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# Mulching has negative impact on fungal and plant diversity in Slovak oligotrophic grasslands

Miroslav Caboň [*a], Dobromil Galvánek [a], Andrew P. Detheridge [b], Gareth W. Griffith [b], Silvia Maráková [a], Slavomír Adamčík [a]

**ORCID:**

Miroslav Caboň * - 1 https://orcid.org/0000-0002-2255-3816

Dobromil Galvánek - 1 https://orcid.org/0000-0001-9248-3318

Andrew P. Detheridge – 2 https://orcid.org/0000-0002-7472-9035

Gareth W. Griffith - 2 https://orcid.org/0000-0001-6914-3745

Silvia Maráková - 1

Slavomír Adamčík - 1 https://orcid.org/0000-0003-2156-5767

**Affiliations**:

[a]: Institute of Botany, Plant Science and Biodiversity Centre, Slovak Academy of Sciences, Bratislava, Dúbravská cesta 9, Bratislava 84523, Slovak republic

[b] : IBERS, Adeilad Cledwyn, Penglais Aberystwyth University, Aberystwyth, SY23 3DD, Wales, UK

* corresponding author. Tel.: 00421 2 5942 6108

E-mail address: miroslav.cabon@savba.sk

## Highlights

Overall fungal species richness and diversity do not change with management in oligotrophic grasslands, but mulching has a negative effect on richness and diversity of CHEGD fungi.

The effect of mulching on CHEGD fungi and on vascular plants is similar but these two groups have contrasting seasonal responses.

The results suggest that mulching affects vascular plant diversity directly by changing their reproductive and competitive ability, but fungal diversity is altered indirectly by changes in soil properties caused by decomposition of dead plant biomass.


## Abstract

Mulching (cutting of vegetation without removal of clippings) is used as a low-cost method for maintaining remote or abandoned grasslands in Slovakia. The likely consequence of mulching is seasonal nutrient enrichment resulting from decomposition of plant litter by saprotrophic organisms. The potential changes in biodiversity of the ecosystem caused by long-term application of mulching are to date only very poorly understood. In order to examine the impact of mulching on soil mycobiota, we compared six different grassland management regimes applied over nine years on a sub-montane oligotrophic *Nardus* pasture in the Central Slovakia. The diversity of soil fungi was assessed using DNA metabarcoding of the ITS2 regions of the nrRNA locus performed by Illumina MiSeq.

We focused on a particular group of macrofungi which is characteristic of traditionally managed and undisturbed European grasslands, and which are often the dominant soil fungi in these habitats. These are collectively known as CHEGD fungi (the acronym of the constituent taxa: Clavariaceae, Hygrophoraceae, Entolomataceae, Geoglossaceae and *Dermoloma*). We compared the relative abundance and diversity of CHEGD fungi with the total fungal and plant diversity. CHEGD fungi were dominant across all treatments. Although there were no statistical effects of treatments on total fungal richness and diversity, CHEGD fungi and vascular plants diversity and richness were lower on plots where mulching or no management were imposed, suggesting that such management regimes would have a negative impact on grassland fungi. However, no single treatment covered the total CHEGD diversity of the study, indicating that the localized use of mulching in addition to traditional managements can enhance overall diversity of grasslands in the area. Our results also suggest that the impact of mulching depends on the season when the grassland is mulched and it might be reduced by combination with other management treatments. The high relative abundance and sensitivity of CHEDG fungi in oligotrophic grasslands to management treatments makes them excellent indicators of grassland natural quality and is consistent with the ecological importance of this fungal group.

***Keywords:*** soil metabarcoding, managements, trophic interactions, functional diversity, biomass degradation, Clavariaceae, Hygrophoraceae, *Entoloma*, Geoglossaceae, *Dermoloma*

# Introduction

Grasslands are recognised as important European habitats highly dependent on agricultural practices (Halada et al. 2011). Grazing and mowing were common traditional management strategies applied in the Western Carpathians, but the dramatic decline of cattle numbers after the political changes in 1989 in Eastern and Central Europe resulted in frequent management cessation (Kanianska et al. 2014). Traditional management, which has maintained grasslands of high conservation value in the Western Carpathians, has become rare (Meyer et al. 2015). Mulching (cutting of vegetation without removal of clippings) is used as a low-cost method for maintenance of abandoned grasslands in Central Europe (Mašková et al. 2009). The purpose is to control scrub encroachment and establishment of trees, which is required for farmers to receive subsidy support from the Common Agricultural Policy of the European Union (Gaisler et al. 2013). Mulching differs from traditional managements (mowing and grazing) in that phytomass is not removed but rather left to decompose *in situ*. As a consequence, it can modify soil properties, for example contribute to maintain phosphorus (P) nutrition (Oelmann et al. 2017), reduce nitrogen (N) requirements (Qian et al. 2003) and/or stabilize carbon (C) sequestration (Werth et al. 2005).

The general purpose of mulching and various traditional management regimes on grasslands is the prevention of succession. Mulching has some similar effects to traditional cutting for hay or sheep/cattle grazing, since taller vegetation is removed, but their effects on functional diversity and community structure may be very different (Moog et al. 2002, Römermann et al. 2009, Doležal et al. 2011). Recent studies suggest that mulching does not substitute for traditional management treatments and may lead to plant diversity decline (Gaisler et al. 2019), change of local ant species composition (Wiezik et al. 2013), butterfly population decline (Schmitt, 2003) as well as a decrease in microbial biomass and microbial metabolic efficiency (Uhlířová, Šimek & Šantrůčková 2005). Mulching of biomass in grasslands of Central Europe is a relatively recent and regionally specific phenomenon that is poorly understood and urgently deserves a study because of possible consequences linked to ecosystem services and conservation concern.

Plant-driven changes in soil properties are strongly associated with the compositional turnover of fungi (Yang et al. 2017, Anthony at al. 2019, Oriol et al. 2019). Specific soil fungi play their particular roles in grassland ecosystems and plant species richness does not affect their diversity directly (Navrátilová et al. 2018). However, individual plant species have different associated endophytic or mycorrhizal fungi (Wearn et al. 2012), suggesting that compositions of fungal soil communities changes with changes in plant community structure. The functions of fungi in soil ecosystem are different from other organisms, for example under mowing management, they play a more important role in nitrogen (N) mineralization than bacteria (Li et al. 2017). Diverse fungal groups with different dispersal mechanisms are able, under changing environmental condition of newly forming soil, to establish diverse communities in a relatively short time (Detheridge et al. 2018). This makes soil fungi a potentially useful indicator group for the study of microbial responses to grassland management practices.

Among fungi occurring in semi-natural grasslands of Europe, macrofungi collectively referred as CHEGD fungi (an acronym of Hygrophoraceae, Entolomataceae, Clavariacaeae, Geoglossaceae and *Dermoloma*) are well known to be typically associated with undisturbed, unfertilised grasslands (Griffith et al. 2013). Natural abundance of $^{15}N$, $^{14}C$ and $^{13}C$ isotopes in basidiomata of Hygrophoraceae, as well as $^{13}C$ pulse label experiments, further suggest that these fungi are not saprotrophs but rather biotrophic endophytes and possibly mycorrhizal symbionts (Halbwachs et al. 2018). The metabarcoding analyses of fungal microbial diversity shown that CHEGD fungi are often the most abundant group in oligotrophic grasslands (Detheridge et al. 2018, Hay, Thorn & Jacobs 2019). Another clue to identify the trophic strategy of CHEGD fungi is proof that *Cuphophyllus* (formerly *Hygrocybe*) *virgineus* is a systemic endophyte of *Plantago lanceolata* (Tello et al. 2014).

The likely consequence of mulching is seasonal nutrient enrichment resulting from decomposition of plant biomass by saprotrophic organisms. Competitive and antagonistic interactions of saprotrophic microorganisms and changes in available nutrients likely reshape the fungal community structure of grasslands. We aimed to study changes in the fungal microbial diversity of Slovak oligotrophic grasslands caused by long-term (eight years) application of six different grassland management regimes. The results of fungal soil microbial diversity assessment by metabarcoding are compared with vascular plant diversity assessed during the ninth year of the experiment. In particular, we want to test the reliability

of CHEGD fungi as indicators of ecosystem changes in grasslands. We also aim to compare the response of CHEGD fungi to various mulching regimes with the responses of vascular plants.

## Materials and methods

### *Study site and sampling*

The study area was located in Central Slovakia (48°42′09.57″ N, 19°22′00.8″ E, 715 m a.s.l.), in the Poľana Mts., a mountainous area of volcanic origin. The experimental grassland area was traditionally maintained through extensive grazing by sheep and occasionally also by young cattle (heifers). It is located on south-west-facing slope with an inclination of 15°. Average daily temperature was 6.5 °C. Average annual precipitation was 852 mm (Cornes et al. 2018). The geological bedrock was classified as ryodacite tufas (http://mapserver.geology.sk/gm50js/) and the soil as a Cambic Umbrisol Endoarenic Skeletic (Sobocká, 2000). Organic carbon (Cox), humus, N and P contents of soil, sampled 7$^{th}$ November 2016, were measured for all individual plots. Organic carbon ranged between 21.58 to 36.92 g/kg, humus 37.2–63.65 g/kg, N 2.69–3.42 g/kg and P 1.59–3.79 g/kg. Only P content showed significant differences between treatments (Appendix A: Tab. 1). The vegetation of this sub-montane oligotrophic *Nardus* grassland was classified into the alliance *Violion caninae* Schwickerath 1944 (Hegedüšová-Vantarová & Škodová 2014).

The field experiment was established in 2009 with six management treatments: 1. grazing (GR) (positive control); 2. traditional mowing by scythe (clippings removed), combined with grazing (MOGR); 3. mulching in autumn (first half of September) (MUAU); 4. mulching in spring (second half of June – first half of July) (MUSP); 5. grazing combined with autumn mulching (MUGR) and 6. no management (NM) (negative control). Each treatment was represented by four randomly distributed replicates (Fig. 1). In total, 24 permanents plots were established, with 12 plots fenced to eliminate grazing. Plots outside the fencing were extensively grazed by sheep (herd of approximately 600–700 individuals, stocking density 0.3–0.4 LU/ha) from May to September each year and occasionally also grazed by young cattle (herd of 80–100 heifers) at the end of summer or in autumn (August to October). Cut biomass for mulching treatments experiments was evenly distributed across the plot whereas for mowing treatments the biomass was removed. Each plot was 2 × 2 m and surrounded by a 1-m buffer zone, maintained the same way as the corresponding core zone.

Presence and cover of vascular plants were sampled in 2 × 2 m plots using the percentage cover estimation. The diversity of vascular plants was recorded in May–June 2018. Soil cores for DNA metabarcoding were collected on 12 Oct 2017 and 3 May 2018. Five soil cores of 2.5 cm diameter to a depth of 10 cm were sampled per plot (approx. 120–170 g of dry soil), one from the center and four at a distance of 1 m along the diagonal axis, and samples were pooled. The organic soil horizon, stones, plant tissues and animal remnants were removed. Soil samples were stored at -80 °C.

***DNA amplification and sequencing***

Environmental DNA (eDNA) was extracted in three replicates following the modified cetyl trimethylammonium bromide (CTAB) protocol of Sagová-Marečková et al. (2008). The diversity of soil fungi was assessed by metabarcoding analysis of the internal transcribed spacer 2 region of the ribosomal DNA operon (ITS2 rDNA) using primers ITS3F, ITS4R (White et al. 1990). PCR amplification, library preparation and amplicon sequencing were performed by Illumina MiSeq using MiSeq reagent kit v2 by SEQme s.r.o. (Dobříš, Czech republic; www.seqme.eu), following standard protocols used by the company. Both autumn and spring samples were sequenced in a single Illumina run. Raw amplicon sequence data are deposited in NCBI as BioProject PRJNA691143 under accession numbers SAMN17282714–SAMN17282761.

***Sequence data processing***

Amplicon sequence data were processed using the SEED 2.0.1. pipeline (Větrovský, Baldrian & Morais 2018). Amplicons were shortened to 250 bp prior to pair-ending. Pair-ended sequences with 20% maximum difference and minimum overlap 20 bp were further analysed applying recommended settings and automatic chimera removing. Sequences with average phred score lesser than 30 were discarded.

Pair-ended sequences were processed as detailed in Detheridge et al. (2016) with the USEARCH clustering of molecular operational taxonomic units (MOTUs) at 98.5% identity and taxonomy assignment by the RDP Naïve Bayesian Classifier (Wang et al. 2007) against a database built from v 8.0 of UNITE (Abarenkov et al. 2010, UNITE community 2019). The most abundant sequences of each cluster were checked using Blastn against UNITE v 8.0

(Kõljalg et al. 2013) and GenBank databases. Non-fungal sequences were excluded from further analysis. Fungal amplicon abundances at each plot are listed in Appendix A: Tab. 2.

Ecological functions were assigned as described in Detheridge et al. (2018), with eight main groups: fungi associated with grasslands (CHEGD), plant mutualists forming arbuscular mycorrhiza (AMF), plant mutualists forming ectomycorrhiza (ECM), dark septate plant endophytes (DSE), parasitic fungi (PAR), pathogenic fungi (PAT), lichenized fungi (LICH), and saprotrophic fungi (SAP).

Special emphasis was given to grassland macrofungi associated with semi-natural habitats known as CHEGD fungi (Griffith et al. 2013). These include members of Clavariaceae (i.e. *Clavaria*, *Clavulinopsis*, *Ramariopsis*, *Camarophyllopsis* and *Hodophilus*), the genus *Hygrocybe* and related genera of Hygrophoraceae (*Cuphophyllus*, *Gliophorus*, *Neohygrocybe*), *Entoloma* (Entolomataceae), *Dermoloma* (Tricholomataceae), and members of the family Geoglossaceae (i.e. *Geoglossum*, *Glutinoglossum*, *Trichoglossum*). We also included genera of the 'green' earth tongue fungi *Microglossum* and *Thuemenidium* (Leotiaceae) because they were previously included in CHEGD fungi and the genus *Pseudobaeospora* (Tricholomataceae) because of its apparent affinity to grasslands with high conservation value (Adamčík, Ripková & Kučera 2007).

Occurrence of CHEGD MOTUs was transferred into a binary matrix based on a minimum threshold 0.01% of all amplicons per sample (two to four sequences per sample). Representative sequences of each CHEGD MOTU from both analyses were aligned within our CHEGD reference datasets built on relevant phylogenetic studies for Clavariaceae (Birkebak et al. 2016), Hygrophoraceae (Ainsworth, Cannon & Dentinger 2013, Lodge et al. 2013, Wang et al. 2018), Entolomataceae (Morgado et al. 2013, Morozova, Noordeloos & Vila 2014), Geoglossaceae (Schoch et al. 2010, Fedosova & Kovalenko 2015, Fedosova et al. 2017) and Tricholomataceae (Sánchez-García et al. 2021). For individual genera, the alignments were trimmed to ITS2 amplicon size and analysed by ML as fasta files using RAxML-HPC2 on XSEDE (8.2.12) (Stamatakis 2014) under the GTR + GAMMA model with 1000 bootstrap iterations. MOTU clustering was reconsidered based on inconsistencies between automatic clustering and accepted taxonomic concepts supported by the phylogenetic analysis (ML>75 is considered as significant support).

### *Statistical analysis*

Spring and autumn sequence data were pooled and analysed together as one entry per plot. Diversity indices (Inverse Simpson and Shannon) were calculated from relative abundance based on amplicon sequence data in PAST version 4.0 (Hammer, Harper & Ryan 2001). All other analyses of CHEGD fungi are based on presence/absence data at four plot replications (Appendix A: Tab. 3). Average percentage cover data were analysed for vascular plants (Appendix A: Tab. 4). Post hoc comparisons (Tukey´s test) were computed by Statistica 12 software. One-way PERMANOVA was computed using PAST version 4.0 (Hammer, Harper & Ryan 2001). Species composition of CHEGD fungi and of vascular plants were evaluated by Principal Components Analysis (PCA) using the program Canoco for Windows 5 (Šmilauer & Lepš 2014), with plant data logarithmically transformed. Non-scaled or row-scaled heatmap analysis was plotted under default settings in R version 3.6.1 (R Core Team 2019) using heatmap.2 function implemented in gplots package (Warnes et al. 2009).

## Results

### *Total fungal diversity assessed by metabarcoding analyses*

DNA was successfully purified, amplified and sequenced by Illumina MiSeq from all 48 samples (spring and autumn sampling of 24 permanent plots), resulted in an average of 66898 unpaired amplicons per sample. On average 21148 pair-ended fungal amplicons (ranging from 8293 to 33577) per sample were retrieved after quality check, chimera detection and elimination of non-fungal sequences. In total, 1191 fungal MOTUs were retrieved. More than half (666 MOTUs), representing 94.8% of all fungal amplicons were identified to family level or better. The average number of fungal MOTUs per treatment was similar across all samples (range 353–402), with species richness highest for MUSP and NM and no significant differences between treatments resulted in Tukey´s test . The highest diversity according to average Shannon and Simpson indexes showed MOGR to be the most diverse, followed by NM and GR, with lower values of all mulching treatments.

More than 80% of all amplicons were identified as Basidiomycota and Ascomycota (Fig. 2), with the former group being consistently more abundant. These two groups were nearly equally represented in GR and MOGR. Glomeromycotina had low relative abundance,

241 ranging from 0.4% to 1%. Blastocladiomycota showed a distinct increase of relative
242 abundance in NM plots (NM1, NM2, NM4).

243 All treatments showed similar general patterns in relative abundance of groups defined
244 by ecological function (Fig. 3). CHEGD fungi were the most abundant functional group in all
245 samples, ranging from 28.8% (NM) to 54.1% (MUAU). On average, NM had the highest
246 relative abundance of ECM (8.7%), PAR (1.1%) and SAP (7.4%). DSE showed an affinity to
247 traditional management with more than 50% of all their amplicons retrieved from GR and
248 MOGR.

249 ***Diversity of CHEGD fungi***

250 We identified 121 MOTUs belonging to the CHEGD fungi: 51 Clavariaceae, 23
251 Hygrophoraceae, 30 Entolomataceae, 10 Geoglossaceae, four Leotiaceae and three
252 Tricholomataceae (Appendix A: Tab. 3). Clavariaceae consistently had the highest species
253 richness, followed by (depending on treatment) Hygrophoraceae or Entolomataceae (Table 2).
254 Also frequent were Geoglossaceae with at least three MOTUs present in all treatments. There
255 were no significant differences in overall CHEGD diversity and richness among treatments,
256 but some individual groups showed differences. NM had significantly lower Entolomataceae
257 (similar to MUAU and MUSP), and significantly higher Leotiaceae representation (similar to
258 MUSP and MOGR). Geoglossaceae were significantly more abundant at MUSP and less
259 abundant at MORG and MUGR.

260 There were no significant differences in relative abundance of individual groups of
261 CHEGD fungi nor their total diversity between treatments (Table 2). In terms of relative
262 sequence abundance, Hygrophoraceae were dominant, ranging from 24.6% (NM) to 42.4% of
263 all fungal amplicons across all treatments. The second most abundant group were, depending
264 on treatment, Clavariaceae or Leotiaceae. Both groups exhibited reduced relative abundance
265 in NM plots, and abundance of Clavariaceae was additionally reduced on MUSP plots.
266 Entolomataceae had higher relative abundance in traditional managements (GR and MORG)
267 and was lower in NM.

268 Within the dominant Hygrophoraceae family, the most abundant species were
269 *Neohygrocybe nitrata,* with average relative abundance per treatment 10.81%, followed by
270 *Hygrocybe chlorophana* (5.96%) and *H. punicea* (5.38%). These three species were the most

abundant taxa across the whole experiment, representing >20% of all the fungal sequences obtained. The most abundant MOTU of Clavariaceae was *Camarophyllopsis schulzeri* (1.53%), of Geoglossaceae *Geoglossum barlae* (1.97%), of Leotiaceae *Microglossum* sp. 2 (1.18%), the most abundant Entolomataceae MOTU was *E.* cf. *bloxamii* (0.3%), of the genus *Dermoloma* was *D.* sp. 2 (0.32%) and there was only one MOTU of the genus *Pseudobaeospora*, *P. pyrifera* (0.02%).

NM and MUSP also showed the lowest total CHEGD species richness per treatment (Table 2). In addition, both Inverse Simpson and Shannon index showed that NM and two mulching treatments (MUSP and MUAU) have the lowest diversity of CHEDG fungi. The relative proportion of total fungal and CHEGD richness was highest in NM and was also high in MUSP (Appendix A: Fig. 1). GR showed the highest (and consistent at all plots) proportion of CHEGD fungi compared to total fungal richness.

There was no single CHEGD MOTU that occurred in all 24 research plots, but 40 MOTUs were recorded in at least one plot of each treatment (Appendix A: Table 3, Appendix A: Figs. 3–4). Ten CHEGD MOTUs were recorded only from a single treatment and nine of them were only present in a single plot.

***Plant diversity***

We recorded 97 taxa of vascular plants. The total species richness per treatment varied from 61 to 76 and it was highest in grazed treatments (GR, MUGR). The average richness per plot was higher in GR, MOGR and MUSP treatments and was significantly lower in NM. Both Shannon and Inverse Simpson indices clearly showed NM as the least diverse treatment (Table 3, Appendix A: Fig. 2).

Plant richness was broadly correlated with CHEGD richness, showing consistently high taxon numbers for grazing. Plant species diversity decrease in the NM treatment was even more apparent than in CHEGD fungi.

***Comparison of fungal and plant communities between different treatments***

Pairwise comparison of treatments based on presences of CHEDG fungal MOTUs on individual plots (Table 4) showed the only significant difference in community structure between GR and NM (PERMANOVA $P<0.05$). The same pairwise analyses of plant

community structure revealed NM as significantly different from all other treatments except of MUSP and the only other significant difference show MUAU and MUSP.

The PCA analysis comparing all treatments in the ordination space (Fig. 4A) revealed similar community structure of CHEGD fungi of the NM and MUSP treatments. These two treatments were correlated with presence of *Hygrocybe citrinovirens*, *H.* sp. 1, *Entoloma prunuloides, Neohygrocybe ovina*, *Pseudobaeospora pyriforme* and *Trichoglossum* cf. *walteri*. The traditional treatments MOGR and GR were placed together in the ordination. GR was correlated with presence of *Clavaria* sp. 3, *Hygrocybe insipida*, *H. phaeococcinea*, *Microglossum* sp. and *Ramariopsis* sp. 8. MOGR was correlated with *Clavulinopsis* sp. 1, *Entoloma griseocyaneum* and *Entoloma* cf. *bloxamii*. MUAU was grouped with MUGR and they were correlated with presence of three Clavariaceae MOTUs: *Camarophyllopsis* sp. 1, *C.* sp. 2 and *Clavaria fumosa*.

The PCA of vascular plant cover showed an isolated position of NM (Fig. 4B). The first axis was correlated to the left with the gradient of managed *Nardus* grasslands represented by the species *Festuca ovina, Pilosella officinarum, Lotus corniculatus* or *Euphrasia rostkoviana*. To the right was the first axis correlated with unmanaged plots (NM) represented by tall species typical for such habitats e.g. *Avenula praeusta* and various woody plants e.g. *Pinus sylvestris, Picea abies* that would otherwise be controlled by mowing, mulching or grazing. The second axis represents the gradient from the low grazed grassland vegetation (GR) with presence of small species like *Genista pilosa* or *Danthonia decumbens* to the taller vegetation of MUAU defined by presence of species *Briza media* or *Ranunculus bulbosus*.

The hierarchical clustering based on relative abundance (amplicons per treatment sequence count) showed the isolated position of NM, whereas MUSP and MUAU were clustered together and all grazing treatments including MUGR form the other cluster (Appendix A: Figs. 4). This hierarchical clustering based on fungal amplicon relative abundances did not agree with PERMANOVA and PCA of CHEGD fungi that analysed only presence/absence data. It should be noted that within the hierarchical clustering, the treatments are found in three groups based on management style (mulching, grazing and no management).

# Discussion

### *Plant and microbial diversity changes with management*

The current biological diversity of grasslands in temperate areas of the Northern Hemisphere is the result of longstanding traditional practices of stock-moving and grazing. In this study, we treated GR and MOGR as positive controls, NM as a negative control and we tested the hypothesis that mulching can be used as an alternative treatment to maintain the diversity and community structure of grasslands. Our study showed similar overall patterns for richness and diversity of vascular plants and CHEGD fungi (Appendix A: Figs. 1–2). NM treatment had the lowest richness and diversity of both plants and CHEGD fungi and it was clearly different from other treatments. This is in agreement with previous studies demonstrating decline of general biological diversity in abandoned grasslands (Mariott et al. 2004, Öckinger, Eriksson & Smith 2006). In contrast to the conclusions of Tälle et al. (2016), we did not find that grazing had a more positive effect than mowing on the diversity of vascular plants and CHEGD fungi in grasslands.

The traditional managements (GR and MOGR) were similar to each other and more diverse in both CHEGD and vascular plants than other treatments. There was a contrasting effect of mulching season on the community structure of fungi and plants. While spring mulching (MUSP) had an effect on CHEGD fungi similar to NM and dissimilar from other treatments (Fig. 4A), the plant community structure was more strongly affected by autumn mulching (MUAU) (Fig. 4B). Some studies have found that the amount of plant biomass left at the end of the season is more important in determining yields in the following year than is the amount removed by grazing during the season (Willms, Smoliak & Bailey 1986). Community turnover of fungi is more similar to plant responses than to bacterial communities (Sayer et al. 2013, Cassman et al. 2016, Egan et al. 2018), and depends only weakly on edaphic factors (Detheridge et al. 2018). The similar responses of plants and CHEGD communities to management regimes can be explained by plant-driven changes in soil properties (Yang et al. 2017, Oriol et al. 2019).

The effect of plants on fungal and microbial diversity and community structure can be indirect. Navrátilová et al. (2018), for example, found no direct correlation between plant and soil microbial diversity. Whilst various grassland management regimes affect plants by changing their reproductive and competitive abilities (Binet et al. 2016), fungi may be more

influenced by available biomass and nutrient availability (Detheridge et al. 2016). As a consequence, the global plant alpha diversity patterns in temperate grasslands are poorly related to those observed for soil microbial groups, but plant beta diversity (compositional dissimilarity between sites) is significantly correlated with the beta diversity of bacterial and fungal communities (Prober et al. 2015).

Cessation of stock-moving/grazing causes either dominance of individual herbaceous plants or succession to scrubland, with both resulting in lower diversity of plants (Binet et al. 2016, Oriol et al. 2019). This is also in agreement with our results (Fig. 4B). The increased input of cut biomass causes an increase in soil C:N ratio or changes in availability of some essential elements that are not beneficial to plant diversity maintenance (Xiong et al. 2016, Oriol et al. 2019).

There is a negative correlation between nitrogen levels and diversity of some functional fungal groups, including CHEGD fungi (Detheridge et al. 2018, Halbwachs et al. 2018). The changes in the abundance and diversity of CHEGD fungi seen between traditional managements and mulching experiments in this study may be the result of increased N levels due to elevated N input from cut vegetation (Fang, Xie & Zhang 2007). We found no significant differences in N content between treatments (Appendix A: Tab. 1) but our measurements are all from a single timepoint. Soil N can change seasonally in response to plant uptake and mobilisation of N by soil microbes but it is likely that mulching, especially in spring when there is high competition for N, would result in a transient increase which would dissipate rapidly as N release from plant debris was taken up by plants and soil microbes (Hooper & Vitousek 1998; Jackson et al. 1988). The contribution of plant residues to available N is linked to the C:N ratio of decomposed plant biomass, and this depends strongly on plants species (Chen et al. 2014; Hooper & Vitousek 1998).

NM and MUGR had significantly reduced P content compared to MOGR and MUAU, but our analyses of plant and CHEGD fungal community structure (Figs. 6 and 7) did not support any similarities linked to P content. The less distinct CHEGD diversity loss and richness of autumn mulching (MOGR, MUGR) was probably also linked to reduced hay nutrition quality of biomass under delayed treatment (Klink van et al. 2017). ~~Fungi may play an important role in N mineralization in grassland ecosystems (Li et al. 2017).~~

Community structure of CHEGD fungi analysed by hierarchical clustering showed different patterns for presence/absence data and relative sequence abundances (Appendix A: Figs. 3–4). The presence/absence clustering was similar to PCA analysis in this study that was also based on the same dataset and it showed three pairs: NM and MUSP, MUGR and MUAU and GR and MOGR. The clustering based on relative abundance grouped treatments into three sets, i.e.no management (NM), grazing (GR, MORG, MUGR) and mulching (MUAU and MUSP).

Amplicon numbers and relative abundance may be influenced by a number of factors including GC contents, variation in nrRNA operon copy number, and the length of the ITS2 spacer region (Fonseca, 2018; Lofgren et al., 2019) but these will be consistent within a given experiment and thus not affect comparisons between samples. Our hierarchical clustering analysis grouped treatments based on grassland management and suggested that a dataset based on multiple, carefully selected MOTUs of several phylogenetic lineages, based on their ecology, can yield reliable results (Appendix A: Figs. 3–4).

***CHEGD community interactions***

Egan et al. (2018) suggested that important functioning of soil microbiota may only be detected at lower taxonomic levels. Here we demonstrate that CHEGD fungi are a reliable group to monitor management effects in grassland ecosystems, and especially relevant since they comprise the majority of the fungal OTUs found in these soils. The high relative abundance of these fungi in all treatments is consistent with previous grassland fungal microbial community studies using different metabarcoding loci (Detheridge et al. 2018, Hay, Thorn & Jacobs 2019). The response of CHEGD species richness to management treatments was very different from that of total fungal richness, for example CHEGD MOTUs declined but total fungal MOTUs increased in NM (Fig. 3, Appendix A: Fig. 1).

The arbuscular mycorrhizal fungi (Glomeromycotina) are often considered to have special importance for grassland environments (Koziol & Bever 2017). In our experiment, they were represented by a low relative abundance compared to CHEGD fungi, as has been reported in various other studies using a range of metabarcoding approaches (Geml et al. 2014, Jumpponen & Jones 2014, Detheridge et al. 2018) but it is possible that despite being present at low levels in terms of biomass that they are highly active.

Ectomycorrhizal fungi, which dominate the soils of forest habitats where relevant host trees are present (Wei, Song & Jia 2020), were also detected in some plots within our experiment. In NM plots, saplings of ectomycorrhizal host trees (*Pinus*, *Picea* and *Betula*) established as the result of succession. However, the relative abundance of ECM fungi was less than 9%, suggesting that colonization of tree roots by ECM fungi was still at a very early stage and ecologically these fungal communities are still dominated by fungi from the pre-existing grassland communities. Toju, Sato and Tanabe (2014) hypothesised that ECM alter other components of the soil ecosystem where they are present but we did not find evidence to support of this hypothesis here, since CHEGD fungi remained the dominant group in NM plots. ECM fungi were also detected at lower levels (<5%) in some other plots, presumably due to the presence of roots from adjacent trees(Kageyama et al. 2008) or presence of host tree seedlings (Lynch & Thorn 2006). However, here too we found no effect of ECM on communities of CHEGD fungi.

***Conservation importance of CHEGD fungi metabarcoding***

Our study confirmed that metabarcoding analysis of CHEGD fungi can result in much higher CHEGD species counts than field surveys (Griffith, Cavalli & Detheridge 2019). 'Traditional' field surveys based on fruitbody collections are highly influenced by seasonality and weather conditions. Our survey included only two visits of the sampling area (October 2017, May 2018), during which we collected only one *Hygrocybe* species, one *Ramariopsis* species and one *Clavaria* species. The paucity of fruitbodies was probably due to lack of rain and humidity in the days preceding our visits. Recording of CHEGD fruiting bodies is currently the main method for assessment of the conservation value of grasslands (Bosanquet et al. 2018). However, even with careful planning and repeated visits on collecting sites, fruitbody data yields fewer species in total and per site, and had larger variance in site richness compared to the metabarcoding approach (Frøslev et al. 2019).

The system for scoring grassland fungi is often based only on the presence of *Hygrocybe* species (Griffith et al. 2013), including other Hygrophoraceae genera recognised by recent phylogenetic studies and previously classified in this genus (Lodge et al. 2013). From our eDNA analyses, we identified a total of 23 Hygrophoraceae MOTUs which correspond to *Hygrocybe* in the traditional broad sense. This number is higher than the best Slovak *Hygrocybe* site scored so far based on a field survey (Adamčík & Kautmanová 2005). Based on Hygrophoraceae species number, our small research area of 16 × 24 m is placed in a

category of international importance (Boertmann, 2010). Clearly this scoring system has to be reconsidered and adapted to eDNA metabarcoding datasets (Griffith, Cavalli & Detheridge 2019).

One usual feature of our plots was the high abundance of *Neohygrocybe nitrata*. In western Europe, where CHEGD fungi have been more intensively studied, this species is very rare, and only very occasionally found in eDNA metabarcoding studies (Griffith et al., 2019). It is globally rare, being classed as Vulnerable by IUCN (Jordal, 2019). It is however more commonly recorded in Scandinavia (https://www.gbif.org/species/2538440), possibly indicating that this species favours high altitude/latitude habitats with very low winter temperatures.

Metabarcoding can also recover cryptic diversity of fungi, since sequence-based identifications are more accurate than morphological ones (because of phenological variation), but the species delimitation depends on reliable reference sequence database which can be problematic in the case of closely related taxa (Rees & Cranston 2017). Our study shows how insufficient taxonomic knowledge in combination with seasonality and cryptic diversity affects the results of field fruiting body-based surveys. Many field surveys reported *Hygrocybe* (in a broad sense) and Entolomataceae as the richest and the dominant grassland fungi (Newton et al. 2003, Genney et al. 2009, McLay, 2016). However, our study detected Clavariaceae as the richest group of CHEGD fungi, in agreement with other studies dealing with metabarcoding of soil fungi in pastures (Marí et al. 2020) and even in successional agricultural grasslands which were previously tilled (e.g. Lynch & Thorn 2006). The total MOTU richness of Clavariaceae recovered in our study was more than twice that of Hygrophoraceae and more than 50% higher than that of Entolomataceae. The majority (79%) of Clavariaceae MOTUs were not identified to species rank, in contrast to only 28% of Hygrophoraceae MOTUs. This is due to poor knowledge of diversity and systematics of the group. Phylogenetic study of agaricoid Clavariaceae members of the genus *Hodophilus* revealed much higher diversity of agaricoid members and suggests urgent need of taxonomic research of clavaroid lineages (Adamčík et al. 2020). Cryptic diversity, low mycelial biomass and difficulties with identification due to taxonomic problems are probable reasons why Clavariaceae were previously overlooked or underrepresented in field surveys.

In this study, we did not recover any sequences of *Hodophilus* spp. a genus of the family Clavariaceae with currently 16 well-defined species in Europe (Adamčík et al. 2020).

The genus *Dermoloma* was represented only by two MOTUs in our study, but the diversity of the genus in Europe is higher than 15 species (Sánchez-García et al. 2021). The genus *Pseudobaeospora* represented in our study by a single MOTU has 20 accepted species known from Europe (Adamčík & Jančovičová 2011). Absence or low representation of some taxomomic groups typical for grasslands indicates that there might be higher CHEGD community variation between different and distant grassland habitats. Members of Hygrophoraceae have probably a special function in grassland ecosystems. Their relative abundance is very high compared to other groups and 25 MOTUs detected in this study represent a relatively high proportion of the known diversity of the group which includes approximately 50 species in *Hygrocybe* s.l. in Northern Europe (Boertmann, 2010).

Several of the CHEGD species recorded during our study are known to be rare and are included in national Red Lists of several countries (https://www.nationalredlist.org), e.g. *Camarophyllopsis schulzeri*, *Cuphophyllus flavipes*, *Entoloma prunuloides*, *Microglossum olivaceum*, *Neohygrocybe ovina* or *Ramariopsis crocea*. Some rare species were correlated with MUSP and NM, for example *Hygrocybe citrinovirens*, *N. ovina*, *E. prunuloides*, *Pseudobaeospora pyrifera* and *Trichoglossum* cf. *walteri*. All the above-mentioned species occurred on all six or at least five treatments and it seems that some of them may benefit from available plant biomass substrate. However, in the long term, the change of ecosystem to forest dominated by ECM trees will probably cause their decline. The research area is situated in a large pasture maintained by traditional forms of management for a long time and this has probably played an important role in the relatively high diversity of CHEGD fungi in all research plots and insignificant decline of richness and diversity with mulching.

## Conclusion and future perspectives

Mulching is not a suitable substitute to replace traditional managements and maintain natural ecosystems of oligotrophic *Nardus* grasslands. Total soil fungal richness of mulching treatments was lower than in traditional and no-management treatments, while vascular plant richness of mulched treatments was similar to traditional treatments. However, even mulching and no-management treatments contributed some unique CHEGD fungi, and some other CHEGD species increased under these treatments. This suggests that combining of all the management regimes within the area would enhance overall levels of CHEGD diversity in these grasslands.

CHEGD fungi proved to be a reliable group to explain the impact of management on changes in soil ecosystem. They showed similar richness patterns to vascular plants, with a decline in NM and an increase in GR. CHEGD fungi showed a more distinctive effect of mulching than vascular plants when compared to traditional treatments. The timing of mulching had contrasting effects on plants and fungi, with spring mulching (MUSP) affecting CHEGD fungi diversity (relative to traditional treatments) and autumn mulching (MUAU) having a greater effect on plants. We hypothesize that changes in fungal community structure and functional group representations may be induced by changes in soil chemistry driven by decomposition of biomass. Future studies should focus on correlation of fungal soil diversity with levels of bioavailable essential elements.

Mulching (often referred to as grasscycling) is also widely practised in amenity grassland (Harivandi & Gibeault 1999, Hartin, Henry & Harivandi 2001), as evidenced by the many varieties of "mulch mower" that can be purchased. Often it is claimed that the return of nutrients is beneficial to the sward (e.g. https://wildseed.co.uk/page/management-of-lawns). However, the harmful effects of eutrophication are not appreciated by the wider public and the evidence from our study suggests that such policies should be reconsidered since they cause reduction in the diversity of both soil fungi and higher plants.

Our study demonstrated high relative abundance of some CHEGD fungi in oligotrophic grassland and our ordination analyses showed interesting links of individual fungal MOTUs to different treatments. This information strongly suggests an important role of CHEGD fungi for the function of oligotrophic grassland ecosystems.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at XXXXX.

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# Figures

Fig. 1. Study design showing arrangement of permanent plots and sampling

Fig. 2. Stacked bar chart showing relative abundance of the different fungal phyla at the different treatments (mean of four plot replications).

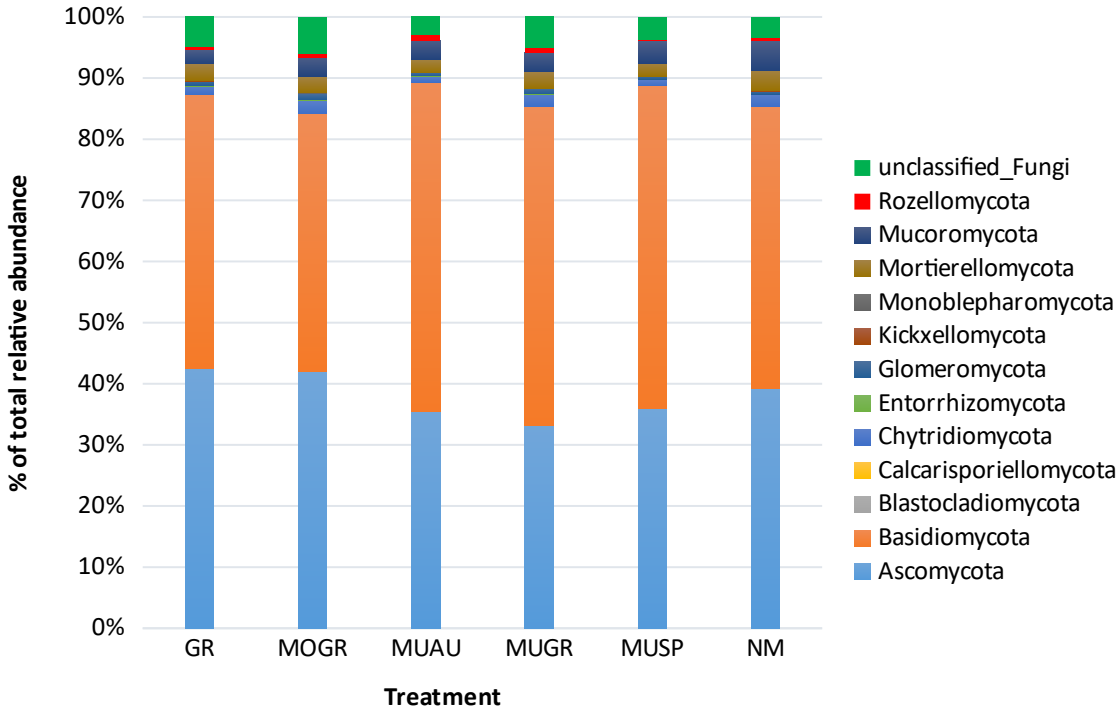

Fig. 3. Stacked bar chart showing relative abundance of the different ecological functional groups at the different treatments (mean of four plot replications). Ecological functions: SAP - saprotrophic fungi, PAT - pathogenic fungi, PAR - parasitic fungi, ECM - plant mutualists forming ectomycorrhiza, DSE - dark septate plant endophytes, AMF - plant mutualists forming arbuscular mycorrhiza, LICH - lichenized fungi, CHEGD – fungi associated with grasslands.

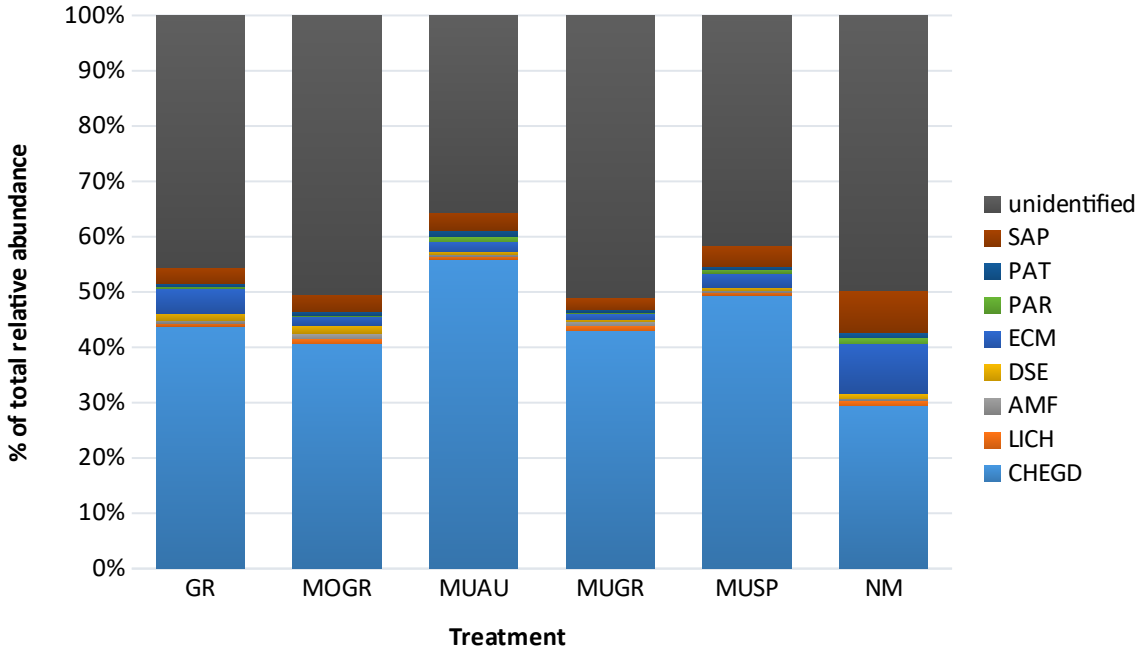


Fig. 4. Community structure of CHEGD fungi and vascular plants analysed by PCA. (A) Ordination diagram of CHEGD fungi based on cumulative numbers of MOTU presences at four plot replications per treatment (Appendix A: Tab. 3). (B) Ordination diagram of vascular plants community based on data are represented as average percentage cover of the four replications (Appendix A: Tab. 4).

893 Table 1. Fungal relative abundance, MOTU richness and diversity within the treatments.
894 Given values are averages of spring and autumn sampling from four replicate plots per
895 treatment. Values in parentheses are standard errors.

| | GR | MOGR | MUAU | MUGR | MUSP | NM | Average all plots |
|---|---|---|---|---|---|---|---|
| **Fungi identified to family [%]** | 94.7 (2.4) | 94.0 (1.8) | 94.8 (3.1) | 95.1 (1.2) | 95.2 (2.9) | 95.1 (1.9) | 94.8 (2.3) |
| **Fungi identified to genus [%]** | 94.3 (2.7) | 93.4 (1.7) | 94.6 (3.2) | 94.7 (1.2) | 94.9 (3) | 94.8 (2) | 94.4 (2.5) |
| **Fungi identified to species [%]** | 86.7 (5.2) | 84.2 (4.3) | 89.5 (4.8) | 88.6 (2.5) | 90.6 (4.8) | 89.3 (2.8) | 88.2 (4.7) |
| **Shannon Index** | 3.88 (0.49) | 4.21 (0.55) | 3.49 (0.73) | 3.71 (0.62) | 3.52 (0.66) | 4.05 (0.6) | 3.81 (0.67) |
| **Inverse Simpson Index** | 17.84 (12.43) | 23.59 (13.05) | 12.03 (9.09) | 13.02 (11.48) | 11.25 (13.58) | 21.88 (13.17) | 16.6 (13.15) |
| **Average fungal MOTU richness** | 353.1 (47.2) | 372.3 (83.9) | 358.1 (56.5) | 385.3 (54.6) | 402 (46.8) | 401.5 (74.5) | 378.7 (65.1) |

896

Table 2. MOTU richness / relative abundance of individual groups of CHEGD fungi per treatment. Given values are averages of spring and autumn sampling from four replicate plots per treatment. In the last three rows; Inverse Simpson index, Shannon index and average of CHEGD MOTU richness are followed by standard errors in parenthesis. If there are any statistically significant differences between treatments resulted from Tukey´s test, they are are labelled by lowercase letters .

| | **GR** | **MOGR** | **MUAU** | **MUGR** | **MUSP** | **NM** | **Average all plots** |
|---|---|---|---|---|---|---|---|
| **Clavariaceae** | 18.3/6.7 | 22.8/7.8 | 21.5/6.1 | 21.5/7.3 | 15/3.1 | 17.8/2.8 | 19.5/5.7 |
| **Hygrophoraceae** | 14.5/25.7 | 12.5/28.7 | 11.8/40.5 | 11.3/30.6 | 13.8/42.4 | 11.8/24.6 | 12.6/32.2 |
| **Entolomataceae** | 12.3b/2.4 | 14b/2.4 | 11.3ab/0.7 | 12.8b/1.2 | 11ab/0.5 | 6.8a/0.5 | 11.3/1.3 |
| **Geoglossaceae** | 4.8ab/3.7 | 3.5a/2.8 | 5ab/2.0 | 3.3a/2.6 | 6.3b/2.5 | 4.3ab/1.4 | 4.5/2.5 |
| **TRICHOLOMATACEAE** | 1.8/0.5 | 1/0.3 | 1.5/0.6 | 1.3/0.3 | 2/0.1 | 1.8/0.2 | 1.5/0.3 |
| **Leotiacae** | 2.8a/5.9 | 2.3ab/0.8 | 3.25a/5.3 | 3a/1.8 | 2.3ab/0.7 | 1.3b/0.1 | 2.5/2.4 |
| **Shannon Index** | 2.74 (0.23) | 2.64 (0.35) | 2.11 (0.19) | 2.53 (0.16) | 1.78 (0.09) | 2.15 (0.12) | 2.33 (0.19) |
| **Inverse Simpson Index** | 8.39 (1.59) | 7.17 (0.87) | 4.63 (0.54) | 7.0 (0.36) | 2.94 (0.29) | 4.32 (0.45) | 5.74 (0.68) |
| **Average CHEGD MOTU richness** | 54.25 (1.79) | 56 (8.8) | 54.25 (7.56) | 53 (4.18) | 50.25 (3.49) | 43.25 (1.5) | 51.88 (6.73) |

Table 3. Species richness and diversity of vascular plants per treatment. Given values are averages of spring and autumn sampling from four replicate plots per treatment. Values in parentheses are standard errors. Statistically significant differences between treatments resulted from Tukey´s test are labelled by lowercase letters

| | GR | MOGR | MUAU | MUGR | MUSP | NM | Average all plots |
|---|---|---|---|---|---|---|---|
| **Shannon Index** | 3.13b (0.17) | 3.25b (0.09) | 3.15b (0.22) | 3.32b (0.15) | 3.16b (0.16) | 2.37a (0.23) | 3.06 (0.36) |
| **Inverse Simpson Index** | 14.21ab (3.81) | 15.62ab (2.39) | 12.50ab (5.48) | 15.40ab (4.11) | 14.74b (4.05) | 5.31a (2.11) | 11.21 (5.25) |
| **Average plant species richness** | 50.8b (1.5) | 50b (4.5) | 45ab (3.2) | 54ab (2.4) | 45b (6.7) | 35.8a (2.5) | 46.8 (7) |

Table 4. Results of one-way PERMANOVA comparisons of plant and CHEDG communities between pairs of treatments. Significant values ($p<0.05$) are labelled with asterisk (*).

| | | | | CHEGD | | |
|---|---|---|---|---|---|---|
| | | | | | | 0.0256 |
| | **GR** | 0.8791 | 1 | 0.8778 | 0.2002 | * |
| | | **MOG** | | | | |
| | 0.7501 | **R** | 0.8516 | 0.7153 | 0.3709 | 0.1138 |
| | 0.0592 | 0.1849 | **MUAU** | 1 | 0.5804 | 0.0541 |
| PLANTS | | | 0.0297 | **MUG** | | |
| | 0.12 | 0.2542 | * | **R** | 0.4814 | 0.0583 |
| | 0.2306 | 0.2852 | 0.9374 | 0.1099 | **MUSP** | 0.0571 |
| | 0.0291 | 0.0287 | | 0.0326 | | |
| | * | * | 0.029* | * | 0.0583 | **NM** |

# Appendix A: Supplementary materials

Appendix A: Table 1. Inorganic composition of the soil at the studied plots

Appendix A: Table 2. List of fungal MOTUs retrieved at all 24 permanent plots arranged based on cumulative relative abundance

Appendix A: Table 3. Presence of CHEGD MOTUs at individual permanent plots

Appendix A: Table 4. Average percentage cover of vascular plants at individual permanent plots recorded in the last (9th) year of the research

Appendix A: Fig. 1. Species richness and diversity of fungi at the different treatments. The large pie chart compares CHEGD (in blue) and all fungi (in red) richness at individual plots. The small pie charts below show species richness, Inverse Simpson and Shannon indices of CHEGD fungi at the individual treatments.

Appendix A: Fig. 2. Species richness and diversity of CHEGD fungi and vascular plants at the different treatments. The large pie chart compares CHEGD fungi (in blue) and vascular plants (in green) richness at individual plots. The small pie charts below show species richness, Inverse Simpson and Shannon indices of vascular plants at the individual treatments.

Appendix A: Fig. 3. Heatmap showing hierarchical clustering of treatments based on presence/absence of CHEGD fungi.

Appendix A: Fig 4. Heatmap showing hierarchical clustering of treatments based on relative abundances of CHEGD species.