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Title

Change in heathland dominant plants strongly increases C mineralization potential despite marginally affecting microbial community structure

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Abstract

Purpose

In many ecosystems, the identity of the dominant plant is changing because of global change. If the new dominant species has different litter and root traits than the one it replaces, it is likely to have an influence on soil microbial communities and the functions they perform. We used a grass-encroached heathland, where dwarf shrubs are replaced by grasses with different ecological traits, as a case study to explore this question. We hypothesized that grass colonization of heathland would improve litter quality, which would favor soil copiotroph microbes and increase C mineralization rate.

Methods

We established a 13-plot field observatory spanning across a 0-100% gradient of grass cover percentage. In each plot, we characterized plant, fungal and bacterial communities, using a combination of ARISA (taxonomic diversity), metabarcoding plus hierarchical modelling of species communities (community structure), FDA assay (metabolic activity) and Biolog ecoplates (functional diversity and rate of C mineralization).

Results

Our results show that microbial taxonomic and functional diversities are not affected by grass colonization. Microbial communities were also similar at high phylogenetic level, including for ericoid mycorrhizas and typical oligo- and copiotrophic species. At a finer phylogenetic level, some abundant extremophilic OTUs (*e.g* *Acidothermus* bacteria) were progressively replaced by fungal black yeasts. Functional response of microbial communities was more obvious. The C mineralization potential significantly increased across the grass gradient.

Conclusion

Change in dominant plant traits may induce drastic functional changes in microbial communities despite having only a very minor effect on their diversity or structure.

Keywords

Heathland, grass encroachment, microbial communities, functional diversity, taxonomic diversity, C mineralization

Introduction

Global change is causing a shift in the identity of the dominant plant species. For example, southern species are moving northwards or up the altitudinal gradient and challenging local plant species because of climate change (Kelly and Goulden 2008). As another example, nitrogen deposition leads to a replacement of dominant grassland species by other species (Isbell et al. 2013). Such a change in the dominant plant in the ecosystem may lead to significant alterations on soil processes, if the new dominant plant differs in its traits from the one it replaces (Brown et al. 2001). Indeed, these new traits may affect litter quality and quantity, or rhizodeposition, both of which are recognized to influence the structural and functional properties of microbial communities (Blagodatskaya and Kuzyakov 2008; Fierer et al. 2009), which are in turn driving many soil processes, such as C and N mineralization.

Grass-encroached heathland in North-Western Europe are a good illustration of this phenomenon. The initially dominant ericaceous shrubs, most often belonging to the species *Calluna vulgaris*, and the invading grasses, belonging to the species *Molinia caerulea* or *Deschampsia flexuosa*, differ in terms of their spatial biomass distribution, their litter quality, and the type of mycorrhizal association they are involved in. These grasses indeed have deeper and denser root systems, while heather shrubs concentrate most of their roots as a mat in the topsoil (Aerts and Heil 1993). Thus root-derived litter input in C, N and P is about one order of magnitude higher for grasses (Aerts et al. 1992). Heather litter is much more lignified than that of grasses, resulting in lower litter quality and twice higher C/N ratios under *C. vulgaris* than under *M. caerulea* (Certini et al. 2015). Finally, *M. caerulea* and *D. flexuosa* form arbuscular mycorrhizal associations, and *C. vulgaris* ericoid ones (Wang & Qiu, 2006). Both mycorrhizal types significantly differ in their functional profiles, with for example ericoid mycorrhizae having higher potential for secretion of organic-matter degrading enzymes (Genney et al. 2000; Read and Perez-Moreno 2003).

These major changes in plant traits are expected to influence both community structure and activity of microbes, especially in the rhizosphere, where plant litter, root necromass and exudates have most impact. For example, in grasslands, switching of the dominant species to one of different productivity increased soil microbial biomass but reduced diversity (Bardgett et al. 1999). In fact, even a switch from dominance by *C. vulgaris* to another ericoid shrub, *V. myrtillus*, led to fungal assemblages in the rhizosphere, with higher frequency of Basidiomycetes under *V. myrtillus* (Bougoure et al. 2007). Even domination by a different ecotype of the same species led to significant changes in soil microbial respiration in a salt marsh ecosystem, probably because of its higher sugar percentage in rhizomes (Seliskar et al. 2002). As the microbial community is affected, so are the functions it performs: change in plant communities have been associated with alterations of soil microbial enzyme activities (Kardol et al. 2010), N mineralization, nitrification, and basal respiration (Massaccesi et al. 2015), even though these results occur due to changes in plant community and not only dominant species.

In the case of heathland, a change in litter input and quality and higher rhizodeposition should improve C availability, which favours microbial copiotrophs (Fierer et al. 2007). These species have a fast growth rate and therefore accelerate mineralization. Hence, grass colonization in heathlands induces significant shifts in ecological traits of the dominant plant, which has high chances to cascade down to a change in microbial community structure and functioning, and eventually in soil processes. This has however never been tested, at least to our knowledge.

The goal of this study is to assess how the change in a dominant plant from shrub to grass affects microbial community structure and functioning. We focused on C mineralization as a crucial microbe-driven soil function. Our hypothesis is that higher litter quality and higher rhizodeposition of the grass improves soil quality, and especially C availability, and that this affects the microbial community structure by favouring copiotrophic species, which increases C mineralisation because of their fast growth rate. To test this, we used a field observatory of 13 plots differing only in terms of grass dominance. There, we assessed microbial community structure using metabarcoding, taxonomic diversity using ARISA, metabolic activity using FDA assay and mineralization of a range of C substrates using Biolog plates.

Material & Methods

Field site

The study was carried out in the National Park Hoge Kempen (Limburg, Belgium), at the Mechelse Heide site (50°59’07.0”N, 5°38’01.7”E), at an altitude of 104 m above sea level. The mean annual temperature is 10.3°C and the average annual precipitation is 839 mm. This area is dominated mainly by the dwarf shrub *Calluna vulgaris*, with local encroachment by the subdominant species *Molinia caerulea*. The site measures 287 500 m^2 and consists of a mosaic of 500-1000m^2 heathland patches that are managed by mowing, burning or sod-cutting in order to maximize spatial heterogeneity, and is characterized by various degrees of grass encroachment. The dominant soil types within this area are albic podzols and brunic-dystric arenosols. We chose 13 of these plots, that were similar in terms of vegetation age (5-10 years old, in the early building-up phase), density, management history (burning), and slope (flat), but varied only in the *Molinia caerulea* / *Calluna vulgaris* ratio, to set up a field observatory of grass encroachment (see Figure S1 for the spatial arrangement of the plots in a map of the site), arranged in a gradient design (Figure 1).

Soil sampling

The soil samples were collected on the 13th of August 2018, after a few days of rain in a summer characterised by intense droughts. In each of the 13 plots, we took soil samples to assess features of microbial communities, with two constraints in mind: i) they had to be taken at a random position in the plot and ii) we wanted to keep track of the vegetation cover at the exact spot where the soil sample was taken. Indeed, the plant cover was heterogeneous at the sub-meter scale, especially in the plots in the middle of the gradient, so there was a significant probability to sample under a heather plant even in a plot with 50% grass cover.

Therefore, we randomly set out three 50 cm quadrats in each plot. In each quadrat, we measured plant cover (*C. vulgaris*, *M. caerulea* in the herbaceous stratum and bare soil and mosses in the ground stratum; we did not observe other vascular plant species in the quadrats). In the middle of each quadrat, we took a 10 cm deep and 10 cm diameter soil core, using an auger, that was sterilized with 70% ethanol between each core. The soil sample was immediately stored at 4°C. Once in the lab, the samples (including the roots) were sieved using a 2 mm mesh sieve and aliquots of the sieved soil were labelled and stored at -20°C. The samples issued from the sieving step therefore included both bulk and rhizospheric soil.

Measurement of microbial diversity by bacterial- and fungal-arisa

Soil DNA was extracted using the Dneasy® PowerSoil® Kit (QIAGEN, Venlo, The Netherlands). The 16S-23S and 18S-28S intergenic spacer region of bacteria and fungi, respectively, were then amplified by PCR. The bacterial and fungal Automated Ribosomal Intergenic Spacer Analysis (ARISA) PCR mastermix (50 µl) contained 5 µl 10x buffer, 1 µl of the primers (10 µM), 5 µl 10x dNTP's, 1µl Advantage polymerase mix, 36 µl PCR grade water (Takara Bio, Kusatsu, Japan) and 1 µl of the DNA-samples. We used the primers S-D-Bact-1522-b-S-20 (5'-TGCGGCTGGATCCCCTCCTT-3') and L-D-Bact-132-a-A-18 (5'-CCGGGTTTCCCCATTCGG-3') for bacteria and 2234C (5'-GTTTCCGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3') for fungi. Both these primer sets were used and recommended for ARISA by (Ranjard et al. 2001). The PCR was executed in a PCR thermocycler Biorad T100 or Biorad C1000 (Biorad, Temse, Belgium). First, a hotstart of 3 minutes was performed at 94°C, followed by 25 cycles at 94°C for 1 minute, 55° for 30 seconds and 72°C for 1 minute. Finally, a terminal elongation step was performed at 72°C for 5 minutes (37).

The resulting PCR-amplicons were used for ARISA to generate an electropherogram. This is used to determine the microbial fingerprint of the soil sample (Ranjard et al. 2001). ARISA on each sample was done using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, US). An Agilent DNA 1000 Kit was used with the Agilent DNA-chips. Amplicons were separated by length through capillary electrophoresis on the microchip.

ROX gel-dye was used as an internal standard. After adjusting the gel-dye mix to room temperature for 30 minutes, 9 µl of gel-dye mix was added to the 3 designated wells on the microchip. One of the wells was firmly put under pressure with the chip priming station. Next, 5 µl marker was added to the wells designated to the ladder and samples. After running the chip in the Bioanalyzer, the results were presented in the form of gels and electropherograms in the Agilent Expert 2100 software. DNA fragments are presented as peaks in the electrophoregram.

Characterization of microbial community structure

The same DNA samples were used as for ARISA. DNA samples were subjected to bacterial 16S rRNA gene and fungal ITS2 region sequencing. For the bacteria, in the first round of 16S rRNA gene PCR, an amplicon of 290 bp was generated, using primers 515F and 806R (Caporaso et al. 2010). For the first round of Fungi-specific PCR, the primers gITS86f and ITS4R generate an amplicon with an average length of 450 bp (Op De Beeck et al. 2014). Using the Q5 High-Fidelity DNA Polymerase system (M0491, New England Biolabs, Ipswich, Massachusetts, US), a reaction volume of 25 µl per sample was prepared containing 1 µl of extracted DNA (final DNA-concentration per reaction 1-10 ng), 1x Q5 Reaction Buffer with 2 mM MgCl2, 200 µM dNTP mix, 1x Q5 High GC Enhancer (for the soil and fungi samples), 0.2 µM forward and reverse primer, and 1.2 U Q5 High-Fidelity DNA polymerase. The PCR program started with an initial denaturation for 3 min at 98 °C, followed by a 30 s denaturation at 98 °C, a 30 s annealing at 53 °C for V4 (58 °C for ITS) and a 1 min extension at 72 °C, all three steps were repeated for a total of 35 cycles. The reaction was ended by a final 7 min extension at 72 °C. The amplified DNA was purified using the AMPure XP beads (Beckman Coulter, Brea, California, US) and the MagMax magnetic particle processor (Thermo Fisher Scientific, Waltham, Massachusetts, US). Subsequently, 5 µl of the cleaned PCR product was used for the second PCR, attaching the Nextera indices (Nextera XT Index Kit v2 Set A (FC-131-2001), and D (FC-131-2004), Illumina, San Diego, California, US). For these PCR reactions, 5 µl of the purified PCR product was used in a 25 µl reaction volume, and prepared following the 16S Metagenomic Sequencing Library Preparation Guide. PCR conditions were the same as described above, but the number of cycles reduced to 20, and 55 °C annealing temperature. PCR products were cleaned with the AMPure XP kit, and then quantified using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific) and the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Once the molarity of the sample was determined, the samples were diluted down to 4 nM using 10 mM Tris pH 8.5 prior to sequencing on an Illumina MiSeq. Samples were sequenced using the MiSeq Reagent Kit v3 (600 cycle) (MS-102-3003) and 15% PhiX Control v3 (FC-110-3001). For quality control, a DNA-extraction blank and PCR blank were included throughout the process, and also the ZymoBIOMICS Microbial Mock Community Standard (D6300) to test efficiency of DNA extraction (Zymo Research, Irvine California, US). Obtained sequences were clustered into operational taxonomic units (OTUs) and annotated using Qiime (Caporaso et al. 2010) within the DADA2 package with standard settings, with the SILVA database for bacteria and the Unite database for Fungi.

Measurement of microbial metabolic activity

The Fluorescein Diacetate (FDA) hydrolysis assay was performed following the protocol of (Schnurer and Rosswall 1982) and (Adam and Duncan 2001). Aliquot soil samples (1g) were placed in a 125 ml Erlenmeyer flask with 50 mL of 60 mM sodium phosphate buffer (pH = 7.6) and 0.5 mL of an FDA stock solution (2 mg fluorescein diacetate per mL of reagent-grade acetone), and incubated for 3 h at 37°C to allow hydrolysis of fluorescein diacetate to fluorescein by a variety of different microbial enzymes (e.g. proteases, lipases, and esterases) present in the soil samples. Negative control flasks without soil samples were included to check for non-specific fluorescein release. After incubation, 2 mL of acetone was added to the suspension and swirled to mix the contents and terminate the reaction. Thereafter, 30 mL of the soil suspension was transferred to a 50 mL centrifuge tube and centrifuged at 3500 g for 10 min. The resulting supernatant was filtered through a Whatman No. 2 filter paper into a new 50 mL centrifuge tube. Finally, the filtrate (200 µL) was transferred to a 96 well plate and the absorbance was measured at 490 nm on a plate reader (FLUOstar® Omega plate reader, BMG LABTECH GmbH, Ortenberg, Germany).

Measurement of microbial C mineralization functions

The C mineralization potential by microbial communities was measured using The functions of C mineralization by soil microbes were investigated using community-level physiological profiling (Biolog PM1 MicroPlate Carbon Sources; BioLog Inc., Hayward, California, US), where an inoculum prepared from a given soil sample was exposed to 32 carbon sources, and the mineralization rate measured spectrophotometrically as the reduction of a tetrazolium dye by microbial cell respiration, integrated over time using trapezoidal estimation. Using sterilized equipment, aliquot soil samples (1g) were dissolved in 10 mL sterile 10 mM PBS buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4) and shaken for 20 minutes at room temperature. After shaking, soil particles were allowed to settle for 30 minutes at 4°C. Subsequently, 130 µL of the supernatant was dispensed into each well of the Biolog MicroPlate. Inoculated plates were placed in self-sealing plastic bags containing a water-soaked paper towel to minimize evaporation from the wells, and incubated at 28-30 °C. Absorbance was measured at 595 nm with a plate reader immediately after inoculation (0 h) and at 3, 6, 18, 24, 48, 72, and 144 h. Raw absorbance values were recorded at each time point and individually standardized by subtracting the corresponding absorbance value measured immediately after inoculation (0 h) (reaction-independent absorbance). Furthermore, to semi-quantitatively evaluate the kinetic Biolog MicroPlate dataset, the net area under the absorbance versus time curve was calculated according to the trapezoidal approximation (Guckert et al. 1996). The resulting value calculated via the trapezoidal approximation summarizes different aspects of the measured respirometric reaction, including differences in lag phases, increase rates, or maximum optical densities (Preston-Mafham et al. 2002). The Shannon-Weaver index (H) was calculated per sample as an indicator of functional diversity, or *range* of C sources used by the community of microbes. The *rate* of C source use by the microbial community was calculated as the average of the area under the curve for all C sources (referred to as AWCD: average well colour development). The C sources could be categorized into 6 different types, based on (Rutgers et al. 2016): carbohydrates (β-methyl-D-glucoside, n-acetyl-D-glucosamine, D-cellobiose, glucose-1-phosphate, α-D-lactose, D-1-α-glycerol-phosphate, D-galactonicacid-1-lactone, D-xylose, 1-erythritol, D-mannitol), amino acids (L-arginine, L-asparagine, L-serine, L-phenylalanine, L-threonine), carboxylic acids (glycyl-L-glutamic acid, D-galactonic acid, γ-hydroxybutyric acid, D-glucosamic acid, itaconic acid, alpha-ketobutyric acid, pyruvic acid methyl ester, D-malic acid), polymers (tween40, tween80, α-cyclodextrin, glycogen), phenolics (2-hydroxy-benzoic acid, 4-hydroxy-benzoic acid), and amines (phenylethylamine, putrescine).

Statistics

The strength of the relationship between grass colonization and both microbial diversity and activity was assessed using correlation tests. Correlation between grass cover and both bacterial and fungal microbial diversity was tested in three ways: with OTU richness (number of peaks in the ARISA electropherogram) using a Kendall rank correlation coefficient test, after normality and homoscedasticity assumptions were tested and not met; and with Shannon and Simpson indices (diversity indices based on OTU richness and abundance, assessed by metabarcoding, see above) using a Pearson's correlation test. The correlation between grass cover and microbial activity (measured as the absorbance at 690 nm in the FDA assay, and where no distinction between bacteria and fungi can be made) was tested using a Pearson's correlation test.

To evaluate the effect of grass colonization on the microbial community structure, we used Hierarchical Modelling of Species Communities (HMSC) (Tikhonov et al. 2020), which is a type of joint species distribution modelling (Wilkinson et al. 2021). The advantage of these models over PERMANOVA, ANOSIM or a Mantel Test is that HMSC explicitly accounts for the effect of species-species interactions on their distribution over an environmental variable (Tikhonov et al. 2020), to reduce the effect of species interactions as a confounding variable. We built three separate mixed models, all with quadrat's grass cover as independent variable and quadrat identity as random variable; only the dependant variable (OTU abundance as counts) differed between the models. Model 1 grouped all OTUs present in at least 40% of the samples (16 of 39), model 2 in 25% (10 of 39), and model 3 in 10% (4 of 39). The rationale behind testing these three models was that we wanted to find a compromise between including as many OTUs in the analysis as possible, on the one hand, and keeping the highest explanatory power, on the other hand. We then fitted all models using Bayesian statistics. For that, we sampled the posterior distribution using two Monte Carlo Markov Chains (MCMC), each of which being run for 5000 iterations, out of which the first 4900 were removed as burn-in and the remaining ones thinned by either 1, 10, or 100. We then evaluated and compared model's explanatory power using the Root Mean Square Error (RMSE), coefficient of determination (SR^2), area under the receiver operating characteristic curve (AUC) and coefficient of discrimination ($Tjur^2$) (see results in Figure S2); and verified that convergence of the Markov chains was achieved by comparing ess.β, ess.V, PSRF.β and PSRF.V parameters (for more details see (Tikhonov et al. 2020)). We chose model 1, which was the best compromise between number of OTUs and explanatory power (Table S1), with a thinning of 100 (Table S2). The post estimates of the model are displayed in Table S2: we selected the ones having a support higher than 0.95. Statistical analyses were done in R (R core team 2019), using the Hmsc package for the joint species distribution modelling part.

Results

Microbial taxonomic diversity

There was no significant influence of grass encroachment on both bacterial and fungal diversity as assessed by number of peaks detected by ARISA in soil samples ($p>0.05$, Figure 2). In both cases, however, only a small number of peaks were detected (0 to 6 for bacteria and 0 to 7 for fungi), which suggests that either many species did not differ in their amplicon lengths, or only the very most abundant species were detected. This non-significant trend was however confirmed by deeper investigation on diversity, this time using metabarcoding data, where diversity was calculated as Shannon or Simpson indices: there was no significant relationship between grass cover in a given quadrat and both bacterial and fungal diversity measured from soil samples taken in the middle of this quadrat (Figure S3).

Microbial community structure

Bacterial and fungal communities were very similar across the grass cover gradient, at least at high phylogenetic level. Bacterial communities were dominated by three phyla, representing 92% of all OTUs: *Proteobacteria* (46%), *Actinobacteria* (31%) and *Acidobacteria* (16%), of which the relative proportion remained unaffected throughout the gradient of grass encroachment (Figure S4, top). Fungal communities were dominated by the *Ascomycetes* phylum (86%), from which *Leotiomycetes* (45%) and *Eurotiomycetes* (23%) were the most abundant classes, and were also unaffected by the degree of grass encroachment (Figure S4, bottom), though a large number of OTUs could not be identified at the phylum level (22% of the total reads).

This broad analysis may however miss many taxonomical responses to grass colonization at a finer phylogenetic level. We therefore investigated the relationship between grass dominance and community structure at the OTU level, by means of a hierarchical modelling of species communities (HMSC), where metabarcoding read counts were the independent variables, the grass cover in the quadrat from which the soil sample community was characterized the dependant variable, and sample the random variable. Results showed that grass cover was significantly positively correlated ($p<0.05$) with the abundance of 5 OTUs, all belonging to the fungal kingdom (1 *Basidiomycete*, 4 *Ascomycetes*), and negatively with 3 OTUs, all belonging to the bacterial kingdom (Figure 3). The 5 fungal OTUs were attributed to the *Tremellaceae* (*Saitozyma podzolica*), *Dermataceae*, *Coniochaetaceae*, *Herpotrichiellaceae*, and *Teratosphaeriaceae* (*Devriesia sp.*) families; the 3 bacterial OTUs all belonged to the genus *Acidothermus*. These 8 OTUs were all abundant to very abundant, accounting altogether for 2% of all reads. Additionally, all ranked among the 15% most abundant OTUs, 4 of them being in the top 5% (*Devriesia sp.*, *Herpotrichiellacaeae*, and two *Acidothermus* species). Additionally, these OTUs were almost always the most abundant among the ones that shared the same phylogenetic assignment: there were in total 5 OTUs attributed to *Devriesia sp.*, 14 to *Coniochaetaceae*, 41 to *Herpotrichiellaceae*, 2 to *Saitozyma podzolica*, 12 to *Dermataceae* and 112 to *Acidothermus*.

Some OTUs were attributed to the *Glomales*, but were present in only one sample. The OTUs belonging to *Helotiales* were by far the most abundant of all microbes. The ones assigned to *Rhizoscyphus* or *Pezoloma ericae* were not correlated with the grass cover.

Microbial metabolic activity

Microbial activity, assessed using FDA assay, was not significantly correlated with grass dominance ($p>0.05$, Figure S5). The absorbance of the FDA solution at 690 nm oscillated between 0.3 and 1.2, with a high variability between samples.

Microbial C mineralization functions

We found that the diversity of C substrates used was not significantly correlated with grass cover ($p>0.05$, Figure S6). However, there was a significant, positive correlation ($p<0.05$) between grass dominance and the rate of C mineralization, with an estimate of 22, which corresponds to a 2.6 factor increase from pure heather to pure grass (Figure S7); moreover, this correlation was significant across all 6 C source types (Figure 4). The estimates of the linear model were the highest for amino acids (Pearson's R^2=0.85, an increase of a factor 2.7 throughout the gradient) and carbohydrates (0.75, 2.3), and to a lower extent, carboxylic acids (0.45, 1.8) and polymers (0.36, 1.6). Mineralization rate of phenolics and amines were not significantly affected by the grass cover (Figure 4).

Discussion

We investigated how replacement of the dominant plant affected microbial community structure and function, using heathland ecosystem as a case study. There, the dominant ericoid plant (*C. vulgaris*) is replaced by grasses (*M. caerulea*). Our hypothesis was that, as grass becomes dominant, i) litter quality improves, which should favour fast-growing species thriving on easily decomposable organic matter, and ii) ericoid mycorrhizal fungi should become less dominant. To test this hypothesis, we set up a field observatory of 13 heathland plots, varying in the levels of grass dominance, and organized them in a gradient. In each plot, we set up 3 quadrats, in which we measured plant cover (in order to determine local plant dominance) and took a soil sample from which we measured microbial metabolic activity, microbial taxonomic diversity, microbial community structure, as well as functional diversity and rates of C mineralization.

Microbial community structure was affected only at the level of some abundant OTUs

We found that a shift in vegetation had only limited impact on microbial community structure: microbial taxonomic and functional diversity were not significantly different, nor were the structure of the communities at high phylogenetic level, and the overall metabolic activity of microbes. This was surprising. We expected a change in litter quality to lead to a change in microbial community structure, since C availability is among the most important structuring factors for microbial communities (Fierer 2017). Increase in C availability has been for example shown to promote abundance of *β-Proteobacteria* and *Bacteroidetes* at the expense of *Acidobacteria* in a grassland ecosystem (Fierer et al. 2007), while the proportion of these phyla remained the same across the grass dominance gradient in our study. However, OTUs belonging to the genus *Acidothermus* were at the same time abundant and negatively correlated with grass dominance. This genus has been investigated for its potential to produce cellulases with high thermal stability, as well as for its thermophilic and acidophilic growth optima (Mohagheghi et al. 1986). This suggests that this genus is particularly sensitive to changes in soil microhabitat induced by replacement of shrubs by grasses.

The response of fungal OTUs to grass colonization was characterized by an increased abundance of black yeasts (*Heprotrichiellaceae*, genus *Devriesia*, and *Saitozyma podzolica*). More precisely, members of *Herpotrichiellaceae* are black yeast anamorphs known for being extremotolerant (Untereiner et al. 1995). The members of the genus *Devriesia*, often found as a plant endophytes (Crous and Groenewald 2011), are soil-borne heat-resistant black yeasts related to the genus *Cladosporium* (Seifert et al. 2004). Finally, the species *Saitozyma podzolica* is a frequent oleaginous basidiomycete black yeast commonly found in soils (Moreira et al. 2020), especially in heathlands (Op De Beeck et al. 2015). It has been several times associated with vegetation change, for example after mining activity, but also in human disturbed forests, where the abundance of this species was negatively correlated with the disturbance (Moreira et al. 2020). In that case the presence of grass was used as a proxy for disturbance. (Yurkov 2018) noted that this species is usually a good marker of acid, well-drained soils. Measurements in soils in nearby plots (data not shown) does however not support the evidence that acidity and soil drainage increased with grass colonization, so the reasons for increase in relative abundance of *S. podzolica* probably lie somewhere else. The two other OTUs that were significantly (and positively) affected by grass colonization belong to the *Dermataceae*, which are most often associated with roots of Ericaceous plants (Obase et al. 2009), and to the *Coniochaetaceae*, which are often plant endophytes or pathogens, and more numerous in extreme habitats (Chen et al. 2021).

Most of microbial OTUs, however, remained unaffected by grass colonization. Many phylogenetic groups known to be associated with easily decomposable C (such as genera *Penicillium* or *Trichoderma*) had the same relative abundance throughout the gradient, as well as oligotrophs such as *Mycena*, *Actinobacteria*, or *Deltaproteobacteria*, which confirms that there was no shift in the oligotrophic / copiotrophic ratio, contrary to what we hypothesized. This result implies that litter quality either did not change significantly across the gradient, or that it had no impact on the abundance of these species. Moreover, we expected at least a shift towards a replacement of ericoid mycorrhizal genera (*Rhizoscyphus*, associated with roots of ericoid plants) by arbuscular ones (*Glomus*, associated with roots of most grasses) (Read et al. 2004). The primer pair we used picked up only 60 reads for Glomales, which may be due to a poor performance at amplifying this phylogenetic group, and therefore we cannot draw conclusions on arbuscular mycorrhizal fungal (AMF) abundance. However, we definitely did not observe a drop in the relative abundance of *Rhizoscyphus* and *Pezoloma* as grasses became more dominant. On a side note, we identified no *Archaeorhizomycetes* sequences in our metabarcoding results, contrary to what was found in similar heathlands (Radujković et al. 2020), or even in samples taken less than a hundred meters away from two of the plots of this study (data not shown). Again, the primer pair used here could be the explanation, as ITS4 mismatches with *Archaeorhizomyces* sequences (Ihrmark et al. 2012).

It seems difficult to synthesize all of these contradictory data in a coherent picture. What is clear is that there is no evidence that grass colonization increased abundance of copiotrophic species, nor of species commonly associated with higher litter or soil quality. Instead, we see a community shift, where some abundant strains of extremotolerant bacterial species (*Acidothermus*) are progressively replaced by others fungal ones (black yeasts), and ericoid mycorrhizal fungi seem unaffected.

Possible influence of the 2018 drought

Heathland soil macrocosms taken from the same area were following ambient conditions measured at an ICOS ecosystem tower located on the same site (Rineau et al. 2019). The response of these 12 macrocosms to 2018 drought was very close to what we observed in the field. We have therefore precise measures of precipitation and of the top 10 cm soil moisture on that date. They show that the soil moisture was low (6% +/- 1 SE, n=36) despite re-wetting events before sampling (3 and 10mm on the 07/08 and 09/08, respectively), because it was preceded by a long 2-month period where there was no rain event (01/06-06/08). The community at the date of sampling is therefore likely affected by this low water availability. Drought is usually reported to select for Gram + bacteria (Firmicutes and Actinomycetes in particular) against Gram – ones (Proteobacteria, Verrucomicrobia and Bacteroidetes), that are less tolerant to stress (Fierer et al. 2003; Naylor and Coleman-Derr 2018). And indeed, we see that the Gram + / Gram - ratio based on the abovementioned bacterial phyla is twice higher in our samples than the ones reported by (Seaton et al. 2021) on a very similar upland heathland. This suggests that the proportion of Gram + bacteria in particular, and of extremophiles in general is higher in this study than what heathland soils may harbour outside of extreme events; but also, that community shifts may be larger in non-extreme weather conditions, as plant properties would proportionally contribute more to shaping soil microbe communities when selection by water availability is relieved.

Was our main hypothesis rejected because of wrong assumptions?

As our hypothesis was rejected, we may need to take a step back and evaluate the accuracy of our assumptions. The basis of our reasoning is that increase in grass dominance improves C availability. Literature supports the assumption that *M. caerulea* litter inputs brings higher amounts of more available C to soil than *C. vulgaris*, through both above (Van Vuuren and Berendse 1993; Certini et al. 2015) and belowground inputs (Aerts et al. 1992). However, we do not know exactly how this biomass interacts with organic matter that has been shaped by decades of dominance by ericoid shrubs. Indeed, litter mixes have different biochemical properties that influence each other's decomposition rate: their dynamics of decomposition is therefore non-additive (Gartner and Cardon 2004). Heathland ecosystem is no exception: *M. caerulea* litter degrades significantly slower under *C. vulgaris* than under itself (Certini et al. 2015). We therefore cannot rule out that the increase in available C coming from the grass litter is inhibited by some compounds originating from long-term accumulated heather litter in the organic topsoil, such as polyphenols (Kraus et al. 2003). The soil properties would then remain the same, and so would most of the microbial community. Moreover, the literature that we use as comparison to understand the effects of soil properties on microbial communities may represent a much more extreme case than grass colonization in our field sites. For example, (Fierer et al. 2007) amended their soil samples with large amounts (up to 800 g of equivalent C/m2/y) of a C source of high quality (sucrose), and (Zhou et al. 2021) with 120 µg/g of soil of glucose carbon. While litter input from a grass species differ from shrubs mostly by the relative abundance of structural compounds, with more crystalline cellulose, and less aromatic compounds (Certini et al. 2015). This improves litter quality, but to a much lower extent than glucose or sucrose, as polysaccharides need to be processed by hydrolytic and/or oxidative enzymes before being incorporated for microbial metabolism, which is energy demanding. Hence it appears logical to expect less drastic shifts in microbial communities in our study than in (Fierer et al. 2007) and (Zhou et al. 2021). Finally, the absence of response of the ericoid genus *Rhizoscyphus* to grass dominance could be caused by a slower necromass or propagule decomposition than other species (Lenaers et al. 2018), which would artificially maintain its relative abundance figures in metabarcoding surveys (since it is based on DNA which can be found in necromass and spores). Alternatively, that could be explained by the ability of these species to persist in soil as saprotrophs after their host disappeared (Read et al. 2004).

Functional responses to grass colonization were more clear-cut than structural ones

Contrary to microbial community structure, C mineralization functions responded significantly to the increase in grass cover. Even though the overall metabolic activity of the microbial communities remained the same, the mineralization rate of tested C sources increased by an estimated factor of 2.7 across the full range of grass cover. In other words, similar microbial assemblages showed almost three times more C mineralization potential under pure grass than under pure heather, and this was not because microbes were just more metabolically active, as shown by the results of the FDA assay. We cannot rule out, however, that the fungal OTUs favoured by grass colonization (belonging to black yeasts, *Dermataceae*, *Coniochaetaceae*) contributed to this higher activity. In fact, the number of read counts of *S. podzolica* was significantly correlated with the mineralization of D-cellobiose, α-ketobutyric acid, 2-hydroxybenzoic acid, and β-methyl-D-glucoside; and the number of read counts of the OTU belonging to *Devriesia sp*. was positively correlated with the mineralization rate of β-methyl-D-glucoside (data not shown). The mineralization rates may nevertheless result from more complex inhibitory or synergistic functional interactions.

Hence, the communities present in this heathland soil were adapted to these soil conditions, and responded to change in litter input by raising their ability to mineralize C. In particular, grass dominance stimulated mineralization rates of carbohydrates, amino acids, carboxylic acids, and polymers. The former three are characteristic of root exudates (Griffiths et al. 1998), and the latter of plant biomass. We therefore speculate that the increase in grass dominance stimulates C mineralization of root exudates, and to a lower extent, litter biomass; but that litter input is in too low quantity relative to the soil organic matter pool to significantly affect its composition, and therefore microbial community structure, before a decade or more. It also implies that the large-scale grass colonization we see in many North-Western European heathlands potentially results in significant increases in C mineralization rates, which has negative consequences for this ecosystem's C sequestration. However, this case study has been conducted in only one site, albeit a large one (287 500 m^2), and different responses may be observed in other heathlands because of site-to-site variability.

Methodological considerations

For practical reasons, samples could not be analyzed fresh, and had to be stored long-term at -20C. Such storage conditions usually do not alter microbial community structure (Wallenius et al. 2010). They are however known to affect soil enzyme activities (Peoples and Koide 2012), decreasing them by about 20-30% (Wallenius et al. 2010), likely through a decrease in viable microbial cells (Shishido and Chanway 1998). Nevertheless, since all samples have been stored the same way, the relative differences between samples may be less biased. The method used to measure the mineralization rates is also not without drawbacks: just like most in vitro soil enzyme assays, it measures only potential activities (because they are measured in optimal conditions of moisture, temperature and substrate availability); it is also influenced by soil water content and inoculum density (Preston-Mafham et al. 2002). The C mineralization and FDA results have therefore to be interpreted with caution. The responses may especially reflect those of bacteria, and may not fully represent field microbial communities. Still, they are apparently large and consistent enough to be detectable despite noise associated with methodology.

Conclusion

Our results show that microbial community structure is only moderately affected by grass colonization, with the exception of a limited number of abundant OTUs. However, this is not the case for community functioning: C mineralization potential significantly increases, almost by a factor 3 overall. This was especially clear for the substrates that were chemically related to root exudates, and to a lower extent the ones related to plant litter. This led us to speculate that the change in dominant plant increased soil C mineralization by microbes through a change in availability and nature of root exudates, and to a lesser extent litter input. Our study also points to some species of both bacteria and fungi who were responsive to such a change in plant traits, thereby extending our knowledge on the otherwise still mysterious ecology of *Acidothermus* bacteria and black yeast fungi. Taken altogether, these results show that a change in dominant plant traits may induce drastic functional changes in microbial communities despite having only a very minor effects on their diversity or community structure. Moreover, our study implies that grass colonization observed in many heathlands in Northwestern Europe leads to higher C mineralization rates, which has potentially negative impact on the C sequestration by this ecosystem.

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Figure captions

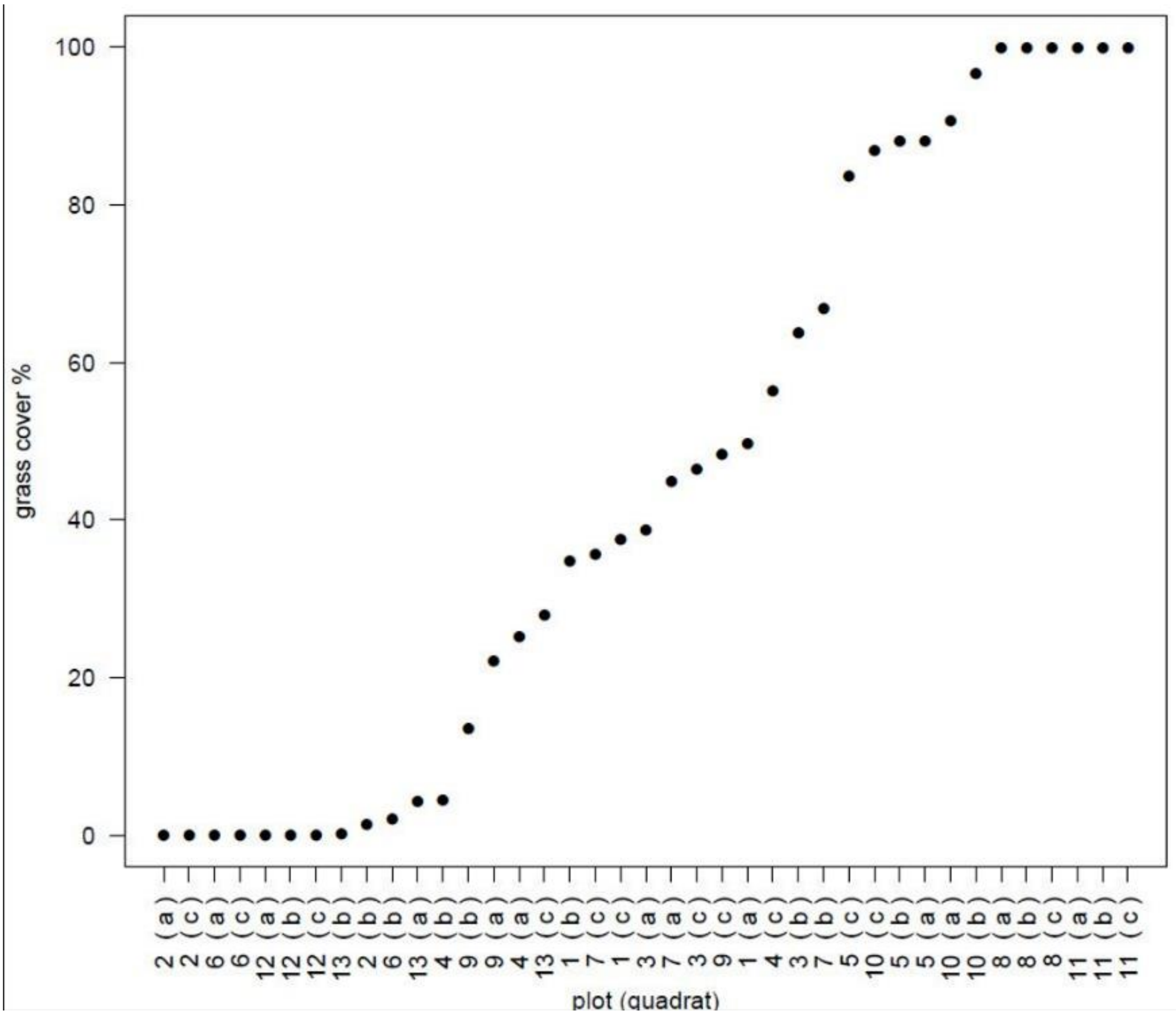

Figure 1. Grass cover (%) in each quadrat ((a), (b), (c)) of the 13 plots (1-13) of the gradient, sorted by increasing order. The heather cover is not represented here for clarity purposes but is the exact opposite of grass cover.

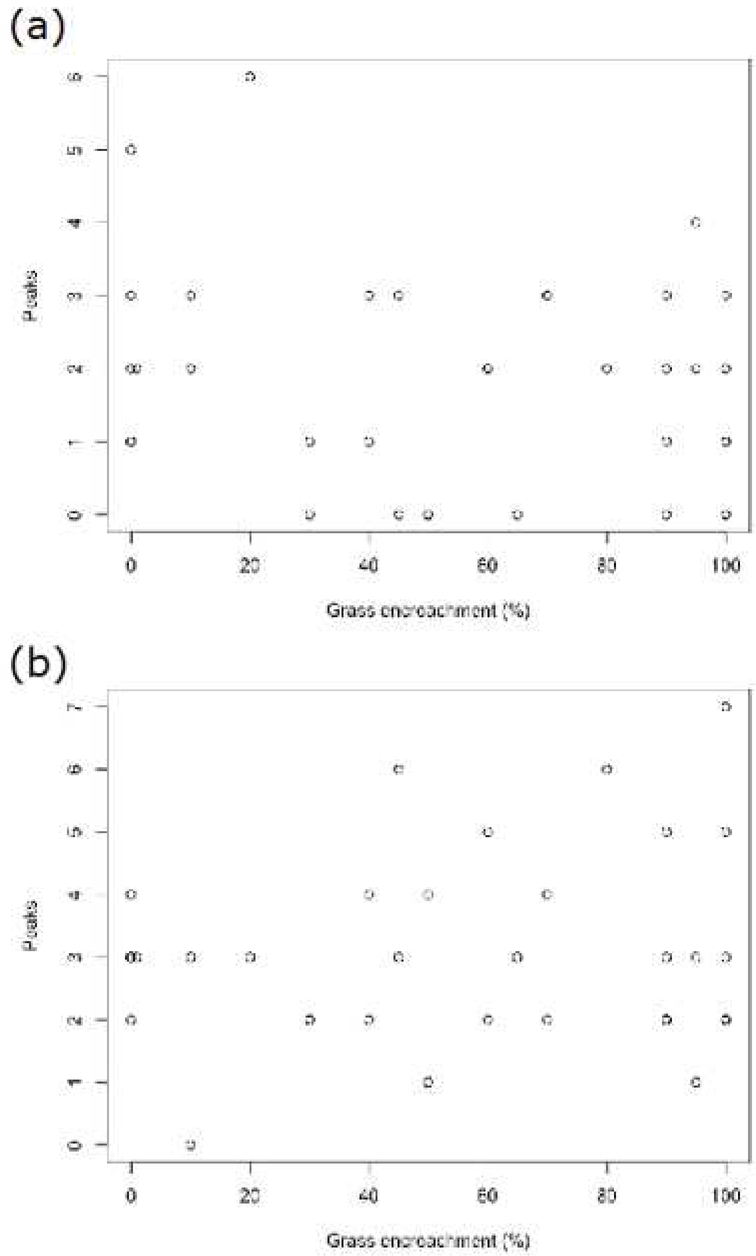

Figure 2: Effect of grass colonization on the bacterial (a) and fungal (b) diversity in function of grass encroachment. Diversity is expressed as the number of OTU-peaks in bacterial and fungal ARISA fingerprints.

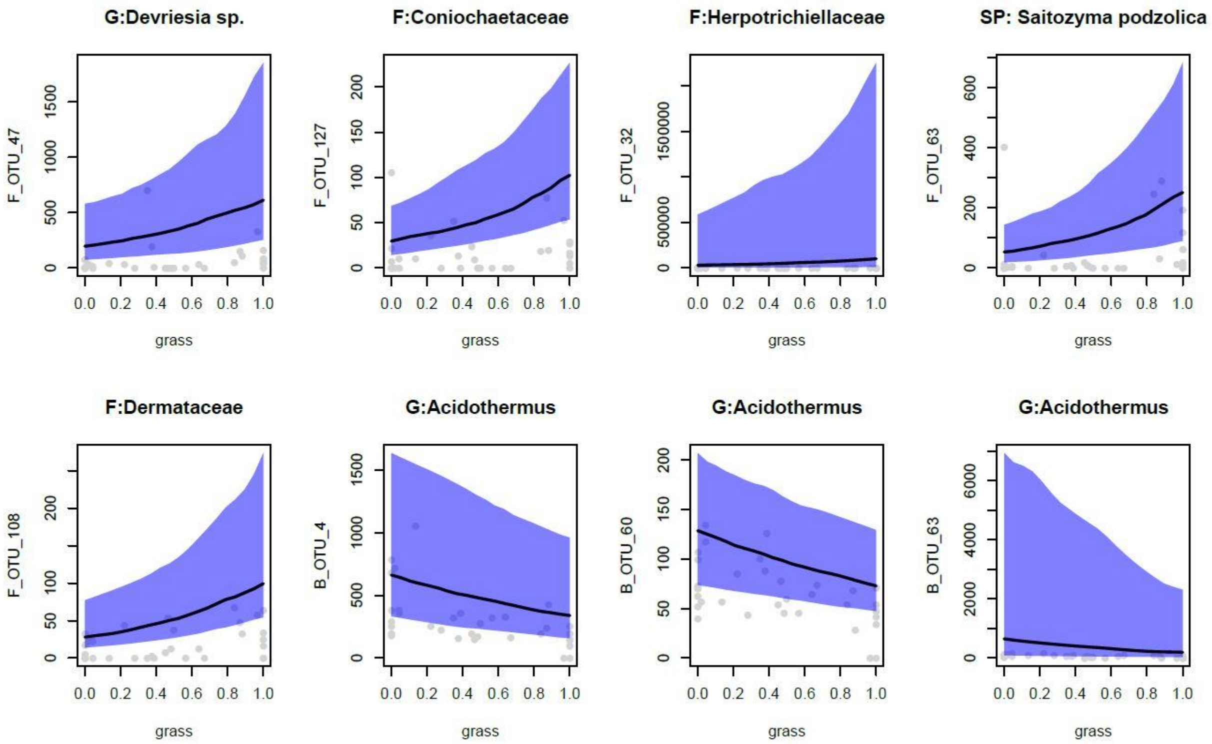

Figure 3: Effect of grass colonization on abundance of microbial species as predicted by hierarchical modelling of species communities. Only the OTUs for which estimates were significant in the model are shown ($p<0.05$). X-axis: grass cover proportion in the quadrat (1=100%). Y-axis: read counts. Black line: model predictions. Blue area: 95% confidence interval of the model. Grey dots: actual data. The title of the graph corresponds to the name of the OTU's best hit according to the BROCC software pipeline (Dollive et al. 2012)at the highest phylogenetic resolution possible (F: Family, G: Genus, SP: Species). The OTU ID appears as the title of the y axis of each graph (F: fungus, B: bacteria; the smaller the OTUs ID number, the more reads).

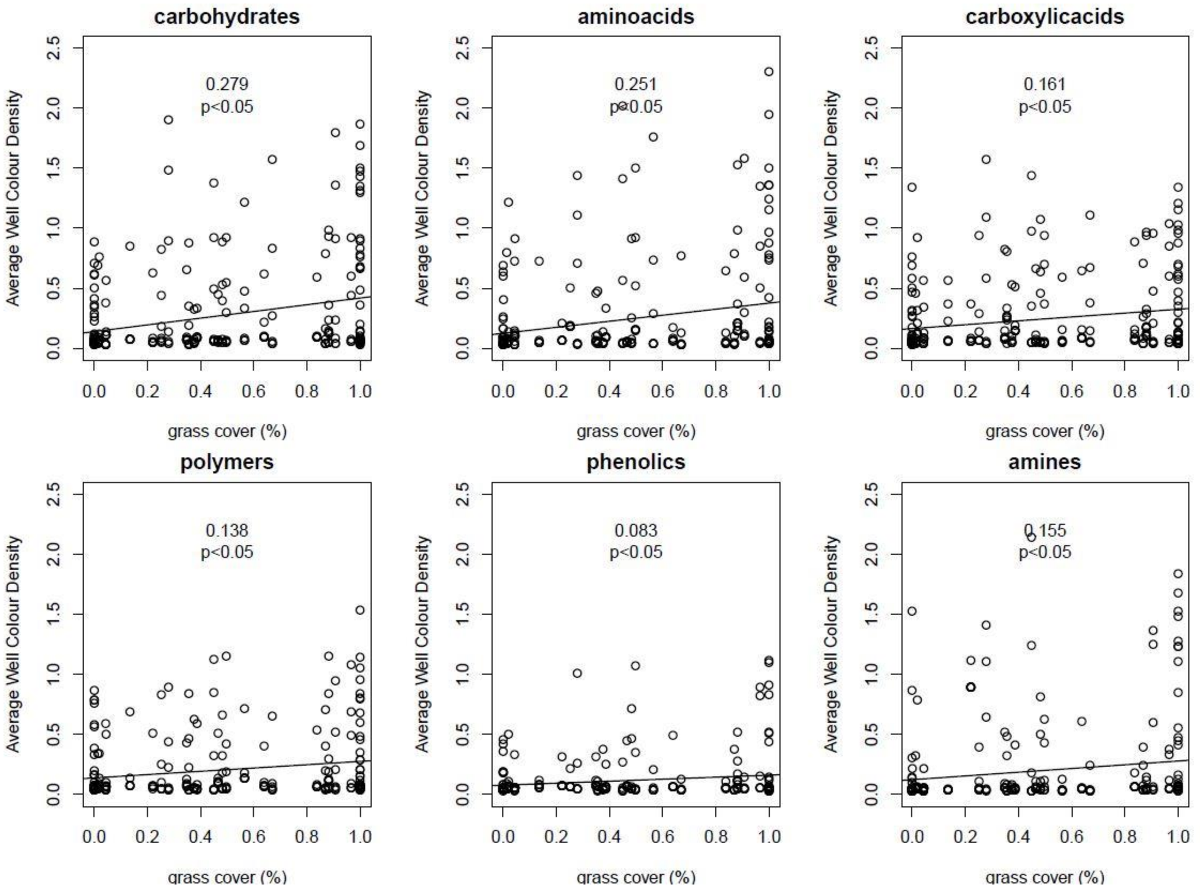

Figure 4. Relationship between grass cover (0=no grass, 1= 100% grass) and mineralization rate of six types of carbon substrates. The mineralization rate is measured spectrophotometrically as the reduction of a tetrazolium dye by the respiration of microbial cells having a single C substrate in a microplate well. The microbial cells have been inoculated from a soil sample. We used a linear model and Bonferroni correction to test the significance of the relationship between the two variables. Significant relationships are labelled with "p<0.05". The slope of the relationship is given above the significance level.

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Statements and declarations

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Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.