

Decades to establish, centuries to assemble: Soil microbial community development following woodland creation on agricultural land

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ABSTRACT

Woodland creation on former agricultural land is a central component of UK and European land-use policy for climate change mitigation and biodiversity restoration. However, the long-term trajectories of soil fungal and bacterial community assembly following woodland creation, and the extent to which they converge toward ancient woodland communities, remain poorly resolved. In this study we examined whether belowground microbial communities progressively converge toward those of ancient woodland, if fungal and bacterial communities follow similar successional trajectories, and whether their development is associated with changes in soil properties, particularly soil organic carbon (SOC) and pH. We investigated soil fungal and bacterial communities across a > 250-year chronosequence in central Scotland (UK), spanning agricultural pastures, planted woodlands of increasing age, and ancient woodland comparators, using fungal ITS1 and bacterial (16S rRNA) metabarcoding at 35 sites. Ectomycorrhizal (EcM) fungi increased across the chronosequence, reflecting the progressive establishment of forest-associated symbioses during woodland development, from a mean of 8.2% in pastures to 36.4% in ancient woodlands. Arbuscular mycorrhizal fungi declined sharply following tree establishment, and SOC was the strongest correlate of both fungal and bacterial community composition. Most notably, fungal communities in planted forests remained compositionally distinct from ancient woodland even after 160 years of development, being more dissimilar to ancient woodland than ancient woodland sites were to one another. The persistence of compositionally distinct fungal communities after 160 years highlights the difficulty of recreating the soil conditions characteristic of ancient woodland, underscoring its irreplaceable conservation value and the need to consider soil condition as a core component of woodland restoration and monitoring alongside aboveground vegetation.

1. Introduction

Forests once covered around 75% of the British landscape, yet centuries of clearance for agriculture and timber extraction reduced woodland cover to just 5% by the start of the twentieth century - among the lowest in Europe (Watts, 2006). The ecological consequences of this loss extend far beyond the removal of trees (FAO, 2020; Millennium Ecosystem Assessment, 2005). Woodland creation on former agricultural land is now a central component of UK and European land-use

policy for climate change mitigation and ecosystem restoration (IPCC, 2018; Lewis et al., 2019). Since 1990, global forest area has increased by approximately 5 million hectares through active tree planting programmes in Europe and beyond, aiming to enhance carbon sequestration, support biodiversity, and promote sustainable land use (FAO, ITPS, GSBI, SCBD and EC, 2020). Critically, most of this new woodland has been created through deliberate planting rather than natural colonisation; in the United Kingdom alone, over 13,000 ha yr⁻¹ are planted under public and private woodland creation schemes (Forestry Commission,

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2023). However, the ecological benefits of woodland creation on former agricultural land remain partly understood: recent evidence suggests that outcomes vary considerably with size, structural complexity, and landscape context (Fuentes-Montemayor et al., 2022; Waddell et al., 2024). While the aboveground benefits of such land-use change are increasingly documented, the development and compositional change of belowground microbial communities remain poorly understood (Guo and Gifford, 2002; Parkhurst et al., 2022). As soil microbiomes drive plant health, biogeochemical cycling, and ecosystem stability (van der Heijden et al., 2008), understanding their assembly following woodland creation is critical for predicting long-term restoration outcomes.

Prior land use leaves deep and lasting legacies on soil structure and biota. Both arable cultivation and managed pasture - the two main agricultural precursors to afforestation in the UK - impose distinct but overlapping pressures on soil communities. Frequent tillage, fertiliser application, and simplified crop rotations in arable systems typically reduce soil organic carbon (SOC), alter pH, and deplete microbial diversity, particularly symbiotic fungi (García-Orenes et al., 2013; Lauber et al., 2008). Managed pasture, while less physically disruptive, maintains simplified plant communities dominated by fast-growing grasses, suppresses forest-associated mycorrhizal taxa, and through sustained grazing pressure and soil compaction reduces the structural heterogeneity that supports specialist soil organisms (Jangid et al., 2011; Rillig et al., 2002). Agricultural disturbance favours fast-growing, copiotrophic microorganisms while suppressing the specialist taxa and complex hyphal networks characteristic of mature forests. This matters for soil quality: copiotrophic-dominated communities can produce microbial necromass efficiently (Cotrufo et al., 2013) but, under continued disturbance, are associated with reduced soil aggregate protection and mineral association of that carbon (Azevedo et al., 2026), so that stabilisation of organic matter and cycling of nutrients bound in recalcitrant organic matter are diminished — functions that take decades to recover following land-use change (Allison et al., 2013; Vivian et al., 2025). The timescales and completeness of microbiome recovery following the transition to woodland remain unresolved, particularly over the centennial scales relevant to forest development (Bremer and Farley, 2010; Castañeda-Gómez et al., 2021, 2023).

Mycorrhizal fungi, forming symbioses with plant roots, are central agents in forest soil development and ecosystem functioning (Baldrian, 2017; Suz et al., 2022, 2021). In grasslands and arable soils, arbuscular mycorrhizal (AM) fungi broadly support herbaceous plant communities, while the establishment of temperate tree species progressively favours ectomycorrhizal (EcM) fungi (van der Heijden et al., 2015). EcM fungi form symbiotic associations with most temperate tree species and facilitate nutrient and water uptake, drought tolerance, pathogen resistance, and organic matter turnover (Baldrian, 2017; Clemmensen et al., 2015; Suz et al., 2021). Changes in EcM community composition have been linked to nutrient imbalances in trees and to potential tipping points in forest ecosystem functioning, making the trajectory of EcM assemblage following afforestation of particular importance for forest resilience (Suz et al., 2022, 2021). Several studies have tracked soil fungal community change across successional gradients spanning up to 80 years, but these are typically restricted to natural succession (unmanaged secondary woodland developing without deliberate planting) rather than the active planting of trees on former agricultural land that characterises most UK woodland creation (Li et al., 2020; Qiang et al., 2023). This distinction matters because naturally regenerating woodlands develop from local propagule pools and under self-selected plant communities, whereas planted woodlands impose specific tree species compositions on soils with strong agricultural legacies, potentially altering the pace and trajectory of mycorrhizal assemblage (Peay et al., 2012). To our knowledge no published study has followed mycorrhizal community succession in deliberately planted forests over centennial timescales using a chronosequence that also includes ancient woodland comparators.

Soil bacteria, alongside fungi, govern decomposition of organic

matter, nitrogen and carbon cycling, and soil structure maintenance (Delgado-Baquerizo et al., 2018; Fierer, 2017). Early in forest establishment, bacterial communities are often dominated by generalist, fast-growing taxa adapted to high resource availability and fluctuating conditions (Fierer et al., 2012; Lladó et al., 2017). Over time, slower-growing, specialist bacteria capable of degrading recalcitrant organic matter, including lignocellulose decomposers and nitrogen cyclers, become more prevalent (Allison et al., 2013). The ecological significance of this shift extends beyond community composition: specialist decomposer communities can accelerate the stabilisation of recalcitrant SOC fractions, though their enhanced organic matter mineralisation may simultaneously reduce the pool of stable carbon stocks, creating a potentially important tension in restoration contexts (Averill et al., 2014). The long-term trajectories and drivers of bacterial community changes following the conversion of agricultural land to planted woodland are not well characterised. Comparing fungal and bacterial communities within the same chronosequence provides a broader test of belowground recovery: if both groups track the same soil gradients, this points to shared edaphic control, whereas divergent trajectories would indicate taxon-specific responses to woodland development.

Aboveground vegetation and habitat structure shape belowground microbial communities through litter quality and quantity, root exudate composition, and microhabitat provision (Chen et al., 2019; Wardle et al., 2004). Tree species identity is a major determinant of soil C:N ratios in European forests, primarily through differences in litter chemistry between deciduous and evergreen species (Cools et al., 2014; Prescott and Grayston, 2013). These tree species effects on soil stoichiometry in turn influence the composition of mycorrhizal and decomposer communities by altering resource quality for soil microorganisms (Tederloo et al., 2020). Coarse woody debris (CWD) and ground cover further influence soil microclimate and decomposer communities (Harmon et al., 1986; Wu et al., 2020). Herbivore grazing modifies plant community composition and can disrupt mycorrhizal networks through soil compaction and root disturbance, effects documented in grassland studies using enclosure experiments (Rillig et al., 2002; Wardle et al., 2004). Several studies have examined the influence of specific aboveground variables on soil fungi in afforested systems. For example, tree diversity has been linked to increased soil fungal diversity through enhanced mycorrhizal host availability and complementary litter inputs (Tederloo et al., 2020), while CWD effects on fungal communities are typically weak relative to soil chemistry drivers in early succession (Baldrian, 2017). It remains unclear whether these patterns hold across the longer timescales and managed plantation contexts relevant to UK woodland creation.

Here we present a chronosequence analysis spanning more than 250 years of woodland development on former agricultural land in central Scotland, including seven ancient woodland comparators. Using ITS1 and 16S rRNA metabarcoding combined with measurements of soil chemistry (pH, soil organic carbon, C:N ratio), vegetation (tree species composition, diversity), and habitat structure (coarse woody debris, ground cover, grazing), we address three research questions:

How do fungal EcM community composition and diversity change across the forest chronosequence?

Hypothesis 1. EcM fungal abundance will increase progressively with forest age, with community composition in older planted forests more closely resembling ancient woodlands.

Which environmental variables are the strongest drivers of fungal and bacterial community composition?

Hypothesis 2. Soil chemistry variables, particularly SOC and pH will be stronger predictors of community composition than aboveground management factors, reflecting persistent agricultural land-use legacies.

Do planted forests achieve fungal communities similar to ancient woodlands, and over what timescale?

Hypothesis 3. Planted forests will show directional compositional convergence toward ancient woodland communities with increasing age, though complete convergence will not be achieved within the planted woodlands.

2. Methods

2.1. Site selection and characteristics

This study used a space-for-time chronosequence approach to examine soil microbial communities across a gradient of woodland maturity. Sampling was conducted between 2019 and 2021 at sites established by the WrEN project (Woodland Creation and Ecological Networks; Watts et al., 2016). All sites were located on brown earth soils (Dystric/Eutric Cambisol); restricting the chronosequence to a single dominant soil type removes texture and parent material as major confounding factors. Sites comprised three agricultural pasture controls and 32 native broadleaf woodlands. Of the woodlands, 25 were planted on former agricultural land and categorised by development stage (Oliver and Larson, 1996): stand initiation ≤ 30 years ($n = 4$), stem exclusion 31–80 years ($n = 6$), and understorey re-initiation 81–160 years ($n = 15$). Seven ancient woodland sites (>250 years, $n = 7$), embedded within the same agricultural landscapes, served as long-term reference communities (Fig. 1). The woodland sites have received minimal active management since planting (no systematic thinning, coppicing, or deliberate deadwood creation has been recorded) and are representative of the small, unmanaged native woodland patches characteristic of lowland agricultural landscapes in Scotland.

Vegetation and habitat characteristics for each site were obtained from the WrEN project vegetation survey described in Fuentes-Montemayor et al. (2022). Briefly, tree surveys were conducted using the point-centred quarter method along edge-to-interior transects with points every 15 m; at each point the nearest tree per quarter was identified to species and its diameter at breast height (DBH; trees ≥ 7 cm only) recorded. From these data, site-level estimates of tree density, species richness, tree species dominance and a Shannon diversity index were calculated. *Acer pseudoplatanus* dominated nine sites, followed by *Betula pubescens*, *Quercus robur*, and *Crataegus monogyna* (four, four, and three sites respectively); four sites showed mixed dominance with no single species exceeding 50% of stems. Ground cover was predominantly grass at 24 sites, herbs at six, leaf litter at three, and bare soil at two. Coarse woody debris (CWD) load was classified into three categories

within 2×2 m quadrats: low (predominantly fine litter and twigs ≤ 1 cm diameter), moderate (branches ≤ 10 cm), or high (CWD >10 cm diameter including fallen trees), corresponding to scores of 1, 2, and 3 respectively used in statistical analyses. Grazing exposure was recorded as a binary variable (Yes/No) based on the presence or absence of livestock and deer-proof fencing, corroborated by visible signs of grazing activity including dung, browsing damage, and bark striping where present. Unfenced sites with observed grazing signs ($n = 8$) were classified as exposed to grazing. This variable is acknowledged as a proxy measure for grazing pressure rather than a direct quantification of grazing intensity or frequency.

2.2. Soil sampling and chemistry

At each site, soil was sampled from the 0–15 cm mineral horizon; loose litter and surface organic (O) layer were removed before coring. In the oldest stands, however, the low bulk density and high carbon concentration indicate that decomposed organic material remained incorporated into the upper mineral horizon and could not be fully separated in the field. For bulk density determination, five cylindrical cores (15 cm height $\times$ 4.5 cm diameter) were collected per site. Cores were sieved through a 2 mm mesh to remove the coarse fraction, then weighed before and after oven drying at 105°C until constant mass; bulk density was calculated as the mass of oven-dry fine earth (<2 mm) per unit volume of fine earth, correcting for the coarse (>2 mm stone) fraction (g cm^{-3}). Gravimetric moisture content was calculated from the same cores as the mass of water lost on drying expressed as a proportion of dry soil mass.

For chemical analysis, ten soil cores were collected per site using a Dutch auger to 15 cm depth. Samples were sieved through a 2 mm mesh to remove coarse fragments and dried at 60°C . Total carbon and total nitrogen concentrations were determined by elemental analysis using a C:N elemental analyser (FLASH EA 1112, Carlo Erba/Thermo Fisher Scientific). Soil pH was measured in a 1:2.5 soil:water suspension. Soil organic carbon stocks (t C ha^{-1}) were calculated by combining SOC concentration (%) with bulk density and the 15 cm sampling depth, assuming negligible inorganic carbon contribution given the non-calcareous nature of the study soils. The C:N ratio was calculated as the mass ratio of total carbon to total nitrogen. For microbial analysis, approximately 20 g subsamples from three replicate cores were stored at 4°C prior to freezing at -20°C , yielding 105 samples. Site-level soil chemistry values were calculated as means across all ten cores per site using 0–15 cm mineral soil samples only. SOC, pH, and C:N ratio were used as environmental predictors.

2.3. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from the same frozen soil subsamples using the CTAB method (Novogene, Cambridge, UK), ensuring that ITS1 (fungi) and 16S rRNA (bacteria) analyses used identical source material. The fungal ITS1 region was amplified using primers ITS1F and ITS2R (White et al., 1990). The bacterial 16S V3-V4 region was amplified using primers 341 F and 806 R (Yu et al., 2005). Sequencing libraries were prepared using the NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit and sequenced on Illumina platforms (Novogene). Fungal taxonomic annotation used the UNITE database (v9); bacterial annotation used SILVA (v138). The microbial analyses were restricted to named taxa, representing the identifiable, most-abundant fraction of the community and diversity metrics and ordination results should be interpreted in this context and do not represent the full breadth of soil microbial diversity. Four sites were excluded from bacterial analyses after rarefaction due to insufficient named-taxon read depth, leaving 31 of 35 sites for bacterial community analyses.

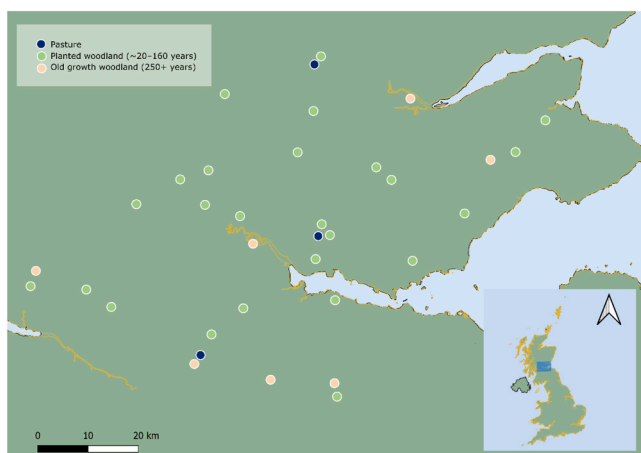


Fig. 1. Location of study sites across central Scotland. Circles indicate the 35 sampling sites: pasture ($n = 3$), planted woodland aged approximately 20–160 years ($n = 25$), and ancient woodland continuously wooded for >250 years ($n = 7$). Sites were established as part of the WrEN project (Woodland Creation and Ecological Networks; Watts et al., 2016). Inset shows the location of the study area within Great Britain.

2.4. Functional annotation

Fungal taxa were assigned to functional guilds (EcM, AM, saprotrophic, pathogenic) using FUNguild. Guild relative abundances were calculated per site as the proportion of total fungal reads assigned to each guild, with unclassified reads retained in the denominator. Note that ITS1 primers have well-documented bias against AM taxa and relative abundances should be interpreted with caution (Öpik et al., 2014). Bacterial taxa were annotated using a genus-level lookup table derived from the FAPROTAX database (Louca et al., 2016). Functional groups were collapsed into five biogeochemically specific relevant to forest soil biogeochemistry: denitrification; nitrification/N-fixation; C mineralisation (cellulolysis, chitinolysis, fermentation); methanogenesis; and sulphur cycling. The methane group comprised methanogenic archaea only (Methanobacterium and Methanoculleus); no methanotrophs were recovered in the dataset. Aerobic chemoheterotrophy was excluded from functional figures as a non-specific background process. Results represent the functional potential inferred from the most abundant, best-characterised genera only.

2.5. Statistical analysis

2.5.1. Data preparation and rarefaction

All analyses were performed in R version 4.3.3 using the vegan (Oksanen et al., 2025), coin (Hothorn et al., 2008), and rstatix (Kassambara, 2023) packages with a fixed random seed for reproducibility. Fungal and bacterial OTU community matrices were rarefied to the minimum library size within each dataset to equalise sampling effort: fungi to 229,479 reads (minimum across 35 sites); bacteria to the 10th percentile of named-taxon read depth (5430 reads) to avoid excessive information loss from one low-depth site. Square-root transformation was applied to rarefied abundance data prior to dissimilarity calculations.

2.5.2. Diversity and functional guild analyses

Differences in EcM and AM relative abundance among age categories were tested using the Jonckheere-Terpstra trend test (coin package, Hothorn et al., 2008), which directly tests the hypothesis of a monotonic increase (EcM) or decrease (AM) across ordered age categories. This approach is more appropriate than a standard ANOVA because the age categories have an inherent ordinal structure (Pasture < 30 < 31–80 < 81–160 < 250 + years) that a standard ANOVA ignores. A Levene test confirmed significant heterogeneity of variance across EcM groups ($p = 0.018$), further supporting the use of a non-parametric approach. One-sided tests were used reflecting the directional biological hypotheses. Spearman rank correlation was used as a complementary measure of monotonic association. Post-hoc pairwise comparisons used the Games-Howell test where relevant. Alpha diversity (Shannon H') was compared across age categories using one-way ANOVA and correlated with continuous forest age using Pearson's r . The sampling design targeted age categories representing developmental stages, and consequently plot ages are clustered rather than evenly distributed along a continuous gradient (Supplementary Figure S5). Moreover, pasture (the pre-woodland baseline) and ancient woodland (recorded only as >250 years) cannot be placed on a continuous age axis, yet both are central to the research questions: an ordinal framework incorporating the rank order of all categories was therefore the most appropriate approach. Continuous plot age was retained only for the convergence analysis of planted forests (Fig. 9), where higher age resolution is both possible and most informative.

2.5.3. Environmental drivers

Community composition was analysed using Bray-Curtis dissimilarity matrices on square-root-transformed rarefied abundance data. NMDS ordinations were performed in two dimensions (vegan::metaMDS, 100 random starts; stress values <0.20 considered acceptable).

Environmental variable importance was quantified using vector fitting to NMDS ordinations (envfit, 999 permutations; R^2 represents proportion of NMDS ordination variance explained) and PERMANOVA with marginal tests (adonis2, 999 permutations; R^2 represents the proportion of total community variance explained). Homogeneity of multivariate dispersion was tested using betadisper (permutest, 999 permutations). To partition community variance between predictor groups, variation partitioning was performed using the varpart function in vegan (Oksanen et al., 2025), with two predictor groups: soil chemistry (SOC, pH, C:N ratio) and vegetation & management (tree diversity, CWD, grazing, ground cover). Adjusted R^2 was used to provide unbiased estimates of explained variance. The significance of each unique fraction was tested using partial RDA permutation tests (999 permutations), conditioning on the other predictor group. For planted woodland sites these variables reflect documented establishment and management history, whereas for ancient woodland sites, where management history is largely unknown, their interpretation is less certain - in particular, whether tree diversity reflects deliberate species selection or natural colonisation.

2.5.4. Convergence analysis

Convergence toward ancient woodland was quantified as mean Bray-Curtis dissimilarity from each site to all seven ancient woodland sites. The convergence threshold was defined as the mean of all 21 pairwise BC dissimilarity among the seven ancient woodland sites (i.e. every unique site-to-site comparison, rather than distance to a single centroid or averaged profile; $BC = 0.693 \pm 0.110$), representing natural compositional variability within the reference state. Linear regression tested the relationship between forest age and mean dissimilarity to ancient woodland in planted forest sites only ($n = 25$). Differences between adjacent age categories were tested using Wilcoxon rank-sum tests.

3. Results

ITS1 metabarcoding identified 2375 fungal taxa and 16S rRNA sequencing identified 557 named bacterial taxa across 35 sites. Fungal rarefaction depth was 229,479 reads per site; bacterial rarefaction depth was 5430 named-taxon reads per site, with four sites excluded due to insufficient depth (31 sites retained). Fungal NMDS stress = 0.165; bacterial NMDS stress = 0.152, both within acceptable bounds.

3.1. EcM accumulation across the forest chronosequence

EcM fungi showed a consistent directional increase with forest age, from a mean abundance of 8.2% (± 7.5 SD) in pastures to 36.4% (± 23.5 SD) in ancient woodlands (Fig. 2A; Table 1). The Jonckheere-Terpstra trend test confirmed a significant monotonic increase across ordered age categories ($z = 2.29$, $p = 0.011$; Spearman $\rho = 0.38$, $p = 0.013$). No individual pairwise Games-Howell comparisons were significant after correction, reflecting high within-category variance particularly in the 31–80 year category (SD = 29.0%, range 1.7–71.1%). AM fungi showed a significant monotonic decrease across ordered age categories (Jonckheere-Terpstra: $z = -2.49$, $p = 0.006$; Spearman $\rho = -0.39$, $p = 0.010$), with mean abundance dropping from 1.9% (± 1.6 SD) in pastures to 0.4% (± 0.2 –0.4 SD) in all woodland categories.

To test whether dominant tree mycorrhizal identity influenced mycorrhizal fungal abundance, woodland stands were classified by the mycorrhizal association of their dominant tree genus (AM-associated: Acer, Prunus, Crataegus, Sorbus, Ulmus, Fraxinus; EcM-associated: Betula, Quercus, Alnus, Picea, Pinus), yielding 17 AM-dominant and 13 EcM-dominant stands. AM fungal abundance was significantly higher under AM-associated trees (mean $0.49\% \pm 0.29$) than under EcM-associated trees ($0.20\% \pm 0.13$; Wilcoxon $p = 0.002$), indicating a detectable tree-species imprint on the residual AM community. However, even under AM-associated trees, AM fungal abundance remained

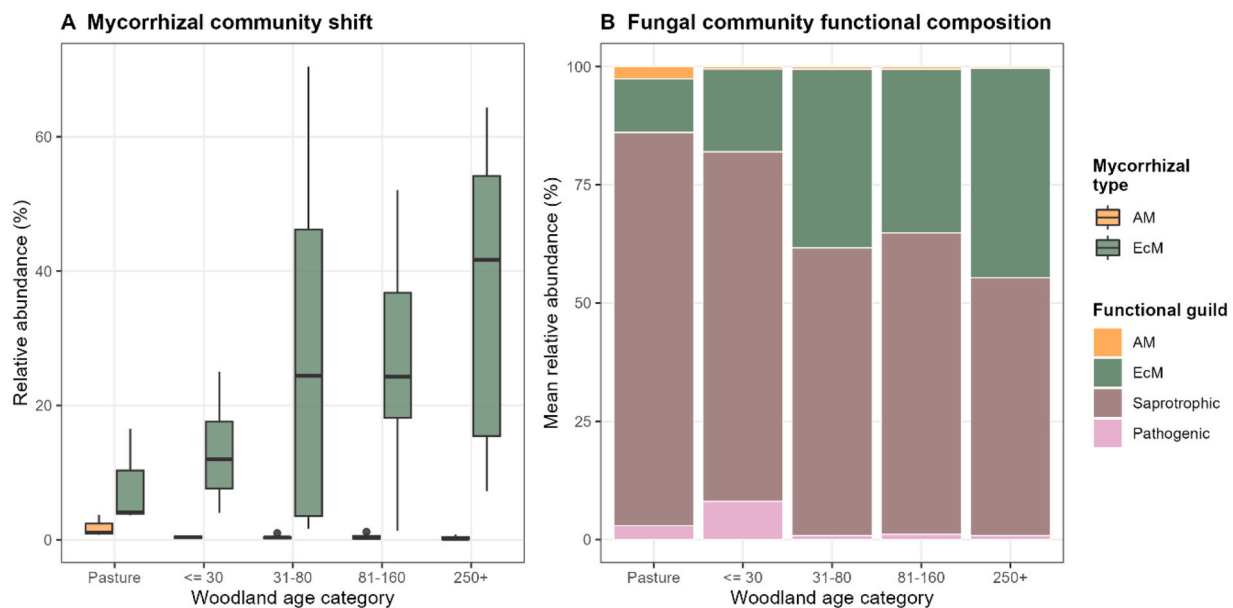


Fig. 2. Fungal functional guild composition across the woodland chronosequence. (A) Boxplots showing relative abundance of ectomycorrhizal (EcM) and arbuscular mycorrhizal (AM) fungi by age category. (B) Mean relative abundance of classified fungal guilds by age category; unclassified taxa excluded and proportions renormalised to 100% within classified reads.

Table 1

Site characteristics and soil microbial community metrics by woodland age category. Values are mean $\pm$ standard deviation. Fungal and bacterial Shannon diversity (H') calculated from rarefied ITS1 and 16S rRNA community matrices respectively. EcM and AM values represent mean relative abundance of ectomycorrhizal and arbuscular mycorrhizal fungi as a proportion of classified fungal reads. SOC = soil organic carbon. pH measured in 1:2.5 soil:water suspension. Four sites excluded from bacterial analysis due to insufficient named-taxon read depth after rarefaction.

	Pasture	≤ 30 yr	31–80 yr	81–160 yr	250 + yr
n (fungal / bacterial)	3/3	4/4	6/5	15 / 13	7/6
Tree richness	0	6.2 $\pm$ 2.4	4.2 $\pm$ 2.3	4.7 $\pm$ 2.2	5.0 $\pm$ 2.6
Tree H'	0	1.41 $\pm$ 0.45	1.01 $\pm$ 0.63	1.05 $\pm$ 0.47	0.89 $\pm$ 0.65
Fungal richness	718 $\pm$ 77	818 $\pm$ 16	616 $\pm$ 128	720 $\pm$ 85	720 $\pm$ 79
Fungal H'	3.71 $\pm$ 0.08	3.61 $\pm$ 0.18	3.03 $\pm$ 0.17	3.40 $\pm$ 0.29	3.50 $\pm$ 0.35
EcM (%)	8.2 $\pm$ 7.5	13.4 $\pm$ 9.2	28.6 $\pm$ 28.8	26.6 $\pm$ 15.7	36.4 $\pm$ 23.5
AM (%)	1.9 $\pm$ 1.6	0.4 $\pm$ 0.2	0.4 $\pm$ 0.3	0.4 $\pm$ 0.3	0.3 $\pm$ 0.3
Bacterial richness	223 $\pm$ 26	216 $\pm$ 21	189 $\pm$ 33	189 $\pm$ 27	199 $\pm$ 23
Bacterial H'	3.52 $\pm$ 0.32	3.68 $\pm$ 0.11	3.27 $\pm$ 0.51	3.43 $\pm$ 0.26	3.46 $\pm$ 0.25
Bulk density (g cm ⁻³)	0.68 $\pm$ 0.16	0.45 $\pm$ 0.10	0.30 $\pm$ 0.19	0.39 $\pm$ 0.13	0.34 $\pm$ 0.16
Moisture (%)	36.9 $\pm$ 8.2	46.0 $\pm$ 8.0	59.7 $\pm$ 20.7	47.7 $\pm$ 11.1	52.7 $\pm$ 15.8
C:N ratio	11.1 $\pm$ 0.9	12.2 $\pm$ 7.7	16.1 $\pm$ 2.8	16.1 $\pm$ 2.8	17.0 $\pm$ 4.0
pH	5.65 $\pm$ 0.59	5.68 $\pm$ 1.04	4.73 $\pm$ 0.39	4.87 $\pm$ 0.27	4.59 $\pm$ 0.54
SOC (g kg ⁻¹)	41.5 $\pm$ 5.4	48.4 $\pm$ 24.3	120.4 $\pm$ 124.3	72.9 $\pm$ 23.4	120.2 $\pm$ 162
SOC (t ha ⁻¹)	28.9 $\pm$ 10.6	20.8 $\pm$ 10.0	20.6 $\pm$ 9.3	27.2 $\pm$ 10.2	26.7 $\pm$ 15.2

far below the pasture baseline (1.9% $\pm$ 1.6), and the nine strongly AM-dominant stands ($\geq 50\%$ dominance) averaged just 0.50% AM. AM fungi therefore declined sharply on woodland establishment regardless of

dominant tree mycorrhizal type, with a smaller tree-identity effect superimposed on this dominant successional decline (Fig. 3).

Saprotrophic fungi comprised the largest guild throughout (44–60% of classified reads), remaining the dominant functional group across all age categories. Saprotrophic relative abundance showed a weak but non-significant negative trend across ordered age categories (Spearman $\rho = -0.26$, $p = 0.132$). Pathogenic fungi were a minor component throughout ($< 7\%$) with no significant trend across the chronosequence (Spearman $\rho = -0.06$, $p = 0.752$) (Fig. 2B).

Fungal Shannon diversity differed significantly among age categories ($F_{4,30} = 4.12$, $p = 0.009$; Fig. 4), with the 31–80 year category showing the lowest mean diversity ($H' = 3.03 \pm 0.19$), compared to 3.71 ± 0.10 in pastures and 3.50 ± 0.38 in ancient woodlands. The full range across all sites was $H' = 2.73$ – 3.90 . No significant linear relationship between diversity and continuous forest age was detected ($r = 0.08$, $p = 0.645$). Bacterial Shannon diversity showed no significant differences among categories ($F_{4,26} = 0.88$, $p = 0.489$), with means ranging from 3.03 to 3.68.

3.2. Environmental drivers of community composition

Soil chemistry variables were the strongest predictors of fungal community composition (Fig. 5). NMDS ordination showed clear compositional gradients associated with the SOC and pH axes. Envfit vector fitting identified SOC ($R^2 = 0.68$, $p = 0.001$), pH ($R^2 = 0.45$, $p = 0.001$), and C:N ratio ($R^2 = 0.42$, $p = 0.001$) as the strongest correlates of ordination space. Tree diversity also showed a weak, non-significant association in envfit ($R^2 = 0.15$, $p = 0.076$; Figure S4). CWD, grazing, and ground cover also showed weak and non-significant associations (all $R^2 < 0.17$, $p > 0.05$; Figure S4).

PERMANOVA (marginal tests) confirmed SOC as the most significant predictor of fungal community composition ($R^2 = 0.049$, $F = 1.82$, $p = 0.008$), with pH additionally significant ($R^2 = 0.041$, $F = 1.52$, $p = 0.033$).

All other variables — CWD, tree diversity, grazing, and ground cover — were not significantly associated with the fungal community composition (all $p > 0.32$). Fungal betadisper revealed significant heterogeneity in community dispersion among age groups ($F_{4,29} = 3.14$, $p = 0.041$), indicating that compositional variability itself changes

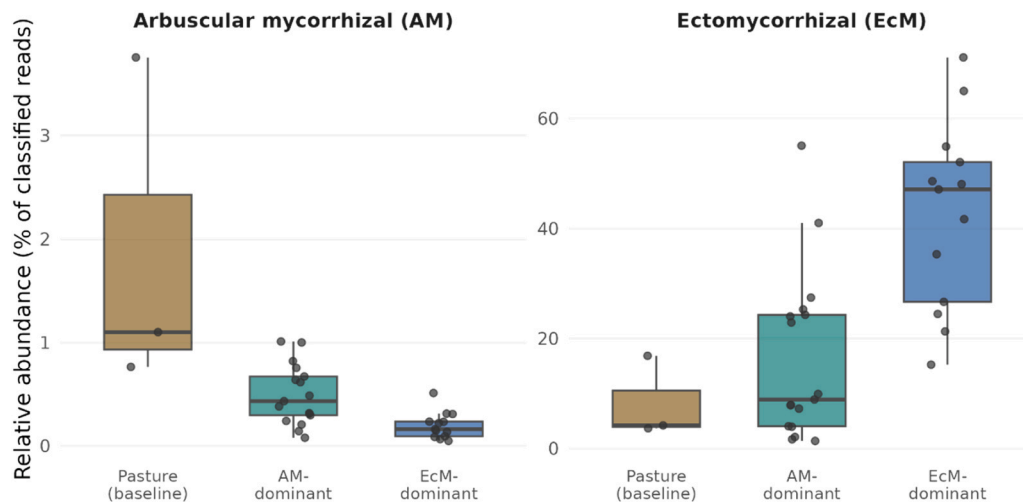


Fig. 3. Relative abundance of arbuscular mycorrhizal (AM) and ectomycorrhizal (EcM) fungi by dominant-tree mycorrhizal type. Fungal guild abundance in pasture (baseline) and woodland stands grouped by mycorrhizal association of their dominant tree (AM-associated: *Acer*, *Prunus*, *Crataegus*, *Sorbus*, *Ulmus*, *Fraxinus*; EcM-associated: *Betula*, *Quercus*, *Alnus*, *Picea*, *Pinus*). AM fungi decline sharply on woodland establishment regardless of dominant-tree type (both woodland groups far below the pasture baseline) but remain significantly higher under AM-associated than EcM-associated trees (0.49% vs 0.20%; Wilcoxon $p = 0.002$). Points are individual sites; boxes show median and interquartile range, and whiskers extend to $1.5 \times$ the interquartile range.

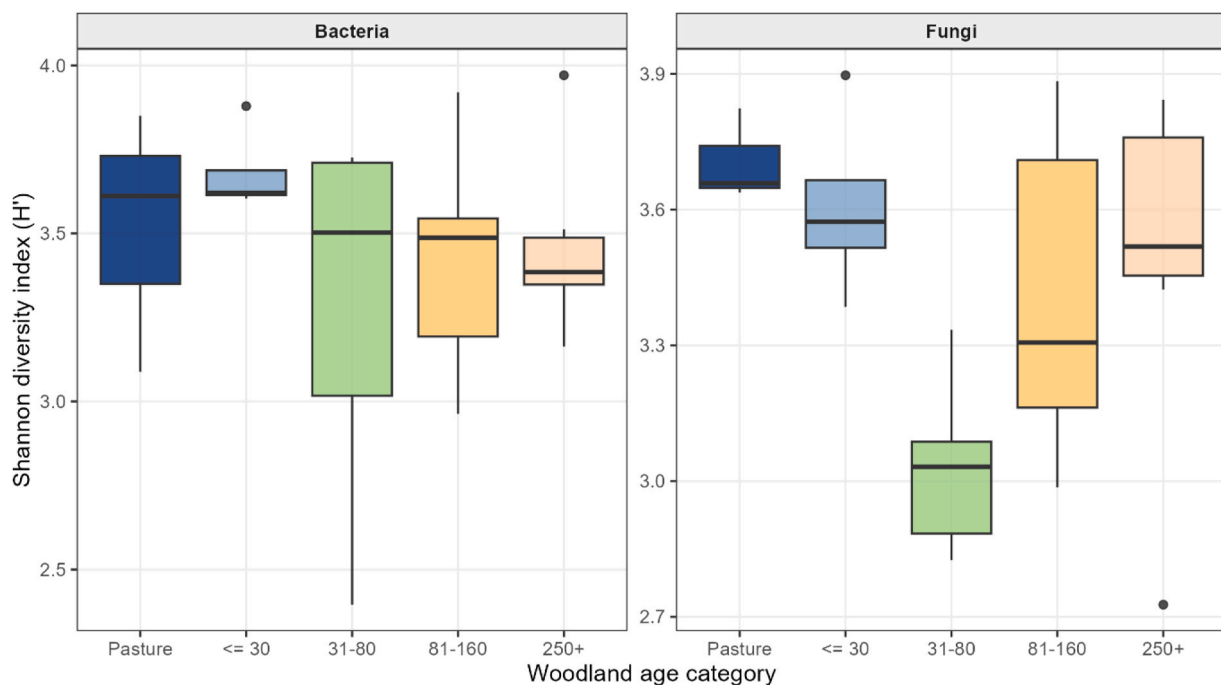


Fig. 4. Alpha diversity (Shannon H') for fungal and bacterial communities across the woodland chronosequence. Boxplots show within-category variation. Fungal diversity based on ITS1 communities rarefied to 229,479 reads (35 sites). Bacterial diversity based on 16S rRNA communities rarefied to 5430 reads (31 sites). Fungal Shannon diversity differed significantly among age categories (one-way ANOVA: $F_{4,30} = 4.12$, $p = 0.009$); bacterial Shannon diversity did not ($F_{4,26} = 0.88$, $p = 0.489$).

across the chronosequence; results should be interpreted considering both centroid location and dispersion effects. Mean SOC concentration increased markedly from pasture to woodland ($41.5 \pm 5.4 \text{ g kg}^{-1}$ in pastures to $120.2 \pm 162 \text{ g kg}^{-1}$ in ancient woodlands; Table 1). However, when expressed as stocks, the gradient flattens considerably (pasture 28.9 ± 10.6 ; $\leq 30 \text{ yr}$ 20.8 ± 10.0 ; 31–80 yr 20.6 ± 9.3 ; 81–160 yr 27.2 ± 10.2 ; 250 + yr $26.7 \pm 15.2 \text{ C ha}^{-1}$), reflecting the substantially lower bulk density of woodland soils relative to pasture soils (Table 1).

Variation partitioning identified soil chemistry as the only group with a significant unique contribution to fungal community composition

(adjusted $R^2 = 0.053$, 5.3%; $p = 0.001$; Fig. 6).

Bacterial community composition showed a similar, though less strong, response to soil chemistry. SOC was the sole significant driver in envfit ($R^2 = 0.58$, $p = 0.003$; Fig. 7). PERMANOVA confirmed SOC ($R^2 = 0.087$, $F = 2.90$, $p = 0.015$) as well as pH ($R^2 = 0.058$, $F = 1.91$, $p = 0.027$) as significant predictors of bacterial community composition. Bacterial betadisper showed no significant dispersion heterogeneity among age groups ($F_{4,25} = 1.03$, $p = 0.373$), indicating that differences in bacterial community composition reflect centroid shifts rather than changes in spread. The convergence of both kingdoms on SOC as the primary driver supports the interpretation that agricultural

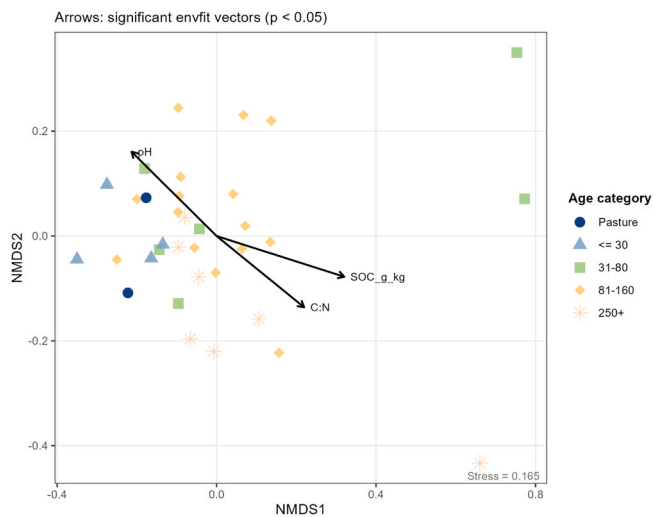


Fig. 5. Fungal community composition across the chronosequence (NMDS ordination, Bray-Curtis dissimilarity). Points represent individual sites coloured and shaped by woodland age category. Arrows show significant envfit vectors ($p < 0.05$): SOC = soil organic carbon, C:N = carbon to nitrogen ratio. Stress = 0.165. Ordination based on square-root transformed rarefied abundance data (229,479 reads per site, 35 sites).

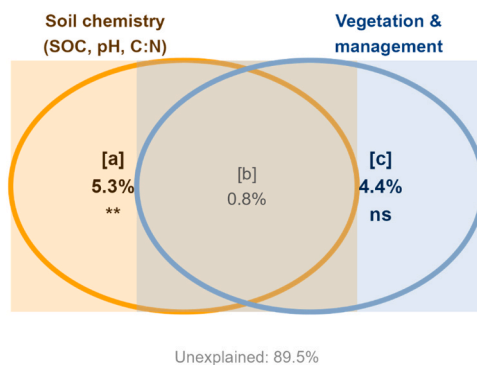


Fig. 6. Variation partitioning of fungal community composition between soil chemistry and vegetation & management variables. Circles show the proportion of community variance (adjusted R^2) attributable to each predictor group: [a] unique to soil chemistry (SOC, pH, C:N ratio); [b] shared between both groups; [c] unique to vegetation & management (tree diversity, CWD, grazing, ground cover). Fractions were estimated using partial RDA (vegan::varpart; Oksanen et al., 2025). Significance of unique fractions tested by permutation test (999 permutations): ** $p < 0.01$, ns = not significant. Negative fractions, indicating negligible unique contribution, are set to zero. For planted woodland sites, the variables in group [c] reflect documented establishment and management history, whereas for ancient woodland sites, where management history is largely unknown, their interpretation is less certain – in particular, whether tree diversity signifies species selection at establishment or natural colonisation. The envfit R^2 bar chart for individual variable contributions is provided in Fig. S4.

soil legacy is the dominant constraint on belowground community recovery.

3.3. Bacterial functional groups

Among the identifiable bacterial functional groups, carbon mineralisation taxa (mean $2.56\% \pm 1.31$ of named reads) and denitrifiers (mean $1.36\% \pm 0.82\%$) dominated the classifiable community, followed by nitrification/N-fixation ($0.33\% \pm 0.13\%$), methanogenesis ($0.28\% \pm 0.52\%$), and sulphur cycling ($0.12\% \pm 0.05\%$) groups (Fig. 8, and Supplementary Figure S3). Denitrification was represented primarily by

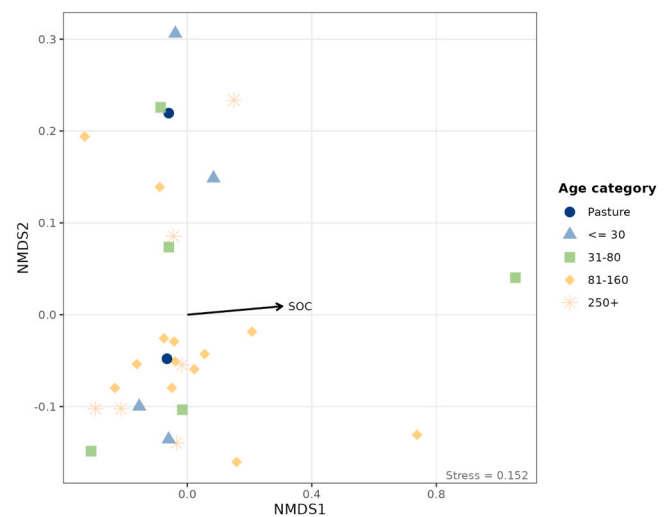


Fig. 7. Bacterial community composition across the chronosequence (NMDS ordination, Bray-Curtis dissimilarity). Points represent individual sites coloured and shaped by woodland age category. Arrow shows the sole significant envfit vector (SOC, $p = 0.003$), all other variables non-significant. Stress = 0.152. Based on named-taxon fraction only; 31 of 35 sites retained after rarefaction.

Pseudomonas and *Rhodanobacter* species, Methane cycling taxa, while low in abundance overall, showed elevated variance in the 81–160 year category relative to other age classes.

3.4. Convergence toward ancient fungal communities

Mean Bray-Curtis dissimilarity from planted forest sites to ancient woodland sites showed a directional decrease with increasing forest age (Fig. 9). Dissimilarity was highest in recently planted forests (≤ 30 years; mean BC = 0.707 ± 0.066) and lowest in ancient woodland pairwise comparisons (mean within-ancient BC = 0.693 ± 0.110). The overall pattern across all age categories was significant (Kruskal-Wallis $\chi^2 = 14.21$, $df = 4$, $p = 0.007$). Mature planted forests (81–160 years; mean BC = 0.688 ± 0.049) remained significantly more dissimilar to ancient woodlands than the within-ancient pairwise mean (Wilcoxon $W = 95$, $p = 0.003$), demonstrating that even the oldest planted forests in this chronosequence differ from ancient woodland community composition.

Linear regression of mean dissimilarity against forest age in planted forests only ($n = 25$) was not statistically significant ($R^2 = 0.09$, $p = 0.145$). The high within-category variance – particularly in the 31–80 year category ($SD = 0.092$) – and the substantial internal heterogeneity of ancient woodland communities (BC range 0.39–0.87) together limit statistical power. The regression line intersects the within-ancient dissimilarity threshold at approximately 127 years. Given the non-significant slope, however, this estimate should be treated as indicative rather than a precise biological prediction.

Notably, the 31–80 year category showed higher mean dissimilarity to ancient woodlands (0.756 ± 0.092) than both the ≤ 30 year category (0.707) and the 81–160 year category (0.688). This non-monotonic pattern likely reflects the high compositional variability within the 31–80 year category, a period of active community reorganisation, rather than genuine compositional divergence from ancient woodland during this phase.

4. Discussion

This study demonstrates that soil fungal and bacterial community change following woodland creation on former agricultural land is driven primarily by soil chemistry legacies, with EcM communities accumulating progressively across decades and both kingdoms responding to the same SOC gradient. Three principal findings emerge:

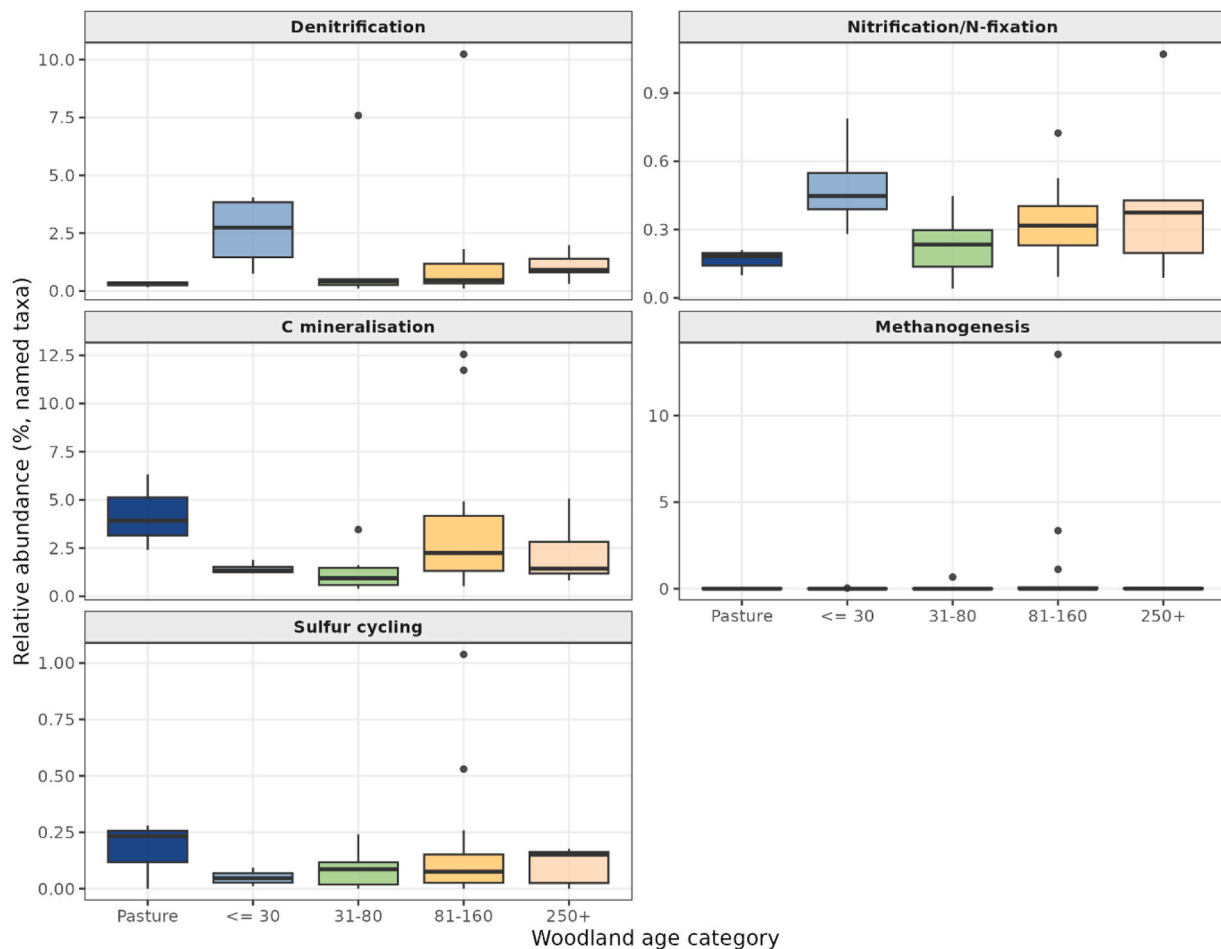


Fig. 8. Bacterial functional groups across the woodland chronosequence. Individual boxplots for each of the five cycling groups by age category. Aerobic heterotrophy excluded as a non-specific background process.

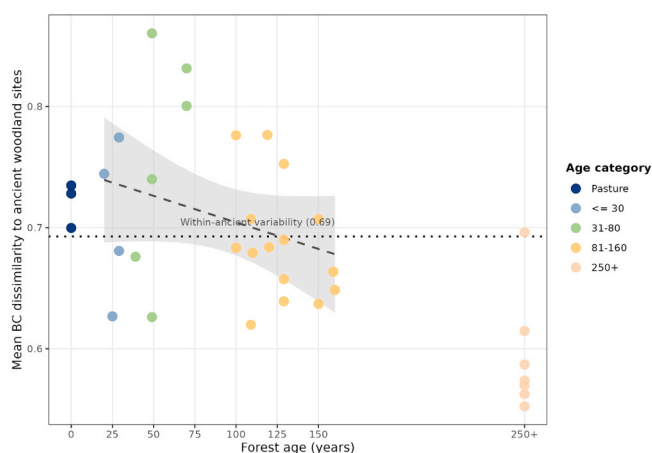


Fig. 9. Convergence of fungal community composition toward ancient woodland. Each point shows the mean Bray-Curtis dissimilarity from a site to all seven ancient woodland sites, plotted against forest age. Dashed line shows linear regression fitted to planted forests only (≤ 160 years; $R^2 = 0.09$, $p = 0.145$); grey shading shows 95% confidence interval. Dotted horizontal line indicates mean pairwise Bray-Curtis dissimilarity within ancient woodland sites (0.693), used as the convergence threshold. Ancient woodland sites are plotted at 250+ years for visualisation. Fungal communities only; bacterial convergence analysis not conducted due to named-taxon fraction limitations.

(i) EcM fungi show a significant directional increase across all woodland

age categories; (ii) SOC is the dominant correlate of both fungal and bacterial community composition, substantially outweighing above-ground management factors; and (iii) mature planted forests (81–160 years) remain compositionally distinct from ancient woodlands.

4.1. Progressive EcM accumulation

The increase in EcM relative abundance from 8% in pastures to 36% in ancient woodlands represents a biologically meaningful community shift across the chronosequence. The significant monotonic trend across age categories is consistent with progressive EcM community development following woodland establishment: as this is a space-for-time chronosequence rather than a longitudinal study (Damgaard, 2019), this temporal trajectory is inferred from sites of different ages rather than directly observed. However, the high within-category variance — particularly in the 31–80 year category, where EcM abundance ranged from 5% to 42% across six sites — indicates that local conditions modulate the pace of EcM accumulation substantially. Site-level factors likely include soil chemistry trajectory, tree species composition, landscape connectivity for EcM spore dispersal, and the agricultural legacy of the specific site (Peay et al., 2013; Tedersoo et al., 2020). Notably, the AM decline appears more abrupt than the EcM accumulation: AM abundance dropped sharply from 1.9% in pastures to around 0.4% in all woodland categories regardless of age, while EcM continued to accumulate progressively across the full chronosequence. This asymmetry suggests that the loss of AM-dominated communities occurs rapidly following tree establishment, while the accumulation of EcM communities characteristic of older woodland is a slower, centennial-scale

process. These contrasting timescales indicate that different fungal guilds assemble at markedly different rates following woodland establishment.

The 31–80-year category also showed the lowest mean fungal Shannon diversity ($H' = 3.03$), coinciding with the period of canopy closure and the early phases of EcM community establishment. This dip in diversity has been observed in other chronosequence studies of forest development and may reflect a temporary environmental filtering or shifts in resource availability during forest development rather than a demonstrated mechanism of competitive exclusion (Fukami, 2015; Hiscox et al., 2015).

4.2. Soil chemistry as the dominant correlate of community recovery

Soil organic carbon emerged as a significant predictor of both fungal and bacterial community composition from the predictors assessed in this study, suggesting it acts as an ecosystem-level integrator of land-use history. Rather than directly shaping microbial community states, SOC accumulates the net outcome of decades of changed litter inputs, reduced disturbance, and shifting pH, simultaneously reflecting resource availability, soil physical structure, and agricultural legacy. That the same variable structures communities across both kingdoms independently, confirmed by envfit and PERMANOVA, suggests that fungi and bacteria are responding to this shared edaphic gradient rather than to distinct predictors. SOC likely exerts its influence through multiple pathways: directly as a carbon resource for saprotrophic decomposers, and indirectly by influencing soil aggregate stability, moisture retention, and the progressive acidification that favours EcM over AM-associated plant communities (Bahram et al., 2018; Carrino-Kyker et al., 2016). Notably, when SOC is expressed as stocks (t C ha^{-1}) rather than concentration (g kg^{-1}), the gradient across age categories flattens considerably (pasture 28.9 ± 10.6 , ancient woodland $26.7 \pm 15.2 \text{ t C ha}^{-1}$). This reflects the substantially lower bulk density of woodland soils relative to pasture soils, which partially offsets the higher SOC concentration in older woodlands. This pattern highlights the importance of reporting both concentration and stock when comparing SOC across land-use types, as concentration alone may overestimate differences in ecosystem carbon storage capacity. Tree diversity showed only a weak, non-significant association with fungal community composition, variation partitioning revealed that this association was not independent of soil chemistry, the individual contribution of the vegetation & management group became non-significant and the vegetation and management group to which it belongs made no significant independent contribution once soil chemistry was accounted for in the variation partitioning. This suggests that tree diversity and soil chemistry track the same underlying gradient, consistent with the known influence of tree species composition on litter quality and SOC accumulation (Cools et al., 2014).

The interpretation of SOC in this chronosequence is complicated by the nature of the soil samples. In the older stands, carbon concentrations were high (mean 11.6% in the 31–80 category and 14.6% in the 250+ category) while bulk densities were low ($0.29\text{--}0.36 \text{ g cm}^{-3}$), the characteristic signature of decomposed organic (forest-floor) material admixed into the shallow mineral soil. This admixture likely explains both the high SOC concentrations and the flat, non-monotonic trend in SOC stocks across woodland age, since the low bulk density of organic-rich woodland soils offsets their higher carbon concentration. While the incorporation of decomposed organic material is appropriate for characterising the microbial communities these soils support, it complicates direct comparison of SOC stocks with chronosequence studies that separated forest floor from mineral soil, and we therefore interpret the SOC-stock gradient with caution. This may also explain why SOC concentration, which more directly reflects the carbon environment experienced by soil microorganisms, was a stronger correlate of community composition than SOC stock.

The finding that the same variable (SOC) is associated with both

fungal and bacterial community composition, and that management variables (grazing, CWD, ground cover) do not, has practical implications for restoration planning. It suggests that soil-focused interventions (organic matter addition, practices that promote SOC accumulation, and avoidance of soil disturbance) are more likely to accelerate microbial community recovery than management of aboveground vegetation structure. This aligns with growing evidence that soil legacy effects represent the primary bottleneck in ecosystem restoration (Jangid et al., 2011; Watson et al., 2022). It should be noted, however, that grazing was characterised here as a binary grazed/ungrazed variable (based on fencing and corroborating field signs of grazing), which is a relatively blunt proxy for grazing intensity or duration. This may have reduced statistical power to detect grazing effects on mycorrhizal communities, and future work incorporating direct measures of grazing pressure would strengthen conclusions about the role of herbivory in below-ground community assembly.

The significant heterogeneity in fungal community dispersion indicates that compositional variability itself changes across the chronosequence: communities in intermediate-aged forests are more variable among sites than those in pastures or ancient woodlands. This has implications for restoration monitoring: point-in-time comparisons between planted and ancient forests may be misleading if they do not account for the increased stochasticity of community assembly during the 30–80 year phase.

4.3. Bacterial functional groups and shared edaphic control

The identification of significant SOC and pH effects on bacterial community composition confirms that the soil chemistry gradient structuring fungal communities also structures bacterial communities. The association of both kingdoms with SOC is consistent with the view that SOC acts as an ecosystem-level integrator of land-use history, simultaneously influencing resource availability, soil physical properties, and pH, which in turn filter both kingdoms toward distinct community states (Bahram et al., 2018).

Among the identifiable bacterial functional groups, carbon mineralisation and denitrification were the most abundant. The methane group in this study comprised methanogenic archaea only. The elevated variance in methane cycling taxa within the 81–160 year category is ecologically plausible: methanogenesis requires anaerobic conditions, and variation in soil moisture and topographic wetness among sites would control the availability of the anaerobic microsites methanogens require. This phase also coincides with maximum SOC accumulation (mean SOC = 76 g kg^{-1}), which would sustain moisture-driven variation in anaerobic microsite availability.

4.4. Slow and uncertain convergence toward ancient woodland

The finding that mature planted forests (81–160 years) remain compositionally distinct from ancient woodlands is the clearest and most policy-relevant result of the convergence analysis. Even after more than a century of woodland development, fungal communities have not achieved the compositional variability range of ancient woodland sites.

The broader implications extend beyond belowground communities. For instance, parallel research at these sites found that larger and structurally complex woodlands provided greater benefits for woodland plants (Waddell et al., 2024). This illustrates that different ecosystem components may recover at different rates and respond to different drivers: aboveground vegetation and belowground microbial communities may follow partially independent trajectories, so there is unlikely to be a single trajectory of woodland recovery. Accordingly, integrated, multi-taxon assessments are needed to evaluate the full ecological value of created woodlands.

The high internal heterogeneity of ancient woodland communities (mean pairwise BC = 0.693, range 0.39–0.87) is itself ecologically important. It indicates that ancient woodland does not represent a single

compositional endpoint but rather a diverse compositional space shaped by centuries of site-specific history, microhabitat differentiation, and stochastic colonisation events. The implication is that convergence toward ancient woodland is not convergence toward a fixed target, but toward a range of community states

The non-monotonic pattern in dissimilarity, with the 31–80 year category showing higher mean BC to ancient woodland than both earlier and later categories, is consistent with the diversity dip observed in the same age class and with increased compositional stochasticity during canopy closure. This phase of high variability and high dissimilarity deserves further attention as a potential bottleneck in forest restoration.

5. Conclusion

Fungal and bacterial community composition following woodland creation on former agricultural land is associated primarily with soil organic carbon legacies rather than with current aboveground management practices. EcM fungi accumulate progressively with forest age, with a significant directional increase across all age categories, though high site-to-site variance indicates that local conditions modulate recovery pace substantially. Mature planted forests (81–160 years) remain compositionally distinct from ancient woodlands. These findings underscore the irreplaceable conservation value of ancient woodland, the importance of soil quality as the strongest correlate of fungal restoration success, and the need for soil-focused management interventions and long-term monitoring to track compositional change under changing environmental conditions.

CRediT authorship contribution statement

Olivia Azevedo: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Vanguelova:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition. **Sietse van der Linde:** Writing – review & editing, Validation, Supervision, Conceptualization. **Frank Ashwood:** Writing – review & editing, Supervision, Resources. **Elisa Fuentes-Montemayor:** Writing – review & editing, Supervision, Resources. **Clare Wilson:** Writing – review & editing, Supervision, Resources. **Kevin Watts:** Writing – review & editing, Supervision, Resources. **Kirsty J. Park:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.foreco.2026.124228](https://doi.org/10.1016/j.foreco.2026.124228).

Data availability

Data will be made available on request.

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