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Vegetation and edaphic factors influence rapid establishment of distinct fungal communities on former coal-spoil sites

Andrew Paul Detheridge, David Comont [1], Tony Martin Callaghan, Jennifer Bussell [2], Graham Brand, Dylan Gwynn-Jones, John Scullion, Gareth Wyn Griffith*

Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Adeilad Cledwyn, Penglais, Aberystwyth, Ceredigion SY23 3DD Wales, United Kingdom

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ABSTRACT

We investigated re-establishment of fungal communities on eight former colliery sites in South Wales following revegetation 22–27 y earlier. Regraded bare coal-spoil was seeded to sheep-grazed grasslands, with saplings planted into coal-spoil for woodlands. Metabarcoding (28S rRNA, D1 region) of soil fungal populations showed that woodland and grassland habitats were clearly divergent but edaphic variables only weakly affected fungal community structure. Root-associated basidiomycetes dominated all habitats, with ectomycorrhizal fungi more abundant in woodlands and Clavariaceae/Hygrophoraceae ('CHEG' fungi) in grasslands. The composition of coal-spoil grassland communities resembled that of a typical upland grassland site, suggesting that propagule immigration was not a limiting factor. However, fungal biomass (ergosterol) was 3-fold lower, reflecting high bulk density and poor structure. Re-establishment of fungal communities in coal-spoil soils represents an important barometer of restoration success. From a fungal conservation perspective, such sites represent important refugia for waxcap fungi subject to habitat loss from agricultural intensification.

1. Introduction

Fungi play a central role in soil as decomposers of organic matter, plant mutualists and pathogens; hence they have a significant impact on plant growth and carbon sequestration (Paul, 2014). The effect on plant growth of mycorrhizal fungi depends on the nutrient status of soils (Jonsson et al., 2001) and their impact can range along a continuum from parasitism to mutualism (Jones and Smith, 2004) although evidence indicates a positive interaction in grasslands (Van Der Heijden et al., 2006). Saprotrophic fungi are important for the mineralisation of organic matter, the nutrients released being made available via mycorrhizal fungi to plants (Lindahl et al., 2002). Therefore, if fungi from the saprotrophic and symbiotrophic guilds do not establish well on newly vegetated land, plant growth will be impacted, affecting litter decomposition and carbon sequestration in soil (Clemmensen et al., 2015). The extent to which fungal populations establish in new habitats and the structure of these populations are, therefore, important questions when considering successional systems or restoration of habitats.

Coal-spoils represent a legacy from the industrial revolution in the UK and during the 1980s several projects looked at regeneration of their soil and habitats. Spoils typically have very limited organic matter and are depauperate of microbial populations, very different from natural communities. For example, Elhottova et al. (2006) found fungi to be present in the lacustrine clays of a post-mining site, primarily microfungi such as *Penicillium* and *Aspergillus*, but these assemblages were very different from soil within semi-natural woodland or grassland. Pedogenesis can occur *de novo* on coal-spoils in a manner not dissimilar to that in post-glacial or post-volcanic areas. In this respect, the restoration may be similar to a primary successional system for soil biota and so provide an indication of how fungal communities develop in new habitats.

Factors driving microbial community development are poorly understood. Soil pH has been shown to correlate with bacterial composition but the correlation with fungal community

* Corresponding author.
E-mail address: gwg@aber.ac.uk (G.W. Griffith).
[1] Current address: Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom.
[2] School of Biosciences, University of Nottingham, Leicestershire, LE12 5RD, United Kingdom.

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composition appears much weaker (Lauber et al., 2008; Rousk et al., 2010). A factor important in the development of soil communities on former coal-spoil is soil structure, since the heterogeneous structure of well-developed soil provides a variety of niches for soil fungi and other organisms (Mummey et al., 2002). Formation of soil starts with the weathering of the parent material (Frouz et al., 2011) followed by biological action such as earthworm activity, root exudation and fungal secretions (Bronick and Lal, 2005). Soil organic carbon (SOC) content has been used as a predictor of microbial biomass (Fierer et al., 2009), but little is known of the influence of these factors on fungal community structure.

Plant community composition impacts fungal community composition via the quantity and quality of the organic inputs, which affects the saprotrophic community structure; and also through the types of root symbioses that are formed (Read and Perez-Moreno, 2003). In the northern hemisphere, succession to woodland leads to the establishment of large populations of ectomycorrhizal fungi (EcM) (Tedersoo et al., 2010), with a consequent decrease in both the abundance and species richness of arbuscular mycorrhizal fungi (AMF) (Johnson et al., 1991b).

A diverse group of macrofungi, known as 'CHEG' fungi (Clavariaceae, Hygrophoraceae, Entolomataceae and Geoglossaceae) are abundant in undisturbed nutrient-poor grasslands (Griffith et al., 2013). Although their ecology is not well understood, many lines of evidence now suggest that they are biotrophic and probably mycorrhizal (Griffith et al., 2002; Seitzman et al., 2011; Halbwachs et al., 2013). Restored grasslands at coal-spoil sites represent potentially suitable habitats for these fungi, since no fertilisers are applied beyond the initial establishment phase and short swards are maintained by sheep grazing.

Here fungal populations are considered at coal-spoil tips in South Wales that were restored to grassland and woodland 25 y previously. The restoration of disturbed land to a self-sustaining grassland or woodland, requires an effective soil fungal community (Sun et al., 2017). Restorations of the spoil tips were undertaken by direct seeding of grass and clover or planting of tree saplings directly into regraded coal shale without any addition of soil. The coal-spoil had previously been 'stored' in large dumps for several decades and contained very little organic matter with limited populations of active microbes (Johnson et al., 1991a). The coal-spoil was re-profiled before restoration so any vegetation and below ground communities that had established naturally would also have been buried.

Metabarcoding of DNA extracted from soil was used to determine the composition of fungal communities hypothesising that vegetation cover (grassland vs. woodland) would be a key determinant of the structure of their communities. It was also hypothesised that the structure of communities of root-associated fungi (e.g. mycorrhizas) would be more affected by vegetation, than those of saprotrophic fungi. The latter would be expected to be influenced more by soil parameters. We also sought to determine whether fungal communities in restored soils would differ from those in more natural undisturbed soils, due to the early stage of soil development, and the resultant differences in the physicochemical characteristics of the soil.

2. Materials and methods

2.1. Study sites and sampling

The eight sites were chosen based on the presence of both woodland and grassland habitats established during restoration. Grassland areas had originally been seeded with *Lolium perenne*, *Festuca* spp. and clovers (*Trifolium* spp.), and had received some initial inorganic fertiliser, although the amounts are unknown. Commonly planted trees in woodland areas included *Alnus glutinosa, Betula pubescens, Corylus avellana, Salix* spp., *Populus* spp., *Larix decidua* and *Sorbus aucuparia*, with some climax species such as *Quercus petraea* (Supplementary data S14). At one site (Cwm Daren) a pure stand of *L. decidua* was also present and this was included as a separate sample to provide a comparison with the mixed woodland at the same site. One younger site (Deep Navigation; 12 y since restoration) was included in our study; woodland and grassland vegetation was also not noticeably less mature than at other sites (Supplementary data S1).

All soil sampling was undertaken in October 2013. At each site and land use combination, five 3×3 m quadrats were randomly located across the area. Nine cores were taken from each quadrat using a 9 mm diameter soil auger to a depth of 10 cm, with all cores pooled into one sample per quadrat (ca. 150 g/sample; total of 80 samples). In woodland quadrats, the uppermost organic (O) layer was removed prior to soil coring. Edge effects from undisturbed land were avoided by sampling at least 20 m from any boundaries. Samples were stored cold and frozen at −80 °C within 12 h of sampling. Ten intact soil cores (5.6 cm diameter, 6 cm deep) were taken from each site 1 cm below the surface of the soil for bulk density measurement.

Cores were also taken (October 2013) from a semi-natural, grazed grassland (Brignant Longterm Experiment; lat/long: 52.3652, −3.8297; 367 m asl.) on Manod soil series. The Brignant experiment was established in 1994 to monitor the effects of contrasting management (haycutting/grazing/liming) on floristic diversity but is used here since its climatic conditions, altitude and soil type are very similar to those in upland grasslands found in areas adjacent to the coal-spoil sites. The Brignant experiment comprises seven triplicated treatments. Fertiliser (60 kg N. ha^{-1} and 30 kg P. ha^{-1}, applied in May) was added on only one treatment to mimic standard management of upland sheep-grazed meadows. Three summer-grazing treatments across three replicates were selected as a comparison for the restored sites and these were sampled at a similar coring density to the coal-spoil grasslands. Cores were combined for each replicate treatment, nine samples in total. No suitable reference woodland of equivalent age and composition could be found for comparison with woodland areas, since remnant woodland around the spoil tips were old (>100 y) oak woodland or mature forested monocultures.

2.2. Soil processing and measurements

Samples for DNA analyses were frozen at −80 °C and freeze-dried. After freeze-drying, soil was passed first through a 4 mm sieve, the stones removed and weighed, and then through a 500 μm sieve. The sieved soil was thoroughly mixed. DNA was extracted from 200 mg of the dried soil sample using PowerSoil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad USA).

Soil organic matter (OM) content was measured through the loss on ignition method (LOI) (Ball, 1964). The restored soils contained coal particles that would inflate the organic matter content if ignited. To determine the ignition point of the coals from the restored sites, particles of coal were ground and ignited at a range of temperatures from 300 to 400 °C for 18 h and then weighed to determine at what temperature weight loss occurred. The highest temperature without weight loss was 320 °C so this was chosen as the temperature for ignition. Freeze-dried soil (ca. 2 g) was ignited for 16 h and the weight loss was recorded. Igniting at this temperature however does not burn all the plant derived OM. Igniting natural grassland soils at 320–400 °C indicated that on average 90% of weight is lost at 320 °C when soils were burnt overnight, so this was used as the conversion factor to determine actual OM content.

Total soil nitrogen and carbon were measured (Vario MACRO

Cube Elementar), using approximately 200 mg of sieved freeze dried soil in duplicates. Organic matter content of the restored soils from LOI was converted to carbon content using a function derived from total carbon and OM data from the reference grassland site. Soil pH was measured in a 5:1 water to soil ratio. 25 ml of distilled water was added to 5 g of freeze dried soil in a 50 ml tube. Tubes were shaken for 1 h in a flatbed shaker and left to stand for 30 min before pH was measured.

For bulk density, the soil from the intact cores was oven dried and weighed and passed through a 2 mm sieve. Stones retained by the sieve were weighed and the volume measured. The weight and volume of the stones was subtracted from the total soil weight and volume and the bulk density of the <2 mm fraction calculated.

2.3. DNA amplification and sequencing

A 200 bp portion of the D1 region of the 28S (large) subunit of the ribosomal DNA operon (rDNA LSU) was amplified and sequenced using the fungal-specific primers D1F2 and NLC2-AF, as detailed in Detheridge et al. (2016). High throughput sequencing with an Ion Torrent PGM and after quality control processes, clustering of OTUs at 97% identity, a taxonomy was assigned to each OTU using a reference database of curated fungal LSU sequences (Ribosomal Database Project; v11), as described by Detheridge et al. (2016). Sequences were rarefied to the lowest number of sequences obtained per sample (23296) across the whole dataset prior to further analyses. The barcodes used and the number of sequences per sample is shown in Supplementary data S2A.

2.4. Sequence data processing

The resulting sequence file was processed as detailed in Detheridge et al. (2016). No evidence was seen of a significant decline in Phred scores over the length of the sequence after quality checking (Supplementary data S2B). An example of the raw output table, showing headline data for samples and the distributions of the most abundant taxa is shown in Supplementary data S3.

Broad ecological function of the fungi was assigned to each taxon (where identified) at genus or family level based on searches of academic literature (Hibbett and Donoghue, 2001; Tedersoo et al., 2010; Mandyam and Jumpponen, 2015; Detheridge et al., 2016). A similar approach to that of Tedersoo et al. (2014) was followed. If different ecological functions were identified within a taxon, a function would only be assigned if > 75% of known species within that taxon could be assigned to a single function. Otherwise the function remained undetermined, approximately 10% of genera in our database had no allocated function. Five main groupings were used here: CHEG fungi (associated with grasslands), EcM (plant mutualists more associated with woody species), AM fungi (plant mutualists associated with most types of plant species), DSE (dark septate endophytes) and saprotrophic fungi (SAP). DSE are ascomycete endophytic fungi with dark melanised septa (mostly in the order Helotiales). The exact function of this group is still to be determined. They may provide a similar function to EcM fungi more prevalent in less fertile ecosystems (Wilberforce et al., 2003; Mandyam and Jumpponen, 2015). More recent research suggests that the plant response to DSE colonisation is not always positive and that the relationship is on a mutualism-parasitism spectrum (Mandyam and Jumpponen, 2015). SAP (saprotrophic fungi) are those soil fungi not associated with plant roots that decompose organic matter. Sequence data have been submitted to the European Nucleotide Archive with **reference number PRJEB9609**.

2.5. Ergosterol determination

Freeze dried soil (2 g) was incubated overnight (shaking incubator; 25 °C) with 3 ml 99% methanol (HPLC grade) containing 5% (w/v) KOH to extract and saponify sterols. Tubes were vortexed, incubated at 80 °C for 30 min, then cooled. Sample volume was made up to 2 ml before partitioning three times in 2 ml petroleum ether. Pooled ether partitions were reduced to dryness under a stream of nitrogen gas. Saponified sterols resuspended in 1 ml methanol and filtered through 0.2 μm PVDF syringe filters. HPLC analysis was used to quantify ergosterol (LDC Analytical constaMetric® 3200/spectroMonitor® 3200 system with a Spherisorb PhaseSep HPLC cartridge column 5 μm ODS2 running an isocratic mobile phase of HPLC-grade methanol [1.5 mL/min flow rate] at 25 °C). Samples were injected via 200 μL loop injection. 7-Dehydrocholesterol (7-DHC) was used as an internal standard. The ergosterol retention time ranged from 10.5 to 11.5 min with absorbance measured at 282 nm. Ergosterol concentration was derived by comparison of peak height against a standard curve, and corrected for extraction efficiency against the relative peak height of the 7-DHC standard. A ratio of 5 μg per mg of dry fungal biomass (1:200) was used, as a general ratio from environmental samples as recommended by several published studies (Ruzicka et al., 2000; Newell, 2001). Data for relative abundance of fungi were converted to biomass using the ergosterol content and this conversion factor.

2.6. Data analysis

Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in community data. Of the 40 grassland and 45 woodland samples one of each had very low sequence counts and were excluded from the analyses. Abundance percentage data were subjected to square root transformation (to reduce the effect of dominant taxa) and Bray-Curtis distance matrices calculated. PERMANOVA was carried out using default settings with 9999 unrestricted permutations and the Monte Carlo P value was calculated. To calculate the contribution of environmental data on fungal communities, distance based redundancy analysis (dbRDA) was used (Legendre and Anderson, 1999), predictor variables were normalised before analysis. Although all sampling was carried out within 3 d, it was not possible to correct for rainfall variations at sampling. Analysis of similarity (ANOSIM) was used to indicate the degree of divergence in communities at site level and similarity percentages (SIMPER) was used to indicate which taxa were contributing most to the differences between soil types. Principal coordinate analysis (PCO) was used as the unconstrained ordination technique against the Bray-Curtis distance matrix. The above analyses were performed in PRIMER 6 & PERMANOVA+ (versions 6.1.12 and 1.0.2 respectively; Primer-E, Ivybridge, UK), analyses of variance (ANOVA) and correlations were performed in SigmaPlot v 12.5. Before ANOVA, normality was tested using Shapiro-Wilk and equal variance using Levene's test. *Post hoc* differences were tested using Fisher's least significant difference (LSD).

3. Results

The eight former colliery spoil sites (Table 1) were selected based on similarities in restoration methods and occurrence of contrasting vegetation types (woodland and grassland) at each site. Restoration of these sites involved re-profiling of coal-spoil, followed by 'ripping' to alleviate compaction and direct planting of tree sapling or grassland seed mixes into the coal-spoil. At the time of soil sampling (October 2013), all sites contained trees exceeding 5 m in height and 10 cm diameter at breast height (DBH) with this

Table 1
Details of the restored coal-spoil sites in south Wales sampled in October 2012.

Site Name	Age	Altitude (m)	Lat.	Long.	Area(ha)	% Woodland
Bargoed	22	210	51.683	−3.222	19.1	60
Bryn Bach	23	375	51.783	−3.268	62.7	40
Cambrian	25	280	51.623	−3.472	83.4	20
Craig y Dyffryn	22	186	51.691	−3.396	21.3	65
Cwm Daren	25	363	51.719	−3.275	60.4	75
Deep Navigation	12	267	51.668	−3.3	28	30
Gelliwion	27	124	51.595	−3.353	14.7	90
Windsor	23	162	51.598	−3.275	12	20

proportion increasing with age (data not shown; Supplementary data S1; S14).

A total of 85 pooled soil core samples were analysed (five replicates per site and land use combination; including samples from the two woodland types at Cwm Daren) and following DNA metabarcoding (D1 region of the 28S rRNA locus), the resulting sequences were assigned to taxa using the RDP database (Cole et al., 2014). The mean number of sequences per sample was 51831 (range 23296-97700); rarefaction curves are shown in Supplementary data S4. It is clear from these curves that at the level of rarefaction used here most samples had not reached a plateau in terms of OTU richness, therefore measures of taxon diversity must be treated with caution. After rarefaction, clustering (excluding clusters containing <5 sequences) and classification, the mean number of sequences per sample was 17469. Some non-fungal sequences (mean 1455 per sample) were obtained and excluded from analyses. Of the fungal sequences, 87% were identified to family and 75% to genus, with Basidiomycota being the dominant phylum (mean 62%; range 12–94%), followed by Ascomycota (mean 29%; range 1–76%; Fig. 1; Supplementary data S3).

3.1. Comparison of fungal populations in grassland and woodland habitats on former coal-spoil sites

Vegetation cover clearly dictates fungal community composition in restored coal-spoils. Unconstrained PCO ordination of fungal communities (Fig. 2A; Table 2), showed a clear separation between grassland and woodland. The PERMANOVA main test was significant (PERMANOVA $F = 14.625$, $p = 0.001$, $p(MC) = 0.001$). A significantly higher proportion of sequences were identified as Basidiomycota (83%) in woodland compared to grassland soils (60%) ($F = 39.407$, $P < 0.001$), whilst for Ascomycota the opposite was found (15% vs 32%; $F = 33.990$, $P < 0.001$). The Shannon and Simpson diversity indices showed that fungal diversity was significantly higher in coal-spoil grassland than woodland (Table 2) and was also apparent in rarefaction curves (Supplementary data S4). Fungal biomass (ergosterol) was two-fold greater in woodland soils ($F = 17.339$, $P < 0.001$), with ergosterol levels (mean 6.55 vs 3.53 $\mu g\, g^{-1}$ dw) corresponding to ca. 1.31 and 0.71 mg fungal biomass $dw.g^{-1}$ soil, using x200 conversion factor (Scheu and Parkinson, 1994; Djajakirana et al., 1996; Newell, 2001).

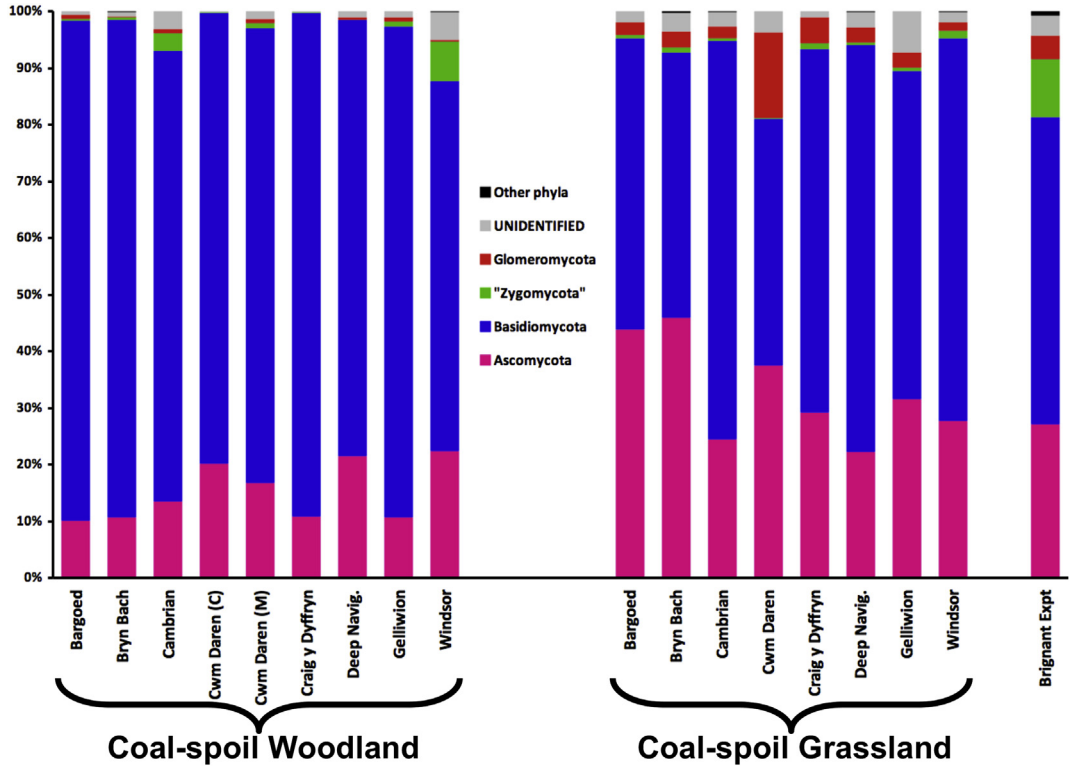

Fig. 1. Stacked barchart showing relative abundance of the different fungal phyla at the different sites (mean of 5 samples). The 'Other phyla' category includes Blastocladiomycota, Chytridiomycota, Entomophthoromycota and Neocallimastigomycota, together comprising 0.81% or less of the total sequences. For the Cwm Daren site data for the coniferous (C) and mixed (M) woodlands are shown separately.

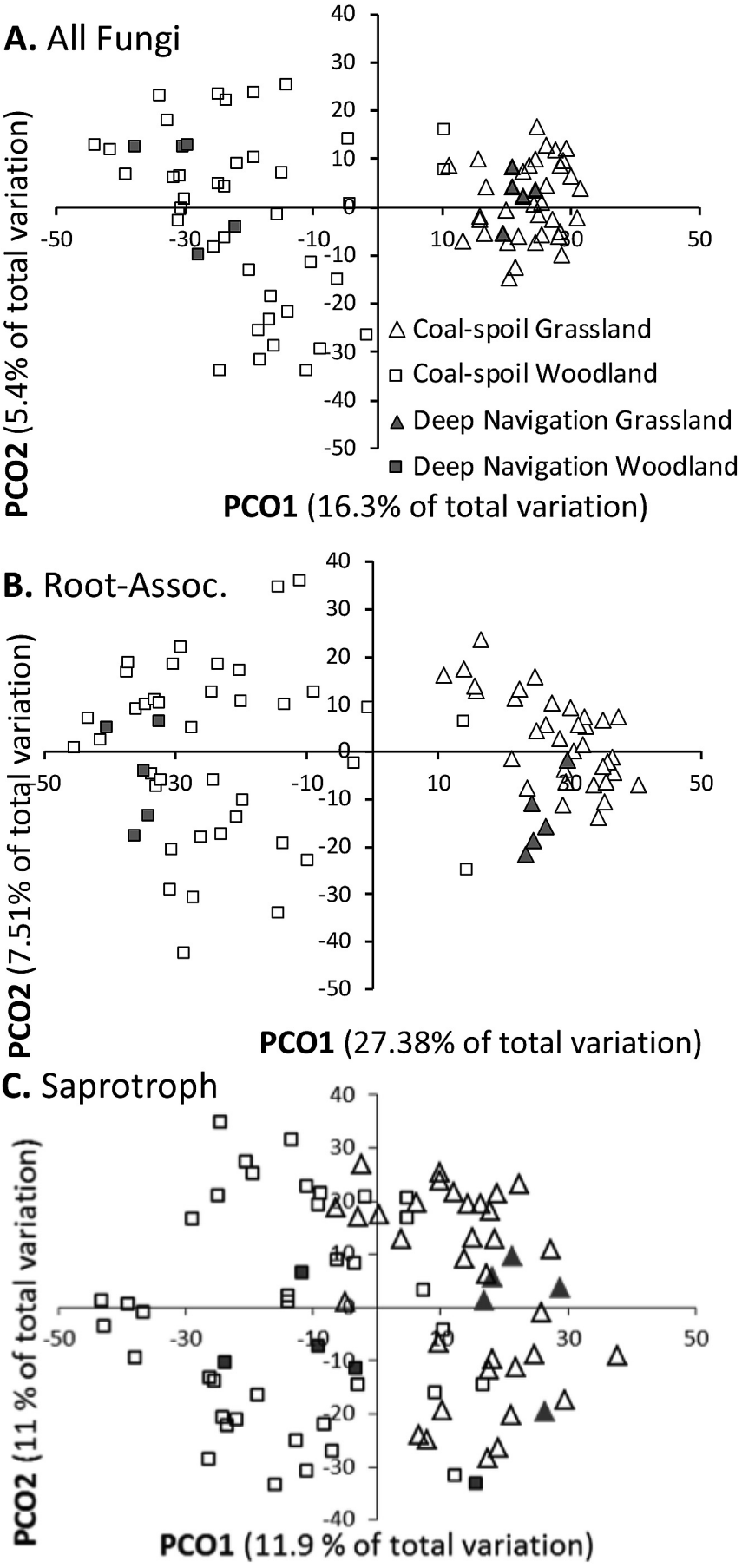

Fig. 2. Unconstrained Principal coordinate ordination using Bray-Curtis dissimilarity matrix on square root transformed abundance data, of coal-spoil woodland and grassland fungal communities. (A) All fungi; (B) Root-associated fungal groups only; (C) Saprotrophs only. The amount of variation explained by the two primary axes is much greater for the root-associated taxa. Grey shaded crosses and diamonds indicate the woodland and grassland quadrats respectively from the younger (12 y old) Deep Navigation site.

Table 2
Results of ANOVA and PERMANOVA analyses on fungal community data comparing coal-spoil grasslands to coal-spoil woodland, and the coals-spoil and Brignant grasslands (*** P < 0.001, ** P = 0.001–0.01, *P = 0.01–0.05). Figures in brackets after means indicate standard deviation.

Test	Means			P Values	
	Coal-spoil Woodland	Coal-spoil Grassland	Brignant Grassland	Coal-spoil Woodland v Grassland	Coal-spoil vs Brignant Grassland
PERMANOVA	n/a	n/a	n/a	P(MC) 0.001	P(MC) 0.001
CHEG Relative abundance %	4.9(11.1)	46.4(24.1)	56.2(21.0)	<0.001(***)	0.122(NS)
SAP Relative abundance %	6.6(9.6)	10.8(11.3)	7.1(7.3)	0.065(NS)	0.219(NS)
AM Relative abundance %	0.4(0.6)	4.2(6.1)	2.9(2.2)	<0.001(***)	0.342(NS)
EM Relative abundance %	54.8(20.3)	4.3(9.3)	0.3(0.3)	<0.001(***)	0.053(NS)
DSE Relative abundance %	5.8(4.8)	10.0(6.3)	7.0(3.9)	<0.001(***)	0.054(NS)
Ergosterol μg g^{-1} soil	6.54(4.79)	3.53(2.74)	11.97(5.11)	0.001(**)	<0.001(***)
CHEG μg biomass g^{-1} soil	47(101)	361(404)	1997(1234)	<0.001(***)	<0.001(***)
SAP μg biomass g^{-1} soil	78(109)	71(83)	194(183)	0.844(NS)	<0.001(***)
AM μg biomass g^{-1} soil	4(7)	31(45)	85(55)	<0.001(***)	<0.001(***)
EM μg biomass g^{-1} soil	680(580)	28(65)	7(6)	<0.001(***)	0.175(NS)
DSE μg biomass g^{-1} soil	78(120)	65(64)	246(222)	0.580(NS)	<0.001(***)
pH	5.10(0.68)	5.23(0.53)	5.28(0.23)	0.346(NS)	0.687(NS)
%N	0.39(0.13)	0.40(0.09)	0.64(0.06)	0.865(NS)	<0.001(***)
%OM	10.0(3.3)	9.4(3.5)	14.6(1.3)	0.363(NS)	<0.001(***)
C:N	12.91(2.62)	11.71(3.13)	11.22(0.52)	0.049(*)	0.495(NS)
BD g cm^{-3}	1.28(0.25)	1.19(0.26)	0.87(0.03)	0.138(NS)	<0.001(***)
Inverse Simpson	8.08(3.84)	11.95(9.32)	15.44(4.97)	0.019(*)	0.159(NS)
Shannon	2.67(0.47)	3.23(0.70)	3.55(0.23)	<0.001(***)	0.062(NS)
OTU Count	144(40)	228(65)	237(32)	<0.001(***)	0.557(NS)

SIMPER analysis was used to identify taxa that contributed to the difference between woodland and grassland, with the top ten taxa identified contributing 16% to the total dissimilarity (Table 3). All of these were root-associated, seven EcM and three CHEG. Total relative abundances of these two functional groups indicate a clear divergence of fungal communities between woodland and grassland soils. EcM fungi (e.g. *Hebeloma, Inocybe* and *Lactarius* spp.) were more abundant in woodland (F = 202.588, P < 0.001; 54.8% vs. 4.3% respectively) whilst CHEG taxa (notably *Hygrocybe* and *Cuphophyllus* spp.) showed the opposite distribution (F = 105.736, P < 0.001; mean 46.4% vs. 5.0% respectively). In addition, Glomeromycota (AMF e.g. *Glomus* and *Rhizophagus*) were less abundant in woodland (F = 17.260, P < 0.001; 0.4% vs. 4.2% respectively).

Pairwise ANOSIM analysis of woodland sites (Supplementary data S5) indicated that the *L. decidua* woodland (Cwm Daren) was most divergent from other woodland sites. Further analysis showed that was due to the dominance (>30%) in several (3/5) of these quadrats of an unidentified OTU (family Atheliaceae; nearest named BLAST match [97%] *Piloderma lanatum* [JQ711873], EcM from *Pinus contorta*, Canada (Jones et al., 2012)). This OTU was also found in mixed woodland at Craig y Dyffryn where *Larix* was also abundant.

The C:N ratio was significantly higher in woodland compared to grassland soils (ANOVA F = 3.975; P = 0.049; 12.91 vs 11.71 respectively) but there were no significant differences in pH, total nitrogen %, organic matter content and bulk density. Correspondence analysis (dbRDA) was undertaken to determine whether changes in fungal communities were associated with any of the edaphic factors measured but did not separate woodland and grassland soils (Supplementary data S6). In contrast, variation partitioning analysis, as used by Krüger et al. (2017), did reveal a contribution by edaphic factors (3% of explained variance) to fungal community composition (Supplementary data S7). However, the effect of vegetation was much greater (12% of explained variance).

To explore the possibility that the host specificity of root-associated fungi was responsible for the differences in fungal community between woodland and grassland sites, ecological functions were ascribed to the taxa identified from the RDP database, as described below, and in Detheridge et al. (2016). The data were re-analysed focusing only on taxa known to be root-associated (i.e. EcM, CHEG, DSE, AM; together comprising a mean of 56% of all sequences across all samples) or saprotrophic (together comprising a mean of 7.2% across all samples). Following this splitting of the data, woodland and grassland communities were still significantly different in the PERMANOVA analysis (Pseudo-F = 5.5363 P(Perm) < 0.001 P(MC) < 0.001 saprotrophic, Pseudo-F = 25.019 P(Perm) < 0.001 P(MC) < 0.001 root-associated) and PCO ordination showed clear separation (Fig. 2B and C), although the degree of separation was reduced (ANOSIM R = 0.243 vs R = 0.789 for root-associated taxa only [R = 0.77 for all taxa]).

According to SIMPER analysis, the main genera of saprotrophic fungi contributing to the differences between grassland and woodland were *Saitozyma, Mycena*, *Penicillium* and *Trechispora* (Supplementary data S8A). Since dbRDA ordination also suggested

Table 3
Results of SIMPER analysis, identifying the top 10 taxa contributing to the difference between coal-spoil woodland and coal-spoil grassland sites.

Genus	Ecology	Coal-Spoil Grassland Ave. Ab.	Coal-spoil Woodland Ave. Ab.	Contribution to Difference	Cumulative Difference
Hygrocybe (*conica* group)	CHEG	12.25%	0.25%	3.03%	3.03%
Hebeloma	EM	0.01%	4.84%	1.96%	4.99%
Inocybe	EM	0.16%	4.84%	1.86%	6.86%
Clavaria (*acuta* group)	CHEG	4.41%	0.16%	1.64%	8.5%
Lactarius	EM	0.04%	2.89%	1.49%	9.99%
Russula	EM	0.00%	2.25%	1.30%	11.30%
uncultured_Thelephoraceae	EM	0.16%	2.56%	1.28%	12.57%
Hyaloscypha	EM	0.09%	2.25%	1.22%	13.79%
Cortinarius	EM	0.01%	1.44%	1.16%	14.96%
Cuphophyllus (*virgineus* group)	CHEG	0.81%	0.09%	1.06%	16.02%

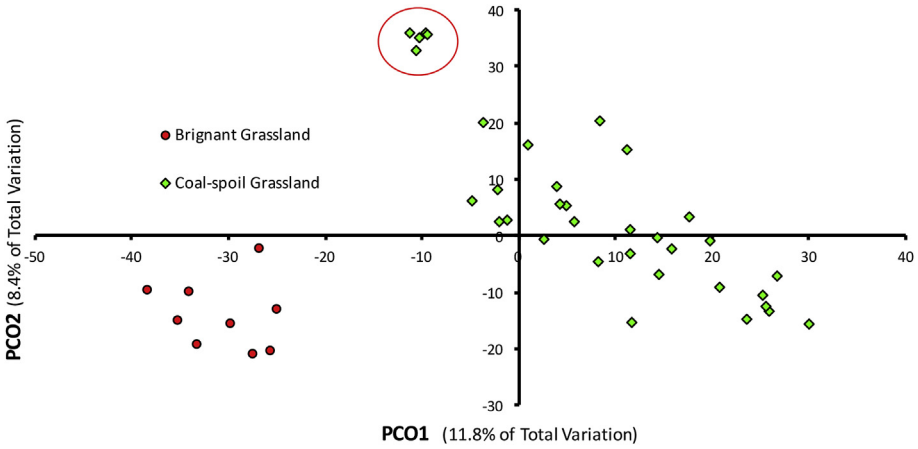

Fig. 3. Unconstrained Principal coordinate ordination using Bray-Curtis dissimilarity matrix on square root transformed abundance data, of coal-spoil grassland and Brignant grassland fungal communities. The red circle highlights the quadrats from the Bargoed site, notably wetter than other sites.

that differences between saprotrophic populations in woodland and grassland were not driven by the measured physicochemical parameters (Supplementary data S9), it is likely that saprotrophic taxa exhibit habitat differentiation due to differences in input plant litter quality.

3.2. Comparison of fungal populations in coal-spoil and Brignant grasslands

Fungal communities in restored grassland soils were compared with those found in more natural undisturbed soils. The eight coal-spoil grassland sites were compared to three triplicated sheep-grazed treatments (nine plots in total) from the Brignant long-term grassland experiment. The Brignant site has a very similar climate (and altitude) to the coal-spoil sites. The soil at Brignant (Manod - brown podzolic) is similar to those found in grasslands adjacent to the coal-spoil sites, and thus representative of the types of soil that were likely to ultimately develop in these spoils. PERMANOVA showed a significant separation between grassland fungal communities in coal-spoil grassland soils and those at Brignant ($F = 8.1486$, $P(Perm) = 0.001$, $P(MC) = 0.001$). The difference in the communities was also clear in PCO ordination plot (Fig. 3).

The relative abundance of sequences identified as Basidiomycota was similar in the Brignant and coal-spoil grasslands (64% vs. 61% respectively; $F = 0.572$ $P = 0.454$), as was the relative abundance of Ascomycota (28% vs. 32%; $F = 0.375$ $P = 0.55$). Analysis of relative sequence abundances of mycorrhizal (CHEG, EcM, AM and DSE) fungi and saprotrophic fungi as groups rather than individual taxa also showed no significant differences between the two grassland types. SIMPER analysis of the two grasslands (Table 4) showed that differences in the relative abundances of CHEG taxa were mainly responsible for the community difference between the two grassland types. *Hygrocybe conica* was the most abundant CHEG species in coal-spoil grassland, whereas other Hygrophoraceae (*Cuphophyllus* spp., *Gliophorus* spp. and *Hygrocybe acutoconica*) were more abundant at Brignant. However, no difference in the overall relative abundance of Hygrophoraceae was observed. Two genera of Clavariaceae also contributed strongly to the differences between these grassland types, with *Clavulinopsis* spp. more abundant at Brignant and *Clavaria* spp. more abundant on coal-spoil grasslands.

Pairwise ANOSIM analysis (Table 5) also showed that fungal populations at Brignant differed from those in coal-spoil grasslands. Of the latter, the fungal populations at the Bargoed site were the most divergent (Fig. 3), mainly due to the presence of three ascomycete OTUs (unidentified Pseudeurotiaceae [OTU4], unidentified Chaetothyriales [OTU5] and unidentified Leotiomycetes [OTU39]) not found elsewhere. This site was the wettest of all the grasslands sampled (36–40% moisture content), with high abundance of *Juncus* spp. in all quadrats. *Entorrhiza casparyana* (phylum Entorrhizomycota), which forms galls on *Juncus* roots (Bauer et al., 2015), was found here, and also in lower quantities at other sites where some *Juncus* was present.

Re-analysis of the data with PCO including only saprotrophic fungi showed a slightly clearer separation between the coal-spoil and the Brignant fungal communities (Supplementary data S10), as also seen from the ANOSIM R statistic ($R = 0.297$ for saprotrophic taxa vs. $R = 0.296$ for all taxa combined). Comparison of root-associated taxa in the two types of grassland (Supplementary data 8B), where plant species composition was similar and dominated by *Agrostis* and *Festuca* spp., revealed a lower degree of

Table 4
SIMPER results for the top 10 taxa contributing to the difference between the Brignant and coal-spoil grasslands.

Genus	Ecology	Brignant Grassland Ave. Ab.	Coal-spoil Grassland Ave. Ab.	Contribution to Difference	Cumulative Difference
Hygrocybe (*conica* group)	CHEG	4.41%	12.25%	2.25%	2.25%
Cuphophyllus (*virgineus* group)	CHEG	2.89%	0.81%	1.72%	3.97%
Clavulinopsis	CHEG	3.61%	0.04%	1.47%	5.45%
Clavaria (*acuta* group)	CHEG	1.44%	4.41%	1.26%	6.71%
Glutinoglossum	CHEG	2.56%	0.64%	1.11%	7.82%
Glomus	AM	3.24%	1.44%	1.06%	8.87%
Gliophorus	CHEG	1.44%	0.16%	1.03%	9.90%
Hygrocybe (*acutoconica*)	CHEG	1.00%	0.09%	0.99%	10.89%
Cuphophyllus (*pratensis* group)	CHEG	1.44%	0.01%	0.99%	11.88%
Trechisporaceae OTU 18	SAP	1.44%	0.25%	0.97%	12.86%

Table 5
ANOSIM results for coal-spoil and Brignant grasslands, showing the R statistic from ANOSIM indicating the degree of divergence. Significant results at a cut off of P = 0.05 are in bold, indicating those sites that are different. The most divergent site is Brignant.

	Brignant	Bargoed	Bryn Bach	Cambrian	Cwm Daren	Craig y Dyffryn	Deep Navigat.	Gelliwion
Bargoed	**0.951**							
Bryn Bach	**0.889**	**0.788**						
Cambrian	**0.957**	**0.956**	**0.464**					
Cwm Daren	**0.908**	**0.984**	**0.532**	**0.756**				
Craig y Dyffryn	**0.966**	**1.000**	**0.550**	**0.850**	**0.875**			
Deep Nav.	**0.966**	**0.968**	**0.576**	**0.584**	**0.684**	**0.300**		
Gelliwion	**0.786**	**0.660**	**0.320**	**0.400**	**0.344**	**0.363**	**0.280**	
Windsor	**0.736**	**0.640**	**0.404**	**0.548**	**0.456**	**0.350**	**0.348**	*0.164*

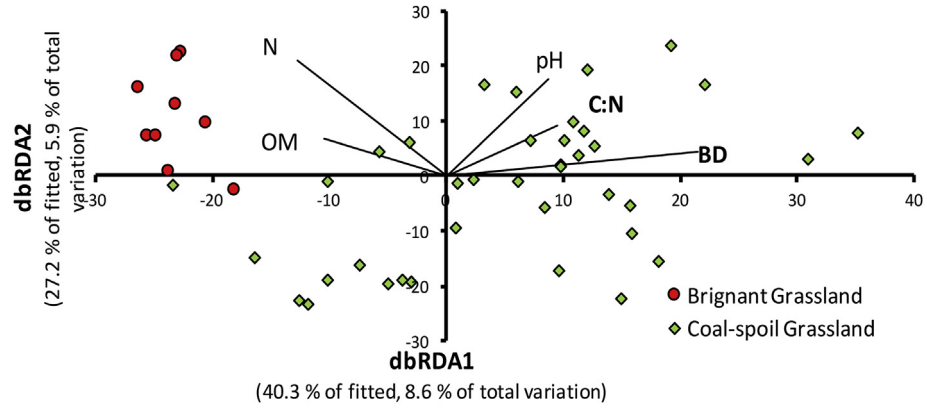

Fig. 4. dbRDA constrained ordination of coal-spoil and Brignant grassland fungal communities. Black arrows show the direction and magnitude of the effect of environmental gradients on the fungal communities. The clearest separation between the two grasslands is along the x-axis dbRDA1, where bulk density is the most strongly correlating of the soil parameters.

separation (R = 0.218). This contrasts with the results of similar comparison of coal-spoil woodland and grassland communities (Fig. 2C), where analysis of saprotrophic fungi alone reduced the degree of separation, whereas analysis of root-associated fungi alone increased the separation.

3.3. Differences in total fungal biomass between coal-spoil and Brignant grasslands

The most pronounced difference between the Brignant and coal-spoil grasslands was in fungal biomass, with Brignant having a 3-fold greater ergosterol content (11.97 vs. 3.53 $\mu g\, g^{-1}$ dw soil; ANOVA $P < 0.001$; $F = 55.142$). Constrained dbRDA analysis also revealed separation between the two grassland types along the primary axis (Fig. 4), with soil bulk density being lower at Brignant (0.86 vs 1.28 $g\, cm^{-3}$; $P < 0.001$, $F = 24.415$). Correlation of bulk density with ergosterol levels for coal-spoil grasslands alone (excluding Brignant samples) showed a significant negative correlation (Fig. 5; Supplementary data S11; Pearson $r = -0.522$; $P = 0.001$), suggesting that more compacted soils sustained lower fungal populations (Supplementary data S12). Other edaphic factors also showed differences between Brignant and coal-spoil grassland soils, with both organic matter content (14.60% vs. 9.41%; $P < 0.001$, $F = 19.114$) and total soil N% (0.64% vs. 0.39%; $P < 0.001$ $F = 63.305$) at Brignant but none showed significant correlations with ergosterol levels when coal-spoil grasslands alone were analysed.

4. Discussion

The processes of soil formation have primarily been studied in post-glacial and post-volcanic sites but restoration programmes at post-industrial sites can also elucidate the dynamics of these processes in a range of climatic and geological contexts. Here we report the first use of DNA metabarcoding to examine how fungal communities have developed on former coal-spoil sites, where plant communities have been re-established without any soil addition. Most investigations of these successional processes have focused on the processes of plant community development and the physicochemical aspects of soil formation (Ruiz-Jaen and Mitchell Aide, 2005). Our study examined sites where revegetation occurred >20 y previously, in contrast to the bulk of published data which have focused on more recently restored sites (Frouz et al., 2013; Li et al., 2014). The strategy used in Wales, establishing mixtures of woodland and grassland and deploying sheep grazing to maintain plagioclimax vegetation in the latter, is relatively uncommon at a global scale (Krüger et al., 2017).

4.1. Vegetation effects on fungal communities

Our first hypothesis was confirmed in that vegetation cover was found to be a key determinant of the structure of fungal communities in these spoils. Fungal communities in both woodland and grassland sites were dominated by fungi known to be associated with the roots of the dominant plant species (ANOSIM R = 0.798), EcM in the case of woodland and CHEG fungi in the case of grassland. The important role of EcM in woodland ecosystems is well-recognised (Courty et al., 2010) and the dominance of EcM taxa in woodland here (mean 54.8% relative abundance) suggests that a typical woodland fungal community had established in the wooded areas (Johnson et al., 2014). The relative abundances of EcM fungi found in the coal-spoil woodlands and the range of taxa present are similar to those associated with temperate deciduous woodland in soils globally (Tedersoo et al., 2014). The EcM taxa were mainly

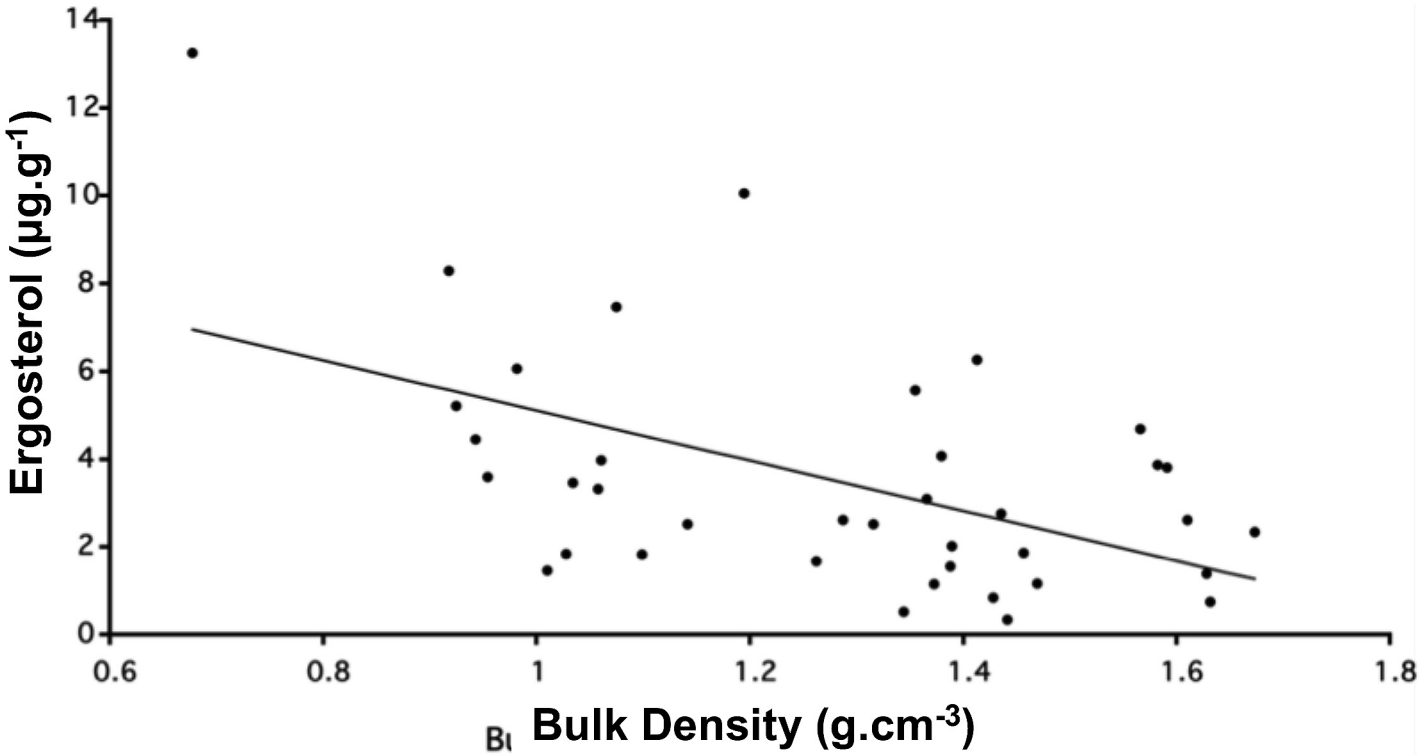

Fig. 5. Pearson correlation of bulk density with ergosterol content of the soils from coal-spoil grassland sites ($r = -0.522$; $P < 0.001$). The Brignant soils (not included in these correlations) contained a mean ergosterol content of 12 $\mu g\, g^{-1}$ and mean bulk density of 0.86 $g\, cm^{-3}$.

those associated with early-mid successional woodlands (e.g *Inocybe*/*Thelephora*/*Tomentella*/*Laccaria*) and EcM associated with mature woodlands (e.g. *Boletus*/*Amanita*) were rare or absent (Bauman et al., 2013; Dickie et al., 2013). Schramm (1966) reported that EcM played an important role in the establishment success of trees planted on coal-spoil, whilst Bauman et al. (2013) demonstrated that ripping of the coal-spoil to alleviate compaction prior to tree planting had a beneficial effect on sapling growth, and also on the species richness of EcM present.

4.2. Immigration of fungi via airborne propagules

The survival of any EcM 'spore' bank from pre-mining vegetation is highly unlikely, not least because these sites were all previously bare or under grass. Some trees may have been naturally infected during nursery cultivation of saplings but it is likely that immigration of EcM was primarily via airborne basidiospores, as has been suggested for the rapid colonisation of early succession glacier forefronts and post-volcanic sites (Jumpponen, 2003; Nara et al., 2003).

Fungal populations of coal-spoil grasslands were very distinct from woodland, being dominated by 'CHEG' fungi which are abundant in low-nutrient grassland soils in northern Europe (Griffith et al., 2013). The basidiocarps of CHEG fungi are most commonly associated with undisturbed grasslands, though their mycelia are detected at low abundance in more disturbed grasslands (Detheridge et al., 2016). The most abundant waxcap species in the soils of coal-spoil grasslands (*H. conica*, *Cuphophyllus virgineus*) are also the two most frequently recorded species (as basidiocarps) across the British Isles (FRDBI; www.fieldmycology.net). Both are observed to fruit in more recently established grasslands, where other waxcap species are absent; as such they are suspected to have less stringent habitat requirements (Griffith et al., 2013). The ecology of waxcap fungi is poorly understood but evidence is accumulating that they form root-endophytic (and very likely mycorrhizal) associations with grassland plants (Seitzman et al., 2011; Halbwachs et al., 2013). Migration patterns for these taxa are also poorly understood but the abundance of suitable low-nutrient grassland habitats in the areas surrounding the mine-spoil sites would suggest airborne basidiospores as the main route of immigration.

Other root-associated taxa such as AMF also showed expected preference for grassland but were present at low abundance. The mean abundance of AMF (Glomeromycota) across the coal-spoil grasslands was 4.2%, comparable with levels observed in other grassland habitats (Geml et al., 2014; Jumpponen and Jones, 2014). AMF have been found to be abundant in the roots of herbaceous plants on coal-spoil within 10–20 y of revegetation by microscopy (Daft and Hacskaylo, 1976) and also genetic analysis (Krüger et al., 2017). Nielsen et al. (2016) using AMF-specific DNA metabarcoding also detected the presence of AMF on the artificial island of Peberholm (Denmark) within 12 y of its construction, with similar levels of root infection and only slightly lower levels of AMF diversity than on a neighbouring natural island. Since AMF form large spores (ca. 200 μm diameter), not well-suited to long-distance airborne dispersal (Nielsen et al., 2016), they suggested that immigration had occurred via the faeces of migratory birds. Mycophagy by mammals and soil invertebrates plays a role in dispersal of both AMF and EcM (Cázares and Trappe, 1994; Reddell et al., 1997), and such vector-borne routes may also have contributed to recolonisation of these coal-spoils.

4.3. Root-associated vs. saprotrophic fungi

The detailed knowledge base of ecological, host specificity and distribution data for many fungi allows taxa to be classified into guilds or functional groups, thus aiding ecological interpretation (Clemmensen et al., 2015; Detheridge et al., 2016; Nguyen et al., 2016). Here we focused on the fungal taxa which were identified as either root-associated or saprotrophic. The former group included EcM and CHEG fungi, as well as AMF, DSE (dark septate endophytes), whilst saprotrophs contained a broader range of taxa including diverse ascomycetes, zygomycetes, ligninolytic basidiomycete and also yeasts.

We reasoned that root-associated taxa abundance would be determined by the plant communities present. Analysis of only root-associated fungi enhanced, as expected, the separation between the coal-spoil woodlands and grasslands (note the x-axis scales on Fig. 2). Less expected was the fact that saprotrophic fungal communities in the two habitat types were also significantly different (albeit with lower ANOSIM R values; Fig. 2C). We ascribe this to the different qualities of plant litter in the two habitat types. Many fungi decaying coarse woody debris are host-specific (Heilmann-Clausen et al., 2015), as are saprotrophs inhabiting leaf litter (Pasqualetti et al., 2014), so the initial stages of litter decay are likely to be mediated by fungi exhibiting some substrate specificity.

Application of the same approach to the comparison of coal-spoil grasslands with a semi-natural grassland (Brignant grassland experiment) showed reduced separation of fungal communities when root-associated fungi alone were examined (Supplementary data S10A), as might be expected given the similar vegetation cover (*Agrostis* and *Festuca* spp.). Also illuminating was the fact that when saprotrophic fungi alone were examined, the separation between the Brignant and coal-spoil grasslands was slightly enhanced (Supplementary data S10B), opposite to woodland vs. grassland. Since litter quality would be expected to be similar in both grassland habitats (due to the similar plant communities), differences in the saprotrophic fungal community are likely to be driven by factors relating to soil organic matter or other edaphic factors. The main saptrotrophic taxa differing between the two grassland types were basidiomycetous yeasts (*Chernovia*, *Solicoccozyma*), zygomycetes (*Mucor*, *Gamsiella, Mortierella*) and members of the order Trechisporales (Supplementary data 8B). These fungi are all commonly detected in DNA-based studies of the soil mycobiota. It is noteworthy that *Trechispora* and allied taxa (order Trechisporales) are all capable of lignin decay (Nagy et al., 2016) and may thus play a role in the cycling of more recalcitrant carbon sources.

4.4. Effect of bulk density on fungal biomass

In addition to the differences in the abundance of some saprotrophic fungi, another clear difference between the coal-spoil and Brignant grasslands was the amount of fungal biomass, more than three-fold higher (3.5 vs. 12.0 $\mu g\, g^{-1}$) on the latter. Ergosterol at all sites was within the range of other grassland soils: Brodie et al. (2003) reporting ergosterol content of 17 $\mu g\, g^{-1}$ in upland soils from Ireland whilst Ruzicka et al. (2000) found ergosterol contents of 0.54–5.21 $\mu g\, g^{-1}$ for grassland restored from arable land and 1.74–18.2 $\mu g\, g^{-1}$ on long-established meadows. Within the coal-spoil grassland quadrats there was considerable variation in ergosterol content (0.33–13.3 $\mu g\, g^{-1}$) and there was a negative correlation with soil bulk density (range: 0.68–1.67 $g\, cm^3$). Despite the ripping that is routinely undertaken prior to revegetation, Haigh et al. (2015) reported that the bulk density of restored coal-spoil grasslands in the south Wales coalfield was significantly higher than in natural soils in adjacent areas (Rudeforth et al., 1984). Reducing compaction is a key objective in restoring coal-spoils but bulk density can remain high for many decades following revegetation (Armstrong and Bragg, 1984).

Bulk density alters the growth of fungal hyphae (Harris et al., 2003), with the continuity of air-filled pores being an important factor (Otten et al., 1999). The formation of pores in soil is the result of complex processes related to soil crumb formation (Moreno de las Heras, 2009). Whilst fungi are important in this process at a fine-scale, the activity of larger soil animals, notably earthworms, is clearly important, not only because of their mucus secretions which can bind fine soil particles but also as a direct result of their burrowing activities, in contrast to fungi which cannot expand pores (Armstrong and Bragg, 1984). Mummey et al. (2002) found that fungal:bacterial ratios increased along a coal-spoil restoration chronosequence and that this correlated with organic matter accumulation, mirroring agricultural soils where the progressive dominance of fungi over bacteria occurs following cessation of disturbance (Vries et al., 2012). Therefore, across diverse chronosequences, as organic matter increases in amount with time, total fungal biomass increases at the expense of bacteria (Jumpponen, 2003; Clemmensen et al., 2015; Liu et al., 2016). This may explain the reduced abundance of the saprotrophic taxa (Table 4) in the less mature coal-spoil grasslands relative to better developed natural soils. In seeking an explanation as to why the coal-spoil grasslands differed so much in bulk density, it was observed that bulk density was positively correlated with altitude (Supplementary data S13; $r = 0.435$; $P = 0.006$), suggesting that climatic differences may account for the differences in soil development across the different sites.

4.5. Conservation value of coal-spoil grasslands

From a fungal biodiversity perspective, low nutrient grasslands have increasing conservation importance due to the spread of intensive mechanised agricultural practices in grassland management (Griffith et al., 2013). Ploughing/reseeding of pastures and application of inorganic fertilisers have led to a drastic reduction in 'haymeadow' habitats across Europe. Therefore, the creation of low-nutrient grassland habitats offers some mitigation against these agricultural losses and creates habitats suitable for colonisation by grassland biota threatened by habitat loss. The importance of former coal-spoil sites as refugia for threatened invertebrates (Eyre et al., 2003) has long been recognised but with increasing awareness of waxcaps and other threatened fungi, such habitats may also play a role in microbial conservation (Griffith, 2012). CHEG fungi formed a large component of the fungal biomass in all the coal-spoil grasslands with a wide range of species represented. Some waxcap basidiocarps were observed (Supplementary data S1), mostly *H. virginea* and *H. conica* (consistent with DNA data) but the presence of a much wider range of CHEG fungi detected in soil DNA indicates the presence of colonies of these fungi at immature stages of development and species forming inconspicuous fruit bodies. Therefore, management of restored coal-spoil sites needs to consider the long-term maintenance of grassland to avert scrub encroachment. From a woodland perspective, it has been suggested that deciduous woodland restoration would be more successful when trees are planted in former coniferous plantations where some EcM species already exist (Johnson et al., 2014). However, our data show that high rates of propagule immigration allow the development of diverse EcM communities in woodlands established *de novo*.

5. Conclusions

We found that distinct grassland and woodland fungal communities have developed in soils derived from former coal-spoil. Furthermore, the communities have developed, linked primarily to the vegetation cover, with natural spore dispersal providing a means for establishment of diverse fungal communities over relatively short timescales. Fungal biomass however was significantly lower in the restored soils, probably linked to the physical conditions. This is in turn linked to the accumulation and ageing of soil organic matter, consistent with our observation that the abundance of several groups of saprotrophic fungi differed between restored and natural grassland soils. The primary focus of studies of soil fungal community structure has been on soils that have formed over millennia with different extents of anthropogenic disturbance. Here we have examined newly forming soils in a temperate climate, showing that a diverse fungal community, with varied dispersal mechanisms, will establish in a short time frame (<30 y). We conclude that fungi across the phylogenetic range have efficient dispersal mechanisms and that dispersal is not a limiting factor in establishing a functioning fungal community.

Authors' contributions

APD collected the data (with assistance from DC/TMC/GB/JB). APD and GWG analysed the data and drafted the manuscript. JS conceived the study with input from APD/GWG/DGJ. All authors edited the manuscript.

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funeco.2018.02.002.

References

Armstrong, M.J., Bragg, N.C., 1984. Soil physical parameters and earthworm populations associated with opencast coal working and land restoration. Agric. Ecosyst. Environ. 11, 131–143.
Ball, D., 1964. Loss-on-ignition as an estimate of organic matter and organic carbon in non-calcareous soils. J. Soil Sci. 15, 84–92.
Bauer, R., Garnica, S., Oberwinkler, F., Riess, K., Weiß, M., Begerow, D., 2015. Entorrhizomycota: a new fungal phylum reveals new perspectives on the evolution of fungi. PLoS One 10, e0128183.
Bauman, J.M., Keiffer, C.H., Hiremath, S., McCarthy, B.C., 2013. Soil preparation methods promoting ectomycorrhizal colonization and American chestnut *Castanea dentata* establishment in coal mine restoration. J. Appl. Ecol. 50, 721–729.
Brodie, E., Edwards, S., Clipson, N., 2003. Soil fungal community structure in a temperate upland grassland soil. FEMS Microbiol. Ecol. 45, 105–114.
Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma 124, 3–22.
Cázares, E., Trappe, J.M., 1994. Spore dispersal of ectomycorrhizal fungi on a glacier forefront by mammal mycophagy. Mycologia 86, 507–510.
Clemmensen, K.E., Finlay, R.D., Dahlberg, A., Stenlid, J., Wardle, D.A., Lindahl, B.D., 2015. Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. New Phytol. 205, 1525–1536.
Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42, D633–D642.
Courty, P.-E., Buée, M., Diedhiou, A.G., Frey-Klett, P., Le Tacon, F., Rineau, F., Turpault, M.-P., Uroz, S., Garbaye, J., 2010. The role of ectomycorrhizal communities in forest ecosystem processes: new perspectives and emerging concepts. Soil Biol. Biochem. 42, 679–698.
Daft, M., Hacskaylo, E., 1976. Arbuscular mycorrhizas in the anthracite and bituminous coal wastes of Pennsylvania. J. Appl. Ecol. 13, 523–531.
Detheridge, A.P., Brand, G., Fychan, R., Crotty, F.V., Sanderson, R., Griffith, G.W., Marley, C.L., 2016. The legacy effect of cover crops on soil fungal populations in

a cereal rotation. Agric. Ecosyst. Environ. 228, 49–61.
Dickie, I.A., Martínez-García, L.B., Koele, N., Grelet, G.-A., Tylianakis, J.M., Peltzer, D.A., Richardson, S.J., 2013. Mycorrhizas and mycorrhizal fungal communities throughout ecosystem development. Plant Soil 367, 11–39.
Djajakirana, G., Joergensen, R., Meyer, B., 1996. Ergosterol and microbial biomass relationship in soil. Biol. Fertil. Soils 22, 299–304.
Elhottova, D., Krištůfek, V., Frouz, J., Novakova, A., Chroňáková, A., 2006. Screening for microbial markers in Miocene sediment exposed during open-cast brown coal mining. Antonie Van Leeuwenhoek 89, 459–463.
Eyre, M.D., Luff, M.L., Woodward, J.C., 2003. Beetles (Coleoptera) on brownfield sites in England: an important conservation resource? J. Insect Conserv. 7, 223–231.
Fierer, N., Strickland, M.S., Liptzin, D., Bradford, M.A., Cleveland, C.C., 2009. Global patterns in belowground communities. Ecol. Lett. 12, 1238–1249.
Frouz, J., Cajthaml, T., Kříbek, B., Schaeffer, P., Bartuška, M., Galertová, R., Rojík, P., Krištůfek, V., 2011. Deep, subsurface microflora after excavation respiration and biomass and its potential role in degradation of fossil organic matter. Folia Microbiol. 56, 389–396.
Frouz, J., Elhottová, D., Baldrián, P., Chronakova, A., Kristufek, V., 2013. Soil Microflora Development in Post-mining Sites. Soil Biota and Ecosystem Development in Post Mining Sites. CRC Press, Boca Raton, pp. 104–131.
Geml, J., Gravendeel, B., van der Gaag, K.J., Neilen, M., Lammers, Y., Raes, N., Semenova, T.A., de Knijff, P., Noordeloos, M.E., 2014. The contribution of DNA metabarcoding to fungal conservation: diversity assessment, habitat partitioning and mapping red-listed fungi in protected coastal *Salix repens* communities in The Netherlands. PLoS One 9, e99852.
Griffith, G.W., 2012. Do we need a global strategy for microbial conservation? Trends Ecol. Evol. 27, 1–2.
Griffith, G.W., Easton, G.L., Jones, A.W., 2002. Ecology and diversity of waxcap (*Hygrocybe* spp.) fungi. Bot. J. Scotl. 54, 7–22.
Griffith, G.W., Gamarra, J., Holden, E., Mitchel, D., Graham, A., Evans, D., Evans, S., Aron, C., Noordeloos, M., Kirk, P., 2013. The international conservation importance of Welsh 'waxcap' grasslands. Mycosphere 4, 969–984.
Haigh, M., Reed, H., Flege, A., D'Aucourt, M., Plamping, K., Cullis, M., Woodruffe, P., Sawyer, S., Panhuis, W., Wilding, G., 2015. Effect of planting method on the growth of *Alnus glutinosa* and *Quercus petraea* in compacted opencast coal-mine spoils, south Wales. Land Degrad. Dev. 26, 227–236.
Halbwachs, H., Dentinger, B.T.M., Detheridge, A.P., Karasch, P., Griffith, G.W., 2013. Hyphae of waxcap fungi colonise plant roots. Fungal Ecology 6, 487–492.
Harris, K., Young, I.M., Gilligan, C.A., Otten, W., Ritz, K., 2003. Effect of bulk density on the spatial organisation of the fungus *Rhizoctonia solani* in soil. FEMS Microbiol. Ecol. 44, 45–56.
Heilmann-Clausen, J., Barron, E.S., Boddy, L., Dahlberg, A., Griffith, G.W., Nordén, J., Ovaskainen, O., Perini, C., Senn-Irlet, B., Halme, P., 2015. A fungal perspective on conservation biology. Conserv. Biol. 29, 61–68.
Hibbett, D.S., Donoghue, M.J., 2001. Analysis of character correlations among wood decay mechanisms, mating systems, and substrate ranges in homobasidiomycetes. Syst. Biol. 50, 215–242.
Johnson, D., Williamson, J., Bailey, A., 1991a. Microbiology of soils at opencast coal sites. I. short-and long-term transformations in stockpiled soils. J. Soil Sci. 42, 1–8.
Johnson, N.C., Zak, D.R., Tilman, D., Pfleger, F., 1991b. Dynamics of vesicular-arbuscular mycorrhizae during old field succession. Oecologia 86, 349–358.
Johnson, J., Evans, C., Brown, N., Skeates, S., Watkinson, S., Bass, D., 2014. Molecular analysis shows that soil fungi from ancient semi-natural woodland exist in sites converted to non-native conifer plantations. Forestry 87, 705–717.
Jones, M.D., Smith, S.E., 2004. Exploring functional definitions of mycorrhizas: are mycorrhizas always mutualisms? Can. J. Bot. 82, 1089–1109.
Jones, M.D., Phillips, L.A., Treu, R., Ward, V., Berch, S.M., 2012. Functional responses of ectomycorrhizal fungal communities to long-term fertilization of lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) stands in central British Columbia. Appl. Soil Ecol. 60, 29–40.
Jonsson, L.M., Nilsson, M.C., Wardle, D.A., Zackrisson, O., 2001. Context dependent effects of ectomycorrhizal species richness on tree seedling productivity. Oikos 93, 353–364.
Jumpponen, A., 2003. Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. New Phytol. 158, 569–578.
Jumpponen, A., Jones, K.L., 2014. Tallgrass prairie soil fungal communities are resilient to climate change. Fungal Ecology 10, 44–57.
Krüger, C., Kohout, P., Janoušková, M., Püschel, D., Frouz, J., Rydlová, J., 2017. Plant communities rather than soil properties structure arbuscular mycorrhizal fungal communities along primary succession on a mine spoil. Front. Microbiol. 8.
Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N., 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 40, 2407–2415.
Legendre, P., Anderson, M.J., 1999. Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. Ecol. Monogr. 69, 1–24.
Li, Y., Wen, H., Chen, L., Yin, T., 2014. Succession of bacterial community structure and diversity in soil along a chronosequence of reclamation and re-vegetation on coal mine spoils in China. PLoS One 9, e115024.
Lindahl, B.O., Taylor, A.F., Finlay, R.D., 2002. Defining nutritional constraints on carbon cycling in boreal forests–towards a less phytocentric perspective. Plant Soil 242, 123–135.
Liu, C., Ding, N., Fu, Q., Brookes, P.C., Xu, J., Guo, B., Lin, Y., Li, H., Li, N., 2016. The influence of soil properties on the size and structure of bacterial and fungal communities along a paddy soil chronosequence. Eur. J. Soil Biol. 76, 9–18.
Mandyam, K.G., Jumpponen, A., 2015. Mutualism–parasitism paradigm synthesized from results of root-endophyte models. Front. Microbiol. 5, 776.
Moreno de las Heras, M., 2009. Development of soil physical structure and biological functionality in mining spoils affected by soil erosion in a Mediterranean-continental environment. Geoderma 149, 249–256.
Mummey, D.L., Stahl, P.D., Buyer, J.S., 2002. Microbial biomarkers as an indicator of ecosystem recovery following surface mine reclamation. Appl. Soil Ecol. 21, 251–259.
Nagy, L.G., Riley, R., Tritt, A., Adam, C., Daum, C., Floudas, D., Sun, H., Yadav, J.S., Pangilinan, J., Larsson, K.-H., Matsuura, K., Barry, K., Labutti, K., Kuo, R., Ohm, R.A., Bhattacharya, S.S., Shirouzu, T., Yoshinaga, Y., Martin, F.M., Grigoriev, I.V., Hibbett, D.S., 2016. Comparative genomics of early-diverging mushroom-forming fungi provides insights into the origins of lignocellulose decay capabilities. Mol. Biol. Evol. 33, 959–970.
Nara, K., Nakaya, H., Wu, B., Zhou, Z., Hogetsu, T., 2003. Underground primary succession of ectomycorrhizal fungi in a volcanic desert on Mount Fuji. New Phytol. 159, 743–756.
Newell, S., 2001. Fungal biomass and productivity. Meth. Microbiol. 30, 357–372.
Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., Schilling, J.S., Kennedy, P.G., 2016. FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20, 241–248.
Nielsen, K.B., Kjøller, R., Bruun, H.H., Schnoor, T.K., Rosendahl, S., 2016. Colonization of new land by arbuscular mycorrhizal fungi. Fungal Ecology 20, 22–29.
Otten, W., Gilligan, C.A., Watts, C., Dexter, A., Hall, D., 1999. Continuity of air-filled pores and invasion thresholds for a soil-borne fungal plant pathogen, *Rhizoctonia solani*. Soil Biol. Biochem. 31, 1803–1810.
Pasqualetti, M., Mulas, B., Rambelli, A., Tempesta, S., 2014. Saprotrophic litter fungi in a Mediterranean ecosystem: behaviour on different substrata. Plant Biosyst. 148, 342–356.
Paul, E.A., 2014. Soil Microbiology, Ecology and Biochemistry. Academic press.
Read, D.J., Perez-Moreno, J., 2003. Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? New Phytol. 157, 475–492.
Reddell, P., Spain, A.V., Hopkins, M., 1997. Dispersal of spores of mycorrhizal fungi in scats of native mammals in tropical forests of northeastern Australia. Biotropica 29, 184–192.
Rousk, J., Baath, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME J. 4, 1340–1351.
Rudeforth, C., Hartnup, R., Lea, J., Thompson, T., Wright, P., 1984. Soils and Their Use in Wales.
Ruiz-Jaen, M.C., Mitchell Aide, T., 2005. Restoration success: how is it being measured? Restor. Ecol. 13, 569–577.
Ruzicka, S., Edgerton, D., Norman, M., Hill, T., 2000. The utility of ergosterol as a bioindicator of fungi in temperate soils. Soil Biol. Biochem. 32, 989–1005.
Scheu, S., Parkinson, D., 1994. Changes in bacterial and fungal biomass C, bacterial and fungal biovolume and ergosterol content after drying, remoistening and incubation of different layers of cool temperate forest soils. Soil Biol. Biochem. 26, 1515–1525.
Schramm, J.R., 1966. Plant colonization studies on black wastes from anthracite mining in Pennsylvania. Trans. Am. Philos. Soc. 47, 331–340.
Seitzman, B.H., Ouimette, A., Mixon, R.L., Hobbie, E.A., Hibbett, D.S., 2011. Conservation of biotrophy in Hygrophoraceae inferred from combined stable isotope and phylogenetic analyses. Mycologia 103, 280–290.
Sun, S., Li, S., Avera, B.N., Strahm, B.D., Badgley, B.D., 2017. Soil bacterial and fungal communities show distinct recovery patterns during forest ecosystem restoration. Appl. Environ. Microbiol. 83 e00966–00917.
Tedersoo, L., May, T.W., Smith, M.E., 2010. Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20, 217–263.
Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Ruiz, L.V., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., 2014. Global diversity and geography of soil fungi. Science 346, 1256688.
Van Der Heijden, M.G., Streitwolf-Engel, R., Riedl, R., Siegrist, S., Neudecker, A., Ineichen, K., Boller, T., Wiemken, A., Sanders, I.R., 2006. The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytol. 172, 739–752.
Vries, F.T., Manning, P., Tallowin, J.R., Mortimer, S.R., Pilgrim, E.S., Harrison, K.A., Hobbs, P.J., Quirk, H., Shipley, B., Cornelissen, J.H., 2012. Abiotic drivers and plant traits explain landscape-scale patterns in soil microbial communities. Ecol. Lett. 15, 1230–1239.
Wilberforce, E.M., Boddy, L., Griffiths, R., Griffith, G.W., 2003. Agricultural management affects communities of culturable root-endophytic fungi in temperate grasslands. Soil Biol. Biochem. 35, 1143–1154.