Microbial diversity of soils of the Sand fynbos

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Summary

The soil environment is thought to contain a lot of the earth's undiscovered biodiversity. The aim of this study was to understand the extent of microbial diversity in the unique ecosystem of the Western Cape's fynbos biome. It is known that many processes give rise to this immense microbial diversity in soil. In addition the aim was to link microbial diversity with the soils physio-chemical properties as well as the plant community's structure. Molecular methods especially automated ribosomal intergenic spacer analysis (ARISA) was used in the study.

The most important property of environmental DNA intended for molecular ecology studies and other downstream applications is purity from humic acids and phenolic compounds. These compounds act as PCR inhibitors and need to be removed during the DNA extraction protocol. The fist goal in the study was to develop an effective DNA extraction protocol by using cationic flocculation of humic acids. The combination of cationic flocculation with $CuCl_2$ and the addition of PVPP and KCl resulted in a high yield of DNA, suitable for PCR amplification with bacterial and fungal specific primers.

Determining the reproducibility and accuracy of ARISA and ARISA-PCR was important because these factors have an important influence on the results and effectiveness of these techniques. Primer sets for automated ribosomal intergenic spacer analysis, ITS4/ITS5, were assessed for the characterization of the fungal communities in the fynbos soil. The primer set delivered reproducible ARISA profiles for the fungal community composition with little variation observed between ARISA-PCR's. ARISA proved useful for the assessment and comparison of fungal diversity in ecological samples.

The soil community composition of both fungal and bacterial groups in the Sand fynbos was characterized. Soil from 4 different Sand fynbos sites was compared to investigate diversity of eubacterial and fungal groups at the local as well as a the landscape scale. A molecular approach was used for the isolation of total soil genetic DNA. The 16S-23S intergenic spacer region from the bacterial rRNA operon was amplified when performing bacterial ARISA from total soil community DNA (B-ARISA). Correspondingly, the internal transcribed spacers, ITS1, ITS2 and the 5.8S rRNA gene from the fungal rRNA operon were amplified when undertaking fungal

ARISA (F-ARISA). The community structure from different samples and sites were statistically analysed. ARISA data was used to evaluate different species accumulation and estimation models for fungal and bacterial communities and to predict the total community richness. Diversity, evenness and dominance were the microbial communities were used to describe the extent of microbial diversity of the fynbos soils. The spatial ordination of the bacterial and fungal species richness and diversity was considered by determining the species area relationship and beta diversity of both communities. The correlation between the soil physio-chemical properties was determined. The plant community structure data was correlated with the fungal and the bacterial community structure. The results indicated that bacterial species numbers and diversity were continually higher at the local scale. Fungi however showed higher species turnover at the landscape scale. Bacterial community structure showed stronger links to the plant community structure whereas the fungi community structure conformed to spatial separation patterns.

To further investigate the diversity of soil microbes the potential of genus specific primes was investigated. The genus *Penicillium* is widespread in the soil environment and the extent of its diversity and distribution is however not. For this reason *Penicillium* was chosen as a model organism. To expand the insight into the diversity of *Penicillium* species in the fynbos soil ecosystem, a rapid group specific molecular approach would be useful. *Penicillium* specific primers targeting the 18S rRNA ITS gene region were evaluated. Fungal specific primers ITS4 and ITS5, targeting the internal transcribed region (ITS) were used to target *Penicillium* specific in the soil sample. Nested PCR, using primer Pen-10 and ITS5, was then utilized to target *Penicillium* species specifically. The discrimination of *Penicillium* species was possible due to length heterogeneity of this gene region. Eight different peaks was detected in the soil sample with ARISA and eight different species could be isolated on growth media. The technique proved useful for the detection and quantification of *Penicillium* species in the soil.

Opsomming

Grond word tans beskou as die setel van meeste van die aarde se onontdekte biodiversiteit. Die doel van die studie was om die omvang van mikrobiese diversiteit in die unieke fynbos bioom van die Wes-Kaap te bepaal. Daar word aanvaar dat heelwat prosesse verantwoordelik is vir die aansienlike mikrobiese diversiteit in die grond. Addisioneel is gepoog om te bepaal of daar ‘n verband tussen mikrobiese diversiteit, fisio-chemiese eienskappe en die struktuur van die plant gemeenskap bestaan. Die studie het gebruik gemaak van molekulêre tegnieke om mikrobiese gemeenskappe te klassifiseer, veral geoutomatiseerde ribosomale intergeniese afstand ontleding (ARISA)

Die belangrikste eienskap van grond deoksiribonukleïensuur DNS wat gebruik word in molekulêre tegnieke is suiwerheid van humiensuur en fenoliese komponente. Hierdie komponente tree op as PKR inhibitore en moet verwyder word tydens die DNS ekstraksie prosedure. Die eerste doelwit van die studie was dus die ontwikkeling van ‘n effektiewe DNS ekstraksie protokol deur gebruik te maak van kationies sedimentasie van humiensuur. Die kombinasie van kationies sedimentasie en die bevoeging van PVPP en KCl lewer DNS geskik vir PKR met bakteriese en fungi spesifieke inleiers.

Die herhaalbaarheid en akkuraatheid van ARISA en ARISA-PKR was bepaal omdat hierdie twee faktore ‘n belangrike invloed het op die resultate wat verkry word met die tegniek. Die universele inleier stel, ITS4 en ITS5, was getoets vir die karakterisering van fungi gemeenskappe in fynbos grond. Die inleiers het herhaalbare ARISA profiele gelewer met geringe variasie tussen ARISA-PKR reaksies.

Die gemeenskapsamestelling van beide die fungus en bakteriese populasies in die Sand fynbos was gekarakteriseer. Grond van 4 verskillende liggings is vergelyk om die diversiteit van fungi en bakterieë op ‘n lokale sowel as ‘n landskap skaal te ondersoek. ‘n Molekulêre benadering was gebruik om die totale grond DNS te isoleer. Die 16S-23S intergeniese spasiëring gebied van die bakteriese rRNA operon was geamplifiseer om die bakteriese gemeenskaps profiel in die grond te verkry (B-ARISA). Ooreenstemmend is die interne getranskribeerde spasieerder area, ITS1, ITS2 en die 5.8S rRNA geen van die fungus rRNA operon ge-amplifiseer vir fungus ARISA (F-ARISA). Die gemeenskaps samestelling van die verskillende

monsters is statisties ontleed. ARISA data is gebruik om verskillende spesie teenwoordigheid en beramings modelle te evalueer en die aantal bakteriese en fungus spesies te bepaal. Diversiteit, gelykheid en dominansie van die mikrobiese gemeenskappe was bepaal en gebruik om die mikrobiese diversiteit van die Sand fynbos te beskryf. Die spasiëring oriëntasie van die bakteriese en fungus spesie rykheid en diversiteit is ondersoek deur gebruik te maak van die spesie-area-verhouding en die beta diversiteit van die twee gemeenskappe. Die korrelasie tussen die grond eienskappe en die fungus en bakteriese gemeenskapstruktuur sowel as die korrelasie tussen fungus en bakteriese gemeenskapstruktuur en die plant gemeenskapstruktuur was bepaal. Die resultate toon dat die aantal bakteriese spesies sowel as bakteriese diversiteit hoër was op ‘n lokale skaal. Fungi aan die ander kant toon ‘n hoër spesies diversiteit op ‘n groter skaal. Bakteriese gemeenskapstruktuur toon ‘n nouer verband met die plant gemeenskap, waar die fungi weer ooreenstem met verwyderings patrone.

Die gebruik van genus spesifieke inleiers vir die toepassing op diversiteit studies was getoets. Die genus *Penicillium* is wydverspreid in grond habitatte, hoewel die omvang van hul diversiteit nie bekend is nie. Die ontwikkeling van ‘n groep spesifieke molekulêre tegnieke sal dus handig wees om verdere kennis oor die diversiteit van *Penicillium* te bekom. Vir hierdie rede is *Penicillium* as model organisme gekies. *Penicillium* spesifieke inleiers wat die 18S rRNA ITS geen area teiken, was ge-evalueer. Die fungus spesifieke inleiers ITS4 en ITS5 was gebruik om grond *Penicillium* te teiken. Addisioneel is nested PKR gebruik met behulp van die inleiers Pen-10 en ITS5 om spesifiek *Penicillium* spesies te teiken. Die uitsondering van *Penicillium* spesies was moontlik as gevolg van die lengte verskille in die geen area. Agt verskillende pieke kon in die grond monster gevind word met ARISA en agt verskillende spesies kon ook met behulp van uitplaat tegnieke op groeimedia gevind word. Die tegniek kan dus suksesvol gebruik word om *Penicillium* in die grond waar te neem en te kwantifiseer.

Chapter 1

Current advances in microbial ecology

1. Microbial soil abundance and diversity

The soil environment is thought to contain a large proportion of the earth's undiscovered biodiversity. The number of fungal species currently described is approximately 77000 of the estimated 1.5 million species in the world (Hawksworth 2001). The number of bacterial species describe is approximately 5422 (Euzeby 2004). The current estimate of bacterial species, however, is anywhere from 400 000 (Groombridge and Jenkins 2002) to 10^6 species (Hawksworth and Kalin-Arroyo 1995). This means that about 3 % of both fungal and bacterial species have been described. The goal of microbial ecology is to understand the extent of microbial diversity and the processes that give rise to this diversity (Torsvik *et al.* 1990, Zhou *et al.* 2004). In addition, microbial ecologists aim to link microbial diversity to ecosystem function (Torsvik and Wardle 2002, Nannipieri *et al.* 2003, Coleman and Whitman 2005, Gutknecht *et al.* 2006, Urich *et al.* 2008).

The soil environment and the microbes living within, present an ideal opportunity to test ecological theories and to develop new ones (Lynch *et al.* 2004). Studying the microbial diversity and processes has positively contributed to the understanding of ecological theory and the understanding of ecosystems. However, researchers for the most part, focused on macro-ecological systems and thus, much more is known about these systems (Dale and Beyeler 2001). This is despite the importance of the soil microbial communities in all the biochemical cycles such as the nitrogen and carbon cycle (Germida 2002, Hayatsu *et al.* 2008).

The inherent difficulties in studying microbial ecological systems is the main reason for the limitation in our understanding of these habitats (Kirk *et al.* 2004). The microscopic nature of microorganisms make the enumeration and identification of these organisms in environmental samples more complex. The growth requirements of microorganisms are also very diverse and most microbial species are non-culturable (Thorn 1997, Van Elsas *et al.* 2000, Jacobs *et al.* 2005, Leckie 2005). It is thought that less than 1 % of all soil microbes can be cultured by traditional culturing methods. Results from both, culturing and nucleic acid-based approaches indicate that soil microbial richness is even higher than previously imagined (Thorn 1997, Van Elsas 2000, Torsvik 2001).

2. Current ecological theories applying to soil microbial communities

Microbial communities are affected by spatial and temporal habitat heterogeneity, variations in soil chemical composition and habitat disturbances, as is the case with above ground communities. The habitat heterogeneity is also influenced by the heterogeneity and diversity of other organisms, especially that of plants. Stress, such as desiccation, has a negative effect on diversity. Factors which have a positive effect on diversity are resource diversity and biological interactions. The result of increased diversity is increased ecosystem stability (Griffiths *et al.* 1997, Nannipieri *et al.* 2003).

The idea that a diverse ecosystem is more resilient and stable was first proposed by MacArther (1955). He stated that within an ecosystem with many energy pathways, changes in the numbers of one species would affect other species less dramatically than would be the case if fewer energy pathways existed. High biodiversity is also associated with high functional redundancy (Yin *et al.* 2000, Nannipieri *et al.* 2003). The loss of species in high diversity soils due to disturbances does not necessarily result in the loss in soil function.

Another aspect of current ecological theory applies to the dispersal patterns of microorganisms. The species-area relationship power-law is one of the few laws that exists in ecological theory. The power-law describes the linear logarithmic relationship between the log number of species and the log of the scale of the area (Kilpatrick and Ives 2003). Although the species-area relationship for microorganisms have not been studied extensively, indications are that soil microorganisms also comply to the power-law (Lawton 1999, Green *et al.* 2004, Honer-Devine *et al.* 2004, Martin and Goldenfeld 2006, Zhou *et al.* 2008).

3. History of soil diversity studies of soil microorganisms

The history of soil microbial ecology started in the 1900's at which time, only culture dependant techniques were used to quantify the major groups of microorganisms (Wall *et al.* 2005). The number of microbial groups which are culturable are limited. The planctomycetes for example are non-culturable and can only be detected with molecular techniques (Kowalchuk and Stephen 2001). Culture-based techniques also results in an underestimate of the number and diversity of bacteria and fungi.

The ecology of soil microorganisms in natural systems was, however, not a priority of any research focus during this era. Initial research conducted in the field of soil microbiology focused mainly on agricultural systems with the emphasis on quantification of plant pathogens (Cutler and Crump 1920). When looking at natural systems, the focus was mainly on the taxonomy and abundance of microorganisms (Hammond 1938). This happened as a result of the development of microscopy techniques which enabled researchers to study the micro-morphology of organisms (Sieracki *et al.* 1985).

Research during the 1960's and 1970's lead to the development of the concept of biodiversity and advancement in the knowledge of the role that species diversity plays in the ecosystem (Hariston *et al.* 1968, Swift *et al.* 1979). The focus of researchers in this era was on nutrient cycling in arable and non-arable land (Lie and Mulder 1971). Before widely-used molecular tools became available, numerous studies were conducted on the cycling of nutrients such as carbon and nitrogen in the soil ecosystem (MacDonald *et al.* 1989). These studies were made possible by the development of isotopic tracers (Schoenheimer and Rittenberg 1935, Verschoor *et al.* 2005). With the aid of laboratory systems, an improved understanding of the role of microbes in the soil food web was established (Turpeinen *et al.* 2002).

Techniques using culture media improved with the development of more specialized isolation media that contain specific substrates, and the addition of metabolic inhibitors like antibiotics (Janssen *et al.* 2002). The development of molecular techniques led to further developments in ecosystem science and to a new understanding of the species concepts. The field of soil ecology was now able to address various issues such as species loss and global climate change (Ingram and Freckman 1998, Heal 1999, Ruess *et al.* 2001). The introduction of bioinformatics, together with molecular techniques, resulted in a shift of focus to the question of biodiversity and the role of biodiversity in the function of ecosystems (Hawksworth and Colwell 1992, Sugawara 1996). The more fundamental ecological questions such as the spatial and temporal patterning of soil microorganisms also received considerable attention in diversity studies (Fisher and Triplett 1999, Green *et al.* 2004, Mummey and Stahl 2006, Carrino-Kyker and Swanson 2008, Dimitriu 2008). Current research still aims to determine the extent of microbial diversity in the soil environment (Torsvik and Øvreås 2002, Grüter *et al.* 2006, Koeppel *et al.* 2008).

4. Alpha diversity of microbial communities

Alpha diversity can be defined as the diversity of a specific group of organisms or communities within a specific area. The most important issue concerning diversity is the way in which it is measured (Lozupone 2008). The development of robust indicators was a problem particularly due to the different scales at which diversity was measured. Some indices take into account not only the number of species but also the evenness of the species distribution. In order to describe α-diversity, a number of indices are used. These include the Simpson index (Simpson 1949), which is essentially a dominance index, and the Shannon-Weaver index (Shannon 1948), which is a measure of chaos or entropy of the community. The Shannon-Weaver is now commonly used to express the level of microbial diversity in a particular habitat or niche (Nübel *et al.* 1999, Rodríguez *et al.* 2007, Srivastava *et al.* 2007). The species richness of especially bacterial communities in soil has received particular attention.

The soil environment is reported to contain up to 5000 species and 10^9 bacteria cells per gram of soil (Schloss and Handelsman, 2006). Determining the exact number of species over larger areas is not practical due to the high number of samples required. For this reason existing ecological species accumulation and species estimations models are applied. These species accumulation models include the Power model, Monod function, Negative exponential model, exponential model, Asymptotic regression, Rational function, Chapman-Richards, Weibull and Beta-P model (Arrhenius 1921, Monad 1950, Mielke and Johnson 1974, Ratkowski 1983, Brown and Mayer 1988, Miller and Weigert 1989, Ratkowski 1990). The fitting of specific models to data allows for the direct comparison of species accumulation curves between samples. Other species estimation models are nonparametric and include 1ste Order Jackknife (Burnham and Overton 1979), Chao (Chao 1984), Bootstrap (Smith and Van Bell 1984) and Michaelis-Menten (Raaijmakers 1987). There is no clear indication which method is superior and these methods seem to give different results within different systems.

The species-area relationship is a good measure of the number of species one can expect in a specific sized area. The number of species is plotted against the size of the area on a logarithmic scale. The gradient of the species area curve is called the z-value. The larger the z-value, the steeper the curve and subsequently the higher

the number of species that can be expected in an area. Numerous studies inferred the z-value for species including plants (Usher *et al.* 1973), protozoa (Hillebrand *et al.* 2000), nematodes (Azovsky 2002), fungi (Green *et al.* 2006) and bacteria (Horner-Devine *et al.* 2004). Some studies reported on bacterial z-values as low as 0.05 (Horner-Devine, 2006) in salt marsh soil while, a high value of 0.47 (Noguez *et al.* 2005) was observed in tropical deciduous forest. Z-values as high as 0.2 to 0.23 were reported for fungal species area relationships (Peay *et al.* 2007). The z-value for microbial communities, although believed by some authors to be much lower than that of plants and animals seem to be of a similar range (Noguez *et al.* 2005, Green *et al.* 2006, Horner-Devine 2006, Peay *et al.* 2007). The z-value is expected to be higher when the ecosystems occur as discreet islands, than would be the case for continuous ecosystems (Usher 1979). The same high species area relationship observed in island ecosystems, is also a feature of isolated managed nature reserves (Miller and Harris 1977, Bell *et al.* 2005, Peay *et al.* 2007).

5. Beta diversity of microbial communities

Beta-diversity is defined as the variation of species composition over space and time (Anderson *et al.* 2006). The measure of beta diversity is usually determined by pair wise comparison of sites. The different similarity indices used include the Jaccard, Sørensen, and Whittaker index (Jaccard 1908, Sørensen, 1948, Whittaker 1952, Real and Vargas 1996, Hewson and Fuhrman 2006). The Jaccard- and Sørensen indices are based on the presence or absence of species and are particularly useful in cases where the relative abundance of species is not known. The Whittaker index, on the other hand, is useful when the relative abundance of species is known within a sample. The Whittaker index makes use of species ratios which means no standardization is necessary before samples are compared. This index can thus be effectively used with techniques such as ARISA and T-RFLP, where the relative abundance of species is shown by the fluorescent intensities and standardization between samples is difficult (Steele *et al.* 2005, Hewson and Furman 2006, Hewson *et al.* 2007). The number of studies evaluating the alpha diversity of soil microbial communities especially on a small scale is numerous, but little has been done on the spatial distribution or beta diversity of soil microorganisms.

Previous studies published on soil microbial diversity suggested that the dispersal of soil microorganisms is fairly homogenous, with the exception of variations occurring due to large differences in physico-chemical properties. The origin of microbial beta diversity has been a contentious issue (Lozupone *et al.* 2006). Firstly, it is accepted that the environment selects for organisms to a certain extent and is only partly responsible for spatial variation patterns. It can thus be said that environmental factors in itself is spatially variable. Environmental factors found to be most important in the regulation of microbial communities and diversity are pH, organic matter, nitrogen, oxygen, carbon, phosphorus, Na, Mg, Cu, and Ca content (Brady 1984, Bending *et al.* 2002, Denton 2007, Nieder and Benbi 2008).

The dispersal history and the subsequent deposited heterogeneity of microorganisms play an important role in the community structure. Dispersal history arises from random dispersal of organisms and the local dynamics of speciation and extinction (Hubble 2001). The balance is seen as a trade-off between the environmental factors and dispersal history. This spatial patterning is typically presented as a distance decay relationship and is now believed to observe the power-law of distance decay. This in essence implies that regardless of the similarity index used to compare samples, the similarity should decrease with the increase in spatial separation. In general, this distance decay relationship was much steeper for soil bacteria than for soil fungi (Green *et al.* 2004).

6. Soil as microbial habitat

Soil is a consolidated mineral or organic substance on the earth's surface that provides a natural medium for growth of land plants and the support of that which live and function within. Soil is a heterogeneous system that is generally relatively poor in nutrients when compared with the nutrient levels found in the rhizophere of plant roots (Davison 1988).

The properties of soil are the result of biotic and abiotic components and the interactions between these components (Aon *et al.* 2001). Most of the soils found on earth are mineral soils primarily composed of mineral particles. The physical properties of the soils are mostly the result of the physical properties of the mineral fraction it contains (Ugolini *et al.* 1996). Sandy mineral soils generally contain

between 2 to 7 % organic matter (Christensen and Sørensen 2006). The organic matter includes humic substances and other decomposing or partially decomposing material, for example plant roots and dead animals. The various soil gasses, eg. CO_2, N_2, O_2 and CH_4, water and dissolved solutes make up the rest of the soil habitat (Fu *et al.* 2005).

The microbial community has the ability to alter the physical properties of the soil (Bond and Harris 1964). Soil determines plant productivity of terrestrial ecosystems and it maintains biogeochemical cycling due to the inhabiting microorganisms. All substances in the soil are degraded over time including organic compounds such as persistent xenobiotics and naturally occurring polyphenolic compounds (Gadd 2004). The living population inhabiting soil includes animals, plants, fungi, protista and bacteria.

7. The mechanisms that determine community structure

The study of soil microbial communities mainly concentrated on for the factors which influence soil microbial diversity (Weiner and Keddy 1999). There are, however, no single rule which govern microbial diversity and community structure. All factors appear to have an influence to a greater or smaller extent depending on the ecosystem or group of organisms studied. In short, these factors are space, time, physical soil properties, chemical properties and interactions with other organisms.

7.1 Spatial distribution

The spatial patterns of some organisms, for example plants and macro fauna can easily be studied compared to the spatial variance of soil microorganisms which are more cryptic in nature (Hernandez-Stefanoni and Ponce-Hernandez 2005, Prasad *et al.* 2006). For this reason the effect of spatial patterns on species interactions and the distribution of soil microorganisms are still in question. Most studies, focusing on spatial patterns in soil, were designed to determine the distribution of abiotic factors for example pH and nutrients (Huston and DeAngelis 1994, Glazebrook and Robertson 1999). Studies in microbial soil ecology also tend to focus on total microbial biomass and other collective parameters such as their capacity to degrade

organic matter. Few studies were conducted that have also considered the variations in the microbial community structures. Designing field experiments to examine the spatial distribution of microorganisms remains difficult due to the large number of samples required to be representative. It was found that using samples of 1 to 5 g of soil will bias the results and favour dominant species (Grundmann and Gourbiere 1999).

The principles of spatial distribution in ecology is well known (Tilman and Kareiva 1997) and more recently the importance of spatial distribution in microbial ecology was studied (Saetre and Bååth 2000, Green *et al.* 2004, Kang and Mills 2005). The concept of space in ecology is important in order to understand the distribution and diversity of species. Despite this, the importance of spatial patterns in soil ecology studies have focused on the aboveground biota. The low degree of resource specialization by microorganism in the soil seems to be contradictory when looking at the exceptionally high degree of soil microbial diversity (Ekschmitt and Griffiths 1998). Spatial heterogeneity is an important contributor in the maintenance of microbial diversity. The soil environment is generally discontinues and largely heterogeneous and spatial effects may form within a community even if the soil environment is completely homogenous.

Gradients of resources and physical and chemical conditions exist in the soil over space. This heterogeneity allows for the co-existence of competing microorganisms due to the partitioning of the niche. The few studies that determined the spatial patterns of soil biota clearly indicated spatially predictable patterns at various scales. The spatial variability of soil biota, in the past were seen as a problem when studying soil biodiversity, but now is thought to be one of the main driving forces for this biodiversity. This spatial variability can be described as patches, or areas of similar species composition. The scale of the biodiversity is highly variable and studies showed highly similar patches of 1 to 3m (Ettema and Wardle 2002). On a finer scale, patches were as little as 5cm (Ettema and Wardle 2002). Studies have measured spatial variance at a single analytical scale and observed autocorrelation ranging from µm's to km's (Peay *et al.* 2007). The autocorrelation observed depended on the scale and the focus of the study. These studies revealed a spatial dependency of 1m and less (Ettema and Wardle 2002, Green and Bohannan 2006). This spatial pattern was also nested within a larger scale nested within a larger scale (Ettema and Wardle 2002, Green and Bohannan 2006).

Studies have shown that many species may co-exist on a single resource when they have relative low mobility (King and Hastings 2003). The spatial separation of microorganisms in a homogenous system may still occur. Intrinsic population processes such as reproduction and limitation on dispersal capabilities leads to spatial patterning and aggregation. The relative immobility of soil microorganisms and the complex soil matrix, limits competition as a community structuring cause. Resource heterogeneity leads to the formation of microhabitats and these different microhabitats enables spatial separation of organisms that may potentially compete. Soil studies in simplified laboratory conditions indicated limited overlapping between species after certain time periods. This was due to the limited heterogeneity in the system (Smith *et al.* 1996).

Species richness is a function of microhabitat diversity. Microbial communities are exposed to environmental gradients which influence their abundance, composition and activity. The abiotic factors that influence microbial community structure and spatial scaling are for example pH, temperature, nutrients and biotic predation and competition. Some of the biotic and abiotic factors may be of microscopic scale. Soil practical size and soil structure are examples of this. Other factors may influence microbial scaling over the larger landscape scale for example vegetation and climatic conditions.

The differences in scale of influence that arise from the various environmental and soil factors results in nested scales of variability and thus promotes diversity. The spatial patterns observed when studying microorganisms can be described as a species-area relationship. The positive power-law relationship is observed between the number of species in an area and the magnitude of the area during studies observing animal and plant spatial patterns. The species-area relationship is important for the understanding of landscape and global biodiversity. It was demonstrated that communities between samples taken closer together are more similar in composition than those taken further apart (Green *et al.* 2004, Green and Bohannan 2006, Noguez 2005, Horner-Devine *et al.* 2004).

7.2 Physico-chemical factors that influence soil as a microbial habitat

7.2.1 Soil organic compounds

Most microorganisms are found in the top layers of the soil profile, usually the top 10 centimetres, since this is typically the location of the major concentrations of organic matter (Barness *et al.* 2008). Organisms may however occur at depths of several kilometres below the soil surface, but the types of organisms that occur this far down are not the same as those close to the surface (Fang 2005). Many organisms in soil are commonly found close to root surfaces in the rhizosphere, within living and dead roots, on soil particles, or amongst aggregates of soil particles (Smalla *et al.* 2001).

Organic carbon compounds, from plant residues and soil organic matter, are used as energy and carbon sources by the heterotrophic microorganisms in soil. The quality of the plant litter reflects the biochemical composition of the substrates and the physical availability of the substrates to the microorganisms (Wardle and Giller 1996, Bending *et al.* 2002). Bacteria tend to respond rapidly to additions of simple carbon compounds such as starch, sugars, and amino acids, while fungi and actinomycetes dominate if complex carbon compounds such as cellulose and more resistant lignin materials are available (Nieder and Benbi 2008). When organic residues are deposited on the soil surface, microbial activity is dominated by fungi (Doran 1980, Hendrix *et al.* 1986, Sá *et al.* 2001). The adsorption of organic compounds by soil colloids retards their microbial degradation and the location of potential substrates inside pores or micro aggregates reduces their accessibility to soil microorganisms (Ladd *et al.* 1996).

7.2.2 Soil texture

The various fractions of the soils are classified according to their size into clay, silt and sand (Naime 2001). The various fractions of the soil provide a surface for the development of the soil microbial communities. Clay and colloid material have the smallest diameter and, therefore, as a whole have the largest overall area for interaction with microbes. The soil texture also determines the levels of aeration in the soil. Finely textured clay soils hamper the movement of air in the soil and these result in higher levels of carbon dioxide due the metabolic activity. Low oxygen levels generally favour the anaerobic and micro-aerophilic organisms (Ferrara-

Guerrero 2007). Soil containing a higher sand fraction on the other hand tends to be better aerated and drained and thus favour the obligate aerobic microorganism (McGechan *et al.* 2005).

7.2.3 Soil atmosphere

Due to the slow aeration of the soil atmosphere and microbial metabolic processes, the main characteristic is the CO_2 component which is on average 100x that of the atmosphere (Zuberer and Wollum 2005). The oxygen available in the soil correlates strongly to the soil moisture content and the level of microbial activity (Schjonning *et al.* 2003). When the soil aggregates are saturated with water, local patches of prevailing anaerobic conditions occur in the soil (Skopp *et al.* 1990). This contributes to habitat heterogeneity. Anaerobic conditions result in a decrease in the redox potential in the soil. This lower redox potential causes anaerobic processes to occur in the soil for example denitrification and iron reduction (Knowles 1982, Korom 1992).

7.2.4 Soil water

The soil water is necessary for life in the soil. Soil water acts as a solvent for the nutrients in the soil, making them accessible for uptake by living organisms. Soil water is also the medium in which many soil biota live and move, for example nematodes, protozoa and bacterial communities. The amount of water that is available to microbes depends on the concentration of solutes, temperature, and the soil texture. Finer textured clay has higher water holding capacity but less water is available for microbes due to adsorption (Voroney 2007). Generally, the soil pores will contain water as well as air. Most of the water is held in pores as films and are adsorbed onto the soil particles (Alexander 1964). After heavy rains, the soil pores are filled with water and the soil is considered to be saturated. The percentage soil water, thus, determines the aeration status of the soil which in turn affects the soil microbial population.

Water is an important medium for microbial movement in the soil and a lack of water will cause a decrease in the movement of microorganisms and a sharp increase in predation on microbes by protozoa (Van Veen and Kuikman 1990). The available

soil water is a more important factor influencing microorganisms than the total soil water. Filamentous fungi are generally capable to withstand much lower water activities than the bacteria, protozoa and the algae (Wollenzien *et al.* 1995). Some bacteria, for example the Streptomycetes, tolerate water activity as low as a_w = 0.90 (Berrocol *et al.* 1996). Filamentous actinomycetes and some fungi tolerate a water activitie as low as a_w = 0.62 (Goodfellow and Williams 1983). The process of nitrogen fixation is completely inhibited at water potentials below -2.1 MPa (Kuo and Boersma 1971). Soil water also has a marked influence on the available carbon and the microbial activity in the soil (Balesdent *et al.* 2000). The amount of organic matter also has a positive influence on the water holding capacity and availability of the soil nutrients (Williams and Rice 2006).

7.2.5 Soil pH

The largest proportion of the earth's soils can be described as acidic. The major base cations that occur in soil are K^+, Mg^+, Ca^+ and Na^+ and any reduction in the concentration of these cations will cause a reduction in the pH (Elias and Cresser 1995, McLaughlin and Wimmer 1998). The base cations may leach out of the soil due to their replacement on cation exchange sites by H^+ and Al^{3+} (McLaughlin and Wimmer 1998). Some of the various sources of soil acidity are carbonic acid, microbial oxidation of NH^{4+} to NO^{3-}, atmospheric pollution for example acid rain and the decomposition of organic matter (Wherry 1920).

Carbonic acid is formed when CO_2 dissolves in water which dissociates to form H^+ ions (Wherry 1920). The pH of the soil is an important feature that determines the nutrient availability the soil (Elias and Cresser 1995, McLaughlin and Wimmer 1998). Acidic soils are characterised by higher amounts of heavy metals for example Al^{3+} (Dijkshoorn *et al.* 2005). At a very low pH the amount of soluble heavy metals may reach toxic levels for plants and microbes (Denton 2007). The available phosphorus also reduces with the decrease in pH due to the formation of ion and aluminium phosphate (Jongbloed *et al.* 1991). Phosphorus is only released when the pH increases. The availability of mineral nitrogen is also depended on the soil pH. Nitrification is impaired by acid pH while the volatilization of NH^{4+} to NH^3 is promoted by alkaline pH conditions (Miller and Cramer 2004, Nordin *et al.* 2004). Soil pH correlates positively with nitrogen mineralization rates (Giesler *et al.* 1998). In

addition, soil pH may influence the composition of the water-soluble soil amino acid pool (Kielland 1994).

The effect of pH on soil organisms is well documented. Most soil microbes prefer a soil pH of between 6 and 7.5, but the acidophiles may grow in a pH as low as 1 and the alkalophiles have the ability to grow in a pH above 9 (Krulwich and Guffanti 1989, Hartel 2005). The pH of the soil is an important factor for the repression of plant pathogens (Haas and Défago 2005). Any change in the pH of the soil may lead to favourable conditions for a different set of soil microorganisms which may lead to a change in the composition of the soil microbial community (Bååth and Anderson 2003).

7.2.6 Soil temperature

The soil temperature is largely a function of the climate. The activity of soil microbes are generally optimal within the temperatures 20–40°C (Roper 1985). The warmer temperatures tend to favour bacteria while colder temperatures are beneficial to fungi (Bassio *et al.* 1998, Lipson *et al.* 2002). The soil temperature influences the rate at which soil chemical and metabolic processes take place. Within a limited range the metabolic rate doubles for every 10°C increase in soil temperature (Price and Sowers 2004). This increase in temperature also corresponds to an increase in microbial biomass and respiration rates when the soil moisture conditions are favourable (Rastogi *et al.* 2002, Wang *et al.* 2003). The soil temperature and the soil moisture levels are thus unavoidably linked. Due to the high amount of energy needed to raise the temperature of water by 1°C, the addition of water has a large influence on the soil temperature (Visher 1923). Soil water also releases its energy far slower than air. Soil depth has a stabilizing effect on the soil temperature, with less fluctuation being observed with an increase in soil depth (Chacko and Renuka 2002).

7.3 The influence of plant diversity on the soil microbial communities

The variety and quantity of components introduced by plants into the soil vary greatly among plant species. The abundance of the plants themselves may differ in different

ecosystems. The differences between plant species has a direct effect on the quality and quantity of soil organic matter and exudates added to the soil.

The carbon from plants and plant residues increases the biodiversity in the soil. The aboveground differences in the plant community type and density can cause variations in soil microbial communities. Thus, plant communities and soil properties are important in shaping the soil microbial community. The indirect effect of a plant community on the composition and structure of the soil microbial community, is manifested by the effect of plant residues. Soil moisture, temperature, and pH may play a role in the decay of plant material (Choi 2006). At the interface between the soil and plant litter, the soil microbial communities become very active. It is reported that the bulk of the residue may be actively under attack by soil microbes. During the decay process, some of the litter may be transported by the soil fauna beneath the soil and mixed with the upper horizon of the soil (Choi 2006).

The total amount of the plant residues play a major role with regards to the microbial community. The total amount of plant residues include plant litter, as well as the root exudates (Kögel-Knabner 2002). The composition of these plant residues has a direct influence on the microbial soil community (Yang *et al.* 2007). Plant residues are comprised of complex polymers like lignin, cellulose and hemicelluloses (Kögel-Knabner 2002, Albrecht *et al.* 2008). The simpler compounds for example, sugars extruded by roots, are more easily decomposed. Generally, the compounds with high energy value are not very resistant to enzymatic degradation (Kögel-Knabner 2002, Albrecht *et al.* 2008). Generally, complex compounds promote microbial diversity because large consortiums of organisms are needed to decompose these compounds (Boopathy 2001). Each organism has a specific niche in the processes in the soil environment.

7.4 Ecological trade-offs

Ecological trade-offs are introduced in many ecological theories to be a driving force for the maintenance of biological diversity (Horn and MacArthur 1972, Armstrong 1976, Hastings 1980, Tilman 1994, Pacala and Rees 1998). Ecological trade-off implies a trade-off between an attribute which may be advantagous and at the same time resulting in a disadvantage to another function. Organisms may have an

increased capacity to utilize one nutrient source but a reduced capacity to use another. Ecological trade-offs are common in nature because there are limited resources for all possible cellular functions (Bohannan *et al.* 2002). The ability to perform all possible cell functions would require a huge genome, which could not be efficiently replicated. The result of ecological trade-offs are populations of organisms with diverse growth requirements. The occurrence of ecological trade-offs in soil ecosystems limits competitive exclusion. This allows organisms competing for the same resource to coexist. The existence of ecological trade-offs has an important effect on the community structure and function (Bohannan *et al.* 2002, Bonsall *et al.* 2002, Walker 2003).

Ecological trade-offs may also be observed when microorganisms in the soil experience periods of adverse conditions. Numerous studies have demonstrated the occurrence of ecological trade-offs in natural ecosystems (Bohannan *et al.* 2002, Arnold and Herre 2003, Grandy *et al.* 2006, Prosser *et al.* 2007 Gudelj *et al.* 2007). The trade-off is made between the organism's ability to effectively and quickly utilize resources and the organism's ability to survive adverse conditions.

7.5 Temporal changes in soil communities

Soil microbial communities are not stagnant but their composition is changing constantly over various timeframes. These changes in the microbial communities may be seasonal and, therefore, dependant on short term climatic conditions. Changes in the plant communities result in changes in microbial community structure (Smit *et al.* 2003). Community structure can also change over a longer timeframe. Succession of plant communities towards maximum plant biomass, results in succession of microbial communities. This succession is around 25 to 30 years for fynbos plots. Disturbances, such as regular fire events, will again change the structure of the soil microbial community (Hart *et al.* 2005, Díaz-Raviña *et al.* 2006, Janzen and Tobin-Janzen 2007).

8. The soil biota

Soil microbes can either follow the r or K selection strategy (MacArther and Wilson 1967, Liebich *et al.* 2006). K-strategists select for traits which result in persistence in the soil under conditions of low nutrient levels or other unfavourable conditions (Fontaine *et al.* 2003). These organisms are slow-growing and utilize substances that are persistent in the soil. These organisms are referred to as oligotrophes, do not perform well on isolation media in the lab and are often not culturable. R-strategists, in contrast, select for traits which make them more competitive under high nutrient conditions (Liebich *et al.* 2006). For example, conditions prevailing after the addition of fertilizers to the soil.

8.1 Fungi

Fungi can form hyphal mats, which can extend centimetres or even meters through the soil (Griffiths *et al.* 1991). They can also form a network of hyphae inside soil aggregates (Tisdall *et al.* 1991). Fungi are generally much more efficient at assimilating and storing nutrients than bacteria (Six *et al.* 2006). One reason for this higher nutrient storage by fungi lies in the chemical composition of their cell walls. Fungal cell walls consist of polymers of chitin and melanin and are very resistant to degradation (Alexander 2004). Bacterial membranes, in comparison, are phospholipids, which are energy-rich and far less recalcitrant (Jastrow *et al.* 2007). They degrade easily and quickly and function as a food source for a wide range of microorganisms. The C: N ratios of fungal biomass is between 7:1 and 25:1. Fungi need a large amount of carbon to grow and reproduce. Fungal biomass may be as much as several hundred meters of hyphae per gram dry weight of soil (Miller 1982).

Fungi are the most energy-efficient organisms in the soil environment (Adu 1978). Filamentous soil fungi bridge across open areas between soil particles. This type of growth exposes the fungi to high levels of oxygen. The hyphae of these fungi tend to darken and form oxygen-impermeable structures including sclerotia and hyphal cords. Terrestrial soil fungi fall pray to insects and a wide variety of animals that are contained in the soil for example earth worms (*Polypheretima elongata*) (Lattaud *et al.* 1998). Predation is an important factor in the reduction of fungal biomass (Kardol *et al.* 2005). Although fungi usually occur in smaller numbers than bacteria, fungi

dominate the biomass and metabolic activity in many soils because of their relatively large size and branching (Ananyeva *et al.* 2006).

8.1.1 Zygomycota

Zygomycetes include common soil born genera *Mucor* and *Rhizopus* (Griffin 1972). Zygomycetes are unique from the other fungi in possessing haploid nuclei and lacks septa between different cells. Zygomycetes are characterized by the composition of its cell wall which contains chitin, chitosan and polyglucuronic acid (Guarro *et al.* 1999). Zygomycetes are specifically adapted for survival in soil and produces thick walled survival spores. These zygospores are formed sexually after the fusion of hyphae of different mating types. When the conditions are favorable, the zygospores germinate to form a sporangiophore which produces sporangiospores asexually (Barnett and Lilly 1956). These spores are easily dispersed by the wind and water. Zygomycetes are important saprophytes in the soil, on animal dung and decomposing fruit (Jackson 1965, Bååth and Söderström 1980, Domsch 1980, Van Elsas 2007).

8.1.2 Glomeromycota

The Glomeromycota is the second oldest phylum of fungi. Thus far about 150 species of the Glomeromycota have been described (Schübler *et al.* 2001). Taxonomy of the group was based on spore structure to describe species, but new molecular techniques may reveal many more species, both culturable and unculturalble (Schübler *et al.* 2001). The Glomeromycota include all the fungi that form arbuscular mycorrhiza with plants and the species *Geosiphon pyrifomis* which has an endosymbiotic relationship with cyanobacteria (Schüβler *et al.* 2001, (Schüβler *et al.* 1996, Schüβler and Kluge 2001). Arbuscular mycorrhizal fungi are obligate symbiotes of plant roots and may grow either inter- or intracellular.

8.1.3 Ascomycota

The ascomycetes are the largest and most diverse phylum of fungi on earth. The

phylum Ascomycetes contains over 50000 species (Van Elsas *et al.* 2007). The Ascomycetes are different from other fungi because they do not have multiple haploid nuclei but one haploid nucleus per cell in their primary mycelium and they are dihaploid. The phylum includes the common soil colonizing genera *Aspergillus*, *Fusarium* and *Penicillium* which dominate soil fungal communities (Griffin 1972). *Penicillium* are typically the dominant species in temperate soil and *Aspergillus* species were shown to dominate in tropical regions (Domsch *et al.* 1980, Christensen 1981). In soil, the Ascomycetes tend to form only asexual spores. Due to the high diversity of ascomycete species in the soil, various species have proved to be important for nutrient cycling in the soil (Osono *et al.* 2003). The soil Asomycetes also include important plant pathogens (Vakalounakis and Fragkiadakis 2003).

8.1.4 Basidiomycota

The Basidiomycetes form the second largest phylum with about 9000 terrestrial species described (Lynch and Thorn 2006). They are characterized by separated mycelium with two haploid nuclei in each cell. The Basidiomycetes include various saprophytes that have the ability to degrade complex polymers such as cellulose, hemicelluloses and lignin (Hibbett and Thorn 2001). They, therefore, play an important role in the degradation of leaf litter and woody debris. The basidiomycetes contains various significant plant pathogens of which most are rusts (Littlefield and Heath 1979, McLaughlin *et al.* 1995) and smuts (Wennström 1999). The phylum also includes some ectomycorrhizal fungi which form symbiotic relationships with plants (Hibbett and Thorn 2001).

8.2 Bacteria

Soil bacteria may reach numbers as high as 10^8 to 10^9 cells per gram of dry weight, with a biomass density of 300 – 30000 kg/ha (Rosello-Mora and Amann 2001). They are perhaps the most complex and diverse group of soil microorganisms with about 500 to 5000 different species per gram of soil and are adapted to most environments (Borneman and Triplett. 1997, Torsvik *et al.*1990, Schloss and Handelsman 2006). Bacteria tend to accumulate inside soil aggregates because they are less likely to be preyed upon by soil macro-organisms such as protozoa and mites in this

environment (Sessitsch *et al.* 2001, Zhang *et al.* 2007). Bacteria can be carried down further into the soil with percolating water, but generally they do not move over large distances. Most bacteria are unable to self propel and hence their dispersion is dependent on water movement, root growth or the activity of soil and other organisms (Lavelle and Spain 2001).

Water and nutrients must be located in their immediate vicinity. The different proportions of C and N of bacteria and fungi might also play a role in the mineralization and immobilization processes of nutrients in the soil. Bacteria, however, have a lower C:N ratio, between 5:1 and 7:1, and a higher nitrogen requirement and take more nitrogen from the soil for their own requirements (Swift *et al.* 1979, Bloem *et al.* 1997).

Soil particles with smaller pore sizes (2 to 6 µm) are generally more suitable for bacteria (Sessitsch *et al.* 2001, Zhang *et al.* 2007). The small pores leave the bacteria less vulnerable to predation from protozoa. Bacteria that are located on the exposed outer surfaces of sand and organic matter fall prey to protozoa very easily. Some bacterial cells produce extracellular polysaccharides interacting with clay particles and these clay–polysaccharide complexes can persist even after the death of the microbes (Chen 1998, Huang and Bollag 1998). The use of traditional and more recent electron microscopy techniques with staining procedures has allowed the visualization of the microbial groups, and inorganic and organic colloids in the soil matrix (Forster 1994, Assmus *et al.* 1995, Assmus *et al.* 1997, Bakken 1997). All bacteria are aquatic and they live free or attached to surfaces, in water films surrounding solid particles, and inside aggregates (Stotzky 1997).

8.2.1 Actinomycetes

Bacteria from this group are characteristically Gram-positive with a high genomic G + C content of usually more than 60 %. Similar to fungi, Actinomycetes are filamentous and often have profusely branched cells, although their mycelia threads are generally much smaller than those of fungi. Actinomycetes were previously classified as fungi but are classified as bacteria (Waksman 1932). They have no nuclear membrane and separate into spores that closely resemble bacterial cells (Stuart 1959). This phylum includes most bacteria that are able to grow under low nutrient conditions. They,

however, have a low growth rate but have a constant presence in the soil. They are, therefore, classified as K-strategists. Actinomycetes usually grow best in moist, warm, well-aerated soils, and are functionally important in arid-, salt-affected soils (Zenova *et al.* 2007). Members of the phylum Actinomycetes which are present in soil include the genera *Rhodococcus*, *Arthrobacter* and *Micrococcus*.

8.2.2 Proteobacteria

The Proteobateria in the soil is a highly diverse group in terms of their metabolism and environment which they are able to inhabit (Liesack and Stackebrandt 1992, Ng *et al.* 2005, Roesch *et al.* 2007, Zhang *et al.* 2007, Lesaulnier *et al.* 2008). The proteobacteria are divided into subclasses α, β, γ, δ and ε (Woese *et al*. 1985, De Ley 1992, Woese *et al.* 1992,).

8.2.2.1 α-Proteobacteria

The α-Proteobacteria incorporates the majority of the oligotrophic proteobacteria, of which some are capable of growing in nutrient poor soils (Farelly *et al.* 1995). *Rhodobacter* spp. are known to be able to fix CO_2 in the soil (Wang *et al.* 1993). Some species of α-proteobacteria have the metabolic capacity to acquire energy from the single carbon compounds (Sy *et al.* 2001, Holmes *et al.* 1997). α-Protobacteria are capable of forming symbiotic as well as pathogenic relationships with plants. *Rhizobium* spp. is capable of forming nitrogen fixing nodules in association with plant roots (Yanni *et al.* 1997), while *Rhizobium tumefaciens* causes crown gall disease (Tarbah and Goodman 1987, Zoina *et al.* 2001).

8.2.2.2 β- and γ-Proteobacteria

β- and γ-Proteobacteria include many r-strategists (Lebaron *et al.* 2006). These bacteria are abundant in various soils but especially in very fertile soil (Hugenholtz *et al.* 1998). They are capable of effectively colonizing plant roots and grow well in the rhizosphere (Tesar *et al.* 2002, Roesch *et al.* 2008). The subclass ß-Proteobacteria includes some well known plant pathogens for example *Erwinia carotovora (*Boureau

et al. 2006*).* Many of these bacteria produce antibiotics and are antagonistic towards other bacteria and fungi in the soil. Members of the genus *Burkholderia* are known to interact with soil fungi by living as intercellular symbionts (Johansson *et al.* 2004). The β-Proteobacteria subclass includes nitrifying bacteria for example *Nitrosomonas* and *Nitrosospira* (Purkhold *et al.* 2003). β-proteobacteria are also well known for their ability to degrade xenobiotic compounds (Pallud *et al.* 2001).

8.2.3 Phylum Firmicutes

Some very common soil bacteria are included in this phylum such as the genera *Bacillus, Paenibacillus and Clostridium* (Gibbons and Murray 1978). These genera are Gram-positive and have a low genomic G+C content (Gibbons and Murray 1978). *Bacillus* and *Paenibacillus* have the ability to produce endospores that may survive for a long period in the soil (Cano and Borucki 1995, Petras and Casida 1985, Vreeland *et al.* 2000). Members of this phylum are r-strategists (Klappenbach *et al.* 2000). They occur at high numbers in the rhizosphere of plant roots and on the surface of plant residues (Roesch *et al.* 2007).

9. The study of soil microbial ecology in laboratory and natural environments.

Understanding microbial ecology involves the use of laboratory systems as well as field studies (Bohannan 1999). Most of the soil microorganisms cannot be cultured using simple culturing methods (Thorn 1997, Van Elsas *et al.* 2000, Leckie 2005, Jacobs *et al.* 2005). This may give a distorted image of microbial diversity and the interactions in the soil. Due to numerous unknown factors, it is difficult to observe specific interactions in the soil for example when organisms are introduced (Van Elsas 1998). In natural field studies it is also very difficult to manipulate the physical conditions such as moisture conditions. Furthermore, field studies are not easily reproducible. Due to the heterogeneity of natural environments it is also difficult to quantify resources and environmental parameters.

The use of laboratory experiments can largely overcome the complexity and uncontrollable parameters of natural ecosystems (Seidl and Tisdell 1999). An artificial laboratory system, however, has various drawbacks. The dispersal of

microorganisms causes difficulties in maintaining heterogeneity at a small scale (Ettema and Wardle 2002, Jessup *et al.* 2004) which is critical for ecological dynamics and rapid evolution can lead to changes in the population dynamics. The large population size of microorganisms in model systems are often clonal, which does not reflect the diversity of the natural environment were the population of specific species may be small and in equilibrium (Carpenter 1996). This is also due to the differences in scale from the small laboratory system to vast natural soil ecosystems. The relative simplicity of artificial models makes it difficult to extrapolate the behaviour of a specific organism to the more complex natural ecosystems.

10. Molecular approaches designed for studying soil microbial communities.

Limitations of culture based techniques can largely be overcome by the development numerous molecular DNA based techniques (Kirk *et al.* 2004). Over a number of years numerous studies on soil diversity were conducted using small subunit rDNA (Borneman and Triplett 1997, Brown *et al.* 2005, Fierer *et al.* 2007). There are numerous advantages in using rDNA in diversity studies. The 16S region is present in all prokaryotes and the 18S rRNA in all eukaryotes which include the fungi. The rDNA regions have well defined regions used in taxonomic classification, which allows for universal primer design with group specificity.

Non-culturing techniques include the extraction of total DNA and amplification with specific primers. These amplicons are then used in techniques such as Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), and Automated Ribosomal Intergenic Spacer Analysis. (ARISA) (Liu *et al.* 1997, Fisher and Triplett 1999, Torzilli 2006). Although these methods provide little direct evidence to the function of organisms in the soil, it has become invaluable to the understanding of soil microbial diversity. Molecular data can be used to determine the community structure which includes the diversity and the evenness of these communities. The direct extraction of total DNA from soil samples also allows for the preparation of clone libraries of and the subsequent identification of these sequences (Brown *et al.* 2005).

The molecular techniques, however, all have their own specific limitations. The DNA extraction method used may result in variation in diversity (Wintzingerode *et al.*

1997). Various methods may also result in different yields. Bias may result form the different lysis properties of cells. Different types of cells found in soil have different lyses efficiencies (Prosser 2002). Gram-negative cells lyse more easily than Gram-positive cells and fungal mycelia more readily than spores. PCR bias can also influence the relative abundance of certain fragments in the product when conducting diversity studies (Wintzingerode *et al.* 1997). This is especially problematic where ecological samples are compared. Soil generally contains high concentrations of humic acids. Humic acids act as inhibitors of PCR and is often co-extracted during DNA isolation. The removal of humic acids necessitates various purification steps and this often results in DNA loss (Moreira 1998, Dong *et al.* 2006).

The most common technique currently used, is rRNA intergenic spacer analysis (RISA). It provides a method for the estimation of the community diversity and community composition. The technique was first applied to examine microbial diversity in soil from the Eastern Amazonian rainforest (Borneman and Triplett, 1997). The RISA method allows one to estimate the microbial diversity without the need to culture organisms. The bias in favour of fast growing organisms and against slow-growing organisms is largely eliminated with the RISA technique.

The technique requires that the total community DNA must be extracted from the environmental sample. The method involves the amplification of the total extracted DNA and the subsequent electrophoreses on a polyacrylamide gel. The RISA technique has been enhanced by the addition of an automated component to the technique (Fisher and Triplett 1999). PCR, when utilizing ARISA, is performed with fluorescently labelled oligonucleotide primers. Commonly used fluorescent markers are ROX and FAM (Fisher and Triplett 1999, Hewson and Fuhrman 2004). The electrophoresis of the total amplified DNA is performed on an automated system for example the ABI 310 genetic analyzer which detects the fluorescent labelled DNA fragments with the aid of a laser. The ARISA method is an effective and rapid method for estimating the diversity and composition of microbial communities. This is especially useful in ecological studies were a large number of samples need to be processed and diversity determined at spatial and temporal scale.

F-ARISA targets the total fungal community DNA of the intergenic spacer region 1, the 5.8S small subunit and the intergenic spacer region 2. This region, especially within the intergenic spacer regions 1 and 2, displays significant heterogeneity in

length and nucleotide sequence between species. B-ARISA targets the total bacterial community DNA of the intergenic region between the 16S and the 23S subunits of the rDNA genes in the rRNA operon. This region also displays size and sequence heterogeneity between species. In general, the ARISA profile is highly reproducible and requires very low concentrations of PCR product in comparison to terminal restriction fragment length polymorphism (T-RFLP) analysis (Jones *et al.* 2007).

The operational taxonomic units revealed by the ARISA technique are substantially more than revealed by (DGGE) and T-RFLP. In a study published by Jones and Thies (2008) an identical sample showed an 3.6 to 4.2 fold increase in operational taxonomic units when using the T-RFLP compared to DGGE for replicate samples. The ARISA technique revealed 60 to 140 OTU's compared to T-RFLP (Jones *et al.* 2007). The current methods, however, do not fully reveal diversity because total bacterial communities are too complex. Re-annealing kinetics, however, shows extremely high species diversity with up to 5000 species per gram of soil (Tosvik *et al.* 1990). Current research is starting to focus on different functional and taxonomic groups instead of total diversity studies (Wellington 2003). This approach will enable better resolution of especially bacterial diversity but also allow better understanding of the function of diversity in the soil.

11. Definition of fynbos and classification Sand Fynbos

Numerous definitions for fynbos have been used over the years, but the most recent definition was described in 2006 (Mucina and Rutherford 2006). The fynbos biome reseive high amounts of winter rain, periodic fire at intervals of 5 to 50 years. Fynbos can be defined by the dominance of low to medium-height shrubland including the fire prone true fynbos and the renosterveld as well as the non-fire-prone strandveld. The area extends from Port Elizabeth and Clanwilliam. Shrubs have isobilateral picophyllous or microphyllous to mesophyllous leaves. Shurbs are evergreen aphyllous and/or narrow-leaved sclerophyllous hemicryptophytes. The soils have various origins and are generally oligotrophic (Mitchell *et al.* 1984, Holmes and Cowling 1997).

The Cape Floristic Region (CFR) is well-known for being the richest and smallest of the six floral kingdoms in the world (Goldblatt 1978, Cowling and Hilton-Taylor 1994, Linder 2003). The CFR includes a land area of 90 000 km^2 and is the only one of the six floral kingdoms that is entirely contained within a single country (Low and Rebelo 1996, Linder 2003). This is less than 6 % of the total land area of South Africa. Despite the small size of the CFR, it has one of the richest plant species diversities on earth and contains approximately one third of South Africa's plant biodiversity (Bond and Goldblatt 1984). An estimate of the number of plant species was calculated to be approximately 9030 vascular plants of which 8920 are flowering plants (Rebelo and Low 1996). The number of endemic plants is high and estimated to be around 68.7 % of the total number of plants (Cowling and Hilton-Taylor 1994, Goldblatt and Manning 2000). This number of endemic plant species is comparable to endemic levels of the wet neotropics (Cowling and Hilton-Taylor 1994, Low and Rebelo 1996, Linder 2003). The species area relationship in this region is also substantial (Cowling *et al.* 1992). The number of species that can be found per square kilometre in the area is similar when compared to the wet tropics, even though the climate is temperate and best described as Mediterranean (Huntley 1984, Gentry 1986, Low and Rebelo 1996).

The Cape fynbos biome is located in the Western Cape, South Africa from Van Rhynsdorp to the Cape Peninsula and Mountains of the Boland. From the Boland area, the region extends east towards Grahamstown (Low and Rebelo 1996). The fynbos region is characterized by a temperate climate with wet winters and warm dry summers with strong prevailing south easterly winds (Lindesay 1998). The Sand fynbos is the second larges area of fynbos and it covers 15 % of the total fynbos area.

11.1 Plant community structure of the Sand fynbos

The Sand fynbos has a large number of restiodes and proteas, with asteraceous fynbos and patches of ericaceous fynbos. Trees are rare in this area but the white milkwood and low candlewood does occur sporadically (Moll *et al.* 1984). Some of the important species are, *Erica mammosa, Leucospermum parile, Phylica cephalantha, Staberhoa distachya* and *Thamnochortus punctatus* (Rebelo and Low 1996).

11.2 Soil of the Sand fynbos region

The soil of the Cape Floristic Region consists of a mixture of sandstone and shale substrata with local areas of limestone (Kruger and Taylor 1979). It has a highly dissected, rugged topography (Kruger and Taylor 1979) and a diversity of climates with rainfall mostly falling in the winter months and varying from 2000 mm locally to less than 100 mm (Goldblatt and Manning 2002). Rainfall is usually associated with frontal conditions in the winter (Deacon 1992). Summer drought is pronounced in the western parts of the fynbos region.

Ecological gradients are steep as a result of abrupt differences in soil, altitude and precipitation (Colwing 1990). These factors combine to form an unusually large number of local habitats for plants. The soil type observed in the Atlantis Sand fynbos is sandy with an aeolian origin. Soils of the Atlantis Sand fynbos ranges from very shallow to extremely deep (Rebelo 1996, Mucina and Rutherford 2006).

The soils of the study sites, however, are all on average 2m in depth. The pH of the soil in the Atlantis Sand fynbos ranges from 3.6 to 4.7. The soil typically has an organic matter content of between 1-3 % and an available carbon content of less than 1 % (Low 1983, Mitchell and Allsopp 1984). This sandy soil is especially poor in phosphorus due to the low amounts of P_2O_5 found in the parental palaeozoic rocks, sandstones, shales, schists and granites, and is characteristically below 1 % of the total soils constitutes (Marchant and Moore 1978).

11.3 Conservation status of the Sand fynbos

The conservation status of the CFR is very sensitive, with approximately three-quarters of all the plants in the South African Red Data Book occurring in the area (Rebelo 1992). There are about 1700 plant species threatened with extinction which is very high considering the area only compromises 6 % of the total area of the country. This highly threatened state of many plants is in part due the localized distribution patterns forming centres of endemism which include a high number of fynbos species found nowhere else (Cowling 1991). Urban expansion is a major threat to the continuous existences of certain centres of endemism (Cowling *et al.* 1994) and other threats include the invasion of alien plant species into large areas of mountains and flats.

The biggest threat to the lowland fynbos is the increase of agricultural farmlands. Incorrect fire management practices, for example the burning of fynbos in spring instead of late summer. Burning fynbos frequently prevent plants form seeding which may eliminate certain species (van Wilgen and Richardson 1985). The Sand fynbos is one of the most critically endangered, poorly conserved vegetation types in South Africa (Rebelo and Low 1996). The main factors that threaten its survival is the establishment of alien vegetation and habitat loss due to agriculture and urban development. The vegetation is currently classified by the National Spatial Biodiversity Assessment (NSBA) of 2005 as endangered (Rouget *et al.* 2004). Only 2 % of this vegetation type is currently conserved, but a conservation target of 38 % was set by the NSBA.

11.4 Topography and geology of fynbos and the Sand Fynbos

The topography of the fynbos region is rugged with numerous features like mountains and valleys. The landscape of the fynbos biome is dominated by mountain formation named the Cape Folded Belt (Lambrechts 1979). The mountains consist mainly of the hard quartzitic rocks of the Table Mountain and Witteberg groups. Shales phyllites, slates, conglomerates and granites are restricted to the valleys between the mountains. The coastal foreland is the zone between the Cape Folded Belt and the ocean. The lowland fynbos occurs on the western forland and is a plain underlain by phyllites and covered by Aeolian sand of a clovelly form (MacVicar *et al.* 1977). The Sand fynbos which include the Atlantis Sand Fynbos is on average 150 m above sea level, which mean no disenable rain shadows occur in the area, although a decrease from the coast to inland can be observed (Cowley 1983). The soil Sand fynbos can be distinguished from the Renosterveld which consists of fine-textured soils derived from Cretaceous mudstone and conglomerates (Witkowski and Mitchell 1987).

11.5 The Ecology of Sand fynbos

The two major vegetation groupings in fynbos are quite distinct and have contrasting ecological systems. Essentially, Renosterveld used to contain the large animals in the CFR, but these are now extinct or else have been reintroduced into conservation

areas. In contrast, fynbos is much richer in plant species, but has such poor soils that it cannot support even low densities of big game. However, most of the endemic amphibian, bird and mammal species in the region occur in fynbos vegetation types (Bond and Goldblatt 1984, Hall and Veldhuis 1985, Cowling 1992, Rebelo 1992, Cowling and Richardson 1995)

Plants in the fynbos ecosystem have made special adaptations to thrive in nutrient poor soil environments (Richardson *et al.* 2007). In general, carbon is not the limiting factor in fynbos ecosystems, but other nutrients, usually nitrogen. Plants thus posses an excess carbon that cannot be utilized for the new plant growth or the development of new tissue or the maintenance of existing tissue. According to studies (Baas 1989) the excess carbon in the system is utilized as various secondary metabolites and mechanical structures. These compounds have important effects on the other biotic and abiotic factors in the above and subsoil systems.

Studies at Pella have shown the largest reserves of nitrogen and phosphorus is in the soil and not in the foliage (O'Callaghan 1981). The process of nutrient cycling is driven by litter fall and the decomposition thereof. The litter fall increases with the increase in biomass and increase in age of the fynbos plant communities due to an increase in senescence of leafs. In general the litter production of the coastal fynbos communities are in the region of 451.0g.m^{-2} which is low compared to other systems in Mediterranean climates. Studies at Pella by O'Callaghan (1981) on a 20 year old plot indicated a litter mass of 792.0 g/m in the soil and a study conducted by Mitchell *et al.* (1986) on an eight year old plot revealed a litter mass of 451.0 g/m. The average age of the vegetation has a strong effect on the litter mass in the soil and thus also on the cycling of nutrients in the ecosystem. Indirectly, the age of the vegetation has a real effect on all living organisms in the ecosystem and this includes the soil microbial communities, which are also the primary decomposers in the soil. Studies conducted on soil microorganisms in fynbos has however mainly focused on a specific group, namely the mycorrhizas *(*Allsopp and Stock 1993, Allsopp and Holmes 2001).

The global trend for patterns of diversity is high species diversity at the tropical equatorial regions with a decline in species richness and diversity with a decline in latitude (Rahbek 2006). The CFR is an exception to the longitudinal pattern with one of the highest diversity levels on earth (Cowling *et al.* 2004). The Cape floral region

also possesses high species densities and high numbers of endemic species. To understand the processes that drive the microbial diversity in the fynbos region it is important to understand what processes are involved in creating the plant diversity (Ehrenfeld *et al.* 2005). Niche differentiation is an important factor which states that the niches of the coexisting plant species are different enough so that competitive exclusion does not occur (Darlington 1972, Shmida and Ellner 1984). The limit to the number of species in the community is limited to the number of niches.

The rainfall and the soil plays an important combined role in defining the plant community composition of fynbos (Wright and Samways 1999). The rainfall may include or exclude the growth of fynbos species in specific areas. Fynbos growing in mountainous areas generally need a rainfall above 700mm/yr and in other vegetation types, such as the Lowland fynbos a minimum annual rainfall of 400mm/yr is needed for the inclusion of fynbos (O'Conner and Bredenkamp 1997). Both soil nutrient and soil moisture are important factors determining the distribution of fynbos (Lechmere-Oertel and Cowling 2001). The rainfall in the lowland fynbos is typically around between 300 mm and 750 mm (Deacon 1992). Due to the plain like nature of the area the environmental gradient in terms of rainfall is not steep. The main contributor to diversity in this area may thus be the subtle change in soil chemistry and geographical distance. These same factors could also have an effect on the distribution of microbial communities associated with fynbos.

The richness of an area is defined as the interactions between the alpha, beta and gamma diversity (Whittaker 1972). The Cape Floristic Region has a relatively moderate alpha diversity even though the total species area ratio is extremely high (Cowling 1992). The fynbos region has an average 66 species per $1000m^2$ on any plot and is relatively low compared to the 140 species per $1000m^2$ in the lowland areas of the neotropics (Cowling 1992). The alpha diversity compares better with the South West botanical province of Australia with an alpha diversity of 69 species per $1000m^2$ (Cowling 1992). The alpha diversity of the fynbos ecosystem is thus not the main contributor to the high diversity, but the combination of the alpha diversity and the extremely high species turnover due to the beta and gamma diversity (Kruger and Laylor 1979, Campbell and Van Der Meulen 1980, Linder 1985, Cowling 1990).

12. Objective of this study

The current approach in microbial soil ecology is to place emphasis on the utilization of molecular non-culturing techniques when studying microbial diversity. This study at the outset aimed to develop a protocol for the extraction of total soil DNA. The properties of an adequate DNA extraction protocol for soil is firstly efficient lyses of cells and subsequent high DNA yield and secondly purity of the sample form PCR inhibitors mainly humic acids. The study also aimed to standardise the automated ribosomal intergenic spacer analysis protocol used to evaluate microbial diversity. The standardization of ARISA included the evaluation of its reproducibility and sensitivity, evaluation of the group specific primer sets used and testing different binning protocols available. The study further aimed to use the ARISA protocols to evaluate the bacterial and fungal diversity in soils of the Sand fynbos (Figure 1). The aim was to observe diversity over space and time by sampling 11 separate plots over 4 seasons. In conjunction with this, data was used to statistically test the link between, fungal community and bacterial community structures with plant community structure and soil physico-chemical properties. Finally the study aimed to adapt the ARISA protocol to specifically target *Penicillium* groups in soil. *Penicillium* was used as model organism due to its dominance in the soil and its potential as a bio-indicator species for fungal diversity in the soil.

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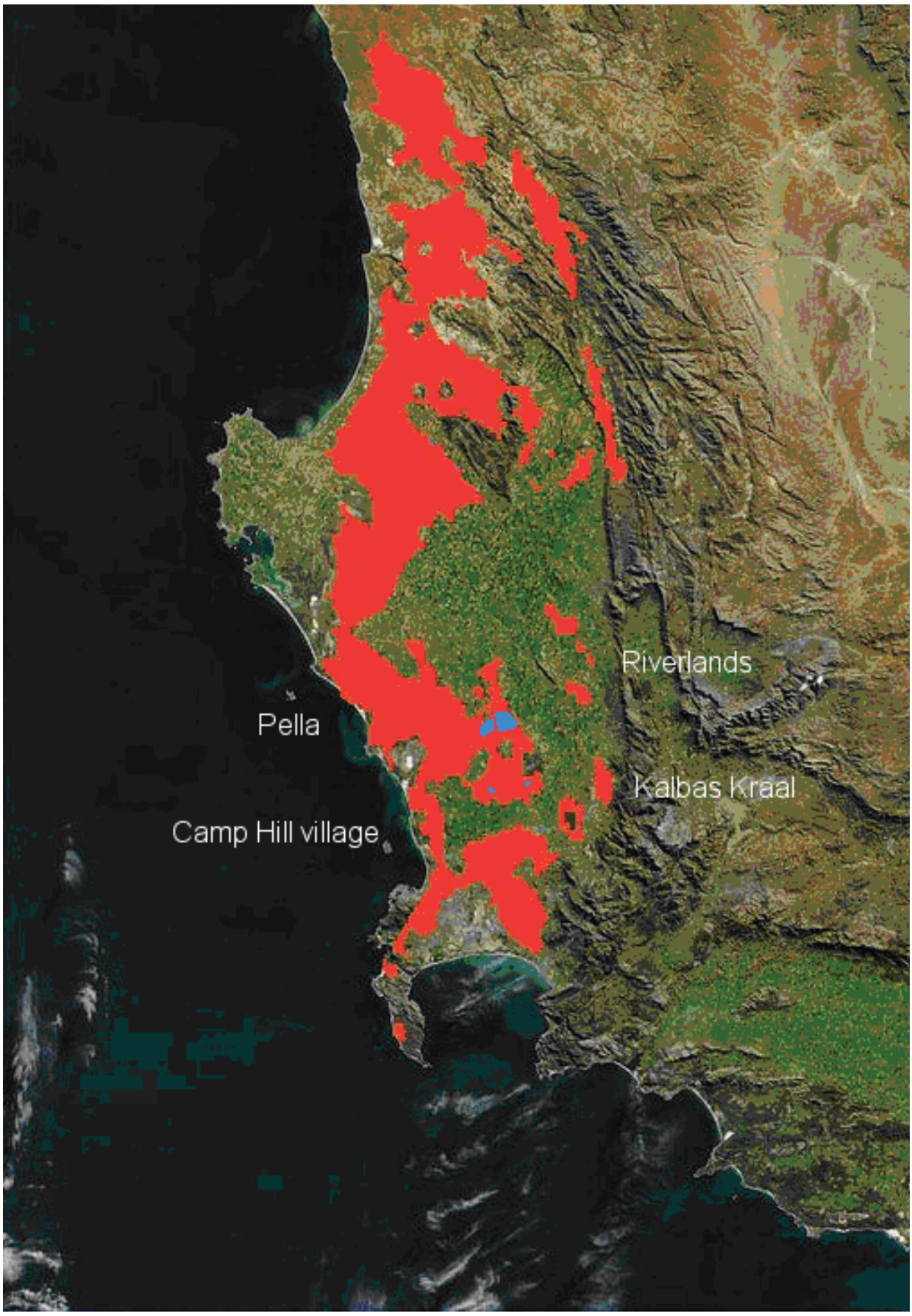

Figure 1: Location of the Sandveld fynbos (red) and the study sites (blue).

Chapter 2

The effective removal of PCR inhibitors from soil DNA by cationic flocculation

Abstract

The most important property of environmental DNA intended for molecular ecology studies and other downstream applications is purity from humic acids and phenolic compounds. These compounds act as PCR inhibitors and need to be removed during the DNA extraction protocol. Effective purification protocols include several types of exclusion columns as well as commercial kits. Previously, aluminium sulphate was shown to effectively remove humic acids from environmental DNA. In this study, the effectiveness of cationic flocculation of humic acids with different cations was evaluated as an aid in the purification method. The combination of cationic flocculation with $CuCl_2$ and the addition of PVPP and KCl resulted in a high yield of DNA, suitable for PCR amplification with bacterial and fungal specific primers.

The following chapter has been submitted to Soil Science

Introduction

Until recently, the study and estimation of microbial diversity in soils was largely dependant on the traditional culturing of microorganisms (Kirk et al. 2004). This is especially true for the filamentous fungi. However, these studies are flawed as significant numbers of taxa in the soil environment are not culturable using standard microbiological techniques (Thorn 1997, Van Elsas et al. 2000, Jacobs et al. 2005, Leckie 2005). The use of modern culture-independent techniques makes the culturing of organisms obsolete, resulting in a more accurate estimate of soil biodiversity (Borneman et al. 1996, Dunbar et al. 2000).

One of the prerequisites of doing molecular studies is the extraction of high quality DNA, free from PCR inhibitors. DNA extractions from soil are particularly difficult, as the soil environment is characterized by many compounds that may inhibit or negatively influence PCR based techniques. The most abundant PCR inhibitors in soil are humic acids and fulvic acids (Stach et al. 2001). Even present in amounts as little as 1 ng per PCR reaction these compounds inhibit the *Taq* polymerase during PCR reactions. (Tsai et al. 1992, Yeates et al. 1997, Watson et al. 2000).

Humic acids are particularly problematic since they have similar physical and chemical properties to those of DNA molecules and are, therefore, co-extracted using standard DNA extraction methods (Cheng et al. 2004). In addition, the presence of humic acids also interferes with the quantification of DNA in spectrophotometric methods. Humic acids show absorbance maxima at 230 nm and 260 nm, while DNA shows a maximum absorbance at 260 nm. The absorbance reading at 230 nm is, therefore, useful for the estimation of DNA purity (Yeates et al. 1998)

The ability of multivalent cationic compounds to precipitate humic acids has been extensively studied. This interaction between multivalent cations and humic acids has established industrial applications in, for example, the removal of humic acid from water and the removal of toxic cations such as Pb^{2+} (Escobar et al. 2006). Metal ions such as $CuCl_2$ have the ability to bind to humic acid to form insoluble chelates (Tipping et al. 1995). Studies by Dong et al. (2006) reported the successful isolation of soil DNA by the cationic flocculation of humic acid with Al_2SO_3 followed by precipitation of Al^{3+} by increasing the pH. This study reports a relatively easy, effective and inexpensive method for the selective precipitation of humic acids with

$CuCl_2$ during the extraction of genomic DNA from soil without an adjustment to pH levels during the extraction protocol.

Materials and methods

Genomic DNA extraction

All DNA isolation experiments were done in triplicate. A random soil sample consisting primarily of either sand or silt, was collected and excess debris removed by sieving through a 2 mm sieve (United wire test sieves, Nigel, South Africa), after which 0.3 g was used for each DNA extraction. DNA isolations were performed without, or in the presence of either 1% Polyvinylpolypyrrolidone (PVPP) or PVPP (1%) and KCl (100 mM). The PVPP was added as a 1% suspension in water or 100 mM KCl, and added to the soil samples prior to the addition of the extraction buffer. For all experimental procedures, 0.3 g of soil was mixed with 500 µl extraction buffer (100mM Tris, 10mM EDTA and 2% SDS; pH 8.5). The extraction buffer additionally contained different concentrations (20mM to 100mM) of cation producing salts ($CuCl_2$, $FeCl_2$, $Al_2(SO_4)_3$ or $MnCl_2$). The samples were then vortexed at 2500 rpm for 20 seconds and exposed to two cycles of freezing at -80°C and thawing in boiling water. Five µg of proteinase K was added and the suspension was incubated for 60 min at 60°C. The salt concentration was adjusted by adding 140 µl NaCl (5M) after which 80 µl 10% CTAB (w/v) was added. The suspension was further incubated for 10 min at 65°C, and then extracted with 1 volume SEVAG (24:1 chloroform: isoamylalcohol) to precipitate proteins. To obtain phase separation, the samples were centrifuged for 10 min at 13000 rpm with a Biofuge 13 centrifuge (Heraeus instruments, Germany) and 500 µl of the supernatant transferred to a clean 1.5 ml eppendorf tube. The DNA was then precipitated with 710 µl cold isopropanol and centrifugation for 10 min at 13 000 rpm. The supernatant was discarded and the pellet was washed twice with 500 µl 70% Ethanol. The pellet was dried and dissolved in 100 µl MilliQ water.

The extracted DNA was separated on a 1% agarose gel and visualized using ultra violet light. DNA concentrations were determined spectrofotometrically (Nanodrop, Thermo Fisher Scientific, USA) at 260 nm. The A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios were measured to determine the purity of the samples. Different DNA isolation methodologies were compared with each other using the unpaired Student *t*-test to test for significant differences in purity.

PCR amplification

PCR reactions were performed on the DNA using a number of different primer sets to evaluate the suitability of the DNA for downstream applications. Bacterial primers ForB and RevB specific for the 16 rDNA (Yeates et al. 1997) and fungal specific ITS4 and ITS5 (White et al. 1990) were used in the reactions. PCR reactions were done using a GeneAmp PCR System 2400 (AppliedBiosystems, USA). The reaction mixture contained 1 μl of the purified genomic DNA (10-20 ng/μl) extracted from soil, 1.75 mM $MgCl_2$, 500 nM of each primer and 5U Taq (Kapa Taq) in a total volume of 25 μl. The PCR conditions consisted of an initial denaturing step of 3 min at 95 °C followed by 42 cycles of 95 °C for 30s, 51 °C for 30s and 72 °C for 30s. The reaction was completed with a final extension at 72 °C for 5 min and then cooled and held at 4 °C. PCR samples were separated and visualized as described above.

Results

The use of PVPP is a well established procedure to remove humic substances and contaminating phenolics when isolating DNA from soil (Zaporozhenko 2006, Arbeli 2007, Lakay 2007). The addition of PVPP alone, however, did not result in pure DNA based on the A_{260}/A_{230} ratio (Table 1). An increase in the ionic strength by the addition of KCl also did not improve the ability of PVPP to remove the impurities. The addition of KCl without PVPP to the extraction protocol actually lead to an increase in the amount of impurities as seen from the A_{260}/A_{230} ratio, which decreased from 0.69 to 0.4 in the presence of 100 mM KCl (Table 1). As reported by LaMontagne et al. (2002), we did observe an increase in the yield of isolated DNA with higher amounts

of KCl added, with an increase from 50 ng/µl without any KCl to 250 ng/ul when 100 mM KCl was added.

Next, the addition of cations as a means of improving the purity of the isolated DNA, was investigated. Extractions which included concentrations of 20 mM to 100 mM $FeCl_3$, $MnCl_2$ or $Al_2(SO_4)_3$ yielded no or very low concentrations of DNA. However, the addition of $CuCl_2$ resulted in substantial amounts of DNA. The presence of PVPP and KCl were, however, found to be essential for increased yield and adequate purity of the isolated DNA. With an increase in $CuCl_2$ concentration, there was a decrease in the DNA yield, accompanied with an increase in the purity of the isolated DNA (Table 1 and Figure 1). At higher concentrations of $CuCl_2$ added, there were no significant differences in the yield and purity. This concentration effect also seems to be dependent on the amount of humic acid content in the soil sample. For the lower humic acid containing sand sample the yield and purity stabilized at a $CuCl_2$ concentration of 60 mM and higher, whereas the silt sample gave stable yield and purity at $CuCl_2$ concentrations of 80 mM and higher (Table 1).

For the sand sample only the genomic DNA isolated using 60, 80 and 100 mM $CuCl_2$ in the presence of 1% PVPP and 100 mM KCl were pure enough to obtain positive PCR products for both the bacterial and fungal specific primers. The silt sample gave PCR products for DNA isolated in the presence of 80 mM $CuCl_2$ or higher. All other DNA extractions did not result in any amplification products. The bacterial specific primers produced PCR products in the size range of 100 bp to 1500 bp, whereas the fungal specific primers produced PCR products in the size range 300 bp to 1500 bp. All the PCR reactions produced smears with the highest peak intensities in the region of 400 bp (Figure 2).

The result of the Student-*t* test between the treatment including 1% PVPP and 1% PVPP + 100mM KCl shows a significant *t* value of 2.55 ($p = 0.02$). The *t* value of the treatment including no PVPP or KCl and the treatment utilizing 1% PVPP + 100mM KCl is 3.03 ($p = 0.013$).

Discussion

In this study we developed an improved method for isolating DNA from soil by exploiting the ability of metal cations to selectively remove humic acid soil

contaminants. The use of $CuCl_2$ in the presence of PVPP and 100 mM KCl was found to be best suited for extracting DNA from soil samples. This method resulted in DNA that could be used on downstream PCR reactions, without interference of contaminants.

Humic acids can be precipitated by adsorption of metal cations, believed to occur through the binding of the cations with negative carboxyl and phenolic sites on the humic acids (Schmitt et al. 1996). Polyvalent cations generally have a higher affinity towards humic acids than monovalent cations. Of the divalent cations, Cu^{2+} has the highest affinity towards humic acids (Wrobel et al. 2003). This would explain why Cu^{2+} cations were more suitable for removing humic substances during the DNA isolation procedure than Mn^{2+}. The affinity and ability to form complexes of metal ions with humic acid increases with a rise in pH, although the solubility of the metal cations decrease (Chang et al. 2005, Crist et al. 2001, Liu et al. 2000). Therefore, an extraction buffer with pH 8.5 was used to cause substantial coagulation of humic acids (Guy et al. 1976), while keeping the Cu^{2+} cations in solution. Although an increase in ionic strength decreases the adsorption of Cu^{2+} to humic acid, the adsorption becomes less sensitive towards a change in ionic strength while still being sufficient for humic acid precipitation to occur (Liu et al. 2000).

Condensation and precipitation of DNA was very efficient for trivalent metals, but monovalent and divalent cations were unable to condense DNA under normal circumstances (Flock et al. 1996). The precipitation of DNA by the trivalent Al^{3+} and Fe^{3+} cations is most probably the reason for the absence or very low levels of DNA, when using these cations to remove humic substances during DNA isolation. Although Dong et al. (2006) could effectively precipitate humic substances with $Al_2(SO_4)_3$, they needed additional steps of pH adjustment to remove the Al^{3+} cations before SDS lysis of the microbes in their isolation protocol.

Certain divalent cations such as Mn^{2+} and Cu^{2+}, form metal complexes with DNA by binding to purine bases, mostly guanine (Kagawa 1991, Ma 1994, Gao 1993, Drevensek 2003, Hackl 2005). The DNA precipitation by these divalent cations is believed to be mainly due to intermolecular cross-linking (Hackl 2005, Gao 1993, Drevensek 2003). Increasing the ionic strength decreased the binding of Cu^{2+} to DNA (Balan 2003, Hackl 2005). In our experiments the addition of 100 mM KCl resulted in higher DNA yield and a decrease in humic acid content. The results indicated an

increased binding and precipitation of the humic substances with Cu^{2+} cations at a higher ionic strength, together with reduced Cu^{2+} binding and precipitation of the DNA. An additional advantage of higher salt concentrations in the extraction buffer was an increase in the concentration of DNA isolated from soil (LaMontagne 2002).

In conclusion, in this study we developed a rapid and inexpensive method for isolating DNA from soil. The method uses the flocculation ability of Cu^{2+} cations in the presence of 1% PVPP and 100 mM KCl to remove humic substances, resulting in soil DNA suitable for downstream applications.

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Table 1: Concentrations and A_{260}/A_{230} absorbance ratios of DNA extracted from sandy soil and silt using different amounts of $CuCl_2$ and with or without PVPP or PVPP and KCl.

	Sand		Silt	
Sample	DNA (ng/µl),mean (SD)	A_{260}/A_{230}, Mean (SD)	DNA (ng/µl),mean (SD)	A_{260}/A_{230}, mean (SD)
0mM $CuCl_2$	34.6 (4.4)	0.64 (0.02)	155.4 (45.3)	0.61 (0.2)
0mM $CuCl_2$ + 1%PVPP	39.8 (4.9)	0.62 (0.021)	158.3 (34.6)	0.64 (0.02)
0mM $CuCl_2$ + 1%PVPP + 100mM KCl	196.3 (39.3)	0.7 (0.03)	230.4 (55.5)	0.6 (0.02)
20mM $CuCl_2$	4.9 (1.2)	0.76 (0)	46.2 (9.77)	0.65 (0.02)
20mM $CuCl_2$ + 1%PVPP	38 (3.91)	0.77 (0.01)	34.6 (6.54)	0.68 (0.01)
20mM $CuCl_2$ + 1%PVPP + 100mM KCl	110 (22.1)	0.77 (0.05)	90.7 (17.6)	0.69 (0.01)
40mM $CuCl_2$	4.3 (1.35)	0.74 (0.03)	15.4 (5.4)	0.75 (0.03)
40mM $CuCl_2$ + 1%PVPP	12 (2.55)	0.8 (0.03)	11.5 (3.6)	0.81 (0.04)
40mM $CuCl_2$ + 1%PVPP + 100mM KCl	44.4 (6.42)	1.01 (0.05)	94.5 (22.3)	0.78 (0.04)
60mM $CuCl_2$	6.7 (2.1)	0.69 (0.02)	10.4 (3.2)	0.71 (0.03)
60mM $CuCl_2$ + 1%PVPP	15.7 (3.12)	0.73 (0.03)	8.2 (2.66)	0.78 (0.03)
60mM $CuCl_2$ + 1%PVPP + 100mM KCl	30.6 (5.97)	1.22 (0.06)	20.2 (6.44)	1.01 (0.06)
80mM $CuCl_2$	5.4 (2.1)	0.74 (0.05)	4.3 (1.6)	0.79 (0.02)
80mM $CuCl_2$ + 1%PVPP	14.6 (4.2)	0.77 (0.02)	7.2 (2.1)	0.9 (0.04)
80mM $CuCl_2$ + 1%PVPP + 100mM KCl	40.6 (7.89)	1.24 (0.1)	33.2 (5.2)	1.14 (0.07)
100mM $CuCl_2$	5.3 (1.55)	0.75 (0.04)	3.2 (1.4)	0.84 (0.04)
100mM $CuCl_2$ + 1%PVPP	16.8 (4.1)	0.87 (0.01)	2.4 (1.1)	0.87 (0.04)
100mM $CuCl_2$ + 1%PVPP + 100mM KCl	33 (6.33)	1.26 (0.07)	21.3 (7.88)	1.12 (0.06)

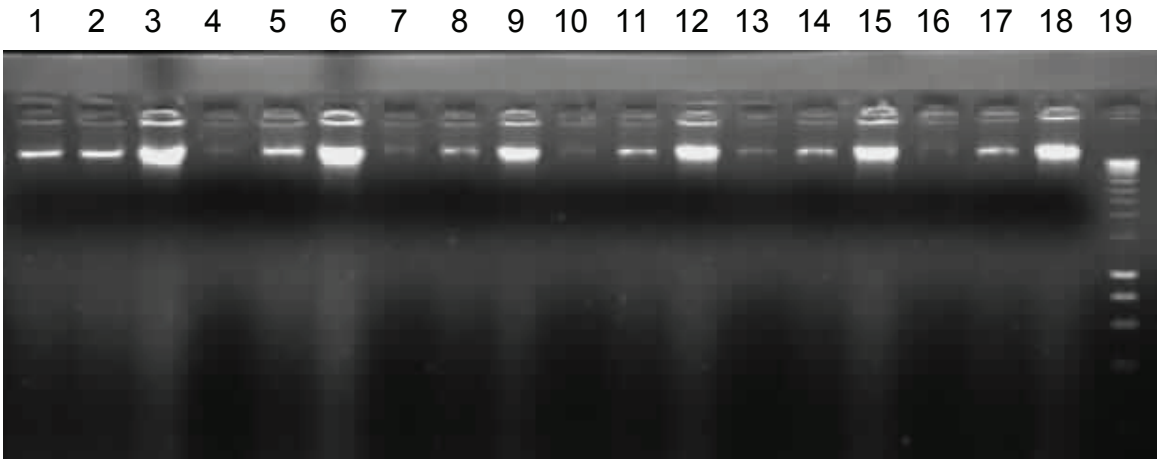

Figure 1: Agarose gel electrophoreses of total DNA extracted from soil sample using different concentrations of $CuCl_2$. Lanes 1, 0mM $CuCl_2$; 2, 0mM $CuCl_2$ + 1%PVPP; 3, 0mM $CuCl_2$ + 1%PVPP 100mM KCl; 4, 20mM $CuCl_2$; 5, 20mM $CuCl_2$ + 1%PVPP; 6, 20mM $CuCl_2$ + 1%PVPP + 100mM KCl; 7, 40mM $CuCl_2$; 8, 40mM $CuCl_2$ + 1%PVPP; 9, 40mM $CuCl_2$ + 1%PVPP + 100mM KCl; 10, 60mM $CuCl_2$; 11, 60mM $CuCl_2$ + 1%PVPP; 12, 60mM $CuCl_2$ + 1%PVPP 100mM KCl; 13, 80mM $CuCl_2$; 14, 80mM $CuCl_2$ + 1%PVPP; 15, 80mM $CuCl_2$ + 1%PVPP 100mM KCl; 16, 100mM $CuCl_2$; 17, 100mM $CuCl_2$ + 1%PVPP; 18, 100mM $CuCl_2$ + 1%PVPP 100mM KCl; 19, HyperLadder I.

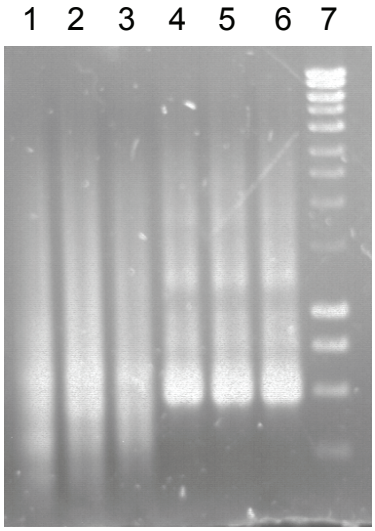

Figure 2: Agarose gel electrophoreses of PCR amplification of total extracted DNA from a sand soil sample. Lane 1 to 3: PCR product using primers RevB and ForB of DNA extracted with 60mM, 80mM and 100mM $CuCl_2$. Lane 4 to 6: PCR product using primers ITS4 and ITS5 of DNA extracted with 60mM, 80mM and 100mM $CuCl_2$. All DNA isolations were done in the presence of 1% PVPP and 100 mM KCl.

Chapter 3

Optimization of Automated Ribosomal Intergenic Spacer Analysis (ARISA) for the estimation of microbial diversity in fynbos soil

Abstract

Automated Ribosomal Intergenic Spacer Analysis (ARISA) has become a commonly used molecular technique for the study of microbial populations in environmental samples. The reproducibility and accuracy of ARISA and ARISA-PCR are important aspects that have an influence on the results and effectiveness of these techniques. Primer sets for ARISA, ITS4/ITS5, were assessed for the characterization of the fungal community composition of two sites situated in the Sand Fynbos. The primer set proved to deliver reproducible ARISA profiles for the fungal community composition with little variation observed between ARISA-PCR's. Variation that occurred in samples due to repeated DNA extractions, were within limits for ecological studies. This made ARISA a useful tool for the assessment and comparison of diversity in ecological samples. Particular observations were made concerning the binning strategy for the analysis of ARISA profiles.

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Introduction

Over a number of years, numerous studies on soil diversity were conducted using small subunit rDNA (Borneman and Triplett 1997, Kuske et al. 1997, Brown et al. 2005, Fierer et al. 2007). Although these methods provide little direct evidence to the function of organisms in the soil, they have become invaluable for the understanding of soil microbial diversity and community composition (Fisher and Triplett 1999, Ranjard et al. 2001, Lerner et al. 2006, Mills et al. 2007). Molecular techniques provide a good indication of the community structure, which include the diversity and the evenness of communities. The method of rRNA intergenic spacer analysis (RISA) provides a technique for the estimation of the community diversity and community composition. The technique was first applied to examine microbial diversity in soil from the Eastern Amazonian rainforest (Borneman and Triplett 1997). This method allows one to estimate the microbial diversity without the need to culture organisms. Culturing methods were shown to reveal only about 1% of the total microbial diversity (Torsvik et al. 1990, Richaume et al. 1993, Tsuji et al. 1995). In addition, the bias in favour of fast growing organisms against slow growing organisms is to a large extent eliminated with the RISA technique. The RISA technique also includes the diversity from non-culturable organisms in the soil, which was lacking in studies using traditional culturing media (Kennedy and Clipson 2003).

The RISA technique requires genomic DNA of the total population to be extracted from the environmental sample. The method further involves the amplification of the selected DNA with universal primers, and the subsequent electrophoreses on a polyacrylamide gel. The RISA technique has been enhanced by the addition of an automated component to the technique by including the automated genetic analyzer (Fisher and Triplett 1999). ARISA-PCR, involves the use of fluorescently labelled oligo-nucleotide primers commonly labelled with the fluorescent markers ROX and FAM (Fisher and Triplett 1999, Hewson and Fuhrman 2004). The electrophoresis of the total amplified DNA is performed on an automated system, for example the ABI 3010xl Genetic analyzer, which detects the fluorescent labelled DNA fragments with the aid of a laser and CCD camera.

The ARISA method is an effective, rapid and fairly inexpensive method for estimating the diversity and composition of microbial communities without bias towards fast growing or dominant species (Ranjard et al. 2001). This is especially useful in

ecological studies were a large number of samples need to be processed and diversity needs to be determined at a spatial and temporal scale. The method can be denoted as F-ARISA, which targets the total fungal community DNA of the intergenic spacer region 1, the 5.8S small subunit and the intergenic spacer region 2. This region, especially within the intergenic spacer regions 1 and 2, displays significant heterogeneity in length and nucleotide sequence between species. The oligonucleotides primers target the conserved region of the 28S rDNA and the 18S rDNA region. B-ARISA targets the total bacterial community DNA of the intergenic region between the 16S and the 23S subunits of the rDNA genes in the rRNA operon. This region also displays size and sequence heterogeneity between species. The primer targets the conserved 16S and 23S genes (Cardinale et al. 2004).

The ARISA-PCR, and subsequently the ARISA profiles, are highly reproducible, as demonstrated by Cardinale et al. (2004) The reproducibility of the ARISA was assessed in terms of peak occurrence, peak height and peak size, and this feature allows direct comparisons between ARISA profiles, even between different studies (Hansgate 2005). The standard deviation for fragments smaller than 1 kbp was shown to be between 1 and 2 base pairs (Fisher and Triplett 1999). The reproducibility of the abundance of the ARISA fragments is also determined to be high, with the peaks with the largest contribution to the total fluorescence being the most reproducible. Studies also showed that ARISA may be a measure of relative abundance of bacteria (Brown et al. 2005). The main advantage of ARISA is not the accurate estimation of the abundance of organisms but the reproducibility. Variation in the number of cycles in PCR does not influence the overall ARISA pattern, but it influences the sensitivity of the assay, with more cycles producing more peaks (Cardinale et al. 2004).

The sensitivity of ARISA is a function of both the DNA extraction and the ARISA-PCR. The detectable limits of an organism in the ARISA profile is firstly determined by the effective extraction of DNA from the sample (Volossiouk et al. 1995). Certain cells may be more resilient to lyses, thus have a higher minimum detection level i.e. fungal spores (Prosser 2002). Secondly, the sensitivity of the PCR and the primer set determines the consistent detection of an organism with ARISA. Low numbers of template is likely to result in variable detection and weak fluorescence. The most abundant organisms present, result in the strongest and most reproducible peaks.

DNA fragments that are in low abundance and near the lower detection limit of the ARISA profile have decreased reproducibility in the subsequent amplification reactions. Some primer sets also exhibit some bias for certain species over others (Jones et al. 2007).

The ARISA technique has a few sources of errors. For this reason cut-off standards are in place for the fluorescent intensity of the ARISA profile. The heterogeneity of the sampling environment and the randomness of the sampling procedure play a role in the standardization of the method. The PCR amplification of the targeted DNA is subjected to low levels of error, and may introduce errors in length of the ITS region. In addition, the ABI system is very precise but not fully accurate. The ABI system is also less accurate with the increase in the size of the fragments (Brown et al. 2005). The possibility exists that two fragments of the same size may be represented as one peak on the ARISA profile. This problem with the ARISA method should be recognized and incorporated into a binning strategy (Ruan et al. 2006).

The literature has proposed various empirical binning strategies. Fisher and Triplett (1999) observed size variation across replicate ARISA profiles. They observed size variation of 1-2 bp for fragment sizes below 1kb and 3-5 bp for fragments up to 1150 bp and up to 13 bp for the largest size fragments. Hewson and Fuhrman (2004) used a bin size of 3 for fragment lengths up to 500 bp and a bin size of 7 for fragments larger then 500 bp. Brown et al. (2005) suggested that windows of 3 bp wide be used from 400 to 700 bp, windows of 5 bp wide from 700 to 1000 bp, and windows of 10 bp wide from 1000 to 1200 bp. Larger fragments do not, however, necessarily result in higher similarities between similar samples due to the splitting of peaks. between adjacent bin windows. The aim of this study was thus to optimize and evaluate the ARISA protocol to determine microbial populations in fynbos soil.

Material and methods

Sensitivity of DNA extraction for ARISA

The sensitivity of DNA extraction for ARISA was tested on a random soil sample collected at Kalbas Kraal, Western Cape (S 33.57061º, E 18.62861º), using the ZR Soil Microbial DNA kit (Zymo Research, USA). *Penicillium* spores are more robust than vegetative cells and were used to provide a stringent test for the extraction

method (Doaré-Lebrun 2006) and this was also found to be the dominant species in these soils. Spores of a *Penicillium* sp. were suspended in phosphate buffered saline (pH 7) solution. The concentration of the cells in the suspension was determined by direct microscope count using a haemocytometer (Superior Marienfeld laboratory glassware, Marienfeld, Germany). Spores were used to prepare a dilution series of 10^4, 10^3, 10^2, 10^1, 1 spores per milliliter. One milliliter of each diluted spore suspension was added to 1g of both autoclaved (121°C, 100kPa, 20min) soil samples and unsterilized soil samples. Total DNA was extracted from the soil using ZR Soil Microbial DNA kit (Zymo research, USA) and quantified spectrophotometrically (Nanodrop). PCR was performed using fungal specific primers ITS4 and FAM labeled ITS5. The reaction mixture contained 1 μl of the purified genomic DNA, 500 nM of each primer and 23 μl of KapaTaq Readymix (Kapa Biosystems, South Africa) in a total volume of 25 μl. The PCR conditions consisted of an initial denaturing step of 3 min at 95 °C followed by 40 cycles of 95 °C, for 30 s, 51 °C for 30 s and 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 5 min and then cooled and held at 4 °C. Samples were run on a 1% agarose gel stained with ethidium bromide and visualised under a UV light for the presence of amplified products.

Sensitivity of ARISA-PCR

Total genomic DNA was extracted for a soil sample collected at Kalbas Kraal as described earlier. Spore suspensions from *Penicillium* spp. were quantified using a heamocytometer as described earlier. The spore suspension was vortexed for 1 min at full speed after the addition of 2 mm steel beads. The number of *Penicillium* spores lysed were determined by counting the spores after lyses. The number of spores lysed, were diluted to 100, 80, 40, 20 and 10 spores per soil DNA extraction and added to the genomic DNA from soil. The number of spores corresponded to the number of genome copies in the PCR. ARISA-PCR was performed using fungal specific primers ITS4 and FAM labeled ITS5.

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The PCR products of every sample were run on an ABI310xl Genetic analyzer to obtain an electropherogram of the different fragment lengths and fluorescent intensities. ARISA-PCR samples were run with the size standard LIZZ 600 containing sizes from 60 bp to 600 bp in length. GeneMapper 4.1 software converted the fluorescence electropherogram data representing operational taxonomic units into peaks indicating the fluorescent intensity. Peak heights were favoured over peak area for further analysis due to concerns that peak areas for larger peaks may be subjected to inaccuracies. Variance of peak area values are higher than is the case for peak height. This may be due to peak smoothing and height algorithms that are simpler and a more consistent measure of peak height. Peaks which represented 1% or more of the total fluorescence were considered for analysis. Similarities between samples were represented by calculating the pair-wise Whittaker similarity index.

Evaluating binning sizes for ARISA

Different PCR reactions from a single DNA extraction were compared to determine the effect of different bin sizes on the similarity of the profiles. Bin sizes ranging from of 1 to 7 were considered. Similarities were compared by calculating the Whittaker similarity index. The number of unique operational taxonomic units was determined for each binning protocol and the number of corresponding and conflicting operational taxonomic units was determined. The test requires lenient peak selection criteria with no peaks under 0.5% of total fluorescent considered for analysis. This was done to include the effect lower intensity peaks had on the binning strategy. Profile similarities increase due to high stringency of peak selection across all binning sizes and this phenomenon should be limited in order to observe the real effect bin size has on the similarities of the profiles. Four different profiles were considered and data of all profiles were normalized to the mean values.

Evaluating the use of ARISA to study environmental samples

The significance of the variability of ARISA was evaluated when environmental samples with the primer set ITS5 and ITS4 were compared. Soil samples were collected from Sandveld fynbos approximately 10 km outside the town of Malmesbury in the Western Cape. Samples were collected in a range of plots on different sites. Profile variability from two different sites at Camphill Village and Kalbas Kraal located approximately 6.5km apart was evaluated by calculating the Whittaker similarity index. Two different profiles of two unique samples within the plot at Kalbas Kraal were compared to evaluated variability of different samples within the same plot. The variability within the sample was compared by performing ARISA on different DNA extractions of the same sample. The variably of PCR within the same DNA extraction was lastly evaluated. The distance relationship of the similarities at the different levels of sampling was illustrated be means of cluster analysis. Cluster analysis was performed by complete linkage of the Pearson-r correlation values of the Whittaker similarity indices between the sites.

Constructing of ROX 1.1

To enable the use of ARISA for species with peaks larger than 600 bp a size standard was created. A ROX labeled M13 forward primer was used in conjunction with 6 other non-labeled primers to generate 6 ROX labeled DNA fragments using the plasmid pGEM-3zf as template. The fragment sizes were calculated by counting the number of bases from the 5' end of the labeled primer to the 5' end of the unlabeled primer. These PCR fragments were mixed with a standard ROX 500 size standard to create a size standard (Called ROX 1.1) ranging from 75 to 1121bps.

Results and discussion

Sensitivity of the DNA extraction on ARISA

The results of the performed sensitivity test indicated that *Penicillium* spores could be detected in the DNA extractions from sterile soil at 40 spores per gram of soil according to the peaks observed in Figure 1. The detection of the spores in the

untreated soil samples with higher peak diversity, however, required a higher concentration of spores (100 spores/g) to distinguish between the spores and the background noise and to compensate for competitive binding to the environmental DNA (Figure 1). This compared favorable to other studies, where, Van Elsas et al. (2000) were able to detect *Trichoderma harzianum* as low as 10^3 spores/g soil on an agarose gel, while Doaré-Lebrun et al. (2006) also found *Aspergilus carbonarius* to be detectable at levels of 10^3 cells in a mixed culture and *Penicillium expansum* in the range of 10^5 cells. The detectable limit of fungal spores in a sample is generally higher than that of vegetative cells.

Sensitivity of the ARISA-PCR

The minimum number of spores that could be detected by PCR in a DNA sample was 40 spores per gram of soil (Figure 1, Table 1). The higher dilution of 20 and 10 were impossible to detect probably due to competitive binding by high concentrations of DNA templates. Peaks that were detected at solutions of 10 to 20 spores per g soil were extremely variable with less then 1 in 4 PCR's detecting the *Penicillium* spp. at very low levels. The result indicates that DNA concentration should be at least the equivalent of 40 spores per gram of soil extracted, to produce a consistent peak on the electropherogram. The sizing of the fragment varied between 479.32 and 480.02 bp. The peaks were thus sized with an error in precision of less then 1 bp.

Evaluating binning sizes for ARISA

The different binning sizes examined for the various samples indicate that the maximum similarity between identical samples was reached at a bin size of 4 bp. The Whittaker index value, however, did not show a significant difference between bin sizes 2, 3 and 4. The bin size of 3 resulted in the highest number of peaks shared namely 23 with 2 peaks not shared. The binning size of 5 bp results in an average of 9 operational taxonomic units that is not shared. The Whittaker dissimilarity index increase at a bin size of 5 bp due to unwanted overlaps that occur at this bin size with 9 operational taxonomic units not shared. Larger binning sizes do not necessarily result in a more analogous profile. Furthermore, an increase in bin size, also result in a decrease of the total number of operational taxonomic units

(Table 2) The ideal binning protocol, thus, require maximum similarity between parallel samples with the maximum number of total and shared operational taxonomic units.

Reproducibility ARISA profiles

The Whittaker index revealed that the ARISA profiles were exceedingly reproducible when considering duplicate samples of ARISA from the same DNA extraction (Figure 2). The duplicate ARISA-PCR showed 98% similarity with a linkage distance of 0.01 after cluster analysis with normalized data and analysed using a bin size of 3. All peaks were reproduced in the ARISA profile and only differed in terms of their relative proportions of fluorescence intensity. The Whittaker distance index revealed high similarity between repeated DNA extractions from the same soil sample. ARISA profiles showed a percentage similarity of 93% with a linkage distance of 0.1. The Whittaker index of similarity decreased when DNA extractions of different samples on the same plot were used for ARISA. The linkage distance value increased to 0.7 with a percentage similarity of 78%. The Whittaker similarity index again decreased when a sample for a different site for Camphill was compared in the cluster analysis. The similarity of the ARISA profile after repeated PCR's with the same sample and high similarity with repeated DNA extractions demonstrated that analysis was reproducible. The findings confirmed those of Ranjard et al. (2006) who found greater variation between sites than within the site and greater variation between PCR reactions of DNA extractions from the same plot than from different plots. The method was found to be robust regardless of the primer set used and the specific ARISA conditions.

The ARISA was also evaluated for bacterial specific primers. However, detected peaks with sizes up to 1000 bps in length were observed. This required a sized standard which incorporated larger fragments. ROX1.1 was constructed and included size fragments (75, 100, 139, 150, 160, 200, 300, 340, 350, 400, 450, 490, 500, 583, 683, 782, 932, 991, 1121 bps). The same binning strategy proved to be sufficient for bacterial ARISA.

Conclusion

The primer set ITS4/ITS5 has not been commonly used for the purpose of automated ribosomal intergenic spacer analysis. The primer set performs well when testing the sensitivity and 40 templates per gram of soil would be sufficient to detect both dominate and minor species in environmental samples. The DNA extraction method tested detected cells at 100 spores per gram of soil and as is the case with most studies looking at environmental samples, the DNA extraction method is the defining step which determines the sensitivity of the method. The reproducibility of ARISA using the primer set ITS4 and ITS5 is very high with little variation even under lenient filtering conditions.

The correct binning strategy is, however, necessary to eliminate inaccuracies in ARISA. The samples evaluated demonstrated that a binning size of 3 was sufficient to confer maximum similarity between parallel samples with maximum detection of operational taxonomic units.

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Table 1: Fluorescent intensities of different spore concentrations added.

Number of Spores	Size	Fluorescent intensity
10	–	–
20	–	–
40	480	286
80	479.55	414
100	480.08	889
1000	479.34	7570

Table 2: Evaluation of the bin sizes of the binning protocol between two different samples for the same plot.

No of base pairs (bin size)	Whittaker index	Shared peaks	Unique peaks	Total peak number
1	0.1484	20	7	27
2	0.0923	21	4	25
3	0.0925	23	2	25
4	0.0921	20	2	22
5	0.1919	17	9	26
6	0.0997	16	3	19
7	0.0934	18	1	19

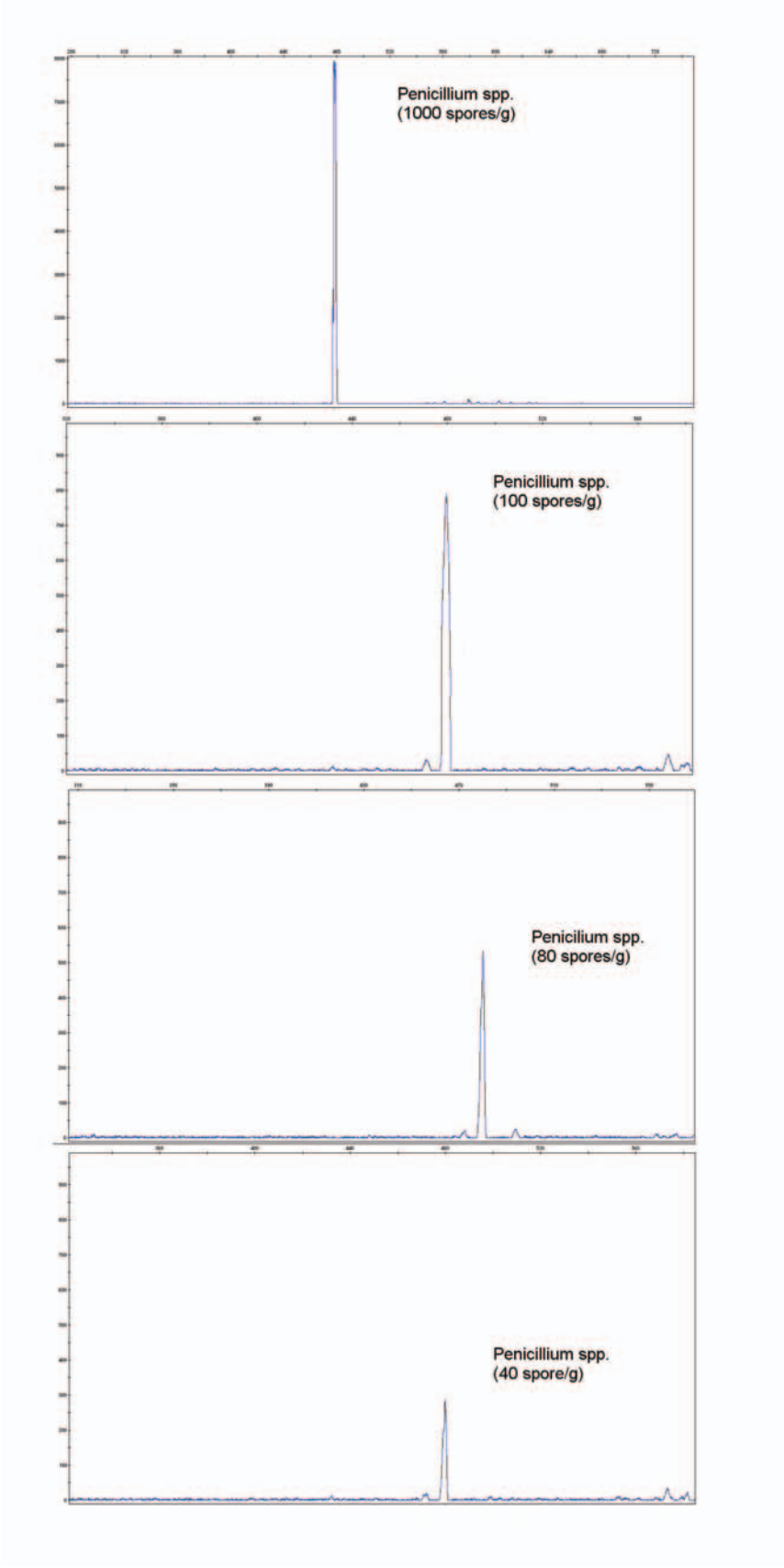

Figure 1: Fluorescent intensities of the various concentrations of *Penicillium* spores added to the DNA extraction

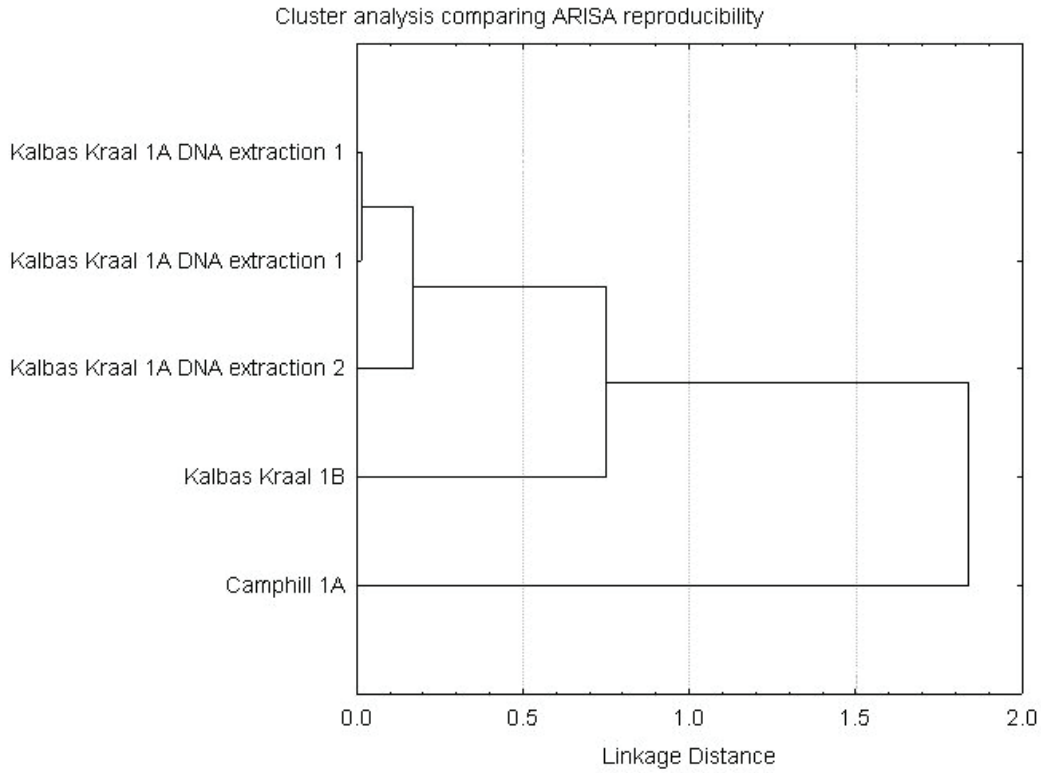

Figure 2: Cluster analysis comparing the variability of ARISA PCR, ARSA from different DNA extractions, different samples and different sites ($p < 0.05$).

Chapter 4

Microbial diversity in soils of the Sand Fynbos

Abstract

The soil community composition of both fungal and bacterial groups in the Atlantis sand fynbos was characterized over space and time. Soil from 4 different sites was compared to resolve to diversity of eubacterial and fungal groups on a local (alpha diversity) as well as a landscape scale (beta diversity). A molecular approach was used based on the isolation of total soil genetic DNA. The 16S-23S intergenic spacer region from the bacterial rRNA operon was amplified when performing bacterial ARISA from total soil community DNA (B-ARISA). Correspondingly, the internal transcribed spacers, ITS1, ITS2 and the 5.8S rRNA gene from the fungal rRNA operon were amplified when undertaking fungal ARISA (F-ARISA). The community structure from different samples and sites were compared with cluster analysis by considering the numbers and fluorescent intensities of peaks between ARISA profiles. ARISA data was used to evaluate different species accumulation and estimation models for fungal and bacterial communities and predict the total community richness. Diversity, evenness and dominance of the microbial communities were expressed by calculating the Shannon Weaver index, evenness and Simpson index. The spatial ordination of the bacterial and fungal species richness and diversity was also considered by determining the species area relationship and beta diversity of both communities. Correlations between microbial community structures and the soil physcio-chemical properties were determined by performing canonical correlation analysis. The plant community structure data was correlated with the fungal and the bacterial community structure by performing a Mantel test and a Partial Mantel test.

Introduction

The Cape Floristic Region is the smallest and one of the richest of the six floral Kingdoms in the world with over 8700 species and 6252 of those endemic (Cowling 1992). Due to this immense plant diversity, the Cape Floristic Region is recognized as one of the world's biodiversity hotspots and centres of endemism (Myers et al. 2003). The fynbos biome is subdivided into 60 vegetation types which include the Sand fynbos. The Sand fynbos is among the most poorly conserved of all vegetation types in South Africa and has been classified as endangered according to the red data list (Cowling et al. 1992, Low and Rebelo 1996). Almost 95% of this vegetation type has been transformed into agricultural land (Low and Rebelo 1996). The result of this development is the fragmentation of the vegetation in the area which now only occurs in isolated pockets (Rebelo 1992, Heijnis et al. 1999). Plant studies have shown that in fynbos, fragments have significantly fewer plant species than areas of the same size located inside a more extensive pristine area (Bond et al. 1988, Kemper et al. 1999). The absences of fires in small fragments are one of the main reasons for species diversity loss along with the occurrence of invasive species. However, the occurrence of uncontrolled wild fires can have a devastating effect on these small fragmented areas (Bond et al. 1984). Smaller fragments of land are also easily disturbed by outside factors compared to larger areas. The effect and significance of the fragmentation of land on microbial populations has thus far not been completely clarified. Specifically the influence on beta diversity in the microbial diversity structure has largely been ignored in diversity studies (Lozupone and Knigh 2008).

The soil environment possibly harbors a large proportion of the earth's undiscovered biodiversity. The diversity of soil micro-organisms is not well-studied and it is believed that only about 1% of the organisms that occurs in soil have been cultured, identified, and characterized (Torsvik et al. 1990, Hawksworth 2001). Studying the microbial diversity and processes have also contributed to the understanding of ecological theory of ecosystems (Jessup et al. 2004, Bertin et al. 2008, Zhou et al. 2008). The soil microbial diversity and viability is known to be essential for the proper functioning of the ecosystem and soil health (Kennedy and Smith 1995). The exact factors that are responsible for this diversity is, however, not well understood (Zhou et al. 2002).

The general factors that influence microbial populations are the soil properties, which may be physical or chemical. Physical properties include soil moisture, aeration, texture, structure and temperature (Gaur and Misra 1978, Fomsgaard and Kristensen 1999, Chen et al. 2007). Chemical properties include the nutrient characteristics and pH (Gaur and Misra 1978, Jonasson et al. 1999, Lipson et al. 1999, Turrión et al. 2002). The third set of factors influencing the microbial community is external factors which include the ambient temperature and levels of precipitation (Norris et al. 2002). Lastly, the above ground vegetation structure and makeup also influences the microbial population within the soil.

The vegetation that can be observed in the Sand fynbos is predominantly fynbos shrubs with the characteristic species being, *Erica mammosa*, *Leucospermum parile*, *Phylica cephalantha*, *Staberhoa distachya* and *Thamnochortus punctatus* (Low and Rebelo 1996). The Lowland coastal fynbos has significant numbers of heaths, restiods, numerous ericas and proteas. More grass species occur in the lowland coastal fynbos compared to mountain fynbos as well as more annual plant species. The beta diversity in the fynbos biome is characteristically high (Cowling et al. 1992). This high species turnover results in a significant variation in the plant community structure between sites. This species turnover may also drive variations in the microbial communities between different locations. Most studies have focused on the effect of plant diversity on biological processes and ecosystem functioning (Richards et al. 1997).

The diversity and variation of soil micro-organisms have been studied in a variety of ecosystems (Saetre and Baath 2000, Bhatnagar and Bhatnagar 2005, Bezemer et al. 2006). This includes studies which looked at soil microbial diversity at a landscape scale and meso-scale (Green et al. 2004). Many studies have, however, focused on the rhizosphere and diversity of mycorrhizal fungi (Allsopp and Stock 1995). The effect of soil micro-organisms on ecological function and productivity of the fynbos biome has also drawn significant attention in previous studies (Spriggs et al. 2003). The indirect effect of a plant community on the composition and structure of the soil microbial communities is manifested by the effect of senescent plant residues which differ in quality, quantity and composition.

The soil type observed in the Sand fynbos is sandy with an Aeolian origin. Soil of the Sand fynbos ranges from very shallow to extremely deep (Lambrechts 1983, Rebelo

1996). The soils of the study sites are all on average 2m in depth. The pH of the soil ranges from 3.6 to 4.7 (Brown et al. 1984). The soil typically has an organic matter content of between 1-3% and available carbon content of less than 1% (Low 1983, Mitchell et al. 1984). Soil derived form sandstone, as seen in the study areas of Riversland, Pella, Camphill and Kalbas Kraal, are characteristically low in nutrients (Kruger 1979, Richards et al. 1997). This sandy soil is especially poor in phosphorus due to the low amounts of P_2O_5 found in the parental palaeozoic rocks, sandstones, shales, schists and granites, and is typically below 1 % (Marchant and Moore 1978, Low and Bristow 1983).

A number of studies have correlated the species richness to the nutritional status of the soil (Van der Heijden et al. 2007). Beta diversity is important when determining the regional species diversity. Beta diversity may differ significantly within similar habitats (Cowling et al. 1992). Studies conducted by Cowling et al. (1990) observed complete species turnover on different soil types. High species turnover rates were also observed between sites with slight differences in the chemical composition of the soil and a turnover rate in the fynbos region (Rouget et al. 2003). The similarity of the habitat and the high turnover rate suggest that a divergence in the evolution occurred between the specialists of the various niches.

In this study the microbial community structure, similarities and relative diversity of fungal and eubacterial groups were investigated and compared with the fragmentation of the area and land use history. The microbial community's relationship with the plant community as well as the physico-chemical environment was evaluated.

Materials and Methods

Study area

Soil samples were taken from four different sites in the Sand fynbos (Western Cape South Africa). The area has a mean annual rainfall of 400 mm. The fynbos region is characterized by a temperate climate with wet winters and warm dry summers with strong prevailing south easterly winds (Lindesay 1998). The study sites were located between 160 and 220 m above sea level and only sloped slightly between 0 and 4 % (Witkowski and Mitchell 1987). The sites were located at Pella, Riverlands, Kalbas

Kraal and Camphill Village (Table 1 and Figure1a -b). The sites at Pella and Riversland are part of a large conservation area administered by Cape Nature. The site at Kalbas Kraal is in the process of being rehabilitated from Port Jackson (Acacia saligna) infestation after its introduction to stabilize the sandy soil. The site at Camphill Village is surrounded by farmland and has not been burned for 20 years and can thus be considered neglected with many mature and senescing plants. Even slow growing fynbos has a lifespan of 12 to 20 years and fire is necessary for population succession and cycling of nutrients (Cowling 1992).

Sampling

On every site, 3 random plots of 10 m by 10 m were located inside an area of 100 m by 50 m. The plots were selected and divided in four quadrants. Ten soil samples were taken randomly in each plot within the first 10 cm of the surface. The ten samples from the quarters were homogenised and sieved using a 2 mm mesh sieve to produce a composite sample (Coutinho et al. 1999). Sampling was conducted in February, March, July and September 2007 to acquire representative samples for each season.

Soil chemistry and physical properties

The homogenised samples were used to determine the average soil water content gravimetrically. Ten grams of each samples was oven dried at 105°C and weighed again after 24 hours. Soil water content was expressed as a fraction of the total soil weight (p). This faction is expressed as $p = \frac{W - D}{D}$ were W is the mass of the sample and D is the mass of the dried sample. The physico-chemical properties of the homogenised samples were analysed (Bemlab, Somerset West, South Africa). The soil pH was determined by the addition of 25 ml KCl to 5g soil followed by incubation at 36°C (McLean 1982). The hydrometer method was used to determine the soil texture (Van der Watt 1966). Soil organic carbon was determined by using the Walkey-Black method (Nelson and Sommers 1982). The phosphate content was determined by the Bray-2 extract (Thomas and Peaslee 1973). Di-ammonium EDTA extract was used to determine the copper zinc and manganese content of the soil

and boron content was determined by using a hot water extract (Beyers and Coetzer 1971). A 1 mol/L ammonium acetate extract was used to determine the exchangeable ione content of calcium, magnesium, potassium and sodium.

Vegetation structure

The plant community structure of the different sites was compared by constructing a data matrix where the presence of a species was expressed by a 1 and the absence as a 0. Fynbos communities at the different sites were compared by calculating the Jaccard's similarity index Sj = W/ (a_1 + a_2 – W) (Fisher and Triplett 1999). *W* is the number of plant species occurring in both populations 1 and 2 and a_1 and a_2 are total numbers of different plants in populations 1 and 2.

DNA extraction

DNA was extracted from 0.35 g of soil. DNA was extracted using the ZR Soil Microbe DNA kit (Zymo research USA). Extracted and purified DNA was separated on a 1% agarose gel stained with ethidium bromide and visualized using ultra violet light.

PCR amplification

PCR reactions were performed on the DNA using fungal and eubacteria specific primer sets to evaluate its application in automated ribosomal intergenic spacer analysis (ARISA) (Table 2). Eubacterial specific primers ITSReub and FAM (carboxy-fluorescein) labelled ITSF specific for the 16 rDNA intergenic spacer region of the bacterial rRNA operon were used to determined to bacterial diversity with ARISA (Cardinale 2004). The fungal diversity was determined by using fungal specific primers ITS4 and FAM labelled ITS5 (White et al. 1990) in the PCR reactions.

PCR reactions were done using a GeneAmp PCR System 2400 (AppliedBiosystems, USA). The reaction mixture contained 1 μl of the purified genomic DNA extracted from soil, 500 nM of each primer and 23 μl of KapaTaq readymix (KapaBiosystems,

South Africa) in a total volume of 25 µl. The PCR conditions consisted of an initial denaturing step of 3 min at 95 °C followed by 40 cycles of 95 °C, for 30 s, 51 °C for 30 s and 72 °C for 30 s. The reaction was completed with a final extension at 72 °C for 5 min and then cooled and held at 4 °C. PCR for each sample was performed in triplicate and pooled to eliminate background noise form the ARISA profile and reduce the PCR variability occurring. PCR samples were separated on a 1% agarose gel, stained with Ethidium Bromide and visualized using ultra violet light.

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The PCR product, using group specific (bacterial and fungi) fluorescently labelled rRNA primers, was used to determine the microbial community structure and diversity by means of automated ribosomal intergenic spacer analysis (ARISA). The PCR products of every sample were run on a ABI 3010xl Genetic analyser to obtain an electropherogram of the different fragments length and fluorescent intensity. F-ARISA PCR samples were run along with LIZZ 600 size standard which contained sizes from 60bp to 660bp in length. B-ARISA samples were run with ROX 1.1 size standard which varied from 20 to 900 bp. The GeneMapper 4.1 software converted fluorescence data to an electropherogram and the peaks which represented fragments of different sizes are termed operational taxonomic units (OTU). The heights of the peaks indicate the relative abundance of the fragments. The lengths was calculated using the size standards by plotting a best fit curve and extrapolating fragment sizes from the sample.

Data analysis

ARISA is an estimate of diversity but according to findings by Crosby and Criddle (2003) the Shannon-Weaver diversity may be inaccurate up to 0.3 index units due to the occurrence of multi-copy 16S fragments in a singular organisms and overlapping of same size fragments from different organisms. This PCR based method is, however, very accurate in estimating diversity and evenness compared to all other high throughput methods available (Crosby and Criddle 2003).

Data were analysed using Genemapper 4.1 software. Only fragment sizes from 100 to 1000 base pairs in length was considered for analysis. The peak heights considered for analysis were 0.5% of the total fluorescents of the profile. The peaks that were considered and counted as OTU's were typically above 150 fluorescent units. A binning strategy to minimise the inaccuracies in the ARISA profiles of was employed with a bin size of 3bp for fragment below 700bp and 5bp for fragment above 700bp's (Brown et al. 2005, Slabbert et al. chapter 3). GeneMapper 4.1 software converted the fluorescence electropherogram data representing operational taxonomic units into peaks indicating the fluorescent intensity. Peak heights were favoured over peak area for further analysis due to inaccuracies of peak areas for larger fragments. Peak area values tend to show more variation than is the case for peak height. This may be due to peak smoothing and height algorithms that are simpler and a more consistent measure of peak height. Peaks which represented 0.5% or more of the total fluorescence were considered for analysis.

Frequency of operational taxonomic units

The frequency of both fungal and bacterial OTU's throughout the year were determined. The different sizes were represented as 1 or 0 based on the presence or absence of peaks. All samples were summated in order to construct a histogram representation of the peak or OTU frequencies. OTU frequencies are based on data observed on all 4 sampling dates.

Species accumulation

The species accumulation curves were plotted for fungi and bacteria for each of the 4 sampling events during the year, by plotting the cumulative number of species against the number of samples taken in the area. Each separate ARISA profile was treated as different sampling event. The order in which the samples were added to the data set was randomized by re-sampling the data using Poptools 2.7 software. When a species accumulation curve is created, the most prevalent variation occurs in the initial samples and reduces towards the tail. Bootstrapping methodology was applied by repeated re-sampling and analysis until the mean value was within acceptable limits of variation ($p < 0.05$).

Twelve species-accumulation models which are commonly observed in literature were tested against the entire species accumulation curve for both bacteria and fungi. All of the models fitted were asymptotic. Species accumulation models were fitted with nonlinear regression using Statistica 8 software (Statsoft 2008) and the Guess Newton estimation method with the concept of generalized least squares. The performance of the models were evaluated by determining the coefficient of determination ($R^{2)}$, which gives an indication of the proportion of the variance that is accounted for as well as the residual sum of squares which indicate the discrepancy between the data and the fitted model. A model with a higher R^2 value and a lower sum of square value will identify the better fit models. After the elimination of non-useful models by graphical examination of the models the Akaike's Information Criterion (AIC) was used to further evaluate the fit of the model. The AIC determine which model is more likely to have generated the data compared to the others considered. When different models performed similarly, the simpler model with the fewest parameters will generally be favoured by the AIC. The species accumulation curves of bacteria and fungi were transformed to 100% of the maximum value in order to visually inspect and compared the shape and curvature of the fungal and bacterial species accumulation curve. The parametric value of the species accumulation models which determine the form of the curve was determined and compared. The fungal and bacterial alpha and beta diversity for each sampling was correlated with the form of the curve by regression analysis.

Species estimation

The occurrence of many rare species causes estimates using species accumulation curves to be distorted (Colwell and Coddington 1994). To determine the estimated number of species in the area, nonparametric methods was shown to be more accurate (Chazdon et al. 1998). The estimation methods that were used included Chao (based on presence and absence data), 1ste order Jack-knife, Bootstrap and Michaelis-Menten models. The performances of most richness estimators are, however, suspect and there are no clear indication which one is superior (Colwell and Coddington, 1995). The various estimators appear to perform different depending on the system. The suitability of the richness estimators needed to be

evaluated before any determination of species richness can be applied. The estimators were tested by reducing each species accumulation data set, to include halve of the number of individuals sampled and comparing the estimators prediction with the known number of species observed (Bartels and Nelson 2007). In the equations (Table 3), S is the number of species predicted, S(obs) is the number of species observed, G is the square of the number of species that only occur in one sample, M is the number of species that occur in two samples, p(j) is the proportion of the samples holding the j th species, L is the number of species only captured in one sample, n the number of samples and B is a parametric constant.

Diversity

The peak heights were used to calculate the diversity indices for each ARISA profile using Microsoft Excel software (Table 4). The Shannon-Weaver (H) index was calculated for each sample and each plot to indicate the disorder in the species destitution of the community. Pi represents the fraction of each peak of the total integrated area and S is the number of OTU's of the profile. The Shannon-Weaver is a good indication of diversity and evenness but is not a linear measure of diversity. By determining the effective number of species, the Shannon-Weaver value can be transformed into a linear measure of diversity and samples directly compared. The Simpson index was calculated for each plot to indicate the influence of dominant species on diversity. The higher the Simpson's index the larger the chance that two species picked randomly will be the same species. The Simpson's index leans to the more abundant species in the sample and is not influenced dramatically by the presence or absence of minor species. In addition to the Shannon-Weaver index the effective number of species was determined.

Community structure

The Whittaker similarity index was calculated between all plots. In total there were 1939 comparisons made between all plots on each sampling date. These comparisons were done for the bacterial and fungal communities individually. The distance relationship between the samples was illustrated by performing a complete linkage cluster analyses using the Pearson-R value of the Whittaker (Sw) similarity

indices. The b_i value represents the fraction of the peak from the first and second sample being compared. The special assemblage of the relationship between plots was illustrated by performing principal component analysis (PCA). The PCA was based on the presence and absence data of the plot profiles. The Whittaker similarity index present the similarities between samples which is expressed as a value between 0 and 1 with 0 being completely dissimilar and 1 being completely similar. $S_W = 1 - \sum_{i=1}^{n} \frac{|b_{i1} - b_{i2}|}{2}$

Spatial scaling of species richness

The species area relationship was determined by plotting the number of species against the logarithmic scale of the area. The power law for the taxa-area relationship is estimated in the form $\mathrm{OTU_a} = \mathrm{OTU_A} \left(\mathrm{A/a}\right)^z$ (Harte 1999). OTU_A and OTU_a are the number of operational taxonomic units in the larger area A and the smaller area a. The z-value is the value of the slope of the species area curve and remains constant.

Beta diversity

The increase of beta diversity was determined over the different samples, plots and sites. The Whittaker (βw) index for beta diversity was determined over all scales and comparisons made between samplings (Whittaker 1972). The Whittaker index is calculated by βw = S/α – 1, where S is the total number of species sampled and α is the average number of species in a sample. When determining the beta diversity in ecological samples it has been shown that the Whittaker (βw) index is generally superior (Wilson and Shmida 1984). The Whittaker index relatively sensitive to changes in the community, while being independent of sample size, as well as alpha diversity, while allowing data from different samples to be added. This is a useful feature when using data originating from ARISA because the need to standardize the data is eliminated. Studies which looked at beta diversity showed discrepancies between beta diversity values, on local scales compared to smaller scale. Thus, the beta diversity of the study area was determined by calculating the βw over increasing

areas and scale dependencies was demonstrated for many organisms (Mena and Vázquez-Domínguez 2005).

Relationship between soil microbial richness, diversity and soil properties.

The possible association between fungal species richness and diversity as well as bacterial species richness and diversity and soil characteristics was tested by canonical correlation analysis. Canonical correlation analysis allows one to investigate the relationship between two sets of variables. Canonical correlation analysis was performed using raw variables of all factors irrespective of the factor loading. Canonical correlation analysis was performed on log transformed bacterial and fungal richness and diversity variables against the additive soil chemistry variables. This was done in order to assess the influence of the environmental factors as a whole instead of using linear regression techniques. The soil variables that were assessed by canonical correlation analysis were soil moisture, pH, resistance, as well as, P, K, Cu, B, Mn, Zn concentrations, exchangeable iones Na, K, Ca, Mg, carbon concentrations as well as base saturation Na, K, Mg, Ca and T-value. In each case, factor analysis was performed to extract the principal component which carries the most importance. This was followed by addressing the effect each environmental factor has by means of multiple regression between bacterial and fungal richness and diversity against soil property variables. The correlations were based on Pearson's correlation analysis which was used to determine whether there was a significantly linear relationship between measured soil chemistry and observed richness and diversity.

Relationship between microbial and plant community structure.

To relate fungal and bacterial community structures with plant community structure, the relationship between the structures of all three data matrices was determined. The bacterial and the fungal community profile data was expressed as a data matrix based on the presence or the absence of operational taxonomic units. The plant data of the larger Whittaker plot was also expressed as a data matrix based on the presence or absence of a specific plant species in the plot. Data was used to calculate the Sorenson similarities (Cn) between different sites for each sampling

date and this resulted in a similarity matrix for each community type. The relationship between the bacterial and fungal community similarity data and the plant community similarity was tested by using a non-parametric partial Mantle test. The partial Mantel test eliminates the influence of spatial patterning on the community structure by controlling of this variable (Sokal and Rohlf 1981). The partial mantel test was performed using ZT-software with a confidence level of 95% (Bonnet and Van der Peer 2002).

Results and Discussion

DNA extraction

Visualization of electrophoresis gels showed substantial concentrations of DNA in all the samples extracted (Figure 2). The DNA extractions proved to be constant with yields persistently above 200ng/µl. Generally, 0.25 grams of soil were adequate to reveal the majority of fungal and bacterial diversity (Kang and Mills 2006).

PCR amplification

The bacterial specific primers produced PCR products in the size range of 100 bp to 1000 bp (Figure 3), whereas the fungal specific primers produced PCR products in the size range 200 bp to 1000 bp (Figure 4). All the PCR reactions produced smears with the highest peak intensities in the region of 600 bp in the case of the fungal specific PCR and in the range of 400 to 600 for the bacterial specific primers.

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Bacterial ARISA electropherogram data produced peaks from 100 to 900 bp as extrapolated by Genemapper from the ROX 1.1 size standard. The fungal ARISA electropherogram results show peaks ranging from 150 to 800bp as extrapolated using the LIZZ 600 size standard. Genemapper detected approximately 150 peaks for F-ARISA and 250 for B-ARISA. The number and intensity of the peaks of the

ARISA profiles were distinct enough to differentiate between sites. The ARISA profiles did, however, demonstrate overlap when comparing plots from the same site.

Frequency of bacterial and fungal OTU's

Figure 5 and 6 summarize all the size data and frequencies at which the specific bacterial and fungal OTU's occurred. Figure 5 indicates the dominance the fragments of between 150 and 500 bp in length for the bacterial population. This size category constitutes 90.6% of the total operational taxonomic units observed for all the samples taken for February to September 2007. Generally a very small number of taxa with very low peak intensities were observed under 100 bp in length. These were considered to be artefacts and not included in further analysis. (Fisher and Tripplett 1999). This finding is consistent with previous work that was done on B-ARISA, which found between 85 and 90% of OTU's to occur in this region (Fisher and Tripplett 1999). These smaller fragments are most likely Gram-positive bacteria, because most Gram-positive bacteria have no tRNA in its spacer region which results in shorter lengths (Gürtler and Stanisich 1996). This would be expected due to the well recognized dominance of Gram-positive bacterial species in soil (Torsvik 1990, Dunbar et al. 1999, Smalla 2001).

The size data based on the frequency of fungal OTU's indicate the dominance of peak length between 550bp and about 650bp in length (Figure 6). This region of the histogram constitutes 45.6% of the total number of peaks observed. The result is similar to findings by Ranjard et al. (2001) who found the majority of peaks in the range of 450 to 550 bp using the fungal specific primer set (2234C, 5'-GTTTCCGTAGGTGAACCTGC-3' and 3126T, 5'-ATATGCTTAAGTTCAGCGGGT-3') which resulted in fragments which on average a 100bp shorter in length compared to fragments resulting from amplification with ITS4 and ITS5. The results are also expected due to the well known dominance of the Ascomycetes in soils located in Mediterranean type climates. *Penicillium* and *Aspergillus* is known to by to be the dominant soil born fungi in heathland vegetation similar to fynbos (Christensen 1989).

Species richness

The number of bacterial OTU's remained significantly higher than fungal OTU's across all plots and was consistent from February to September. The number of different bacterial species is thus higher than the number of fungal species in Sand fynbos soil. The number of bacterial OTU's in each sample in February ranged from 42 to 68, April from 40 to 56, June from 40 to 51 and in September from 43 to 49. The species richness observed with ARISA in this study is very high when compared to studies using media culturing methods. Other studies which explored soil bacteria in different agricultural soils only observed 9 to 22 different species (Silva and Nahas, 2002). Cardinale et al. (2004) found 41 OTU's when investigating bacterial diversity of polluted soil. Meier et al. (2007) detected 42 to 46 OTU's from soil collected in a agricultural field. Studies investigating the bacterial OTU richness in a variety of different ecosystems found scrubland to be the richest in species numbers with 35 to 115 species commonly detected using DNA fingerprinting methods (Cardinale et al. 2004, Fierer and Jackson 2006).

The number of unique fungal OTU's in each sample were consistently less than the number of bacterial OTU's, and varied between 12 and 34 in February, 21 to 37, in April, from 21 to 35 in June and from 27 to 36 in September. This species diversity value is significantly lower when compared to the fungal in tropical rainforest ecosystems where an average number of fungal species in an observation has been observed to be about 56 (Persiani et al. 1998).

The numbers of bacterial and fungal OTU's were expressed as a percentage of the total number of unique OTU's detected with ARISA. This percentage is an indication of the contribution each subplot makes to the total species richness in the plot. This percentage is also an indication of the similarity of the subplots and the heterogeneity of the species distribution. The differences in species richness between the plots and the sites illustrate the effect that spatial scale has on the observed species richness. The heterogeneity of both the bacterial and fungal communities was high with the lowest mean percentage for bacteria of 46.16 being observed in June and a high of 55.1 being observed in September. The lowest percentage for the fungi was 43.38 and a high of 54.79. These percentages are an indication of the high degree of patchiness in the dispersal of both fungal and bacterial communities. The values

observed for bacteria and fungi, however, showed no significant difference according to the T-test performed.

The number of bacterial operational taxonomic units detected at each site was consistently higher than the number of fungi detected over all sites and samplings. The number of bacterial operational taxonomic units on the sampling in September 2007 was significantly lower than during any other sampling month. The fungal: bacterial ratio also showed the highest values during the month of September. The scale at which species richness is compared, has proved to be important. The bacterial richness per plot did not appear to be significantly lower during September when observing species richness of plots (Figure 8) but did show a significant difference at the larger scale (Figure 9). This lower species richness is also evident when examining the species area relationship (Figure 14). The species accumulation models also confirmed that the least number of unique bacterial species was observed during the month of September (Figure 11).

The fungi: bacteria ratio ranged from a minimum of 0.37 for Kalbas Kraal in February 2007 and as high as 0.82 for CampHill during September. These values are typical for moderate heathland vegetation (Fierer et al. 2006). The fungi: bacteria ratio at the CampHill site was statistically higher throughout the year in comparison to the other three sites (T-test, $p < 0.05$). The moisture content of the site at CampHill was also significantly higher than that observed at the other sites. The structure of the vegetation may also play a role in the fungal: bacterial ratio. The vegetation on the site was observed to be typically denser compared to the other sites in the study which leads to a lower mean soil temperature. The denser vegetation results in an increase in the amount of plant input into the soil. The increased canopy density and lower soil temperature presumably leads to the preservation of soil moisture. The lowest fungal: bacterial ratios were observed at all the other sites and did not differ significantly form one another. Sites where the plant cover is less dense will lead to lower input and quality of plant litter which causes the fungal: bacterial ratio to be lower. Wet cool conditions also prevailed during sampling in the month of September and an increase in the fungal: bacterial ratio is seen for all sites in the month of September. The colder conditions offer an advantage to soil fungi over that of eubacteria (Seiter et al. 1999).

Species accumulation

The species accumulation curves of bacteria and fungi have a tendency to become more asymptotic with an increase in the number of samples. All the models differed in goodness to fit (R^2). Table 8 show the mean sum of square and R^2 values of the species accumulation models evaluated, the sum of square, mean sum of square values as well as the AIC value. The AIC value is an indication of the likeliness that the model evaluated is the right one, were the data from the correct model probably originating from the one with the lowest AIC value. The performance of most of the bacterial species accumulation models varied significantly between sampling events. The 3 parametric Morgan–Mercer–Flodin model and the 4 parametric Beta-P model showed consistent good fit over all 4 sampling date with the lowest AIC value throughout all sampling events. No significant difference was observed between the two models. The data indicates that these models can thus be useful to describe bacterial species accumulation in the Sand fynbos. In addition to the Morgan-Mecer-Flodin model and the Beta-P, the 3 parametric Weibull also showed significant fit with fungal species accumulation data.

None of the species accumulation curves for bacterial or fungal species plateaued completely. This is expected since the study site does not comprise a closed system (Scheiner, 2004). It is also evident from the fungal species accumulation curves that the number of species is still increasing relatively rapidly compared to the bacterial equivalents. This relatively steep increase towards the tail of the curve reveals the occurrence of very rare fungal species which requires a more considerable sampling effort to detect. The initial part of the fungal curve is less steep compared to bacterial species accumulation curves and this is an indication of the patchiness in the fungal species distribution and thus high beta diversity. The diversity data are expected to reveal that bacteria are more evenly distributed than soil fungi. The relatively steep increase of the initial part of the curve point towards the dominance of certain bacterial species. The relative flatter tail of the curve on the other hand illustrates the lack of rare species. The Beta-P model showed a good fit for both the fungal and bacterial accumulation curves. The added advantage of the Beta-P model is the manner in which the variables are able to describe the shape of the curve. The b value describe rate of increase in species accumulation and thus the degree that abundant species occur. A high b-value indicates a steep initial rise in the curve. The d-value is the component of the equation which describes the curvature of the

species accumulation curve. This d-value was successfully correlated to the Shannon Weaver diversity index in a previous study (Thompson et al. 2003). The fungal and bacterial diversity indices here also showed a significant correlation with the d-value ($r^2 = 0.8044$, $p = 0.006$). Some authors have proposed the use of species accumulation curves as an indicator of diversity, and the result of this study provide added evidence for this statement.

Species estimation

The histograms in Figure 6 indicate that the Bootstrap method performed better than the other species estimation models tested. The variation between the predicted value using half the number of individuals sampled, and the real value observed was constantly smallest when using the Bootstrap estimation model. The different methods have been showed to be effective for specific applications and specific niches (Bartels and Nelson 2007), but the Bootstrap method proved most useful to estimate total soil microbial species (Figure 12). The Bootstrap method was furthermore used to obtain the total number of predicted species for both fungi and bacteria. The results predicted a detection level of around 90% to 92.6% for bacterial species and around 87.5% for fungi. This indicates a relatively larger number of rarer species of fungi than would be the case for bacteria (de Boer 2005). These levels hold true for both bacteria and fungi when using the data to directly evaluate ecological samples. The predicated values for bacteria are higher than is the case for fungi and the fungi: bacteria ratio using predicted values are similar compared with ratios calculated from observed values.

Species diversity

The species diversity was calculated for all 4 sampling dates. The Shannon-Weaver index, Simpson index and the effective number of species of the fungal and bacterial communities of all four plots are expressed in Table 10. Due to the relatively higher species number, the bacterial diversity is expected to be higher than the fungal diversity and that is in fact the case. The variation in the bacterial diversity was less than was the case for the fungal community. The Shannon-Weaver index for the bacterial communities remained very high throughout the year with a value of 3.60

being the minimum value obtained and 4.4 the maximum. These values of above 4 are high since the Shannon-Weaver index for typical soil bacterial communities are in the range of 3.5 to 4. The minimum fungal Shannon-Weaver value was at it lowest point at 2.9 at Kalbas Kraal and highest at 4.3 at Riverlands during the same sampling in February 2007. Although diversity indices varied spatially and over time there was no clear significant difference between sites and over time. The range of Shannon-Weaver values and thus the diversity of bacteria and fungi in Sand fynbos remained relatively constant within the indicated levels. The exact factors influencing the high alpha diversity is, however, unclear. The effective number of species compares the Shannon-Weaver on a linear scale and allows direct comparisons to be made. These values indicated that bacterial diversity is 1.3 to 4.3 times higher than fungal diversity based on the Shannon-Weaver values.

The Simpson index is primarily a dominance index. The higher the Simpson index the higher the effect of dominance in the community. Table 10 indicates that the Simpson index for bacteria is significantly higher than that of fungi. The dominance of certain species of bacterial in a community is thus more pronounced than in the fungal community. In other words, the likelihood that two random species from a bacterial community are the same, is higher compared to the fungal community. This dominance of a few species is also apparent upon visual inspection of the fungal ARISA profiles.

Species-area-relationship

The species area relationship has proven to be an important characteristic, when considering microbial diversity especially on a landscape or meso-scale (1 to 100 m^2) (Horner-Devine et al. 2004). Only a few studies, however, have considered the species area relationship of bacteria or fungi (Horner-Devine et al. 2004, Green et al. 2004). The indication is, however, that the two groups abide by the power-law relationship between the number of species in an area and the size of that area. The power-law for species area relationship has been observed for both plant and animal communities (Arhennius, 1921). The nature of microbial species area relationships has, however, been an illusive characteristic.

The species area relationship curve shows a logarithmic relationship between the number of species and the area. This is an indication that a power law for species accumulation is adhered to by both fungal and bacterial communities. The gradient of the curve corresponds to the z-value of the species turnover. The z-value of the bacterial community in the fynbos soil over the 4 sampling dates remained relatively stable and ranges from 0.1201 to 0.1292 for the first 3 samples. During sampling in September the z-value decreased to 0.0965. This indicates a reduction in bacterial richness that could be detected during this sampling time.

The z-value for both bacteria and fungi is expected to be low (< 0.1). Bacteria, in fact, were thought have some of the lowest z-values of any organism which indicates homogenous distribution in the soil environment with a low turnover (Horner-Devine et al. 2004). Many resent studies, however, has revealed much higher values in various ecosystems like forest soils where z-values as high as 0.47 was observed (Noguez et al. 2005). This study also supports the occurrence of high species turnover rate for bacteria in soil and contradicts studies which assigned definite z-values for soil bacteria.

The species area relationship is bound to the specific ecosystem. The z-value of the fungal species richness against the area is lower than z-values of the bacterial communities. This value is, however, high when compared to other ARISA studies where z-values in the region of 0.074 were observed in a desert soils (Green et al. 2004). During the first 3 sample events the z-value ranges between 0.1021 and 0.1109. The month of September shows a dramatic increase in the z-value with coincides with the decrease seen in the bacteria spatial turnover. This indicates a high microbial diversity exhibited by the bacterial community as well as the fungal community. The turnover for bacterial and fungal communities in the fynbos soil is higher than is generally the case for terrestrial ecosystems.

Community structure

Although the diversity between the plots in most cases did not differ considerably, the peak composition of the ARISA profiles did indicate large differences according to the Whitaker similarity indices. The cluster analysis was performed to illustrate the similarity relationship between the intra-fungal and bacterial communities according

to occurrence and abundance of operational taxonomic units. The tree diagram for bacteria and fungal communities display high similarity between plots from the same sites. This is expected when considering that regressions of communities are known to occur over distance. This is especially the case when looking at the fungal community structure.

There is a strong indication when observing the Mantel and Partial Mantel test that space plays an important role. The indication is that although bacterial are more closely linked to plant communities, it does, however, follow a similar clustering pattern than the fungal communities during all four samplings. The cluster analysis of fungal communities showed higher similarities between sites on the main reserve namely Pella and Riverlands, which is expected when the community structure leans heavily towards spatial patterns. In contrast, the bacterial communities at Riverlands showed higher similarity within the communities. It does, however, reflect a similar clustering pattern to fungal communities during all four samplings. The similarities between plots, as well as the ordination pattern remained constant for both bacterial and fungal species over all four sample events. This is an indication that the relationship between communities remained relatively stable for both bacterial and fungal communities.

The tree diagrams in figure 16 and 17 is also a confirmation that the distribution of microbial communities is not random and that strong spatial patterns exist even if environmental gradients are regarded as relatively insignificant. The tree diagram also suggests that the fragmentation of land has an influence on the structure of both the bacterial and fungal communities due to the consistently strong grouping of plots within the same site. The diagram does, however, not elucidate to the exact factors caused by the fragmentation of the area. The two main factors which may play a role in soil microbial community structure is the variation of plant communities over the area or spatial separation due to the physical isolation of the sites.

Beta diversity

The particularly species turnover demonstrated by the ARISA profiles is an important primary factor which contributed to the diversity of fungal and bacterial species of the Sand fynbos. Botanists have long recognized that the primary factor influencing

fynbos diversity is not its alpha diversity at a specific site but the beta diversity which describe the number of species shared between sites. The beta diversity of bacterial and fungal samples can be regarded as very high, with values above 4.0 being observed except for bacteria in the month of September when it only reach 3.6. The beta diversity is influenced by the scale with a decline with increasing scales. The beta diversity of both the fungal and bacterial communities' decreases with an increase in scale due to the increase likelihood of species overlap occurring. The spatial separation between sites and the scale at which beta diversity is observed proved to be important factors when looking at the beta diversity of bacteria and fungi in the Sand fynbos. When comparing beta diversity at small scale the difference seems insignificant. The comparative beta diversity for fungi is significantly higher at larger scales during each sampling event. The trend is seen across all the larger scales considered. The description of beta diversity just by determining the similarities between similar sized plots has thus proven not to be sufficient.

The beta diversity of soil fungi in Sand fynbos soil is generally higher than that of the soil bacteria. The data indicate that the fungi would be less evenly distributed in the area as would be the case for soil bacteria. The beta diversity between sites indicates limitations in the dispersal between plots due to the physical isolation and fragmentation of the plots or as result of the diversity influence of above ground plant cover. The influence of climatic or substrate differences are largely eliminated due to the uniformity of these factors over the study area. The high beta diversity which is the main contributing factor resulting in the high biodiversity seen in fynbos, appear to play an important role in the biodiversity of bacterial and, especially the fungal communities.

The fragmentation of Sand fynbos and the loss of land area have caused great loss in the diversity of plant species (Rouget et al. 2003). The large species turnover seen in fynbos is of vital importance when considering the conservation of fynbos (Cowling et al.1990, Rouget et al. 2003). Locally existing microbial species represent a large proportion of the total regional richness. The sharp reduction in the beta diversity with an increase in scale is an indication of the rapid rate at which the most dominant species is detected and not necessarily due to extremely heterogeneous microbial community composition.

The physical isolation of species does not play an important role in the diversity of microorganism in the Sand fynbos. The role of spatial distribution is not as important on these scales. However, the microbial communities are by no means homogenous. The distinctive community structures are not due to the influence of space only but also due to other factors. The fungal community has significantly higher beta diversity, even though its dispersal by aerial spores poses less of a limitation to dispersal than is the case for bacteria. The environmental factors which determine the growth of fungi in the soil, other than the spatial dispersion plays a role in the higher beta diversity of fungi on a landscape level.

Relationship between soil microbial richness, diversity and soil properties

The Canonical correlation analysis depicted a weak relationship between the bacterial richness and the soil properties considered and between bacterial diversity and soil properties. The variation in bacterial richness and diversity at these scales cannot be explained by the soil properties and other factors must have a superior influence at these scales. Pearson's correlation analysis also reveals little importance in the linear correlation between any specific factor and soil bacterial richness and diversity.

The fungal species richness and diversity shows a strong relationship with the environmental factors considered in the multiple regression. The specific combination of soil characteristics has a large influence on the fungal species richness as well as the diversity. When considering the relationship between species diversity and soil properties the results suggest a fairly strong overall relationship between the variables in the two sets even thought the p-value suggest that the relationship between the two sets of data is marginally non-significant. However, when considering the redundancy, the values can be interpreted such that, based on all canonical roots, the soil properties you can account for, 76.43% of the variance in the soil fungal diversity variable set.

The fungal species numbers and diversity correlate positively with a decrease in the available potassium and thus the nutrient status in the soil (Pande and Tarafdar, 2004). Factor analysis indicated that potassium content and base saturation are the most important variables in the analysis. Previous studies also have observed the

negative correlation between fungal biomass and potassium concentrations (De Vries et al. 2007). The relative insignificant movement of the other variables provide an opportunity to observe the relationship between fungal diversity and soil potassium concentrations. The reason for the variation in potassium levels is undetermined but it is known that potassium leaches easily from sandy soils (Lodge et al. 1993). Due to its importance in plant as well as fungal biomass, potassium immobilization may also play an important role (Lodge 1993). Due to the largely positive correlation between biomass and diversity, the acquisition of potassium is most likely the reason for the negative correlation between potassium concentrations and fungal richness. Sodium and carbon also showed significant negative correlation with fungal richness and diversity. The model showed a positive correlation for copper, zinc and boron with the species richness but not with fungal diversity. Copper in contrast correlated negatively with bacterial richness.

Relationship between plant and microbial communities

The partial Mantel test showed a strong to very strong correlation between the bacterial community structures and the plant community. The high beta diversity of the aboveground plant population can thus be expected to directly influence the diversity of soil bacteria. The fungal community showed a strong relationship when performing the Mantel test. When controlling for the influence of space on the relationship it is seems that the relationship becomes less evident.

The structure of the cluster analysis of the fungal communities reflects the history and state of the different sites. The plots on the main reserve showed more similarities to each other than those found at Kalbas Kraal and Camp Hill Village. The effect of Port Jackson's on the fungal community is evident by its significant linkages distance from the other plots. The relationships between the bacterial communities were also consistent but were different from the relationship exhibited by the fungal communities. Riverlands which is located inside the main reserve is more similar over the entire year of sampling. This relationship suggests that the relationship did not correlate with the plant community data. The exact reason for the relationship between bacterial communities between the sites is unclear.

The correlation between plant and microbial communities exists but the exact causes of the relationship remains unclear. A few possibilities may explain the linked community structure between plants and micro-organisms. The maintenance of biodiversity within a system is a dynamic feedback system. Both the plant and the microbial populations have an effect on the abiotic environment and directly towards each other. The other possibility is that the same prevailing soil and environmental conditions that affects the species composition of the fynbos affect the microbial population structure and the two does not affect each other to a very high degree. The last possibility is a one-way interaction between the plant communities and the soil micro-organism where the plant litter and exudates is the main contributing factor and driver of microbial community structure.

Conclusion

The differences in communities where a plant population vary greatly, showed large variations in the microbial soil communities. The study indicates that the more subtle differences like that seen within the Sand fynbos also reflect on the soil microbial population. The effect of spatial distribution on microbial communities cannot be discounted and seem to play a bigger role in the fungal compared to the bacterial communities. The fynbos areas considered had little or no differences in structure during the entire year of sampling. Although bacterial communities correlated better with the plant communities and fungal community structure appeared linked with spatial regression and physico-chemical properties. No one characteristic appears to be the overriding factor which determines community structure. The soil environment is too complex. Factors which appear less important may become significant in another ecosystem if adequate variation occurs. The species accumulation characteristics categorise these communities as having a high risk of local extinction since reduced levels of genetic variation will probably affect the species ability, particularly in the smaller populations, to adapt to changes in its habitat.

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Table 1: Location of the study area.

Site	Location of site (GPS)
Pella	
plot 1	S 33,51022 E 18,55236
plot 2	S 33,50960 E 18,54925
plot 3	S 33,52011 E 18,54766
Riverlands	
plot 1	S 33,49795 E 18,58931
plot 2	S 33,49788 E 18,58608
plot 3	S 33,49608 E 18,58388
Camphill Village	
plot 1	S 33,59701 E 18,56554
plot 2	S 33,59787 E 18,56433
plot 3	S 33,59774 E 18,56327
Kalbas Kraal	
plot 1	S 33,57061 E 18,62861
plot 2	S 33,57133 E 18,62773

Table 2: The sequences of the bacterial and fungal specific primer sets.

Primer	Sequence of Oligo nucleotide	Tm (ºC)
Fungal specific primer		
ITS4	5' - TCCTCCGCTTATTGATATGC - 3'	57.2
ITS5	5' - GGAAGTAAAAGTCGTAACAAGG - 3'	55.6
Eubacterial specific primer		
ITSF	5' - GTCGTAACAAGGTAGCCGTA-3'	59.7
ITSReub	5′-GCCAAGGCATCCACC-3′	54.3

Table3: The species estimation models evaluated.

Estimation Model	Equation	
Chao - for presence - absence data	$S = S(obs) + G/2M$	Chao (1984)
Bootstrap	$S = S(obs) + Sum[1-p(j)]$	Smith and Van Bell (1984)
1ste Order Jackknife	$S = S(obs) + L((n-1)/n)$	Burnham and Overton (1979)
Michaelis-Menten	$S(n) = (Smax.\ n) / (B + n)$	Raaijmakers (1987)

Table 4: Equations representing the Shannon-Weaver index, Simpson index and Evenness of ecological communities.

Index	Equation	
Shannon-Weaver index	$H = \sum_{i=1}^{n} Pi \log(Pi)$	Shannon, C.E, 1948 Krebs C. J., 1989
Effective number of species	$E = e^{H}$	
Simpson index	$\frac{1}{D} = \frac{1}{\sum_{i=1}^{n} P_i^2}$	Simpson E. H., 1949

Table 5: The mean number of bacterial OTU's of each subplot expressed as a percentage of the total unique OTU's in the plot.

	Mean number of bacteria as a percentage of the total			
Site	**February 2007**	**April 2007**	**June 2007**	**September 2007**
Pella 1	43.81	38.22	48.56	57.53
Pella 2	54.05	48.67	43.51	59.81
Pella 3	44.02	36.95	47.8	63.96
Riverlands 1	63.86	38.46	48.81	50.57
Riverlands 2	50.82	57.01	40.69	54.76
Riverlands 3	40.49	53.91	39.84	53.57
Camp Hill 1	52.68	52.75	50.26	53.44
Camp Hill 2	50.54	59.24	46.75	55
Camp Hill 3	39.02	61.08	49.27	55.98
Kalbas Kraal 1	46.5	45.12	47.99	51.06
Kalbas Kraal 2	43.75	49.18	44.23	50.54
Mean	48.14	49.14	46.16	55.11
Max	63.86	61.08	50.26	63.96
Min	39.02	36.95	39.84	50.54

Table 6: The mean number of fungal OTU's of each subplot expressed as a percentage of the total unique OTU's in the plot.

	Mean number of fungi as a percentage of the total			
Site	**February 2007**	**April 2007**	**June 2007**	**September 2007**
Pella 1	40.82	61.31	48.84	57.35
Pella 2	52.38	62.96	46.43	62.79
Pela 3	28.88	53.65	51.83	50.46
Riverlands 1	38.81	58.51	52.59	58.33
Riverlands 2	56.05	69.77	54.27	48.13
Riverlands 3	45.96	56.4	48.11	49.65
Camp Hill 1	41.15	46.15	51.49	47.06
Camp Hill 2	48.02	40.56	34.48	51.54
Camp Hill 3	43	49.62	52.65	49.66
Kalbas Kraal 1	33.33	53.75	53.8	48.18
Kalbas Kraal 2	48.75	50	43.4	46.88
Mean	43.38	54.79	48.9	51.82
Max	63.86	61.08	50.26	63.96
Min	28.88	40.56	34.48	46.88

Table 7: The ratio of bacterial and fungal operational taxonomic units at each site.

	Ratio of fungal and bacterial operational taxonomic units			
Date	Pella	Riverlands	Camp Hill	Kalbas Kraal
February 2007	0.47	0.61	0.63	0.37
April 2007	0.4	0.45	0.72	0.38
June 2007	0.46	0.54	0.73	0.55
September 2007	0.63	0.72	0.82	0.68

Table 8a: The sum of square, mean sum of square, coefficient of determination (R^2) and AIC value for the different species accumulation models for bacteria and fungi for each sampling.

Bacteria								
Parameters (n)	Model		Feb	April	June	Sept	Average AIC	Std. error
	Number of species observed		276	240	262	215		
	Number of individuals observed		2064	2204	2089	2054		
2	Asymptotic regression	SS	1554.64	867.24	1020.54	1306.69		
		Mean SS	37.9	20.17	23.73	31.87		
		R 2	0.99	0.99	0.99	0.98		
		AIC	0.03	-27.72	-20.56	-9.9	-14.54	12.17
2	Negative exponential	SS	6519	4467.92	4621.75	4808.53		
		Mean SS	155	101.54	105.04	114.49		
		R 2	0.95	0.95	0.97	0.93		
		AIC	59.7	41.09	42.58	46.37	47.43	8.47
2	Clench	SS	1818	687.06	845.89	1158.03		
		Mean SS	43	15.62	19.22	27.57		
		R 2	0.99	0.99	0.99	0.98		
		AIC	5.59	-38.98	-29.83	-13.96	-19.3	19.55
2	Exponential	SS	872.1	222.33	477.87	60.36		
		Mean SS	20.76	5.05	10.86	1.44		
		R 2	0.99	1	1	1		
		AIC	-26.44	-88.62	-54.96	-143.95	-78.49	50.5
2	Power	SS	1883	2925.95	3314.57	1401.56		
		Mean SS	45	66.5	75.33	33.37		
		R 2	0.99	0.97	0.98	0.98		
		AIC	7.59	24.77	30.26	-5.57	14.26	16.37
3	Weibull	SS	267	74.91	134.9	254		
		Mean SS	6.5	1.74	3.14	6.2		
		R 2	1	1	1	1		
		AIC	-75.12	-133.05	-107.17	-77.23	-98.14	27.49
3	Morgan–Mercer–Flodin	SS	195	36.03	98.92	170.08		
		Mean SS	4.7	0.84	2.3	4.15		
		R 2	1	1	1	1		
		AIC	-89.39	-165.26	-120.82	-94.88	-117.59	34.61
3	Chapman-Richards	SS	nf	166.08	nf	nf		
		Mean SS	nf	3.86	nf	nf		
		R 2	nf	1	nf	nf		
		AIC	nf	-98.02	nf	nf		
3	Rational	SS	625	145.84	259.54	506.96		
		Mean SS	15.2	3.39	6.04	12.36		
		R 2	1	1	1	0.99		
		AIC	-37.74	-103.74	-78.38	-46.82	-66.67	30.23

Bacteria Parameters (n)	Model		Feb	April	June	Sept	Average AIC	Std. error
3	Hill	SS	1883.24	2925.95	3314.57	1401.56		
		Mean SS	45.93	68.05	77.08	34.18		
		R 2	0.99	0.97	0.98	0.98		
		AIC	10.92	28.21	33.7	-2.08	17.69	16.37
3	Logarithmic B	SS	nf	nf	nf	nf		
		Mean SS	nf	nf	nf	nf		
		R 2	nf	nf	nf	nf		
		AIC	nf	nf	nf	nf		
3	Asymptote	SS	1554.64	867.24	1020.54	1306.69		
		Mean SS	37.92	20.17	23.73	31.87		
		R 2	0.99	0.99	0.99	0.98		
		AIC	2.48	-25.3	-18.14	-5.16	-11.53	12.52
4	Beta-P	SS	107	35.13	96.58	84.95		
		Mean SS	2.7	0.84	2.3	2.12		
		R 2	1	1	1	1		
		AIC	-111.22	-162.78	-118.29	-121.78	-128.52	23.26

* nf – indicated that no fit could be established using the model

Table 8b: The sum of square, mean sum of square, coefficient of determination (R^2) and AIC value for the different species accumulation models for bacteria and fungi for each sampling.

Fungi Parameters (n)	Model		Feb	April	June	Sept	Average AIC	Std. error
	Number of species observed	179	159	154	180			
	Number of individuals observed	845	1303	1156	1364			
2	Asymptotic regression	SS	337	nf	307.82	380.64		
		Mean SS	8.6	nf	7.7	9.28		
		R 2	0.9953	nf	0.99	0.99		
		AIC	-62.8012	nf	-70.12	-61.86	-65.99	5.84
2	Negative exponential	SS	1231.9	2531.58	2371.8	3429.23		
		Mean SS	30.8	55.03	57.85	81.65		
		R 2	0.9829	0.96	0.94	0.94		
		AIC	-6.66806	16.45	18.64	33.8	22.96	9.45
2	Clench	SS	371.5	1393.59	965.71	1486.52		
		Mean SS	9.3	30.3	23.55	35.39		
		R 2	0.9948	0.98	0.98	0.98		
		AIC	-59.3581	-9.82	-20.9	-2.98	-11.23	9.04
2	Exponential	SS	1333.5	2835.25	848.77	1418.5		
		Mean SS	33.3	61.64	20.7	33.77		
		R 2	0.9815	0.96	0.98	0.98		
		AIC	-5.65982	21.43	-26.57	-5.04	-3.39	24.04
2	Power	SS	830.3	200.06	148.65	138.17		
		Mean SS	20.8	4.35	3.63	3.29		
		R 2	0.9885	1	1	1		
		AIC	-23.9408	-95.22	-103.23	-107.51	-82.4764	6.24
3	Weibull	SS	89	130.42	25.17	23.52		
		Mean SS	2.3	2.9	0.63	0.57		
		R 2	0.9988	1	1	1		
		AIC	-120.831	-110.66	-177.86	-181.93	-147.82	40.03
3	Morgan–Mercer–Flodin	SS	77.3	134.27	22.04	21.37		
		Mean SS	2	2.98	0.55	0.52		
		R 2	0.9989	1	1	1		
		AIC	-126.98	-109.38	-183.69	-186.15	-151.551	43.63
3	Chapman-Richards	SS	nf	nf	nf	nf		
		Mean SS	nf	nf	nf	nf		
		R 2	nf	nf	nf	nf		
		AIC	nf	nf	nf	nf		

Fungi Parameters (n)	Model		Feb	April	June	Sept	Average AIC	Std. error
3	Rational	SS	114.9	143.4	152.98	192.82		
		Mean SS	3.7	3.19	3.82	4.7		
		R 2	0.998	1	1	1		
		AIC	-99.9121	-106.48	-98.46	-89.36	-98.5518	8.57
3	Hill	SS	830.3	200.06	148.65	138.17		
		Mean SS	21.3	4.45	3.72	3.37		
		R 2	0.9885	1	1	1		
		AIC	-22.8956	-91.83	-99.72	-104.02	-79.6174	6.18
3	Logarithmic B	SS	Nf	Nf	nf	nf		
		Mean SS	Nf	Nf	nf	nf		
		R 2	Nf	Nf	nf	nf		
		AIC	Nf	Nf	nf	nf		
3	Asymptote	SS	337	175.09	307.82	380.64		
		Mean SS	8.6	3.89	7.7	9.28		
		R 2	0.9953	1	0.99	0.99		
		AIC	-62.8012	-97.7	-67.69	-59.43	-71.9066	20.14
4	Beta-P	SS	73.3	135.8	20.18	19.09		
		Mean SS	1.9	3.09	0.52	0.5		
		R 2	0.999	1	1	1		
		AIC	-129.237	-105.34	-183.92	-185.07	-150.892	45.71

* nf – indicated that no fit could be established using the model

Table 9a: The performances of the Bootstrap model based on halve the number individuals sampled, the estimated number of species and the percentage of the estimate observed with ARISA.

	Bacteria					
	Number of Samples used	Model	Number observed	Estimation using half the individuals	Estimate number of species	Percentage observed with ARISA
Feb	21	Bootstrap	276	275.03	306	90.19608
April	25	Bootstrap	240	242	259	92.66409
June	23	Bootstrap	265	259	290	91.37931
September	23	Bootstrap	215	214	239	89.95816

Table 9b: The performances of the Bootstrap model based on halve the number individuals sampled, the estimated number of species and the percentage of the estimate observed with ARISA.

	Fungi					
	Number of Samples used	Model	Number observed	Estimation using half the individuals	Estimate number of species	Percentage observed with ARISA
Feb	18	Bootstrap	179	168	205	87.31707
April	24	Bootstrap	164	163.75	188	87.23404
June	19	Bootstrap	154	145	176	87.5
September	23	Bootstrap	180	177	205	87.80488

Table 10: Summery of the Shannon-Weaver indices, Effective number of species and Simpson index for the months February to September 2007 for bacteria and fungi.

		Shannon-Weaver		Effective number of species		Simpson	
Date	Site	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Feb 2007	Pella site 1	3.136275	2.78745	23.01797	16.23956	13.078	10.412
	Pella site2	3.8764	3.04275	48.2502	20.96281	49.942	14.356
	Pella site3	3.73215	3.093863	41.76881	22.06213	58.088	9.5292
	Riverlands site1	3.729375	2.802675	41.65307	16.4887	40.36	15.518
	Riverlands site2	3.7441	3.003575	42.27095	20.15747	46.185	24.426
	Riverlands site3	3.58495	3.253963	36.05156	25.89274	45.442	51.699
	Camp Hill site1	3.513925	2.9366	33.57981	18.85164	36.302	21.254
	Camp Hill site2	3.641725	3.232663	38.1576	25.34705	39.254	53.67
	Camp Hill site3	3.335125	2.57435	28.08189	13.12278	42.262	3.6985
	Kalbas Kraal site1	3.591	2.242025	36.27033	9.412372	40.96	3.7939
	Kalbas Kraal site2	3.4058	2.7098	30.1384	15.02627	34.11	7.574
April 2007	Pella site 1	3.50605	2.771575	33.31641	15.98379	37.245	13.1
	Pella site2	3.739225	2.45855	42.06538	11.68785	24.068	8.1371
	Pella site3	3.734375	2.48375	41.86185	11.98613	35.565	7.7451
	Riverlands site1	3.2089	2.901025	24.75184	18.19278	55.813	15.191
	Riverlands site2	3.49385	3.038975	32.91242	20.88383	29.746	16.53
	Riverlands site3	3.451825	2.686125	31.55793	14.6747	48.505	17.542
	Camp Hill site1	3.713975	3.011867	41.01652	20.32531	30.905	25.363
	Camp Hill site2	3.42115	2.948733	30.60459	19.08177	30.021	24.457
	Camp Hill site3	3.38006	2.969175	29.37253	19.47585	32.376	26.547
	Kalbas Kraal site1	3.690375	2.23135	40.05987	9.312429	53.538	8.111
	Kalbas Kraal site2	3.38644	2.308425	29.56053	10.05857	33.395	9.3333

Table 10: Continue

		Shannon-Weaver		Effective number of species		Simpson	
Date	Site	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Jun-07	Pella site 1	3.66674	2.383475	39.12415	10.84252	42.258	8.3609
	Pella site2	3.3153	2.345725	27.53065	10.44084	38.272	6.2765
	Pella site3	3.346425	2.369125	28.40102	10.68804	22.287	8.8577
	Riverlands site1	3.63625	3.008125	37.94926	20.2494	36.636	22.468
	Riverlands site2	3.378725	2.253525	29.33335	9.521239	33.102	8.0235
	Riverlands site3	3.27395	2.8496	26.41547	17.28087	29.161	13.928
	Camp Hill site1	3.63628	3.052575	37.9504	21.16979	37.764	25.258
	Camp Hill site2	3.527425	2.882167	34.03621	17.85291	39.811	29.813
	Camp Hill site3	3.682	3.047025	39.72577	21.05262	36.09	15.341
	Kalbas Kraal site1	3.40855	2.492775	30.22139	12.09479	26.974	13.084
	Kalbas Kraal site2	3.3975	2.22745	29.88928	9.276182	32.798	6.9628
Sep-07	Pella site 1	3.534825	2.383475	34.28901	10.84252	32.892	11.233
	Pella site2	3.6124	2.345725	37.05488	10.44084	32.831	9.0395
	Pella site3	3.65705	2.369125	38.74687	10.68804	33.347	8.7056
	Riverlands site1	3.48235	3.008125	32.53609	20.2494	35.829	20.074
	Riverlands site2	3.580025	2.253525	35.87444	9.521239	33.011	22.342
	Riverlands site3	3.54075	2.8496	34.49278	17.28087	30.538	26.03
	Camp Hill site1	3.333375	3.052575	28.03279	21.16979	22.448	29.863
	Camp Hill site2	3.5789	2.882167	35.8341	17.85291	31.215	22.465
	Camp Hill site3	3.642125	3.047025	38.17287	21.05262	30.8	24.419
	Kalbas Kraal site1	3.5964	2.492775	36.46672	12.09479	38.764	16.93
	Kalbas Kraal site2	3.5178	2.22745	33.71018	9.276182	40.711	28.039

Table 11: The Z-value for bacterial and fungal communities

	Z-Value	
	Bacteria	Fungi
February 2007	0.1292	0.1109
April 2007	0.1201	0.1099
Jun-07	0.126	0.1021
Sep-07	0.0965	0.1245

Table 12: Summery of the Whittaker beta diversity index over the three are sizes considered.

	Whittaker (Bw)		
	Sample	Plot	Site
Bacteria			
February	5.0965	1.7154	0.67683
April	4.0091	1.3848	0.43928
June	4.8353	1.6719	0.68521
September	3.6056	1.543	0.67315
Fungi			
February	5.647	2.1989	1.1232
April	5.0414	2.2043	1.0892
June	4.7284	1.7455	0.85542
September	4.8423	1.9685	0.87013

Table 13: Summery of canonical correlation analysis between soil properties, bacterial richness, bacterial diversity, fungal richness and fungal diversity.

Community property	Canonical R	P value	Redundancy
Bacterial richness	0.72747	0.727	52.9217%
Bacterial diversity	0.71285	0.78197	50.8157%
Fungal richness	0.89419	0.02789	79.9585%
Fungal diversity	0.87425	0.6446	76.4313%

Table 14: Multiple regression analysis between individual soil properties, bacterial richness, bacterial diversity, fungal richness and fungal diversity. Highlighted values indicate significant R^2 values ($p < 0.05$).

Soil characteristic	Bacterial richness Total	Bacterial diversity	Fungal richness Total	Fungal diversity
pH	-0.001	-0.244	-0.146	-0.158
Resistance (ohm)	-0.376	-0.108	0.642	0.302
H+ (cmol/kg)	0.001	-0.076	-0.154	-0.255
P (mg/kg)	0.083	0.054	-0.086	-0.097
K (mg/kg)	0.021	-0.116	-0.549	-0.386
Na (cmol/kg)	0.325	0.015	-0.413	-0.317
K (cmol/kg)	0.027	-0.087	-0.538	-0.367
Ca (cmol/kg)	-0.395	-0.256	0.285	0.108
Mg (cmol/kg)	-0.208	-0.205	-0.018	-0.167
Cu (mg/kg)	-0.321	-0.226	0.357	0.062
Zn (mg/kg)	-0.232	0.092	0.379	0.253
Mn (mg/kg)	-0.117	-0.035	0.036	-0.047
B (mg/kg)	-0.468	-0.138	0.349	-0.024
C %	-0.1	0.007	-0.368	-0.182
Na %	0.401	0.072	-0.45	-0.288
K %	0.158	-0.008	-0.63	-0.382
Ca %	-0.328	-0.179	0.427	0.282
Mg %	-0.126	-0.08	-0.068	-0.174
T-value	-0.308	-0.282	0.044	-0.147

Table 15: Results for the Mantel test and Partial Mantel test.

	Fungi		Bacteria	
	Mantel test	Partial Mantel test	Mantel test	Partial Mantel test
Feb-07	0.706	0.212	0.59	0.6
Apr-07	0.442	0.112	0.37	0.62
Jun-07	0.398	0.091	0.24	0.57
Sep-07	0.659	0.124	0.11	0.49

Figure 1a: Site at Pella.

Figure 1b: Site at Riverlands.

Figure 1c: Site at Camp Hill Village.

Figure 1d: Site at Kalbas Kraal

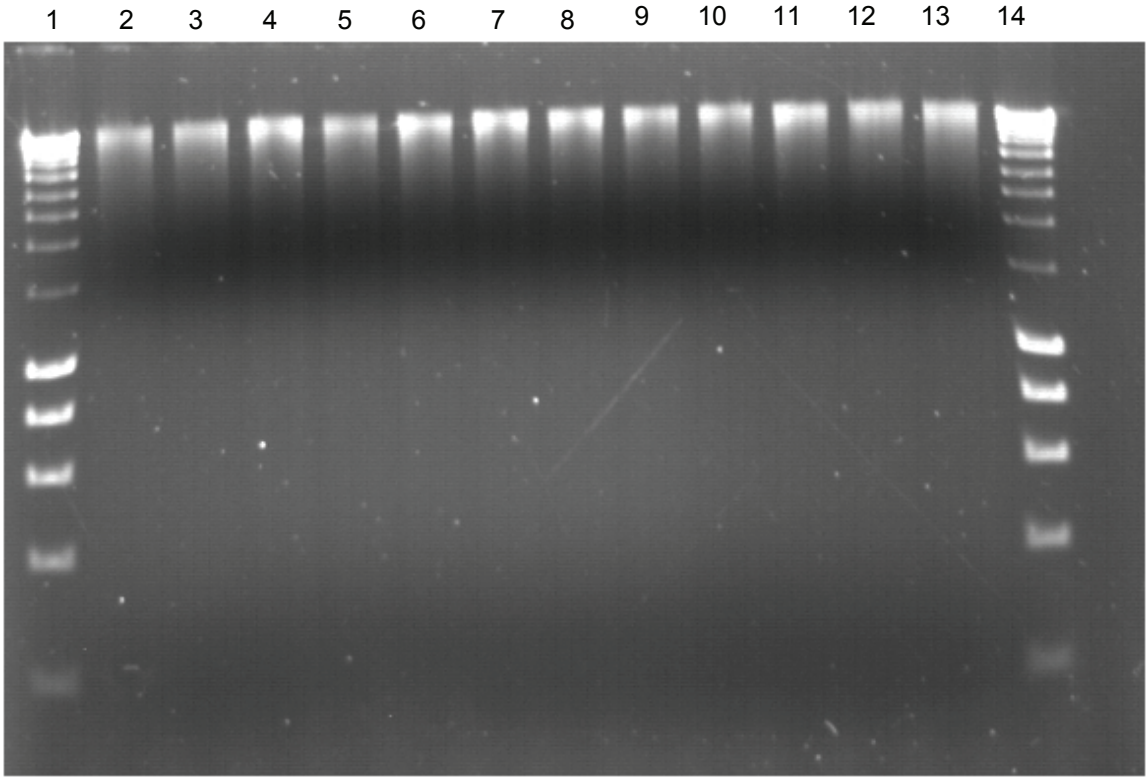


Figure 2: Agarose gel electrophoreses total extracted DNA from Riverlands on Feb 2007. Lane 1 and 14: Hyper Ladder I, Lane 2-5: Riverlands plot1A - D, Lane 6-9: Riverlands plot A-D, Lane 10-13 Riverlands plot A-D PCR amplification.

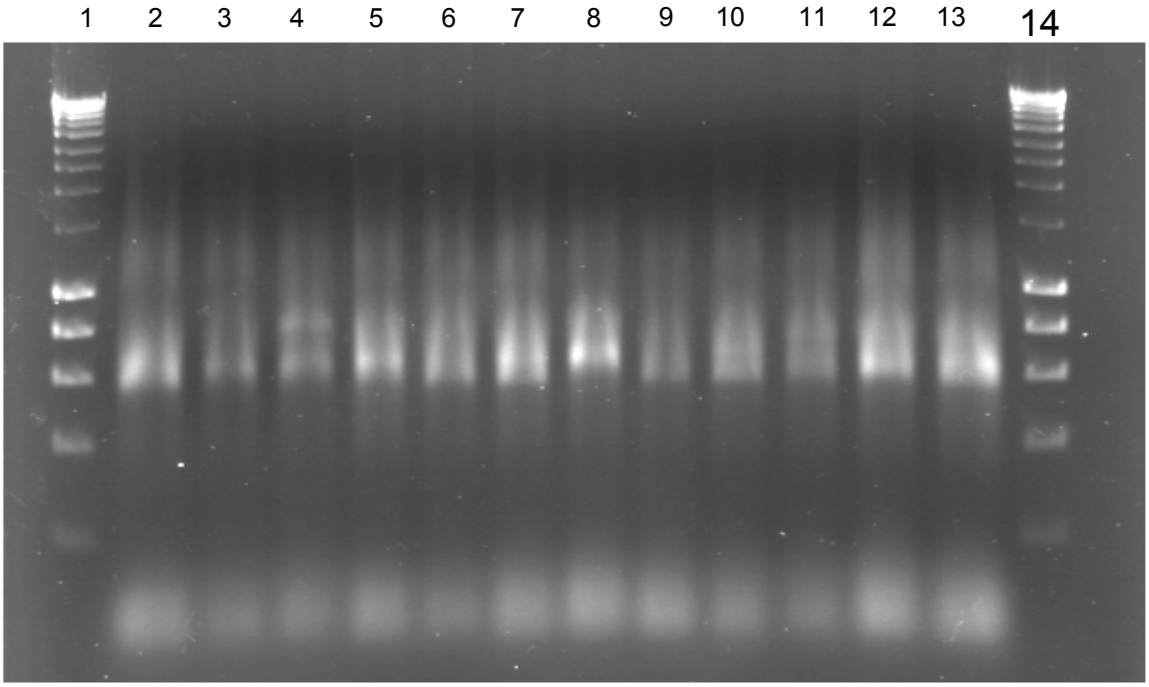


Figure 3: Agarose gel electrophoreses of PCR amplification of total extracted DNA from Riverlands on Feb 2007 with fungal specific primer set ITS5 (FAM) and ITS4. Lane 1 and 14: Hyper Ladder I, Lane 2-5: Riverlands plot1A - D, Lane 6-9: Riverlands plot A-D, Lane 10-13 Riverlands plot A-D PCR amplification.

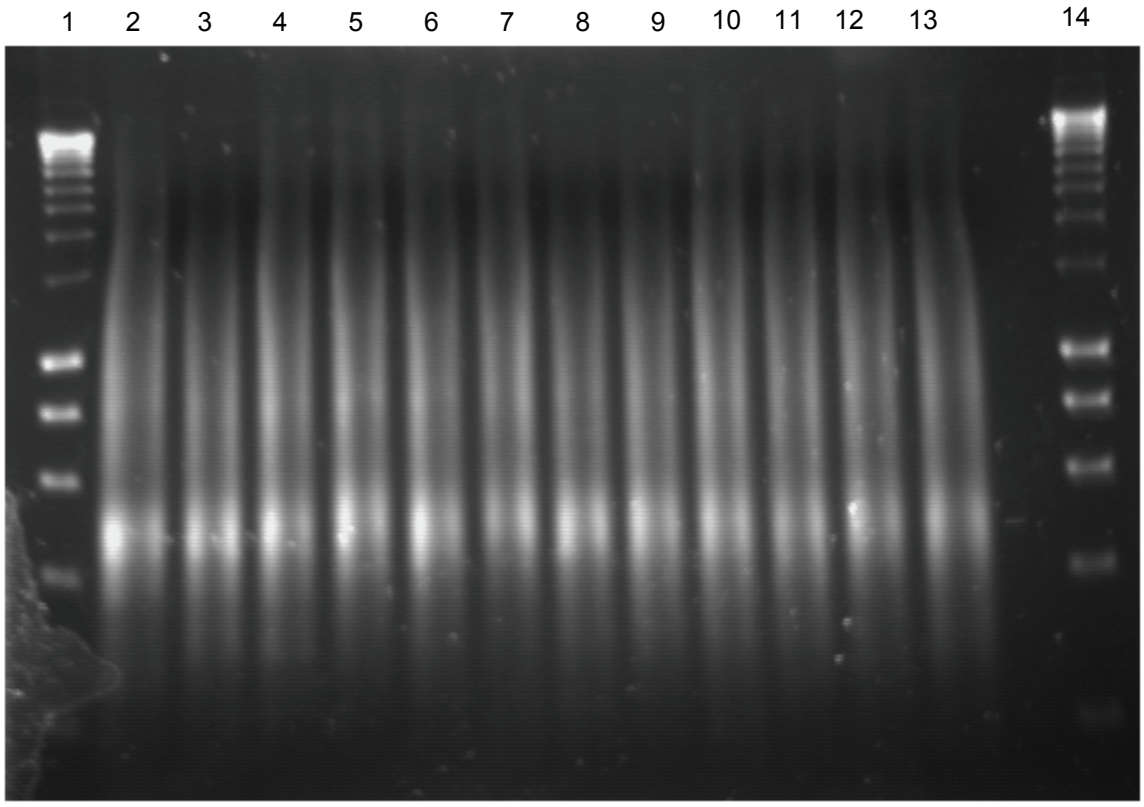


Figure 4: Agarose gel electrophoreses of PCR amplification of total extracted DNA from Riverlands on Feb 2007 with bacterial specific primer set ITSF (FAM) and ITSR. Lane 1 and 14: Hyper Ladder I, Lane 2-5: Riverlands plot1A - D, Lane 6-9: Riverlands plot A-D, Lane 10-13 Riverlands plot A-D PCR amplification.

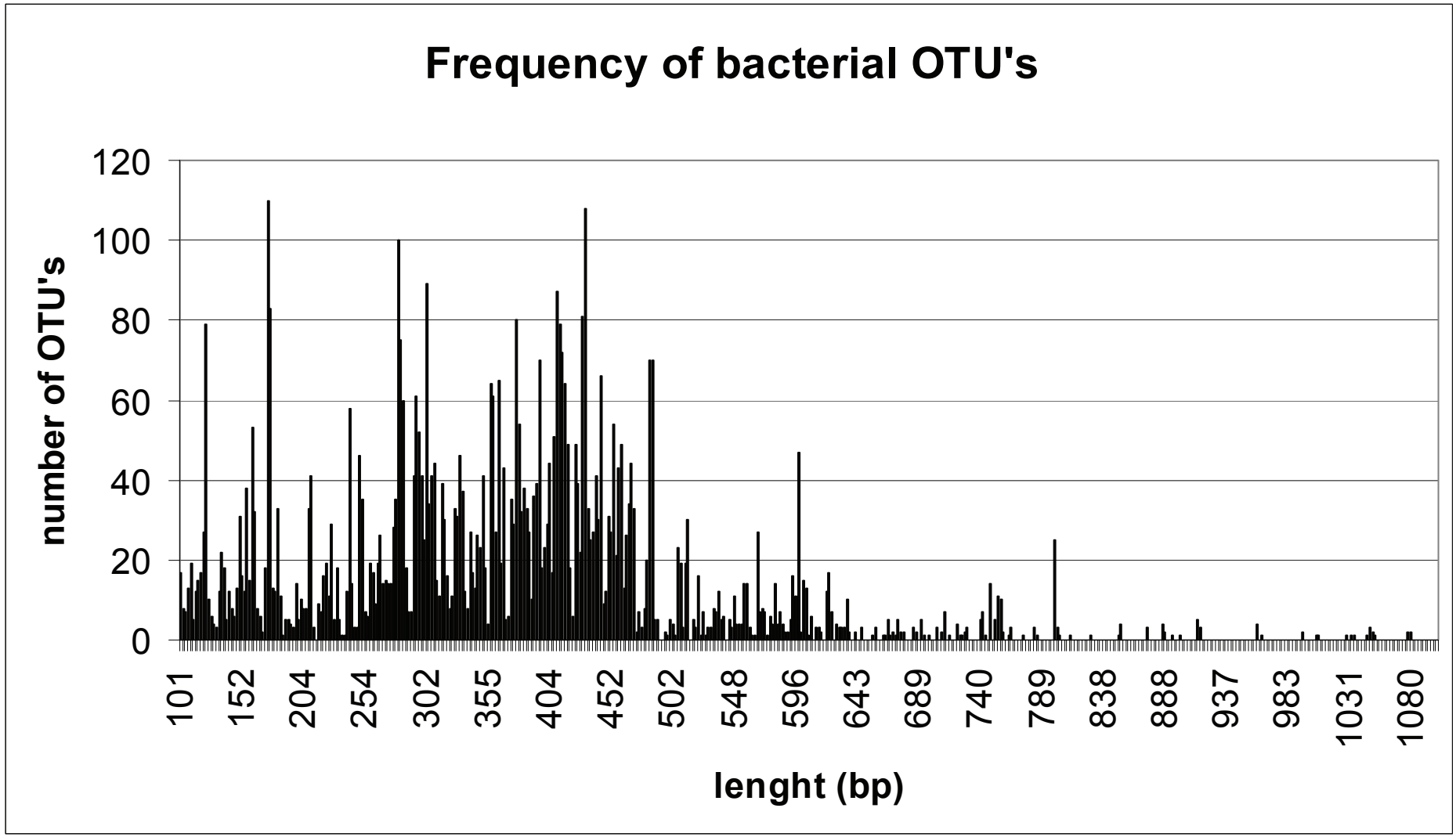
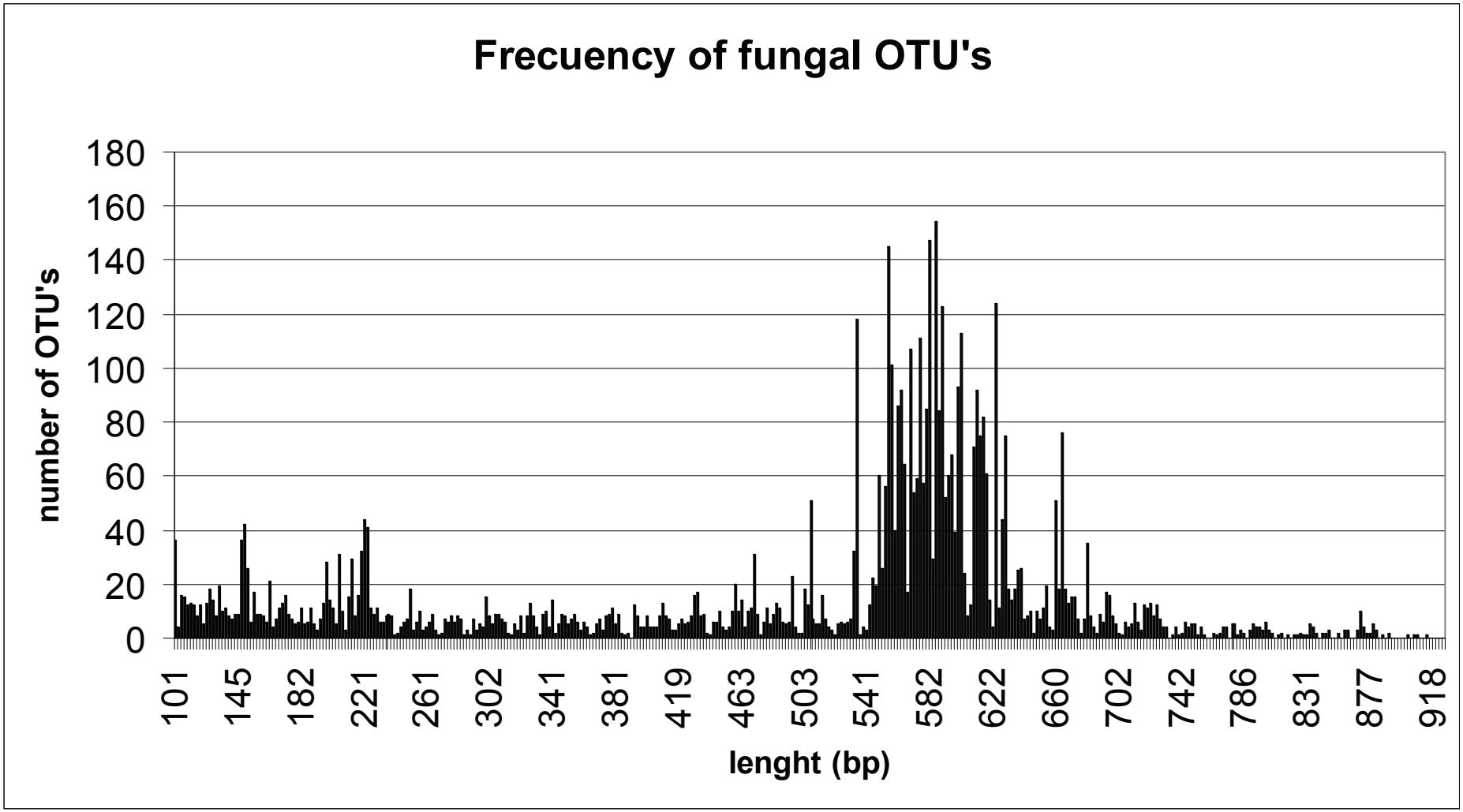


Figure 5: Summery of the number of bacterial operational taxonomic units detected of each size category.



. **Figure 6**: Summery of the number of fungal operational taxonomic units detected for each size category.

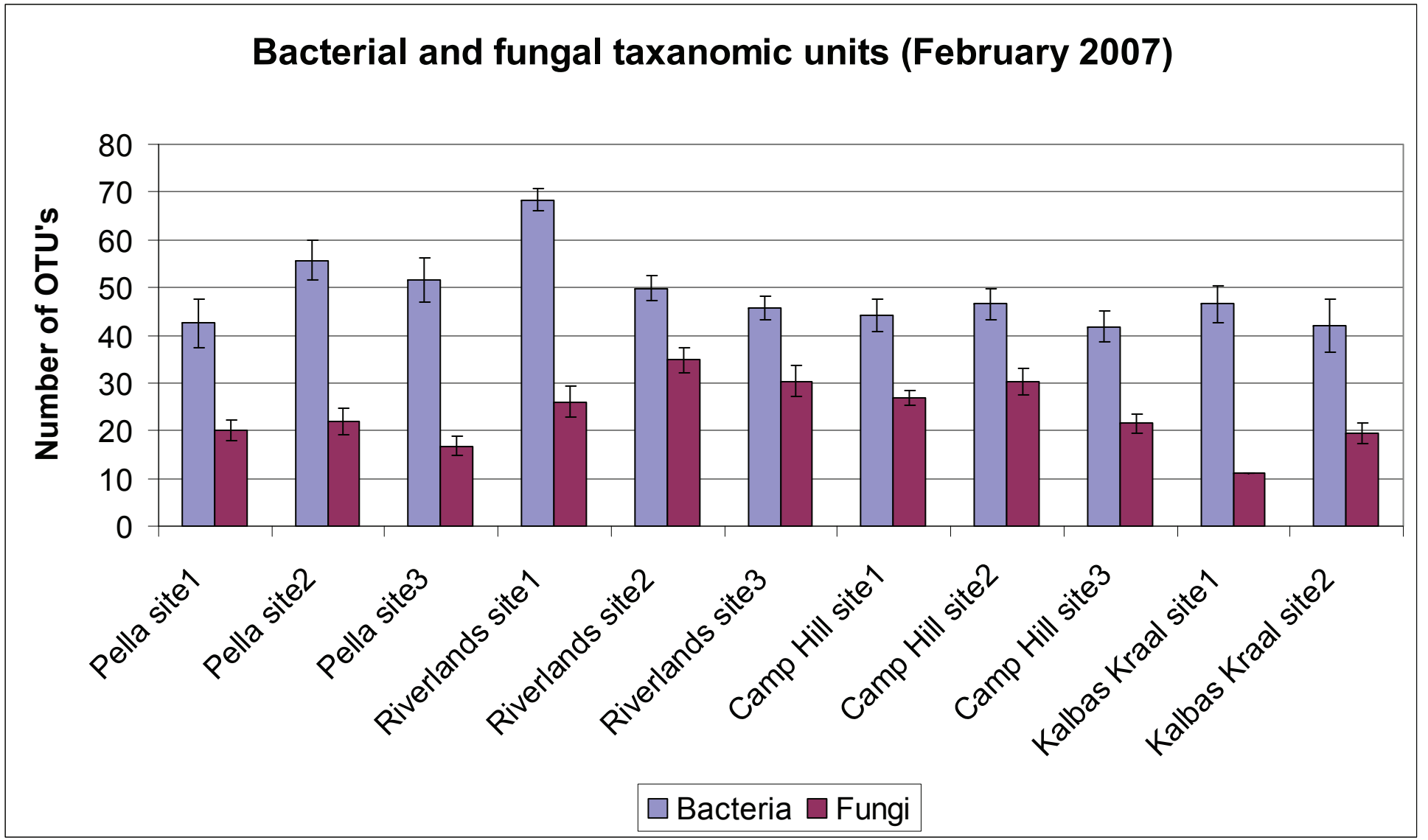


Figure 7a: The number of bacterial and fungal OTU's detected in each plot during February 2007.

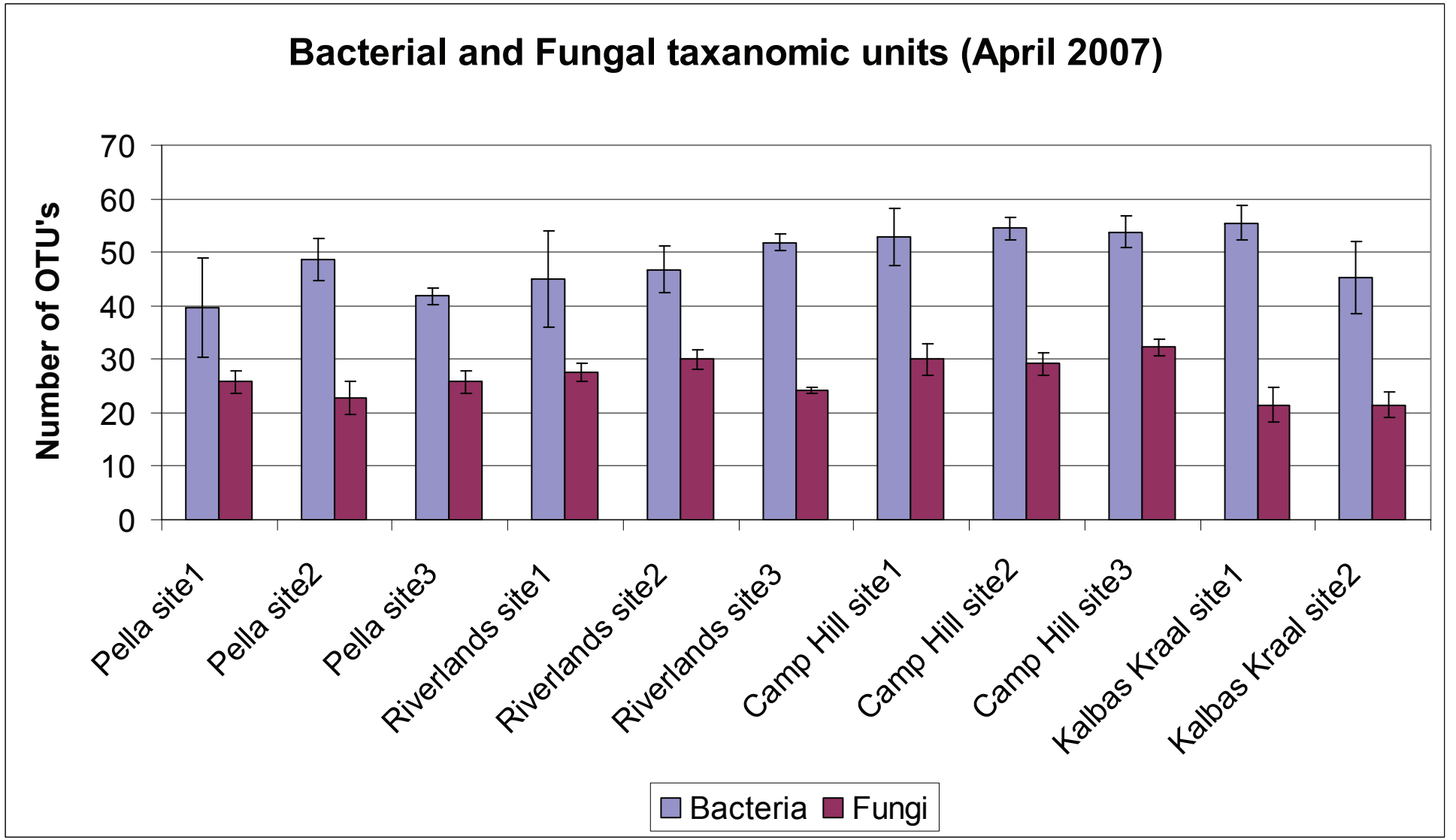


Figure 7b: The number of bacterial and fungal OTU's detected in each plot during April 2007.

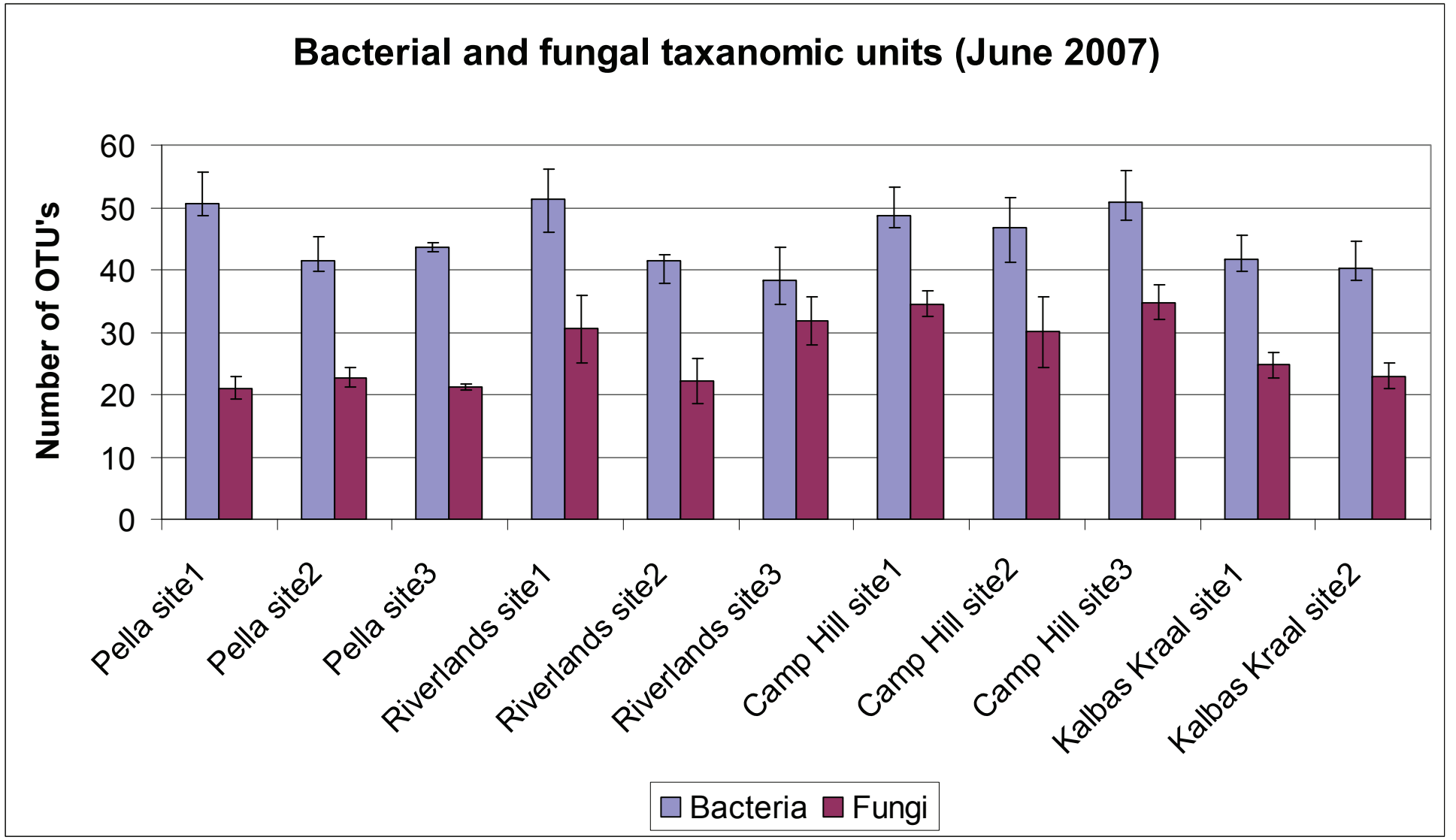


Figure 7c: The number of bacterial and fungal OTU's detected in each plot during June 2007

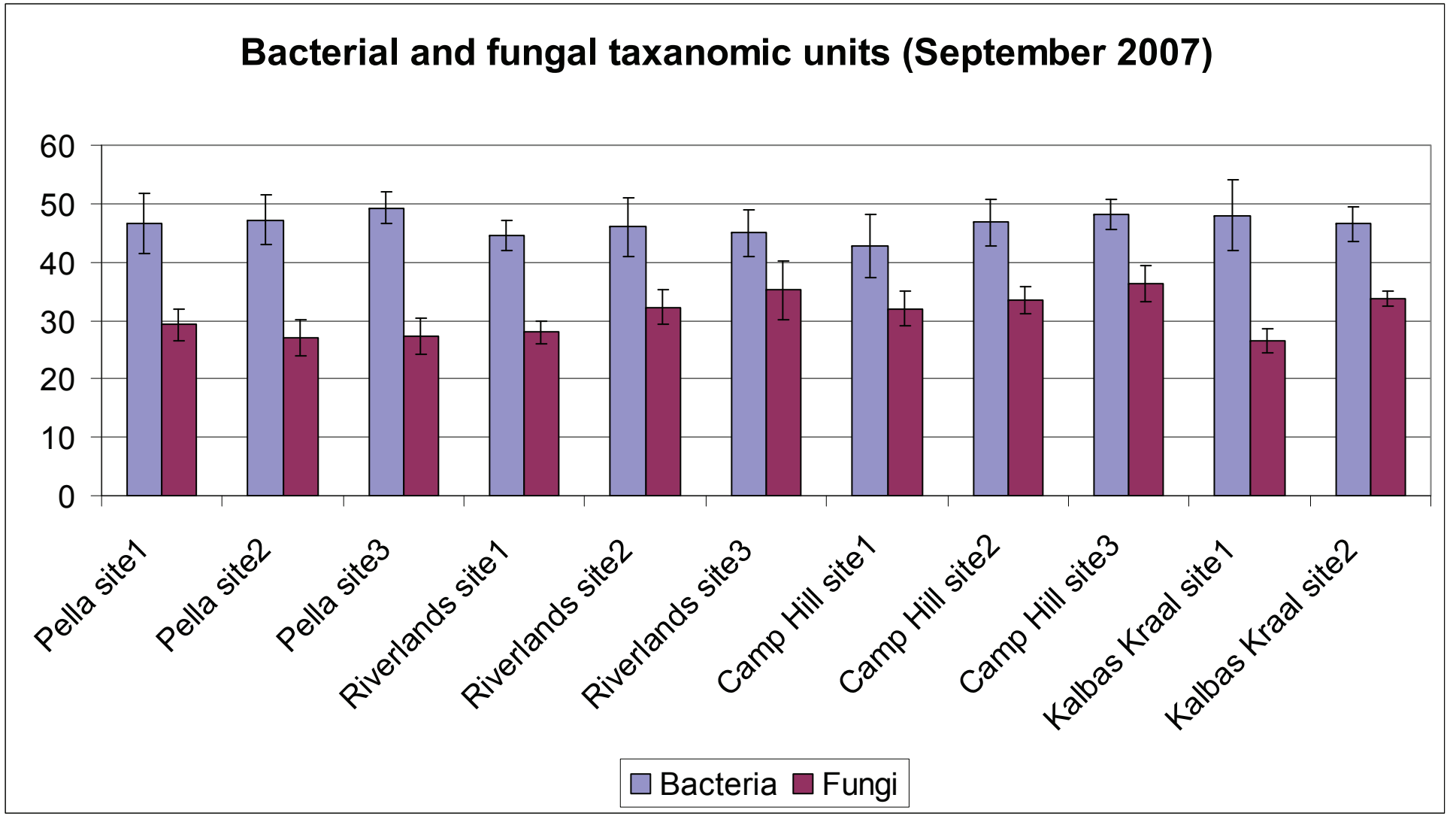


Figure 7d: The number of bacterial and fungal OTU's detected in each plot during September 2007.

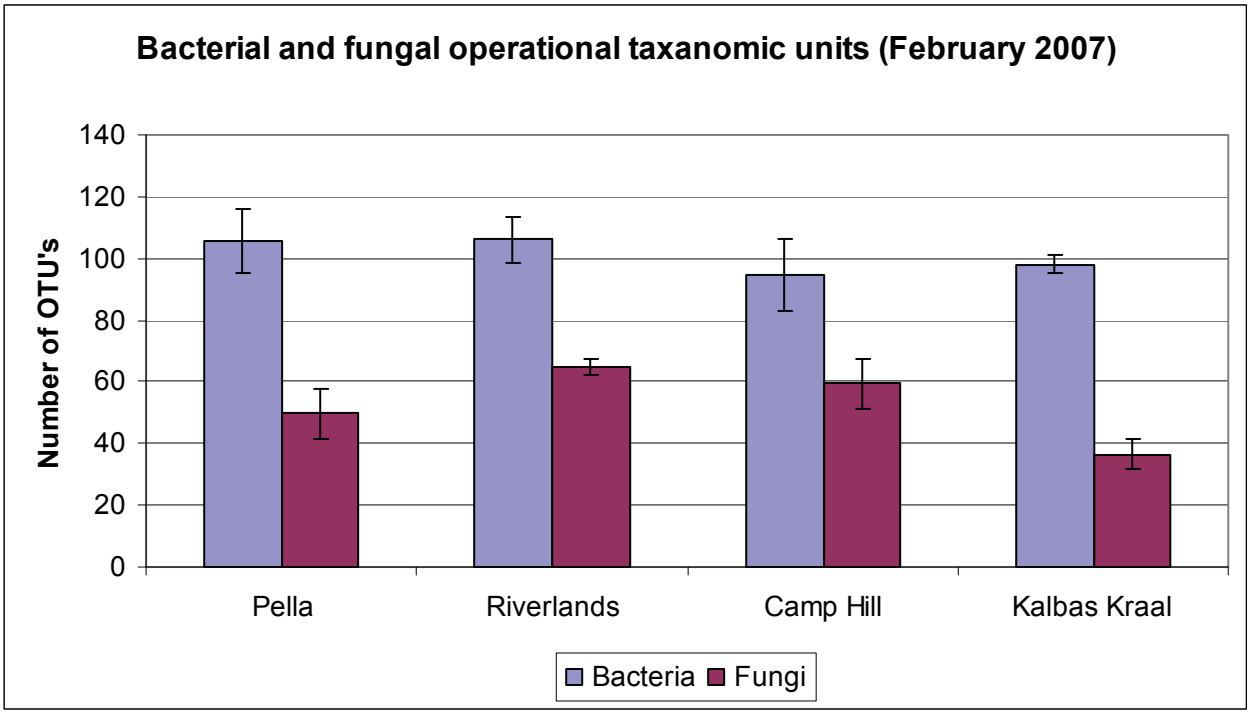


Figure 8a: The mean number of fungal and bacterial operational taxonomic units detected at each site during February 2007.

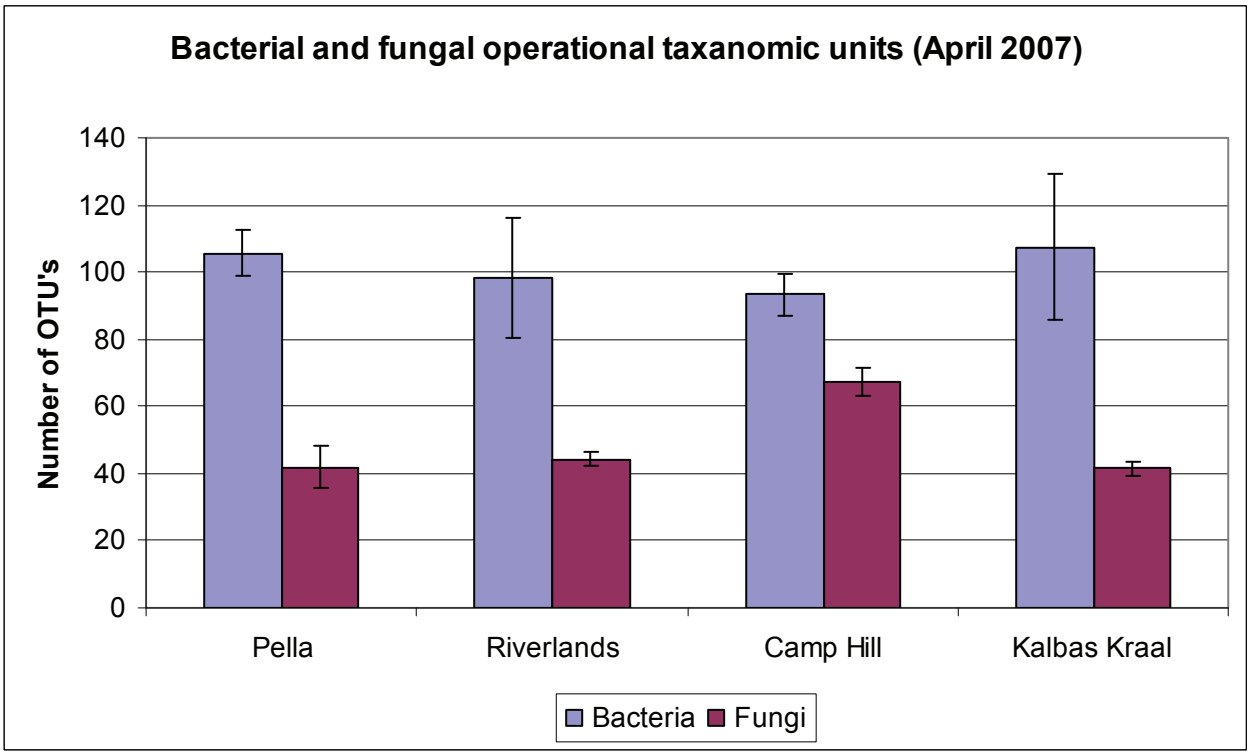


Figure 8b: The mean number of fungal and bacterial operational taxonomic units detected at each site during April 2007.

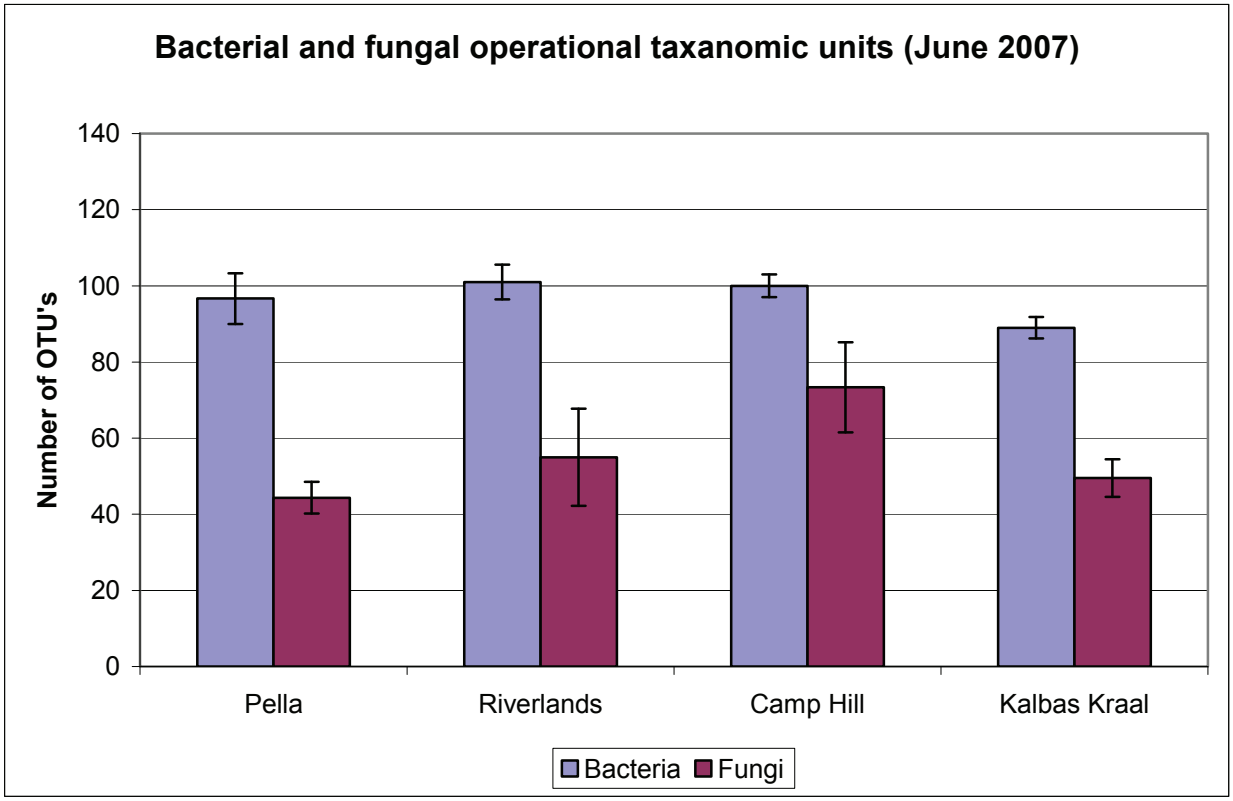


Figure 8c: The mean number of fungal and bacterial operational taxonomic units detected at each site during June 2007.

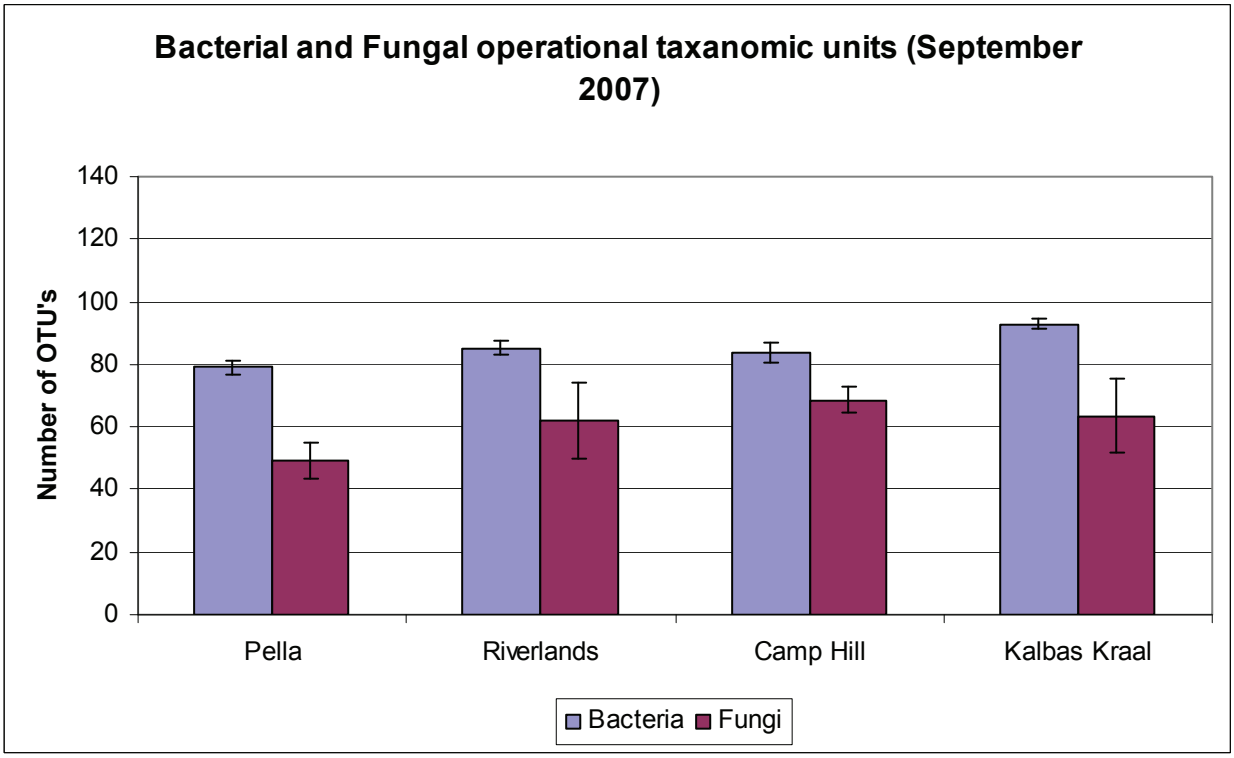


Figure 8d: The mean number of fungal and bacterial operational taxonomic units detected at each site during September 2007.

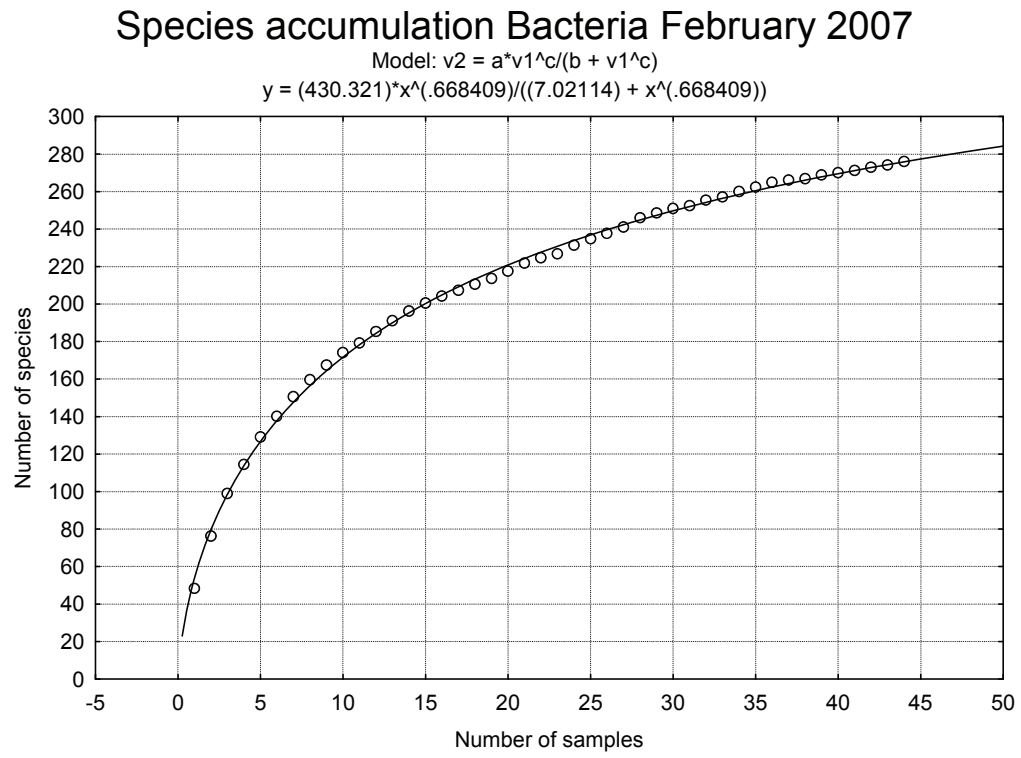


Figure 9a: The species accumulation curves of bacterial operational taxonomic units for February 2007.

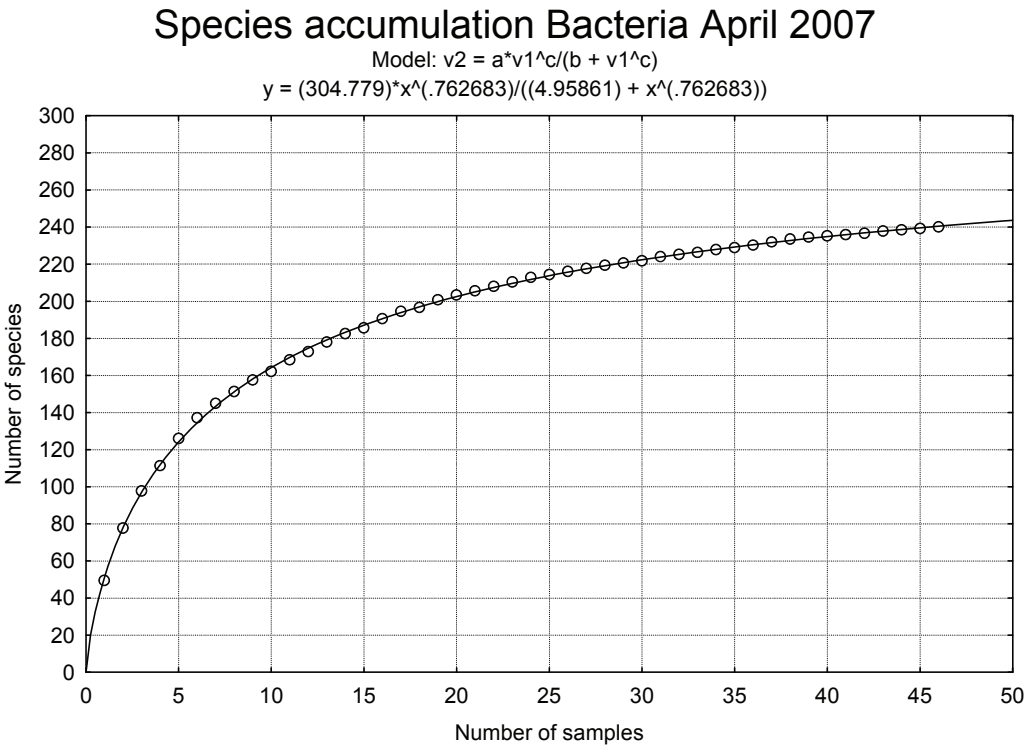


Figure 9b: The species accumulation curves of bacterial operational taxonomic units for April 2007.

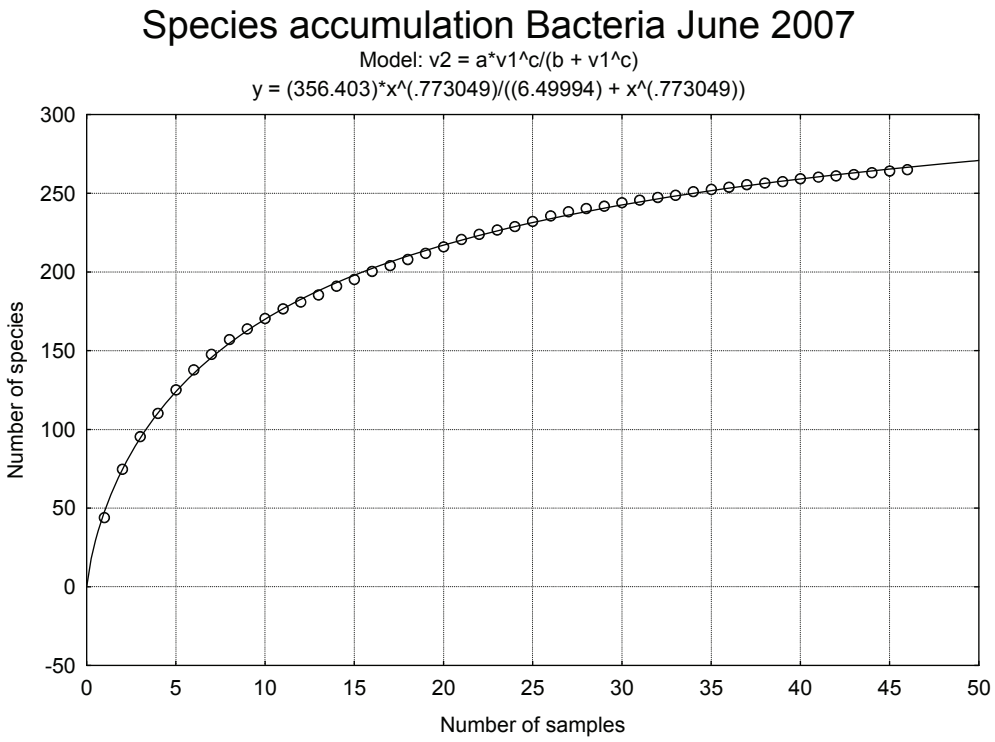


Figure 9c: The species accumulation curves of bacterial operational taxonomic units for June 2007.

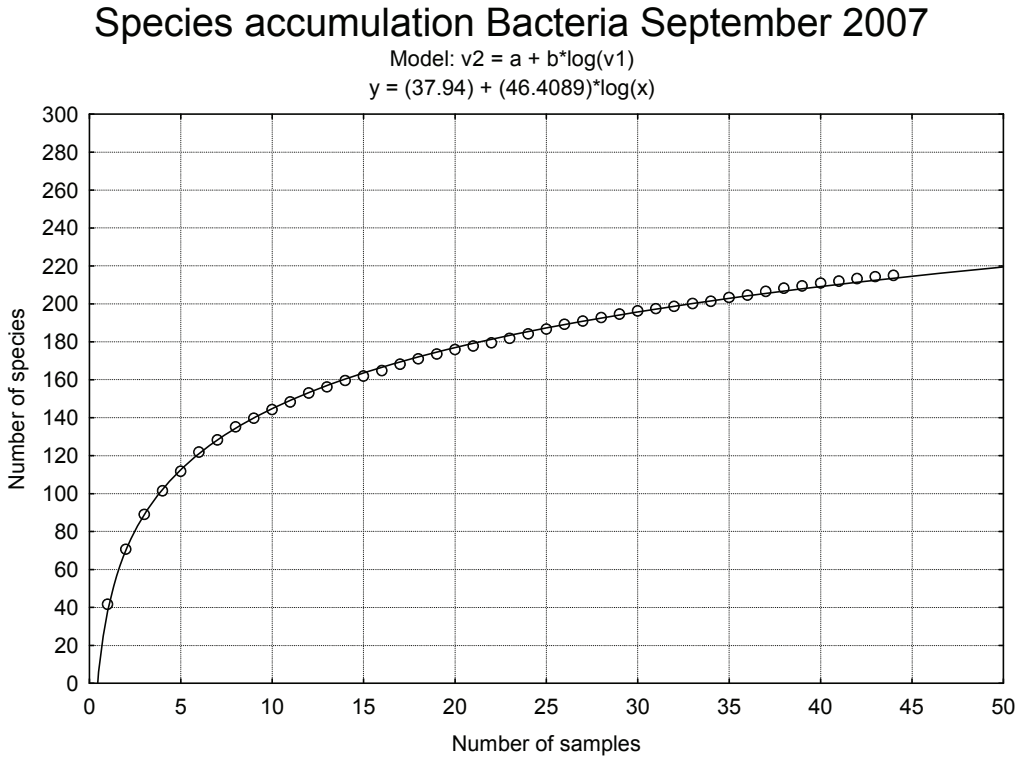


Figure 9d: The species accumulation curves of bacterial operational taxonomic units for September 2007.

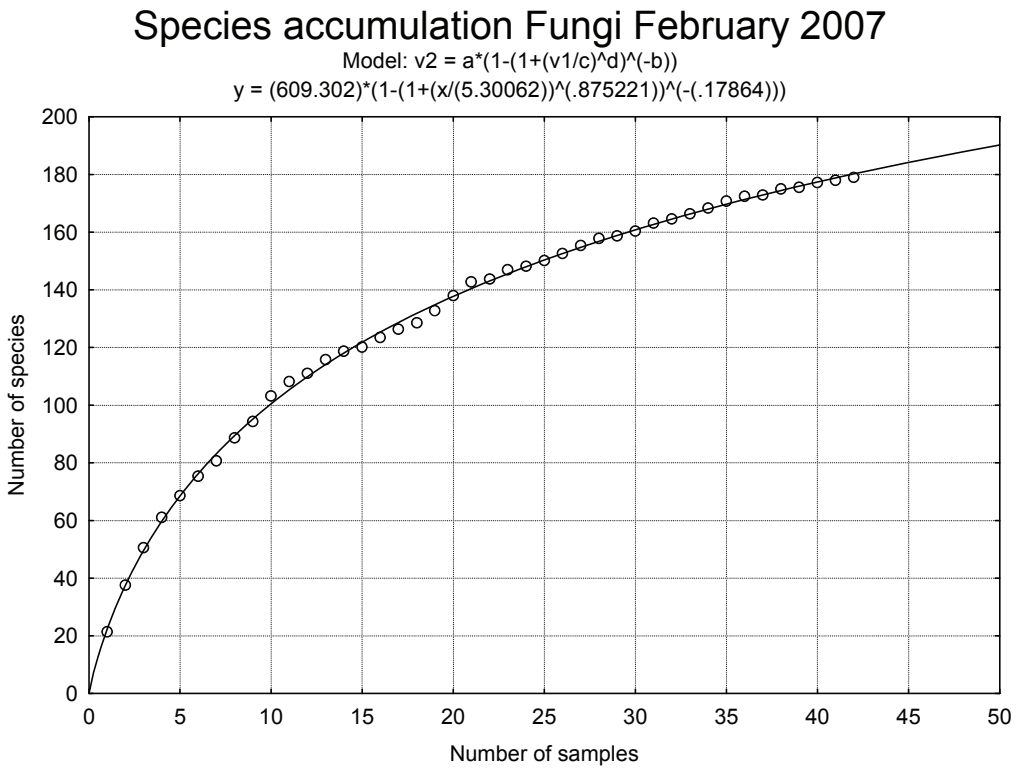


Figure 10a: The accumulation curves of bacterial operational taxonomic units for February 2007.

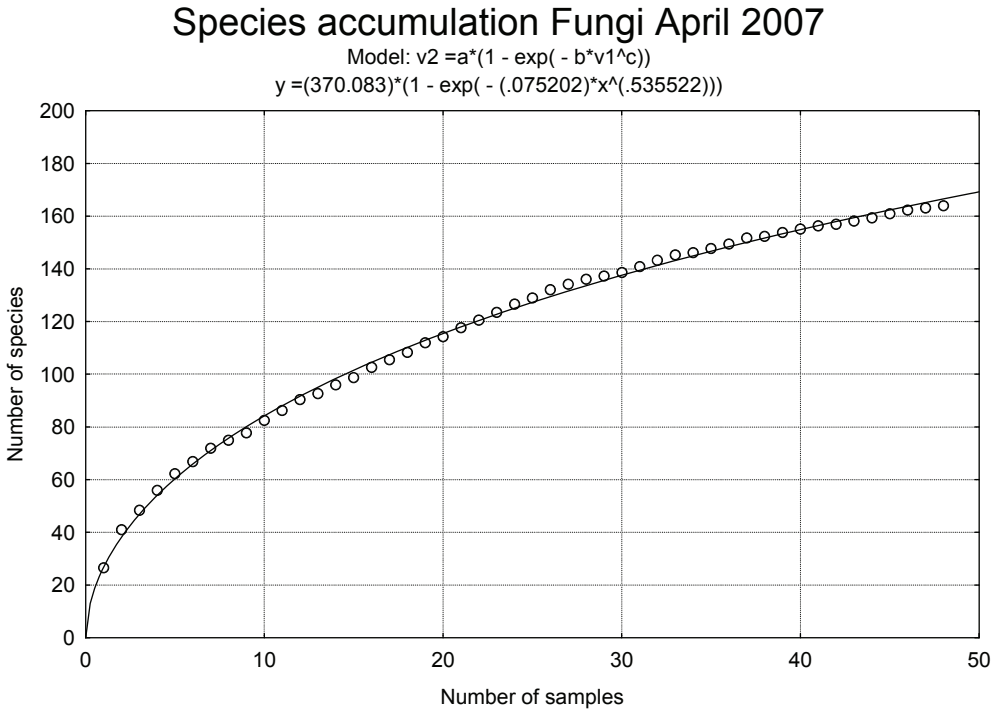


Figure 10b: The accumulation curves of bacterial operational taxonomic units for April 2007.

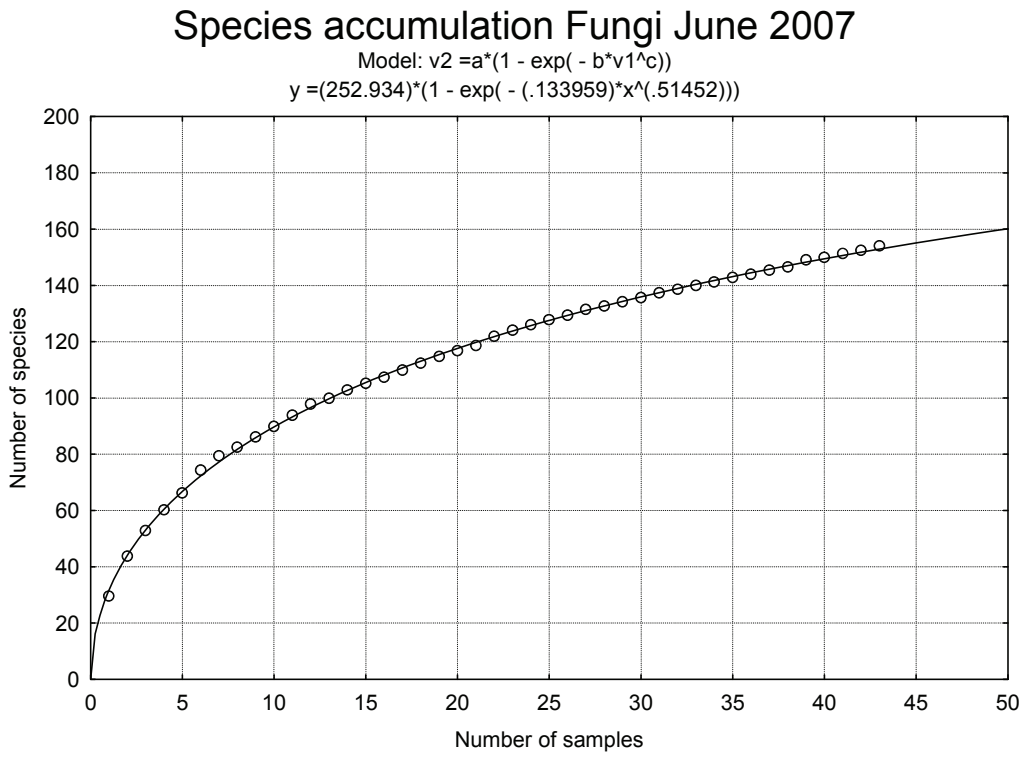


Figure 10c: The accumulation curves of bacterial operational taxonomic units for June 2007.

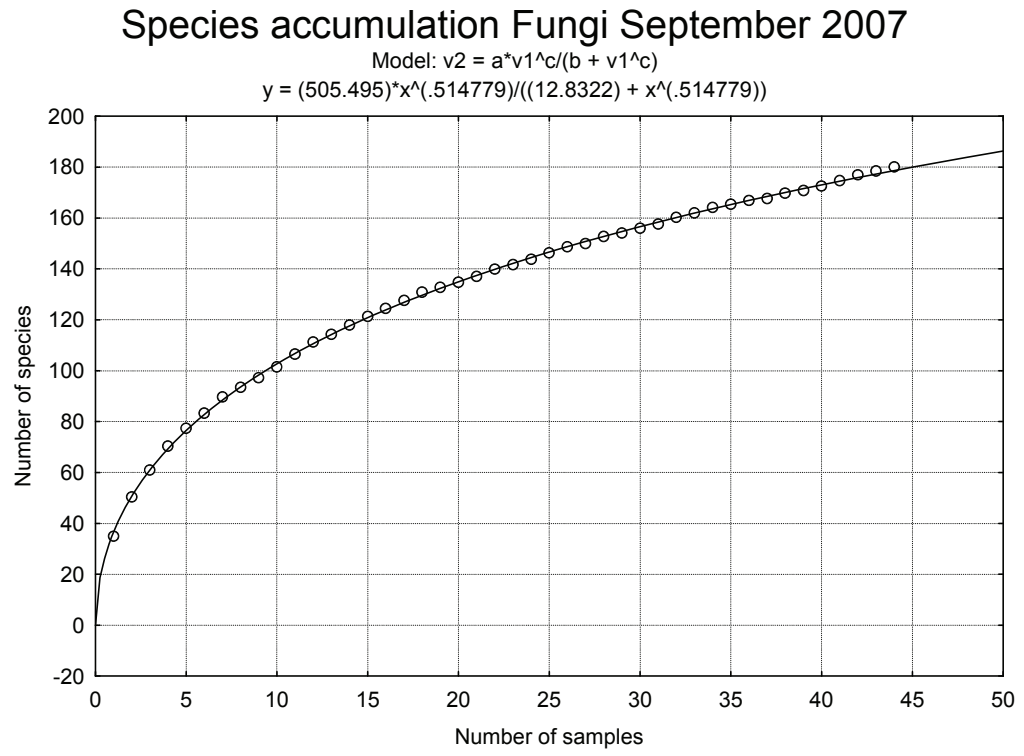


Figure 10d: The accumulation curves of bacterial operational taxonomic units for September 2007.

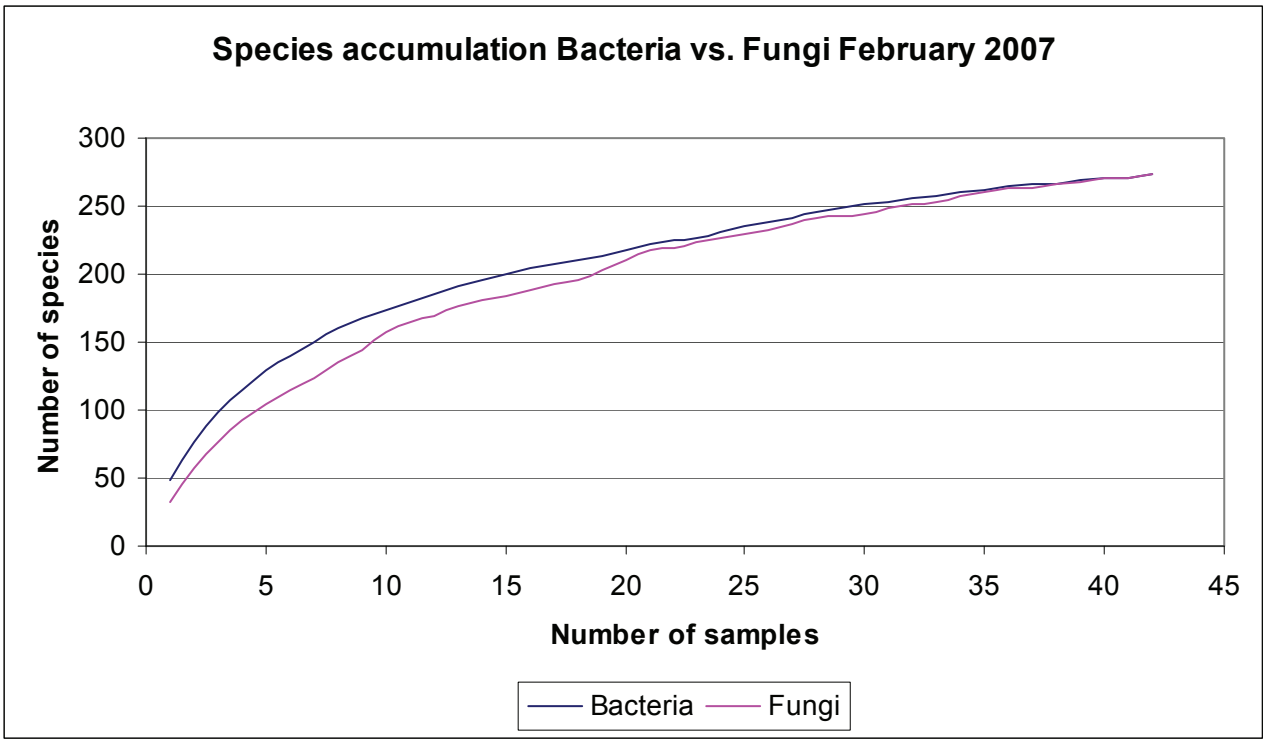


Figure 11a: The refraction curves of fungal and bacterial OTU's for February 2007.

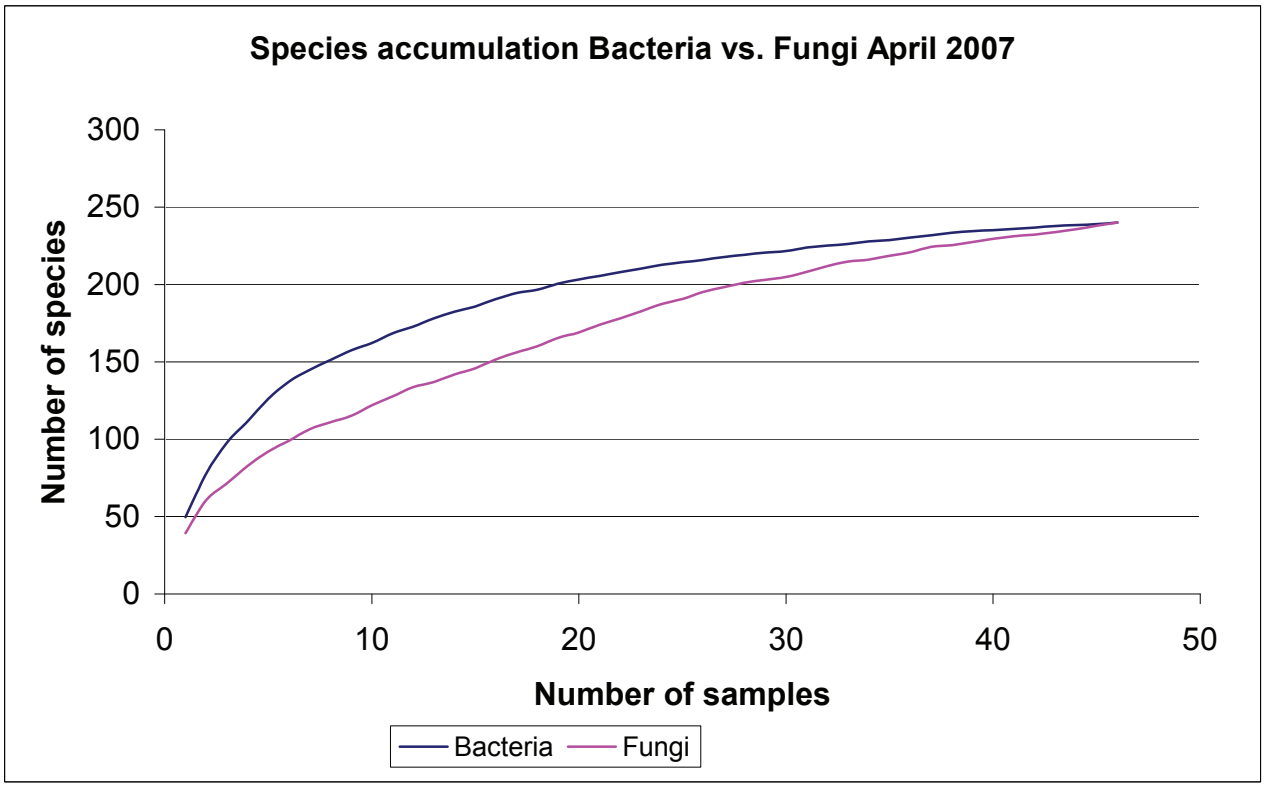


Figure 11b: The refraction curves of fungal and bacterial OTU's for April 2007.

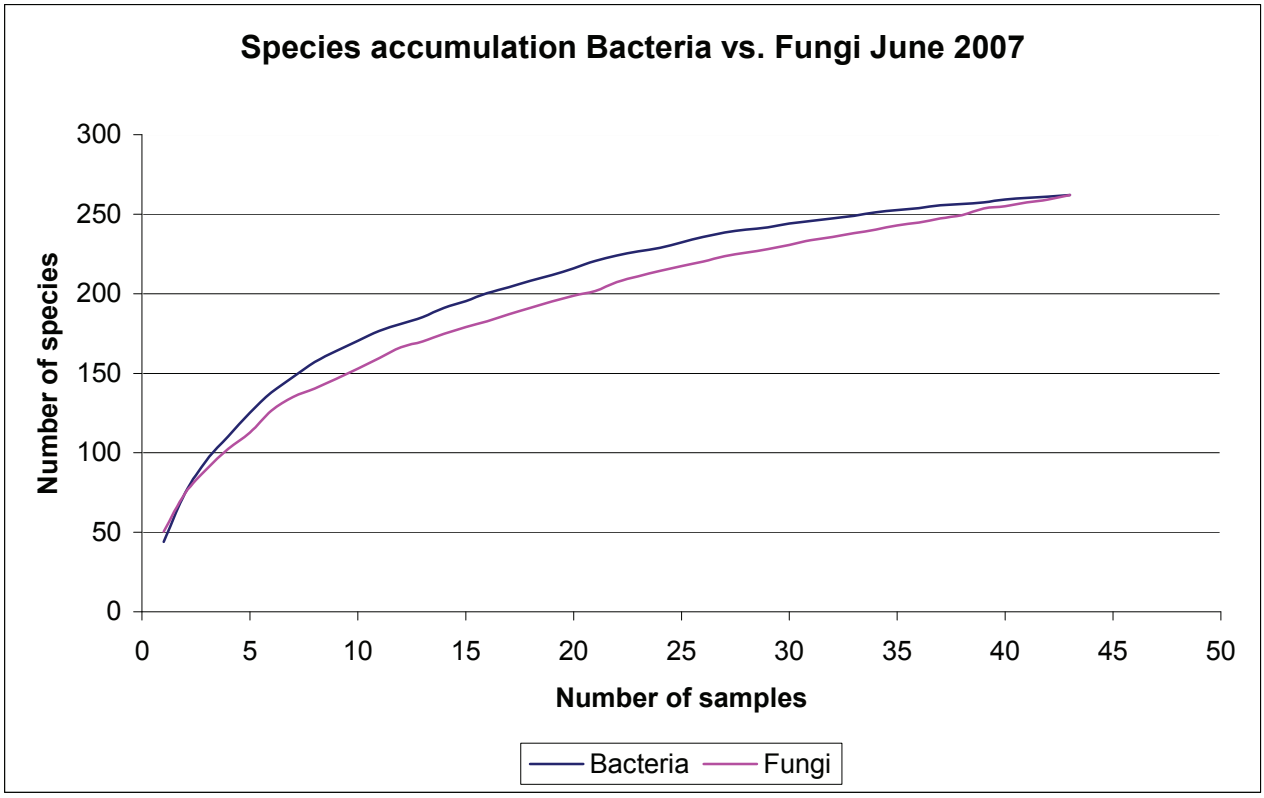


Figure 11c: The refraction curves of fungal and bacterial OTU's for June 2007.

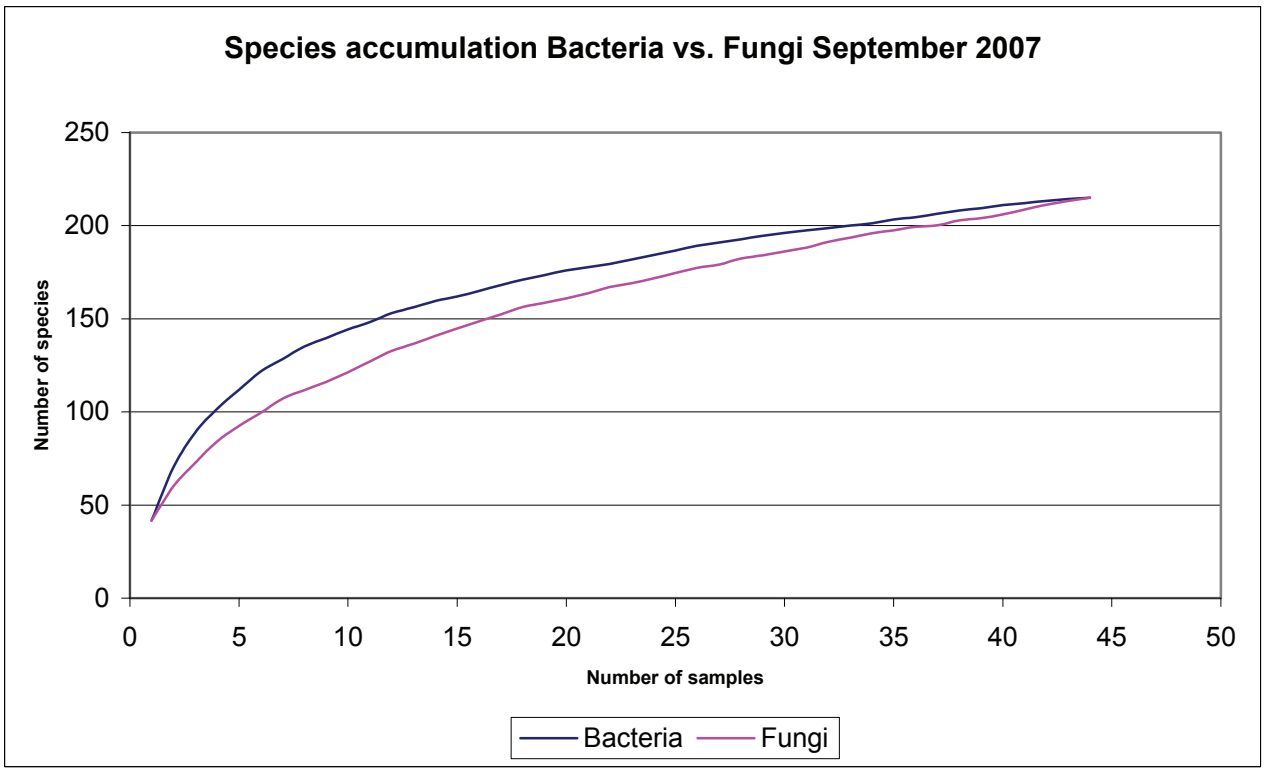
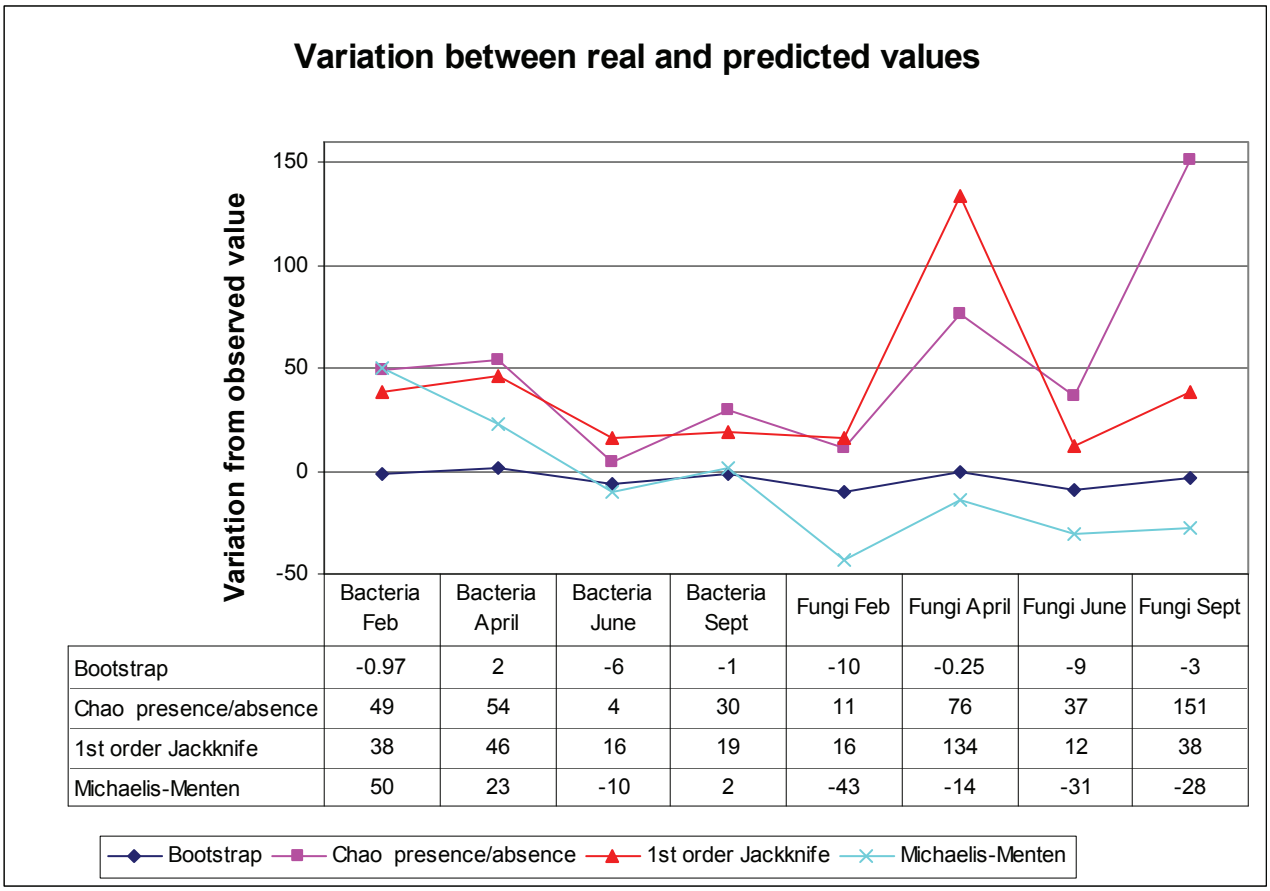


Figure 11d: The refraction curves of fungal and bacterial OTU's for September 2007.



	Bacteria Feb	Bacteria April	Bacteria June	Bacteria Sept	Fungi Feb	Fungi April	Fungi June	Fungi Sept
Bootstrap	-0.97	2	-6	-1	-10	-0.25	-9	-3
Chao presence/absence	49	54	4	30	11	76	37	151
1st order Jackknife	38	46	16	19	16	134	12	38
Michaelis-Menten	50	23	-10	2	-43	-14	-31	-28

Figure 12: Performance of the Bootstrap, Chao presence/absence, 1ste Order Jackknife and Michaelis-Menten estimation models based on the variation observed between observed and predicted values.

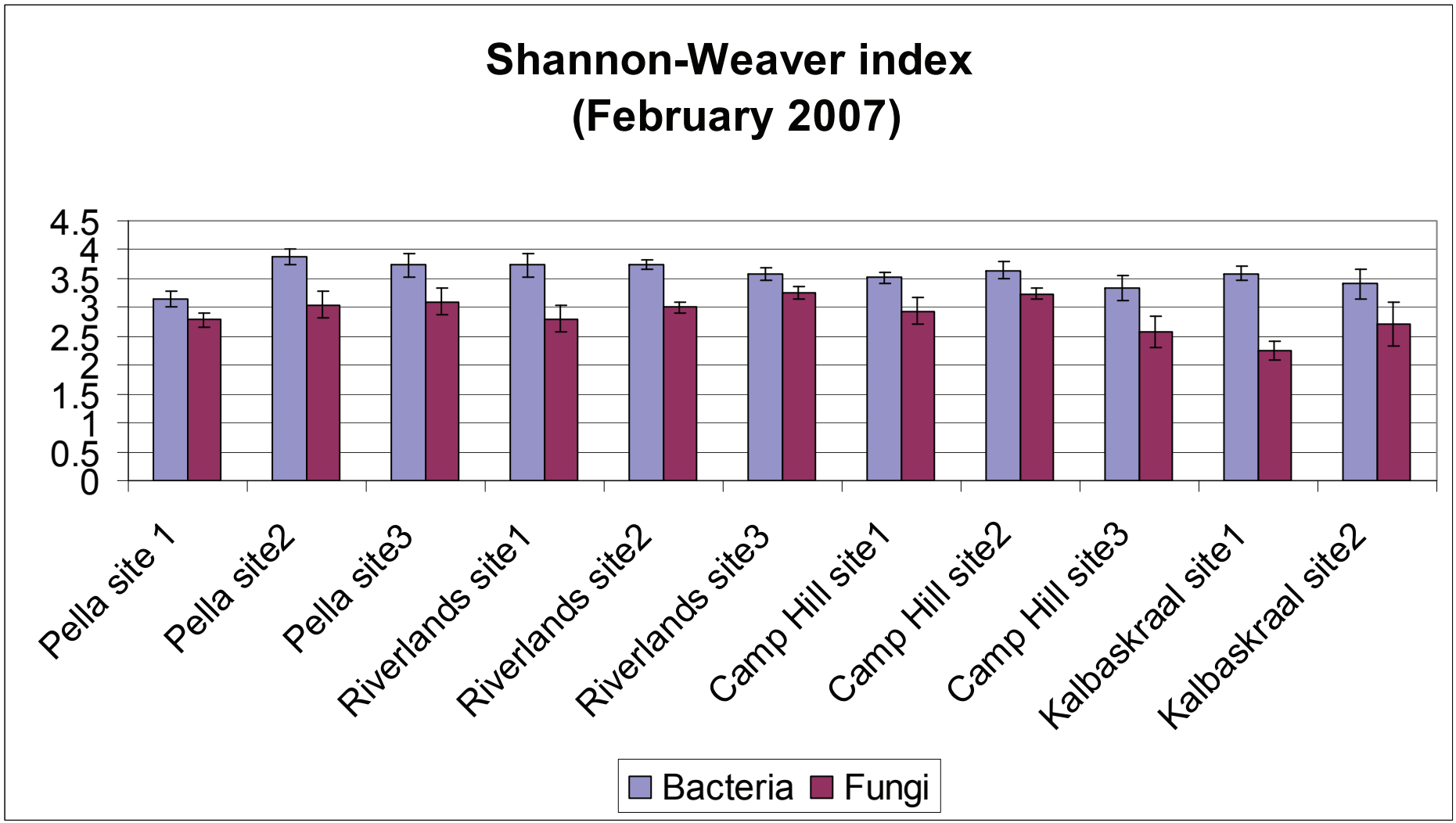


Figure 13a: Comparative Shannon-Weaver diversity between fungi and bacteria for February 2007

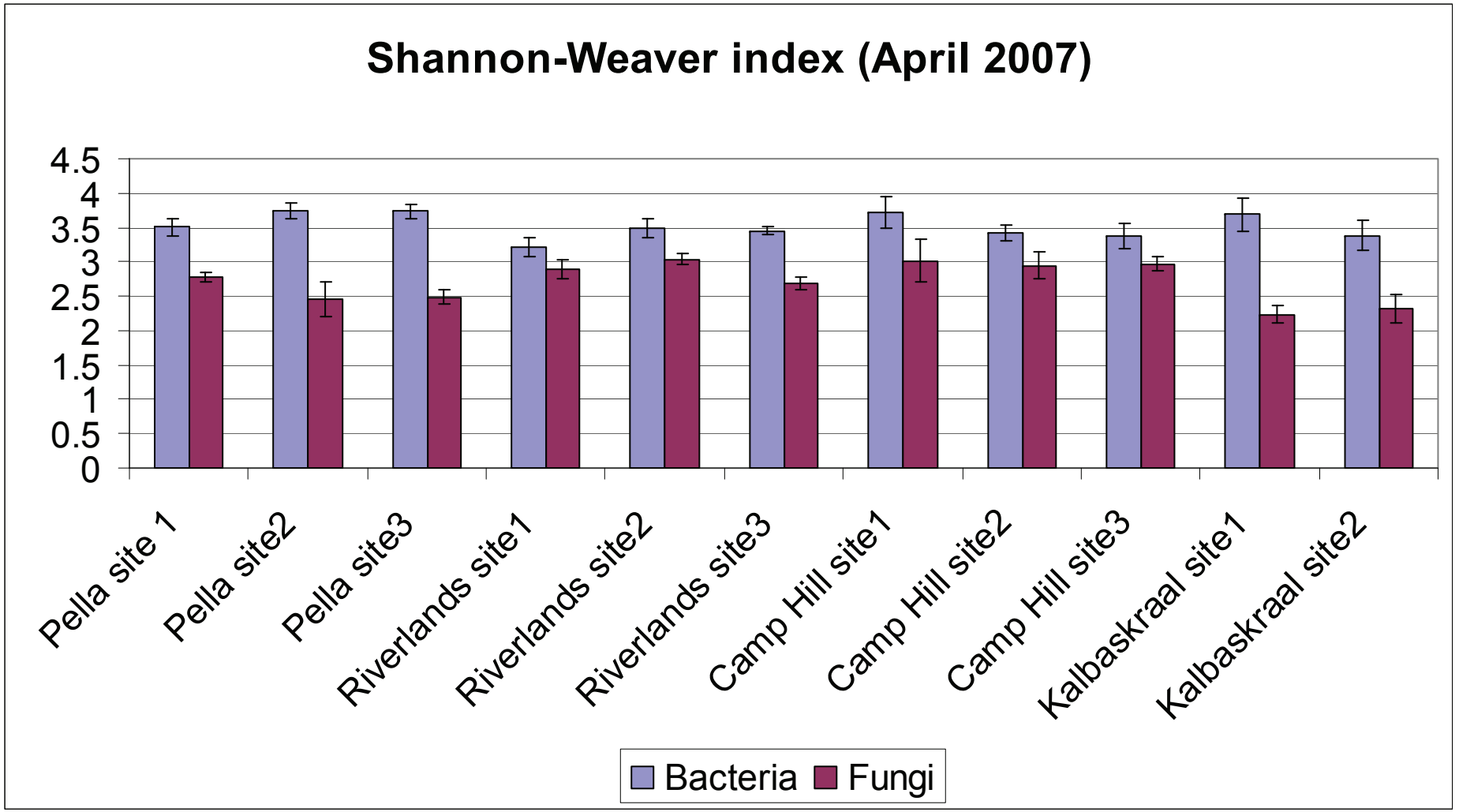


Figure 13b: Comparative Shannon-Weaver diversity between fungi and bacteria for April 2007.

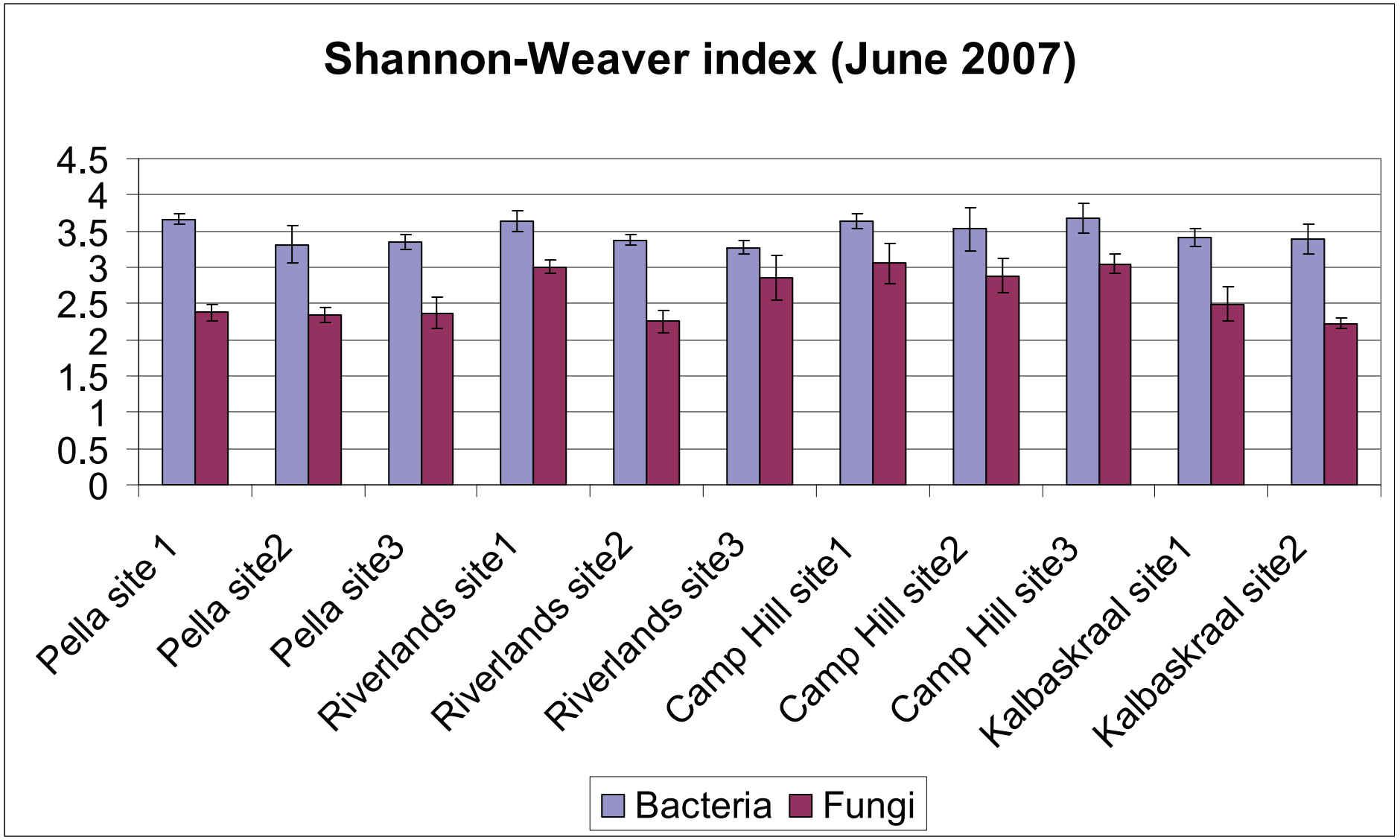


Figure 13c: Comparative Shannon-Weaver diversity between fungi and bacteria for June 2007.

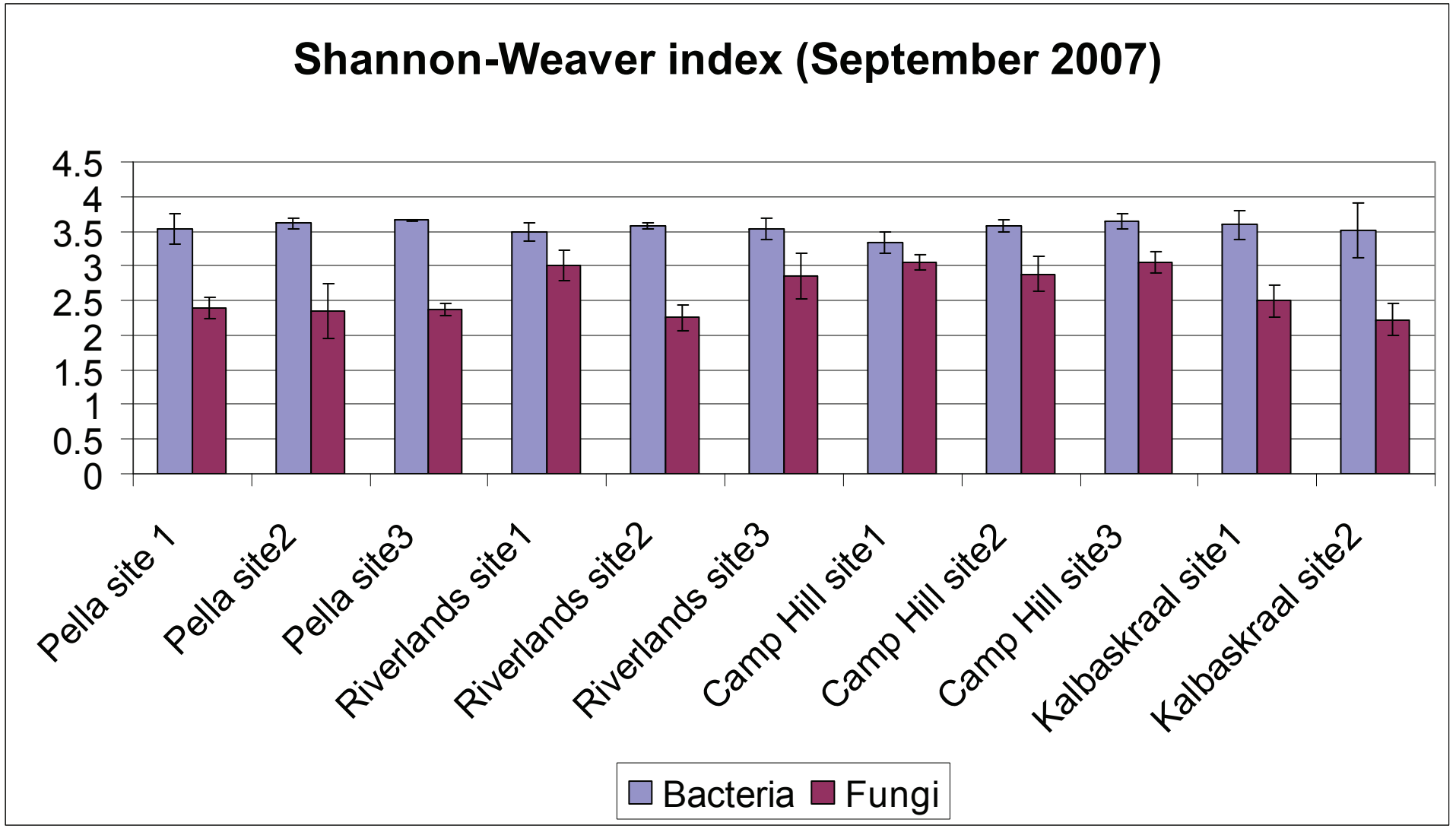


Figure 13d: Comparative Shannon-Weaver diversity between fungi and bacteria for September 2007

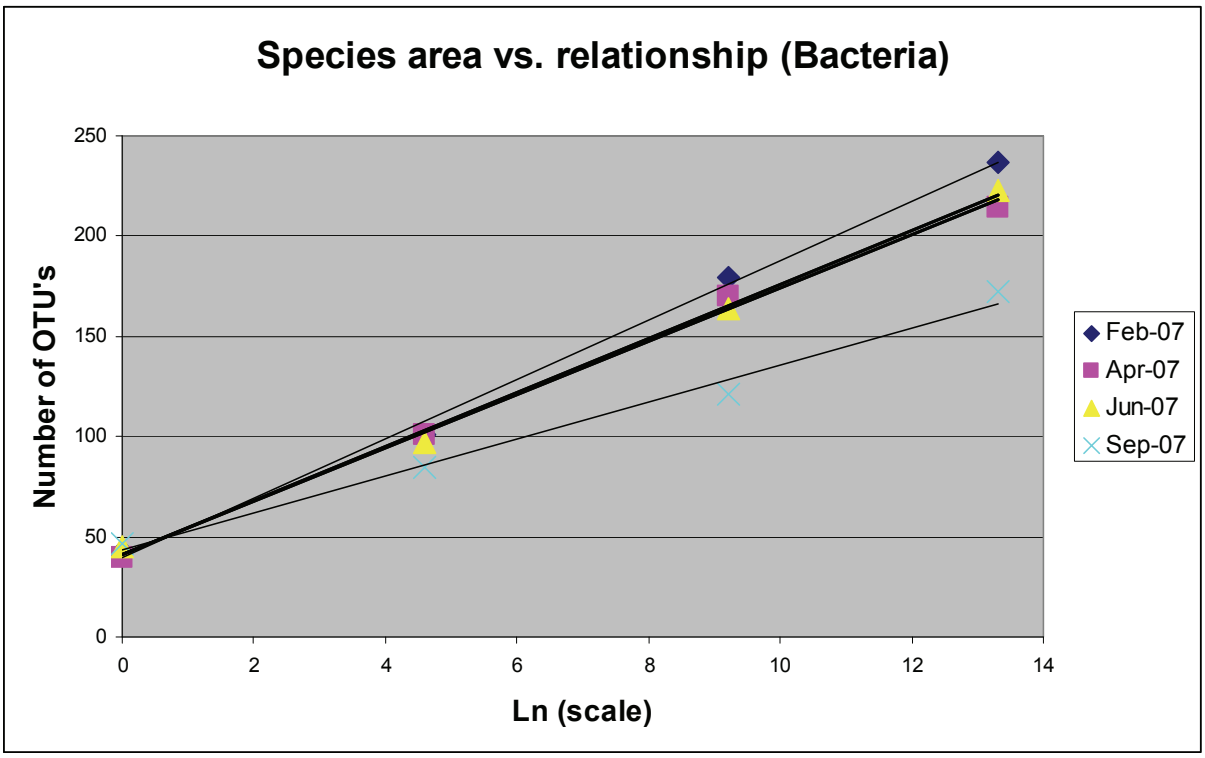


Figure 14: Species area relationship for bacterial species observed during each sampling.

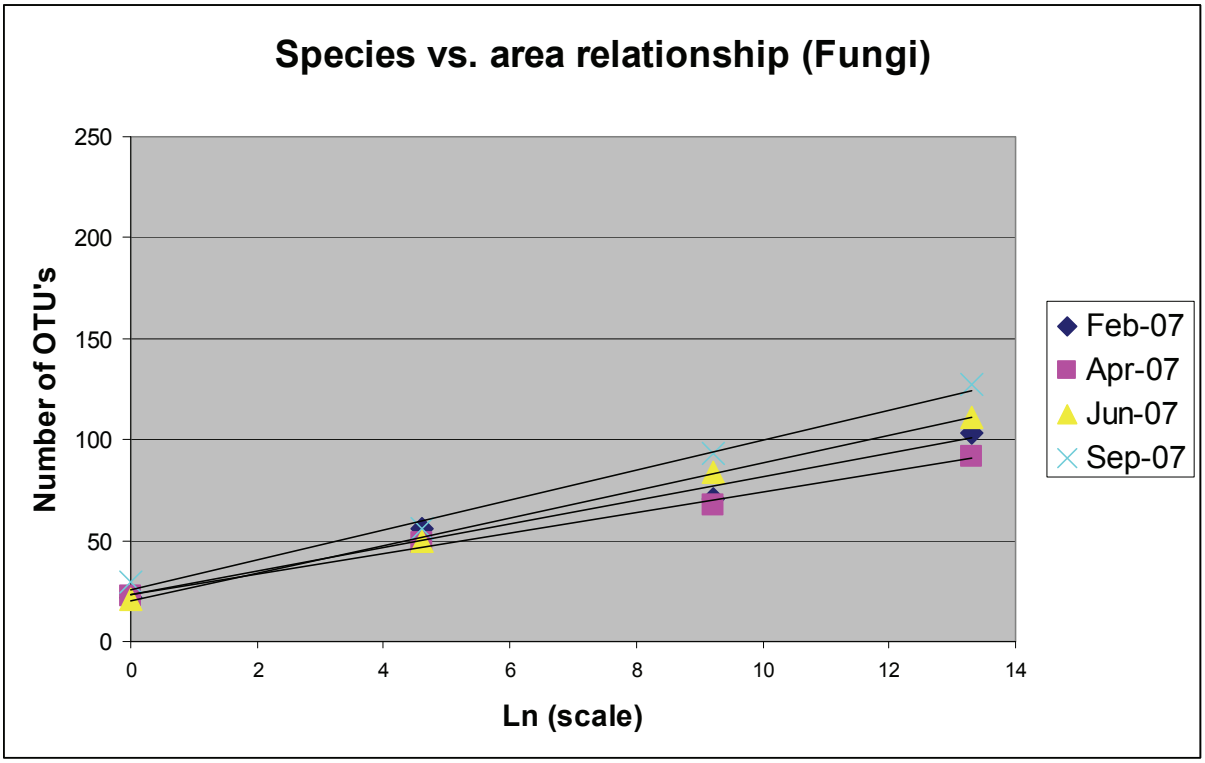


Figure 15: Species area relationship for fungi species observed during each sampling.

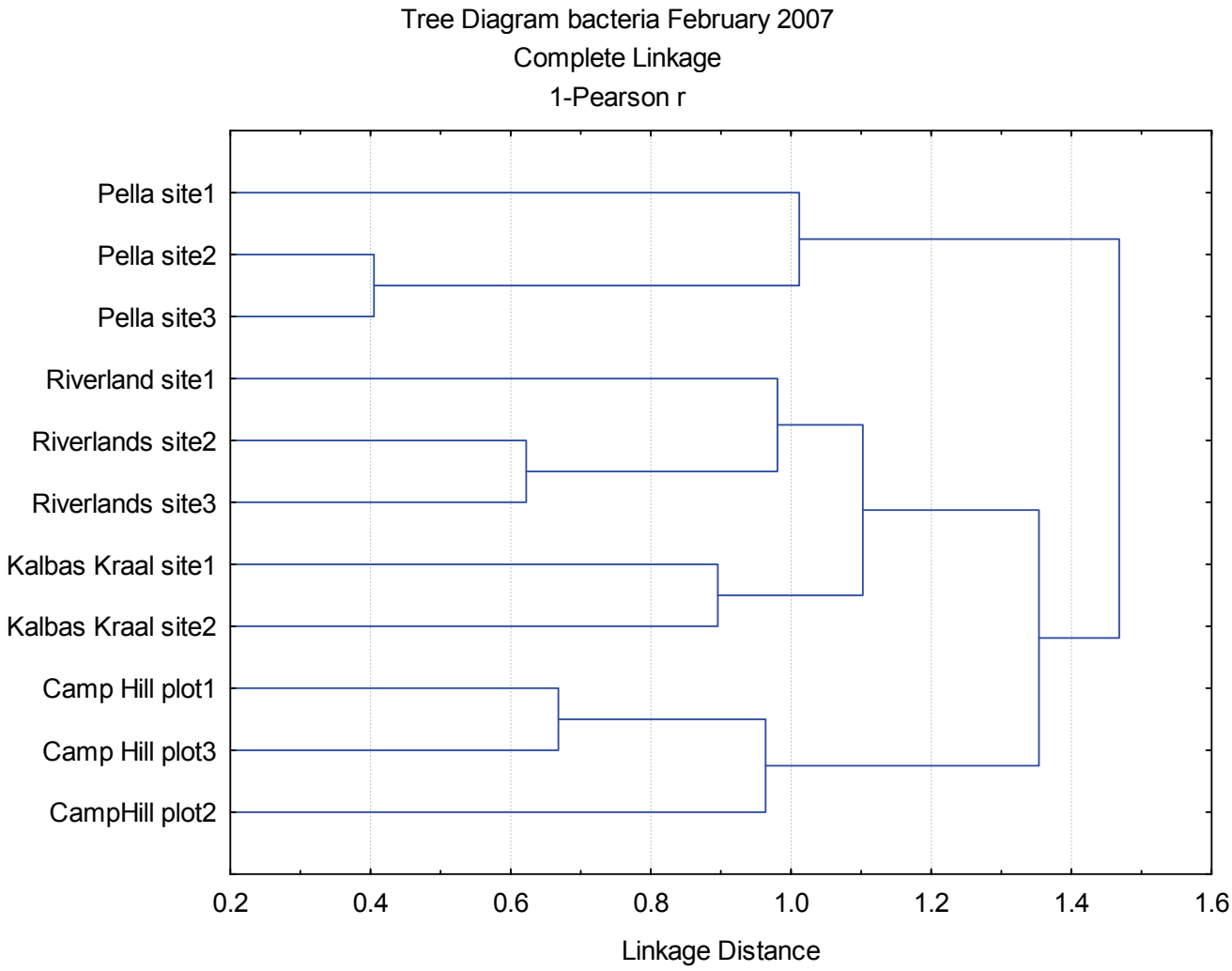


Figure 16a: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the bacterial OTU's during February 2007.

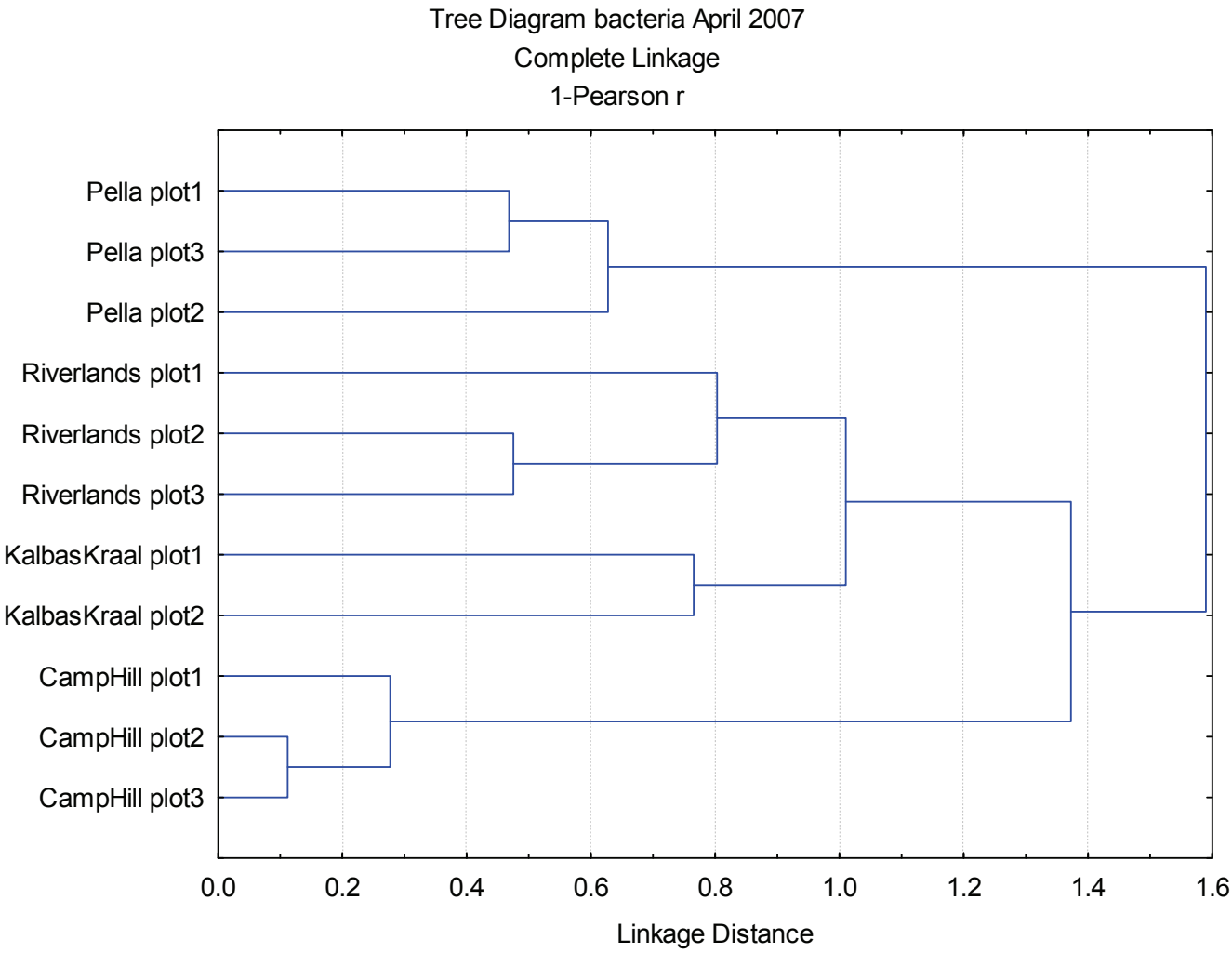


Figure 16b: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the bacterial OTU's during April 2007.

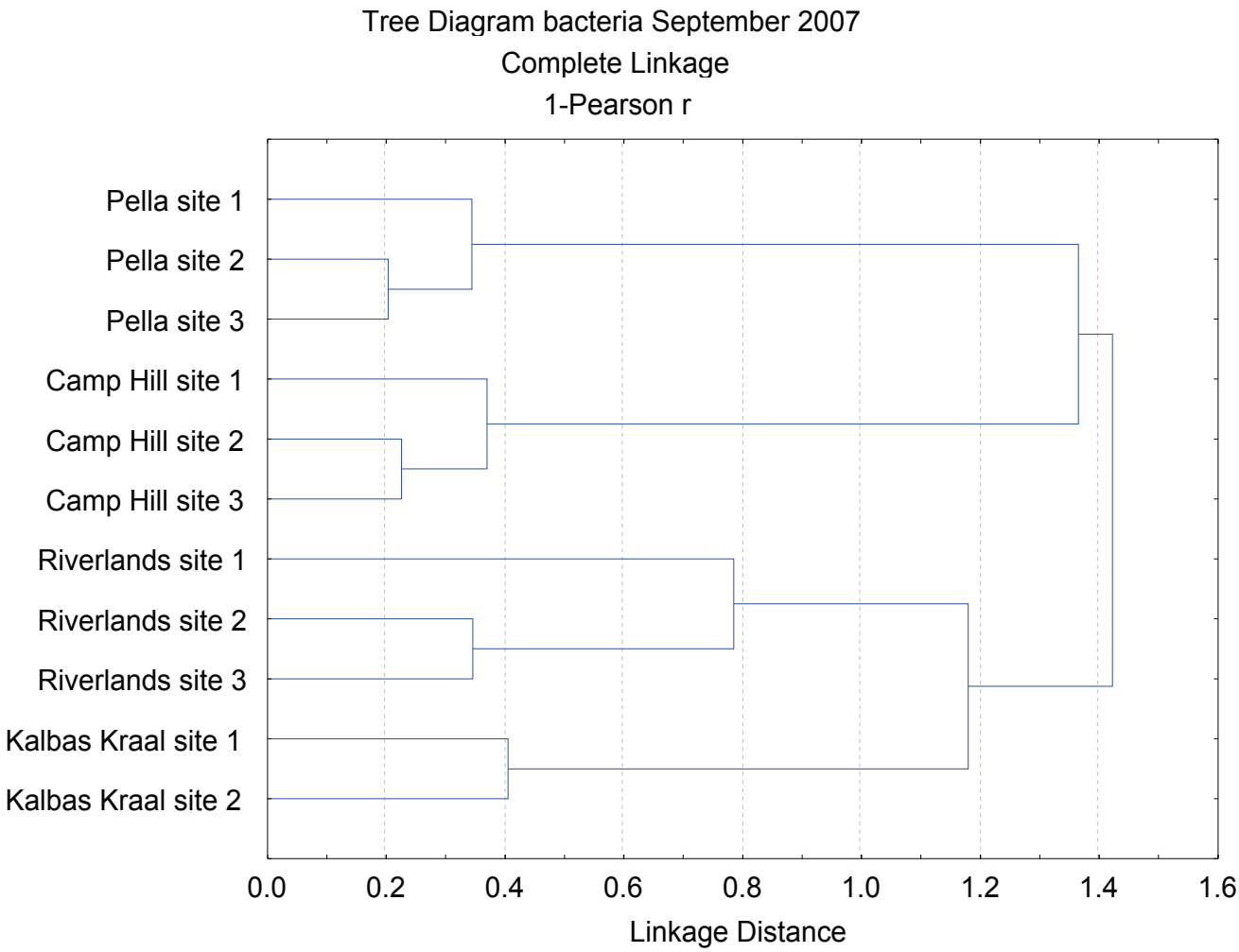


Figure 16c: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the bacterial OTU's during June 2007.

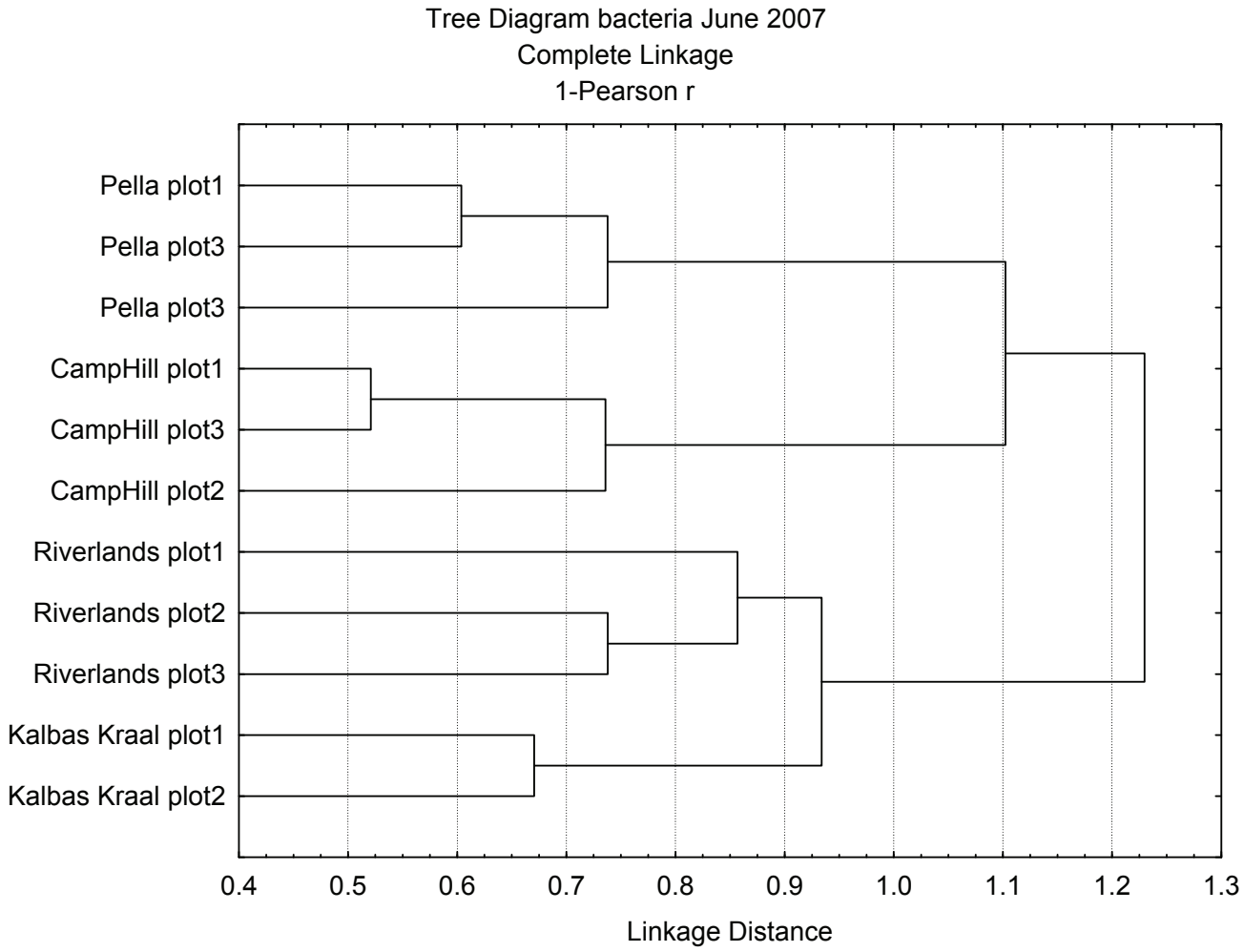


Figure 16d: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the bacterial OTU's during September 2007.

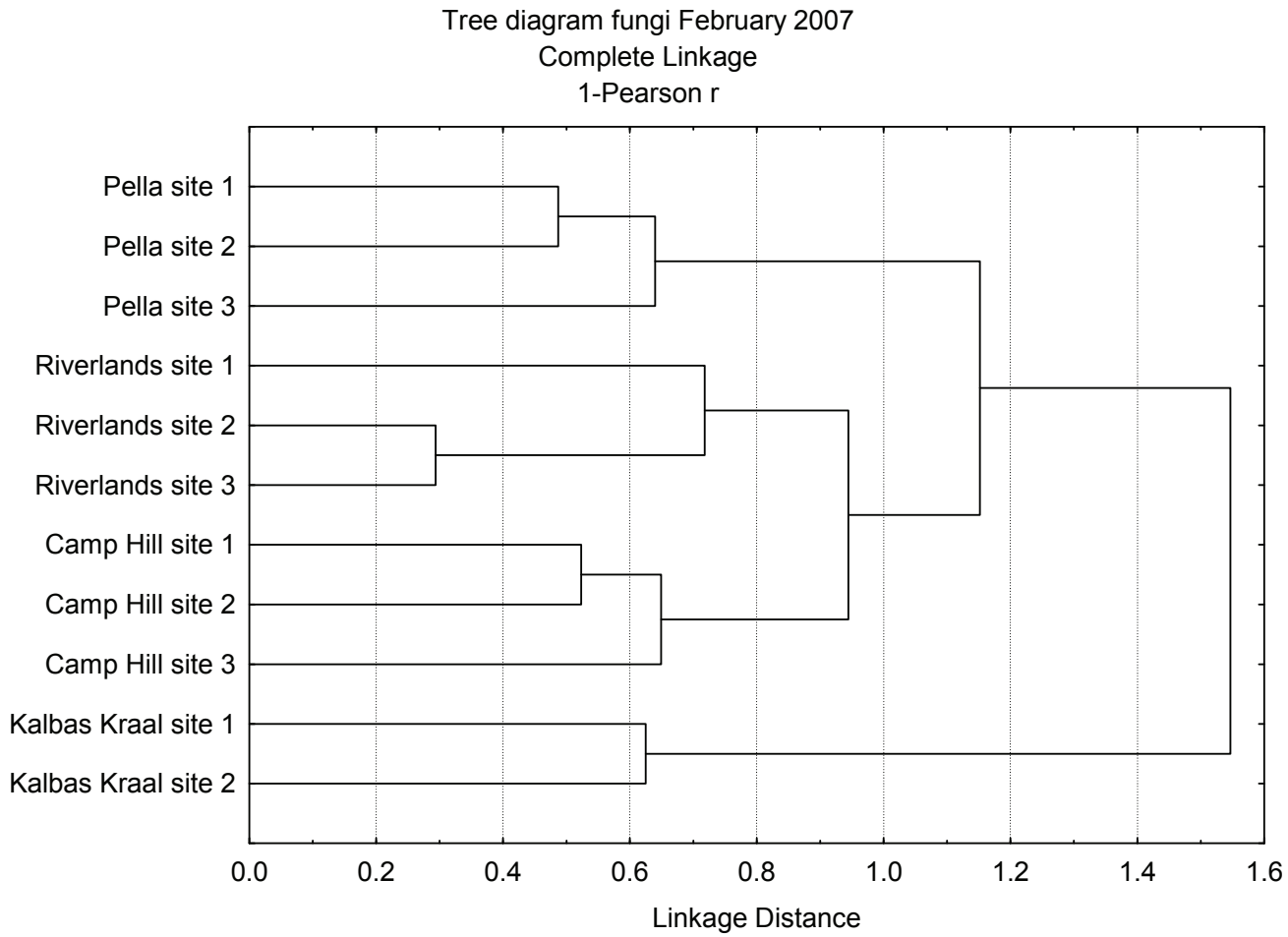


Figure 17a: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the fungal OTU's during February 2007.

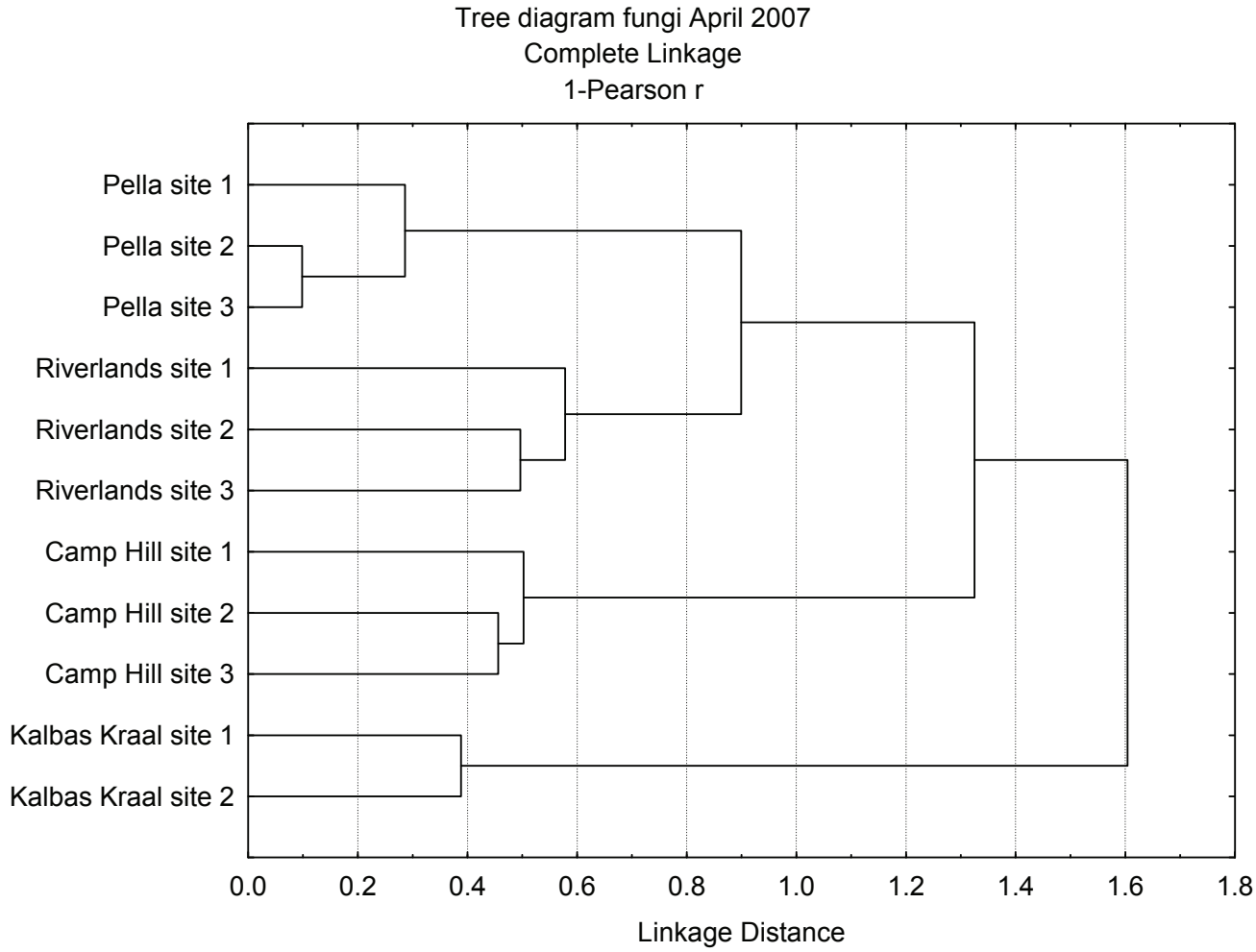


Figure 17b: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the fungal OTU's during April 2007.

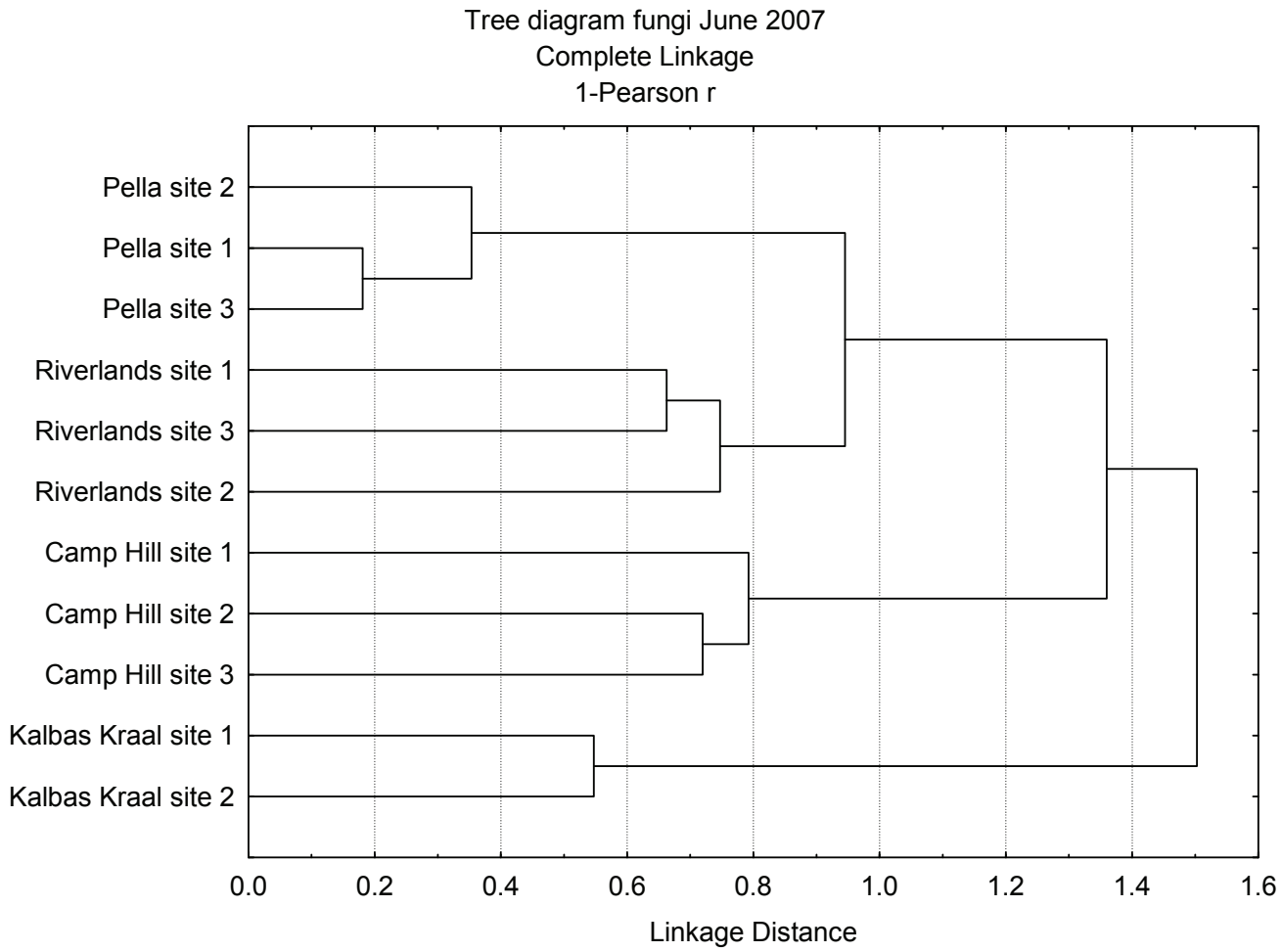


Figure 17c: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the fungal OTU's during June 2007.

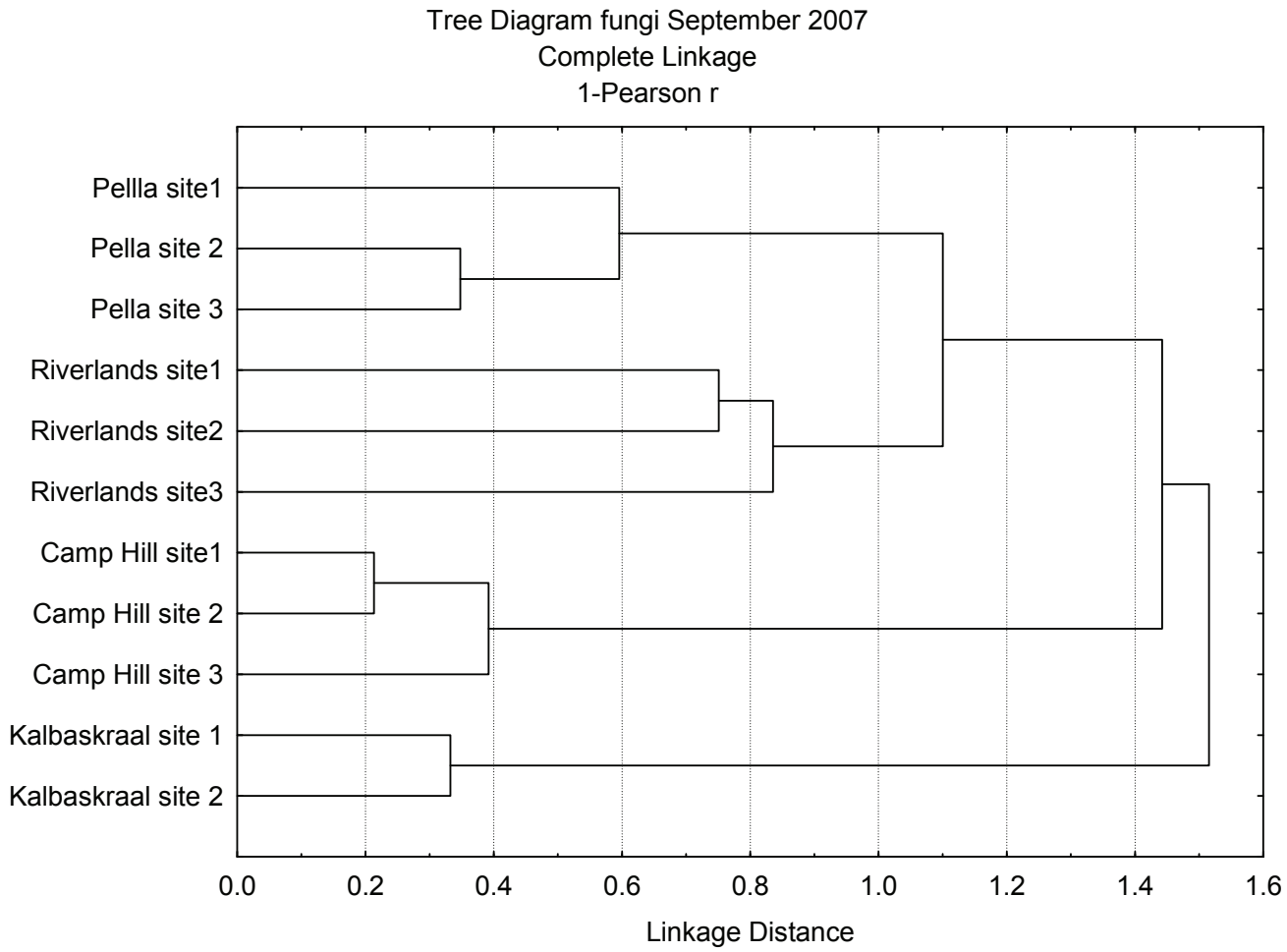


Figure 17d: Tree diagrams of the Cluster analysis of the Whitaker similarity analysis for the fungal OTU's during September 2007

Chapter 5

Automated ribosomal intergenic spacer analysis (ARISA) as a screening tool for *Penicillium* species

Abstract

The genus *Penicillium* is widespread in the soil environment, but the full extent of its diversity and distribution is not known. To expand the insight into the diversity of *Penicillium* species in the fynbos soil ecosystem, a rapid group specific molecular approach would be useful. *Penicillium* specific primers targeting the 18S rRNA ITS gene region were evaluated. Fungal specific primers ITS4 and ITS5, targeting the internal transcribed region (ITS) were used to target *Penicillium* specific in the soil sample. Nested PCR, using primer Pen-10 and ITS5, was then utilized to target *Penicillium* species, specifically. The discrimination of *Penicillium* species was possible due to length heterogeneity of this gene region. All culturable *Pencillium* proved to be detectable using ARISA.

Introduction

Automated Ribosomal Intergenic Spacer Analysis (ARISA) is commonly used to analyze and characterize numerous fungal and bacterial communities from soil samples (Fisher and Triplett 1999, Ranjard et al. 2001, Brusetti et al. 2004, Jouquet et al. 2005, Ranjard et al. 2006). ARISA is an automated system and this property makes it a suitable technique to analise and compare a large number of samples. ARISA is a relatively sensitive, reliable and reproducible. The data obtained from ARISA are often complex especially when analyzing bacterial communities (Crossby and Criddle 2003). Although ARISA performs better than other high throughput methods such as Terminal Ristriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) when resolving diversity, it is still expected to be an underestimation of diversity (Jones and Thies 2007).

Studies using re-association kinetics estimate the number of unique species in the soil to be at least 5000 while ARISA will reveal 38 to 232 unique peaks (Torsvik et al. 1990, Cardinale et al. 2004). This discrepancy is the result of a number of reasons. The sizes of peaks when using ARISA can only range from 100 to 1000 bp. Depending on the environment studied, a certain number of overlap of peaks can be expected. The sensitivity of ARISA is also limited due to PCR bias as well as the ineffective amplification of minor species (Von Wintzingerode et al. 1997, Jones et al. 2007). ARISA data of total fungal or bacterial communities provide a good indication of the total diversity of communities, however, data cannot be related to soil function. To avoid the complexity of total community profiles, primers specific for taxonomic and functional groups are suggested (Thies et al. 2001).

Fynbos soil, being acidic and containing limited amounts of carbon, nitrogen and phosphorous, is considered to be a low nutrient environment. Soil, however, is not heterogeneous, varying in its physical and chemical properties and, therefore, also creating spatial differences in microbial communities (Ettema and Wardle 2002, Mummey et al. 2006, Slabbert and Jacobs 2008). This creates a problem when searching for specific organisms and results in the need to screen through a large number of samples to detect them. Traditional culturing methods are labor intensive and time consuming and it would, therefore, be advantageous to have a fast and effective screening method for specific groups in the soil. *Penicillium* is one of the dominant fungal genera in soil and was for this reason selected as the model

organism for this study. *Penicillium* is easy to culture and this allows for comparisons to be made between culturing methods and molecular detection techniques. The aim of this study was to develop ARISA as a screening tool for *Penicillium* species in environmental samples.

Materials and methods

Soil samples were collected from a Sandveld Fynbos site at Kalbaskraal in the Western Cape, South Africa (S 33,57061º, E 18,62861°). *Penicillium* spp. were isolated from soil dilution plates and single spore cultures incubated on Czapek Yeast Agar (CYA) and Malt Extract Agar (MEA), at 25°C for 7 days in the dark (Samson and Pitt 1985). Isolates were characterized and placed into morphological groups. Total DNA was extracted from the soil with the ZR Soil microbial DNA kit (Zymo Research, USA), while DNA was isolated from the *Penicillium* strains using the method of Möller et al. (1992). A nested PCR was performed on the soil sample using reaction mixture containing 0.5 μl of the purified genomic DNA extracted from the soil, 1.75 mM $MgCl_2$, 1X PCR buffer, 500 nM of *Penicillium* specific primer Pen-10 (Wu et al. 2003) and ITS5 (White et al. 1990) and 5U Taq. The step-up PCR conditions consisted of an initial denaturing step of 3 min at 95 °C followed by 15 cycles of 95 °C, for 30 sec, 51 °C for 30 sec 72 °C for 30 sec and 25 cycles of 95 °C, for 30 sec, 51 °C for 30 sec 72 °C for 30 sec, 72 °C for 5 min and then cooled and held at 4 °C.

The PCR product from the soil sample and that of the genomic DNA from the fungi were amplified for ARISA using the primer pair, ITS4 / FAM labelled ITS5 (White et al. 1990, Fisher et al. 1999). The reaction volume of 15 μl contained 0.5 μl initial PCR product, 5U Taq 1.75 mM $MgCl_2$, 1x PCR buffer, 500 nM of each primer. The PCR conditions consisted of an initial denaturing step of 3 min at 95 °C followed by 38 cycles at 95 °C, for 30 sec, 54 °C for 30 sec 72 °C for 30 sec, 72 °C for 5 min and then cooled and held at 4 °C. ARISA was performed using the ABI 310 genetic analyser (Fisher and Triplett 1999) and run with ROX 1.1 size standard. ARISA profiles were analysed using Genemapper software. Sensitivity of the ARISA-PCR was tested by preparing a dilution series of a known number of *Penicillium* spores and adding it to DNA extractions with the ARISA-PCR done as described above. The

profiles were analyzed to determine the lowest number of spores per gram of soil needed for the ARISA-PCR to successfully detect the spores.

Results

Eleven *Penicillium* strains were isolated from a single Fynbos soil sample and grouped into 8 morphological groups (Table 1). The *Penicillium* specific ARISA profiles examined from DNA extracted directly from the soil sample also detected 8 operational taxonomic units. The PCR products that were obtained were of different lengths due to the heterogeneity of the ITS region of the *Penicillium* species evaluated. The individual ARISA profiles (Figure 1a-h), obtained from DNA extracted from the 11 cultures, correlated with the peak-sizes of the ARISA performed from the extracted soil sample (Figure 2). The ARISA performed with the specific primer sets was, therefore, able to detect all of the culturable *Penicillium* species in the soil sample.

Discussion

The members of the genus *Penicillium* are generally accepted to be easily culturable on growth media. The corresponding results using ARISA as well as culturing is an indication that all culturable species form the genus could be resolved with ARISA. The profiles of the individual isolates showed only one distinct band each. This eliminates the overestimation of diversity due to the occurrence of multiple bands. The specificity of the primer for *Penicillium* resulted in a relative uncomplicated profile. Focusing on specific genera or functional groups reduces the possibility of more than one species occurring on the same size fragment. This allows for more accurate estimation of the diversity within groups than is the case when observing total fungal communities. The length variation allowed the generation of fingerprints of *Penicillium* communities in the environmental samples. The size fragment heterogeneity was also sufficient to discern between morphologically distinct species. This method allows for rapid comparisons of *Penicillium* communities and is thus a useful tool to study *Penicillium* communities in different soil and environmental samples. This technique may also be utilized as a screening tool for *Penicillium* species from a large number of environmental samples.

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Table 1: The size fragments of the *Penicillium* species isolated for the soil sample according to electropherogram data.

Isolate	Fragment size (bp)	Alleel size (bp)
P. citrinum	562.94	563
*P. cumulacinatum** isolate 1	591.87	592
*P. cumulacinatum** isolate *2*	593.55	593
P. minioluteum	598.16	598
P. canescens	600.38	600
*P. subturcoseum**	603.41	603
Penicilium spp.	606.3	606
*P. occultum**	617.2	617

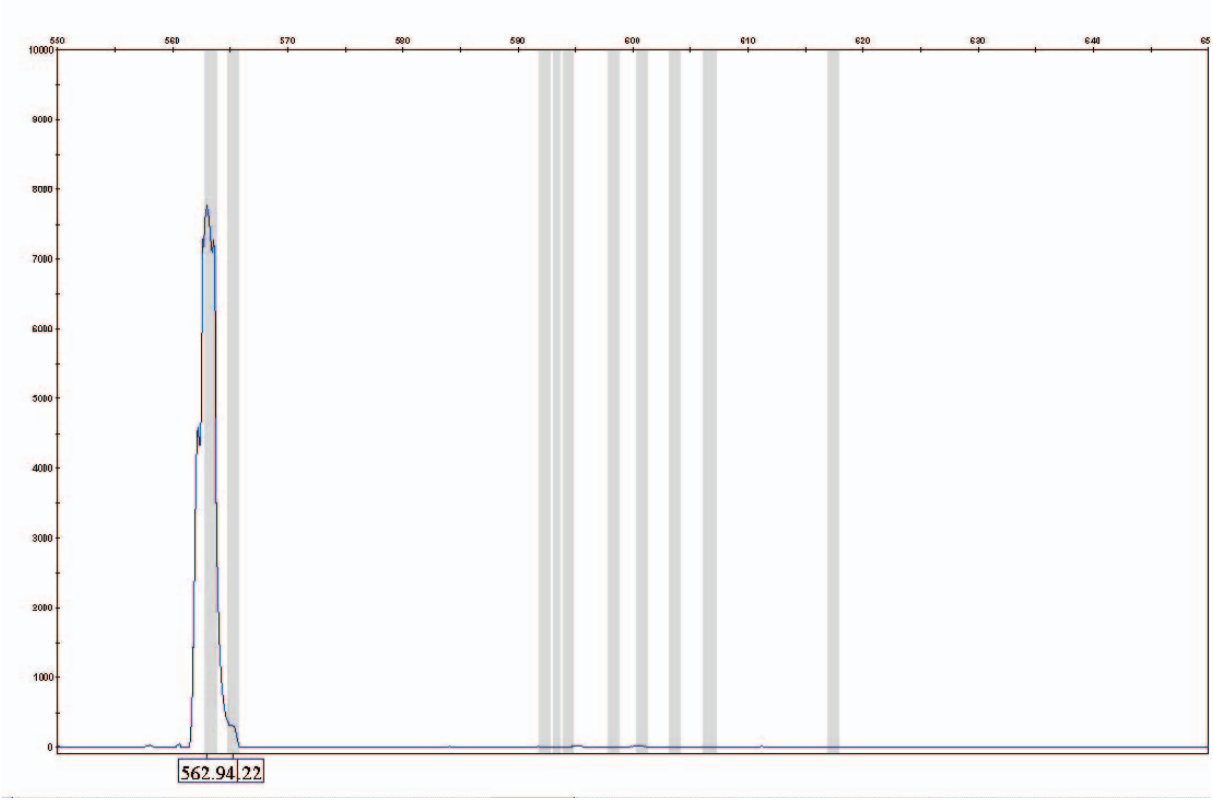

Figure 1a: *P. citrinum*

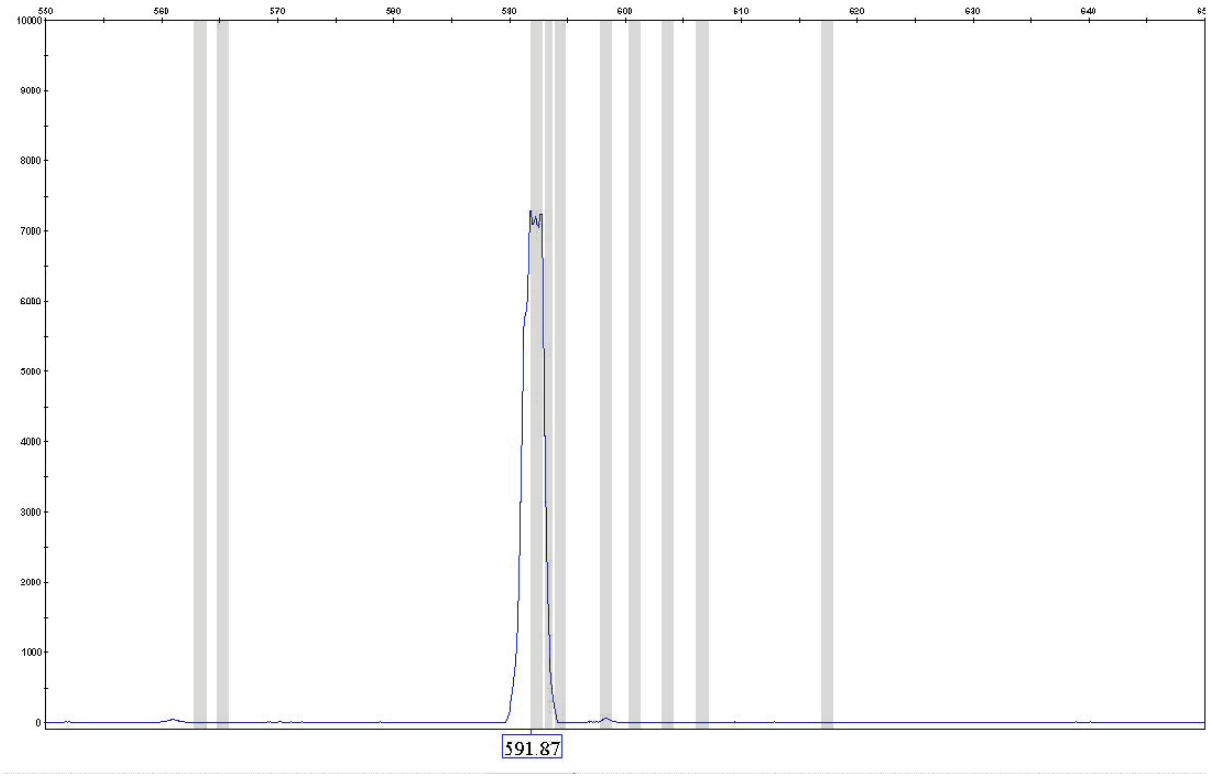

Figure 1b: *P. cumulacinatum* isolate 1

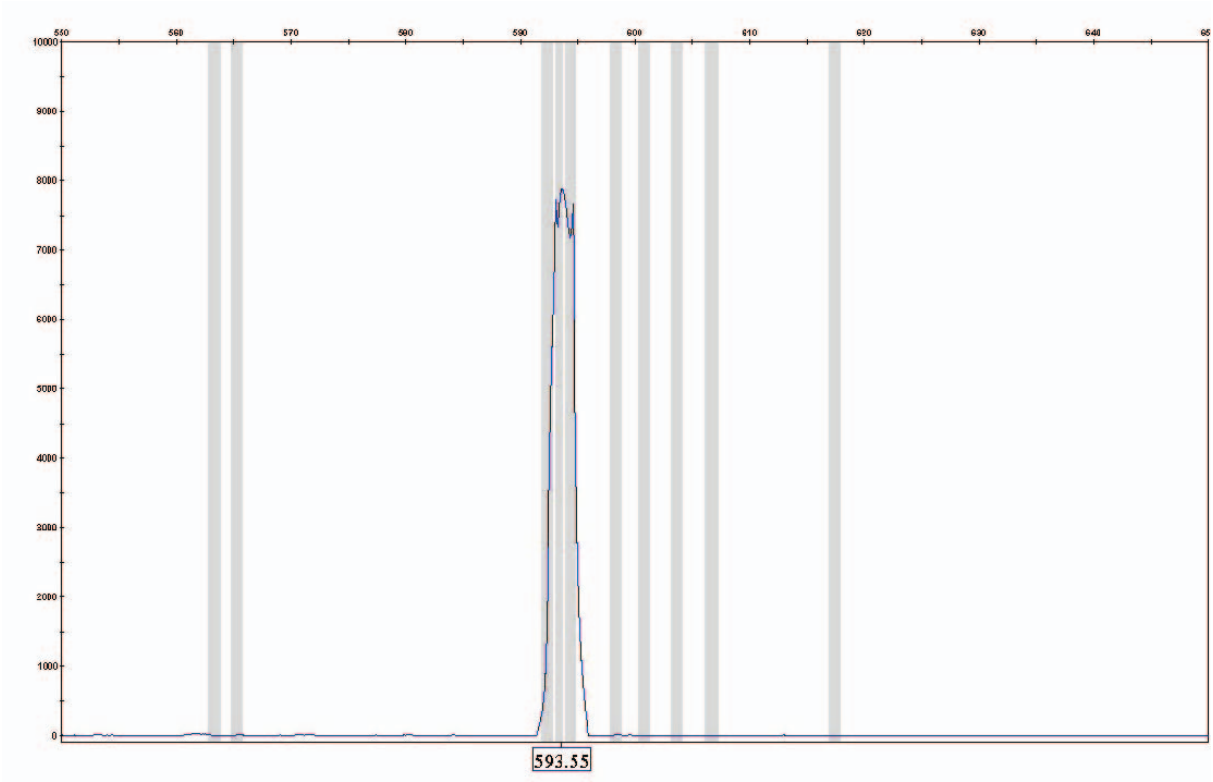

Figure 1c: *P. cumulacinatum* isolate 2

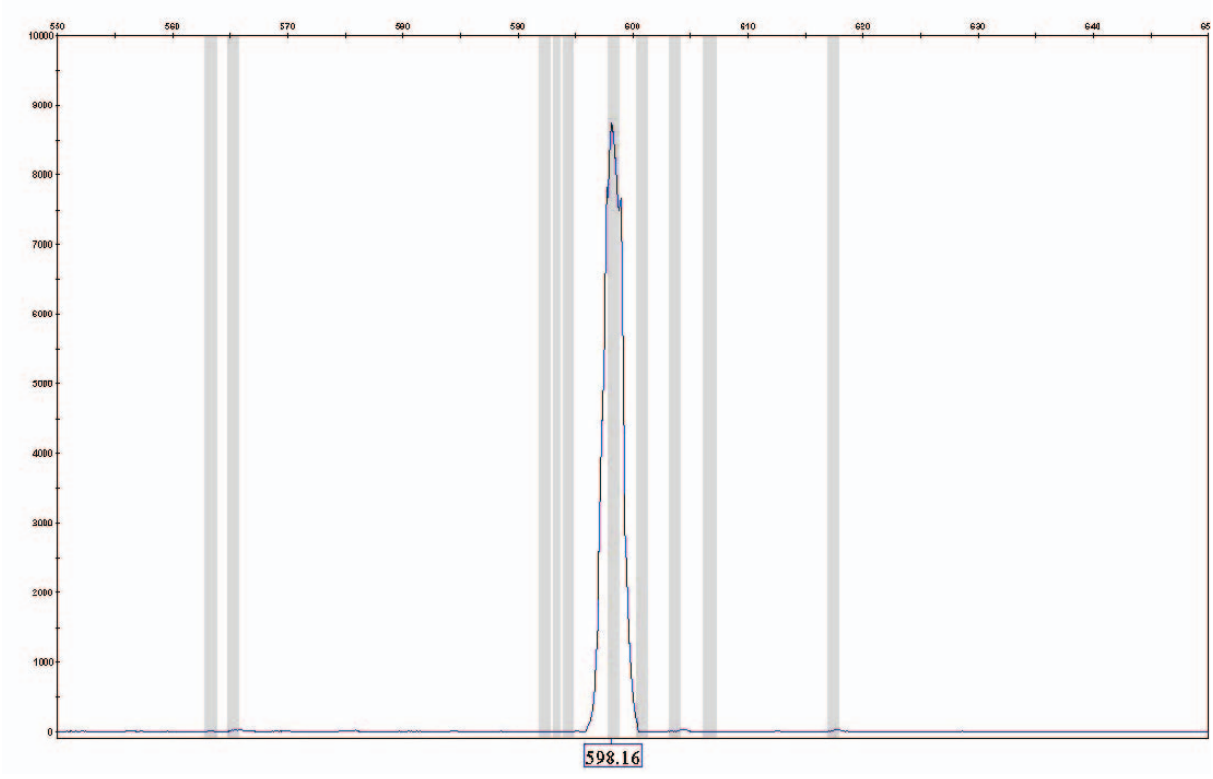

Figure 1d: *P. minioluteum*

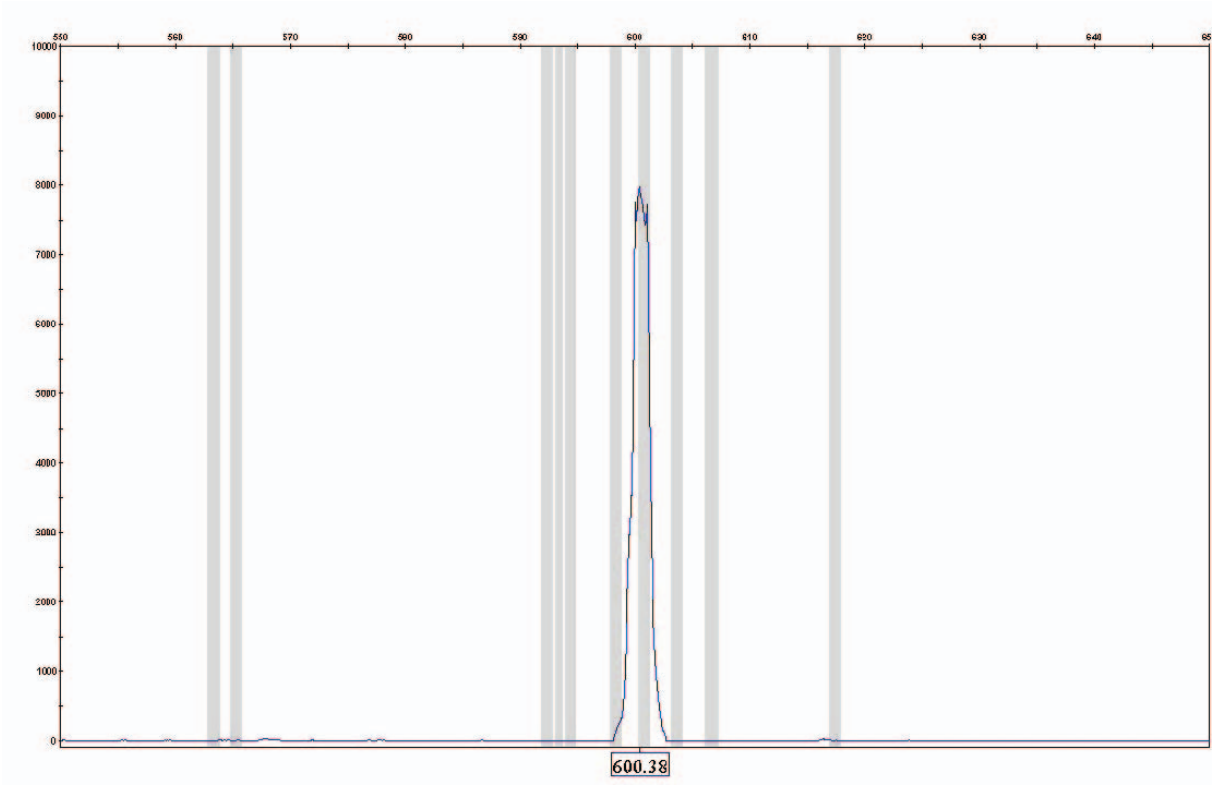

Figure 1e: *P. caescenes*

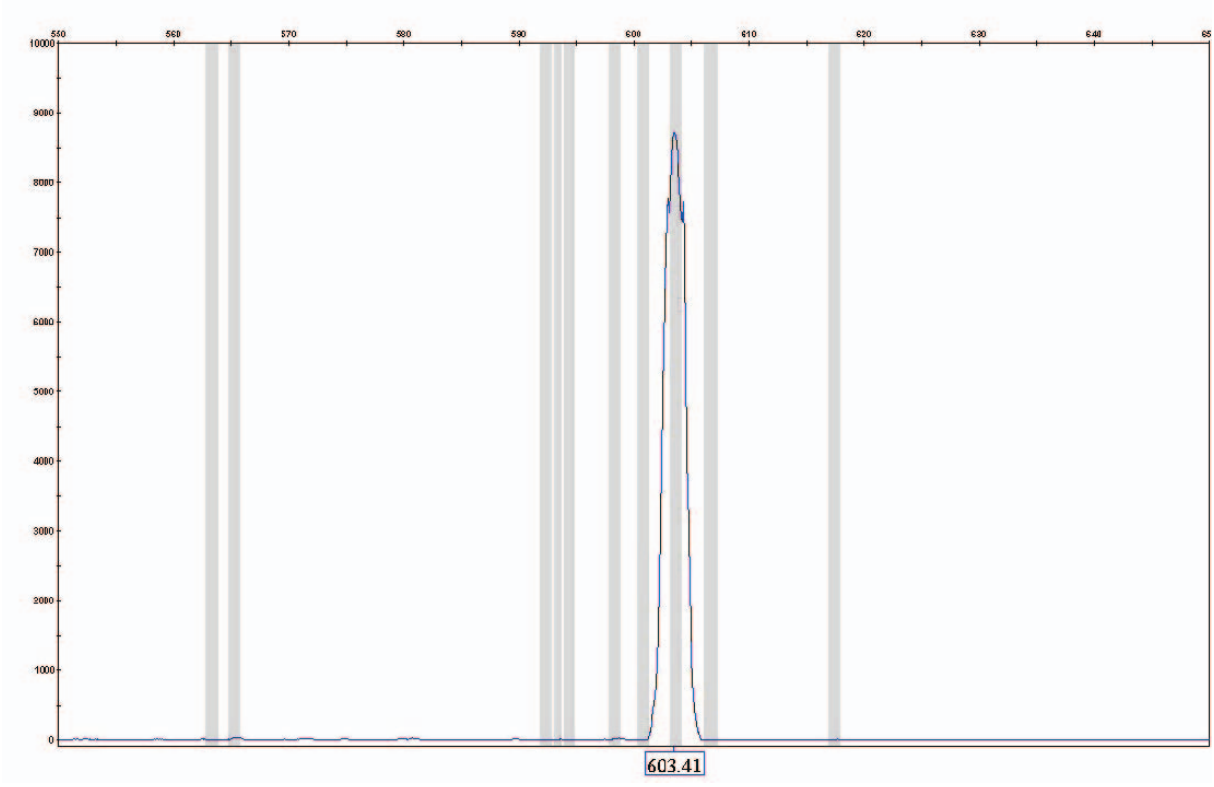

Figure 1f: *P. subturroseum*

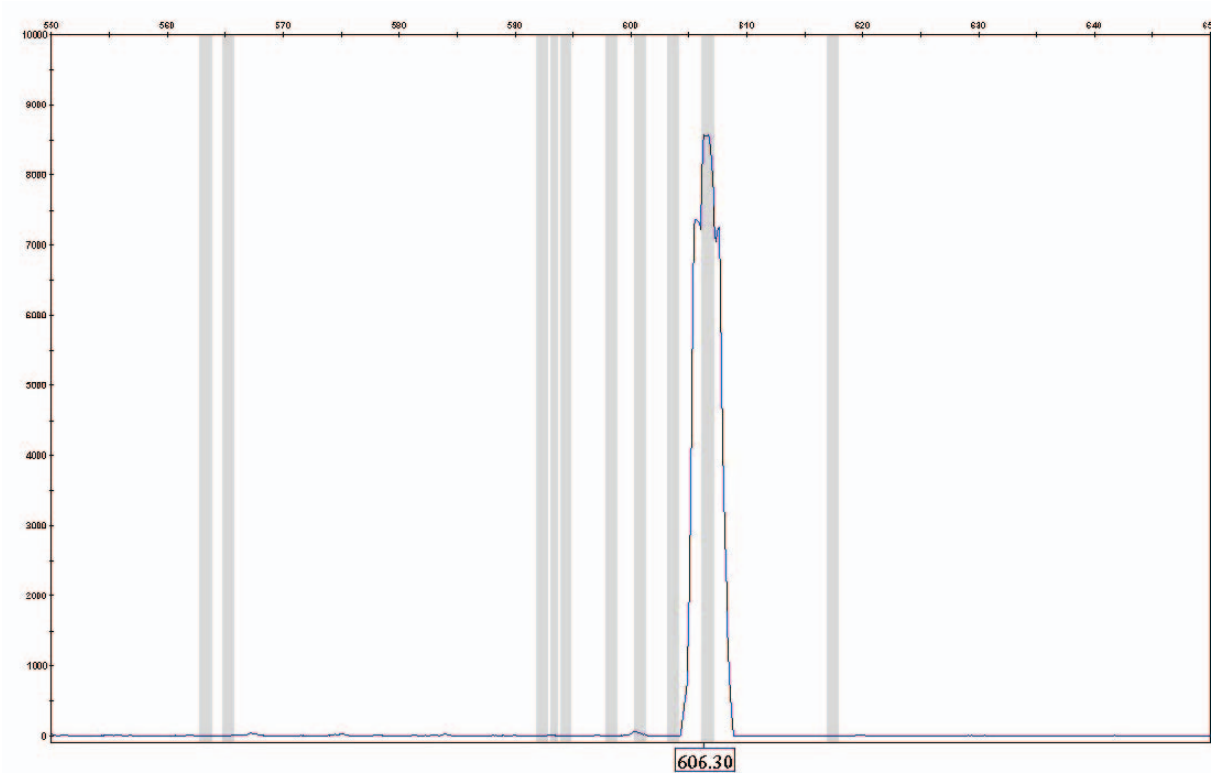

Figure 1g: *Penicilium spp.*

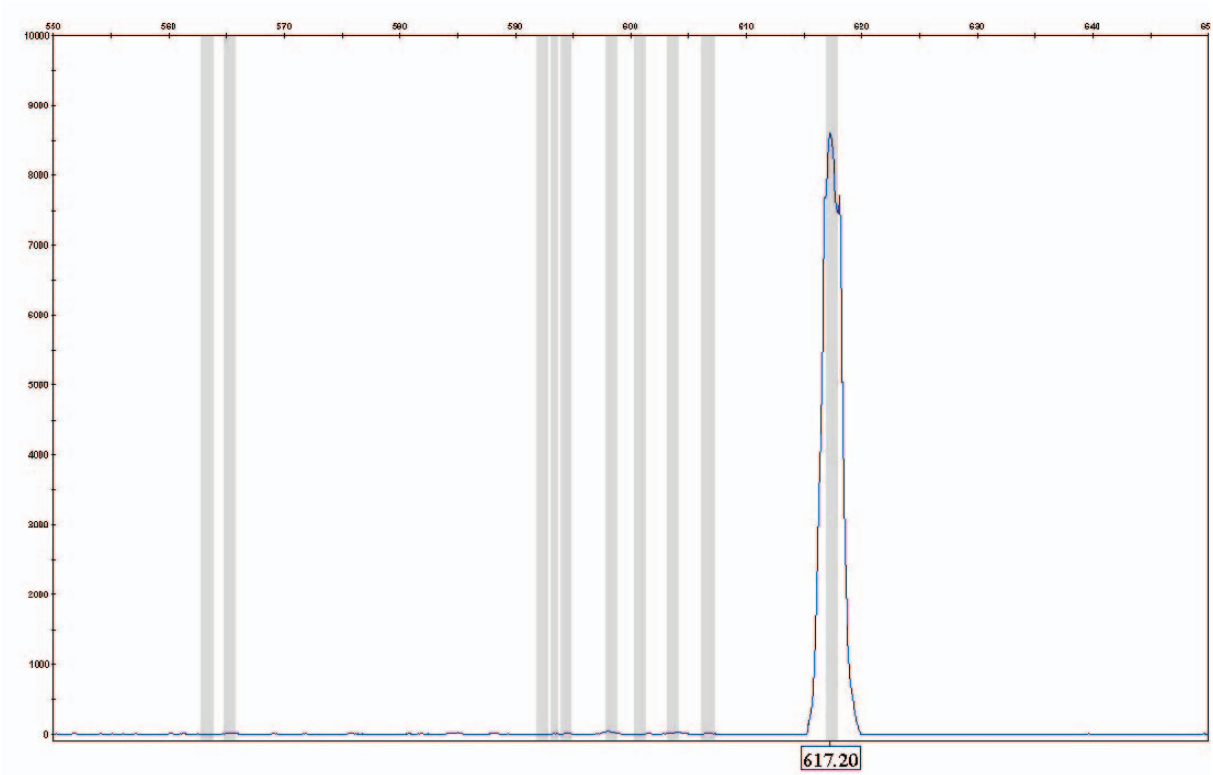

Figure 1h: *P. occultum*

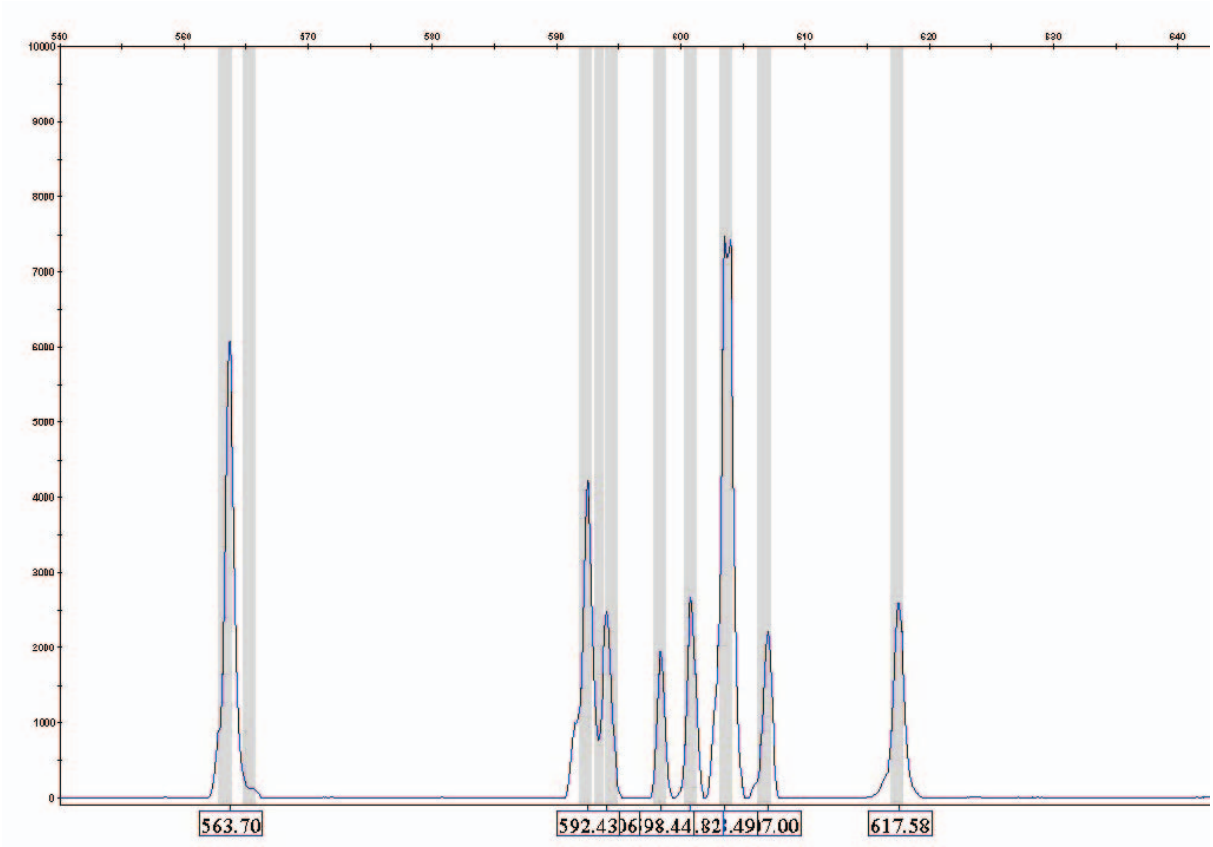

Figure 2: ARISA profile of the total DNA extracted for the sol sample.

Conclusion and future research

Primer sets for automated ribosomal intergenic spacer analysis (ARISA), ITS4/ITS5, delivered reproducible ARISA profiles. This proved useful for the assessment and comparison of fungal diversity in ecological samples. The molecular profiles obtained by using ARISA showed that the number of bacterial operational taxanomic units were significantly higher than the number of fungi. Although the number of OTU's did not differ significantly over the sampling year or between samples. The fungal and bacterial community structure within the soils did differ between the different sites. The similarity between the sites differed in terms of the bacterial and the fungal communities. The structure of both bacterial and fungal communities remained similar over the year. The differences in communities where a plant population varied and showed differences in the microbial soil communities. The study indicates that the more subtle differences like that seen within the Sand fynbos also reflect on the soil microbial population. The effect of spatial distribution on microbial communities and seem to play a bigger role in the fungal compared to the bacterial communities. Bacterial communities correlated better with the plant communities and fungal community structure appeared linked with spatial regression and physico-chemical properties. No one characteristic appears to be the overriding factor which determines community structure. ARISA revealed that the effect of species dominance on the diversity was greater in the case of bacteria than fungi.

With the use of Penicillium specific primers it was possible to detect all possible culturalble soil Penicillium. Future research will aim to use this to determine the diversity of Penicillium specie in soil. Group specific primers will not only be used to detect Penicillium groups but other phylogenetic microbial groups. The focus will specifically fall on taxnomic groups performing a specific function in the soil, for example bacterial groups responsible for denitrification. This will aim to link the diversity of certain groups of microorganism with the function they perform in the soil.