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Microbial drivers of plant richness and productivity in a grassland restoration experiment along a gradient of land-use intensity

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Summary

- Plant–soil feedbacks (PSFs) underlying grassland plant richness and productivity are typically coupled with nutrient availability; however, we lack understanding of how restoration measures to increase plant diversity might affect PSFs. We examined the roles of sward disturbance, seed addition and land-use intensity (LUI) on PSFs.
- We conducted a disturbance and seed addition experiment in 10 grasslands along a LUI gradient and characterized plant biomass and richness, soil microbial biomass, community composition and enzyme activities.
- Greater plant biomass at high LUI was related to a decrease in the fungal to bacterial ratios, indicating highly productive grasslands to be dominated by bacteria. Lower enzyme activity per microbial biomass at high plant species richness indicated a slower carbon (C) cycling. The relative abundance of fungal saprotrophs decreased, while pathogens increased with LUI and disturbance. Both fungal guilds were negatively associated with plant richness, indicating the mechanisms underlying PSFs depended on LUI.
- We show that LUI and disturbance affect fungal functional composition, which may feedback on plant species richness by impeding the establishment of pathogen-sensitive species. Therefore, we highlight the need to integrate LUI including its effects on PSFs when planning for practices that aim to optimize plant diversity and productivity.

Introduction

Biodiversity is considered to be a key driver of primary productivity in semi-natural systems (Duffy *et al.*, 2017). However, studies that investigate the relationship between biodiversity and productivity are mainly focused on aboveground processes while the role of belowground plant and microbial diversity and processes has been less studied (Hannula & Träger, 2020). In herbaceous systems such as grasslands, most of the plant biomass is belowground, concentrating substantial amounts of resources in the soil (Mokany *et al.*, 2006; Träger *et al.*, 2019). This suggests that plant–soil feedbacks (PSFs) likely play an essential role in the biodiversity–productivity relationship (Schnitzer *et al.*, 2011; Li *et al.*, 2014; Guerrero-Ramírez *et al.*, 2019). The feedback process involves two steps: first, plants change abiotic or biotic soil conditions (e.g. the composition of the soil community); second, this change affects the rate of growth of the plant or population (Bever *et al.*, 1997). A positive feedback increases plant biomass or richness, while a negative feedback may decrease both. Therefore, the complex interplay between soil microbes and plants influences the relationship between biodiversity and productivity through many pathways such as enhancing resource acquisition as well as affecting competitive relations between plant species (van der Heijden *et al.*, 2008). Plant and microbial community composition and functioning are known to be influenced by nutrient availability (Reich, 2014; Cavicchioli *et al.*, 2019; Fox

et al., 2021). However, little is known on how gradients in land-use intensity (LUI), e.g. in terms of fertilization, influence the mechanisms underlying the PSFs (Lekberg *et al.*, 2021).

In semiarid grasslands, Chen *et al.* (2020) showed that the positive plant richness–productivity relationship was mediated by different biotic factors, namely different fungal functional guilds, depending on nutrient availability. Under high nutrient availability, both mycorrhizal symbionts and decomposers (saprotrophs) contributed positively to plant richness and productivity. The authors suggest that mycorrhizal fungi helped decrease the differences in nutrient acquisition between dominant and subdominant species, increasing plant species richness by enhancing niche partitioning and species coexistence, in both high- and low-nutrient sites (van der Heijden *et al.*, 2008). Decomposers are usually associated with facilitating PSFs due to their role in organic matter mineralization (van der Heijden *et al.*, 2008; Bardgett & van der Putten, 2014; Francioli *et al.*, 2021), but litter decomposition can also release phytotoxic compounds, and produce negative PSFs (van de Voorde *et al.*, 2012; Mazzoleni *et al.*, 2015; van der Putten *et al.*, 2016). Greater plant richness can lead to a host-dilution effect, decreasing the abundance of pathogens (Collins *et al.*, 2020). However pathogens can also decrease plant species richness and productivity and produce negative PSFs, such as found under low-nutrient conditions (Chen *et al.*, 2020). In addition, fertilization favors fungal pathogens at the expense of mutualistic symbionts (Lekberg *et al.*, 2021). However, the plant richness–productivity relationship is not always positive (Adler *et al.*, 2011; Hagan *et al.*, 2021) and we still lack understanding of the PSFs underlying it when this relationship is negative or insignificant (Hannula & Träger, 2020), which depicts a significant research gap.

In temperate grasslands of Central Europe, fertilizer addition is used to increase fodder production, which often leads to a decrease in plant species richness (Humbert *et al.*, 2016). In these grasslands, we normally find a negative relationship between plant productivity and species richness (Socher *et al.*, 2012). In low-diversity sown grasslands and biodiversity experiments that control for environmental variability, positive effects of plant diversity on aboveground productivity were found at the plot scale, due to e.g. complementarity among species. At the landscape scale, this relationship is commonly overruled by site environmental conditions and increasing productivity with fertilization. This increased productivity induces competition among plant species, resulting in a negative relationship between diversity and productivity in observational studies (Klaus *et al.*, 2020; Freitag *et al.*, 2022).

Fertilization is also linked to a shift in the plant community composition from the slow to the fast end of the plant economic spectrum (Reich, 2014; Allan *et al.*, 2015). The increased nutrient availability stimulates the growth of rapidly-growing plants, less dependent on mycorrhizal associations, which release more carbon (C) into the soil and allow for a larger soil microbial biomass dominated by copiotrophic (fast-growing) bacteria (Fontaine *et al.*, 2011; de Vries *et al.*, 2012). Nutrient- and C-cycling rates can be assessed through the activities of extracellular enzymes involved in C-, nitrogen (N)- and phosphorus (P)-cycling, which are good indicators of the status of nutrient availability (Allison & Vitousek, 2005; Trivedi *et al.*, 2016). Activities of β-D-glucosidase, β-D-xylosidase, *N*-acetyl-β-D-glucosaminidase increase at high concentrations of their corresponding substrate, while others (e.g. acid phosphomonoesterase) decrease at high concentration of their corresponding product (e.g. phosphate; Allison & Vitousek, 2005). Fertilized grasslands usually have lower C : N ratios in the soil and in the microbial biomass (C_{mic} : N_{mic}; Wardle, 1992; Bittman *et al.*, 2005). In contrast, in N-poor grasslands, oligotrophic fungi that efficiently use recalcitrant C compounds with high C : N ratios and that immobilize nutrients longer typically dominate the soil microbial community, leading to greater fungal : bacterial (F : B) ratios (Bardgett *et al.*, 2003). Therefore, nutrient availability mediates PSFs that influence primary productivity, microbial functions, and plant as well as microbial community composition.

Sustainable management and restoration of biodiversity in grasslands represent a major goal of nature conservation, as documented by the United Nations 'Decade on Ecosystem Restoration 2021–2030', which attempts to restore ecosystem services and stem the rapid decline of biodiversity (UNEP/FAO, 2020). A popular way to restore plant species diversity in grasslands is seed addition (Kiehl *et al.*, 2010), combined with sward disturbance such as by harrowing or tillage to facilitate the establishment of sown species (Donath *et al.*, 2007; Klaus *et al.*, 2017). However, soil disturbance can lead to nutrient losses due to leaching (Klaus *et al.*, 2018; Schäfer *et al.*, 2019) and disrupt soil nutrient cycling and redistribution by shifts in the microbial community, especially fungi, due to breaks in the hyphal network that prevent nutrient redistribution (Helgason *et al.*, 2009). Therefore, disturbance and seed addition can lead to major changes in PSFs, due to potential shifts in microbial community composition. These shifts are also expected to depend on LUI.

The aims of our study were to understand the belowground mechanisms underlying the plant richness–productivity relationship and address the following research questions:

(**1**) How are plant biomass, plant species richness and microbial properties (biomass, community composition and soil enzyme activities) affected by LUI?
(**2**) How are microbial properties affected by disturbance and seed addition as a measure to increase plant diversity for ecological restoration?
(**3**) How do LUI, disturbance and seed addition affect PSFs, that is, how do plant species richness and productivity affect enzyme activities?
(**4**) How do the different fungal guilds affect plant productivity and species richness under different management intensities during 4 yr after disturbance and seed addition in a restoration experiment?

To address these questions, we conducted a two-factorial sward disturbance and seed addition experiment along a gradient of LUI in 73 grasslands in Germany (Klaus *et al.*, 2017; Freitag *et al.*, 2021). Of these, 10 grasslands, i.e. five fertilized and five unfertilized grasslands, were selected for this study. As such, our

manuscript aims to unveil PSFs underlying a negative or non-significant richness–productivity relationship in temperate grasslands, which depicts a significant research gap.

Materials and Methods

Study region and experimental design

Our study was carried out in temperate grasslands in the Biodiversity Exploratories project in Germany (Fischer *et al.*, 2010). In the region of the Schwäbische Alb (southwest Germany), we chose 10 permanent agricultural grasslands. The study region is a UNESCO biosphere reserve on calcareous bedrock with karst phenomena. Soils are shallow Leptosols with bedrock 10–15 cm below the soil surface and loess-derived loamy Cambisols. The sites are located at 460–860 m above sea level with a mean annual temperature between 6°C and 7°C and mean annual precipitation of 700–1000 mm (Fischer *et al.*, 2010).

In October 2014, we established a full-factorial disturbance and seed addition experiment. Each site had four 7 m × 7 m plots: control, disturbed, seed addition, and both disturbed and seed addition. The topsoil was disturbed by tilling the topsoil to a depth of 10 cm with a rotary harrow. Sward fragments were left on the disturbance plots. A region-specific seed mixture of 66 plant species (15 grasses, seven legumes, 44 nonlegume forbs) was used. The seed mix, mixed with crushed soybean to ease a homogeneous seed spreading, was added twice to the seeding plots, of which two-thirds in November 2014 and one-third in March 2015. Crushed soybean was also added in the nonseeded plots. In total, we sowed 5.37 g m^{-2} (see Klaus *et al.*, 2017, for further details).

We selected five sites with manure and mineral fertilization with high LUI, and five unfertilized sites with low LUI (Tables 1, S1). We calculated the LUI index for the years 2006–2017 using the index by Blüthgen *et al.* (2012), based on information from the landowners on mowing, grazing and fertilization intensities (Vogt *et al.*, 2019; Ostrowski *et al.*, 2020). The studied sites with low LUI were unfertilized sheep pastures and single-cut meadows and the high LUI sites were fertilized meadows or mown pastures with up to three cuts per year or intensive grazing (Fischer *et al.*, 2010).

Table 1 Mean land-use intensities of the 10 temperate permanent grasslands calculated for 2006–2017 using the index developed by Blüthgen *et al.* (2012), which integrates quantitative information on grazing, mowing and fertilization.

Site	Land-use intensity index	Grazing intensity (livestock unit days of grazing ha^{-1} yr^{-1})	Mowing frequency (cuts yr^{-1})	Fertilization (kg N ha^{-1})
AEG07	0.61	35.4	0	0
AEG09	0.85	67.5	0	0
AEG10	0.91	1.8	1	0
AEG49	1.14	134.8	0	0
AEG33	1.17	162.2	0	0
AEG06	2.09	294.2	1	49.2
AEG18	2.31	0.0	2.6	124.5
AEG15	2.55	0.0	3.0	181.6
AEG02	2.77	0.0	2.9	249.0
AEG21	3.31	829.3	0.5	156.6

Plant community composition surveys, belowground and aboveground biomass

The plant community composition was surveyed yearly from 2015 to 2018 and the percentage cover of all vascular plant species on a 2 m × 2 m area inside the 7 m × 7 m plots of all grasslands between May and early June (Klaus *et al.*, 2017).

Together with the vegetation records, aboveground community biomass was harvested as a proxy of productivity in four quadrats of 0.25 m^2 inside the experimental (7 m × 7 m) plot. Aboveground plant material was dried for 48 h at 80°C and weighed to the nearest gram. Belowground plant biomass was collected from two soil augers per plot, 10 cm deep, and by sieving the soil at 5 mm. The augers were the same as the ones used for soil sampling. The soil attached to the roots was washed off and the roots dried at 60°C for 72 h and weighed.

Soil sampling

We collected soil samples each year in May from 2015 to 2018 on each treatment plot with a soil auger of 5.4 cm diameter, 10 cm depth. We recorded the precise core depth to calculate soil bulk density. Four soil cores per plot in the first year and two cores per plot in the following years were mixed and a subsample was frozen in dry ice for molecular analyses (DNA isolation and amplicon sequencing) and transported to a −80°C freezer. The remaining pooled sample was cooled, frozen at −20°C within 8 h, then thawed overnight and sieved (< 2 mm) and refrozen at the same temperature within 24 h. The remaining pooled sample was used for microbial biomass, C and N, extractable organic carbon (EOC), extractable nitrogen (EN), NH_4^+, NO_3^-, P, phospholipid fatty acids (PLFAs) analyses and enzymatic assays. We obtained 160 samples, consisting of 10 sites, four plots per site, over 4 yr.

We measured the concentration of labile inorganic phosphorus (P_i) and total phosphorus (total P) in the soil (Methods S1) in samples from 2018 using the Olsen-P method (Olsen *et al.*, 1954). Olsen organic phosphorus (P_{org}) was calculated as the difference between total P and P_i.

For pH, in May 2017, we took a composite sample of 14 soil cores (10 cm depth) in the direct vicinity of the experiment (one pooled sample per site), dried the soil and measured pH in a 0.01 M calcium chloride ($CaCl_2$) solution (1 : 2.5 soil : solution ratio).

Microbial biomass C and N, EOC, EN, NH_4^+ and NO_3^-

Soil microbial biomass carbon (C_{mic}) and soil microbial biomass nitrogen (N_{mic}) were determined using the chloroform-fumigation-extraction method (Vance *et al.*, 1987) as modified

by Keil *et al.* (2011), see Methods S1 for details. Extractable organic carbon and EN were measured on a C/N analyzer (Multi N/C 2100(S); Analytik Jena AG, Jena, Germany). The C_{mic} and N_{mic} were calculated as EOC or EN of fumigated samples minus EOC or EN of nonfumigated samples divided by k_{EOC} of 0.45 for C_{mic} (Joergensen, 1996) or by k_{EN} of 0.54 for N_{mic} (Brookes *et al.*, 1985). Concentrations of NH_4^+ and NO_3^- were measured colorimetrically in nonfumigated, undiluted extracts with a Bran & Luebbe autoanalyzer (Bran & Luebbe, Norderstedt, Germany), see Methods S1 for details.

Phospholipid fatty acids and neutral lipid fatty acids

Phospholipid fatty acids were extracted as described by White *et al.* (1979) with the modifications introduced by Frostegård *et al.* (1991; Methods S1). The PLFAs i15:0, a15:0, i16:0 and i17:0 were used as biomarkers for Gram-positive bacteria and cy17:0 and cy19:0 for Gram-negative bacteria, and 16:1ω7 as a widespread bacterial marker (Frostegård & Bååth, 1996; Zelles, 1999; Ruess & Chamberlain, 2010). Total bacterial PLFAs were obtained by adding Gram-positive, Gram-negative bacteria and 16:1ω7. The PLFA 18:2ω6,9 was used as a biomarker for saprotrophic and putative pathogenic fungi, but not for arbuscular mycorrhizal fungi (AMF; Larsen *et al.*, 1998; Olsson, 1999; Ruess & Chamberlain, 2010) because the amounts of the 18:2ω6,9 were found to be negligible in AMF (Larsen *et al.*, 1998) Total microbial PLFAs were obtained by adding the concentrations of all the bacterial and the fungal PLFA biomarkers. The neutral lipid fatty acid (NLFA) 16:1ω5 was used as a biomarker for AMF (Ruess & Chamberlain, 2010).

Molecular analyses: DNA isolation and amplicon sequencing

Genomic DNA from freeze-dried soil samples stored at −80°C was extracted from the 160 samples using 0.25 g of soil using PowerSoil DNA Isolation Kit (Mobio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The bacterial and fungal amplicon libraries were prepared as previously described in Schöps *et al.* (2018) and Nawaz *et al.* (2019). Briefly the V4 region of the bacterial 16S SSU rRNA gene was amplified using the primer pair 515f and 806r (Caporaso *et al.*, 2011). For fungi, a semi-nested PCR was performed to amplify the ITS2 rDNA region using initial primer combination of ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990) followed by fITS7 (Ihrmark *et al.*, 2012) and ITS4. Amplicons were cleaned using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany) and indexed through index PCR using Illumina Nextera XT Indices for sample multiplexing. The equimolar pool of bacterial and fungal libraries were mixed together in a 3 : 1 ratio and paired-end sequencing of 2 × 300 bp was performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) using MiSeq Reagent kit v.3 at the Department of Environmental Microbiology, UFZ, Leipzig, Germany (for details see Methods S1).

Bioinformatic processing

Demultiplexed forward and reverse raw reads from Illumina MiSeq were further processed using MOTHUR (Schloss *et al.*, 2009) and OBI Tools (Boyer *et al.*, 2016) software suits as previously explained in Schöps *et al.* (2018) and modifications introduced by Marjanović *et al.* (2020). At a threshold of 97% sequence similarity, the reads were then clustered into operational taxonomic units (OTUs) using the VSEARCH algorithm (Rognes *et al.*, 2016). Using naïve Bayesian classifier (Wang *et al.*, 2007), the representative sequences of fungal and bacterial OTUs were taxonomically classified against the UNITE (v.7.0; Kõljalg *et al.*, 2013) and SILVA databases (v.128; Quast *et al.*, 2013), respectively. Fungal reads were quality filtered using ITSx (v.1.0.11; Bengtsson-Palme *et al.*, 2013) to remove 5.8S and 28S fragments and any nonfungal reads from the dataset.

All taxonomically assigned fungal OTUs were used for functional annotation against the FUNGuild database (Nguyen *et al.*, 2016) to assign putative functional groups. The fungi were assigned to pathotrophs (putative plant pathogens), saprotrophs or symbiotrophs (mycorrhizal symbionts) with confidence levels of 'highly probable' and 'probable'. The fungal ITS2 and bacterial 16S raw sequences are deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB56102.

Enzymatic assays

The activities of the enzymes β-D-glucosidase (cleaving oligosaccharides of cellulose to glucose, EC 3.2.1.21), *N*-acetyl-β-D-glucosaminidase (cleaving chitin to *N*-acetylglucosamine, EC 3.2.1.52), β-D-xylosidase (cleaving hemicelluloses like plant-derived xylan, EC 3.2.1.37) and acid phosphomonoesterase (hydrolyze the ester bonds binding P to C in organic P, EC 3.1.3.2) were analyzed according to Marx *et al.* (2001; see Methods S1). Biomass-specific enzyme activities were obtained by relating each enzyme activity to C_{mic}. Greater biomass-specific enzyme activities indicate higher enzyme production by a similar microbial biomass.

Statistical analyses

To understand the relationship between aboveground and belowground (root biomass) plant productivity during early summer (May–June) and plant species richness, we fitted linear mixed-effects models with aboveground biomass as response variable, with plant species richness, year, LUI, disturbance and seed addition, with all possible interactions as fixed predictor variables. The selection of the random structure was made by fitting a maximal random effects structure, which includes random slopes for year (coded as integer) nested in site (random = ~ year|site; Piepho & Edmondson, 2018).

To test whether microbial properties (C_{mic}, N_{mic}, PLFAs, proportion of each fungal guild or enzyme activities) were affected by the treatments (i.e. disturbance and seed addition) and LUI, and whether these effects changed over time, we fitted linear

mixed-effects models with the earlier-mentioned microbial properties as response variables, and disturbance, seed addition, LUI and year (as a factor) as fixed effects with all the interaction terms. We fitted a maximal random effects structure, including random slopes for each year as described earlier. We used the lme function from the NLME package (Pinheiro *et al.*, 2019). We fitted a maximal random effects structure, including random slopes for each year as described earlier. We used model selection based on the Akaike information criterion (AIC) to find the most parsimonious model describing our data for all linear mixed-effect models (see Methods S1 for details).

We checked whether the fungal and bacterial community compositions (rarefied number of reads per sample) shifted with treatments and fertilization using nonmetric multidimensional scaling (NMDS; metaMDS function from the PHYLOSEQ package, McMurdie & Holmes, 2013) with two axes, the Bray–Curtis dissimilarity index (Faith *et al.*, 1987) and 100 restarts from random configurations to avoid local minima (Oksanen *et al.*, 2019). Shepard plots were used to assess the appropriateness of the NMDS results by comparing the dissimilarities among objects in the ordination plot with the original dissimilarities (Borcard *et al.*, 2018). Using the community-by-species matrix, we tested how the relative abundance of fungal and bacterial OTUs were affected by fertilization, disturbance, seed addition and plant species richness, grouped by year. We performed nonparametric permutational multivariate analyses of variance (PERMANOVA), with 999 permutations, that allowed for multivariate hypothesis testing and a direct additive partitioning of variation (Anderson, 2001). We used the adonis2 function of the VEGAN package also using the Bray–Curtis dissimilarity index (Oksanen *et al.*, 2019). Finally, in order to understand which soil and plant properties helped explain community composition, we used the envfit function of the VEGAN package to find vectors averages of environmental variables, with 999 permutations, with year as strata to constrain the permutations within years. The envfit function finds directions in the ordination space towards which the continuous environmental vectors change most rapidly and to which they have maximal correlations with the ordination configuration; and finds averages of ordination scores for factor levels of categorical variables. We ran the envfit function with LUI, root biomass, EOC, EN, NH_4^+, NO_3^-, N_{mic}, soil pH, total P (Olsen), soil organic carbon (C_{org}) and $P_{org(Olsen)}$ as continuous explanatory variables and year, disturbance and seed addition as categorical explanatory variables.

We used multigroup structural equation modeling to calculate standardized path coefficients for each year using multi-group structural equation models (SEMs), with year as a grouping variable and site as random effect (Shipley, 2016), which is a powerful statistical tool to infer direct and indirect relationships between variables. We built two models. In the first SEM, we evaluated the effects of LUI, and disturbance and seed addition on plant species richness and plant biomass. In addition, we evaluated the feedback effect of plant biomass and richness on β-D-glucosaminidase: C_{mic}. We did not add all enzymes because their activities were co-linear. As such, we chose β-D-glucosaminidase because it represented the effect of disturbance on soil microbes.

In the second SEM, we evaluated the effects of LUI, disturbance, and seed addition, as well as the proportion of reads belonging to each fungal guild (symbiotic (mycorrhizal), pathogenic and saprotrophic fungi) on plant species richness and the effect of plant species richness on plant biomass. In both SEMs, the variable values were scaled and centered before the SEM analyses. We performed a backward selection of variables and stopped when the removal of a variable from the model led to an invalid model (C-statistic P value < 0.05). We fitted all SEMs with the PIECEWISESEM package in R (Lefcheck, 2016). All analyses were done in R v.4.1.2 (R Development Core Team, 2022).

Results

Biodiversity and plant biomass production

The fertilized sites, with high LUI, produced more aboveground biomass (244 g m^{-2}) and contained 10–30 plant species (mean = 25 species), while the unfertilized sites with low LUI produced less aboveground biomass (65 g m^{-2}), and had 20–60 species (mean = 40 species) in the 2 m × 2 m control plots. Disturbance negatively affected aboveground biomass production (linear mixed-effect models standardized coefficient: $\beta = -0.93$, $P < 0.0001$), while seed addition did not affect biomass ($\beta = 0.05$, $P = 0.51$; Fig. 1a; Tables S2, S3). The effect of disturbance on aboveground biomass depended on the year (significant interaction) because aboveground biomass was significantly reduced in the first year after disturbance but recovered in following years (Fig. 1a; Tables S2, S3). Our sites exhibited a positive relationship between aboveground biomass and LUI ($\beta = 0.70$, $P = 0.002$; Table S2) which decreased with plant species richness (plant richness × LUI interaction; $\beta = -0.11$, $P = 0.03$).

Root biomass of the upper 10 cm was lower in sites with high LUI (466 g m^{-2}) as compared to sites with low LUI (973 g m^{-2}; $\beta = -0.42$, $P = 0.004$) and decreased with disturbance to 245 and 507 g m^{-2} in high-, and low-LUI sites, respectively, in the first year after disturbance, but recovered after 1 yr (year × disturbance interaction; $\beta = 1.01$, $P = 0.007$ for 2016; Fig. 1b; Tables S2, S3).

Microbial biomass carbon and nitrogen

A greater LUI supported a larger microbial biomass, which varied between years. The C_{mic} increased from 1640 to 2145 μg C g^{-1} soil DM (dry mass; $\beta = 0.69$, $P = 0.008$; Fig. 2a) and N_{mic} from 206 to 305 μg N g^{-1} soil DM ($\beta = 0.48$, $P = 0.03$; Tables S2, S3) with increasing LUI. The C_{mic} was also differently affected by disturbance according to LUI (LUI × disturbance interaction $\beta = -0.21$, $P = 0.008$), where with the increase in LUI, there was a decrease in the effect of disturbance on C_{mic}. The C_{mic} : N_{mic} ratio ranged from 5 to 12 over the years and decreased with disturbance ($\beta = -0.41$, $P = 0.003$; Fig. 2b; Tables S2, S3). The EOC was mostly negatively affected by disturbance and this effect varied over the years (year × disturbance interaction: EOC decreased from 226 to 212 μg C g^{-1} soil DM in 2015 but increased from 211 to

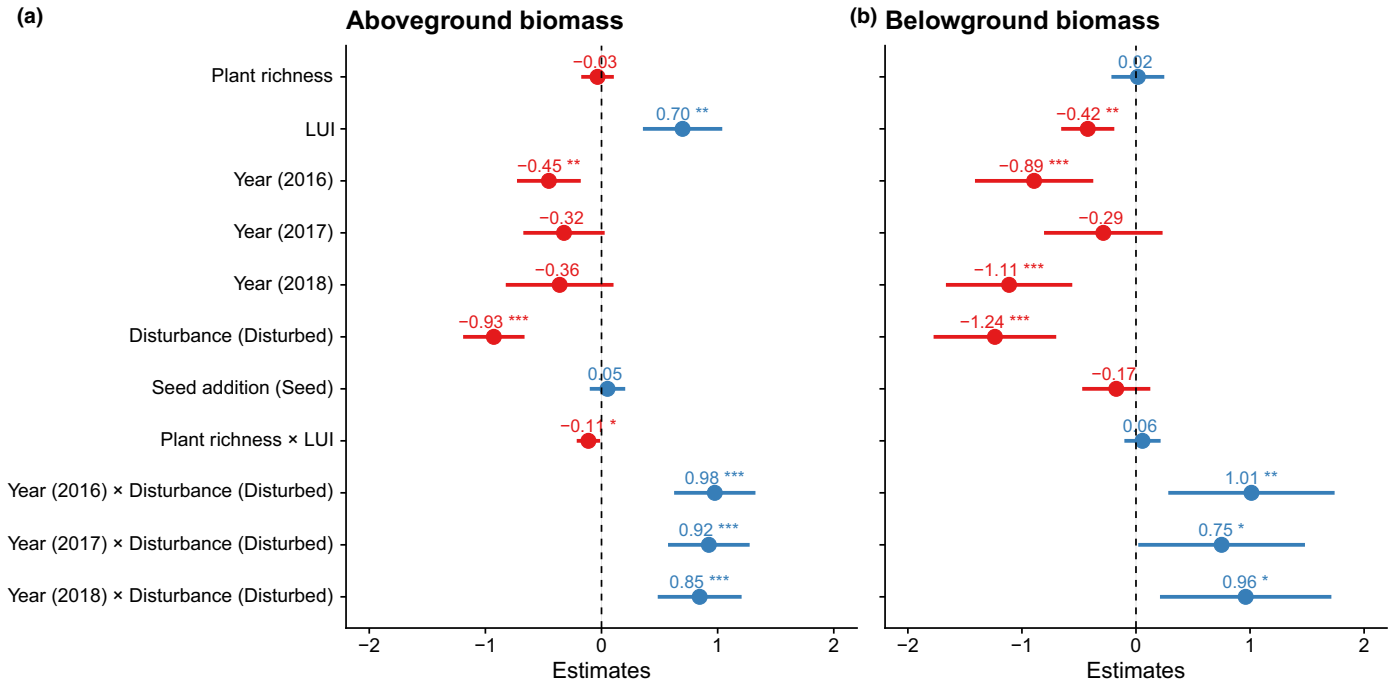

Fig. 1 Aboveground (a) and belowground (b) plant biomass of temperate grasslands along a gradient of land-use intensity (LUI) under disturbance and seed addition treatments (as a measure to increase plant diversity) between 2015 and 2018. The dashed vertical line represents the estimate for samples from 2015, unfertilized, undisturbed, nonseeded plots, within the mixed-effect model analyses. The circles are the fixed-effects standardized model coefficients, the whiskers are 95% confidence intervals, and the numbers above the circles are standardized coefficients. Significant fixed effects are marked with asterisks: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Positive estimates (in blue) represent an increase, and negative estimates (in red) represent a decrease in the predicted values of biomass. The standardized estimates presented were obtained from model selection, where predictor variables that did not contribute to explain the response variables were removed from the model.

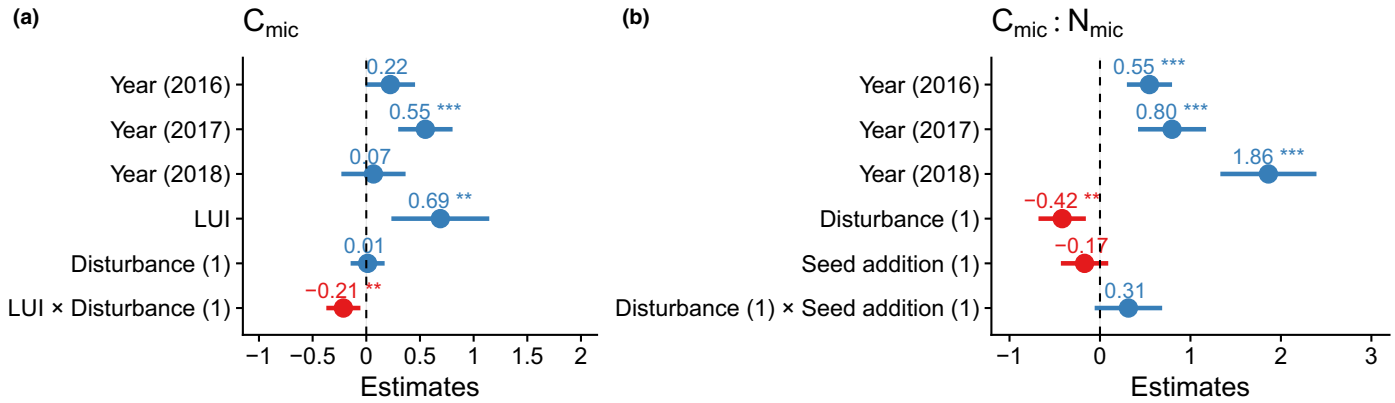

Fig. 2 Effects of year, land-use intensity (LUI) and experimental treatments (disturbance and seed addition) on (a) microbial biomass carbon (C_{mic} in μg C g^{-1} soil dry matter (DM)); and (b) C_{mic} : N_{mic} ratios in temperate grasslands between 2015 and 2018. The dashed vertical line represents the estimate for samples from 2015, undisturbed, nonseeded plots, within the mixed-effect model analyses. The circles are the fixed-effects standardized model coefficients, the whiskers are 95% confidence intervals, and the numbers above the circles are standardized coefficients. Significant fixed effects are marked with asterisks: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Positive estimates (in blue) represent an increase, and negative estimates (in red) represent a decrease in the predicted values of C_{mic} and C_{mic} : N_{mic}. The standardized estimates presented were obtained from model selection, where predictor variables that did not contribute to explain the response variables were removed from the model.

231 in 2016). The EN increased by 7% with seed addition (from 65 to 70 μg N g^{-1} soil DM; $\beta = 0.24$, $P = 0.05$; Tables S2, S3) and the effect of disturbance varied over the years. The soil NO_3^- concentration was 5.5 times greater at high LUI (33.1 mg N g^{-1} soil DM) than at low LUI sites (5.9 mg N g^{-1} soil DM; $\beta = 0.51$, $P = 0.001$) and increased with disturbance ($\beta = 0.23$, $P = 0.009$) whereas soil NH_4^+ concentration varied over the years and was halved with increasing LUI (from 26.2 at low LUI to 14.0 mg N g^{-1} soil DM at high LUI, $\beta = -0.47$, $P = 0.0006$).

Absolute abundance estimates for broad microbial groups – phospholipid fatty acids and neutral lipid fatty acids

The effect of disturbance on total microbial PLFAs decreased with LUI (average = 126.1 nmol g^{-1} DW (dry weight), LUI × disturbance interaction: $\beta = -0.17$, $P = 0.03$; Fig. 3a). Fungal to bacterial ratios decreased by 30% with LUI (from 0.10 at low to 0.07 at high LUI; $\beta = -0.52$, $P < 0.03$) and decreased with disturbance by 21% (from 0.10 to 0.08, $\beta = -0.41$, $P < 0.0001$; Fig. 3b; Tables S2, S3). The effect of disturbance on fungal to bacterial ratios increased with LUI ($\beta = 0.33$, $P < 0.0001$; Fig. 3b; Tables S2, S3). The bacterial PLFAs mostly only varied among years (Fig. 3c); therefore, the changes in fungal: bacterial ratios were mainly due to a decrease in (nonarbuscular mycorrhizal) fungal PLFAs with disturbance ($\beta = 0.40$, $P < 0.0001$; Fig. 3d; Tables S2, S3). There was, however, a decreasing effect of disturbance on bacterial PLFAs with increasing LUI ($\beta = -0.18$, $P < 0.01$; Fig. 3c; Tables S2, S3). The concentration of the NLFA used as a biomarker for AMF decreased by 19% in disturbed plots (37 nmol g^{-1} DW in disturbed, as compared to 44 nmol g^{-1} DW in undisturbed plots; $\beta = 0.77$, $P = 0.0005$; Fig. 3e; Tables S2, S3).

Microbial community and functional composition – molecular data

Fungal community composition We detected 113 015 fungal OTUs, ranging from 301 to 4728 OTUs per sample, and a total of 3857 060 reads, ranging from 12 658 to 54 870 reads per sample. Therefore, the dataset was rarefied to minimum sequencing depth of 12 000 reads per sample representing 74 244 fungal OTUs ranging from 214 to 2540 OTUs per sample.

We obtained a stress value of 0.20 in the fungal NMDS ordination, which indicates a good representation of the fungal community composition in reduced dimensions. The fungal communities had the strongest differentiation in composition between fertilized and unfertilized sites along the first NMDS axis ($P < 0.001$; Fig. 4a), where the largest proportion of the variance (*c.* 14%) was explained by fertilization (Table S4) and another 1% of the variance was explained by disturbance. Seed addition was not significant in the PERMANOVA. When we calculated the regression of variables to ordination axes (envfit), we found that the first NMDS axis was related to LUI as well as to concentrations of soil total P, P_{org} and mineral N in the form of NO_3^- (Fig. 4a). Values along the first NMDS axis were related to unfertilized sites, where the highest amounts of belowground biomass were obtained, together with highest NH_4^+ concentrations. The second NMDS axis separated samples according to soil pH (Table S5). Among the categorical explanatory variables tested (year, disturbance, seed addition and fertilization), only the presence of fertilization helped to explain fungal community composition ($R^2 = 0.5$, $P = 0.001$; Table S5). When assigned to fungal functional groups, we observed a large proportion of uncertainty (Fig. S1).

Bacterial community composition We detected 723 463 bacterial OTUs, ranging from 10 211 to 42 851 OTUs per sample, and a total of 7100 035 reads, ranging from 21 517 to 142 041 reads per sample. Therefore, the dataset was rarefied to minimum sequencing depth of 21 517 reads per sample representing 409 191 bacterial OTUs, ranging from 5798 to 12 957 OTUs per sample.

We obtained a stress value of 0.23 in the NMDS ordination, which indicates a poor representation of the bacterial community composition in reduced dimensions. The bacterial community composition was separated between fertilized (high LUI) and unfertilized (low LUI) sites (Fig. 4b; Table S4), where the largest proportion of the variance (*c.* 5%) was explained by fertilization (PERMANOVA $P < 0.001$; Table S4) and another < 1% of the variance was explained by disturbance ($P = 0.03$). Seed addition was not significant in the PERMANOVA. When we calculated the regression of variables to ordination axes (envfit), we found that the first NMDS axis was related to soil pH, soil NO_3^-, and soil total P (Table S5). The second NMDS axis was related to soil P_{org}, C_{org}, and LUI and to EOC, NH_4^+ and belowground biomass (Fig. 4b). None of the categorical explanatory variables tested (year, disturbance, seed addition and fertilization) helped explain bacterial community composition ($P > 0.05$; Table S5).

Biomass-specific enzyme activities

The potential biomass-specific activity of β-D-glucosidase increased 13% with disturbance (from 0.57 to 0.64 nmol MUF h^{-1} μg^{-1} C_{mic}; $\beta = 0.23$, $P = 0.05$; Fig. S2a; Tables S2, S3). The biomass-specific activity of *N*-acetyl-β-glucosaminidase, β-D-xylosidase and acid phosphomonoesterase (phosphatase) were not affected by any treatment (averages 0.17, 0.10 and 0.61 nmol MUF h^{-1} μg^{-1} C_{mic}, respectively; Fig. S2b–d; Tables S2, S3).

Biodiversity, plant biomass production and plant–soil feedbacks

In the first SEM, which aimed to test the feedback of the plant biomass and species richness on the labile-C enzyme activity (Fischer' $C = 17.25$, $P = 0.069$; Table S6), we found that disturbance and seed addition positively affected plant species richness, while LUI decreased plant richness. Plant aboveground biomass was also affected by disturbance and LUI, but not seed addition. Finally, microbial functioning, measured in terms of the biomass-specific activity of a labile-C-cycling enzyme (β-D-glucosidase: C_{mic}), was positively related to disturbance and negatively to plant species richness (Fig. 5a).

In the second SEM (Fischer's $C = 26.65$, $P = 0.086$; Table S6), sorting the fungi into trophic modes (according to Nguyen *et al.*, 2016) allowed us to investigate detailed effects of LUI, disturbance and seed addition on the fungal functional composition (proportion of pathogens, saprotrophs and mycorrhizal symbionts) and how these influenced plant biomass and richness. The proportion of mycorrhizal symbionts had a variable and nonsignificant effect on plant biomass, where the path removal invalidated the model (Fig. 5b). The proportion of saprotrophic fungi was negatively affected by disturbance (second and third year after disturbance) and negatively affected by LUI. The

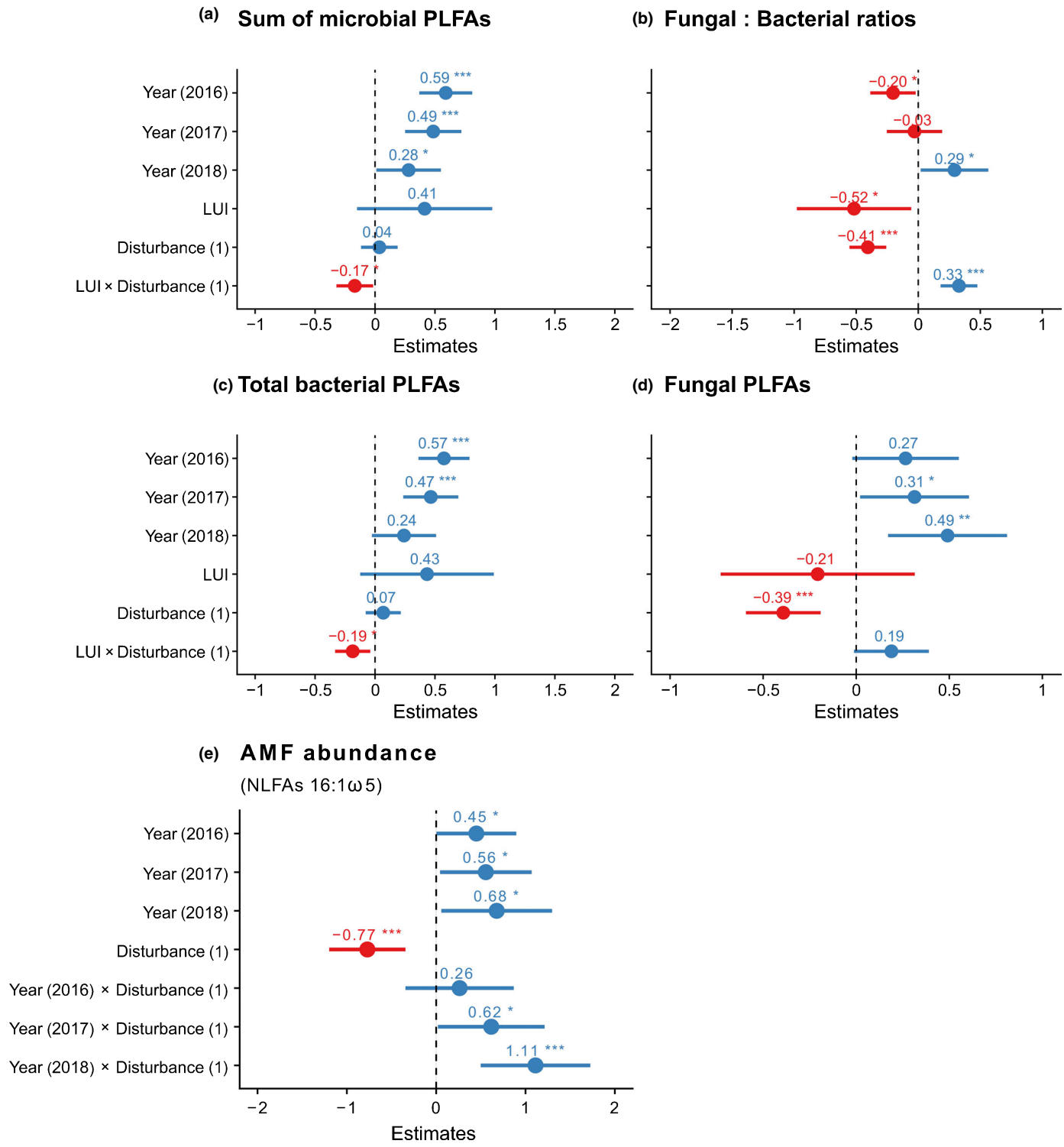

Fig. 3 Effects of year, land-use intensity (LUI) and experimental treatments (disturbance and seed addition) on the abundance of (a) total microbial phospholipid fatty acids (PLFAs), (b) fungal to bacterial PLFA ratios, (c) total bacterial PLFAs, (d) fungal PLFAs and (e) arbuscular mycorrhizal fungi (AMF) measured as neutral lipid fatty acids (NLFAs 16:1ω5) in temperate grasslands. The circles are the fixed-effects standardized model coefficients within the mixed-effect model analyses, the whiskers are 95% confidence intervals, and the numbers above the circles are standardized coefficients. Significant fixed effects are marked with asterisks: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Positive estimates (in blue) represent an increase, and negative estimates (in red) represent a decrease in the predicted values of PLFA and NLFA concentrations or ratios. The standardized estimates presented were obtained from model selection, where predictor variables that did not contribute to explain the response variables were removed from the model.

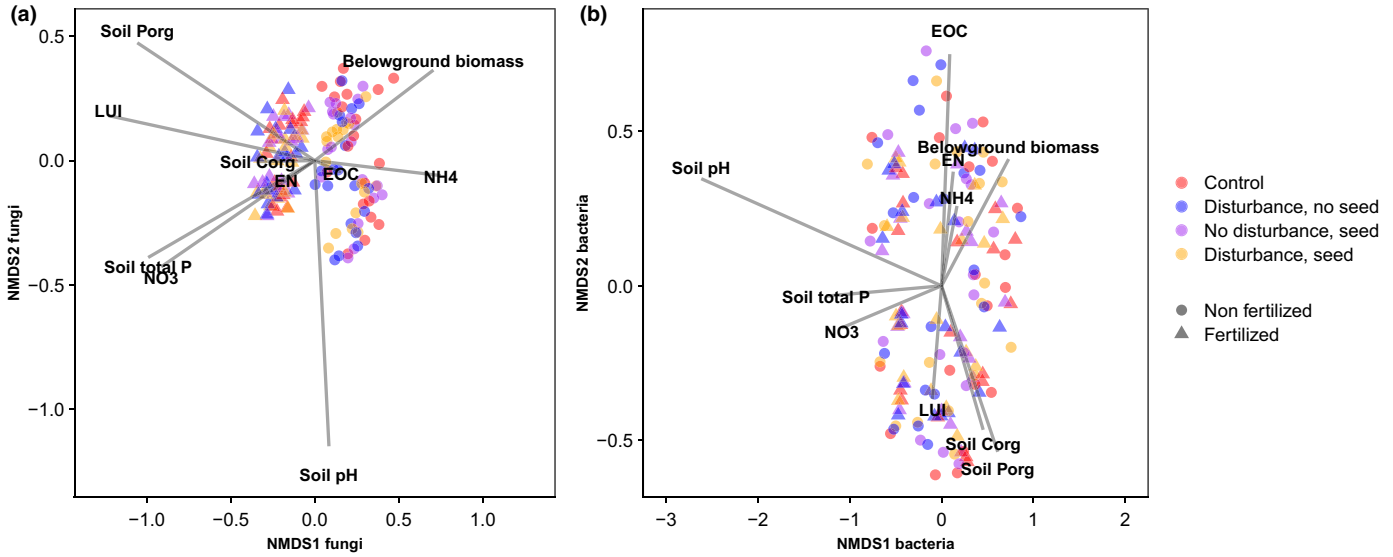

Fig. 4 First and second axes of nonmetric multidimensional scaling (NMDS) of the (a) fungal and (b) bacterial community compositions of temperate grasslands under disturbance and seed-addition treatments. Unfertilized sites are represented by circles and fertilized sites are represented by triangles. Red shapes represent control plots, blue represents disturbance without seed addition, purple represents seed addition without disturbance, and orange represents both disturbance and seed addition. Gray lines represent environmental variables and land management indexes that are correlated with the ordination (overlay). Belowground biomass, root biomass; C_{org}, organic carbon; EN, extractable organic nitrogen; EOC, extractable organic carbon; P_{org}, organic phosphorus.

negative effect of LUI on the proportion of saprotrophic fungi was not significant but was also necessary to maintain model validity. Finally, the proportion of pathogenic fungi was positively affected by disturbance, LUI and seed addition (Fig. 5b). The path coefficients that represent these negative PSFs are rather low, but significant. As such, they only explain a small proportion of the variation in plant species richness. We also found that plant species richness was negatively affected by LUI and by the proportion of pathogenic and saprotrophic fungi (Fig. 5b). Plant aboveground biomass, in turn, was positively affected by LUI, and negatively affected by disturbance, in the first year only, as in the first SEM.

Discussion

Mechanistic understanding of PSFs underlying biodiversity–productivity relationships in managed ecosystems in response to measures to restore plant species richness is still limited. The grasslands included in this study showed a richness–productivity relationship that depended on LUI, showing a negative relationship at fertilized and no relationship at unfertilized sites. Fertilization increased plant biomass production, decreased plant richness and increased microbial biomass, with the microbial community being dominated by bacteria. We also found that disturbance and increasing LUI enhanced negative PSFs and were related to a decrease in plant richness, but not to a change in plant aboveground biomass. Our study allowed us to better understand the role of the PSFs on plant diversity in grasslands with variable biodiversity–productivity relationships and its changes along a fertilization gradient.

Effects of land-use intensity on plant species richness, biomass, and microbial properties

We observed an increase in plant aboveground biomass and a decrease in belowground biomass with LUI (Fig. 1a,b). In the same experiment, Schäfer *et al.* (2019) observed that fertilized grasslands, with greater LUI, exhibited a different plant composition, towards fast-growing plants, related to greater resource inputs into the soil (Fry *et al.*, 2017). Accordingly, microbial biomass increased with LUI, as shown by increasing C_{mic} (Fig. 2a). Furthermore, an increasing LUI lead to a decrease in PLFA F : B ratios (Fig. 3b). However, we found no indication that plants relied more on AMF for nutrient acquisition at unfertilized sites with lower LUI (Figs 3e, 5b). Therefore, we observed a microbial gradient from acquisitive communities at fertilized sites to conservative, fungal-dominated, communities at unfertilized sites, strongly dependent on plant inputs into the soil.

Fertilization shifted the fungal and bacterial communities in terms of OTU composition, regardless of sampling year (Fig. 4). Microbes from low-nutrient (low-LUI) sites were tightly associated with higher belowground plant biomass and higher NH_4^+ concentrations in the soil, opposing fertilization at high-nutrient sites, which increased NO_3^- and total soil P (Fig. 4). This is in line with findings by de Vries *et al.* (2012).

Effects of sward disturbance and seed addition on plant species richness, biomass, and microbial properties

In accordance with the aim of the experimental treatment, sward disturbance combined with seed addition increased plant species richness in established grasslands (Freitag *et al.*, 2021). In the

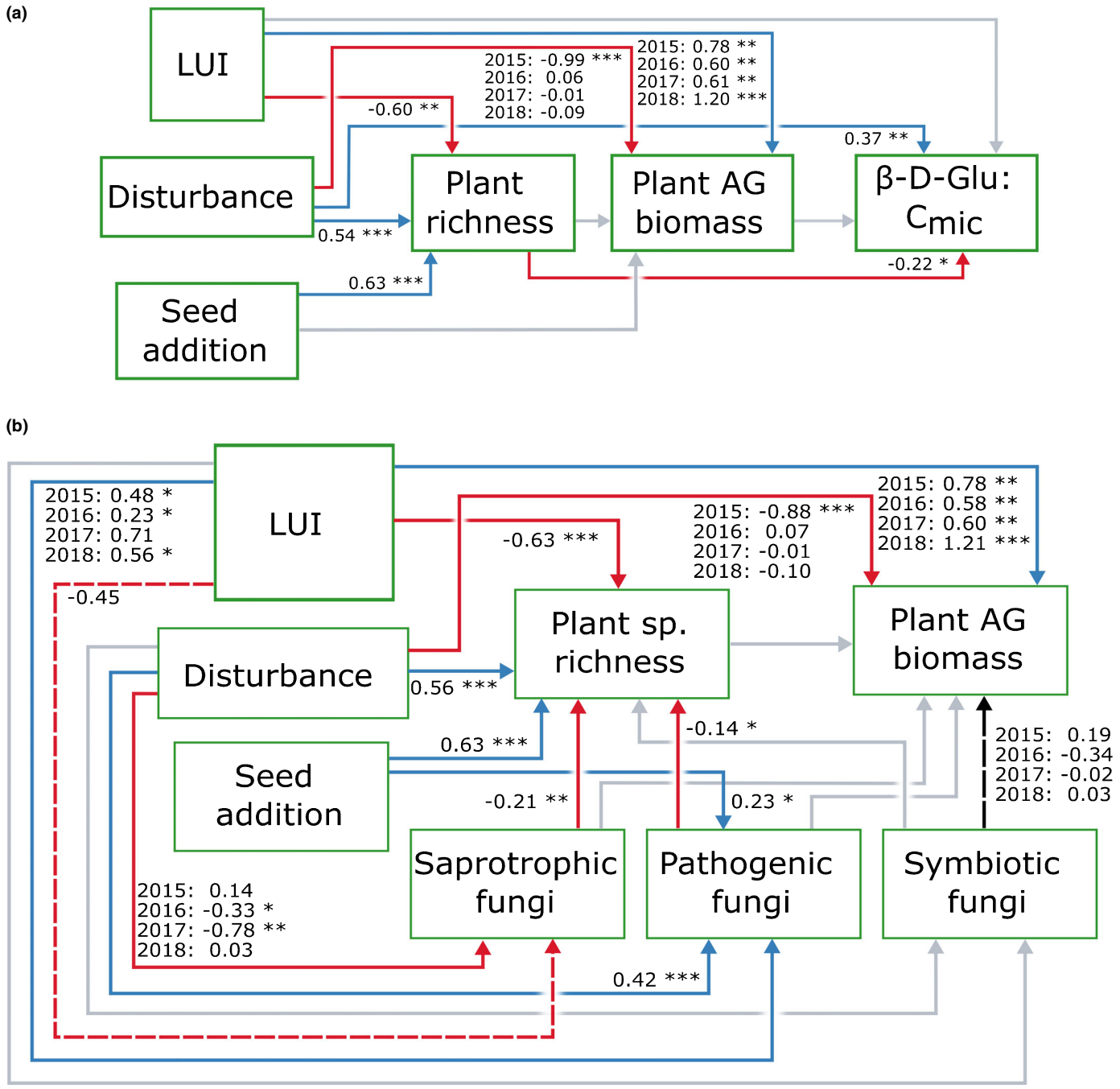

Fig. 5 (a) Path coefficients of the final model of the first structural equation model (SEM) testing the effects of land-use intensity (LUI), treatments (disturbance and seed addition) on plant species richness and aboveground (AG) biomass in temperate grasslands from 2015 to 2018 and the plant feedbacks on microbial functioning assessed via biomass-specific enzyme activity of β-D-glucosidase (β-D-Glu: C_{mic}). Multilevel SEM, with year as grouping variable. (b) Path coefficients of the final model of the second SEM that tested the effects of the proportion of different fungal guilds on plant species richness and aboveground (AG) biomass of temperate grasslands, along a gradient of LUI under the effect of disturbance and seed addition from 2015 to 2018 (multigroup SEM, with year as grouping variable). Significant path coefficients are marked with asterisks: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. The blue arrows represent positive and red arrows represent negative path coefficients. Dashed arrows represent nonsignificant path coefficients without which the models are invalid. Black arrows changed from negative to positive path coefficients over time. The gray arrows represent relationships that are not present in the final model after model simplification.

same experiment using a larger selection of sites (73, including the 10 sites in this study), plant richness increased on average by 8.3 species 5 yr after sward disturbance combined with sowing (Freitag *et al.*, 2021). The increase in species richness was negatively related to plant aboveground productivity, which increased with fertilization intensity.

Mechanical sward disturbance affected bacteria and fungi in different ways. Disturbance broke plant roots, homogenized the

distribution of organic matter in the topsoil and thus provided the microbiota with fresh litter to decompose. However, this kind of disturbance also presumably breaks fungal hyphae, especially the AMF hyphae, as shown by the drastic reduction in the concentration of AMF-derived NLFAs after disturbance (Fig. 3e) and leads to soil nutrient leaching (Klaus *et al.*, 2018). Therefore, to maximize soil nutrient retention and suppressive effects against pathogenic microorganisms performed by mycorrhizal fungi, grassland sward renewal involving soil disturbance, as it is still a widespread agricultural practice in many countries (Velthof *et al.*, 2010; Creighton *et al.*, 2011), should be avoided as much as possible.

The disturbance also limits the fungal capacity to redistribute limiting nutrients (Helgason *et al.*, 2009), reducing their competitive advantage over soil bacteria (Frey *et al.*, 1999; Strickland & Rousk, 2010). For instance, in our study, both lower F : B ratios (Fig. 3b) and lower C_{mic} : N_{mic} ratios (Fig. 2b), at low LUI, indicate that, like fertilization, disturbance favored more bacterial dominated systems, especially in the first year after disturbance. However, the negative effect of disturbance on F : B increased with LUI, indicating a disturbance-sensitive fungal-dominated community at fertilized sites. As such, our experiment showed that manure applications selected for a more bacteria-dominated system that was more resistant to disturbance (Yang *et al.*, 2018). A similar pattern was observed for the plant communities in the experiment, where fast-growing plants in fertilized sites recovered faster from disturbance than slow-growing plants in the unfertilized sites (Schäfer *et al.*, 2019), indicating a potential synergism between the recovery of plant and bacterial functions.

Changes in bacterial and fungal functional compositions were linked to changes in microbial functions. We only observed a higher biomass-specific for β-D-glucosidase activity with disturbance (Fig. S2a), representing an increase in the enzyme production at high substrate availability.

Biodiversity, plant biomass production and plant–soil feedbacks

Unfertilized grasslands had a higher plant species richness but produced significantly less biomass due to low nutrient availability. To understand the PSFs underlying the plant richness–productivity relationship observed, we tested how plant species richness and productivity affected microbial functioning (enzyme activities), and, in turn, which of the microbial properties (i.e. fungal guilds) were related to plant richness and productivity.

Interestingly, the biomass-specific β-D-glucosidase activity did not increase with plant biomass (Fig. 5a), as we would expect, but decreased with plant richness. Our sites with greater species richness are also the low-LUI sites, where plants grow slowly and produce more recalcitrant litter, which could explain the lower β-D-glucosidase activity, involved in labile-C cycling.

The proportion of the different fungal guilds is known to be influenced by plant species richness, biomass and functional traits (Sweeney *et al.*, 2021), but the aim of the current study was to investigate the relationship the other way around. We observed negative PSFs on plant richness related to an increase in the proportion of saprotrophic and pathogenic fungi (Fig. 5b). The negative feedbacks of saprotrophs were greater at low LUI, and undisturbed soils, while those of pathogens increased with LUI and disturbance. In contrast to our results, fungal saprotrophs are thought to be involved in interspecific plant facilitation processes (van der Putten *et al.*, 2016), potentially promoting plant coexistence and diversity in grasslands (Chen *et al.*, 2020). It is possible that in our study, some saprotrophs switched to pathogens, but this is known to occur mainly under low plant diversity (Stergiopoulos & Gordon, 2014; Semchenko *et al.*, 2018), and in our study the negative effect of saprotrophs on plant richness was stronger at low LUI, where we found greater plant diversity. It is also possible that litter feedback effects enhanced a potential competitor's advantage, leading to a decrease in plant species coexistence and reducing diversity (Miki & Kondoh, 2002). Alternatively, it could also be that litter decomposition released phytotoxic compounds into the soil, which limited plant growth and reproduction, reducing plant richness. Among the candidates, extracellular self-DNA (conspecific DNA) is believed to inhibit root functionality and increase the plant susceptibility to pathogens (Mazzoleni *et al.*, 2015; Cartenì *et al.*, 2016).

We observed an increased proportion of fungal pathogens with fertilization. Animal manure amendment may add manure-sourced exogenous species, and among them, potential plant pathogens to the soil (Sun *et al.*, 2020). As such, fertilization may shift the ratio of detrimental to beneficial soil fungi, potentially excluding plant species that are sensitive to specific pathogens (Maron *et al.*, 2011; Makiola *et al.*, 2019; Chen *et al.*, 2020; Ke & Wan, 2020; Ebeling *et al.*, 2022). In fact, we observed a significant negative effect of the proportion of fungal pathogens on plant species richness (Fig. 5b), indicating negative PSFs, where pathogens might limit the plant richness by selecting for fewer resistant plant species in the community (Mommer *et al.*, 2018; Ke & Wan, 2020). This illustrates the complex interplay between plant species richness, productivity, microbial community composition and its fertility (Chung *et al.*, 2007; Chen *et al.*, 2019).

Conclusions

The gradient of LUI in our study allowed us to test which microbial components interact with plant richness and productivity at different richness and productivity levels and how these feed back on microbial properties. We found that the microbial functional composition (in terms of fungal guilds) was an important predictor of plant richness; however, these mechanisms depended on LUI. At low LUI, saprotrophs mediated negative PSFs, while at high LUI, the negative PSFs were mediated by pathogens. As such, we must underline the need to integrate PSFs and LUI in biodiversity-ecosystem function studies (Hagan *et al.*, 2021) as well as in the development of management strategies aiming to enhance biodiversity and aboveground and belowground biodiversity and ecosystem functioning.

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Author contributions

EK, SM, AA, NH, TK, UH, VHK and TW conceived the ideas and designed the methodology with contributions from MF, HL and RSO; AA, RSB, MF and AN collected data; AA and AN analyzed the data; AA led the writing of the manuscript. All authors contributed to the drafts and gave final approval for publication.

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Data availability

The molecular data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB56102. The remaining data are available in the Biodiversity Exploratories Information System (Abrahão *et al.*, 2022a,b,c,d,e): doi: 10.25829/bexis.31348-12, 10.25829/bexis.31349-9, 10.25829/bexis.31350-6, 10.25829/bexis.31352-8, and 10.25829/bexis.31353-7.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Proportion of fungal guilds under different fertilization, disturbance, and seed addition treatments between 2015 and 2018.

Fig. S2 Standardized coefficient estimates of potential biomass-specific soil enzyme activities under different fertilization, disturbance, and seed addition treatments between 2015 and 2018.

Methods S1 Details of laboratory and bioinformatics procedures.

Table S1 Type of fertilizer applied to the fertilized grasslands between 2015 and 2018.

Table S2 Summary statistics of the linear mixed-effects models to test the effects of experimental manipulations on microbial and plant properties.

Table S3 Estimates of the linear mixed-effects models to test the effects of experimental manipulations on microbial and plant properties.

Table S4 Permutational multivariate analysis of variance (PERMANOVA) of the experimental variables on the fungal and bacterial community compositions.

Table S5 Correlation of environmental variables, management and treatments with the nonmetric multidimensional scaling (NMDS), grouped (permuted) by year, for the bacterial and fungal communities.

Table S6 Marginal and conditional R^2 values for the endogenous variables of the structural equation models.

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