Understanding the biodiversity impacts of invasive species: Investigating changes in below- and above-ground mutualistic networks in response to invasions

by

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Declaration

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Abstract

Invasive non-native plant species threaten global biodiversity, and significantly impact on economic, agricultural, and ecosystem services. Specifically, invasive plants impact on native communities by altering ecological interactions between native species and by altering soil conditions, eventually impacting on whole ecosystems. For example, invasive nitrogen (N) fixing species such as legumes (Fabaceae) are some of best-known examples to cause such ecosystem-level impacts by elevating soil N content and altering soil bacterial community diversity and functionality. Considering that soil bacteria are essential for the health and diversity of plant communities, and ultimately to the functioning of ecosystems, such native system impacts ultimately lead invasive species in becoming ecosystem engineers, to the detriment of recipient environments.

Considered a global biodiversity hotspot, South Africa's Core Cape Subregion (CCR) is an area of international significance and is home to exceptional plant diversity. The generally strong link between above- and belowground community diversity implies that soil microbial diversity might mirror plant diversity in the CCR, e.g. like its unique fynbos vegetation. Despite this, virtually nothing is known about communities of CCR soils. Moreover, several invasive plants, notably Australian acacias, have severe impacts on CCR ecosystems. Thus, the aim of this thesis was to study the diversity and structure of CCR (fynbos) soil bacterial communities, and to investigate the impacts that invasive acacias have on them, together with impacts on soil nutrients, that ultimately lead to alteration in soil functioning. Furthermore, it is believed that the mutualistic associations that acacias form with nitrogen-fixing bacteria, known as rhizobia, might give them a competitive advantage when establishing, colonizing, and invading new environments. Thus, I also aimed to investigate whether differences in invasiveness between various acacias in South Africa can be explained by differences in the effectiveness of mutualistic rhizobial associations.

To address the aims outline above, I made use of next-generation DNA sequencing (NGS) techniques and a paired design consisting of various sites with heavily acacia-invaded areas (as treatments) in

close proximity to pristine, uninvaded fynbos areas. This allowed me to generate baseline data of the diversity and community composition of pristine fynbos soil bacterial communities, and how these relate to spatial and environmental attributes across different seasons. I then determined how invasive acacias alter fynbos soil bacterial communities, specifically in terms of community composition and diversity, and how impacts relate to the main spatial and environmental patterns of soil bacterial community turnover. Thereafter I investigated the impacts of acacias on soil chemistry and function (carbon, nitrogen, and phosphorus cycling), and determined what the links are between soil function, soil nutrient loads, and bacterial community composition, and whether acacia-induced changes translate into altered soil functionality. Finally, I shifted focus to differences between various acacia species in terms of their mutualistic rhizobial partnerships under field conditions, and asked whether there are differences in the rhizobial mutualistic associations and their effectiveness between widespread and invasive acacias, and localised non-invasive acacias.

I found fynbos soils to be characterised by high bacterial diversities and unique bacterial assemblages characterised by specific dominant taxa. Turnover in pristine fynbos soil bacterial communities was mainly due to replacement, with little nestedness. Furthermore, turnover itself was largely driven by differences in abiotic soil conditions, specifically pH and NH_4^+, as well as spatial separation. Together with these soil abiotic and spatial drivers, I found seasonality to play a significant role in shaping fynbos soil bacterial communities. Upon introducing the invasion component, I found acacias to significantly alter soil bacterial community composition, but not diversity, and that the presence of invasive acacias reduced the spatial variability across soil communities, such that community turnover could no longer be predicted by geographical distance, as was the case for pristine soils. This compositional change in bacterial communities was primarily driven by acacia-induced changes of soil pH and NH_4^+. Furthermore, I found acacias to significantly increase levels of soil nitrogenous compounds (NO_3^-, NH_4^+, and total N), C and pH, and although such impacts were not consistent across all invaded sites, the direction of impacts were. Acacias significantly impacted on key aspects of soil functioning, as demonstrated by elevated activities of enzymes involved in nitrogen (urease) and phosphorous (phosphatase) cycling, but such impacts were site-specific. Changes in soil nitrogen

and phosphorous content were correlated with changes in the activities of enzymes linked to their cycling, i.e. urease and phosphatase, respectively. For one of these enzymes (phosphatase), changes in soil bacterial community composition was correlated with enzymatic activity, suggesting that altered soil functionality is a direct result of acacia induced changes in soil nutrients, and an indirect result of alteration in bacterial community composition. Finally, I did not find any differences in richness, diversity and rhizobium community composition between localised and widespread invasive acacias in fynbos, and also did not find consistent differences in their ability to fix atmospheric nitrogen, except for some species by site comparisons, indicating differential symbiotic effectiveness between these species at specific localities. Thus, differential invasiveness of acacias in South Africa is likely linked to attributes other than mutualistic bacterial interactions, such as differences in propagule pressure, introduction pathways (e.g. forestry vs. ornamental) and intensity of plantings in the country.

Opsomming

Uitheemse indringer plant spesies bedreig wêreldwye biodiversiteit, en het merkwaardige impakte op ekonomiese, landboukundige, en ekosisteem dienste. Indringer plante het spesifiek 'n impak op inheemse gemeenskappe deurdat hulle ekologiese interaksies tussen inheemse spesies verander, sowel as grondkondisies, wat op die uiteinde lei na 'n impak op ekosisteme as a geheel. Byvoorbeeld, indringer spesies wat atmosferiese stikstof (N) kan fikseer, soos peulplante (Fabaceae), is van die beste voorbeelde van spesies wat sulke ekosisteem-vlak impakte tot gevolg het en kan merkwaardige verskille veroorsaak in die ekosisteme waar hulle indring, soos byvoorbeeld om grond N vlakke te verhoog, asook om grond bateriële gemeenskaps diversiteit en funksie te verander. Sienende dat bakterieë noodsaaklik is vir die gesondheid en diversiteit van plant gemeenskappe, en uiteindelik die funksionering van ekosisteme as a geheel, lei sulke impakte van indringer akasias op inheemse sisteme daartoe dat hulle uiteindelik ekosisteem ingenieurs word, tot die nadeel van sulke inheemse sisteme.

Suid Afrika se Kaapse Kern Subarea (KKS) word erken as biodiversiteits warmpunt (‘hotspot’) van internasionale belang en is die tuiste vir buitengewone plant biodiversitiet. Die sterk verband tussen bo- en ondergrondse gemeenskaps diversiteit impliseer dat grondmikrobiese diversiteit die plant diversiteit in die KKS kan weerspieël, soos in die unieke fynbosplantegroei. Ten spyte daarvan is feitlik niks bekend oor ondergrondse gemeenskappe van die KKS nie. Daarbenewens het verskeie indringerplante, veral Australiese akasias, 'n ernstige impak op KKS ekostelsels. Die doel van hierdie proefskrif was dus om die diversiteit en struktuur van KKS (fynbos) grondbakteriese gemeenskappe te bestudeer en die impakte wat indringer akasias op hulle het, te ondersoek, tesame met die impakte op grond nutriente wat uiteindelik tot verandering in grondfunksionering kan lei. Verder word daar geglo dat die mutualistiese assosiasies wat akasias met stikstofbindende bakterieë genaamd rhizobia vorm, hulle 'n mededingende voordeel gee wanneer hulle in nuwe omgewings vestig en indringer populasies vorm. Ek het daarop gemik om te ondersoek of verskille in indringer potensiaal tussen verskillende akasias in Suid-Afrika verklaar kan word deur verskille in die effektiwiteit van rhizobiale assosiasies.

Om die bogenoemde doelstellings aan te spreek, het ek gebruik gemaak van volgende generasie DNA- basis volgorde bepalings (NGS) tegnieke en 'n gepaarde ontwerp wat bestaan uit verskillende terreine waar akasias in hoë digthede voorkom (as behandelings) in die nabyheid van ongerepte fynbosgebiede. Dit het my toegelaat om verwysingsdata te genereer van die diversiteit en samestelling van die ongerepte fynbos grondbakteriese gemeenskappe, en hoe dit verband hou met ruimtelike en omgewingskenmerke oor verskillende seisoene. Ek het toe vasgestel hoe indringer akasias fynbos grondbakteriese gemeenskappe verander, spesifiek in terme van gemeenskap samestelling en diversiteit, en hoe dié impakte verband hou met die belangrikste ruimtelike en omgewingspatrone van grondbakteriese gemeenskapsomset. Daarna het ek die impakte van akasias op grond chemie en funksie (koolstof, stikstof, en fosfor siklusse) bestudeer, met die doel om te bepaal of grond funksie gekoppel is aan grond nutrient inhoud en bateriële gemeenskap samestelling, en of sulke veranderinge wat veroorsaak is deur akasias kan lei na veranderinge in grond funksie. Laastens het ek my fokus geskuif na die verskille tussen verskeie akasia spesies in terme van hul mutualistiese assosiasies met rhizobia onder natuurlike omstandighede en gevra of daar verskille bestaan tussen die rhizobiale assosiasies, en hul doeltreffendheid, tussen wydverspreide indringer akasias en gelokaliseerde en wydverspreide akasias.

Ek het ek gevind dat fynbos grond gekenmerk word deur hoë bateriële diversiteit en dat sulke gronde unieke bateriële samestellings het wat gekenmerk word deur spesifieke dominante taxa, en dat omset in grondbakteriese gemeenskappe grootliks as gevolg van vervanging was, met lae nestedness. Verder was omset self hoofsaaklik gedryf deur verskille in abiotiese grond kondisies, spesifiek pH en NH_4^+, tesame met ruimtelike verdeling. Tesame met hierdie grond abiotiese en ruimetlike drywers, het ek gevind dat seisoene 'n merkwaardige rol speel in die vorming van fynbos grond bateriële gemeenskappe. Ek het gevind dat indringer akasias die samestelling van grond bateriële gemeenskappe merkwaardig verander, maar nie die diversiteit nie, en dat hulle die geografiese variasie van grond gemeenskappe verminder, sodanig dat gemeenskap omset nie meer voorspel kon word die geografiese afstand nie, soos wat die geval was vir ongerepte fynbos gronde. Die verandering in samestelling van grond grondbakteriese gemeenskappe as gevolg van akasias was

hoofsaaklik gedryf deur veranderinge in grond pH en NH_4^+. Verder het ek gevind dat akasias 'n merkwaardige verhoging in grond stikstofagtige verbindings (NO_3^-, NH_4^+, en totale N), C en pH veroorsaak het, en alhoewel die impakte nie ooreenstemmend was oor al die ingedringde areas nie, was die rigting van sulke impakte wel. Akasias het 'n merkwaardige impak gehad op sleutel aspekte van grond funksie, soos bewys deur verhoogde vlakke van aktiwiteite van ensieme wat betrokke is by stikstof (urease) en fosfor (fosfatase) sirkulering, maar sulke impakte was area-spesifiek. Veranderinge in grond stiksof en fosfor inhoud was gekorreleer met veranderinge in ensiem aktiwiteite vir urease en fosfatase, onderskeidelik. Die verandering in grondbakteriese samestelling was vir een van hierdie ensieme (fosfatase) gekorreleer met ensiem aktiwiteit, wat daarop dui dat veranderinge in grond funksie 'n direkte resultaat is van veranderinge in grond nutriente as gevolg van akasias, en 'n indirekte resultaat van die verandering in grondbakteriese gemeenskap samestelling. Laastens het ek geen verskille gevind in die rykheid, diversiteit, en rhizobium gemeenskap samestelling tussen gelokaliseerde en wydverspreide indringer akasias in fynbos nie. Ek het ook nie ooreenstemmende verskille gevind in hulle vermoëns om atmosferiese stikstof te bind nie, behalwe vir sekere spesie by area vergelykings, wat daarop dui dat symbiotiese effektiwiteit tussen die spesies by spesifieke lokaliteite verskil. Dus, die verskille in indringingsvlakke van akasias in Suid-Afrika is moonltlik gekoppel aan eienskappe anders as mutualistiese bateriële interaksies, soos byvoorbeeld verskille is propaguledruk, redes vir vrystelling (bv. bosbou teenoor tuinbou) en die intensiteit van aanplantings in die land.

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" The grass withereth, the flower fadeth: but the word of our God shall stand for ever" – Isaiah 40:8

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CHAPTER 1: General introduction and synthesis

1.1. Ecological impacts caused by invasive plants

Plant species have been moved by humans and cultivated throughout the world for centuries. In some instances, such introductions have resulted in invasive populations, i.e. those species spreading over vast distances and causing measurable environmental impacts (Richardson *et al.* 2000a). Today biological invasions pose the second largest threat to global biodiversity after direct habitat destruction (Vitousek *et al.* 1997; Mack *et al.* 2000) and have devastating economic impacts on agriculture and ecosystem services (van Wilgen *et al.* 2008; de Lange and van Wilgen 2010). Alarmingly, the rate of introductions shows no signs of decreasing (Tye 2001; Hulme 2009; Van Kleunen *et al.* 2015; Seebens *et al.* 2017). In South Africa alone, annual losses in ecosystem services due to invasive non-native plant species have been estimated to amount to 6.5 billion South African Rand (de Lange and van Wilgen 2010). As such, invasive plants are a major cause for concern and understanding their impacts on the environment remains a high research priority.

Major challenges to biodiversity conservation include how to best control plant invasions, how to quantify and prevent their impacts, and how to restore the ecosystems they invade. The latter requires a detailed understanding of the extent and types of impacts caused by them. However, impacts on many important ecosystem processes remain poorly understood. For example, how invasive plants influence belowground (i.e. soil) conditions within and between the communities they invade, and its consequences for biodiversity, remains poorly understood.

An important mechanism by which invasive plants impact communities is by altering ecological interactions between native species (Vitousek 1990), with important, and sometimes unpredictable, multi-trophic ecosystem impacts. Processes such as primary or secondary productivity (e.g. increasing system biomass [Ehrenfeld *et al.* 2001; Chapuis-Lardy *et al.* 2006]), hydrology (e.g. changing the rate or timing of evapotranspiration or runoff [Levine *et al.* 2003]), nutrient cycling/availability (e.g. changing soil fungal communities that in turn influence nutrient status [Corbin and D'Antonio 2012];

elevation of soil N_2 content [Yelenik *et al.* 2004]), soil development (e.g. directly altering the physical soil environment [Wolfe and Klironomos 2005]) or disturbance frequency (e.g. increasing the frequency and intensity of fire cycles [D'Antonio and Vitousek 1992; Mack and D'Antonio 1998]) can all be altered by the mere presence of one or two high-density invasive plants. Ultimately these changes can lead to invasive species becoming ecosystem engineers (Vitousek 1990; Vitousek *et al.* 1997). Invasive nitrogen fixing plants, especially legumes, are some of best known examples to cause such severe ecosystem-level impacts (Gordon 1998). For example, the volcanic island of Hawaii is characterised by nitrogen poor soils, but subsequent invasion by nitrogen-fixing *Morella faya* has lead soil changes allowing a higher abundance of species that were previously unable to establish in these nutrient-poor soils (Walker and Vitousek 1991). Another example is nitrogen-fixing Australian acacias (genus *Acacia* Mill.) invading Cape fynbos in South Africa. These legumes drastically increase fuel loads in dense stands, resulting in altered fire regimes (Gaertner *et al.* 2009) and also increase soil nitrogen, which indirectly impact on native fynbos species that are adapted to the naturally nutrient-poor soils of the region (Yelenik *et al.* 2004). Invasive plants can also impact interactions between native species, including their mutualists (Bronstein 2009; Traveset and Richardson 2014). Furthermore, invasive plants are often able to infiltrate native mutualistic interaction networks because they are capable of forming generalist interactions with mutualists found in their new environments (Bartomeus *et al.* 2008; Bascompte 2009; Aizen *et al.* 2012). These include aboveground interactions such as pollination (Bartomeus *et al.* 2008; Aizen *et al.* 2012), seed dispersal (Spotswood *et al.* 2012; Heleno *et al.* 2013) and rhizobial interactions (Rodríguez-Echeverría *et al.* 2007, 2012). Again, legumes provide a good example of such impacts. For example, invasive legumes are often co-introduced with their native nitrogen-fixing rhizobia (i.e. bacteria that are also non-native to the recipient environment) (Ndlovu *et al.* 2013). Thus, invasive legumes can potentially significantly alter associations between native legumes and their native rhizobia, especially when invasive legumes occur in dense stands that might lead to the rapid build-up of non-native, co-introduced bacteria in soil (Le Roux *et al.* 2016, 2017). These examples illustrate that invasive plants may not only alter the physical composition of the environments they invade, but also the biotic interactions between native species and their mutualists.

1.2. Invasive legumes in South Africa's Core Cape Subregion

Southern Africa has an exceptionally high botanical diversity. Here, more than 10% of the world's vascular flora occurs in an area that represents less than 2.5% of earth's land surface (van Wyk and Smith 2001). Moreover, about 60% of the species that occur within southern Africa are endemic, representing some of the highest levels of endemism globally (van Wyk and Smith 2001). South Africa's Core Cape Subregion (CCR) (previously known as the Cape Floristic Region and now forming part of the Greater Cape Floristic Region) is home to most of South Africa's unique flora (Manning and Goldblatt 2012). It is host to approximately 9 400 plant species, around 70% of which are endemic to the region (Manning and Goldblatt 2012), but that figure may well be as high as 80% (van Wyk and Smith 2001). The CCR is thus home to almost half (~46%) of the ± 20 300 vascular plant species of southern Africa, representing about 20% of the entire flora of sub-Saharan Africa (Klopper *et al.* 2007; Manning and Goldblatt 2012).

The legume family (Fabaceae) comprises almost 10% of the total CCR flora (Manning and Goldblatt 2012), with about 83% of CCR legumes being endemic (Manning and Goldblatt 2012). For example, the genus *Aspalathus* L., comprising almost 300 species, is almost entirely restricted to the CCR (Goldblatt and Manning 2002; Manning and Goldblatt 2012). *Aspalathus* is the second largest genus in the CCR following *Erica* L. and represents about 36% of all CCR Fabaceae (Manning and Goldblatt 2012). Thus, not only is the CCR extremely rich in species, it is also rich in specific taxa. The uniqueness of the CCR is also reflected in other community components, like invertebrates. For example, insect diversity in the CCR is equally high, sharing an intimate link with native plant community distribution patterns, with high insect beta diversity at local scales appearing to be correlated with plant community turnover (Kemp *et al.* 2017). Lastly, while large spatial scale data is lacking, there is some support to suggest that below-ground communities (soil bacteria) in the CCR may also show high levels of uniqueness and diversity, possibly tracking aboveground plant community diversity (Slabbert *et al.* 2010).

Interestingly, some of the world's worst invasive plants are restricted to only a few plant families (Mack *et al.* 2000), including the Fabaceae (Daehler 1998). Furthermore, some invasive plant species appear to have more severe impacts than others, as is the case for nitrogen-fixing species such as legumes (Castro-Díez *et al.* 2014). In the CCR, Australian legumes in the genus *Acacia* Mill. are considered to be the most damaging invasive plant species (Witkowski 1991; Yelenik *et al.* 2004; Le Maitre, Gaertner, *et al.* 2011; Galloway *et al.* 2017; Mostert *et al.* 2017; Nsikani *et al.* 2017). Acacias form dense monospecific stands that cover tens of thousands of hectares and have marked environmental impacts in the region (Gaertner *et al.* 2009; Le Maitre, de Lange, *et al.* 2011; Richardson *et al.* 2011). Other than the direct impacts on native biodiversity (competitive exclusion), acacias also lead to reductions in river flow and consequent reduction in water availability (Le Maitre *et al.* 2002; Le Maitre 2004), and increased fire severity (as a result of a higher fuel load). The latter leads to drastic declines of native resprouting species and the decimation of the seed banks of re-seeding species (Holmes 2001). In addition, increases in soil nitrogen content causes impaired establishment of native species during restoration, following clearing of the acacia invasions (Stock *et al.* 1995), often resulting in secondary invasions by nitrophyllic species (Yelenik *et al.* 2004).

1.3. The legume-rhizobium symbiosis and its relevance in legume invasion ecology

Rhizobia encompass a diverse array of endosymbiotic bacteria belonging to the *Proteobacteria* division and are associated with legumes (Franche *et al.* 2009). The term rhizobia *sensu stricto* historically included only members of the genus *Rhizobium*, but nowadays refer to bacteria belonging, entirely or in part, to the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Neorhizobium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium* and *Shinella* (all members of the α-Proteobacteria), *Burkholderia*, *Caballeronia*, *Cupriavidus* and *Paraburkholderia* (members of the β-Proteobacteria) (Moulin *et al.* 2001; Willems 2006; Mousavi *et al.* 2014; Dobritsa and Samadpour 2016). Thus, the term is broadly used for all bacteria that are capable of fixing atmospheric nitrogen (N_2), often inside specialised structures called root nodules, on the roots of the host legume (Willems

2006; Stacey 2007; Franche *et al.* 2009). Biological nitrogen fixation is catalyzed by the bacterial-expressed enzyme nitrogenase, an oxygen-labile complex that is highly conserved in free-living and symbiotic diazotrophs (N_2-fixing microorganisms) (Franche *et al.* 2009). Atmospheric nitrogen fixed by rhizobia is converted to organic ammonia (NH_4^+) (Stacey 2007; Franche *et al.* 2009), which host legumes can utilise. Legumes, in exchange, provide their rhizobial symbionts with carbohydrates (Franche *et al.* 2009).

The ability to fix N_2 is not unique to rhizobia and legumes, and is in fact a trait associated with representatives of most bacterial phyla as well as methanogenic *Archaea* (Young 1992). For example, the bacterial genus *Frankia* (Actinobacteria) is capable of forming nodules in association with a small number of non-leguminous plant families (namely Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rosaceae and Rhamnaceae; the so-called actinorhizal plants) (Gualtieri and Bisseling 2000; Vessey *et al.* 2005). Also, cyanobacteria are known to form nitrogen-fixing associations in some instances with cycads (Cycadaceae, Stangeriaceae and Zamiaceae), the genus *Gunnera* (Gunneraceae), ferns (Pteridophytes), mosses (Bryophytes), as well as fungi, but nitrogen fixation is not carried out within specialised structures such as root nodules and the associated hosts therefore do not provide a specialised environment for these symbionts (Gualtieri and Bisseling 2000; Meeks and Elhai 2002). Currently, the only non-leguminous plant genus that is known to nodulate with rhizobial symbionts is *Parasponia* (Ulmaceae) (Gualtieri and Bisseling 2000; Vessey *et al.* 2005).

The phenomenal invasion success of legumes globally has, in part, been attributed to their ability to fix atmospheric N_2 (Parker 2001; Rodríguez-Echeverría *et al.* 2011; Le Roux *et al.* 2017). This may be particularly true when invasions occur in environments that are characterised by nutrient poor soils such as sand dunes and fynbos soils. However, some introduced legumes may fail to establish in the absence of appropriate rhizobia (Richardson *et al.* 2000b), analogous to reduced legume crop performance in agricultural settings in the absence of appropriate rhizobial inocula (Hafeez *et al.* 2001; Kiers *et al.* 2007; Hayat *et al.* 2010). It is therefore evident that the establishment of

belowground mutualistic associations in novel environments may be crucial to the establishment and spread of not only invasive legumes (Le Roux *et al.* 2017), but invasive species in general. For example, pines (genus *Pinus*) only became invasive in the Southern Hemisphere after the introduction of their specific mutualistic mycorrhizal fungi (Nuñez *et al.* 2009; Pringle *et al.* 2009; Dickie *et al.* 2010). It is also expected that the relative abundance of invasive legumes can lead to positive feedback loops via the modification of soil microbial communities (Callaway *et al.* 2004; Le Roux *et al.* 2017), leading in turn to easier establishment of successive generations (Thrall *et al.* 2007a). Furthermore, legumes are often co-introduced with their co-evolved rhizobia, either directly as inoculants for agroforestry, or as hitchhikers on the plants themselves (Marques *et al.* 2001; Weir *et al.* 2004; Rodríguez-Echeverría 2010; Porter *et al.* 2011; Crisóstomo *et al.* 2013; Ndlovu *et al.* 2013). Such invading plant-mutualist complexes are expected to exacerbate positive feedback loops (Rodríguez-Echeverría *et al.* 2009; Nuñez and Dickie 2014; Le Roux *et al.* 2017). Thus, invasive legumes possess the capacity to dramatically re-engineer mutualistic rhizobial interactions in the habitats that they invade, to their own benefit and possibly to the detriment of native species.

1.4. Whole soil microbial communities and their relevance in invasion ecology

The type, total abundance, and community composition of mutualistic soil bacteria play an important role in the growth, health and reproduction of plants (Hayat *et al.* 2010; Berendsen *et al.* 2012). In contrast, pathogens and commensal soil microbes play a role in hindering or, indirectly, promoting plant performance (Berendsen *et al.* 2012). Ultimately soil microbial communities are important mediators of above-ground plant community composition (Klironomos 2002; Slabbert *et al.* 2010, 2014). Native soil microbes often have weaker inhibitory effects on invasive plant growth compared to microbial effects from the species' native ranges (Callaway *et al.* 2004). In fact soil biota in novel regions might even aid invasive plants (Marler *et al.* 1999; Simberloff and Holle 1999; Richardson *et al.* 2000b; Callaway *et al.* 2001). For example, in Canada Klironomos (2002) found invasive plants to exhibit strong positive plant-soil feedbacks while co-occurring and rare native species showed strong negative feedbacks. This, in part, reflects a lack of specialist soil pathogens in association with these

invasive species in their new range. Thus, invasive plants potentially stand to benefit greatly by modifying soil microbial communities to suite their own needs (Callaway *et al.* 2004).

Soil bacteria play crucial roles in regulating nutrients and organic matter and therefore play an essential role in the functioning of soil ecosystems (Brussaard *et al.* 1997; Fisk and Fahey 2001). For example, extracellular enzymes secreted by soil bacterial communities break down complex organic and non-organic substrates (e.g. carbon, nitrogen and phosphorous, Allison and Vitousek 2005). Furthermore, there is an intricate link between soil bacterial diversity and composition, and soil function (Nannipieri *et al.* 2017), and since the functional capacity of soil microbiomes is strongly dependent on the dominant plant species present (Waldrop *et al.* 2000), the presence of dense, near monotypic stands of invasive plants can potentially alter soil microbiomes and thus also their underlying functions (van der Putten *et al.* 2007; Gibbons *et al.* 2017; Xiang *et al.* 2018). For example, invasive acacias often reduce whole soil rhizobial community diversity and change soil microbial community diversity, composition, and function (Souza-Alonso *et al.* 2014, 2015; Kamutando *et al.* 2017; Le Roux *et al.* 2018). Inferences of links between invader abundances and soil microbial communities, such as mentioned for acacias above, are usually observational and lack spatial and environmental context (also see Slabbert *et al.* 2014; Le Roux *et al.* 2018). There is an urgent need to determine the predictability of below-ground impacts by invasive species, that include environmental (e.g. soil nutrients), spatial (e.g. localised vs. widespread), temporal (e.g. seasonal), biotic (e.g. soil microbial community composition) and functional (e.g. bacterial soil enzymes) components.

1.5. Research questions

The combination of a unique native legume flora, unique soil bacterial diversity and predominance of invasive Australian acacias in fynbos represents an ideal system to explore how invasive acacias alter belowground soil community composition and function (Figure 1.1). In this study, by making use of a paired design consisting of various sites with heavily acacia-invaded areas (as treatments) in close proximity to pristine, uninvaded fynbos areas, I aimed to first determine how invasive Australian

acacias change the belowground soil microbial community. Specifically, with reference to pristine habitats, I wanted to determine whether invasive acacias change the identity and composition of whole soil microbial communities and whether there are clear patterns and changes in alpha and beta diversity across habitats. Thus I first generated baseline data of soil microbial community diversity and composition in fynbos using pristine sites only (Chapter 2). I also wanted to determine whether there are changes in community diversity and composition between seasons and whether specific biomarker bacterial taxa are associated with such pristine sites. Secondly, I aimed to determine how the presence of dense acacia invasions impact on soil microbial community structure and composition at different spatial scales in fynbos (Chapter 3). Again, I infer whether there are seasonal patterns in turnover in these inferences. Thirdly, I aimed to determine what the functional impacts of invasive acacias are in fynbos, using soil bacterial enzyme activities as proxies for soil microbial functions (Chapter 4). Specifically, I investigate enzymes involved in the primary nutrient cycles in soils, i.e. carbon, nitrogen and phosphorous. I also determined to what extent altered soil nutrient levels due to acacia invasion influences the composition of microbial communities, and whether there are correlations between certain soil abiotic variables and bacteria as a result of invasion. Finally, I shift my focus to differences between various acacia species in terms of their mutualistic rhizobial partnerships under field conditions, and ask whether there are differences in symbiotic generalisation (promiscuity) between widespread, invasive acacias, and localised, non-invasive acacias (Chapter 5). Specifically, by making use of a paired study design, next generation sequencing approaches, and nitrogen isotopes, I ask whether there are differences in the genetic makeup of rhizobial communities that inhabit nodules of invasive and non-invasive acacias, and whether there are differences in symbiotic effectiveness between these two groups of acacias.

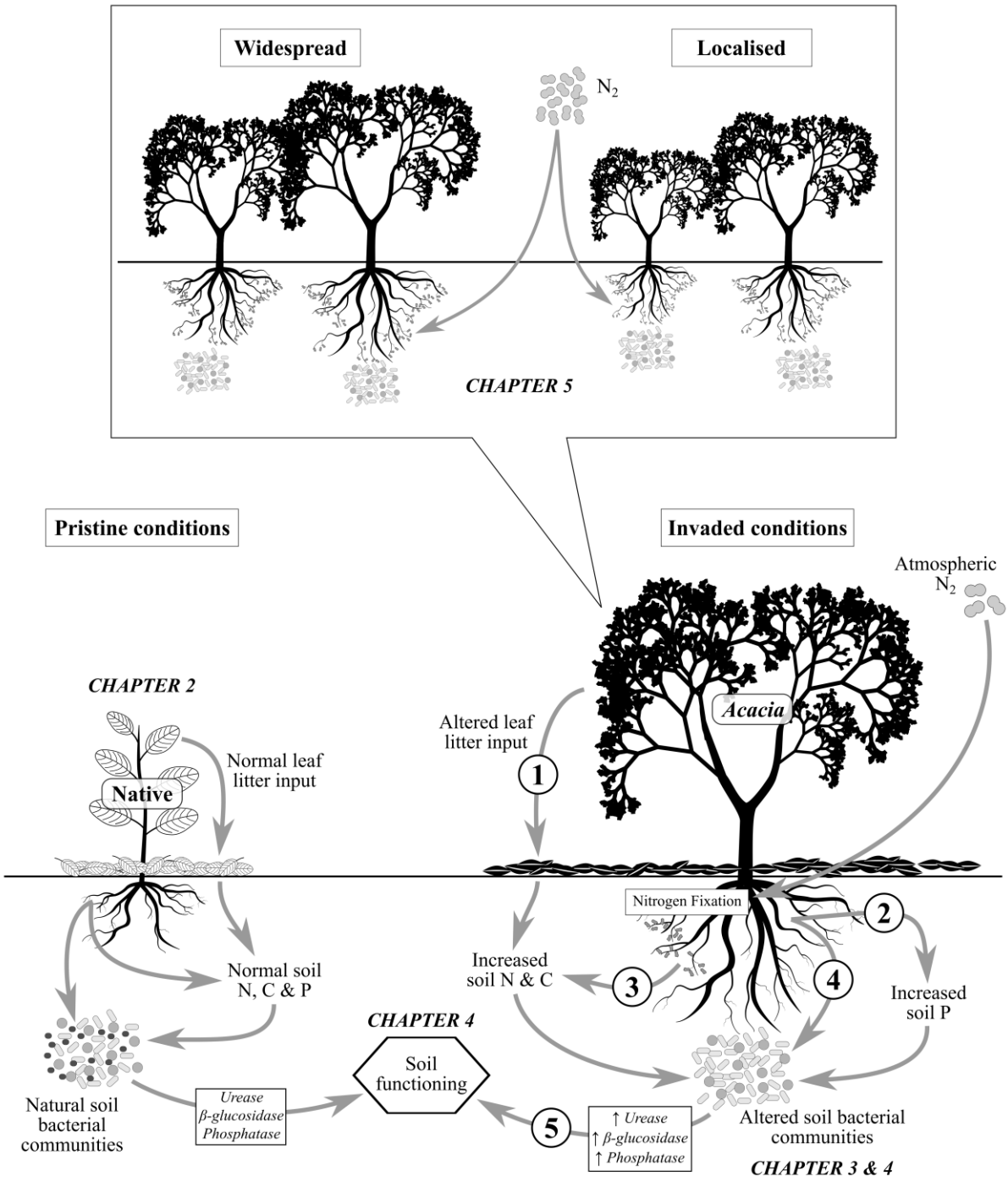


Figure 1.1: Thesis conceptual figure. Influences of invasive acacias on native systems are as follows (not intended to be chronological): 1) dense invasive stands input increased amounts of litter in the system, which also differs in quality compared to native species, leading to altered nutrient levels due to differences in decomposition rates; 2) root exudates can increase availability of certain unavailable nutrients, such as phosphorous (P); 3) nitrogen fixation can alter biogeochemical processes; 4)

differences in root architecture and function, as well as mutualistic microbial associations, can lead to changes in local environments, and together with altered nutrients lead to 5) altered soil functionality as a result of increased secretion of extracellular enzymes involved in nutrient cycling, such as glucosidase (carbon), phosphatase (phosphorous), and urease (nitrogen) (see Wolfe and Klironomos [2005] for detailed overview). In Chapter 2, I address differences in pristine (i.e. uninvaded) sites to get a baseline of differences in soil bacterial communities across various fynbos sites. I then build upon this in Chapter 3 where I introduced the invasion component, and using a pairwise study design of invaded and pristine fynbos sites, I investigate how invasive Australian acacias alter soil bacterial communities, and how such alterations are linked to spatial and environmental conditions. Hereafter, in Chapter 4 I take an in depth look at the impacts of acacias on soil nutrients and functioning, and investigate whether such changes in nutrients and soil bacterial community composition is linked to changes in soil functioning. Finally, in Chapter 5 I shift focus to acacias only, and ask whether there are differences in the root nodule rhizobial communities between various widespread, invasive acacias, and localised, non-invasive species, and whether such difference translates into differences in nitrogen fixing effectiveness.

CHAPTER 2: Insights into spatial and temporal turnover of soil bacterial communities in South Africa's hyperdiverse fynbos biome

Candidate Journals: New Phytologist; Journal of Ecology, Applied and Environmental Microbiology

2.1. Abstract

Soil bacteria are pivotal for ecosystem functioning and have important roles in regulating the health and diversity of plant communities. However, despite the large amount of information on above-ground dynamics and diversity of plant communities, surprisingly little is known about their associated below-ground bacterial communities. For example, South Africa's fynbos is a highly threatened and fragmented vegetation type, a global biodiversity hotspot with a well-characterised flora, but where almost nothing is known about the below-ground communities that underlie the region's exceptional botanical diversity. Here, using next-generation sequencing I investigated the diversity and composition of soil bacterial communities between different seasons across various fynbos sites, and how these relate to spatial and environmental attributes. I found fynbos soils to be characterised by high bacterial diversities and to harbour unique bacterial assemblages characterised by specific dominant taxa (e.g. Acidobacteria for acidic soils and Actinobacteria for alkaline soils). Turnover in soil bacterial communities was largely driven by differences in abiotic soil conditions (particularly pH and NH_4^+), and spatial separation. Finally, I found that seasonality plays a significant role in shaping fynbos soil bacterial communities. This study highlights the need to explore soil biodiversity in biodiversity hotspots before such areas ultimately disappear due to human-induced habitat loss.

KEYWORDS: 16S rDNA, alpha diversity, community composition, fynbos, next-generation sequencing, soil bacteria, soil microbial ecology, temporal diversity.

2.2. Introduction

Soils effectively form the basis for all life on Earth, and their biotic components are vital for optimal functioning of terrestrial ecosystems (Gibbons and Gilbert 2015). For example, soil biota are responsible for the decomposition of organic matter (Van Der Heijden *et al.* 2008), biogeochemical cycling (Falkowski *et al.* 2008), bioturbation, and the suppression of soil borne pests and diseases (Brussaard *et al.* 1997). Microorganisms like bacteria, are the unseen majority in soils, and are responsible for many of these, and other, ecosystem functions and properties (Van Der Heijden *et al.* 2008).

Soil bacteria represent an impressive amount of genetic diversity, and therefore functional diversity, in soils. One gram of soil can contain upwards of 50 000 unique genomes (Roesch *et al.* 2007), potentially representing billions of individual bacteria (Horner-Devine *et al.* 2004). Soil bacteria are important regulators of nutrient cycling and organic matter turnover (Fisk and Fahey 2001). They also represent a considerable portion of above-below ground interaction networks, such as plant-symbiont interactions (Coats and Rumpho 2014), and can therefore impact on the structure of aboveground communities (O 'Connor *et al.* 2002; Fitzsimons and Miller 2010; Slabbert *et al.* 2010). For plants, soil bacterial communities are important drivers, not only of community composition, but also diversity (Van Der Heijden *et al.* 2008). This may be due to the fact that growth rates of many bacteria are host plant-dependent, and therefore the identity of the local hosts will lead to specific soil bacterial community signatures (Reynolds *et al.* 2003; Elgersma *et al.* 2012). Conversely, the relative abundance of plant species within communities are highly influenced by soil organisms and their associated feedback effects (Klironomos 2002). These feedbacks manifest in plant community structure as important mediators of co-existence through the interactions with plant competitiveness and microbial-induced plant-soil feedbacks. For example, a recent meta-analysis suggests that highly competitive plants often experience stronger negative plant-soil feedbacks than less competitive plants (Lekberg *et al.* 2018).

In addition to above-ground community structure, many abiotic factors contribute to the diversity and structuring of soil bacterial communities. Factors such as pH, temperature, salinity, organic carbon and general nutrient input, soil moisture, and geographic distance, all seem to be important drivers of soil bacterial community diversity and composition (Lauber *et al.* 2009; Fierer *et al.* 2012; Gibbons and Gilbert 2015; Thompson *et al.* 2017). Of these factors, pH is widely recognised as one of the most important drivers of soil microbial community richness, diversity, and composition (Fierer and Jackson 2006; Lauber *et al.* 2009; Fierer *et al.* 2012). For example, soils with more extreme pH conditions, i.e. highly acidic or alkaline soils, are usually less diverse than neutral soils (Fierer and Jackson 2006; Lauber *et al.* 2009; Zhou *et al.* 2016; Wu *et al.* 2017). Furthermore, the relative abundances of certain bacterial phyla seem to be highly correlated with soil pH (Lauber *et al.* 2009). Although temperature is an important factor influencing soil bacterial communities, it seems less important than pH and salinity, even though richness generally increases in soil up to a around 26°C (Lozupone and Knight 2007; Wang *et al.* 2018). However, temperature and pH can exert a combined effect on bacterial community diversity, such as highest richness in cool neutral soils (Thompson *et al.* 2017). Substrate type and salinity are also important drivers of bacterial community diversity, and in certain instances can even exceed the effects of pH and temperature (Lozupone and Knight 2007). Unsurprisingly, temporal variation in soil abiotic conditions is an important driver of soil bacterial communities dynamics. Bacterial communities in the same soils can vary in diversity and composition over years (Buckley and Schmidt 2003), seasons (Lipson 2007) and even days (e.g. following rain events) (Grundmann 2004; Zhang *et al.* 2011; Prosser 2012). For example, seasonal changes in alpha diversity levels of soil bacterial communities can sometimes exceed differences between land use types (Lauber *et al.* 2013) or habitats (Samaritani *et al.* 2017). Previous work has suggested that beta diversity of soil bacterial communities is less impacted by temporal variation than alpha diversity (Lauber *et al.* 2013). Thus, although seasonality can significantly influence soil bacterial community diversity and structure, the degree of change seems to be context specific.

As one of the world's most diverse biodiversity regions, South Africa's fynbos biome has received much botanical research attention (Myers 2003; Manning and Goldblatt 2012), being home to

approximately 7500 plant species, at least 60% of which are endemic (Goldblatt 1997; Mucina and Rutherford 2006; Manning and Goldblatt 2012). As such, fynbos vegetation is characterised by high levels of alpha and beta diversity. Fynbos is the most characteristic vegetation type of the Core Cape Subregion (CCR) (Mucina and Rutherford 2006; Manning and Goldblatt 2012), and has a Mediterranean type climate, with hot, dry summers, and cold, wet winters, and a mean annual precipitation of about 500 mm (Rebelo *et al.* 2006). Rainfall seasonality itself in fynbos is highly variable, with a strong east to west trend of strict winter rainfall to more evenly distributed rainfall during the year (Rebelo *et al.* 2006). Temperatures are also highly variable depending on altitude, but lower lying areas generally experience a mean annual temperature of 16°C (Rebelo *et al.* 2006). The exceptional plant diversity and turnover in the CCR is primarily governed by soil characteristics, diverse geology, and rainfall patterns, which together produce a diverse array of habitats that are sharply defined and in close proximity to one another (Linder 1991; Manning and Goldblatt 2012). Similar to plant diversity, insect beta diversity in the CCR is also high, primarily determined by plant distribution patterns, that is, plant community turnover is correlated with high insect beta diversity, at least at local scales (Kemp *et al.* 2017).

Despite the large amount of information available on plant diversity and structure across fynbos communities, little attention has been given to other components of fynbos diversity (but see Kemp *et al.* 2017 for insects), and how they may impact plant diversity. For example, to date, only two studies have explored the structure of fynbos bacterial communities on local scales (Slabbert *et al.* 2010, 2014). Given the importance of soil abiotic conditions in driving fynbos plant abundance and distributions (Ellis *et al.* 2014), and the known link between below- and aboveground diversity, this lack of information, certainly over large spatial scales, is surprising (but see Stafford *et al.* 2005; Slabbert *et al.* 2010, 2014; Miyambo *et al.* 2016; Moroenyane *et al.* 2016; Postma *et al.* 2016). Most soil related studies in the region have focused on the impacts of invasive plants on soil chemistry (Yelenik *et al.* 2004), nutrient cycling (Witkowski 1991), localised bacterial dynamics (Slabbert *et al.* 2014), and associated implications for restoration (Holmes and Cowling 1997; Nsikani *et al.* 2017). However, the extent to which bacterial communities of fynbos soils vary over large spatial and

environmental scales remains unexplored. Given the role of below-ground communities in plant health and performance (Hayat *et al.* 2010; Berendsen *et al.* 2012; Pieterse *et al.* 2016)the need for descriptions of belowground biodiversity is critical, not only to better understand the factors driving exceptional above-ground biodiversity, but also to gather baseline data to measure the impacts of future disturbances on these ecosystems, e.g. those caused by plant invasions and climate change.

Here I compared whole soil bacterial community diversity and composition between pristine fynbos habitats spanning various spatial scales, and across different seasons. Specifically, I wanted to determine: 1) the spatial structure of bacterial community turnover (i.e. local within-site vs. regional between-site turnover) and to what extent bacterial species replacement occurs across space, 2) if identified, whether differences in soil bacterial communities can be attributed to spatial and/or environmental (soil) components, 3) whether seasonal changes play a significant role in shaping fynbos bacterial communities, and 4) whether specific taxa are characteristic of specific sites. I hypothesised that, due to the high plant species diversity turnover of fynbos systems, and its perceived intricate link with belowground diversity, soil bacterial communities will show high beta turnover between sites. I expect that soil characteristics should predict a large portion of soil bacterial community variability, and that fynbos bacterial communities should exhibit strong seasonal turnover, especially in alpha diversity.

2.3. Materials and methods

2.3.1. Study sites and soil collections

Soil conditions can display considerable geographical variability (Wandrag *et al.* 2013); a phenomenon common in South Africa's fynbos vegetation (Cowling 1990). To investigate such variability, I selected five study sites spanning a wide geographic range within fynbos vegetation (Figure 2.1A): Vergelegen Wine Estate (VG; approximate coordinates: 34.056°S, 18.934°E), Vermaaklikheid (VM; 34.358°S, 21.038°E), Koude Vlakte Conservancy (KV; 34.475°S, 19.455°E), Walshacres (WA; 34.420°S, 19.442°), and Flower Valley (FV; 34.559°S, 19.470°E). These sites were

between 6 km and 280 km apart. Sites were chosen based on the absence of invasive species and signs of human-mediated disturbance (clearing, grazing, etc.).

Soil sampling took place during the austral autumn and spring seasons of 2016. At each site four random plots of 1 m x 1 m were identified (Supplementary Figure S2.1). In each of these plots five soil subsamples of approximately 50 g each were taken randomly within the first 10 cm of the soil surface. All samples were collected away from plants to avoid roots. Where present, the top layer of litter/organic material was removed before soil collection (Roesch *et al.* 2007). For each plot, the five collected soil subsamples were bulked and mixed, leading to four independent replicates per site per season (total n = 40: 4 replicates x 5 sites x 2 seasons). Soil samples were kept on ice during transport and were immediately stored at -80°C upon arrival at the lab.

2.3.2. Soil abiotic variables

The following soil variables were analysed for all collected samples: pH, Olsen phosphorous (P), total carbon (C), nitrate content (NO_3^-), ammonium content (NH_4^+), and total available nitrogen (N). Analyses were conducted at BemLab (SANAS Accredited Testing Laboratory, Somerset West, South Africa), according to standard quality control procedures (SSSA 1996).

2.3.3. Soil DNA extraction and sequencing

For whole soil microbiome analysis total genomic DNA was extracted from 0.25g of each soil sample using the PowerSoil® DNA extraction kit (MO BIO laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. The 16S rRNA gene was amplified using the primers 799F (5'-AAC MGG ATT AGA TAC CCK G-3') and 1391R (5'- GAC GGG CGG TGW GTR CA-3'), with sample-specific barcodes in the forward primer. Amplification was done using a 30 cycle PCR and the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following PCR conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, followed by a final elongation at 72°C for 5 minutes. After amplification, PCR products were checked on a 2% agarose gel to determine the success of amplification and the relative intensity

of bands. Multiple PCR samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, Beverly, MA, USA) and used to prepare DNA libraries by following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) following the manufacturer's guidelines.

2.3.4. Bioinformatics

All raw MiSeq DNA sequence data were processed following standard procedures as described in Schloss *et al.* (2011) using mothur version 1.37.1 (Schloss *et al.* 2009). Briefly, after removal of low quality sequences and optimizing sequence lengths (to between 383 and 395 bp), unique sequences were aligned to the SILVA-ARB (release 123) reference database. Sequences were aligned to the same region of the 16S rRNA gene and columns containing only gaps were removed. All chimeric sequences were removed independent of a reference database using the uchime algorithm (Edgar *et al.* 2011) and the template as self, i.e. *de novo* removal. Sequences were subsequently clustered into Operation Taxonomic Units (OTUs) at the 97% sequence similarity level. Representative sequences for OTUs were chosen as those that were most abundant in each cluster. The ribosomal database project (RDP) Classifier (Q Wang *et al.* 2007) was used to determine the taxonomic identity of each OTU, and all sequences classified as chloroplast, mitochondria, and archaea, were removed. For standardization I subsampled an equivalent number of sequencing reads from each of the 40 replicates. Finally, singleton and doubleton OTUs (i.e. OTUs with only one or two sequence reads for the entire dataset) were removed, leading to a final of 564 346 total reads and 39 501 OTUs. A limitation on the classification of bacterial OTUs from next-generation sequencing techniques results from the incompleteness of current sequence databases (Thompson *et al.* 2017). However, it does not detract from the usefulness of using OTUs in the calculation of various diversity metrics (both alpha and beta diversity).

2.3.5. Statistical analyses

All statistical analyses were conducted in R statistical environment (version 3.5.1) (R Core Team 2017), unless otherwise specified. For diversity analysis I used the sample x OTU matrix to calculate species richness (S), the exponent of Shannon diversity (H), Inverse Simpson diversity (Si) and evenness (J; OTU abundance equality) (Hill 1973). I specifically made use of the exponent of Shannon diversity and Inverse Simpson diversity since these represent true diversities (i.e. "effective species"), in contrast to other diversity indices (Jost 2006, 2010). These were calculated using the `renyi` function in the `vegan` R package (version 2.3-3) (Oksanen *et al.* 2016), which calculates true diversities as a set of specified Hill numbers (${}^{0}D$ = S, ${}^{1}D$ = H, ${}^{2}D$ = Si) (Hill 1973). I calculated evenness as H/ln(S) (Hill 1973). Diversity metrics were then analyzed with Two-way ANOVAs (factors: site and season and their interaction) and Tukey HSD post hoc tests (corrected for multiple comparisons).

I used the package `betapart` (Baselga and Orme 2012) to describe beta diversity within and between sites, and between seasons. Specifically, I was interested in disentangling the two components of total beta diversity (calculated as Bray-Curtis [BC] dissimilarity, β_{bray}), which are the OTU balanced variation component of BC dissimilarity (β_{bal}) and the abundance gradient component of BC dissimilarity (β_{gra}) (Baselga 2017). These are the abundance equivalents of OTU replacement and nestedness derived from presence-absence (i.e. richness) turnover data (Baselga 2010). Firstly, I used the function `beta.multi.abund` to assess overall multiple-site dissimilarity. Secondly, I used the function `beta.pair.abund` (which calculates pairwise dissimilarities between all samples) to assess the effect of geographic distance on whole soil bacterial OTU dissimilarity and to disentangle the two components of total beta diversity turnover (Baselga 2010). Dissimilarity distances necessarily lack independence between observations, which precludes testing significance by means of traditional regression procedures. Therefore, I used Mantel permutation tests (9999 permutations) with the function `mantel` in package `vegan` to test for significance of the Pearson correlations between dissimilarity values and geographic distances. I also fitted linear models to each of the dissimilarity components to assess rates of turnover. Lastly, I used the function `beta.temp`

(which inputs a presence-absence matrix) to assess turnover for the same sites across two seasons (i.e. temporal turnover). Beta diversity is then computed as overall Sørensen dissimilarity (β_{sor}) with the components OTU replacement (β_{sim} = Simpson dissimilarity) and nestedness (β_{sne} = nestedness component of Sørensen dissimilarity) (Baselga 2010). In order to visualise community composition I used function `metaMDS` to create non-metric multidimensional scaling (nMDS) plots based on Horn similarity values (Jost 2007), created with the function `sim.table` in package `vegetarian` (Charney and Record 2012), and subsequently tested variation in community composition by Permutational Multivariate Analyses of Variance (PERMANOVA) with 9999 permutations with function `adonis` in `vegan` and factors site and season. As confirmation of whether variation was higher between sites than within, I used the dissimilarity matrix together with a null-matrix (Rundle and Jackson 1996) in a mantel test with 9999 permutations (Supplementary Figure S2.4).

I was also interested in the extent to which spatial and environmental variables drive soil bacterial community composition and structure; all functions hereafter were used from the `vegan` R package (Oksanen *et al.* 2016). For this I first used longitude–latitude coordinates of each sample to generate a set of spatial variables (S) using principal coordinates of neighbour matrices (PCNM) (Borcard and Legendre 2002; Griffith and Peres-Neto 2006) with the function `pcnm`. The advantage of using PCNM variables is that they represent all perceivable spatial scales in the sampling scheme (Ramette and Tiedje 2007), with the order of variables corresponding to decreasing spatial scales (Borcard *et al.* 2004). Since the community data table contained many instances of very low abundance OTUs (Figure S2.2), I only used OTUs that represented a cumulative contribution of 80% and also used a Hellinger transformation to account for the presence of many zero abundances (Legendre and Gallagher 2001). To find the set of variables that explained the variation in the community data the best I applied a forward selection of the environmental (soil) and spatial variables, respectively (Ramette 2007; Stomeo *et al.* 2013), with the `ordistep` function. I used the PCNM variables together with the set of environmental (E) variables and subset OTU table in a distance based redundancy analysis (db-RDA) variation partitioning (Borcard *et al.* 1992; Legendre and Andersson 1999; Peres-Neto *et al.* 2006) with function `varpart`; the output given is RDA-adjusted R^2 values,

which are unbiased (Peres-Neto *et al.* 2006). This establishes the extent to which either pure environmental (E|S) or pure spatial (S|E) variable fractions, or their intersection (E∩S), explain variation in bacterial community composition. The analysis also indicates the amount of variation that remains unexplained (U = 1 - [E + S]). I tested the significance of fractions E|S and S|E, respectively, with 9999 Monte Carlo permutations; fractions U and E∩S are not testable. Unfortunately, `varpart` cannot handle missing data, and since I did not have soil data for one of the replicate samples, I was obliged to remove it from the `varpart` analysis.

Finally, I aimed to identify bacterial taxa that characterise bacterial communities in the various fynbos soils by using linear discriminant analysis (LDA) effect size (LEfSe) (Segata *et al.* 2011) to discriminate between markers of soils across sites, using the mothur software. Such taxa are those having both high relative abundances and frequencies across replicate samples within each site. In order to visualise such biomarker taxa I plotted their relative abundance and frequency of occurrence across each sample type. Using site as "class", the method identifies bacterial biomarkers via a non-parametric Kruskal-Wallis rank sum test to detect significant features, after which an LDA is performed for effect size estimation (Segata *et al.* 2011). Biomarkers were identified using an alpha value of 0.05 and an effect size threshold (i.e. LDA score) of 2.

2.4. Results

I obtained 564 346 high-quality reads after data filtering, which resulted in 39 501 OTUs representing 318 genera, 134 families, 78 orders, 44 classes and 19 phyla. Approximately 72.1% (406 680) of sequences could not be classified to genus level, 59% (332 786) to family level, 35.6% (201 153) to order level, 9.5% (53 707) to class level, and 7.1% (40 276) to phylum level. No OTUs could be classified to species level as the short sequencing reads associated with NGS data normally do not allow such fine taxonomic resolution. The most abundant phyla across all sites were Proteobacteria, followed by Actinobacteria and Acidobacteria (Table 2.1).

Each of the sites had a high number of unique OTUs, aggregated for both seasons, although there was a core set of 750 OTUs (229 894 reads or 40.7% of total sequencing reads) that were shared among all sites (Figure 2.1B). A total of 10 723 OTUs (25 673 sequences or 4.55% of total) were unique to the Autumn season, whereas 11 654 OTUs (29 749 sequences or 5.27% of total) were unique to Spring (Figure 2.2).

All alpha diversities (S, H, Si and J) were significantly higher in spring (Figure 2.3, Table 2.2), and I observed no interaction effects between season and site for any of the diversity metrics included here. Furthermore, Flower Valley had significantly higher OTU richness (S) compared to Vergelegen ($p_{bonferonni}$ = 0.005) and Walshacres ($p_{bonferonni}$ = 0.04), while Koude Vlakte had significantly higher values for H ($p_{bonferonni}$ = 0.01) and J ($p_{bonferonni}$ = 0.006) compared to Vergelegen. Even though ANOVA indicated Si to be marginally significant for factor site, Tukey HSD indicated that no pairwise site comparisons were significant.

The high number of unique OTUs observed for each season at the respective sites was reflected in the analysis of overall temporal beta diversity, with mean β_{sor} = 0.685 (± 0.012 SE). Furthermore, I observed the same trend for temporal turnover as for geographic turnover, i.e. that overall total temporal dissimilarity was almost completely due to OTU replacement (β_{sim} = 0.671 ± 0.012 SE) instead of nestedness (β_{sne} = 0.014 ± 0.002 SE) (Figure 2.4A). Overall beta dissimilarity indicated a high level of turnover among sites (β_{bray} = 0.960). This turnover was almost completely due to OTU balanced variation (β_{bal} = 0.956), with almost no abundance gradients observed (β_{gra} = 0.004). I also observed high levels of turnover between replicates within site/season combinations (Supplementary Table S2.2). Furthermore, at site level I observed a significant positive correlation for both β_{bray} and β_{bal} with geographical distance (r_{Mantel} = 0.83, p = 0.025 and r_{Mantel} = 0.82, p = 0.025, respectively), whereas there was non-significant negative correlation for β_{gra} (r_{Mantel} = -0.23, p = 0.792) (Figure 2.4B). Total dissimilarity β_{bray} increased with geographic distance (slope = 0.0014, p < 0.01), but this increase was due to the β_{bal} component of dissimilarity (slope = 0.00115, p < 0.01), whereas β_{gra}

decreased (slope = -2.01 x 10^{-5}, p = 0.53). There was a near-complete turnover of OTUs (β_{bray} = 0.955) at the highest end of geographic distance included in the analysis (ca. 200 km).

High beta diversity between sites was reflected in the nMDS plot which indicated clear separation between both sites and seasons (Figure 2.5), both factors accounted for a significant component of variation in composition between samples (Table 2.3). The mantel test confirmed that replicates within sites were more similar to each other than replicates between sites (r_{Mantel} = 0.6282, p < 0.001).

After forward selection I retained pH and NH_4^+ as significant predictors of bacterial community composition for the environmental component, and PCNM's 1, 3, and 4 as significant predictors for the spatial component. I retained season as a factor due to the high number of unique OTUs found at each site for each season. Variation partitioning indicated that environmental variables explained about 30% of the variation in the species (OTU) matrix (Figure 2.6). Roughly half of this (~17%) could also be predicted by the information from the geographical coordinates, meaning that bacterial OTUs and environmental data have a fairly similar spatial structuring (Borcard *et al.* 1992). The spatial component (S|E) uniquely explained about 18% (p = 0.001) of the variation. Local effects, i.e. variation solely due to environment (E|S) (Borcard *et al.* 1992), explained a significant amount of variation (13.5%, p = 0.001). However, a large amount of variation remained unexplained (~52%) indicating that there are other intrinsic processes responsible for community structuring that are unaccounted for here, e.g. plant community composition.

Finally, the analyses of biomarker taxa highlighted numerous taxa that are characteristic to the sites. Specifically, the genera *Acidocella*, *Bacillus*, Acidobacteria_Gp1, and Betaproteobacteria were characteristic of the sites Flower Valley, Koude Vlakte, Walshacres, and Vermaaklikheid, respectively (Figure 2.7, Table S2.1). The class Acidobacteria_Gp1 was highly abundant at Vergelegen.

2.5. Discussion

Until now, no studies have investigated the influences of environmental and spatial variables on the broad scale structure and composition of fynbos bacterial communities. The data illustrate that fynbos soil bacterial communities possibly mimic the high spatial turnover characteristic of many of the above-ground components in the region like plants and insects (Linder 2005; Manning and Goldblatt 2012; Kemp *et al.* 2017). It is well known that belowground bacterial community diversity and composition are strongly dependent on aboveground plant community diversity and composition (Reynolds *et al.* 2003; Elgersma *et al.* 2012), and that they are intricately linked by feedback loops (Klironomos 2002). This below/above-ground link should be particularly strong for plant-associated core microbiomes. Indeed, some fynbos plants harbour unique rhizosphere bacterial communities, even when occurring in sympatry (Miyambo *et al.* 2016). These findings suggest that this link may persist, even over large spatial scales and provides indirect support for the expectation that fynbos soil bacterial communities will mimic the diversity and spatial turnover of above-ground plant communities. Although many of our bacterial taxa remained unclassified, having a certain number of unclassifiable sequences is common since full length sequence databases do not yet capture large swathes of bacterial community diversity (Thompson *et al.* 2017). As sequence databases continually expand, it can be expected that a higher number of bacterial (potentially unknown) species and communities will be captured in the future.

Although many of the bacterial taxa remained unclassified at genus level some informative taxonomic patterns were identified. Acidobacteria were the most important biomarker taxa within soils at three of the sites (Flower Valley, Vergelegen and Walshacres) (Supplementary Table S2.1). Members of the Acidobacteria are known to thrive under oligotrophic soil conditions (Dion and Nautiyal 2003), characteristic of fynbos soils (Manning and Goldblatt 2012). Furthermore, an abundance of Acidobacteria are generally indicative of acidic soil conditions (Philippot *et al.* 2010; Bardhan *et al.* 2012; Sun *et al.* 2014), with their relative abundance generally declining as soil pH increases (Jones *et al.* 2009). Unsurprisingly, these three sites had acidic soils. The absence of Acidobacteria at Vermaaklikheid and Koude Vlakte sites may reflect the alkaline (pH ~ 8) or slightly acidic (pH ~ 6)

soils found at these sites, respectively. Under field conditions Acidobacteria are important regulators of iron (Coupland and Johnson 2008; Lu *et al.* 2010) and the biogeochemical cycling of sulphur (Rowe *et al.* 2007). At the Koude Vlakte site, the genus *Bacillus* was the most characteristic taxon. Some members of the genus *Bacillus* are known for their role in promoting plant growth (Bulgarelli *et al.* 2013), specifically due to their ability to solubilise mineral phosphorus and thus promote phosphorus availability/uptake. Microorganisms that have the capacity to solubilise mineral phosphorus are abundant in most soils (Bulgarelli *et al.* 2013), but the characteristic presence of *Bacillus* at Koude Vlakte is not surprising given that the site has high levels of phosphorous content (Supplementary Figure S2.5). Finally, Actinobacteria is an important indicator taxon of the high-pH soils collected at Vermaaklikheid. Actinobacteria are known to act as plant growth promoters (e.g. by solubilising rock phosphate) and disease suppressors (e.g. having antimicrobial activities) in natural ecosystems (Palaniyandi *et al.* 2013). In contrast to Acidobacteria, the abundance of Actinobacteria are positively correlated with pH (Lauber *et al.* 2009), and they are known to be rich in diversity in limestone derived soils (Nimaichand *et al.* 2015). As such their characteristic presence in the alkaline limestone derived soils of Vermaaklikheid is not surprising. Although Betaproteobacteria were the most characteristic taxa at Vermaaklikheid, they are indifferent towards changes in soil pH (Lauber *et al.* 2009), indicating that some other conditions modulate their abundance at this site. The characteristic biomarker taxa of the various soils might serve a predictive value due to their preference for specific soil conditions (e.g. oligotrophic, acidic soils). For example, upon colonising and spreading in new environments, invasive plants are known to alter soil nutrients (Yelenik *et al.* 2004; Marchante *et al.* 2008; Lorenzo *et al.* 2010a; González-Muñoz *et al.* 2012; Souza-Alonso *et al.* 2014, 2015) and microbial communities (Klironomos 2002; Callaway *et al.* 2004). Thus, soil biomarker taxa might potentially serve as useful indicators of changes in soil conditions under such invasions.

Bacterial community composition itself is influenced by differences in vegetation type and succession (Carney and Matson 2006; Yu *et al.* 2012). One of the factors contributing to high plant diversity and vegetation types in fynbos, is high levels of plant beta diversity (Manning and Goldblatt 2012). In fact, near-complete turnover of plant diversity has been reported for sites with different soil properties

but with similar topography and climate (Cowling 1990). I sampled five different vegetation types, namely Canca Limestone Fynbos (Vermaaklikheid), Overberg Sandstone Fynbos (Flower Valley), Agulhas Limestone Fynbos (Koude Vlakte), Boland Granite Fynbos (Vergelegen), and Elim Ferricrete Fynbos (Walshacres). The latter two are listed as Endangered vegetation types due their high levels of plant endemism (especially Proteaceae species) and low extent of remaining habitat (Mucina and Rutherford 2006). All these vegetation types vary considerably in both abiotic soil conditions and associated plant communities (Mucina and Rutherford 2006). Thus, the high soil bacterial community turnover found in this study might be reflective, in part, of the high levels of plant community turnover between sites (Manning and Goldblatt 2012). Disentangling the effects of above-ground community composition vs. soil abiotic conditions on soil microbial community diversity and structure from observational data is challenging. Specifically, factors such as soil pH, moisture availability, and organic carbon input from plants are known to shape soil bacterial communities (Fierer *et al.* 2012), may also be important regulators of plant community composition in fynbos environments (e.g. soil pH). I attempted to disentangle the role of soil abiotic factors in the high beta diversity turnover of the fynbos bacterial communities. I found that pH and NH_4^+ were significant predictors of community composition. It is known that many bacterial species are sensitive to fluctuations in pH (Bartram *et al.* 2014), and its role as significant predictor is no surprise given the large variation in soil pH conditions among sites (range 3.8 - 8.2). The fact that the effects of pH on taxon sorting is evident even at coarse taxonomic levels (Lauber *et al.* 2009), means that pH might serve as a primary driving force of the observed differences in soil bacterial community composition between sites. For example, the most unique soil bacterial communities identified were from Vermaaklikeid and Vergelegen soils, the former characterised by the typical limestone substrates of the Aghulhas plain and associated alkaline soils, while the latter is characterised by highly acidic soils (Mucina and Rutherford 2006).

Previous work suggested limited temporal change in the structure of fynbos soil bacterial communities within the same vegetation type and over small spatial scales (Slabbert *et al.* 2010). In contrast, I found strong differences in both alpha and beta soil bacterial diversities between seasons,

despite the stability of the aboveground plant communities at these sites. This indicates that factors other than plant community composition alone can drastically effect soil microbial communities (Carney and Matson 2006; Yu *et al.* 2012). The results are in accordance with what has been found elsewhere for soil bacterial communities, namely that they can undergo significant changes in community composition on various temporal scales (years, seasons, and even days) (Buckley and Schmidt 2003; Lipson 2007; Zhang *et al.* 2011). In fact, season itself can be a significant driver of soil bacterial community structure (Samaritani *et al.* 2017). The high temporal diversity observed here should not be surprising, since fynbos systems are characterised by large seasonal variation associated with the prevailing Mediterranean type climate experienced by the region, i.e. hot, dry summers, and cold, wet winters (Manning and Goldblatt 2012; Ellis *et al.* 2014). Soil bacterial community composition is often strongly linked with such dramatic fluctuations in temperature and soil moisture (Fierer and Jackson 2006; Fierer *et al.* 2012; Thompson *et al.* 2017). Finally, more spatially heterogeneous communities, such as different vegetation types included here, also generally seem to be temporally more heterogeneous (Collins *et al.* 2018). It should be noted, however, that alpha diversity of soil bacterial communities can vary significantly depending on the month of sampling (Lauber *et al.* 2013), and thus it remains to be explored what the effects of finer scale temporal sampling would be on bacterial communities of fynbos soils (e.g. monthly sampling vs. seasonal sampling).

Although fynbos soil bacterial community composition was explained by both spatial and environmental variables (~48%), a vast amount of variation remained unexplained (~52%). Although it is not clear whether this reflects factors not included here or stochastic processes, the hyper diverse nature of fynbos habitats means that sampling of more sites, or more sampling effort per site (seeing as there are high levels of unique taxa even between soil replicates within sites, Supplementary Figure S2.3), would account for a larger amount of diversity and might shed light on such factors/processes. Both stochastic and deterministic processes are important in structuring bacterial diversity (Gibbons and Gilbert 2015) and the approaches in this study do not account for other confounding factors that are important for structuring these communities, such as, among others, competition and predation,

historical events (e.g. fires, which are particularly important in fynbos), and dispersal limitations related to the mode of reproduction (Borcard *et al.* 1992). Spatial isolation has been suggested as a factor in promoting high soil bacterial turnover (Zhou *et al.* 2002). Therefore, the high variation in soil conditions between fynbos vegetation types (Mucina and Rutherford 2006) might serve as environmental barriers, effectively and indirectly isolating bacterial populations spatially, resulting in the observed high levels of diversity. In fact, high spatial turnover (i.e. species replacement) as observed in this study, in contrast to nestedness, suggests that barriers or selective differentiation exist between fynbos communities (Baselga 2010). Such barriers are in fact well established for plant communities of the fynbos, e.g. between the southern and south-western geographical units (across which sampling was conducted) (Manning and Goldblatt 2012; Ellis *et al.* 2014), and could thus explain the turnover in soil bacterial communities observed. Soil fungal community diversity in fynbos soils seems to be even higher than bacterial diversity (Slabbert *et al.* 2010). It remains to be explored whether similar temporal and spatial trends to findings presented here exist for fungal communities across environmentally heterogeneous fynbos sites.

Finally, there were many unique bacterial species even within single soil replicates (Supplementary Figure S2.3), as well as high turnover levels between replicates within sites (Supplementary Table S2.2, S2.3). A potential explanation for this is micro-scale changes in the bacterial community, e.g. associated with rapid microbial responses to plant root inputs (Bach *et al.* 2018). In fact, recent evidence suggests that different soil microaggregates support very diverse and distinct microbial and fungal communities (Bach *et al.* 2018), and it is likely that the large micro-scale diversity can be found in single soil replicates. Thus it seems that distinct bacterial communities at fine spatial scales reflect much of the aboveground fine scale patterns that have been observed for plants (Manning and Goldblatt 2012) and insects (Kemp *et al.* 2017) in fynbos, i.e. high specificity to local conditions.

The findings presented here suggest that fynbos soil bacterial community diversity and structure may mirror the unique diversity and endemism of their aboveground counterparts. Thus, considering the extremely high levels of plant diversity and endemism in fynbos (Manning and Goldblatt 2012),

together with its distinct and diverse soil types which lead to the formation of a multitude of unique microhabitats (Linder 2003, 2005; Cowling *et al.* 2009), there remains much to be discovered for the belowground components of this globally important biodiversity hotspot. For example, the causal pathways between above-ground communities, soil bacterial, and abiotic soil conditions provide unique and interesting future research directions. Furthermore, considering the threatened nature of many fynbos habitats (Mucina and Rutherford 2006) and the rate at which these environments are transformed (either by invasive non-native species or direct anthropogenic effects such as urbanization and agriculture), we advocate that the belowground components of these systems must be explored before they are lost. I argue that such unique biodiversity might represent a novel, but hitherto overlooked genetic resources (Stafford *et al.* 2005) that might prove invaluable towards the monitoring and rehabilitation of highly threatened habitats, especially given the fact that interaction between plants and soil biota are critical for the restoration of disturbed ecosystems (Philippot *et al.* 2013).

2.6. Acknowledgements

This research was funded by the South African National Research Foundation (Nos. 76912 and 89967 to JLR).

2.7. Tables and figures

Table 2.1: Top contributing taxa across all sites for various taxonomic levels. Values are percentage of classified sequences.

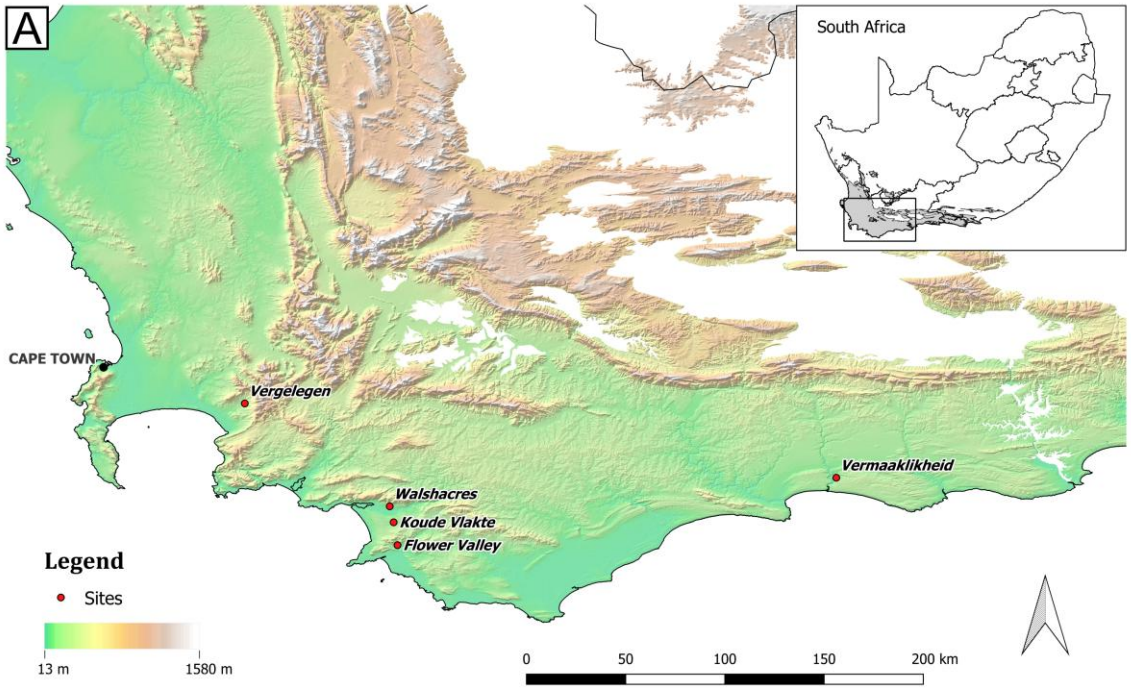
Level	Taxon	%	Level	Taxon	%
Phylum	Proteobacteria	37.11	Family	Mycobacteriaceae	11.17
	Actinobacteria	32.17		Acetobacteraceae	11.14
	Acidobacteria	25.63		Sphingomonadaceae	8.64
	Bacteroidetes	1.44		Bradyrhizobiaceae	7.08
	Firmicutes	1.32		Geodermatophilaceae	5.47
Class	Actinobacteria	32.37	Genus	*Mycobacterium*	16.36
	Alphaproteobacteria	23.02		*Bradyrhizobium*	8.60
	Acidobacteria_Gp1	13.12		*Gaiella*	6.95
	Betaproteobacteria	9.95		*Burkholderia*	5.61
	Acidobacteria_Gp3	8.32		*Blastococcus*	4.50
Order	Actinomycetales	27.34			
	Rhizobiales	14.44			
	Rhodospirillales	7.78			
	Burkholderiales	5.91			
	Sphingomonadales	5.81			

Table 2.2: Two-way ANOVA results for various diversity metrics of fynbos soil bacterial communities sampled during austral autumn and spring seasons. Significance indicated in bold and as follows: * – p < 0.05, ** – p < 0.01.

Diversity	Factor	df	Mean Sq	F	p
Richness	Site	4	185790	4.64	**0.005****
	Season	1	284766	7.11	**0.012***
	Site x Season	4	36211	0.90	0.474
	Residuals	30	40062		
Exponent of Shannon	Site	4	134966	3.58	**0.017***
	Season	1	492782	13.07	**0.001****
	Site x Season	4	30997	0.82	0.521
	Residuals	30	37711		
Inverse Simpson	Site	4	23530	2.75	**0.046***
	Season	1	75571	8.83	**0.006****
	Site x Season	4	8108	0.95	0.451
	Residuals	30	8561		
Pielou's Evenness	Site	4	0.0017	3.70	**0.015***
	Season	1	0.0058	12.50	**0.001****
	Site x Season	4	0.0004	0.91	0.47
	Residuals	30	0.0005		

Table 2.3: Permutational multivariate analysis of variance (PERMANOVA) results for soil bacterial communities of various pristine fynbos sites sampled during austral autumn and spring seasons. Significance indicated in bold and as follows: * – p < 0.05, *** – p < 0.001.

Factor	df	Mean Sq	F	p
Site	4	1.679	14.53	**<0.001*****
Season	1	0.278	2.41	**0.0298***
Site x Season	4	0.169	1.46	0.0781
Residuals	30	0.116		



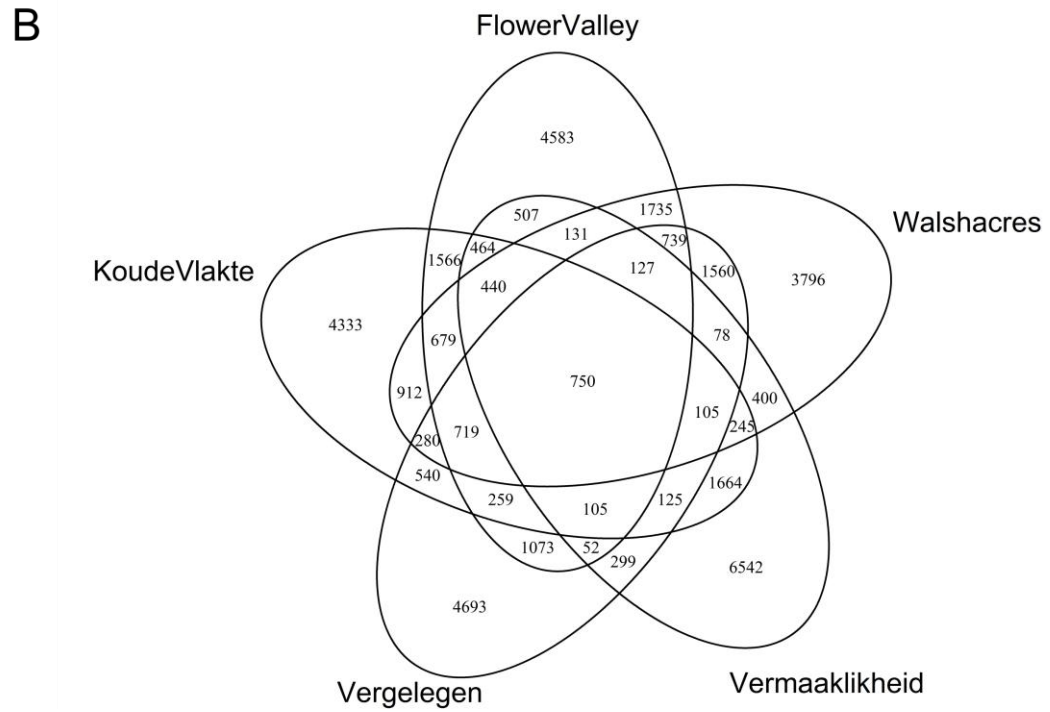


Figure 2.1: Site and soil bacterial OTU richness details. A) Five sites were selected within the boundaries of the Fynbos biome of South Africa's Core Cape Subregion for this study. B) Venn

diagram showing the distribution of soil microbial OTUs for all sites included here, with OTUs aggregated for season. Numbers in figures represent OTU richness (total number of OTUs for the dataset = 39 501). All sites shared a core number of OTUs (750 in total), but had a high number of unique OTUs.

Figure 2.2: Venn diagrams for each site and season (i.e. austral autumn or spring) combination. Numbers indicate OTUs that are unique or shared between seasons for each site (i.e. richness).

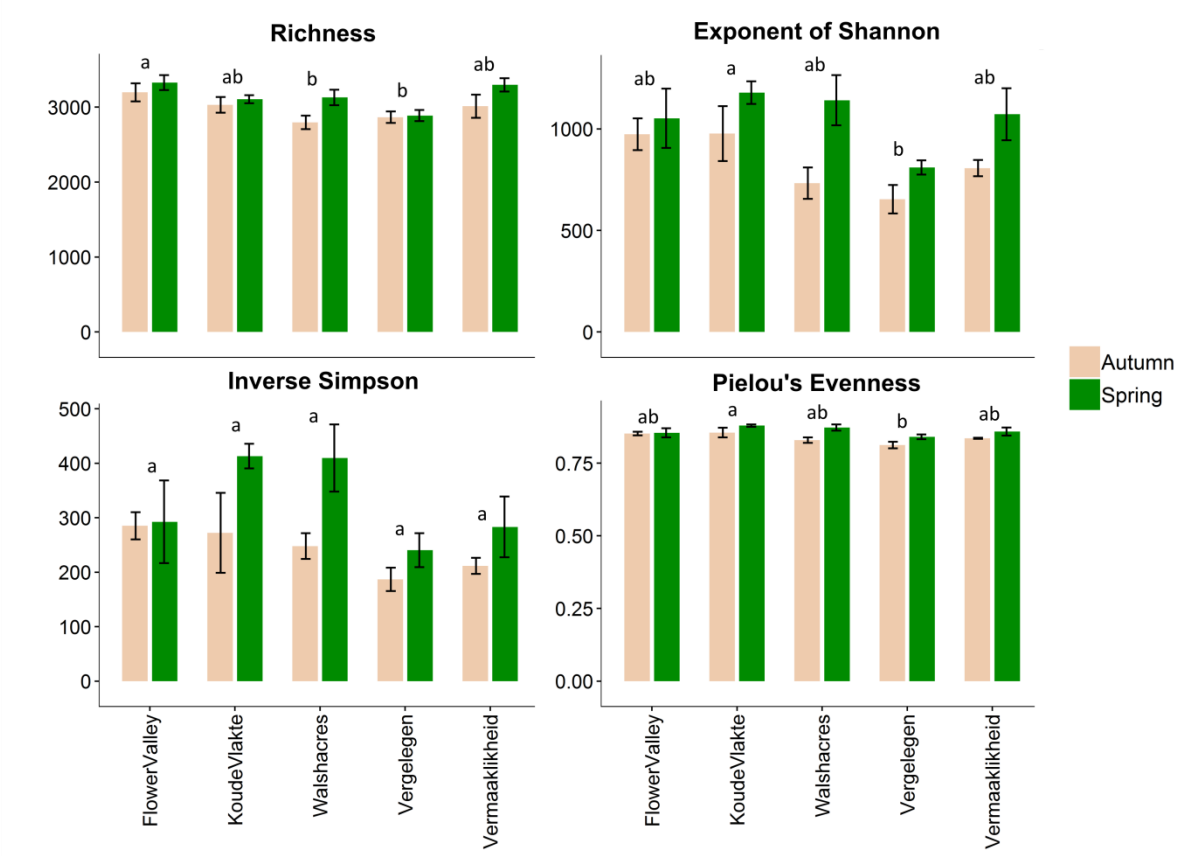


Figure 2.3: OTU alpha diversities for whole soil bacterial communities at various pristine fynbos sites sampled during austral autumn and spring seasons. All diversities were significantly higher in the spring season according to a two-way ANOVA. Letters indicate significant differences at $p < 0.05$ between sites from a Tukey HSD post hoc test.

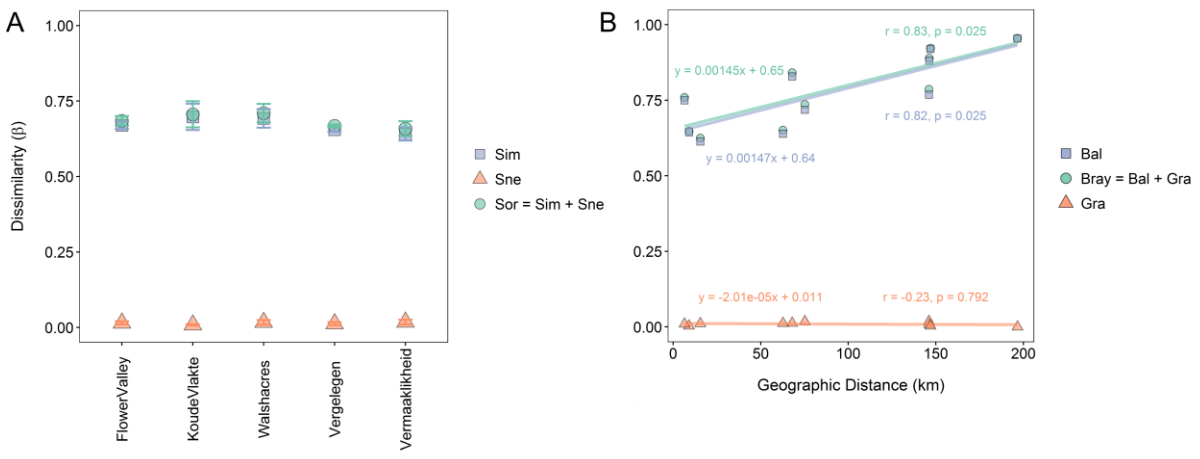


Figure 2.4: Soil bacterial beta diversity turnover for the five sites in this study was high and was almost completely due to soil bacterial OTU replacement instead of nestedness in the case of A) presence-absence temporal data. The same applied for abundance data B) where turnover was mostly due to balanced variation (Bal) component of Bray-Curtis dissimilarity instead of abundance gradient (Gra). A) temporal turnover between autumn and spring samples from each site, and B) Spatial turnover between sites (using community data aggregated across both seasons) plotted against geographic distance between sites. Both the balanced variation component of Bray-Curtis dissimilarity and overall Bray-Curtis dissimilarity increased with geographical distance (slopes: 1.47×10^{-3} and 1.45×10^{-3}, respectively). Also indicated in B) are Mantel correlations (Pearson r with corresponding p-values) between dissimilarities and geographic distance. Abbreviations: Sim = Simpson dissimilarity; Sne = nestedness component of Sørensen dissimilarity; Sor = Sørensen dissimilarity; Bray = Bray-Curtis dissimilarity; Bal = balanced variation component of Bray-Curtis dissimilarity; Gra = abundance gradient component of Bray-Curtis dissimilarity.

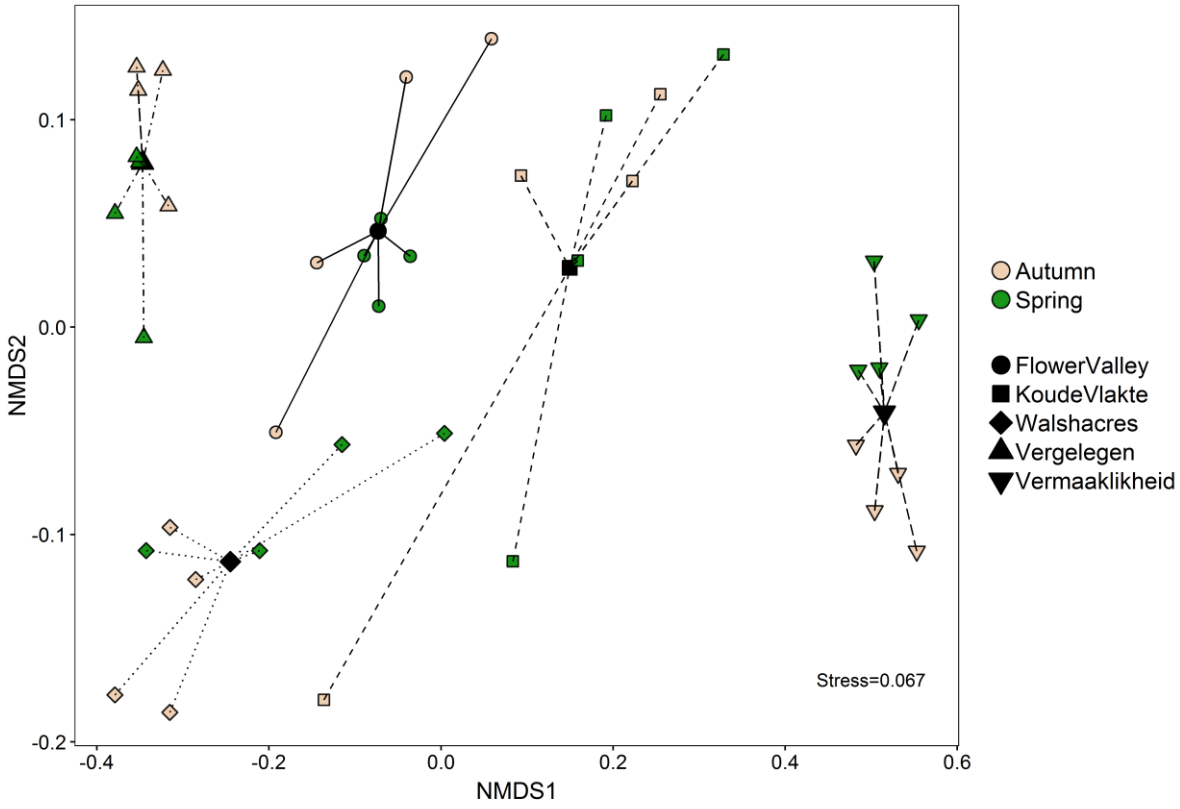


Figure 2.5: Non-metric multidimensional scaling (nMDS) plot of whole soil bacterial communities from various pristine fynbos sites sampled during austral Autumn and Spring seasons, based on Horn similarity values (Jost 2007). Lines indicate distances from individual samples (coloured symbols) to their respective site centroids (black symbols). The low stress value indicates that the plot is a good representation of community differences (i.e. not much variation is unaccounted for) (Clarke 1993).

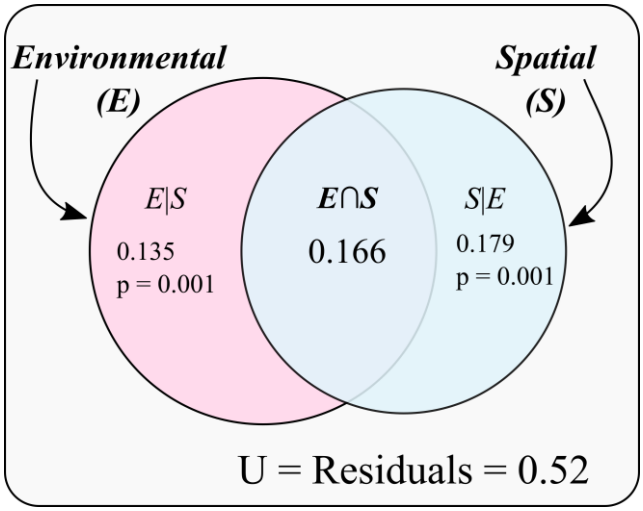


Figure 2.6: Variation partitioning of environmental (E) and spatial (S) components in explaining whole soil bacterial community structure. The bounding box indicates all variation in the community. Variation explained only by fraction E is indicated as E|S and that explained only by S as S|E. Shared variation (i.e. intersection) among E and S is indicated by E∩S. All unexplained variation is indicated by fraction U. Values indicated are RDA-adjusted R^2 values with significance added. Fractions U and E∩S cannot be tested for significance.

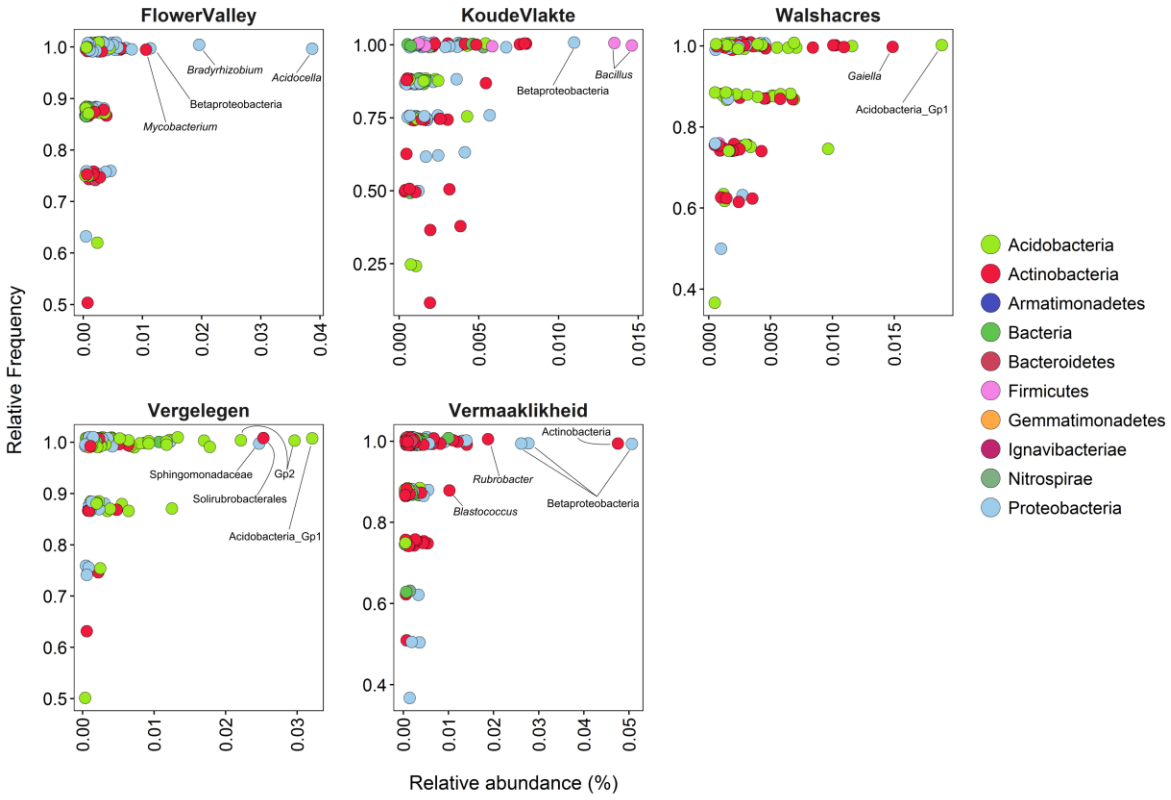


Figure 2.7: Linear Discriminant Analysis Effect Size (LEfSe) for biomarker taxa. Taxa that occurred in both high relative abundances and frequencies are the most important taxa that distinguish sites, corresponding to taxa that have the highest LDA scores. (Note: a small amount of jitter was added to the graph to give more clarity to overlapping dots).

2.8. Supplementary information

Table S2.1: Top ten biomarker taxa for each site. OTUs were selected by having both high relative abundances and frequencies across replicate samples within each site and then classified by the consensus taxonomy. Taxa are ordered from high to low in terms of relative abundance (all taxa listed occurred in all samples within each site, thus giving them relative abundances of 1). The "Highest Level" column indicates the finest scale to which individual OTUs could be classified. LDA scores listed are all significant at $p < 0.05$.

Site	LDA	Taxon	Highest Level
Flower Valley	4.10	*Acidocella*	Genus
	3.79	*Bradyrhizobium*	Genus
	3.50	Betaproteobacteria	Class
	3.50	*Mycobacterium*	Genus
	3.41	*Acidisoma*	Genus
	3.37	*Acidocella*	Genus
	3.29	*Gaiella*	Genus
	3.27	*Burkholderia*	Genus
	3.27	*Gaiella*	Genus
	3.22	Betaproteobacteria	Class
Koude Vlakte	3.59	*Bacillus*	Genus
	3.55	*Bacillus*	Genus
	3.48	Betaproteobacteria	Class
	3.31	Actinobacteria	Class
	3.30	*Aeromicrobium*	Genus
	3.35	*Kribbella*	Genus
	3.23	*Sphingomonas*	Genus
	3.12	Bacillaceae_1	Family
	3.18	Candidatus_Solibacter	Order
	3.17	*Nitrospira*	Genus
Vergelegen	4.01	Acidobacteria_Gp1	Class
	3.98	Gp2	Order
	3.90	Solirubrobacterales	Order
	3.87	Sphingomonadaceae	Family
	3.88	Gp2	Order
	3.77	Acidobacteria_Gp1	Class
	3.74	Acidobacteria_Gp1	Class
	3.61	Acidobacteria_Gp1	Class

Table S2.1 continued...

Site	LDA	Taxon	Highest Level
	3.59	*Bradyrhizobium*	Genus
	3.62	Acidobacteria_Gp1	Class
Vermaaklikheid	4.12	Betaproteobacteria	Class
	4.13	Actinobacteria	Class
	3.85	Betaproteobacteria	Class
	3.85	Betaproteobacteria	Class
	3.72	*Rubrobacter*	Genus
	3.59	*Solirubrobacter*	Genus
	3.60	Sphingomonadaceae	Family
	3.55	Sphingomonadaceae	Family
	3.55	Micromonosporaceae	Family
	3.44	Solirubrobacterales	Order
Walshacres	3.72	Acidobacteria_Gp1	Class
	3.67	*Gaiella*	Genus
	3.56	Acidobacteria_Gp3	Class
	3.50	*Mycobacterium*	Genus
	3.51	*Mycobacterium*	Genus
	3.49	Actinobacteria	Phylum
	3.42	*Mycobacterium*	Genus
	3.34	Acidobacteria_Gp1	Class
	3.31	Solirubrobacterales	Order
	3.31	Acidobacteria_Gp3	Class

Table S2.2: Within-site and within-season soil bacterial community turnover (i.e. between replicates). Values represent dissimilarities, i.e. a value of 1 would indicate complete turnover among all replicates. Abbreviations: Bal – Balanced variation, Gra – Abundance gradients, Bray – Bray-Curtis dissimilarity.

	Autumn			**Spring**		
Site	**Bal**	**Gra**	**Bray**	**Bal**	**Gra**	**Bray**
Flower Valley	0.72	0.01	0.73	0.74	0.02	0.75
Koude Vlakte	0.69	0.01	0.69	0.53	0.04	0.58
Walshacres	0.79	0.00	0.79	0.65	0.01	0.66
Vergelegen	0.74	0.01	0.76	0.66	0.02	0.68
Vermaaklikheid	0.59	0.05	0.64	0.62	0.06	0.68

Table S2.3: Between-site, and between-season, soil bacterial community turnover. Values represent dissimilarities, i.e. a value of 1 would indicate complete turnover among all replicates. Abbreviations: Bal – Balanced variation, Gra – Abundance gradients, Bray – Bray-Curtis dissimilarity.

Site	**Bal**	**Gra**	**Bray**	**Season**	**Bal**	**Gra**	**Bray**
Flower Valley	0.80	0.01	0.81	Autumn	0.93	0.01	0.93
Koude Vlakte	0.84	0.01	0.84	Spring	0.93	0.00	0.93
Walshacres	0.78	0.03	0.81				
Vergelegen	0.75	0.02	0.76				
Vermaaklikheid	0.75	0.04	0.79				

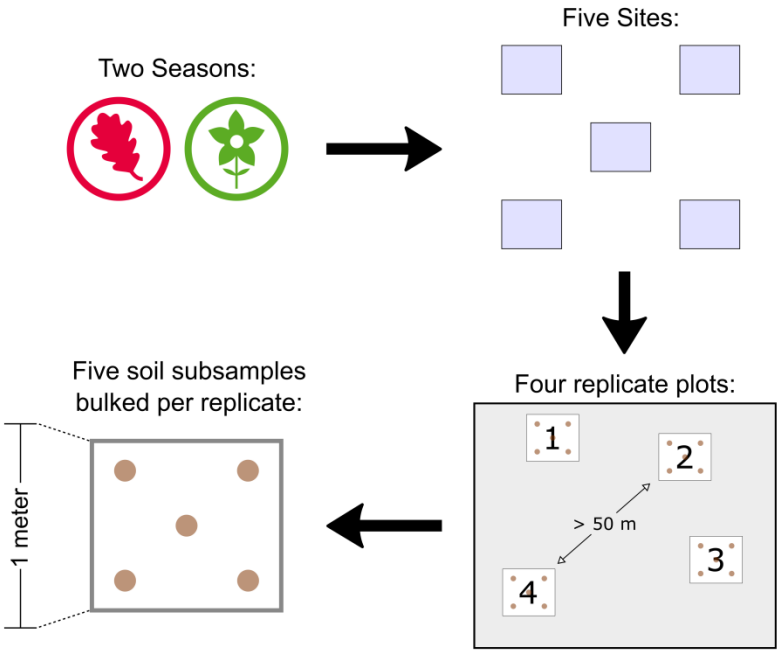


Figure S2.1: Sampling setup for soil sample collections. Each site comprised pristine fynbos vegetation. At each site four plots (1 m x 1 m) were randomly identified at least 50 m apart and within each plot five soil subsamples were collected. The subsamples for each plot were bulked to form one independent replicate, thus yielding a total of four independent replicates for each site. This was repeated for both austral Autumn and Spring, respectively. See Materials and Methods for detailed overview.

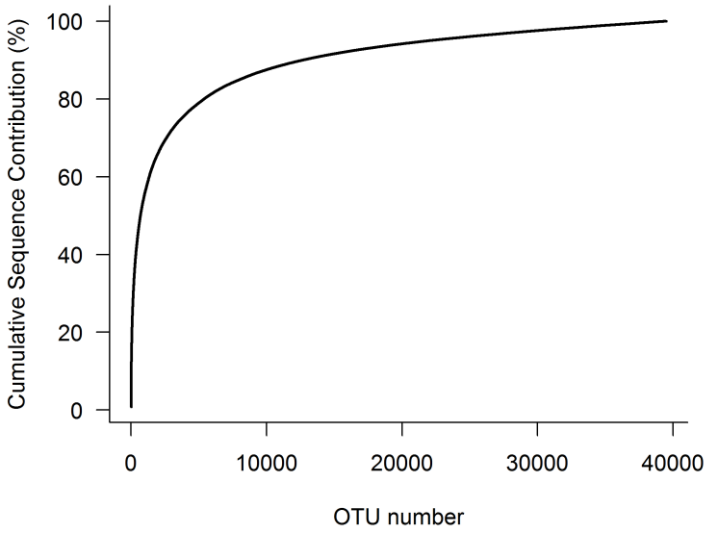


Figure S2.2: Cumulative percentage contribution of OTUs. A cumulative contribution of 80% marked a total of 5 371 OTUs. The dataset consisted of a total of 39 501 OTUs (564 346 sequence reads).

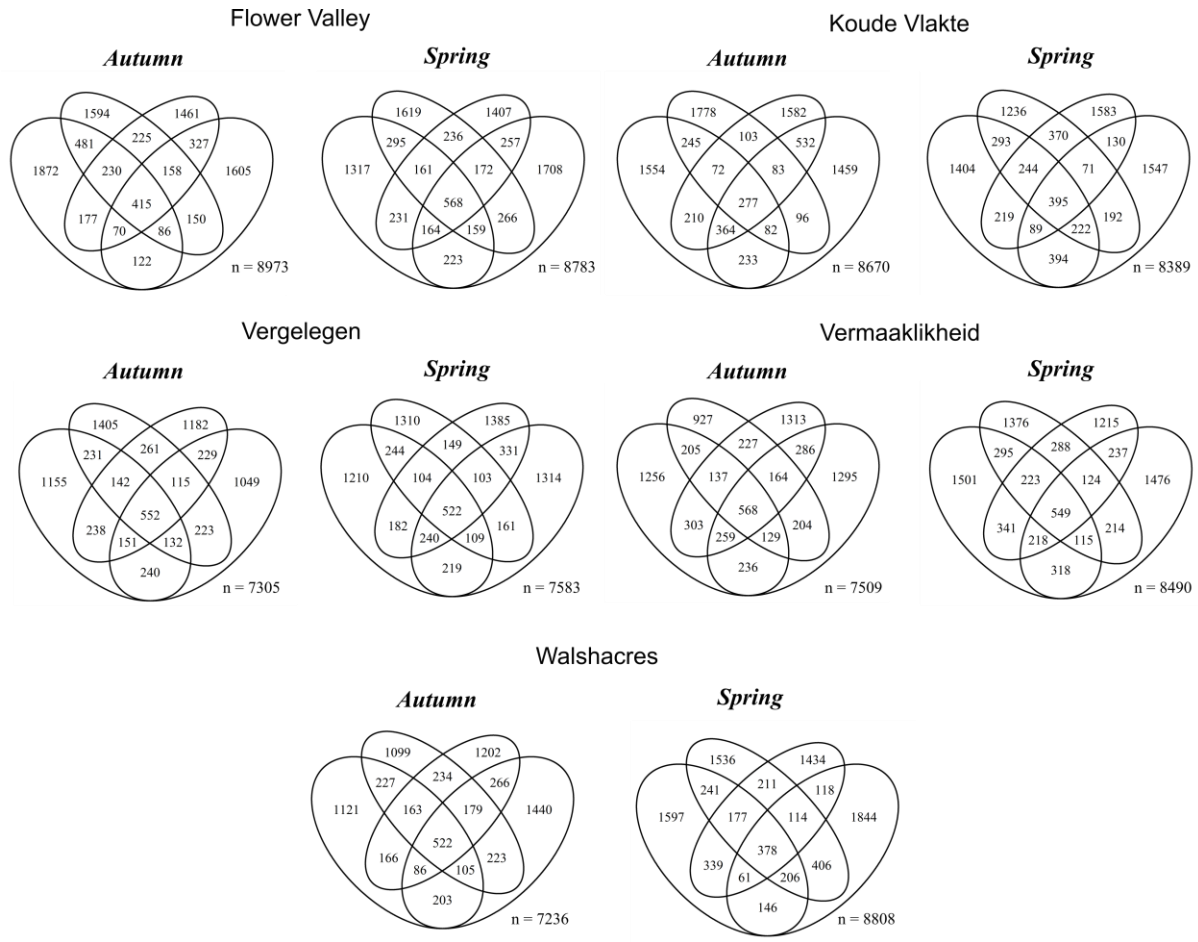


Figure S2.3: Venn diagrams of individual samples across sites and seasons. Numbers in diagrams are number of OTUs (i.e. richness). Each site and season combination consisted of four samples (n = 40 samples total).

		SITE1				SITE2				SITE3			
		Rep1	Rep2	Rep3	Rep4	Rep1	Rep2	Rep3	Rep4	Rep1	Rep2	Rep3	Rep4
SITE1	Rep1	0											
	Rep2	0	0										
	Rep3	0	0	0									
	Rep4	0	0	0	0								
SITE2	Rep1	1	1	1	1	0							
	Rep2	1	1	1	1	0	0						
	Rep3	1	1	1	1	0	0	0					
	Rep4	1	1	1	1	0	0	0	0				
SITE3	Rep1	1	1	1	1	1	1	1	1	0			
	Rep2	1	1	1	1	1	1	1	1	0	0		
	Rep3	1	1	1	1	1	1	1	1	0	0	0	
	Rep4	1	1	1	1	1	1	1	1	0	0	0	0

Figure S2.4: Example of the pairwise design matrix used to confirm whether replicates between sites were more different than replicates within sites. A value of one indicates complete turnover, whereas a value of zero indicates no turnover. Using the Horn dissimilarity matrix of OTUs and the design matrix in a mantel test to evaluate the hypothesis that replicates within sites are more similar than replicates between sites, a significant positive correlation would confirm higher between replicate values than within replicate values, whereas a negative correlation would indicate the opposite, and zero correlation would indicate no difference.

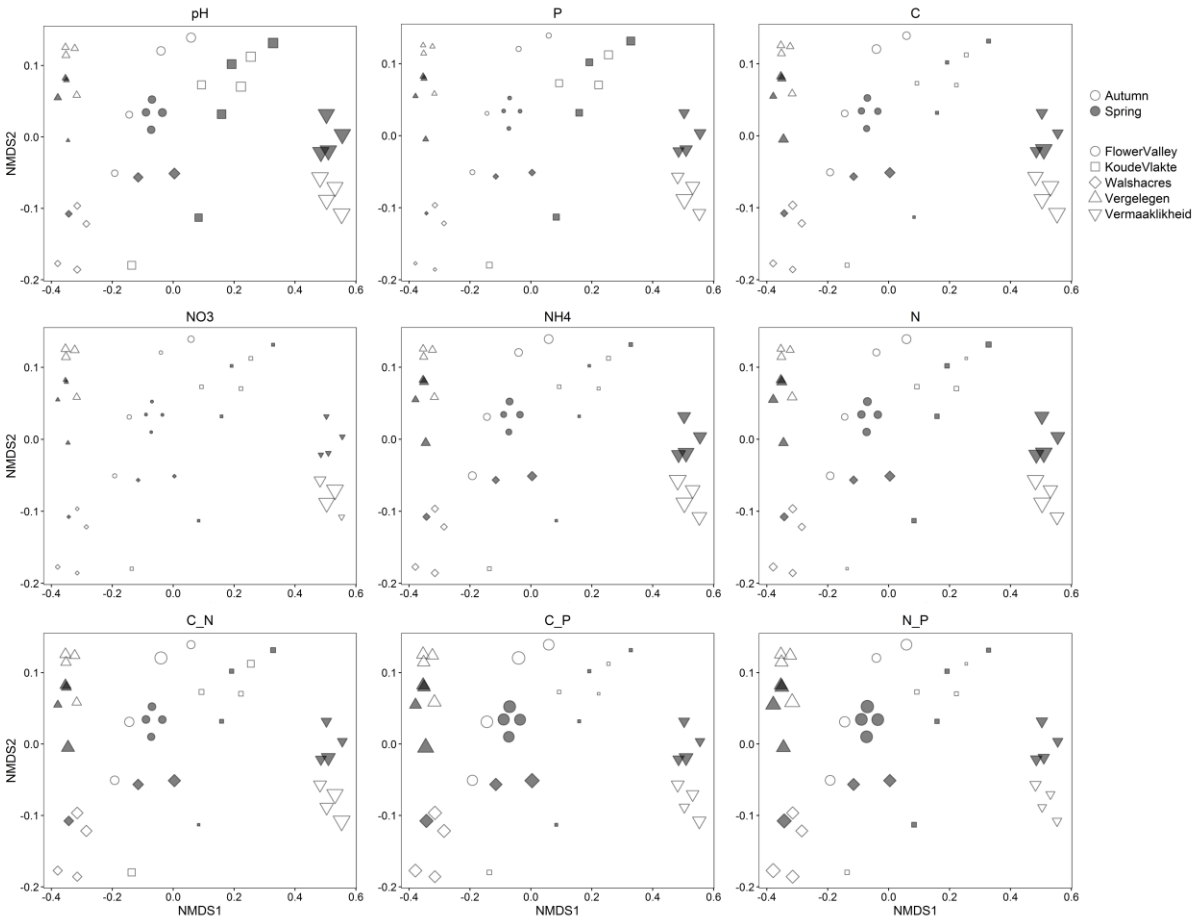


Figure S2.5: Non-metric multidimensional scaling (nMDS) plots of whole soil bacterial communities of pristine fynbos soils. nMDS scores (i.e. coordinates) of all plots are the same, but in each plot the size of symbols is proportional to the environmental variable assigned to that plot (e.g. pH, NO_3^-, etc.). Note there are only three replicates shown for Walshacres during spring as one sample did not have associated soil data. nMDS stress value = 0.067.

CHAPTER 3: Invasive Australian acacias significantly alter whole soil bacterial communities in South Africa's hyper-diverse fynbos

Candidate Journals: New Phytologist; Frontiers in Microbiology, FEMS Microbiology Ecology

3.1. Abstract

Although the impacts of invasive non-native plants on aboveground components of the communities they invade have been well studied, the situation regarding soil bacterial communities remains less well understood. This is surprising given the pivotal roles these belowground communities play in the functioning of ecosystems. South Africa's Cape fynbos is highly fragmented and threatened, and although much is known about the impacts invasive plants have on fynbos plant communities, their impacts on below-ground communities are largely unknown. Here, by utilising next-generation sequencing techniques, I aimed to investigate the impacts of invasive Australian *Acacia* spp. on soil bacterial components of fynbos, such as bacterial community diversity and composition. I was also interested in determining invader effects on the main spatial and environmental patterns of soil bacterial community turnover in fynbos. I found acacias to significantly alter soil bacterial community composition, but not diversity. This compositional change in bacterial communities was primarily driven by acacia-induced changes of soil pH and NH_4^+. I also found acacias to reduce spatial variability across soil communities, such that community turnover could no longer be predicted by geographical distance, as was the case for pristine soils.

KEYWORDS: 16S rDNA, Australian acacias, community composition, fynbos, invasion, next-generation sequencing, soil microbial diversity.

3.2. Introduction

Impacts by invasive non-native plants on aboveground components of the communities they invade are easily observed, and not surprisingly, have received much research attention (Brussaard *et al.* 1997). These impacts, amongst others, often include the alteration of fire regimes (Mack and

D'Antonio 1998), replacement of native vegetation (Ehrenfeld 1997), prevention of access to watercourses (Rahman *et al.* 2003), serving as hosts for pests and diseases (Jin 2011), and even acting as drivers of ecosystem regime shifts (Gaertner *et al.* 2009, 2014). Contrary to this, the belowground impacts of invasive plants, such as changes in soil chemistry, microbiota and soil bacterial communities (Yelenik *et al.* 2004; Yelenik and D'Antonio 2013; Checcucci *et al.* 2016; Broadbent *et al.* 2017; Carey *et al.* 2017; Xiang *et al.* 2018; Zhang *et al.* 2018) have received comparatively little attention. This is surprising given the known importance of soils in the functioning of ecosystems and therefore, their usefulness as a proxy for invader impacts.

Soil microorganisms are pivotal in the functioning of ecosystems (Gibbons and Gilbert 2015). They are responsible, for example, for the decomposition of organic matter, cycling of nutrients (Fisk and Fahey 2001), and the suppression of soil borne pests and diseases (Brussaard *et al.* 1997). Soil bacterial communities also represent a considerable portion of plant-symbiotic interaction networks (Coats and Rumpho 2014), and therefore can directly and indirectly influence the diversity and structure of aboveground communities through plant-soil feedbacks (Slabbert *et al.* 2010, 2014). For example, microbial community composition in South Africa's hyper diverse fynbos soils is strongly correlated with aboveground plant community composition at small spatial scales (Slabbert *et al.* 2010; Miyambo *et al.* 2016). This is partly because soil bacterial community composition has been found to directly relate to the identity of plant species present in communities (Elgersma *et al.* 2012), i.e. growth rates of microbes are host-dependent and the identity of the local hosts will therefore determine soil community composition (Reynolds *et al.* 2003). Conversely, the relative abundance of plant species within communities is influenced by soil microorganisms and their associated feedback effects (Klironomos 2002), either as positive or negative feedback loops. It has been suggested that a key strategy for the success of certain invasive plants lies in the mediation of changes to soil microbiota and resulting competitive advantages over native plants (Malinich *et al.* 2017). Invasive plants often lack their specialist enemies, including soil pathogens, so that negative plant-soil feedbacks do not impact their competitiveness under new environmental conditions (Lekberg *et al.* 2018).

Invasive plants can modify biotic and abiotic components of soils in various ways (Yelenik *et al.* 2004; Lorenzo *et al.* 2010a; Weidenhamer and Callaway 2010; Rodríguez-Echeverría *et al.* 2011). For example, they can drastically influence nutrient and water cycling, and their availability in soils (Bohlen 2006). The timing and amount of water used by invasive plants can lead to soil water depletion (especially in seasons when low soil water recharge is experienced), in turn impacting on the soil bacterial community and nutrient turnover (Enloe *et al.* 2004). High densities of invasive plants are also often correlated with higher organic (leaf litter) inputs and decomposition rates with subsequent increases in soil mineralization, nitrification and organic carbon content (Liao *et al.* 2008). In addition, nitrogen-fixing species, such as legumes, can modify soil nitrogen accumulation and cycling rates (Corbin and Antonio 2004; Rice *et al.* 2004). Many invasive plants also alter soil chemistry through the release of novel biochemical secondary metabolites (Weidenhamer and Callaway 2010). Taken together, invasive plants therefore possess the capacity to significantly alter soil bacterial communities.

The numerous effects that invasive plants can have on soils is well-illustrated by invasive Australian acacias (genus *Acacia* Mill.). In many invasive ranges, acacias have been found to alter soil nitrogen, carbon and phosphorous content (Witkowski 1991; Yelenik *et al.* 2004; Souza-Alonso *et al.* 2015), as well as microbial community structure and function (Lorenzo *et al.* 2010b; Rodríguez-Echeverría *et al.* 2011; Souza-Alonso *et al.* 2015; Kamutando *et al.* 2017; Le Roux *et al.* 2018). Such soil alterations can lead to so-called legacy effects, e.g. elevated soil nutrient levels that can persist, even for decades, after the removal of invasive biomass (Marchante *et al.* 2009; Elgersma *et al.* 2012; Nsikani *et al.* 2017). These legacy effects may explain why native species are usually slow to recover in sites cleared of acacias (Holmes and Cowling 1997; Daehler 2003). Acacias have also been shown to reduce microbial diversity and to enrich soils for certain bacterial genera (Slabbert *et al.* 2010, 2014; Le Roux *et al.* 2018). In South Africa's hyper diverse fynbos, acacia invasions also tend to homogenise soils or certain bacterial groups, even over large spatial scales (e.g. mutualistic rhizobial communities, Le Roux *et al.* 2018). Fynbos soils have high soil bacterial beta diversity (high rates of species turnover between sites), which is a result of high levels of unique bacterial species associated

with different soil conditions and aboveground communities (Chapter 2). Thus, acacia invasions can potentially reduce, or possibly even eliminate, unique bacterial species from these floristically diverse sites.

South Africa's Core Cape Subregion has long been recognised as a biodiversity hotspot of international significance (Myers *et al.* 2000; Myers 2003), with high levels of plant diversity and endemism (Manning and Goldblatt 2012), primarily driven by a mosaic of different soil types and habitats across a relatively small geographic area (Linder 2003, 2005; Cowling *et al.* 2009). Specifically, fynbos is the most distinctive vegetation type of the Core Cape Subregion, and also highly fragmented and thus threatened (Manning and Goldblatt 2012). As discussed, much is known about the impacts invasive acacias have on fynbos aboveground plant communities (Holmes and Cowling 1997; Le Maitre, Gaertner, *et al.* 2011; Krupek *et al.* 2016; Mostert *et al.* 2017; Fill *et al.* 2018), but their impacts on belowground communities are largely unknown (but see Slabbert *et al.* 2014; Le Roux *et al.* 2018). Here I made use of the situation in fynbos by comparing whole soil bacterial communities of neighbouring acacia-invaded and uninvaded pristine habitats, spanning a large geographic range throughout fynbos vegetation. The overall goal was to investigate the impacts of invasive *Acacia* spp. on soil bacterial components of fynbos. Specifically, I aimed to determine whether invasive acacias: 1) reduce fynbos soil bacterial alpha diversity, 2) alter fynbos microbial community composition and structure (beta diversity), and 3) alter the main spatial and environmental patterns of soil bacterial community turnover in fynbos. I hypothesised that, as a result of the nitrogen-fixing ability, in conjunction with high organic litter input of dense acacia stands, soil bacterial diversity and composition will differ significantly between invaded and pristine sites.

3.3. Materials and methods

3.3.1. Study sites

To limit within-site variability in soil conditions and other confounding factors, I selected five study sites where areas heavily invaded by Australian acacias occurred in close proximity (<500 m) to pristine areas (also see Chapter 2). These paired sites spanned a wide geographic range (Figure 3.1):

Vergelegen Wine Estate (VG; 34.056°S, 18.934°E), Vermaaklikheid (VM; 34.358°S, 21.038°E), Koude Vlakte Conservancy (KV; 34.475°S, 19.455°E), Walshacres (WA; 34.420°S, 19.442°), and Flower Valley (FV; 34.559°S, 19.470°E). *Acacia cyclops* A.Cunn. ex G.Don was the dominant invasive species at VM, KF, FV, while *A. saligna* (Labill.) H.L.Wendl. and *A. longifolia* (Andrews) Willd. were dominant species at WA and VG, respectively.

3.3.2. Field collections and sample preparation

Soil sampling took place during the Austral Autumn and Spring seasons of 2016. I sampled two seasons since whole soil bacterial diversity and composition are known to display seasonal variation (Slabbert *et al.* 2014) which may be particularly pertinent in regions experiencing strong seasonality like South Africa's Core Cape Subregion. At each site four random plots of 1 m x 1 m were identified within each treatment: invaded vs. pristine (Supplementary Figure S3.1). In each of these plots five soil subsamples of approximately 50 g each were taken randomly within the first 10 cm of the soil surface. All samples were collected away from plants to avoid roots. Where present, the top layer of litter/organic material was removed before soil collection. For each plot, the five collected soil subsamples were bulked and mixed, resulting in four independent replicates per invaded and pristine treatment (n_{total} = 80: 4 replicates x 2 treatments [acacia-invaded vs. pristine] x 5 sites x 2 seasons). Soil samples were kept on ice during transport and were immediately stored at -80°C upon arrival at the lab.

3.3.3. Soil abiotic variables

The following soil variables were analysed for all collected samples: pH, Olsen phosphorous (P), total carbon (C), nitrate content (NO_3^-), ammonium content (NH_4^+), and total available nitrogen (N). Analyses were conducted at BemLab (South African National Accreditation System Accredited Testing Laboratory, Somerset West, South Africa), according to standard quality control procedures (SSSA 1996).

3.3.4. Soil DNA extraction and sequencing

For whole soil microbiome analysis total genomic DNA was extracted from 0.25g of each soil sample using the PowerSoil® DNA extraction kit (MO BIO laboratories Inc., Carlsbad, CA, USA) and following the manufacturer's protocol. The 16S rRNA gene was amplified using the primers 799F (5'-AAC MGG ATT AGA TAC CCK G-3') and 1391R (5'- GAC GGG CGG TGW GTR CA-3'), with sample-specific barcodes in the forward primer. Amplification was done using a 30 cycle PCR and the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following PCR conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, followed by a final elongation at 72°C for 5 minutes. After amplification, PCR products were checked on a 2% agarose gel for amplification success and the relative intensity of bands. Multiple PCR samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, Beverly, MA, USA) and used to prepare DNA libraries by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) following the manufacturer's guidelines.

3.3.5. Bioinformatics

All raw MiSeq DNA sequence data were processed following standard procedures as described in Schloss *et al.* (2011) using the mothur version 1.37.1 software (Schloss *et al.* 2009). Briefly, after removal of low-quality sequences and optimizing sequence lengths (to between 383 and 395 bp), unique sequences were aligned to the SILVA-ARB (release 123) reference database. Sequences were aligned to the same region of the 16S rRNA gene and columns containing only gaps were removed. All chimeric sequences were removed independent of a reference database using the uchime algorithm (Edgar *et al.* 2011) and the template as self, i.e. *de novo* removal. Sequences were subsequently clustered into Operation Taxonomic Units (OTUs) at the 97% sequence similarity level. Representative sequences for individual OTUs were chosen as those that were most abundant in each OTU cluster. The ribosomal database project Classifier (Q Wang *et al.* 2007) was used to determine

the taxonomic identity of each OTU, and all sequences classified as chloroplast, mitochondria, and archaea, were removed. In order to standardise the number of reads across all replicates I subsampled an equivalent number from each of the 80 replicate samples. Finally, singleton and doubleton OTUs were removed, leading to final read count of 1127770 representing 48169 OTUs. A limitation on the classification of bacterial OTUs from next-generation sequencing techniques results from the incompleteness of current sequence databases (Thompson *et al.* 2017). However, it does not detract from the usefulness of using OTUs in the calculation of various diversity metrics (both alpha and beta).

3.3.6. Statistical analyses

All statistical analyses were conducted in R statistical environment (version 3.5.1) (R Core Team 2017), unless otherwise specified. For diversity analyses I used the sample x OTU matrix to calculate species richness, the exponent of Shannon diversity, Inverse Simpson diversity and evenness (OTU abundance equality) (Hill 1973). I specifically made use of the exponent of Shannon diversity and Inverse Simpson diversity since these represent true diversities (i.e. "effective species"), in contrast to other diversity indices (Jost 2006, 2010). These were calculated with the function `renyi` in the R package `vegan` (version 2.3-3) (Oksanen *et al.* 2016), which calculates true diversities as a set of specified Hill numbers (^0D = richness, ^1D = exponent of Shannon, ^2D = Inverse Simpson) (Hill 1973). I calculated evenness as H/log(S) (Hill 1973), with log being the natural logarithm. Diversity metrics were then analyzed with two-way ANOVAs (factors: treatment and season) with site as random factor.

In order to visualise community composition I used function `metaMDS` to create non-metric multidimensional scaling (nMDS) plots based on Horn similarity values (Jost 2007), created with the function sim.table in `vegetarian` R package (Charney and Record 2012), and subsequently fitted environmental variables as smoothing surface with function `ordisurf` to the NMDS ordination. I tested variation in community composition by Permutational Multivariate Analyses of Variance

(PERMANOVA) with 9999 permutations using the `adonis` function in the `vegan` R package, with invasion treatment and season as fixed factors, and site as a random factor.

I used the package `betapart` (Baselga and Orme 2012) to describe beta diversity between sites and seasons for invaded and pristine sites. Specifically, I was interested in disentangling the two components of total beta diversity (calculated as Bray-Curtis [BC] dissimilarity, β_{bray}), which are the OTU balanced variation component of BC dissimilarity (β_{bal}, akin to the replacement component of presence-absence data) and the abundance gradient component of BC dissimilarity (β_{gra}, akin to the nestedness component of presence-absence data) (Baselga 2017). These reflect the abundance equivalents of OTU replacement and nestedness, respectively (Baselga 2010). Disentangling such components is important for conservation purposes, since the two components would require antithetic management strategies: if turnover is a result of nested subsets, then conservation can focus on a small number of the richest sites, whereas if turnover is a result of replacement, then focus shifts to conservation of a large number of different sites (which are not necessarily the richest ones) (Baselga 2010). First, I used the function `beta.multi.abund` to assess overall multiple-site dissimilarity. I then used the function `beta.pair.abund` (which calculates pairwise dissimilarities between all samples) to assess the effect of geographic distance on whole soil bacterial OTU dissimilarity and to disentangle the two components of total beta diversity turnover (Baselga 2010). Dissimilarity distances necessarily lack independence between observations, which precludes testing significance by means of traditional regression procedures. I therefore used Mantel permutation tests (9999 permutations) with the function `mantel` in the `vegan` R package to test for significance of the Pearson correlations between dissimilarity values and geographic distances. I also fitted linear models to each of the dissimilarity components to assess rates of turnover. To detect significant differences in intercepts and slopes between invaded and pristine treatments for the different similarity measures, I used bootstrapping to estimate the frequency distributions of the parameters. For this, I used the `boot` R package (Canty and Ripley 2017) to create frequency distribution of 9999 linear model slopes and intercepts. To assess whether the significance of one parameter was larger in one treatment over the

other, I used the estimated distributions of parameters to empirically compute the probability of obtaining the opposite result by chance.

To determine the extent to which spatial and environmental variables drive differences in soil bacterial community composition between invaded and pristine soils I used functions from the `vegan` R package (Oksanen *et al.* 2016). First, I converted longitude–latitude coordinates to a set of spatial variables (S) using principal coordinates of neighbour matrices (PCNM) (Borcard and Legendre 2002; Griffith and Peres-Neto 2006) with the `pcnm` function. Since the community data table contained many instances of very low abundance OTUs, I only used OTUs that represented a cumulative contribution of 80%, I also used Hellinger transformation to account for the presence of many zero abundances (Legendre and Gallagher 2001). I used the `ordistep` function with forward selection to find the set of environmental and spatial variables, respectively, that explained the variation in the community data the best (Ramette 2007; Stomeo *et al.* 2013). Since the sites spanned a wide geographic range, I conditioned out the effect of site in the model selecting procedure (i.e. partial distance-based redundancy analysis [db-RDA]). I used the PCNM variables together with the set of environmental (E) variables and subset OTU table in db-RDA variation partitioning (Borcard *et al.* 1992; Legendre and Andersson 1999; Peres-Neto *et al.* 2006) with the `varpart` function; I did this analysis for both invaded and pristine communities. This establishes the extent to which either pure environmental (E|S) or pure spatial (S|E) variable fractions, or their intersection (E∩S), explain variation in microbial community composition. This analysis also indicates the amount of variation that remains unexplained (U = 1 - [E + S]). I tested the significance of fractions E|S and S|E with 9999 Monte Carlo permutations; fractions U and E∩S are not testable. Unfortunately, the `varpart` function cannot handle missing data, and since I did not have soil data for one of the replicate samples, I removed it from this analysis.

Finally, I wanted to identify bacterial taxa that characterise soil bacterial communities between invaded and pristine areas in the study sites. For this I used linear discriminant analysis (LDA) effect size (LEfSe) (Segata *et al.* 2011), implemented in mothur software (using "site" as subclass to correct

for natural variation between sites). In order to visualise such biomarker taxa I plotted their relative abundance and frequency of occurrence across each sample type. The LEfSe method identifies bacterial biomarkers via a non-parametric Kruskal-Wallis rank sum test to detect significant features, after which an LDA is performed for effect size estimation (Segata *et al.* 2011). Biomarkers were identified using an alpha value of 0.05 and an effect size threshold (i.e. LDA score) of 2.

3.4. Results

In the analysis of the soil bacterial communities associated with acacia-invaded and pristine areas across all sites, I obtained 1127770 high-quality sequencing reads after data cleaning, which resulted in 48169 OTUs (97% cut-off) representing 331 genera, 134 families, 78 orders, 44 classes and 19 phyla. Approximately 72.7% (820435) of sequences could not be classified to genus level, 60.5% (682031) to family level, 37.7% (424974) to order level, 10.4% (117840), and 7.8% (87655) to phylum level. Unfortunately, the short reads associated with next generation sequencing data often preclude identification to species level.

The most abundant phyla across all replicates were Proteobacteria (34.8%), followed by Actinobacteria (28.7%) and Acidobacteria (23.2%). At class level Actinobacteria (28.1%) were most abundant, followed by Alphaproteobacteria (20%), Acidobacteria_Gp1 (11.9%), Betaproteobacteria (9.9%), and Acidobacteria_Gp3 (6.3%). Compared to pristine soils, invaded soils were significantly depauperate for Alphaproteobacteria (Kruskal-Wallis $\chi^2 = 4.7$; $P < 0.05$), whilst enriched for Gammaproteobacteria (Kruskal-Wallis $\chi^2 = 9.5$; $P < 0.01$) and Nitrospirae (Kruskal-Wallis $\chi^2 = 5.0$; $P < 0.05$) (Figure 3.2).

Acacia-invaded soils harboured 39829 OTUs while pristine soils had 39501 OTUs. 8668 OTUs (35204 sequences or 3.12% of total) were unique to invaded soils while 8340 (35440 sequences or 3.14%) were unique to pristine soils. Invaded and pristine soils shared 31161 OTUs (1057126 sequences or 93.7%, Figure 3.3A). When considering seasonal variation, I found a total of 7037 OTUs (24839 sequences or 2.2%) to be unique to Autumn while 7953 OTUs (29983 sequences or

2.66%) were unique to Spring. Both seasons shared 33179 OTUs, representing 1072948 sequences or 95.1% of all sequences (Figure 3.3B). Across both seasons, all five sites shared a core number of 1625 OTUs (587433 sequences or 52.1% of total, Figure 3.3C), while they collectively harboured 15385 site-specific OTUs (70162 sequences or 6.22% of total).

All alpha diversities were significantly higher in Spring, irrespective of invasion treatment (Figure 3.4, Table 3.1). Invasion did not significantly alter any of the diversity metrics, and I observed no interactive effects between season and invasion. The random site factor, however, was significant for all diversities and thus I explored the invasion treatment and seasonal effects further for each site separately. At site level, acacia invasion significantly lowered all diversities at site Vergelegen, irrespective of season (Supplementary Table S3.1). However, for site Vermaaklikheid the presence of invasive acacias increased both soil bacterial richness and exponent of Shannon diversity.

Acacia invasion significantly, and consistently, altered soil bacterial community composition ($F_{PERMANOVA}$=2.10, p=0.0001) (Figure 3.5, Table 3.1, Supplementary Figure S3.2); microbial community composition was also significantly affected by seasonal changes ($F_{PERMANOVA}$=1.27, p=0.0002).

At site level I observed a significant positive correlation for both β_{bray} and β_{bal} with geographical distance for pristine soil bacterial communities (r_{Mantel} = 0.83, p = 0.025 and r_{Mantel} = 0.82, p =0.025, respectively), whereas there was non-significant negative correlation for β_{gra} (r_{Mantel} = -0.23, p = 0.792) (Figure 3.6). I observed positive correlations for β_{bray} and β_{bal} diversity components for invaded communities, however they were not significant (β_{bray}: r_{Mantel} = 0.18, p = 0.175; β_{bal}: r_{Mantel} = 0.17, p = 0.175; β_{gra}: r_{Mantel} = 0.00, p = 0.508). Overall, beta diversity of soil bacterial communities showed a higher increase with geographical distance for pristine communities (β_{bray} slope = 0.00145, intercept = 0.65) compared to invaded (slope = 0.000435, intercept = 0.74). However, these were not significantly different (p_{slope} = 0.105, $p_{intercept}$ = 0.823). Beta dissimilarity was almost completely due to balanced variation for both invaded and pristine soils (β_{bal} invaded: slope = 0.000435, intercept =

0.73; pristine: slope = 0.00145, intercept = 0.65), with very little abundance gradient contributions (β_{gra} invaded: slope = 4.29 x 10^{-8}, intercept = 0.0061; pristine: slope = -2.01 x 10^{-5}, intercept = 0.011). Again, both these components were not significantly different between invasive and pristine soils (β_{bal}: p_{slope} = 0.115, $p_{intercept}$ = 0. 830; β_{gra}: p_{slope} = 0.663, $p_{intercept}$ = 0.095). Note that intercept and slope values for β_{bray} are the sum of intercept and slope values of by β_{gra} and β_{bal}, respectively.

After forward selection of environmental components to determine bacterial community composition I retained pH and NH_4^+ as significant predictors of community composition in pristine sites and pH and season as significant predictors in invaded sites. I determined PCNM's 1, 3, and 4 to be significant predictors for the spatial component for both pristine and invaded sites. Variation partitioning results were similar for both invaded and pristine soils for the total environmental contribution (E), each explaining about 30% of the variation in the species matrix (Figure 3.7), while total spatial contributions (S) explained more variation in pristine than invaded soils (34.5% vs. 25.7%). The proportions of environmental and spatial components that uniquely explained variation in pristine and invaded soils (i.e. E|S and S|E) were opposite in trend, with the environmental component explaining less variation in pristine soils (13.5% pristine vs. 18.4% invaded), whereas the spatial component explained more variation in pristine soils (17.9% pristine vs. 14.6% invaded). Overall, there was also less variation shared between environmental and spatial components for pristine compared to invaded soils (16.6% pristine vs. 11.1% invaded). A large amount of variation remained unexplained for both soil types (52% pristine vs. 56% invaded) indicating that there are other intrinsic processes responsible for community structuring that are still unaccounted for, e.g. plant community composition.

Finally, analyses of biomarker taxa highlighted numerous taxa that are characteristic to invaded and pristine areas at the sites included here (Figure 3.8, Supplementary Table S3.2). For Flower Valley, pristine soils were characterised by *Acidocella* and *Acidisphaera*, and invaded soils to be characterised by *Gemmatimonas*. For Koude Vlakte, pristine soils were characterised by Betaproteobacteria while invaded soils were characterised by Actinobacteria. For Vergelegen,

Solirubrobacterales and *Sphingomonas* were the characteristic taxa of pristine soils, and Acidobacteria_Gp1 and *Burkholderia* were characteristic of invaded soils. Vermaaklikheid pristine soils were characterised by *Rubrobacter* and invaded area by *Solirubrobacter*. Finally, Walshacres pristine soils were characterised by *Acidocella* and Acidobacteria_Gp1, while invaded soils were characterised by Burkholderiales_incertae_sedis.

3.5. Discussion

I expected soil bacterial alpha diversity to be higher in pristine fynbos soils compared to acacia-invaded soils, an observation that has been repeatedly made (Slabbert *et al.* 2014; Kamutando *et al.* 2017; Le Roux *et al.* 2018). However, the data do not support this general notion and, instead, I observed little differences in diversities between invaded and pristine soils as a whole and the effects of invasion appeared to be site specific and not consistent across sites (Table 3.1, Figure 3.4). The discrepancy between these findings and those reported previously may stem from the small spatial scales (e.g. Slabbert *et al.* 2014; Le Roux *et al.* 2018), bacterial taxonomic level (e.g. rhizobia only, Le Roux *et al.* 2018), or sampling design (e.g. comparing rhizosphere soils to soils >5m away, Kamutando *et al.* 2017) used in previous studies. Scale is an important factor in soil bacterial biogeography (Fierer and Jackson 2006; Martiny *et al.* 2011; O'Brien *et al.* 2016). Bacterial communities can show heterogeneity (both high alpha and beta diversity) even at very small spatial scales, i.e. centimetre scale or rhizosphere level (O'Brien *et al.* 2016), which is usually related to soil micro-structure and organic matter distribution (Vos *et al.* 2013). Various biotic factors can also impact on bacterial community structures and diversities at these scales, such as roots (alter physical environment and resource availability, associate with fungi, Paterson *et al.* 2007; O'Brien *et al.* 2016) and competition and predation by fungi (O'Brien *et al.* 2016). In contrast, microbial biogeography over large spatial scales seems to be controlled more by edaphic factors (e.g. pH and climate) (Fierer and Jackson 2006; Ranjard *et al.* 2013). Acacia invasion significantly altered alpha diversity at two sites, albeit with opposite effects (Supplementary Table S3.1). In their native ranges, some acacias show increased soil bacterial diversity related to plant size, and thus age (Dinnage *et al.* 2018). A potential reason for the observed increase in bacterial richness and diversity at site Vermaaklikheid is

that acacias actively participate in niche construction for local soil biota, which is not surprising since invasive plants can modify soil communities to their own benefit (Klironomos 2002; Callaway *et al.* 2004). A similar scenario has been observed for mycorrhizal fungi, whereby dense plant invasions increase the abundance and diversity of their associated fungi (Lekberg *et al.* 2013). However, since the ages of invasive stands and their density were not quantified during this study, the aforementioned idea remains speculative, and there is no reason why such niche construction should not be plausible at other sites too.

The genus *Gemmatimonas* was dominant in invaded soils at Flower Valley. *Gemmatimonas* belongs to the phylum Gemmatimonadetes, which are among the top 2% of bacteria found in soils (Janssen 2006). The phylum is adapted to low-moisture soil conditions, and usually have higher abundances in soils with near-neutral pH, compared to acidic, soils (DeBruyn *et al.* 2011). Interestingly, pH was not significantly influenced by invasion at Flower Valley (Chapter 4), thus it seems unlikely that altered pH led to a proliferation of *Gemmatimonas*. Acacias are however, are known to lower water availability (Dye and Jarmain 2004), and this could be a reason for the proliferation of low moisture-loving *Gemmatimonas* under invasion. Thus, it might serve as a predictor of such altered water availability. However, the phylum does have a cosmopolitan distribution in terrestrial systems (DeBruyn *et al.* 2011). Pristine soils at Flower Valley were enriched for *Acidisphaera*, which are characteristic of acidic soil conditions (Hiraishi *et al.* 2000; Hamamura *et al.* 2005). At Koude Vlakte invaded soils were characterised by Actinobacteria, which are known plant growth promoters (e.g. through rock phosphate solubilisation) and disease suppressors (e.g. having antimicrobial activities) in natural ecosystems (Palaniyandi *et al.* 2013). The abundance of Actinobacteria is usually positively correlated with soil pH (Lauber *et al.* 2009), and have a high diversity in limestone derived soils (Nimaichand *et al.* 2015). It is possible that geological differences between the invaded and pristine areas of Koude Vlakte could have led to differences in indicator taxa, since the site is characterised by deep sandy soils overlying limestone, with some areas having shallow limestone outcrops. The proliferation of Actinobacteria might also be linked to the significantly elevated pH levels under acacia invasion (Chapter 4), thereby serving as a predictor of invasion induced pH regime shifts.

However, Actinobacteria are also known to have a generally high abundance in soil (Janssen 2006). Pristine soils at Vergelegen were characterised by Solirubrobacterales and *Sphingomonas*. The order Solirubrobacterales usually show high abundance in soils with low soil organic carbon, but can also favour systems with high levels of physical disturbance (Shange *et al.* 2012). Invaded soils at Vergelegen were characterised by Acidobacteria_Gp1, which usually have high abundance in acidic soil conditions (Philippot *et al.* 2010; Bardhan *et al.* 2012; Sun *et al.* 2014). Interestingly, invaded soils were also characterised by *Burkholderia*, which are symbionts of native legumes in fynbos (Lemaire *et al.* 2015). However, the genus *Burkholderia* has been noted to be a highly abundant component of soils in general (Janssen 2006). Pristine soils in Vermaaklikheid were characterised by *Rubrobacter*, which are apparently tolerant to high salt concentrations, whereas *Solirubrobacter*, which were characteristic of invaded soils, is not (Singleton *et al.* 2003). However, *Rubrobacter* was also a biomarker taxon of invaded soils, presumably different genetic strains, and thus conclusion as to its enrichment in both soils is difficult. Biomarker taxa might potentially serve a predictive value of expected shifts following invasion or under specific environmental conditions, however the ubiquitous nature of many of these bacterial groups in soils makes it difficult to ascribe such changes to environmental change under invasion without direct testing.

Invasive acacias are known to alter soil bacterial community composition (Slabbert *et al.* 2014; Kamutando *et al.* 2017; Le Roux *et al.* 2018), and thus I expected to see community differences between invaded and pristine soils. Despite limited evidence for impacts on overall soil bacterial alpha diversity, I found some evidence that invasive acacias, irrespective of season, significantly alter soil bacterial community structure and composition (Figure 3.5, S3.1, Table 3.2). Although the precise mechanisms whereby acacias are able to alter soil bacterial community composition remain unknown, evidence suggests that invasive nitrogen-fixing plants may do so in specific ways. For example, as nitrogen-fixers legumes may affect certain functional groups such as ammonia-oxidizing microbes, a key group involved in soil nitrogen cycling (Malinich *et al.* 2017). Furthermore, changes in microbial communities seem to be strongly dependent on proximity to and local density of the invasive N-fixing species (Malinich *et al.* 2017). Although I did not specifically set out to document the dependence of

changes in microbial communities on invader density, I observed acacias to occur in moderate to high densities at all invaded sites, while neighbouring pristine sites had few native legumes present.

I was also interested in the degree to which soil abiotic variables are responsible for the observed differences in soil bacterial communities between invaded and pristine sites. I expected pH to explain differences in soil bacterial communities between invaded and pristine sites, since invasive acacias often influence soil pH (Yelenik *et al.* 2004; González-Muñoz *et al.* 2012; Lazzaro *et al.* 2014; Souza-Alonso *et al.* 2014, 2015; Nsikani *et al.* 2017), and because pH is a strong predictor of bacterial community composition and structure in general (Gibbons and Gilbert 2015; Thompson *et al.* 2017). Soil bacterial communities are also sensitive to disturbances such as changes in carbon, nitrogen, and phosphorous content (Allison and Martiny 2008). Thus, I expected carbon and nitrogenous compounds (i.e. NO_3^- and NH_4) to contribute to differences in soil bacterial communities due to high carbon inputs through acacia leaf litter, and high nitrogen input as a result of biological nitrogen-fixation by acacias (Liao *et al.* 2008; Marchante *et al.* 2008; Lorenzo *et al.* 2010b; Lazzaro *et al.* 2014). Forward variable selection confirmed pH and NH_4^+ to be significant discriminatory factors in explaining differences between acacia-invaded and pristine soil bacterial community composition and structure. Thus, I confirm that invasive acacias may alter whole soil bacterial communities via plant-mediated soil chemical changes. It should be noted however, that in some instances soil abiotic variables can be more important in structuring soil bacterial communities than the dominant plant species identity of the region (Erlandson *et al.* 2018). Interestingly carbon was not observed to be a major driver that separates invaded and pristine soil bacterial communities.

Variation partitioning between soil bacterial communities indicated that abiotic soil variables explained similar amounts of variation for both invaded and pristine sites. In contrast, the amount of variation explained solely by environmental factors (E|S) was higher in invaded sites, while the amount of variation explained solely by spatial variation (S|E) was higher in pristine sites. Thus, it seems that the presence of invasive acacias reduces spatial variability in soil bacterial communities of fynbos. However, this breakdown of the distance decay seems to result from increased dissimilarity of

nearby invaded sites relative to the pristine sites, i.e. upon invasion even closer sites are more dissimilar than without invasion (Figure 3.6). In contrast to this, it has been observed that on smaller spatial scales dominant invasive acacias homogenise soil nitrogen-fixing bacterial communities via strong host selection, possibly for compatible rhizobial symbionts (Le Roux *et al.* 2018).

I also found strong seasonal effects on both alpha and beta diversities of fynbos soils, irrespective of invasion treatment (Table 3.1 and Table 3.2). These patterns are maybe to be expected as microbial communities are known to change over years (Buckley and Schmidt 2003) and seasons (Lipson 2007). In fact, soil bacterial community composition can even change over months or even days, for example after rain events (Grundmann 2004; Zhang *et al.* 2011; Prosser 2012; Lauber *et al.* 2013). Season can also be an important driver of soil bacterial community structure especially when seasonality is strong, such as seasonal floodplain habitats that may experience extreme dry and wet periods (Samaritani *et al.* 2017). Considering that fynbos is characterised by a Mediterranean-type climate with high seasonal variation in temperature and rainfall (Manning and Goldblatt 2012), the significant seasonal effects found on microbial communities is not surprising. The results suggest that such seasonal variation should be incorporated in invasive species-soil feedback studies in order to capture most changes in soil bacterial community composition in response to invasion.

The implication of the findings of this study is important as the composition of soil bacterial communities in early plant successional stages influence plant community structure in later stages (Kardol *et al.* 2007; Jordan *et al.* 2008; Elgersma *et al.* 2011). Thus, when acacias change microbial communities they may create positive feedback loops, to the detriment of native species. For example, Le Roux *et al.* (2018) recently found acacias grown in invaded fynbos soils to always outperform those grown in pristine soils. Furthermore, invasive plants are known to change native plant community structure, and their legacy effects are evident even decades after their removal (Maclean *et al.* 2018), including impacts on soil chemistry (Nsikani *et al.* 2017). Together with the findings presented here, these observations point to the possibility that such legacy effects may also hold up for microbial communities. That is, acacia-induced changes in soil pH and NH_4^+ may persist over long

periods, in turn impacting on microbial community composition. Such legacy effects may have long-term implications for the restoration of native communities following the clearing of invasive acacias.

3.6. Acknowledgements

This research was funded by the South African National Research Foundation (Nos. 76912 and 89967).

3.7. Tables and figures

Table 3.1: Two-way ANOVA results including interactions for different soil bacterial community alpha diversity metrics between invaded and pristine fynbos sites. Factor "site" was included as an interaction specifically to investigate whether impacts of invasion was dependent on site effects. Significance indicated in bold and as follows: ** – p<0.01; *** – p<0.001.

Diversity	Factor	df	Mean Sq	F	p
Richness	Invasion	1	155761	2.87	0.095
	Season	1	548136	10.10	**0.002****
	Invasion x Season	1	205	0.004	0.951
Exponent of Shannon	Invasion	1	35331	1.01	0.317
	Season	1	943563	27.08	< **0.001*****
	Invasion x Season	1	457	0.01	0.909
Inverse Simpson	Invasion	1	2795	0.34	0.559
	Season	1	170213	20.96	< **0.001*****
	Invasion x Season	1	566	0.07	0.792
Pielou's Evenness	Invasion	1	0.000005	0.01	0.911
	Season	1	0.012904	30.52	< **0.001*****
	Invasion x Season	1	0.00003	0.07	0.792

Table 3.2: Permutational multivariate analysis of variance (PERMANOVA) results for whole soil bacterial communities of invaded and pristine fynbos sites sampled during autumn and spring seasons. Factor "Site" was included as a random variable in the model to account for differences in soil properties (see Materials and Methods for details). Significance indicated in bold and as follows: *** – $p < 0.001$

Factor	df	Sum of Sq	F	p
Invasion	1	0.7264	2.10	**< 0.001*** **
Season	1	0.4397	1.27	**< 0.001*** **
Invasion x Season	1	0.2649	0.77	0.1072
Residuals	76	26.2198		

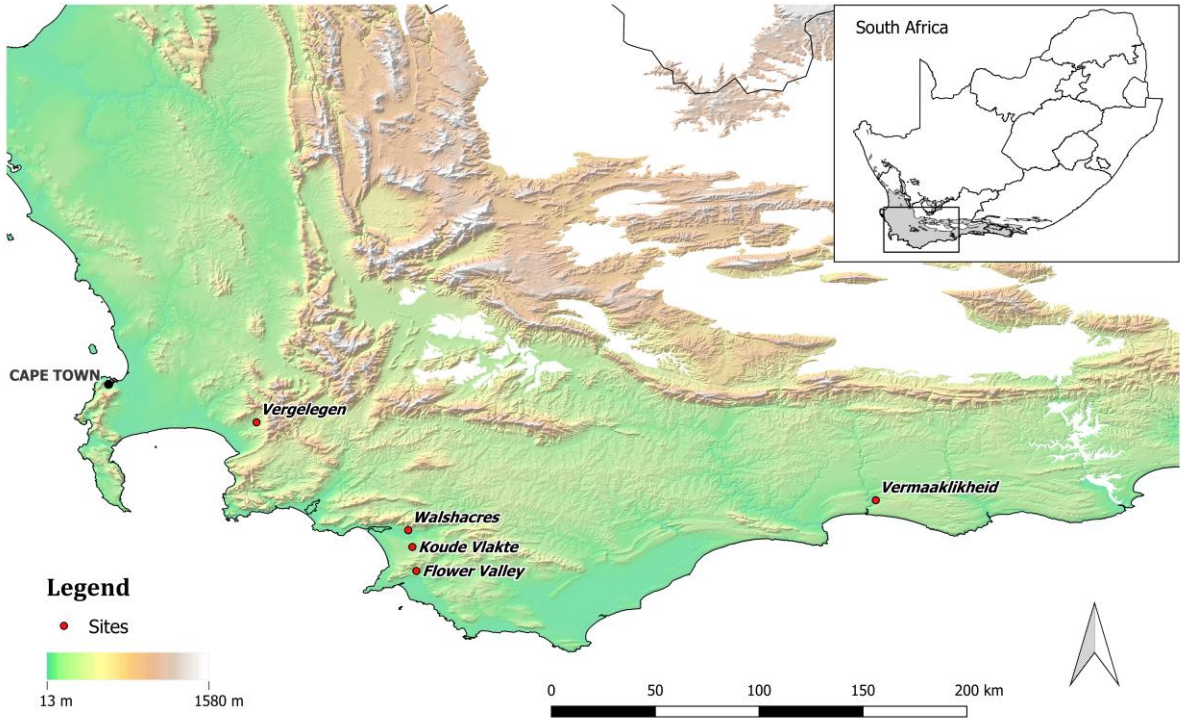


Figure 3.1: Maps showing locations of five sites within the Fynbos biome of South Africa's Core Cape Subregion included in this study (extent of biome indicated in gray in map inset; only areas that are within the biome are displayed on the main map). Each site consisted of a densely acacia-invaded and neighbouring pristine area. Sites were selected so that acacia-invaded and pristine areas were in

close proximity (<500 m) to each other in order to eliminate as many confounding factors (e.g. soil structural and chemical variation, vegetation type, climatic conditions etc.) as possible.

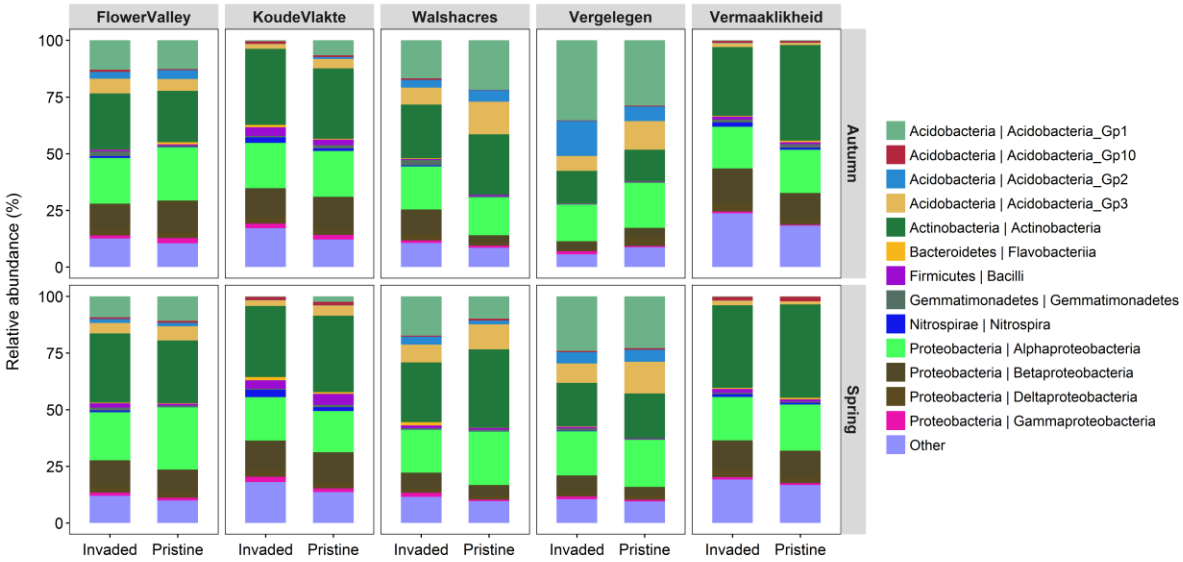


Figure 3.2: Relative abundances of taxa (Phylum | Class) for each site, season, and invasion treatment combination. Class level relative abundances were calculated using the number of sequences for each taxon as a percentage of the total sequences for each site/season combination. The "Other" category includes taxa that were unclassified at Class level together with classes representing less than 0.5% of the total number of sequences.

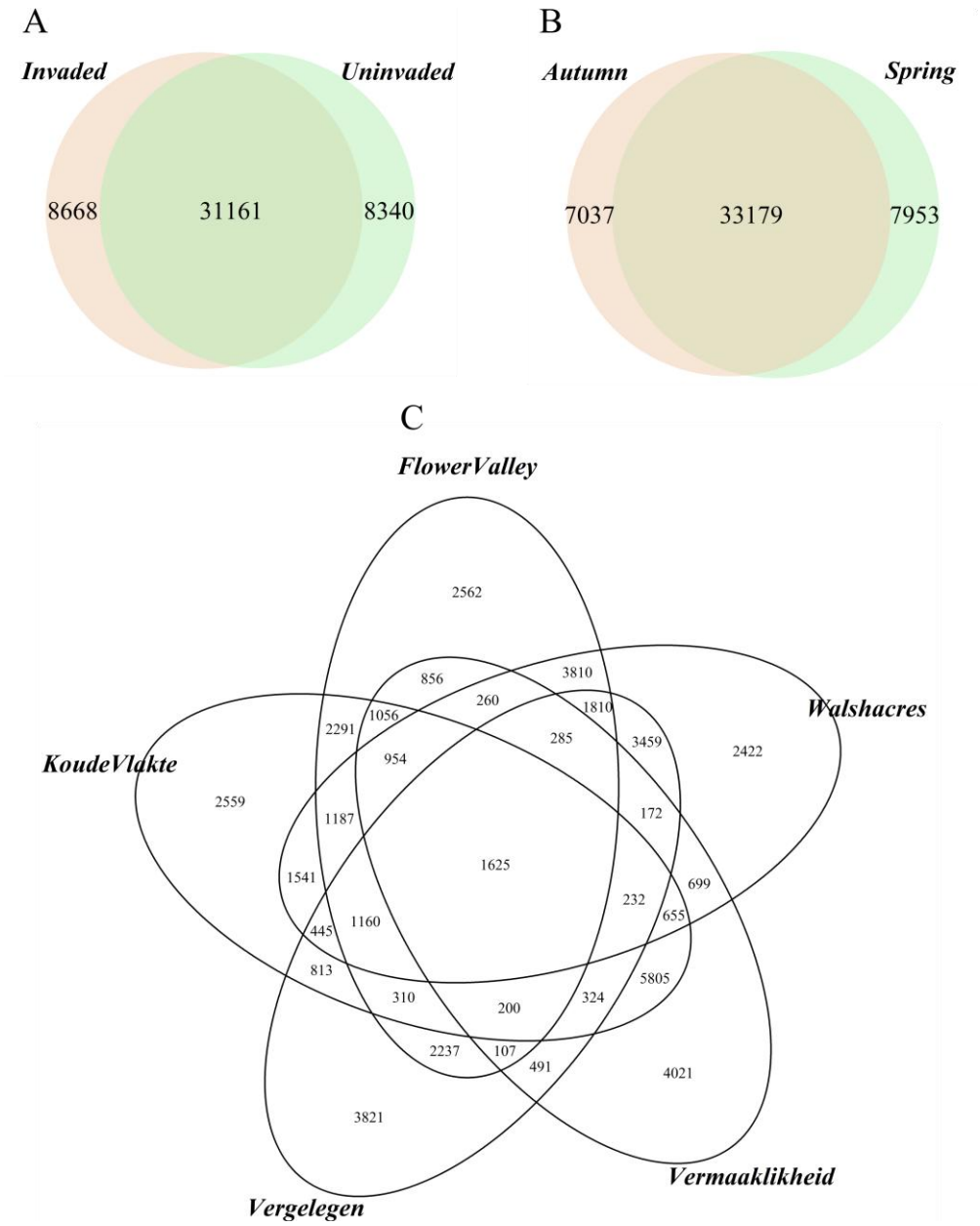


Figure 3.3: Venn diagrams showing the distribution of OTUs for each factor (with its subsequent levels) in this study, namely A) invasion treatment, B) season, and C) site. Numbers in figures represent OTU richness, and not read abundances (total number of OTUs for the dataset = 48169).

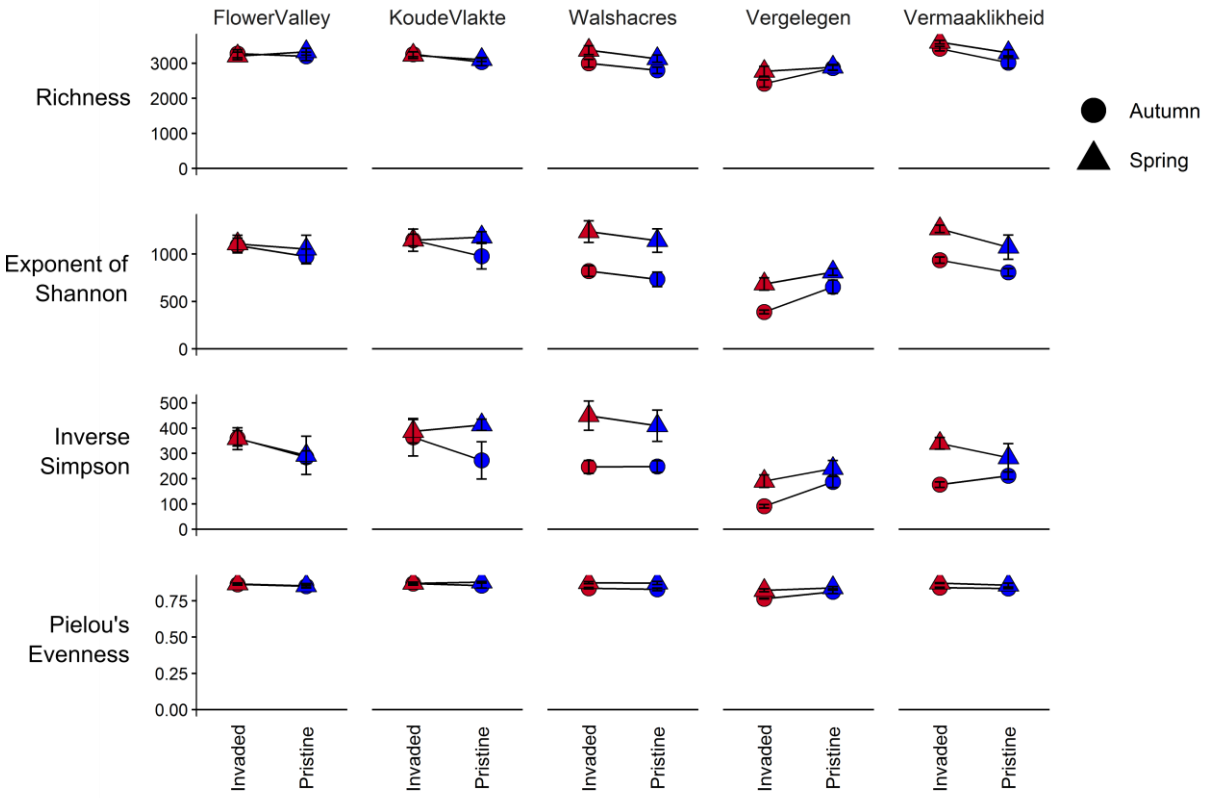


Figure 3.4: OTU alpha diversities for microbial communities at various fynbos sites between Acacia invaded and pristine soils across autumn and spring seasons. All diversities were significantly higher in spring (two-way ANOVA with season and invasion as factors). Error bars indicate mean ± SE.

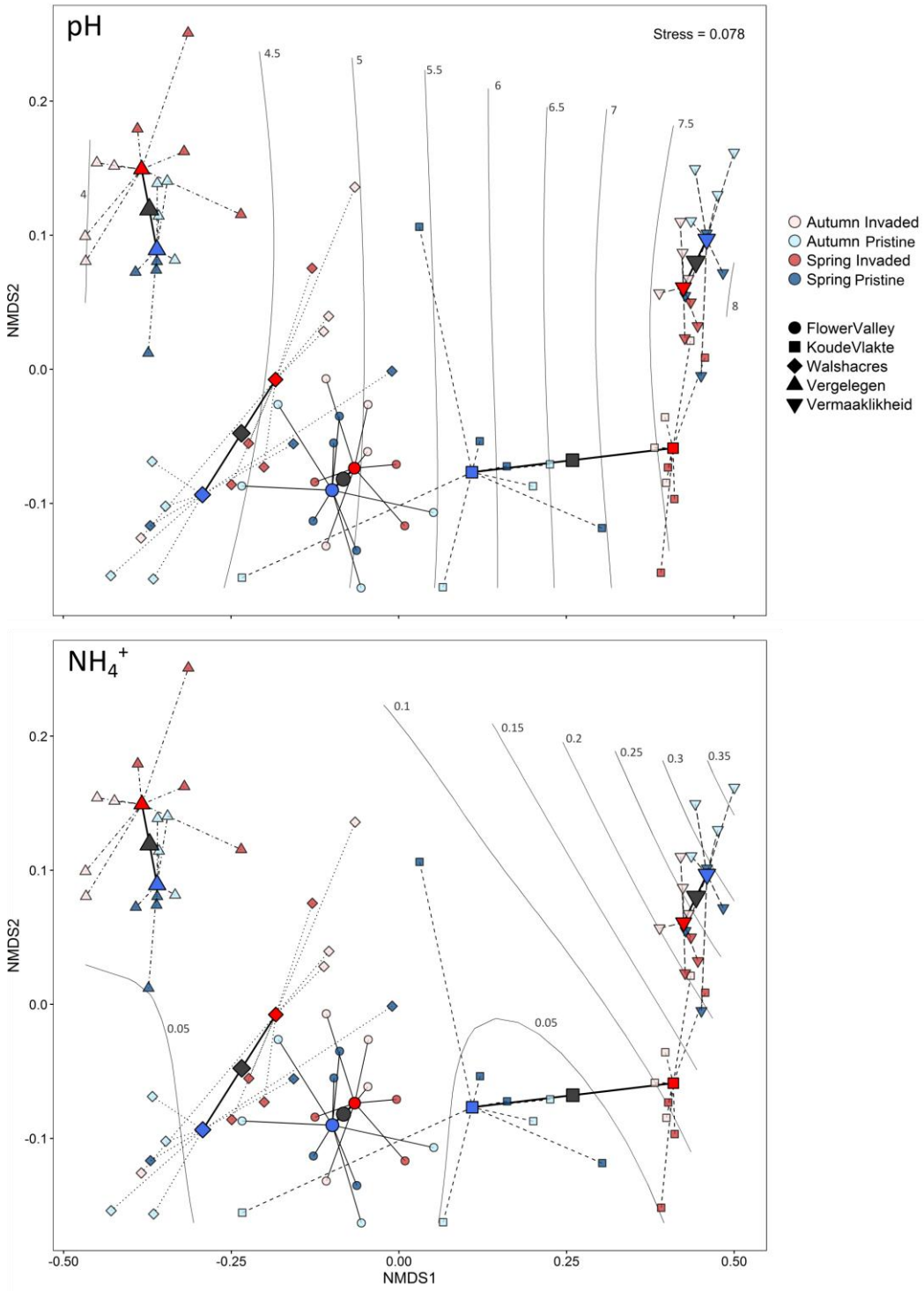


Figure 3.5: Non-metric multidimensional scaling (nMDS) of whole soil bacterial communities from various invaded and pristine fynbos sites across two seasons (austral autumn and spring), based on Horn similarity values (Jost 2007). Large black symbols indicate site centroids; red and blue medium symbols indicate invaded and pristine treatment centroids, respectively (connected to site centroids by

medium solid lines). Individual samples for both seasons are connected to their respective invasion treatment centroids within each site. Contours in each plot, respectively, represent the two most significant contributing environmental variables added with smoothing spline surface (top and bottom plots are the same ordination; values represent respective environmental parameters). The low stress value indicates that the plot is a good representation of community differences (i.e. not much variation is unaccounted for) (Clarke 1993).

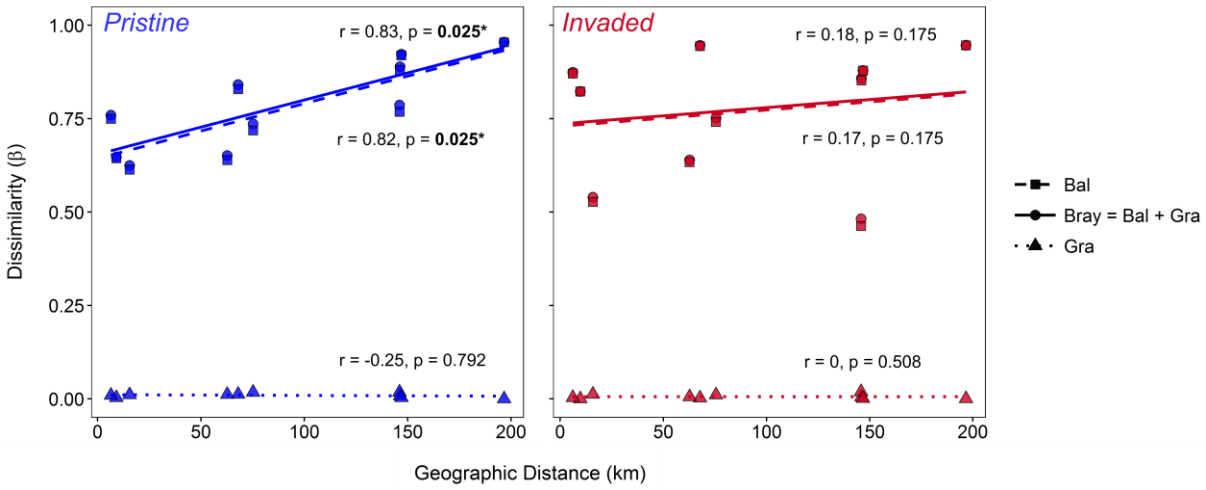


Figure 3.6: Beta diversity turnover for the five sites in this study indicating partitioning of beta diversity along geographical distance (Bray = Bray-Curtis dissimilarity; Bal = balanced variation component of Bray-Curtis dissimilarity; Gra = abundance gradient component of Bray-Curtis dissimilarity). Also indicated are regression lines together with Mantel correlations (Pearson r with corresponding p values) between dissimilarities and geographic distance. A dissimilarity value of 1 indicates complete turnover.

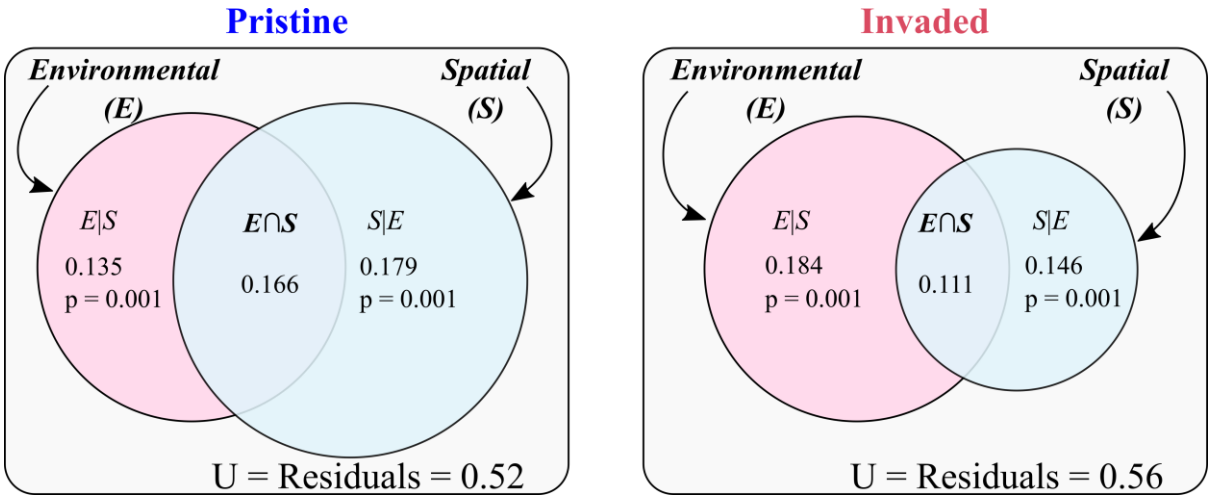


Figure 3.7: Variation partitioning (environmental component E – left circle; spatial component S – right circle) for invaded and pristine fynbos sites explaining whole soil bacterial community structure. The bounding boxes indicate all variation in the respective communities. Variation explained only by fraction E is indicated as E|S and that explained only by S as S|E. Shared variation (i.e. intersection) among E and S is indicated by E∩S per community. All unexplained variation is indicated by fraction U. Values indicated are RDA-adjusted R^2 values with significance added. Fractions U and E∩S cannot be tested for significance.

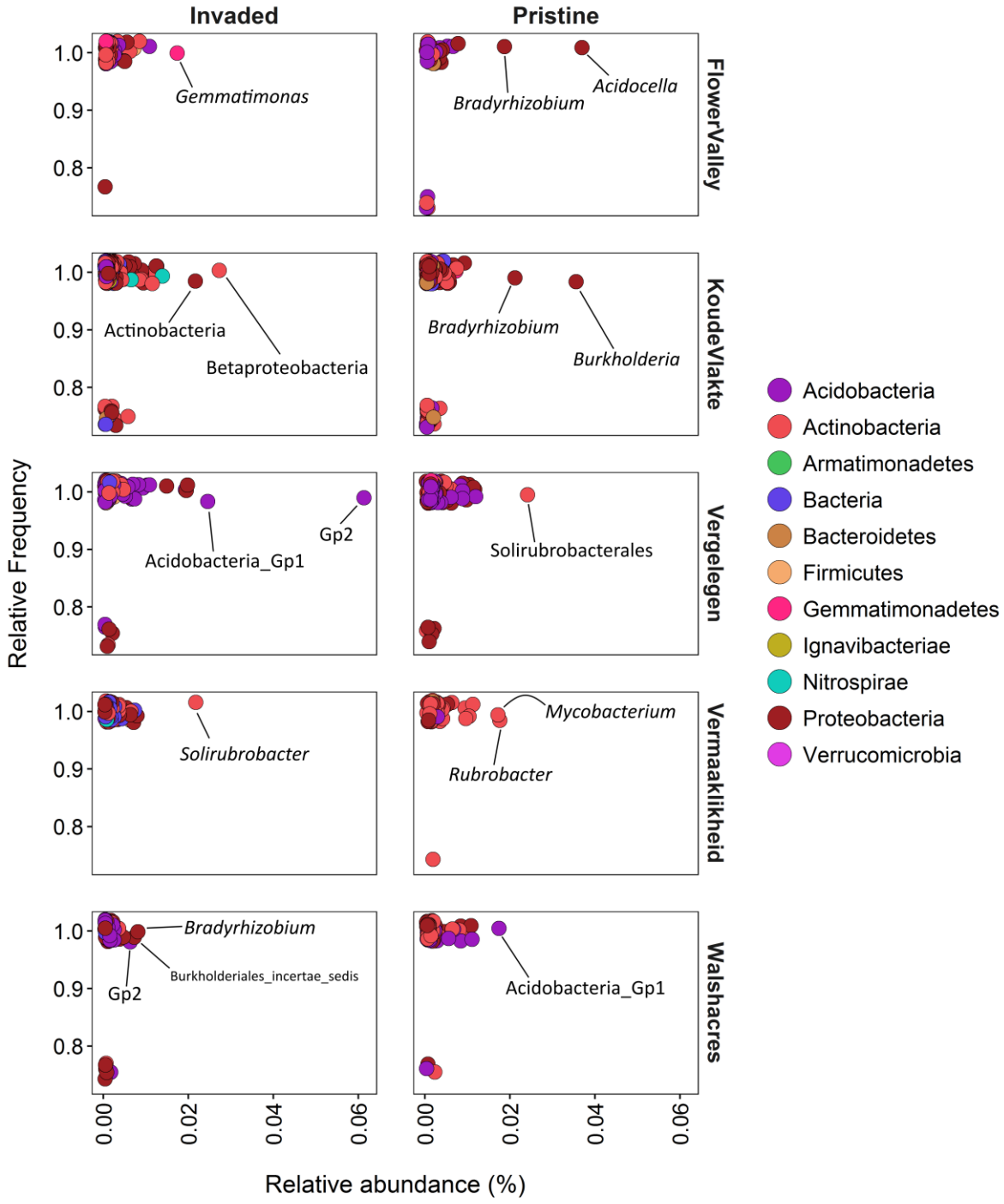


Figure 3.8: Biomarker taxa that discriminate between invaded and pristine areas in each site (i.e. taxa that are highly characteristic of each area) were determined using Linear Discriminant Analysis Effect Size (LEfSe). Taxa that occur in both high relative abundances and frequencies are the among the most important taxa that distinguish invaded and pristine areas, however they do not necessarily have

the highest LDA scores. (Note: a small amount of jitter was added to the graph to give more clarity to overlapping dots).

3.8. Supplementary information

Supplementary Table S3.1: Two-way ANOVA p-values for different bacterial community alpha diversity metrics between invaded and pristine fynbos soils for each site. Abbreviations: FV – Flower Valley, KV – Koude Vlakte, VG – Vergelegen, VM – Vermaaklikheid, WA – Walshacres. Significance indicated in bold and as follows: * – p<0.05, ** – p<0.01; *** – p<0.001.

Diversity	Factor	FV	KV	VG	VM	WA
Richness	Invasion	0.835	0.062	**0.016***	**0.004****	0.065
	Season	0.778	0.789	0.092	**0.034***	**0.007****
	Invasion x Season	0.394	0.544	0.130	0.615	0.853
Exponent of Shannon	Invasion	0.419	0.503	**0.003****	**0.044***	0.366
	Season	0.642	0.323	**0.001****	**0.001****	**0.001****
	Invasion x Season	0.776	0.331	0.204	0.662	0.972
Inverse Simpson	Invasion	0.170	0.584	**0.008****	0.741	0.686
	Season	0.953	0.184	**0.006****	**0.003****	**0.002****
	Invasion x Season	0.930	0.334	0.336	0.170	0.660
Pielou's Evenness	Invasion	0.223	0.769	**0.003****	0.222	0.534
	Season	0.751	0.217	**< 0.001*****	**0.003****	**< 0.001*****
	Invasion x Season	0.934	0.317	0.119	0.580	0.750

Table S3.2: Top five biomarker taxa for each site and invasion treatment (invaded vs. pristine soils) combination. OTUs were selected by having both high relative abundances and frequencies across replicate samples within each site. These were classified by the consensus taxonomy. Taxa are ordered from high to low in terms of relative abundance (all taxa listed occurred in all samples within each site, thus giving them relative abundances of 1). The "Taxon Level" column indicates the finest scale to which individual OTUs could be classified. Linear discriminant analysis (LDA) scores listed are all significant at $p < 0.05$.

Site	Treatment	LDA	Taxon	Taxon Level
Flower Valley	Invaded	3.62	*Gemmatimonas*	Genus
		3.44	Acidobacteria_Gp1	Class
		3.23	Actinobacteria	Class
		3.13	*Bacillus*	Genus
		3.08	Actinobacteria	Phylum
	Pristine	3.98	*Acidocella*	Genus
		3.07	*Acidisphaera*	Genus
		2.70	Myxococcales	Order
		2.47	Alphaproteobacteria	Class
		2.42	*Phenylobacterium*	Genus
Koude Vlakte	Invaded	3.84	Actinobacteria	Class
		3.71	Betaproteobacteria	Class
		3.47	*Nitrospira*	Genus
		3.42	Betaproteobacteria	Class
		3.06	*Variovorax*	Genus
	Pristine	3.07	Betaproteobacteria	Class
		3.04	Bacteria	Domain
		2.88	Solirubrobacterales	Order
		2.58	*Bacillus*	Genus
		2.38	Betaproteobacteria	Class
Vergelegen	Invaded	3.75	Acidobacteria_Gp1	Class
		3.65	*Burkholderia*	Genus
		3.47	Acidobacteria_Gp1	Class
		3.22	Acidobacteria_Gp1	Class
		3.14	Gp2	Order
	Pristine	3.72	Solirubrobacterales	Order
		3.24	*Sphingomonas*	Genus
		3.23	Actinobacteria	Class

Table S3.2 continued...

Site	Treatment	LDA	Taxon	Taxon Level
		2.98	*Sphingomonas*	Genus
		2.73	Candidatus_Solibacter	Order
Vermaaklikheid	Invaded	3.40	*Solirubrobacter*	Genus
		3.13	Betaproteobacteria	Class
		3.09	*Rubrobacter*	Genus
		3.04	*Bacillus*	Genus
		2.91	Bacteria	Domain
	Pristine	3.26	*Rubrobacter*	Genus
		3.23	Solirubrobacterales	Order
		3.22	Micromonosporaceae	Family
		2.99	Acidimicrobiales	Order
		2.91	Caulobacteraceae	Family
Walshacres	Invaded	3.08	Burkholderiales_incertae_sedis	Family
		2.97	Xanthomonadaceae	Family
		2.72	Bacteria	Domain
		2.70	Candidatus_Solibacter	Order
		2.48	Rhizomicrobium	Family
	Pristine	3.55	Acidobacteria_Gp1	Class
		3.36	*Acidocella*	Genus
		3.17	*Mycobacterium*	Genus
		3.04	Rhodospirillales	Order
		2.61	Acetobacteraceae	Family

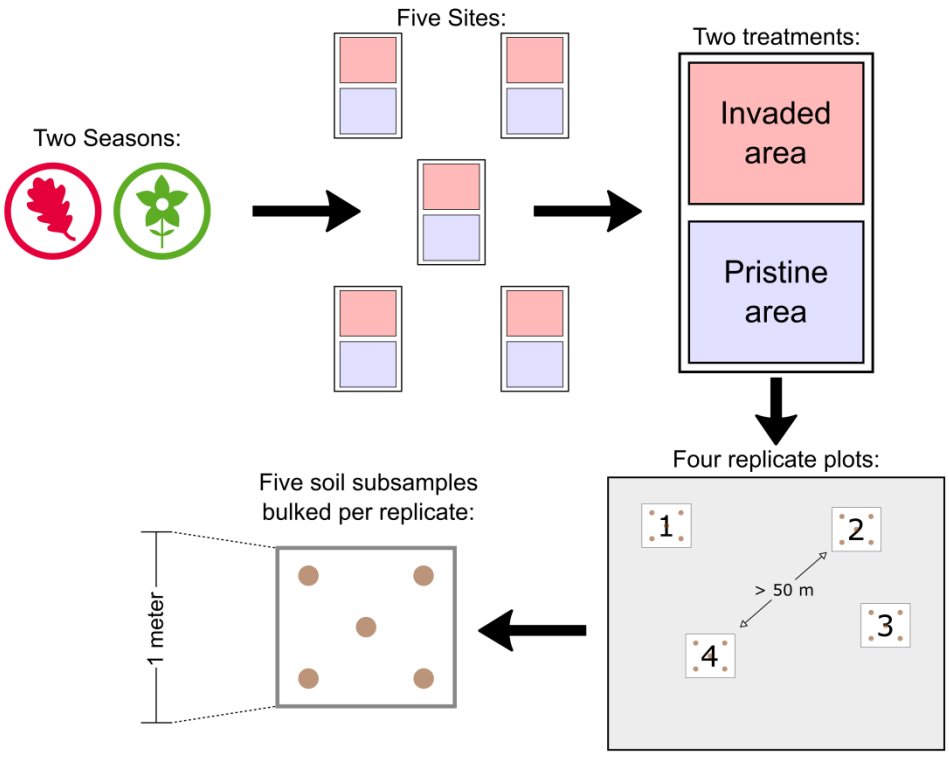


Figure S3.1: Sampling setup for soil collections. Each site had an *Acacia* invaded and a pristine treatment in close proximity to each other. In each treatment four plots (1 m x 1 m) were randomly placed at least 50 m apart and within each plot five soil subsamples were collected. The subsamples for each plot were bulked to form one independent replicate, thus yielding a total of four replicates for the invaded area and four replicates for the pristine area. This was repeated for a total of five sites, for both austral Autumn and Spring. See Materials and Methods for detailed overview.

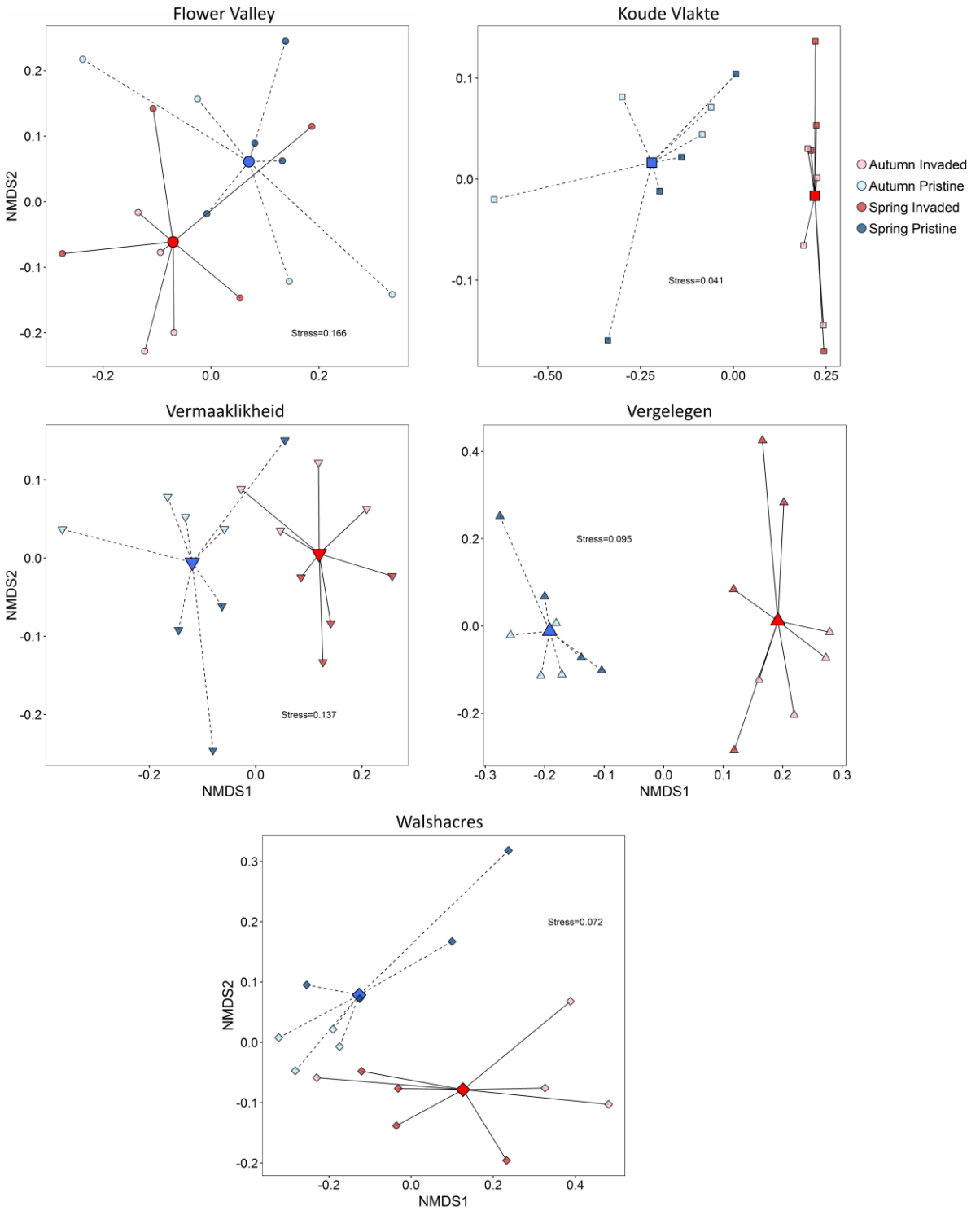


Figure S3.2: Non-Metric Multidimensional Scaling plots (NMDS) on site level. Large symbols represent centroids of invasion treatments (red symbols = invaded, blue symbols = pristine).

CHAPTER 4: Changes in soil functioning and nutrient composition mediated by invasive acacias in a Mediterranean type ecosystem are context specific

Candidate Journals: Applied and Environmental Microbiology, Plant and Soil

4.1. Abstract

Invasive nitrogen-fixing species such as legumes often impact the soils they invade, notably through changes in soil chemistry (for example soil N and P content), as well as microbial community composition. This in turn can lead to alterations in soil functionality, such as carbon, nitrogen, and phosphorous cycling, as expressed through soil microbial enzyme activities. Here, using Australian *Acacia* invasions in South Africa's Core Cape Subregion (CCR), I aimed to determine the impacts these invaders have on soil chemistry and function (carbon, nitrogen, and phosphorus cycling). I also wanted to determine whether soil function is linked to soil nutrient content and bacterial community composition, and whether acacia-induced changes in these parameters translate into altered soil functionality. I found acacias to significantly increase levels of soil nitrogenous compounds (NO_3^-, NH_4^+, and total N), C and pH. However, I found that such impacts were not consistent across all invaded sites, although the directions of impacts for these parameters were consistent. Furthermore, I found acacias to significantly elevate activities of enzymes involved in nitrogen (urease) and phosphorous (phosphatase), but not carbon (β-glucosidase) cycling. These impacts, however, were also site-specific. Acacia invasions induced changes in soil nutrients that, in turn were correlated with changes in enzyme activities for urease and phosphatase. Changes in soil bacterial community composition due to the presence of invasive acacias was also correlated with phosphatase enzymatic activity. Thus, while I found evidence for acacias altering soil functions via changing soil nutrients and bacterial community composition, the mechanistic feedbacks between these impacts appear to be context specific.

KEYWORDS: 16S rDNA, Australian acacias, enzyme activities, fynbos, invasion, next-generation sequencing, soil bacteria, soil function, soil microbial ecology.

4.2. Introduction

The extensive movement of, and trade in, plants during the past few centuries has seen the unprecedented introductions of non-native species globally (Vitousek *et al.* 1997; Mack *et al.* 2000; Meyerson and Mooney 2007), with no saturation in the rate of new introductions in sight (Tye 2001; Hulme 2009; Van Kleunen *et al.* 2015; Seebens *et al.* 2017). In many instances these introductions have led to biological invasions. The impacts caused by biological invasions ramify deeply into the ecosystems they invade. Following human-mediated habitat destruction, invasive species are considered the second largest threat to global biodiversity (Vitousek *et al.* 1997; Mack *et al.* 2000). For plant invasions, many of these impacts have been documented on the aboveground components of invaded ecosystems (e.g. reduction in native species richness and diversity, altering native plant community structure and homogenising of floras, soil nutrient enrichment etc.) (Schwartz *et al.* 2006; Hejda *et al.* 2009; Souza-Alonso *et al.* 2014, 2015; Michelan *et al.* 2018). Recently, an appreciation for the impacts of invasive plants on below-ground ecosystem processes, such as soil nutrient cycling and soil microbial functions, have emerged (Ehrenfeld *et al.* 2001; Kourtev *et al.* 2002, 2003; Caldwell 2006; Li *et al.* 2006; Souza-Alonso *et al.* 2014, 2015). Communities dominated by one or a few invasive plants are thus expected to experience impacts on one of the most important biological components of soils, namely bacteria (Kourtev *et al.* 2002; Gibbons *et al.* 2017).

Ecosystem functioning is highly dependent on microbes. Specifically, soil bacteria are important regulators of nutrient cycling and organic matter turnover in ecosystems (Brussaard *et al.* 1997; Fisk and Fahey 2001), as they represent a considerable portion of plant-symbiotic networks (Coats and Rumpho 2014). The decomposition and nutrient mineralization of complex organic and non-organic substrates (e.g. carbon, nitrogen and phosphorous) begins with the secretion of extracellular enzymes by soil microbial communities and subsequent catalytic breakdown of such substrates (Allison and Vitousek 2005). Furthermore, soil bacterial diversity and function are intricately linked (Nannipieri *et al.* 2017). Some belowground diversity components may be more closely linked with above ground communities than others. It is now widely accepted that higher organisms, such as plants, are not made up of autonomous individuals, but rather represent organised biological units comprised of

many closely associated organisms (e.g. plant and its rhizosphere microbiome), or so-called holobionts (Bordenstein and Theis 2015). Associated microbiomes can have strong and evident impacts on the physiology, anatomy, reproduction, and overall health and fitness of their hosts (Berendsen *et al.* 2012; Bordenstein and Theis 2015). Given these strong links between above- and belowground biodiversity, the presence of dense, near monotypic stands of invasive species is expected to dramatically alter the make-up and underlying functions of soil microbial communities (van der Putten *et al.* 2007; Gibbons *et al.* 2017; Xiang *et al.* 2018).

It is easy to understand how invasive plants can impact soil functions. Dense invasions could lead to alterations in litter fluxes (i.e. leaf litter input), whereby the presence of the invader leads to the deposition of organic material that differ in chemistry, quantity, and quality from litter inputs prior to invasion (Ehrenfeld 2003; Liao and Boutton 2008; Liao *et al.* 2008). Additional chemical changes to soil may result from the excretion of volatile chemical compounds released by aboveground plant tissues, so-called allelopathic compounds, and/or root exudates that mobilise nutrients, e.g. phosphorous and iron (BL Wang *et al.* 2007; Berg and Smalla 2009; Ens *et al.* 2009; Tharayil *et al.* 2009; Weidenhamer and Callaway 2010; Coats and Rumpho 2014). Furthermore, invasion by certain plant functional groups may have distinct impacts on soil organisms. For example, nitrogen-fixing species can potentially modify soil nitrogen accumulation and cycling rates (Corbin and Antonio 2004; Rice *et al.* 2004). These changes often lead to positive invader-soil feedbacks, and therefore higher competitiveness (Lekberg *et al.* 2018).

The genus *Acacia* Mill. has been extensively introduced around the world for various reasons, and has become invasive in many regions, especially those with Mediterranean-type climates (Richardson and Rejmánek 2011; Richardson *et al.* 2011). Like most legumes, acacias are capable of fixing atmospheric nitrogen because of their associations with symbiotic bacteria known as rhizobia (Franche *et al.* 2009). Acacias often attain extremely high densities in their invasive ranges, associated with significant increases in leaf litter loads (Gaertner *et al.* 2014). Unsurprisingly, acacias have been found to alter soil nitrogen and carbon content, as well as phosphorous levels (Witkowski 1991;

Yelenik *et al.* 2004; Souza-Alonso *et al.* 2015). Acacias have also been shown to alter rhizosphere and soil microbiomes (Slabbert *et al.* 2014; Kamutando *et al.* 2017), and can lead to changes in soil function as manifested by altered soil microbial enzymatic activities (Souza-Alonso *et al.* 2014, 2015). Moreover, these alterations may lead to so-called legacy effects, whereby changes to soil conditions often persist, even several years after clearing of invasive populations (Marchante *et al.* 2009; Corbin and D'Antonio 2012; Elgersma *et al.* 2012; Nsikani *et al.* 2017). These impacts, and their consequent legacy effects, are likely responsible for the observed slow recovery of native species following invasive acacia removal (Holmes and Cowling 1997; Daehler 2003).

Using acacia invasions in fynbos of South Africa's floristically hyper-diverse Core Cape Subregion, I aimed to explore their impacts on soil microbial community diversity and composition, function and soil nutrient loads over a large geographic range. I did this by investigating whether invasive acacias induce consistent changes in: 1) soil nutrients, 2) soil microbial function (as expressed by enzyme activities), and 3) whether these changes can be linked to alterations in soil microbial communities across geographically isolated sites in fynbos. I hypothesised that invasive acacias would increase soil nutrient loads as a result of high leaf litter input and nitrogen fixing ability. Similarly, I expected that acacias would elevate soil enzyme activities, specifically urease (urea degrading) and β-glucosidase (carbon degrading) as a result of increased nutrient loads (i.e. nitrogen and carbon). Finally, I hypothesised that alterations in soil nutrient levels and bacterial community composition under invasion will translate into changes in soil function.

4.3. Materials and methods

4.3.1. Study sites

Soils can show considerable geographical variability (Wandrag *et al.* 2013), even over fine spatial scales, such is the case in the CCR (Cowling 1990). I selected five study sites in the CCR that captures some of this variation: Vergelegen Wine Estate (VG; approximate coordinates: 34.056°S, 18.934°E), Vermaaklikheid (VM; 34.358°S, 21.038°E), Koude Vlakte Conservancy (KV; 34.475°S, 19.455°E), Walshacres (WA; 34.420°S, 19.442°), and Flower Valley (FV; 34.559°S, 19.470°E)

(Figure 4.1). At each site (soil type) I identified areas heavily invaded by Australian acacias occurring in close proximity (<500 m) to pristine areas. *Acacia cyclops* A.Cunn. ex G.Don was the dominant invasive at VM, KF, FV, while *A. saligna* (Labill.) H.L.Wendl. and *A. longifolia* (Andrews) Willd. were dominant at WA and VG, respectively.

Soil samples were collected for all four seasons in 2016 (Supplementary Figure S4.1). At each site six random plots of 1m x 1m were identified within each of the invaded and pristine areas (hereafter referred to as treatment). In each of these plots five soil subsamples of approximately 50g each were taken randomly within the first 10 cm of the soil surface. All samples were collected away from plants to avoid roots. Where present, the top layer of litter/organic material was removed before soil collection (Roesch *et al.* 2007). For each plot, the five collected soil subsamples were bulked and mixed, leading to six independent replicates (~250 g each) per invaded and pristine treatment (n = 240: 6 replicates x 2 treatments x 5 sites x 4 seasons). Each of the bulked sample replicates were divided into three parts for different analyses: soil nutrients, microbial enzyme activity, and microbiome sequencing. Soil samples were kept on ice during transport and were immediately stored at 4°C (for enzyme analysis), or -80°C (for DNA extractions), or room temperature (for nutrient analyses) upon arriving at the lab. For nutrient and soil enzymatic analyses, soil samples were first sieved with a 2 mm diameter sieve before storage to remove root fragments and organic debris.

4.3.2. Soil nutrients

For soil nutrient analyses I randomly selected four replicates per site for each invasion treatment X season combination (n = 160: 4 replicates x 2 treatments x 5 sites x 4 seasons). The following chemicals and nutrients were analysed for all samples: pH, Olsen phosphorous (P), total carbon (C), nitrate content (NO_3^-), ammonium content (NH_4^+), total available nitrogen (N). All analyses were performed by Bemlab Pty Ltd (SANAS Accredited Testing Laboratory, Cape Town, South Africa) according to standard quality control procedures (SSSA 1996). Briefly, pH was determined in saturated soil extracts. Total N and C were measured by combustion at 1350 °C. Total phosphorus was extracted using HCl-HNO_3 after combustion (3 h, 550°C), followed by quantification

by inductively coupled plasma optical emission spectrometry (ICP-OES). Ammonium and nitrate were extracted with 2 M KCl and diluted prior to determination by a flow injection analyser (FIA).

4.3.3. Soil bacterial enzymatic activities

Approximately 10g of soil per replicate was analysed for soil microbial enzymatic activities (n = 240: 6 replicates x 4 seasons x 5 sites x 2 treatments). I analysed three enzymes that play key roles in soil nutrient cycling: β-glucosidase (BG, E.C. 3.2.1.21, hereafter only referred to as glucosidase), involved in carbon degradation through the release of glucose from cellulose; urease (Ur, E.C 3.5.1.5), involved in the release of nitrogen by degrading urea to ammonium; and phosphatase (AP, E.C. 3.1.3.1), involved in the release of phosphate from organic matter by hydrolyzing phosphate ester bonds (Novoa *et al.* 2014). The substrates for BG, Ur and AP were p-nitrophenol (pNP) β-d-glucopyranoside, urea, and pNP-phosphate, respectively (Novoa *et al.* 2014). Substrates were made up in Modified Universal Buffer (20.14 mmol L^{-1} Tris-hydroxymethyl aminomethane; 17.3 mmol L^{-1} Malic acid; 14.6 mmol L^{-1} Citric acid; 20.3 mmol L^{-1} Boric acid and 1 mol L^{-1} NaOH). For each replicate, I included a control. Controls were used to eliminate the effects of impurities on absorbance values (i.e. absorbance values of controls were subtracted from non-controls so that absorbance values are solely due to the effects of substrate/product quantities). Absorbances of the three enzymes were quantified according to the methods as described by Novoa *et al.* (2014). Some potential caveats of soil enzyme activity analysis include storage conditions and substrate concentrations. With regards to the former, it is recommended that enzyme assays be conducted as soon as possible after soil collections, since the disturbance of soil can cause changes in enzyme activity as a result of changes in microbial activity, enzyme immobilization, and availability of substrate (Burns *et al.* 2013). It is however, acceptable to store soil samples temporarily at 5°C for temperate regions, as in this study, which minimizes external impacts (Burns *et al.* 2013). With regards to substrate concentrations, it is recommended that concentrations be optimized via substrate saturation curves, except for cases where soils are nutrient limited (Burns *et al.* 2013), as is the case with fynbos soils.

4.3.4. Soil DNA extraction and Next Generation Sequencing

For whole soil microbiome analysis total genomic DNA was extracted from 0.25g of each soil sample using the PowerSoil® DNA extraction kit (MO BIO laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. Here, the 16S rRNA gene was amplified using the primers 799F (5'-AAC MGG ATT AGA TAC CCK G-3') and 1391R (5'- GAC GGG CGG TGW GTR CA-3'), with sample-specific barcodes in the forward primer. Amplification was done using a 30 cycle PCR and the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following PCR conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, followed by a final elongation at 72°C for 5 minutes. After amplification, PCR products were checked on a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple PCR samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, Beverly, MA, USA) and used to prepare DNA libraries by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) following the manufacturer's guidelines.

4.3.5. Bioinformatics of NGS data

All raw MiSeq DNA sequence data were processed following standard procedures as described in Schloss *et al.* (2011) using mothur version 1.37.1 (Schloss *et al.* 2009) Briefly, after removal of low quality sequences and optimizing sequence lengths (to between 383 and 395 bp), unique sequences were aligned to the SILVA-ARB (release 123) reference database. Sequences were aligned to the same region of the 16S rRNA gene and columns containing only gaps were removed. All chimeric sequences were removed independent of a reference database using the uchime algorithm (Edgar *et al.* 2011) and the template as self, i.e. *de novo* removal. Sequences were subsequently clustered into Operation Taxonomic Units (OTUs) at the 97% sequence similarity level. Representative sequences for OTUs were chosen as those that were most abundant in each cluster. The ribosomal database project (RDP) Classifier (Q Wang *et al.* 2007) was used to determine the taxonomic identity of each

OTU, and all sequences classified as chloroplast, mitochondria, and archaea, were removed. In order to standardise the amount of reads across all replicates I subsampled an equivalent number of reads from each of the 80 replicates. Finally, I removed singleton and doubleton OTUs and had a final of 1 127 770 total reads and 48 169 OTUs. A limitation on the classification of bacterial OTUs from next-generation sequencing techniques results from the incompleteness of current sequence databases (Thompson *et al.* 2017). However, it does not detract from the usefulness of using OTUs in the calculation of various diversity metrics (both alpha and beta).

4.3.6. Statistical analyses

All statistical analyses were conducted in R statistical environment (version 3.5.1) (R Core Team 2017), unless otherwise specified. Soil nutrient and enzyme activity datasets were analyzed with ANOVAs with season and invasion as fixed effects. I was specifically interested in whether the impacts of acacias were consistent irrespective of local site conditions, or whether such impacts were site (i.e. context) specific. Thus, I also included site as a fixed effect including a site x invasion interaction to specifically test for the influence of site on invasion impacts. Where I found significant site x invasion interaction terms, I did ANOVAs on a per site bases.

In order to investigate compositional differences for soil abiotic variables and enzyme activities (i.e. whole soil activities), I conducted a principle components analysis (PCA) for each dataset, respectively. PCA inputs a correlation matrix and is thus well suited to dimension reduction for variables that are measured on different scales. I used the function `prcomp` in R base package for PCAs. Significant differences in whole soil nutrient and enzyme activities were tested, respectively, by Permutational Multivariate Analyses of Variance (PERMANOVA) with 9999 permutations using the `adonis` function in the `vegan` R package; I used invasion treatment and season as fixed factors, and site as a random factor.

In order to investigate whether or not changes in enzyme activities were driven by acacia-induced soil nutrient changes, I calculated the mean difference between invaded and pristine areas for each enzyme

and nutrient pair, respectively (i.e. Δphosphatase vs. ΔP, Δglucosidase vs. ΔC, and Δurease vs. ΔN), for each site and season combination. I then used Pearson's r^2 to determine whether or not there were significant correlations between these enzyme-nutrient pairs. A high correlation would suggest elevated enzyme activity levels as a result of invasion induced elevated soil nutrient levels. In a similar fashion, I investigated whether or not changes in enzyme activities were driven by acacias induced changes in soil bacterial community composition. For this I first obtained a measure of soil bacterial community composition by performing a principle coordinates analysis (PCoA) with the function `cmdscale` in R base package based on Horn similarity values (Jost 2007) for the OTU table, created with the function `sim.table` in `vegetarian` R package (Charney and Record 2012). I then used the first axis of the PCoA (which captured the most variation) in a correlation analysis with enzyme activity (as abovementioned for soil nutrients, i.e. mean difference in invaded between pristine values for composition and enzymes, respectively). Thus, a high correlation would similarly suggest elevated enzyme activity levels as a result of invasion induced soil bacterial community compositional changes.

To confirm which factors were the strongest drivers of soil bacterial community compositional and functional changes between invaded and pristine sites, I performed multiple regressions on distance matrices (MRM) (Sauvadet *et al.* 2017). MRMs are an extension of partial Mantel tests, and have the ability to test several distance matrices concurrently as explanatory variables (soil nutrient, geographical, and compositional dissimilarity matrices in this study) and their effects on a response distance matrix (e.g. bacterial community composition or function) (Lichstein 2007). Thus, I performed MRMs to explain which effects had the highest influence on compositional and functional changes, respectively, by using the following formulas: `Composition ~ Nutrients + Invasion + Geographic distance` for influences on community composition, and `Function ~ Nutrients + Composition + Invasion + Geographic distance` for influences on enzyme functioning. Geographical distance was included to test for the influence of space on response variables. Predictors and response variables were distance matrices, and for invasion influence I coded a design matrix with 0 for invaded/invaded and pristine/pristine pairs (i.e.

no difference), and 1 for invaded/pristine pairs (i.e. maximal difference). MRMs were performed with the function `MRM` in the `ecodist` package (Goslee and Urban 2007).

4.4. Results

4.4.1. Effects of acacias on soil abiotic variables

Overall analysis of soil abiotic variables indicated highly significant site x invasion interactions for all variables (Supplementary Table S4.1, Figure 4.2, Figure 4.3). Thus, although I detected the influence of invasion on some variables (e.g. pH, N, NH_4^+), these effects were not consistent across sites, and thus generalizations regarding the overall directionality of invasion impacts is difficult.

Site-level ANOVAs indicated that the only parameters that were consistently elevated by acacia invasions were pH, NO_3^-, NH_4^+, and total N (Table 4.1). Thus, although the magnitude of invasion effects on pH and nitrogenous compounds varies between sites, the direction of change is consistent (i.e. elevated under invasion). Furthermore, I found P to be significantly higher in the presence of invasive acacias at two sites, but significantly lower at another site, thus the invasion differences were not consistent and differed in directionality between these sites. Finally, C was significantly higher for only one of the sites included here (KV).

The overall PERMANOVA model indicated that invasion influences soil nutrient composition (Supplementary Table S4.3, Figure 4.5), however the strong site x invasion interaction indicates that such effects are not consistent across sites. Site-level PERMANOVAs confirmed that invasion had a significant influence on overall soil nutrient composition. Thus, although I found evidence that acacias alter soil abiotic variables in fynbos soils, the extent of such alterations seem to be highly context dependent.

4.4.2. Effects of acacias on soil function

I expected to see large difference in enzyme activities between invaded and pristine areas, but surprisingly only found invasion to be a significant main effect for phosphatase (Supplementary Table

S4.2, Figure 4.4). However, I also found highly significant site x invasion interactions for phosphatase, indicating that the influence of invasion is not consistent across sites.

Site level two-way ANOVAs indicated invasion to significantly increase phosphatase activity at four of the five sites (Table 4.2). Furthermore, I found urease actively to be significantly different at two sites, however activity increased with acacias invasion at one site (KV), and decreased significantly at the other site (VG).

The overall PERMANOVA model indicated that invasion influences overall soil enzyme function (Table S4.3, Figure 4.5), however the strong site x invasion interaction indicates that such effects are not consistent across sites. Site-level PERMANOVAs confirmed that invasion had a significant influence on overall enzyme functioning at two sites (Table 4.4). Thus, I do not find strong evidence that acacias alter soil functioning in fynbos soils.

4.4.3. Effects of season on soil abiotic variables and function

I found seasonality to play a significant role on four of the soil abiotic variables included here (pH, N, NO_3^- and NH_4^+) (Table 4.1, Supplementary Table S4.1). I also observed seasonality to play a significant role in the activities of all three enzymes analysed (Table 4.2, Supplementary Table S4.2).

4.4.4. Relating impacts of invasion to soil bacterial community composition and function

When I compared mean differences between invaded and pristine areas for the three relevant major nutrient-enzyme pairs (i.e. P-phosphatase, C-glucosidase, and N-urease, respectively), I found strong correlations between ΔP and Δphosphatase (r^2 = 0.40, p = 0.003), as well as ΔN and Δurease (r^2 = 0.248, p = 0.026) (Figure 4.6). This suggests that acacia-induced differences in soil nutrient levels are directly related to changes in soil functioning for P and N. Interestingly, I did not find such a correlation for ΔC and Δglucosidase (r^2 = 0.001, p = 0.89). These results agree with the findings that acacias elevated soil nitrogen levels.

For mean differences between invaded and pristine areas for enzyme activities and soil bacterial community composition (PCoA axis 1 explaining 47.1% of variation) I also found a strong correlation between ΔPCoA1 and Δphosphatase (r^2 = 0.49, p = 0.025), suggesting that acacia induced differences in soil bacterial communities directly are associated with changes in phosphatase activities (Figure 4.6). However, there was no correlation between change in bacterial community composition and change in glucosidase (r^2 = 0.19, p = 0.20) or urease (r^2 = 0.084, p = 0.42) activity.

Finally, MRM analyses confirmed that invasion had a significant influence on soil bacterial community composition (Table 4.5). However, the effect of nutrients and geographical distance was much larger (coefficients of 0.43 and 0.29, respectively, compared to 0.07 for autumn, and 0.42 and 0.48, respectively, compared to 0.08 for spring), suggesting that invasion plays a lesser role in shaping soil bacterial communities compared to nutrient differences and geographical distance. For soil functioning, I found invasion to only be a significant influence on enzyme activities in spring, and again I found geographical distance to be larger influence (coefficients of 0.68 and 0.53 for autumn and spring, respectively), suggesting that invasion plays a lesser role in influencing soil functioning compared to geographical distance.

4.5. Discussion

This study suggests that acacia-mediated invasion impacts (on soil nutrients and function) may be highly context-specific, that is, significant impacts were only evident in certain fynbos habitats. When considering these site-specific impacts, I found acacia invasions to always elevate pH, NO_3^-, NH_4^+, and total N. Similarly, although invasion did not affect soil functionality (microbial enzymatic activities) consistently across all sites, it did consistently lead to elevated phosphatase activities where site-specific effects were evident. Unsurprisingly, invasion-induced changes in soil phosphorous and nitrogen were correlated with changes in soil enzymes related to their cycling, i.e. phosphatase and urease activity, respectively. In contrast, invasion-induced changes in soil bacterial community composition was only significantly correlated with phosphatase activity.

The influence of invasive acacias on soil nutrient levels found here is in agreement with what is known from other invasive ranges of these plants around the world (Yelenik *et al.* 2004; Marchante *et al.* 2008; Lorenzo *et al.* 2010a; González-Muñoz *et al.* 2012; Souza-Alonso *et al.* 2014, 2015). Similarly, while many previously-documented impacts caused by invasive acacias appear to be context specific, their presence usually cause increased levels of soil total N, NO_3^-, NH_4^+, and available P (Yelenik *et al.* 2004; Marchante *et al.* 2008; Lorenzo *et al.* 2010a; González-Muñoz *et al.* 2012; Souza-Alonso *et al.* 2014, 2015). Nitrogen and carbon content is expected to be higher in areas heavily invaded by acacias due to the combined effects of symbiotic nitrogen-fixation by the acacias, and their high densities, leading to high leaf litter inputs that are rich in carbon and nitrogen (Liao *et al.* 2008; Lorenzo *et al.* 2010b; Lazzaro *et al.* 2014). Although I did not find a consistent effect of invasion on nitrogenous compounds (NO_3^-, NH_4^+, and total N), when evident, invasion always led to elevated levels of these compounds. These instances are in agreement with what has been found for other acacia-invaded fynbos soils (Yelenik *et al.* 2004, 2007), and in general for invasive legumes (Castro-Díez *et al.* 2014). Surprisingly though, I only found evidence for higher total soil C at one of the invaded study sites. This finding largely supports evidence from elsewhere around the world. For example, soil C levels are lower in soils recently invaded by acacias compared to uninvaded soils in Portugal (Marchante *et al.* 2008), while soils under well-established acacia invasions have similar C content than uninvaded soils in Italy (Lazzaro *et al.* 2014). Evidence from many acacia-invaded regions from around the world suggest, similar to this study, that the impacts of invasion on soil pH are highly context specific. For example, differences in pH levels in acacia-invaded forests and shrublands in Spain are opposite in directions, with pH being elevated in forest but lowered in shrublands (Souza-Alonso *et al.* 2014). Similarly, González-Muñoz *et al.* (2012) found pH to be lowered in sites heavily invaded by acacias, whereas Lorenzo *et al.* (2010a) found elevated soil pH levels in invaded pine forests, but lowered pH in invaded shrublands and grasslands, while others have found no influence of acacia invasion on soil pH levels (Yelenik *et al.* 2007; Hellmann *et al.* 2011). The age of invasive stands also seems to influence pH levels under acacia invasion (Marchante *et al.* 2008). Contrary to findings of this study, acacia invasions in equivalent Mediterranean-type ecosystems to fynbos habitats have led to a decrease in soil pH (Lazzaro *et al.* 2014), seemingly as a

means to increase available soil P (Weidenhamer and Callaway 2010). Finally, I also found the impact of invasion on soil P to be context specific and without consistent directionality, being higher under invasion at two sites, but lower at another. Again, evidence from elsewhere suggests that impacts on soil P may vary in magnitude and direction between habitats. For example, Lorenzo *et al.* (2010a) found acacia invasions to increase P in pine forests, but decrease P in grasslands, whereas others have failed to detect difference in soil P levels under invasion (Marchante *et al.* 2008; Hellmann *et al.* 2011). Recent evidence suggests that surface soil P (0–5 cm depth) to not be influenced by the presence of dense stands of N-fixing woody invaders, but rather that P concentration in deeper soil layers (15–120 cm depth) increases in response to woody encroachment (Zhou *et al.* 2018). This could, in part, explain the lack of evidence for impacts on soil P under acacias invasion since deeper soil layers were not sampled. However, considering the similar inconsistencies from other studies regarding the influence of acacia invasion of soil P, this seems unlikely.

High levels of standing above-ground biomass and leaf litter inputs by invasive acacias are expected to impact soil microbial enzyme activities as novel or highly-abundant substrates become available. For example, N-fixing and invasive *Falcateria moluccana* in Hawaii has been shown to increase phosphatase activity (phosphate cycling) (Allison *et al.* 2006). Similarly, invasive *Solidago gigantea* in Belgium increases phosphate activity levels, leading to improved release of soil phosphorus (Chapuis-Lardy *et al.* 2006). Similarly for acacias, *Acacia dealbata* invasions in Spain has been found to increase phosphatase levels depending on phenological stage (i.e. reproductive vs. vegetative), but these effects seem to be dependent on vegetation type (e.g. mixed forest vs. shrublands) (Souza-Alonso *et al.* 2014), as well as age of the invasive stand (Souza-Alonso *et al.* 2015). This study supports the elevation of phosphatase levels under acacia invasion, however, similar to data from elsewhere these impacts appear to be context dependent. Despite this, when present, impacts on phosphatase activity had clear directionality, i.e. being higher under invasion. I expected that activities of β-glucosidase (carbon cycling), and urease (nitrogen cycling) would increase under acacia invasion, an indication of accelerated carbon and nitrogen mineralization (Souza-Alonso *et al.* 2015). However, I found no evidence to suggest that invasive acacias impact glucosidase activities, and for

the two sites where invasion impacted on urease activity, impacts were in the opposite direction, i.e. elevated at one site and lowered at the other. In general, the effects of acacia invasion on glucosidase also appear to be highly context dependent, with invasion having no influence on glucosidase levels in mixed forests in Spain (Souza-Alonso *et al.* 2014), and being linked to age of the invasion stand (Souza-Alonso *et al.* 2015). The response of urease activity to acacia invasion also fits this general trend, in that impacts are dependent on invasive stand age and seasonal variation (Souza-Alonso *et al.* 2015).

Abiotic soil conditions are known to be a strong driving force in shaping soil bacterial community composition (Gibbons and Gilbert 2015; Malinich *et al.* 2017; Thompson *et al.* 2017), and therefore, dense invasive populations are expected to alter such communities indirectly via alteration of soil nutrients and root exudates (Wolfe and Klironomos 2005). For example, dense invasive N-fixing species can affect soil microbial communities in specific ways, such as enriching certain functional groups (e.g. ammonia-oxidizing microorganisms) which are key controllers of the N cycle (Malinich *et al.* 2017). This is because microbial communities seem to be strongly dependent on proximity to and local density of the invasive species (Malinich *et al.* 2017). Furthermore, such alterations in community composition has been linked to alteration in soil functioning (Kourtev *et al.* 2002, 2003), which is not surprising since soil enzyme activity (i.e. function) is a result of the excretion of extracellular enzymes from soil microbes. Seeing as soil abiotic conditions have a profound impact on soil bacterial community composition and function, it is conceivable that invasive acacias alter soil functioning either directly via altering soil nutrients, or indirectly via altering bacterial community composition. By correlating mean differences in invasion between nutrients and the respective enzymes that influence their functioning, I show that changes in soil functioning for phosphatase and urease are influenced by invasion induced changes in their soil substrates, P and N, respectively. However, I did not find such a correlation between changes in glucosidase activities and change in soil C under invasion. Intuitively, soil bacterial enzyme activities should reflect the diversity and composition of soil bacterial communities that secrete them. For example, phosphatase activity has been found to be linked to soil bacterial community composition (Waldrop *et al.* 2000). Indeed, I find

evidence to support the notion that acacia-induced changes in soil bacterial community composition is linked with changes in phosphatase activity, implicating that acacia invasion alters soil phosphatase activity as a result of altered soil bacterial communities, but potentially also altered fungal communities. However, I do not find soil glucosidase and urease activities to be linked to acacia-induced changes in bacterial community composition, even though such links have been demonstrated elsewhere (Waldrop *et al.* 2000). This is maybe not surprising since I did not detect any impact of acacia invasion on glucosidase activity and, for urease activity, identified only two instances of impacts, but which were in opposite directions. Such functional and compositional alterations under acacia invasion, as illustrated by phosphatase activities, may reflect the feedbacks created between acacia litter inputs and the structure and composition of soil microbial communities associated with these (Sauvadet *et al.* 2017), since acacia leaf litter quality differs substantially from that of native fynbos species (Yelenik *et al.* 2007).

From the data it also emerges that seasonality is a significant environmental factor influencing not only soil conditions, but also microbial community composition and their functions. This is maybe unsurprising, since microbial communities are known to differ in their abilities to break down substrates depending on season (Koranda *et al.* 2013), and also that soil bacterial community composition is highly sensitive to changes in abiotic factors that vary with seasons, for example soil moisture and temperature (Lauber *et al.* 2009; Fierer *et al.* 2012; Gibbons and Gilbert 2015; Thompson *et al.* 2017). Interestingly, enzymatic activities have been suggested as sensitive indicators of changes in soil function owing to their fast responses to environmental changes and disturbances, including invasions (Souza-Alonso *et al.* 2014). However, from the observations in this study, I argue that such enzyme activities might in fact be too sensitive to environmental change and disturbance, and that the major influence of seasonality on their functioning potentially confounds other factors that might be perceived as influencing such activities (e.g. invasion). Ultimately, the results here show that the impacts of invasive species may be highly context dependent, i.e. influenced by native/invasive species composition and traits, together with other biotic and abiotic factors of the invaded range (e.g. *Parthenium*, Osunkoya *et al.* 2017).

4.6. Acknowledgements

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4.7. Tables and figures

Table 4.1: Site level two-way ANOVA p-values for soil nutrients. Significance indicated as follows: * – p<0.05; ** – p<0.01; *** – p<0.001.

Site	Factor	pH	C	P	N	NO_3^-	NH_4^+
Flower Valley	Invasion	0.159	0.221	**0.009****	0.067	**0.015***	**0.018***
	Season	0.903	0.694	**0.049***	0.310	**0.038***	**0.014***
Koude Vlakte	Invasion	**< 0.001*****	**< 0.001*****	**0.001****	**< 0.001*****	**0.002****	**< 0.001*****
	Season	0.151	0.107	0.644	0.368	**0.001*****	0.590
Vergelegen	Invasion	0.415	0.469	0.526	0.097	**0.004****	**0.001****
	Season	**0.009****	0.741	0.050	**0.001****	**< 0.001*****	**< 0.001*****
Vermaaklikheid	Invasion	**0.009****	0.090	**0.016***	0.675	0.490	0.631
	Season	**0.023***	**0.038***	0.690	0.255	**0.014***	0.207
Walshacres	Invasion	0.890	0.215	0.690	**0.004****	**0.026***	**0.006****
	Season	0.990	**0.019***	0.973	**0.002****	0.062	**< 0.001*****

Table 4.2: Two-way ANOVA p-values for enzyme activities at each site. Significance indicated in bold and as follows: ** – p<0.01; *** – p<0.001.

Site	Factor	Phosphatase	Glucosidase	Urease
Flower Valley	Invasion	**0.008****	0.152	0.324
	Season	0.247	**0.035***	**< 0.001*****
Koude Vlakte	Invasion	**< 0.001*****	0.189	**0.005****
	Season	**0.005****	**< 0.001*****	**< 0.001*****
Vergelegen	Invasion	**0.001****	0.809	**< 0.001*****
	Season	**< 0.001*****	**< 0.001*****	**< 0.001*****
Vermaaklikheid	Invasion	0.959	0.152	0.903
	Season	**< 0.001*****	**0.035***	**< 0.001*****
Walshacres	Invasion	**0.007****	0.604	0.978
	Season	0.547	**< 0.001*****	**< 0.001*****

Table 4.3: Site-level permutational multivariate analysis of variance (PERMANOVA) results for soil nutrients. Significance indicated in bold and as follows: * – p<0.05; ** – p<0.01; *** – p<0.001.

Site	Factor	df	SS	R^2	F	p
Flower Valley	Invasion	1	21.81	0.12	4.30	**0.005****
	Season	3	27.35	0.15	1.80	0.056
	Residual	27	136.84	0.74		
	Total	31	186.00	1.00		
Koude Vlakte	Invasion	1	87.73	0.47	30.09	**< 0.001*****
	Season	3	19.56	0.11	2.24	0.053
	Residual	27	78.71	0.42		
	Total	31	186.00	1.00		
Vergelegen	Invasion	1	13.72	0.07	3.27	**0.014***
	Season	3	58.94	0.32	4.68	**< 0.001*****
	Residual	27	113.34	0.61		
	Total	31	186.00	1.00		
Vermaaklikheid	Invasion	1	14.22	0.08	2.83	**0.036***
	Season	3	35.11	0.20	2.33	**0.017***
	Residual	26	130.67	0.73		
	Total	30	180.00	1.00		
Walshacres	Invasion	1	14.88	0.08	3.12	**0.020***
	Season	3	41.17	0.23	2.88	**0.003****
	Residual	26	123.95	0.69		
	Total	30	180.00	1.00		

Table 4.4: Site-level permutational multivariate analysis of variance (PERMANOVA) results for soil enzymes. Significance indicated in bold and as follows: *** – p < 0.001

Site	Factor	df	SS	R^2	F	p
Flower Valley	Invasion	1	0.028	0.02	3.13	0.058
	Season	3	0.837	0.67	31.32	**< 0.001*** **
	Residual	43	0.383	0.31		
	Total	47	1.248	1.00		
Koude Vlakte	Invasion	1	0.462	0.29	94.47	**< 0.001*** **
	Season	3	0.950	0.59	64.75	**< 0.001*** **
	Residual	41	0.200	0.12		
	Total	45	1.612	1.00		
Vergelegen	Invasion	1	0.027	0.02	3.31	**0.048***
	Season	3	1.176	0.76	47.39	**< 0.001*** **
	Residual	41	0.339	0.22		
	Total	45	1.543	1.00		
Vermaaklikheid	Invasion	1	0.027	0.01	2.18	0.125
	Season	3	1.585	0.75	42.87	**< 0.001*** **
	Residual	40	0.493	0.23		
	Total	44	2.105	1.00		
Walshacres	Invasion	1	0.027	0.02	2.11	0.106
	Season	3	0.635	0.53	16.76	**< 0.001*** **
	Residual	42	0.530	0.44		
	Total	46	1.192	1.00		

Table 4.5: Spearman's Rank Correlations between soil nutrients, enzymatic activities, geographical distance, and bacterial community composition for autumn and spring. A design distance matrix (with invaded/invaded and pristine/pristine pairs coded as 0, and pristine/invaded pairs coded as 1) was included to test the effect of invasion on community composition. Multiple regression on distance matrices (n=9999 permutations) were used to calculate correlations. Significance indicated in bold and as follows: * – $p<0.05$; ** – $p<0.01$; *** – $p<0.001$.

	Correlate	**Autumn**	**Spring**
Composition	Intercept	84.401	6.192
	Nutrients	**0.29*** **	**0.48*** **
	Invasion	**0.07***	**0.08***
	Geographic Distance	**0.43*** **	**0.42*** **
	Model R^2	**0.37*** **	**0.50*** **
Function	Intercept	189.713	63.234
	Nutrients	**-0.12***	0.063
	Composition	-0.051	**0.19****
	Invasion	0.010	**0.036***
	Geographic Distance	**0.68*** **	**0.53*** **
	Model R^2	**0.38*** **	**0.46*** **

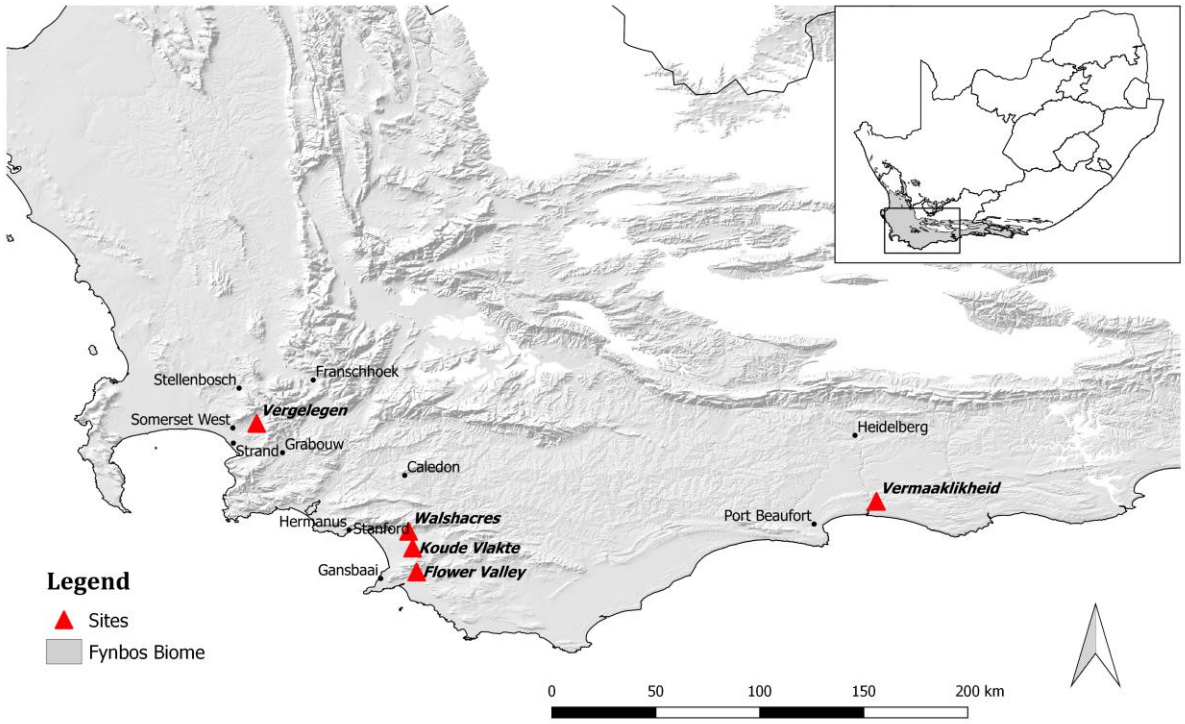

Figure 4.1: Five sites were used during this study, all of which were within the boundaries of the Fynbos biome of South Africa's Core Cape Subregion. Each site consisted of a densely invaded (by Australian *Acacia* spp.) and pristine treatment. Sites were selected so that treatment areas were in close proximity (<500 m) to each other so as to eliminate as many confounding factors (e.g. soil structural and chemical variation, vegetation type, climatic conditions etc.) as possible.

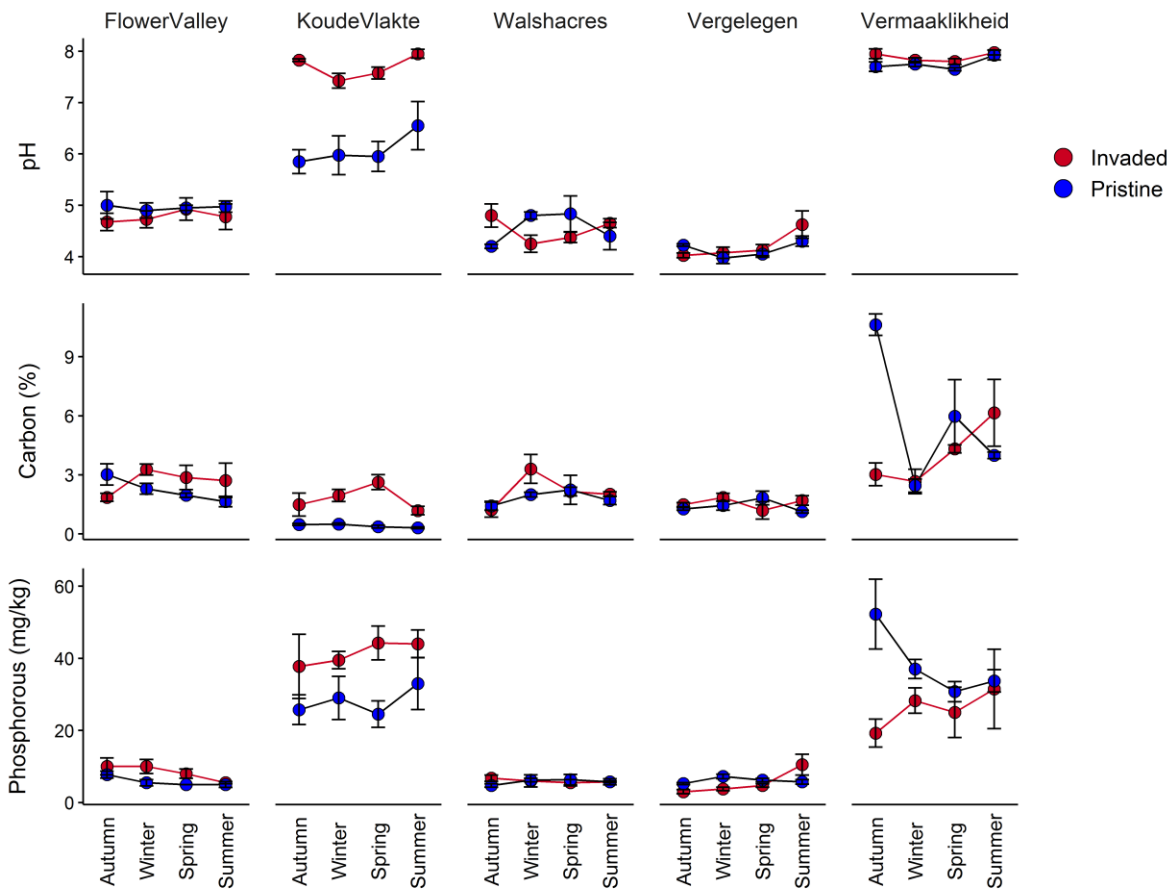

Figure 4.2: Soil abiotic variables of various invaded and pristine sites in the CCR: pH, Carbon and Phosphorous content of soils. Error bars represent standard errors. Note: the grouping of sites are according to sampling trips in each season, i.e. the first three sites (Flower Valley, Koude Vlakte, and Walshacres) and the last two sites (Vergelegen and Vermaaklikheid) were sampled together each time, respectively.

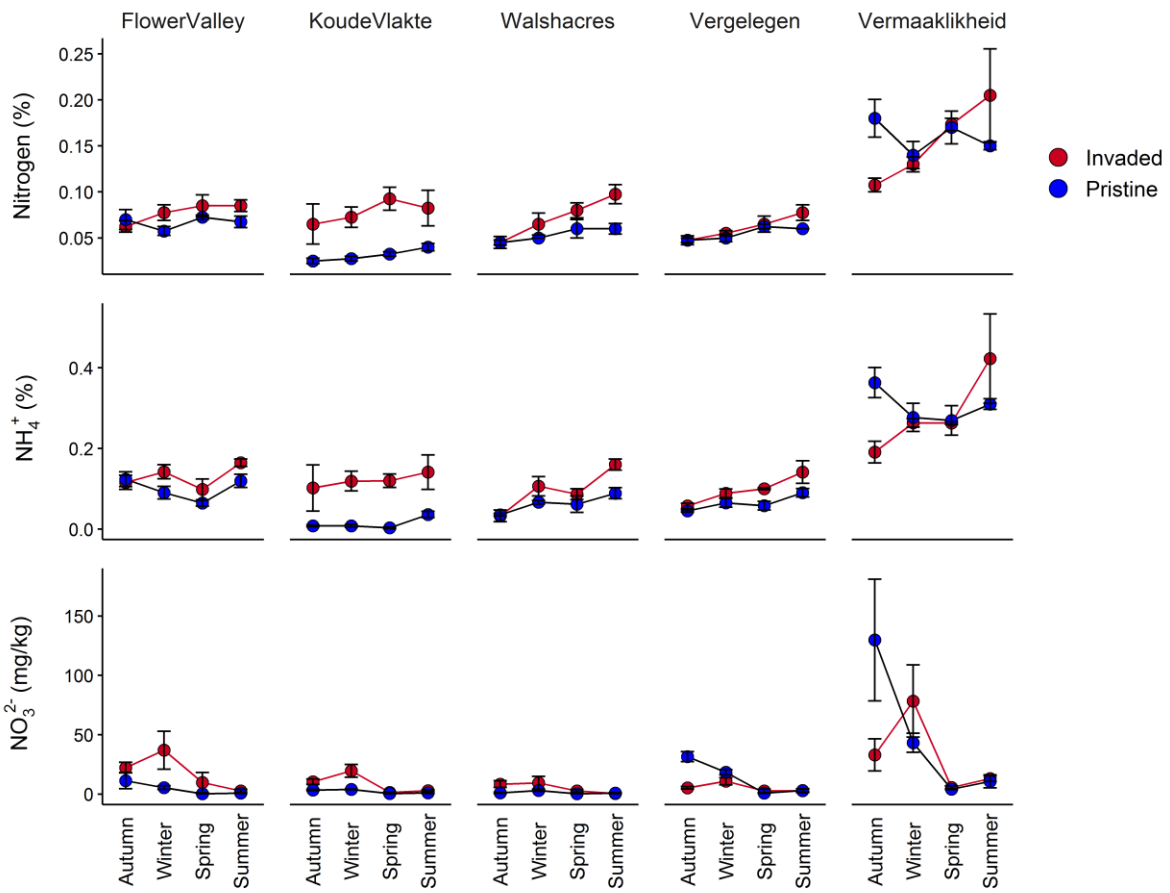

Figure 4.3: Soil abiotic variables of various invaded and pristine sites in the CCR: nitrogenous content of soils. Error bars represent standard errors. Note: the grouping of sites are according to sampling trips in each season, i.e. the first three sites (Flower Valley, Koude Vlakte, and Walshacres) and the last two sites (Vergelegen and Vermaaklikheid) were sampled together each time, respectively.

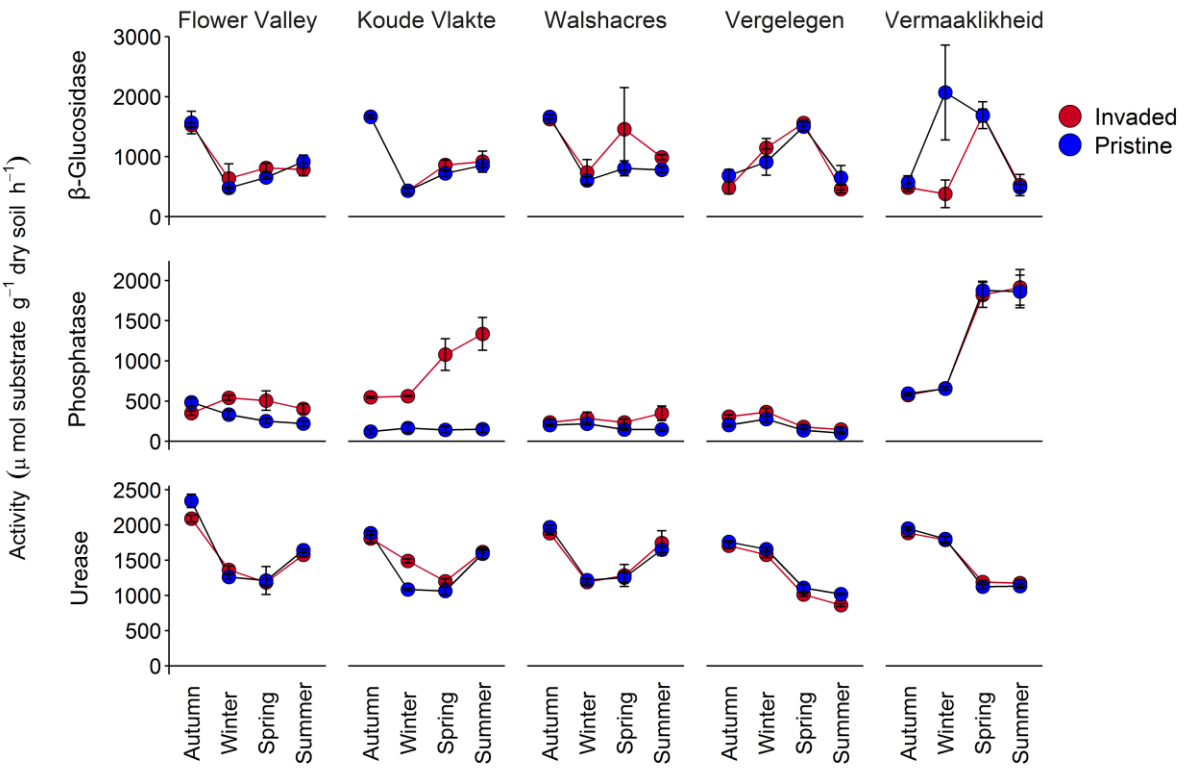

Figure 4.4: Activities of three soil bacterial extracellular enzymes that were analysed in this study, namely β-glucosidase, phosphatase, and urease. Error bars represent standard errors. Note: the grouping of sites are according to sampling trips in each season, i.e. the first three sites (Flower Valley, Koude Vlakte, and Walshacres) and the last two sites (Vergelegen and Vermaaklikheid) were sampled together each time, respectively.

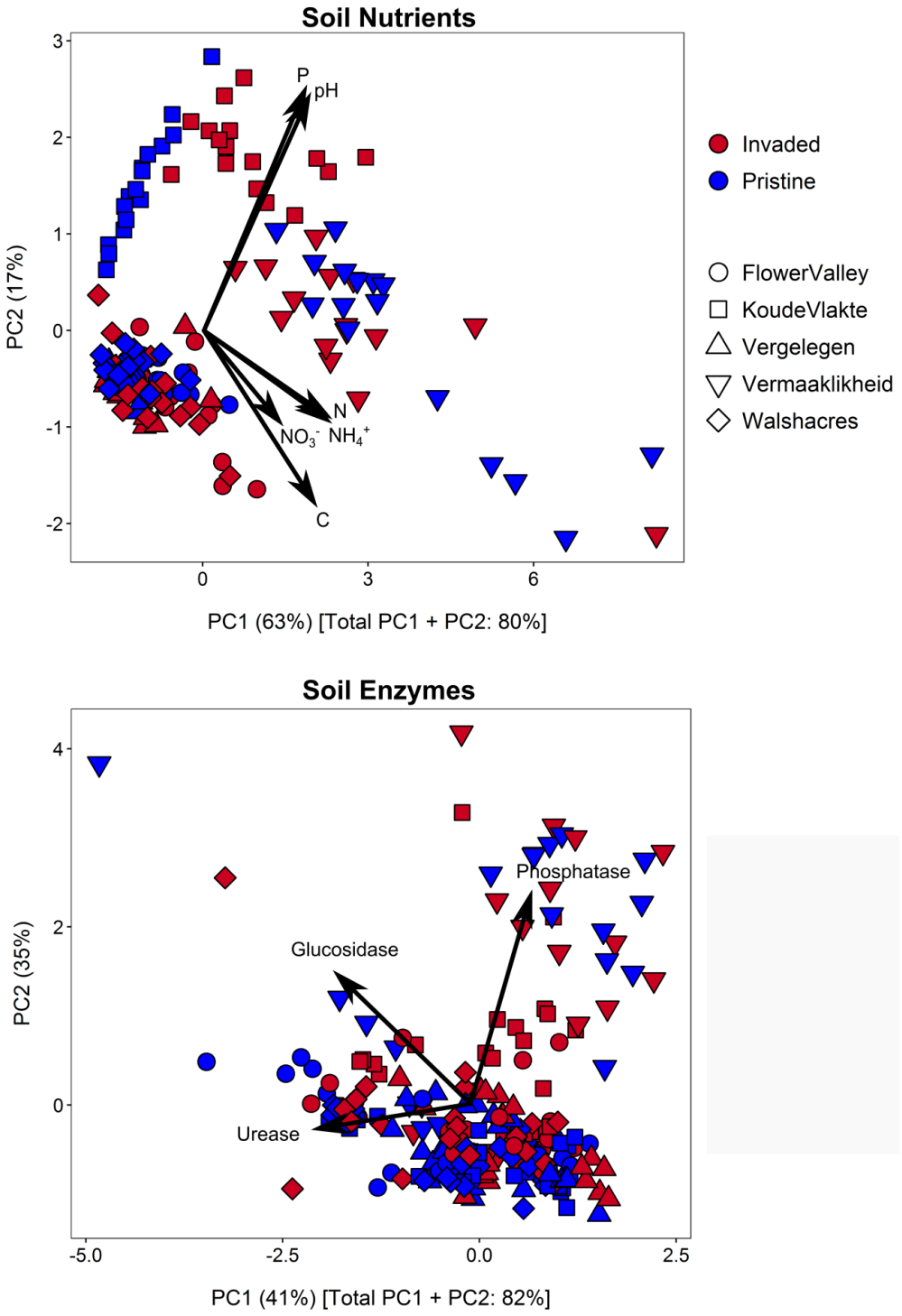

Figure 4.5: Principle components analyses of soil nutrients and enzyme activities (i.e. function) from various invaded and pristine sites in the CCR.

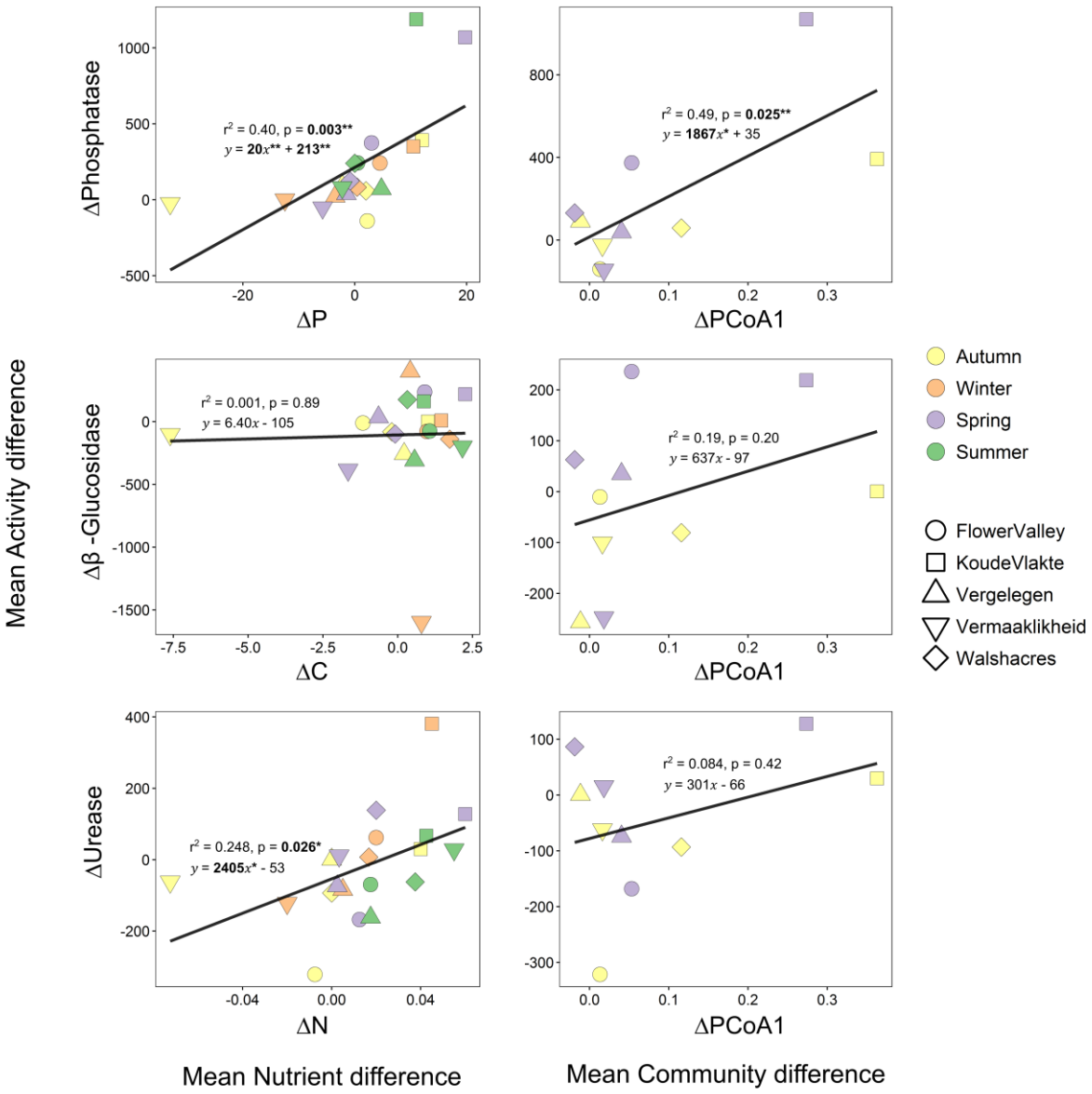

Figure 4.6: Relationships between mean differences in nutrients (left panels) and community composition (as expressed by the first axis of a principal coordinates analysis [PCoA]; right panels), and enzyme activities. All values are expressed as differences between mean invaded and pristine areas.

4.8. Supplementary information

Table S4.1: ANOVA results for different soil nutrients. Significance indicated in bold and as follows: * – p<0.05; *** – p<0.001.

Nutrient	Factor	df	Mean Sq	F	p
pH	Invasion	1	3.230	25.3	< **0.001*****
	Season	3	0.500	3.9	**0.011***
	Invasion x Site	8	42.690	334.0	< **0.001*****
C	Invasion	1	0.350	0.1	0.700
	Season	3	1.600	0.7	0.563
	Invasion x Site	8	40.410	17.3	< **0.001*****
P	Invasion	1	6.000	0.1	0.757
	Season	3	26.000	0.4	0.723
	Invasion x Site	8	3886.000	65.3	< **0.001*****
N	Invasion	1	0.008	11.6	**0.001*****
	Season	3	0.005	7.7	< **0.001*****
	Invasion x Site	8	0.030	46.2	< **0.001*****
NH_4^+	Invasion	1	0.047	14.5	< **0.001*****
	Season	3	0.031	9.5	< **0.001*****
	Invasion x Site	8	0.151	46.5	< **0.001*****
NO_3^-	Invasion	1	1.000	0.0	0.966
	Season	3	5837.000	9.0	< **0.001*****
	Invasion x Site	8	4078.000	6.3	< **0.001*****

Table S4.2: Overall ANOVAs for enzyme activities. Significance indicated in bold and as follows: *** – p<0.001.

Enzyme	Factor	df	Mean Sq	F	p
Phosphatase	Invasion	1	2517185	21.93	< **0.001*** **
	Season	3	1453495	12.67	< **0.001*** **
	Invasion x Site	4	1090811	9.50	< **0.001*** **
Glucosidase	Invasion	1	15870	0.04	0.841
	Season	3	3366982	8.58	< **0.001*** **
	Invasion x Site	4	648761	1.65	0.162
Urease	Invasion	1	2920	0.05	0.828
	Season	3	6182372	100.12	< **0.001*** **
	Invasion x Site	4	83533	1.35	0.251

Table S4.3: Overall permutational multivariate analysis of variance (PERMANOVA) results for soil nutrients and enzymes, respectively. Significance indicated in bold and as follows: *** – p < 0.001.

	Factor	df	SS	R^2	F	p
Nutrients	Invasion	1	27.58	0.049	6.77	<**0.001*** **
	Season	3	74.63	0.132	6.11	<**0.001*** **
	Invasion x Site	4	111.69	0.198	6.86	<**0.001*** **
	Residual	86	350.10	0.621		
	Total	94	564.00	1.000		
Enzymes	Invasion	1	0.18	0.019	8.25	<**0.001*** **
	Season	3	2.32	0.240	35.11	<**0.001*** **
	Invasion x Site	4	2.35	0.243	13.32	<**0.001*** **
	Residual	219	4.82	0.499		
	Total	231	9.66	1		

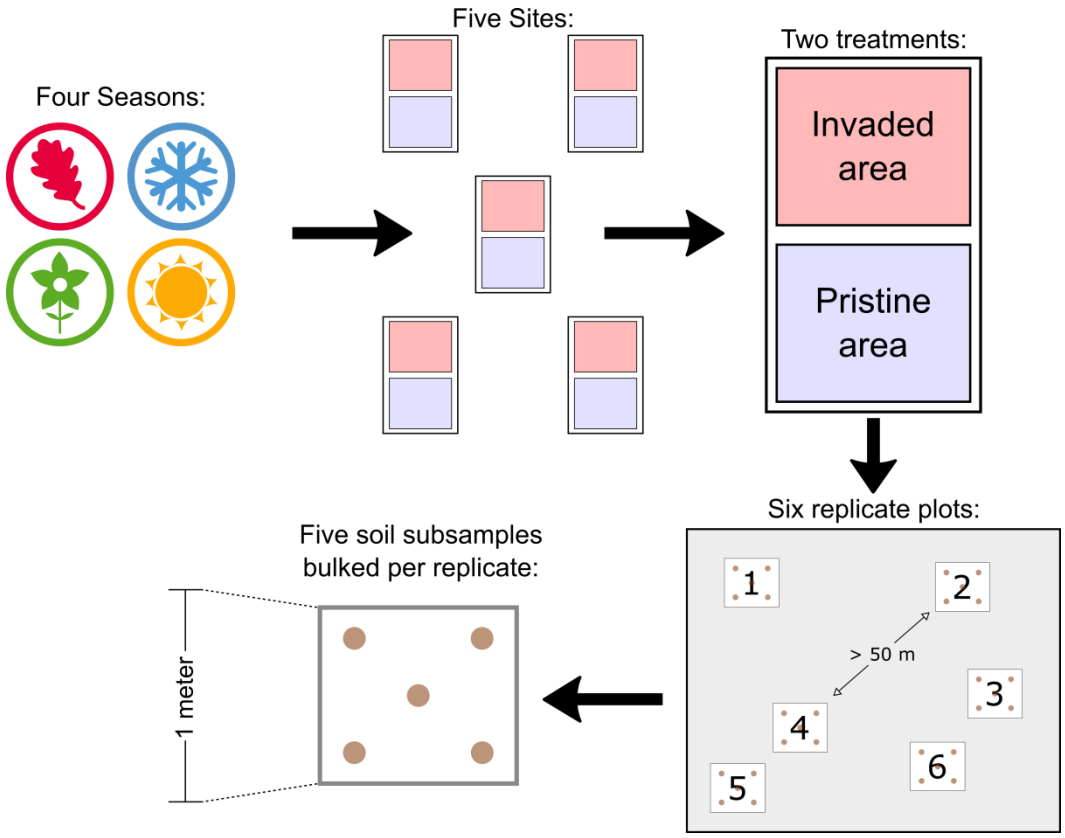

Figure S4.1: Sampling setup for soil collections. Each site had an *Acacia* invaded and a pristine treatment in close proximity to each other. In each treatment six plots (1 m x 1 m) were randomly placed at least 50 m apart and within each plot five soil subsamples were collected. The subsamples for each plot were bulked to form one independent replicate, thus yielding a total of four replicates for the invaded area and four replicates for the pristine area. This was repeated for a total of five sites, for both for all four seasons. See Materials and Methods for detailed overview.

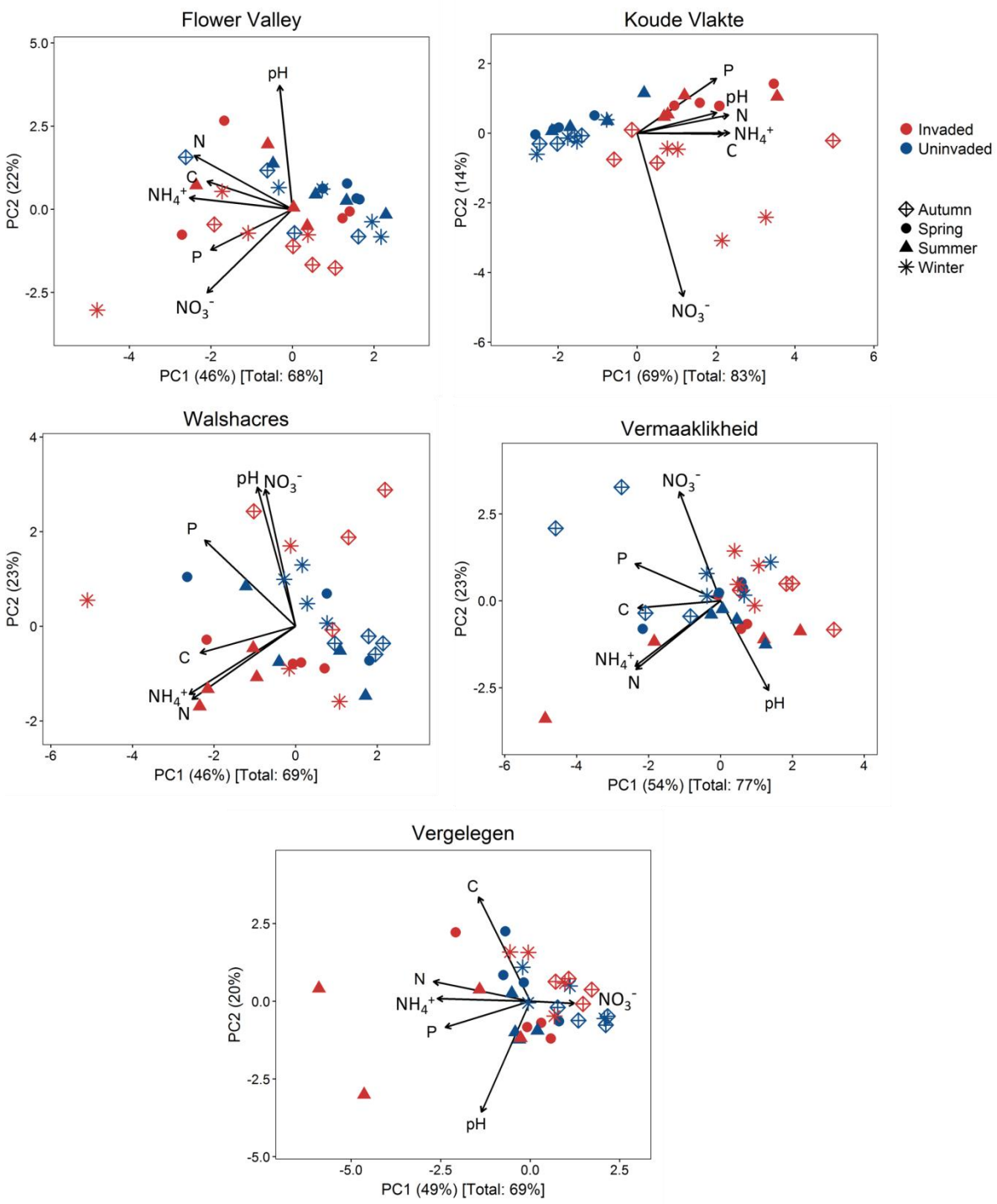

Figure S4.2: Site level principle components analyses (PCA) biplots of soil abiotic variables from various invaded and pristine fynbos sites.

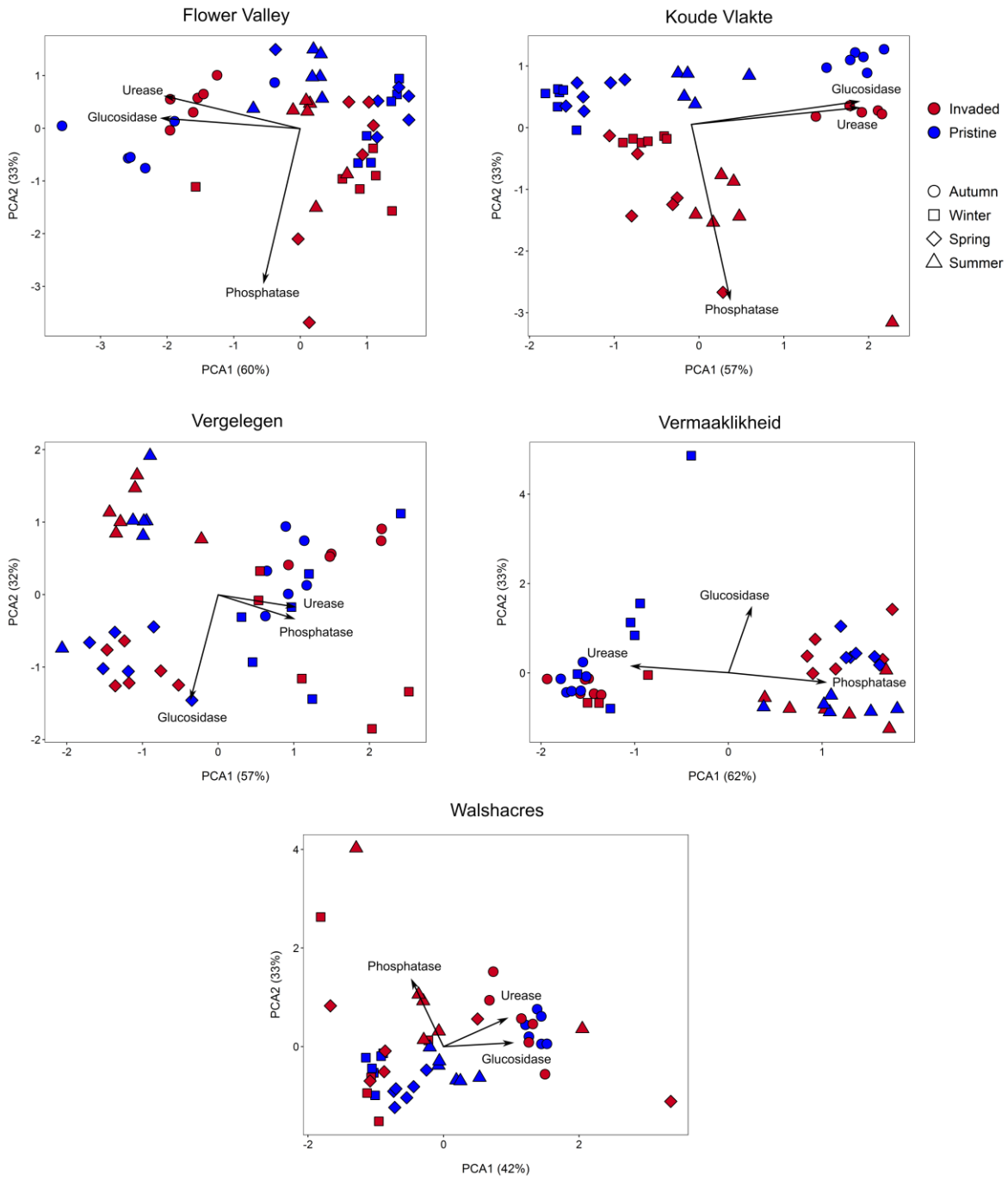

Figure S4.3: Site level principle components analyses (PCA) biplots of enzyme activities (β-glucosidase, phosphatase, and urease) of soils from various invaded and pristine fynbos sites.

CHAPTER 5: Legume-rhizobium symbiotic promiscuity and effectiveness do not affect plant invasiveness

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5.1. Abstract

The ability to fix atmospheric nitrogen is thought to play an important role in the invasion success of legumes. Interactions between legumes and nitrogen fixing bacteria (rhizobia) span a continuum of specialisation and promiscuous legumes are thought to have higher chances of forming effective symbioses in novel ranges. Using Australian *Acacia* species in South Africa it was hypothesised that widespread and highly invasive species will be more generalist in their rhizobial symbiotic requirements and more effective in fixing atmospheric nitrogen compared to localised and less invasive species. To test these hypotheses eight localised and eleven widespread acacias were examined using next generation sequencing data for the nodulation gene, *nodC*, to compare the identity, species richness, diversity and compositional similarity of rhizobia associated with these acacias. Stable isotope analysis was also used to determine levels of nitrogen obtained from the atmosphere via symbiotic nitrogen fixation. No differences were found in richness, diversity and community composition between localised and widespread acacias. Similarly, widespread and localised acacias did not differ in their ability to fix atmospheric nitrogen. However, for some species by site comparisons significant differences in $\delta^{15}N$ isotopic signatures were found, indicating differential symbiotic effectiveness between these species at specific localities. Overall, the results support recent findings that root nodule rhizobial diversity and community composition do not differ between acacias that vary in their invasiveness. Differential invasiveness of acacias in South Africa is likely linked to attributes like differences in propagule pressure, reasons for (e.g. forestry vs. ornamental), and extent of, plantings in the country.

KEYWORDS: *Acacia*, invasive, mutualism, nitrogen fixation effectiveness, rhizobia, root nodule, symbiotic promiscuity

5.2. Introduction

The ability to establish mutualistic interactions is thought to be an important determinant of colonisation success and spread of non-native plants (Traveset and Richardson 2014). These interactions result in higher fitness as afforded by increased nutrition (e.g. biological nitrogen fixation), reproduction (e.g. pollination) and spread (e.g. seed dispersal). Like most biological interactions, plant mutualisms fall along a continuum of specialisation, with plants at one end of the spectrum capable of forming mutualisms with a wide range of partners (i.e. exhibiting high levels of symbiotic promiscuity or generalism), but at the other end only associating with one or a few partners (i.e. specialisation) (Bascompte 2009). Promiscuity may allow plants to more easily utilise potential mutualists found in their new ranges (Aizen *et al.* 2012; Heleno *et al.* 2013). Intuitively then, promiscuity on the part of either plant or potential mutualists should enhance colonisation probability of introduced plants that are often unaccompanied by their own mutualists (Parker *et al.* 2006; Stanton-Geddes and Anderson 2011; Wandrag *et al.* 2013).

The establishment success of plants, specifically invasive species, has been increasingly linked to their interactions with mutualistic soil microbes (Inderjit and Cahill 2015; Vestergård *et al.* 2015). For example, most legumes form symbioses with bacteria called rhizobia resulting in biological nitrogen fixation, a physiological adaptation that has been linked to their success as invasive species (Rodríguez-Echeverría *et al.* 2009, 2011). Legumes benefit from the acquisition of fixed atmospheric nitrogen from rhizobia in specialised structures called root nodules, while simultaneously providing rhizobia with carbon resources (Franche *et al.* 2009). The formation of root nodules involves complex signalling pathways between plants and bacteria (Stacey 2007). For example, various rhizobial nodulation genes (*Nod* genes) respond to plant root exudates (typically flavonoids) by producing nodulating factors (so-called nod factors), leading to root nodule formation (Hopkins and Hüner 2009). *Nod* genes, which are important determinants of specialization in legume-rhizobium

interactions (Spaink 2000), are located on symbiotic plasmids or highly mobile 'symbiotic islands', which can be transferred between different bacterial species, and even genera, through conjugation (Ding and Hynes 2009). This means that bacteria with the same identity based on core genes (e.g. 16S rDNA) might not be able to nodulate the same legume species if they carry different symbiotic genes.

Nodulation per se (i.e. establishment of an interaction) does not always translate into effective symbiosis benefiting the plant, as a single plant individual can be colonised by multiple strains of bacteria differing in nitrogen-fixing effectiveness (Mårtensson *et al.* 1989; Kiers *et al.* 2006). Different strains of the same rhizobial species can differ in their effectiveness (Dwivedi *et al.* 2015), even in association with the same host legume species (Thrall *et al.* 2000, 2011; Klock *et al.* 2015). Like other mutualisms, legume-rhizobium interactions are susceptible to cheating strategies (Franche *et al.* 2009; Klock *et al.* 2015), whereby the less effective 'cheater' strains act as free riders providing limited or no benefits to the host plant (Franche *et al.* 2009; Barrett *et al.* 2015), for example certain strains of *Bradyrhizobuim japonicum* and *Rhizobium meliloti* (Amarger 1981; Singleton and Stockinger 1983; Kiers *et al.* 2003). Moreover, contrary to the widely held view that individual nodules typically comprise a single strain of rhizobia, it is now known that single nodules can, in some instances, harbour multiple strains (Denison 2000; Kiers *et al.* 2006; Checcucci *et al.* 2016), including non-rhizobial endophytes whose functions are not yet fully understood (Hoque *et al.* 2011; Birnbaum *et al.* 2016). Thus, cheating behaviour should theoretically be possible within individual nodules (Checcucci *et al.* 2016) and may therefore impact on overall symbiotic effectiveness. To counter the effects of cheating some legumes have acquired the ability to select for more "cooperative" rhizobia depending on their nitrogen-fixing effectiveness (Kiers *et al.* 2003).

Congeneric legumes that differ in their levels of invasiveness are excellent study systems to investigate how changes in diversity, composition and effectiveness of root nodule-associated rhizobial communities impact plant invasion. For example, trees in the genus *Acacia* Mill. have been extensively transported around the globe to regions outside their native Australian ranges for various reasons (e.g. forestry, fuel, ornamental) (Kull and Rangan 2008; Carruthers *et al.* 2011; Kull *et al.*

2011). Globally acacias differ in invasiveness and their introduction histories (Richardson *et al.* 2011; Rodríguez-Echeverría *et al.* 2011). For example, in South Africa, widespread acacias are considered to be some of the country's most damaging invasive species (e.g. *A. dealbata*, *A. decurrens*, *A. mearnsii*, Le Maitre *et al.* 2011, 2015) while others are restricted to a single locality and are found in relatively low abundance (e.g. *A. paradoxa*, Zenni *et al.* 2009). Differences in invasiveness of acacias have been attributed to varying propagule pressure and residence times (Richardson and Rejmánek 2011). Differential invasiveness may, however, also reflect differences in the effectiveness of their mutualistic relationships with rhizobia. Globally, highly invasive acacias appear to be promiscuous rhizobial hosts, predominantly nodulated by various *Bradyrhizobium* strains (Rodríguez-Echeverría *et al.* 2007, 2011; Crisóstomo *et al.* 2013; Le Roux *et al.* 2016). In some instances these rhizobia have been co-introduced with acacias into their new ranges (e.g. Rodríguez-Echeverría 2010; Crisóstomo *et al.* 2013; Ndlovu *et al.* 2013) while in other instances they appear to form associations with novel rhizobia (e.g. Ndlovu *et al.* 2013). Co-introduction means that introduced plants may not be limited by their ability to find compatible rhizobia in their new ranges, which might also be less effective. There is some evidence suggesting that invasive acacias are generally more promiscuous than naturalised or non-invasive acacias (Klock *et al.* 2015), but such generalism appears to be constrained by geographical scale (Klock *et al.* 2016).

Here, using next-generation sequencing data for the nodulation gene, *nodC*, obtained from root nodule communities from various introduced acacias that differ in their degree of invasiveness in South Africa, together with stable isotope analysis of nitrogen, I tested the hypothesis that mutualist promiscuity enhances symbiotic effectiveness and that promiscuity is linked to invasion success. Specifically, I hypothesised that widespread acacias (i.e. successful invaders) will show higher levels of symbiotic promiscuity and effectiveness, compared to localised acacias (those that have not spread extensively). I tested three predictions arising from these hypotheses 1) that widespread acacias should associate with more diverse rhizobial communities than co-occurring localised acacias, 2) that widespread acacias should exploit a compositionally different rhizobial community than localised

species, and 3) that widespread acacias should form associations with more effective rhizobial strains and will therefore accumulate more fixed atmospheric nitrogen.

5.3. Materials and methods

5.3.1. Study species

I sampled rhizobial communities from populations of all known naturalised Australian *Acacia* species found in South Africa (with the exception of *A. decurrens*), i.e. 19 species in total. Wild populations were recently discovered of two species, *A. cultriformis* and *A. piligera* (JRU Wilson, personal communication), not previously recorded in the South African Plant Invaders Atlas (SAPIA) database (Henderson 1998), but which are suspected to have been introduced long ago to the country (Poynton 2009), and were included here. To classify taxa according to spread status, i.e. as widespread or localised, I extracted all distribution records from the SAPIA database (Henderson 1998). These were used to determine the number of quarter degree squares (QDS: 15 min x 15 min grids cells in the WGS84 geodetic datum) occupied by each species (Figure 5.1). I then used the natural break in QDS occurrences to classify species as widespread (QDS≥38) or localised (QDS<38). This approach identified 8 localised species and eleven widespread species (Table S5.1). Importantly, pairs of localised and widespread species were sampled at each sampling site, with the geographic location of sites determined by the distribution of localised species (Figure 5.2). This site-level paired sampling design allowed us to investigate differences in rhizobial communities associated with localised and widespread *Acacia* species at the local/site scale, thus controlling for strong spatial compositional turnover often found in microbial communities (Slabbert *et al.* 2010). For five widespread taxa (distributed over three sites) I could not find populations co-occurring with a localised species and thus they were sampled alone. These species were not included in paired comparisons, but were included in the visualization of bacterial community composition between species.

5.3.2. Root nodule collections, DNA isolation and next generation sequencing

Root nodules were collected from July – November 2015 by excavation of acacia saplings and removal of nodules from roots. Between five and 10 root nodules per individual plant were collected

from at least five individuals per species at each site. The individuals were at least five meters apart from each in order prevent potential sampling of co-infected plants. Root nodules were kept on silica until needed for DNA extraction. For DNA extraction I pooled five root nodules per individual plant and five replicate plants for each species at each site. Pooled desiccated root nodules for each plant were tissue-lysed to create a homogenous mixture for DNA extraction. DNA was extracted from tissue-lysed root nodules using the DNeasy® Plant Mini Kit (Qiagen, supplied by White Head Scientific, Cape Town, South Africa) following the manufacturers protocol. Samples were nanodropped to determine DNA quality and concentration.

The nodulation gene, *nodC*, was amplified for nodule-extracted DNA using the primers nodCF12F (5'-CCG GAT AGG MTG GKB CCR TA-3') and nodCRI2R (5'-GTG CAC AAS GCR TAD RCC TTC AH-3') and with sample-specific barcodes in the forward primer. This barcode has been successfully applied across rhizobia in both the Alpha- and Betaproteobacteria (Le Roux *et al.* 2016). Amplification was done using a 30 cycle PCR and the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following PCR conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, followed by a final elongation at 72°C for 5 minutes. After amplification, PCR products were checked on a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple PCR samples (each sample representing the contents of five nodules from an individual plant) were barcoded first and then pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled PCR samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA) and used to prepare DNA libraries by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at the Molecular Research LP next generation sequencing service (www.mrdnalab.com, Shallowater, TX, USA) on a Illumina MiSeq instrument following the manufacturer's guidelines. The approach of utilizing next generation sequencing techniques has the advantage of including multiple rhizobial OTUs per nodule (Checcucci *et al.* 2016), since conventional methods of culturing are not able to detect all of these endophytes (Birnbaum *et al.* 2016).

5.3.3. Bioinformatics

I used mothur version 1.36.1 (Schloss *et al.* 2009) to perform downstream analyses of raw MiSeq sequence data. Briefly, I removed all DNA sequences that had low quality scores (<25% quality) and which had more than two differences to the barcode primer sequences. I screened out all sequences that had any ambiguous bases and a maximum of six homopolymers. I optimised the minimum and maximum lengths of sequences by choosing those sequences that started and ended in positions that were occupied by 90% of all sequences, resulting in sequences of between 333 and 336 bp long. Because individual root nodules can comprise multiple strains of rhizobia (Checcucci *et al.* 2016) I subsampled 1000 sequences from each replicate (i.e. 5 pooled nodules from an individual plant), yielding a total of 5000 sequences for every 25 nodules from each species. For one species, *A. cyclops*, only 3000 sequences were obtained as only three replicates yielded good quality sequencing data. This approach also allowed us to account for the usually strong correlation between the number of observed operational taxonomic units (OTUs) and the number of sequences obtained, thus reducing the biases these measures introduce to alpha and beta diversity measures (Schloss *et al.* 2011). Note that although 'bacterial strain' commonly refers to a group of genetically identical individuals and 'OTU' to a certain level of genetic similarity, I use these terms interchangeably here. From these subsamples I removed all chimeric sequences independent of a reference database using the uchime algorithm (in mothur) (Edgar *et al.* 2011) and the template as self, i.e. *de novo* removal. Since no reference database is available for *nodC*, I computed pairwise sequence similarities with the Needleman-Wunsch algorithm and then clustered the sequences into root nodule rhizobial OTUs (RNR OTUs) at the 97% DNA sequence similarity with the nearest neighbour algorithm. I then removed singleton and doubleton OTUs (i.e. OTUs with only one or two reads across all 98000 *nodC* sequences). The resulting RNR OTU table (individual acacia tree by RNR OTU count matrix) had many instances of extremely low RNR OTU abundances (e.g. < five DNA sequence reads across all 98 replicates – 0.1% of total sequences), which likely represent data generation errors. I removed these extremely low abundance OTUs from the dataset in two ways. First, after inspection of a cumulative sequence contribution curve (Figure S5.1) I only retained RNR OTUs representing the majority of sequences across all replicates for further downstream analyses. This approach identified

25 RNR OTUs that accounted for 99.1% of all DNA sequence reads. Next, all cells in the OTU x replicate matrix that contained less than 1% of sequences for that replicate were converted to zero. To determine the taxonomic affinity of OTUs I blasted their associated sequences against the NCBI's Genbank database (http://blast.ncbi.nlm.nih.gov/Blast).

5.3.4. Phylogeny

Representative DNA sequences for each RNR OTU were aligned, and a Bayesian inference phylogeny reconstructed using Mr Bayes v 3.2 (Ronquist and Huelsenbeck 2003). Sequence data from the genus *Mesorhizobium* were included as outgroup taxa. jModelTest (Posada 2008) and the Akaike information criterion (Akaike 1973) were used to determine the best fit model for the data. The Bayesian model was run for four million iterations sampling every 1000th generation and a consensus tree was built, discarding the first 25% of trees as burn-in specifying the GTR + G substitution model. Posterior probabilities (PP) were calculated using a majority rule consensus method to assess tree topology support.

5.3.5. Root nodule rhizobial community diversity

I aggregated RNR OTUs across replicates to determine diversity at the population level, i.e. I summed all replicates for each species per site that was sampled. In order to determine whether sampling effort was adequate I performed rarefaction analyses in mothur version 1.36.1 (Schloss *et al.* 2009). From the aggregated RNR OTU matrix I calculated species richness (S; total number of RNR OTUs per species, giving equal weight to both abundant and rare RNR OTUs), Shannon diversity (H; diversity measure that takes into account the abundance differences between dominant and rare RNR OTUs), Inverse Simpson diversity (Si; diversity measure that weights the abundance of dominant RNR OTUs higher than rare ones) and evenness (J; which measures how equally the abundances of RNR OTUs are spread in the sample) (Hill 1973; Chao *et al.* 2014). I calculated evenness as the natural logarithm of H/S (Hill 1973). Diversity indices were calculated using the R package `vegan` (version 2.3-3, Oksanen *et al.* 2016) and the function `renyi`. Together, these three metrics account for the influence of both common and rare RNR OTUs. I also calculated Faith's Phylogenetic Diversity (PD, Faith

1992) based on the retrieved RNR OTU phylogeny using the package `picante` (Kembel *et al.* 2010). This measure includes phylogenetic relatedness when calculating diversity for a sample of various abundances.

In order to investigate differences between localised and widespread species I conducted paired t-tests to compare the various metrics described above across pairs of species sampled at each site.

5.3.6. Root nodule rhizobial community composition

To visualise acacia species-associated root nodule community compositions I ran a Principal Coordinates Analysis (PCoA) based on a Bray-Curtis dissimilarity matrix, calculated with the function `vegdist` in the R package vegan (Oksanen *et al.* 2016). I did this for all acacia species that were sampled and not just for co-occurring localised and widespread species. I then used a Permutation Multivariate Analysis of Variance (PERMANOVA) (Anderson 2001) with 9999 permutations in PRIMER v7 (Clarke *et al.* 2014) to test for the influence of status (widespread vs. localised) on differences in RNR OTU composition; I used site as a random factor and status as a fixed factor. To determine whether localised and widespread species were significantly over- or under-dispersed in terms of their group centroids (i.e. multivariate homogeneity of group dispersions/variances) I used the function `betadisper` in the vegan package with 9999 permutations. I also wanted to see whether acacias within sites are associating with compositionally more similar rhizobial communities compared to acacias from other sites. For this I made a design matrix with zeros (0) coding for within-site distances and ones (1) coding for between-site distances (Rundle and Jackson 1996). I then used a Mantel test, also in package vegan, with 9999 permutations to test the correlation between the Bray-Curtis dissimilarity matrix and the design matrix. Finally, in order to visualise the OTU abundances I created a heat map with four abundance categories (excluding zero): 10 – <100, 100 – <1000, 1000 - <2500 and ≥2500 using the package gplots (version 2.17.0) and function `heatmap.2` (Warnes *et al.* 2015).

5.3.7. Stable isotope analysis

The ratio of N^{15}/N^{14} ($\delta^{15}N$) of plant leaves/shoots has been used as a proxy for biological nitrogen fixation (BNF) (Hobbie *et al.* 1998; Rodríguez-Echeverría *et al.* 2009; Lötter, Valentine, *et al.* 2014; Lötter, Van Garderen, *et al.* 2014). This is because nitrogenase preferentially incorporates the lighter isotope of N_2 gas (Sra *et al.* 2004; Unkovich 2013), and has the effect of diluting the overall isotopic signature, leading to decreased $\delta^{15}N$ values. Thus, smaller values of δ are indicative of added N from biological nitrogen fixation (Rodríguez-Echeverría *et al.* 2009; Lötter, Valentine, *et al.* 2014; Lötter, Van Garderen, *et al.* 2014). Conversely, soil available nitrogen generally has a higher value of 15N compared to the atmosphere (Unkovich *et al.* 2008), leading to increased values of $\delta^{15}N$ in plants exploiting soil-derived nitrogen as the primary source of nitrogen. Thus, to infer whether widespread and localised acacias differed in their abilities to obtain nitrogen from the atmosphere, and therefore the effectiveness of their rhizobial mutualists, I used stable isotope analyses (Environmental Isotope Laboratory, iThemba Labs, WITS University, Johannesburg). The first three to five fully expanded leaves were collected from the same individuals from which root nodules were collected. All leaves were immediately oven-dried for one week at 45°C to prevent moulding after collection. Dried leaf samples were crushed into a fine powder and nitrogen isotopic analyses carried out using a Flash HT Plus integrated via a ConFlo IV system with a Delta V Plus Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). Samples were combusted at 1,020°C and the nitrogen isotope values corrected against an in-house standard (Merck Gel $\delta^{15}N = +6.80‰$).

Isotope values were expressed in parts per thousand (‰) following Lötter *et al.* (2014a; b) and Rodríguez-Echeverría *et al.* (2009):

$$\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000‰$$

where $\delta^{15}N$ is the heavy N isotope, and R is the ratio of heavier to light isotopes ($^{15}N/^{14}N$) for the sample and standard (being atmospheric nitrogen), respectively. $\delta^{15}N$ values were used in t-tests for

each site, comparing each widespread with co-occurring localised species pair. For site TK, which had two localised species, I did a pairwise t-test with Bonferroni correction.

All statistical analyses (Paired t-tests, ANOVAs, and PCoA) were performed in the R programming language (version 3.2.2) (R Core Team 2017) with functions from the base package.

5.4. Results

5.4.1. Root nodule rhizobial community diversity

From the 98000 *nodC* sequences selected for downstream analyses 2480 (2.5%) were removed as chimeric. After clustering at 97% sequence similarity, a further 1256 sequences representing singleton or doubleton RNR OTUs were removed. A total of 170 RNR OTUs were recovered from the remaining 94264 DNA sequences. Not surprisingly, the first 25 RNR OTUs accounted for 99.1% (93444 DNA reads) (see supplementary Figure S5.1), all of which are representative of the genus *Bradyrhizobium*. Rarefaction analyses showed nodule bacterial taxon accumulation curves to reach asymptotes in all instances (Figure S5.2), indicating that sampling was representative after removal of singletons/doubletons and low frequency interactions (i.e. representing <0.1% of sequences for an individual tree).

RNR OTU richness, Shannon diversity, Inverse Simpson diversity, Evenness, or Phylogenetic Diversity did not differ significantly between pairs of co-occurring widespread/localised acacias (Figure 5.3; Table 5.1, S5.2). While overall diversity of nodule communities did not differ significantly between widespread and localised acacias across sites (paired t-tests in Table 5.1), the trend was towards higher bacterial diversity associated with widespread acacias for all metrics (Table 5.1, Figure 5.3). For six of the eight sampled widespread/localised acacia pairs the widespread species housed more diverse bacterial communities (Figure 5.3).

5.4.2. Root nodule rhizobial community composition

The first two components of the PCoA explained 62% of the variation in bacterial community composition (PC1 – 39%, PC2 – 23%). There were no consistent compositional differences in the communities of bacteria with which widespread and localised acacias are associated with (F-value=0.31, p=0.865). There was substantial variation in the composition of bacterial communities associated with both widespread and localised acacias (Figure 5.4) and the dispersion of communities did not vary with status (F value=0.288, p=0.601). Finally, while there was a tendency for compositional differences within sites (i.e. between widespread and localised acacia pairs) to be lower than differences between acacia species across sites (r^2=0.0087, p=0.0597) this trend was not significant. Interestingly, one ubiquitous RNR OTU (OTU1) associated with all acacias, although to various extents (Figure 5.5), while three OTUs were unique to widespread species (OTU20, 24 and 25) and one OTU was unique to a localised species (OTU23, associated with *Acacia fimbriata*).

5.4.3. Stable isotope analysis

For two pairs of widespread and localised acacias there were significant differences in $\delta^{15}N$ values, and in both cases the localised species had lower values (*A. adunca* p<0.05, and *A. paradoxa* p<0.01), indicating that it had received more nitrogen from atmosphere through fixation (Figure 5.6A) (Rodríguez-Echeverría *et al.* 2009; Lötter, Valentine, *et al.* 2014; Lötter, Van Garderen, *et al.* 2014). A mixed trend was observed at site TK where one of the localised species (*A. implexa*) differed significantly (p<0.05) from the widespread species (*A. baileyana*), and also from the other co-occurring localised species *(A. piligera*). Thus, here the two localised species differed significantly in their BNF abilities. Apart from these sites, overall there were no differences in BNF between localised and widespread species. It is interesting to note that at sites DP, G, and KF, there seems to be an increased uptake of atmospheric nitrogen by acacias. One possibility for this might be lower level of soil nitrogen available for use by these acacias, or that these might represent cases of more effective mutualisms.

5.5. Discussion

I found no support for the prediction that mutualist promiscuity or effectiveness influences invasiveness of acacias in South Africa. Counter to expectations under this hypothesis, rhizobial communities of widespread and localised acacias do not exhibit consistent diversity differences. Furthermore, analyses of RNR community composition between widespread and localised species indicate no consistent preference for particular community assemblages between these two groups. These findings are in agreement with Klock *et al.* (2016) who found rhizobial richness and community composition to be similar for Australian acacias that have differential invasiveness in California. In the native Australian ranges of acacias, narrowly distributed species also have similar levels of rhizobial associations to extremely widespread species (Murray *et al.* 2001), i.e. range restricted species do not show greater levels of specialization. Interestingly, such patterns have been demonstrated for other legume species, for example non-native *Trifolium* species that associate with equally diverse rhizobial communities in their introduced (New Zealand) and native (UK) ranges (McGinn *et al.* 2016). Although there seems to be a general paucity of data regarding the rhizobial symbionts nodulating acacias in their native Australian environment, there seems to be little difference in the promiscuity of invasive and non-invasive acacias (Rodríguez-Echeverría *et al.* 2011). For example, based on 16S rRNA genetic data, *A. dealbata*, *A. longifolia*, *A. mearnsii*, *A. melanoxylon* and *A. saligna* are able to nodulate with both *Bradyrhizobium* and *Rhizobium* strains (Lawrie 1983; Barnet *et al.* 1985; Marsudi *et al.* 1999; Lafay and Burdon 2001; Yates *et al.* 2004), while *A. pycnantha* nodulates with both *Bradyrhizobium* and *Burkholderia* strains, suggesting that these acacias are all generalist in their symbiotic requirements.

Similar to native Australian regions (Burdon *et al.* 1999; Stępkowski *et al.* 2012) and other introduced ranges (Weir *et al.* 2004; Rodríguez-Echeverría *et al.* 2011; Crisóstomo *et al.* 2013; Ndlovu *et al.* 2013), I found naturalised acacias in South Africa to be primarily associated with slow-growing rhizobia from the genus *Bradyrhizobium*. *Bradyrhizobium* is a cosmopolitan genus and is associated with a diverse range of legumes globally (Weir *et al.* 2004; Andam and Parker 2008; Birnbaum *et al.* 2012). Without data from the native ranges, and the short sequence reads employed here, it is difficult

to elucidate whether rhizobial symbionts have been co-introduced with acacias to South Africa. Such co-introductions are a common phenomenon among woody invaders and their microbial mutualists (Nuñez and Dickie 2014). It is conceivable that the high propagule pressure underlying many acacia introductions to South Africa (Poynton 2009) may have facilitated co-introductions of compatible rhizobia, as has been demonstrated for *A. pycnantha* in South Africa (Ndlovu *et al.* 2013). Co-invasions of acacias and their associated rhizobia have been found for *A. longifolia* and *A. saligna* in Europe (Rodríguez-Echeverría 2010; Crisóstomo *et al.* 2013) and for *A. longifolia* in New Zealand (Weir *et al.* 2004).

Recent studies have found native legumes of South Africa's Greater Cape Floristic Region to nodulate predominantly with bacterial symbionts other than *Bradyrhizobium* (e.g. *Burkholderia* and *Mezorhizobium* strains, [Kock 2004; Lemaire *et al.* 2015]), while invasive acacias preferentially nodulate with *Bradyrhizobium* (Ndlovu *et al.* 2013; Le Roux *et al.* 2016). A similar pattern was observed for acacias in New Zealand where they are nodulated only by *Bradyrhizobium* strains while native legumes associate predominantly with strains of *Mesorhizobium* (Weir *et al.* 2004). Therefore, considering the general rarity of *Bradyrhizobium* in the Cape, and their prominent association with Australian acacias in this study, it is unlikely that they represent mutualists with which native legumes frequently nodulate. An intriguing possibility for the ubiquitous nature of *Bradyrhizobium* in association with all acacias studied to date, and specifically with regards to localised species included in the current study, is that soil bacterial communities have been transformed to such an extent by the widespread species that their strains now dominate the below ground environment, i.e. facilitated proliferation of certain strains upon acacia invasion. Indeed, a recent study found that rhizosphere soils of invasive *A. dealbata* in South Africa are significantly enriched for *Bradyrhizobium* strains compared to soils where the species is absent (Valverde *et al*. submitted). Such transformations of soil community composition could therefore potentially create opportunities for localised species to encounter, and associate with rhizobial strains utilised by widespread acacias. Overall I found no evidence that a lack of generalism characterises localised acacias in South Africa. Instead, I found that both localised and widespread acacias associate equally well with a range of bacterial OTUs and thus

appear not to be constrained by locating compatible rhizobia. This is in agreement with observations from California where non-native acacias differing in invasiveness showed similar levels of symbiotic promiscuity and performances when grown in non-native soils (Klock *et al.* 2016). Although it is possible that certain strains have been co-introduced with some of the acacias studied here, the cosmopolitan status of *Bradyrhizobium* makes conclusions regarding this difficult. To conclusively determine whether co-introductions of rhizobia occurred with acacias to South Africa would require a phylogeographic study of Australian and South African rhizobia (e.g. Ndlovu *et al*. 2013).

The abundance of compatible rhizobia in soils seems to be an important factor for determining growth responses of acacias (Thrall *et al.* 2007a) as well as their invasiveness (Parker 2001). It therefore seems that the successful establishment and spread of acacias in new environments might be dependent on the density of compatible rhizobia (Thrall *et al.* 2007a; Thrall *et al.* 2008). This means that the first arriving acacias would induce a local proliferation of their preferred symbionts and that later arriving species might end up acquiring symbionts that might not necessarily have been the preferred genotypes had such species been the first to have colonised the site. This also means that it is possible that certain species might not have been capable of colonising a site if the initial species did not cause a local proliferation of compatible symbionts.

The similarity in rhizobial diversity found between localised and widespread acacias in this study may reflect similar levels of host promiscuity and invasive potential, and therefore that localised species have not yet reached their full potential ranges in South Africa. However, this is unlikely to be the case as most localised species included here have never been recorded as invasive (Richardson and Rejmánek 2011, but see Zenni *et al.* 2009; Kaplan *et al.* 2012).

Rhizobial diversity and community structure are important factors influencing the productivity of acacias, but increased diversity alone does not always translate into higher plant fitness (Barrett *et al.* 2015). That is in part because different rhizobial strains may differ in their symbiotic effectiveness (Franche *et al.* 2009; Klock *et al.* 2015). I found no consistent differences in rhizobial community

composition between localised and widespread acacias (also see Klock *et al.* 2016) and this may reflect high frequency associations of all acacias with one or a few OTUs of rhizobia. For example, RNR OTU1 made up the vast majority of the associations with most acacias (average read count per associated acacia species=2522) albeit at different frequencies (Figure 5.5) and may represent one of the most effective and preferred acacia strains. In New Zealand Bever *et al*. (2013) found extensive variation in symbiotic effectiveness of acacia-associated rhizobial strains; with some strains broadly effective across all species tested, some strains varying in effectiveness depending on host species, and some strains being relatively ineffective across all acacias tested. At site level, I only found two instances where symbiotic effectiveness differed between widespread and localised acacias, albeit in the opposite direction than I hypothesised. Widespread *A. mearnsii* showed significantly less accumulated atmospheric nitrogen compared to co-occurring localised species (*A. adunca* [site BD] and *A. paradoxa* [site DP]) (Figure 5.6). This could indicate that *A. mearnsii* is less reliant on atmospheric nitrogen and is better able to utilise already-scarce nutrients, or that it had less effective associations than co-occurring localised species. Interestingly in both these instances all three species shared OTU1 at extremely high frequencies (Figure 5.5). This suggests that shared OTUs between different acacias may indeed, dependent on host species, differ in their symbiotic effectiveness. Such differential effectiveness of a single rhizobium strain on different hosts/genotypes is well documented (e.g. Checcucci *et al.* 2016). Overall, however, it appears that nitrogen fixation effectiveness is similar for widespread and localised acacias in South Africa.

The sampling design might suffer from the fact that inferences are restricted to local scales, since shifts in the distribution of rhizobial genotypes across the landscape is likely and the capacity to utilise different partners across a landscape may be critical for the success of widespread legumes. Thus, it is not clear whether these patterns will hold at landscape levels. Also, there could be many additional factors that might influence the invasive capacity of acacias in South Africa. Firstly, propagule pressure and introduction history have been implicated as being important factors determining invasiveness (Wilson *et al.* 2007). Widespread and localised species have shared similar residence times in South Africa ($t = -1.1771$, $df = 13$, $p>0.05$), but do differ substantially in propagule

pressure and number of introductions ($t = -2.6819$, $df = 14$, $p<0.05$; data from Poynton 2009), with widespread species often characterised by extremely high propagule pressure. Then, soil microbes other than rhizobia might influence plant performance, including pathogens and other mutualistic organisms (Thrall *et al.* 2007b), as well as the abiotic conditions of soils (Klock *et al.* 2016). Although the data suggest that rhizobial diversity and community composition to not translate into differential mutualistic effectiveness for acacias and therefore their varying degrees of invasiveness in South Africa, there are a wide variety of local environmental factors that may influence the relative amount of symbiotic nitrogen gained. The inherent ability of plants to obtain nitrogen and other important nutrients from the soil, the nature of the recipient environment and its conditions (e.g. nutrient status), the ubiquitous nature of some cosmopolitan strains of compatible rhizobia, together with the potential for co-introduction of these strains, indicate that multiple factors could aid acacias to become successful invaders. The potential modification of whole soil bacterial communities through facilitated proliferation of invasive plants means that, in terms of restoration, soil microbiome composition should be considered a major impact by invasive plants. Restoration may need to realign soil microbial communities to native plant communities in order to maximise restoration potential (Harris 2009). Future work should address the effectiveness of these mutualistic associations on finer and more precise scales and under common garden conditions, for example, by inoculating various acacias with specific rhizobial strains and calculating symbiotic responses using fitness correlates such as growth kinetics (Thrall *et al.* 2011; Klock *et al.* 2015), in conjunction with isotope studies.

5.6. Acknowledgements

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5.7. Tables and figures

Table 5.1: Summary statistics for paired t-tests of RNR OTUs for co-occurring localised and widespread *Acacia* species pairs for Richness (S), Shannon diversity (H), Inverse Simpson diversity (Si), Evenness (J) and phylogenetic diversity (PD).

Metric	t	df	p
S	-0.893	7	0.402
H	-1.960	7	0.091
Si	-1.791	7	0.117
J	-1.593	7	0.155
PD	-0.994	7	0.353

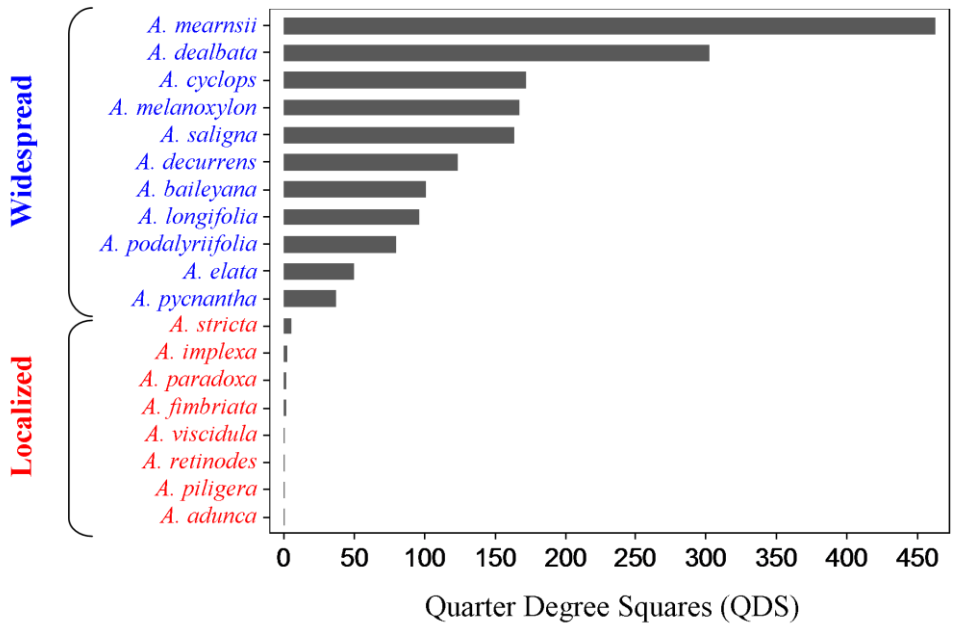

Figure 5.1: Occurrence records of Australian *Acacia* species extracted from the South African Plant Invaders Atlas (SAPIA) database. Following the natural break in the occurrence records, all species present in 38 Quarter Degree Squares or more were considered widespread, while the remaining taxa were considered localised.

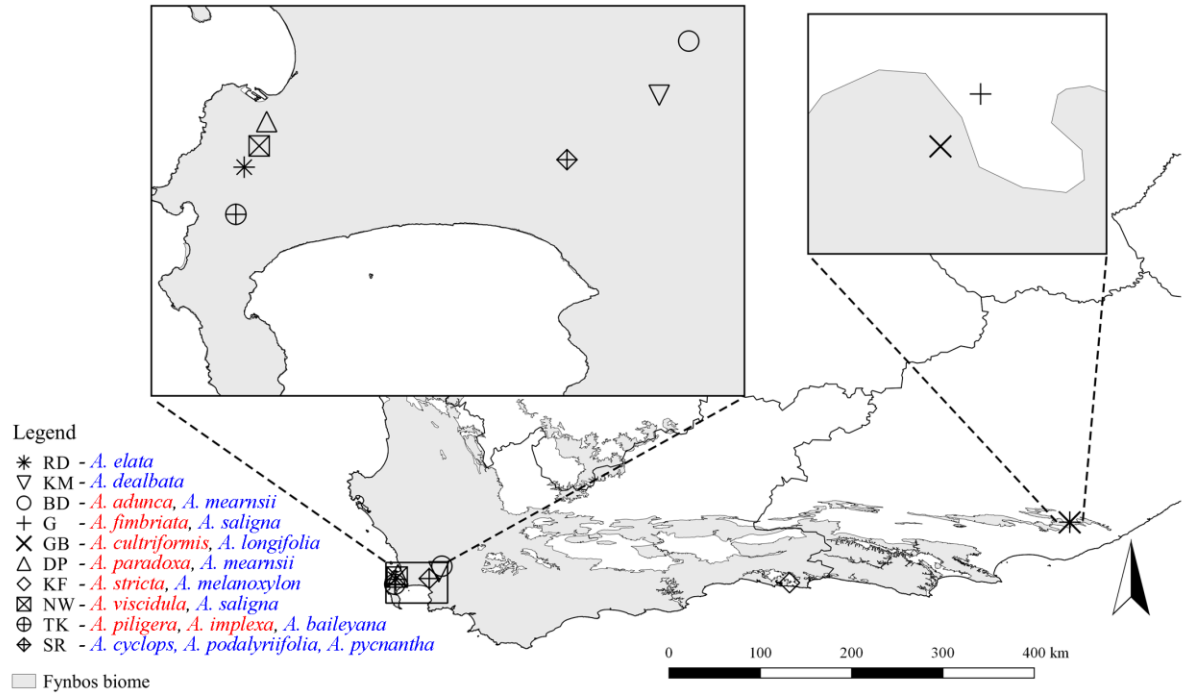

Figure 5.2: Map illustrating site locations in South Africa and *Acacia* species sampled at each site during this study. Colours indicate the status of the species (red – localised, blue – widespread). Acacias from sites RD, KM and SR were not used in paired bacterial diversity and community comparisons of localised and widespread species, but were included in the PCoA plot in order to investigate how their bacterial communities relate to other acacias.

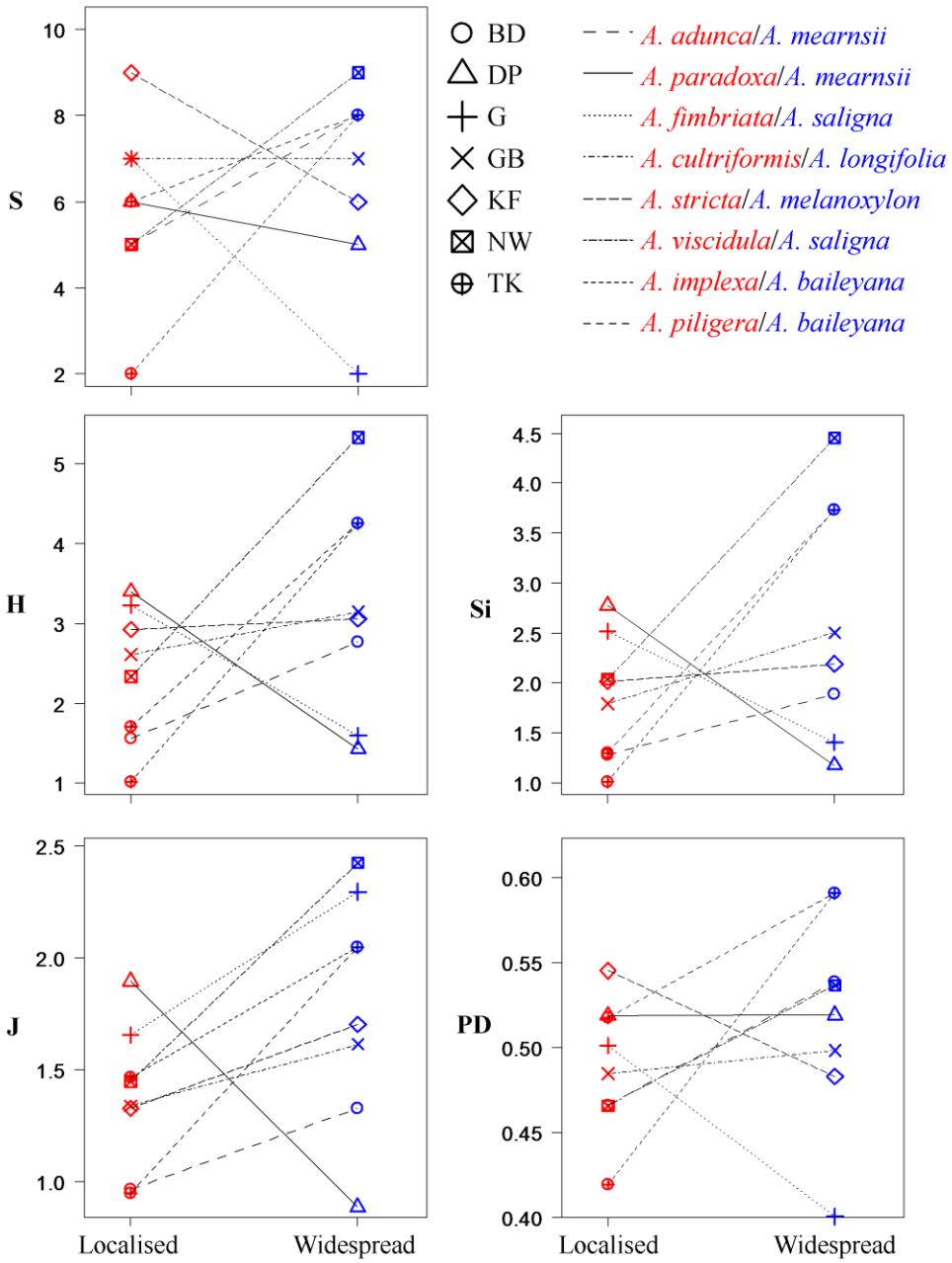

Figure 5.3: Diversity metrics (S: Richness, H: Shannon diversity, Si: Inverse Simpson diversity, J: evenness, PD: Faith's Phylogenetic Distance) for co-occurring widespread and localised acacia species pairs (lines) at different sites (symbols).

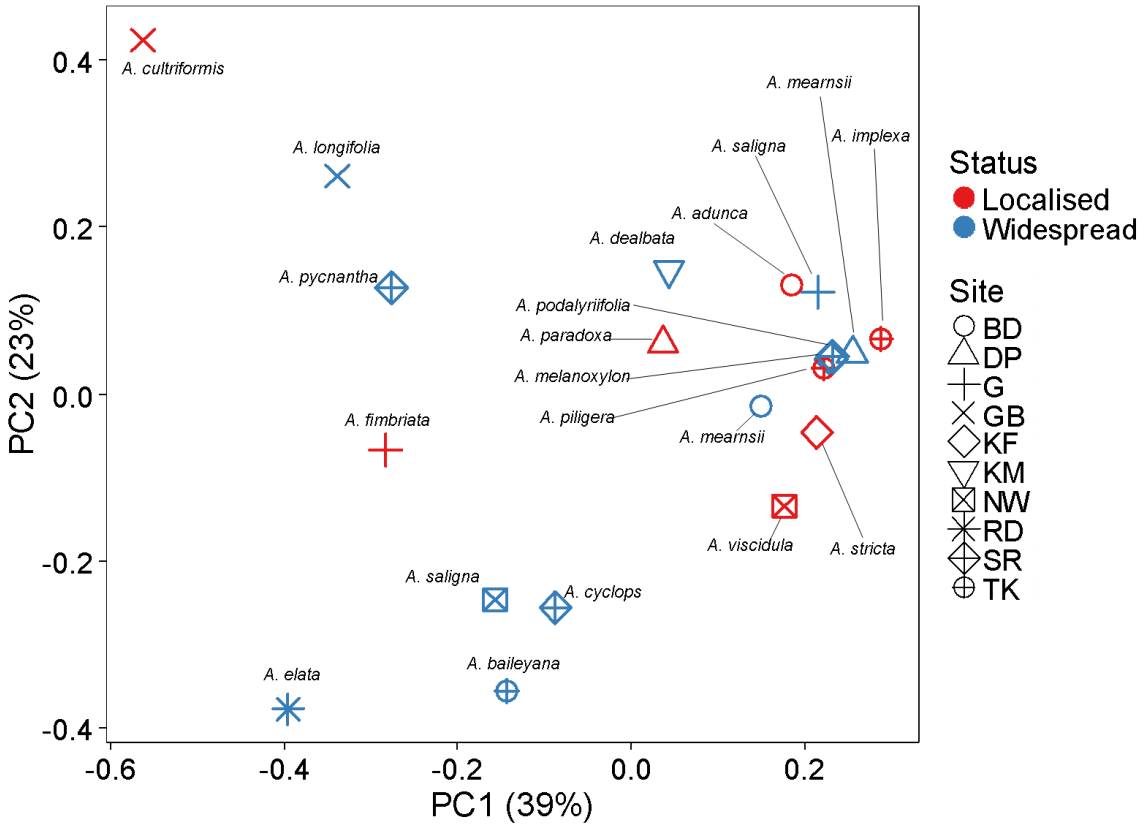

Figure 5.4: Principal Coordinates Analysis (PCoA) plot of *Acacia* RNR OTU community associations across all sites. A PERMANOVA model indicated no significant difference in community composition between localised and widespread acacias, when considering site as a random factor.

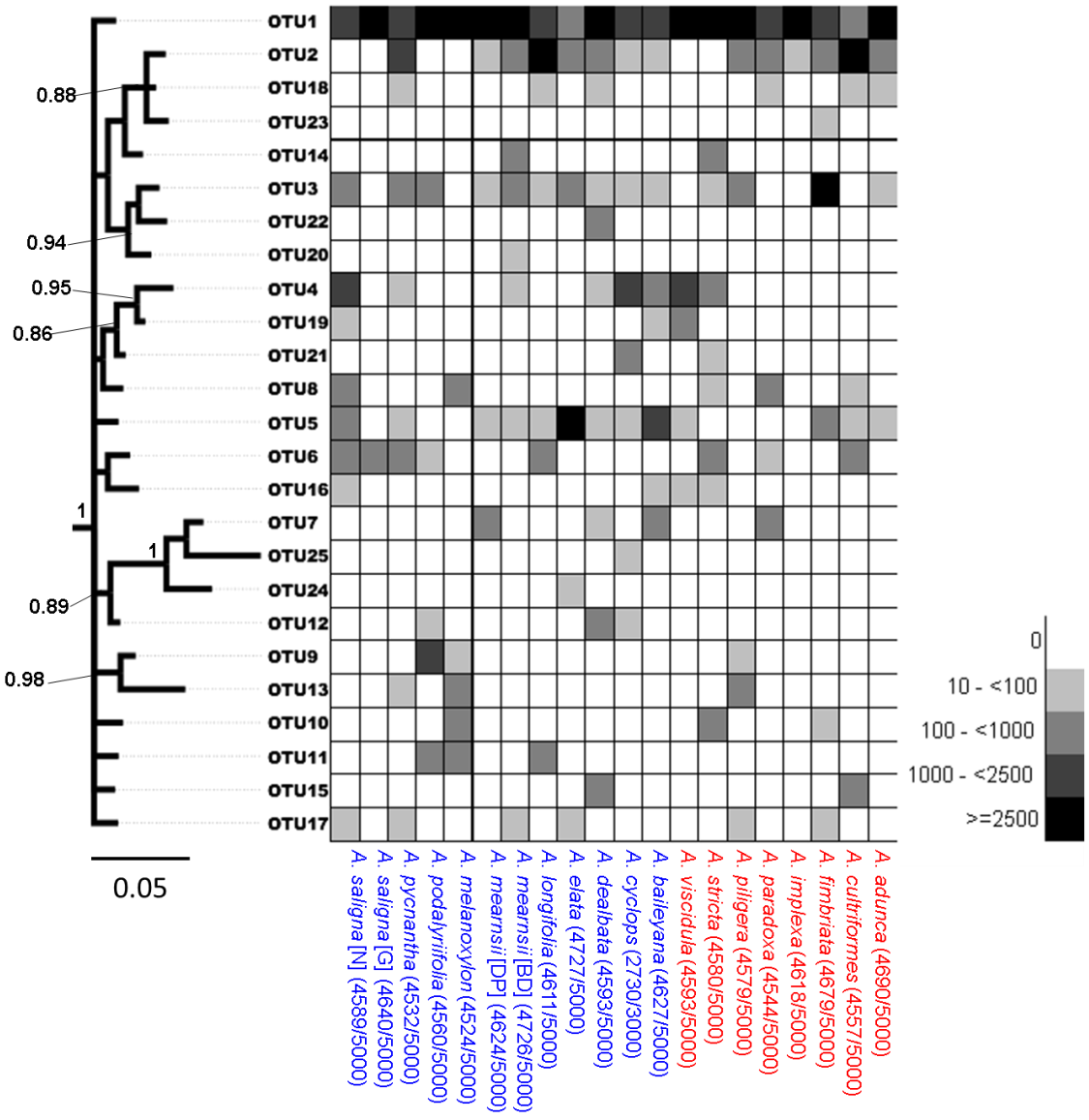

Figure 5.5: Heat map indicating RNR OTU abundance and associations with each *Acacia* species (blue=widespread, red=localised). Numbers in brackets next to species names indicate total number of sequence reads used after quality filtering. The left hand side RNR OTU tree represents the retrieved Bayesian topology based on *nodC* DNA barcodes. Tree topology support is indicated as posterior probabilities at nodes and bar represents number of substitutions per site.

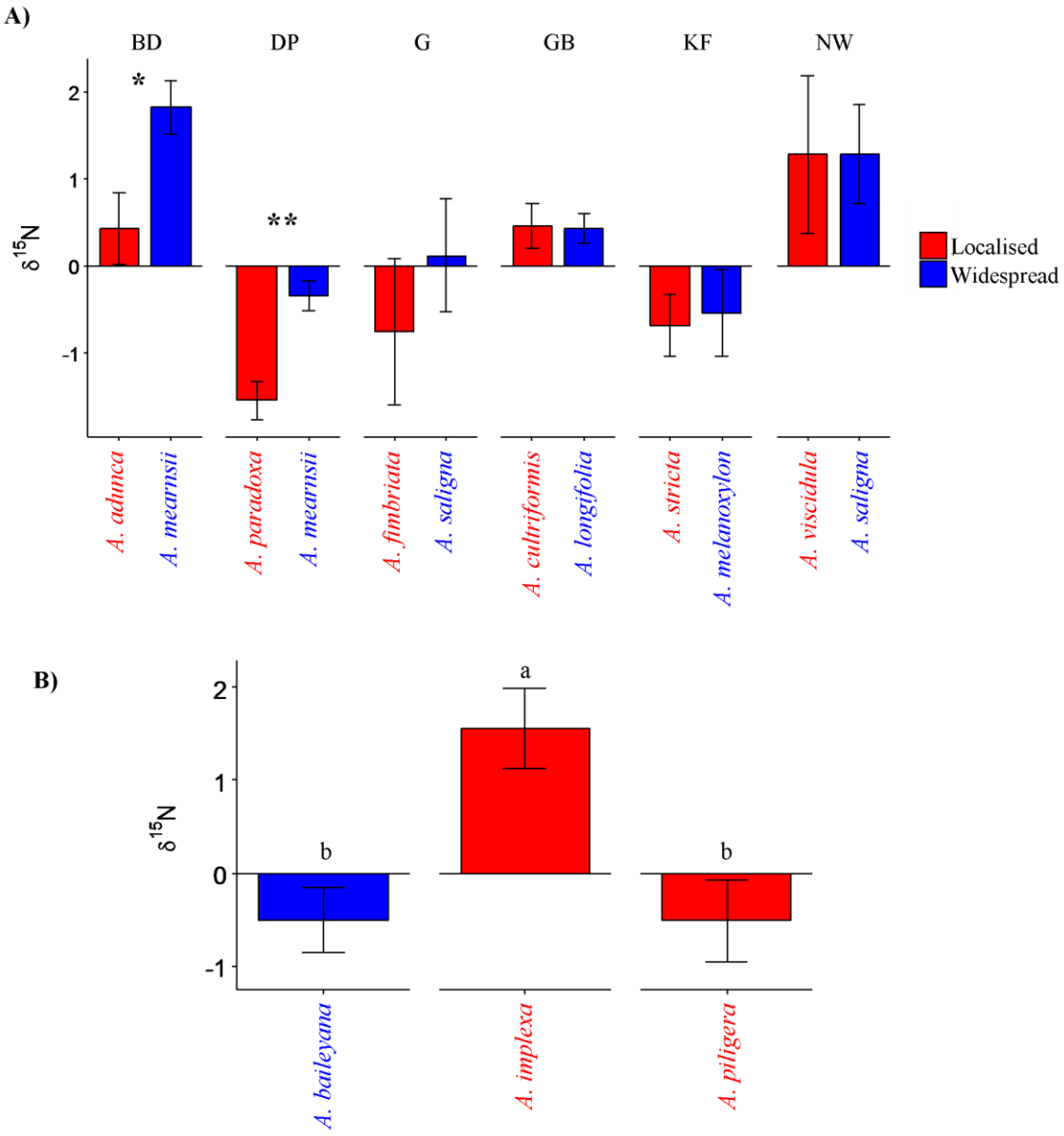

Figure 5.6: Variation in stable nitrogen (N) isotopic signatures for widespread and localised *Acacia* species (mean ± S.E.) for A) sites which had pairs of co-occurring widespread and localised species, and B) site TK which had two localised and one widespread species. For A) significance indicated as: * – $p < 0.05$, ** – $p < 0.01$; for B) the same letter above bars indicates that the treatments are not significantly different from each other ($p \leq 0.05$, pairwise t-test with Bonferroni correction). Positive values indicate a higher sample abundance of ^{15}N compared to atmosphere, while negative values indicate a lower abundance of ^{15}N compared to the reference value.

5.8. Supplementary information

Table S5.1: Locality details of all *Acacia* species included in this study.

Species	Status	Site (abbreviation)	Latitude	Longitude
Acacia adunca	Localised	Bienne Donne (BD)	-33.84425	18.98177
Acacia cultriformis	Localised	Grahamstown Botanical Garden (GB)	-33.31782	26.52868
Acacia fimbriata	Localised	Grahamstown Botanical Garden (GB)	-33.31782	26.52868
Acacia implexa	Localised	Tokai (TK)	-34.06017	18.41537
Acacia paradoxa	Localised	Devil's Peak (DP)	-33.94452	18.45396
Acacia piligera	Localised	Tokai (TK)	-34.06017	18.41537
Acacia stricta	Localised	Kruisfontein (KF)	-34.03794	23.15760
Acacia viscidula	Localised	Newlands (NW)	-33.97450	18.44383
Acacia baileyana	Widespread	Tokai (TK)	-34.06017	18.41537
Acacia cyclops	Widespread	Stellenrust (SR)	-33.99269	18.83059
Acacia dealbata	Widespread	Kylemore (KM)	-33.91134	18.94369
Acacia elata	Widespread	Rhodes Drive (RD)	-34.00215	18.42519
Acacia longifolia	Widespread	Grahamstown outskirts (G)	-33.31782	26.52868
Acacia mearnsii	Widespread	Bienne Donne (BD); Devil's Peak (DP)	-33.844245; -33.944516	18.981769; 18.453964
Acacia melanoxylon	Widespread	Kruisfontein (KF)	-34.03794	23.15760
Acacia podalyriifolia	Widespread	Stellenrust (SR)	-33.99269	18.83059
Acacia pycnantha	Widespread	Stellenrust (SR)	-33.99269	18.83059
Acacia saligna	Widespread	Grahamstown outskirts (G); Newlands (NW)	-33.317816; -33.9745	26.52868; 18.44383

Table S5.2: Richness (S), diversity (Shannon – H; Inverse Simpson – Si), evenness (J) and phylogenetic diversity (PD) metrics for all species. Values are means. Acronyms: n – number of replicates; BD – Bienne Donne; DP – Devil's Peak; G – Grahamstown; N – Newlands.

Species	**n**	**S**	**H**	**Si**	**J**	**PD**
A. adunca	5	5	1.559	1.276	0.968	0.466
A. baileyana	5	8	4.255	3.735	2.046	0.591
A. cultriformis	5	7	2.609	1.791	1.341	0.484
A. cyclops	3	8	3.110	2.519	1.496	0.603
A. dealbata	5	10	3.709	2.583	1.611	0.599
A. elata	5	6	2.626	1.949	1.465	0.537
A. fimbriata	5	7	3.225	2.516	1.657	0.501
A. implexa	5	2	1.018	1.005	1.469	0.419
A. longifolia	5	7	3.143	2.503	1.615	0.498
A. mearnsii BD	5	8	2.767	1.884	1.330	0.538
A. mearnsii DP	5	5	1.430	1.176	0.888	0.519
A. melanoxylon	5	6	3.055	2.191	1.705	0.483
A. paradoxa	5	6	3.400	2.773	1.897	0.519
A. piligera	5	6	1.706	1.298	0.952	0.517
A. podalyriifolia	5	6	2.487	1.935	1.388	0.485
A. pycnantha	5	9	4.300	3.646	1.957	0.590
A. saligna G	5	2	1.592	1.408	2.296	0.400
A. saligna N	5	9	5.328	4.451	2.425	0.537
A. stricta	5	9	2.923	2.013	1.330	0.545
A. viscidula	5	5	2.333	2.036	1.449	0.465

Table S5.3: Stable $\delta^{15}N$ isotope values for the various *Acacia* species used in this study. Values are means (± standard errors). Pairs of localised and widespread species whose means were significantly different are indicated in bold and as follows: * – p < 0.05; ** – p < 0.01; ***– p < 0.001.

Species	Status	Site	$\delta^{15}N$
Acacia adunca	Localised	BD	**0.426 (±0.412) ***
Acacia mearnsii	Widespread	BD	**1.822 (±0.309) ***
Acacia paradoxa	Localised	DP	**-1.55 (±0.219) ****
Acacia mearnsii	Widespread	DP	**-0.346 (±0.167) ****
Acacia fimbriata	Localised	G	-0.76 (±0.844)
Acacia saligna	Widespread	G	0.118 (±0.646)
Acacia cultriformis	Localised	GB	0.45 (±0.255)
Acacia longifolia	Widespread	GB	0.425 (±0.168)
Acacia stricta	Localised	KF	-0.688 (±0.353)
Acacia melanoxylon	Widespread	KF	-0.54 (±0.502)
Acacia dealbata	Widespread	KM	-0.083 (±0.495)
Acacia viscidula	Localised	NW	1.277 (±0.901)
Acacia saligna	Widespread	NW	1.284 (±0.566)
Acacia elata	Widespread	RD	-0.327 (±0.053)
Acacia cyclops	Widespread	SR	0.535 (±0.41)
Acacia podalyriifolia	Widespread	SR	1.268 (±0.464)
Acacia pycnantha	Widespread	SR	-0.415 (±0.507)
Acacia implexa	Localised	TK	**1.553 (±0.426)***
Acacia piligera	Localised	TK	-0.512 (±0.438)
Acacia baileyana	Widespread	TK	-0.505 (±0.345)

Table S5.4: Introduction histories of the *Acacia* species analysed in this study. Abbreviations: Date – first recorded date, Years – years since first introduction, PP – Propagule pressure (kg), #Intro – Number of introductions, Scale – Scale of dissemination.

	Status	**Date**	**Years**	**PP**	**#Intro**	**Scale**
Acacia adunca	Localised	1900	116	NA	3	narrow
Acacia cultriformis	Localised	1858	158	2.2	NA	wide
Acacia fimbriata	Localised	1900	116	NA	2	narrow
Acacia implexa	Localised	1886	130	NA	NA	narrow
Acacia paradoxa	Localised	1858	158	NA	2	narrow
Acacia piligera	Localised	NA	NA	NA	1	narrow
Acacia stricta	Localised	NA	130	NA	1	narrow
Acacia viscidula	Localised	NA	NA	NA	1	narrow
Acacia baileyana	Widespread	1898	118	3.3	4	wide
Acacia cyclops	Widespread	1845	171	at least 90000	2	extremely wide
Acacia dealbata	Widespread	1858	158	at least 4.5	4	extremely wide
Acacia elata	Widespread	1904	112	at least 25	11	wide
Acacia longifolia	Widespread	1827	189	at least 1.4	6	extremely wide
Acacia mearnsii	Widespread	1858	158	at least 23	8	extremely wide
Acacia melanoxylon	Widespread	1848	168	at least 10	10	extremely wide
Acacia podalyriifolia	Widespread	1894	122	NA	2	narrow
Acacia pycnantha	Widespread	1865	151	NA	5	extremely wide
Acacia saligna	Widespread	1850	166	at least 5100 + 90000	2	extremely wide

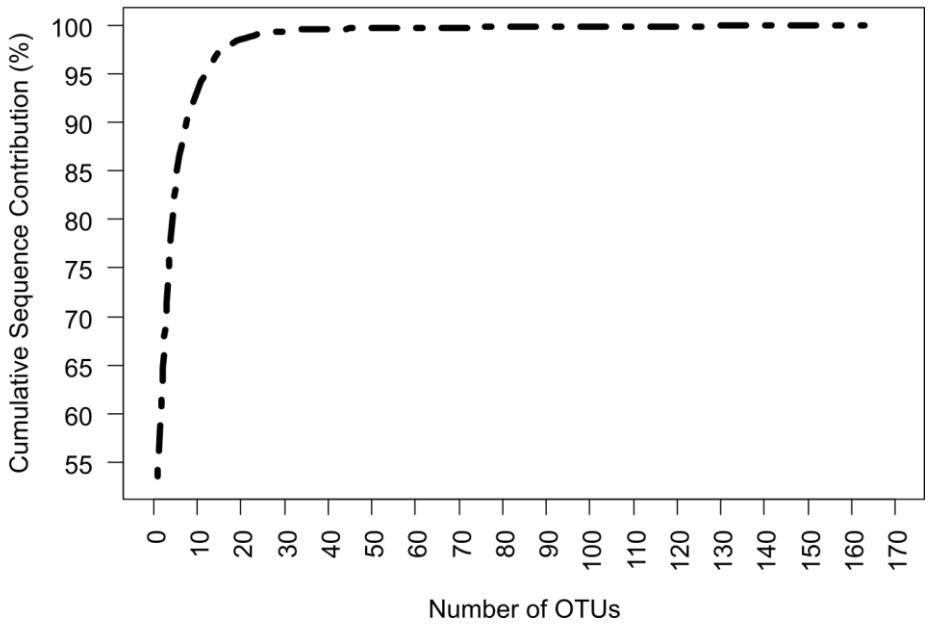

Figure S5.1: Cumulative distribution of sequence reads across identified OTUs showing that 25 OTUs account for the majority of sequences (99.1%).

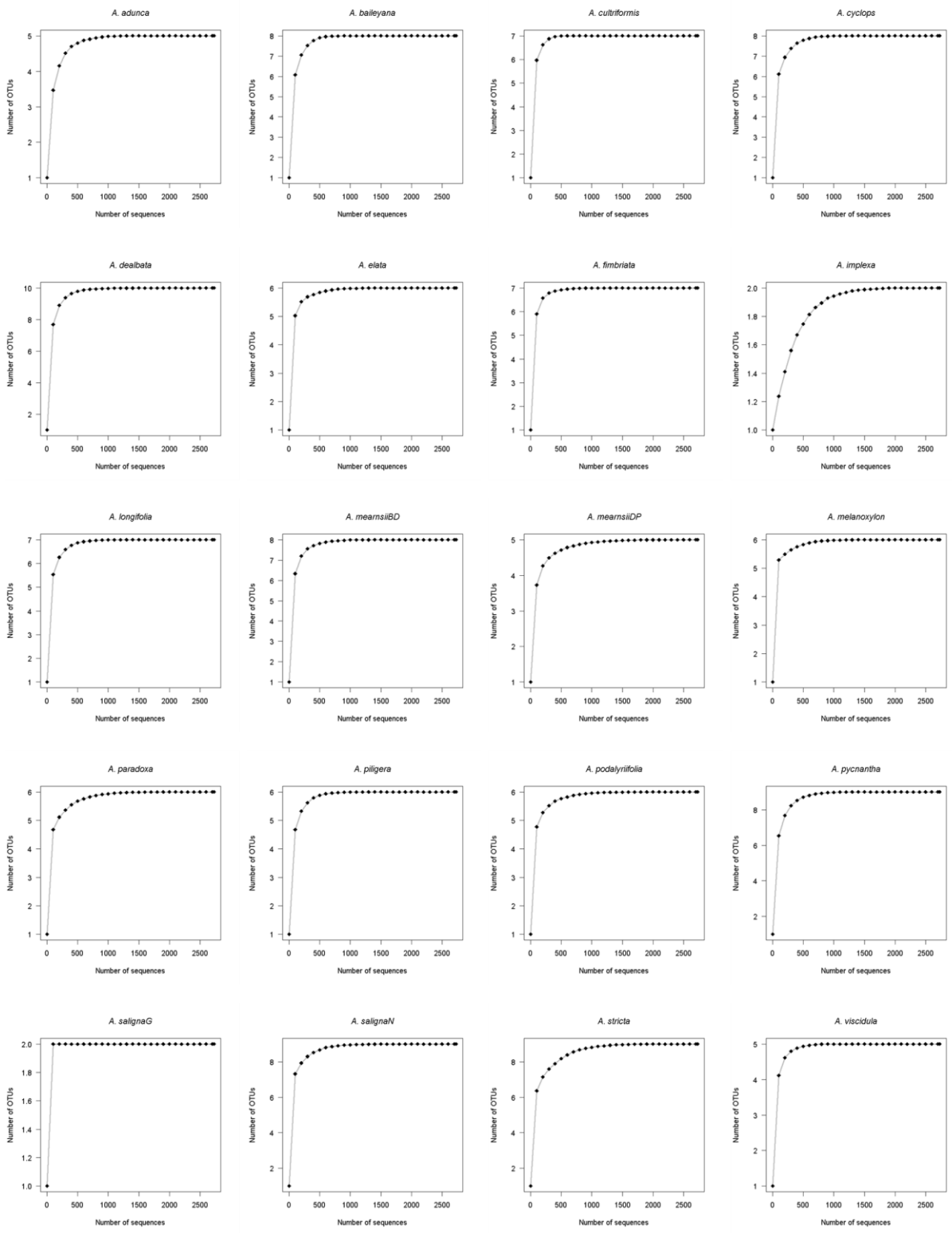

Figure S5.2: Rarefaction curves for all acacia species.

CHAPTER 6: Conclusion

This research contributed new insights into the ecology of soil bacterial communities of fynbos soils, and the impacts that invasive species have on such soils and their associated bacterial communities. To date, only one study has investigated whole soil bacterial communities in South Africa's fynbos vegetation (Slabbert *et al.* 2010), and only one other study has investigated the impacts of invasive acacias on such soil communities (Slabbert *et al.* 2014). This is surprising given the status fynbos as an important global biodiversity hotspot (Myers *et al.* 2000), with exceptional diversity not only in terms of plants and insects (Cowling *et al.* 2009; Manning and Goldblatt 2012; Kemp and Ellis 2017), but also bacteria (Lemaire *et al.* 2015). The overall aim of this thesis was therefore to investigate the dynamics of fynbos soil bacterial communities across a large geographical and environmental gradient, and to elucidate how invasive acacias impact on these communities. Furthermore, given the known impacts that acacias have on ecosystems, not only in South Africa (Musil and Midgley 1990; Holmes and Cowling 1997; Yelenik *et al.* 2004), but across the world (Richardson *et al.* 2011, 2015), I also asked a fundamentally important question generally related to invasiveness of species, namely: can differences in invasiveness between various acacias in South Africa be explained by difference in mutualistic associations and subsequent competitive advantage gained by such associations?

My study, for the first time across a wide geographic range, illustrated that fynbos soils are characterised by diverse bacterial communities with distinct taxa, and that these communities are structured primarily by soil pH and NH_4^+ content, with spatial variability also being a notable driver of community composition. Furthermore, I have shown that turnover between pristine fynbos soil bacterial communities can almost entirely be attributed to replacement, with very low levels of nestedness. This finding has important conservation consequences since microbial turnover due to nestedness implicates that only a small number of the richest sites need to be prioritised for conservation of soil communities and processes, since all other sites would only contain species subsets of the richest sites. However, when turnover is a result of replacement, which I have shown to be the case for fynbos soils, then conservation of a large number of different sites (which are not

necessarily the richest) is required, since sites do not share similar sets of species and the loss of even single sites (e.g. habitat destruction or degradation) would thus result in the loss of unique species (Baselga 2010). I have further shown that soil bacterial communities of fynbos are significantly affected by seasonality, most likely as a result of the Mediterranean-type climate characteristic of the region. These new findings compliment what is already known regarding high diversity and endemism of plants and insects of fynbos (Manning and Goldblatt 2012; Kemp and Ellis 2017).

I have also shown that invasive acacias impact on fynbos soil bacterial communities by significantly changing their composition, but not diversity. This compositional change in bacterial communities was primarily driven by acacia-induced changes of soil pH and NH_4^+. I also found acacias to reduce spatial variability across soil communities, such that community turnover could no longer be predicted by geographical distance, as was the case for pristine fynbos soils. In addition, I have shown that acacia invasions in fynbos significantly increase levels of soil nitrogenous compounds (NO_3^-, NH_4^+, and total N), as well as carbon and pH. However, these changes appear to be context specific. Furthermore, I showed that acacias significantly increase activities of enzymes involved in nitrogen (urease) and phosphorous (phosphatase), but not carbon (β-glucosidase) cycling. As with the impacts of acacias on soil nutrients, their impacts on enzyme activities were also context specific. The context specificity of soil impacts as a result of acacias invasion has direct management implications: sites that have soil conditions affected by invasive acacias might need active restoration approaches (e.g. soil rehabilitation) upon clearing of invasive biomass, vs. passive approaches at sites that do not have significant acacia impacts. I also showed that increased nutrient levels as a result of acacia invasion were correlated with increases in enzyme activities for urease and phosphatase. Acacia-induced changes in soil bacterial community composition was likewise correlated with elevation in phosphatase enzymatic activity.

Finally, I showed that for acacias in South Africa there are no differences in rhizobial richness, diversity and community composition between localised and widespread species and that they do not differ in their ability to fix atmospheric nitrogen. Thus, different levels of invasiveness for acacias in

South Africa is more likely to be the result of other factors, such as differences in propagule pressure, reasons for, and extent of, plantings in the country.

Although this thesis has answered some fundamental questions related to fynbos soil bacterial community ecology and the impacts of invasive species on these communities, knowledge gaps remain and much work remains to be done. Some of the remaining questions are:

- By which mechanisms do invasive acacias affect soil bacterial community composition? It is known that invasives can alter soil communities as a result of exudates such as allelopathic compounds, nitrogen fixation, nutrient solubilisation etc., but the precise mechanisms underlying such alterations of fynbos soil bacterial communities under invasion remains to be explored.
- What are the consequences of altered soil bacterial communities for the restoration of sites cleared from invasive acacias? Since initial soil conditions are crucial for the establishment of seedlings, changes due to invasion might serve as a barrier to the establishment of native plant species following the removal of invasive acacias, i.e. due to so-called legacy effects. This has serious management implications, since focus would shift from passive to active (e.g. the deliberate inoculation of soils with native soil microbes) strategies.
- Do soil fungal communities follow the same diversity and community patterns as soil bacterial communities? Fungal mutualistic associations are equally crucial for the successful establishment and growth of plant species, especially in fynbos systems where many plants depend on mycorrhizae (e.g. the genus *Erica*). It remains to be determined how invasive plants impact on these fungal communities and what the consequences are for soil and ecosystem functionality.

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