The effects of urban forest restoration and environmental heterogeneity on microbial diversity and ecosystem functioning

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Abstract

Increasing global urbanisation poses extensive environmental threats, including the loss of biodiversity and associated ecosystem functions. Ecosystem restoration interventions like native plantings can assist in halting and reversing the degradation of forest patches in urban settings, which is particularly relevant for the United Nations' decade of ecosystem restoration. While the benefits of restoration plantings for aboveground communities have been well established, belowground community responses remain understudied. It is assumed that the soil microbiome will self-assemble to a pre-disturbance state following restoration. However, as microorganisms are rarely the target of restoration interventions themselves, various questions remain regarding the extent to which microbial communities will respond and recover with aboveground plantings, and how environmental variability might influence trajectories of microbial community reassembly. In this thesis, I contribute to this knowledge gap by investigating the impacts of forest age since restoration alongside a suite of environmental covariates on microbial community responses. My fieldwork took place in a New Zealand-wide chronosequence of restored urban forest sites. I analysed the diversity and taxonomic composition of bacterial, archaeal, fungal, and protist communities from these soil samples using metabarcoding data in chapter 2. Furthermore, I investigated changes in total microbial biomass, bacterial and fungal taxonomic group biomass via PLFA, total basal respiration, the spatial variability of microbial biomass and respiration, and carbon metabolism on substrates of varying recalcitrance using MicroResp™ in chapter 3. My synthesised findings show that environmental variability is typically more explanatory of microbial responses than forest age alone. Plant community factors in particular—such as native woody seedling density and tree species richness—were most often linked with enhanced microbial diversity and respiration, as well as driving increased local spatial variability. Specific phylum-level shifts from metabarcoding data indicated microbiome recovery on a trajectory towards mature forest soil communities, yet PLFA data showed that community composition does not yet reflect fungal dominance as would be expected in a mature or fully recovered forest soil. This thesis provides a holistic assessment of microbial community responses to urban forest restoration interventions, by assessing both

diversity and microbial functions at multiple taxonomic resolutions. In conclusion, results from my thesis highlight the importance of taking into account the recovery of the soil microbiome and its associated ecosystem functioning to improve both aboveground and belowground success in future forest restoration projects.

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Chapter 1

Introduction and literature review

1.1 Introduction

The global human population is increasingly congregating in cities, with 68% expected to live in urban areas by 2050 (United Nations, 2018). Urbanisation has led to vast declines in native forest cover, biodiversity losses (McKinney, 2002), and increased environmental pollution (Bai et al., 2017), including functional extinctions of native species and the ecosystem functions they provide (Luna et al., 2018). Ecological restoration is a central strategy for reversing the current global trajectory toward ecosystem degradation (Breed et al., 2020). The purpose of ecological restoration is to recreate or advance the recovery of disturbed environments via human intervention (Young, 2000). For example, forest restoration may imply planting native species to create new forests, or enrichment planting in remnant forest fragments (Wallace & Clarkson, 2019). The objective of ecosystem restoration is to restore an ecosystem to a reference or pre-disturbance state (Gann et al., 2019), and increasingly, restoration projects aim to recover lost biodiversity and ecosystem functions in the process (Aerts & Honnay, 2011).

Restoration practices have more recently extended to urban ecosystem restoration, a sub-discipline concerned with restoring nature and its associated benefits to urban areas (Clarkson & Kirby, 2016). Urban areas generate dynamic and unique challenges. For example, urban "heat island" effects mean that heatwaves can impact densely populated areas, leading to human health issues and even death (Douglas, 2012; Fouillet et al., 2006). Urbanisation also supports radically altered, concentrated populations of exotic species and decreased diversity of native species, mainly because environmental disturbance and pollution lead to a decline in habitat suitability for rare, specialist taxa, instead facilitating simplified populations of generalist invaders (Faeth et al., 2011; Santana Marques et al., 2020). The presence of forests in urban spaces has demonstrable benefits, such as removal and filtering of air pollutants by trees (Yang et al., 2005), mitigation of urban heat island effects via tree shading and

evapotranspirational cooling (Meier, 1990; McPherson et al., 1997), hence also buffering against climate change (Bastin et al., 2019), especially as urban trees sequester carbon (Nowak & Crane, 2002). Diverse tree assemblages, in particular, produce greater woody biomass per unit area than monoculture plantations or grasslands, thus storing greater amounts of carbon (Huang et al., 2018). Even minor increases to areas lacking in forest cover are associated with large boosts in native species (Ruffell & Didham, 2017). Furthermore, urban forests benefit people by improving their social and cultural ties to the land (Wehi & Wehi, 2010), creating a feedback loop whereby people are more willing to value nature in the urban settings where they live (Baur et al., 2020). Ecosystem restoration in Aotearoa New Zealand has traditionally focused on national parks and offshore islands (Clarkson & Kirby, 2016). As new policies emerge in Aotearoa New Zealand (i.e., the National Policy Statement on Indigenous Biodiversity) that require cities to meet >10% indigenous forest cover, studying aspects of urban forest restoration will be of increasing importance to understand long-term forest dynamics in urban environments (McPhearson et al., 2010; Ministry for the Environment, 2019).

The effects of restoration on the soil microbiome are still poorly characterised in the scientific literature (Lance et al., 2020). The incredible array of biodiversity harboured in the soil supplies vital ecosystem services, including and not restricted to water purification, climate regulation, and supporting primary production (Bardgett and van der Putten, 2014; MEA, 2003). Ecosystem functioning includes processes and properties – such as nutrient cycling and organic matter decomposition – that enable a forest to supply these vital ecosystem services and support the human population (Aerts & Honnay, 2011; Duffy, 2009). Losses of microbial diversity pair with losses in ecosystem functions and services (Delgado-Baquerizo et al., 2016). Restoration studies that do exist commonly focus on plant community outcomes, Northern hemisphere locations, and young (<5 years) restored forests (Doroski et al., 2018). As Wallace & Clarkson (2019) proposed, a scientific, evidence-based approach is necessary for the success of urban forest restoration due to the significant costs and unique challenges involved. As cities around the world invest substantial resources into planting self-sustaining urban forests, it is necessary to understand whether restoration work thus far is having desired impacts on the recovery of soil microbiota, which is essential for restoration success (Harris, 2009). Forest restoration aims to restore degraded ecosystems, and integral to that goal

is the reinstatement of ecosystem functioning (Aerts & Honnay, 2011; Hobbs & Norton, 1996). The endpoint of forest restoration is reached when forest structure and function are reinstated (Wallace & Clarkson, 2019). Yet belowground communities and their associated functions are typically ignored in restoration studies (Strickland et al., 2017). Aboveground-belowground interactions are hugely important to ecosystem functioning; therefore we cannot know if forest ecosystems are truly restored without understanding how soil organisms respond (Eviner & Hawkes, 2008).

1.1.1 The dark side of restoration: restoring belowground microbial biodiversity

Despite soil being one of the most biodiverse and speciose environments on the planet, belowground communities are commonly overlooked in restoration research (Bardgett & Van Der Putten, 2014). Land-use changes or restoration plantings that impact aboveground communities also impact belowground community structure (Marian et al., 2020). Recognising and integrating aboveground and belowground linkages can improve our scientific understanding of forest restoration and improve chosen intervention outcomes – restoring an aboveground component may require manipulation of a belowground component and vice versa, due to ecological networks that span the interface between plant and microbial communities (Kardol & Wardle, 2010), particularly as restoration projects are susceptible to failure (Thomas et al., 2014). Studies that have been carried out on soil microbiota often take place in natural systems but anthropogenic influences now impact many ecosystems; we do not currently know enough to elucidate the differences structuring natural or urban soil ecosystems, but we know soil microorganisms are typically adversely impacted by urbanisation and land-use change (Ramirez et al., 2014). A rather despondent view is that aboveground restoration cannot restore the soil microbiome because it remains an untested assumption that recovery of aboveground plant communities will beget belowground recovery (Strickland et al., 2017). The possibility that microbial recovery is not coupled with aboveground restoration interventions is a direct contradiction of the core “field of dreams” concept that is central to restoration ecology, which states that biotic systems can self-assemble if we recreate the physical structure of a target system (“if you build it, they will come”) (Hilderbrand et al., 2005). Belowground communities will be top priority subjects of soil ecological studies in the future and require global-

scale research efforts to address our lack of knowledge on the drivers of species distribution patterns, especially in anthropogenically transformed environments (Eisenhauer et al., 2017).

Soil microbiota comprise around one-third of Earth's biomass and play vital ecological roles in nutrient cycling and decomposition (Mendes et al., 2017). The functional diversity and activity of the soil microbial community have far-reaching effects on the functioning of a whole ecosystem (Berkelmann et al., 2020). Soil microbial communities include bacteria, archaea, fungi and protists. The bacteria are the best studied of these groups (Angel et al., 2010). Bacteria are abundant prokaryotes with pivotal roles in ecosystem functioning, like nutrient cycling and symbiotic associations with plants (Yan et al., 2020). Archaea are often associated with extreme or unusual environments but are in fact widespread prokaryotes in soils (Bintrim et al., 1997) that function as key players in carbon and nutrient cycling (Baker et al., 2020). Fungi are eukaryotic organisms that are most diverse in forests, functioning as crucial decomposers of organic matter, and symbiotes that form mycorrhizal associations with plant roots that are key for plant community survival (Origazzi et al., 2016). Protists are a diverse group encompassing all non-fungal microbial eukaryotes and are involved in nutrient cycling as well as regulating microbial populations (Geisen et al., 2017; Urich et al., 2008). Protists can function as consumers of bacteria and fungi (Seppey et al., 2017), saprotrophs that degrade soil carbon, primary producers, or as symbiotes (including parasites) of plants (Dai et al., 2021; Oliverio et al., 2020). Less than 1% of all microorganisms can be grown in agar, therefore we know the direct biology of very few microbial species and can only infer functions indirectly by community-level activities (Harris, 2009). More recently, metagenomics has allowed us to more precisely determine soil microbes' functional roles by detecting genes associated with particular functions (Epp Schmidt et al., 2019). We must also remain aware that our attempts to extrapolate patterns in microbial community assembly are limited due to scale. Macrobial biogeography patterns have been established from data collected at a vastly different spatial and temporal scale compared to microorganisms in even one gram of soil, which may create statistical complications and sampling errors that confound patterns between plants and microbes (Nemergut et al., 2013).

Biotic homogenisation is often discussed in urban community ecology research. This term describes how urban systems display low diversity in plant, animal, and microbial assemblages. The merging of abiotic conditions which characterise urban areas worldwide is thought to merge the functional capacity of biota, summarised as the "urban convergence hypothesis" (Epp Schmidt et al., 2019). Species that comprise this global, homogenised, urban meta-community can withstand high stress and succeed in distributing far and wide (Gutiérrez-Cánovas et al., 2013; Lôbo et al., 2011). In addition to between-site similarity in urban soil communities, biotic homogenisation also describes within-site similarity, as functional traits are not spatially varied as expected in natural, heterogeneous forest soil (Mori et al., 2015; Olden, 2006). This phenomenon is expected to contribute to the next great extinction (Epp Schmidt et al., 2019). Restoration is a strategy that can allow soil communities to re-differentiate and recover from biotic homogenisation (Mori et al., 2015).

Forest soils are home to incredibly dynamic and diverse microbial communities assembled from bacteria, fungi, archaea, and protists, which makes characterising soil community composition very challenging (Ross-Davis et al., 2014). Bioinformatic tools can help us understand soil microbial community composition and ecosystem functions. The recovery of microbial biomass and functional diversity can accelerate the restoration of the soil ecosystem and plant community (Sansupa et al., 2021). Soil microbial communities are challenging to identify, but recent innovations in DNA sequencing technology have allowed greater access to the study of microbial life forms (Baker et al., 2020). For example, metabarcoding is a technique used to identify taxa from DNA material found in the environment, producing an assessment of diversity in a sample (Taberlet et al., 2012). Metabarcoding, via high-throughput amplicon sequencing, uses the highly conserved small-subunit RNA (SSU RNA) to interpret changes in species richness, diversity, and community composition between samples and over time, without requiring the technical expertise of time-intensive morphological identification (Borrell et al., 2017; Hugerth & Andersson, 2017). The emerging use of metabarcoding data in a practical sense, rather than a strictly academic sense, enables us to unlock more information on the biodiversity of understudied groups than would be achieved with other, non-DNA-based methods (Pawlowski et al., 2016). Therefore, metabarcoding is a promising tool in the restoration management toolbox for

demonstrating the effects of aboveground planting on microbial community compositions (Gellie et al., 2017). Microbial taxa are most commonly clustered into OTUs, as determined by a DNA sequence identity threshold cut-off at a conserved gene locus. OTUs function as an approximation of "species", given a lack of consensus on what defines a microbial species unit, and groups identified as belonging to the same OTU show high ecological consistency across habitats (Koeppel & Wu, 2013). Metabarcoding may become an increasingly relevant tool for our holistic understanding of restoration effects on understudied belowground systems as we enter the United Nations' decade of ecosystem restoration (United Nations General Assembly, 2019).

1.1.2 Forest belowground successional dynamics

Forest succession causes abiotic and biotic variables to change over time. As microclimatic conditions, nutrient concentrations, and plant compositions change with the progression of forest development (Thuille et al., 2000; Walker et al., 2007), therein also arrives differences in soil physicochemical properties and accumulation of organic matter that changes microbial biomass and respiration (Jia et al., 2005). Forest restoration techniques are intentionally employed to drive the succession of an ecosystem towards a desired endpoint (Walker et al., 2007). Forest successional development along a restoration trajectory can be shown by aboveground attributes such as overhead canopy closure or increased herbaceous ground cover (Wallace et al., 2022). Forest succession is shown belowground by increased carbon and nutrient cycling (Teixeira et al., 2020). While aboveground indicators of forest restoration are common, soil indicators – especially soil microbial indicators – are vastly underreported (Lozano-Baez et al., 2021).

A range of deterministic and stochastic factors determine soil microbial community structure dissimilarity but the exact mechanisms governing microbial reassembly in forest restoration remain poorly understood. Deterministic factors include abiotic and biotic factors, which create environmental selection (or filtering) and interspecies interactions to determine which microbes can survive, as stipulated in niche-based theory (Zhao et al., 2019). Evidence suggests a greater influence of environmental filtering on processes affecting microbial assembly related to local environmental characteristics (Calderón et al., 2017); however, the influence of drivers for re-colonisation can vary depending on the ecological context. Neutral-based theory states

that stochastic factors such as historical contingencies (i.e., prior land-use), dispersal limitation (due to local propagule size and abundance), and priority effects (where organisms that colonise first are more abundant) culminate in random microbial community assembly, independent of other species' traits (Fargione et al., 2003; Hubbell, 2001). For example, neutral assembly may dominate fungal community structure due to greater priority effects from antagonistic interactions of early-arriving fungi (Fukami et al., 2010), whilst bacteria may be driven more by deterministic factors and less susceptible to priority effects thanks to a higher abundance and lower likelihood of dispersal limitation (Jiang et al., 2018; Powell et al., 2015). Priority effects may also have greater or lesser influence depending on soil type and nutrient availability (Fry et al., 2017). It is generally thought that a combination of neutral and niche-based processes integrate to drive microbial community assembly (Powell et al., 2015).

1.1.3 Environmental drivers of microbial community structure and functioning

The literature is inconsistent on the main drivers structuring microbial communities, where the most influential drivers of microbial community and activity will vary depending on the environmental context (Li et al., 2021). Commonly cited environmental variables important for determining microbial diversity, biomass, activity, and community structure include pH (Tripathi et al., 2013) and nutrient status (Wan et al., 2015). Microbes have an optimum intracellular pH to maintain, hence microbial diversity peaks where pH is suitable for a broader range of microbes (Booth, 1985). Nutrient availability may also be limiting for microbes, i.e., microbes that respire via nitrification are inhibited by decreased availability of nitrogen sources (Jiménez et al., 2019). The nitrogen form that is most available may favour either bacteria or fungi, for example, thereby altering the microbial community structure (Li et al., 2021). Many of these environmental factors will covary with each other. pH can also influence microbes via regulating available nutrient and metal ions in the soil (Lauber et al., 2008). Soil moisture content can also alter the availability of soil organic matter and hence soil nutrients, impacting microbial biomass and respiration (Bell et al., 2008). Temperature is another important variable as it can alter soil physical properties by freeze-thaw cycles to impact soil metabolism or create sub-optimally low or high temperature conditions that reduce microbial diversity (Cui et al., 2019). Biotic factors determine microbial communities as well. Plant diversity impacts microbial community structure via

symbiotic interactions with specific taxa (Yan et al., 2020) and plants' transferral of carbon resources to the soil (De Deyn et al., 2011). Certain litter or root carbon compounds of varying structural complexity may require specific enzymes for their degradation, causing differences in microbial assemblages and benefitting specialist taxa (Baldrian et al., 2019; De Deyn et al., 2011). The overhead forest canopy closure can filter waterfall and microclimatic conditions that alter stress and the structure of belowground microbial communities (Rosier et al., 2014). Changes in microbial community structure brought about by a range of abiotic and biotic variables will also affect the ecosystem functions microbes carry out, such as nitrogen fixation or carbon mineralisation, and thus functional diversity (Ruuskanen et al., 2018). Studies have shown that manipulating vegetation via restoration interventions can alter plant communities, soil physicochemical properties, and these may interact to drive changes in microbial activity, abundance, diversity within (α-diversity) and between (β-diversity) sites, microbial community and functional group composition (Hu et al., 2022; Qu et al., 2020). The critical roles played by microbial communities suggests further research is necessary to untangle the relative influence of various environmental drivers on microbial community reassembly.

1.1.4 Microbes as "old friends" for human health

Evidence shows that maintaining biodiverse spaces in urban settings is crucial for not only ecosystem health but human health (Liddicoat et al., 2020; von Hertzen et al., 2015). Environmental microbiome exposure helps to shape immune system development, where a lack of contact with naturally diverse green areas compromises immunoregulation (Baruch et al., 2020; Rook, 2013). Microbial diversity in the soil provides greater resistance to the establishment of pathogenic invaders, and their loss links with public health concerns such as the rise of immune-related diseases like allergies, inflammatory conditions, and asthma (Marczylo et al., 2021; van Elsas et al., 2012). Therefore, some microbial species ("old friends") from biodiverse environments provide immunomodulating functions that are lacking in degraded and disturbed environments (Liddicoat et al., 2019; Rook, 2013). For city-dwellers, a local park may be the best or only opportunity for connection with nature, as urban areas are often distinctly lacking in native forest cover (Wallace & Clarkson, 2019). However, parks and residential gardens alone do not restore key ecosystem functions or components (e.g.,

ectomycorrhizal fungi) compared with natural forests and are, in fact, linked with greenhouse gas emissions as well as human pathogen transmission (Delgado-Baquerizo et al., 2021). Humans will derive many benefits from restored urban forest ecosystems – they alleviate mental illness, stress, and disease whilst correlating positively with increased physical exercise and wellbeing benefits via cultural and recreational spaces (Alcock et al., 2014; Coombes et al., 2010; Elmqvist et al., 2015; Maas et al., 2009; Taylor et al., 2017). Human engagement in environmental stewardship is known to correlate with support for pro-environmental and conservation-related initiatives throughout their life, which is increasingly important for forming political decisions in a changing world (Woolley et al., 2021). Considering that urban expansion increases physical and mental risks to human health – due to a loss of human-nature connection – the marriage between ecological degradation and human health crises is clear (Breed et al., 2020). Soil microbiome recovery is, therefore, a very relevant area of research due to its benefits for ecosystem functioning, and human health.

1.2 Research aims and thesis structure

This thesis addresses the need to assess microbial community responses to urban forest restoration. To my knowledge, this research presents the first of its kind to take a multivariate analysis approach uncovering changes in microbial taxonomic composition, diversity, activity, and biomass occurring in microbial communities over a New Zealand-wide chronosequence, with remnant sites for reference. The field sites used in this study were created under the interdisciplinary People, Cities & Nature (PCaN) research programme. Lab analyses were carried out in collaboration with the Integrative Biodiversity Research Centre (iDiv) in Leipzig, Germany. The overarching question of this thesis is: what is the relative importance of forest age since restoration versus environmental variables on soil microbiome responses to a nationwide urban forest restoration chronosequence?

Chapter 2 is an enquiry into the shifting taxonomic composition, diversity, and dissimilarity of bacterial, archaeal, fungal, and protist communities, using metabarcoding data applied to a subset of 19 urban forest sites. I tested the relative importance of forest age since restoration versus a suite of environmental covariates in determining these responses. I sought to find out whether there are notable shifts in

taxonomic composition that could infer a shift in ecosystem function; whether microbial communities become more, less, or variably diverse and dissimilar across the chronosequence; and whether different microbial groups differ in their responses to restoration, considering that lesser-known microbial groups (i.e., archaea, protists) are understudied.

Chapter 3 uses data taken from soil samples across 66 forest sites to measure microbial basal respiration (BR), biomass (Cmic), phospholipid fatty acid analysis (PLFA), and respiration across 15 carbon substrates. My objective was to assess overall microbial activity and broad-scale community composition changes in the biomass of bacterial and fungal communities in response to forest age and environmental heterogeneity within the context of urban forest restoration. In doing so, this chapter elucidates how urban forest restoration efforts drive changes in biomass production of belowground microbial communities, as well as their metabolism of different carbon sources as a measure of microbial ecosystem functioning.

In unison, these chapters shed light on whether forest age since restoration or environmental variability is more important for determining microbial responses to an urban forest restoration chronosequence. Overall, this research presents a holistic assessment of restoration impacts on the soil microbiome and contributes towards under-studied knowledge voids that lay at the intersection of microbial ecology and restoration ecology.

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Chapter 2

Forest age alone does not predict soil microbial recovery after urban forest restoration

2.1 Abstract

Urban areas are heavily modified, degraded natural environments. Restoring forests is vital for maintaining biodiversity and ecosystem service provision in urbanised landscapes. Soil microbiota contribute essential functions that often dictate the survival of native restoration plantings. Yet, our understanding of microbial community responses lags far behind knowledge of aboveground processes during urban forest restoration. In this study, I sampled soils from 19 urban forest sites in eight cities throughout New Zealand, representing a chronosequence of restoration sites ranging from 10 to 48 years of age since planting, including three urban remnant forests for baseline comparisons. I analysed DNA metabarcoding data from my soil samples to quantify the diversity and taxonomic composition of bacteria, fungi, archaea, and protist communities. My analyses investigate the effects of urban forest restoration, alongside a suite of environmental covariates, on microbial community structure and functional group composition. Key findings indicate that restored sites generally showed taxonomic shifts that illustrate forests progressing toward later successional stages. While forest age was important, environmental covariates were more explanatory of trends in microbial diversity. In particular, native woody seedling density and soil pH. My findings suggest that soil microbial community compositional changes are still progressing decades after restoration planting. Specific soil and vegetation traits are more important than forest age alone, and thus can be managed to aid the respective recovery of bacteria, fungi, archaea, and protists in a restored urban forest setting.

2.2 Introduction

Land-use change and degradation caused by anthropogenic pressures can have detrimental impacts on soil microorganisms, leading to biodiversity losses (Bardgett & van der Putten, 2014). Urban settings come with particularly heightened disturbances and alterations in soil physicochemical properties which alter microbial abundance and community composition (Huot et al., 2017; Zhao et al., 2013). Aboveground restoration actions such as forest planting can boost belowground microbial diversity (Liddicoat et al., 2019). Studies have also found that replanting native forest species in urban settings leads to increased abundance and richness of soil microbes (Baruch et al., 2020). This is important because microbial diversity buffers against ecosystem disturbances and maintains ecosystem functions (Singh & Gupta, 2018). Soil microbial biodiversity and composition has reciprocal benefits for the performance of restoration plantings (de Araujo et al., 2018). It is implicitly presumed that the growth of aboveground restoration plantings and increased forest successional time will lead to microbial community recovery (Hart et al., 2020). However, covariations in abiotic and biotic conditions in the highly heterogeneous soil medium have distinct influences on microbial communities that may potentially override the sole effect of forest age since restoration planting (Banning et al., 2011).

Soil microbial communities provide key ecosystem functions that can facilitate native planting success in forest restoration via aboveground-belowground linkages (Harris, 2009). The soil microbiome encompasses myriad organisms in groups such as the bacteria, fungi, archaea and protists (Dunlap, 2001). These soil microorganisms mediate nutrient cycling, decompose organic matter, degrade pollutants, influence soil structural formation, and regulate plant growth via symbiotic relationships (Aislabie et al., 2013; Feeney et al., 2006; Harris, 2009; Selosse et al., 2004; Yan et al., 2020). Therefore, the soil microbial loop is vital for aiding the restoration of plant communities through succession towards a mature or 'climax' ecosystem (de Araujo et al., 2018). Simultaneously, plant communities also affect microbial assemblages via their influences on soil moisture, nutrient content, and through root exudates (Ehrenfeld et al., 2005). The result is a complicated network of ecological linkages and plant-symbiont molecular communication (Ehrenfeld et al., 2005; Singh & Gupta, 2018; Yan et al., 2020). The cascading effects of interactions between aboveground

biota, belowground biota, and ecosystem processes are still being elucidated (Heneghan et al., 2008). Further studies are needed to understand the combined recovery of aboveground-belowground linkages with aboveground restoration plantings (Kardol & Wardle, 2010).

Our knowledge of soil microbial community reassembly following ecological restoration remains opaque, lagging far behind restoration studies of plant communities (Strickland et al., 2017). Plant responses have been studied thoroughly, shedding light on how aboveground restoration interventions determine the trajectory of plant community reassembly (Christian et al., 2015). The soil microbiome itself is rarely the target of restoration. It inherently assumed that in response to restoration plantings, microbial communities will also be restored, reassembling themselves to pre-disturbance ecosystem functioning and taxonomic compositions (Hart et al., 2020; Strickland et al., 2017). This reassembly assumption should be challenged, as microbial communities are highly dynamic and responsive to environmental heterogeneity characteristics of the soil (Beare et al., 1995). Soil heterogeneity can be especially high in urban areas (McPhearson et al., 2010; Pouyat et al., 2007) undergoing restoration, and may largely drive belowground microbial community responses to aboveground forest restoration. Much like plant communities, environmental parameters can predict some of this spatial variation in microbial community composition and diversity (Oliverio et al. ,2020). However, direct comparison of biogeographical patterns in microbes and plants is difficult due to vast differences in size, spatial scaling, taxonomic unit definition – i.e., whether the microbial operational taxonomic unit (OTU) is equal to the definition of species – and sampling accuracy (Martiny et al., 2006; Meyer et al., 2018).

A variety of deterministic and stochastic drivers can individually or conjointly determine microbial colonisation following disturbance (Dini-Andreote et al., 2015). Influential environmental variables explaining microbial community composition and diversity often include plant species composition (Kourtev et al., 2003), climatic factors (Tedersoo et al., 2014), soil properties such as nutrient availability (Zhang et al., 2021a), moisture content (Stefan et al., 2014), and most frequently of all, soil pH (Bartram et al., 2014). Unexplained variation may be attributable to the role of stochastic factors, such as random dispersal,

priority effects (organisms that arrived first are more abundant), previous land use, and propagule size (Estrada-Villegas et al., 2020; Fukami et al., 2010; Powell et al., 2015)

The continuum hypothesis suggests that an integration of niche and neutral drivers contributes toward microbial community assembly (Gravel et al., 2006). Mechanisms of microbial reassembly are difficult to tease apart given the complex interplay between deterministic and stochastic filters occurring at a microscopic scale and the enormous number of possible resultant microbial community configurations (Goss-Souza et al., 2017).

Measures of soil microbial diversity and activity using high-throughput amplicon sequencing may serve as ecological indicators in forest restoration. The identification of microbial OTUs via metabarcoding data can be analysed to approximate functional groups, therefore indicating the provision of soil-related ecosystem functions that are integral to successful terrestrial restoration (Hart et al., 2020; Muñoz-Rojas et al., 201; Yan et al., 2020). The presence of specific microbial taxa can indicate natural versus disturbed ecosystems, where disturbed systems favour opportunistic and pathogenic bacteria that may be harmful to humans (Liddicoat et al., 2019). Restoration chronosequence studies tend to find that as the chronosequence progresses, forest succession is indicated by a shift in taxa from those which can tolerate disturbed conditions to those which require mature forest conditions, i.e., oligotrophic to copiotrophic organisms (Liang et al., 2021). We must further decode the patterns and mechanisms driving microbial community reassembly, especially in an urban context, for microorganisms to be useful as restoration indicators.

Forest restoration variables impact bacteria, fungi, archaea, and protists differently. Though widespread, archaeal communities are less well-studied than bacteria or fungi in restoration studies as they are commonly considered extremophiles (Buée et al., 2009). Archaeal diversity in restored forest soils is often linked to pH and nitrate due to their role in elemental cycling – in comparison to bacteria and fungi, archaea do not tend to be involved in litter decomposition (Manerkar et al., 2008; Yan et al., 2020). Protists have essential roles in forest soils in nutrient turnover and as microbial predators, thus they are likely to increase in abundance as other abiotic and biotic forest components recover (Dai et al., 2021; de Araujo et al., 2018). Protists are also closely linked to soil water holding

capacity (WHC) (Geisen et al., 2017). Bacterial communities tend to become more diverse with forest restoration and be highly influenced by variables like pH and nutrient concentrations (Ma et al., 2021). Fungal diversity tends to increase as the vegetative community matures due to their association with net primary productivity (NPP) as the most common symbionts of trees and decomposers of wood (Aerts & Honnay, 2011; Baldrian, 2017). For that reason, later successional systems tend to show a shift from bacterial to fungal dominance (Ohtonen et al., 1999). Bacterial diversity changes often happen earlier in successional time while fungal diversity changes happen later (Ren et al., 2019) hence microbial groups can display differential responses to restoration plantings. Forest successional age can produce different microbial responses depending on the context; for example, Liu et al. (2019) found bacterial diversity decreased with time since active restoration planting, while Zhang et al. (2016a) found bacterial diversity increased under natural succession. In general, forest restoration enhances soil microbial biodiversity and thus multifunctionality (Crouzeilles et al., 2016; Delgado-Baquerizo et al., 2020; Meli et al., 2017; Shi et al., 2021).

For this chapter, I assessed soil microbial community development along a planting chronosequence using a space-for-time approach, whereby different sites represent various ages along a restoration chronosequence (Walker et al., 2010). My research looks at 16 urban forest sites where complete forest ecosystem reconstruction had occurred 10-48 years prior. I also included three reference urban forest patches for comparison, which were remnant forest patches. Forest sites were also distributed spatially across the latitude of Aotearoa New Zealand in order to incorporate a wide range of environmental heterogeneity along a spatial and temporal chronosequence.

This chapter investigates 1) whether diversity and taxonomic composition (i.e., relative abundance of OTUs) of microbial communities change with time since restoration, and 2) whether environmental properties (abiotic and biotic) influence the successional development of microbial communities over time. I hypothesise that different microbial groups will exhibit different responses to aboveground restoration planting due to their different ecological niches. Specifically, I predict that microbial diversity will increase non-linearly with forest age (as measured in time since initial restoration planting) in bacteria,

fungi, archaea, and protists, as an indication of reduced biotic homogenisation and greater niche specialisation in microbial communities as forests mature and become more complex (Goss-Souza et al., 2017). I hypothesise that fungi, in particular, will show increased diversity with forest age, as they are later-successional microorganisms (Morriën et al., 2017). Likewise, I predict that community compositional changes in bacteria, fungi, archaea, and protists will shift from taxa known to inhabit disturbed areas, towards an increasing relative abundance of taxa known to prefer less degraded, mature forest conditions (Liang et al., 2021). Furthermore, I expect to find strong effects of environmental properties (abiotic and biotic) on microbial diversity. I predict that pH and nutrient data will drive bacterial and archaeal diversity, fungal communities will be strongly predicted by plant community factors, and protists will be associated with soil water-holding capacity (WHC). Studying these microbial groups together allows us to compare the responses of different taxa under the same biotic and abiotic conditions in forest age. This information will further basic knowledge of succession theory for soil microbial communities and can be applied by restoration managers in understanding the impact of management of the belowground community.

2.3 Methods

2.3.1 Study sites and experimental design

I carried out a field survey in 16 actively restored urban forest patches aged from 10 – 48 years, and three urban forest remnants. Over the Aotearoa New Zealand summer of November 2019 – February 2020, soil samples were taken from these forest patches, which had been fully replanted with native plantings (Table 2.1). My study sites were located throughout eight cities across both the North and South Island of the country, with two or three sites per city (Fig. 2.1; Table 2.1). Each study site was a forest patch that hosted a rectangular forest plot 10 x 20m (200m^2 area), established by the Ministry of Business, Innovation and Employment-funded research programme People, Cities, and Nature (see Busbridge, 2020). Plots were placed so that the edge of the rectangular plot was always >1m from the edge of the forest patch and slope not exceeding 10 degrees. The sampling design accounted for spatial variability in the soil. Each rectangular plot contained five

square subplots at each of the four corners and the centre point of the plot, which were used to collect environmental data (Fig. 2.2). My soil sampling took place from three subplots spaced evenly along a diagonal transect, including a corner, central point, and the opposite corner of each plot.

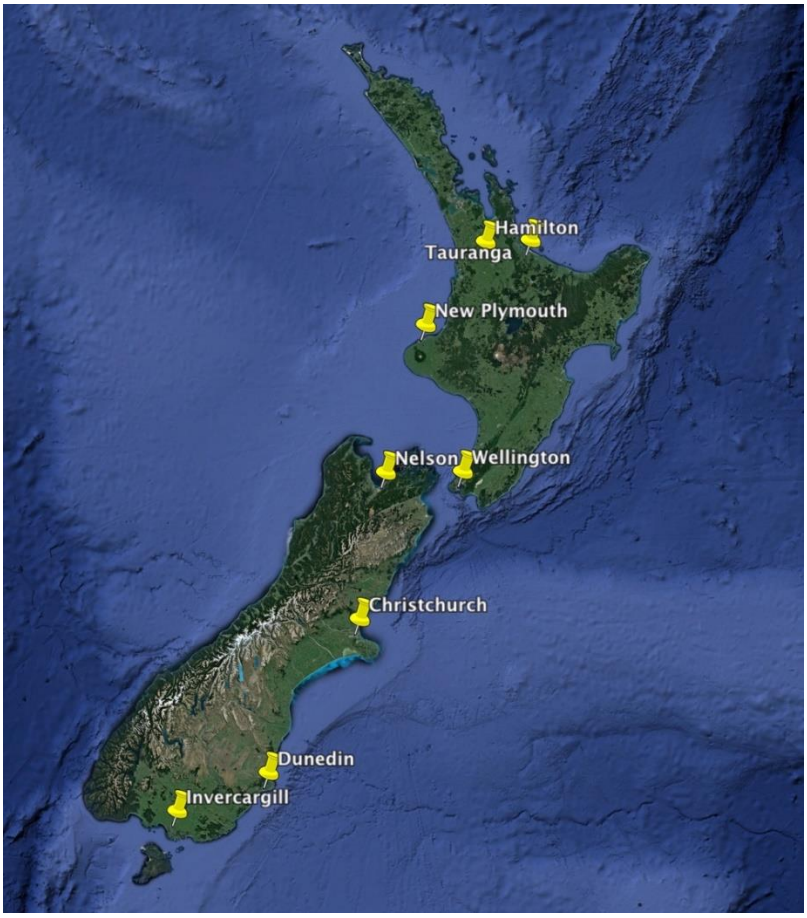

Figure 2.1. The 8 New Zealand cities selected for site establishment. Image captured from Google Earth.

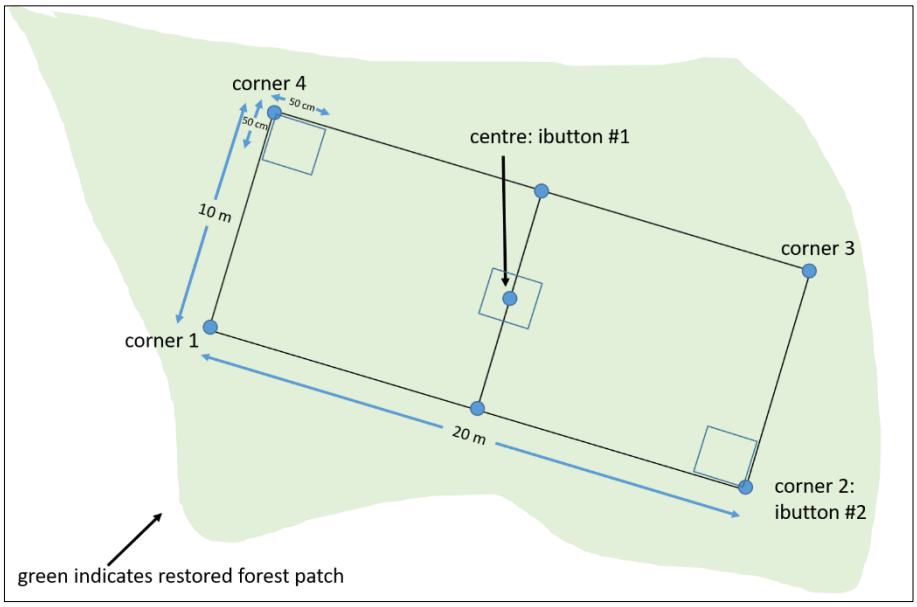

Figure 2.2. People, Cities, & Nature research plot layout within a hypothetical restored forest patch. Small squares represent subplot locations for soil core sampling.

Table 2.1. New Zealand restored urban forest study sites used for field observations and relevant site information.

Forest age (years)	Site name	City	Year planted	Patch size (ha)	GPS coordinates (latitude-longitude)
10	McCardles Bush	Tauranga	1987	9.86	-37.6963222 S 176.1472382 E
10	Murphy Reserve	Nelson	2010	1.65	-41.2851917 S 173.2636194 E
10	Old Chest Hospital	Wellington	2010	3.19	-41.3074833 S 174.7861389 E
11	Carmichael Playground	Tauranga	2009	1.36	-37.68802054 S 176.1214438 E
11	Island Park	Dunedin	2009	77.30	-45.92413584 S 170.4107913 E
13	Waihopai River	Invercargill	2007	0.10	-46.3891000 S 168.3498556 E
31	Signal Hill	Dunedin	1989	148.00	-45.85738857 S 170.5480194 E
14	Avalon Drive	Hamilton	2006	0.46	-37.7704167 S 175.2414111 E
14	Peringa Park	New Plymouth	2006	1.19	-39.0424722 S 174.1118167 E
14	Marshland Road	Christchurch	2006	0.49	-43.4511917 S 172.6603583 E

23	Estuary Walkway	Invercargill	1997	0.99	-46.4274222 S 168.3432583 E
28	Mt Albert	Wellington	1992	7.34	-41.3291861 S 174.7820889 E
31	Bob's Track	Nelson	1989	5.00	-41.2824778 S 173.2571056 E
40	Minogue Park	Hamilton	1980	2.44	-37.7738167 S 175.2497306 E
41	Riccarton Bush	Christchurch	1979	9.65	-43.5293472 S 172.5943528 E
48	Huatoki Walkway	New Plymouth	1972	30.00	-39.0836139 S 174.0763111 E
NA	Claudelands Bush	Hamilton	Remnant	6.05	-37.462743 S 175.172492 E
NA	Kew Bush	Invercargill	Remnant	3.79	-46.262653 S 168.212956 E
NA	Otari Wilton Bush	Wellington	Remnant	237.00	-41.160020 S 174.453053 E

2.3.2 Aboveground data

Forest age was the number of years between initial restoration planting and the year data was collected (2020). Vegetation surveys were carried out beforehand to collect data on the plant community in each plot (Busbridge, 2020). Adult tree species richness/diversity in the plots ranged from 1 to 15. Seedling density was recorded in 2019 as the total number of woody species under 135cm height within ten circular subplots of 1.5m radius, covering a total 70.7m^2 of the plot, and scaled up to approximate seedling density per 200m^2. Adult tree species richness was a tally of all the different native and exotic adult tree species present in each plot which had a diameter at breast height (DBH) >2.5cm. Mean tree basal area per plot was summed as the cross-sectional trunk area measured by DBH in centimetres squared (cm^2). Tree density was a tally of trees occurring in each plot (200m^2), calculated per 100m^2. Herbaceous cover encompassed the plot mean percentage of herbaceous cover, calculated by visually estimating the % cover from 8 subplots which added up to the entire plot (200m^2). Leaf litter depth (i.e., the dead layer on the forest floor) was measured to the nearest centimetre at five points in all four corner subplots and the centre subplot. Canopy openness was measured as an index of light availability and microclimate formation and shown as a mean percent value per plot. Canopy openness was calculated as a single plot value from measurements at all four plot corners and the centre

three times over 12 months (at 0, 6, and 12 months) with a convex Spherical Crown densiometer at breast height (~1.3m above the ground). Plot mean air temperature was recorded every four hours for approximately 12 months using HOBO data loggers (model MX2301A; Onset, Cape Cod, Massachusetts, USA) contained in a radiation shield and placed on a tree ~1m above the ground at the centre point of each plot.

2.3.3 Belowground data

Soil pH and nutrient data were measured at the German Centre for Integrative Biodiversity Research (iDiv) in Leipzig, Germany. pH was measured using the Orion Star™ A211 pH meter. The Orion™ ROSS Ultra™ Triode™ pH/ATC combination electrode was calibrated with three buffers at pH 4, 7, and 10 prior to use. Ten grams of air-dried soil was shaken with 25mL of calcium chloride, left to settle for ≥1 hour, and immersed in the pH-electrode until a stable measurement was displayed and recorded. Carbon and nitrogen concentrations were measured as per the methods of Ferlian et al. (2017). Soil samples were first dried at 60°C for 72 h, ground using a ball mill, then dried for a further 24 h, and deposited in tin capsules. Analyses were run on an elemental analyser (Vario EL II, Elementar Analysensysteme GmbH, Hanau, Germany), with C and N concentrations recorded as relative mass (%) per sample mass, respectively. Mass proportions of C and N were used to calculate C:N ratios. A soil corer of 2.7cm diameter was used to take five core samples to a depth of 10cm from each subplot to collect soil microorganisms. The resulting 15 cores were pooled to create a composite sample representing each plot's microbial community heterogeneity. From each pooled sample 150g of fresh soil was sieved through 2mm metal mesh sieves and stored at -80°C. Samples were shipped on dry ice at -80°C to iDiv for further lab analyses.

2.3.4 Metabarcoding via high-throughput amplicon sequencing

For analysing taxonomic composition of the urban restored forest soil microbiome, metabarcoding data was collected from all 19 samples at the Helmholtz Centre for Environmental Research in Halle, Germany. DNA was extracted from samples using the Qiagen PowerSoil kit. Microbial DNA was amplified via PCR and identified via an Illumina MiSeq sequencer using marker ribosomal RNA gene regions, including 16S rRNA (for

bacteria and archaea), ITS1 (fungi), and 18S rRNA (protist) regions. Bacterial 16S (SSU) rRNA was amplified using 7.5 µl of 2x KAPA HiFi HotStart Ready Mix dissolved in 5.9 µl nuclease-free water with 1 µl template DNA and 0.3 µl respectively of 10 µM 515f forward primer (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 reverse primer (5'-GGACTACHVGGGTWTCTAAT-3'). The thermal programme was as follows: initial denaturation of 95°C for 5 minutes, 25x PCR cycles were run with a denaturation temperature of 98°C for 20 seconds, annealing at 55°C for 15 seconds, and elongation at 72°C for 15 seconds, followed by 5 minutes of final extension at 72°C and storage at 10°C. The ITS1 region of the fungal rRNA operon was amplified with a PCR mixture containing 10.00 µl of 2.5x 5 Prime Hot Master Mix dissolved in 13 µl nuclease free water, 1.00 µl template DNA and 0.5 µl of 10 µM ITS1f forward primer (5'-CTTGGTCATTTAGAGGAAGTAA-3') combined with 0.5 µl of 10 µM ITS2 reverse primer (5'-GCTGCGTTCTTCATCGATGC-3'). The PCR program amplified sequences at an initial denaturation of 94°C for three minutes, followed by 35x cycles of 94°C denaturation for 45 seconds, 50°C annealing for 1 minute, 72°C elongation for 1.5 minutes, and concluded with extension at 72°C for 10 minutes. Protist 18S (SSU) rRNA regions were amplified with a PCR mix containing 10 µl of 2x KAPA HiFi HotStart ReadyMix and 1 µl of diluted DNA extract in 13 µl nuclease-free water, with 0.5 µl of 10 µM 1319f forward primer (5'-GTACACACCGCCCGTC-3') and 0.5 µl of 10 µM 1510r reverse primer (5'-TGATCCTTCTGCAGGTTCACCTAC-3'). PCR amplification was conducted with an initial denaturation of 94°C for 3 minutes, followed by 35x cycles of 94°C denaturation for 45 seconds, 50°C annealing for 1 minute, 72°C elongation for 1.5 minutes, and finally, extension at 72°C for 10 minutes. Finally, the 16S (SSU) rRNA region of archaea was amplified with a PCR mixture containing 10.00 µl of 2x KAPA HiFi HotStart ReadyMix dissolved in 8.6 µl nuclease-free water, 1.00 µl template DNA, 0.2 µl of 10 µM SSU1ArF forward primer (5'-TCCGGTTGATCCYGCBRG-3'), and 0.5 µl of 10 µM SSU520R reverse primer (5'-GCTACGRRYGYTTTARRC-3'). The PCR program amplified sequences at an initial denaturation of 95°C for 2 minutes, 30x cycles of 95°C denaturation for 20 seconds, 55°C annealing for 15 seconds, 72°C elongation for 30 seconds, and finally, extension at 72°C for 5 minutes.

2.3.5 Bioinformatic processing

Sequencing reads were rarefied to an even depth (Fig. S1) and then processed to amplicon sequence variants (ASVs) using the DADA2 (Callahan et al., 2016) based pipeline dadasnake, version 0.10 (Weißbecker et al., 2020) (Fig. S1). The consensus sequence of each ASV was aligned by BLASTn against databases accessed from NCBI (Table S1). Consensus taxonomy was parsed using BASTA (Kahlke & Ralph, 2019). In addition, taxonomic assignments were performed using the mothur implementation of the Bayesian classifier (Schloss et al., 2009) against suitable databases. Reads of ≥97% sequence similarity were clustered for analyses.

The Mothur.SILVA database outputs were used to determine bacteria and archaea taxonomy, whilst fungi and protists were characterised using mothur.unite and mother.PR, respectively. All outputs were edited to remove any non-target taxa.

2.3.6 Statistical analysis

Statistical analyses were performed in R version 4.0.5 (R Core Team, 2021). All analyses were repeated for bacteria, fungi, archaea, and protists. The phyloseq package (Bioconductor v3.12) was used to create phyloseq objects composed of an OTU matrix, taxonomy table, and environmental data to analyse and visualise the influence of planted urban forest restoration age and environmental variability on microbial community composition and diversity.

I quantified the mean relative abundance of bacteria, archaea, fungi and protists OTUs to compare taxonomic shifts across the restoration chronosequence. OTUs with no assigned phylum were removed before generating graphs of mean relative abundance at the phylum level. I then removed the top 9, 2, and 4 most common phyla of the bacteria, archaea, and protists (respectively) to visualise trends in lesser phyla that were masked by the dominant phyla.

Non-metric multidimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarity were used to visualise microbial community composition among restoration sites of varying ages, with the three remnant sites displayed for reference. To statistically test for the effects of forest age alongside the effects of environmental characteristics of restoration sites on

the composition of microbial communities, I used permutational multivariate analysis of variance (PERMANOVA) set to 1000 permutations using the 'adonis' function in the 'vegan' R package (v2.5-7). PERMANOVA outputs allowed me to interpret the β-diversity (i.e., microbial community dissimilarity) between sites. This was done for each of the four major microbial taxa (bacteria, archaea, fungi, protists) by first constructing a maximal model that included forest age along with ten measured environmental variables as predictors: soil pH, soil C:N, soil water-holding capacity (WHC), plot seedling density, tree species richness, mean plot tree basal area, tree density per $100m^2$, herbaceous (herb) cover, canopy openness, and mean annual plot air temperature. Model simplification was then performed by removing non-explanatory predictors based on the adjusted R^2 values, until a minimal adequate model was reached.

Four indices were used as assessments of α-diversity on OTUs for all four microbial groups, including observed OTU richness, Shannon diversity, Chao1, and Simpson diversity. I constructed general linear models (glms) whereby the minimal adequate models included only forest age as a key predictor, and the maximal models included the ten aforementioned environmental variables in addition to forest age. Non-explanatory environmental variables were removed in succession via R^2 values to reach a minimal adequate model. All models testing observed OTU richness were modelled on a quasipoisson distribution to account for overdispersed count data.

2.4 Results

2.4.1 Microbial diversity across the restoration chronosequence

A total of 17,735 quality-filtered sequences belonging to 39 phyla were found for bacteria, 14,010 OTU reads from 8 phyla for archaea, 5056 quality-filtered sequences from 4 phyla for fungi, and 9865 sequences across ten phyla were found for protists. Forest age since aboveground restoration planting was not a significant predictor for bacteria or archaea across all four measures of diversity (Fig. 2.3; Table 2.2). Protist Shannon (P = 0.00171) and Simpson (P = 0.0135) diversity had an inverse relationship with forest age. In fungi, forest age was associated with a decrease in Chao1 (P = 0.0123), Shannon (P = 0.0428) and

Simpson (P = 0.0352) diversity indices (Table 2.2). The highest OTU richness across the chronosequence was observed in bacteria, followed by protists, fungi, and then archaea.

In response to testing environmental covariates, I found that an increase in pH was a significant predictor of increased diversity in bacteria (P = 0.0185), fungi (P = 0.0233), and protists (P = 0.000137) (Table 2.2). Archaeal diversity was also predicted by increased mean plot air temperature (P = 0.00199) and seedling density (P = 0.0154) (Table 2.2). Higher tree density, mean tree basal area, and tree species richness as well as lower canopy openness and lower seedling density were significant predictors of fungal diversity. Protist diversity was also predicted by decreased WHC, canopy openness, and increased soil C:N.

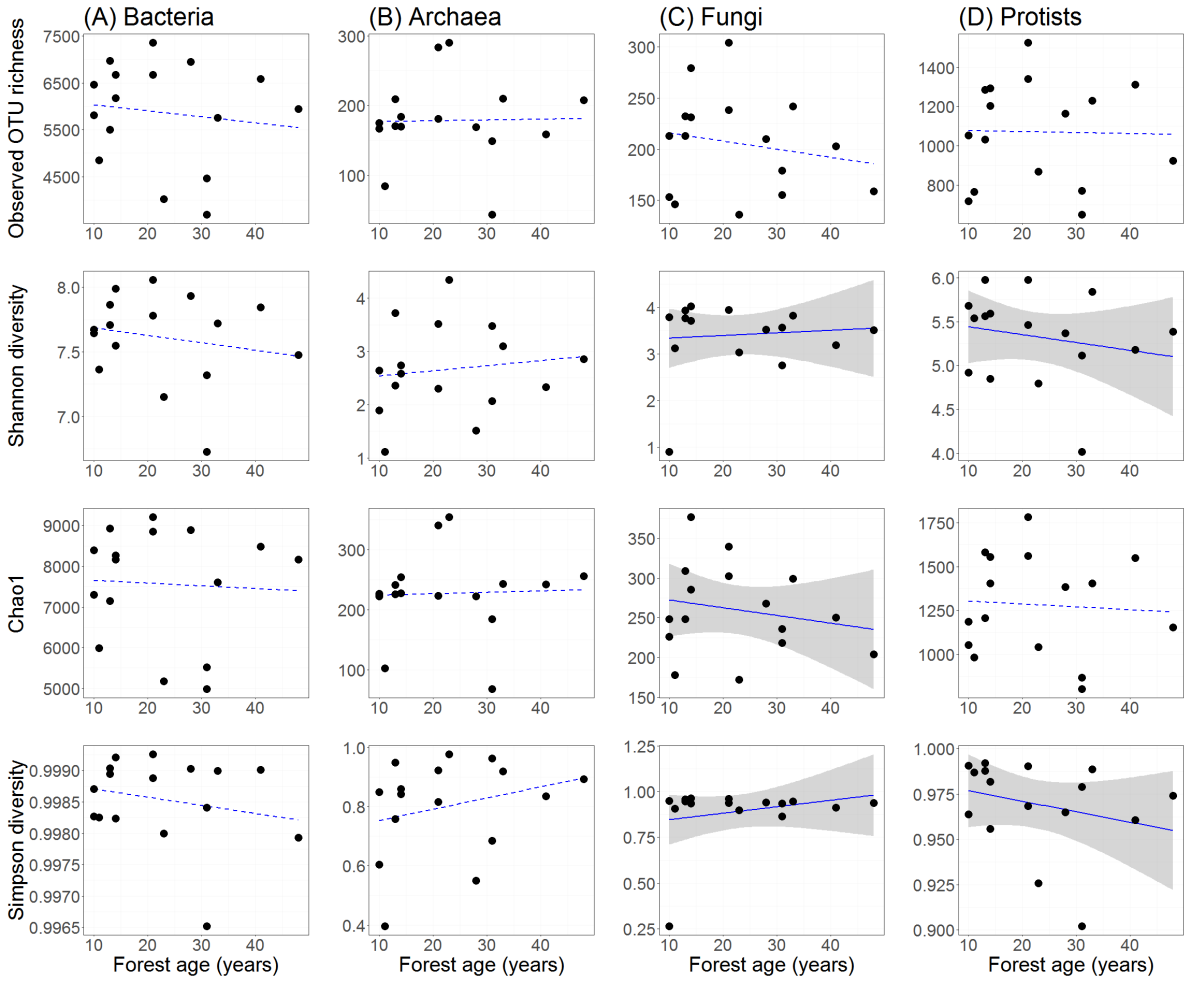

Figure 2.3. Four measures of alpha diversity of microbial communities in response to forest age since restoration. Columns show (left to right) bacteria, archaea, fungi, and protists, whilst rows show (top to bottom) Observed OTU richness, Shannon, Chao1, and Simpson indices of diversity. Dotted lines denote non-significant relationships while solid lines denote significant relationships

between forest age and diversity. Shaded areas show the 95% confidence interval for the significant relationships.

Table 2.2. Minimal general linear model summary statistics for a) bacteria, b) archaea, c) fungi, and d) protists for the relationship between forest age and each of four diversity measures: observed OTU richness, Shannon, Chao1, and Simpson. All minimal adequate models retain forest age in order to test the main hypothesis. Bold values indicate statistically significant relationships ($P < 0.05$).

Model	Estimate	Std. Error	t value	*P*
a) Bacteria				
Observed OTU richness				
Forest age	-0.002206	0.004295	-0.514	0.616
Shannon				
Forest age	-0.007291	0.005315	-1.372	0.195
pH	0.200730	0.073747	0.29860	**0.0185**
Chao1				
Forest age	17.31	34.63	0.500	0.626
Herb cover	18.38	12.06	1.524	0.151
Simpson				
Forest age	-1.284e-05	9.587e-06	-1.339	0.205
pH	3.201e-04	1.330e-04	2.407	**0.0331**
b) Archaea				
Observed				
Forest age	0.001227	0.005715	0.215	0.833
Air temp	0.146666	0.038042	3.855	**0.00199**
Shannon				
Forest age	8.907e-03	1.561e-02	0.571	0.578
Seedlings	1.462e-04	5.244e-05	2.789	**0.0154**
Chao1				
Forest age	0.3552	1.1891	0.299	0.77
Air temp	27.1129	7.6390	3.549	**0.00356**
Simpson				
Forest age	0.006157	0.003564	1.727	0.108
Canopy openness	0.004039	0.002148	1.880	0.0827
c) Fungi				
Observed				
Forest age	-0.0049408	0.0044559	-1.109	0.291
pH	0.1554928	0.0590594	2.633	**0.0233**
Tree density	0.0021376	0.0008547	2.501	**0.0295**
Shannon				
Forest age	-7.974e-02	3.436e-02	-2.320	**0.0428**
Seedlings	-3.127e-04	1.401e-04	-2.233	**0.0496**
Tree species richness	1.341e-01	5.707e-02	2.350	**0.0406**
Canopy openness	-3.456e-02	1.329e-02	-2.601	**0.0265**
Basal area	1.504e+02	6.264e+01	2.402	**0.0372**
Chao1				
Forest age	-4.269e+00	1.401e+00	-3.048	**0.0123**
Seedlings	-2.277e-02	7.735e-03	-2.944	**0.0147**
Tree species richness	7.784e+00	2.744e+00	2.837	**0.0176**
Basal area	8.496e+03	3.363e+03	2.526	**0.0301**
Tree density	6.437e-01	2.558e-01	2.517	**0.0306**

Model	Estimate	Std. Error	t value	*P*
Simpson				
Forest age	-1.588e-02	6.524e-03	-2.435	**0.0352**
Seedlings	-5.961e-05	2.659e-05	-2.242	**0.0488**
Tree species richness	2.846e-02	1.084e-02	2.627	**0.0253**
Canopy openness	-9.098e-03	2.523e-03	-3.606	**0.0048**
Basal area	3.265e+01	1.189e+01	2.745	**0.0206**
d) Protists				
Observed				
Forest age	-0.003307	0.004870	-0.679	0.5100
pH	0.161723	0.065864	2.455	**0.0303**
Shannon				
Forest age	-0.022323	0.005068	-4.405	**0.00171**
WHC	-0.025407	0.008036	-3.162	**0.0115**
pH	0.614049	0.097065	6.326	**0.000137**
Canopy openness	-0.010669	0.002930	-3.641	**0.00539**
C:N	0.169319	0.030048	5.635	**0.000320**
Chao1				
Forest age	-4.760	5.622	-0.847	0.413
pH	196.794	78.004	2.523	**0.0268**
Simpson				
Forest age	-0.0009825	0.0003209	-3.062	**0.0135**
WHC	-0.0017227	0.0005088	-3.386	**0.00805**
pH	0.0185430	0.0061456	3.017	**0.0145**
Canopy openness	-0.0004749	0.0001855	-2.560	**0.0307**
C:N	0.0048460	0.0019024	2.547	**0.0313**

2.4.2 Microbial taxa relative abundance across the restoration chronosequence

Visual inspection of plotted relative abundances indicated a decline in the mean relative abundance of Bacteroidota and less obviously in Chloroflexi, Actinobacteriota, and Myxococcota with increasing forest age (Figure 2.4A). In contrast, Acidobacteriota and rarer taxa (>1% relative abundance, grouped as 'Other') appeared to become more relatively abundant across the forest age chronosequence (Figure 2.4A). The most dominant bacterial phyla across all sites included the Proteobacteria, Actinobacteriota, and Acidobacteriota.

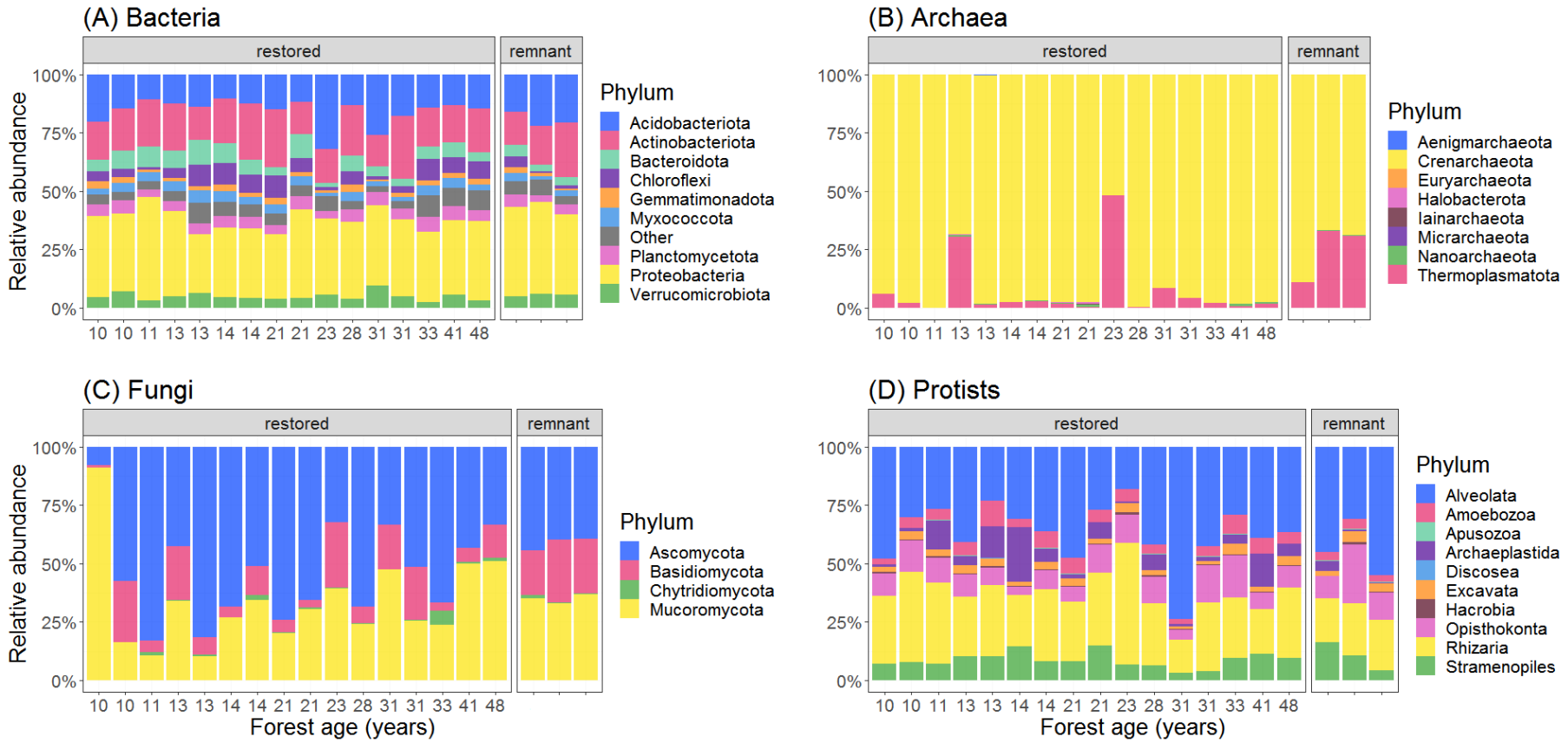

Figure 2.4. Relative abundance of A) top 10 most numerous bacterial phyla (with the 10th category classified as 'Other' to encompass all remaining taxa), B) all 8 detected archaeal phyla, C) all 4 detected fungal phyla, and D) all 10 detected protist phyla across the restored urban forest chronosequence. Time since aboveground restoration planting is shown on the x axis, increasing in age from left to right, with remnant forest values shown on far right for comparison.

The Crenarchaeota phylum was dominant for archaea, generally representing over 90% of reads in younger sites, but far less abundant remnant stands (Fig. 2.4B). Thermaplasmatota represented a much greater proportion of archaeal community composition in remnant forests compared to the restored forests (Fig. 2.4B). The remaining six phyla detected made up a small (<1%) proportion of the total reads.

The dominant fungal phyla across all sites were the Ascomycota, Mucoromycota, and Basidiomycota, while the less relatively abundant Chytridiomycota phylum encompassed any remaining taxa (Fig. 2.4C). Mucoromycota showed a general trend of increasing in relative abundance in older and remnant forests. However, a particularly high relative abundance of Mucoromycota dominated most fungal sequences at a 10-year site (NelMRY) (Fig. 2.4C). The Ascomycota dominated OTU reads in younger sites, became less abundant relative to other taxa with increasing forest age, and then relative abundance of different phyla (Ascomycota, Basidiommycota, and Mucoromycota) approximated a more even composition across remnant forests than compared to the rest of the chronosequence (Fig. 2.4C). The Basidiomycota varied but tended to generally increase across the chronosequence, whilst the Chytridiomycota represented a small percentage of total reads that did not show a clear relationship with age (Fig. 2.4C).

Alveolata and Rhizaria were the most common protist phyla across our restoration chronosequence (Fig 2.4D). The Opisthokonts tended to show a general increase in relative abundance with increasing age, whereas Archaeplastida and Rhizara decreased. The Alveolata, Amoebozoa, Hacrobia, and Stramenopiles were represented fairly evenly across the chronosequence with slight variations in relative abundance (Fig 2.4D). Apusozoa and Excavata were slightly more relatively abundant in older and remnant forests, but significant changes were not obvious on a whole community level visualisation (Fig. 2.4D).

A variety of trends were notable from the minor bacterial, archaeal, and protist phyla, when the more dominant phyla were removed (Fig. 2.5). In the bacteria, Armatimonadota, Bdellovibrionotam, Dependentiae, Elusimicrobiota, and Fibrobacterota appeared to decrease, on average, in abundance across the chronosequence (Fig. 2.5A). The Dadabacteria appeared in a younger forest, while Campilobacterota appeared in both a

young and remnant forest. Calditrichota, Entotheonellaeotama, and Zixibacteria appeared in older forests, and the Firmicutes were most relatively abundant around the 14-year mark (Fig. 2.5A). GAL15 appeared in older and remnant forests, while the Methylomirabilota, NB1-j, Nitospirota phyla were most relatively abundant in older sites and decreased again in remnant sites (Fig. 2.5A). Cyanobacteria, Desulfobacterota, FCPU426, Latescibacterota, MBNT15, Sumerlaeota, WPS-2, WS2 were, similarly, variable across the chronosequence (Fig. 2.5A). Patescibacteria were dominant in younger forests and became less dominant in oldest and remnant forests (Fig. 2.5A). Finally, RCP2-54 increased in relative abundance across the chronosequence and was more commonly represented in remnant forest patchess (Fig. 2.5A).

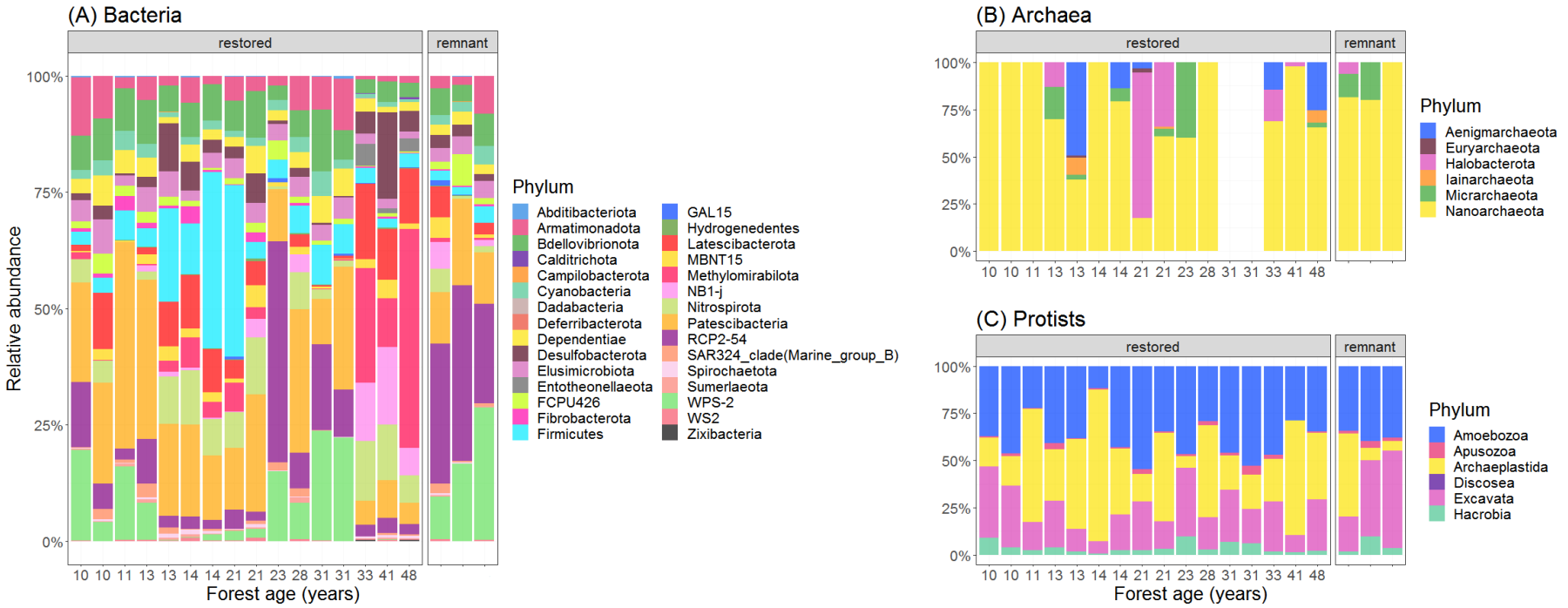

Figure 2.5. Relative abundance of the less-detected A) bacteria, B) archaea, and C) protist phyla (dominant phyla removed) across the restored urban forest chronosequence. Fungal phyla are not visualised here because all fungal phyla are clearly represented in Figure 3. Time since aboveground restoration planting is shown on the x axis, increasing in age from left to right, with remnant forest values shown on far left for comparison.

Nanoarchaeota was the third most common archaeal taxa and was present in all sites except the two 31-year-old sites (Fig. 2.5B). Halobacterota appeared in sites over 13 years and decreased across the chronosequence, whilst the Micrarchaeota appeared in sites over 13 years, and then became more relatively abundant in older and remnant sites (Fig. 2.5B). The Euryarchaeota was only detected in one site, and likewise, the Iainarchaeota was only obvious in a few sites (Fig. 2.5B). The Aenigmarchaeota decreased in relative abundance from 13-year to 48-year-old sites and were not represented in remnant sites (Fig. 2.5B).

The protist group Archaeplastida appeared less relatively abundant in remnant sites compared to the restored sites (Fig. 2.5C). The Amoebozoa, Apusozoa, and Hacrobia did not show obvious trends in mean relative abundance with forest age (Fig. 2.5C). Discosea were barely represented, whilst Excavata were more relatively abundant in two of the three remnants compared to the restored sites (Fig. 2.5C).

2.4.3 Microbial community composition across the restoration chronosequence

Microbial communities did not appear to show distinct clustering related to forest age (Fig. 2.6). However, the remnant mature forest communities were more clustered (Fig. 2.6). Soil pH had a significant effect on the dissimilarity of bacterial ($P = 0.0001$, $R^2 = 0.015$), fungal ($P = 0.014$, $R^2 = 0.099$), and protist ($P = 0.0001$, $R^2 = 0.11$) communities among restoration sites (Table 2.3). Seedling density per plot was also found to explain a significant proportion of variation in bacteria ($P = 0.0001$, $R^2 = 0.12$), archaea ($P = 0.004$, $R^2 = 0.12$), and protist community composition ($P = 0.009$, $R^2 = 0.095$) (Table 2.3). Forest age was explanatory for bacteria ($P = 0.023$, $R^2 = 0.11$) and archaea ($P = 0.025$, $R^2 = 0.13$), marginally significant as a predictor for fungi ($P = 0.065$, $R^2 = 0.087$), and not related to protists ($P = 0.321$, $R^2 = 0.068$) (Table 2.3).

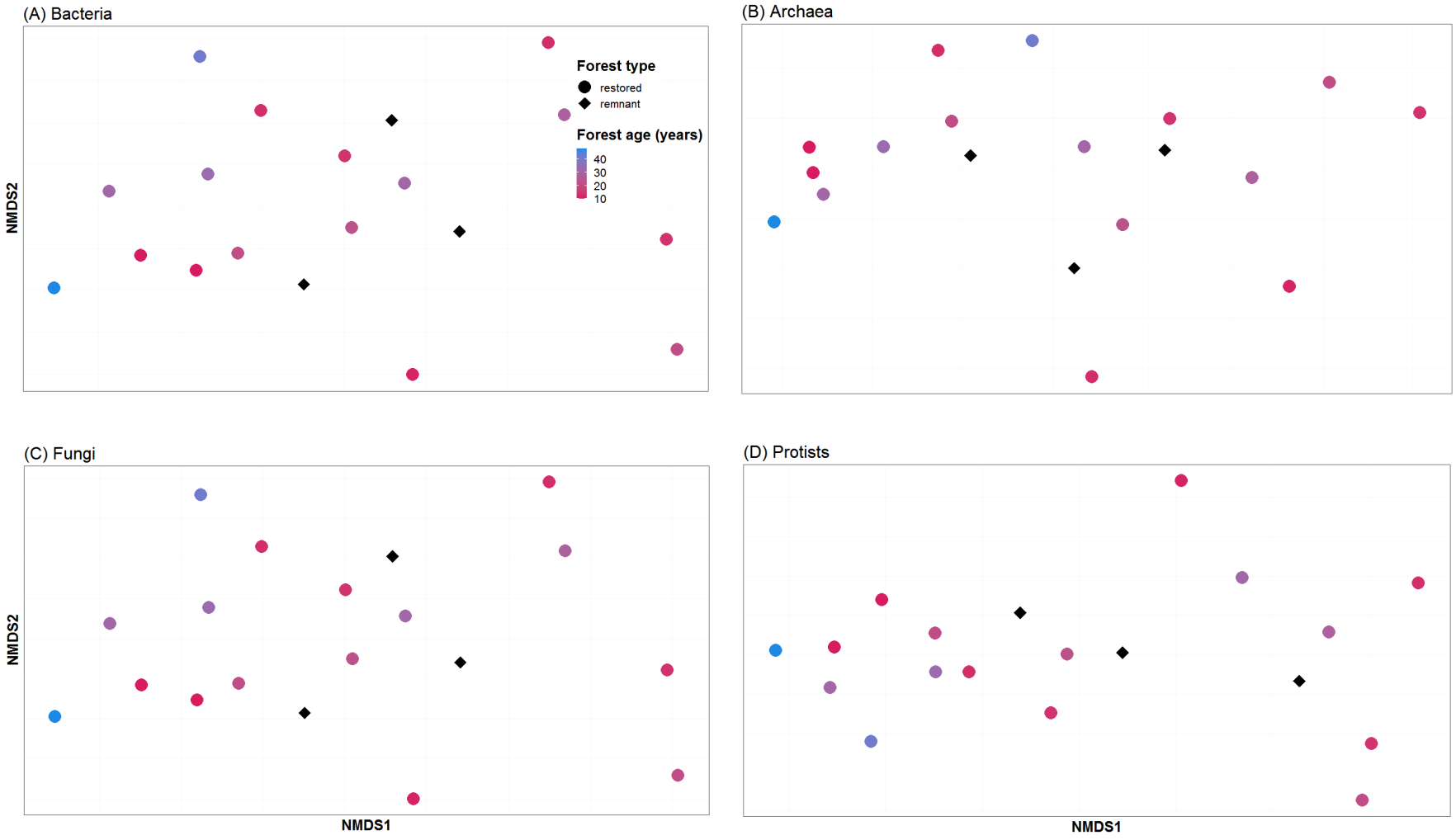

Figure 2.6. NMDS ordination figures of A) bacteria, B) archaea, C) fungi, and D) protist community compositions as related to forest age (shown by a blue (young) to pink (old) colour gradient, denoted with circles). Remnant forest patches are denoted with diamonds.

Table 2.3. Permutational multivariate analysis of variance of OTUs derived from 16S, 18S, and ITS gene data of bacteria, archaea, fungi, and protists. Bold values indicate statistically significant relationships ($P < 0.05$).

Model	df	SS	MS	F-value	R^2	*P*
Bacteria						
Age	1	0.3910	0.39099	1.95	0.104	**0.0229**
Seedlings	1	0.4734	0.47344	2.37	0.13	**0.000999**
Plot mean air temperature	1	0.3329	0.33293	1.66	0.088	**0.0489**
pH	1	0.5752	0.57522	2.88	0.15	**0.000999**
Residuals	10	1.9993	0.19993		0.53	
Total	14	4.1418			1.00	
Archaea						
Age	1	0.6503	0.65030	2.01	0.13	**0.025**
Seedlings	1	0.6454	0.64539	2.00	0.12	**0.004**
Residuals	12	3.8819	0.32349		0.75	
Total	14	5.1776			1.00	
Fungi						
Age	1	0.4118	0.41182	1.4	0.087	0.0649
Plot mean air temperature	1	0.4379	0.43786	1.49	0.092	**0.37**
Herb cover	1	0.4812	0.48118	1.63	0.101	**0.00799**
pH	1	0.4680	0.46798	1.59	0.099	**0.014**
Residuals	10	2.9472	0.29472		0.62	
Total	14	4.47461			1.00	
Protists						
Age	1	0.2986	0.29860	1.05	0.069	0.321
Seedlings	1	0.4152	0.41518	1.45	0.095	**0.00899**
pH	1	0.5023	0.50233	1.76	0.12	**0.000999**
Residuals	11	3.1416	0.28560		0.720	
Total	14	4.3577			1.00	

df = degrees of freedom, SS = sums of squares, MS mean squares.

2.5 Discussion

My research reveals the responses of soil bacteria, fungi, archaea, and protists to a nationwide urban forest restoration chronosequence. Overall, the chronosequence exhibited a soil microbiome that showed some shifts with increasing forest age. I observed phylum-level microbial community shifts as aboveground planted conditions changed from more recently replanted habitats to maturing plant communities. I speculate that as time after initial restoration plantings increased, organic matter accumulated and enhanced the soil carbon profile to produce mature forest soil conditions, as occurs in most native forest successional trajectories (George et al., 2010; Liddicoat et al., 2019).

Interestingly, time since initial restoration plantings did not appear to be the main factor determining microbial community composition and diversity. Environmental factors such as soil pH were often found to be strong drivers of microbial community structure, suggesting that restoration management and key environmental variables could be good indicators of soil microbiota responding to restoration interventions (Wallace et al., 2022). Finally, remnant forest community composition generally differed from restored sites, suggesting that other factors such as land-use legacies or forest maturity levels also drive successional processes in microbial community composition.

2.5.1 Soil microbial diversity in maturing restored forests

I analysed soil microbial α-diversity and β-diversity along the forest restoration chronosequence to determine whether microbial diversity trends are related to forest age or other environmental variables. Fungal and protist diversity increased with forest age, whereas bacterial and archaeal diversity showed no relationship. Bacterial and archaeal community dissimilarity was predicted by age, while fungal and protist communities were not. It is clear that certain environmental factors like soil pH and seedling density drove the diversity of major microbial taxa, which suggests that soil variables and restoration interventions to the plant community also affect microbial responses (Zhang et al., 2021b). Soil pH has consistently been the best predictor for microbial community composition

(Bartram et al., 2014; Fierer & Jackson, 2006). Microbial community structure is more often linked to environmental and soil physicochemical properties than climatic factors or geographic distance between communities, and this has been noted in urban and non-urban ecosystems (Ramirez et al., 2014).

Native woody seedling density was positively related to bacteria, archaea, and protists on the β-diversity level and remained related to increased archaea and fungi communities at the α-diversity level. Seedling regeneration and recruitment is a pivotal process in forest succession. There is likely a cyclical relationship whereby microbial communities positively influence seedling density while seedling density impacts the abundance and spread of soil microbes (Bagchi et al., 2014; Bell et al., 2006; Chanthorn et al., 2013; Long et al., 2021; Packer & Clay, 2000). Though further studies are needed to elucidate the mechanisms between soil microbes and seedlings, we know that seedling roots provide an interface for microbial activity by supplying habitat for rhizosphere-inhabiting microorganisms (Yi et al., 2020).

Interestingly, seedling density was associated with decreased fungal alpha diversity. Fungal diversity only slowly increased with maturing forest metrics (i.e., adult tree species richness, tree density, and basal area), likely indicating a lagging succession and growth strategy of fungal communities in general. This agrees with the tendency of increased fungal taxa richness and functional composition to be associated with more established forests and mature trees (Cline et al., 2005; Dejene et al., 2021). Forests in later stages of succession generate large amounts of carbon and lignin in forms such as deadwood, and therefore more microhabitats and new niches for wood-inhabiting fungi, such as the lignin-digesting Basidiomycetes (Menkis et al., 2020). This is again a symbiotic association, where the adult trees living with more established, diverse soil fungal networks reap benefits such as accelerated tree growth, structural stability, and carbon storage (Birch et al., 2020).

I observed a positive relationship between the diversity of bacteria, fungi, archaea, and air temperature. Warmer temperatures can trigger microbe propagation, and microbial communities tend to peak in diversity in summer seasons (Luo et al., 2019). Furthermore, this connection implies that changing temperature regimes due to climate change could

seriously impact soil microbial communities, with further cascading impacts on ecosystem services and functioning (Zhou et al., 2016). Unsurprisingly, soil pH was another important factor predicting bacterial α-diversity and β-diversity. Soil pH is widely recognised as the most important determinant of bacterial communities (Chu et al., 2010; Griffiths et al., 2011; Shen et al., 2013). pH is thought to either indirectly affect bacterial communities, as an index of general soil condition and nutrient status or affect bacteria directly on a physiological level that may or may not suit their intracellular pH preference (Lauber et al., 2009).

Forest successional shifts due to restoration actions may support fungi more than bacteria as forests mature. Bacterial diversity did not show a relationship with forest age, whilst fungal Shannon and Simpson diversity increased, agreeing with Liu et al. (2019). As Liu et al. (2019) theorised, this could be due to root exudates from a growing plant community negatively impacting bacterial establishment, whilst the successional plant community positively influences the development of fungi in the forest rhizosphere. Root exudation is one of the ways plants can manipulate root-associated microbial community assembly, hence plants can be both symbionts and inhibitors of select microbes (Pascale et al., 2020). This contradiction of the microbial loop, where microbes can also become pathogenic of plants, needs more experimental evidence to disentangle its exact mechanisms (Kudoyarova et al., 2019). As fungi are the most common plant symbionts, the increase in fungal diversity compared to other groups may reflect their increasing importance and specific relationships formed with plant species as a forest develops (Sun et al., 2017). In urban greenspace types, the most remarkable mycobiome differences occur between lawns and forests (Marczylo et al., 2021). Planting urban greenspaces is strongly linked with increasing fungal diversity (Baruch et al., 2020), as has been found in my research context. The links between fungal dissimilarity and diversity with other ecosystem attributes such as higher soil pH, herb cover, tree density, tree species richness, and a more closed canopy support Baruch et al. (2020) and Song et al. (2019) in suggesting that soil and vegetation traits shape fungal communities. Land management strategies such as restoration planting can reverse damage done by urbanisation, meaning that urban forests have tremendous potential for restoring fungal diversity and function (Newbound et al., 2012).

Archaeal diversity was low compared to bacterial diversity – a common finding in soil archaea studies (Tripathi et al., 2013). Forest age since restoration was a significant predictor of dissimilarity in archaeal communities between sites, as seen in an Australian eucalypt restoration study by Yan et al. (2020). Hence archaeal communities are more similar within than between ecosystem types (Angel et al., 2010; Auguet et al., 2009). Both α-diversity and β-diversity in archaea were influenced by soil pH, contrary to other studies which clearly define pH as the most important predictor of archaeal diversity and community composition (Hu et al., 2015; Tripathi et al., 2013; Yan et al., 2020; Zheng et al., 2013). The influence of pH on archaeal communities is still not well understood (Hu et al., 2013), although it may relate to substrate availability for processes such as ammonia oxidation (Nicol et al., 2008). The other significant predictor of archaeal communities' dissimilarity was seedling density. It remains unclear why archaea in the study sites showed strong associations with woody seedling density and not trees, for example – although vegetation cover in general is known to affect archaea (Angel et al., 2010). It may be that the rhizosphere associated with late successional seedling species differs from that of the early successional adult trees initially planted to jump-start these forests.

Increasing forest age led to a decrease in protist Shannon and Simpson diversity. Protist α-diversity was predicted by soil pH, water holding capacity (WHC), C:N ratio, and canopy openness. In contrast, only soil pH and native woody seedling density predicted protist beta diversity. As protists live in the aqueous part of the soil, where they obtain dissolved nutrients, protist functioning is largely dictated by water-filled soil pore space (Geisen et al., 2018; Stefan et al., 2014). My findings are supported by the literature, where soil WHC typically determines protist communities (Geisen et al., 2018). Specifically, I found protist diversity decreased with increased WHC. WHC values were within 40.2-78.2% across the forest chronosequence, which falls within the optimum range for protists where community abundance can fluctuate, hence my results were similar to those of Stefan et al. (2014). Theoretically, it may be that increasing WHC leads to increased flow and "chemical warfare" from the arsenal of bacteria resisting their protozoan predators, or it may relate with nematode predator-prey dynamics (Clarholm, 1981; Matz & Kjelleberg, 2005; Stefan et al., 2014).

The soil C:N fits into the picture where the patchiness of nutrient resources may constrain protist distribution (Geisen et al., 2018). To my knowledge, this is the first study to document a relationship between increasing protist diversity with decreasing canopy openness, although the exact mechanism is unclear. I hypothesise that this mechanism either functions by either a) harsher soil-drying and drought effects under more open canopy conditions, b) canopy closure is accepted as a key threshold in forest development that bolsters native plant regeneration, or c) a cascading effect of both (Doroski et al., 2018; Wallace & Clarkson, 2019). For example, a similar cascading effect was found in a Canadian post-wildfire chronosequence, where the reassembly of protist communities was influenced by vegetation succession, associated soil property changes, and changes in bacterial communities (Dai et al., 2021). Similarly, Oliverio et al. (2020) state that the best predictors of protist communities are distinct from those shaping bacterial and archaeal communities. Therefore, protist community reassembly cannot be generalised based on knowledge gleaned from studies of bacteria or archaea community structure. However, β-diversity patterns were strongly predicted by pH in protists just as they were for bacteria, similar to findings of Bates et al. (2013).

Seppey et al. (2017) concluded that protist Shannon diversity is lower in forests than open grass or croplands, supporting my findings that protist diversity decreased with the progression from grassed areas to maturing forests. While the exact mechanism for why this occurs is difficult to discern, it may be that other taxa were able to outcompete protist communities (Zhao et al., 2019) or bottom-up effects occurred via changes in bacterial and fungal communities, which are prey for phagotrophic protists (Zhao et al., 2019). It is alternatively possible that forest restoration and increased tree dominance led to increased ingestion of autotrophic protists (i.e., algae) by soil invertebrates or other microbial grazers, which constitute an underestimated carbon source for the soil ecosystem (Seppey et al., 2017).

Protists are widely distributed yet may be more susceptible to dispersal limitation due to their larger size and narrower habitat breadth compared to bacteria, according to some theories (Wu et al., 2018), and they are constrained by the restriction of passive mass flow of water in soils (Geisen et al., 2018). It is unknown how much we can predict factors

shaping protist communities from larger spatial scales such as this nationwide chronosequence (Oliverio et al., 2020). Protists are also not thought to have particularly cosmopolitan distributions; hence the impacts of urban settings on forests may have unaccounted-for influences on protist diversity (Bates et al., 2013). My findings help answer the questions posed by Geisen et al. (2017) on the abiotic and biotic determinants of protist communities, which remain understudied, and the diversity patterns of protists along a restored urban forest chronosequence. This research is one of very few studies directly comparing soil protist biogeography with distributions of soil bacteria, archaea, and fungi (Geisen et al., 2017; Oliverio et al., 2020).

Microbial community structure in restored and remnant forests did not appear highly dissimilar when visualised in an NMDS ordination. The NMDS ordination generally suggests overlaps in microbial community similarity and perhaps timeframes for microbial recovery differ by taxonomic group. The chronosequence sampled in this study began at ten years' forest age, which may have been enough time for vegetation growth and organic matter accumulation to enhance microbial diversity. For example, Yan et al. (2020) identified community shifts in archaea culminating in the resemblance of a mature forest ecosystem state after a decade of eucalyptus woodland restoration. Further, ecological restoration via native planting has been shown to result in soil bacterial community shifts after eight years, according to Gellie et al. (2017). A study of fungal community change after restoration by Yan et al. (2018) found that ten years of replanting on ex-pasture was enough to produce complete functional recovery. However, studies propose longer timeframes for microbial diversity recovery, i.e., 30 years (Zhang et al., 2016b). It may be that microbial assemblages do not need to completely return to a reference state for an ecosystem to recover (Hart et al., 2020; Van Nuland et al., 2019). The intermediate disturbance hypothesis postulates that diversity will be low after initial soil disturbance, increase to a maximum as resources increase, and then decrease again and level out as certain species become highly dominant and suppress others (Zhang et al., 2016b). Further research should investigate these temporal dynamics in sites planted <10 years prior and in unrestored grassed reference sites to see broader chronosequence shifts.

2.5.2 Taxonomic composition of microbial communities along the urban forest restoration chronosequence

Microbial phyla that appeared most frequently in my metabarcoding results matched what I would expect to find in forest soils (de Araujo et al., 2018; Liu et al., 2019; Yan et al., 2020). Taxonomic composition changed with increasing forest age across all four major soil microbial groups, which shows there is some coherence between microbial group responses to restoration, even at high taxonomic ranks (i.e., the phylum level) (Philippot et al., 2010; Sun et al., 2017). However, bacteria, archaea, fungi, and protists showed independent community shifts, indicating that each major microbial group occupies separate niches. Bacteria and fungi, for example, have often been found in the literature to follow different restoration trajectories due to their vast ecological differences (Sun et al., 2017). Typically, fungal diversity tends to increase with restoration planting age, while bacterial diversity may decrease (Liu et al., 2019). General shifts in microbial OTU compositions may suggest community development towards mature forest conditions (Fang & Peng, 1997).

Notable changes in bacterial taxonomic composition in this study appeared to reflect a successional gradient along the urban forest chronosequence. As per the findings of Gellie et al. (2017), I found that the Acidobacteriota expanded in relative abundance across the restoration chronosequence, which is indicative of a transition to an increasingly forest-like state with more complex sugar substrates available in the soil (George et al., 2010; Shen et al., 2013). The Acidobacteriota are considered *K* strategists, with slow growth rates, whilst the Bacteroidetes are considered *r* strategists, with rapid growth rates (Fierer & Jackson, 2006; Philippot et al., 2010). Thus, the rise of Acidobacteriota dominance concurrent with the decrease in Bacteroidota dominance along the chronosequence is consistent with known natural forest successional dynamics. The decrease in the relative abundance of Firmicutes phylum members with increasing forest age also indicates a shift to soils reflecting native forest soil communities, as the Firmicutes encompasses human pathogens and commensals, which tend to be higher in pasture than forest soils (Gellie et al., 2017; Jesus et at al., 2009). Interestingly, bacterial taxonomic shifts in response to forest age were complex and varied, which supports the conclusion that abiotic environmental properties

such as pH more directly drive bacterial community composition, rather than forest age alone.

The majority of archaea I identified belong to the Crenarchaeota and Thermoplasmatota. Crenarchaeota are a ubiquitous group in soils and likely a dominant phylum in grasslands (Auguet et al., 2009; Nicol et al., 2003), possibly remaining from pre-restoration conditions, when these sites were pasture or mowed grassy parks. Crenarchaeota members may be involved in nitrification, carbon metabolism, and some can colonise plant roots in the rhizosphere but may compete with other microbial symbionts (Kemnitz et al., 2007; Simon et al., 2000). The Crenarchaeota phylum is ecologically diverse, yet its ecology remains poorly known and largely speculative (Buckley et al., 1998; DeLong, 1998; Kemnitz et al., 2007). The receding dominance of Crenarchaeota in the remnant forest reference sites points to known mature forest soil archaeal community composition (Nicol et al., 2005). The Euryarchaeota were only visible in one 21-year restored forest soil; as a phylum largely composed of methanogens, their virtual absence from the rest of the chronosequence may be decreasing under forest conditions which are less involved in methane production (Jurgens et al., 1997). Thermoplasmatota was the second most common phylum found in the study sites. Surprisingly, archaea belonging to the Thermoplasmatota are typically documented as abundant planktonic archaea in oceans (Li et al., 2021) or associated with methane metabolism (Bräuer et al., 2020). However, my findings are consistent with Zhao et al. (2021), who found a dominance of Crenarchaeota, followed by Thermoplasmatota, and little representation of other phyla in a cropland conversion gradient of Northern China. Archaeal communities were linked with soil properties influencing nitrogen cycling and showed more minor change than bacterial communities at the same sites (Zhao et al., 2021). Reclamation of mudflats also led to an increase in Thermoplasmatota, according to Wan et al. (2021), due to their role in carbon and nitrogen cycling. Microbial ecologists still do not fully comprehend the environmental factors that regulate the abundance of these common groups in the archaea, let alone in the urban forest restoration context hence it is an area requiring further study (Hu et al., 2013).

Dominant fungal phyla are known to change depending on the environment (Liu et al., 2019). The fungal relative abundance results showed variation over the restoration

chronosequence, culminating in a more stable, even composition of the four major fungal phyla (Ascomycota, Basidiomycota, Chytridiomycota, and Mucoromycota) found in the remnant forest sites. Mucoromycota fungi are generally saprobic (consumers of dead matter), thriving in polluted and cosmopolitan environments. They are soil fungi that often occur as parasites or pathogens of plants, animals, and humans (Vega et al., 2012; Wijayawardene et al., 2018), which may explain their drastically higher relative abundance in a younger 10-year-old forest. Mucoromycota can also function as beneficial mycorrhizal fungi, decomposers, and root endophytes, which would explain why they remain established in older restored and remnant sites (Bonfante & Venice, 2020). My findings match with Dang et al. (2018) and Song et al. (2019) by revealing a decrease in the relative abundance of Ascomycetes paired with an increase in Basidiomycetes over the chronosequence, which would indicate ecological restoration of the topsoil. Seppey et al. (2017) showed that Basidiomycetes were more common in forests than grassland or cropland plots. The Basidiomycota phylum contains many slow-growing, late-successional species that can digest recalcitrant compounds, are negatively affected by disturbance and are commonly associated with forests (Frankland 1998; Osono, 2020). Ascomycetes are more common in monoculture plantations, and in contrast with the Basidiomycota, ligninolytic enzymes are essentially absent in the phylum (Osono, 2020; Song et al., 2019).

Alveolata and Rhizaria made up the major fraction of protist phyla detected, which agrees with other studies of soil protist taxa (Bates et al., 2013; Urich et al., 2008). It is unsurprising that the Alveolates would dominate the protist taxa due to their ecological significance, i.e., as parasites or endosymbionts (Tikhonenkov et al., 2014). Their fairly consistent importance across the chronosequence may mean that we can assume, as per Pellegrino et al. (2021), that Alveolates play a significant role in small soil macroaggregate formation and the rate of decomposition of soil organic matter. Further, I could infer that parasitic Alveolates persist due to a diversity of arthropod hosts, as mentioned by de Araujo et al. (2018). Rhizarians also remained relatively constant across the chronosequence, where they are large protists that function as predators and contribute to biogeochemical cycling (Mansour et al., 2021). Archaeplastida contains green algae, whilst Stramenopiles are golden algae (Bates et al., 2013). Hence the Stramenopiles and Archaeplastida contain many

photoautotrophic species (Geisen et al., 2018). As protists are vastly understudied and are the subject of few restoration chronosequence studies compared to other taxa, it remains challenging to identify the mechanisms behind taxonomic shifts during restoration stages. More work is needed to document protist community compositional changes and ascertain their functional significance in forest restoration.

2.6 Conclusion

Soil bacterial, archaeal, fungal, and protist communities showed taxonomic shifts along the urban forest restoration chronosequence that indicate aboveground forest succession is progressing microbial communities towards increasing dominance of taxa known to inhabit mature forest soils. Recovery appeared to be developing closer to reference remnant forest assemblages, in line with my hypothesis. As predicted, environmental properties did indeed predict diversity trends in microbial groups. Plant community factors explained fungal community diversity trends, protist diversity was explained by water-holding capacity, soil pH affected bacterial and archaeal diversity. However, I was surprised to find that nutrient data was not explanatory for any taxa, and native woody seedling density was relevant to all groups in at least one diversity metric. Furthermore, microbial diversity did not increase with forest age since restoration, contradicting my hypothesis. While it remains unclear whether pre-disturbance microbial assemblages can be achieved, these findings imply that restoration managers can recover later successional microbial forest soil communities and their associated ecosystem functioning by creating mature aboveground forest communities that supply understorey seedling growth.

2.7 References

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2.8 Appendix

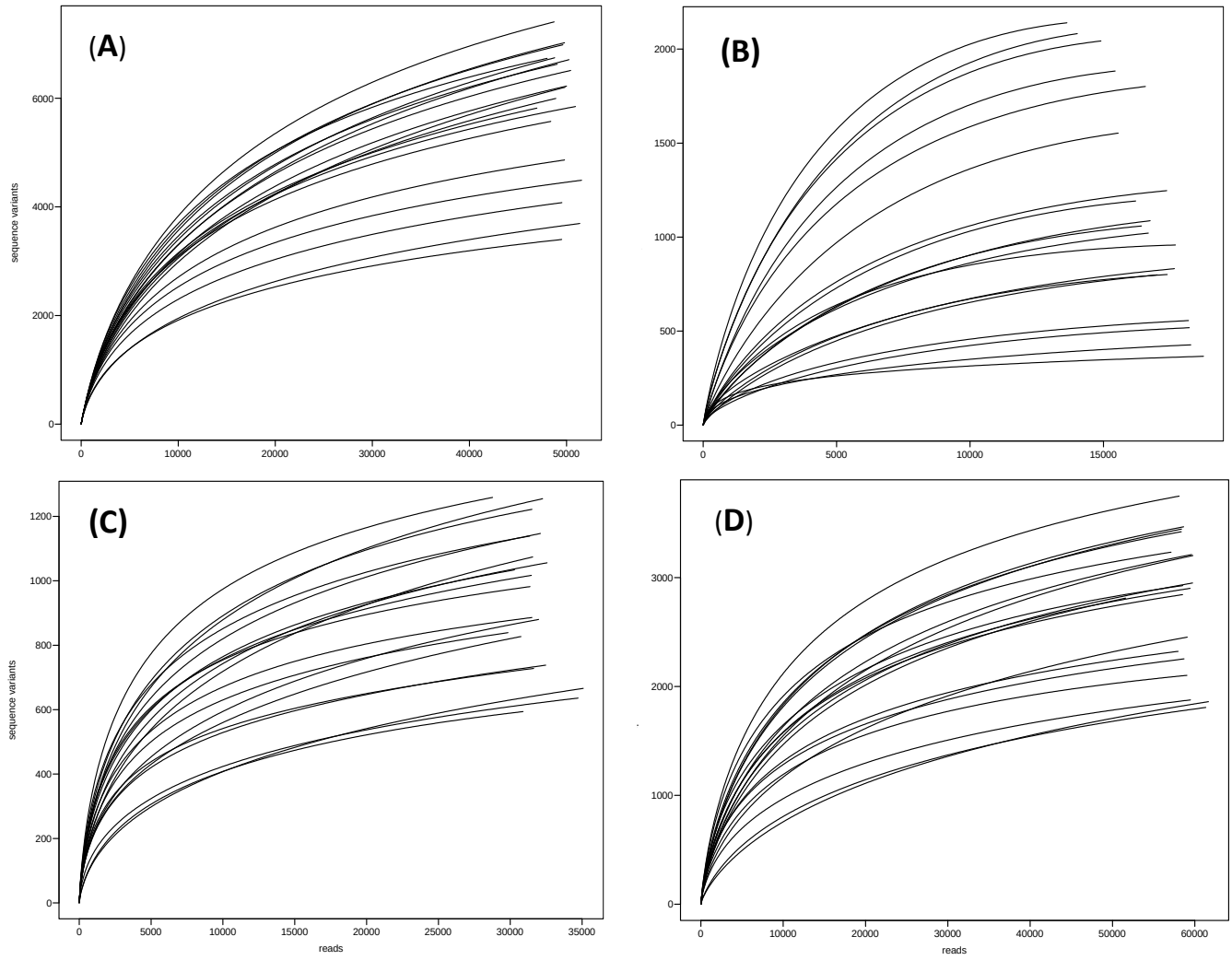

Figure S1. Rarefaction curves for (A) bacteria, (B) archaea, (C) fungi, and (D) protists.

Table S1. Databases used for taxonomic assignment of amplicon sequence variants.

Target	Mothur database name(s)	Database	Database selection	Blast database
16S - bacteria/archaea	SILVA_138_SSURef_NR99_prok.515F.806R	SILVA_138_SSURef_NR99	only prokaryotes, cut to match amplicon	16S_ribosomal_RNA
18S - eukaryotes	pr2_version_4.13.0_18S_mothur.1391F.1510R	PR2 4.13.0	cut to match amplicon	SSU_eukaryote_rRNA
18S - eukaryotes	SILVA_138_SSUParc_spec_euk.1391.1510	SILVA_138_SSUParc	only eukaryotes, cut to match amplicon	SSU_eukaryote_rRNA
ITS - fungi	unite_8.2_fungi.ITS1f.ITS2	unite_8.2	only fungi, cut to ITS1	ITS_RefSeq_Fungi
Archaea	SILVA_138_SSURef_spec_prok.SSU1ArF.SSU520R	SILVA_138_SSURef	only prokaryotes, cut to match amplicon	16S_ribosomal_RNA

Chapter 3

The effects of urban forest restoration on biomass, carbon metabolism, and spatial variability of soil microbial communities

3.1 Abstract

Land degradation leads to decreased soil microbial biomass, activity, and the essential ecosystem functions microbes provide. Soil microbial communities may recover with native restoration planting in response to aboveground and belowground changes that occur through forest succession, which leads to an accumulation of carbon of varying structural complexity to simulate microbial growth. However, microbial responses to planting interventions require further elucidation. Soil samples were taken from 66 restored urban forest sites along a New Zealand-wide chronosequence. I tested the effects of forest age since restoration and key environmental covariates on total microbial biomass, taxonomic group biomass via PLFA, total basal respiration, and carbon metabolism on substrates of varying recalcitrance using MicroResp™. My results showed that forest age was not particularly significant for predicting trends in microbial biomass and activity, though it was related to the dissimilarity of substrate-induced respiration over the chronosequence. Microbial basal respiration and respiration on carbon compounds was strongly linked with plant community ameliorations such as native woody seedling density and tree species richness. Similarly, microbial biomass became more spatially variable when average tree size increased, implying large trees stimulated microbial abundance via soil microhabitat creation. Finally, the chronosequence sites showed bacterial community dominance rather than fungal dominance, indicating that the soil microbiome lags behind aboveground plant succession and further recovery needs to occur. The necessity of soil microbial biomass, respiration, and composition for supporting aboveground restoration plantings survival via their contributions to ecosystem functioning creates an important context for investigating

soil microbial responses to restoration interventions. Findings from this research demonstrate that soil microbial communities and their associated functions were not fully restored – although soil microbial functioning was enhanced by plant community ameliorations – in an urban forest restoration chronosequence. It suggests that longer timeframes and specific environmental parameters may be needed to enhance soil microbiome recovery in forest restoration.

3.2 Introduction

Soils represent the largest terrestrial reserves of carbon and biodiversity on Earth (Crowther et al., 2019; Dixon et al., 1994). Respiration by the soil microbiome dominates total ecosystem respiration, which regulates net carbon exchange in forests (Huang et al., 2016; Valentini et al., 2000), as soil respiration is the primary channel for the biochemical release of CO_2, fixed by autotrophs, to the atmosphere (Schlesinger & Andrews, 2000). Therefore, soil respiration is a major function carried out by microbes (Beugnon et al., 2021). The heterogeneous structure of microhabitats and variations in biological, chemical, and physical properties in the soil means that microbial activity can be substantially variable, even at small scales (Xu & Qi, 2001). Anthropogenic activities also impact soil properties through both direct and cascading impacts of disturbances (Schmidt et al., 2011). Soil microbial biomass represents an index of organic matter turnover and nutrient cycling, which is highly sensitive to land-use change (Singh & Gupta, 2018). Disturbances can influence microbial community structure, activity, and associated ecosystem services such as decomposition and soil carbon cycling (Schmidt et al., 2011). Microbial biomass may be a “keystone” driver of soil ecosystem functioning and its loss is linked to decreased ecosystem productivity (Singh & Gupta, 2018). Although microbial biomass and activity are crucial for the soil carbon cycle (Smith et al., 2021) as well as plant-soil feedbacks (Connell et al., 2021), the recovery of microbial biomass and respiration during aboveground planted forest restoration is not well understood.

Aboveground forest restoration interventions have been found to enhance microbial biomass, including the biomass of specific microbial taxonomic groups from PLFA (Zhang et

al., 2019). Positive microbial biomass responses to native forest restoration plantings have been linked to plant diversity and increasing forest age, which promote an accumulation of carbon compounds (Araújo et al., 2013; Martucci do Couto et al., 2016; Zhang et al., 2019). Studies have found that small-scale spatial variability of microbial biomass and taxonomic group biomass patterns has been induced by the spatial arrangement of trees and their contributions to organic matter quality (Saetre & Bååth, 2000), which create heterogeneous soil spatial biological, physical and chemical properties (Baldrian, 2017). Soil heterogeneity can be especially high in natural forest soil (Štursová et al., 2016) and sustains soil biodiversity (Safford & Vallejo, 2019). Microbial taxonomic composition likely impacts carbon compound degradation because certain enzymes are restricted to particular microbial assemblages (Hammel, 1997; Padmanabhan et al., 2003). The ratio of bacteria to fungi is a good example; forests tend to have higher fungal dominance than bacteria (Smith et al., 2021). The hyphal growth network of fungi translocates nutrients between microsites in the soil (Frey et al., 2003), hence conveying slow-growth advantages that would be eliminated under physical disturbance (Helgason et al., 2009). Fungi can also degrade more recalcitrant litter compounds, like lignin and cellulose, compared to bacteria (de Boer et al., 2005). In contrast to fungal strategies, bacteria generally occur as individual cells which tend to be more efficient soil colonisers after disturbance (Strickland & Rousk, 2010) due to their fast growth strategy that decomposes labile substrates, supports rapid nutrient cycling and fast-growing plant species (Crowther et al., 2019). Gram-positive (GP) and gram-negative (GN) bacteria tend to be more abundant in forests than in converted land (He et al., 2017). GN bacteria are deemed fast-growing, copiotrophic r-strategists and consumers of labile carbon compounds (Bai et al., 2021), whilst GP bacteria are considered slow-growing, oligotrophic K-strategists (Wang et al., 2018). Natural forest restoration increases the proportion of GP and decreases the proportion of GN biomass measured by PLFA biomarkers (Cai et al., 2022). In general, bacteria favour more easily degradable substrates, while fungi are the primary decomposers of more complex polymeric substrates (Koranda et al., 2014). Arbuscular mycorrhizal (AM) fungi are thought to be more prolific with higher plant cover due to the host plant benefits they gain from root symbiosis (Yu et al., 2017). Similarly, free-living saprotrophic fungi are abundant in undisturbed forests (Shi et al., 2019) and are negatively impacted by open canopies with fewer litter inputs (Newbound, 2009).

Hence microbial group biomass should increase and change in composition under restoration plantings, with the accumulation of decomposable material as the forest ages (Cai et al., 2022).

The amount of soil microbial respiration in a system is highly related to microbial biomass (Wei et al., 2015) and taxonomic composition, which are in turn affected by carbon substrate availability (Wang et al., 2003). Microbial biomass carbon can be a useful ecological indicator of restoration planting efficacy, as planting pioneer species enhance soil habitats that support microbial abundance (An et al., 2009). Forest plantings can modify microbial community structure and function (Bastida et al., 2006). However, interpreting microbial biomass and respiration as ecological indicators can be problematic because microbial activity may also increase in response to disturbance and stress (Schipper & Sparling, 2000; Wardle & Ghan, 1995). Land-use change can have varied effects on microbial life. In some cases, microbial activity has been shown to increase in response to disturbances like increased nutrient loads from urban pollution (Mgelwa et al., 2019), forest thinning (Concilio et al., 2006), and experimental soil warming to mimic climate change (Schindlbacher et al., 2011). On the other hand, perturbations such as forest to agricultural land-use conversion has been shown to decrease microbial biomass by 75% (Waldrop et al., 2000). Furthermore, the microbial biomass remaining is typically of a different community composition, with altered physiological capacity (Waldrop et al., 2000). Forest clearance has been shown to negatively impact soil microbes, decreasing microbial basal respiration and biomass (Bastida et al., 2006). In general, disturbance and forest clearance have detrimental effects for soil microbial functioning, whilst restoration activities like tree planting are beneficial for microbial respiration, abundance, and enhancing ecosystem functions (Holden & Treseder, 2013; Yan et al., 2006; Yan et al., 2009).

Microbial respiration is typically measured as an indication of microbial activity and is an important ecosystem function in soils (Beugnon et al., 2021; Fang et al., 1998). Aerobic and heterotrophic microorganisms respire by decomposing organic compounds to obtain energy and carbon for biomass (Waldrop et al., 2000). The soil CO_2 efflux is affected by abiotic variables and their seasonal variations, such as soil moisture, woody and leaf litter inputs, pH, and soil temperature (Cleveland et al., 2007; Creamer et al., 2016; Davidson et

al., 2006; Yan et al., 2009). Microbial respiration is spatially variable over small-scale gradients in forest soil conditions, where soil heterogeneity contributes to “hotspots” for microbial activity, such as in the rhizosphere of large trees (Saetre, 1999). The same environmental factors affecting respiration should co-determine microbial biomass and composition. However, there is no universal trend that ordains which drivers will most prominently elicit microbial responses across large spatial scales (Hendershot et al., 2017). While the traditional view holds that microbial activity is passively controlled by abiotic variables alone, evidence now shows that these abiotic variables are not universal predictors of decomposition rates, and that microbial community composition has independent effects on carbon degradation rates (Waldrop et al., 2000; Zogg et al., 1997). In addition, plant community factors such as diversity and plant density can provide carbon resource inputs and microclimatic conditions that stimulate microbial metabolic activity and carbon storage in soils (Lange et al., 2015). Further research of ecological relationships between soil microbes and other biotic ecosystem attributes is warranted.

Soil microbial communities associated with different plant communities show distinctive patterns in their utilisation of carbon sources due to unique capabilities for carbon source degradation (Buyer & Drinkwater, 1997; Waldrop et al., 2000). Carbon sources for soil microorganisms range in structural complexity from simple sugars to complex compounds. Forest succession leads to carbon compound accumulation, accompanied by shifts in soil microbes from fast-growing, generalist communities to specialists capable of degrading recalcitrant substrates from mature forests (Herzog et al., 2019). Substrate availability is under-studied as a determinant of respiration, yet the dynamic temporal and spatial variations in substrate supply may account for much of the variation in respiration across soils (Davidson et al., 2006). Measurements of microbial respiration using carbon compounds of varying complexity offers a measure of ecological functional capacity in the microbial community. It can indicate responses of microbial communities and their multiple associated ecosystem functions to changes in land management practices and soil properties (Creamer et al., 2016). This is based on the knowledge that changes in soil microbe respiration during forest maturation can predict how efficiently microbes can sequester carbon reserves in soil (Huang et al., 2016). Carbon degradation is spatially

variable in forest soils following small-scale heterogeneity in carbon substrate availability and the presence of microbes with differing enzymatic capabilities (Baldrian et al., 2010). Land-use and soil properties tend to have a large effect on microbial distributions and aboveground resource inputs, impacting substrate utilisation (Creamer et al., 2016). By quantifying the use of soil carbon substrate by soil microbes during forest restoration, we can shed light on how restoration efforts may enhance the functional diversity and capacity of microbial communities.

To understand how the restoration of urban forests impact the recovery of soil microbial biomass and ecosystem functioning across varying environmental conditions, I took soil samples from 66 urban forest patches in an Aotearoa New Zealand nationwide spatial and temporal ecosystem restoration chronosequence. Using substrate-induced respiration via the MicroResp™ method and phospholipid and neutral lipid fatty acid analysis (PLFA and NLFA) as an index of microbial taxonomic group presence, I assessed changes in total microbial biomass and composition of broad bacterial and fungal taxa biomasses across the restoration chronosequence. Furthermore, I quantified responses in total microbial community respiration and the respiration of microbes on 15 different carbon compounds that appear commonly in forest soils. I also look into the local spatial variability of microbial respiration and biomass using the coefficient of variation. I predict that older restored forest sites with greater tree density and diversity will enhance microbial biomass, basal respiration, and the spatial variability of these processes. I also hypothesise that functional group biomass changes will reflect an increased dominance of fungi relative to bacteria over the chronosequence. Finally, I predict that substrate-induced respiration will indicate increasing utilisation of carbon compounds, especially substrates of greater recalcitrance, as the chronosequence progresses.

3.3 Methods

3.3.1 Study sites and experimental design

I carried out a field survey in 66 actively restored urban forest patches aged 6 – 60 years. Over the Aotearoa New Zealand summer of November 2019 – February 2020, soil samples were taken from these forest patches which had been restored from scratch. Our study

sites were located throughout eight cities across both the North and South Island of the country, with two or three sites per city (Fig. 3.1). Each study site was a forest patch that hosted a rectangular forest plot 10 x 20m (200m^2 area), established by the Ministry of Business, Innovation and Employment-funded research programme People, Cities, and Nature (see Busbridge, 2020). Plots were placed so that the edge of the rectangular plot was always >1m from the edge of the forest patch and slope not exceeding 10 degrees. The sampling design accounted for spatial variability in the soil. Each rectangular plot contained five square subplots at each of the four corners and the centre point of the plot, which were used to collect environmental data (Fig. 3.2). My soil sampling took place from three subplots spaced evenly along a diagonal transect, including a corner, central point, and the opposite corner of each plot.

Figure 3.1. Major New Zealand cities selected for fieldwork. Image from Google Earth.

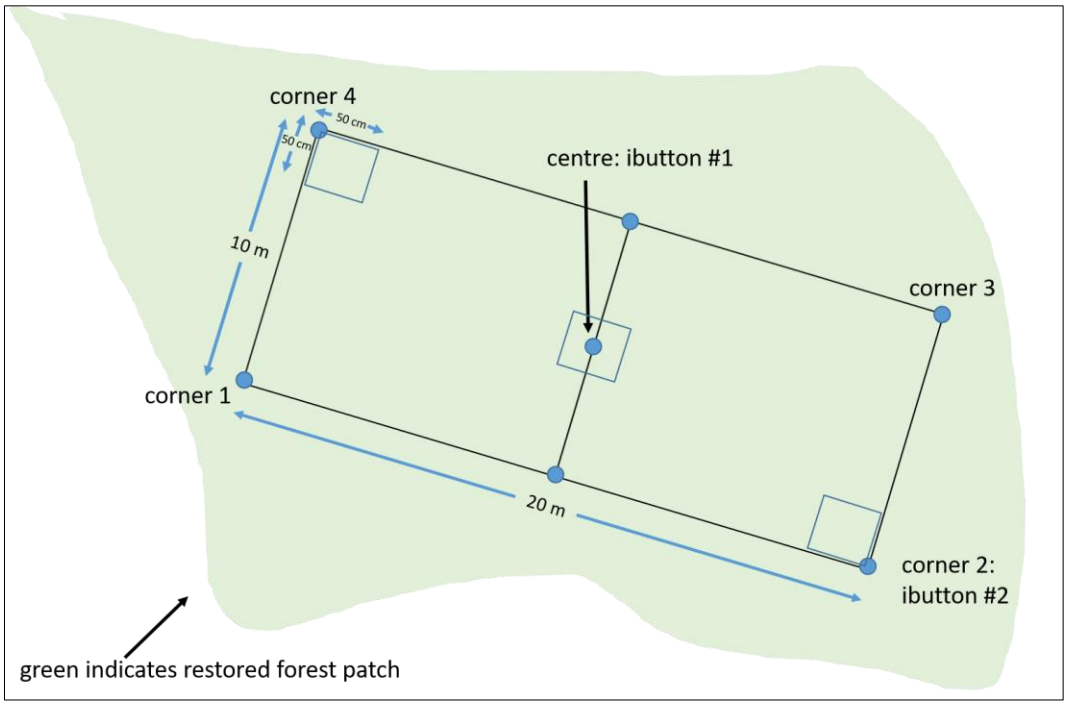

Figure 3.2. People, Cities, & Nature research plot layout within a hypothetical restored forest patch. Small squares represent subplot locations for soil core sampling.

3.3.2 Aboveground data

Forest age was the number of years between initial restoration planting and the year data was collected (2020). Vegetation surveys were carried out beforehand to collect data on the plant community in each plot (Busbridge, 2020). Adult tree species richness/diversity in the plots ranged from 1 to 18. Seedling density was recorded in 2019 as the total number of woody species under 135cm height within ten circular subplots of 1.5m radius, covering a total 70.7m^2 of the plot, and scaled up to approximate seedling density per 200m^2. Adult tree species richness was a tally of all the different native and exotic adult tree species present in each plot which had a diameter at breast height (DBH) >2.5cm. Mean tree basal area per plot was summed as the cross-sectional trunk area measured by DBH in centimetres squared (cm^2). Tree density was a tally of trees occurring in each plot (200m^2), calculated per 100m^2. Herbaceous cover encompassed the plot mean percentage of herbaceous cover, calculated by visually estimating the % cover from 8 subplots which added up to the entire plot (200m^2). Leaf litter depth (i.e., the dead layer on the forest floor) was measured to the nearest centimetre at five points in all four corner subplots and the

centre subplot. Canopy openness was measured as an index of light availability and microclimate formation and shown as a mean percent value per plot. Canopy openness was calculated as a single plot value from measurements at all four plot corners and the centre three times over 12 months (at 0, 6, and 12 months) with a convex Spherical Crown densiometer at breast height (~1.3m above the ground). Plot mean air temperature was recorded every four hours for approximately 12 months using HOBO data loggers (model MX2301A; Onset, Cape Cod, Massachusetts, USA) contained in a radiation shield and placed on a tree ~1m above the ground at the centre point of each plot.

3.3.3 Microbial sample collection

A soil corer of 2.7cm diameter was used to take five core samples to a depth of 10cm from each subplot to collect soil microorganisms. The resulting 15 cores were pooled to create a composite sample representing each plot's microbial community heterogeneity. From each pooled sample 150g of fresh soil was sieved through 2mm metal mesh sieves and stored at -80°C. Samples were shipped on dry ice at -80°C to the German Centre for Integrative Biodiversity Research (iDiv) in Leipzig, Germany, for further lab analyses. Subplot soil samples were used for PLFA and Cmic analyses whilst samples were homogenised to plot-level for calculating basal respiration (BR).

3.3.4 Lab analyses

3.3.4.1 Microbial total biomass and PLFA and NLFA analysis

Microbial biomass (µg C/g soil dry mass) was calculated via the substrate-induced respiration (SIR) technique, performed on an O_2-microcompensation apparatus. 8mg/g of glucose in water solution was added to dry soil and incubated. Over the 24-hour incubation period, metabolically active microorganisms were detected by recording O_2 consumption.

Phospholipid fatty acid (PLFA) analysis represents microbial community composition by estimating the biomass of different microbial functional groups according to their differing PLFA patterns (Harris, 2003). This study uses PLFA biomarker data to estimate gram-positive (GP) bacteria, gram-negative (GN) bacteria, saprophytic fungi, arbuscular mycorrhizal fungi, total bacteria, total fungi, and the bacteria:fungi ratio. Neutral phospholipid fatty acid

(NLFA) analyses were also used to identify arbuscular mycorrhizal fungi. Protocols were based on the methods of Frostegård et al. (1991). 3g of fresh soil were shaken with 18.5mL of Bligh & Deyer solution over 2 hours. The mixture was centrifuged, then chloroform and citrate buffer were mixed with the organic phase. Approximately 4mL of the lower phase was purified with glass wool and sodium sulphate and evaporated at 30°C under nitrogen. The lipid phase was dissolved and fractionated in chloroform or in methanol for NLFAs and PLFAs, respectively, over silica columns. Lipids were purified, dissolved, and stored at -80°C in hexane. Fatty acids (FA) were identified with gas chromatography-mass spectrometry (GC-MS), and raw data was manually verified with TotalChrom software. If necessary, peaks were modified to fit the right FA, using two current standards and the internal standard 19:0. The NLFA peaks were revised for the FA 16:1ω7, 16:1ω-5 and 18:1ω9c+a19:0, which refer to arbuscular mycorrhizal fungi (AMF), where PLFA and NLFA assignments were based on Ruess et al. (2010) (Table S2).

3.3.4.2 Microbial basal respiration

The O_2-microcompensation apparatus was used to estimate BR as an index of restoration potential (Ivashchenko et al., 2014). BR is steady-state respiration without substrate addition, which therefore investigates microbial decomposition in dormant conditions. After 8–14 hours, a stable plateau in BR occurred for the remainder of the experiment, from which an average BR value over three hours was calculated.

3.3.4.3 Substrate-induced respiration (SIR)

The MicroResp™ method (Campbell et al., 2003) was used to determine the microbial metabolic profile. Colorimetric CO_2 traps measured variation in CO_2 production over six hours on 16 different complex carbon substrates known to occur in forest soils (Banning et al., 2012). Soil samples were sieved, soil moisture content (%) was determined, and samples were dissolved in 25mL deionised water for storage at 4°C. Samples were filled homogenously into 96-well deep-well plates via a filling device and incubated over seven days in dark conditions. Detection plates were filled with 3% purified agar and indicator solution in a 1:2 (agar:indicator) ratio, and quality control checks were run on Omega analysis software 48 hours after plate preparation. Each carbon substrate was made up as

30 mg per gram of soil water. After the 7-day incubation period, 25µL of each desired substrate was pipetted into deep-well plates, sealed and placed into the spectrophotometer. CO_2 production was visible by colorimetric detection and recorded from samples, starting from t0 using Omega software.

3.3.5 Statistical analysis

Statistical analyses were performed in R version 4.0.5 (R Core Team, 2021). I aggregated plot-level data to calculate mean microbial basal respiration (BR) and biomass (Cmic) and used sub-plot data to calculate the coefficient of variation (CV) for both BR and Cmic as a measure of spatial variability in microbial communities. I tested BR and Cmic mean and CV, PLFA bacterial and fungal groups (i.e. total bacteria, total fungi, gram-positive bacteria, gram-negative bacteria, saprophyte fungi, arbuscular mycorrhizal fungi, log bacterial:fungal ratio), and SIR data for each of the 15 substrates (arabinose, lignin, glucose, trehalose, oxalic acid, malic acid, alpha-ketobutyric acid, citric acid, cysteine, syringic acid, alanine, lysine, acetyl-glucosamine, ascorbic acid, and mannitol) as responses in linear mixed-effects models. For each response variable, a maximal was constructed that included forest age and a set of eight key environmental variables as predictors, with 'city' specified as a random effect to account for the experimental grouping of sites across cities. Assumptions of normality were checked, and log-transformations were applied where necessary to ensure homogeneity of variance and normality of model residuals. Minimum adequate models were determined by stepwise backward selection with the Akaike Information Criterion (AIC) using the 'stepAIC' function, always retaining forest age in the minimal adequate model to test the relative importance of forest age versus the eight other environmental variables. Marginal effects from the models were then plotted using the package 'sjPlot'.

I constructed a distance matrix via Bray-Curtis dissimilarity based on the relative biomasses of microbial taxa from the PLFA analysis. I then constructed an NMDS ordination and tested for effects of canopy openness, tree density per $100m^2$, mean plot tree basal area, water-holding capacity (WHC), plot native woody seedling density, tree species richness, herbaceous (herb) cover, and mean annual plot air temperature on the composition of

microbial relative biomass using a PERMANOVA with the 'adonis' function from the package 'vegan'. I began with eight key variables and removed non-explanatory predictors in a stepwise fashion based on the adjusted R^2 values, until I was left with only significant response variables. I created model fits for each of the seven functional groups in the PLFA data and plotted their partial effects within sjPlot.

Similar to the multivariate analysis of microbial relative biomass based on PLFAs, I tested the dissimilarity of microbial community metabolic profiles (i.e., respiration on different C substrates) using PERMANOVA with the 'adonis' function and based on Bray-Curtis dissimilarity. I then calculated evenness of respiration across substrates using the formula J = H'/ln(S), where H' represents Shannon diversity and S represents the number of "species" (in this case, C substrates) in the community. Evenness was tested as a response variable in a linear mixed-effects model using the same procedure as explained above for linear mixed-effects models on all other microbial community response variables.

3.4 Results

3.4.1 Soil microbial biomass (Cmic)

I found a general trend of mean and spatial variability (CV) of microbial biomass increasing with forest age since restoration, but both relationships were non-significant (Fig. 3.3A, 3.3B). Furthermore, none of the tested environmental variables predicted mean biomass. However, average tree basal area per plot had a significant effect on the spatial variability of microbial biomass (P = 0.0322) (Table 3.1).

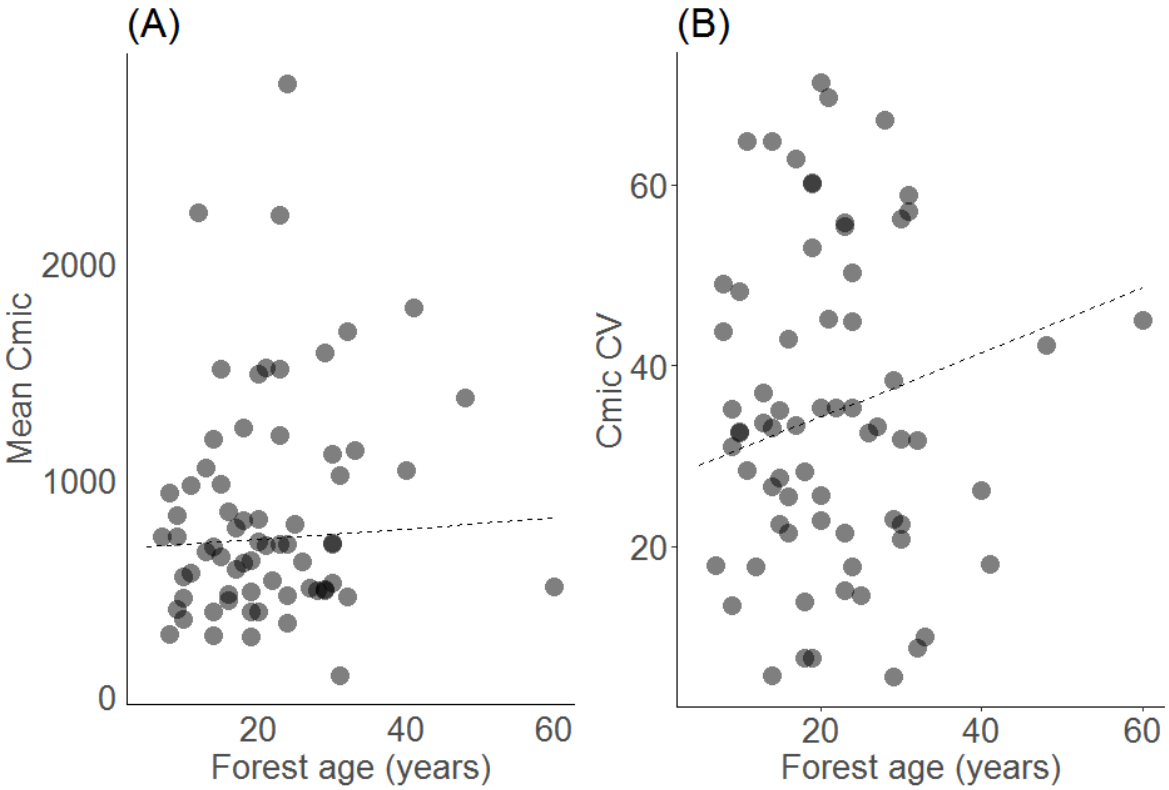

Figure 3.3. Mean soil microbial biomass (Cmic) (A) and spatial variability (CV) of microbial biomass within restoration plots (B) in response to forest age since restoration. Dotted lines denote non-significant relationships between forest age and Cmic.

Table 3.1. Results from mixed-effect models with mean and spatial variability (CV) of microbial biomass (Cmic) in response to forest age since restoration and environmental covariates. Bold values indicate statistically significant relationships ($P < 0.05$).

	Value	Std. Error	df	t-value	*P*
Log Cmic mean					
Age	0.003287	0.007461	58	0.44052	0.6612
Mean basal area	10.793024	12.874975	58	0.83829	0.4053
Cmic CV					
Age	0.01177	0.009511	57	1.237835	0.2209
WHC	0.01330	0.008719	57	1.525903	0.1326
Mean basal area	-36.12002	16.450822	57	-2.195636	**0.0322**

3.4.2 PLFA community data

The marginal effects of age and key environmental variables had no significant effects on the biomass of different bacterial and fungal groups from PLFA data (Fig. 3.4; Table 3.2). Decreasing canopy openness had a marginally significant positive effect on gram-negative bacteria (P = 0.0753), whilst higher tree density had a marginally significant positive effect on the biomass of saprophytic fungi (P = 0.0502) and fungi in total (P = 0.0607) (Table 3.2).

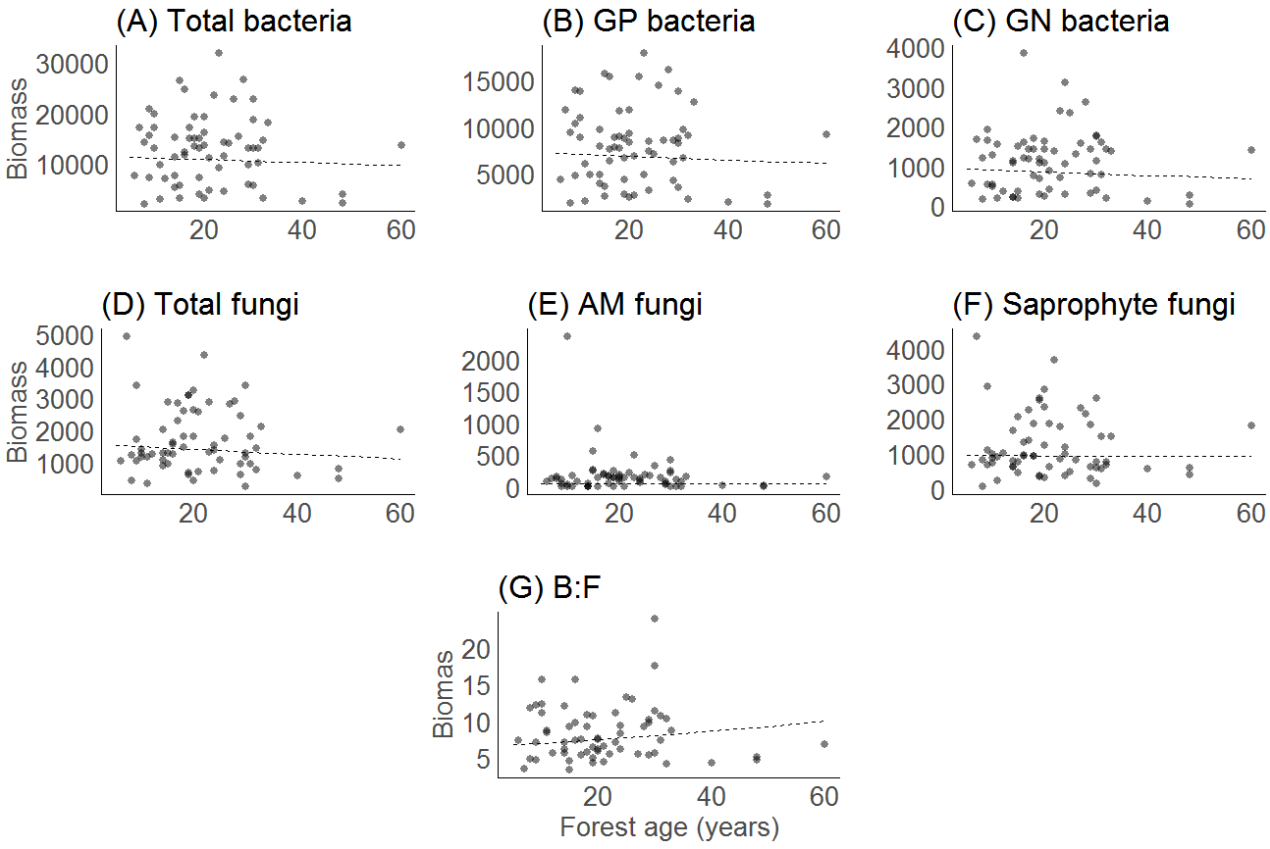

Figure 3.4. Marginal effects of forest age since restoration for A) total bacteria, B) gram-positive bacteria, C) gram-negative bacteria, D) total fungi, E) arbuscular mycorrhizal fungi, F) saprotrophic fungi, and G) the bacteria:fungal ratio. Dotted lines denote non-significant relationships between forest age and functional group biomass.

Table 3.2. Results from mixed-effect models of microbial taxa PLFA data in response to forest age since restoration and other covariates.

	Value	Std. Error	df	t-value	*P*
Log total bacteria					
Age	-34.460	75.0016	57	-0.459452	0.6477
Seedlings	-0.405	0.4512	57	-0.897990	0.3730
Log GP bacteria					
Age	-0.005791	0.00674295	57	-0.85876	0.3941
Seedlings	-0.000055	0.00004058	57	-1.35471	0.1809
Log GN bacteria					
Age	-0.009007	0.0096591	56	-0.932479	0.3551
Seedlings	-0.000092	0.0000569	56	-1.621834	0.1105
Canopy openness	-0.010322	0.0056955	56	-1.812331	0.0753.
Log total fungi					
Age	-9.9306	11.1453	57	-0.891018	0.3767
Tree density	5.5967	2.9244	57	1.913785	0.0607.
Log AM fungi					
Age	-4.9657	3.84563	57	-1.2912690	0.2018
Tree density	-0.7131	0.97397	57	-0.7322066	0.4670
Log saprophyte fungi					
Age	-5.7799	9.8414	57	-0.587307	0.5593
Tree density	5.1612	2.5794	57	2.000941	0.0502.
Log B:F					
Age	0.05062	0.05763	56	0.8783470	0.3835
Mean basal area	-158.53373	104.24352	56	-1.5208018	0.1339
Mean air temperature	0.49100	0.28614	56	1.7159394	0.0917

3.4.3 PLFA community dissimilarity

Sites did not appear significantly dissimilar based on forest age, with sites of different ages overlapping in ordination space without any clear grouping according to similar forest ages (Fig. 3.5; Table 3.3). Seedling density was the most significant predictor of dissimilarity in microbial relative biomass ($P = 0.032$, $R^2 = 0.0544$), closely followed by herb cover ($P = 0.044$, $R^2 = 0.055$).

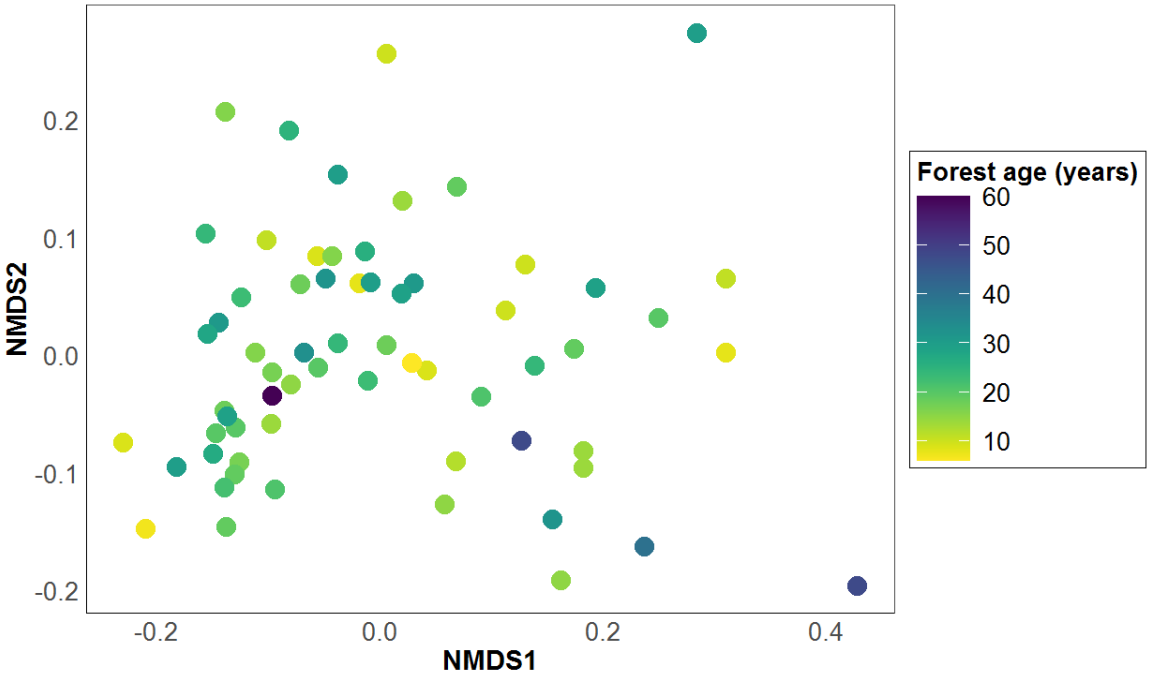

Figure 3.5. NMDS ordination displaying dissimilarity of relative microbial taxa biomass in response to forest age since restoration.

Table 3.3. Results from Permutational multivariate analysis of variance (PERMANOVA) of microbial taxa PLFA data in response to forest age since restoration and environmental covariates. Bold values indicate statistically significant relationships ($P < 0.05$).

	df	SS	MS	F-value	R^2	*P*
Age	1	0.0681	0.068119	0.9381	0.0137	0.35
Seedlings	1	0.2703	0.270288	3.7223	0.0544	**0.032**
Herb cover	1	0.2755	0.275502	3.7941	0.0554	**0.044**
Residuals	60	4.3568	0.072614		0.8765	
Total	63	4.9707			1.00000	

df = degrees of freedom, SS = sums of squares, MS mean squares.

3.4.4 Response of microbial respiration and carbon metabolism to restoration

Average BR appeared to increase slightly across the chronosequence but was not significantly predicted by forest age (Fig. 3.6A). In contrast, I found a significant effect of forest age on spatial variability of BR across the forest restoration chronosequence (*P* = 0.0281) (Fig. 3.6B; Table 3.4). Average BR increased in plots where seedling densities were higher (*P* = 0.0163). Looking at individual marginal effects of significant environmental variables, spatial variability of microbial respiration increased when soil water holding capacity was greater (*P* = 0.0194), where forest canopy openness was higher (*P* = 0.0073), and also increased where plots hosted a greater density of trees (*P* = 0.0115) (Table 3.4).

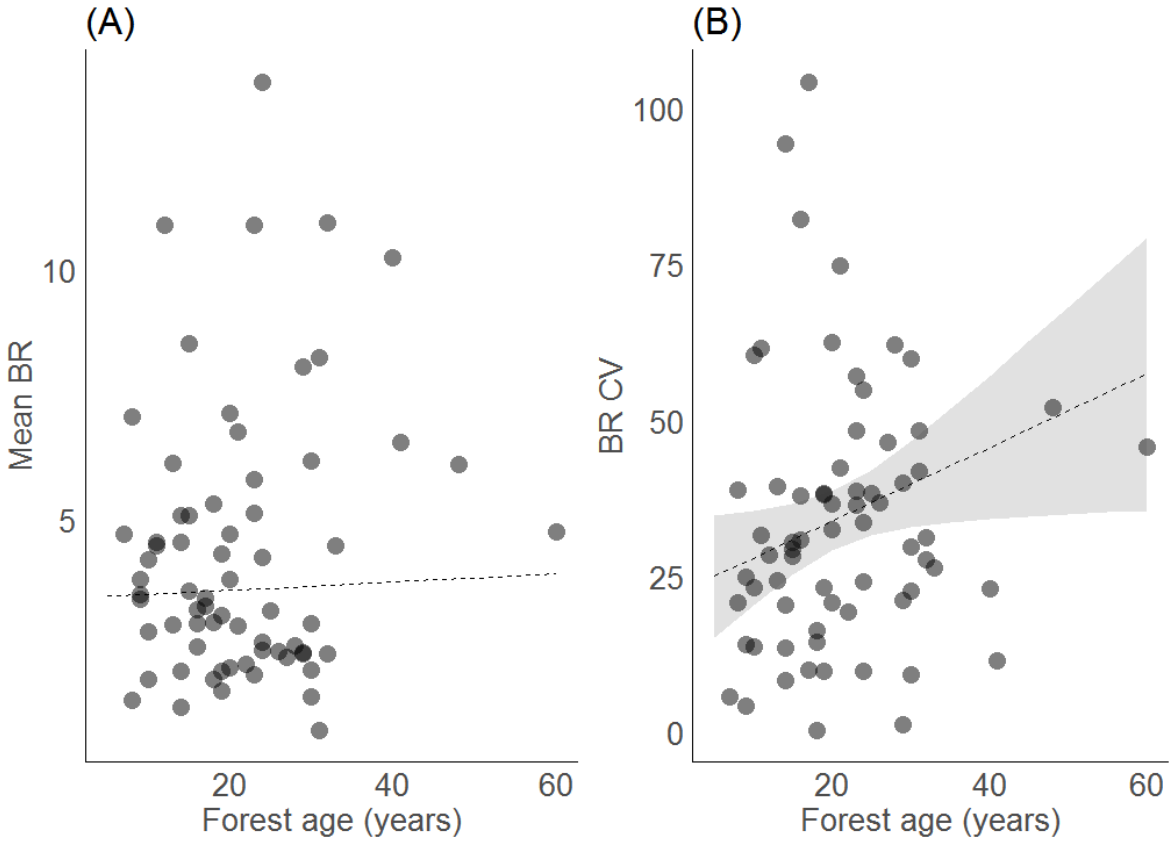

Figure 3.6. Mean soil microbial basal respiration (BR) (A) and spatial variability (CV) of microbial basal respiration within restoration plots (B) in response to forest age since restoration. Dotted lines

denote non-significant relationships while solid lines denote significant relationships between forest age and BR. Shaded areas show the 95% confidence interval for the significant relationships.

Table 3.4. Results from mixed-effect models with mean and spatial variability (CV) of microbial basal respiration (BR) in response to forest age since restoration and environmental covariates. Bold values indicate statistically significant relationships ($P < 0.05$).

	Value	Std. Error	df	t-value	*P*
Log BR mean					
Age	0.0022340	0.0053718	57	0.4158762	0.6791
Seedlings	0.0000786	0.0000318	57	2.4750692	**0.0163**
WHC	0.0098965	0.0060842	57	1.6266003	0.1093
BR cv					
Age	0.02712529	0.0120256	55	2.2556249	**0.0281**
Seedlings	-0.00008794	0.0000655	55	-1.3418702	0.1852
WHC	0.03055567	0.0126917	55	2.4075283	**0.0194**
Canopy	0.02097587	0.0075298	55	2.7857271	**0.0073**
Tree density	0.00813748	0.0031131	55	2.6139651	**0.0115**

The evenness of microbial respiration over 15 carbon substrates did not significantly differ with forest age (Fig. 3.7). Instead, tree species richness had a significant positive effect on the evenness of respiration across C substrates ($P = 0.0190$), and herbaceous cover had a marginally significant effect on evenness ($P = 0.0698$) (Table 3.5). In mixed-effects models testing responses of C metabolism on individual substrates, I found that respiration on alpha-ketobutyric acid decreased under herbaceous cover ($P = 0.0254$), while respiration on citric acid ($P = 0.0290$), syringic acid ($P = 0.0438$), ascorbic acid ($P = 0.0384$), and especially alanine ($P = 0.0063$) all responded positively to increasing tree species richness (Table S3).

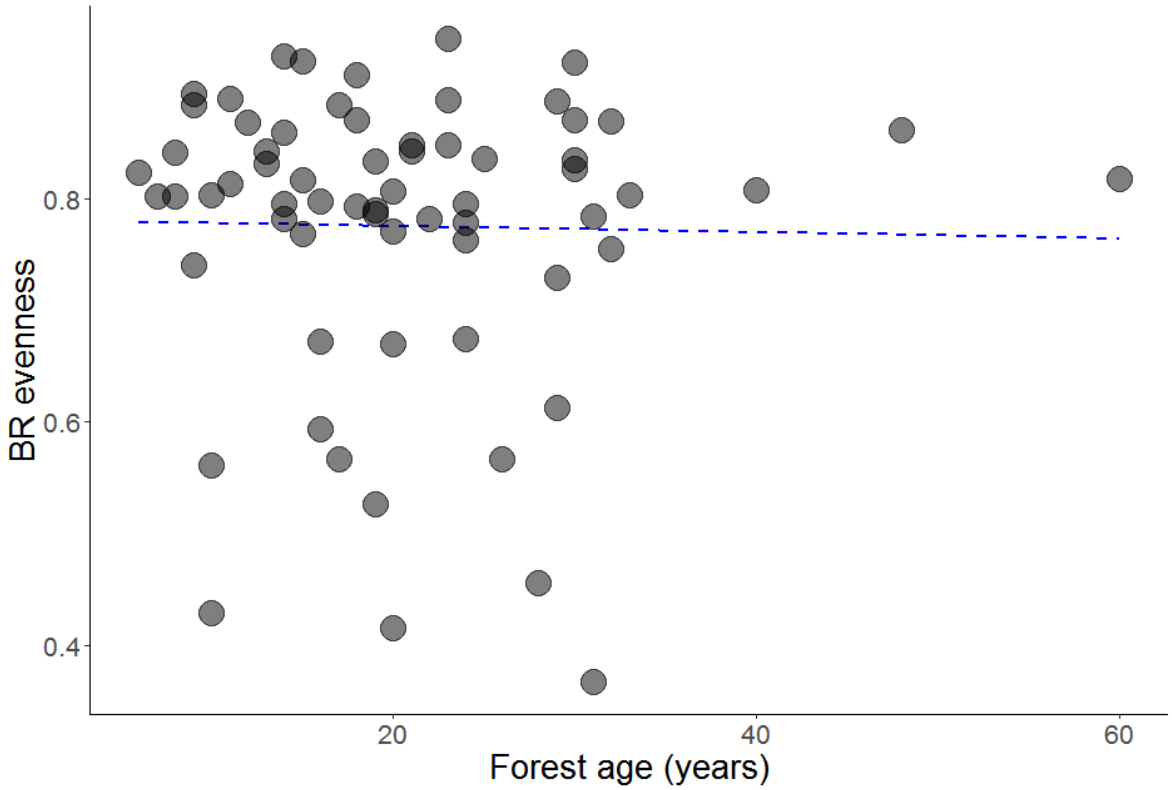

Figure 3.7. Evenness of microbial substrate-induced respiration (SIR) in response to forest age since restoration. Dotted lines denote non-significant relationships between forest age and Cmic.

Table 3.5. Results from mixed-effects model of evenness of substrate-induced respiration from MicroResp™ data in response to forest age since restoration and environmental covariates. Bold values indicate statistically significant relationships ($P < 0.05$).

	Value	Std. Error	DF	t-value	*P*
Age	0.0000666	0.00150208	55	0.044342	0.9648
Herb cover	0.0009765	0.00052800	55	1.849448	0.0698.
Tree species richness	0.0092308	0.00381836	55	2.417491	**0.0190**

3.4.5 Compositional shifts in carbon metabolism along the restoration chronosequence

NMDS ordination showed some clustering of sites from MicroResp™ data in ordination space due to forest age, with younger sites tending to congregate closer to each other and older sites displaying greater dispersion along NMDS axis 1 (Fig. 3.8). These differences in

dissimilarity were corroborated by PERMANOVA results showing forest age was a highly significant predictor of community dissimilarity (P = 0.000999, R^2 = 0.326) (Table 3.6). Tree species richness also predicted dissimilarity of substrate-induced respiration between sites (P = 0.011, R^2 = 0.048) (Table 3.6).

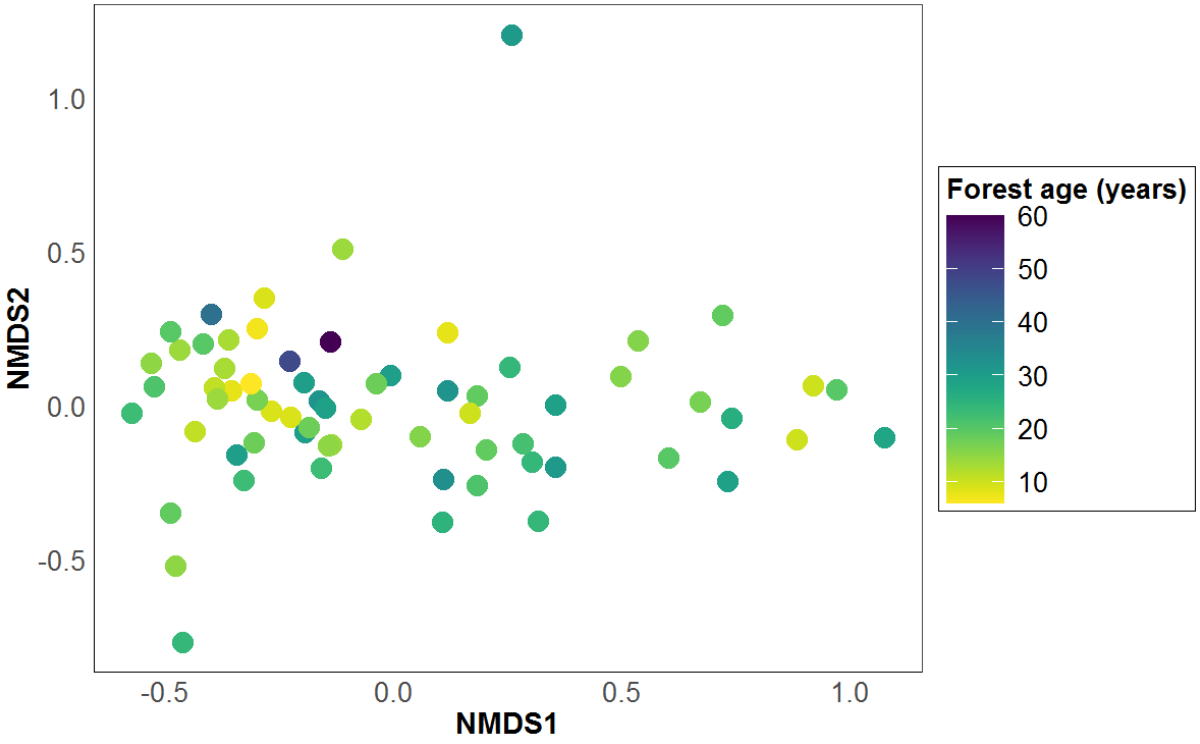

Figure 3.8. NMDS ordination displaying dissimilarity of substrate-induced respiration (SIR) in response to forest age since restoration.

Table 3.6. Results from Permutational multivariate analysis of variance of substrate-induced respiration from MicroResp™ data in response to forest age since restoration and environmental covariates. Bold values indicate statistically significant relationships (P < 0.05).

	df	SS	MS	F-value	R^2	*P*
Age	1	1.1174	1.11743	26.600	0.326	**0.000999**
Tree species richness	1	0.1642	0.16417	3.908	0.048	**0.011**
Residuals	51	2.1425	0.04201		0.626	
Total	53	3.4241			1.00000	

df = degrees of freedom, SS = sums of squares, MS mean squares.

3.5 Discussion

Restored forest sites along the chronosequence displayed differing responses in total microbial biomass, taxonomic group biomass, total microbial respiration, and carbon metabolism on substrates of varying recalcitrance. Overall, my results suggest that vegetative properties in restored forest patches substantially impact microbial respiration and biomass, likely due to increased C sequestration by growing trees (Katayama et al., 2009). For example, Yan et al. (2006) showed that increased litter carbon inputs from later successional forests could increase respiration, indicating that this could be an important driver of microbial activity in our study. Spatial variability of microbial basal respiration and biomass in the soil was increased by measured environmental variables, indicating that local-scale environmental changes cause soil heterogeneity (Stoyan et al., 2000), thereby influencing the biomass production and respiration of soil microbes. Contrary to my predictions, microbial biomass and respiration did not show clear age-related trends, nor did fungal dominance or lignin utilisation. I expected that my findings might not follow patterns seen in other studies due to complex responses of soil microbes to the highly variable environmental conditions across our restoration chronosequence that spanned the latitudinal extent of New Zealand (Hendershot et al., 2017).

3.5.1 Trends in total microbial biomass in maturing restored forests

I did not detect any significant effect of forest age on microbial biomass, and there were no significant effects of measured environmental variables on mean biomass across the chronosequence. These findings contradict those from other studies, such as from An et al. (2009), who found that microbial biomass increased with forest restoration planting until 23 years, after which biomass remained stable. I would have expected that as forests mature along the chronosequence, microbial biomass would increase, as plant community ameliorations can stimulate biomass production of soil microorganisms (Wardle et al., 2004; Zak et al., 1994). Several possible explanations may individually or jointly explain why I did not find an effect of forest age in this chronosequence. It could be possible that our

results are showing already stabilised microbial biomass following dynamic changes in microbial biomass occurring in the early stages of forest restoration. In future, studies may capture these changes by including more sites under ten years of age. Insam & Domsch (1988) also found no significant correlation between microbial biomass and forest age, concluding that in tracking restoration changes, a combination of measurements has more practical value than basing conclusions on changes in one variable. A combination of other data that was not measured (i.e., nutrient data) may have contributed substantially to unknown variation in my results (Wardle et al., 1999). My data also did not contain prior land use information, which could yield legacy impacts that may have long-enduring effects and could additionally obscure microbial responses in the chronosequence (Connell et al., 2021). Furthermore, although belowground root production is difficult to measure and was therefore not included in my study, it can influence carbon availability to microbes and may have been a determinant for microbial biomass in my sites (Wardle et al., 2004; Zak et al., 1994).

When the average tree size (mean basal area) increased, microbial biomass was more spatially variable, indicated by the coefficient of variation. Chroňáková et al. (2019) found that spatial variability of microbial communities increased due to the diversification of microhabitats by different plant species, and Saetre (1999) similarly attributed microbial spatial variability to the spatial arrangement and species identity of trees. As average basal area increased in plots during progression towards “climax” stands, the increased plant biomass likely created larger C pools in the soil, where root biomass and detritus arrive from larger trees as substrate to stimulate microbial activity and abundance (Fang et al., 1998; Luo et al., 2012; Raich & Tufekciogul, 2000). Therefore, the effect of increased average tree size on increasing spatial variability in microbial biomass that I found in the chronosequence is understandable and aptly describes that microbial communities thrive in response to microhabitats created by tree growth and maturation following aboveground restoration plantings.

3.5.2 Taxonomic composition of bacterial and fungal communities along the urban forest restoration chronosequence

Differences in the community biomass composition of major microbial taxa derived from PLFA analyses were linked to seedling density and herb cover in urban forest restoration sites. These vegetative properties likely predicted differences in taxonomic group biomass due to the differences in litter inputs, root exudates, and carbon accumulation created from changes in aboveground biomass, which also affects microbial biomass (An et al., 2013; Fu et al., 2020; Han et al., 2020). A large amount of unexplained variation remained in the PERMANOVA (R^2 = 0.876), which may have been attributable to unmeasured parameters such as soil pH and nutrient status (Han et al., 2020), precipitation (Fu et al., 2020), or pH (Dimitriu et al., 2010).

I did not find any significant influence of forest age or environmental variability on microbial taxonomic group biomass in the chronosequence. The ratio of bacterial to fungal biomass between sites did not significantly change with forest age or any environmental predictors included in the models. This was a surprising result as there is a range of environmental variables which, based on a number of studies, are expected to affect the bacterial to fungal ratio, such as water holding capacity and soil temperature (Strickland & Rousk, 2010). It is possible that soil pH or the soil carbon to nitrogen ratio – which were not measured across all restoration sites in this study – could have influenced the relative composition of bacteria to fungi, which was found to occur in a German study investigating bacteria to fungi ratios in spruce and beech forest soils (Blagodatskaya & Anderson, 1998). However, conflicting results have also been found in other studies (e.g., Bardgett et al., 1996). The lack of significant effects of forest age or other predictors on microbial group biomass could have occurred because my sites all shared the same urban restored forest biome type, which may support more similar community compositions than, for example, an agricultural-forest conversion gradient, which has been found to cause more pronounced changes in relative microbial group composition (Waldrop et al., 2000). Alternatively, it could be that no sites in this study have yet reached later stages of forest succession where fungi have often been found to dominate over bacteria (Smith et al., 2021), which is typical of soils that have not fully recovered from disturbance (MacKenzie & Quideau, 2012). Hence, while

aboveground plant communities can resemble pre-disturbed forests within 25 years (Rowland et al., 2009), belowground microbial recovery may lag far behind.

3.5.3 Shifting microbial functions with urban forest restoration

Average microbial respiration over the chronosequence was not predicted by forest age, but it was influenced by seedling density. This positive relationship between seedling density and microbial respiration could be due to reciprocal plant-microbe benefits, as fungal colonisation is crucial for seedling development (Asmelash et al., 2016). Additionally, Shabaga et al. (2015) found that post-harvest forest understorey seedling regrowth led to increased basal respiration, inferring that increased understorey seedling growth and fine woody debris created rhizospheric substrate pools that stimulated microbial activity. Based on findings from other studies, I had expected other covariables to be important predictors of microbial respiration. Katayama et al. (2009) found forest structure and total tree basal area were important for soil microbial respiration in a Bornean tropical rainforest, inferring that larger trees would pass on more root surface area and greater litterfall for microbial activity (Katayama et al., 2009). By contrast, Doff Sotta et al. (2004) found soil moisture was important for soil microbial respiration in an Amazonian tropical forest, which they suggested may be because soil and litter characteristics were not influenced by tree trunks as such, but rather by the forest crown. Additionally, in a Chinese forest succession gradient, Yan et al. (2006) found that litter inputs of the previous month are strongly linked with soil respiration. Overall, there appears to be no overriding consensus in the literature on the main drivers of microbial respiration (Cleveland et al., 2007), which makes it difficult to draw meaningful comparisons of results from this study to other previous findings.

Average microbial basal respiration rates were high, indicating increasing carbon allocation from belowground competition due to forest succession (Yan et al., 2006) and hence high rates of soil biological activity due to greater effectiveness of restored areas (Tufekcioglu et al., 2001). However, soil respiration is not necessarily an indicator of forest development under restoration due to a lack of defined thresholds for desirable values, as high respiration may also indicate soil disruption or stress (Padmanabhan et al., 2003; Schipper & Sparling, 2000).

The spatial variability of microbial basal respiration was attributed to a range of environmental predictors. Spatial variability increased with forest age, water holding capacity, tree density, and canopy openness. It is expected that a range of abiotic and biotic factors can affect the spatial variability of soil respiration (Epron et al., 2004). In a study on primary forest succession across the forehead of a temperate glacier, Luo et al. (2012) deduced that increased soil moisture determined increased spatial variability of soil microbial respiration by reducing water limitation and improving plant productivity with increasing forest age, enabled by the ameliorated soil structure and porosity of later successional forest soils. Though it appears contradictory that spatial variability should increase under greater tree density but decrease under greater canopy closure, it is important to note that these are marginal effects of predictors while holding all other predictors constant at their means. Therefore, this result suggests that there is a decline in the variability of basal respiration with an increasingly closed canopy for a given level of tree density.

I found some discernible trends in microbial respiration on 15 carbon compounds of varying recalcitrance across the chronosequence. I expected to find that respiration on more structurally complex compounds (e.g., syringic acid) would increase with forest age as conditions began to approach recovered or natural forest soil microbe communities (Banning et al., 2012). I found that forest age strongly predicted dissimilarity in substrate-induced respiration between microbial communities. However, my results did not show any trends in microbial community capacity for degradation of individual carbon substrates, simple or complex, through forest age. I found that tree species richness appeared to positively influence the metabolism of ascorbic acid, alanine, syringic acid, and citric acid, whilst reduced herb cover at restoration sites led to increased respiration on alpha-ketobutyric acid. Tree species richness also determined the evenness of carbon source utilisation across sites. Altogether, these results show that increased tree species richness leads to greater physiological potential for microbial respiration on different carbon substrates. A number of different factors have been shown to influence the metabolism of more easily available carbon sources (Banning et al., 2012); for example, alanine is preferentially utilised by microbes under greater tree cover (Andersen et al., 2013), and

citric acid is generally utilised more often under restored and remnant forest areas (Strickland et al., 2017). Rajapaksha et al. (2004) found that microbial respiration increases with ascorbic acid addition in contaminated soils and attributes this to fungi dominating ascorbic acid decomposition. Syringic acid – a complex phenolic acid – is utilised by microbes that are able to degrade recalcitrant senesced leaves (Stanek et al., 2021). I am not aware of other studies which have observed or explained the mechanism by which microbial utilisation of these compounds increases with greater tree species richness, in particular, under forest restoration. Broadly speaking, greater tree species richness likely supported greater microbial biomass (Beugnon et al., 2021), a greater diversity of root-secreted compounds (Grayston et al., 1998), and enhanced plant productivity (Liu et al., 2017). Furthermore, this may reflect the known relationships between plant diversity and enhanced microbial ecosystem functioning (Beugnon et al., 2021). It also remains unclear why alpha-ketobutyric acid was utilised less as herb cover increased; I would have expected to find the opposite, as this substrate has been found to elicit a greater catabolic response in forest soils compared to pasture (Stevenson et al., 2004). Moscatelli et al. (2018) supported the use of substrate-induced respiration as an ecological indicator of functional diversity in soil microbe but showed that the MicroResp™ method used here is more sensitive in sites where organic matter is lower. I could therefore speculate that overall, my restored sites had an organic matter content that rendered the MicroResp™ method less applicable (Moscatelli et al., 2018). Buyer & Drinkwater (1997) detected shifts in PLFA composition but not in carbon source utilisation between experimental farm site treatments, which implies that different microbial communities may not necessarily differ in their functional capabilities.

3.6 Conclusion

Study findings showed that plant community factors were particularly important for enhancing microbial biomass and respiration. Unlike my prediction, I did not find a prominent effect of forest age since restoration on total microbial taxonomic group biomass or that of functional groups. Bacterial communities were more dominant than fungal communities and the utilisation of recalcitrant compounds did not increase across the chronosequence, which contradicted my hypotheses and suggests that restored urban

forest sites had not yet reached a later successional stage. I also found that plant community factors correlated with increased basal respiration, greater evenness of substrate-induced evenness, and higher spatial variability of microbial biomass and respiration, supporting my hypothesis and likely reflecting the role of tree plantings in creating small-scale heterogeneity in microhabitats and resource pools. My results therefore suggest that aboveground planting restorations have led to partial recovery of microbial functioning. Moving forward, it may be necessary to specifically look into restoration interventions that target soil microbiome recovery and succession.

3.7 References

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3.8 Appendix

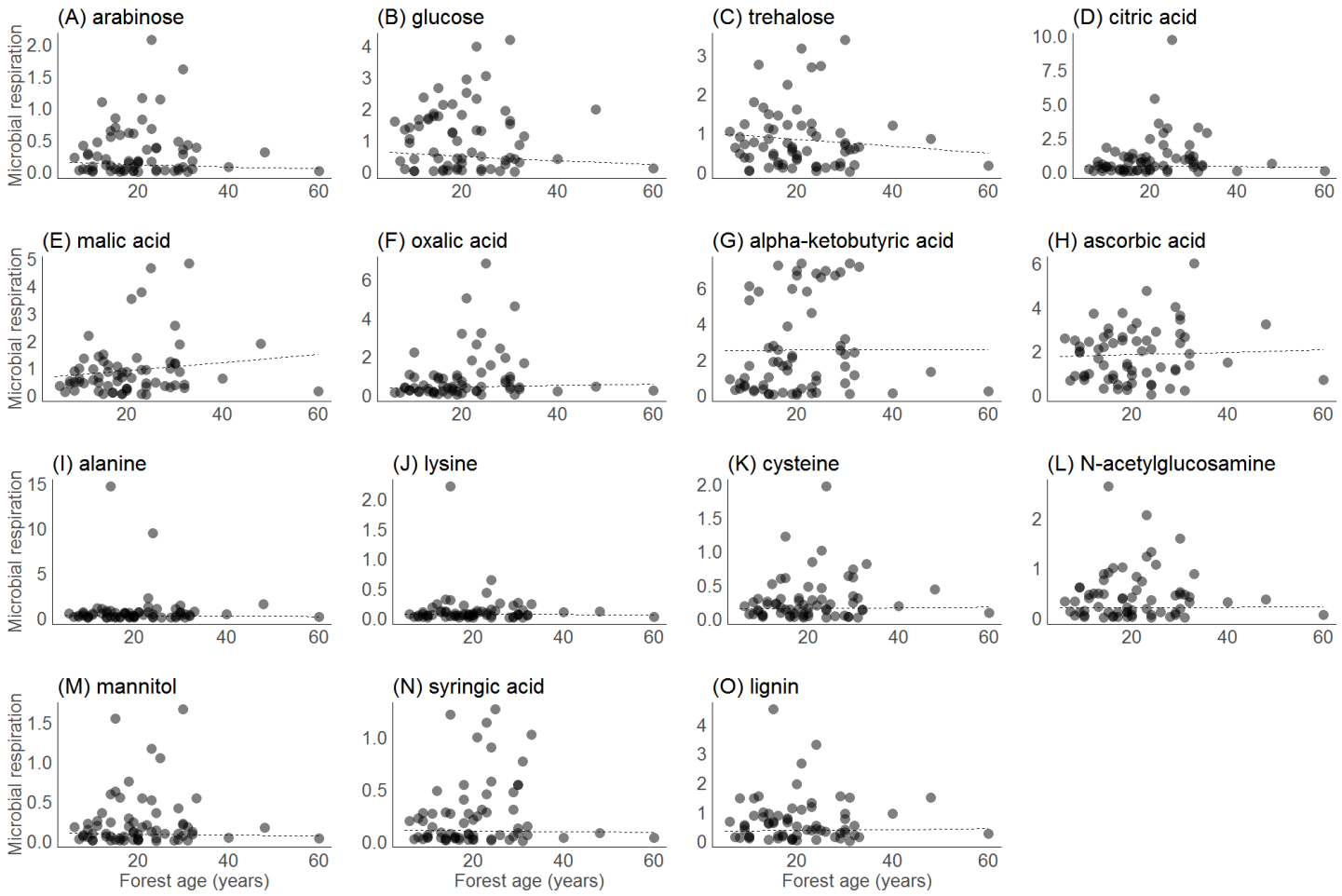

Figure S2. Marginal effects of forest age since restoration on respiration per substrate for 15 substrates commonly found in forest soils.

Table S2. Fatty acid markers used to distinguish bacterial and fungal functional groups via PLFA and NLFA.

Fatty acid markers	Lipid fraction	Functional group origin
i15:0, a15:0, i16:0, i17:0	PLFA	Gram-positive bacteria
cy17:0, cy19:0	PLFA	Gram-negative bacteria
16:1ω5, 16:1ω7	PLFA	Bacteria
20:01	PLFA	Arbuscular mycorrhizal fungi
16:1ω5	NLFA	Arbuscular mycorrhizal fungi
18:2ω6c	PLFA	Saprophytic fungi
18:1ω9c, 18:3ω6	PLFA	Fungi

Table S3. Results from mixed-effect models for respiration on 15 forest soil substrates.

	Value	Std. Error	DF	t-value	*P*
Log arabinose					
Age	-0.0196566	0.0212647	56	-0.924375	0.3593
Tree species richness	0.1073552	0.0547847	56	1.959585	0.0550
Log lignin					
Age	0.00400	0.02116	54	0.1888713	0.8509
Seedlings	0.00021	0.00012	54	1.7963683	0.0780.
Tree species richness	0.03134	0.04017	54	0.7800214	0.4388
Mean basal area	-55.24093	46.34712	54	-1.1918958	0.2385
Log glucose					
Age	-0.0169603	0.0198064	56	-0.8563026	0.3955
Tree species richness	0.0835967	0.0510630	56	1.6371277	0.1072
Log trehalose					
Age	-0.0088197	0.00999181	56	-0.8826973	0.3812

	Value	Std. Error	DF	t-value	*P*
Tree species richness	0.0354734	0.02556984	56	1.3873130	0.1708
Log oxalic acid					
Age	0.0076260	0.0171023	56	0.445907	0.6574
Tree species richness	0.0621194	0.0435442	56	1.426583	0.1593
Log malic acid					
Age	0.01469496	0.0130384	56	1.1270548	0.2645
Tree species richness	0.04260097	0.0327793	56	1.2996306	0.1991
Log alpha-ketobutyric acid					
Age	0.000815	0.0329964	55	0.024702	0.9804
Seedlings	-0.000272	0.0001742	55	-1.561306	0.1242
Herb cover	-0.025106	0.0109282	55	-2.297378	0.0254
Log citric acid					
Age	-0.0028040	0.0205245	56	-0.136617	0.8918
Tree species richness	0.1156665	0.0515998	56	2.241608	0.0290
Log cysteine					
Age	0.0034768	0.0141327	56	0.246011	0.8066
Tree species richness	0.0664111	0.0364464	56	1.822156	0.0738
Log syringic acid					
Age	-0.0042150	0.0194928	55	-0.216235	0.8296
Tree density	-0.0078994	0.0049660	55	-1.590704	0.1174
Tree species richness	0.1106609	0.0536410	55	2.062990	0.0438
Log alanine					
Age	-0.012967	0.02902	53	-0.4467687	0.6569
Seedlings	0.000137	0.00017	53	0.8167868	0.4177

	Value	Std. Error	DF	t-value	*P*
Mean basal area	-13.163016	70.43977	53	-0.1868691	0.8525
Tree species richness	0.156340	0.05494	53	2.8455921	0.0063
Tree density	-0.008661	0.00598	53	-1.4479541	0.1535
Log lysine					
Age	-0.0007977	0.0151473	57	-0.052665	0.9582
Log acetyl-glucosamine					
Age	0.001359	0.0179893	57	0.075544	0.9400
Log ascorbic acid					
Age	0.0054713	0.0149554	55	0.3658416	0.7159
Tree species richness	0.0882095	0.0415714	55	2.1218813	0.0384
Tree density	-0.0073750	0.0038817	55	-1.8999566	0.0627
Log mannitol					
Age	-0.007873	0.0213996	56	-0.367913	0.7143
Tree species richness	0.097764	0.0550236	56	1.776772	0.0810

df = degrees of freedom, SS = sums of squares, MS mean squares.

Chapter 4

Thesis synthesis

4.1 Discussion

My thesis aimed to investigate soil microbial responses to an urban forest restoration chronosequence. I sought to test if forest age can explain patterns in microbial community diversity, activity, and composition across a New Zealand-wide restoration chronosequence, or these restoration effects might be obscured by environmental heterogeneity among sites. In Chapter 2, I analysed taxonomic composition and diversity patterns for bacteria, fungi, archaea, and protists using metabarcoding of amplicon sequence variants and environmental data. To delve deeper into microbial activity, chapter 3 looked at patterns in broad-scale microbial respiration and biomass using various techniques such as substrate-induced respiration, PLFA biomass, as well as mean and spatially variable total microbial biomass and respiration, due to their utility in detecting microbial functional diversity and capacity. Global interest in forest restoration continues to grow (Fagan et al., 2020). Yet, belowground communities have often been overlooked in restoration studies, with most research focusing on aboveground plantings (Strickland et al., 2017). Therefore, the findings from these chapters contribute towards a research area that requires further exploration by providing insight into the recovery of soil microbial community structure, activity, and ecosystem functioning following urban forest restoration.

4.1.1 Restoring microbial biodiversity by reforesting urban areas spaces

Urbanisation creates altered and disturbed environments, leading to losses in soil microbial biodiversity (Zhao et al., 2013). The loss of microbes and their vital ecosystem functions such as nutrient cycling, organic matter decomposition, and symbiotic rhizospheric interactions is cause for concern (Aislabie et al., 2013; Dominati et al., 2010). Native restoration plantings have been shown to increase microbial abundance and richness

(Baruch et al., 2020), which in turn enhances aboveground plant performance (de Araujo et al., 2018). It is presumed that aboveground planting interventions will be sufficient to produce belowground microbial recovery over increasing time since restoration (Hart et al., 2020). This assumption is a token of the lack of studies on soil microbiome recovery following restoration plantings, whilst aboveground plant community responses are well-established in the literature (Strickland et al., 2017). It is known that a range of niche and neutral-based processes drive microbial community assembly, but the contribution of different drivers is variable depending on the study context (Gravel et al., 2006). Bacteria, archaea, fungi, and protists are the major microbial groups, and each may show different responses to abiotic and biotic conditions under forest successional dynamics (Ohtonen et al., 1999). Studying all four major microbial groups can shed light on how their diversity and taxonomic composition shifts throughout an urban forest restoration chronosequence. My results thereby draw attention to the responses of the crucial yet overlooked soil microbial component of the ecosystem following aboveground restoration plantings.

In Chapter 2, I assessed diversity trends and taxonomic composition of soil bacteria, archaea, fungi, and protists throughout 16 restored sites in an urban forest restoration chronosequence. I incorporated metabarcoding data, four indices of α-diversity, Bray-Curtis β-diversity (dissimilarity), and ten important environmental parameters into analyses of bacterial, fungal, archaeal, and protist OTUs. My results showed that environmental parameters such as native woody seedling density and soil pH tended to be more informative than forest age of shifts in diversity and dissimilarity. The positive reciprocal relationship between microbes and seedling development is mentioned (Asemelash et al., 2016; Shabaga et al., 2015) but, to my knowledge, is not well-documented in forest restoration. Soil pH, however, is an established driver of microbial community diversity trends and compositional shifts (Bartram et al., 2014). There was a large amount of unexplained variation driving differences in microbial community diversity between sites that remains to be explored. I would postulate that the effects of historical land-use legacies could in part drive this unknown variation (Connell et al., 2021). At phylum-level classification, specific taxonomic shifts resembled a transition across the chronosequence away from disturbed conditions and toward reference microbial communities that are

typical of mature forest soils. An example of this was the decreasing relative abundance of fungal Ascomycotes paired with an increasing proportion of Basidiomycetes, indicating a shift from homogenous, disturbed conditions to later successional forest soil topsoil (Seppey et al., 2017; Song et al., 2019). Three remnant sites, used as references, shows that microbial communities in the restoration chronosequence are progressing towards – but do not yet resemble – the taxonomic composition of original forest. Results from this chapter add to our growing understanding of microbial responses to restoration interventions in an urban forest context. It would appear that microbial community compositional shifts continue to progress along a successional trajectory decades after restoration planting. My study shows that forest age since restoration should not be relied upon as the best driver of microbial community change. Instead, active monitoring of soil microbiome recovery via measurable environmental drivers – i.e., native woody seedlings –may be more beneficial for predicting belowground community succession.

4.1.2 The restoration of microbial community biomass and carbon metabolism

Microbial biomass and respiration are integral to organic matter turnover and net forest carbon exchange in an ecosystem, yet their recovery is not well understood in forest restoration (Huang et al., 2016; Singh & Gupta, 2018). Microbes and their associated functions are sensitive to land-use change and disturbance (Ramirez et al., 2014). Aboveground restoration plantings have been positively linked with enhancing microbial biomass (Zhang et al., 2019). Restoration plantings can also cause microbial functional group shifts that reflect forest successional dynamics, such as a shift from bacterial to fungal dominance as forests mature (Araújo et al., 2013; Smith et al., 2021; Wang et al., 2003; Zhang et al., 2019). As soil carbon compounds accumulate in forest succession, soil microbes may shift from fast-growing, generalist communities to specialists capable of degrading recalcitrant substrates (Herzog et al., 2019). Forest maturation also creates local-scale spatial variability in soil microbial communities, especially around large trees (Saetre, 1999; Šťursová et al., 2016). Microbial biomass and respiration are generally co-determined by various environmental factors, though there is seemingly no universal trend governing which variables are consistently most influential (Hendershot et al., 2017).

Chapter 3 explored trends in microbial activity and biomass over 66 sites in an urban forest restoration chronosequence. I investigated this by testing the effects of forest age and key environmental variables on total microbial biomass, taxonomic group biomass, total microbial respiration, and carbon metabolism on substrates of varying recalcitrance. I found no effect of forest age on total biomass, but I did find that as average tree size increased, total microbial biomass became more spatially variable. Similarly, plant community ameliorations including native woody seedling density and herb cover determined microbial basal respiration, whilst basal respiration became more spatially variable with forest age, water holding capacity, tree density, and canopy openness. Tree species richness influenced the evenness and dissimilarity of carbon source respiration by microbes across sites and had a positive effect on the utilisation of four carbon substrates. Together, my results suggest that restoration planting maturation created soil microhabitats that stimulated microbial growth. I inferred this occurred based on similar studies that showed plant species diversity impacts microbial spatial variability (Saetre, 1999) and plant biomass inputs create larger carbon pools that stimulate microbial growth from larger trees (Fang et al., 1998) as well as from seedlings (Shabaga et al., 2015). Findings thus appear to show that mature trees and understorey seedling growth are important parameters for enhancing microbial activity.

Although I hypothesised forest age would have a more influential effect on microbial activity in chapter 3, I did find that forest age influenced the dissimilarity of substrate-induced respiration between microbial communities across the chronosequence. Forest age did not predict group biomass changes across the chronosequence for any microbial taxa measured by PLFA, nor did any environmental variables. This is particularly interesting given that research in other regions has shown that variations in soil properties like water holding capacity have led to shifts in taxonomic group biomass (Strickland & Rousk, 2010) or that fungi become more prevalent than bacteria in later successional forests (Ohtonen et al., 1999). I suggest that my results show that microbial recovery has not yet been achieved and may lag behind aboveground plant recovery in the chronosequence. I theorise that unmeasured parameters like soil pH, nutrient data, or legacy impacts may have contributed to unexplained variation and would have helped to inform patterns of microbial

activity in the chronosequence. In general, it appeared that vegetative properties were influential for stimulating microbial growth and activity in the chronosequence therefore restoration managers can aid microbiome recovery by aiming to establish large, mature trees with understory seedling growth. I conclude that aboveground restoration plantings are successfully guiding soil microbial communities along a restoration trajectory but may not have yet led to a fully recovered, natural forest microbiome.

4.1.3 Research outcomes and future directions

My thesis shows that forest age since restoration plantings does not lead to microbial community recovery alone, but vegetative properties are important for soil microbiome restoration in a New Zealand urban forest restoration chronosequence. Together, my chapters demonstrate how whole-community activity and biomass, functional group composition, diversity, and phylum-level taxonomic composition shifts in response to forest age and a defined set of important environmental predictors. Therefore, this thesis presents a comprehensive assessment of the soil microbiome, where results from both chapters unitedly point to the importance of both restoration efforts and the influence of the environmental properties of the areas where these efforts are carried out. While I found that microbes may respond to forest age, more often I found that environmental variability among sites elicits particularly strong microbial responses that likely override the effects of forest age alone. The takeaway for restoration managers is that aboveground restoration plantings can ameliorate conditions for soil life; in particular, soil pH changes alongside increasing seedling density, herb cover, tree species richness, and average tree basal area can promote soil microbial recovery. It remains unclear whether restored urban forests can achieve a “climax” microbial community (Strickland et al., 2017). However, specific microbial taxa or microbial properties could be used as ecological indicators that suggest a return of microbial functioning along a soil restoration trajectory (Hart et al., 2020), such as the bacterial Acidobacteriota or fungal Basidiomycota (Chai et al., 2019). To my knowledge, my results present the most comprehensive study to date of microbial community responses along an urban forest restoration chronosequence, especially as studies on soil archaeal and protist responses to restoration are scarce (Geisen et al., 2018; Yan et al., 2020).

The outcomes of my research offer promising avenues for further investigations. With regard to chapter 2, more information is needed on the ecology and function of microbial species in forest soils, including the use of tools that can connect microbial diversity to function, i.e., metagenomics (Mendes et al., 2017). Microbial ecology remains limited by a lack of knowledge on the properties of individual microbial taxa, which restricts our accuracy in linking individual microbial taxa with ecosystem functioning (Baldrian, 2019). Although microbes are a promising indicator of forest restoration recovery, their universal use as a metric of success remains questionable until further evidence can predict their response to restoration interventions and elucidate the functioning associated with specific microbial taxa (Hart et al., 2020). In relation to chapter 3, repeated sampling events that study seasonal variations in addition to soil pH and nutrient changes between restoration sites may yield more information on trends in microbial respiration and biomass. Microbes fluctuate with seasonal change, which may give a better idea of observed trends in activity throughout the year (Yokobe et al., 2018) and their sensitivity to soil pH and nutrient status (Han et al., 2020) could have contributed toward unexplained variation in my findings. With more time, I could have added repeated samplings, and a deeper analysis of metabarcoding or plant species composition data may have yielded interesting patterns to my results. Urban forests are vastly understudied compared to other restored forest types, despite their importance and proximity to human life. In particular, I believe there is a need for more studies on microbial life in urban restored forests, particularly in more populous cities across the globe and with a greater focus on older restored urban forest sites. Soil microbial communities both respond to and facilitate plant growth (Harris, 2009). Forest soil microbiome recovery is essential for the success of aboveground forest restoration plantings (Harris, 2009), providing ecosystem resilience for buffering against future perturbations (Allison & Martiny, 2008) and for human health (von Hertzen et al., 2015), yet it has been vastly understudied in urban forest restoration. Therefore, with the increasing push for green infrastructure in urban spaces to create resilience against global change (Vargas-Hernández & Zdunek-Wielgołaska, 2021), it would be in the best interest of city councils and restoration managers to understand whether aboveground restoration interventions are indeed working as intended to recover fully functional soil microbial communities.

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