Bacterial and archaeal taxa are reliable indicators of soil restoration across distributed calcareous grasslands

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Running Title: Microbial responses to grassland restoration

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Abstract

Land use intensification can reduce soil carbon stocks and changes microbial community biodiversity and functionality. However, there is a lack of consensus on whether management consistently affects microbial biodiversity across geographic scales, and how this relates to altered soil function. From a regulatory and monitoring perspective, there is a need to identify functionally relevant indicators of land use in order to evaluate the progress of soil restoration approaches. We performed a landscape scale survey of unimproved calcareous grasslands paired with local arable contrasts, and assessed the consistency of responses in a variety of soil, biotic and functional measures. In addition, adjacent grasslands undergoing restoration were assessed to identify soil microbial indicators of recovery. Organic matter content was consistently larger in grasslands than in arable fields, and increased with time in the restoring sites. Molecular comparisons of grassland versus arable soils revealed numerous bacterial, archaeal and fungal indicators, with more representatives of Ca. *Xiphinematobacter, DA101, Bradyrhizobium, Rhodoplanes, Mycobacteria* and *Mortierella* in old grassland soils, while *Nitrososphaera, Sporosarcina* and *Alternaria infectoria* were more abundant in arable soils. Extracellular enzymatic responses were more variable with none of the eight investigated enzymes being consistent indicators of grassland or arable soils. Correlation analyses, incorporating the molecular and enzymatic responses across all surveyed soils, revealed that molecular indicators were more strongly correlated with soil organic matter increases with restoration of arable soils. Our results highlight that microbial taxa are among the most sensitive indicators of soil restoration, and we identify consistent responses of specific taxa to management across geographic scales. This discovery will be important for both the instigation and monitoring of the soil restoration.

Keywords: soil monitoring, microbial community, NGS, grassland, arable soil, restoration, land use indicator, soil organic matter

Highlights:

- Soil microbes are key drivers of soil ecosystem services, and are affected by management

- Calcareous grassland exhibited abundant Verrucomicrobia; cropping increased Nitrososphaera

- These taxa responded to SOM increases with grassland restoration, more so than enzymes and fungi

- Microbes provide consistent, site-independent indicators for calcareous grassland soil function restoration

Introduction

Microorganisms play a major role in delivering soil ecosystem services, including nutrient cycling, soil aggregate stability, plant productivity and biodiversity (Fierer et al., 2017; Kallenbach et al., 2016). For example, as plant pathogens or symbionts, soil bacteria and fungi can significantly influence crop yields in agriculture; and recent evidence is emerging regarding the central role of microbes in increasing soil carbon stocks (Cotrufo et al., 2015; Cotrufo et al., 2013). Differences in land management are known to have strong effects on microbial biodiversity (Griffiths et al., 2011), yet we are still some way from synthesising how land management affects the abundances of specific microbial taxa, precluding wider understanding of functional effects. Better understanding of the resistance and resilience of soil microbial communities and their functions to land use change might provide novel approaches for future sustainable agriculture as well as for restoring ecosystems (Griffiths & Philippot, 2013). In addition, policymakers and land users require reliable indicators of soil function in order to monitor soil state and the efficacy of ameliorative practices (Orgiazzi et al., 2015; Stone et al., 2016).

Grasslands cover about one quarter of the world's ice-free area, and make up 70% of global agricultural land, storing 20% of global soil carbon (Smith et al., 2016). More than 90% of English and Welsh unimproved, species-rich grasslands were converted to more intensive agriculture between 1932 and 1984 (Ridding et al., 2015). The associated cultivation has dramatically modified soil organic matter (SOM) stocks (Deng et al., 2016; Thomson et al., 2015). To prevent further loss of soil C and vulnerable habitats, efforts have been made to restore degraded landscapes and abandoned fields to grassland in the UK (Bullock, 2011), but to date there has been little information on how soil C and wider microbial communities and features recover. Their ability to rapidly adapt, makes microorganisms potential early indicators of succession during the regeneration progress (Bouchez et al., 2016; Griffiths et al., 2016). Past research has identified that microbial biomass and activity is reduced under intensive arable management, and it is thought that intensification leads to a general reduction in fungi compared to bacteria (Emmerling et al., 2001; Lauber et al., 2008; Nunes et al., 2012; Potthoff et al., 2006). New molecular methods now permit a more detailed examination of the responses of individual soil microbial taxa (Hirsch et al., 2010; Vogel et al., 2009), though we are some way from synthesising whether there are geographic consistencies in taxonomic responses to management. Identifying such taxa, and particularly those taxa associated with soil organic matter (SOM) content increases, will advance new functional understanding of the roles of

microbes in soil processes, as well as providing functionally relevant indicators to assess soil recovery.

The effect of land management on soil microbial communities has been assessed at a range of scales, from local studies assessing the impacts of specific managements, to broader landscape scale surveys. At the local scale, one study of bacterial and archaeal communities identified that across three sites there was some consistency in specific indicators of grassland versus arable communities (Zhalnina et al., 2013). This study found that specific archaeal taxa were associated with arable sites, whereas Bradyrhizobia were more abundant in grassland/abandoned arable fields. At the regional scale, a distributed study of bacterial and fungal taxa across arable and grassland sites, focussed on assessing broad diversity effects, but also noted key increases in dominant bradyrhizobial taxa in grasslands. Notably, neither of these studies specifically examined the specific relationships between these taxa and SOM. A critical issue in identifying microbes responsive to SOM changes has been identified in several studies examining intensification effects on microbial communities. Since soil microbes, and bacteria in particular, are primarily structured along gradients of pH (Griffiths et al., 2011); land use driven change in other edaphic properties can often obfuscate direct relationships between intensification, SOM and microbial taxa (Lauber et al., 2008; Thomson et al., 2015). It is therefore likely that constraining contrasts to land use comparisons on soils of similar pH may help identify specific indicators relating to SOM and the lack of disturbance from cultivation.

We therefore seek to determine the consistency of microbial indicators across distributed sites in the South of England, each containing three land management contrasts. Each site selected comprised three contrasting land use categories; including a contemporary intensively managed arable field, ancient grassland and a restoring former arable field established 3 - 65 years ago (Fagan et al., 2008; Wagner et al., 2019). These calcareous grasslands are typically characterised by high levels of plant and faunal diversity and are considered the most diverse habitats in Europe (Poschlod & WallisDeVries, 2002). Here, we specifically focus on calcareous soils to minimise wider confounding effects of soil pH on microbial communities, and consequently hypothesise that consistent microbial indicators of land use change in pristine versus arable contrasts will be apparent across the distributed sites. Relatedly, across all soils assessed we hypothesise that microbial communities will be

dominantly structured across gradients of organic matter and not pH. Finally, we predict that key microbial taxa found to be indicators of pristine grasslands, will increase proportionally with SOM improvements through restorative management. The performance of microbial indicators will additionally be contrasted with enzymatic functional measures to test the utility of such metrics for informing on soil status under a restoration context.

Materials and Methods

Sampling sites

14 undisturbed calcareous grasslands (henceforth "Pristine") were identified in the South of England (**Figure 1**), which were not ploughed, nor improved for grazing for at least 100 years (Fagan et al., 2008; Redhead et al., 2014). Arable fields near each site were used as a control or contrast, which is the land use that replaced the calcareous grassland. At each location, a reverting, ex-arable grassland ("Restoration") was sampled to test for the response of identified indicators to recovery over time. Both the pristine and restoring grasslands were subject to livestock (sheep and/or cattle) grazing at low stocking density and without agricultural improvements. Details of actual stocking rates and grazing dates were unavailable. Dates when reversion of arable land to grassland started are based on past data which investigated land use history utilising historic maps (Fagan et al., 2008; 2010; Redhead et al., 2014; Ridding et al., 2015). Grassland age in the restoring fields differs strongly between sites so that "Restoration" is not considered a defined land use or treatment. Instead, we focus on statistical comparisons between Arable and Pristine. To ensure comparable soil properties, the sample sites were situated on a chalk, lime-rich bedrock material, with the "Pristine" site classified as NVC habitat Calcareous Grassland. Sampling was conducted in summer 2016, with plant cover assessed in five quadrats at each site, and co-located soil cores (20 cm depth, 5 cm diameter) sampled for further analysis. A sub-sample of each of the five cores was stored at -20 °C for microbial diversity and enzymatic analyses. The remaining soil from each of the five cores was pooled for standard chemical analysis of SOM (as loss-on-ignition, 16 hours at 430 °C), total C using the Walkley-Black method, total N, C to N ratio, Olsens's P, K, Mg (NRM Laboratories, Bracknell, UK) and pH (10 g soil in 25 ml distilled water).

Extracellular enzyme activity and bacterial biomass

Three of the five soil cores were randomly selected for extracellular enzymatic activity assays and the same soil solution was used to extract total DNA and measure bacterial biomass (see below). Potential activity of hydrolytic exoenzymes acetase (acetyl esterase, ACE), α-

glucosidase (α -GLU), β-glucosidase (β-GLU), chitinase (N-acetyl-b-glucosaminidase CHIN), phosphatase (PHO), sulphatase (arylsulphatase, SUL), and peptidase (leucine-aminopeptidase, LEU) was assessed with methylumbelliferyl (MUB) and 7-amino-4-methylcoumarin (AMC) conjugated substrates. Enzyme assays were performed on 1.5 g of frozen homogenized soil mixed with 20 ml deionized water in sterile falcon tubes. Samples were shaken for twenty minutes at 400 rpm to obtain a homogeneous soil solution. 30µl soil solution was added to a 96-well microplate containing 170 µl substrate solution at 300 mM (saturated concentration). Reaction plates were incubated in the dark for three hours at 28℃ with one fluorometric scan every 30 minutes (BioSpa 8 Automated Incubator). Fluorescence intensity was measured using a Cytation 5 spectrophotometer linked to the automated incubator and set to 330 and 342 nm for excitation and 450 and 440 nm for emission for the 4-MUB and the 7-AMC substrate, respectively. For each sample, three technical replicates (soil solution + substrate + water) and a quenching curve (soil solution + water + 4-MUB or 7-AMC) were measured. For each substrate, a control including the 4-MUB- or 7-AMC-linked substrate and water alone were used to check the evolution of fluorescence without enzyme degradation over the duration of the assay. All enzyme activities were calculated in nkat, the amount [nmol] of catalysed product per second and normalized by g of dry soil (Marx et al., 2001).

To assess bacterial biomass, 250µl of the soil slurry was mixed with 750µl water, centrifuged at 1000 g for 5 min and 500µl of the supernatant fixed with 500µl 0.5% Paraformaldehyde solution for storage at -20℃. All samples were run at the Accuri® Flow Cytometer in deep-well plates after SYBR Green staining and five minutes incubation in the dark as described in Bressan et al., 2015.

Molecular analyses of microbial communities

For DNA extractions, a 200 µl aliquot of the soil-water slurry used for the enzyme analyses was transferred into 96-well plates and extracted using the PowerSoil® DNA Isolation Kit. Illumina 2-step amplicon sequencing was conducted according to the protocols of the Earth Microbiome Project (Thompson et al., 2017). In brief, amplicons were prepared using established primers for the ITS region GTGARTCATCGAATCTTTG and TCCTCCGCTTATTGATATGC (Ihrmark et al., 2012); and 16S rRNA regions (V4-5 region) 515f GTGYCAGCMGCCGCGGTAA and 806r GGACTACNVGGGTWTCTAAT and PCR protocols (Walters et al., 2016) using high fidelity DNA polymerase (Q5 Taq, New England Biolabs). Amplicon sizes were determined using an Agilent 2200 TapeStation system. For purification, PCR products were treated according to manufacturer's instructions with Zymo

DNA Clean up Kit. In a second round of PCR, Illumina adapters were added and all samples normalized using the SequalPrep ™ Normalization Kit (Invitrogen), pooled and concentration verified spectrophotometrically with Qubit. Illumina high throughput sequencing was performed with MiSeq® Reagent Kit V3 capable of producing 2 x 300 bp paired-end reads.

Illumina sequencing output was analysed with DADA2 (Callahan et al., 2016) in R (R Core Team, 2017), to demultiplex raw sequences and trim paired sequences to uniform lengths. The core sequence-variant inference algorithm was applied with the dada function to dereplicated data before paired-end sequences were merged and chimeras were removed. Taxonomic data was assigned from GreenGenes (DeSantis et al., 2006) for bacterial and UNITE (Koljalg et al., 2005) for fungal taxonomy. The 16S OTU table was rarefied to 4590 reads, while the ITS table was rarefied to 2000 reads to account for differences in sampling depth before assessing β-diversity in non-metric multidimensional scaling ordinations and running PERMANOVAs with the functions in vegan (Oksanen, 2008). Significant (p < 0.05) indicator OTUs for grassland and arable soil were determined using the indval routine in labdsv (Dufrene et al., 2011) and wider statistical analysis and visualisation was performed in R version 3.6.0 using the packages ggplot2 (Wickham, 2016), circlize (Gu et al., 2014), labdsv (Roberts et al., 2019) and igraph (Csardi & Nepusz, 2006).

Results

Soil properties

To assess the effects of land use on soil variables at each location, we quantified soil pH, SOM, P, K, Mg, as well as total C and total N; and present data grouped by management in Figure 2. SOM content in pristine grasslands was significantly greater than in arable soils, with mean of 22.16 % and only 6.76 %, respectively (t-test, p< 0.001). Phosphorous determined by the Olsen method, and soil C: N ratio were less in old grassland soil compared to arable, whilst all other tested parameters with the exception of potassium were significantly greater in pristine. With respect to pH, arable soils were slightly less acidic (pH 7.9 vs. pH 7.7 in pristine grassland, t-test p-value 0.0016). All reverting soils showed attributes intermediate between grassland and arable (**Figure 2, Table SI1**).

Soil extra-cellular enzyme activity Soil extra-cellular enzyme activities did not respond as consistently to land use change as did the soil properties (**Figure 4**). From the eight evaluated enzymes only ACE and CHIN were affected by land use, while variance in PHO, HEM and β-GLU were completely independent from land use. Comparison of pristine and

arable soils show mean α-GLU was most active in arable samples, but not significantly different between land use categories (Table SI2, p= 0.08). ACE activity increased with decreasing land use intensity and was significantly stronger in pristine than in arable soils (p = 0.048). CHIN and SUL mean activities were twice as high in pristine soils as in arable, with CHIN being significantly affected by land use (p =0.024), while differences in SUL activities were not significantly different between land use categories (p > 0.05). Interestingly, LEU showed more potential activity in restoration sites than in pristine grasslands.

Land use effects on plant and microbial community structure

Multivariate assessment of bacterial and fungal communities revealed samples grouped clearly according to land use, as assessed by nonmetric MDS ordination of ASV relative abundances (**Figure 3**). The plant community ordination, based on presence absence data from surveyed quadrats, expectedly showed that arable communities were highly dissimilar to the grasslands. Further significance testing using PERMANOVA revealed all grassland communities were significantly different from arable (Table 1, PERMANOVA p < 0.01, F > 0.5). Restoration sites were situated between grassland and arable, and the variance within this group likely reflects different times since arable abandonment. We also fitted the soil chemical and enzymatic data to the NMDS plots to examine specific relationships with microbial community composition (**Table 2**). For both bacterial and fungal communities, soil organic matter and age (time since cultivation) were highly related to community composition, and importantly, these variables were stronger than pH. In accordance with the results shown in figure 2, enzymatic responses were more weakly associated with microbial communities, though it is noteworthy that CHIN was jointly the third strongest linear fit with fungal community structure.

Molecular indicators of land use change

Indicator analysis revealed 440 prokaryote, and 139 fungal taxa significantly associated with pristine grassland; and 401 prokaryote and 168 fungal taxa associated with arable land use. A full list of these indicators taxa is provided in the Supplementary Materials, whilst dominant taxa are shown in **Figure 5**. Strikingly, the seven most abundant prokaryotic taxa indicative of pristine grassland soils all belong to the phylum Verrucomicrobia (genera: Candidatus *Xiphinematobacter* and *DA101*), with other notable taxa occurring in the top 20 abundance ranked indicators including several α-Proteobacteria (genus: Bradyrhizobia, Rhodoplanes, Mesorhizobium); Actinobacteria (genus: Gaiellaceae, Solirubrobacterales and Mycobacteriaceae). Prokaryotic indicators were abundant in arable soils and highly dominated

by archaeal Candidatus *Nitrososphaera* taxa, as well as several other acidobacterial (iii1-15), firmicute (Sporosarcina, Planococcacaea, Bacillales) and actinomycete phyla (Arthrobacter) (**Figure 5a**). Another notable taxa in the top 20 most abundant arable indicators included a Nitrosomonad (β-Proteobacteria).

Fungal communities were dominated by *Mortierella minutissima*, which was abundant in both land use types but was a significant indicator of arable soils, while *Mortierella exigua* was dominant in pristine grassland (**Figure 5b**). Other abundant and significant fungal taxa in pristine grassland soils were *Pseudeurotium, Preussia flanaganii, Fusarium solani* and *F. oxysporum* and *Clavaria*. Other dominant arable soils indicators, aside from *Mortierella minutissima* included *Gibellulopsis nigrescens, Cladosporium exasperatum, Mycosphaerella tassiana* and a member of the Nectriaceae family.

Indicator relationships with SOM restoration

In order to assess the performance of the arable and pristine grassland indicators in predicting SOM recovery with restoration management, we performed a pairwise Pearson correlation analyses of all microbial indicators, broader plant and microbial biodiversity metrics (diversity indices and ordination scores) together with soil abiotic and enzymatic responses. The correlation matrix is presented in **Figure 6,** displaying only those variables highly correlated with SOM (positive correlation in **Figure 6a**, negative in **Figure 6b**). SOM is positively correlated with the highly abundant Chthoniobacterales, an order of Verrucomicrobia, as well as with members of Rhizobiales and Syntrophobacterales.

The fungal OTU73 and Sordariales were also positively related, though they were found at lower relative abundance. As anticipated, there is a strong positive correlation of SOM with soil C, N, moisture and grassland age. In contrast, soil pH and C to N ratio are negatively correlated to organic matter and likewise with the highly abundant archaeal Nitrososphaerales, Actinomycetales, acidobacterial iii1-15 and RB41 taxa. We further visualise the specific relationships between SOM and the most dominant indicators of both land use, and SOM restoration in **Figure 7**. The selected prokaryotic taxa *Nitrososphaera*, *Ca. Xiphinematobacter* and *Bradyrhizobium*, which were determined as indicative for arable or pristine land use, respectively, are more strongly correlated to SOM ($R^2 > 0.5$, p-value < 0.001) than the most abundant fungal specimen or extracellular acetase potential activity ($R^2 < 0.3$, p-value > 0.001) (Figure 7).

Discussion

In this distributed survey of paired land use contrasts, we found clear differences in plant, fungal and prokaryotic communities between historically undisturbed calcareous grassland soils and intensively managed arable land. Distinct bacterial, fungal and archaeal taxa were identified as highly indicative for each land use, and furthermore, a number of prokaryotic taxa were found to be the most strongly associated with grassland restoration age-related increases in SOM. The abundances of these specific taxa were found to be more sensitive indicators of SOM than any of the functional enzymatic responses, or broader community metrics describing plant or microbial biodiversity.

Amongst the top bacterial indicators for pristine soils are several taxa of the phylum Verrucomicrobia. Our findings are consistent with previous studies which have demonstrated that members of the Verrucomicrobia are dominant across soils in different habitats and ecosystems (Bergmann et al., 2011), with a preference for grassland soils (Brewer et al., 2017). Our findings uniquely demonstrate that members of this phyla, also strongly respond to increases in soil organic matter brought about by grassland restoration. Whilst the lack of cultured representatives means we know little about the functionality of Verrucomicrobia in soils, recent metagenomic reconstruction found evidence of heterotrophy with putative amino acid auxotrophies compensated by efficient mechanisms for amino acid uptake, and abilities to store surplus C (Brewer et al., 2017). Additionally a reduced genome size was noted, which is thought to be a common phenomenon in free-living auxotrophic bacteria, which efficiently assimilate a wide range of compounds at low substrate concentration.

The arable soils were characterised by a dominance of several archaeal Nitrososphaerales taxa. Cultivated soils tend to contain elevated levels of nitrogen as a result of fertilizer application, which ammonia-oxidisers oxidise to nitrate in the first step of the nitrogen cycle (Boddy, 2016; Madigan, 2010). A functionally similar ammonia-oxidising bacteria (AOB), a Nitrosomonad, was also found to be indicative of arable soils, but this was less abundant. AOB and AOA, esp. *Candidatus Nitrososphaera,* were previously defined as signature organisms for agriculture in long-term experiments at one (Rothamsted Park Grass Experiment) or multiple locations and across a range of edaphic conditions (UK, Florida, Michigan), in which soil pH and ammonium concentrations were clearly correlated with AOA abundance. These studies also noted that the abundances of Nitrososphaera were negatively related to *Bradyrhizobium* which was elevated in relatively unimproved plots (Zhalnina et al., 2013, 2014). This is also consistent with our findings, as a bradyrhizobial taxon was also highly

related to increases in organic matter, though less abundant overall than the Verrucomicrobia in these calcareous soils. Previously, it was considered that the opposing abundances of these taxa in relation to N availability reflects differences in N capture, either archaeal ammonia oxidation in improved soils, or bradyrhizobial N fixation in unimproved soils (Zhalnina et al., 2013). Whilst this may be true also in our soils, we also note that the recent metagenomics evidence suggests the Nitrososphaera are able to fix inorganic carbon from bicarbonate (HCO_3) or CO_2 (Berg et al., 2010) which also may be a factor underlying their competitiveness in C depleted arable soils. Moreover, the slow-growing, free-living members of genus Bradyrhizobium were described to be genetically highly heterogeneous, with certain taxa being unable to fix N in symbiosis with legumes, but different functions and carbon metabolisms depending on land use (Jones et al., 2016) .

Whilst we found several fungal indicators of grassland versus arable management, when we included the restoration site data these did not respond as well as the bacterial indicators with respect to relationships with increasing SOM. *Mortierella*, a widely distributed soil fungus was highly abundant across the soils, and was also sensitive to land use change. While *Mortierella minutissima* dominated arable soils, *M. exigua* was found to be elevated in grassland soils. Previous studies on fungal communities under different land management systems found *Mortierella* positively correlated to nitrate-N, but negatively to soil P (Detheridge et al., 2016), with *M. elongata* supporting crop performance by its contribution to the P cycle and increased activity of β-glucosidase and contributing to stable soil C pools via production of recalcitrant C compounds (Li et al., 2018). We also found *Fusarium oxysporum* and *F. solani* as strong indicators for old calcareous grasslands and the potential plant pathogenic *Fusarium merismoides* as an indicator for arable. Other potential plant pathogenic taxa from the classes Leotiomycetales and Dothideomycetales were amongst the top indicators for old grasslands (Sigler et al., 2000) confirming previous work showing uncertainties in the delineation between pathogenic and harmless saprotrophic fungi (Detheridge et al., 2016; Thornton, 1965). The investigated ITS marker gene targets identification of fungi, but picked up unicellular algae as indicative of croplands, too, which are likely to form lichens and soil crusts. Using light as an energy source, they are able to grow on nutrient deficient, bare surfaces (Watkinson, 2016). More specific to croplands were a lichen, *Trebouxia decolorans*, and several green algae, as well as the crop pests *Alternaria infectoria* and *Stemphylium vesicarium*, the cause of spots on certain pears and a saprophyte in soil (Rossi et al., 2005). *Neoascochyta* species cause leaf scorch on wheat (Golzar et al., 2019) and were also more abundant in

croplands. Interestingly, we detected the crop pathogen *Pythium* as arable indicator when analysing the bacterial 16S sequencing output, where it came up as mitochondrial DNA sequence in the order α-Proteobacteria, which are ancestors of eukaryotic mitochondrial cells with their own genetic system (Bevan & Lang, 2004). As fungi are, like plants, spatially more variable than bacteria, their larger variance in soil molecular analysis is likely to be representative and reduces their potential as land use indicators compared to the determined prokaryotic ones.

Extracellular enzyme activities in this study did not react consistently to land use, since responses within land use classes were highly variable. Previous work has shown enzymatic responses can be highly affected by management, and in particular have been shown to be repressed with nutrient addition (Ramirez et al., 2014). However in our study we have to consider not only the impact of fertilizer amendments, but tillage, pesticides, grazing and other plant growth stimulators, as well as the contrasting vegetation cover which may have had unmeasured effects on the enzymatic responses. Other studies have also shown more variable responses across different enzymes across a chronosequence relating to specific nutrient limitations; but identified that correcting enzymatic responses to biomass better reflected efficiency in relation to successional changes in P acquisition (Allison et al., 2007). We also note that soil enzyme responses are known to be sensitive to temperature, season and assay pH (Nottingham et al., 2016; Puissant et al., 2019; Turner, 2010), factors we did not consider in our workflow of multiple substrate degradation assays from a single sampling point.

Conclusions

Soils provide fundamental services to humans and sustainable land management and restoration are crucial for maintaining soil multifunctionality in a changing world. Biological indicators are used widely for monitoring, though typical vegetation surveys are problematic since indicators may not be transferable between different sites and regions, due to differences in environmental factors (Karlík & Poschlod, 2019). Additionally, the relevance of plant indicators for soil services remains uncertain. Our findings demonstrate that, across these calcareous soils, specific phylotypes of soil microbial taxa are the most consistent indicators of both land use change and SOM recovery. We therefore advocate that specific microbial taxa, and not broad taxonomic groups, be strongly considered amongst suites of indicators for soil monitoring (Bouchez et al., 2016; Griffiths et al., 2011). However, we note that our analysis

was purposely limited to high pH soils, and so specific indicators for other geo-climatically defined soils remain to be defined. More generally the specific identification of microbial taxa responding to land use change, and SOM improvement, should guide wider attempts to understand the functional capacity of these enigmatic organisms and their roles in driving soil formation and soil service delivery.

Authorship

RF designed the survey and carried out sampling and field work, MA and TG carried out laboratory analysis, and analysed the data with RG. MA wrote a first draft and all co-authors contributed to the final version of the paper. KF identified and surveyed the original sites.

Funding

Data Sharing and Data Accessibility statement

OTU tables are available as Supplementary Information.

Conflict of Interest Statement

None.

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Table .

Table 1 PERMANOVA results of soil microbial community composition in bacterial, fungal and plant cover as a response to the land use types undisturbed grassland vs. cropland.

	Degrees of freedom	Sums Of Squares	Mean Squares	F value	R^2	p
bacterial 16S	1	1.330	1.330	8.492	0.279	0.001***
Residuals	22	3.445	0.157		0.721	
Total	23	4.774			1.000	
fungal ITS	1	1.374	1.374	5.650	0.176	0.001***

|						
Residuals	26	6.449	0.248		0.821	
Total	27	7.823			1.000	
plant cover	1	2.955	2.955	25.440	0.495	0.001***
Residuals	26	3.020	0.116		0.505	
Total	27	5.975			1.000	

Table 2 Linear fit of environmental variables to the non-metric multidimensional scaling ordination for bacterial (left) and fungal (right) soil communities. ACE = acetase, α-glu = α-glucosidase, β-glu = β-glucosidase, CHIN = chitinase, HEM = Hemicellulase, PHO = phosphatase, LEU = Peptidase, age = years since reconversion from arable to grassland, SOM = Soil Organic Matter content

| bacteria	NMDS1	NMDS2	R^2	p value		fungi	NMDS1	NMDS2	R^2	p value	
SOM	0.97	0.26	0.85	0.001	***	age	0.97	0.24	0.65	0.001	***
total N	1.00	0.09	0.78	0.001	***	SOM	1.00	-0.03	0.53	0.001	***
age	0.99	0.14	0.66	0.001	***	CHIN	0.48	0.88	0.44	0.001	***
pH	-0.87	-0.50	0.66	0.001	***	total N	0.99	-0.12	0.44	0.001	***
m...	0.83	0.56	0.62	0.001	***	Mg	0.82	0.58	0.40	0.001	***
C to N	-0.99	-0.14	0.60	0.001	***	C to N	-0.98	-0.18	0.38	0.001	***
Mg	0.73	0.68	0.53	0.001	***	pH	-0.85	-0.52	0.36	0.001	***
total C	0.89	-0.45	0.50	0.001	***	moisture	0.91	0.41	0.27	0.002	**
bact. biomass	-0.34	0.94	0.38	0.001	***	total C	0.77	-0.64	0.25	0.006	**
ACE	0.61	0.79	0.35	0.001	***	bact. biomass	-0.63	-0.77	0.24	0.008	**
CHIN	0.47	0.88	0.27	0.003	**	ACE	0.60	0.80	0.23	0.007	**
P	-0.50	-0.87	0.21	0.024	*	P	-0.96	-0.29	0.21	0.007	**
LEU	-0.11	0.99	0.16	0.048	*	HEM	0.39	0.92	0.13	0.063	.
α-glu	-0.89	-0.46	0.08	0.259		α-glu	-0.85	0.52	0.11	0.110	
PHO	-0.33	-0.94	0.03	0.581		LEU	-0.19	-0.98	0.06	0.110	
K	-0.80	-0.60	0.03	0.613		K	-0.68	-0.73	0.05	0.345	
β-glu	-0.78	-0.63	0.01	0.820		β-glu	-0.71	0.70	0.04	0.426	
HEM	-0.11	0.99	0.00	0.973		PHO	-0.97	0.23	0.02	0.643	
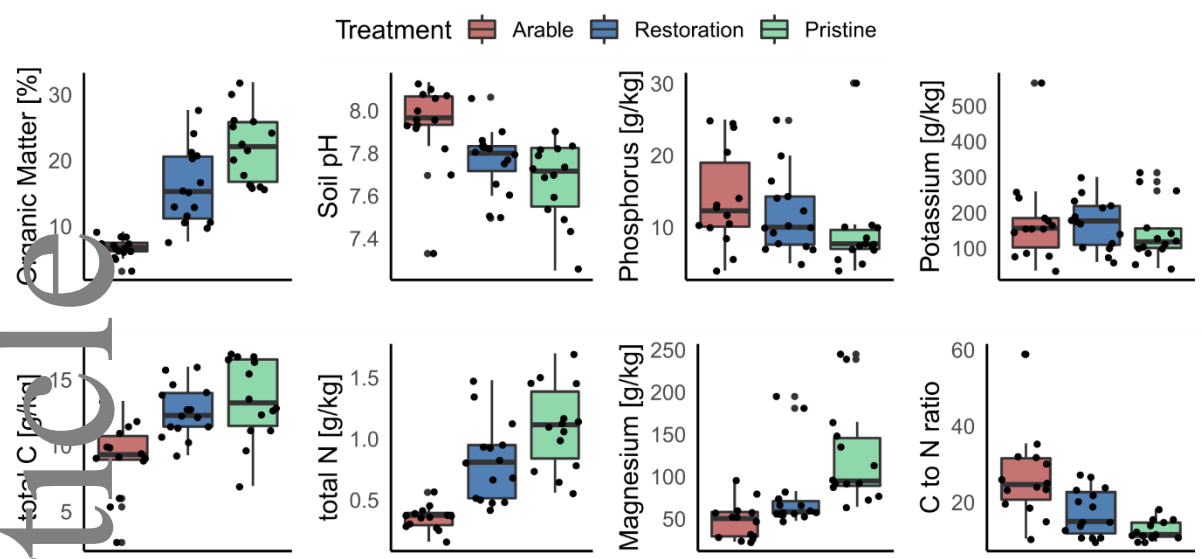
Figures

Figure 1

Figure 2

bacterial 16S
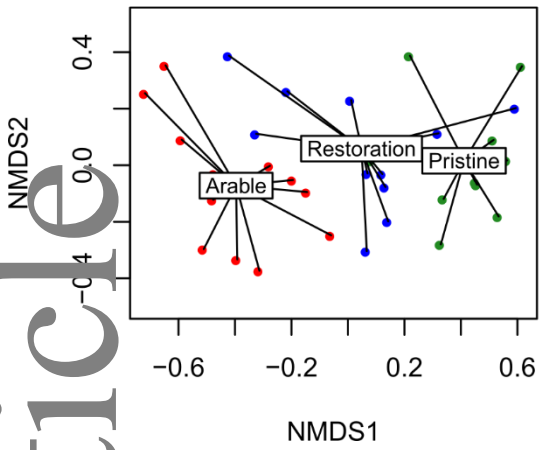
fungal ITS
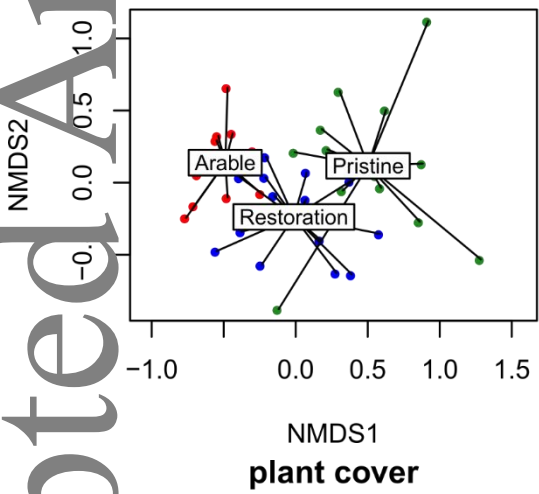
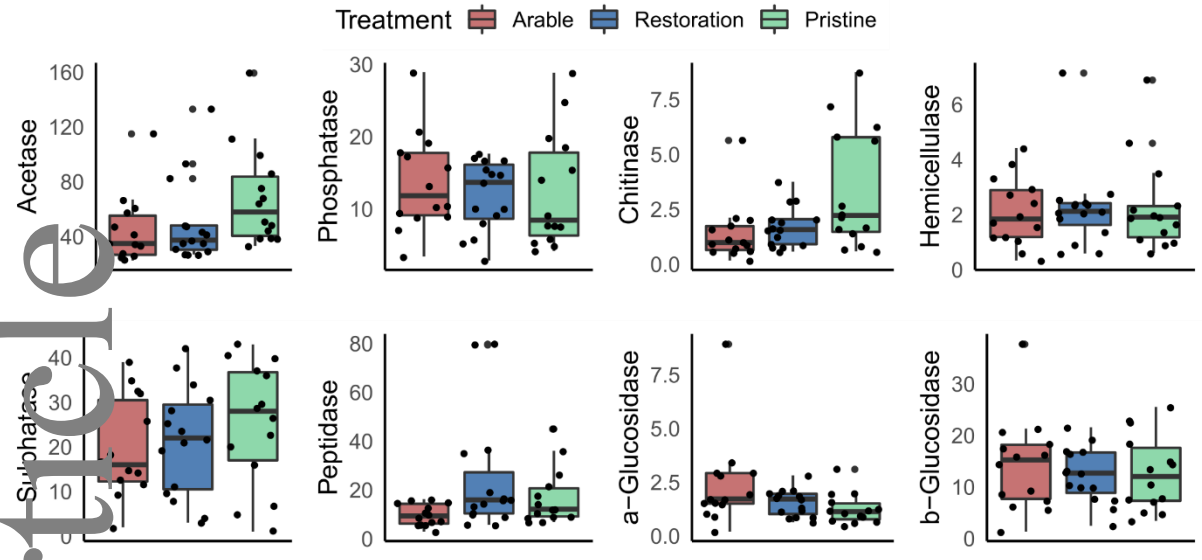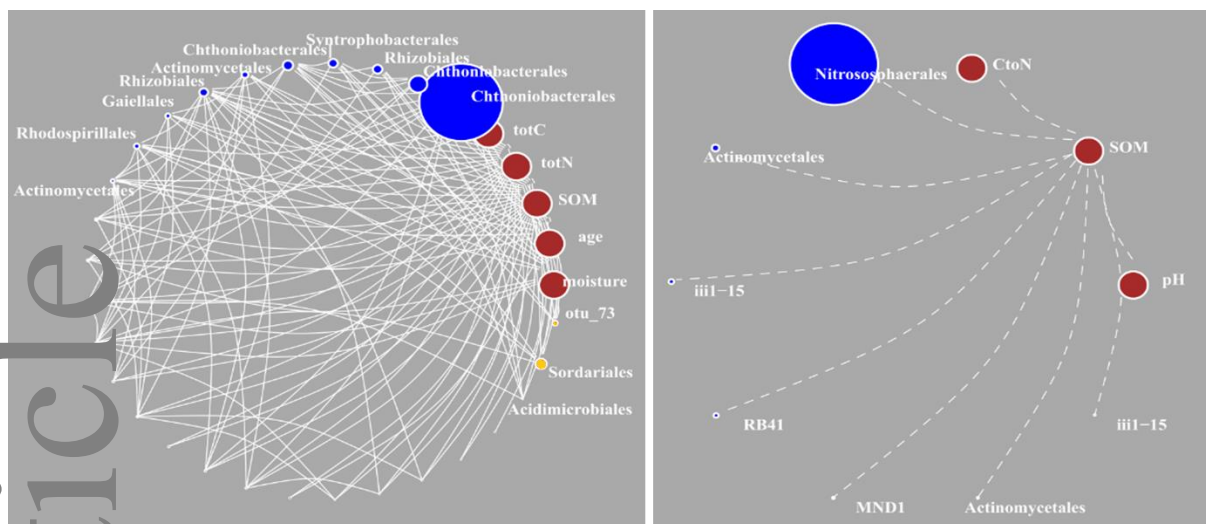**plant cover**
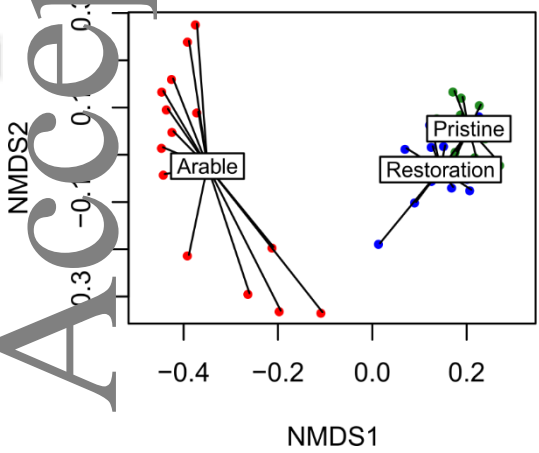
Figure 3

Figure 4

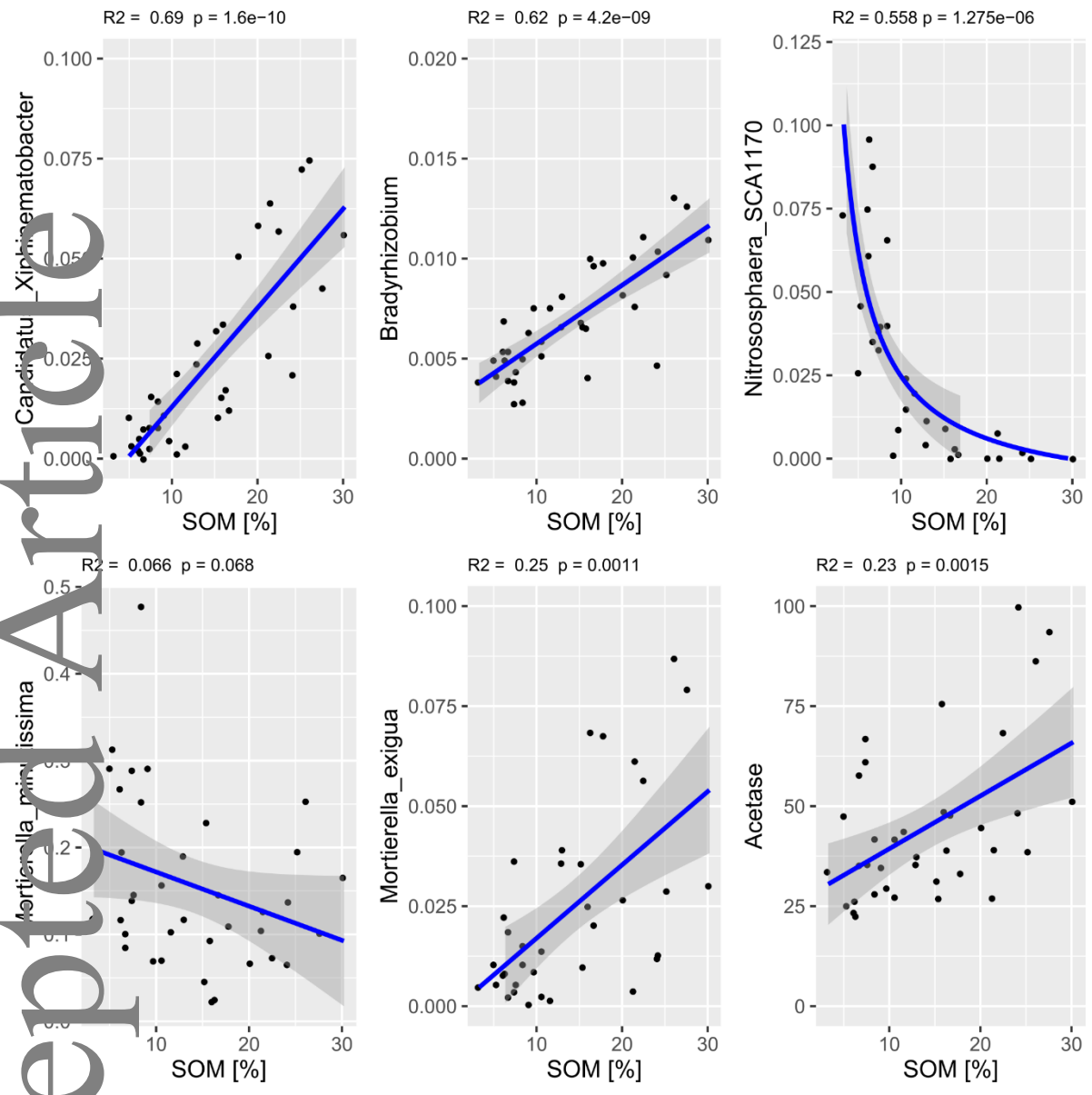**Figure 5**

Figure 6

Figure 7

Figure 1: Location of sampling sites on chalk rich parent material in South England. At each site, a land use contrast of unimproved grassland vs. intensive agriculture vs. reconverted, former arable grassland (3 to 65 years of regeneration time) was surveyed for plant assemblage, soil chemistry, soil bacterial and fungal diversity.

Figure 2: Boxplots of soil properties and plant available nutrients per land use across 14 sites. Arable soils are conventional croplands with elevated levels of P and greater C to N ratio. Pristine soils were not ploughed nor fertilized for at least 100 years, but maintained as species-rich grasslands with high levels of SOM, C and N. Soil nutrient levels of ex-arable fields are recovering with time.

Figure 3: Non Metric Dimensional Scaling plots showing differences in microbial and plant community composition between treatments. Bacterial, fungal and plant communities were all significantly different in grassland compared to arable soils (PERMANOVA, $p < 0.01$), with restoration sites having an intermediate centroid.

Figure 4: Eight hydrolytic soil extracellular enzymatic activities in [nkat] (nanomol substrate degraded per minute, normalised per gram dry soil) as response to land use in a calcareous grassland restoration chronosequence. Acetase, Chitinase, α- and β-Glucosidase, Hemicellulase activities are considered to be relevant for carbon compound degradation, while Phosphatase (aryl-phosphatase) is involved in P cycling and peptidase (leucine-aminopeptidase) catalyses degradation of nitrogen compounds (peptides).

Figure 5: Circle diagram of a) bacterial and b) fungal indicators of grassland and arable soils. The mean relative abundance of 16S and ITS amplicons is plotted in red for arable and green for pristine grassland. Only dominant OTUs are labelled with red text denoting significant arable indicators, green denoting grassland indicators, and black text identifying taxa abundant taxa which are not affected by management.

Figure 6: Network analysis of full dataset (soil chemistry, functional and biodiversity indicators) showing only strong correlations with soil organic matter content. The left panel shows variables positively correlated with SOM (> 0.7) and the right panel shows negative correlations ($< - 0.7$). For the molecular indicators the size of nodes is scaled to relative OTU abundance, and only the more abundant taxa are labelled. Blue nodes represent bacterial taxa, red nodes, soil properties and yellow nodes are fungal taxa.

Figure 7: Top row: relative abundance of the three most dominant bacterial indicator taxa identified in the network analysis, bottom row: Other fungal and functional indicators were clearly related to SOM, but to a lesser extent than prokaryots. *Ca. Xiphinematobacter* and *Bradyrhizobia* are indicative for old grassland soils, while ammonia-oxidising archaeal *Nitrososphaerales* indicate arable land use. Grassland indicators increase in relative abundance with recovery of SOM in the restoration soils, *Nitrososphaerales* decrease. Acetase potential activities [nkat] and the abundance of indicator fungi *Mortierella exigua* are increasing with SOM, while *Mortierella minutissima* abundance decreases.