Investigation of the microbial community composition and functional potential in Namib Desert soils

by

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SUMMARY

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Deserts constitute one-fifth of the Earth's total surface area and represent one of the harshest environments. Soil microbial communities are considered the dominant ecological drivers of these ecosystems as they are major contributors to several processes that are vital for carbon and nutrient cycling. Changes in precipitation regimes have been shown to alter soil microbial communities by causing shifts in community composition. Therefore, gradients of precipitation have been suggested as good systems to evaluate the impact of precipitation on microbial communities, which is largely unexplored in in desert ecosystems. Using 16S rRNA gene high-throughput sequencing and shotgun metagenome sequencing, the taxonomic composition and functional potential of soil prokaryotic communities across two zones with contrasting precipitation history in the Namib desert was investigated. Alpha-diversity of both taxonomic and functional profiles were not impacted by precipitation across the two zones. However, beta-diversity patterns differed significantly between the two zones. Interestingly, a small set of microbial taxa, many of which were present in low abundance, were responsible for these changes. Altogether, these results indicate that precipitation is an important factor in shaping taxonomic and functional attributes of the arid soil microbiome.

Microbes in soil are known to produce antibiotics that are used to inhibit the growth of or to kill off other microbes. This gives antibiotic producers an advantage to compete for nutrients and other limited resources. Antibiotic producers encode antibiotic resistance genes that protect them from the molecules they produce. Soil is therefore the most prominent reservoir of resistance genes, harbouring up to 30% of the genes that confer resistance to antibiotics, metals and biocides, which together make up the soil resistome. Many studies have focused on the resistomes of grassland, agricultural and even cold desert soils. However, little is known about the resistome of hot deserts. With the use of shotgun metagenomics, the resistome and the mobilome in Namib Desert soils across the two above mentioned zones were identified and characterized. A variety of antibiotic resistance genes (ARGs) (e.g., *inh*A, *kat*G, *rpo*B) were detected in low abundance including those that were horizontally acquired (e.g., *AAC (3')*). The presence of metal/biocide resistance genes (MRGs) (e.g., *ars*C*, Mex*K) in close proximity to ARGs indicated a potential co-selection of resistance to antibiotics and metals/biocides. A decoupling between bacterial community composition and ARG profiles was identified, most likely attributed to the presence of mobile genetic elements and horizontally acquired ARGs. These results showed that bacterial communities in Namib Desert soils host a number of resistance elements and that horizontal gene transfer, rather than phylogeny, could play an essential role in their dynamics.

The One Health concept is a holistic and interdisciplinary approach based on the idea that human and animal health are linked to the health of the environment. Indeed, the exhaustive use of antibiotics in humans, animal farming and other agricultural practices has resulted in the frequent appearance of antibiotic resistant bacteria in human-impacted habitats. However, antibiotic resistance in less impacted habitats (e.g., Deserts) is not well understood. A more in-depth investigation of the acquired ARGs reported earlier revealed the presence of a clinically significant extended spectrum β-lactamase (bla_{TEM}-116). This ARG was carried on a ColE1-like plasmid also hosting a metal resistance gene coding for arsenate reductase (*ars*C). The co-selection of resistance to antibiotics and metals encoded on a single mobile genetic element increases the probability of dissemination of these resistance determinants and the potential selection of multiple resistance mechanisms. In addition to these two resistance genes a P7 entero-bacteriophage was found on the same plasmid. This bacteriophage may represent a new vehicle for the propagation of the gene cluster (bla_{TEM}-116 and *ars*C) in these soil communities. These results highlight the importance of less impacted environments in the One Health initiative.

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work and has not been submitted by me towards a degree at this or any other tertiary institution.

Yashini Naidoo November 2020

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"When you want something, ***all the universe conspires*** *in helping you to achieve it."~ Paulo Coelho*

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CHAPTER 1: General Introduction

1.1 The Soil Microbiome

Soil microbial communities are central to life on Earth, as they drive several processes that are vital for carbon and nutrient cycling and to sustain plant growth (Bahram et al., 2018; Jansson and Hofmockel, 2020). Indeed, estimates suggest that approximately 20,000 plant species are completely dependent on microbial symbionts for growth and survival (Van Der Heijden et al., 2008).

Research into the soil microbiome has revealed that soils possess an extensive range of taxa from all three domains of life (Archaea, Bacteria and Eukarya) (Islam et al., 2020). Bacterial species form the biggest group by number and diversity, most of which remain undescribed because they cannot be cultured in a laboratory. A recent study using high-throughput sequencing has found that soil bacterial communities are typically dominated by a relatively small group of phylotypes (Figure 1.1) belonging to the phyla Proteobacteria, Actinobacteria, Acidobacteria, Planctomycetes and few others such as the Firmicutes (Delgado-Baquerizo et al., 2018). Although, it has been shown that these groups are not equally abundant in all soils. For instance, Actinobacteria, Bacteroidetes and Cyanobacteria seem to be more abundant in semi-arid and desert soils compared to grassland, forest and tundra soils (Fierer et al., 2012); as opposed to Proteobacteria, Verrucomicrobia and Acidobacteria that showed the opposite trend (Noronha et al., 2017).

Considerable effort has been invested into identifying the biotic and abiotic factors that shape soil microbial communities, which will allow us to predict how microbes react to the changing environment. Soil pH has been described as the strongest factor driving bacterial diversity in soils (Bartram et al., 2014; Fierer and Jackson, 2006; Wu et al., 2017), with higher bacterial diversity in neutral soils and lower diversity in more acidic or alkaline soils. Soil moisture has also been shown to be an important driver of overall microbial activity, since soil water content helps to determine soil texture, oxygen availability and connectivity within soils (Brockett et al., 2012; Mansson et al., 2014). In addition to influencing microbial activity, changes in soil moisture can also cause shifts in both bacterial and fungal community structures in several terrestrial ecosystems (Brockett et al., 2012; Cregger et al., 2012; Y. Li et al., 2017). For

example, flooding creates physical and chemical barriers to aerobic respiration selecting for anaerobic microorganisms (Balser et al., 2010; Zhou et al., 2018). On the other hand, soil desiccation creates a nutrient-poor environment with negative effects on the metabolic state of microorganisms thus decreasing microbial activity (Wang et al., 2018; Zhou et al., 2016).

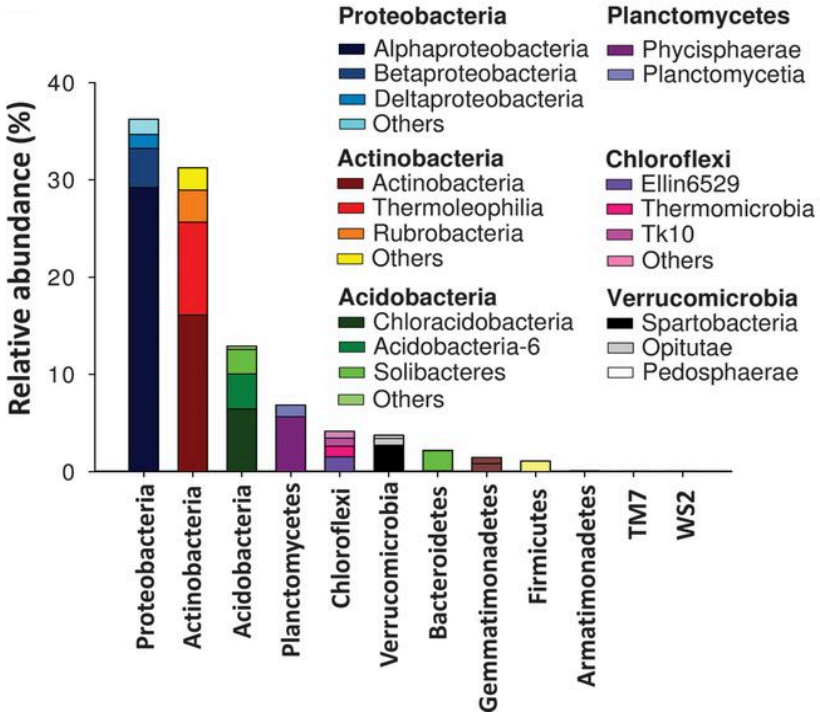

Figure 1.1 The taxonomic composition of the dominant phylotypes in soils across the globe. Re-printed from (Delgado-Baquerizo et al., 2018).

Other major factors that also influence bacterial community composition and diversity are carbon (Rasche et al., 2011), land use (Lauber et al., 2013) and the application of fertilizers (Herzog et al., 2015). Overall, it is clear that there are important disparities in the patterns of diversity and composition of soil microbial communities across different climates and historical contexts at both spatial and temporal scales (Hendershot et al., 2017).

The assembly of microbial communities depends largely on four fundamental processes: diversification, selection, dispersal and ecological drift (reviewed by Zhou and Ning, 2017). Diversification, dispersal and drift are considered to be stochastic processes, whereby communities are assembled randomly (Johnson et al., 2017). In contrast, selection (niche partitioning, environmental filtering) is a deterministic process, whereby microorganisms are

present wherever a suitable niche exists (Shi et al., 2018). It is now widely accepted that stochastic and deterministic processes can act concurrently to regulate community assembly (Yuan et al., 2019). However, the relative importance of these two processes may vary in different environmental contexts and with time (Zhao et al., 2019). In soil ecosystems, it has been repeatedly demonstrated that microbial assembly is principally driven by deterministic processes (Gombeer et al., 2015; Johnson et al., 2017; Ronca et al., 2015), although deterministic disturbances may promote random community assembly. Studies have shown that disturbance promotes a time-dependent shift in the stochastic/deterministic balance (Dini-Andreote et al., 2015; Ferrenberg et al., 2013; Zhou et al., 2014). For instance, a wildfire event was shown to reset assembly processes by drastically altering bacterial community structure and diversity, thereby creating an environment in which stochastic processes briefly governed community assembly at initial succession stages with deterministic selection becoming stronger toward later successional stages (Ferrenberg et al., 2013).

A central goal of microbial ecology is to understand how changes in microbial community structure and composition relates to ecological functions (Bell et al., 2009; Fetzer et al., 2015; Kuang et al., 2016; O'Brien et al., 2017). Indeed, the relationship between microbial community composition and function remains controversial because microorganisms are suggested to be functionally redundant. It is therefore unclear whether soil functioning is influenced by microbial diversity and composition (Zhou et al., 2020). As a few grams of soil can contain hundreds of thousands of bacterial taxa, it is usually thought that the loss of a few species will not generally affect soil functions because the same functions can be performed by multiple species (Jia and Whalen, 2020). Therefore, in soil systems, redundancy may be high enough to assume that community composition and diversity are uncoupled from function (Bardgett and van der Putten, 2014; Maron et al., 2018).

This decoupling was corroborated, for example, by a study carried out in soils across forest ecosystems (Purahong et al., 2014), which showed a disconnection between microbial community structure and function due to a high degree of functional redundancy. In contrast, several studies support the notion that soil microbial diversity is positively associated with ecosystems functioning, with no evidence of functional redundancy (Delgado-Baquerizo et al., 2018). Nevertheless, despite the growing amount of data supporting the positive microbial diversity-function relationship, there is evidence that not all soil microorganisms are equally important for maintaining ecosystem functioning. For instance, network analyses have

revealed that co-occurring species are often organized into groups or modules of functional significance (Banerjee et al., 2018 and references therein). For example, microbial taxa from the same module were involved in specific functions such as C and N-cycles and organic contaminant degradation in contaminated soils from five oil refineries (Jiao et al., 2016). Similarly, functional modules in Antarctic aquatic systems contained key metabolic potential capacities including photoheterotrophy (Vick-Majors et al., 2014).

1.2 The desert soil microbiome

More than 45% of the Earth's terrestrial surfaces are permanently or seasonally arid and are considered deserts (Guerra et al., 2020; Šťovíček et al., 2017). Deserts are characterized by high levels of solar radiation, low levels of rainfall, extreme temperatures, low abundance of nutrients and high salt concentrations, and, therefore, are considered one of the harshest terrestrial ecosystems on the planet (Eida et al., 2018).

The soil communities of deserts have been increasingly studied over the last decade with specific interest in microbial biodiversity (Andrew et al., 2012; Belov et al., 2018; van der Walt et al., 2016), their adaptive mechanisms (McHugh et al., 2017; Šťovíček et al., 2017) and their biotechnological applications (Maza et al., 2019). There are a few variables that are frequently implicated as the main drivers of microbial diversity in deserts such as salinity (Rath et al., 2019; Zhang et al., 2019), plant cover (Fernández-Gómez et al., 2019; Marasco et al., 2018) and soil chemistry (Gombeer et al., 2015; Ronca et al., 2015). Several studies have also shown that water availability is a key factor shaping microbial community composition and function (Neilson et al., 2017; She et al., 2018; Šťovíček et al., 2017). A survey of global dryland soils revealed that increasing aridity reduces microbial abundance and diversity (Maestre et al., 2015). Similarly, studies in the Atacama desert revealed that while low levels of microbial activity occur under extreme hyper-arid conditions, there was a rapid response in metabolic activity following episodic increases of water (Jones et al., 2018; Schulze-Makuch et al., 2018). Nevertheless, our understanding of the shifts in desert soil bacterial communities in response to changing environmental factors remains limited (Rath et al., 2019).

The adaptation of microbial communities to environmental stress in arid soils may include survival at the surface of hot arid soils (temperatures up to 60 °C), desiccation and intense ultraviolet (UV) radiation, which would require specific and/or unusual adaptive mechanisms

(Heulin et al., 2017). For example, members of the genus *Modestobacter* (phylum Actinobacteria), isolated from the surface of rocks in the Tunisian desert, have the ability to produce black melanin-like pigments, which serve as a source of sunscreens enabling the bacteria to tolerate high solar radiation (Essoussi et al., 2010). Interestingly, *Modestobacter* isolates from the Atacama desert soils were found to have genes that encode a 'carbon starvation protein A', which allows for the uptake of peptides during energy starvation enabling the bacteria to use alternative energy sources (Busarakam et al., 2016).

One of the most extensively documented adaptations to UV radiation is in reference to bacteria from the order Deinococcales, which interestingly also have extreme tolerance to desiccation and other oxidative stress conditions (Heulin et al., 2017). Several species of *Deinococcus* isolated from different desert soils and hot springs (reviewed by Cox and Battista, 2005; Daly, 2009) encode DNA repair proteins which are key to enabling extreme UV radiation tolerance (Yuan et al., 2012). These proteins, as the name suggests, allow the repair of stress-induced DNA damage including hundreds of DNA double-strand breaks (Slade and Radman, 2011; Yuan et al., 2012).

Water is essential to all cellular processes and microorganisms experiencing long periods of desiccation need to be adapted to moisture stress (Pointing and Belnap, 2012). Bacterial dormancy (where energy is directed at persistence rather than growth) and the production of bacterial spores (endospores of Firmicutes, exospores of Actinobacteria) and akinetes (Cyanobacteria) have been well-documented mechanisms implicated in bacterial desiccation tolerance (Bay et al., 2018; Heulin et al., 2017; Lebre et al., 2017 references therein). Indeed, Actinobacteria and the Firmicutes are among the more dominant bacterial communities in desert soils globally (Leung et al., 2020). Furthermore, it has been shown that genes relating to dormancy and sporulation are commonly found in high abundance in arid soils (Fierer et al., 2012; Noronha et al., 2017; Tripathi et al., 2017).

Soil creates an environment for microbes where competitive mechanisms are used as strategies for survival (Stubbendieck and Straight, 2016). In soils with high levels of functional redundancy, bacterial species may use the same energy sources for growth and persistence, therefore increasing the inter-specific competition for resources (Tyc et al., 2014). An important competitive strategy is the production of secondary metabolites (e.g., antimicrobials,

toxins and biosurfactants). The high level of antimicrobials in the soil environment allows microorganisms to inhibit competitors that occupy the same niche (Yan et al., 2018). This differential inhibition of the growth of certain soil microorganisms can influence community composition, which can result in alterations of ecological functionality (as reviewed by Cycon et al., 2019). For example, the soil bacterium *Pseudomonas protegens* produces many antibiotics and toxins (Yan et al., 2017) that are known to suppress soil-borne plant pathogens and promote plant growth (Ortíz-Castro et al., 2009). Furthermore, a recent study showed strong fungal-bacterial antagonism inferred from the abundance of antibiotic resistance genes detected in topsoil (Bahram et al., 2018). Many fungal taxa secrete antibiotics to outcompete bacteria which increases the relative abundance of antibiotic resistance genes in the environment (Bahram et al., 2018).

1.3 Antibiotic resistance in the environment

Antibiotic resistance (AR) is the inherited ability of bacteria to grow in the presence of antibiotics, it is also a mechanism of bacterial self-defence, either against naturally occurring antibiotics or antibiotics introduced by human activities (Holmes et al., 2016; Pallecchi et al., 2008). However, there has been little evidence to suggest that antibiotics produced naturally in environmental settings are the key drivers in the selection of resistant microorganisms in the environment, specifically because they exist in very low concentrations (Gillings et al., 2017). More likely, the key drivers in the development of AR can be attributed to the co-selection of resistance with other compounds such as heavy metals and biocides as well as the incorrect use and overuse of antibiotics (Vikesland et al., 2019) in those environments that are highly impacted by human activities. The overuse of antibiotics in the health and agricultural sectors has seen the global consumption of antibiotics rise by a substantial 65% over the last two decades (Klein et al., 2018; Ventola, 2015). Antibiotics used as animal growth promoters have been frequently detected in animal manure (Jechalke et al., 2014) and it has been demonstrated that land application of animal manure increases the diversity and abundance of ARGs in agricultural soils (Wichmann et al., 2014; Zhao et al., 2017). Altogether, the overuse of antibiotics not only increases the pool of resistance genes but also enhances resident soil antibiotic-resistant microorganisms (Udikovic-Kolic et al., 2014).

1.3.1 The origins and mechanisms of antibiotic resistance

Resistance to antibiotics often occur shortly after the clinical use of the drug, although in the case of penicillin, resistance was reported before the widespread use of the antibiotic (Abraham

and Chain, 1940). It has since been established that antibiotic resistance has an ancient origin and evolution (Perry et al., 2016; Song et al., 2005). Most antibiotics used in clinical and agricultural sectors today are produced or have been derived from soil-dwelling bacteria from the phylum Actinomycetes, especially from members of the genus *Streptomyces* (Perry and Wright, 2014; Rangseekaew and Pathom-aree, 2019). Actinomycetes are prolific producers of secondary metabolites, including several classes of antibiotics such as aminoglycosides, β-lactams, glycopeptides, tetracyclines and the very important β-lactamase inhibitor, clavulanic acid (Barka et al., 2016). In order to protect themselves these organisms must be resistant to the antibiotics they produce, and thus may have been the origin of the resistance genes and mechanisms found in clinical pathogens today (Finley et al., 2013).

Resistance in bacteria can be intrinsic or acquired. Intrinsic resistance occurs naturally as a result of structural or functional characteristics (Blair et al., 2015). For example, *Pseudomonas aeruginosa*, a gram negative pathogen is intrinsically resistant to several antibiotics (e.g., aminoglycosides, erythromycin, polymyxin) due to a relatively impermeable outer membrane (OM), which surrounds a thin peptidoglycan layer (Cox and Wright, 2013). In contrast, bacteria can acquire or develop resistance due to changes in the genetic material as a result of spontaneous mutation or via horizontal gene transfer (HGT) by transformation, transduction and conjugation (Munita and Arias, 2016). Acquired resistance via mutation is mediated by several mechanisms such as reduced permeability, increased efflux, changes in antibiotic targets and inactivation of the antibiotic (Blair et al., 2015). Since horizontal gene transfer occurs between different bacterial clones and taxa, the acquisition of resistance determinants via this mechanism is one of the most important drivers of antibiotic resistance (Blair et al., 2015; Munita and Arias, 2016).

1.3.2 A One Health approach to antibiotic resistance

Surveillance strategies tracking AR on local and global scales help to deepen our understanding of resistance and find ways to circumvent it (Thakur and Gray, 2019). Most of this work has been carried out in hospital and community settings as well as in food-producing animals (including fish), companion animals and wildlife (Queenan et al., 2016). Therefore, current surveillance programs of AR have largely neglected the environment (Thakur and Gray, 2019). The World Health Organisation has recognised that antibiotic resistant bacteria may spread across global borders between the human, animal and environmental sectors (World Health

Organisation, 2015). In response to this, the One Health concept was created. This is a holistic and interdisciplinary approach based on the idea that human and animal health are linked to the health of the ecosystems that they are a part of (Figure 1.2) (Hernando-Amado et al., 2019).

The importance of a One Health approach to AR is clear especially with respect to the rapid dissemination of antibiotic resistance genes that have crossed habitat boundaries. One of the first examples relates to the New Delhi metallo-beta-lactamase 1 (NDM-1), which was first discovered in 2008 in a clinical isolate of *Klebsiella pneumonia* (Yong et al., 2009). The NDM-1 gene was subsequently detected in environmental samples in Bangladesh (Islam et al., 2017) and in pristine Arctic soil (McCann et al., 2019), indicating the worldwide spread of this gene and its variants (White and Hughes, 2019). A more recent example follows the first plasmid-mediated polymyxin resistance mechanism (MCR-1), conferring resistance to the 'last resort' antibiotic colistin, which was isolated from chickens in China in late 2015 (Liu et al., 2016).The MCR-1 gene has since been observed in a variety of plasmids isolated from environmental samples in China (Yang et al., 2017) and Brazil (Fernandes et al., 2017) despite these two countries banning the use of colistin in agriculture and hospital settings (Wang et al., 2018). Finally, Hedman et al., (2019) described a spill-over of ARGs from commercially bred chickens treated with high levels of antibiotics to free-grazing animals with no previous exposure to antibiotics. The study highlights the pivotal role of the environment in the transmission of AR since the environment may act as a reservoir of resistance determinants (Hedman et al., 2019).

1.3.3 The soil resistome

Soil is the most prominent reservoir of resistance genes, harbouring up to 30% of the genes that confer resistance to antibiotics, metals and biocides (Nesme and Simonet, 2015). These resistance genes, originating from diverse groups of bacteria, constitute the soil resistome (Cytryn, 2013; Nesme and Simonet, 2015). Therefore, soil has been recognized as a reservoir of antibiotic resistance genes that can be transferred to clinical pathogens (D'Costa et al., 2006; Forsberg et al., 2012). For example, it has been shown that three of the four known clusters of plasmid-mediated class A β-lactamases (i.e., CTX-M-1, CTX-M-2 and CTX-M-9) originated from different species belonging to the genus *Kluyvera*, which were rarely reported in clinical settings (Decousser et al., 2001; Humeniuk et al., 2002; Poirel et al., 2002). Members of *Kluyvera spp*. are usually found in soil and sewage systems and are generally not present in

high enough numbers in the human body to be considered pathogenic (Mutoh et al., 2019). Therefore, an understanding of resistance in the environment might help to discover potential new ARGs that could emerge as clinically relevant and to evaluate antibiotic resistance at the community level (Wang et al., 2020).

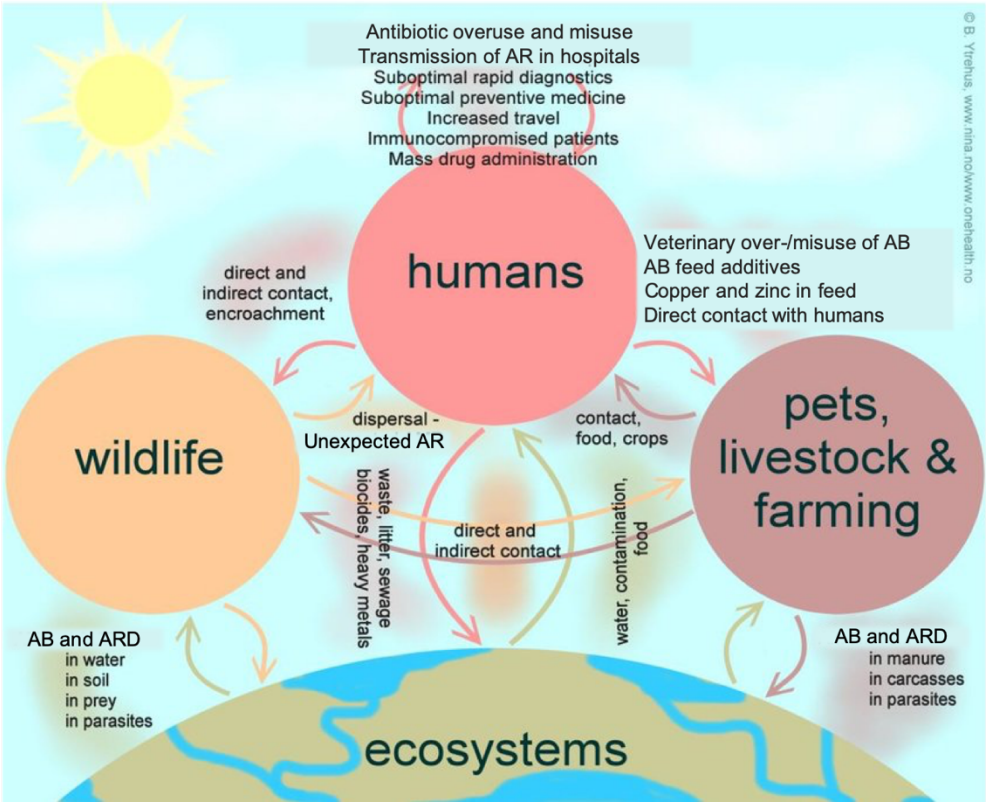

Figure 1.2. The One Heath concept illustrating that human, animal and environmental health is intrinsically linked and processes that affect one sector have an effect on all components of this triad. The figure describes some of the key factors that are involved in the transmission of resistance determinants. AB – antibiotic, AR – antibiotic resistance, ARD – antibiotic resistance determinants. Reprinted from (Nielsen et al., 2018).

Antibiotic resistance genes have been detected in 30,000-year-old permafrost from the Canadian high north (D'Costa et al., 2011), in arctic soil from 5,000 years ago (Perron et al., 2015) and in the microbiome of a cave that was isolated for over four million years (Bhullar et al., 2012). However, there is evidence that antibiotic resistance in the environment has increased significantly since the 1940s (Knapp et al., 2010) concomitantly with the rapid

production of antibiotics to treat bacterial infections (Dungan et al., 2019) and the introduction of antibiotic use in the agricultural and animal farming sectors (Cytryn, 2013; Davies, 1996).

Anthropogenic antibiotic resistance genes have even reached the pristine polar areas (McCann et al., 2019; Hernández and González-Acuña, 2016; Higuera-Llantén et al., 2018; Wang et al., 2016), probably mediated via hosts such as birds. Indeed, studies have revealed the presence of human associated bacteria carrying multi-drug resistant genes in seals, penguins and other birds from Antarctica and sub-Antarctic islands (Griekspoor et al., 2009; Palmgren et al., 2000), indicating that ARGs have reached a high level of global distribution. Over the past 10 years, research into the environment as a source and dissemination route of resistance has increased widely (Bengtsson-Palme et al., 2014; Finley et al., 2013; Larsson et al., 2018; Pal et al., 2016; Pruden et al., 2013; Scott et al., 2020; Willms et al., 2019; Wright, 2010). However, environmental resistomes are still largely unexplored and it remains unclear how resistance determinants are retained and transferred (Pal et al., 2016).

The mobilization of resistance genes is enabled by the mobilome, the compendium of mobile genetic elements (MGEs) such as plasmids, integrons and bacteriophages (Gaze et al., 2013). While much attention has been focused on the conjugative transfer of plasmids carrying ARGs, recent work has implicated bacteriophages as a vehicle of dissemination of resistance (Ross and Topp, 2015). Antibiotic resistance genes have been found in the bacteriophage DNA fraction in various ecosystems such as urban sewer and river water (Colomer-Lluch et al., 2014, 2011), hospital waste effluent and wastewater treatment plants (Marti et al., 2014) and in agricultural soils (Ross and Topp, 2015). Taking into account the abundance and global distribution of bacteriophages and phage-like particles and their flexible DNA-package mechanisms, phages are well suited for the acquisition, maintenance and transfer of resistance in several ecosystems (Calero-Cáceres et al., 2019). A recent study demonstrated the presence of a relatively high abundance of ARG-containing phage particles in agricultural soils (Larrañaga et al., 2018). Using propagation experiments, the authors found that these phage particles were infectious, suggesting that phage particles may act as vehicles for the transfer of resistance in the soil environment.

It has been reported that there is scope for mobilization of resistance genes within soils that have a high abundance (Pérez-Valera et al., 2019) and high diversity (Lu et al., 2020) of ARGs, as well as in those soils that have higher levels of anthropogenic input (Hall et al., 2017).

Conversely, it appears that, the soil resistome of pristine environments (Van Goethem et al., 2018), and less anthropogenically impacted soils (Saenz et al., 2019) have a limited potential for horizontal gene transfer of ARGs (Forsberg et al., 2014). However, resistance genes could be mobilized, in theory, in any environment. Although this would require a strong selection pressure for the resistance gene/s to remain on a mobile genetic element long enough to be successfully transferred between bacterial hosts (Bengtsson-Palme et al., 2018).

The presence of heavy metals and/or biocides in the soil environment provides the selection pressure required for successful gene acquisition. Therefore, bacterial resistance to antibiotics and metals or biocides are frequently linked (Hu et al., 2016). For instance, low levels of arsenic in paddy field soils gave rise to arsenic resistance and antibiotic resistance to sulphonamide and streptomycin, even though there was no evidence of direct input of these antibiotics into the soil (Cao et al., 2020). These resistance genes were found on genomic islands, indicating a co-selection of resistance. The co-selection of resistance occurs via three mechanisms: 1) co-resistance (i.e., genes encoding resistance to metals and antibiotics are located on the same mobile genetic element); 2) cross-resistance (i.e., a single resistance mechanism responsible for the resistance to metals and antibiotics simultaneously) and 3) co-regulation (i.e., expression of resistance mechanisms to antibiotics and metals are controlled by a common regulator), although co- and cross-resistance have been more commonly described (Cao et al., 2020; Pal et al., 2017). Studies have shown that metals (even at very low concentrations) can act as a selection pressure for ARGs in soil environments in the absence of antibiotic pressure (Knapp et al., 2017, 2011). The particular concern is that metals/biocides in soils exert a long-standing co-selection pressure, so that ARGs may persist in environments without obvious exposure to antibiotics (Li et al., 2017).

1.3.4 The desert soil resistome

Many soil resistome studies have focused on anthropogenically impacted soils such as agricultural and grassland soils (Demaneche et al., 2008; Nesme et al., 2014; Xie et al., 2018; Zhao et al., 2017). Consequently, little research has been undertaken in less impacted or pristine soils, specifically deserts, although it is known that both cold (Van Goethem et al., 2018; Wang et al., 2016; Wei et al., 2015; (Diaz et al., 2017; McCann et al., 2019) and hot desert soils (Belov et al., 2018; Saenz et al., 2019; Nowrotek et al., 2019) do harbour resistance genes.

Desert soils have only very recently been suggested as promising sources of antibacterial compounds (Rateb et al., 2018). Bacteria adapted to stressful conditions such as those that prevail in desert ecosystems could have developed unique physiological features such as novel antibiotics and ARGs to ensure their survival under those conditions. Indeed, bacteria inhabiting harsh environments have the capacity to produce a large number of secondary metabolites, including antibiotics (Nunez-Montero and Barrientos, 2018). For example, in Saharan desert soils, researchers identified a novel *Nocardia* species encoding antimicrobial compounds against a broad range of indicator strains (Selama et al., 2014). Furthermore, several actinobacterial strains from desert soils of Saudi Arabia showed broad spectrum antimicrobial activity (Nithya et al., 2015).

Since antibiotic resistance can be linked to both natural and anthropogenic sources, data on background (i.e., ARGs in environments devoid of anthropogenic influence) and baseline (i.e., ARGs in environments before the application of antibiotics or before the beginning of research studies) levels of ARGs in the environment are necessary to understand the evolution and ecology of ARGs in the environment (Rothrock et al., 2016). It is less likely that acquired resistance determinants would accumulate in desert soils due to the minimal anthropogenic input making deserts an ideal platform to characterize background or baseline levels of resistance and effectively assessing the spread of antibiotic resistance (McCann et al., 2019).

1.4 Research questions

It has been established that microbial life in desert ecosystems is highly diverse with strong links to ecosystem functions (Fierer et al., 2012; Guerra et al., 2020; Noronha et al., 2017; Song et al., 2019). Nevertheless, studies linking microbial diversity and ecosystem functions in deserts and other drylands are rare (Guerra et al., 2020; Maestre et al., 2015), particularly those on how this relationship is influenced by environmental gradients (i.e., precipitation).

The Namib is a coastal desert that is characterized by a west/east increasing precipitation gradient, which results in three xeric zones (fog, low/mid-rainfall and high-rainfall). A previous study has shown that soil chemistry, different in the three zones, seems to be important in shaping soil microbial community structure (determined using T-RFLP based profiling) and function (determined using extracellular activities of five enzymes) (Scola et al., 2018). However, the methodology used in this study did not allow an in-depth investigation of the composition and functional attributes of those communities. Consequently, we still do not

know if the composition and functional potential of these communities are influenced by precipitation history.

Therefore, this study was aimed to test, using targeted and shotgun metagenomics (Figure 1.3), whether possible changes in microbial community composition between two rainfall zones (mid- and high-rainfall zones) across a precipitation gradient, within the Namib desert, alter the overall functional attributes and specific functions (i.e., the resistome) of the communities.

More specifically, the following were investigated:

1) If overall microbial diversity and composition changed across the two rainfall zones.
2) If specific microbes were indicative of these two zones.
3) If changes in composition and diversity related to changes in microbial function.
4) The diversity and composition of the ARGs.
5) If environmental variables and precipitation history influenced the composition of the resistome.
6) The possible links between microbial composition and the resistome.
7) Whether or not horizontal gene transfer played a role in the distribution of the resistome.
8) If there was evidence that resistance to antibiotics co-selects for resistance to metals.
9) The presence of acquired clinically relevant ARGs and the potential vehicles of dissemination of these resistance genes.

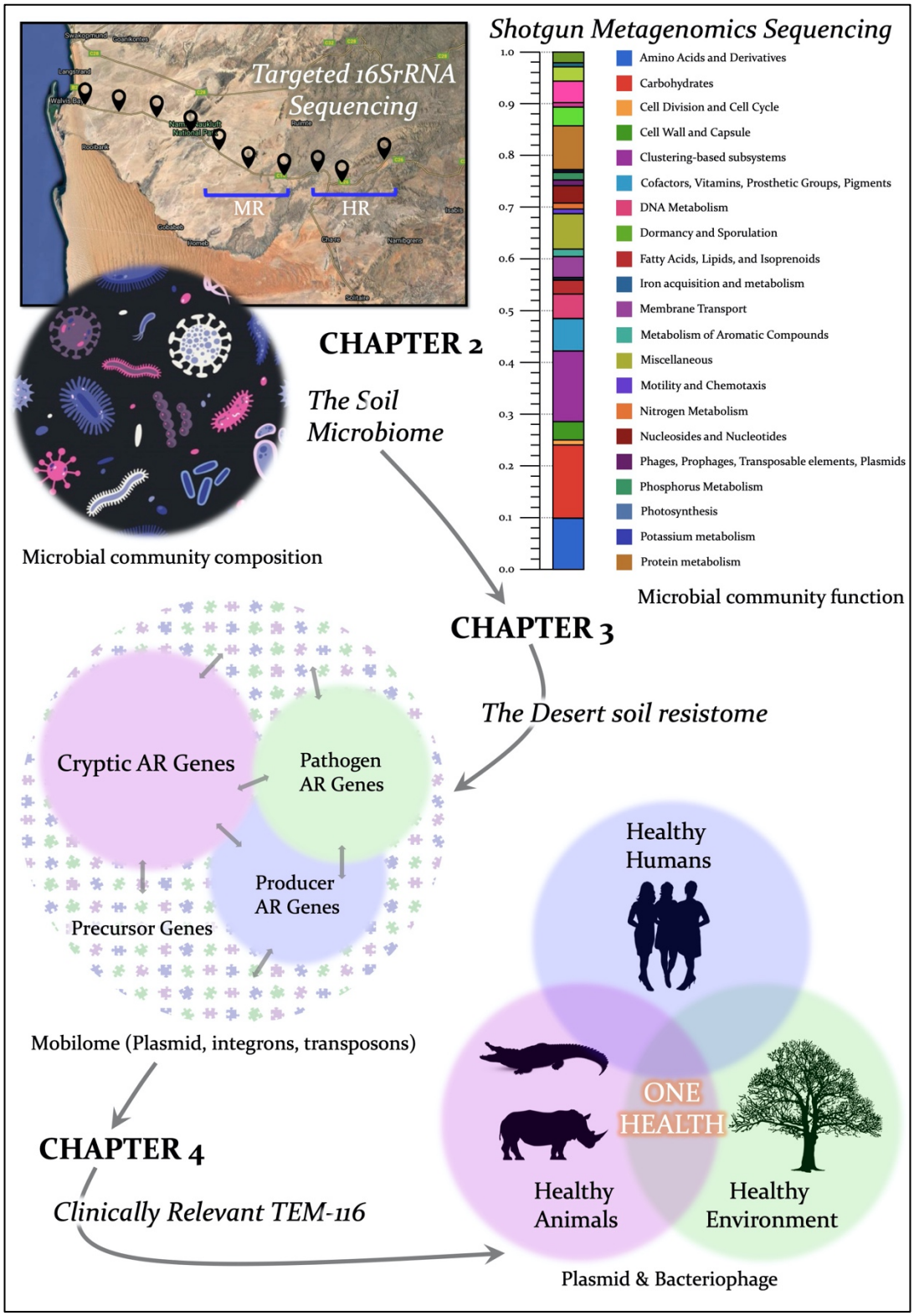

Figure 1.3. Thesis conceptual figure outlining the methodology used in the study and highlighting the main themes of each experimental chapter.

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CHAPTER TWO: HISTORICAL DIFFERENCES IN PRECIPITATION SHAPE MICROBIAL COMPOSITION AND FUNCTION IN NAMIB DESERT SOILS.

Chapter Two: Historical differences in precipitation shape microbial composition and function in Namib desert soils.

2.1 Abstract

The effect of changes in precipitation on soil microbial communities remains unresolved, especially in desert ecosystems. Using 16S rRNA gene high-throughput sequencing (to investigate taxonomic composition) and shotgun metagenome sequencing (to investigate functional potential) across two zones in the Namib Desert with contrasting precipitation, it was found that taxonomic and functional diversity remained unchanged across the two zones. Conversely, both microbial community composition and function differed significantly between the two zones. For instance, Acidobacteriota and 'virulence, disease and defence' related genes were more abundant in the high-rainfall zone. These changes were largely due to a small set of microbial taxa, some of which were present in low abundance (that is, members of the rare biosphere). Overall these results indicate that key climatic factors (i.e., precipitation) shape the taxonomic and functional attributes of the arid soil microbiome. It is proposed that changes in precipitation associated with global climate change will have important impacts on microbial community structure and function in desert soils.

2.2 Introduction

Deserts constitute one-fifth of the Earth's total surface area (Makhalanyane et al., 2015), and represent one of the harshest environments as they are characterised by scarce and irregular rainfall combined with very high temperatures (Heulin et al., 2017). Considering the current climate change scenarios, deserts and other dryland areas have been projected to increase by 11-23% by the end of this century (Jansson and Hofmockel, 2020; León-Sobrino et al., 2019). Notwithstanding these hard conditions, deserts harbour a surprisingly high biodiversity, including some of the most threatened species in the world (Durant et al., 2012).

Microbial communities are considered the dominant ecological drivers of these ecosystems (Quoreshi et al., 2019) because they regulate, among others, organic matter decomposition, carbon cycling, nitrogen cycling, and mediate nutrient acquisition (Zheng et al., 2019). Desert microbiomes display a generally lower species diversity and are phylogenetically and functionally distinct from those of other biomes (Fierer et al., 2012; Noronha et al., 2017). This

suggests that their responses to fluctuating environmental changes might also be distinct (She et al., 2018). Microbial diversity, composition and activity in desert soils is highly dependent on factors such as temperature, moisture and the availability of organic carbon (Andrew et al., 2012). Of these, water availability is the primary factor limiting microbial activity (Aslam et al., 2016) and therefore the ability of the arid soil microbiome to sustain, among others, nutrient cycling functions (Neilson et al., 2017).

The Namib Desert is a coastal desert on the western side of Southern Africa. It is the oldest hyper-arid desert on the planet (ca. 5 million years) with a highly variable surface temperature (0 – 60 °C) (Frossard et al., 2015; Pointing and Belnap, 2012). The Namib Desert has scarce and unpredictable rainfall inputs, receiving an average of 5 to 18 mm in the central zone, increasing gradually from the coast inland (Eckardt et al., 2012; Scola et al., 2018). An important ecological factor of the Namib Desert is the frequently occurring coastal fog, reaching as far as 75 km inland. The fog is influenced by the cold Benguela current and the southwest trade winds on the coast (Wassenaar et al., 2013). The contribution of rainfall and fog has led to a well-defined gradient of xeric stress across the Namib desert (Scola et al., 2018).

Gradients of precipitation have been suggested as good systems to evaluate the impact of precipitation on microbial communities (Neilson et al., 2017). Initial studies analysing soils across low (Bachar et al., 2010) and steep (Angel et al., 2010) precipitation gradients found that microbial diversity is not constrained by precipitation, but that water availability had an effect on microbial community composition. More recent studies, however, found that both microbial diversity and community composition are shaped by steep preciptation gradients (Crits-Christoph et al., 2013; Neilson et al., 2017). These contrasting results call for further investigation on how precipitation history shapes microbial community composition and function.

In an earlier study across the Namib Desert xeric gradient (Scola et al., 2018), it was shown that both microbial community structures and activities differed significantly between the three xeric zones (fog, mid- and high-rainfall). Microbial community structures were inferred using T-RFLP analysis, and their functional capacities were determined using extracellular enzyme activity assays. The combination of these methods does not, however, provide detailed

information on the taxonomic compositions and functional attributes of those communities. This information is important because both taxonomy and function can influence the stability and resilience of microbial communities and their functions in the context of climate change. For instance, highly functional redundant microbial communities should be more functionally stable, as functional redundancy should act as a buffer against changes in diversity and composition (Fetzer et al., 2015).

Here, with the use of high-throughput amplicon sequencing (targeting 16S rRNA genes), the bacterial community diversity and composition in two Namib Desert rainfall zones (medium rainfall and high rainfall) across the intrinsic xeric gradient were analysed. In addition, the functional potential of those communities was assessed using shotgun metagenomics. The working hypotheses were: 1) that the taxonomic and functional diversities would increase from the mid-rainfall zone to the high-rainfall zone due to an increase in water availability, which also can influence nutrient availability and 2) that the taxonomic and functional composition between the two zones would be distinct. To investigate this hypotheses, three basic questions were adressed: 1) Is precipitation history and other environmental variables associated with specific microbial taxa? 2) Is microbial diversity and composition altered across the two rainfall zones? 3) If there are changes in community composition, do they have a direct effect on community function?

2.3 Materials and Methods

2.3.1 Study site and sampling

Eighteen surface soil (0 to 5 cm) samples were collected across a west-east transect (23°11'76.1"S 15°16'69.2"E), which spans three xeric zones (fog, mid- (MR) and high-rainfall (HR)). Samples were collected from the mid (n = 9) and high rainfall (n = 9) zones. Using sterile methods, four aliquots of approx. 50g of soil were taken at each site at 100 m spacing to make a composite sample, and stored in sterile 50 ml polypropylene Falcon tubes (Grenier, Bio-One). Soils were transported to the laboratory within 5 days of collection and stored at -80°C for molecular analysis.

2.3.2 Sample preparation and DNA sequencing

Soils were analysed for soil pH, total carbon, nitrogen, phosphorous and major cations (K, Na, Mg, Ca) at Bemlab, South Africa, using standard analytical procedures. Rainfall data were

accessed from two weather stations of the SASSCAL network (http://www.sasscalweathernet.org/) in the mid-rainfall area (Vogelfederberg station) and the high-rainfall area (Ganab station) (Table1, Appendix 1). Metagenomic DNA was extracted from the soil samples (n = 18), using the DNeasy Powersoil Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. Samples were submitted for sequencing at a commercial supplier (MR DNA Lab, Shallowater, TX, USA, http://www.mrdnalab.com). Shotgun metagenomic sequencing was performed on a HiSeq 2500 Ultra-High-Throughput Sequencing system (IlluminaInc., San Diego, CA, USA) using paired-ends (2 × 250 bp) for 500 cycles as per the manufacturer's instructions.

Targeted sequencing of the 16S rRNA gene amplicons were amplified using primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'). Paired-end 2 × 250 bp sequencing was performed on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina Inc., San Diego, CA, USA) with the parameters as described (https://support.illumina.com/16s-metagenomic-library-prep-guide-15044223-b.pdf). The metagenome sequence data and 16S amplicon sequence data are available on NCBI (PRJNA592367).

2.3.3 Metagenome assembly and functional annotation

Raw reads were quality filtered using FastQC (Andrews, 2010), and trimmed using PrinSeq (Schmieder and Edwards, 2012). Reads were assembled using SPAdes v3.12 (Bankevich et al., 2012), with default settings and the "meta" parameter specified. The quality of each assembled metagenome (n = 18) was assessed using QUAST v5.0.2 (Mikheenko et al., 2018). Gene prediction was performed using Prodigal v2.6.3 (Hyatt et al., 2010) with the "meta" parameter specified. The protein files from the gene prediction were then used for functional annotation. The functional annotation of was carried out using two approaches. First, assembled contigs were uploaded and annotated with the Metagenomics Rapid Annotation using SEED Subsystems (MG-RAST) pipeline version 4 (Meyer et al., 2008). Broader functional pathways were generated using the SEED database (SEED levels 1 to 3), and frequency values of the hits of each individual subsystem for each metagenome were normalized before statistical analysis. Since MG-RAST does not apply very stringent thresholds (e.g., e-value <1.0e-5 and identity >60%), annotation lacks specificity at the individual functional level (An et al., 2014). Therefore, protein families were annotated using the pFam database (Finn et al., 2014). For

this approach, the prodigal protein files were scanned against Interpro's database (which houses member database signatures such as pFam) using the InterProScan software (Jones et al., 2014), which uses Hidden Markov Models (HMM) allowing for a high quality alignment.

2.3.4 Amplicon sequencing analysis

Sequence reads were demultiplexed using Sabre (https://github.com/najoshi/sabre) and primers and barcodes were removed using Cutadapt (Martin, 2011). Amplicon sequence variants (ASVs) were resolved using DADA2 version 1.14 (Callahan et al., 2016) in R version 3.6.2 (R Core Team, 2013). Quality filtering was done using the following parameters: MaxEE = 2, truncLen = 220, 200, with all other parameters were set to default. The error rates were estimated by learnErrors and sequences were dereplicated using derepFastq with default parameters. removeBimeraDenovo was used to remove chimeric sequences. Taxonomy was assigned against the Silva non-redundant database version 138 (https://www.arb-silva.de). The resulting taxonomy and read-count tables constructed in DADA2 were imported into phyloseq (McMurdie and Holmes, 2013) for downstream analysis.

2.3.5 Statistical analyses

The analyses were done in R (R Core Team, 2013) mainly using the packages phyloseq (McMurdie and Holmes, 2013), microbiome (Lahti et al., 2019), tidyverse (Wickham et al., 2019), vegan (Oksanen et al., 2007) and metacoder (Foster et al., 2017). Richness and Shannon, diversities were calculated using the vegan package (Oksanen et al., 2007). Phylogenetic Diversity (PD) was calculated using the picante package (Kembel et al., 2010). Taxonomic (ASVs) and functional (SEED level 3) community data were Hellinger-transformed and the Bray-Curtis distance measure was used to generate dissimilarity matrices. The correlation between taxonomic and functional dissimilarities was assessed using a Mantel test. The differences in community composition and function, and environmental conditions were visualised using Principal Coordinates Analysis (PCoA) and Principal component analysis (PCA), respectively. A permutational analysis of variance (PERMANOVA) was carried out to test for differences in composition between habitats (mid-rainfall and high-rainfall) using the 'adonis' function in vegan. In order to test for differences in composition within habitats, a test for homogeneity of multivariate dispersions (PERMDISP) was done using the 'betadisper' function in vegan. The effect of environmental conditions in explaining variation in microbial community structure was assessed by distance-based Redundancy Analysis (db-RDA). To

assess the ASVs that differed in relative abundance between the two rainfall zones, DeSeq analysis was used. For the analysis of ecotypes, the ASVs were clustered into 97% similarity OTUs using the Opticlust algorithm (Westcott and Schloss, 2017) with Mothur (Schloss et al., 2009).

To determine the statistical differences between the functional profiles of the two climatic zones, the Statistical Analysis of Metagenome Profiles (STAMP) software was used (Parks et al., 2014). The table of frequency of hits generated by the SEED database was used as the input file (at levels 1 and 2). The P-values were calculated using the Welch's t-test (Welch, 1947, 1938) and corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (Benjamini and Hochberg, 1995). The alpha diversity metrics for the functional profiles were calculated using SEED level 3. In order to better understand the relationship between taxonomy (class level) and function (SEED level 2), network analyses were performed. To this end, all possible Spearman's rank correlation coefficients were calculated. Only correlations with $\rho > 0.8$ and *P*-values < 0.01 were selected. The nodes in the reconstructed network represent taxonomical or functional groups and the edges represent significant correlations between the nodes (Mendes et al., 2014). The networks were visualized using Igraph (Csardi and Nepusz, 2005). Core functions were assessed using protein domain families generated by the pFam database and defined as those present in 95% of samples and at ≥ 0.01% of cumulative relative abundance. The core functional profile was represented by a heatmap that was created using the ComplexHeatmap (Gu et al., 2016) package in R.

2.4 Results

2.4.1 Soil chemistry and climate

Physicochemical analysis showed low nutrient levels in all samples for most of the measured parameters (Figure 2.1). Soil pH, K, Mg and NO_3 levels did not differ statistically between the two zones. Statistically significant changes were observed for Ca and Na, which were both higher in the mid-rainfall zone (Kruskal-Wallis $\chi^2 = 7.7\text{-}10.1$; $p < 0.05$); while C, P, NH_4 and mean annual rainfall were higher in the high-rainfall zone (Kruskal-Wallis $\chi^2 = 5.7\text{-}13.1$; $p < 0.05$). PERMANOVA analysis showed a clear distinction in abiotic factors between the two rainfall zones (PERMANOVA: *F* ratio = 7.97, $P < 0.001$).

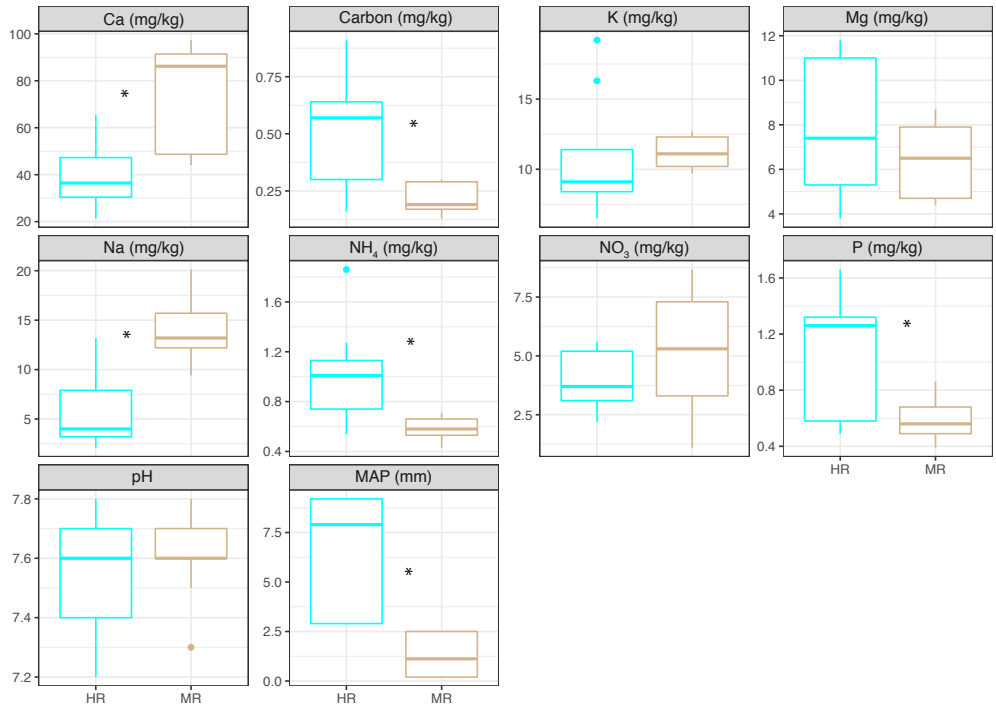


Figure 2.1. Environmental characterization of high-rainfall (HR) and mid-rainfall (MR) zones. Asterisks indicate statistically significant differences between means using Kruskal-Wallis tests ($P < 0.05$).

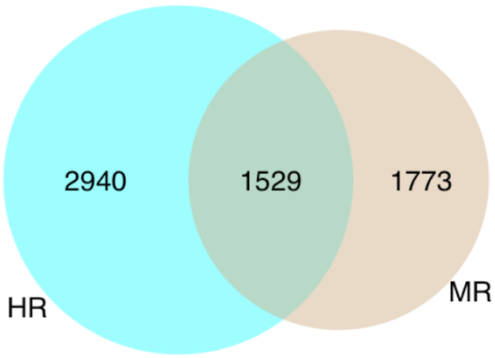
2.4.2 Soil taxonomic profiles

To assess the diversity and composition of the soil microbiomes, amplicon sequences were processed using DADA2. A total number of 667 353 reads were obtained (averaging 37 075 reads per sample), resulting in 4 366 ASVs. Of those, 1 047 (24% of the ASVs) were shared between the two zones, while 2 043 (47%) were unique to the high-rainfall zone and 1 276 (29%) were unique to the mid-rainfall zone (Figure 2.2a). The shared ASVs comprised 78% of the reads, the unique to high-rainfall 14% of the reads and mid-rainfall 8% of the reads.

The overall taxonomic analyses showed a total of seventeen phyla present across the two zones, with thirteen phyla showing relative abundances greater than 1% (Figures 2.2b and 2.3a, b). *Proteobacteria* with a mean relative abundance of 28% (± 5% SD) dominated the soils in both rainfall zones, followed by *Actinobacteriota* (22% ± 8%), *Bacteroidota* (19% ± 8%) and *Acidobacteriota* (7% ± 3%). A relatively low abundance of *Firmicutes* (4% ± 5%), *Verrucomicrobiota* (3% ± 1%), and *Abditibacteriota* (3% ± 2%) was found; with *Crenarchaeota* (6% ± 3%) as the dominant archaeal group. A significant increase in the relative

abundance of *Acidobacteriota* (Figures 2.3b and 2.4) from the mid-rainfall to the high-rainfall zone was noted (Kruskal-Wallis $\chi^2 = 10.4$; $P < 0.05$), while the other phyla were equally distributed between the two rainfall zones.

a)



b)

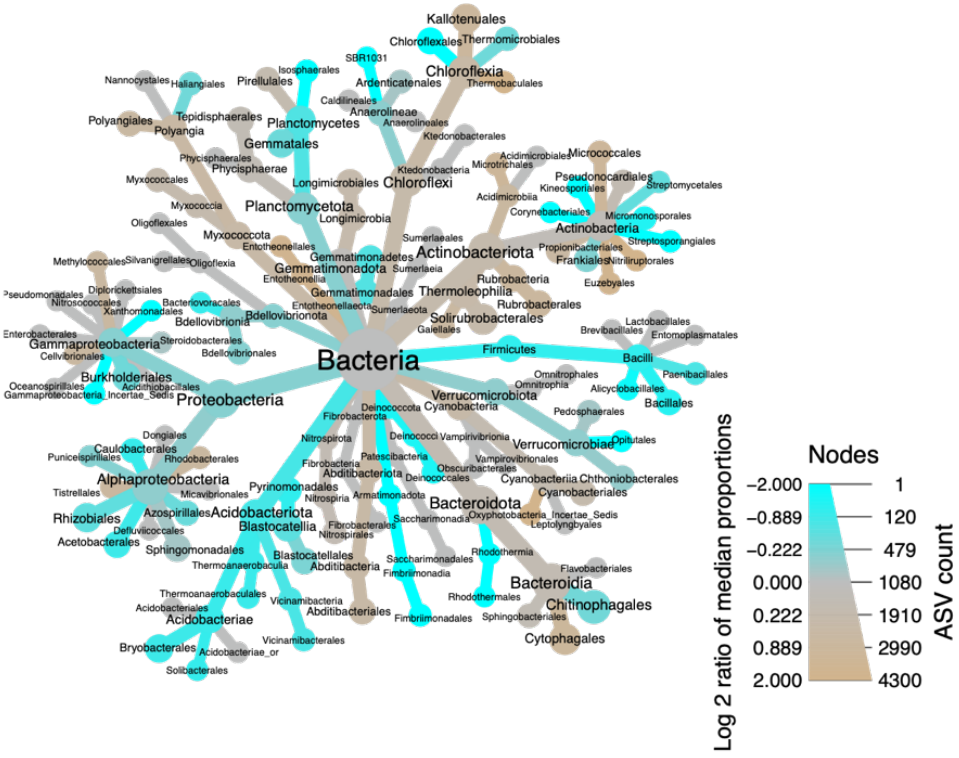


Figure 2.2. Venn diagram (a) showing the number of shared and the number of unique OTUs in the high-rainfall zone (HR) and the mid-rainfall (MR) zone respectively and a taxonomic heat tree (b) displaying the differences in taxon abundance between the two zones.

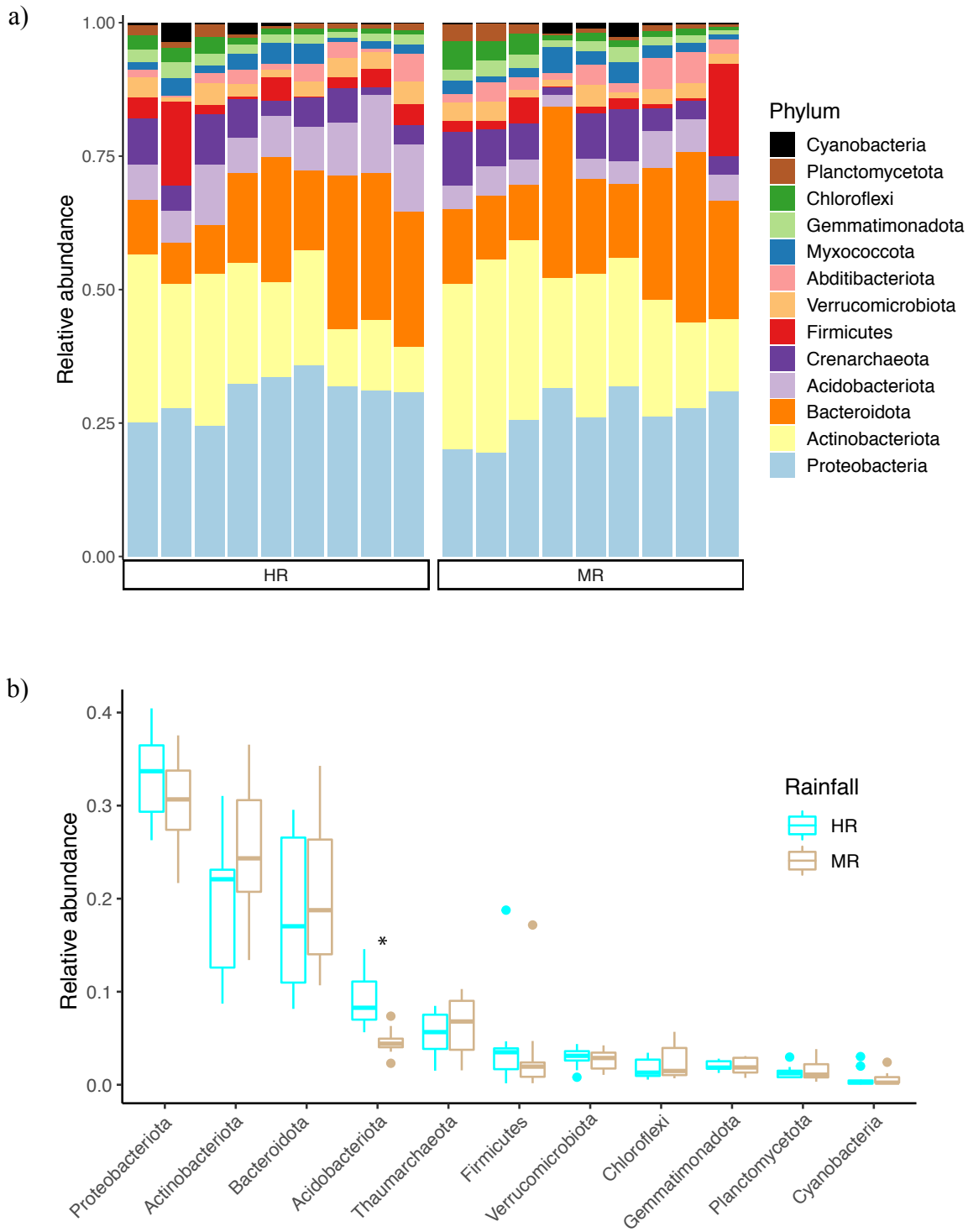


Figure 2.3. Relative abundance of the major phyla based on 16S rRNA gene sequences in both rainfall zones (a), showing the significant increase of *Acidobacteriota* ($P < 0.05$) in the high-rainfall zone (b). HR, high-rainfall; MR, mid-rainfall.

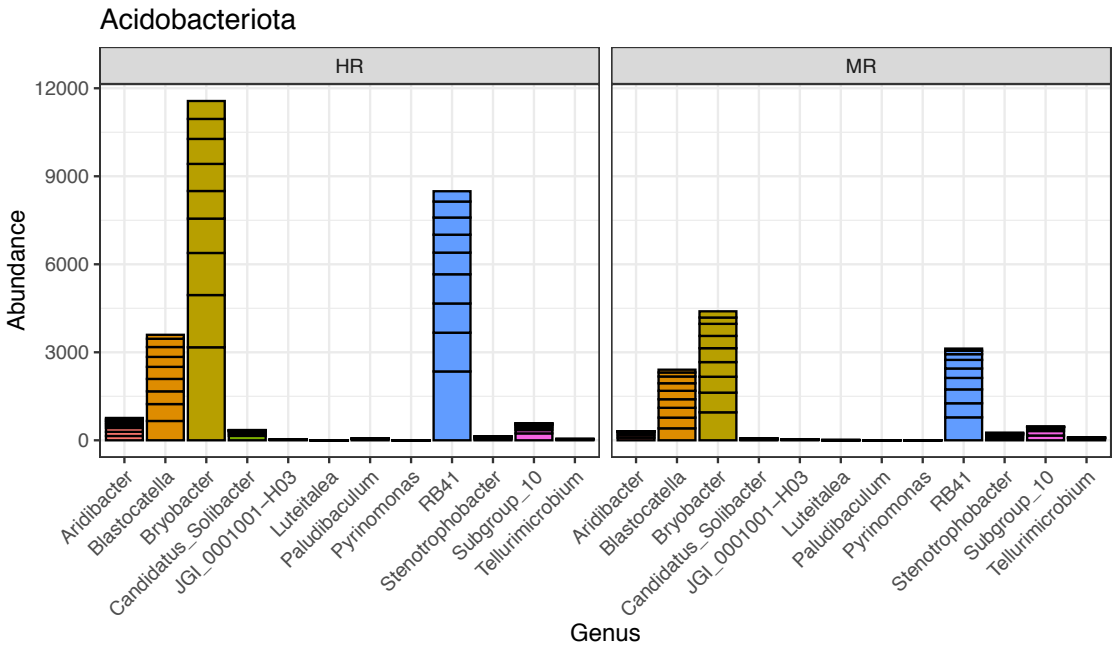


Figure 2.4. The distribution of *Acidobacteriota* at the genus level between the high and mid-rainfall zones. HR, high-rainfall; MR, mid-rainfall.

DESeq analysis was used to identify the ASVs that differed in relative abundance between the two rainfall zones. These results showed that 86 ASVs differed in abundance between the two zones (Figure 2.5). 53 ASVs increased significantly in the high-rainfall zone compared to the mid-rainfall zone. The largest increase (a 9.76 log-fold change) belonged to "*Candidatus* Nitrosphaera", phylum Crenarchaeota. Other ASVs that were more abundant in the high-rainfall zone were members of the genera *Bryobacter* (Acidobacteriota) (Figure 2.4) and *Chlorogloeopsis* PCC−7518 (Cyanobacteria). In contrast, 33 ASVs decreased significantly in the high-rainfall zone compared to the mid-rainfall zone. The largest decrease (a 10.02 log-fold change) was seen within the genus *Adhaeribacter*, phylum Bacteroidota.

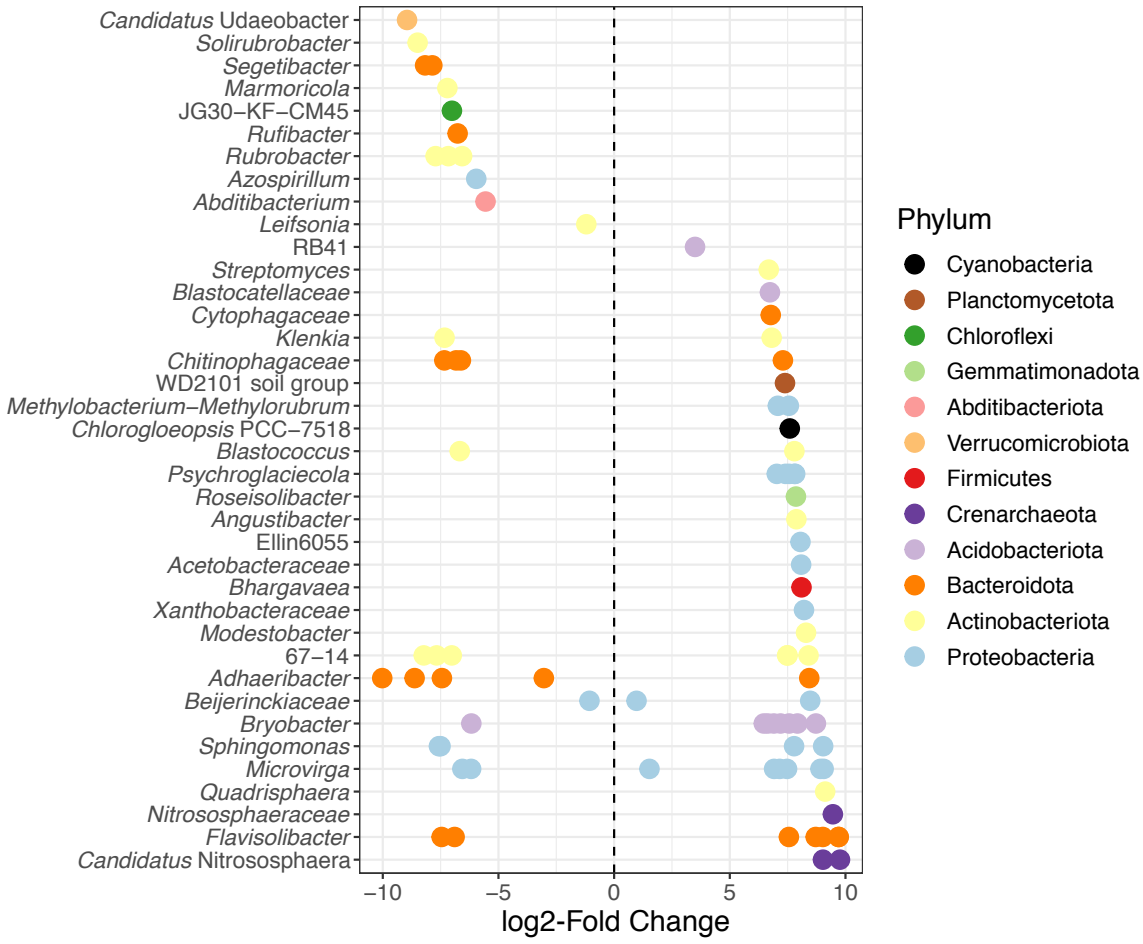


Figure 2.5. Amplicon sequence variants (ASVs) in soil communities that significantly increased (positive log2-fold change values) or decreased (negative log2-fold change values) in relative abundance in high-rainfall compared to the mid-rainfall zone.

By clustering of the 86 ASVs into OTUs (97% identity cut-off), it was found that several of these OTUs were composed of different ASVs with specific preferences for one of the two rainfall zones (Appendix 1, Table 2). For instance, OTU-02 (belonging to the genus *Adhaeribacter*, phylum Bacteriodota) was represented by 3 different ASVS: ASV-170 and ASV-57, more abundant in the mid-rainfall zone; and ASV-307, more abundant in the high-rainfall zone.

2.4.3 Sources of community variation

Based on the 16S rRNA gene sequence data, alpha-diversity (richness, Shannon and phylogenetic diversity) was not significantly different between the two zones (Kruskal-Wallis; $P > 0.05$) (Figure 2.6). The Bray-Curtis distance (beta-diversity) metric after Hellinger transformation was determined for the soil microbiomes, ordinated through Principal Coordinate Analysis (PCoA) (Figure 2.7) and the differences between the two zones tested using PERMANOVA analysis. The results showed that the high-rainfall zone harbored different prokaryotic communities than the mid-rainfall zone (PERMANOVA: F ratio = 3.6, $P < 0.001$).

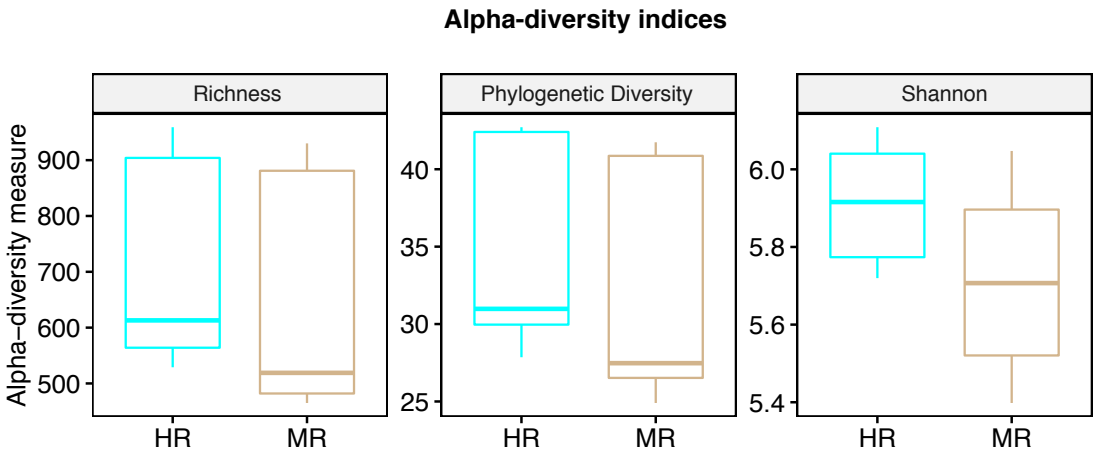


Figure 2.6. Alpha diversity measures (richness, phylogenetic diversity and Shannon diversity) for the two rainfall zones (Kruskal-Wallis; $P > 0.05$). HR, high-rainfall; MR, mid-rainfall.

Importantly, communities from the high-rainfall zone were considerably more variable in their ASVs composition than communities from the mid-rainfall zone (PERMDISP: F ratio = 8.5, $P < 0.01$). Using distance-based redundancy analysis (db-RDA), it was observed that community composition was largely driven by the rainfall zone, and the levels of phosphorous and nitrate, which together explained 38% (18% rainfall zone, 11% Phosphorus and 9% nitrate) of community variation (Figure 2.8).

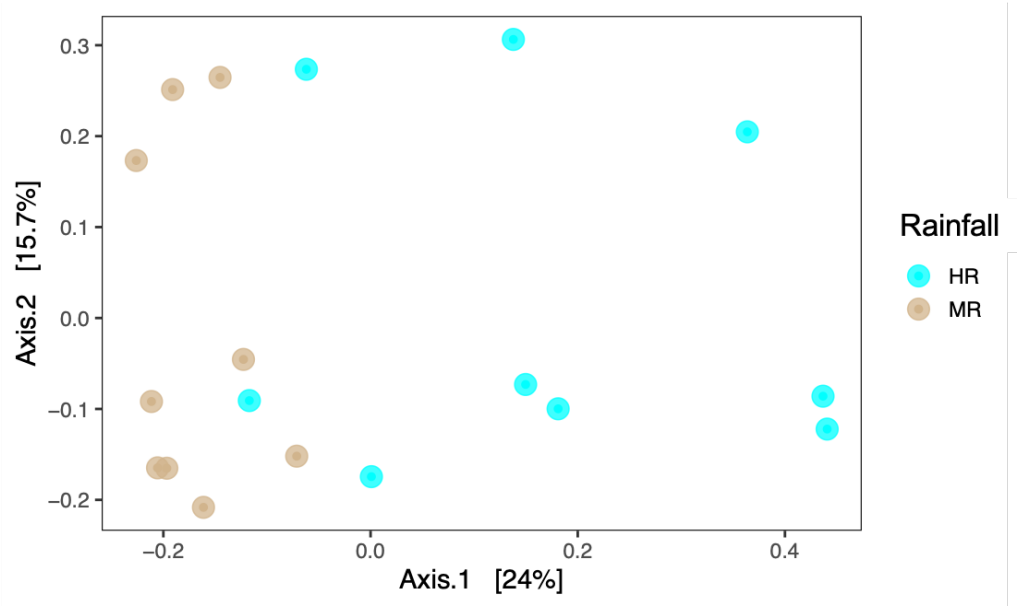


Figure 2.7. PCoA ordination of Bray-Curtis distances (after Hellinger transformation) between microbial communities in the mid-rainfall (MR) and high-rainfall (HR) zones (PERMANOVA: F ratio = 3.6, $P < 0.001$).

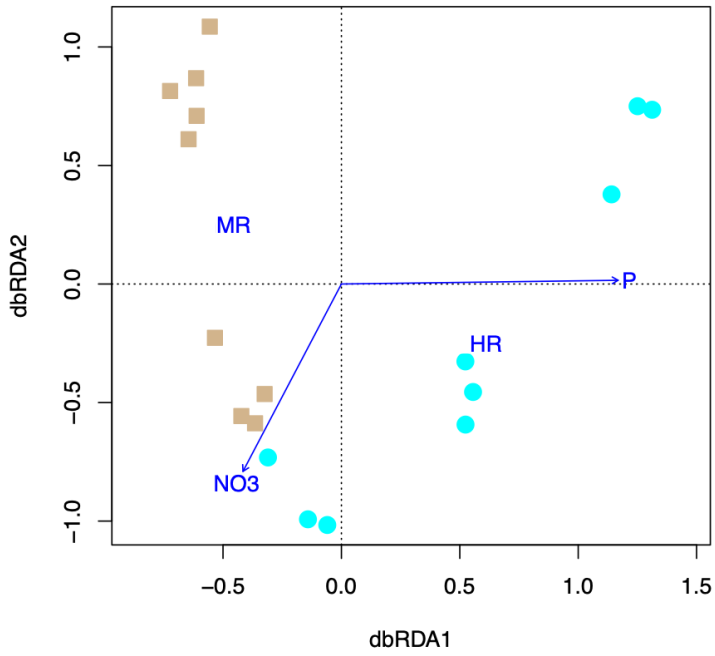


Figure 2.8. Distance-based redundancy analysis (db-RDA) plot of the prokaryotic communities in the high (HR) and mid-rainfall (MR) zones. The two significant environmental variables explaining the variability in microbial community structure are represented by the arrows.

2.4.4 Soil functional profiles

The number of sequence reads per metagenome ranged from 9,868,998 to 11,726,910 (10,632,908 on average). Using MG-RAST SEED level 3 data, alpha-diversity (richness) was not significantly different between the two zones (Kruskal-Wallis; $P < 0.05$); however, Shannon diversity, which includes richness and evenness information, was higher in the high-rainfall zone (Kruskal-Wallis; $P > 0.001$) (Figure 2.9).

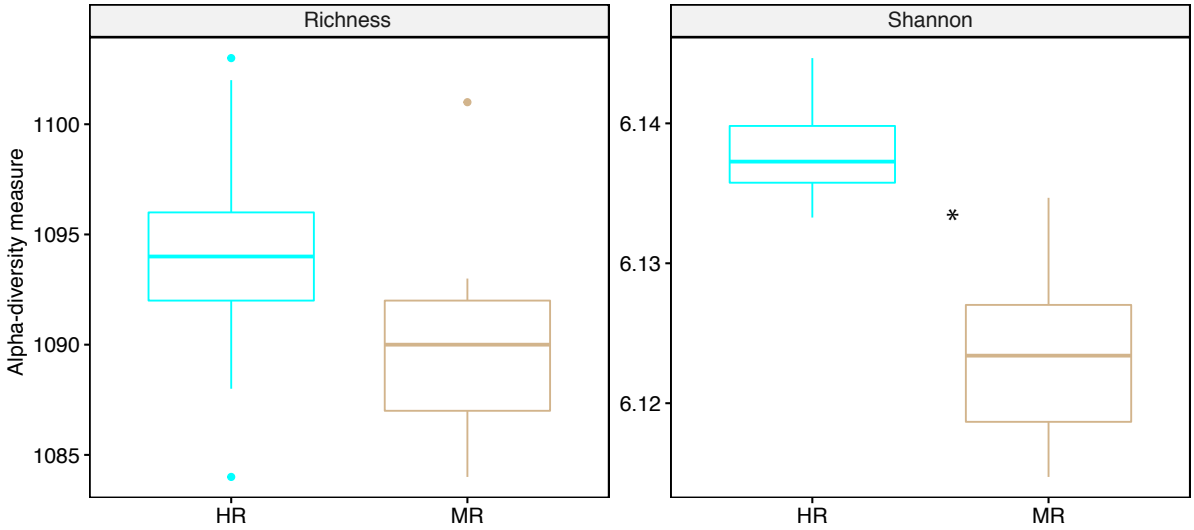


Figure 2.9. Alpha diversity measures (richness and Shannon) for the two rainfall zones. Asterisks indicate statistically significant differences between means using Kruskal-Wallis tests ($P < 0.05$). HR, high-rainfall; MR, mid-rainfall.

The Bray-Curtis distances (beta-diversity) after Hellinger transformation were determined for the functional profiles (SEED level 3), ordinated through Principal Coordinate Analysis (PCoA) (Figure 2.10) and the differences between the two zones were tested using PERMANOVA analysis. The results showed that the high-rainfall zone harbored different functional profiles than the mid-rainfall zone (PERMANOVA: F ratio = 3.5, $P < 0.005$).

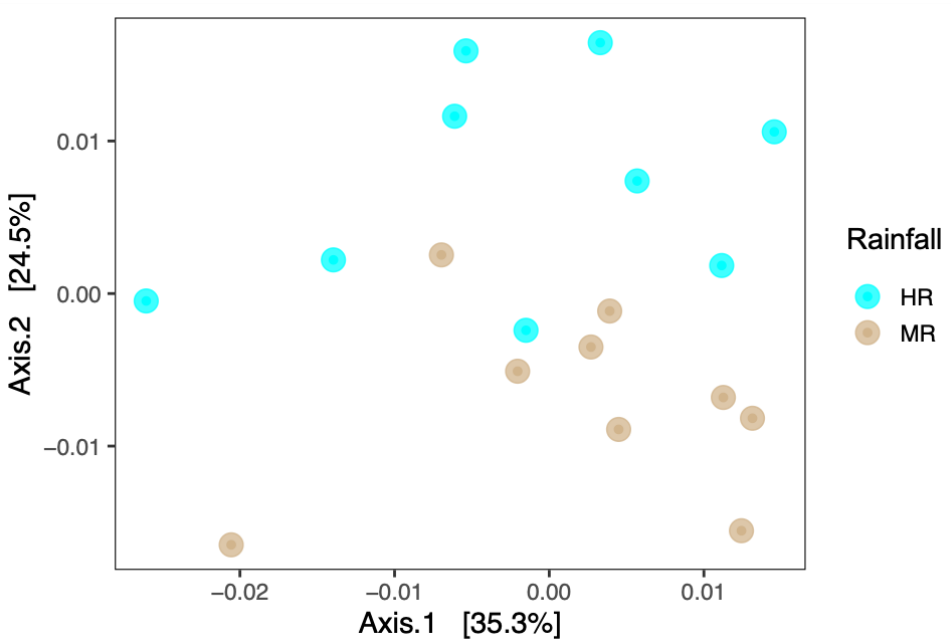


Figure 2.10. PCoA ordination of Bray-Curtis distances (after Hellinger transformation) between functional profiles (SEED level 3) in the mid-rainfall (MR) and high-rainfall (HR) zones (PERMANOVA: F ratio = 3.5, $P < 0.005$).

To determine the functional capacity of the soil, metagenomes were analyzed with the SEED database on MG-RAST. At the top SEED level (level 1), the subsystems 'sulphur metabolism', 'metabolism of aromatic compounds', 'regulation and cell signalling', 'motility and chemotaxis', and 'virulence, disease and defence' were more abundant in the high-rainfall zone compared to the mid-rainfall zone (Welch's t-tests $P < 0.05$, Figure 2.11). Conversely, the subsystems 'Nucleosides and nucleotides' and 'clustering based subsystems' (i.e., protein biosynthesis, ribosomes and recombination related clusters) were more abundant in the mid-rainfall zone (Welch's t-tests $P < 0.05$). Subsystems relating to dormancy and sporulation were present in very low abundance and did not differ between the two zones. In addition, several subsystems related to stress were identified in higher abundance but they did not differ significantly between the two zones.

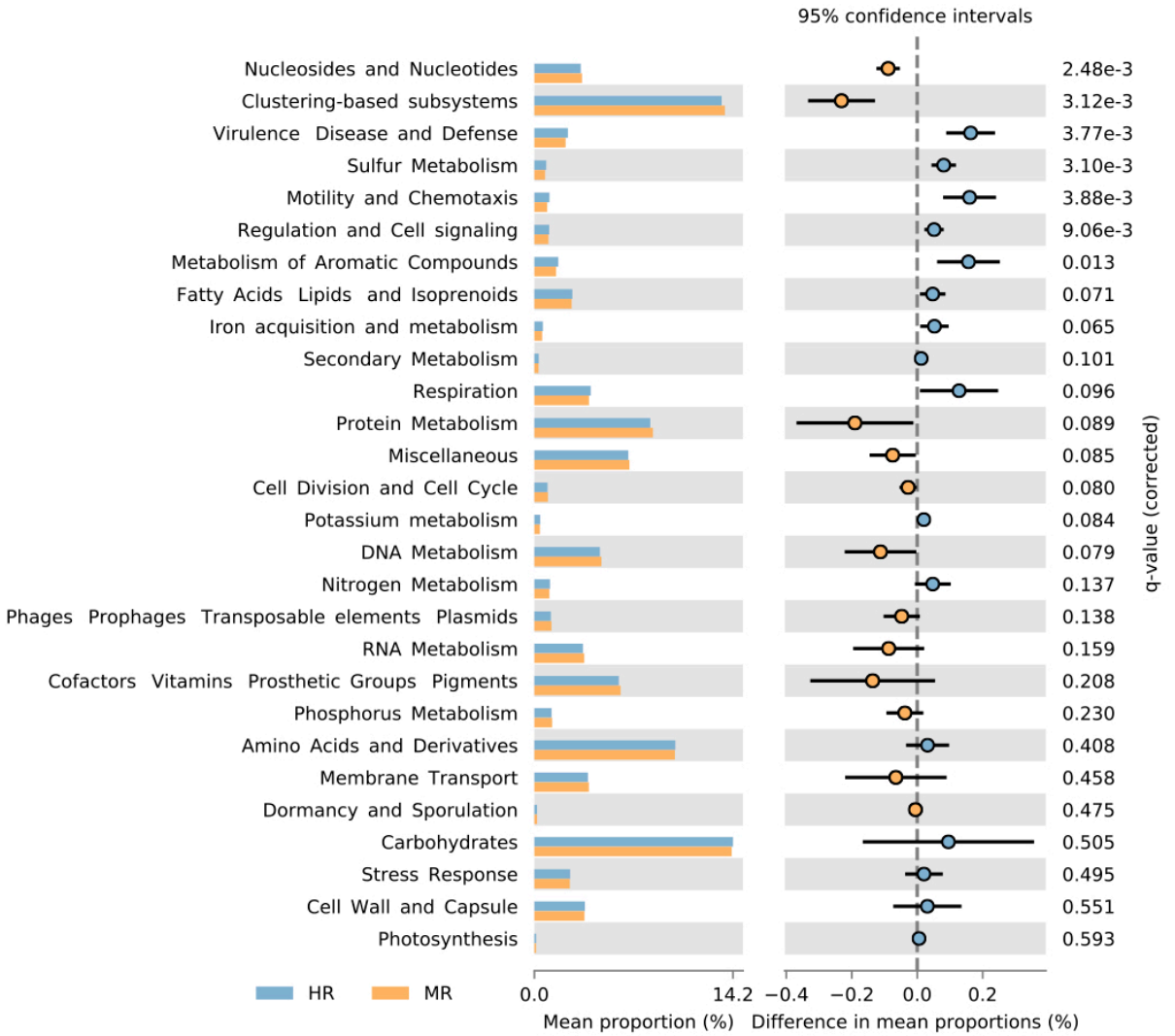


Figure 2.11. Relative abundance of functional categories (SEED subsystem level 1) for the high-rainfall (MR) and mid-rainfall (HR) zones. Statistical differences of the functional profiles were determined by STAMP (Welch's t-tests). Corrected P-values were calculated using the Benjamini-Hochberg false discovery rate.

At SEED level 2 (Figure 2.12), the mid-rainfall microbiomes showed an increased abundance of functional groups responsible for carbohydrate fermentation and secondary functions such as resistance to antibiotics and toxic compounds (Welch's t-tests, $P < 0.05$), while the high-rainfall microbiomes were more enriched in DNA replication and protein synthesis functions (Welch's t-tests, $P < 0.05$).

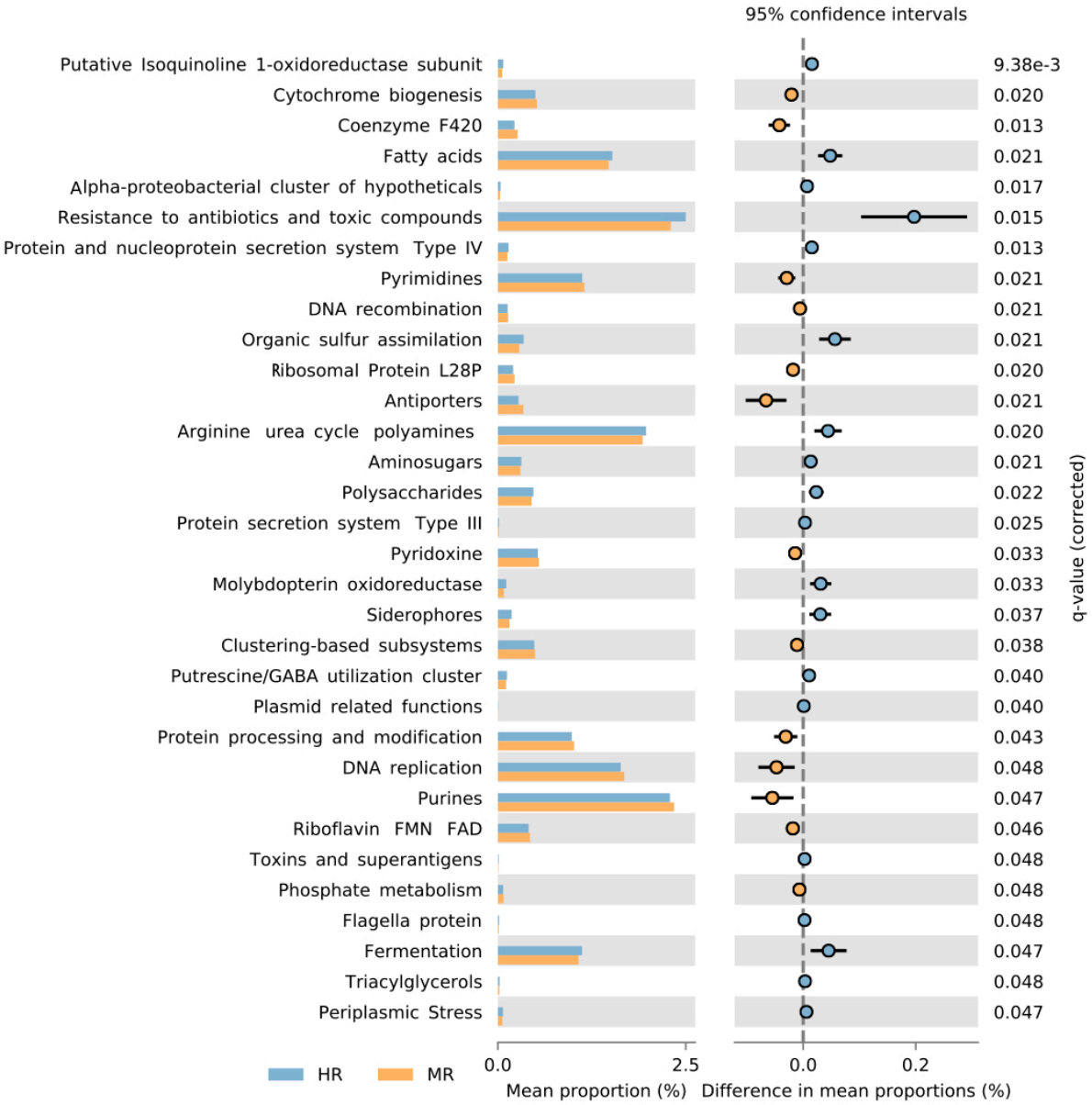


Figure 2.12. Relative abundance of functional categories (SEED subsystem level 2) for the high-rainfall (HR) and mid-rainfall (MR) zones. Statistical differences of the functional profiles were determined by STAMP (Welch's t-tests). Corrected P-values were calculated using the Benjamini-Hochberg false discovery rate.

2.4.5 Core functional profile

Individual functional roles were generated using the pFam (Protein domain Families) database. The results from the pFam database indicate that the most abundant protein families were related to DNA transposition (transposase), protein synthesis initiation (Translation initiation factor 1A /IF−1), energy metabolism, oxidative stress (thioredoxin, glutaredoxin) and general

stress responses (Csbd-like), metabolism of aromatic compounds and proteins responding to stresses like heat shock (GrpE) (Figure 2.13). Several protein families relating to nutrient cycling (nitrogen metabolism, phosphorous metabolism, iron acquisition and metabolism) were found in high and low abundance across the rainfall regimes, as well as families relating to competitive traits (e.g., Beta-lactamases).

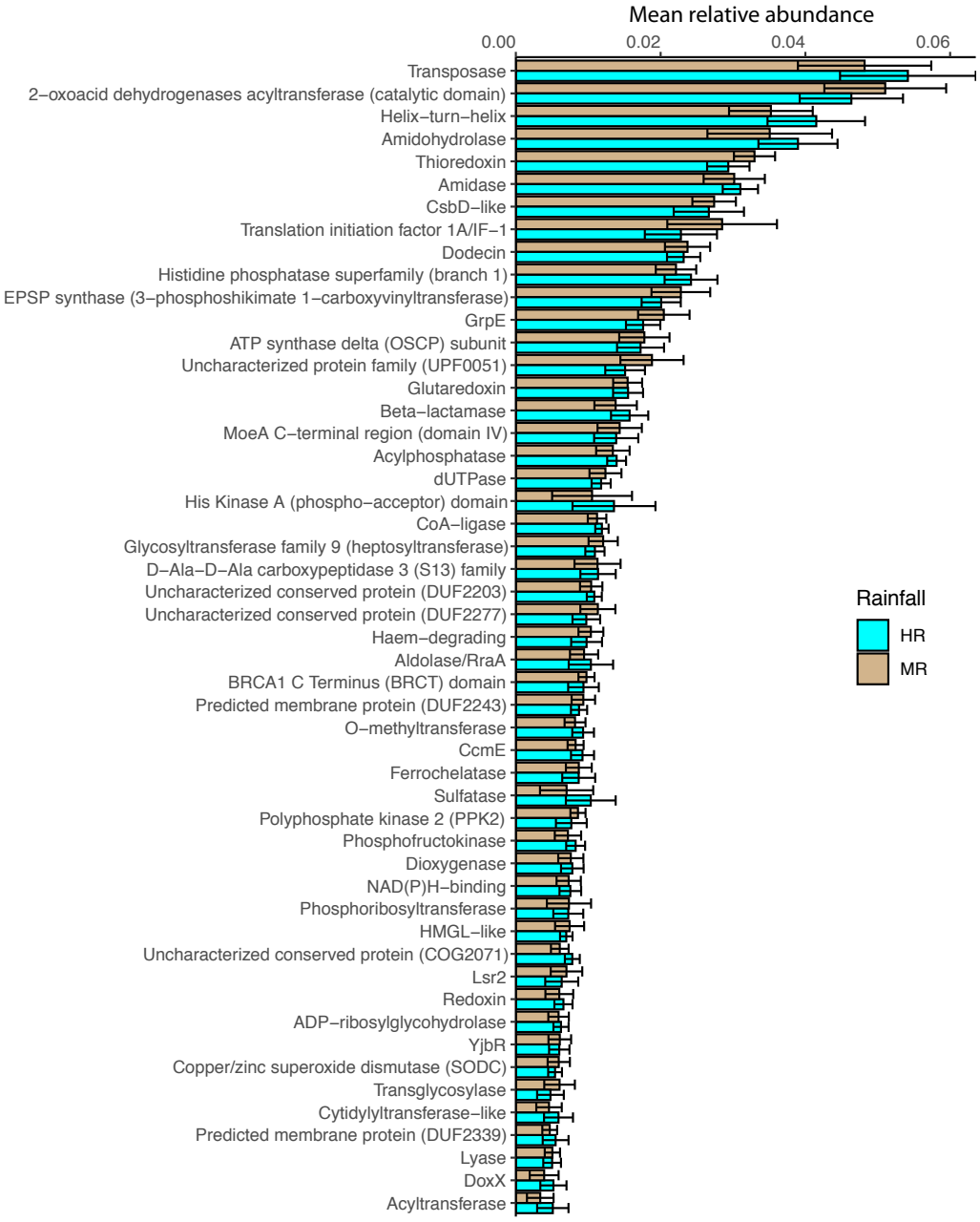


Figure 2.13. Mean relative abundance of proteins/protein domain families (pFam) in mid-rainfall (MR) and high-rainfall (HR) zones. Error bars represent the standard error of the mean.

Functional genes in soil systems can be categorized into core and accessory components. Core functions were defined as those present in 95% of the samples and at ≥ 0.01% of cumulative relative abundance. As expected, the predicted core functional profile (Figure 2.14) revealed a relatively high abundance of protein families linked to general housekeeping functions (i.e., DNA repair, cellular regulation, protein biosynthesis). Proteins involved in general homeostasis functions of the soil system (nutrient and energy metabolism) were also detected as well as the presence of general stress response and oxidative stress protein families, which were detected in relatively high abundance.

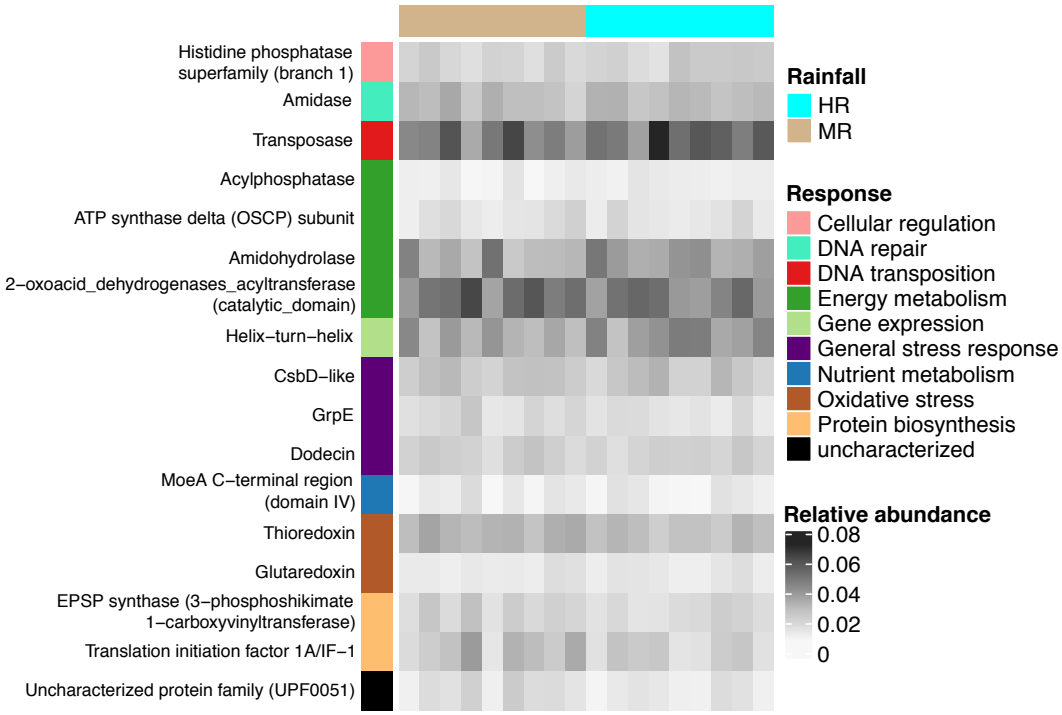


Figure 2.14. Heatmap that shows the core functional profile generated by pFam data in the mid-rainfall (MR) and high-rainfall (HR) zones.

2.4.6 Relating microbial taxonomy and function

To explore the relationship between microbial taxonomy (ASVs) and function (protein-coding gene categories), correlation analysis was performed using both alpha and beta-diversity metrics. For alpha-diversity, taxonomic richness and phylogenetic diversity, but not Shannon diversity, were positively correlated with functional diversity (Spearman $\rho = 0.7$, $P < 0.001$ in both cases). Likewise, changes in microbial community composition were positively correlated with changes in microbial community function (Mantel $\rho = 0.6$, $P < 0.001$).

Network analysis showed that the networks for the two rainfall zones were substantially different (Figure 2.15). Overall, the number of positive correlations was higher in the mid-rainfall compared to high-rainfall zone. In the mid-rainfall zone the network presented 97 nodes and 153 edges; while for the high-rainfall zone, the network had 104 nodes and 118 edges (positive correlations). For the mid-rainfall zone, *Planctomycetes* (0.9 % relative abundance), *Rubrobacteria* (9.7%), *Chloroflexia* (1.9 %) and *Vicinamibacteria* (0.1%) were the classes that were positively correlated with more functions, whereas *Alphaproteobacteria* (24.7 %), *Entotheonellia* (0.04 %), *Actinobacteria* (8.2 %) and *Rubrobacteria* (6.3%); were the microbial classes that were positively correlated with more functions in the high-rainfall zone.

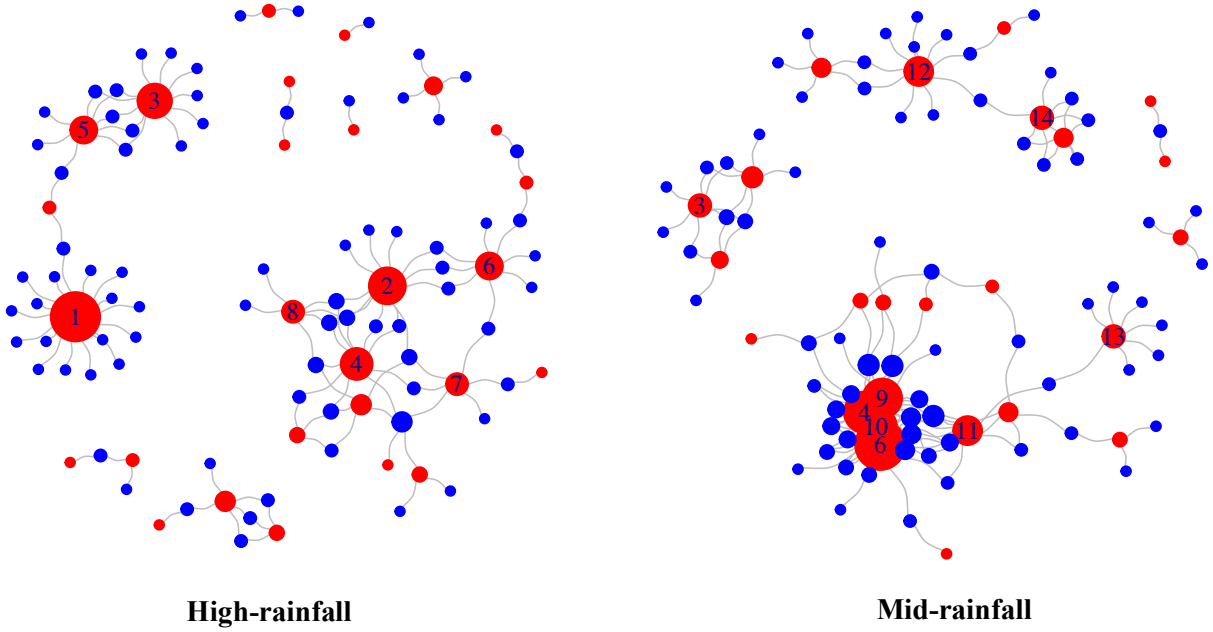


Figure 2.15. Networks based on correlation analysis (Spearman's $\rho > 0.8$, $P < 0.01$) with the taxonomic and functional profiles in the mid-rainfall (MR) and high-rainfall (HR) zones. Red nodes indicate taxonomic affiliation (at the class level) and blue nodes indicate functional categories based on subsystems at level 2 (SEED database). 1 – *Alphaproteobacteria*, 2 – *Entotheonella*, 3 - *Actinobacteria*, 4 – *Rubrobacteria*, 5 – *Myxococcia*, 6 – *Planctomycetes*, 7 – *Longimicrobia*, 8 – *Thermoleophilia*, 9 – *Chloroflexia*, 10 – *Vicinamibacteria*, 11 – *Nitrospiria*, 12 – *Gammaproteobacteria*, 13 – *Acidimicrobiia* and 14 – *Bacteroidia*

2.5 Discussion

The Namib desert is characterized by a precipitation gradient along an East-West transect, where the average annual precipitation increases around 20-fold from the coast inland (Scola et al., 2018). Here, using next generation sequencing (NGS), both the prokaryotic community composition (using targeted high-throughput sequencing of the 16S rRNA genes) and function (using shotgun metagenomic sequencing) of soils from two zones (high and mid-rainfall) were characterized along this gradient.

2.5.1 The effect of the environment on the diversity of the soil microbiome

The physicochemical soil analysis revealed low nutrient levels, with values comparable with other studies performed in the region (Armstrong et al., 2016; Frossard et al., 2015; Scola et al., 2018). The levels of Ca and Na decreased significantly from the mid-rainfall to the high rainfall zone. This was expected since the Namib is a coastal desert and salt aerosol deposition would decrease from the coast inland (Liang et al., 2016). Conversely, mean annual precipitation and the content of carbon, P and NH_4 were higher in the high-rainfall zone. This, together with the PERMANOVA results, supports the conclusion that the two zones have contrasting environmental conditions and that the high-rainfall zone appears to support more benign conditions.

The ubiquitous presence of phyla such as *Proteobacteria*, *Actinobacteriota*, *Bacteroidota* and *Acidobacteriota* across the two zones was expected, as these phyla have been consistently reported in studies across the Namib desert (Gunnigle et al., 2017; Ronca et al., 2015; Valverde et al., 2015; van der Walt et al., 2016) and seem to be the most dominant phyla detected in soil bacterial communities worldwide (Delgado-Baquerizo et al., 2018). Interestingly, *Acidobacteriota* were overrepresented in the high-rainfall zone compared to the mid-rainfall zone (Figure 2.3). This pattern has been observed in other studies (Barnard et al., 2013; Maestre et al., 2015; Zhang et al., 2018) and appears to be related to the alterations in the nutrient pools in the soil after precipitation events (Barnard et al., 2013). Certain species of the phylum *Acidobacteriota* reflect a fast-response life strategy displaying a water-related opportunistic behavior (Barnard et al., 2013; Zhang et al., 2018). After re-wetting, some *Acidobacteriota* seem to increase ribosome synthesis to maximize growth (Barnard et al., 2013). This seems to contradict the classification of *Acidobacteriota* as slow-growing oligotrophic bacteria (e.g., Fierer et al., 2007). However, in addition to nutrient availability, other factors such as microbial interactions (e.g., competition) may contribute to the abundance of *Acidobacteriota*.

There were no significant differences (at the ASV level) in alpha-diversity (richness, Shannon and phylogenetic diversity) between the two zones. Thus, rejecting the initial hypothesis that microbial diversities would increase from the mid-rainfall zone to the high-rainfall zone. These findings are in agreement with those from a recent experimental study (Wu et al., 2020), suggesting that an increase in precipitation does not always result in higher microbial diversity. However, these results were in contrast with results from Scola et al., (2018), who found that alpha diversity increased from the coast inland. This study employed T-RFLP analysis which, although a reliable technique, may have not detected fine scale changes in bacterial community diversity (Carrino-Kyker et al., 2013). The results presented here also indicate that the microbial diversity of these soils is not affected by salt levels. More likely, the narrow range in precipitation differences in these study sites contributed to the pattern observed. However, the structure and composition (beta-diversity) of the microbial communities was significantly different between the two zones.

Using distance-based redundancy analysis it is shown that, in addition to precipitation history, phosphorous and nitrate were other important factors shaping microbial community composition (Figure 2.6). This is not unexpected, as it is well known that the levels of nitrogen and phosphorous commonly shape the composition and structure of microbial communities in soils (Austin et al., 2004; Šťovíček et al., 2017) and other ecosystems (Lee et al., 2017). Furthermore, the decrease in beta-diversity among the microbial communities from the mid-rainfall zone might be an indication of biotic homogenization (Rodrigues et al., 2013), the process by which the similarity of communities increases over time and/or space. Biotic homogenization can occur, for instance, in harsher environmental conditions because a substantial proportion of the regional species pool can be filtered out due to niche-selection (Chase, 2007). Indeed, the mid-rainfall zone seems to contain fewer microbial ASVs compared to the high-rainfall zone (2 323 $ASVs_{MR}$ vs 3 090 $ASVs_{HR}$). In addition, higher productivity due to higher nutrient levels and/or heterogeneities in one or more environmental factors might have led to more divergent communities in the high-rainfall zone (Langenheder et al., 2011; Rodrigues et al., 2013).

Several ASVs that were more abundant in one of the two zones were identified. For instance, seven ASVs belonging to the genus *Bryobacter* (phylum Acidobacteriota) were more abundant in the high-rainfall zone compared to the mid-rainfall zone. This could be due to the higher

levels of carbon in these soils compared to mid-rainfall soils, as members of this genus are known to degrade plant-derived polymers such as cellulose (Kielak et al., 2016). *Chlorogloeopsis* PCC−7518 (Cyanobacteria) was also more abundant in the high-rainfall zone. Many cyanobacteria fix nitrogen, which requires high levels of energy and therefore P (e.g., ATP). Phosphorus levels were also higher in this zone. Conversely, the mid-rainfall zone was enriched with ASVs within the genus *Rubrobacter* and *Solirubrobacter* (both from the phylum Actinobacteriota). Members from these genera were also abundant in the driest locations in other desert studies (Crits-Christoph et al., 2013; Wu et al., 2020). Several strains of extremophilic *Rubrobacter* have been shown to express classic phenotypes of UV- and γ-radiation and desiccation resistant bacteria (Bull, 2011). Several strains of *Rubrobacter* have mechanisms which aid in the resistance of water deficits in soils, such as the increased production of enzymatic systems that counteract the production of reactive oxygen species (ROS) under drought (Starke et al., 2017).

Interestingly, ecotypes (ASVs adapted to a specific ecological niche) were found within the same OTU (e.g., belonging to the genus *Adhaeribacter*, phylum Bacteriodota). These ecotypes seem to be adapted to different values of environmental factors such as available water, carbon and phosphorus concentrations. The presence of ecotypes within a species (microdiversity) has been proposed as a mechanism to promote community survival in changing environments (García-García et al., 2019).

2.5.2 Soil functional profile

Overall, the functional profile of these soils is consistent with reports from other studies carried out in hot deserts (Le et al., 2016; Saenz et al., 2019). Expectedly, the core functional profile included a relatively high abundance of protein families linked to general housekeeping functions (i.e., DNA repair, cellular regulation), which play important roles in maintaining the basic metabolism of bacterial cells for survival. Core protein families responsible for nutrient and energy metabolism were also reported, suggesting that these communities have a higher degree of metabolic flexibility which could result in communities that are more resilient (Allison and Martiny, 2008). Unexpectedly, genes relating to dormancy and sporulation in these soils were in very low abundance. This is in contrast to other studies that detected a high abundance of gene categories relating to dormancy and sporulation due to the ecological selection of moisture stress and frequent drying and rewetting cycles typical to desert

environments (Fierer et al., 2012; Tripathi et al., 2017). A possible explanation for this observation is that those taxa associated with functions relating to dormancy and sporulation may be present in low abundance.

In hot desert soils, microbial community members are exposed to high levels of irradiation, low levels of water, wide temperature fluctuations and desiccation (León-Sobrino et al., 2019; Makhalanyane et al., 2015), which results in high levels of biotic stress. Stress forces microbes to shift resource allocation, which can alter the C and N flows imposing considerable influences on ecosystem functioning (Schimel et al., 2007). Indeed, several core protein families relating to stress response and subsystems (SEED level 2) involved in coping with stress (i.e., oxidative, osmotic, heat and cold shock stress) were found in the soil metagenomes of the two zones and in the same proportion. This suggests that the microbes from the two zones have probably developed similar adaptation mechanisms to thrive in these stressful environments (Belov et al., 2018), for instance cold shock proteins which are usually produced in response to a rapid decrease in temperature can also contribute to osmotic and oxidative stress tolerance (Keto-Timonen et al., 2016).

One of the most noticeable differences between the microbial communities of the high-rainfall and mid-rainfall zones was the differential abundance of genes involved in 'resistance to antibiotics and toxic compounds' (SEED level 2). These genes were more abundant in the high-rainfall zone compared to the mid-rainfall zone. This pattern might reflect increased competition in the high-rainfall zone as this zone presents higher moisture and nutrient levels, which results in a more benign environment. Competition is hypothesized to be more intense in more benign environments, compared to more stressful environments in which cooperation should be more common (Van Horn et al., 2014). Several of those microbial classes that showed more positive correlations with functional categories, such as *Entotheonella* and *Nitrospira*, did not belong to the most abundant groups. Thus, it seems that relatively rare taxa might have an important role in these soil microbial communities. Rare soil microbes have been shown to drive key processes in biogeochemical cycles (Jousset et al., 2017).

2.5.3 Coupling between taxonomy and function

The relationship between microbial community composition and function is largely unknown (Escalas et al., 2019). One of the first comparative metagenomics studies of soil microbial

communities (Fierer et al., 2012) showed that functional profiles were highly correlated with taxonomic profiles; that is, that taxonomy and function were coupled. In contrast, other studies (Pan et al., 2014; Purahong et al., 2014) point out to a decoupling between these two components of microbial diversity, due to high functional redundancy (the coexistence of multiple distinct taxa capable of performing the same biochemical function) in soil microbial communities. Adaptive gene loss, convergent evolution and lateral gene transfer can result in the wide distribution, phylogenetically speaking, of many traits (Louca et al., 2018 and references therein).

In this study, a significant correlation (coupling) between microbial and functional diversity was observed, at both alpha and beta diversity levels. Therefore, in this system the microbial functional potential appears to be largely determined by microbial community composition. Although individual functions may not necessarily be correlated with community structure (e.g., due to horizontal gene transfer), these results indicate that the overall functional profiles of these microbial communities seem to be predictable, at least to a certain extent, from the taxonomic community profiles.

2.6 Conclusion

The main objective of this part of the research program was to understand how precipitation history affected microbial communities in terms of diversity, composition and function. Precipitation history had no effect on microbial taxonomic alpha-diversity which rejects the initial hypothesis that microbial diversities would increase from the mid-rainfall zone to the high-rainfall zone. However, the composition and function (beta-diversity) of microbial communities differed between the two zones in response to precipitation history, confirming the second hypothesis. Additionally, the decrease in beta diversity in the mid-rainfall zone could be due to biotic homogenization in response to the harsher environmental conditions (i.e., lower rainfall and nutrients). The most noticeable difference between the two rainfall zones were the increase in functions relating to competition (e.g., resistance to antibiotics (SEED level 2) and β-Lactamases (pFams)) in the high-rainfall zone, more likely as a result of increased moisture and higher nutrient levels compared to the mid-rainfall zone.

Furthermore, the changes in microbial community and function were governed by a narrow range of phylogenetic taxa including several that were rare, pointing out to an important role

of the rare biosphere. Overall, this study demonstrates that microbial functional potential appears to be largely determined by microbial community composition, and that the taxonomic and functional profiles of desert soil microbial communities are strongly influenced by precipitation. This is important in the context of climate change, which is expected to alter precipitation patterns (precipitation events are predicted to become more extreme but less frequent). This, in turn, will likely affect the biogeochemical processes linked to desert soil microbial communities.

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CHAPTER THREE: CHARACTERIZATION OF THE SOIL RESISTOME AND MOBILOME IN NAMIB DESERT SOILS

CHAPTER 3: Characterization of the soil resistome and mobilome in Namib Desert soils

3.1 Abstract

The study of the soil resistome is important in understanding the evolution of antibiotic resistance, and its dissemination between the clinic and the environment. However, very little is known about the resistome of hot deserts. Soil was sampled in the Namib Desert across a gradient that gradually increased in precipitation from the coast in-land. With the use of shotgun metagenomics, the resistance mechanisms of the resistome and the mobilome in these soils were identified and characterized. Resistance genes were also linked to potential microbial hosts. A variety of antibiotic resistance genes (ARGs) were detected, including those that were acquired horizontally. The presence of metal/biocide resistance genes (MRGs) in close proximity to ARGs indicated a potential for co-selection of resistance to antibiotics and metals/biocides. Mobile genetic elements (MGEs) that may contribute to the mobilization of both ARGs and MRGs were detected. The presence of MGEs and horizontally acquired ARGs most likely contributed to a decoupling between bacterial community composition and ARG profiles. Overall, this study indicates that soil bacterial communities in Namib Desert soils host a number of resistance elements and that horizontal gene transfer, rather than phylogeny, could play an essential role in their dynamics.

3.2. Introduction

The misuse and overuse of antibiotics in the human, animal and agricultural sectors is an important selective force in the evolution and the dissemination of antibiotic resistance (Bhullar et al., 2012; von Wintersdorff et al., 2016), which is one of the major global challenges of the 21st century as it poses serious risks to human health. However, antibiotic resistance genes (ARGs) have been uncovered in 30 000 year old permafrost metagenome samples (D'costa et al., 2011), 10 000 year old cold-seep sediments (Song et al., 2005) and in soil samples from an isolated cave with no anthropogenic activity for four million years (Bhullar et al., 2012). This is an indication that antibiotic resistance is ancient and long predates the use of antibiotics by humans (Perry et al., 2016).

Soils are perhaps the most significant reservoir of ARGs as many soil microbes produce natural antibiotics to outcompete other microbes (Nesme and Simonet, 2015; Zhu et al., 2019). Indeed, most clinically relevant antibiotics such us streptomycin, tetracycline and vancomycin originate from soil-dwelling actinomycetes (Cytryn, 2013; D'Costa et al., 2006). Microorganisms have developed a variety of resistance mechanisms (encoded by resistance genes) to prevent them from succumbing to these toxic metabolites (Jiang et al., 2017; Thaker et al., 2013). Many bacteria are naturally resistant to a broad spectrum of antibiotics (Allen et al., 2009; Demaneche et al., 2008), which may reflect their ability to produce more than one antibiotic, or be a by-product of their evolution in proximity to antibiotic-producing neighboring bacteria (Perry and Wright, 2013). Consequently, it is now clear that the soil environment harbors a plethora of both discovered and undiscovered resistance genes, which together constitute the soil *resistome* (Dantas and Sommer, 2012; Gillings et al., 2017).

Most types of soils contain heavy metals, some of which (at low concentrations) contribute to the biochemical health of microorganisms (Chen et al., 2019; Knapp et al., 2017). For example, at low concentrations zinc plays a significant role in cell division, protein synthesis and nucleic acid metabolism; while at high concentrations Zn can be toxic to microbes by inactivating proteins (Oves et al., 2016). Therefore, resistance to metals is also thought to be an ancient phenomenon (Pal et al., 2017). Interestingly, metals can act as co-selective forces contributing to the increase in antibiotic resistance (Knapp et al., 2017). The two major mechanisms that are involved in co-selection of resistance are co-resistance and cross resistance (Murray et al., 2019). Co-resistance is the genetic linkage of resistance genes, meaning that genes responsible for resistance to two or more compounds are close to each other, most likely on a mobile genetic element (i.e., plasmid, transposon or integron) (Pal et al., 2017; Seiler and Berendonk, 2012). Cross resistance occurs when a resistance gene or a single resistance mechanism simultaneously encodes for resistance to different compounds (Imran et al., 2019; Pal et al., 2017).

The relative importance of co-selection of resistance to both antibiotics and metals/biocides is likely to be different in different environments. For instance, some desert soils are known to have low anthropogenic antibiotic input but relatively high levels of metals over long periods of time (Knapp et al., 2011). Consequently in deserts, even in the absence of antibiotics, metals

may provide a stronger and more persistent selective pressure for the environmental selection of antibiotic resistance (Zhao et al., 2018).

Genes encoding traits, including antibiotic and metal resistance genes, can move between soil microbial taxa via mobile genetic elements, collectively termed the *mobilome* (Carr et al., 2020). The mobilome facilitates the acquisition of traits between bacteria through any of the mechanisms collectively referred to as horizontal gene transfer (HGT); that is, via transformation (involving free DNA), transduction (involving bacteriophages) or conjugation (involving plasmids and integrative conjugative elements) (Peterson and Kaur, 2018). Soil bacteria undergo higher rates of gene transfer in 'hotspots' (i.e., areas of higher nutritional content) such as the rhizosphere and manure-treated soil (Perry and Wright, 2013). However, the prevalence of horizontal gene transfer in native soil microbial communities and the effects it may have on soil processes are largely unknown and require further investigation (Fierer, 2017).

The study of the soil resistome has attracted considerable attention (Han et al., 2016; Ibrahim et al., 2016; McCann et al., 2019; Van Goethem et al., 2018) because it has been hypothesized that many ARGs found in clinical isolates can originate from soil or vice versa (that is, the soil acts as a natural environmental reservoir for ARGs). Therefore, investigating the soil resistome could enable the detection of clinically-relevant antibiotic resistance mechanisms (Forsberg et al., 2012; McCann et al., 2019; Walsh and Duffy, 2013). As it is known that the abundance of ARGs increases with human activity, most of these investigations have been conducted in anthropogenically impacted soils (Demaneche et al., 2008; Forsberg et al., 2012; Pehrsson et al., 2013). Tracking antibiotic resistance genes in less impacted or unimpacted (pristine) soils is also important because this would allow for the detection of background or intrinsic levels of antibiotic resistance in soil in order to estimate potential human health risks (Scott et al., 2020). Additionally, it might help to elucidate the extent of contamination of ARGs due to anthropogenic activity. For instance, studies in both Antarctica (Hernández and González-Acuña, 2016; Wang et al., 2016) and the high Arctic (McCann et al., 2019) revealed human-related ARGs showing that some of these soils are more impacted than expected. Conversely, a recent study in Antarctic soils of the remote Mackay Glacier region found that the ARGs present most likely represented functional background (historical) genes transferred vertically over generations (Van Goethem et al., 2018).

Much less is known about the antibiotic resistome of hot desert soils than of mesic and agricultural soils, with most previous studies limited to the screening, using cultured-based approaches, of antibiotic resistant bacteria (Belov et al., 2018; Rateb et al., 2018, and references therein). A recent metagenomic study (Saenz et al., 2019) revealed the presence of ARGs in Atacama Desert soils, although the main focus of the work was on functional traits such as genes responsible for oxidative stress co-occurring with mobile genetic elements.

Here, using shotgun metagenomics, we characterized the soil resistome and mobilome from two different zones (mid vs high rainfall) in the Namib Desert. The aims of this part of the study were to investigate the following: 1) the diversity and composition of ARGs and MRGs, 2) whether the diversity and composition of the resistome (ARGs and MRGs) was influenced by rainfall regime (i.e., water availability/aridity), 3) if there is a link between microbial community composition and the antibiotic resistome, 4) whether or not horizontal gene transfer (HGT) affected the distribution of the resistome and 5) if there is co-selection of resistance with metals/biocides and antibiotics.

3.3. Materials and Methods

3.3.1 Sampling, soil chemistry and climate data

Eighteen surface soils (0 to 5 cm deep) were collected in two zones (mid-rainfall (MR, 9 samples) and high-rainfall (HR, 9 samples)) of the xeric gradient across the Namib desert. The 18 sampling sites were spaced 5-10 km apart and at each site four aliquots of 50g of soil were taken at 100 m spacing and combined in a composite sample. Soils were collected using sterile methods and stored in sterile 50 ml polypropylene Falcon tubes (Grenier, Bio-One) at -80°C within 5 days after collection. Soils were analysed for soil pH, total carbon, nitrogen phosphorous and major cations (K, Na, Mg, Ca) at Bemlab, South Africa, using standard procedures. Rainfall and soil temperature data were accessed from two weather stations of the SASSCAL network (http://www. sasscalweathernet.org/) in the mid-rainfall area (Vogelfederberg station) and the high-rainfall area (Ganab station) (Table 1, Appendix 1).

3.3.2 DNA extraction and sequencing

Metagenomic DNA was extracted from the soil samples using the DNeasy Powersoil Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. DNA samples were submitted for sequencing at a commercial supplier for both metagenome and 16SrRNA sequencing (MR DNA Lab, Shallowater, TX, USA, http://www.mrdnalab.com). Shotgun metagenomic sequencing was performed on a HiSeq 2500 Ultra-High-Throughput Sequencing system (IlluminaInc., San Diego, CA, USA) using paired-ends (2 × 250 bp) for 500 cycles as per the manufacturer's instructions.

Targeted sequencing of the 16S rRNA gene amplicons were amplified using primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'). Paired-end 2 × 250 bp sequencing was performed on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina Inc., San Diego, CA, USA) with the parameters as described (https://support.illumina.com/16s-metagenomic-library-prep-guide-15044223-b.pdf). The metagenome sequence data and 16S amplicon sequence data are available on NCBI (PRJNA592367).

3.3.3 Metagenome assembly and ARG annotation

Raw reads were quality filtered using FastQC (Andrews, 2010), and trimmed using PrinSeq (Schmieder and Edwards, 2012). Quality reads were assembled using SPAdes v3.12 (Bankevich et al., 2012), with default settings and the "meta" parameter specified. The quality of each assembled metagenome (n = 18) was assessed using QUAST v5.0.2 (Mikheenko et al., 2018). Gene prediction was performed using Prodigal v2.6.3 (Hyatt et al., 2010) with the "meta" parameter specified. To identify antibiotic resistance genes that may have been acquired by HGT, predicted genes were compared against the ResFinder database (Zankari et al., 2012) by means of BLASTn with an E-value threshold of $1x10^{-6}$. The filtering parameters used were: 100% similarity and a minimum query length of >50 %. Genes predicted by prodigal were also compared to the comprehensive antibiotic resistance database (CARD) (McArthur et al., 2013) by means of BLASTp with an E-value threshold of $1x10^{-6}$. Results were filtered for hits with a minimum percentage similarity of 87% and a minimum query length of >40%. These parameters were set with BLAST against all other databases used subsequently.

3.3.4 Mobile genetic elements, metal resistance genes and ARG host phyla annotation

To identify mobile genetic elements flanking ARGS, contigs were compared to the Mobile Genetic Elements Database (Pärnänen et al., 2018) by means of BLASTn. Detected plasmids were confirmed with the PlasmidFinder database (Carattoli et al., 2014). Metal and biocide resistance genes were detected by running BLASTp with these contigs against the BacMet database (Pal et al., 2014). The BacMet database is a manually curated database of antibacterial biocide and metal resistance genes, collectively abbreviated as MRGs in this study. In order to link the ARGs detected to specific taxonomy (i.e., inferring ARG hosts) the assembled contigs were compared against the proGenomes Database V2 (Mende et al., 2017) using DIAMOND v0.7.9.58 with the previously mentioned parameters. Only those contigs that were larger than 1,000bp were annotated.

3.3.5 16S rRNA amplicon sequence analysis

Sequence reads were demultiplexed using Sabre (https://github.com/najoshi/sabre) and primers were removed with cutadapt 2.10 (Martin, 2011). Amplicon sequence variants (ASVs) were resolved using DADA2 version 1.14 (Callahan et al., 2016) in R version 3.6.2 (R Core Team, 2013). Quality filtering and trimming were done using MaxEE = c(2,2), truncLen = c(220, 200), all other parameters were set to default. The error rates were estimated by learnErrors and sequences were dereplicated using derepFastq with default parameters. removeBimeraDenovo was used to remove chimeric sequences. Taxonomy was assigned against the SILVA non-redundant database version 138 (https://www.arb-silva.de).

3.3.6 Data analyses

The analyses were done in R version 3.6.2 using the packages phyloseq (McMurdie and Holmes, 2013), microbiome (Lahti et al., 2019), tidyverse (Wickham et al., 2019) and vegan (Oksanen et al., 2007). ASV alpha-diversity (richness, Shannon, Inverse Simpson, Chao1) were calculated using the vegan package in R. Community data matrices were centre log-ratio transformed and the Euclidian distance measure was used to generate an Aitchison dissimilarity matrix. In addition to comparing the assembled contigs against the proGenomes database (with the use of BLASTp), probable ARG and MRG hosts were inferred using correlation analysis with sparCC (Friedman and Alm, 2012). To reveal the relationship between microbial

composition and the resistome the pairwise Pearson's rank correlation (correlate alpha-diversity) was calculated and a Mantel test was conducted directly from the distance matrices (correlate beta-diversity). A permutational analysis of variation (PERMANOVA) (Anderson, 2006) was carried out to test for differences in composition between samples from the two desert rainfall zones using the 'adonis' function in vegan.

3.4. Results

3.4.1 Characterization and distribution of ARGs

Using shotgun metagenomics, a sequence depth of 5GB with an average of 660, 000 ORFs per sample was obtained. Resulting assemblies had an average N_{50} of 620 bases. The annotation of those ORFs against the CARD database resulted in a total of 6045 ORF hits with the identification of 46 ARGs. The identified ARGs spanned 26 ARG families exhibiting 17 resistance mechanisms (Table 3.1). Most of the ARGs detected were rare, with 39% (18 of 46) of them occurring in a given sample (Figure 3.1a). A total of 48 % of ARGs were shared between the two rainfall zones, with 30 % unique to high rainfall and 22% unique to mid rainfall (Figure 3.1b). There were no significant differences in the diversity and composition of ARGs between the two zones.

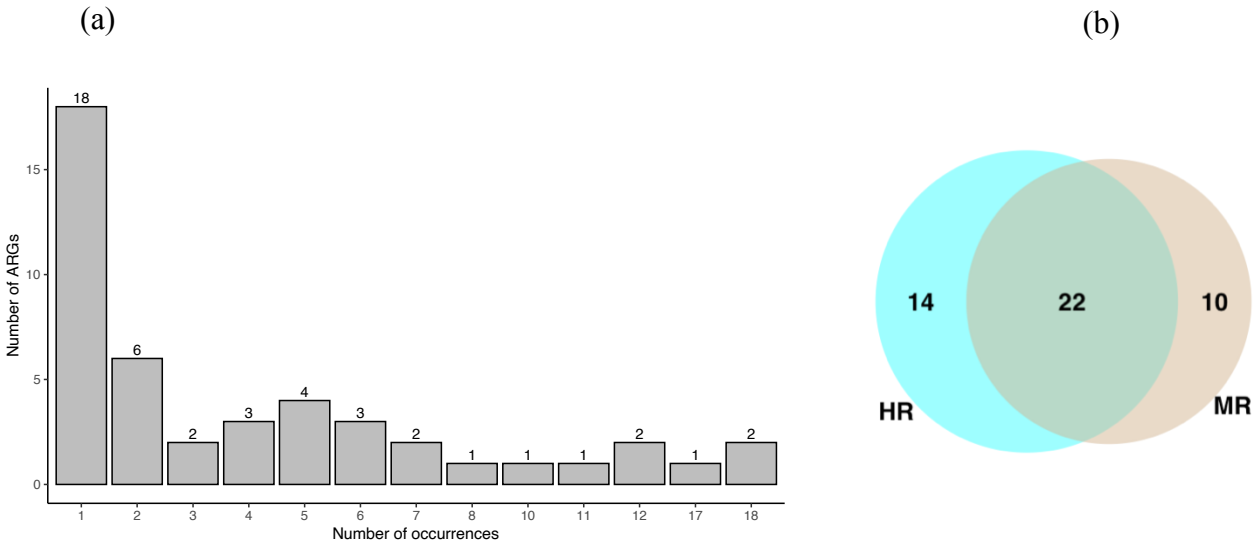

Figure 3.1. The number of occurrences of unique ARGs at each sample site (a) and a Venn diagram showing the distribution of the ARGs between the high-rainfall (HR) and mid-rainfall (MR) zones (b).

Table 3.1. The number of each ARG detected in the study according to the antibiotic resistance gene family and the corresponding resistance mechanism.

ARG family Description	Resistance mechanism	Number of ARGs
rifampin phosphotransferase	antibiotic inactivation by phosphorylation	1
antibiotic resistant isoleucyl-tRNA synthetase (ileS)	antibiotic target alteration or replacement	1
rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	antibiotic target alteration or replacement	3
aminosalicylate resistant dihydrofolate synthase	antibiotic target alteration by mutation	1
antibiotic resistant inhA	antibiotic target alteration by mutation	1
antibiotic resistant kasA	antibiotic target alteration by mutation	1
antibiotic resistant ndh	antibiotic target alteration by mutation	1
antibiotic resistant rpsL	antibiotic target alteration by mutation	1
daptomycin resistant beta prime subunit of RNA polymerase (rpoC)	antibiotic target alteration by mutation	1
elfamycin resistant EF-Tu	antibiotic target alteration by mutation	4
elfamycin resistant EF-Tu kirromycin self-resistant EF-Tu	antibiotic target alteration by mutation	1
ethionamide resistant ethA	antibiotic target alteration by mutation	1
flouroquinolone resistant gyrA	antibiotic target alteration by mutation	7
fluoroquinolone resistanct gyrB	antibiotic target alteration by mutation	3
fluoroquinolone resistant parE	antibiotic target alteration by mutation	1
isoniazid resistant katG	antibiotic target alteration by mutation	1
murA transferase	antibiotic target alteration by mutation	1
RbpA bacterial RNA polymerase-binding protein	antibiotic target protection	1
rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	antibiotic target replacement gene duplication	1
TEM β-lactamase	class A β-Lactamase	1
RND antibiotic efflux pump	efflux pump complex	1
AAC(3')	inactivation by acetyltransferase	1
AAC(6')	inactivation by acetyltransferase	1
rifampin phosphotransferase	inactivation of rifampin	1
RND antibiotic efflux pump	metal and multidrug efflux	1
MFS antibiotic efflux pump	MFS efflux	2
RND antibiotic efflux pump	multidrug efflux	1
glycopeptide resistance gene cluster vanR	restructuring of bacterial cell wall	1
van ligase glycopeptide resistance gene cluster	restructuring of bacterial cell wall	1
elfamycin resistant EF-Tu	Ribosomal alteration	1
tetracycline-resistant ribosomal protection protein	ribosomal protection proteins	1
RND antibiotic efflux pump	RND efflux transporter	1

The most common resistance mechanism was target alteration by mutation, followed by target protection, inactivation mechanisms and various efflux mechanisms (Figure 3.2). The most abundant group of ARGs, based on the drug resistance class, were those that are known to confer resistance to aminoglycosides (Figure 3.3), followed by those ARGs known to confer resistance to the elfamycins, glycopeptides, rifamycins and those that were multi-drug-resistant. Analysis of the contigs against the ResFinder database (Zankari et al., 2012) revealed the presence of horizontally acquired ARGs (i.e., *aac3'-la*) conferring resistance to aminoglycosides at two of the sample sites (Appendix 2, Table 1). However, mobile genetic elements flanking these ARGs were not detected.

Many of the multi-drug resistant ARGs conferred putative resistance to fluoroquinolones, tetracycline and cephalosporins. Class A β-lactamases were detected on a plasmid containing a metal resistance gene (MRG) encoding arsenate reductase and a bacteriophage in four of the sample sites (see chapter 4). MexK (an ARG that has also been classified as an MRG) was detected in 5 of the sample sites (Appendix 2, Table 1).

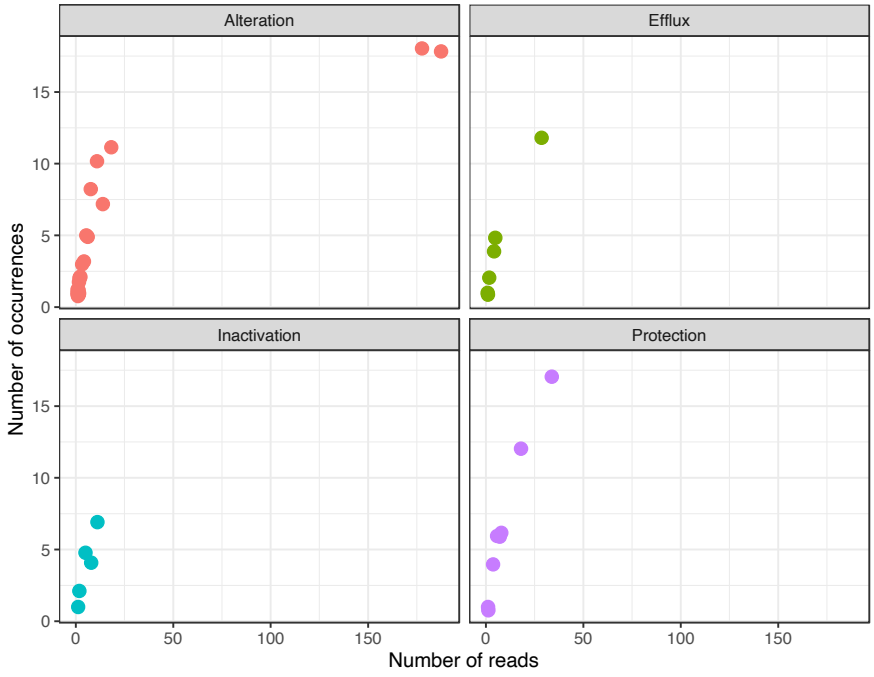

Figure 3.2. Prevalence plot for ARG resistance mechanisms. Each point corresponds to a different ARG, the y-axis measures the number of occurrences while the x-axis measures the number of reads.

3.4.2 Linking the microbiome and the antibiotic resistome

The number of ARGs (richness) did not differ between the two rainfall zones. However, the ARGs Shannon diversity, which includes richness and evenness information, was higher in the high rainfall zone (Figure 3.4). Similarly, there were no significant changes in ARG composition (beta-diversity) between the two zones (Appendix 2, Figure 1). There were no significant correlations, either for alpha-diversity (Pearson r = 0.061 $P > 0.05$) or for beta-diversity (Mantel r = 0.2 $P > 0.05$) between the resistome (ARGs) and microbiome (ASVs derived from 16S rRNA gene sequences).

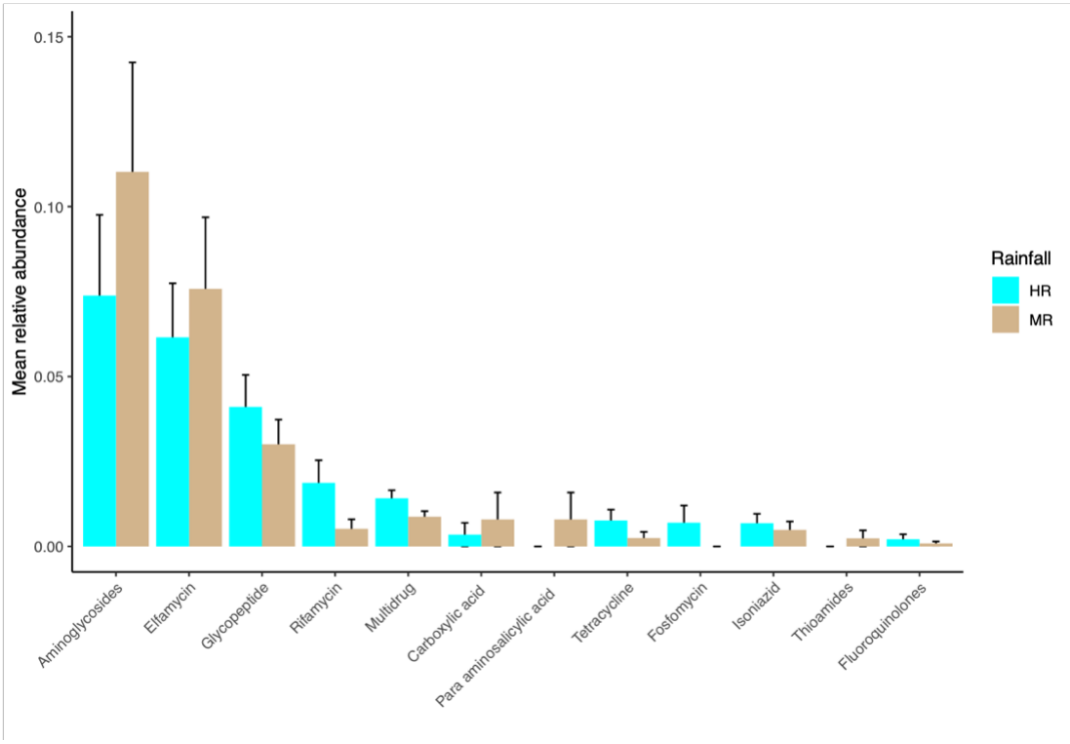

Figure 3.3. The relative abundance and distribution of ARGs between the two rainfall zones. The error bars represent the standard error of the mean. HR – high-rainfall, MR – mid-rainfall.

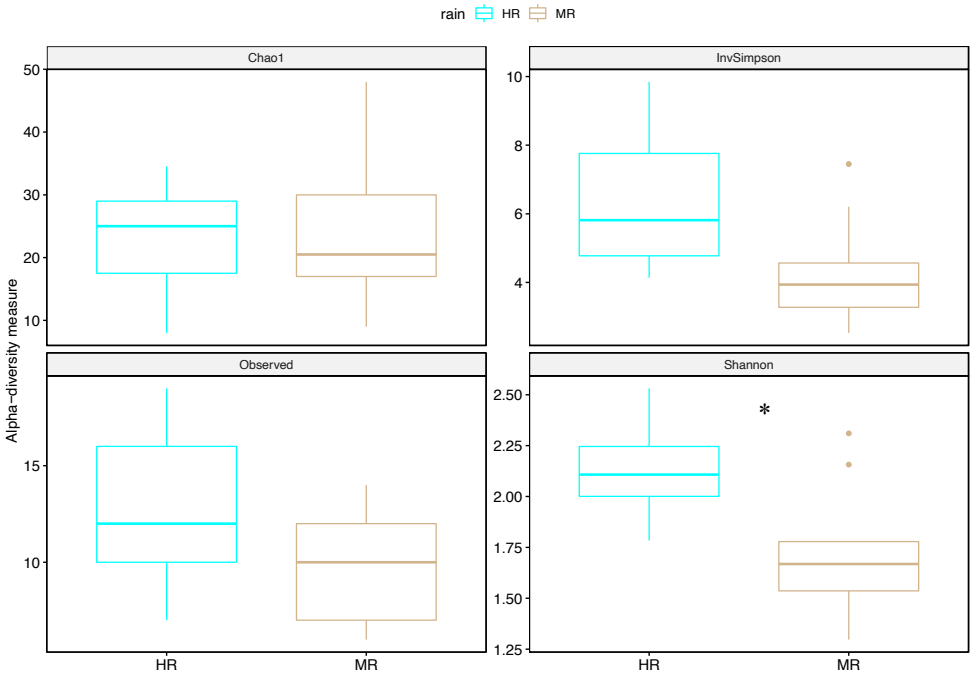

Figure 3.4. The observed ARG alpha diversity indices measured between the two rainfall zones. The asterisk represents significance between the high-rainfall (HR) and mid-rainfall (MR) zones

3.4.3 Identification of probable ARG hosts

To identify potential ARG hosts in the desert soil samples, assembled contigs were compared to the proGenomes database. Unfortunately, most contigs could not be annotated as they were less than 1000 bp in length.

Correlation analysis was then performed between the relative abundances of ARGs and the major phyla in these soils. ARGs conferring resistance to two drug classes (i.e., aminoglycosides (ARO:3003395) and elfamycins (ARO:3003361)) showed strong positive correlations with *Actinobacteriota and Chloroflexi* (Figure 3.5). In addition, *Proteobacteria* had positive correlations with ARGs conferring multi-drug resistance (ARO:3003368) and *Acidobacteriota* showed a positive correlation with ARGs conferring resistance to rifamycin antibiotics (ARO:300444). No positive correlations with the abundances of *Bacteriodota* and *Firmicutes* for any of the ARGs detected were observed.

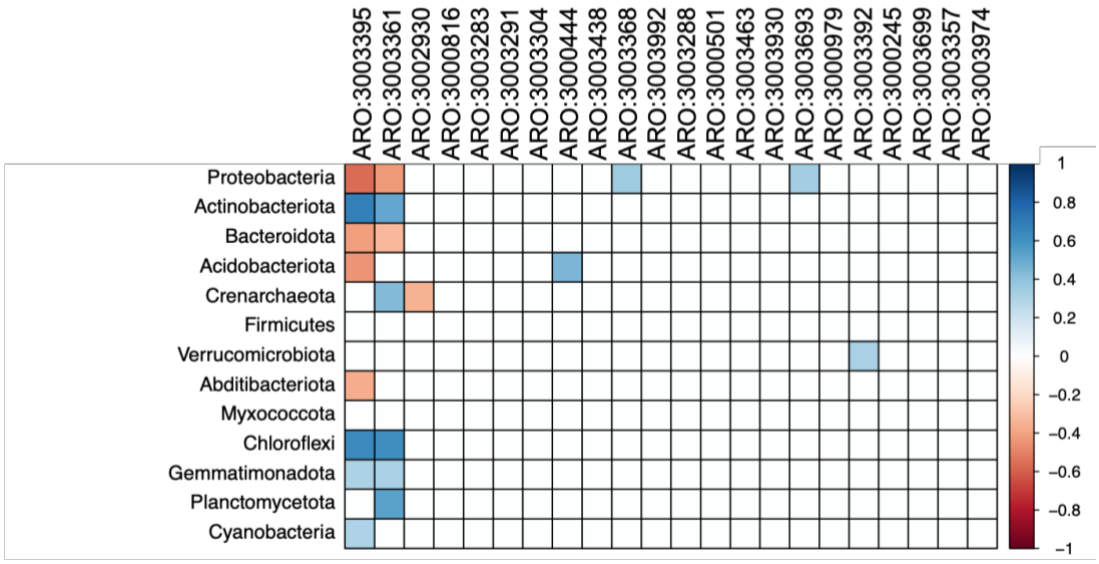

Figure 3.5. Plot displaying the correlation of relative abundance of ARGs and relative abundance of phyla inferred by 16S rRNA data.

3.4.4 Metal and biocide resistance genes (MRGs)

The annotation of prodigal predicted ORFs against the BacMet database resulted in a total of 143 ORF hits with the identification of 29 MRGs. The MRGs spanned 16 families with 4 known resistance mechanisms (Table 3.2). The most abundant MRGs were those that are known to confer resistance to iron (Fe) and Triclosan (biocide) (Figure 3.6). This was closely followed by MRGs that are known to confer resistance to three or more compounds (multi-compound resistance).

Five different MRGs (*arsM*, *arsC*, *arsB*, *arsH* and *arsT*) conferring resistance to arsenic were also identified. Correlation analysis (Figure 3.7) between the MRGs and the major phyla showed that *Proteobacteria* were positively correlated with MRGs conferring resistance to iron (BAC003), arsenic (BAC0584), triclosan (BAC0242) and copper (BAC0205) and was negatively correlated with MRGs conferring resistance to silver (BAC0077) and multi-compound resistance (BAC0565, BAC0203). The *Firmicutes* and *Myxococcota* showed positive correlations with multi-compound resistance and iron resistance, respectively.

Table 3.2. The number of each MRG detected in the study according to the metal/biocide resistance gene family and the corresponding resistance mechanism.

Metal/biocide resistance gene family	Resistance mechanism	Number of MRGs
ABC superfamily	Enhanced Efflux	1
*ars*B family	Enhanced Efflux	1
RND superfamily	Enhanced Efflux	5
Aconitase family	Enzymatic detoxification	1
*ars*C family	Enzymatic detoxification	1
Cation transport ATPase	Enzymatic detoxification	5
Cation transport ATPase (P-type) family	Enzymatic detoxification	1
Methyltransferase family	Enzymatic detoxification	1
Multi-copper oxidase family	Enzymatic detoxification	3
NADPH-dependent FMN reductases family	Enzymatic detoxification	1
RuvB family	Enzymatic detoxification	1
Sodium-solute symporter (SSF) family	Membrane transporters	1
Contains 1 HTH arsR-type DNA-binding domain	Regulatory transporters	2
Contains 1 HTH dtxR-type DNA-binding domain	Regulatory transporters	1
Contains 1 response regulatory domain	Regulatory transporters	1
RND superfamily	Regulatory transporters	2
*ars*T family	Not yet determined*	1

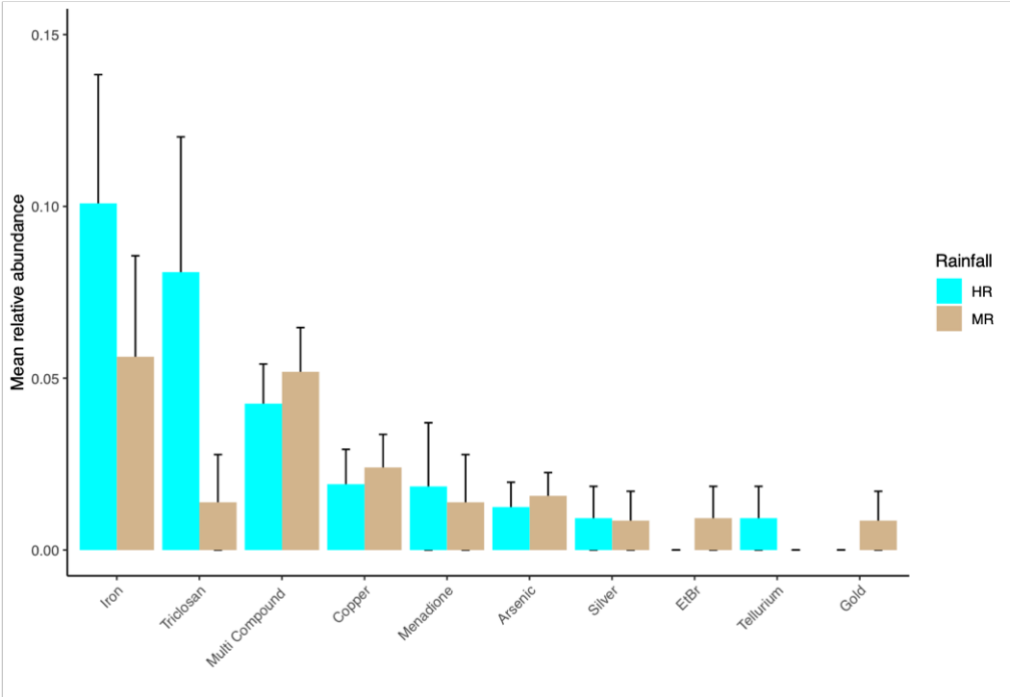

Figure 3.6. The mean relative abundance and distribution of MRGs between the two rainfall zones HR – high-rainfall, MR – mid-rainfall.

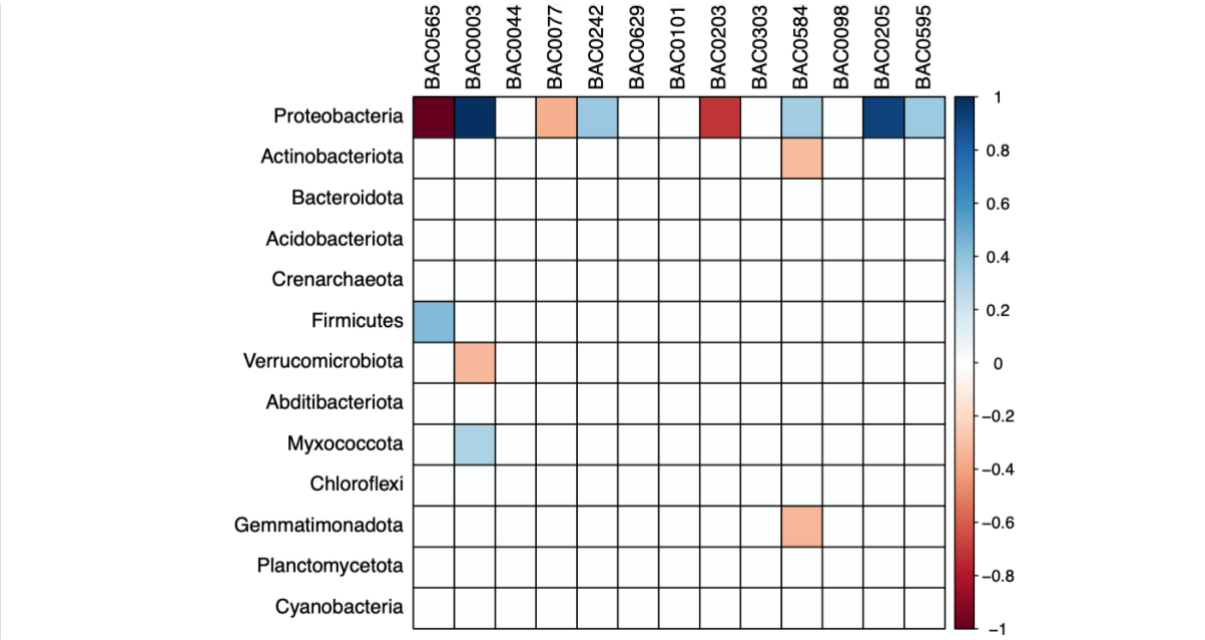

Figure 3.7. Plot displaying the correlation of relative abundance of MRGs and relative abundance of phyla inferred by 16S rRNA data.

3.4.5 The Mobilome

A total of 146 ORF hits were detected with the Mobile Genetics Elements database and 13 of those were identified as mobile genetic elements (MGEs). In all metagenome sequence datasets, insertion sequences were the most abundant MGEs (61%), followed by transposases (34%), integrons (2.5%) and plasmids (2.5%). The insertion sequences detected were from two families (i.e., *IS91* and *IS10*) and a variety of transposases were identified. The presence of class 1 (int1) and class 2 (int2) integrons and ColE1-like plasmids were detected. The only MGEs linked to any of the ARGs were plasmids (e.g., ColE1 with TEM-116).

3.5. Discussion

3.5.1 Low diversity and abundance of ARGs across the transect

Shotgun metagenome sequencing was used to characterize the soil resistome across two zones of a natural rainfall gradient in the Namib Desert. Shotgun metagenomics allows the deep interrogation of resistomes, which can be very important in understanding the increase in

antimicrobial resistance among clinically relevant isolates (Forsberg et al., 2014; Noyes et al., 2016). The results show that Namib Desert soils appear to have a limited number of ARGs, which were in low abundance and did not differ between the two zones. This was not unexpected, as similar results have been found in other desert studies (McCann et al., 2019; Van Goethem et al., 2018). There are different factors that may be contributing to the low diversity and abundance of ARGs in these environments. First, it has been hypothesized that microbial competition is less important in desert microbes due to the allocation of most of the resources to their survival in stressful environmental conditions and/or to their response to moisture availability (Fierer et al., 2012). This is in agreement with the stress gradient hypothesis that states that facilitation (positive interactions) should be more common in high stress environments, compared with benign environments (Bertness and Callaway, 1994).

Second, compared with other edaphic environments, desert areas generally have a very low density human (and animal) occupation and are less impacted by human activities, which have been shown to increase the diversity and abundance of elements of the soil resistome (Wang et al., 2016). Third, while shotgun metagenomics provides the advantage of being unbiased (i.e., by avoiding the use of gene-specific primers), it has been shown to be less sensitive than culture-based and other molecular methods (e.g., Sanger sequencing) in identifying ARGs (Bengtsson-Palme et al., 2017; Gweon et al., 2019). Fourth, sequencing depth may also have affected the profiling of ARGs in this study, as it has recently been shown that the number of sequences and profiling method critically affect the diversity of ARGs from environmental samples (Gweon et al., 2019). Furthermore, the parameters (minimum identity and minimum match length) used in the analysis were more stringent than in other desert studies (Saenz et al., 2019; Van Goethem et al., 2018), although the strategy used here leads to datasets that are more consistent and robust (Graham et al., 2020).

The most common mechanism of resistance detected was target alteration by mutation (Table 3. 1), which typically arises by chromosomal mutations and is therefore not laterally transferred. Mutations in the target-encoding genes often confer multidrug-resistance (D'Costa et al., 2006) and resistance to antibiotics such as cephalosporins (Demaneche et al., 2008) and fluoroquinolones (Riesenfeld et al., 2004). This resistance mechanism is essential for the continued evolution of ARGs to natural and synthetic antibiotics (Woodford and Ellington, 2007), especially in the case of clinically relevant isolates (Munita and Arias, 2016).

Several of the ARGs encoded proteins involved in the modification of antibiotics, most commonly, enzymatic modification of aminoglycosides. The production of enzymes capable of chemically changing antibiotic molecules is often a trait acquired by horizontal gene transfer in both Gram positive and Gram negative bacteria (Munita and Arias, 2016). Enzymatic modification (i.e., inactivation) usually results in high levels of antibiotic resistance (Riccio et al., 2003). Using the ResFinder database, horizontally acquired ARGs (e.g., AAC'3-*la*) encoding acetyltransferases that mediate enzymatic inactivation of aminoglycosides were detected. This database has been designed to detect the presence of resistance genes acquired via horizontal transfer and not those acquired via mutation (Zankari et al., 2012). The presence of bacteriophages or MGEs were not found nearby these genes, and therefore the vehicle for dissemination could not be established. A possible reason for this may be related to the contig assemblies which showed relatively small N_{50} values (ranging from 600 to 650 bases). The length of the contigs is critical for co-localization analysis of ARGs and MGEs, as it is more likely that longer stretches of DNA produce a large enough sequence space to identify multiple co-localized elements (Slizovskiy et al., 2020).

The use of rifamycin antibiotics, such as rifampin (RIF), is a frontline treatment of tuberculosis and many other mycobacterial infections (Stogios et al., 2016). Interestingly, resistance to RIF was conferred by two different mechanisms (i.e., antibiotic target protection (RbpA) and enzymatic inactivation). The mechanism of action of rifamycin involves binding of the RbpA protein to RNA polymerase (RNAP) in bacteria, thereby disrupting transcription, rendering the bacteria resistant to rifamycin antibiotics (Xu et al., 2005). The enzymatic inactivation of RIF occurs via phosphorylation by rifampin phosphotransferases (RPH), which are widespread in pathogenic and non-pathogenic bacteria (Stogios et al., 2016). Rifampin phosphotransferases have the capacity to inactivate natural and semi-synthetic derivatives of the rifamycin family of antibiotics, which poses a threat to other environments should they be mobilized (Spanogiannopoulos et al., 2014).

Neither of the two mechanisms found in the Namib soil resistome has been associated with resistance to rifampin in clinical environments (Tomlinson et al., 2016). In clinical environments, resistance to rifampin is commonly due to point mutations in the drug target (Stogios et al., 2016). However, resistance to RIF in these desert soil samples demonstrates that environmental bacteria may also possess multiple mechanisms of resistance to the same

antibiotic (Spanogiannopoulos et al., 2012), which is an indication of bacterial adaptation to a rapidly evolving environment. Furthermore, it has been shown that bacteria adapting to high temperatures develop resistance to the rifamycin family of antibiotics (Grenni et al., 2018).

Another important group of ARGs are the β-lactamases, which inactivate β-lactam antibiotics by destroying the amide bond in the β-lactam ring and rendering the antibiotic ineffective (Munita and Arias, 2016). Several β-lactamases have been reported as native in both impacted (Donato et al., 2010; Forsberg et al., 2012; Udikovic-Kolic et al., 2014) and non-impacted soils (Allen et al., 2009; Bhullar et al., 2012; Durso et al., 2015), including those from polar deserts (McCann et al., 2019; Van Goethem et al., 2018; Wang et al., 2016). In contrast to these previous studies, native β-lactamases were not detected in this study. Since sequence depth plays such a large role in the profiling of ARGs, it is very possible that this could be the reason that native β-lactamases were not detected. However, the presence of an acquired β-lactamase TEM-116 (see chapter 4), which was encoded on a ColE1-like plasmid was detected. TEM-116 is a second-progenitor of the TEM β-lactamases and has been described in both clinical and non-clinical environments (Zeil et al., 2016).

3.5.2 Potential co-selection of resistance to metals/biocides and antibiotics

Microorganisms have evolved mechanisms of metal/biocide tolerance to avoid cellular damage (Seiler and Berendonk, 2012), and this tolerance can aid in the development and maintenance of antibiotic resistance (Pal et al., 2016). However, unlike antibiotics, which can be easily be degraded by a wide range of different mechanisms, metals are essentially non-degradable and therefore pose a long-term selection pressure. It has been suggested that metals and biocides might even exert a stronger selection pressure for antibiotic resistance than antibiotics themselves (Pal et al., 2017).

One of the key mechanisms by which bacteria become resistant to metals is via enzymatic reduction of metal ions (Ianeva, 2009; Oves et al., 2016). Here, enzymatic reduction was the most common mechanism, putatively encoded by *Proteobacteria*, mostly conferring resistance to Fe, Cu and As (Figure 3.7). Reportedly, several members of the phylum *Proteobacteria* carry a large number of genes associated with heavy metal tolerance and have been extensively studied, under heavy metal stress conditions, in bacterial genomes from different environments

(Johnson et al., 2019) and in metal-contaminated river sediments (Chen et al., 2018). It was found that *Proteobacteria* were also hosts of ARGs encoding multidrug resistance. Multi-drug resistant genes conferring resistance to clinically important antibiotics have been detected in several members of *Proteobacteria* (Jiang et al., 2017). This observation is consistent with the fact that *Proteobacteria* include several known clinical pathogens (e.g., *Pseudomonas aeruginosa*, *Escherichia coli*) (Forsberg et al., 2014). Interestingly, it has been demonstrated that the enrichment of multi-drug resistant *Proteobacteria* in soil increases the detection of shared resistance between the clinic and soil, suggesting that members of *Proteobacteria* could be a conduit through which ARGs and MRGs move between these environments (Forsberg et al., 2014, 2012) and making them important hosts in co-selection of resistance.

One of the most interesting findings was the detection of the resistance gene *Mex*K, which is part of a two-component resistance nodulation cell division (RND) efflux system known as MexJK. This system is responsible for the efflux of triclosan and antibiotics such as macrolides and tetracycline (Chuanchuen et al., 2002). Resistance genes to triclosan were the second most abundant MRGs present in these soils however, resistance to tetracycline was also moderately present. Triclosan is a phenolic compound used in many personal care products as a biocide to stop bacterial spoilage. Triclosan is a compound that is not usually found in desert soils, which indicates a moderate anthropogenic impact in this environment. Since triclosan is a recalcitrant compound with antimicrobial activity in soils (Zaayman et al., 2017), it has the potential to compromise soil health (Dhillon et al., 2015). Alternatively, the presence of other compounds such as tetracycline would also explain the presence of *MexK*.

Evidence of co-selection between antibiotics and metals/biocides by both co-resistance and cross resistance were found. An example of co-resistance was the presence of an *ars*C gene on a plasmid that also contained the clinically relevant TEM-116 β-lactamase, suggesting possible co-selection of resistance with antibiotics and arsenic in *Rhodococcus* (Actinobacteria) (Naidoo et al., 2020). In this study, *Actinobacteriota* putatively host ARGs that also conferred resistance to aminoglycosides and elfamycins (Figure 3.5). This is not uncommon since actinomycetes produce a wide variety of antibiotics (Krause et al., 2016), and contain resistance genes to the compounds they produce as self-protecting mechanisms (Jiang et al., 2017). In addition to their antimicrobial activities, members of *Actinobacteriota* have also shown high

tolerance to a wide range of metals, potentially providing them with a competitive advantage in harsh environments (Tomova et al., 2015)

Cross resistance could occur via the MexJK system, which effluxes both triclosan and antibiotics (tetracycline and macrolides). While the ARGs conferring resistance to tetracycline were moderately abundant, co-selection of resistance (triclosan and tetracycline) would very likely enable the persistence of these ARGs. The levels of metals and triclosan in these samples were not measured, however an earlier report of metal concentrations at sites very close (<100 m) to our sampling sites, indicated that these soils contain high levels of metals such as Fe (17 290), Ni (29.35), Cu (25.36), Zn (59.63), As (3.02), Ag (0.20) and U (2.99) (mean concentration of metals were measured as mg/kg dry weight in soil) (Conti et al., 2018). Thus, it is possible that the metals and biocides present in this environment may add a selection pressure that could increase the antibiotic tolerance level of microbes via these co-selection mechanisms (Imran et al., 2019), which is a rising environmental concern.

3.5.3 The occurrence of mobilome-related antibiotic resistance determinants in desert soils

The most abundant MGEs identified were the insertion sequences (distributed in two different families, *IS91* and *IS10*) followed by transposons, integrons and plasmids (discussed in detail in the next chapter). Insertion sequences (IS) and transposons (Tn) are able to move themselves and their associated resistance genes randomly within a cell, while integrons use site-specific recombination to carry resistance genes between species and lineages over evolutionary time frames (Gillings, 2014; Partridge et al., 2018). Interestingly, among the two IS families, the relative abundance of *IS91* was much higher (92%), which might explain the scarcity of MGEs flanking ARGs. Members of the *IS91* family of bacterial insertion sequences have been reported to very rarely flank ARGs, as they are not suited for rapid dissemination mechanisms (Garcillan-Barcia and De la Cruz, 2002). Conversely, members of the *IS10* family are more rapidly disseminated and are known to affect efflux mechanisms resulting in increased antibiotic resistance (Siguier et al., 2014). Mobile integrons have been a major driver in the spread of antibiotic resistance, specifically in clinical environments (Gillings, 2014). In addition, Class 1 integrons have been proposed as markers of anthropogenic pollution due to their common association with resistance to antibiotics and metals in both pathogenic and non-pathogenic bacteria (Gillings, 2014).

Although several mobile genetic elements were detected, their overall abundance was low. This is consistent with previous reports (Saenz et al., 2019; Wang et al., 2016), suggesting that only a fraction of resistance determinants may be mobilized. The horizontal acquisition of resistance genes is hypothesized to be rare in environments with both low anthropogenic influence and low nutrient levels (Forsberg et al., 2014). Furthermore, regulation of the genetic machinery is highly responsive to the environment, and therefore, parameters such as soil moisture and temperature, pH and soil type may affect the rates of horizontal gene transfer (Aminov, 2011). Desert environments experience extreme fluctuations in temperature and erratic precipitation patterns, and have low nutrient levels with low anthropogenic influence, compared to other edaphic environments (Makhalanyane et al., 2015). Thus, all these factors can be contributing factors to the low observed abundance of MGEs. Nevertheless, the presence of horizontally acquired resistance genes indicates that mobilization of ARGs might occur in Namib Desert soils. Like all deserts, the Namib also is affected by transient wildlife and birds that can act as propagators for the spread of these resistance determinants (Allen et al., 2010). It is also noted that over the past few decades there has been a substantial growth of tourism in the Namib Desert (Woyo and Amadhila, 2018), which may increase the possible routes of transmission for acquired ARGs.

3.5.4 Decoupling between the microbiome and the resistome

In the previous chapter it was shown that the two rainfall zones of the Namib Desert supported distinct microbial communities, more likely as a consequence of historical differences in water regime. In contrast, in this chapter it is evident that the abundance and type of ARGs detected did not show any spatial pattern. This suggests it is unlikely that environmental factors are driving the structure of ARGs in Namib Desert soils. Previous studies have led to proposals that bacterial community composition is a key driver that shapes the distribution and abundance of ARGs, for instance, in surface soils (Forsberg et al., 2014), underground coal mine soils (Dunivin and Shade, 2018) and in sewage sludge (Su et al., 2015). However, the results of the Mantel test showed that microbial community composition was not a major contributor to the variance in ARGs in Namib Desert soils. Since bacterial community composition may not be the key factor influencing ARG profiles, it is very likely that variation in ARG abundance across soils might be largely driven by other factors, possibly such as the mobilome. Mobile genetic elements play a very important role in shaping ARG dynamics, as they transfer genes

even between distantly related taxa, thereby causing a decoupling of bacterial community and ARG profiles (Fang et al., 2019).

Thus, although only a small variety of mobile genetic elements were detected and they were in low abundance the mobilome might be playing an important role in explaining the composition of ARGs. Alternatively, a decoupling between the microbiome and the resistome can also be expected if most of the mobile genetic elements are hosted by a limited number of taxa widely distributed across the soil. Further research would be necessary to confirm this. Culture-based methods remain the absolute standard for such analyses, although these methods overlook the majority of the microbial diversity in soils. In addition, since there are limitations associated with only using metagenomics, we suggest a multiphasic approach. Molecular methods in combination with culture-based methods can conclusively identify the taxa hosting MGEs in this population (Rice et al., 2020).

3.6. Conclusion

With the use of metagenomics, ARGs, MRGs and MGEs have been detected in low abundance in Namib desert soil. The co-selection of resistance with metals and/or biocides could allow for these ARGs to persist in the soil. Importantly and in contrast with other resistome studies from both impacted and non-impacted soils, which suggest a coupling between bacterial community composition and the antibiotic resistome. Evidence from this study points to a decoupling of bacterial community composition and the antibiotic resistome, possibly originating from horizontal gene transfer. The presence of acquired resistance genes supports the assumption that vectors such as animals, birds and humans are partly involved in the spread of resistance determinants in these soils. Overall, this study shows that antibiotic resistance genes are substantially distributed in these soils. The presence of these acquired genes does not necessarily pose a significant health threat to humans and animals living in or visiting this environment. However, the concern is that, like in other environmental settings, the mobilization of these resistance determinants and their expression in bacterial pathogens could present difficulty in treatment options.

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CHAPTER FOUR: A CLINICALLY IMPORTANT, PLASMID-BORNE ANTIBIOTIC RESISTANCE GENE (β-LACTAMASE TEM-116) PRESENT IN DESERT SOILS

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Short Communication

A clinically important, plasmid-borne antibiotic resistance gene (β-lactamase TEM-116) present in desert soils

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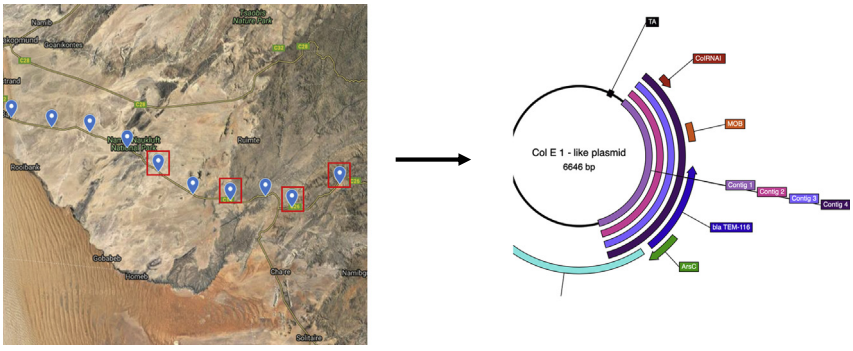
HIGHLIGHTS

- Shotgun metagenomics was used to investigate antibiotic resistance genes (ARGs) in desert soils.
- A clinically relevant plasmid-borne ARG was detected.
- The plasmid also carries a metal resistance gene and a P7 entero-bacteriophage.
- These findings have important implications for the One Health initiative.

GRAPHICAL ABSTRACT



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ABSTRACT

The exhaustive use of antibiotics in humans, animal farming and other agricultural practices has resulted in the frequent appearance of antibiotic resistant bacteria in human-impacted habitats. However, antibiotic resistance in natural (less-impacted) habitats is less understood. Using shotgun metagenomics we analysed soils from relatively low anthropogenic impact sites across the Namib Desert. We report the presence of a clinically significant extended spectrum β-lactamase (TEM-116), on a ColE1-like plasmid also carrying a metal resistance gene (arsC). The co-occurrence of resistance to antimicrobial drugs and metals encoded on a single mobile genetic element increases the probability of dissemination of these resistance determinants and the potential selection of multiple resistance mechanisms. In addition, the presence of a P7 entero-bacteriophage on the same plasmid, may represent a new vehicle for the propagation of TEM-116 in these soil communities. These findings highlight the role of the environment in the One Health initiative.

1. Introduction

The One Health approach refers to the multidisciplinary collaboration on a local, national and global scale to ensure optimal health for people, animals and the environment. It recognises that antimicrobial resistance (AMR) has clear links to all components of this triad (Robinson et al., 2016). Most initiatives in understanding and combatting AMR have focused solely on human and animal sectors, while the extent of antibiotic resistance in the environment is poorly understood (Essack, 2018; Finley et al., 2013). However, recent studies have

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reported that many of the resistance genes found in clinical pathogens originate from soil-borne bacteria (Finley et al., 2013; Martínez et al., 2015) and that soils act as a reservoir of resistance genes (Larsson et al., 2018). The potential mobilization (e.g., through plasmids and other mobile elements) of resistance genes within the soil reservoir and the extent to which soils provide transmission routes for the propagation of resistance determinants to humans and animals is therefore a matter of immediate concern (Bengtsson-Palme et al., 2017; Bengtsson-Palme and Larsson, 2016).

The persistent exposure of bacterial strains to a multitude of β-lactam antibiotics has led to the production and mutation of β-lactamases (Bush, 2018; Pitout and Laupland, 2008), known as extended-spectrum β-lactamases (ESBLs). ESBLs are generally plasmid-borne and have the ability to induce host cell resistance to newer β-lactam antibiotics, specifically to third and fourth generation cephalosporins and monobactams, but not cephamycins and carbapenems (Ur Rahman et al., 2018). ESBLs were primarily found in *Klebsiella pneumonia* and *Escherichia coli* in the 1980's, but have since been reported in several other species of bacteria, including *Pseudomonas*, *Shigella* and *Salmonella* (Mesa et al., 2006; Tissera and Lee, 2013). More recently, with the application of metagenomics screening methods, ESBLs have been reported to be ubiquitous in different natural environments, such as ancient soils (Allen et al., 2009; Perron et al., 2015), deep terrestrial sub-surfaces (Brown and Balkwill, 2009), isolated caves (Bhullar et al., 2012) and in cold deserts (Segawa et al., 2013; Wei et al., 2015). However, little is known about the presence of ESBLs in hot deserts, which represent 20% of the global land area (Ayal, 2007), are major and consistent sources of long-distance airborne particulates (Belov et al., 2018) and therefore of particular significance as reservoirs of these genes.

TEM-116 is an ESBL characterized by two amino acid substitutions at residues 84 and 184, (Usha et al., 2008) and thought to have evolved directly from the first-discovered plasmid mediated β-lactamase, TEM-1 (Usha et al., 2008). It has been demonstrated that TEM-116 has given rise to over 50 TEM variants (Jacoby and Bush, 2016; Zeil et al., 2016), and has been postulated that TEM-116 acts as a second-generation progenitor of TEM variants. TEM-116 is widespread in clinical (Jeong et al., 2004; Naiemi et al., 2005; Usha et al., 2008; Vignoli et al., 2005) and non-clinical environments (Balsalobre et al., 2010; Forcella et al., 2010; Maravić et al., 2016; Mondal et al., 2019), including agricultural soils (Anand et al., 2016; Demaneche et al., 2008). The reports describing TEM-116 in soils, suggest that this ESBL is native to soil resistomes and is chromosomally mediated (Jacoby and Bush, 2016). In this study we provide evidence, for the first time, of the clinically relevant plasmid-borne TEM-116 in desert soils.

2. Materials and methods

2.1. Study site and sampling

The central Namib is a hyper-arid desert located on the south west coast of Africa. The Namib Desert has scarce and unpredictable rainfall patterns with rainfall increasing gradually from the coast, inland, producing a variable west-east rainfall gradient (Eckardt et al., 2013). Previously, anthropogenic influences across the desert have been minimal and were mostly limited to scientific expeditions. However, nature based tourism has increased drastically in the last decade (Dowling and Grunert, 2018), possibly increasing the anthropogenic pressure in the Namib desert. Surface soils (0 to 5 cm) were collected across this west-east transect on the 10th April 2018 (23°11′76.1″S 15°16′69.2″ E). The transect spans three xeric zones (fog, arid and rainfall), samples were collected from the arid (n = 3) and rainfall (n = 3) zones. Four aliquots of 50 g of soil were taken at each site at 100 m spacing, using sterile methods and stored in sterile 50 ml polypropylene Falcon tubes (Grenier, Bio-One). Soils were stored at −80 °C for molecular analysis.

2.2. Sample preparation and DNA sequencing

Metagenomic DNA was extracted from the soil samples (n = 6), using the DNeasy Powersoil Kit (Qiagen) as per the manufacturers' instructions. Samples were submitted for sequencing at a commercial supplier (MR DNA Lab, Shallowater, TX, USA). Sequencing was performed on a HiSeq 2500 Ultra-High-Throughput Sequencing system (Illumina) using paired-ends (2 × 250 bp) for 500 cycles, as per the manufacturers' instructions.

2.3. Metagenomic assembly and acquired ARG identification

The number of reads per sample ranged from 9,868,998 to 11,726,910 (10,632,908 on average), with a sequence depth of 5GB. Raw reads were quality filtered using FastQC (Andrews, 2010), and trimmed using PrinSeq (Schmieder and Edwards, 2011). Reads were assembled using SPAdes v3.12.0 (Bankevich et al., 2012), with default settings and the "meta" parameter specified. The quality of each assembled metagenome (n = 6) was assessed using QUAST v5.0.2 (Mikheenko et al., 2018). The contigs were aligned using EPSript 3.0 (Robert and Gouet, 2014) to identify regions of similarity. To identify the acquired antibiotic resistance genes, contigs were compared against the ResFinder (Zankari et al., 2012) database by means of BLASTn with an E-value threshold of 1×10^{-6} and the following parameters: minimum identity 87% and minimum match length 25 bp. Similar cut-off values were used in a study carried out in Antarctic soil (Van Goethem et al., 2018) and in a more recent study analysing antibiotic resistance in sewage impacted environments (Karkman et al., 2019). In this study, these values were used to identify genes that do not stretch over the entire contig.

2.4. Mobile genetic elements and taxonomic identification

To identify mobile genetic elements associated with ARGS, contigs were compared to the Mobile Genetic Elements Database (Pärnänen et al., 2018) by means of BLASTn with an E-value threshold of 1×10^{-6} and the following parameters: minimum identity 87% and minimum match length 25 bp. The plasmid detected was confirmed with the PlasmidFinder database (Carattoli et al., 2014) using the same parameters. To identify taxonomic markers associated with ARGs, gene prediction was performed using Prodigal v2.6.3 (Hyatt et al., 2010). The genes predicted by Prodigal were compared against the ProGenomes database (Mende et al., 2017) using blastp in DIAMOND v0.7.9.58 (Buchfink et al., 2014) at an E-value cut off of 1×10^{-5}. Only contigs larger than 1000 bp were annotated.

2.5. Phage identification and metal resistance gene annotation

To identify bacteriophage sequences, contigs were compared to the MetaPhinder database, which contains whole bacteriophage genome sequences. BLASTn was run with an E-value threshold of 1×10^{-6}. Metal resistance genes were detected by running BLASTp with these contigs against the BacMet database (Pal et al., 2014) using DIAMOND v0.7.9.58 at an E-value cut off of 1×10^{-6} and the following parameters: minimum identity 87% and minimum match length 25 bp. The raw sequence data and contigs are available on NCBI (PRJNA592367).

3. Results and discussion

We used de novo assembled shotgun metagenomic sequences (contigs) from six surface soil sample sites in the Namib Desert. Analysis using ResFinder (Zankari et al., 2012) revealed the presence of an acquired ESBL, TEM-116, at four of the sample sites (Table S1). The length of TEM-116 reported here was 861 bp and showed 100% similarity to the reference TEM-116 gene (accession no: AY425988.1) (Table S2). TEM-116 has been globally reported in a number of gram negative

organisms, it was first identified in clinical strains of *K. pneumonia* and *E. coli* in Korea (Jeong et al., 2004). Thereafter, on conjugate plasmids in *E. coli* isolates in Uruguay (Vignoli et al., 2005) and later, in *Acinetobacter baumanii* of both clinical and non-clinical origin (Maravić et al., 2016; Naiemi et al., 2005), indicating the widespread dissemination of this ARG. TEM-116 has an extended spectrum of activity against several third and fourth generation cephalosporins (Song et al., 2005; Usha et al., 2008), for this reason it is considered an ESBL.

A comparison of our contigs (Fig. S1) against the Mobile Genetic Elements (Pärnänen et al., 2018) and PlasmidFinder (Carattoli et al., 2014) databases revealed the presence of a ColRNAI replicon (with a length of 131 bp and 90% similarity to the reference replicon) (Table S2). Mapping of the contigs to the Col E1 plasmid pIGMS32 (accession no: DQ298019) indicates that TEM-116 reported here is carried on a plasmid similar to pIGMS32 (Fig. 1). pIGMS32 was initially identified as a narrow host range plasmid. However, it was later reported (Smorawinska et al., 2012) that this plasmid can contain different mobilization (MOB) modules which enables its dissemination among evolutionarily distinct bacterial species (that is, this plasmid has a wider host range than previously thought). Since then, pIGMS32 has been found in clinically relevant Gram-negative organisms, such as a *K. pneumoniae* strain implicated in a carbapenem-resistant infection outbreak (Espedido et al., 2013), and in *Serratia marcescens*, in a clonal multidrug resistant outbreak (Moradigaravand et al., 2016). Consequently, it has been suggested that pIGMS32 is a carrier of resistance mechanisms (e.g., efflux pumps) of clinical significance (Ares-Arroyo et al., 2018), and of genes conferring resistance to several antibiotics including β-lactams (Papagiannitsis et al., 2015).

Gene transfer and acquisition in nature occurs mostly between taxonomically homogenous groups (Courvalin, 1994; Kelly et al., 2009). However, the taxonomic annotation of all TEM-116 identified in the Namib Desert indicated that TEM-116 was found in *Rhodococcus ruber* strain Chol-4. *Rhodococcus* strains are considered good candidates for processes such as bioremediation and biocatalysis since they display wide metabolic versatility with an increased tolerance to varying stress conditions (Guevara et al., 2019). The Chol-4 strain in particular is a model organism for studying biodegradation of steroids to produce pharmaceutically active steroid drugs (Guevara et al., 2019). As far as we know, *R. ruber* has not been previously associated with antibiotic resistance to β-lactams. The presence of this ESBL in *Rhodococcus ruber*, a Gram-positive bacterium, indicates an "inter-Gram" gene transfer event. The first report of inter-Gram transfer was in 1987, where a plasmid present in *E. coli* was successfully transferred to several Gram-positive bacterial strains (Trieu-Cuot et al., 1987). Initially, it was thought that this transfer could only occur via conjugation (Kelly et al., 2009), but most recently it has been demonstrated that it can also occur via transformation (Jiang et al., 2017). The presence of TEM-116 in a gram-positive organism implies that inter-Gram transfer in soil bacteria may be more common than previously thought (Popa et al., 2011). Soils are highly diverse and can act as hot spots for gene acquisition from phylogenetically distant groups (Popa et al., 2011), and therefore, facilitate a direct connection of a large part of the bacterial gene pool (Klümper et al., 2015).

Antibiotic resistance genes have been reported as wide-spread in both hot and cold deserts (Belov et al., 2018; Fierer et al., 2012; Van Goethem et al., 2018), and the ubiquity of TEM-116 in soil environments cannot be disputed (Jacoby and Bush, 2016). However, to the best of our knowledge, resistance genes of clinical relevance on conjugative plasmids have not been reported in desert soils. Hence, the presence of TEM-116 on a conjugative plasmid in this desert soil is unexpected since such soils are thought to be subject to low anthropogenic impact. However, the Namib Desert harbors highly transient populations of wildlife (Hedman et al., 2014), such as Springbok (*Antidorcas marsupialis*), Oryx (*Oryx gazella*), and several native and migratory species of birds (Stein et al., 2008). Consequently, these animals and birds in such natural environments are potentially at risk for the acquisition

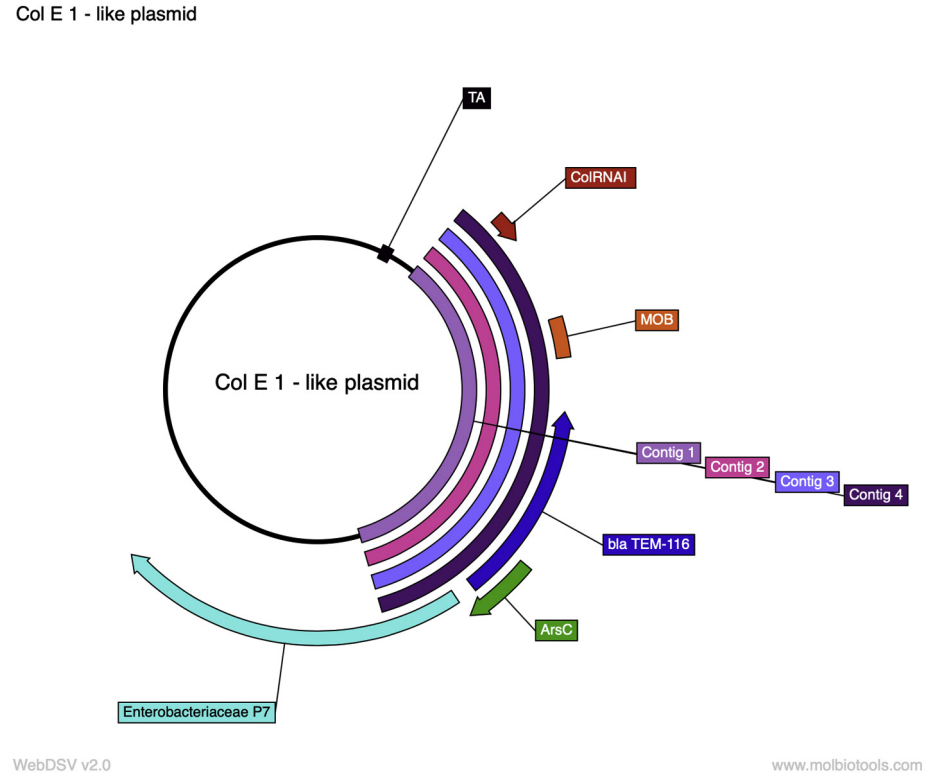

Fig. 1. Graphical representation of the part of the contigs (~90% of the total length) that contain the overlapping sequences for the ARG TEM-116, metal resistance gene ArsC and Bacteriophage P7 mapped to the ColE1-like plasmid profile (accession no: DQ298019). The ColE1 plasmid contains putative genetic modules (MOB: mobilization system, ColRNAI: origin of replication and TA: toxin-antitoxin stabilization system) which, are depicted in the figure. The figure showing the plasmid profile was determined using WebDSV2.0 and can be found at (www.molbiotools.com).

of antibiotic resistant bacteria (Allen et al., 2010; Literak et al., 2010). Although the routes of transmission are difficult to establish, direct contact with domestic livestock, via sharing drinking water and food sources, may be highly significant in transmission flows (Radhouani et al., 2014; Van den Honert et al., 2018). The spread of environmental ARGs mediated specifically by migratory birds, has been well recognized (Wellington et al., 2013; Wu et al., 2018). We note that, following sporadic rain events in the Namib Desert and the resulting growth and seeding of the dominant grass species *Stipagrostis ciliate* (Dean and Williams, 2004), very large numbers (millions) of Gray-backed Sparrow-larks (*Eremopterix verticalis*) invade the region. As groundfeeders, these populations have the capacity to support regional transport of soil microorganisms (and ARGs) via fecal deposits. High proportions of resistance determinants have been detected in feces of migratory birds across countries (Foti et al., 2011; Guenther et al., 2012; Radhouani et al., 2012), including pristine areas (Hernández and González-Acuña, 2016; Rabbia et al., 2016).

There is strong evidence that levels of human and animal fecal pollution correlate positively with the presence of antibiotic resistant organisms (Karkman et al., 2019; Sapkota et al., 2007). However, these studies are largely limited to aquatic environments, which are highly susceptible to anthropogenic contamination (Karkman et al., 2019). Detecting markers for fecal indicator bacteria (FIB) using metagenomics is problematic because of the low abundance of these marker bacteria in microbial communities (Bengtsson-Palme et al., 2017; Karkman et al., 2019). An alternative approach is the use of bacteriophages as indicators of fecal pollution (Ballesté et al., 2019; McMinn et al., 2017; Muniesa et al., 2018). Using MetaPhinder (Jurtz et al., 2016) we detected enterobacteria S-13 phages in these soil bacterial communities and a partial construct of an enterobacteria P7 phage encoded in the plasmid (Fig. 1).

The role of bacteriophages in the dissemination of ARGs in aquatic environments has recently been highlighted (Balcazar, 2014; Colavecchio et al., 2017; Ross and Topp, 2015). For example, the phage-mediated mobilization of quinolone resistance genes (qnrA) in urban wastewater (Colomer-Lluch et al., 2014) and their capacity to facilitate inter-species transfer of β-lactamase genes in hospital effluents (Marti et al., 2014) have both recently been reported. However, the extent to which bacteriophages facilitate the dissemination of ARGs in soil environments is currently unknown. S-13 bacteriophages are somatic coliforms which have been used as indicators of fecal contamination in domestic and municipal sewage as well as in primary and activated sludge (Syngouna and Chrysikopoulos, 2014). However, these phages have the ability to multiply in several species of Enterobacteriaceae, which means that their presence in these soil communities could be unrelated to fecal contamination (Leclerc et al., 2000). The P7 bacteriophage has the ability to replicate in its host as independent plasmid-like elements and reportedly harbors β-lactamases conferring resistance to various β-lactam antibiotics (Billard-Pomares et al., 2014). The presence of P7 on this Col E1-like plasmid may represent a new vehicle for the dissemination of TEM-116 in these communities. Although we cannot explicitly correlate the presence of wildlife to the impact of ARGs in the Namib Desert, our data does imply a link between fecal inputs and the acquired TEM-116 gene, probably mediated by birds.

Antibiotic resistance frequently co-occurs with resistance to heavy metals (Li et al., 2017). This is a result of the colocalization or comigration of genes conferring multiple resistance mechanisms (e.g., target inactivation, target protection, efflux pumps) (Li et al., 2017). Using the BacMet database (Pal et al., 2014), which contains metal resistance genes (MRGs), we demonstrated the presence of arsC genes (coding for arsenate reductase) in the Namib Desert soil metagenome contigs. The arsenate reductase reported here was 113 amino acid residues long (85% of the length of the reference arsC) with a 94% similarity to the reference protein (accession no: BAA24824.1) (Table S2). Interestingly, the arsC gene was encoded within the plasmid. Co-resistance to arsenic and β-lactams has been reported to frequently occur on the same mobile element (Pal et al., 2017). The co-selection potential of arsC and TEM-116 may have functional significance in this particular plasmid due to the presence of a toxin-antitoxin (TA) system (Fig. 1), which stabilizes the plasmid in the host by killing off any of the daughter cells that do not inherit the plasmid (Pal et al., 2015). Therefore, plasmids with ARGs and MRGs, together with a TA system would likely be more persistent in the environment even in the absence of antibiotic selective pressure (Di Cesare et al., 2016). Additionally, if resistance genes for both antibiotics and metals are physically located on the same plasmid, horizontal transfer of the entire gene cluster to other bacteria is likely (Knapp et al., 2011; Pal et al., 2015).

In summary, this work supports the evidence that clinically significant ARGs are widespread even in environments without obvious anthropogenic exposure to antibiotics. It is likely that in bacterial communities, the mobilization of existing resistance determinants happens continuously, although very few of these determinants are selected for and remain in the community. Consequently, resistance genes such TEM-116, which are already circulating between the clinic and the environment, may easily re-emerge in the clinic during antibiotic therapy further reducing treatment options. The likely exchange of resistance genes between soil dwelling bacteria and clinical pathogens highlights the crucial role of the environment in the emergence and re-emergence of these resistance determinants. The value of the One Health approach is undisputed, considering the dynamics of ARGs as they move from clinical environments to natural environments.

CRediT authorship contribution statement

Yashini Naidoo Conceptualization, Investigation, Writing - original draft. **Angel Valverde:** Writing - review & editing, Supervision, Funding acquisition. **Errol D. Cason:** Software, Writing - review & editing. **Rian E. Pierneef:** Software, Validation, Writing - review & editing. **Don A. Cowan:** Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137497.

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CHAPTER FIVE: CONCLUSION AND FUTURE DIRECTION

Chapter 5: Conclusion and future direction

Previous research has shown that the structure (assess using T-RFLP analysis) and activities (assess using five extracellular enzymes) of soil microbial communities in the Namib Desert are affected by a precipitation gradient. Using both targeted (16S rRNA genes) and shotgun metagenomics this research provides a more detailed analysis on the taxonomic and functional attributes of these communities. The bacterial communities were dominated by *Proteobacteria*, *Actinobacteriota*, *Bacteroidota* and *Acidobacteriota* that together represented 76% of the sequences. The precipitation gradient shaped the functional diversity, and both the taxonomic composition and functional potential of these communities. Furthermore, the changes in microbial community composition and function were governed by a narrow range of phylogenetic taxa including several that were rare. Interestingly, the most noticeable difference between the rainfall zones was the increase in functions relating to competition in the high-rainfall zone, more likely as a result of increased moisture and higher nutrient levels. In spite of these differences the core functions such as housekeeping and general homeostasis functions were evenly distributed. Microbial functional potential was shown to be largely determined by microbial community composition. Based on these results, it can be concluded that precipitation history alters both the composition and functional potential of bacterial communities in desert soils, which could help to predict the response of these communities to climate change.

Very little is known about the soil resistome of hot desert ecosystems and the present study, to the best of my knowledge, is the first to characterize the resistome and mobilome of Namib Desert soils. The analysis of soil metagenomes revealed that antibiotic resistance genes, metal/biocide resistance genes and mobile genetic elements were present in low abundance in Namib Desert soil. This was not unexpected since the direct anthropogenic impact in this soil is low. However, the detection of resistance to biocides such as triclosan, an antibacterial commonly used in personal care products, suggests a moderate human impact which could be as a result of increased tourism to the area. Evidence points to a potential co-selection of resistance with metals and/or biocides (*Mex*K, *ars*C) which could allow for these antibiotic resistance genes to persist in the soil even in the absence of antibiotic selection pressure. The results suggest a decoupling between bacterial community composition and the antibiotic resistome, possibly originating from horizontal gene transfer in these soils which is further

supported by the presence of horizontally acquired resistance genes. This seems to indicate that vectors such as birds and humans might be partly involved in the spread of resistance determinants in these soils while highlighting the role that less impacted environments play in assessing the spread of antibiotic resistance globally.

The presence of a clinically significant ARG (i.e., TEM-116) in this environment with no obvious anthropogenic exposure to antibiotics provides evidence for the interchange of ARGs between human and environmental sectors at international scales. Furthermore, resistance genes which are already circulating between the clinic and the environment, may easily re-emerge in the clinic during antibiotic therapy which has a direct effect on treatment options. Overall, this study emphasizes the need for better surveillance of ARGs in low impacted environments as part of the One Health Initiative.

Although this research has provided valuable information on the composition and function of microbial communities in desert soils, there are several questions that require further investigation, for example:

1. Do other microbes (e.g., fungi, archaea) in the soil communities follow similar or different patterns across this gradient? It remains to be determined how other members of these microbial communities will react to the precipitation gradient and what the consequences will be for ecosystem functionality.

2. While metagenomics provides information on which microorganisms are present (i.e., *who is there?*) and what their potential functions are (i.e., *what might they be doing?*), an important question remains: how do the activities of microorganisms relate to ecosystem function? In an attempt to determine whether these communities are active, future research should include RNA analysis since RNA extracted from environmental samples provides valuable information in revealing active versus dormant communities.

3. Are there areas in the Namib Desert that can be classified as ARG hotspots? ARG hotspots can occur in any environment that is subjected to anthropogenic pressure. Given the increase in desert tourism, it would be interesting to analyze soil from those areas that are

frequented by tourists and compare it to areas that receive minimal human traffic to assess the impact in the Namib Desert. Additionally, since the levels of contaminants in these soils (e.g., heavy metals, biocides, antiseptics, biosurfactants) were not measured. Analyzing the levels of contaminants would also provide insight into the levels of anthropogenic pressure exerted in this environment.

4. How often are ARGs successfully conferred via horizontal gene transfer in these soils? New bioinformatics tools have enabled the accurate detection of horizontal gene transfer events, however the identification of the donor and acceptor candidates in these events has proven to be rather complex. The experimental verification of HGT events (e.g., conjugation assays) will ensure higher levels of accuracy and present a better understanding of how, when and why bacteria exchange ARGs.

APPENDIX 1

Table 1. Physicochemical analyses for sample sites including rainfall and soil temperature measurements.

Sample site	Zone	Co-ordinates	Rainfall (mm)	Soil Temp (°C)	pH	P (mg/kg)	Na (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	NH4 (mg/kg)	NO3 mg/kg	% C
C142017sample10	MR	23°11'76.1"S 15°16'69.2"E	1.12	25.6	7.8	0.49	19.2	10.2	91.4	4.5	0.58	4.2	0.19
C142017sample12	MR	23°17'47.2"S 15°26'56.2"E	1.12	25.6	7.6	0.52	9.4	10.1	88.4	5.1	0.54	3.3	0.29
C142017sample14	MR	23°19'22.1"S 15°37'39.7"E	1.12	25.6	7.6	0.86	12.8	11.1	44.1	7.9	0.66	8.67	0.17
C142017sample16	HR	23°18'12.6"S 15°47'42.9"E	7.9	26.8	7.7	0.49	13.2	8.9	47.3	11.8	1.27	3.6	0.91
C142017sample18	HR	23°20'63.2"S 15°55'16.5"E	7.9	26.8	7.6	1.26	3.6	16.3	21.4	7.4	1.86	2.2	0.16
C142017sample20	HR	23°14'71.8"S 16°08'59.9"E	7.9	26.8	7.3	1.32	3.2	11.1	33.7	6.14	0.74	5.2	0.57
C142018sample10	MR	23°11'34.2"S 15°16'56.1"E	2.5	24.7	7.8	0.56	13.2	9.7	97.3	4.7	0.71	5.3	0.18
C142018sample12	MR	23°18'42.2"S 15°26'73.2"E	2.5	24.7	7.3	0.46	13.3	12.7	86.2	8.3	0.43	1.1	0.29
C142018sample14	MR	23°19'33.4"S 15°36'29.4"E	2.5	24.7	7.6	0.56	15.7	12.3	47.2	8.7	0.53	7.1	0.16
C142018sample16	HR	23°18'52.8"S 15°47'55.2"E	9.2	26.3	7.6	0.56	8.4	7.9	65.4	11.2	1.13	5.6	0.64
C142018sample18	HR	23°19'47.2"S 15°56'17.5"E	9.2	26.3	7.8	1.66	4.5	11.4	28.0	5.3	1.01	3.1	0.30
C142018sample20	HR	23°11'67.8"S 16°07'53.1"E	9.2	26.3	7.4	1.08	2.1	6.5	44.8	3.8	0.54	3.7	0.62
C142019sample10	MR	23°11'57.2"S 15°16'35.3"E	0.2	25.7	7.7	0.39	20.12	12.3	95.4	4.4	0.63	2.7	0.22
C142019sample12	MR	23°17'65.3"S 15°26'78.5"E	0.2	25.7	7.5	0.68	12.2	10.8	77.2	6.5	0.45	7.3	0.30
C142019sample14	MR	23°19'22.1"S 15°36'42.7"E	0.2	25.7	7.6	0.78	11.6	11.4	48.7	7.2	0.71	8.4	0.13
C142019sample16	HR	23°18'63.3"S 15°47'57.2"E	2.9	26.1	7.7	0.58	7.9	8.4	59.8	11.0	1.11	4.5	0.38
C142019sample18	HR	23°19'53.2"S 15°56'27.5"E	2.9	26.1	7.2	1.31	4.0	19.2	30.4	8.2	1.0	2.4	0.26
C142019sample20	HR	23°15'67.8"S 16°08'35.7"E	2.9	26.1	7.5	1.32	2.5	9.1	36.46	4.7	0.64	5.2	0.67

Table 2. Clustering of ASVs into OTUs showing the different ecotypes at the Genus level.

ASVs	log2FoldChange	Pvalue	Padj	Phylum	Genus	OTU (97%)
ASV1351	-5.557.700.148	0.00188863	0.022496537	Abditibacteriota	Abditibacterium	OTU47
ASV170	-8.624.350.128	7.75E-06	0.000533968	Bacteroidota	Adhaeribacter	OTU02
ASV307	8.435.301.273	0.00010348	0.003989733	Bacteroidota	Adhaeribacter	OTU02
ASV57	-1.002.387.741	7.07E-20	4.91E-17	Bacteroidota	Adhaeribacter	OTU02
ASV447	-7.443.811.792	0.00234992	0.025481965	Bacteroidota	Adhaeribacter	OTU08
ASV52	-3.037.371.741	0.00356632	0.033000919	Bacteroidota	Adhaeribacter	OTU08
ASV467	7.878.556.731	0.00036789	0.008802526	Actinobacteriota	Angustibacter	OTU34
ASV998	-5.958.935.784	0.00381971	0.034426994	Proteobacteria	Azospirillum	OTU51
ASV386	8.100.113.991	0.00109604	0.016903372	Firmicutes	Bhargavaea	OTU20
ASV510	7.793.483.203	0.00162064	0.020994777	Actinobacteriota	Blastococcus	OTU32
ASV723	-667.327.968	0.00478944	0.041035222	Actinobacteriota	Blastococcus	OTU37
ASV1026	6.469.105.239	0.00601348	0.048527125	Acidobacteriota	Bryobacter	OTU11

ASV439	7.924.458.799	0.00124586	0.018396402	Acidobacteriota	Bryobacter	OTU12
ASV675	7.194.339.537	0.00289598	0.029127665	Acidobacteriota	Bryobacter	OTU13
ASV287	8.723.238.877	5.82E-05	0.00252357	Acidobacteriota	Bryobacter	OTU14
ASV880	6.642.245.509	0.00513202	0.043434473	Acidobacteriota	Bryobacter	OTU15
ASV513	7.563.370.868	0.00188785	0.022496537	Acidobacteriota	Bryobacter	OTU16
ASV872	6.903.442.751	0.00409788	0.035998813	Acidobacteriota	Bryobacter	OTU17
ASV1029	-6.172.149.347	0.00319827	0.03082778	Acidobacteriota	Bryobacter	OTU61
ASV161	9.027.863.954	8.94E-06	0.000533968	Crenarchaeota	CandidatusNitrososphaer	OTU10
ASV86	9.764.208.628	1.63E-08	3.78E-06	Crenarchaeota	CandidatusNitrososphaer	OTU10
ASV144	-8.951.007.554	9.37E-06	0.000533968	Verrucomicrobiota	CandidatusUdaeobacter	OTU46
ASV468	7.593.605.443	0.00218553	0.024864917	Cyanobacteria	ChlorogloeopsisPCC-	OTU43
ASV402	8.055.803.783	0.00101077	0.016310257	Proteobacteria	Ellin6055	OTU52
ASV280	8.713.467.109	0.00025952	0.006927396	Bacteroidota	Flavisolibacter	OTU03
ASV504	-745.626.453	0.00061453	0.012185318	Bacteroidota	Flavisolibacter	OTU03

ASV518	7.554.872.067	0.00048728	0.010567932	Bacteroidota	Flavisolibacter	OTU03
ASV114	9.702.695.164	6.10E-07	0.000105751	Bacteroidota	Flavisolibacter	OTU05
ASV196	9.021.944.496	6.80E-09	2.36E-06	Bacteroidota	Flavisolibacter	OTU05
ASV644	-6.895.353.724	0.00389242	0.034632625	Bacteroidota	Flavisolibacter	OTU05
ASV113	970.713.387	0.00016530	0.004987895	Bacteroidota	Flavisolibacter	OTU26
ASV463	-7.328.995.525	0.00254983	0.026811935	Actinobacteriota	Klenkia	OTU06
ASV806	6.810.089.968	0.00138488	0.019827162	Actinobacteriota	Klenkia	OTU06
ASV12	-1.202.178.341	0.00025332	0.006927396	Actinobacteriota	Leifsonia	OTU35
ASV502	-7.207.834.559	0.00075478	0.012776035	Actinobacteriota	Marmoricola	OTU30
ASV601	7.552.738.577	0.00266575	0.027612474	Proteobacteria	Methylobacterium	OTU66
ASV742	7.076.849.883	0.00025664	0.006927396	Proteobacteria	Methylobacterium	OTU69
ASV177	9.056.765.351	1.00E-05	0.000533968	Proteobacteria	Microvirga	OTU01
ASV182	8.918.555.994	0.00013070	0.004471246	Proteobacteria	Microvirga	OTU01
ASV2	1.527.695.855	2.94E-06	0.000340176	Proteobacteria	Microvirga	OTU01

ASV937	-6.180.520.627	0.00015237	0.00480665	Proteobacteria	Microvirga	OTU09
ASV775	-6.559.330.281	0.00546198	0.045126356	Proteobacteria	Microvirga	OTU58
ASV765	6.915.299.199	0.00381535	0.034426994	Proteobacteria	Microvirga	OTU59
ASV691	7.154.190.819	0.00308195	0.03024589	Proteobacteria	Microvirga	OTU60
ASV577	7.475.465.138	0.00223038	0.024914392	Proteobacteria	Microvirga	OTU71
ASV372	8.295.817.404	0.00068680	0.012543214	Actinobacteriota	Modestobacter	OTU36
ASV268	-8.229.465.201	1.32E-05	0.000654396	Actinobacteriota	NA	OTU04
ASV340	8.403.517.665	0.00011636	0.004250329	Actinobacteriota	NA	OTU04
ASV612	7.496.848.776	0.00064923	0.01241893	Actinobacteriota	NA	OTU04
ASV559	7.478.541.671	0.00212096	0.02453249	Actinobacteriota	NA	OTU07
ASV642	-7.018.275.827	0.00348468	0.032680732	Actinobacteriota	NA	OTU07
ASV10	0.968837379	0.00241243	0.025757345	Proteobacteria	NA	OTU09
ASV575	7.386.712.367	0.00226168	0.024914392	Planctomycetota	NA	OTU18
ASV129	9.456.386.788	2.26E-06	0.000313502	Crenarchaeota	NA	OTU21

ASV832	6.770.665.791	0.00147459	0.02006611	Bacteroidota	NA	OTU23
ASV646	-6.830.087.267	0.00422138	0.03662055	Bacteroidota	NA	OTU24
ASV648	7.297.200.522	0.00075072	0.012776035	Bacteroidota	NA	OTU25
ASV792	-6.623.306.592	0.00058606	0.011962696	Bacteroidota	NA	OTU27
ASV600	-7.010.006.089	0.00103408	0.016310257	Chloroflexi	NA	OTU29
ASV411	-7.672.213.113	0.00041526	0.009296485	Actinobacteriota	NA	OTU41
ASV481	-7.338.886.645	0.00066210	0.01241893	Bacteroidota	NA	OTU45
ASV804	6.735.572.177	0.0013999	0.019827162	Acidobacteriota	NA	OTU48
ASV406	8.084.518.901	0.00100741	0.016310257	Proteobacteria	NA	OTU50
ASV359	8.207.643.783	0.00073949	0.012776035	Proteobacteria	NA	OTU62
ASV281	8.476.349.542	5.48E-06	0.000475323	Proteobacteria	NA	OTU65
ASV15	-1.065.445.622	0.00587510	0.047968488	Proteobacteria	NA	OTU70
ASV709	7.030.952.937	0.00332679	0.031627307	Proteobacteria	Psychroglaciecola	OTU57
ASV451	779.444.607	0.00145202	0.02006611	Proteobacteria	Psychroglaciecola	OTU63

ASV655	7.408.746.585	0.00275515	0.028118742	Proteobacteria	Psychroglaciecola	OTU64
ASV524	7.569.383.544	0.00052942	0.011134027	Proteobacteria	Psychroglaciecola	OTU67
ASV516	7.825.403.349	0.00155321	0.020729421	Proteobacteria	Psychroglaciecola	OTU68
ASV176	9.123.431.455	6.68E-06	0.000514944	Actinobacteriota	Quadrisphaera	OTU33
ASV71	3.497.859.615	0.0012116	0.01827935	Acidobacteriota	RB41	OTU49
ASV452	7.859.349.339	0.00033163	0.008524141	Gemmatimonadota	Roseisolibacter	OTU19
ASV389	-7.716.973.231	0.00039760	0.009197917	Actinobacteriota	Rubrobacter	OTU38
ASV568	-7.166.044.301	0.00309432	0.03024589	Actinobacteriota	Rubrobacter	OTU39
ASV751	-6.571.252.705	0.00538196	0.045000991	Actinobacteriota	Rubrobacter	OTU40
ASV662	-6.765.090.733	0.00183513	0.022496537	Bacteroidota	Rufibacter	OTU22
ASV275	-8.170.626.328	0.00013529	0.004471246	Bacteroidota	Segetibacter	OTU28
ASV369	-7.858.933.945	0.00035644	0.008802526	Bacteroidota	Segetibacter	OTU44
ASV213	-8.494.581.118	4.28E-06	0.000424301	Actinobacteriota	Solirubrobacter	OTU42
ASV433	-7.572.910.795	0.00191253	0.022496537	Proteobacteria	Sphingomonas	OTU53

ASV219	9.035.643.333	1.86E-05	0.000860438	Proteobacteria	Sphingomonas	OTU54
ASV545	7.779.084.243	0.00166385	0.020994777	Proteobacteria	Sphingomonas	OTU55
ASV443	-7.503.883.142	8.83E-05	0.003605056	Proteobacteria	Sphingomonas	OTU56
ASV906	6.683.697.747	0.00165798	0.020994777	Actinobacteriota	Streptomyces	OTU31

APPENDIX 2

Table 1. Acquired antibiotic resistance genes (ARGs) and the metal resistance genes (MRGs) involved in co-selection of resistance determinants

ARG	Resistance mechanism	Original ARG host	Number of ARGs	Number of occurrences
AAC(3')-*Ia*	inactivation by acetyltransferase	clinical strain of Pseudomonas aeruginosa	1	2
TEM-116 β-lactamase	class A β-lactamase, hydrolyses the peptide bond of β-lactam antibiotics	clinical strain of Escherichia coli	1	5
MRG family Description	**Resistance mechanism**	**Co-selection compounds**	**Number of MRGs**	**Number of occurrences**
*Mex*K	enhanced Efflux	triclosan, tetracycline and macrolides	1	5
*ars*C	enzymatic detoxification	arsenic, 3rd generation cephalosporins	1	5

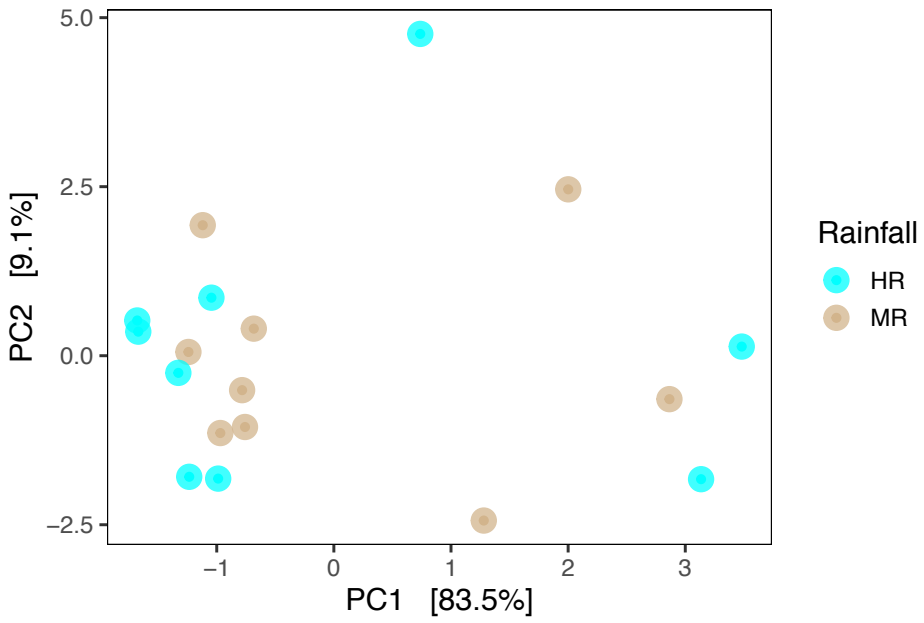

Figure 1. PCA (principle component analysis) ordination of Euclidian distances between ARGs in the mid-rainfall (MR) and high-rainfall zones (HR).

APPENDIX 3

Supplementary material for:

A clinically important, plasmid-borne antibiotic resistance gene (β-Lactamase TEM-116) present in desert soils.

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This file contains:

Table S1: TEM-116 and total ARG frequencies and relative abundances found across samples

Table S2. BLAST output values for antibiotic resistance gene TEM-116 (accession no: AY425988.1), metal resistance gene arsC (accession no: BAA24824.1) and plasmid replicon ColRNAI.

Figure S1: Blast Ring Image Generator (BRIG) representation of the contig alignment from all the samples reporting the acquired TEM-116 gene.

Table S1. TEM-116 and total ARG frequencies and relative abundances found across samples

Sample	Predicted genes*	*bla*TEM-116	Total ARGs	Relative abundance TEM-116[a]	Relative abundance ARGs[b]
S102018	653 463	7	121	0.057	1.85e-04
S142018	655 042	5	159	0.031	2.42e-04
S182018	758 240	22	172	0.127	2.26e-04
S202018	749 007	9	135	0.067	1.80e-04

*Genes were predicted using Prodigal v2.6.3.
[a]Relative abundance is calculated as the total number of blaTEM-116 divided by the total number of ARGs per sample.
[b]Relative abundance is calculated as the total number of ARGs divided by the total number of genes predicted per sample.

Table S2. BLAST output values for antibiotic resistance gene TEM-116 (accession no: AY425988.1), metal resistance gene arsC (accession no: BAA24824.1) and plasmid replicon ColRNAI.

Sample	Query (contig)	Subject	% Identity	Alignment length (bp)	Mismatches	Query length (bp)	Subject Length (bp)	% Query Length aligned	% Subject gene aligned	E-value
S102018	Contig 1	*bla*TEM-116	100	861	0	3425	861	25	100	0
	Contig 1	arsC	93.8	339[a]	7	3425	393	9.8	86	5.12e-59
	Contig 1	ColRNAI	90	131	11	3425	131	4	100	4.34e-35
S142018	Contig 2	*bla*TEM-116	100	861	0	3374	861	25	100	0
	Contig 2	arsC	93.8	339[a]	7	3374	393	10	86	5.4e-59
	Contig 2	ColRNAI	90	131	11	3374	131	3.83	100	4.27e-35
S182018	Contig 3	*bla*TEM-116	100	861	0	3072	861	25	100	0
	Contig 3	arsC	93.8	339[a]	7	3072	393	11	86	5.27e-59
	Contig 3	ColRNAI	90	131	11	3072	131	4.26	100	3.89e-35
S202018	Contig 4	*bla*TEM-116	100	861	0	3425	861	25	100	0
	Contig 4	arsC	93.8	339[a]	7	3425	393	9.8	86	5.12e-59
	Contig 4	ColRNAI	90	131	11	3425	131	3.82	100	4.34e-35

[a] Metal resistance genes were compared against a protein database, however the blast output for arsC was converted into nucleotides for the purpose of this table.

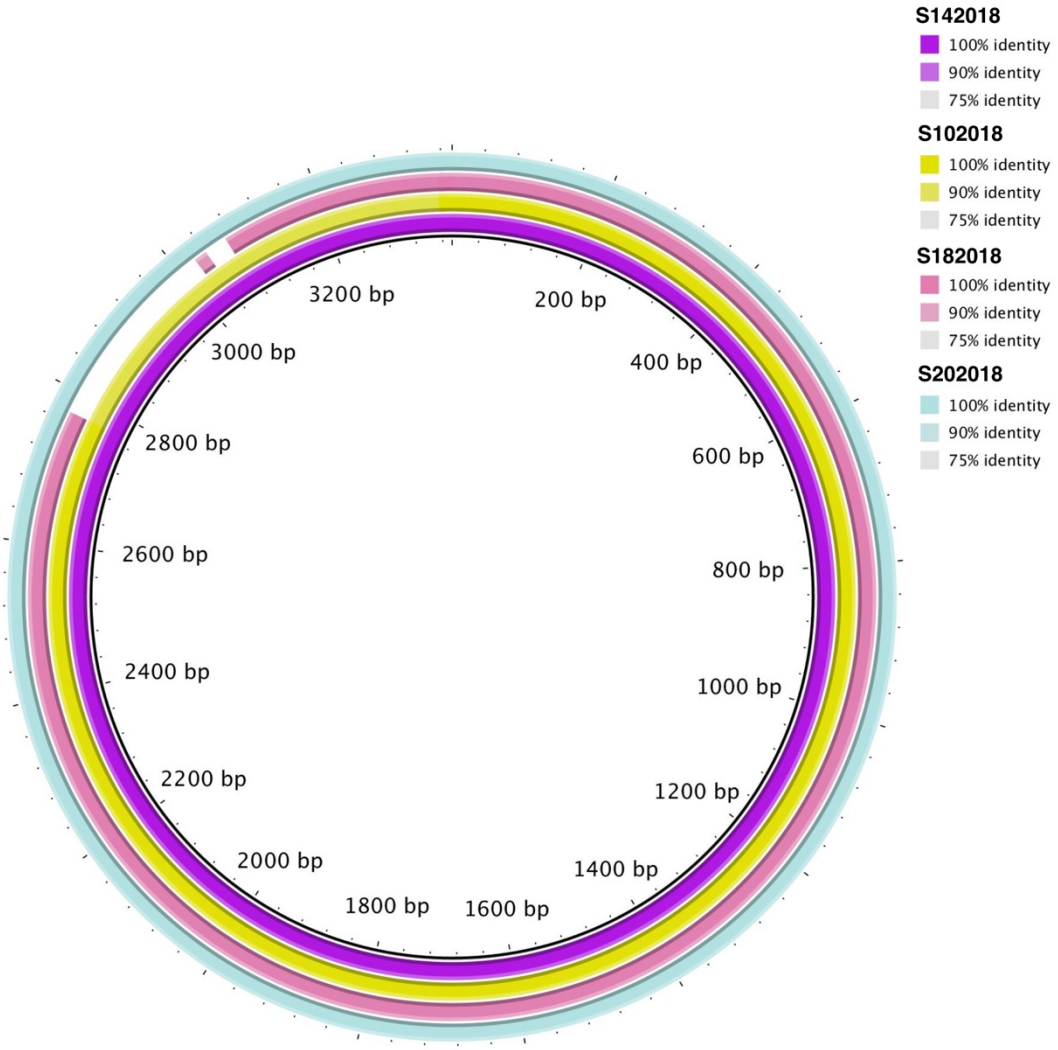

Figure S1. Blast Ring Image Generator (BRIG) representation of the contig alignment from all the samples reporting the acquired TEM-116 gene. The labels of the contigs in comparison to Figure 1. are as follows (S102018 – Contig 1, S142018 – Contig 2, S182018 – Contig 3, S202018 – Contig 4).