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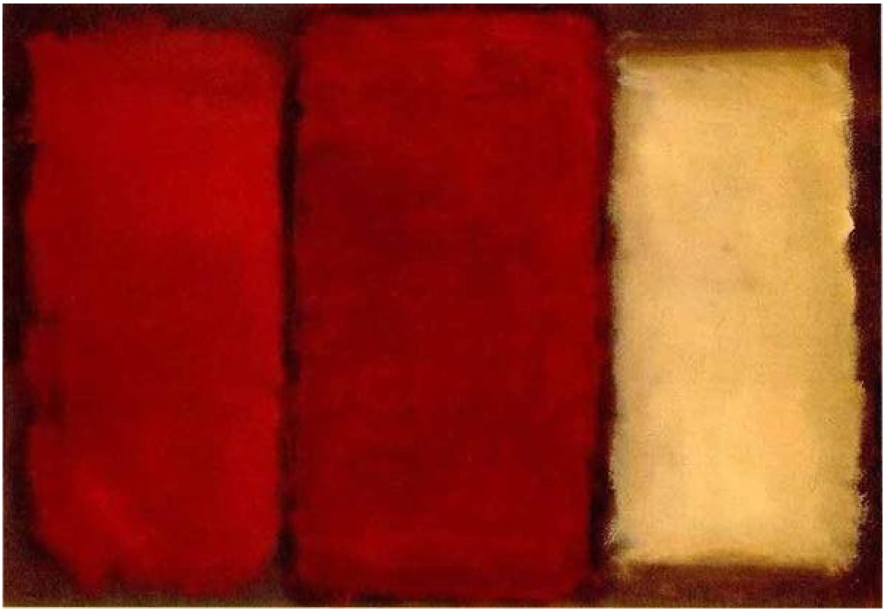
Transient landscapes, changing ecosystems

Microbial implications of land-use change across spatiotemporal scales

Tord Ranheim Sveen



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Transient landscapes, changing ecosystems. Microbial implications of land-use change across spatiotemporal scales

Abstract

Land-use changes lie at the heart of ongoing ecological crises and pose a criticial threat to biodiversity and the functioning of ecosystems. However, the responses of communities and ecosystems to land-use changes are not uniform and vary across space and time. In this thesis I investigate the effects ongoing and historical land-use changes have on soil microbial communities, including plant-soil interactions and potential consequences for carbon cycling. I employ a large-scale successional gradient comprising paired grasslands and forest sites to examine the impacts of land abandonment and afforestation. In addition, I build on historical land-use maps to explore the lingering legacy effects of historical land use on present-day microbial communities. I find that soil microbes are generally resilient to succession in grasslands but undergo marked changes in diversity and functioning when compared to adjacent forest sites. Specifically, microbial communities shift from being more functionally redundant to become more functionally specialized and diverse with land-use change, with shifts in functional diversity and metabolic efficiency potentially posing constraints on soil carbon accumulation during afforestation. I also find that legacy effects of historical land use continue to shape present-day microbial communities. Still, these effects are transient and mostly relate to free-living microbes and fungal pathogens. Overall, the results highlight that the repercussions of land-use changes extend spatially and temporally and affect structural and functional components of microbial communities, which may have significant implications for carbon cycling and ecosystem functioning.

Keywords: land-use change, soil microbes, succession, functional diversity, niche specialization, carbon sequestration, assembly processes, legacy effects.

De förändrade landskapens ekosystem: Mikrobiella konsekvenser av ändrad markanvändning genom tid och rum

Sammanfattning

Förändrad markanvändning är en starkt bidragande orsak till förlusten av biologisk mångfald och utarmning av ekosystemfunktioner. Hur arter och artsamhällen påverkas av förändrad markanvändning varierar dock mycket i tid och rum. I denna avhandling undersöker jag hur pågående och historiska förändringar av markanvändning påverkar marklevande mikrober, innefattande interaktioner med växter och möjliga konsekvenser på den mikrobiella kolcykeln. Jag använder mig av en storskalig successionsgradient med matchade gräs- och skogsmarker för att undersöka effekten av igenväxning och förskogning. Därutöver undersöker jag även spår av historisk markanvändning på nuvarande sammansättningar av marklevande mikrober. Resultat från successionsgradienten visar att mikrober i gräsmarker generellt är motståndskraftiga mot igenväxning, men skiljer sig betydligt från mikrober i näraliggande skogsmarker. Mikrobsamhällena genomgår vidare en förändring från hög funktionell redundans till hög specialisering och funktionell mångfald under igenväxning, och detta kan komma att begränsa markens kolinlagringskapacitet i skogsmarker genom minskad mikrobiell metabolisk effektivitet. Historisk markanvändning fortsätter att påverka nuvarande mikrober, men dessa effekter är övergående och huvudsakligen relaterade till frilevande mikrober och patogena svampar. Tillsammans pekar resultaten på att konsekvenserna av förändrad markanvändning breder ut sig i tid och rum och har stor påverkan på marklevande mikrober, och kan medföra betydande förändringar i kolcykeln och i ekosystemfunktioner.

Nyckelord: Förändrad markanvändning, marklevande mikrober, succession, funktionell mångfald, funktionell nisch, kolinlagring, historisk påverkan.

Dedication

To the myriad of microbes born from Something, and to the Something born from Nothing.

> *”Om man slarvar med förarbetet, får man betala efter”*
> Spolpatrullen, Uppsala 10e april 2024

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Ranheim Sveen, T., Viketoft, M., Bengtsson, J. & Bahram, M. (2024). Core taxa underpin soil microbial community turnover during secondary succession. *Environmental Microbiology*, 26(1), e16561,

II. Ranheim Sveen, T., Gustavsson, E., Cousins, S., Buegger, F., Hildebrand, F., Fritscher, J., Osburn E.D., Pritsch, K., Bengtsson, J., Viketoft, M. & Bahram, M. Historical land use disrupts plant-microbe interactions**.** (manuscript).

III. Ranheim Sveen, T., Viketoft, M., Bengtsson, J., Lejoly, J., Buegger, F., Pritsch, K., Strengbom, J., Fritscher, J., Hildebrand, F., Hannula, E.S. & Bahram, M. Functional diversity and functional redundancy of soil microbial communities trade off during succession**.** (manuscript)

IV. Ranheim Sveen, T., Bengtsson, J., Courcoux, E., Viketoft, M. & Bahram, M. Microbial diversity-functioning limits carbon accumulation during succession**.** (manuscript).

The contribution of Tord Ranheim Sveen to the papers included in this thesis was as follows:

I. Led the development of the ideas and design of the study, performed the field work, performed the sample processing of soil samples, led the data analysis, created the figures and led the writing of the manuscript.

II. Led the development of the ideas and design of the study, performed the field work, led the data analysis, created the figures and led the writing of the manuscript.

III. Led the development of the ideas and design of the study, performed the field work, led the data analysis, created the figures and led the writing of the manuscript.

IV. Led the development of the ideas and design of the study, performed the field work, led and carried out the experimental work in collaboration with others, led the data analysis, created the figures and led the writing of the manuscript.

During this doctorate, Tord Ranheim Sveen contributed to the following papers not included in the thesis:

I. Ranheim Sveen, T., Netherway, T., Juhanson, J., Oja, J., Borgström, P., Viketoft, M., Strengbom, J., Bommarco, R., Clemmensen, K., Hallin, S. & Bahram, M. (2021). Plant-microbe interactions in response to grassland herbivory and nitrogen eutrophication. *Soil Biology & Biochemistry* 156, 108208.

II. Ranheim Sveen, T., Hannula, S.E. & Bahram, M. (2023). Microbial regulation of feedbacks to ecosystem change. *Trends in Microbiology*, 32(1), 68-78.

Abbreviations

AMF	Arbuscular mycorrhizal fungi
C	Carbon
CMF	Carbon multifunctionality
DNA	Deoxyribonucleic acid
EcM	Ectomycorrhiza
ITS	Internal transcribed spacer
LUS	Land-use sequence
MAP	Mean annual precipitation
MAT	Mean annual temperature
MMQ	Microbial metabolic quotient
N	Nitrogen
OTU	Operational taxonomic unit
perMANOVA	Permutational multivariate analysis of variance
PPB	Plant beneficial bacteria
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid

1. Introduction

1.1 The mother meadow

"Ängen är åkerns moder"

The meadow is the mother of the field, goes an old Swedish saying. From early Iron Age settlements (~ 500 BC) until the late 19th century, meadows, or semi-natural grasslands (SNG) with a more technical term, were the backbones of the northern European agricultural landscapes. Because of the generally harsh climate in these areas, fodder (hay) had to be gathered over summer by cutting marginal grasslands and mires, thereby ensuring enough feed for the animals to survive the winter. At the same time, during summertime, animals were herded to graze marginal lands across landscapes so that the more fertile fields could be reserved for growing crops. These practices shaped the surrounding landscapes into a biodiversity-rich mosaic of open and semi-open SNGs. In 1891, it was estimated that around 1.9 Mha were actively managed as grasslands in Sweden (Nyemad et al., 2011). By 2013, the corresponding figure was 0.4 Mha, out of which about half (i.e. 0.2 Mha) was managed similarly to historical SNGs.

Grasslands continue to disappear at super-regional scales, with an estimated 0.8 % lost every year (Wikberg et al., 2023). The dramatic decline of open land over a period spanning roughly 150 years reflects Sweden's transformation from an agrarian to an industrialized nation. With the modernization and intensification of agriculture through fertilizers, mechanization, and imports, marginal lands could be improved to yield more, or conversely abandoned and afforested when no longer needed. With this, SNGs went from being a vital to a largely superfluous component of the landscape.

Concomitant with the disappearance of grasslands, the area of cover and productive capacity of forests has increased (Auffret et al., 2018), leading to a large-scale land-use change from grasslands to forests. This can be grasped with the use of historical cadastral maps, where changes in the proportional cover of grasslands, arable land, and forests are displayed across centuries (Fig. 1A). Conversely, as a forest hiker in Sweden, you sometimes stumble across strangely located stonewalls in the middle of a dense forest (Fig. 1B), which once served to enclose a grazed and (semi)open grassland or an arable field. Together, these remnants from past landscapes tell the story of how the mother of fields, over a hundred brief years, became the mother of forests.

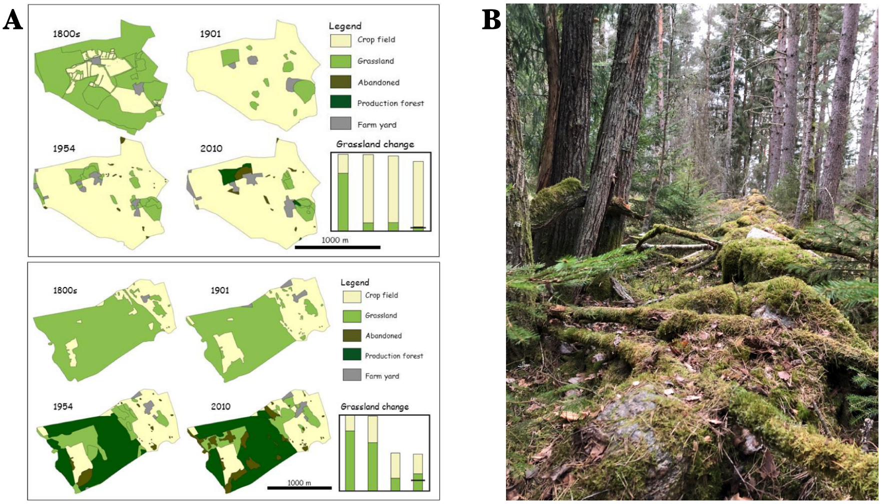

Figure 1. Remnants of historical landscapes

Panel (**A**) shows changing land uses since the 1800s in two Swedish landscapes reconstructed using historical land-use maps. Barplots show the proportion of grasslands and crop fields. The figure was reprinted from Eriksson & Cousins (2014) under the Creative Commons CC BY license. Panel (**B**) shows a remnant stone wall enclosure indicating that this was once a grazed and open semi-natural grassland. Photo by Tord Ranheim Sveen.

1.2 Spatiotemporal effects of land-use change

So landscapes and ecosystems have drastically changed, but what does this have to do with life today? After all - why hold on to or care about landscapes of the past, when mass extinctions threaten our common future? To answer this question, we need to consider how historical land use and landscapes relate to present-day ecosystems and from this try to understand how current land uses will affect future ecosystems. In other words, we need to consider how land use and land-use change affect ecosystems across multiple spatiotemporal scales.

The changes to Swedish landscapes described above are situated within a larger trend in Europe (Huang et al., 2020), and the Global North (Winkler et al., 2021), where afforestation of previously managed agricultural land is the single largest ongoing process of land-use change. Far from being a regional phenomenon, the disappearance of grasslands and arable fields occur at continental scales, and ultimately links to global terrestrial biodiversity depletion (Newbold et al., 2016). For instance, the latest assessment of threats to biodiversity in Sweden (The Swedish Red List), identified the overgrowth of open habitats, coupled with clear-cutting of forests (the standard practice of forest management in Sweden) as the two main drivers negatively impacting biodiversity (Eide et al., 2020). Yet, the ways species and communities respond to land-use changes are rarely direct and linear. Besides spatial landscape changes, which can be measured as the proportion of habitat changes at local, regional, national, or global scales, there is also a temporal component to ecosystem change which extends far beyond the present. For instance, historical landscape configurations and land-use related modifications of soil properties can lead to significant lags in species' response to habitat destruction (Tilman et al., 1994), and generate non-linear trajectories in community development after land-use change (Kimberley et al., 2019). These *legacy effects*, where influences of past land use continue to shape present-day species and ecosystems, can extend across centuries (Gustavsson et al., 2007) or even millennia (Dupouey et al., 2002).

Microbes

The extended spatiotemporal scales and the intricacies of community responses to land-use change, means that there is still much to learn and understand about these in the context of basic ecology. For instance, biodiversity assessments are typically restricted to macroorganisms such as vascular plants, beetles, lichens, butterflies, and fungi exhibiting macrostructures (i.e. fruiting bodies). By contrast, very little is known about the impacts on species and communities residing belowground in the soil, particularly when it comes to the planet's tiniest inhabitants: microbes. A classic statement in ecology has it that insects are the largest and most diverse groups of organisms on Earth. This is likely to be true, if we count only the species we can see. If we instead start counting also microbes, the "unseen majority" (Van Der Heijden et al., 2008), it soon transpires that these dwarf any other organismal group in all aspects of diversity. The problem is that microbes are in many ways different from macroorganisms, and it is still unclear whether we can even classify them as species in any sensible way (Rosselló-Mora and Amann, 2001). Current means of classifying microbes into species from environmental DNA or RNA sequences only allows for putative assignations into "species hypotheses" or operational taxonomic units (OTU), out of which around 70 % typically cannot be classified past the Class or Phylum levels in the taxonomic hierarchy. In short, microbes comprise the most abundant (Giovannoni et al., 2005), the most diverse (Locey and Lennon, 2016), and the most ancient (Nutman et al., 2016) forms of life, and yet we know preciously little about them. This conundrum means that microbes have been typically valued more for what they do (i.e. their functions) than for who they are (i.e. their taxonomic identity) (Inkpen et al., 2017).

With the rapid developments in sequencing technology during the past three decades, we can now "see" into the world of microbes in ways that were previously unimaginable. From this, we can start to fully appreciate their ubiquity – microbes proliferate across virtually all environments on Earth (Liu et al., 2019; Shen et al., 2021) – and their roles as the "engines" driving biogeochemical cycles across ecosystems (Falkowski et al., 2008). Microbes also respond to and are affected by management and land-use change, taxonomically as well as functionally (Bissett et al., 2011). For instance, bacteria and fungi may show differing legacy effects related to the clear cutting of a forest (Osburn et al., 2019), because they typically respond differently to disturbances (Powell et al., 2015). Yet, these generalizations only transmit knowledge on the very coarsest of taxonomic levels, and there are good reasons to believe that legacy effects may differentially impact differing functional groups of microbes both within and across domains.

1.3 Succession

When a grassland is kept open through grazing or hay cutting, a specific flora adapted to continuous biomass removal with high light and low nutrient requirements is benefitted. When management ceases, taller-growing plants gradually replace the species adapted to management and overshadow these, resulting in a shift in the vegetation community (Eskelinen et al., 2022). As time goes on, the taller-growing plants will themselves gradually be replaced by other plants with a differing set of traits better suited to partial shading and changed nutrient levels (McCook, 1994). The resulting sequential replacement of plant species after a disturbance, which is a more appropriate term for the effects of management, is referred to as the process of *succession*, and is one of the most debated and well-studied phenomena in ecology. In the classic view of succession, the sequential changes of plants over time will ultimately steer the grassland into an entirely different forest ecosystem (Fig. 2), where only a few, if any, of the original grassland species remain.

A similar process of succession occurs belowground (Fierer et al., 2010), and typically involves a gradual shift from bacterial- to fungal-dominated energy and carbon channels (Morriën, 2016; Hannula et al., 2017), along with increased nutrient flows (Holtkamp et al., 2011) and microbial biomass (Zak et al., 1990). There is also accumulating evidence that succession above- and belowground, i.e. between plants and soil communities, is interlinked. For instance, shifts within groups of plant-associated microbes (pathogens, mycorrhiza) can feedback to influence plant community dynamics and alter successional trajectories (Kardol et al., 2007; Koziol and Bever, 2019).

Changes in the litter quality of plant communities undergoing succession are an important driver of fungal community development (Cline and Zak, 2015), whereas bacteria appear to be more responsive to changes in soil properties such as pH and C:N (Bahram et al., 2018) and hence also less prone to change with vegetation. Yet, microbial successional developments are still severely understudied compared to studies on plant community changes, especially with regards to functional responses that link to overall changes in ecosystems functioning with succession.

Figure 2. Successional gradient

Conceptual figure of the successional gradient established in **Papers I**, **III** & **IV** which ensues after the cessation of grassland management and goes through successional phases based on changes in the plant community. Photos by Tord Ranheim Sveen.

1.4 Successional problems

Ideally, succession should be studied as repeated measures from single or multiple sites as they develop over time. However, measurements from the same plots over successional timescales, ranging from decades to millennia or even millions of years (Teste and Laliberté, 2019), are exceedingly rare, not least in the fairly novel field of microbial ecology. Instead, researchers are left to infer temporal changes across spatial gradients (space-for-time substitution, also referred to as "chronosequences"), based on the key assumption that sites will develop similarly within a landscape (Walker et al., 2010). This method is not void of problems and has been criticized for yielding unreliable inferences of biodiversity patterns when compared to time series data from permanent plots (Johnson and Miyanishi, 2008).

Because succession occurs everywhere a disturbance has altered an environment, studying how communities recover and assemble afterwards is a natural starting point for formulating ecological theories (Simberloff and Wilson, 1969). Succession was consequently also one of the earliest phenomena used to develop ecological theory in the early 20th century (Clements, 1916; Gleason, 1917, 1926). Plant ecologists Frederic and Edith Clements (Clements, 1916) observed how vegetation communities recuperated and developed after exposure to disturbances, and posited that this was a largely deterministic and progressive process resulting from the interaction of site-specific climatic factors and an infamous "complex organism" steering the community toward increasing complexity (Clements, 1928). The idea of a deterministic, orderly, and fully predictable succession was nonetheless quickly contested by other ecologists who instead found community changes to be a result of stochastic processes whereby individual plant species competed against each other for light and nutrients (Gleason, 1926; Simberloff, 1980). It is noteworthy, that the notion of the *ecosystem*, first coined by British botanist Arthur Tansley in 1935 (Tansley, 1935), was developed as a critical rejoinder to another implication of the Clementian view of succession: the idea of the "monoclimax" state at the end of the successional trajectory (Clements, 1936). To make a long story very short, the Clements' had posited that succession culminates in a mature ("climax") forest, characterized by maximal biomass and a general homeostasis in nutrient cycling, from which no further succession was possible. At least not until another disturbance, for instance a fire, would sweep over the forest and initiate a new cycle of succession. Five decades later, American ecologist Dan Simberloff (Simberloff, 1980) would lament the conception of the monoclimax succession theory and brand it as an unfortunate relic of ancient Greek platonic metaphysics, where empirical processes are understood only in relation to their putative ideal states. Few ecologists today value the notion of the superorganism or believe that successional trajectories are inherently deterministic. Still, it is hard to overestimate the epistemological influence of the Clementian theory of succession over ecology even today. Most study systems applying space-for-time substitution - that is, probably 99 % of studies on secondary succession, including the ones in this thesis – implicitly operate with the understanding that succession can be categorized into differing stages along the chronosequence (i.e. early- mid- and late-stage successional phases) based on characteristic changes in plant communities. Although this may ultimately be an accurate reflection of sequential succession both in theory and practice (McCook, 1994), it nevertheless introduces a risk of circular inference: a chronosequence is established in the field based on current vegetational patterns which are divided into differing stages along the assumed successional trajectory; observations are then gathered and processed according to their location in the chronosequence and used to infer patterns within the successional trajectory already established. In other words, the successional trajectory is established *a priori*, and then used to infer patterns and processes during succession. I mention this extensively here because it constitutes a core problem and source of theory in ecology, and because the results I obtained in the spatial gradient can only be understood against the backdrop of an *a priori* established successional gradient. Encouraging evidence from successional trajectories after glacier retreats indicates that the gap between chronosequence-inferred succession and permanent plot time-series is small (Cantera et al., 2024). However, these time series only extend across two decades at most, whereas chronosequences can be inferred across millions of years (Turner et al., 2024). Thus, chronosequences and succession will continue to be inextricably bound for a long time yet.

2. Aims & Objectives

2.1 Aim

In this thesis, I have aimed to examine the impacts of large-scale landscape transformations across Sweden over the past three centuries on soil microbial communities. This involves the impacts of ongoing land-use changes from grasslands to forests (**Papers I-IV**) and the consequences of historical land-use changes (**Paper II**). To this end, I addressed the following questions

1) What are the structural and functional implications of land abandonment and succession on soil microbial communities? (**Papers I**, **III** & **IV**)

2) What processes drive microbial community assembly during succession? (**Paper I**)

3) How do legacy effects from historical land use impact soil microbial communities in relation to their functional characteristics? (**Paper II**)

4) How does land abandonment affect soil microbial carbon cycling and its relation to the carbon sequestration potential with grassland afforestation? (**Papers III-IV**)

3. Methods

3.1 Site descriptions

For **Papers I**, **III**, and **IV**, the same land-use change gradient from extensively managed grasslands to production forests was used. This gradient was established from grasslands and forest sites that had been inventoried within two environmental monitoring programs run by SLU. Grassland sites were gathered from the REMIIL [*Regional övervakning av småbiotoper, gräsmarker och våtmarker*] database according to their geographic location and management status (managed vs abandoned). Forest sites were obtained from the *National Forest Inventory* [*Riksskogstaxeringen*] and selected based on their proximity to the grassland sites and productivity. The resulting land-use change gradient spanned across the whole of Sweden and covered managed, early- and late successional grasslands (Fig. 3). A total of 101 grasslands and 105 forest sites were selected, out of which 100 sites from each category were paired based on geographical proximity (median distance between paired sites = 3.63 km, maximum distance = 6.5 km). The pairing of grassland and forest sites was done to minimize topographic and environmental variation within the successional gradient. I hereafter refer to this study system as the “spatial gradient”. Note, however, that the terminology for the differing grasslands differ between **Paper I** and **Papers III and IV**, but these refer to the same sites and same categories. For **Paper II**, where I examined temporal aspects of land-use change, sites with documented long-term land-use histories in two regions of Sweden were selected based on data from two previously published studies (Cousins, 2001; Gustavsson et al., 2007). The sites were divided into land-use sequences (LUS) based on historical land-use changes across 300 years. Sites documented as uninterrupted SNG (i.e. not having undergone any land-use changes) were selected as the baseline category to infer legacy effects of land-use changes. I hereafter refer to this study system as the “temporal gradient”.

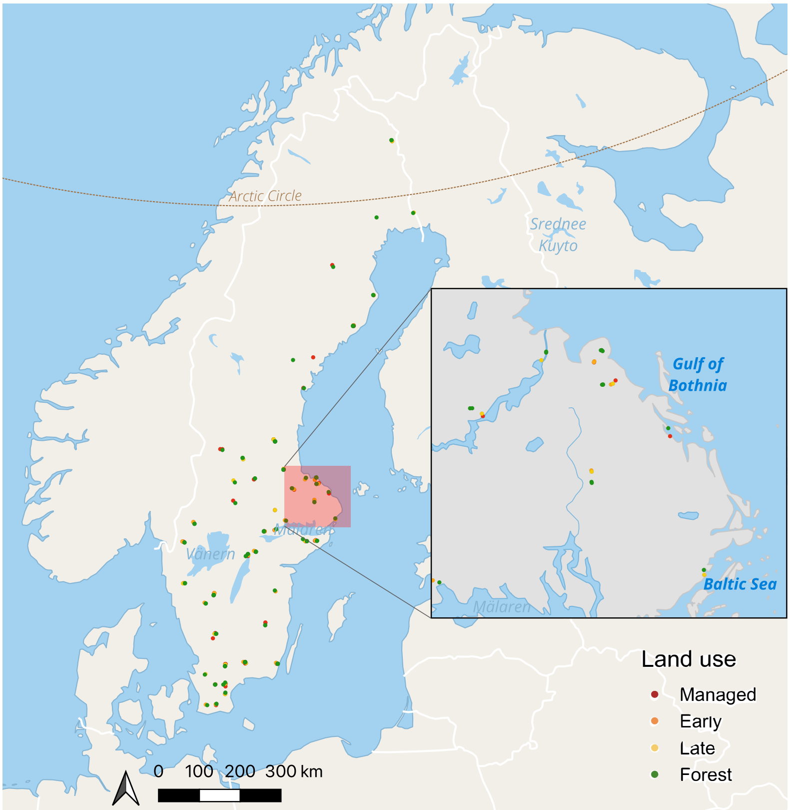

Figure 3. The spatial gradient

Map of sampling sites and their distribution across Sweden used for **Papers I**, **III**, and **IV**. The zoomed-in quadrant shows an example area of paired grassland-forest sites.

3.2 Sampling method

The spatial gradient was sampled during two consecutive summers in 2020 and 2021. At each site, circular areas (30 – 200 m^2) were sampled by pooling 16 cores (diameter = 3 cm, depth = 10 cm) at 2 m distance intervals in every direction from the centre coordinate. No separation of organic and mineral layers was done, but any loose and undecomposed litter was removed prior to sampling. Sampling of the temporal gradient occurred in summer 2021 similarly.

3.3 Soil and vegetation properties

3.3.1 Vegetation data

Vegetation data for all sites in the spatial gradient (**Papers I**, **III** & **IV**) were derived from each respective environmental monitoring program (i.e. REMIIL and NFI) and consisted of presence-absence and cover matrices. For the temporal gradient (**Paper II**), plant species richness and cover (%) was investigated in 25m^2 plots in each site, with all vascular plants recorded to species level. These surveys were conducted in 2015 (Nynäs) and 2021 (Källstorp). Plant functional traits (i.e. leaf nitrogen and dry matter content) were derived from the TRY database (Kattge et al., 2020) (Ver. 6.0) and converted to community-weighted means for each site using the *FD* package (Laliberté and Legendre, 2010).

3.3.2 Soil properties

Soil chemical analyses were performed on subsets of bulk samples for each site in both the spatial and temporal gradient. For pH and soil nutrient analyses, approximately 15 g of the refrigerated soil was used to analyse pH, P, and K using the ammonium lactate and acetic acid method (Egnér et al., 1960). pH was measured in a 1:5 soil: water suspension. Soil ^{13}C natural abundances, along with total soil C and N contents were determined using an Isotope Ratio Mass Spectrometer/IRMS (delta V Advantage, Thermo-Fisher Scientific, Dreieich, Germany) coupled to an Elemental Analyzer (Euro EA, Eurovector, Milano, Italy) on aliquots (1–20 mg) of air-dried soil. For details, see the respective **Papers I-IV**.

3.4 Carbon cycling functions and indices

For **Paper III** and **Paper IV**, two classes of soil-driven ecosystem functions were measured to assess the functional implications of microbial community changes during land-use change and succession.

3.4.1 Catabolic profiling

Microbial carbon cycling was inferred from substrate-induced respiration (SIR) (Strickland et al., 2017). Briefly, 8 mL solutions of glucose, glycine, oxalic acid, autolyzed yeast, lignin, and chitin were added to 4 g dry-weight equivalent of fresh soil (1 analytical replicate per solution). An additional amendment consisting of 8 mL ddH_2O was used to calculate basal respiration rates (BR). After 1 h of pre-incubation, soils were incubated for 4 h at 20 °C except for lignin and chitin which were incubated for 24 h. After incubation, respiration for each amendment was determined using Gas Chromatography (Trace CG Ultra Gas Chromatograph (Thermo Fisher Scientific, Milan, Italy)).

3.4.2 Standardized decomposition

We measured decomposition and litter stabilization in pot mesocosms of surface soils (0-10 cm, $n = 168$) under standardized temperature (22 °C) and humidity (50 % RH) in greenhouse conditions. Pot size was 1L, and standardized litter in the form of red and green tea was used following the protocol of Keuskamp et al. (Keuskamp et al., 2013). After 90 days of incubation, each litterbag was extracted and weighed. Based on the mass loss between pre- and post-incubation weights, decomposition (k) and litter stabilization (s) constants were calculated according to (Keuskamp et al., 2013). Soils were adjusted to 50 % WHC (w/w) prior to incubation and watered when necessary.

3.5 Sequencing and bioinformatics

3.5.1 DNA extraction

Extraction of DNA from dried and pulverized soil samples (~200 mg) was done using PowerMax extraction kits following standard instructions.

3.5.2 Metabarcoding

For bacteria (**Papers I-IV**), the V4-V5 regions of the 16S ribosomal rRNA gene were amplified using polymerase chain reaction (PCR) with the primers 515F and 926R (Walters et al., 2015). Fungi in the spatial gradient (**Papers I, III, IV**), were similarly amplified with the ITS9mun and ITS4ngsuni primers (Tedersoo and Lindahl, 2016) targeting the partial 18S rRNA gene and full-length internal transcribed spacer (ITS) and sequenced on the PacBio Sequel System. For fungi in the temporal gradient (**Paper II**) amplification was done using the ITS4ngsUni-fITS7 primers targeting the second internal transcribed spacer region (ITS2) (Ihrmark et al., 2012; Tedersoo and Lindahl, 2016), and the products were sequenced on an Illumina MiSeq platform (2 × 250 bp paired-end chemistry). Sequences were processed using the LotuS2 version 2.22 pipeline (Özkurt et al., 2022).

3.5.3 Shotgun metagenomics

Shotgun metagenomics was performed on equimolar amounts of DNA from a subset of samples ($n = 94$) in the spatial gradient, and the complete set of samples ($n = 146$) of the temporal gradient. Metagenomic reads were processed using the MATAFILER pipeline (Hildebrand et al., 2021) with a workflow optimized for complex environmental metagenomes (Bahram et al., 2021). More workflow details are found in the method section of **Paper III**.

3.5.4 Abundance estimates

Abundance estimates for fungi and bacteria in **Paper IV** were determined by real-time quantitative PCR (qPCR) using the fITS7 ITS4/ITS4a primers for fungi, and the 515F and 926R primers for bacteria, respectively. The amplifications were validated by melting curve analyses and agarose gel electrophoreses. Potential inhibition of PCR reactions was checked by amplifying a known amount of the pGEM-T plasmid (Promega, Madison, WI, USA) with the plasmid specific M13F/M13R primer set and the addition of 4 ng of template or non-template controls. No inhibition was detected with the amount of DNA used.

3.6 Data analysis

All analyses and data visualizations were done using R and RStudio, with *p*-values involving multiple comparisons adjusted by Benjamin-Hochberg corrections. For **Paper I**, phylogenetic turnover to infer the relative influence of assembly processes was calculated according to the framework developed by Stegen et al. (2013, 2015), based on null-model analyses with 999 randomizations. Tests for differences in the relative influence of these processes between land uses were performed using pairwise comparisons of proportions. Mantel tests with Spearman correlations were used to test the relationship between phylogenetic turnover and soil pH. Core microbiome analyses were done within the zeta diversity framework (Hui and McGeoch, 2014), and additional details on these analyses can be found in the method section of **Paper I**.

Tests for differences in taxonomic (**Papers I-III**) or functional (**Paper III**) community composition between land uses were conducted using permutational analyses of variance (perMANOVA) with Bray-Curtis distance matrices using the *vegan* package (Oksanen et al., 2022). Prior to analyses for differences in alpha diversity, either through Shannon's *H'* or through richness estimates, sequence matrices were standardized to equal sample size by scaling with ranked subsampling (Beule and Karlovsky, 2020) (**Papers I, III & IV**) or with multiple rarefactions (**Paper II**). Functional matrices (**Papers III-IV**) were rarefied to minimum sample depth. Subsequent tests for differences in alpha diversity were based on mixed linear models with paired sites, geographic distance between sites, and climate variables (MAT, MAP) as random factors, depending on comparisons. Analogous models were used when analysing trends in carbon-related functions and indices and microbial abundance estimates with succession (**Paper IV**), but with managed grasslands set as the reference levels for other land uses. Similarly, SNGs were used as the reference baselines for inferences of legacy effects from historical land-use changes on present-day plant and soil microbial diversity estimates (**Paper II**). Conversely, the relative importance of land use across time was inferred through ANOVA Type II tests and dominance analyses using the *car* (Fox and Weisberg, 2019) and *domir* (Luchman, 2023) packages, respectively. Tests for differences in relative abundances between functional groups of microbes were performed using the ANCOM-BC method (Lin and Peddada, 2020). Soil edaphic or climatic variables related to alpha and beta diversity were inferred through forward selection models using Z-transformed predictor variables. In the case of high collinearity (VIF > 5), one of the colinear variables was omitted from subsequent analyses.

Niche specialization and functional redundancy in **Paper III** were inferred by measuring Levin's niche overlap (Hurlbert, 1978) of *CAZymes* within land uses and comparing their average overlap using pairwise Wilcoxon Rank-Sum tests. Niche overlaps were calculated with the *MicroNiche* package (Finn et al., 2020) and *CAZymes* were partitioned into their associated classes of carbon substrates based on tables provided in Piton et al. (2023). In addition, functional redundancy stemming from decoupling between taxonomic and functional composition was inferred using co-correspondence analyses with the *cocorresp* package (Alric et al., 2020).

Relationships between carbon-related functions and indices (**Papers III-IV**) were inferred by ordinary least-square regression. In **Paper IV** we standardized all carbon cycling function, indices and diversity estimates of taxonomic and functional richness and biomass using Z-transformation, and related average Z-scores of fungal and bacterial diversity to carbon multifunctionality with linear regression (Maestre et al., 2012).

4. Results and discussion

4.1 Grassland resilience and successional gaps

Results from the spatial gradient indicate that developments in the soil microbial community is partly decoupled from the successional trajectory in the plant communities aboveground. Plant community composition differed between managed and abandoned grasslands in differing successional stages (Table 1), and complementary analyses of the indicator species contributing to compositional differences aligned well with the expected successional patterns in plant communities after land abandonment in Sweden (Ekstam and Forshed, 1992).

Table 1: Combined tests for differences in composition and indicator species contributing to observed differences between plant communities. All indicator species listed were significant ($p < 0.05$). All grasslands differed from forest sites in terms of plant community composition (perMANOVA, $p < 0.05$).

Comparison	*SES*	*p-value*	*Indicator species*
Managed vs Early	1.20	0.0072	*Taraxacum;* *T. repens; P. tremula*
Managed vs Late	2.59	< 0.0001	*R. arcticus; R. ideaus; F. ulmaria; C. purpurea; C. fontanum; Taraxacum; A millefolium;* *E. sylvaticum; P. lanceolata; L. vulgaris ; T. repens ; P. tremula ; B. pendula ; P. abies*
Early vs Late	1.53	0.0012	*C. purpurea; A. millefolium; P. abies*

In **Paper I** and **Paper III**, I nonetheless found that microbial communities remained remarkably similar across differing stages of grasslands. However, when compared to adjacent paired forest sites, microbial communities invariably changed drastically both in terms of community composition and alpha diversity (Fig. 4). While these results go against the notion of a smooth and linearly progressing successional trajectory, they could be due to either methodological or biological factors, or possibly both. Firstly, the space-for-time substitution methods used to infer succession may be too coarse to capture the rate of diversity change in microbial communities. Despite most of the late-stage successional grasslands having > 60 % tree cover in the sites I examined, the understory was still dominated by grasses and saplings and contrasted markedly with the fully afforested sites used as the successional endpoint. Similar gaps between late successional stages and the designated successional endpoint have been found in other studies (Cline and Zak, 2015), and indicate that several stages are “missing” in the study design to bridge the transition from grasslands to forests. Conversely, successional stages are typically designated based on plant community changes, but these may not be paralleled by similar changes belowground (van der Putten et al., 2009). In other words, successional trajectories above- and belowground may be partly decoupled, so we should strive to develop study designs that better capture belowground trajectories independently of aboveground changes. Recent evidence showing strong resilience of microbial communities to first abandonment and then re-introduced cattle grazing of alpine meadows (Vidal et al., 2020) also point in the direction of decoupled successional trajectories above- and belowground.

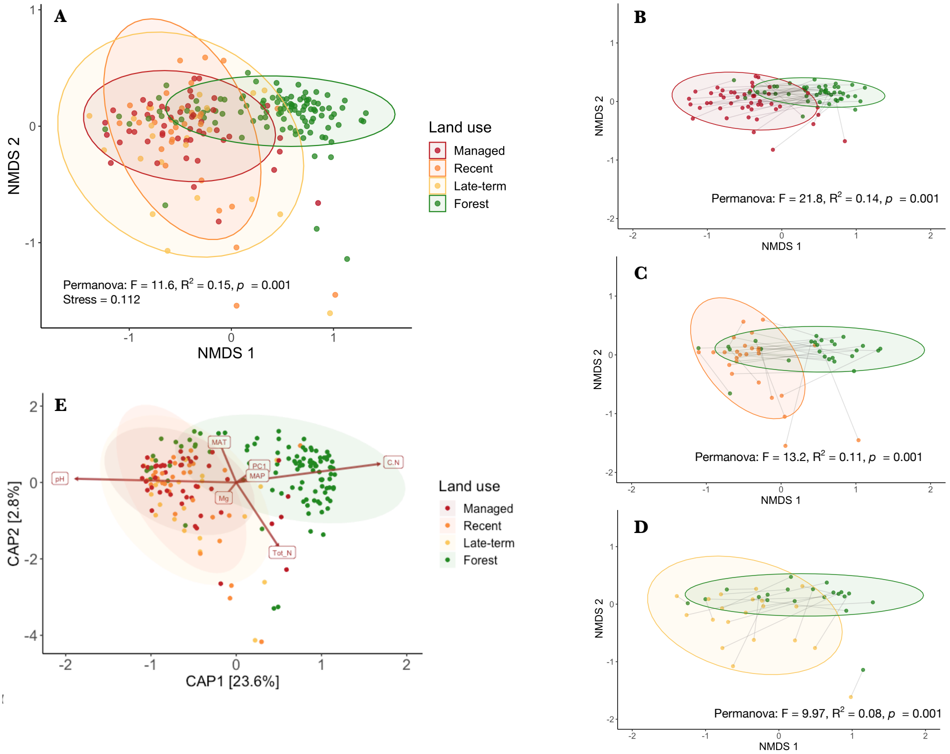

Figure 4. Bacterial community composition across the spatial gradient
Differences in bacterial community composition between land uses across the spatial gradient. Lines connect paired grassland and forest sites in panels (**B-D**).

4.2 Community assembly links to soil heterogeneity

Pioneering studies of microbial succession have successfully related community development to changes in the assembly processes after disturbance (Ferrenberg et al., 2013; Dini-Andreote et al., 2015), and suggested that these processes change predictably from stochastic to deterministic assembly with ecosystem recovery. In **Paper I**, I examined the assembly processes shaping bacterial communities along the spatial gradient. I found no evidence of a similar pattern in the relative influence of deterministic and stochastic assembly in microbial communities after land abandonment (Fig. 5A). Instead, bacterial assembly was mainly related to the heterogeneity of soil variables (especially pH) within the differing successional stages (Fig. 5B). The conclusion from this is that while soil pH is the primary soil property steering deterministic bacterial community development during succession (Tripathi et al., 2018), considering soil heterogeneity within successional stages is an important link to understand patterns observed across successional seres. Specifically, lower pH heterogeneity was linked to higher levels of homogenous selection and thereby more similar communities. However, it is also important to consider the underlying differences in the causal factors (i.e. disturbances) when comparing results across studies of successional trajectories. In the case of heavy disturbances, such as fire (Ferrenberg et al., 2013) or glaciation (Kearns and Shade, 2018), community assembly patterns may be reasonably predictable, as they start from a “blank slate” environment where large parts of the microbial community has been wiped out. With succession ensuing after land abandonment, contingencies from differing management practices may instead influence development and lead to less predictable trajectories (Cramer et al., 2008).

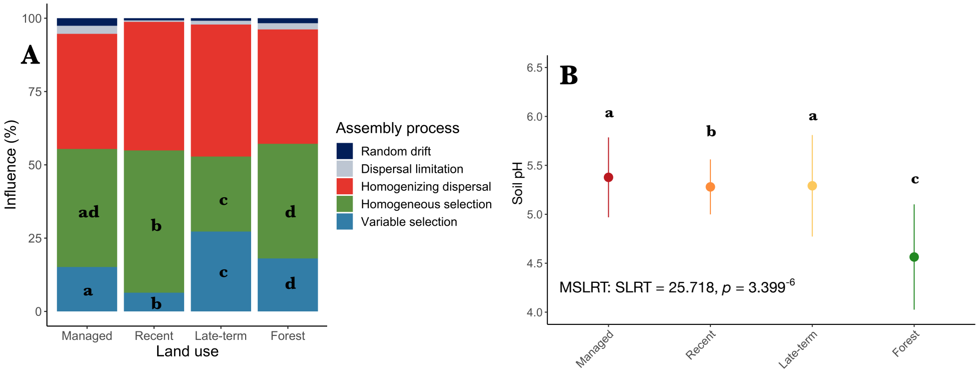

Figure 5. Assembly processes and pH heterogeneity in the spatial gradient
Panel (**A**) shows the relative influence of deterministic (homogenous selection, variable selection) and stochastic (random drift, dispersal limitation, homogenising dispersal) assembly processes within each successional stage along the spatial gradient. Significant differences ($p < 0.05$) based on pariwise comparisions of proportions. Panel (**B**) shows the mean and standard deviation of soil pH along the successional gradient, along with

letters denoting differences in their respective coefficient of variation (CV) based on modified signed-likelihood ratio tests (MSLRT) for equality of CV.

4.3 Legacies of historical land-use change

To further examine possible contingencies of historical land use on present-day microbial communities, I established a temporal gradient (**Paper II**) with sites found in two Swedish regions where land-use changes have been documented in cadastral maps at different time steps over the past 300 years. Previous research from these regions have shown that historical land use has a lingering influence on present-day plant communities (Cousins, 2001, 2009; Gustavsson et al., 2007), but little is known about whether these effects extend also to soil microbial communities. Our results show that legacy effects were generally transient (i.e. they typically disappeared within a century) and varied between differing functional groups of microbes (Fig. 6). Bacteria were more affected by historical land use than fungi, something we attribute to their higher responsiveness to changes in soil abiotic properties (Bahram et al., 2018). Permanently altered soil properties is the arguably best known mechanism of biotic legacy responses (Dupouey et al., 2002; Cramer et al., 2008) and is likely to conserve microhabitats suitable for specific groups or taxa which would otherwise be outside their niche preferences after subsequent land-use changes. In line with this hypothesis, we also found that legacy effects were stronger for free-living than for plant-associated microbes.

An important exception to the general absence of legacy effects in fungal communities was the higher richness and relative abundances of fungal plant pathogens in fields with a history of arable land use when compared to sites characterized by continuous grassland management (Fig. 6C). We attribute these effects to the build-up of plant pathogens in arable fields characterized by cropping practices (Morriën, 2016). This interpretation is supported by recent evidence showing that legacy effects stemming from fungal pathogens found within plant roots amplify with monoculture and create subsequent negative plant-soil feedbacks (Hannula et al., 2021). Over time, the pathogen richness in these fields diminished and eventually disappeared altogether, and this is likely due to increased plant diversity in grasslands (Bever et al., 2015). Notably, afforestation of grasslands entailed a marked decrease of fungal pathogens (Fig. 6C), which could be due linked to the concomitant increase in ectomycorrhizal fungi (Fig. 6B), as these have been shown to increase plant defences toward pathogens (Marx, 1972).

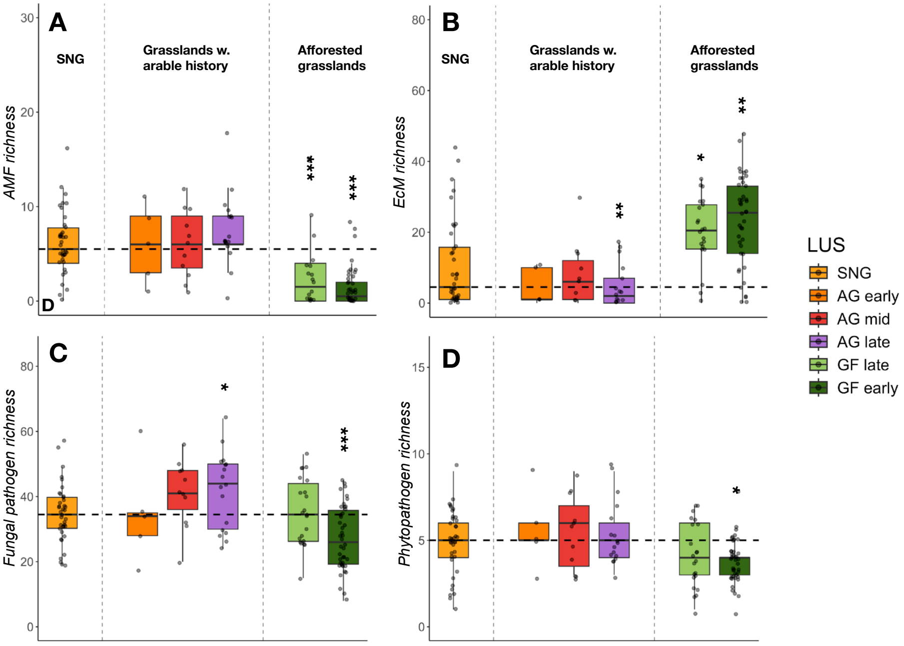

Figure 6. Legacy effects of land-use changes in microbial functional groups
The figure shows the richness of (**A**) arbuscular mycorrhiza (AMF), (**B**) ectomycorrhiza (EcM), (**C**), fungal pathogens, and (**D**) bacterial phytopathogens across each land-use sequence (LUS). Semi-natural grasslands (SNG) is the baseline of sites with a long-term (> 300 years) of uninterrupted grassland management (dashed horizontal line); AG early, AG mid, and AG late are sites with a history of arable land use but currently managed as grasslands. GF early and GF late encompass sites with a history of grassland management but since abandoned and afforested. Asterisks (*) denotes statistically significant differences in diversity compared to the SNG baseline based on generalized linear models with the following significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Although these and other findings contribute to deepen our understanding of how past land use continues to influence today's microbial communities, there is still a range of questions left to answer. For example, legacy effects appear to be generally stronger for habitat specialists than for generalists across trophic levels aboveground (Le Provost et al., 2020). Should we expect the same patterns to hold true for soil microbes? Investigating this would require a deeper understanding of the particular ecology and niche preferences of individual microbial taxa, which is still elusive for most OTUs found in soils today. Traditional measures of specialization, such as a taxon's niche breadth (Feinsinger et al., 1981) can be used to infer specialization statistically. Still, these should ideally be linked to habitat characteristics and management in ways that make ecological and not just statistical sense (Blanchet et al., 2020).

4.4 Implications of land-use change for carbon cycling

4.4.1 Niche specialization and functional redundancy

In the last two chapters of my thesis (**Papers III & IV**), I examined the consequences of land-use change from grasslands to forests, via succession, on microbial carbon cycling. In **Paper III,** this was done by examining whether microbial communities undergoing succession would become increasingly specialized in their capacity to produce carbohydrate-degrading enzymes (*CAZymes*). Niche specialization indeed increased during succession, which is in line with predictions of classical succession theory (Odum, 1969). This was, moreover, true for both bacteria and fungi, and is likely related to the fact that plant litter becoming increasingly complex and difficult to degrade during succession (Zak et al., 1990). When substrates become more recalcitrant, their degradation typically involves several sequential steps performed by differing taxa, each specialized in that particular step (Prescott and Vesterdal, 2021). In other words, succession leads to a gradual opening of new niches within the overall framework of microbial carbon cycling, and this is accompanied by increasing niche specialization (Macarthur, 1965) and functional diversity. Interestingly, our results show that this was not accompanied by increasing taxonomic richness. On the contrary, as seen also in **Paper II**, the taxonomic diversity of bacteria and fungi decreased between grasslands and forests. Additional analyses of the microbial community functional profiles (*CAZymes*) showed that functional diversity increased relative to taxonomic diversity with afforestation, indicating that decomposition and carbon cycling is partitioned across fewer taxa in forests. This suggests that the community-wide functional breadth (also referred to as the "community niche space" (Sánchez González et al., 2023)), is decoupled from, or even negatively related to taxonomic diversity. In **Paper III**, we speculate that the divergence between taxonomic and functional diversity is tantamount to a shift from high levels of functional redundancy, i.e. where many taxa capable of performing the same steps in the carbon decomposition process (Louca et al., 2018), to high levels of functional specialization but low redundancy. To test this hypothesis more explicitly, we assessed trade-offs between functional redundancy and functional diversity for bacterial communities and found that grasslands and forest sites indeed diverged in opposite directions of the redundancy-functional diversity dichotomy (Fig. 7).

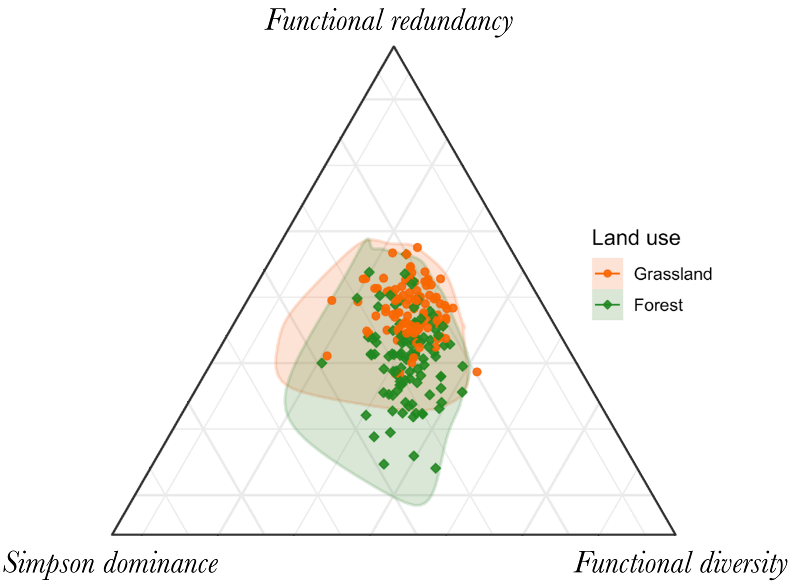

Figure 7. Trade-offs between functional diversity and redundancy
The figure shows a ternary plot over bacterial communities in grassland and forest sites from the spatial gradient. The sites are distributed in relation to three axes of diversity: Functional redundancy, functional diversity, and taxonomic diversity (Simpson dominance). Grassland sites have higher functional redundancy but lower functional diversity than forest sites.

4.4.2 Limited carbon sequestration with afforestation

Currently, much hope is attached to the role of soil carbon sequestration for climate-change mitigation (Minasny et al., 2017), and active or passive afforestation of marginal land is touted as a key strategy within these efforts (Bastin et al., 2019). Yet, a bundle of meta-analyses conducted around two decades ago all pointed to limited carbon accumulation with grassland afforestation (Post and Kwon, 2000; Guo and Gifford, 2002; Paul et al., 2002). In my last thesis chapter (**Paper IV**), I examined whether the changes observed within microbial communities during the afforestation of grasslands could pose eco-physiological constraints to the carbon sequestration potential by analysing the trends in five interrelated carbon cycling functions and indices and relating these to microbial community changes. Results showed that while carbon stocks remained similar between grasslands and forests, the individual carbon cycling functions diverged between the two types of ecosystems. Specifically, forest soils had higher levels of recalcitrant carbon (as inferred by $\delta^{13}C$) and microbial metabolic quotient (MMQ), whereas rates of decomposition and litter transformation were higher in grassland soils. When aggregating these functions under the umbrella term of carbon multifunctionality (CMF) and relating this to microbial diversity, we could show that the functional richness of bacteria and fungi were inversely related to CMF (Fig. 8). Notably, increased MMQ in forests imply that the metabolic costs involved in organic matter decomposition increases, likely as a consequence of higher abiotic stress levels due to low soil pH and increased carbon and nitrogen recalcitrance (Anderson and Domsch, 1993). This could provide a microbially mediated constraint to carbon accumulation in boreal forests but should be considered in tandem with other factors such as the presence of specific decomposer groups with known mechanistic links to organic matter degradation (Lindahl et al., 2021). Bacterial diversity appeared to be most strongly related to carbon cycling along the successional gradient, which is in line with emerging evidence on the importance of bacteria as decomposers in high-latitude environments (Glassman et al., 2018; Huang et al., 2021). Notably, these results concern soil carbon stocks, and does not include standing biomass. Including also this into the analyses may well lead to a net carbon sequestration with afforestation of abandoned grasslands (Thibault et al., 2022).

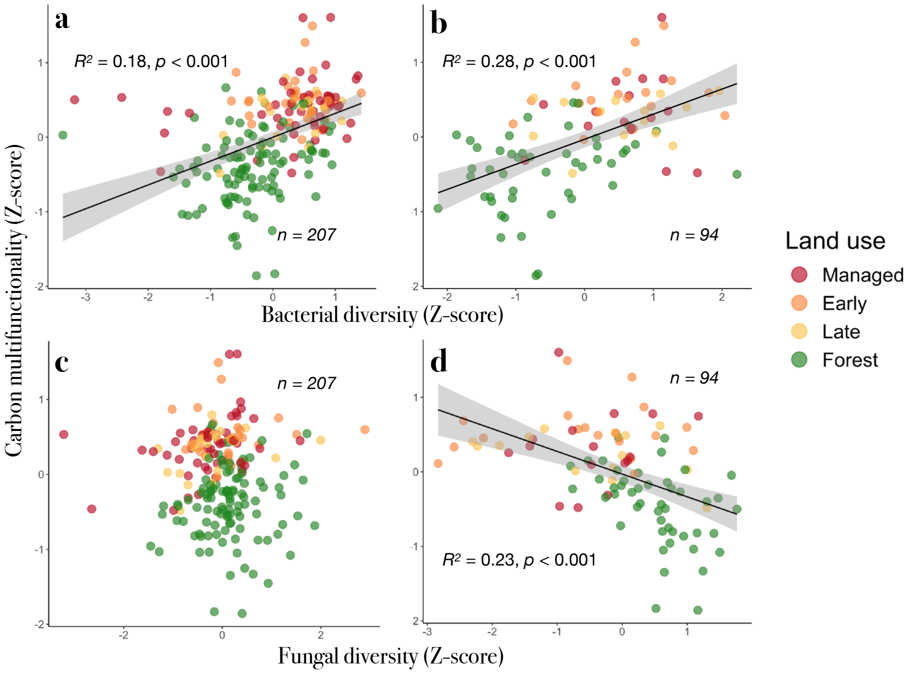

Figure 8. Diversity and carbon multifunctionality

Standardized bacterial (**a-b**) and fungal (**c-d**) diversity in relation to carbon multifunctionality. Panels (**a,c**) show taxonomic (i.e. richness and abundance) diversity and panels (**b,d**) show functional richness.

4.5 Final reflections on succession

Mark Rothko was a Latvian-born American abstract painter who made it into his hallmark to paint with rectangular regions of discrete or contrasting sets of colours, as seen throughout this thesis. Rothko allegedly said of his art that "*A painting is not a picture of an experience, but is the experience*". This statement echoes Simberloff's critique of idealism in ecology (Simberloff, 1980), in asserting that the theory of succession is not succession. Based on these assertions, we should doubt, if not outright abandon the use of chronosequences altogether, as they are inextricably anchored to the assumption of a "reasonably predictable" trajectory of ecosystem development (Odum, 1969). The results and readings I have come across during my work with this thesis has made me think that Simberloff's critique is probably more relevant for the study of microbial succession than for plant communities. It is problematic to *a priori* establish a successional gradient aboveground and expect this to reflect a concurrent succession belowground, because, as seen in **Paper I** and **Paper II** here and elsewhere (Wardle et al., 2004) the links between plants and microbes vary enormously. Thus, community changes in plant-associated bacteria and fungi may reflect successional developments aboveground better than their free-living counterparts. Traits that are unique for microbes may also introduce additional mechanisms into microbial succession that have no equivalent counterpart in plants. For example, dormancy and sporulation may both speed up or slow down community changes after disturbance (Kearns and Shade, 2018; Barnett and Shade, 2024), but these traits are rarely accounted for in classic resource-based succession scenarios. Overall, trait-based approaches have served to advance plant successional theory considerably since the early 20th century (McCook, 1994), and similar approaches could be employed to examine nuances in soil microbial succession more closely, with the aim of testing relative degrees of decoupling between above- and belowground communities.

This is not to say that we should wholly abandon plant successional gradients if they fail to yield any sensible results for belowground communities. On the contrary, these theories represent excellent starting points for belowground inquiries, which should nonetheless be considered also on their own terms, independent of plants. The cover of this thesis is a reproduction of one of Rothko's paintings titled *Red, 1964*, which I think provides a fitting illustration of my findings from the spatial gradient. I see the shifting nuances of red in this painting as the very similar yet distinct stages of grasslands in a successional seres, which contrast with the distinct yellowish of the adjacent forest. Notably, the stages don't blend into each other in a continuous transitional gradient but are separated by discontinuities - discrete borders. Do these borders reflect a flawed methodology or are they real and reflect a tangible biological phenomenon? Perhaps an infinite amount of time series from an infinite amount of permanent plots could provide a general and definite answer to this question, but until we have these, we need to keep inferring ecological patterns across time and space based on flawed theories and the historical artefacts we find scattered across maps and landscapes.

5. Conclusion and future perspectives

Current tendencies of land abandonment and afforestation are likely to continue in Sweden as well as in Europe and the Global North over the foreseeable future, and further transform landscapes and ecosystems continental scales. Meanwhile, we are only beginning to unravel the consequences of present and past land-use changes on soil microbial communities. In this thesis, I have examined some of the contingencies that modulate soil microbial responses during succession and historical land use. By combining field-based soil sampling with vegetation surveys and lab experiments on microbial carbon cycling, I have attempted to address the issues of land-use change on a whole-ecosystem level, from the micrometre scale of microbes to the nationwide pattern of land abandonment. My results and their interpretations are therefore necessarily blunted and quite descriptive, in the sense that there are few identified mechanistic links between the functions of individual microbial taxa or functional groups and changing ecosystems. Instead, I have attempted to situate the development of soil microbial communities in the framework of classic successional theory and its predictions about ecological processes during ecosystem development. Some of the results obtained follow in line with both theory and previously observed patterns (e.g. niche specialization, and the differential responses of fungi and bacteria to land-use change), whereas some findings appear to be more novel and contrast with expected patterns (e.g. the multi-layered development of carbon cycling during succession). Future studies should attempt to isolate, elucidate, and corroborate these findings by for example investigating the transitions between late-stage successional grasslands and forests more closely. Similarly, microbial niche specialization across differing land uses could be investigated at a higher taxonomic resolution to further advance our understanding of the specific links between soil habitat, management factors, and microbial ecology.

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Popular science summary

What is the most universal characteristic of an ecosystem? It always is, and always will be, in the process of change. However, since the onset of the industrialization about 200 years ago, ecosystems and landscapes have been changing at an increasing scale and rate. In Sweden, what was once large open or semi-open landscapes have gradually turned more and more into forests, changing the canopy, the flora, the microbes and arguably also the people that inhabit them. This "closing" of open landscapes is a key threat to biodiversity, because many of the species that thrive in open lands are outcompeted and replaced as tree species progressively change the light and soil acidity levels, the substrate quality, and the nutrient cycling dynamics in the soil.

In general, we know very little about how these changes affect soil microbial communities and the functions they perform. Most biodiversity assessments are targeted at the flora and fauna aboveground, and we tend to ignore the organisms we cannot directly perceive. Soil microbes are especially understudied, both because they play a crucial role in upholding vegetation and nutrient cycling in all ecosystems, but also because they are incredibly diverse – far more diverse than any other known groups of organisms. In this thesis, I have examined how soil microbes respond to the abandonment and gradual overgrowth and afforestation of grasslands. That is, how they change in terms of their diversity and functioning, and how these changes in their turn affect carbon cycling. I relate this to some fundamental concepts developed within the theory of succession, where it has long been thought that ecosystems develop in an orderly and predictable way when management ceases and the afforestation process begins.

I found that microbial communities were quite resilient to abandonment and overgrowth, in that they did not change so much in terms of diversity and community composition. However, when I compared each grassland to an adjacent forest site, microbes changed drastically. Specifically, bacterial diversity decreased considerably, whereas fungal diversity remained quite stable across both grasslands and forests. By contrast, when examining the microbes' genetic profiles, I found that bacterial genetic diversity remained similar during afforestation while fungal genetic diversity increased. I interpret this as a shift on the whole microbial level going from redundancy to specialization, meaning that there are many taxa performing the same tasks and functions in grasslands whereas forests contain fewer microbial taxa doing more specialized tasks. In terms of carbon cycling, what microbes do appear to be of more importance than who they are, and this could be related to the carbon sequestration potential when afforesting grasslands. Specifically, grassland microbes, especially bacteria, appear to be more efficient in transforming carbon sources than forest microbes, and this could be due to the fact that forest soils are harsher and more stressful environments so that microbes need to spend more energy on coping with stress and in accessing resources. We think that this could be a potential mechanism explaining why the conversion of grasslands into forests often fails to increase soil carbon stocks.

When humans change ecosystems from one land use to another, e.g. by turning an arable field into a grassland or a grassland into a forest, some of the species thriving under the former land use will hang on and persist in the new one despite this being a generally unfavourable habitat for them. We found that these so-called *legacy effects* of previous land uses can continue to shape special groups of microbes for about a century after the land-use change happened. In general, the legacy effects we found were stronger for bacteria than for fungi, but we also saw clear effects of previous arable land use on the fungal pathogen loads in what are now grassland sites.

Together the results contained in this thesis indicate that microbes are strongly affected by the large-scale land-use change from grasslands to forests in Sweden, both in terms of diversity and functioning. Further studies should investigate whether these patterns are similar across other regions and continents where climate and biodiversity differ from the Swedish context. We should also strive to examine more how changing microbial communities affect plant communities and ecosystem functioning more broadly than just in terms of carbon cycling.

Populärvetenskaplig sammanfattning

Ekosystem förändras hela tiden och kommer alltid att göra det. Under de senaste dryg 200 åren har ekosystem och landskap dock genomgått förändringar i allt högre omfattning. I Sverige har det som en gång var öppna landskap gradvis vuxit igen och förskogats över hela landet, och med denna förskogning har växtsamhällen, fauna, människor, men även marklevande mikrober genomgått också förändrats. Eftersom många arter trivs bäst i öppna landskap och är beroende av dessa för sin fortlevnad, försvinner dessa gradvis i takt med att markerna växer igen och överskuggas. Förskogningen förändrar dessutom även kemin och näringskretsloppen i marken, eftersom barrträd har en försurande effekt och förnan tar längre tid att bryta ner.

Vi vet dock generellt sett ganska lite om hur igenväxning påverkar marklevande mikrobsamhällen och deras funktionella egenskaper. De allra flesta miljöövervakningsprogram som följer tillståndet för mångfalden i landet fokuserar nästan uteslutande på flora och fauna, medan mikroberna helt ignoreras. Detta trots den stora betydelse mikrober har för hur växtsamhällena utvecklas och för näringskretsloppen som styr b.la. kolinlagringen i marken. Kanske är det för att mikrober är nästan oöverskådligt artrika och mångfaldigt mer mångfaldiga än några andra organismgrupper vi känner till. I denna avhandling har jag undersök hur marklevande mikrober påverkas av förändrad markanvändning och igenväxningen av öppna gräs- och naturbetesmarker, dvs hur de förändras med avseende på mångfald och funktionella egenskaper. Jag använder mig av kärnbegrepp ur litteraturen om ekologisk succession, där det länge har antagits att ekosystem utvecklas i ett stadigt och förutsägbart förlopp när hävden upphör och igenväxningen tar sin början.

Resultaten av dessa undersökningar visar att mikrobsamhällena var förvånansvärt resilienta mot igenväxning och förskogning, eftersom varken mångfald eller funktionella egenskaper förändrades särskilt mycket. När jag jämförde dessa marker mot närliggande skogsmarker som representerar slutstadiet av förskogningsprocessen, hade dock stora förändringar skett, och då särskilt i bakteriesamhällena som minskade mycket i mångfald med förskogning, medan svampsamhällena påverkade i något mindre grad. När jag sedan även undersökte även mikrobsamhällenas genetiska, fann jag att dessa visade ett motsatt mönster med mindre förändringar hos bakterier medan den genetiska mångfalden hos svampar ökade markant i skogsmarker jämfört med gräsmarker. Jag tolkar dessa resultat som att mikrobsamhällena överlag ändras från att präglas av hög redundans i gräsmarker, dvs många mikrober har ungefär samma funktionella egenskaper och kan därmed avlösa varandra, till att präglas av hög specialisering i skogsmarker. Gräsmarksmikroberna verkar även vara mer effektiva i nedbrytningen av organiskt material, vilket kan bero på att skogsmarker är tuffare miljöer med fler faktorer som påverkar mikroberna negativt (t.ex. lågt pH och svårnedbrytbara ämnen), och kräver att mer energi läggs på att överleva dessa miljöer. Det är tänkbart att detta kan vara en bakomliggande faktor som förklarar varför förskogning sällan leder till någon större kolinlagring i marken än gräsmarker.

När markanvändning förändras och åkermark blir till gräsmark eller gräsmark blir till skogsmark, återfinns ofta arter från de gamla habitaten i de nya, trots att de egentligen inte borde trivas där. Vi undersökte gräs- och skogsmarker som historiskt använts som åkermark och fann spår av sådana resteffekter även hos mikrobsamhällen. Bakterier var speciellt påverkade av historisk markanvändning, men vi såg även att andelen patogena svampar var högre i gräsmarker som tidigare skötts som åkermark även om denna effekt verkade upphöra med tiden.

Överlag visar resultaten i denna avhandling på att mikrobsamhällen påverkas starkt av förändrad markanvändning, både vad gäller mångfald och funktionella egenskaper. I framtida studier skulle dessa effekter kunna undersökas närmare och jämföras med hur det ser ut i andra regioner och kontinenter, eftersom olika klimat, jordarter och mångfald kan variera mycket från hur det ser ut i Sverige. Det kunde även vara intressant att undersöka hur kopplingen mellan marklevande mikrober och växtsamhällen ser ut och hur detta kan påverka andra kretslopp än det relaterat till kol.

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I

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RESEARCH ARTICLE

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Core taxa underpin soil microbial community turnover during secondary succession

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Abstract

Understanding the processes that underpin the community assembly of bacteria is a key challenge in microbial ecology. We studied soil bacterial communities across a large-scale successional gradient of managed and abandoned grasslands paired with mature forest sites to disentangle drivers of community turnover and assembly. Diversity partitioning and phylogenetic null-modelling showed that bacterial communities in grasslands remain compositionally stable following abandonment and secondary succession but they differ markedly from fully afforested sites. Zeta diversity analyses revealed the persistence of core microbial taxa that both reflected and differed from whole-scale community turnover patterns. Differences in soil pH and C:N were the main drivers of community turnover between paired grassland and forest sites and the variability of pH within successional stages was a key factor related to the relative dominance of deterministic assembly processes. Our results indicate that grassland microbiomes could be compositionally resilient to abandonment and secondary succession and that the major changes in microbial communities between grasslands and forests occur fairly late in the succession when trees have established as the dominant vegetation. We also show that core taxa may show contrasting responses to management and abandonment in grasslands.

INTRODUCTION

It is well known that habitats with different types of vegetation cover, climate and management practices harbour distinct microbial communities (Bahram et al., 2018; Hartmann et al., 2015). Understanding how these communities vary across space and time can inform us about the potential mechanisms underlying microbial community assembly and functioning. For example, processes driving turnover between habitats can be attributed to either deterministic or stochastic assembly (Dini-Andreote et al., 2015), where stochastic factors are primarily random dispersal and drift (Ferrenberg et al., 2013; Zhou & Ning, 2017), while deterministic assembly is typically driven by selection imposed by abiotic factors through their impacts on competition and performance (Stegen et al., 2013). Successional gradients resulting from colonisation of novel habitat (primary succession) or through the process of ecosystem recovery following a disturbance (secondary succession), provide unique opportunities to examine patterns of community assembly for a more mechanistic understanding (Fierer et al., 2010; Nemergut et al., 2013). For microbes, the relative influence of stochastic and deterministic assembly processes is strongly mediated by abiotic properties such as pH and Na^+ which exert selection on bacterial community composition (Dini-Andreote et al., 2015; Tripathi et al., 2018). Increasing habitat heterogeneity of the selective environment (i.e., pH) within successional stages is thus expected to lead to community divergence and compositional differences as succession progresses, due to variable selection dominating assembly (Dini-Andreote et al., 2015). Conversely, if the selective environment within differing successional stages is spatially homogenous, we would instead expect homogenous selection to dominate microbial assembly and lead to community divergence only as selective factors change enough to require significant niche adaptations.

Community turnover (i.e., beta diversity) between distinct stages along a successional gradient may reflect either replacement (spatial turnover) or loss of taxa (nestedness) (Koleff et al., 2003; Legendre, 2014). For spatial turnover, variation in taxonomic composition is caused by the replacement of one or several taxa by other taxa while the overall species richness remains comparable. Conversely, nestedness results from compositional differences driven by the loss of taxa, as richness decreases. Since spatial turnover and nestedness result from antithetic processes, they can be partitioned according to their relative contribution to the total community turnover (Baselga, 2013). So far, however, little is known about the relative contribution of nestedness and spatial turnover of microbial communities along successional gradients. Moreover, because most microbial communities are dominated by low-abundance species or populations (Lynch & Neufeld, 2015), the turnover between two communities is likely to be driven by rare taxa (McGeoch et al., 2019). Conversely, core taxa that occur persistently within a given habitat type are often of disproportionate importance for the maintenance of that system's basic ecosystem properties (Umaña et al., 2017), and could be sensitive indicators of ecosystem changes. In the zeta diversity framework (Hui & McGeoch, 2014; McGeoch et al., 2019), the contribution of common and rare taxa to compositional change can be inferred within or across habitat types through the construction of species decline curves across multiple assemblages (referred to as zeta orders). However, despite the conceptual advances inherent to beta diversity partitioning and the zeta diversity framework, their application to microbial communities during succession remains limited.

Here, we examined bacterial community turnover across a large-scale successional gradient from managed grasslands to forests using beta- and zeta-diversity analyses complemented with models of phylogenetic turnover to study assembly processes. We evaluated abiotic and biotic drivers by examining differences in the selective environment between each set of paired grassland-forest sites in relation to turnover patterns. Our gradient consisted of managed ($n = 49$) and abandoned grassland sites in two stages of succession (recently abandoned, $n = 30$, and late-term successional, $n = 22$), paired with adjacent fully forested sites representing their successional and land-use change endpoint ($n = 104$). We hypothesised that (i) spatial heterogeneity in the selective environment would increase along the successional gradient and that gradual community divergence would be underpinned by spatial turnover (as opposed to nestedness). We further hypothesised that (ii) deterministic assembly processes would shift in their relative influence from homogenous to variable selection. Last, we expected that (iii) zeta diversity analyses would reveal core taxa inherent to each successional stage and that these taxa would exhibit the same divergent turnover patterns as the whole community.

EXPERIMENTAL PROCEDURES

Sampling and design

We selected 101 grassland sites distributed across the whole of Sweden (Figure 1) and categorised them into three differing successional stages (managed, recently abandoned and late-term successional) based on vegetation characteristics (Table S1). We next paired the grassland sites ($n = 93$, see Table S1 for the distribution of paired and unpaired samples across grassland stages) with an adjacent fully afforested site representing the putative end point of land abandonment and succession (Cline & Zak, 2015). We do not have full historical records on the age and management practices of the grassland and forest sites visited, but forest sites in southern Sweden were likely afforested from grassland, heathland, or grazed forest during the 20th century (Cousins et al., 2015). Forests in the central and northern parts of the country were most likely full forests and as such harvested with various intensity from the mid- or late 1800's and onwards. For more detailed discussions on the historical and ongoing afforestation of open landscapes in Sweden, including regional differences, see (Ericsson et al., 2000; Wei et al., 2021). The sites consisted of a circular area of ∼200 m^2 and were sampled by taking four cores (diameter = 3 cm, depth = 10 cm) at 2 m distance intervals in every direction from the coordinate indicating the centre of the plot ($n = 16$ subsamples for each site). All sites were sampled between July and September 2020. Subsamples were pooled into a site-specific bulk sample. A subset of the bulk sample was air-dried (<40°C) within 24 h of sampling and stored in a zip-lock plastic bag with silica gel to minimise humidity and prevent the development of moulds during transit (Tedersoo et al., 2014). All equipment was sterilised with 95% ethanol between sites. The air-dried soil was milled to a fine powder for molecular analysis, and the remaining bulk samples were sieved (2 mm) and stored refrigerated (4°C) before further chemical analyses.

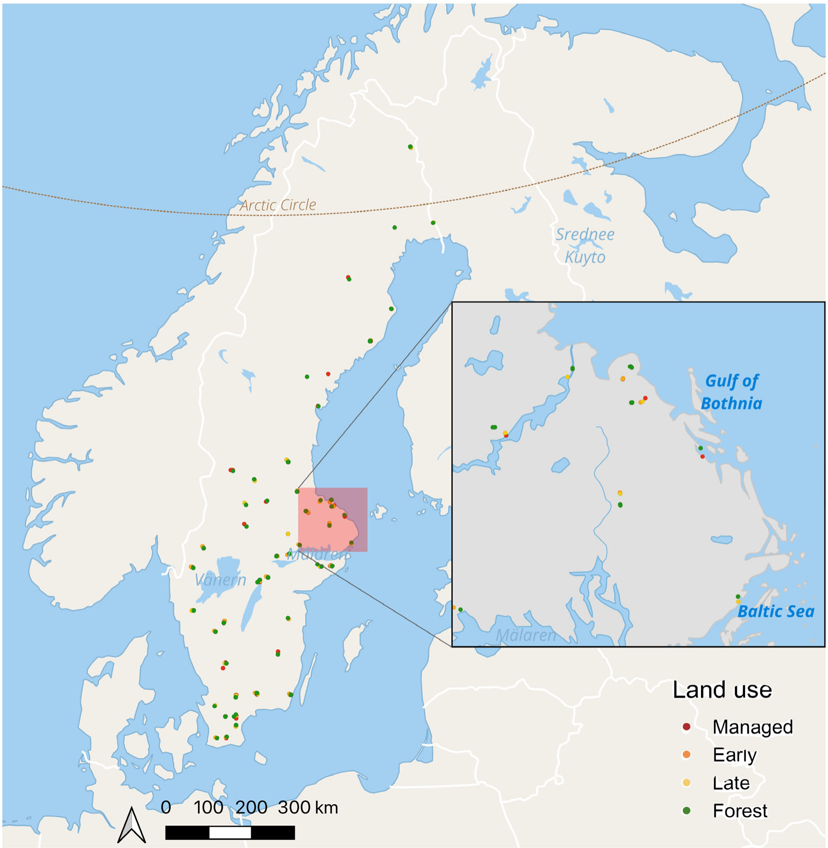

FIGURE 1 Map of sampling sites. Map of sampling sites and their distribution across Sweden. The zoomed-in quadrant shows an example area of paired grassland–forest sites.

Laboratory methods

Soil chemical analyses

Approximately 15 g of the refrigerated soil was used to analyse pH (1:5 soil: water suspension). Available phosphorus (P-AL) and potassium (K-AL) were extracted using ammonium lactate and acetic acid at pH 3.75 (Egnér et al., 1960) and analysed using the stannous chloride-molybdate procedure (P-AL) and inductively coupled plasma atomic emission spectroscopy (ICP-AES), respectively. Exchangeable calcium and magnesium concentrations were measured in ammonium acetate extract ($\text{pH} = 7.0$) using ICP-AES at Agrilab Uppsala, Sweden. Total C and N contents were determined on aliquots (1–20 mg) of air-dried soil using an Elemental Analyser (Euroa EA, Eurovector, Milano, Italy).

DNA extraction, PCR amplification, library preparation and sequencing

DNA was extracted from 200 mg of dried and milled soil samples using the PowerMax Soil DNA Isolation Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Extraction occurred within 3 weeks of sampling. We are aware that storage of air-dried samples can lead to compositional changes of bacterial communities compared to fresh or frozen storage, but these are likely to be few and minor in relation to differences between land uses (Lane et al., 2022).

The extracted DNA was quality-checked based on the 260/280 and 260/230 nm wavelength ratios using a NanoDrop™ (Thermo Scientific, Massachusetts, USA) and stored at −20°C until sequencing. For the production of amplicons for sequencing, the universal prokaryote primers 515F and 926R were used to amplify the 16S V4 subregion of the rRNA gene (Walters et al., 2015). DNA samples were amplified using the following conditions in three replicate runs: 95°C for 15 min, followed by 26 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min with a final extension step at 72°C for 10 min. The 25 μL PCR mix consisted of 18 μL sterilised H_2O, 5 μL 5 × HOT FIREPol Blend MasterMix 0.5 μL of each primer (20 μL), and 1 μL template DNA (final concentration of 400 nM). The amplicons from the replicates were pooled, and purified using a purification kit containing agarose gel (FavourPrep Gel/PCR Purification mini Kit-300 Preps; Favourgen) and shipped for library preparation in the sequencing service facility of the University of Tartu (the Estonian Biocenter). DNA libraries were sequenced on two runs using an Illumina MiSeq platform (2 × 250 bp paired-end chemistry). Blanks containing ddH_2O instead of DNA template were used as negative controls in the library preparation. The raw sequences derived from all soil samples have been deposited at NCBI under accession PRJNA994701.

Bioinformatics

We used the LotuS2 version 2.22 (Özkurt et al., 2022) pipeline to quality-filter, demultiplex and process the filtered reads into operational taxonomic units (OTUs). Chimera detection and removal was done using Uchime (Edgar et al., 2011) with all singletons and sequences shorter than 100 bp discarded. Clustering of sequences was done using a de-novo clustering algorithm in UPARSE (Edgar, 2013) based on a 97% similarity threshold. Taxonomy was assigned against the SILVA database (Quast et al., 2013) (ver. 138.1) for prokaryotic sequences. For inferring a phylogenetic tree from the sequences needed for analysis of assembly processes, multiple sequence alignment of OTUs was done using MAFFT (Katoh & Standley, 2013), and from these a maximum likelihood phylogeny was constructed using fasttree2 (Price et al., 2010). The resulting dataset was manually curated to remove contaminant sequences based on negative controls, and by omitting all OTUs not assigned to Bacteria. This resulted in a total of 13,901 OTUs and 8,893,038 reads across all samples, with a median sample coverage of 0.979.

Statistical analyses

All statistical analyses were conducted using R (ver. 4.1.3) (R Core Team, 2023). In the case of multiple comparisons, test results were adjusted with Benjamin-Hochberg's corrected *p*-values.

Rarefaction and links to vegetation community

All taxa unassigned at the phylum level were omitted from the dataset. We used coverage-based rarefaction to standardise samples to equal completeness (Chao & Jost, 2012) prior to alpha- and beta-diversity analyses, whereas zeta diversity, core microbiome and assembly process analyses were done using raw (i.e., unrarefied) matrices. The coverage depth opted for (Good's coverage = 0.8) was chosen as reflecting a balance between sampling breadth and sequencing depth, and we note beta diversity patterns for many environments including soils are robust even at low sequencing depths after rarefaction (Kennedy et al., 2018). One sample was discarded due to too low sequencing coverage, and a total of 378,840 sequences remained after rarefaction (mean sample depth = 1839 sequences). To test whether bacterial community composition was related to plant community composition along the successional gradient, we first conducted Mantel tests between bacterial and vegetation distances matrices using the *mantel* function in *vegan* (Oksanen et al., 2022). Tests were based on Spearman correlations between Bray–Curtis distance matrices with 9999 permutations. We next extracted the first axes from a PCA comprising the full vegetation community found at each site and used the resulting coordinates as a constraining variable of bacterial community composition in Permanova and RDA analyses.

Species richness, community composition and soil heterogeneity

Taxonomic richness was computed using *Hill* numbers of the order $q = 0$ (Chao et al., 2014) with the *hill_div* function in the *hilldiv* package (Alberdi & Gilbert, 2019). We used pairwise Wilcoxon rank-sum tests to test for differences between successional stages. Compositional differences between successional stages were tested based on Bray-Curtis distance matrices using global permanova tests (9999 permutations). For differences between grasslands and forests, these analyses were done with permutations restricted to paired sites using the *strata* argument of the *adonis2* function in *vegan* (Oksanen et al., 2022). For differences between grassland stages, the same function was applied omitting the *strata* argument. Results were visualised using NMDS plots separated for each set of grassland-forest comparisons. Soil and environmental variables influencing bacterial community composition were extracted using forward selection with the *forward.sel* function in the *adespatial* package (Dray et al., 2023), and fitted to a distance-based redundancy biplot (db-RDA, Bray-Curtis distances) using the *microViz* package (Barnett et al., 2021). We also computed the coefficient of variation (CV) for the two variables explaining most of the community variation (pH and

C:N) and tested for differences in the heterogeneity of these between successional stages using a modified signed-likelihood ratio test (MSLRT) (Krishnamoorthy & Lee, 2014). To assess the contribution of species spatial turnover and nestedness to the community turnover, as well as the influence of variation in taxa abundances, we computed and partitioned dissimilarities using the *betapart* approach (Baselga, 2013, 2017). Spatial turnover and nestedness were computed on presence–absence matrices based on Sørensen dissimilarities, whereas the contribution of balanced or unbalanced abundances to turnover was computed on abundance matrices based on Bray–Curtis dissimilarities. Because the partitioned metrics were quantified between paired sites, we used the absolute difference in soil properties between the same paired sites in linear regressions to analyse the abiotic drivers of the observed patterns.

Zeta diversity and analyses of core taxa within successional stages

While beta diversity partitioning captures spatial turnover and nestedness with compositional change, it is insensitive to occupancy changes in common taxa. The zeta diversity framework instead analyses turnover across multiple sites (referred to as zeta orders), and discriminates diversity patterns across common, intermediate and rare taxa. The steep decline in zeta values, corresponding to the number of shared taxa at n zeta orders (i.e., sites) therefore implies turnover driven by rare taxa, whereas a gentler decline implies a pool of core taxa common to all sites shared within a given habitat. Here, we used the zeta diversity framework to assess turnover within successional stages. Zeta values were computed across 20 zeta orders (i.e., 20 sites) using the function Zeta.decline.mc in the *zetadiv* package (Hui & McGeoch, 2014) with 1000 bootstraps. As rarefaction can alter the number and identities of rare and common taxa (Neu et al., 2021), we used raw (i.e., unrarefied) matrices as input for the zeta diversity and core microbiome analyses (see below), but discarded all samples with fewer than 3000 reads ($n = 3$) to account for the worst biases of uneven sequencing depth. Zeta decline curves were also calculated using both raw and normalised zeta values (Jaccard normalisation).

We also computed zeta values across the full set of grasslands ($n = 101$) and forest sites ($n = 104$), respectively. Patterns of zeta decline were evaluated based on their fit to an exponential or power law form with the best fit determined with AIC criteria (Hui & McGeoch, 2014). Taxa retention curves, showing the degree to which common taxa are more likely to be retained across sites, were constructed from the zeta ratio values obtained across the full range of zeta orders (McGeoch et al., 2019).

To test for the presence of core microbiomes, we first confirmed that zeta decline patterns showed evidence of shared taxa within each successional stage across 20 zeta orders. This was interpreted as indicating the presence of core taxa within the successional stages. Next, we derived these putative core microbiomes for further analyses by prevalence filtering based on occupancy thresholds (Custer et al., 2023). For this, we considered taxa occupying a minimum of 80% of the sites within any given successional stage to be part of the core microbiome. Lastly, we tested for differences in the community composition of core microbiomes using the same approach as for the whole community (see above) and performed indicator species analyses to derive indicator taxa for specific or shared successional stages. Indicator analyses were done using the *multipatt* function from the *indicspecies* package (Cáceres & Legendre, 2009), with 10^4 permutations.

Assembly processes

We used the framework developed by Stegen et al. (2013, 2015) to measure phylogenetic turnover between communities. For full details on the framework and its underlying assumptions, see Stegen et al. (2013, 2015). Briefly, phylogenetic turnover is compared to a null distribution generated through randomizations where taxa names and abundances are shuffled across the phylogeny tips. The difference between the observed and mean null value is referred to as the β-nearest taxon index (β-NTI) and placed in units of standard deviation in relation to the null distribution so that ($|\beta\text{-NTI}| > 2$) indicates domination of deterministic assembly either through homogenous ($\beta\text{-NTI} < -2$) or heterogeneous ($\beta\text{-NTI} > +2$) selection. If there is no significant deviation from the null expectation, differences in phylogenetic community composition are assumed to result from dispersal limitation, homogenising dispersal, or random drift, and partitioned accordingly based on Raup-Crick Bray–Curtis dissimilarity (RC_{Bray}). We used the R scripts provided by Barnett et al. (2020) to calculate pairwise β-NTI and null-model generations based on 999 randomizations. Differences in the relative influence of the differing assembly processes were tested using pairwise comparisons of proportions. Phylogenetic null-model analyses assume that closely related taxa are also ecologically similar, that is, they share the same ecological niches (Stegen et al., 2013). To confirm the validity of this assumption, we tested the presence of phylogenetic signal with pH and C:N (these being the selective variables explaining most of bacterial community composition) using Mantel correlograms as implemented in *vegan* (Oksanen et al., 2022) with the codes provided by Barnett et al. (2020). We found a significant positive correlation between phylogenetic distance and the difference in optimum soil property (i.e., OTU niche preference) across a range of phylogenetic distance classes (Figure S1). Lastly, we analysed the influence of pH on phylogenetic turnover (i.e., β-NTI) through Mantel tests (Spearman correlation, 9999 permutations) across all successional stages combined and separately, and analysed differences in the relationship between pH and β-NTI for each successional stage by comparing their slopes of linear regression with the *lstrends* function in the *emmeans* package.

RESULTS

Microbial communities in grasslands are compositionally stable and differ from forest sites

Beta diversity analyses did not show any differences between the three grassland successional stages (unrestricted Permanova, $p > 0.05$; Table S2), contrary to our hypothesis of a gradual divergence. Differences in taxonomic richness followed a similar pattern with no evident differences between the stages (Wilcoxon, $p > 0.05$), but markedly higher richness in all grassland communities than in forest sites (Figure S2; Wilcoxon, $p < 0.05$). By contrast, when comparing bacterial community composition between grasslands and their paired forest sites, we found that the grasslands differed markedly from forests for every grassland successional stage (Permanova with strata restricted to paired sites, $p = 0.0001$) (Figure 2B–D). The results reflected the overall small differences in soil edaphic properties between grassland stages but large differences between grasslands and forests (Table S3), but contrasted with the differences observed in the community composition of plants between grasslands stages (db-MANOVA, $p < 0.05$; Table S1). As expected, pH was the main variable explaining community composition (Permanova, $R^2 = 0.22$, $p < 0.001$) with C:N, total N, Mg, mean annual precipitation and mean annual temperature together with the first PCA axis of plant community composition explaining an additional 4.6% of variation (Figure 2E). Plant community composition was further only weakly related to bacterial community composition across the whole successional gradient (Mantel test, $r = 0.035$, $p = 0.036$).

Beta diversity between grasslands and forests dominated by spatial turnover

In line with our first hypothesis, species spatial turnover contributed more to total grassland-forest beta diversity than nestedness, in line with our first hypothesis, but we found little variation across the different grassland stages (Figure 3A). The nestedness contribution to beta diversity was higher in recently abandoned grasslands than in late-term successional grasslands (Figure 3A; Wilcoxon, $p = 0.02$). These patterns were reflected also in analyses of the abundance variation, with overall few differences in the contribution of balanced variation to Bray–Curtis dissimilarities between grassland stages (Figure S3A). Analyses on raw (i.e., unrarefied) matrices produced the same patterns of beta diversity partitioning (Figure S4A,B). Differences in soil pH and C:N between the paired grassland-forest sites were the main drivers of the overall community dissimilarity, explaining 46% and 39% of the variation in the Sørensen dissimilarity, respectively (Figure 3B,C). Similar values were obtained when regressing the soil variables against Bray–Curtis dissimilarities (Figure S3B,C). When examining the partitioned components of beta diversity separately, we found that the soil properties better fitted the spatial turnover than the nestedness component (Figure 3D–G), indicating that differences in soil pH and C:N between grasslands and forests drive species spatial turnover more than nestedness in the community turnover. Similarly, the contribution of balanced variation in the abundances to Bray-Curtis dissimilarities between the paired sites was driven by pH and C:N, whereas differences in abundances of bacterial taxa (i.e., abundance gradients) were more related to differences in pH only (Figure S3D–G). By contrast, differences in community composition between sites were not related to the geographic distance separating them (Figure S5A–D).

Core microbiomes reflect whole-community turnover

All land uses showed similar patterns of rapid community turnover over the first ∼5 zeta orders and reached asymptotic levels around zeta orders ∼10 (Figure 4A). The patterns of zeta decline fitted a power law considerably better than the exponential form for all land uses (Table S5), indicating that community assembly is predominantly deterministic since communities with clear niche differentiation are expected to show a power-law form of zeta diversity decline (Hui & McGeoch, 2014). Taxa retention curves, constructed from plotting zeta ratios against zeta orders, also approached asymptotic levels of around 95% retention after ∼15 zeta orders (Figure 4B), indicating the presence of a core set of taxa in line with our third hypothesis. When merging all grasslands to a single category, zeta decline curves for these and all forest sites (104 zeta orders), we again found the same asymptotic patterns and retention curves (Figure S6C,D), indicating that core taxa in these habitats represent generalists common to all grasslands independent of succession. Similarly, when looking at the asymptotic normalised (Jaccard) zeta

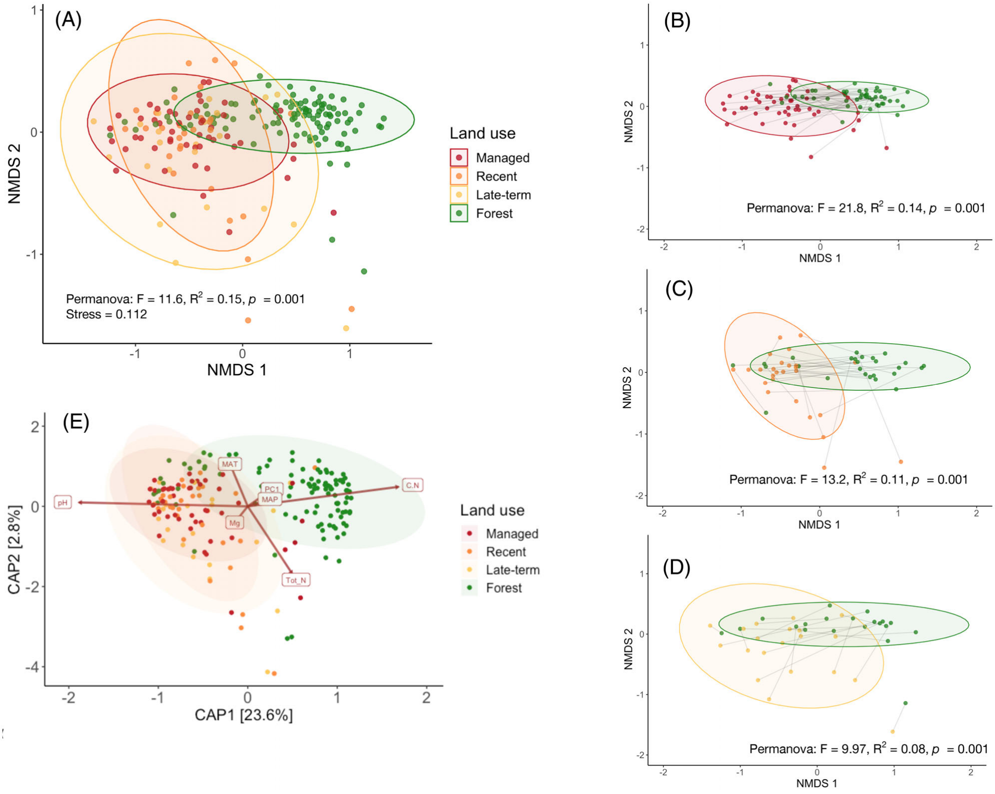

FIGURE 2 Community composition across the successional gradient. NMDS plots showing differences in community composition between (A) all grassland and forest sites based on unpaired permanova tests (See Table S1 for the number of sites in each successional category), (B) managed ($n = 47$), (C) recently abandoned ($n = 26$), and (D) late-term successional grasslands ($n = 20$) displayed along with their respective paired forest sites and test results based on permanova tests accounting for the paired structure. All permanova tests were run with 9,999 permutations with the strata term restricted to within grassland-forest pairs for paired comparisons. The NMDS projections for paired comparisons are based on the whole-community analysis in A). (E) Distance-based redundancy biplot (db-RDA) of all grassland and forest sites along with significantly fitted soil and climatic variables after forward selection ($p < 0.05$).

values at higher orders, we found that 0.7–1.3% of OTUs were shared across 20 sites depending on the land-use category (Figure S6A,B and Table S4).

We next examined the core microbiomes further by occupancy filtering retaining only the microbes present at 80% or more in sites within the different successional stages, and tested if they reflected the same patterns of turnover as the entire microbial communities. Occupancy filtering resulted in a total of 917 OTUs, with most taxa found in managed grasslands and fewest in late-stage successional grasslands (Table S5). We again found a clear separation between grassland and forest core microbiomes (Figure 4C), reflecting the whole-community differences found previously (Figure 2A) but also a difference between the core microbiomes in managed compared to abandoned grasslands (permanova, $p < 0.05$; Table S6). In light of these differences, we next examined the core taxa in each successional stage using indicator species analyses. In total, 745 out of the 917 (81%) identified core taxa were classified as indicator taxa for any or a combination of the successional stages. Fully afforested sites had the highest proportion of specialist indicator taxa (i.e., taxa not shared with any other successional stage), amounting to 45% of all OTUs singled out as indicator taxa (Figure 4D; Table S5). By contrast, the late-term successional stage had the fewest overall number of indicator taxa and the lowest proportion of specialist indicator taxa (Figure 4D; Table S5). The three grassland successional stages shared 183 indicator taxa between them, reflecting a high degree of compositional stability among the grassland core

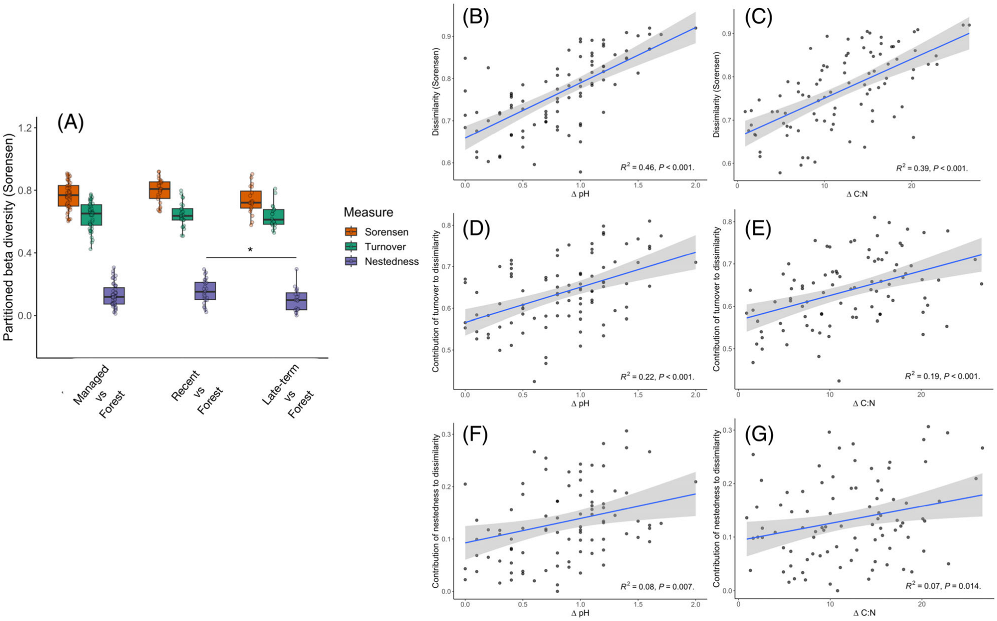

FIGURE 3 Beta diversity components and their abiotic drivers. Boxplots showing (A) beta diversity based on Sørensen dissimilarity and its compartmented turnover and nestedness fractions between paired grassland-forest sites along the differing grassland successional stages. The asterisk (*) denotes a significant difference ($p < 0.05$) in nestedness between successional stages of grasslands when compared to their respective paired forest sites. Tests are based on pairwise Wilcoxon tests. Scatter plots displaying the relationship between absolute differences in pH and C:N and (C,D) overall Sørensen dissimilarity, (E,F) turnover, and (G,H) nestedness, for each pair of grassland-forest sites.

microbiomes, although managed grasslands also had a high proportion of stage-specific indicator taxa (15%; Table S5).

Assembly processes reflect heterogeneity of soil pH within successional stages

Phylogenetic null-modelling showed significant variations in mean β-NTI values between successional stages (Figure 5A), with deterministic assembly processes (|β-NTI| > 2) generally higher than stochastic assembly and differing significantly in relative influence across the different successional stages (Figure 5A). Notably, Homogenous selection was the dominant deterministic assembly processes, whereas homogenising dispersal dominated among stochastic processes (Figure 5A). As expected, differences in pH between sites was the best predictor of assembly (Mantel test, $r = 0.536$, $p < 0.001$; Figure S7A). Differences in C:N were also positively correlated with β-NTI (Mantel test, $r = 0.288$, $p < 0.001$; Figure S7B), but weaker so than pH. We did not find the expected shift from homogenous to variable selection with increasing succession. Instead, variable selection was highest in late-successional grasslands and lowest in recently abandoned grasslands (proportion test, $p < 0.001$), with managed grasslands and forest sites exhibiting similar overall profiles (Figure 5A). The relative influence of homogenous and variable selection correlated well with pH variability within the successional stages. Recently, abandoned grasslands had lower pH variability (MSLRT, $p < 0.001$; Figure 5B) than all other stages, and also a significantly weaker relationship between pH and β-NTI (Figure 5C–F; Table S7). Conversely, increased pH variability between sites resulted in stronger relationships between pH and assembly, and to higher relative influence of variable selection (Figure 5; Table S7).

DISCUSSION

In this study, we examined soil bacterial community turnover and assembly across a large-scale successional gradient comprising managed and abandoned grasslands paired with fully afforested sites. Contrary to our initial expectations, our results do not support a gradual community divergence along the gradient of different grassland successional stages. Instead,

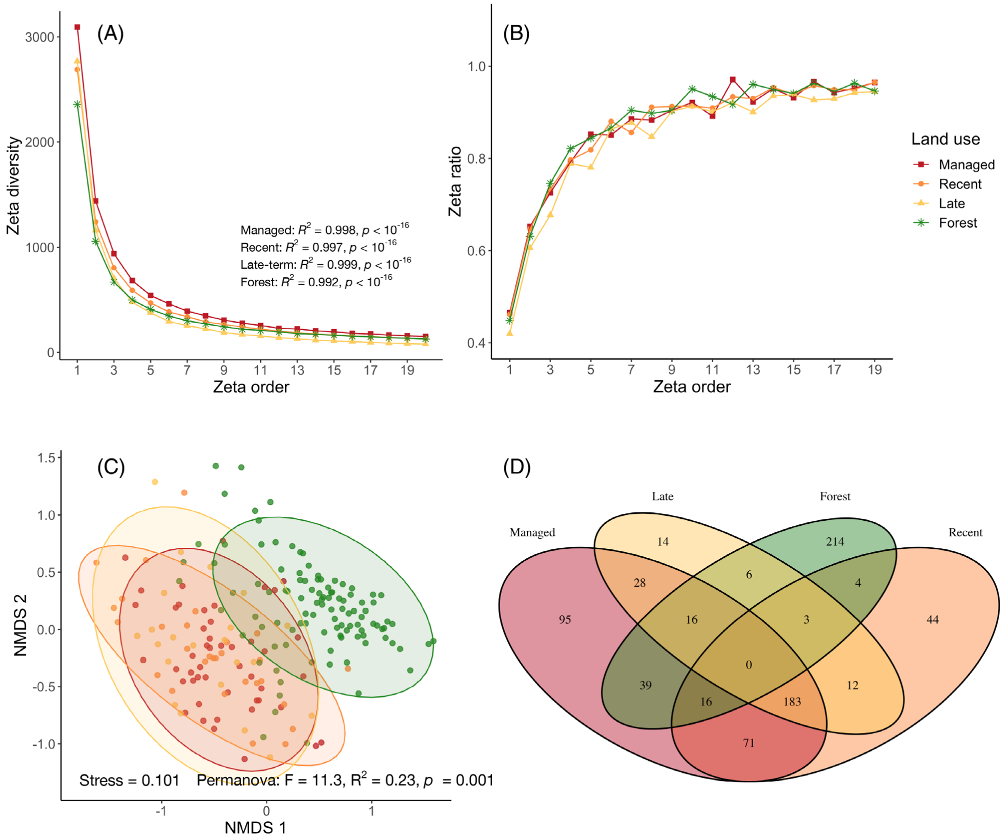

FIGURE 4 Patterns of zeta decline and community turnover of core taxa. Patterns of zeta diversity of the four successional stages across 20 assemblages showing (A) zeta decline curves with power law model fits, and (B) species retention curves showing the ratio of retained taxa between between each successive pair of zeta orders. A ratio of 0 indicates no retained taxa (complete turnover), and a ratio of 1 indicates full taxa retention (zero turnover). (C) NMDS plot showing differences in the composition of core microbiomes of the differing successional stages. Permanova tests for differences in composition were run with 9999 permutations. Results of pairwise comparisons can be found in Table S6. (D) Venn diagram displaying the number of stage-specific and shared indicator species. Only OTUs with a *p*-value <0.05 based on 9999 permutations are included in the analysis.

bacterial communities appeared to be compositionally stable across all grassland stages independent of successional stage and differed only in relation to their paired forest reference sites (Figure 2A). While these results contrast to other observations of clear successional patterns in soil microbial turnover during secondary succession (Cline & Zak, 2015; Gellie et al., 2017), they nonetheless concur with a meta-analysis of microbial development patterns during secondary succession globally (Zhou et al., 2017). The rate of community change between stages in a secondary successional system is related to the severity of the initial disturbance (Kearns & Shade, 2018) and factors such as dormancy, priority effects and the development of soil edaphic properties influencing both the trajectory and speed of microbial succession (Debray et al., 2022; Dini-Andreote et al., 2015). Successional trajectories are likely linked to the biotic and abiotic legacies of cultivation and management (Cramer et al., 2008), and a plausible explanation for the high compositional stability of microbes found here is that semi-natural grasslands represent a low-intensive form of agricultural management rather far from the blank slate environments created during, for example, forest fires (Ferrenberg et al., 2013). Interestingly, a recent study found high levels of soil microbial resistance in the context of re-introduced grazing on abandoned alpine meadows (Vidal et al., 2020). This indicates that microbial communities may be compositionally stable to both abandonment and subsequent re-grazing. In this context,

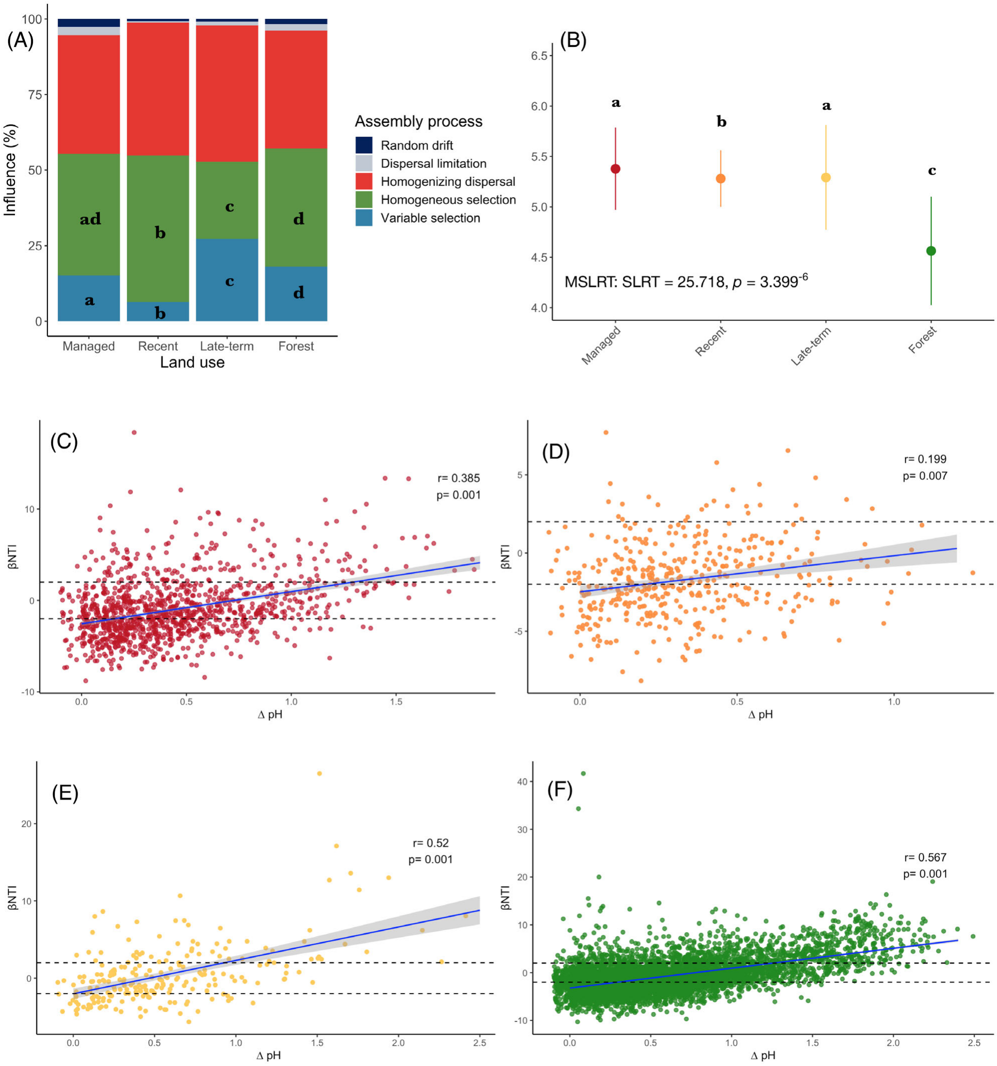

FIGURE 5 Relative influence of assembly processes and their link to pH variability. Barplot showing (A) the relative influence of deterministic (homogeneous selection, variable selection) and stochastic (random drift, dispersal limitation, and homogenising dispersal) assembly processes within each successional stage. Significant differences between successional stages ($p < 0.05$) based on pairwise comparisons of proportions. (B) Mean and standard deviation of soil pH along the successional gradient, along with letters denoting differences in their coefficient of variation (CV) based on modified signed-likelihood ratio tests (MSLRT) for equality of CV's. Relationship between differences in pH and β-NTI values for sites within (C) managed grasslands, (D) recently abandoned grasslands, (E) late-term successional grasslands and (F) forests. Test values derive from Mantel tests with 9999 permutations where *r* indicates Pearson correlation coefficient.

interesting questions can be posed as to the overall resistance and resilience of grassland microbiomes under shifting land-use intensities that contrast with the idea of a global trajectory of grassland degradation (Bardgett et al., 2021).

Soil pH was the main determining abiotic variable structuring bacterial communities (Figure 2E) and the main variable underpinning phylogenetic turnover (Figure S7). Contrary to our expectation of a gradual increase in the within-stage pH heterogeneity, we found that land abandonment initially led to the opposite pattern of less variation in soil pH between the sites in recently abandoned grasslands, only to increase considerably in the next successional stage (Figure 5B). As expected, this was followed by a concomitant shift in the relative influence of deterministic assembly from homogenous to variable selection between early and late-term successional stages (Figure 5A). In the two-stage conceptual model proposed by Dini-Andreote et al. (2015), the authors suggest that homogenous selection results either due to spatially homogenous environmental filtering within each successional stage or because of increasingly extreme filtering in later stages. The latter scenario certainly applies to our results, with the more acidic forest soils exerting a homogenous filtering of bacterial communities to diverge from those in grasslands (Figure 2). However, in line with the former scenario, the higher pH heterogeneity and relative influence of variable selection in late-stage compared to early-stage grasslands should also translate into higher niche differentiation and community turnover, something we did not find.

The whole-community compositional stability of grassland communities was reflected also at the core taxa level, with marked differences between grassland and forest core microbiomes (Figure 4C). Forests had the highest proportion of specialist indicator taxa (45% or 214 taxa) of all successional stages, whereas 183 indicator taxa were shared between all three stages of grasslands (Figure 4D). Core microbiomes can be characterised by bacterial taxa that are highly enriched across certain environments or host plant species, as defined through prevalence or occupancy thresholds (Custer et al., 2023), and can be of disproportionate relevance for host fitness or ecosystem function (D Ainsworth et al., 2015; Toju et al., 2018). Based on our results, the asymptotic convergence of zeta decline and taxa retention curves for all land-use categories into core sets of taxa shared across multiple sites provides compelling complementary evidence for core microbiomes in both grasslands and forests, that generally reflect the overall community turnover patterns. That is, core taxa occupy a broad range of niches that remain relatively stable within grasslands despite vegetation succession. Conversely, non-core taxa with rapid turnover within the differing successional stages are likely to represent taxa with high habitat specificity (Jiao & Lu, 2020). Interestingly, the composition of core taxa in managed grasslands differed from those found in abandoned grasslands (Figure 4C) and managed grasslands had more OTUs classified as indicator species, and a higher proportion of stage-specific indicator taxa than the two abandoned grassland successional stages (Figure 4D; Table S4). Overall, bacteria are emerging as indicators of perturbations and land-use change (Gschwend et al., 2021; Hermans et al., 2016), especially when perturbations cause substantial variation in physicochemical properties. With the improved capacity to describe and characterise unculturable microbes and their life history strategies in detail, the current rather blunt classifications surrounding bacterial habitat preferences could also give way to more nuanced views at higher taxonomic resolution (Stone et al., 2023). It is further likely that transient and core taxa within the same community exhibit differing assembly patterns (Jiao & Lu, 2020), and a further question to pursue is whether and if so what abiotic drivers differentially shape generalist and specialist taxa in the context of succession.

Little is known about the relative contributions of species spatial turnover and nestedness to the turnover of microbial communities during secondary succession. We found that most of the turnover between grasslands and forests was due to species spatial turnover rather than nestedness (Figure 3A), in line with our first hypothesis. However, we also found that species spatial turnover between grasslands and forests was higher in recently abandoned fields compared to late-stage successional fields despite similar levels of taxonomic richness (Figure S2). These patterns were reproduced using raw (i.e., not rarefied) data, and therefore independent of rarefaction (Figure S4A,B). Dynamics of species spatial turnover and nestedness play a fundamental role in plant successional theory, and recent evidence suggests that the proportion of nestedness in plant community turnover increases with successional age in old-field systems (Ladouceur et al., 2023). However, the relative dominance of spatial turnover or nestedness is related to the number of rare taxa in the communities compared (Shade et al., 2013), with species spatial turnover increasing in importance with decreasing number of rare taxa. As our partitioning analyses were based on the turnover of whole communities, the dominance of spatial turnover likely reflected a species spatial turnover of transient taxa between the communities. Similarly to the overall community composition (Figure 2E), soil pH and C:N were the main factors underpinning increased turnover and species spatial turnover between paired sites (Figure 3C–F).

Finally, our study suggests that the major changes in microbial communities after grassland abandonment and succession to forests may occur rather late in the successional trajectory. This highlights the need to follow succession from grasslands to forests for a long time or to design studies that explicitly include stages with young and mid-aged forests. However, such sites are rare in the Swedish monitoring programmes, since it is a common practice among landowners to plant forest trees like Norway spruce or birch once a grassland has been abandoned, effectively short-cutting the natural succession pattern. Hence another approach to studying the transition from grassland to forest microbial communities may be needed, following individual abandoned grassland sites for a long time, that is, decades or even longer.

CONCLUSION

In this study, we examined the patterns of bacterial community composition and assembly in a large-scale secondary successional gradient using three complementary perspectives on assembly and turnover: partitioned beta diversity, zeta decline and the relative influence of assembly processes. Contrary to our hypotheses, we found that community composition remained compositionally stable across grasslands independent of the successional stage and that these patterns were underpinned by persistently occurring core taxa and indicator species. Similarly, deterministic assembly patterns were marginally higher than stochastic processes at all stages of the gradient, with differences in homogenous and variable selection reflecting the degree of pH variability. We also found that spatial turnover was the main process driving pairwise community turnover between grassland and forests. Further research is needed to unravel the link between core microbiomes and whole-community composition in natural transient systems, as well as the assembly processes that shape them. Similarly, additional analyses are required to elucidate the genotypic and phenotypic characteristics of core indicator taxa to differing successional stages, including their link to ecosystem functioning and plant–soil feedback.

AUTHOR CONTRIBUTIONS

Tord Ranheim Sveen: Conceptualization; writing – original draft; writing – review and editing; formal analysis. **Maria Viketoft:** Conceptualization; methodology; writing – review and editing; supervision. **Jan Bengtsson:** Conceptualization; writing – review and editing; supervision. **Mohammad Bahram:** Conceptualization; methodology; writing – review and editing; supervision; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the NCBI Sequence Read Archive (SRA) under BioProject reference number PRJNA994701: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA994701.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supplementary material Paper I

Table S1: The number and classification of grassland and forest sites along the successional gradient. Classification of differences in vegetation community composition based on combined distance-based multivariate ordination and indicator species analyses (Ricotta et al., 2021).

Land use	*N (paired)*	Monitoring program	Classification (db-MANOVA)
Managed grasslands	49 (47)	*Remiil*	a
Recently abandoned	30 (26)	*Remiil*	b
Late-term successional	22 (20)	*Remiil*	c
Forest	104 (93)	*NFI*	d

Table S2: Permanova tests for differences in bacterial community composition between land uses based on Bray-Curtis distances with 9 999 permutations. Comparisons noted with the asterisk* denote tests with permutations restricted to paired grassland-forest sites.

Land use	*Df*	*SoS*	*R2*	*F*	*p*
Global	3	7.875	0.147	11.6	0.001
Managed vs Recent	1	0.243	0.014	1.08	0.306
Managed vs Late-term	1	0.294	0.018	1.27	0.143
*Managed vs Forest**	1	4.73	0.136	21.8	< 0.001
Recent vs Late-term	1	0.200	0.016	0.791	0.809
*Recent vs Forest**	1	2.94	0.101	13.2	< 0.001
*Late-term vs Forest**	1	2.26	0.082	9.97	< 0.001

Table S3: Soil edaphic variables of the differing successional stages (mean ± SD). Letters indicate significant differences ($p < 0.05$) based on pairwise Wilcoxon tests.

Variable	*Managed (n = 49)*	*Recent (n = 30)*	*Late-term (n = 22)*	*Forest (n = 104)*
pH	5.38 (0.41)^{a}	5.28 (0.28)^{a}	5.29 (0.52)^{a}	4.56 (0.54)^{b}
Total C (%)	9.25 (10.4)^{a}	9.06 (10.5)^{a}	11.0 (10.5)^{a}	17.9 (13.3)^{b}
Total N (%)	0.60 (0.60)	0.65 (0.73)	0.74 (0.64)	0.70 (0.52)
C:N	14.4 (2.87)^{a}	13.9 (2.96)^{a}	14.4 (2.22)^{a}	25.3 (6.66)^{b}
Available K (mg kg^{-1})	19.1 (16.7)	19.7 (13.0)	17.1 (6.37)	22.3 (13.1)
Available P (mg kg^{-1})	6.82 (6.15)	5.45 (3.29)	7.54 (8.66)	5.55 (3.12)
Available Fe (mg kg^{-1})	50.3 (43.3)^{a}	71.3 (86.7)^{ab}	100 (97.3)^{b}	45.7 (35.1)^{a}
Available Mg (mg kg^{-1})	15.7 (10.7)	17.5 (13.0)	16.9 (12.2)	18.2 (12.0)
Available Al (mg kg^{-1})	70.0 (60.7)	56.1 (32.8)	81.0 (47.0)	61.1 (47.2)

Table S4: Model fits of zeta decline curves over zeta orders 2:20 for each land-use stage.

Land use	*Model*	*R2*	*AIC*
Managed	Power law	0.998	-102
Managed	Exponential	0.825	-16.0
Recently abandoned	Power law	0.997	-100
Recently abandoned	Exponential	0.824	-15.7
Late-term successional	Power law	0.999	-108
Late-term successional	Exponential	0.839	-11.1
Forest	Power law	0.992	-81.1
Forest	Exponential	0.798	-15.6

Table S5: Species richness of whole communities and core microbiomes across the successional stages, along with the richness and proportion of specialist indicator taxa.

Land use	*Managed*	*Recently abandoned*	*Late-term successional*	*Forest*
Whole-community richness (mean, sd)	2748 (1107)	2354 (902)	2426 (1212)	2129 (1006)
Core microbiome richness	620	505	434	470
Core microbiome indicator taxa	448	333	262	298
Indicator taxa (%)	15.3	8.72	3.23	45.5

Table S6: Permanova tests for differences in bacterial community composition of core microbiomes between land uses. Based on Bray-Curtis distances with 9999 permutations.

Land use	*Df*	*SoS*	*R2*	*F*	*p*
Managed vs Recent	1	0.483	0.033	2.57	0.009
Managed vs Late-term	1	0.518	0.039	2.79	0.009
Managed vs Forest	1	6.96	0.204	38.7	0.001
Recent vs Late-term	1	0.334	0.032	1.63	0.08
Recent vs Forest	1	4.87	0.166	26.2	0.001
Late-term vs Forest	1	3.92	0.145	21.2	0.001

Table S7: Comparisons of the slopes obtained from linear regressions between differences in pH and β-NTI within each successional stage.

Land use	*Estimate*	*SE*	*Df*	*t ratio*	*p*
Managed vs Recent	1.213	0.674	7113	1.800	0.983
Managed vs Late-term	0.806	0.478	7113	1.686	0.331
Managed vs Forest	0.644	0.261	7113	2.469	0.065
Recent vs Late-term	2.019	0.749	7113	2.696	**0.035**
Recent vs Forest	1.857	0.632	7113	2.936	**0.018**
Late-term vs Forest	-0.162	0.418	7113	-0.387	0.273

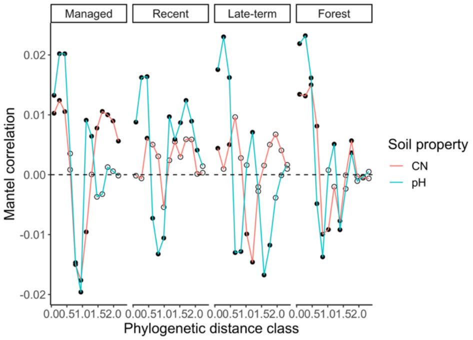

***Fig. S1:** Phylogenetic Mantel correlogram showing significant phylogenetic signal across a range of phylogenetic distances. Solid and open symbols denote significant and nonsignificant correlations, respectively. Significantly positive correlations indicate that ecological niche distance between OTUs increases with their phylogenetic distance.*

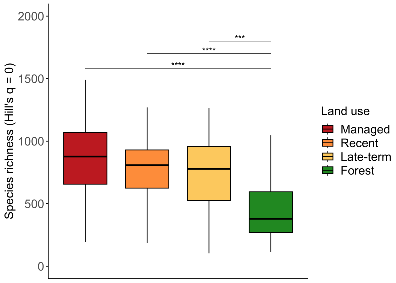

***Fig. S2:** Taxonomic richness (Hill's q = 0) across the successional gradient.*

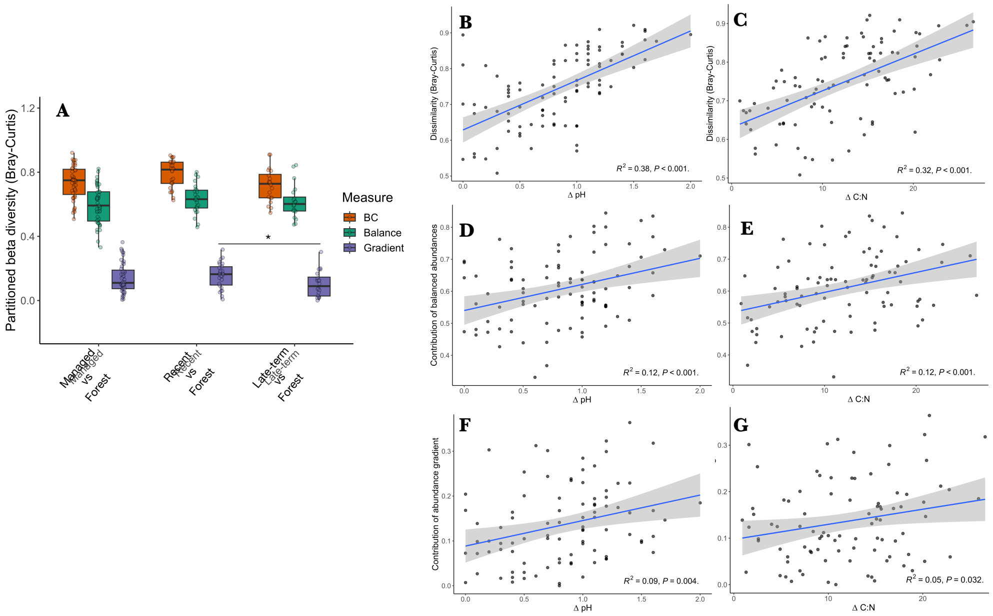

Fig. S3: A) *beta diversity based on Bray-Curtis dissimilarity and its compartmented balanced variation and abundance gradient fractions between paired grassland-forest sites along the differing grassland successional stages. Significant differences (p < 0.05) based on pairwise Wilcoxon tests. Scatter plots displaying the relationship between absolute differences in pH and C:N and* ***C-D)*** *Overall BC dissimilarity,* ***E-F)*** *Balanced variation in abundances, and* ***G-H)*** *Abundance gradients, for each pair of grassland-forest sites.*

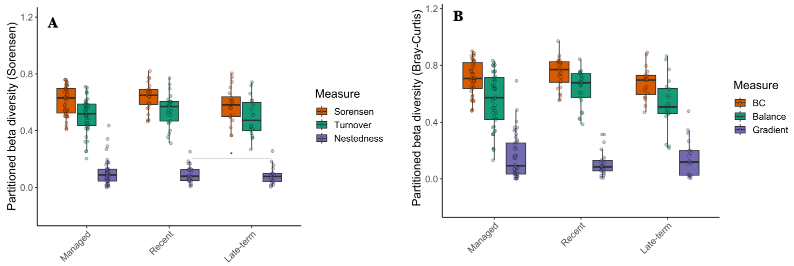

Fig. S4: *Boxplots showing* ***A)*** *beta diversity based on Sørensen dissimilarity and its compartmented turnover and nestedness fractions, and* ***B)*** *beta diversity based on Bray-Curtis dissimilarity and its compartmented balanced variation and abundance gradient fractions between paired grassland-forest sites along the differing grassland successional stages. Analyses done using raw (i.e. unrarefied data). The asterisk (*) denotes significant difference (p < 0.05) in nestedness between successional stages of grasslands when compared to their respective paired forest sites. Tests are based on pairwise Wilcoxon tests.*

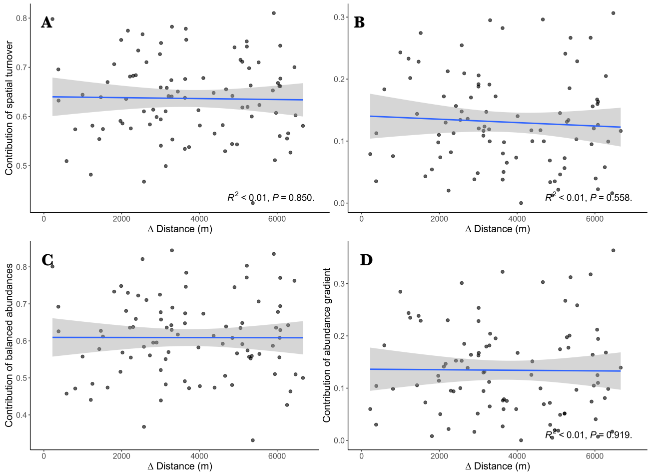

Fig. S5: *Scatter plots displaying the relationship between absolute differences in distance (in meters) between* ***A)*** *Spatial turnover,* ***B)*** *Nestedness,* ***C)*** *Balanced variation, and* ***D)*** *Abundance gradients, for each pair of grassland-forest sites.*

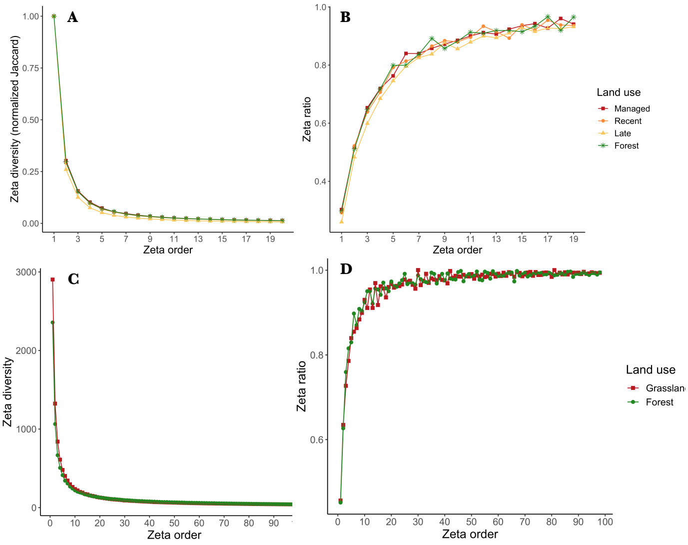

Fig. S6: *Zeta diversity decline and species retention curve based on* ***A-B)*** *normalized zeta diversity values (Jaccard) across 20 zeta orders, and* ***C-D)*** *raw zeta values across 100 zeta orders for grassland and forest ecosystems*

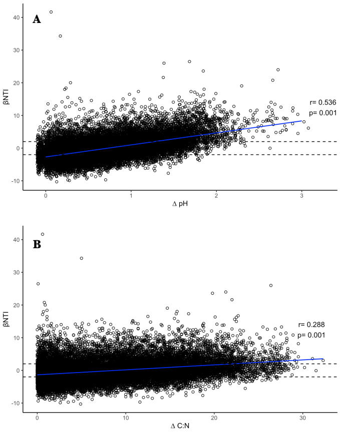

Fig. S7: *Differences in* ***A)*** *soil pH and* ***B)*** *C:N explains significant variation in phylogenetic turnover across all grasslands and forest sites. Correlation value and significance derived from Mantel test (9999 permutations).*

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Land-use changes during the past 150 years have profoundly altered landscapes and biodiversity and this thesis explores how they affect soil microbial communities across time and space. Microbial communities are remarkably resilient to grassland abandonment and overgrowth but change drastically when compared to forests as successional endpoints, thereby affecting carbon cycling. Still, traces of historical land use can be found in functional groups of microbes a century after the land-use change. These findings suggest strong but time-lagged responses of soil microbes to landscape changes both in diversity and functioning.

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