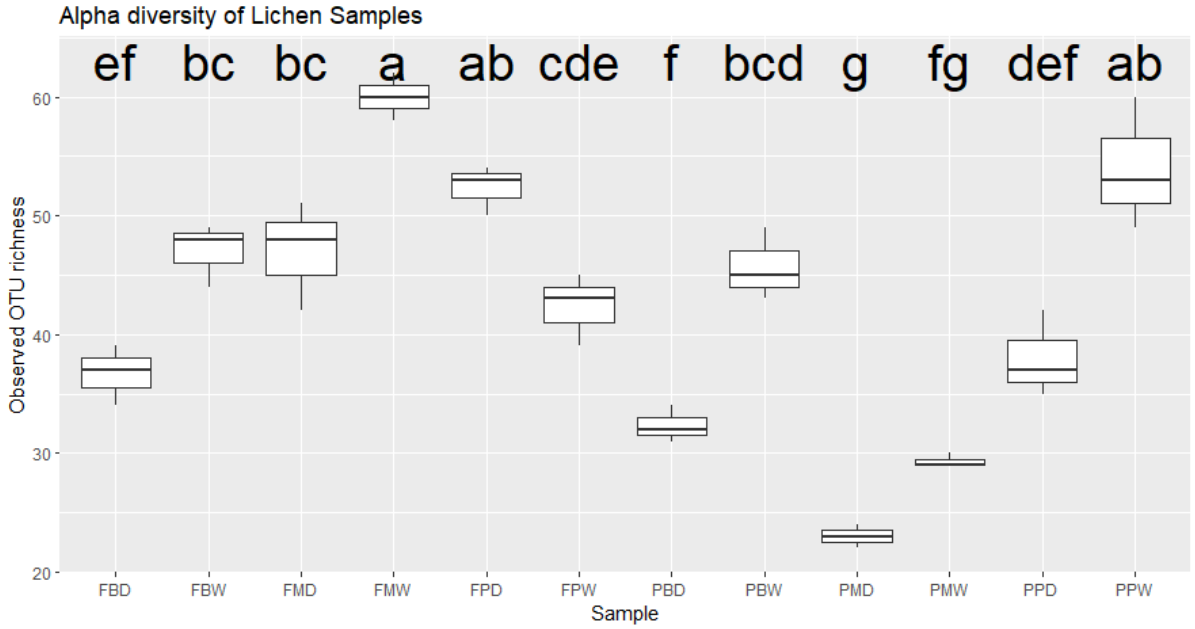


Figure 3. 7. Analysis of Variance of the bacterial alpha diversity within lichen samples using the Observed Operational taxonomic unit richness index. Sample replicates were grouped together making a boxplot (n=3). The first letter in the three letter codes denotes the lichen genus – *Flavopunctelia* (F) or *Parmotrema* (P). The second letter denotes the sample location – Bryanston (B), Melville Koppies (M) or Pretoria (P). The third letter denotes the season – Winter/dry season (D) or summer/wet season (W).

The Inverse Simpson is a measure of richness with a constant evenness (Simpson, 1949), thus the greater the Inverse Simpson, the greater the richness. In contrast to the bacteria, fungal alpha diversity does not follow a clear trend with respect to the summer and winter sampling

(**Fig. 3.8**). This would suggest that season does not play as much of a role in determining fungal diversity as it does bacterial diversity.

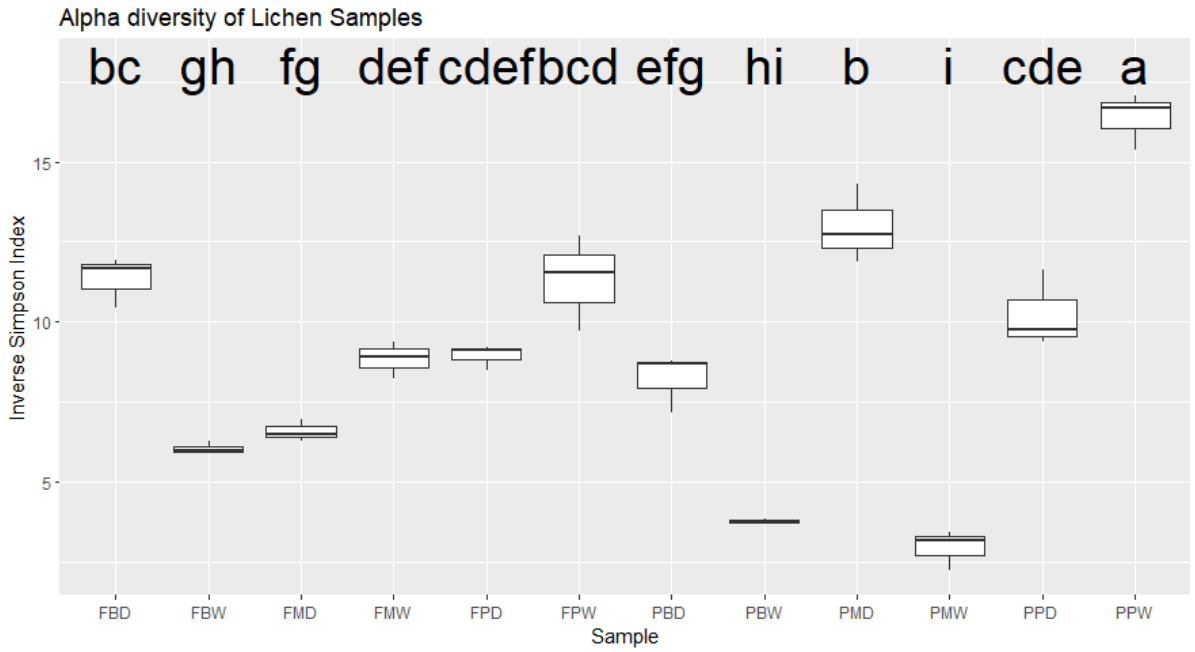


Figure 3. 8. Analysis of Variance of the fungal alpha diversity within lichen samples using the Inverse simpson index. Sample replicates were grouped together making a boxplot (n=3). The first letter in the three letter codes denotes the lichen genus – *Flavopunctelia* (F) or *Parmotrema* (P). The second letter denotes the sample location – Bryanston (B), Melville Koppies (M) or Pretoria (P). The third letter denotes the season – Winter/dry season (D) or summer/wet season (W).

The Kruskal-Wallis rank sum test, the non-parametric equivalent of the ANOVA (Xia, 2020), was used to further determine if there were differences between the lichen genera, sampling location and season based off the alpha diversities that followed a non-normal distribution, and to determine whether it corroborates the findings of the ANOVA analyses. The Inverse Simpson Index was evaluated for the bacterial data (**Table 3.2**) while observed OTU richness was evaluated for the fungal counterpart (**Table 3.3**).

From the analysis (**Table 3.2**), a significant difference between summer and winter ($p = 0.01359$, $p < 0.05$) was observed for the bacteria, while the other factors, the lichen genus with which bacteria are associated and sampling location do not exhibit significant differences ($p > 0.05$).

The heatmaps of the twenty most abundant bacterial genera (Figure 3.5) also show that, with the exception of the *Parmotrema* samples from Melville collected in summer, the summer samples cluster together, and the winter samples cluster together, indicating that the core microbiome is structured according to season. This clustering according to season corroborates the observed increase in diversity in the summer samples compared to the winter samples (**Fig. 3.7**) and the significant difference between the seasons calculated from the Kruskal-Wallis test (**Table 3.2**). The samples were also seen to cluster according to location, which is not supported by the ANOVA and Kruskal-Wallis test, whereby the Bryanston samples within the same season cluster together, the Melville samples from winter cluster together and the Pretoria samples from winter cluster together.

Table 3. 2. Kruskal Wallis test of bacterial alpha diversities using the Inverse Simpson Index. Sample refers to each individual sample with the triplicates combined. Genus refers to the two different lichen genera (mycobionts) collected, namely *Parmotrema* and *Flavopunctelia*. The location is between the three sampling locations which are the Melville Koppies, Bryanston and Pretoria, and season is between summer and winter. Df – degrees of freedom.

Parameter	**chi-squared**	**Df**	**p-value**
Sample	33.084	11	0.00051
Genus	1.0901	1	0.2965
Location	2.7673	2	0.2507
Season	6.0901	1	0.01359

The lichen genus ($p = 0.004144$, $p < 0.05$) and the sampling location ($p = 0.008849$, $p < 0.05$) were found to have statistically significant effects on the fungal alpha diversity. No significant difference was observed between the summer and winter samples, which corroborates the ANOVA analysis, where there was no specific trend observed in the diversity between the seasons.

From Figure 3.6, season is seen to largely cluster together however, it is offset by the Pretoria winter samples of which cluster with the summer samples from Pretoria This shows the impact that the sampling location has on the fungal counterpart of the microbiome, which is supported by the Kruskal Wallis test (**Table 3.3**).

Table 3. 3. Kruskal Wallis test of fungal alpha diversities using the Observed OTU richness index. Sample refers to each individual sample with the triplicates combined. Genus refers to the two different lichen genera (mycobionts) collected which are *Parmotrema* and *Flavopunctelia*. The location is between the three sampling locations which are the Melville Koppies, Bryanston and Pretoria, and season is between summer and winter. Df – degrees of freedom.

Parameter	**chi-squared**	**Df**	**p-value**
Sample	30.785	11	0.001192
Genus	8.2196	1	0.004144
Location	9.4549	2	0.008849
Season	0.072509	1	0.7877

3.7. Bacterial and fungal beta diversities between lichens

Multidimensional scaling (MDS) plots using the Bray-Curtis dissimilarity index were constructed to evaluate bacterial (**Fig. 3.9**) and fungal (**Fig. 3.10**) beta diversity. The Bray-Curtis index is used to determine whether different groups have the same composition (Koleff *et al.*, 2003). For both fungal and bacterial beta diversity, it can be observed that sample replicates (n=3) cluster together. In general, the winter season samples cluster on the left-hand side while the summer season samples cluster on the right-hand side. One exception is observed for both bacterial and fungal beta diversity. The winter samples of both *Flavopunctelia* and *Parmotrema* from Pretoria cluster with those of the other summer samples from both lichen species and all three locations. This corroborates previous results finding that the winter samples from *Pretoria* clustered with the summer samples from Pretoria. The Melville samples are located towards the top of the plots and the Bryanston samples are located towards the bottom for both fungal and bacterial beta diversity, showing a distinction between the Melville and Bryanston samples.

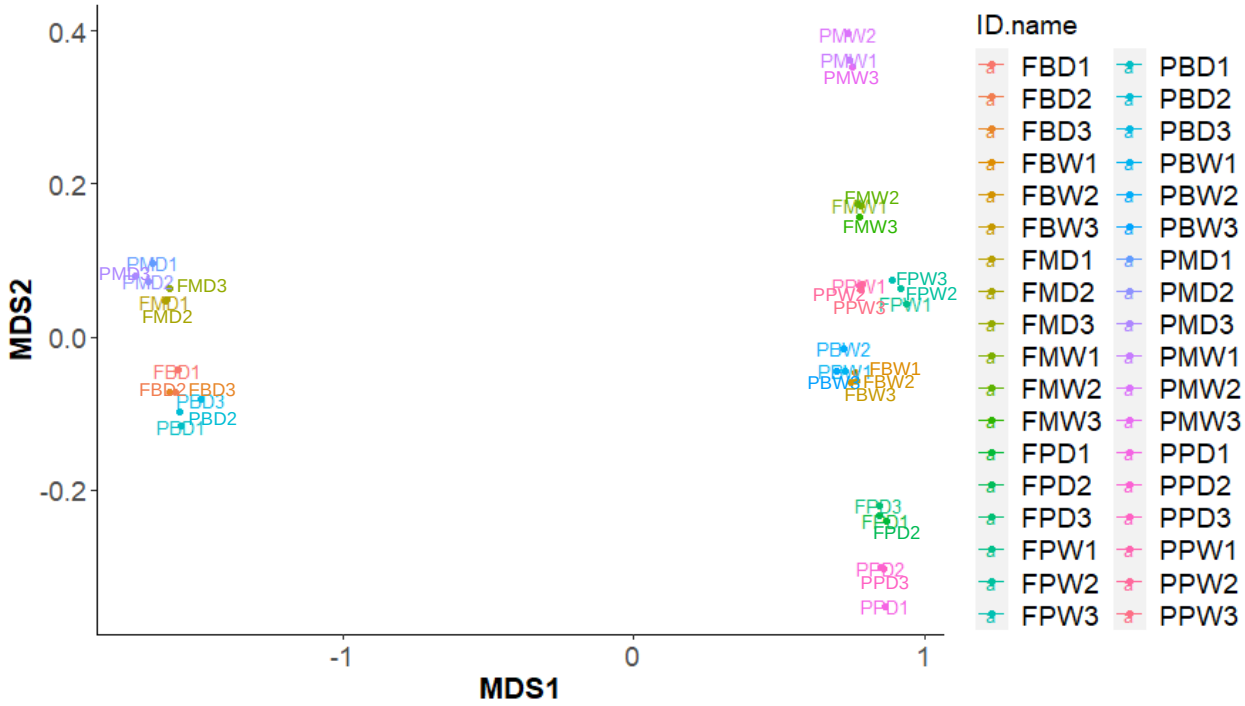


Figure 3. 9. Beta Diversity using Bray-Curtis index of bacterial OTUs across lichen samples and their sample replicates. MDS – Multidimensional scaling. The first letter in the three letter codes denotes the lichen genus (mycobiont) – *Flavopunctelia* (F) or *Parmotrema* (P). The second letter denotes the sample location – Bryanston (B), Melville Koppies (M) or Pretoria (P). The third letter denotes the season – Winter/dry season (D) or summer/wet season (W). The numbers 1, 2 and 3 indicate the sample triplicates.

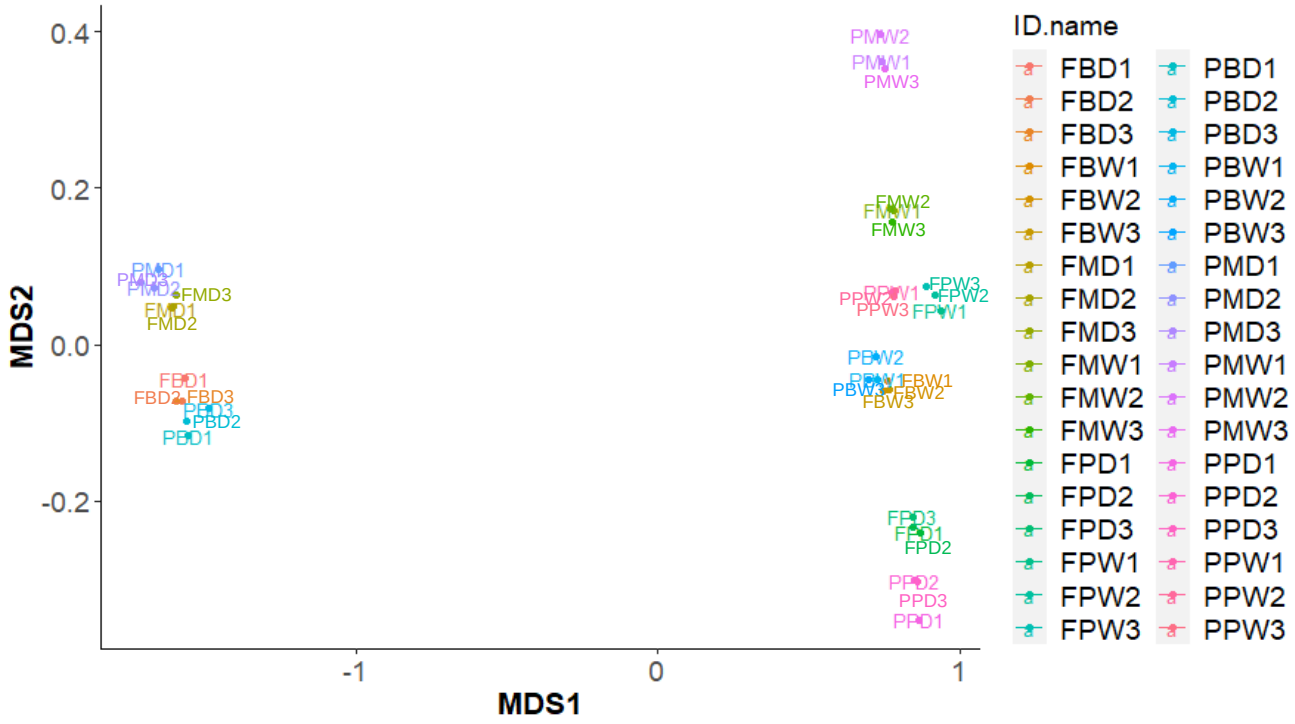


Figure 3. 10. Beta Diversity using Bray-Curtis index of fungal Operational Taxonomic Units across lichen samples and their sample replicates. MDS – Multidimensional scaling. The first letter in the three letter codes denotes the lichen genus (mycobiont) – *Flavopunctelia* (F) or *Parmotrema* (P). The second letter denotes the sample location – Bryanston (B), Melville Koppies (M) or Pretoria (P). The third letter denotes the season – Winter/dry season (D) or summer/wet season (W). The numbers 1, 2 and 3 indicate the sample triplicates.

Ellipses were drawn over the lichen genus (mycobiont), sampling location, and season groups to highlight the difference in the bacterial (**Fig. 3.11**) and fungal (**Fig. 3.12**) beta diversities between these parameters. For both the bacterial (**Fig. 3.11A**) and fungal (**Fig. 3.12A**) beta diversities, the lichen genera were found to overlap, suggesting that the lichen genus from which they originate does not have much influence on the microbial diversity. Clear distinctions between the locations are observed, with some points of overlap, for both the bacterial (**Fig. 3.11B**) and fungal (**Fig. 3.12B**) beta diversities, which highlights the difference in beta diversities between locations. There are clear distinctions with the seasons for both the bacterial (**Fig. 3.11C**) and fungal (**Fig. 3.12C**) beta diversities, with the only point of overlap occurring where the winter samples from Pretoria cluster closer with the summer samples from

Pretoria. This shows both how the change in season can influence the beta diversity but also how location can contribute to the diversity of the lichen microbiome.

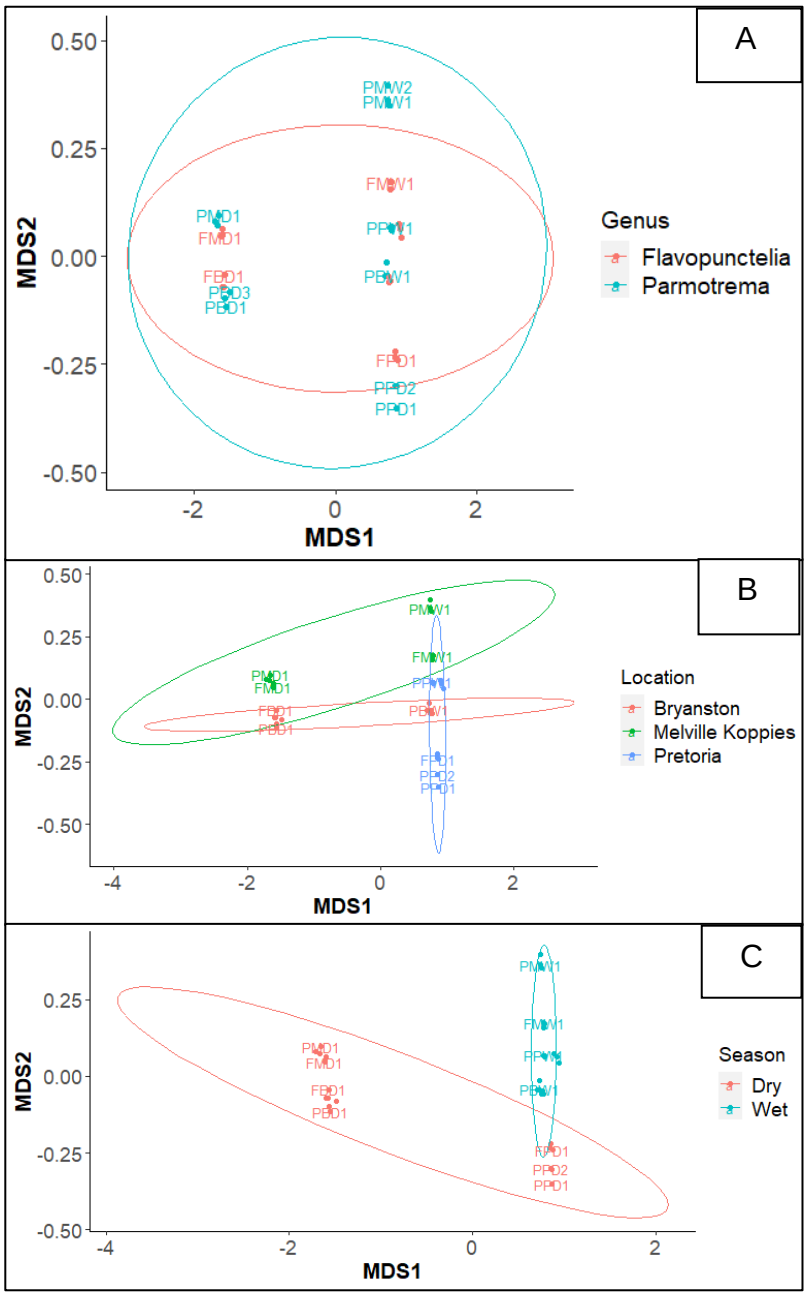


Figure 3. 11. Multidimensional scaling plot of bacterial beta diversities using the Bray-Curtis index with ellipses drawn around the lichen genera, sampling locations and seasons. A – Ellipses drawn around the lichen genera (mycobionts). **B –** Ellipses drawn around the sampling locations. **C –** Ellipses drawn around the seasons. MDS – Multidimensional scaling. The first letter in the three letter codes denotes the lichen genus – *Flavopunctelia* (F) or *Parmotrema* (P). The second letter denotes the sample location – Bryanston (B), Melville Koppies (M) or Pretoria (P). The third letter denotes the season – Winter/dry season (D) or summer/wet season (W). The numbers 1, 2 and 3 indicate the sample triplicates.

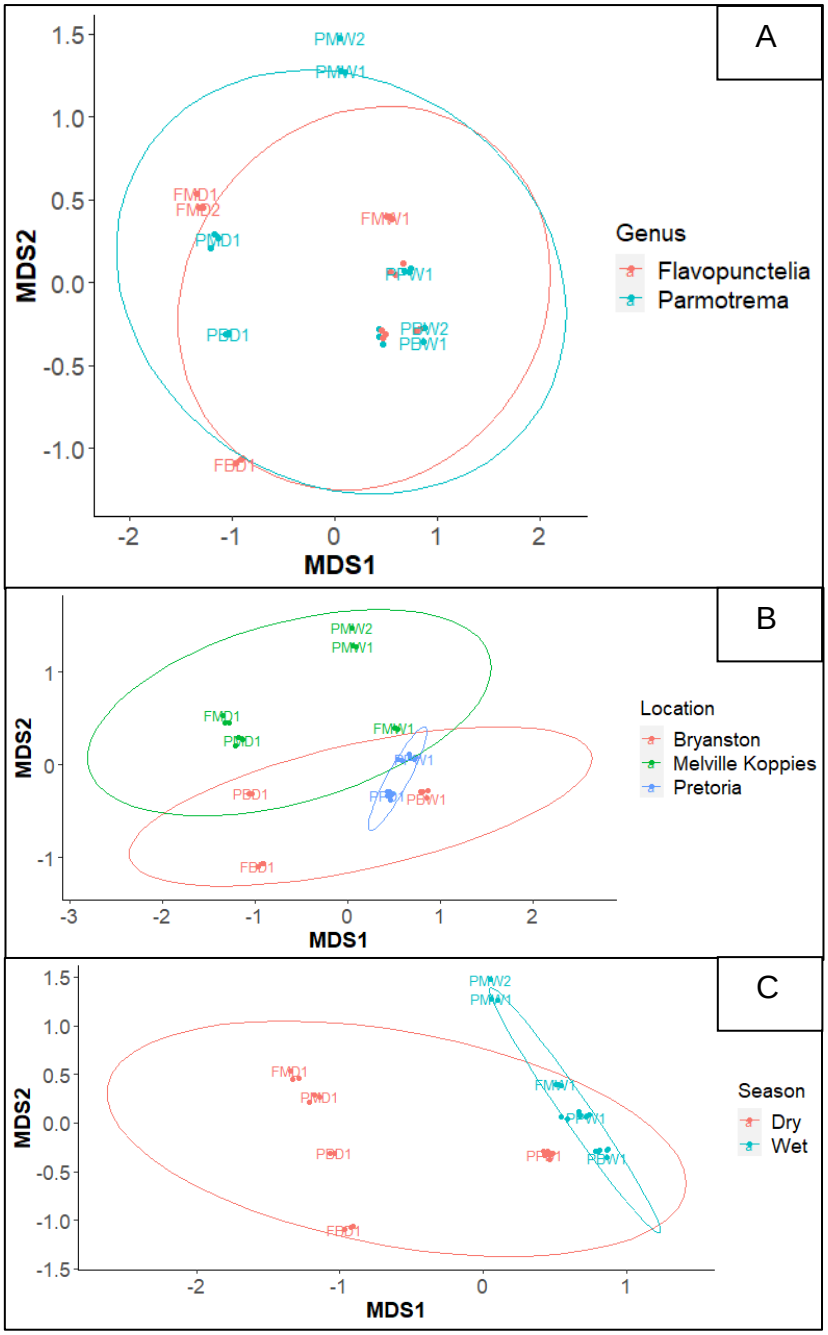


Figure 3. 12. Multidimensional scaling plot of fungal beta diversities using the Bray-Curtis index with ellipses drawn around the lichen genera, sampling locations, and seasons. A – Ellipses drawn around the lichen genera (mycobiont). **B –** Ellipses drawn around the sampling locations. **C –** Ellipses drawn around the seasons. MDS – Multidimensional scaling. The first letter in the three letter codes denotes the lichen genus – *Flavopunctelia* (F) or *Parmotrema* (P). The second letter denotes the sample location – Bryanston (B), Melville Koppies (M) or Pretoria (P). The third letter denotes the season – Winter/dry season (D) or Summer/wet season (W). The numbers 1, 2 and 3 indicate the sample triplicates.

Beta dispersion measures the average distance to the centroid which is used to determine variations in the microbiome within different parameters when analysing beta diversity

(Anderson, 2006). The beta dispersion was calculated using the Bray Curtis Index (Bray and Curtis, 1957). According to the beta dispersion analyses, significant differences were observed between the sampling locations ($p = 0.000999$, $p < 0.05$ for bacteria; $p = 0.01698$, $p < 0.05$ for fungi) and seasons ($p = 0.04496$, $p < 0.05$ for bacteria; $p = 0.04496$, $p < 0.05$ for fungi) for both the bacterial (**Table 3.4)** and fungal microbiomes (**Table 3.5)**, while the lichen genus (mycobiont) was not found to have any significant effect. The lack of influence of the lichen genus, and the influence of the sampling location and the season on the diversity is consistent with the Multidimensional Scaling plot of the bacterial (**Fig. 3.9**) and fungal (**Fig. 3.10**) beta diversities.

Table 3. 4. Beta dispersion of bacterial microbiome using the Bray-Curtis dissimilarity index. Genus refers to the two different lichen genera (mycobionts) collected which are *Parmotrema* and *Flavopunctelia*. The location is between the three sampling locations which are the Melville Koppies, Bryanston and Pretoria, and season is between summer and winter. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	Pr(>F)	Significance
Genus	0.818	
Location	0.000999	***
Season	0.04496	*

Table 3. 5. Beta dispersion of fungal microbiome using the Bray-Curtis dissimilarity index. Genus refers to the two different lichen genera (mycobionts) collected which are *Parmotrema* and *Flavopunctelia*. The location is between the three sampling locations which are the Melville Koppies, Bryanston and Pretoria, and season is between summer and winter. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	Pr(>F)	Significance
Genus	0.4136	
Location	0.01698	*
Season	0.04496	*

To further determine variations in the microbiome between the lichen genera (mycobionts), sampling locations and seasons, Permutational multivariate analysis of variance (PERMANOVA) tests were performed (McArdle and Anderson, 2001). Both the bacterial

(**Table 3.6**) and the fungal microbiomes (**Table 3.7**) were found to exhibit significant differences for the sampling locations and between the seasons, while the lichen genera did not have a significant difference. These results correspond with the beta dispersion results (**Tables 3.4 and 3.5**) and the Bray-Curtis plots (**Fig. 3.9 and 3.10**).

Table 3. 6. Permutational multivariate analysis of variance of bacterial microbiome using the Bray-Curtis dissimilarity test. Genus refers to the two different lichen genera (mycobionts) collected which are *Parmotrema* and *Flavopunctelia*. The location is between the three sampling locations which are the Melville Koppies, Bryanston and Pretoria, and season is between summer and winter. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	**R^2**	**F**	**Pr (>F)**	**Significance**
Genus	0.0402	1.4242	0.1938	
Location	0.29765	6.9927	0.000999	***
Season	0.32799	16.594	0.000999	***

Table 3. 7. Permutational multivariate analysis of variance of fungal microbiome using the Bray-Curtis dissimilarity test. Genus refers to the two different lichen genera (mycobionts) collected which are *Parmotrema* and *Flavopunctelia*. The location is between the three sampling locations which are the Melville Koppies, Bryanston and Pretoria, and season is between the summer and winter. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	**R^2**	**F**	**Pr (>F)**	**Significance**
Genus	0.03009	1.0549	0.3407	
Location	0.34209	8.5795	0.000999	***
Season	0.2214	9.6703	0.000999	***

To determine the impact of the climatological parameters (average maximum and minimum temperatures (°C) and total rainfall (mm)) on the diversity of the lichen microbiome, the beta dispersion test as well as the PERMANOVA tests were calculated. For both the lichen bacteria and fungi, the weather conditions were found to have significant differences for the beta dispersion tests (**Tables 3.8 and 3.9**) and the PERMANOVA analyses (**Tables 3.10 and 3.11**).

Table 3. 8. Beta dispersion of lichen bacteriome using the Bray-Curtis dissimilarity index in terms of weather conditions over six months prior to lichen collections. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	Pr(>F)	Significance
Average Daily Maximum Temperature (°C)	0.000999	***
Average Daily Minimum Temperature (°C)	0.000999	***
Total Rainfall (mm)	0.000999	***

Table 3. 9. Beta dispersion of lichen mycobiome using the Bray-Curtis dissimilarity index in terms of weather conditions over six months prior to lichen collections. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	Pr(>F)	Significance
Average Daily Maximum Temperature (°C)	0.001998	**
Average Daily Minimum Temperature (°C)	0.002997	**
Total rainfall (mm)	0.002997	**

Table 3. 10. Permutational multivariate analysis of variance of lichen bacteriome using the Bray-Curtis dissimilarity index in terms of weather conditions over six months prior to lichen collections. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	R^2	F	Pr (>F)	Significance
Average Daily Maximum Temperature (°C)	0.39606	22.297	0.000999	***
Average Daily Minimum Temperature (°C)	0.42649	25.284	0.000999	***
Total Rainfall (mm)	0.42464	25.094	0.000999	***

Table 3. 11. Permutational multivariate analysis of variance of lichen mycobiome using the Bray-Curtis dissimilarity index in terms of weather conditions over six months prior to lichen collections. Significance codes with p-values: "***" - [0, 0.001], "**" - (0.001, 0.01], "*" - (0.01, 0.05], "." - (0.05, 0.1], " " - (0.1, 1].

Factor	R^2	F	Pr (>F)	Significance
Average Daily Maximum Temperature (°C)	0.25725	11.776	0.000999	***
Average Daily Minimum Temperature (°C)	0.27244	12.731	0.000999	***
Total rainfall (mm)	0.27501	12.897	0.000999	***

Chapter 4: Discussion

4.1. Lichen identification

The lichens that were sampled were morphologically identified as *Parmotrema* and *Punctelia*, two lichen genera commonly observed in South Africa (Fryday, 2015). Through sequencing, the identity of *Parmotrema* was confirmed but *Punctelia* was identified as *Flavopunctelia* (Fryday, 2015). *Parmotrema* has been observed to comprise thirty-two species in South Africa while *Flavopunctelia* has been observed to incorporate two South African species (Fryday, 2015). Both lichen genera are foliose lichens abundant on trees (Jain *et al*., 2016) and are within the Parmeliaceae family (Fryday, 2015; Hale, 1984). *Flavopunctelia* is separated from *Punctelia* based off the differences in the morphology of the conidia and chemistry (Hale, 1984). Identification of lichens using molecular techniques are the most reliable and accurate lichen identification methods (Singh *et al*., 2019). This is because identifying lichens through morphology alone often results in inaccurate results as there may be different lichens that morphologically seem the same, but due to slight genetic differences between them may produce different chemicals (McMullin and Allen, 2022; Singh *et al*., 2019). Due to the inaccuracy of the morphological identification of lichens, the sequencing results in this study were seen to be more accurate and were used in characterising the lichen genus. *Parmotrema* has been used for multiple biomonitoring studies in South Africa such as the biomonitoring of manganese and lead pollution in Pretoria (Forbes *et al*., 2009), and the biomonitoring of atmospheric pollution of trace elements in the Western Cape (Ndlovu *et al*., 2019), while no known South African studies have been found that used *Flavopunctelia.* However, *Flavopunctelia* has been studied in other countries, such as for the biomonitoring of trace elements in Morelia, Mexico (Rangel-Osornio *et al*., 2022).

The main photobiont of both lichen genera in this study was found to be *Trebouxia aggregata*, a green alga from the genus *Trebouxia.* This genus is commonly associated with lichens, being estimated to be associated with 80% of lichens in temperate regions and 20% of lichens around the world (Muggia *et al*., 2020; Nelsen *et al*., 2021).

4.2. The core bacteriome of *Parmotrema* and *Flavopunctelia*

The core microbiome is defined as the set of microorganisms that are specific to a host or environment (Neu *et al*., 2021; Risely, 2020; Turnbaugh *et al*., 2007). Identifying the core microbiome assists with determining what functions the core microorganisms may contribute

to the host (Risely, 2020). Thus, a major objective of this study was to identify the core microbiome and its possible roles in the lichen.

The top three phyla of bacteria were found to be Proteobacteria, Acidobacteria and Bacteroidetes (**Fig. 3.5**). Actinobacteria, Cyanobacteria and Verrucomicrobia were also present at lower abundances. These results correspond with previous studies which have found Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria, and Verrucomicrobia to be the dominant bacterial phyla found in lichens (Sierra *et al.*, 2020). The most abundant and prevalent bacterial genera were found to belong to the phyla Proteobacteria and Acidobacteria. The observation of the same types of bacteria being present in lichens in most studies may be because lichens have been previously described and proved to be an example of convergent evolution (Muggia *et al*., 2011; Watson, 1929), meaning that they have evolved separately but have ended up with similar characteristics (Hall, 2003). This convergent evolution theory could explain why the same types of bacteria form symbiotic relationships with lichens.

The core bacterial genera of the lichen microbiome were observed to be *Methylosinus*, *Terriglobus*, *Granulicella*, *Methylobacterium*, *Sphingomonas*, *Acidisphaera*, *Beijerinckia*, *Rhodopila* and *Candidatus Solibacter* (**Fig. 3.1**). *Methylosinus*, the most abundant bacterial genus that took up 14% of the entire bacteriome, and *Methylobacterium,* which took up 6% of the entire bacteriome, are methylotrophic bacteria that can oxidise methane (Patt *et al*., 1976; Whittenbury *et al*., 1970). *Methylosinus* is widely distributed around the world, occurring in most habitats (Conrad, 1996), while *Methylobacterium* is also widely distributed being present in soil, air, fresh and aquatic water sediments (Hiraishi *et al*., 1995). To date, *Methylosinus* has not been observed in lichens, but *Methylobacterium* has been isolated from lichens in a few instances, with a novel *Methylobacterium* isolate identified from the lichen *Lepraria sp.* from China (Jiang *et al*., 2020), and also being isolated from Antarctic lichens (Noh *et al*., 2021). Other than *Methylosinus* and *Methylobacterium,* high levels of methane oxidising bacteria have been observed in lichen-dominated areas (Belova *et al*., 2020) and methane oxidation that occurs in high-latitude locations are directly related to the lichen associated methane-oxidising bacteria (Evgrafova, 2019). Lichens have been found to produce methane (Lenhart *et al*., 2015) which most likely results in the colonisation of lichens by methanotrophs, which in turn provides carbon dioxide (CO_2) to lichens (Evgrafova, 2019). The lichen consequently utilises CO_2 to build biomass (Seminara *et al*., 2018). It can be assumed that methane production by lichens may have been developed specifically to form relationships with methanotrophs to

increase the lichens' CO_2 sources to adjust to nutrient poor environments. In addition to its roles in nutrient cycling, *Methylobacterium* produces indole-3-acetic acid (IAA) (Noh *et al*., 2021), which promotes increased water up-take in plants, cell growth and biomass production (Mohite, 2013). IAA has been found to be produced in lichens (Pichler *et al*., 2020) and other lichen-associated bacteria (Noh *et al*., 2021).

There is evidence to suggest that *Terriglobus, Granulicella, Beijerinckia, Rhodopila* and *Candidatus Solibacter* are involved in nitrogen cycling (Bates *et al.*, 2011; Männistö *et al.*, 2012; Pearce *et al.*, 2012; Rawat *et al.*, 2012; Reis and Teixeira, 2015) with all genera except for *Candidatus Solibacter* being previously observed in lichens (Panosyan and Nikogosyan, 1966; Pankratov and Dedysh, 2010; Park *et al.*, 2016; Tzovaras *et al.*, 2019; Alonso-García and Villarreal A., 2021). Methanotrophs such as *Methylosinus* and *Methylobacterium* have also been observed to contribute to nitrogen fixation (Cui *et al*., 2022; Madhaiyan *et al*., 2015). Nitrogen fixation is an important process that occurs in lichens as it provides a much needed nutrient source to the lichen in nutrient poor environments (Antoine, 2004; Gunther, 1989; Holub and Lajtha, 2004), but also contributes to processes in the environment such as rock weathering (Seneviratne and Indrasena, 2006). It provides a fixed nitrogen source to the environment when rain leaches nitrogen from lichens and transfers it to plants and soil (Millbank, 1978). Chlorolichens have a higher diversity of nitrogen fixing bacteria than cyanolichens since cyanobionts fix nitrogen for the lichen symbiosis (Almendras *et al*., 2018). This explains the high level of nitrogen fixing bacteria in the lichens sampled for this study since the main photobiont was *T. aggregata,* a green alga. *Granulicella* was the most abundant genus of the nitrogen fixing bacteria, other than *Methylosinus,* as it composed 7% of the lichen bacteriome. In addition to contributing to nitrogen fixation, *Granulicella* has been found to produce exopolysaccharides (Pankratov and Dedysh, 2010) of which the exact nature is unknown but may contribute to an exopolysaccharide matrix that creates a partially anaerobic environment in which nitrogen fixation can occur (Spribille *et al*., 2020). These exopolysaccharides have potential applications for the environment, bioremediation and biotechnology (Kielak *et al.*, 2016). *Rhodopila* also has additional roles which are beneficial to the lichen symbiosis, this includes providing products from photosynthesis, and protecting the lichen against pathogens and oxidative stress (Cernava *et al*., 2017).

Sphingomonas was the second most abundant bacterial genus in the bacteriome, composing 10%, and has been previously isolated from lichens in locations such as the rocky outcrops of

northern Colorado and the sub-arctic tundra of Iceland (Bates *et al.*, 2011; Klarenberg *et al.*, 2020) and is well known for its ability to degrade aromatic hydrocarbons (Baraniecki *et al*., 2002). It may contribute to the lichen symbiosis through the production of IAA (Noh *et al*., 2021). *Sphingomonas* may also inhabit lichens due to the production of ribitol by the algal photobiont *Trebouxia* (Richardson and Smith, 1968) and mannitol by the lichen mycobiont (Lewis and Smith, 1967) which *Sphingomonas* can grow on (Kono *et al*., 2017). *Acidisphaera* has been isolated from lichens (Klarenberg *et al*., 2020) and produces bacteriochlorophyll (Hiraishi *et al*., 2000) which may contribute to the lichen symbiosis through photosynthesis (Tahon and Willems, 2017). Thus, the core bacterial members of the lichen microbiome contribute to nitrogen and methane fixation, participate in photosynthesis, and protect against harmful factors.

4.3. The core mycobiome of *Parmotrema* and *Flavopunctelia*

The fungal component of the lichen microbiome comprises the phylum Ascomycota, and to a far lesser extent Basidiomycota (**Fig. 3.6**). This corroborates previous studies finding that the lichen mycobiome is mainly composed of Ascomycota, as well as Basidiomycota (Smith *et al.*, 2020). At extremely low levels Chytridiomycota and Glomeromycota also occurred, which is again consistent with previous studies which have found the lichen mycobiome to comprise of these phyla at much lower levels (Smith *et al.*, 2020). The core fungal genera of the lichen microbiome all belonged to the phylum Ascomycota. Convergent evolution may yet again explain why these fungal types are so predominant in lichens, whereby in the process of lichens adapting to the environment, they have formed beneficial symbioses with these types of fungi.

The core fungal genera found in the lichens sampled in this study were *Curreya, Microcera, Cladophialophora, Austroafricana, Knufia, Physconia* and *Penidiella* (**Fig. 3.2**). *Cladophialophora, Austroafricana, Knufia,* and *Penidiella* are black fungi (Crous *et al.*, 2007; Harutyunyan *et al.*, 2008; Selbmann *et al.*, 2013), of which *Cladophialophora* (Harutyunyan *et al.*, 2008) and *Austroafricana* (Harutyunyan *et al.*, 2008) have previously been found in lichens. Black meristematic fungi are extremotolerant and can occur in environments with poor nutrient conditions (Sterflinger *et al.*, 1999) due to the melanised cell walls and meristematic structure (Selbmann *et al*., 2013), but are often found in lichens which occur in stressful environments (Onofri *et al*., 2007). It has been suggested that black fungi protect lichens from harsh environmental pressures through the ability to keep the lichen hydrated and protect the

lichen photobiont from harmful levels of sunlight (Selbmann *et al*., 2005). *Cladophialophora*, which was the second most abundant fungal genus constituting 10% of the mycobiome, may also associate with lichens because *Cladophialophora* strains have been found to degrade aromatic hydrocarbons (Prenafeta-Boldú *et al.*, 2006), and lichens are rich sources of aromatic secondary metabolites that could be utilised by *Cladophialophora* (Harutyunyan *et al.*, 2008). *Knufia*, the most abundant genus of the mycobiome comprising 13%, has also been reported to corrode extracellular polysaccharide matrices (Breitenbach *et al*., 2018) which lichen associated bacteria can produce (Spribille *et al*., 2020). Thus, a substantial portion of the core mycobiome constitute black fungi, often associated with lichens occurring in nutrient poor-environments and provide nutrient sources to these lichens. *Coniosporium* is another black fungus which was observed to occur in lichen samples, although it was not identified as a member of the core mycobiome (Sterflinger *et al.*, 1999).

The most prevalent fungal genus (81% prevalence) in the lichens sampled in this study was *Curreya* (comprised 5% of the total mycobiome), which has not previously been observed in lichens. *Curreya* is from the family *Cucurbitariaceae* (Wijayawardene *et al*., 2018) which are commonly found on the bark of trees (Jaklitsch *et al*., 2018; Mirza, 1969). Lichens from this study were sampled from trees, so *Curreya* may have been found in the lichen samples due to its associations with the trees that the lichens were collected from. *Microcera* is a parasite of insects (Gräfenhan *et al*., 2011), which may have also been found in the lichens due to its possible association with insects that live in lichens. Besides *Curreya* and *Microcera,* the core fungal components of the lichen microbiome are composed of black fungi.

To determine what factors affected the distribution of the core microbiome and larger microbiome in general, the alpha and beta diversities were calculated and the effects of the lichen genus, the lichen sampling location, and the season in which the lichens were collected on the lichen microbiome composition and diversity were determined.

4.4. The lichen genus and its effect on the microbiome

Comparison of the fungal read datasets for the two lichen genera sampled here showed that the *Parmotrema* samples had 34% more fungal reads than the *Flavopunctelia* samples. However, the bacterial reads were quite consistent between the *Flavopunctelia* and *Parmotrema* samples. Despite this and despite previous reports finding that the lichen microbiome is heavily determined by the mycobiont (Grube and Berg, 2009; Grube *et al.*, 2009; Bates *et al.*, 2011),

this study found that the lichen mycobionts *Flavopunctelia* and *Parmotrema* had minimal to no influence on the composition and diversity of the bacterial and fungal components of the microbiome. No significant differences were observed in the bacterial and fungal beta diversities between the two lichen genera. This may be due to the fact that the lichens belong to the same family (Fryday, 2015; Hale, 1984), as similar lichen genera have been found to have similar lichen mycobiome constructions (Smith *et al.*, 2020). Thus, it may be a case where South African lichens from different families may affect more dramatic differences in the lichen microbiome and should be studied further.

4.5. The impact of location and season on the lichen microbiome

Microbial communities have been found to vary between seasons, characterised by changes in temperature and moisture (Luo *et al*., 2019). This shift in microbial compositions between seasons has been observed within the soil microbiome where the shift has been attributed to excessively high temperatures and rainfall experienced in the summer (Luo *et al*., 2019). Increased diversity and richness have also been observed in the soil microbiome in summer compared to spring and autumn. Since major differences in temperature and rainfall can impact the composition of a microbiome between seasons, it can be assumed that major differences in temperature and rainfall between locations can also result in a varied microbiome.

The daily average maximum and minimum temperatures (°C) and total rainfall (mm) over the six months prior to lichen collections had a significant impact on the bacterial and fungal beta diversities for both the *Parmotrema* and *Flavopunctelia* lichens (**Tables 3.8-3.11**). This is consistent with the ANOVA analysis (**Fig. 3.7**) where an increase in the bacterial diversity occurred in summer compared to winter. However, fungal alpha diversities were largely the same (**Fig. 3.8**). Bacterial and fungal beta diversities also differ significantly between our sampling locations, Melville Koppies and Bryanston in Johannesburg, and Pretoria (**Tables 3.4-3.7**). This may also be linked to difference in climatological patterns. As such, Pretoria samples collected in winter clustered with the Pretoria samples collected in summer rather than the other winter samples (**Figures 3.5 and 3.6**). This is likely linked to the fact that Pretoria experienced over double the amount of winter rainfall than Johannesburg. Apart from the Pretoria samples, all the other lichen samples were found to cluster according to season. Thus, it can be deduced that major differences in temperature and rainfall between seasons and locations can influence the microbial composition and diversity within the lichens.

Major differences in the abundance of taxa were observed between seasons for the bacterial genera *Granulicella* and *Acidisphaera* (**Fig. 3.5**), and the fungal genera *Cladophialophora*, *Knufia*, *Microcera* and *Coniosporium* (**Fig. 3.6**). *Granulicella* was more abundant in summer while *Acidisphaera* was more abundant in winter. *Cladophialophora* and *Microcera* were more abundant in winter than summer, but *Cladophialophora* was also prevalent in Pretoria samples from summer, which shows both the effect of season and location on the composition of the mycobiome. In contrast, *Knufia* was abundant in the summer samples but also the winter sample from Pretoria. *Knufia* may have been present in only the winter samples from Pretoria because of the higher amount of rainfall in Pretoria compared to Johannesburg. *Coniosporium* was observed in the winter samples from Bryanston and Melville but not from Pretoria. This suggests that *Coniosporium* can become more abundant in drier climates, since it was prevalent in winter in Johannesburg where the rainfall was quite low compared to Pretoria. This may be due to less microorganisms being able to withstand the drier environment, allowing *Coniosporium* to grow.

4.6. Additional factors affecting the lichen microbiome

The *Parmotrema* lichen samples collected from Melville in summer were found to be primarily composed of the fungal genus *Paecilomyces* (accounting for 57% of the fungal counts in the sample) (**Fig. 3.6**) and the bacterial genus *Methylosinus* (accounting for 49% of the bacterial counts in the sample) (**Fig. 3.5**). These samples also had lower bacterial (**Fig. 3.7**) and fungal (**Fig. 3.8**) alpha diversity compared to the winter counterpart and were found to cluster separately from all the other lichen samples (**Figures 3.5 and 3.6**).

Paecilomyces is a parasitic fungi that is known for parasitising nematode eggs (Toyota and Watanabe, 2013) and has been used as a biocontrol agent where *Paecilomyces lilacinus* is the main component in the biocontrol agents BioAct WG® and MeloCon® (Anastasiadis *et al*., 2008). *Paecilomyces* has been found to not only work against nematodes, but also bacteria and phytopathogenic fungi (Piątkowski *et al.*, 2003; Suárez-Estrella *et al.*, 2013). This could underpin the difference in composition in the samples containing *Paecilomyces* and the low read counts for these samples. *Paecilomyces* inhibits bacterial growth through the production of secondary metabolites including alkaloids, phenolic compounds and steroids (Mousa and Raizada, 2013; Lugtenberg *et al.*, 2016). The bacterial component of this lichen sample also clustered separately from the other lichen samples and had an increased abundance of

Methylosinus compared to other samples. It is possible that other bacteria were killed off by the secondary metabolites produced by *Paecilomyces*, but *Methylosinus* was resistant to them and able to increase in abundance. This illustrates the major impact some microorganisms within a microbiome can have on that microbiome.

4.7. Application of results and future studies

As seen from this study, the lichen location and season in which lichens were sampled, which could be attributed to major differences in temperature and rainfall, heavily influenced the composition and diversity of the lichen microbiome. In contrast, rather unexpectedly, very little difference in the composition and diversity in the lichen microbiome between the two lichen genera were observed. It is recommended that future studies focus on investigating a broader range of lichen genera to determine their influence on the lichen microbiome. Studying the lichen microbiome over longer periods of time and across multiple locations that are widely distributed could help further identify the factors that cause these major variations in the lichen microbiome. This study found that the major constituents of the core microbiome contribute to nutrient cycling and protection of the lichen in harsh environmental conditions. Going further, conducting a multi-omics approach to investigating the core microbiome could help characterise the functional contributions of the lichen-associated bacteria and fungi.

4.9. Conclusion

In conclusion, the core microbiome of two South African lichens is composed of bacteria involved in nutrient cycling process which may provide additional benefits to the lichen symbiosis, and black meristematic fungi that provide protection to lichens under harsh environment conditions. The composition and diversity of the lichen microbiome were mainly influenced by seasonal shifts and differences in sampling locations, which may have been attributed to major fluctuations in the average temperature and total rainfall. Minimal differences in the lichen composition and diversity were observed between the lichen mycobionts, which may be because the lichen mycobionts are too similar. Further studies would need to focus on characterising the lichen microbiome between many different mycobiont types, but also between more locations in South Africa over a longer period to investigate the exact factors that influence the microbiome. The functional aspect of the lichen

microbiome must also be further investigated to help gain a better perspective of the individual roles within the lichen symbiosis.

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Chapter 5: Summary

The South African lichen microbiome has not been studied. There is an abundance of lichen taxa distributed throughout the nine biomes of South Africa which have varying climates. Due to climate change, lichens may not evolve fast enough to adapt to the major shifts in the climate that are occurring. The South African lichen microbiome needs to be investigated to determine the effects of climate change on the lichen microbiome and their response. The South African lichen microbiome may also be abundant in novel microorganisms that produce therapeutically relevant secondary metabolites.

This study investigated two lichens found within South Africa, *Parmotrema* and *Flavopunctelia* across three locations in Gauteng namely the Melville Koppies, and residential areas with Bryanston and Pretoria, across winter and summer. The main goals of the study were to identify the core microbiome, and to determine the biotic and abiotics effects on the microbiome.

The core microbiome was found to mainly be comprised of bacteria that are involved in nutrient cycling (nitrogen and methane fixation) and black fungi that contribute to the survival of lichens in harsh environments.

Differences in the microbiome composition and diversity were consistent between the two lichen genera (mycobionts). This suggests that the two lichen genera may have been too similar, and varied microbiomes may be found between lichen families. To determine the influence of the lichen mycobiont on the lichen microbiome, future investigations will need to determine the microbiome between many different lichens.

This study found major differences in the lichen microbiome between lichen locations and seasons which are attributed to major differences in the average temperatures and total rainfall between the locations and seasons. These major differences are supported by the bacterial and fungal genera that varied between season and location. Going further, it is suggested that lichens be sampled widely across the country, and that their microbiomes are monitored over a longer time period, to determine more factors that affect the microbiomes across locations and seasons.

The influence of individual microorganisms on the lichen microbiome were observed. This was seen where one fungal parasite, *Paecilomyces*, constituted a major proportion of one lichen sample, and the bacterial and fungal alpha diversities were decreased due to that. The bacterial

genus *Methylosinus* was found to increase in abundance in that sample, possibly because it was able to withstand secondary metabolites produced by *Paecilomyces* that would antagonise bacteria. Studying the lichen microbiome may further assist to determine the inter-species relationships within the lichen microbiome and how they affect the composition and diversity of the microbiome.

Appendix

forestry, fisheries & the environment
Department:
Forestry, Fisheries and the Environment
REPUBLIC OF SOUTH AFRICA

Private Bag X447, Pretoria, 0001, Environment House, 473 Steve Biko Road, Pretoria, 0002 Tel: +27 12 399 9000, Fax: + 27 86 625 1042

Ref: BABS/001521N
Enquiries: Thindeka Sibanda
Tel: 012 399 9838 **Email:** Tsibanda@environment.gov.za

Ms. Angela Botes

The University of the Witwatersrand

School of Molecular and Cell Biology

1 Jan Smuts Avenue

Braamfotein

JOHANNESBURG
2050

Email: angela.botes@wits.ac.za

Dear Ms. Angela Botes,

RE: APPLICATION FOR DISCOVERY PHASE NOTIFICATION SUBMITTED BY THE UNIVERSITY OF THE WITSWATERSRAND, JOHANNESBURG ACTING THROUGH ITS SCHOOL OF MOLECULAR AND CELL BIOLOGY, REFERENCE NUMBER: BABS/001521N

The Department of Forestry, Fisheries and the Environment (DFFE) acknowledges receipt of the email submitted application for the Discovery Phase Notification from The University of the Witwatersrand, Johannesburg acting through its School of Molecular and Cell Biology on 15 September 2021. This application was submitted in compliance with the National Environmental Management: Biodiversity Act, 2004 (Act No. 10 of 2004) (NEMBA) and the Bioprospecting, Access and Benefit Sharing Amendment Regulations of 2015 (BABS Regulations). The title of the project is: *"Identify the core microbiome of South African lichens-a potential source of novel antifungals"*

The following documents were received**:**

1. Annexure 1: Completed Discovery Phase Notification form;
2. Internal memo from the Gauteng Department of Agriculture and Rural Development

3. Proposal document for identifying the core microbiome of South African lichens-a potential source of novel antifungals;
4. Investigation / application form: Permit for Bioprospecting Project;
5. Certified ID copy of Miss Angela Botes;
6. Proof of payment of an amount of R200.00 paid to Gauteng Department of Agriculture and Rural Development;
7. Annexure F: Discovery Phase BABS checklist.

**APPLICATION FOR DISCOVERY PHASE NOTIFICATION SUBMITTED BY THE UNIVERSITY OF
THE WITSWATERSRAND, JOHANNESBURG ACTING THROUGH ITS SCHOOL OF MOLECULAR AND CELL BIOLOGY, REFERENCE NUMBER: BABS/001521N**

Kindly note that an internal assessment on the above-mentioned documents was conducted to ascertain whether all the required information have been submitted in accordance with the provision of NEMBA and the BABS Regulations. In addition, your discovery phase notification has been captured in the Departmental database and has been assigned a reference number: BABS/001521N. Please quote this reference number in any future correspondence in respect of this application.

Furthermore, the proposed timeframe is May 2021 to December 2026, you are requested to submit on an annual basis, a status/progress report of the project to the Department. You are also reminded to comply with the section 81(1) (a) of NEMBA, once the project reaches commercialization.

Once again, the Department wishes to express its sincere appreciation for the efforts made by The University of The Witwatersrand to ensure compliance with the requirements of NEMBA and the BABS Regulations of 2015.

If you have any enquiries relating to your permit application, please do not hesitate to contact the responsible official in the department as detailed in the enquiries section above or Mr Ntambudzeni Nepfumembe, email: NNepfumembe@environment.gov.za or tel: 012 399 9612, cell: 078 598 3056 or Mrs Lactitia Tshitwamulomoni, e-mail: LMabadahane@environment.gov.za or tel: 012 399 9611 or cell: 082 593 9893

Yours sincerely



Ms Nomfundo Tshabalala
Director-General
Department of Forestry, Fisheries and the Environment
Letter signed by: Ms Natalie Feltman
Designation: Director: Bioprospecting and Biodiversity Economy
Date: 5 October 2021

Friends of Melville Koppies

(administered by Melville Koppies Management Committee)

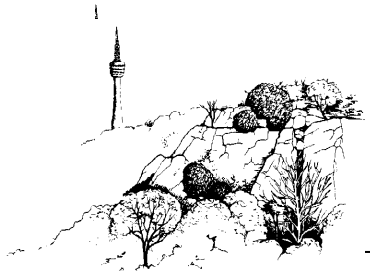
(038-274-NPO, PBO 930027329)

125 Third Avenue, Melville 2092

Phone: 011-482-4797

Website: www.mk.org.za

Melville Koppies



60th Jubilee 1959-2019

13th May 2021

Collecting lichen samples from Melville Koppies

University of the Witwatersrand student, Pascale Naude is hereby given permission to collect samples of lichen for an MSc project on Molecular and Cell biology.

Dr Angels Botes is the supervisor.

Pascal will be responsible for own security.

Wendy Carstens

Conservation Manager

079 532 0083 / 011 482 4797

The provision of the data is subject to the User providing the South African Weather Service (SAWS) with a detailed and complete disclosure, in writing and in line with the requirements of clauses 1.1 to 2.4 (below), of the purpose for which the specified data is to be used.

1 **Should the User intend using the specified data for commercial gain then the disclosure should include the following**:

1.1 the commercial nature of the project/funded research project in connection with which the User intends to use the specified data;

1.2 the names and fields of expertise of any participants in the project/funded research project for which the specified data is intended; and

1.3 the projected commercial gains to the User as a result of the intended use of the specified data for the project/funded research project.

2 **Should the User intend using the specified data for the purposes of conducting research, then the disclosure should include the following**:

2.1 the title of the research paper or project for which the specified data is to be used;

2.2 the details of the institution and supervisory body or person(s) under the auspices of which the research is to be undertaken;

2.3 an undertaking to supply SAWS with a copy of the final results of the research in printed and/or electronic format; and

2.4 the assurance that no commercial gain will be received from the outcome from the research.

If the specified data is used in research with disclosure being provided in accordance with paragraph 2 and the User is given the opportunity to receive financial benefit from the research following the publication of the results, then additional disclosure in terms of paragraph 1 is required.

The condition of this disclosure statement is applicable to the purpose and data requirements of the transaction recorded in Schedule 1 below.

SCHEDULE 1

Please note: The South African Weather Service will only act upon customer requirements noted on this disclosure statement and not from any other correspondence.

FULL PERSONAL DETAILS OF USER

Full Names	Pascale Josine Naude
University/school/organisation	University of the Witwatersrand in the School of Molecular and Cell Biology
Student Number (if applicable)	1635131
Email address	1635131@students.wits.ac.za
Cellphone	0645396818
Supervisor	Dr Angela Botes and Prof Pieter De Maayer
Project/Thesis Title	Identifying the core microbiome of two South African lichens
Current registered degree (e.g. BSc)	MSc
Expected finalization date (MM-YYYY)	12-2022

The South African Weather Service reserves the right to request, at any time, from the student proof of registration for the Degree at the University.

THE PURPOSE (*Please indicate a detailed description of the purpose for which the data will be used)*

For my MSc research I am working with lichens, trying to identify the core microbiome of lichens and if various factors including the lichen species, geographical location, season, and weather play a part in the structure and diversity of the microbiome. I have found that the season plays a major role in the structure of the microbiome as lichen samples have been collected in August (the dry season) and February (the wet season), so I would like to delve more into this by looking at the weather and its effect on the microbiome.

DATA REQUIRED *(Indicate weather elements (e.g. rain, temp), place/s, time period and resolution (e.g. daily, hourly)*

I would like weather data (rain, temperature, and if possible pollution levels) from 1 August 2021 to 1 September 2022 from around these locations: the Melville Koppies in Emmarentia, Hamilton Park in Bryanston and Jan Cilliers Park in Pretoria in the form of daily readings.

I hereby accept that:

- SAWS will be acknowledged in the resulting thesis/project or when published, for the data it provided.
- SAWS will be provided with a copy of the final results in printed or electronic format.
- The data received shall not be provided to any third party.

Signature of the User:  Date: 14/09/2022

(*Please sign the document and do not type your name in as this is a legal document and requires a signature*.)