



Earthworm and soil microbial communities in flower strip mixtures

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Abstract

Aims Incorporation of flower strips is an agricultural measure to increase aboveground biodiversity and ecosystem services. Although soil communities are key components of terrestrial biodiversity and drive important ecosystem services, their abundance, diversity, and composition in flower strips remain largely unexplored. Here, we shed light on earthworms and soil microorganisms in flower strips.

Methods We sowed a grassy field margin vegetation as well as two annual and two perennial flower strip mixtures in fully randomized plots of 9 × 28 m in three different types of soil in Germany. Two years following sowing, we determined earthworm communities using chemical extraction and investigated the soil microbiome using real-time PCR (archaea, bacteria, fungi, and soil-N-cycling genes) and amplicon sequencing (bacteria and fungi).

Results Different plant mixtures (i.e. field margin, annual and perennial flower strips) harbored distinct earthworm and soil microbial communities. Earthworm density and biomass declined or remained unaffected in annual flower strips but increased in perennial flower strips as compared to the field margins. Arbuscular mycorrhizal fungi showed greater diversity and relative abundance in non-tilled (i.e. field margin and perennial flower strips) than in tilled plant mixtures (i.e. annual flower strips).

Conclusions We attribute changes in earthworm and microbial communities mainly to the effect of tillage and plant diversity. Overall, we suggest that perennial flower strips serve as refugia. Future studies should compare soil biota in perennial flower strips to those in adjacent fields and investigate whether the promotion of soil communities extends into adjacent fields ('spillover').

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(AMF)

Introduction

The global loss of biodiversity has far-reaching negative impacts on ecosystem functions (Tilman et al. 2014) and consequently humanity (Cardinale et al. 2012). Agricultural intensification significantly contributes to the loss of biodiversity in agroecosystems (e.g. Kleijn et al. 2009). In the European Union, one of the financially supported measures to mitigate the loss of aboveground biodiversity and associated ecosystem services in agroecosystems is the integration of semi-natural habitats such as uncultivated herbaceous or woody strips within or along arable fields (EU Regulation No 1307/2013). Among semi-natural habitats, incorporation of flower strips along field edges is a common practice. For example, in Germany, flower strips accounted for approximately 1% of the total arable land in 2018 (Schütz et al. 2022). Flower strips are known to increase, maintain or restore aboveground biodiversity and its related ecosystem functions in agroecosystems. For example, flower strips provide habitat and food resources for pollinators and therefore promote their abundance and diversity (e.g. Geppert et al. 2020). The magnitude of the effects of flower strips on pollination services and crop yield in adjacent croplands is variable and depends on the age of the flower strip and its plant diversity (i.e. perennial and old flower strips with high plant diversity promote pollination services most effectively) (Albrecht et al. 2020). Furthermore, flower strips can increase the abundance of natural enemies of pests and promote pest control services (e.g. Tschumi et al. 2015). A recent data synthesis revealed that flower strips enhance pest control services in adjacent croplands by 16% on average (Albrecht et al. 2020).

Although soil communities are a key component of terrestrial biodiversity and their diversity and composition determine ecosystem multifunctionality (Wagg et al. 2014), soil biota in flower strips remain largely unexplored. However, several studies investigated the effect of other types of semi-natural habitats on soil biota. For example, compared to adjacent arable fields, grassy field margins have been shown to harbor greater abundance as well as diversity of earthworms (e.g. Smith et al. 2008; Crittenden et al. 2015) and certain groups of soil-dwelling insects (e.g. Smith et al. 2008). Furthermore, a study conducted by Sechi et al. (2017) revealed differences in trait-dependent

(i.e. eco-physiological, behavioral, and morphological traits) responses of belowground microfauna (i.e. nematodes) and mesofauna (i.e. collembolans, mites, and enchytraeids) to semi-natural field margins as compared to adjacent arable fields. Furthermore, Sechi et al. (2017) showed that semi-natural field margins favor fungal and bacterial biomass. The findings of D'Acunto et al. (2016) further suggest that compared to a conventionally managed soybean field, adjacent herbaceous field margins harbor functionally distinct microbial communities. Besides the effect of plant composition of semi-natural habitats on soil biota, management strategy (i.e. tillage) of semi-natural habitats has been shown to alter the abundance and diversity of soil macrofauna (Smith et al. 2008). Considering the known effects of semi-natural habitats and their management on soil biota, it is reasonable to assume that flower strips alter the belowground biota as well.

In their role as ecosystem engineers, earthworms contribute to several beneficial soil functions (e.g. water infiltration (e.g. Ehlers 1975; Ernst et al. 2009; Capowiez et al. 2015), suppression of phytopathogens (e.g. Wolfarth et al. 2011; Euteneuer et al. 2019; Plaas et al. 2019), and cycling of nutrients (e.g. Reichle 1977; Blouin et al. 2013; Medina-Sauza et al. 2019)) and enhance soil fertility (e.g. Tomati and Galli 1995; Bhadauria and Saxena 2010; Ahmed and Al-Mutairi 2022). Overall, earthworms are suitable biological indicators for sustainable soil management in agriculture (Paoletti 1999). More than two decades ago, Kohli et al. (1999) conducted one of the first studies on earthworms in flower strips. The authors showed that conversion of a tilled maize field into a non-tilled wild flower strip increased the abundance of earthworms already after one year and reached a plateau after two years of absence of tillage (Kohli et al. 1999). Besides tillage, the impacts of plant diversity and biomass on earthworm communities have frequently been studied in grasslands. While some studies revealed a positive impact of plant diversity and biomass on earthworm density and biomass (Zaller and Arnone 1999; Spehn et al. 2000; Eisenhauer et al. 2013), other studies were not able to confirm this (Wardle et al. 1999; Hedlund et al. 2003). These discrepancies among studies may be related to, *inter alia*, interactions with other soil biota (Milcu et al. 2006) and plant community composition (Gastine et al. 2003; Milcu et al. 2006, 2008; Eisenhauer et al.

2009). Yet, comprehensive experimental data on the effects of flower strips on earthworm communities and their functions are lacking.

Soil microbial communities regulate fundamental biogeochemical cycles (e.g. Rousk and Bengtson 2014) and drive plant productivity (e.g. van der Heijden et al. 2008) and therefore provide existential functions for agriculture. Although flower strips are widely applied and cover large areas, to our knowledge, data on microbial communities in flower strips are missing in the scientific literature. With respect to diversification of agroecosystems, the question of whether flower strips promote beneficial soil microorganisms arises. For example, Burrows and Pflieger (2002) found positive relationships between plant diversity and spore number and volume of arbuscular mycorrhizal fungi (AMF). Thus, it is reasonable to assume that higher plant diversity in flower strips benefits AMF. Furthermore, as reduced tillage promotes AMF (e.g. Bowles et al. 2017), it can be expected that non-tilled perennial flower strips benefit AMF more than tilled annual flower strips. AMF form symbiotic associations with the majority of terrestrial plants and, *inter alia*, enhance nutrient acquisition by associated plants (Clark and Zeto 2000). Whether flower strips promote AMF remains yet to be tested.

In this work, we shed light on representatives of soil biota under flower strips. For the first time, we investigated soil archaea, bacteria, fungi, and earthworms under a grassy field margin vegetation versus four different types of flower strip mixtures (two annual and two perennial flower strip mixtures comprising 11 to 13 and 30 to 51 plant species, respectively). The five plant mixtures were sown in fully randomized plots of 9 × 28 m and soil biota were studied two years following sowing. Our experimental design was replicated on three study sites with three different soil types to evaluate whether belowground responses to flower strip mixtures are soil type-specific. We hypothesized that i) flower strip mixtures increase the abundance and alter the composition of soil microbial communities as well as increase the population size of earthworms compared to grassy field margin vegetation. We further expected that ii) perennial flower strip mixtures promote soil biota more effectively than annual flower strip mixtures due to the absence of soil management (annual flower

strip mixtures were re-established) and larger plant richness.

Materials & methods

Study sites and study design

Our study was conducted at three study sites (near Lippetal on a Gleyic Podzol, at the experimental research station of the South Westphalia University of Applied Sciences near Merklingsen on a Gleyic Luvisol, and near Ense on a Stagnic Cambisol (IUSS Working Group WRB 2015); Fig. 1; see Table SI 1 for site description and general soil properties)) in the federal state of North Rhine-Westphalia, Germany. We refer to the study sites by their soil group (i.e. Podzol, Luvisol, and Cambisol soil).

In 2020, five different plant mixtures were sown at each site at a seeding rate of 10 kg ha⁻¹. One of the mixtures was a field margin vegetation comprising four grasses commonly found in field margins at our study region (referred to as ‘field margin’). This mixture was sown in autumn 2020. In spring 2020, four different flower strip mixtures were sown. The flower strip mixtures comprised two annual flower strip mixtures (comprising 11 and 13 plant species, referred to as ‘annual flower strip 1’ and ‘annual flower strip 2’, respectively) and two perennial flower strip mixtures (comprising 30 and 51 plant species, referred to as ‘perennial flower strip 1’ and ‘perennial flower strip 2’, respectively) (Fig. 1). The floral composition of the five different plant mixtures at sowing (2020) and during our year of sampling (2022) are given in Table SI 2 and File SI 1, respectively. At each site, each plant mixture was sown in three replicate plots of 9 × 28 m in a completely randomized design (3 study sites × 5 plant mixtures × 3 replicate plots = 45 replicate plots across sites) (Fig. 1). Prior to the experiment, the sites were conventionally managed croplands (Podzol and Cambisol soil) or fallow (Luvisol soil). Prior to sowing, soils were tilled twice using a grubber and rotary harrow due to weed pressure.

At each site, the annual flower strips were re-established (flower strips were mulched and the soil was tilled twice (grubber and rotary harrow) prior to resowing) in April 2021 and 2022. The field margin and perennial flower strips were topped at 15 cm height in March 2022 and not further managed, except

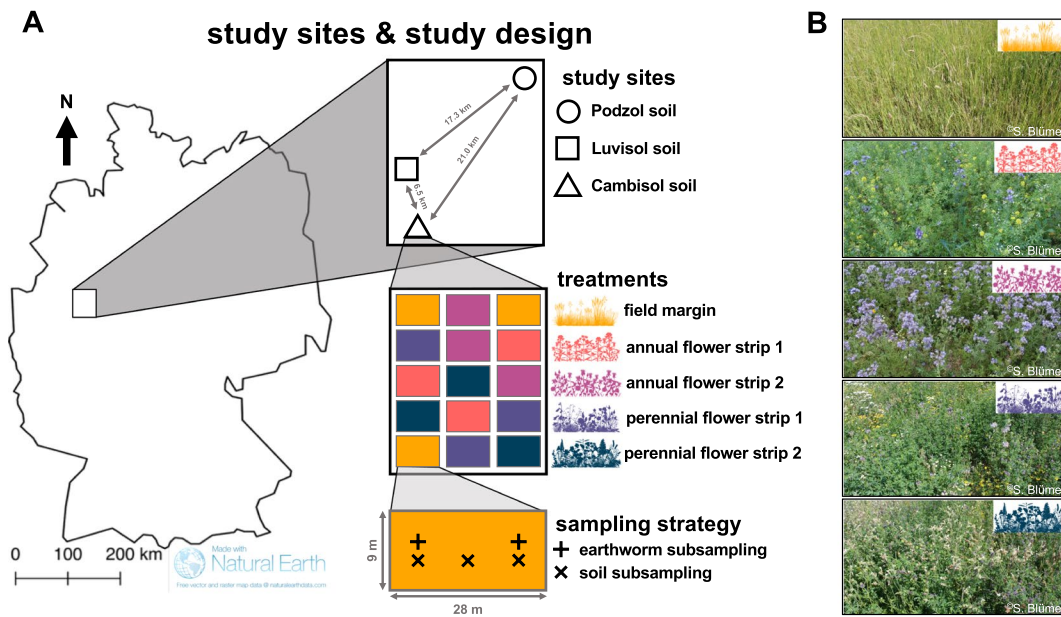


Fig. 1 Study sites and study design. Study sites and study design (A) and photos of the flower strips taken in July 2022 at the study site on the Cambisol soil (B). Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library)

in the Luvisol soil where all flower strips had to be re-established in spring 2021 due to high weed pressure. None of the replicate plots received fertilizer or plant protection products during the experiment.

Determination of general soil properties

Soil samples for the analysis of general soil properties were collected from July 15 to 16 2022. Soil bulk density was determined at 0–5 cm soil depth with 250 cm³ stainless steel cylinders using the soil core method (Blake and Hartge 1986). Soil pH, soil organic C (SOC), total N, double lactate-extractable P (P_{DL}) and K (K_{DL}), calcium chloride-extractable Mg (Mg_{CaCl_2}), effective cation exchange capacity (CEC_{eff}), and soil texture were determined at 0–30 cm soil depth. Samples at 0–30 cm soil depth were collected using a stainless-steel auger (\varnothing 3.5 cm). Three soil subsamples were collected and thoroughly homogenized in a sterile polyethylene bag to obtain one composite soil sample for 0–30 cm soil depth for each replicate plot. Composite soil samples were air-dried and sieved to <2 mm. Soil pH was determined in demineralized H₂O at a ratio of 1:2.5

(soil:water (w/v)). Prior to the determination of SOC, carbonates were removed from the samples using acid fumigation as per Harris et al. (2001). SOC and total N were determined using a CNS elemental analyzer (Vario EL Cube, Elementar, Germany). P_{DL} and K_{DL} were determined as per (VDLUF 1991a) and Mg_{CaCl_2} as per (VDLUF 1991b). Soil texture and CEC_{eff} were determined as per DIN 19683–2 (1997) and DIN ISO 11260 (1997), respectively.

Earthworm extraction and species identification

Earthworm communities were sampled from October 16 to 18 2022 using Allyl isothiocyanate (AITC) expulsion as described previously (Vaupel et al. 2023). Briefly, within each replicate plot, earthworms were expelled from two subplots in order to account for spatial heterogeneity. Squared aluminum frames (50×50 cm) were embedded approx. 5 cm into the soil and 5 L of a 0.01% (v/v in tap water) AITC solution were poured into the frames. Emerging earthworms were collected from the soil surface for 30 min, washed with tap water, and stored in tap water. In total, 2,250 earthworms were collected

within 72 h. Within 12 h post sampling, earthworms were weighed (including gut content), species were determined based on morphology as per (Krück 2018), and all collected individuals were released. Earthworm counts and biomass from the two subplots were added up. Although the remaining gut content of earthworms may add bias to our earthworm biomass data, the large number of individuals restricted us from allowing earthworms to empty their gut prior to weighing. Seven different earthworm species were found across the three study sites: *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea longa*, *Aporrectodea rosea*, *Aporrectodea trapezoides* (also referred to as a subspecies of *Aporrectodea caliginosa*), *Lumbricus rubellus*, and *Lumbricus terrestris*. Earthworm species were classified into three ecological groups: epigeic (*Lumbricus rubellus*), endogeic (*Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea rosea*, and *Aporrectodea trapezoides*), and anecic earthworms (*Aporrectodea longa* and *Lumbricus terrestris*), which were introduced by Bouché (1972). Earthworm data (i.e. count of individuals as well as total biomass per square meter) has been deposited at the BonaRes Repository (<https://doi.org/10.20387/bonares-gx1f-bh69>).

Soil DNA extraction

Soil samples for the analysis of soil microorganisms at 0 – 30 cm soil depth were collected on the same day as those for general soil properties (July 15 to 16 2022). An aliquot of approximately 50 g fresh soil from the composite samples of 0 – 30 cm soil depth (see *Determination of general soil properties*) was stored at -20 °C in the field. Upon arrival at the laboratory, frozen soil samples were stored at -20 °C until freeze-drying. Frozen soil samples were freeze-dried for 72 h and thoroughly homogenized using a vortexer as described previously (Beule et al. 2019). DNA was extracted from 50 mg finely ground soil using a cetyltrimethylammonium bromide (CTAB)-based protocol as per (Beule et al. 2021). Quantity and quality of the DNA extracts were assessed on 1.7% (w/v) agarose gels stained with SYBR Green I solution (Thermo Fisher Scientific GmbH, Dreieich, Germany).

Quantification of soil microbial groups using real-time PCR

Prior to real-time PCR, DNA extracts were diluted 1:50 (v/v) in double distilled H₂O (ddH₂O) to overcome PCR inhibition (Guerra et al. 2020). Soil bacteria and fungi were quantified as described previously (Beule et al. 2020). Soil archaea were quantified using the primer pair 340F / 100R (Gantner et al. 2011) using the identical master mix composition as for fungi (Beule et al. 2020). The thermocycling conditions of archaea were as follows: initial denaturation at 95 °C for 120 s followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 68 °C for 30 s, and final elongation at 68 °C for 5 min. Genes involved in soil nitrogen (N)-cycling (nitrification: ammonia-oxidizing archaea (AOA) and bacteria (AOB) *amoA* genes; denitrification: *nirK*, *nirS*, and *nosZ* clade I and II genes) were quantified to estimate the population size of N-cycling microorganisms as per (Beule et al. 2019). All reactions were carried out in 4 µL reaction volumes in a Pqstar 96Q thermocycler (PEQLAB, Erlangen, Germany). Melting curves were generated as described previously (Beule et al. 2019).

Amplicon sequencing of the soil microbiome

Soil bacteria and fungi were amplified using the primer pair 341F (5'-CCTACGGGNGGC WGCAG-3') / 785R (5'-GACTACHVGGGTAT CTAACKC-3') (Klindworth et al. 2013) and ITS1-F_KYO2 (5'-TAGAGGAAGTAAAAGTCGTA-3') (Toju et al. 2012) / ITS86R (5'-TTCAAAGATTCG ATGATTCA-3') (Vancov and Keen 2009), respectively. Prior to PCR, DNA extracts were diluted 1:50 (v/v) in ddH₂O to overcome PCR inhibition (Guerra et al. 2020). Amplification was carried out in 25 µL reaction volume in an Eppendorf Mastercycler EP Gradient S thermocycler (Eppendorf, Hamburg, Germany). Bacteria and fungi were each amplified within one PCR run using the same mastermix for all samples. The reaction volume contained 18.75 µL mastermix and 6.25 µL template DNA or ddH₂O for a negative control. The mastermix comprised ddH₂O, buffer (10 mM Tris-HCl, 50 mM KCl, 2.0 mM MgCl₂, pH 8.3 at 25 °C), 100 µM of each deoxynucleoside triphosphate (New England Biolabs, Beverly, Massachusetts, USA), 0.5 µM of

each primer, 1 mg mL⁻¹ bovine serum albumin, and 0.03 u μL⁻¹ Hot Start *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts, USA). Each primer was a mixture of primer with (50%) and without (50%) Illumina TruSeq 5'-end adapters (5'-GACGTGTGCTCTTCCGATCT-3' for the forward primer and 5'-ACACGACGCTCTTCCGATCT-3' for the reverse primer). Bacteria and fungi were amplified using a touch-up PCR protocol (Beule and Karlovsky 2021) with initial denaturation at 95 °C for 2 min, 3 touch-up cycles (95 °C for 20 s, 50 °C for 30 s, and 68 °C for 60 s), 22 or 25 cycles (95 °C for 20 s, 58 °C for 30 s, and 68 °C for 60 s) for bacteria and fungi, respectively, and final elongation at 68 °C for 10 min. Amplification success was verified on 1.7% (w/v) agarose gel stained with SYBR Green I solution (Thermo Fisher Scientific GmbH, Dreieich, Germany) and libraries were shipped to LGC Genomics (Berlin, Germany). A second amplification with standard i7- and i5- sequencing adapters was performed at the facilities of LGC Genomics. Libraries were multiplexed and sequenced on an Illumina MiSeq (V3 chemistry, 2 × 300 bp) (Illumina, Inc., San Diego, CA, USA). Amplicon sequencing data have been deposited at NCBI's Short Read Archive (BioProject PRJNA905898 for bacteria and PRJNA905904 for fungi).

Bioinformatic processing of amplicon sequencing data

Paired-end sequencing data of bacteria and fungi were demultiplexed using Illumina's bcl2fast version 2.20 (Illumina, San Diego, CA, USA). One-sided and conflicting barcodes as well as barcodes containing more than two mismatches were removed. Sequencing adapter and primer sequences were clipped and reads with < 100 bp were discarded. Afterwards, sequencing reads were processed in QIIME 2 version 2022.2 (Bolyen et al. 2019). Quality scores were manually inspected using the 'q2-demux' plugin. Sequence reads were quality filtered (allowing two expected errors), merged, and cleaned from chimeric sequences and singletons using DADA2 (Callahan et al. 2016). Obtained amplicon sequencing variants (ASVs) of bacteria and fungi were taxonomically classified against the SILVA ribosomal RNA gene database version 138 (Quast et al. 2013) and UNITE database version 8.3 QIIME developer release

(Abarenkov et al. 2021), respectively. Classification was achieved utilizing a scikit-learn Naive Bayes machine-learning classifier ('q2-fit-classifier-naive-bayes' and 'q2-classify-sklearn' plugin) in the 'balanced' configuration ([7,7]; 0.7 for bacteria and [6,6]; 0.96 for fungi as suggested by (Bokulich et al. 2018)). Following classification, non-bacterial and non-fungal sequence reads were discarded from the bacterial and fungal data sets. Scaling with ranked subsampling (SRS) (Beule and Karlovsky 2020) using the 'SRS' R package version 0.2.3 (Heidrich et al. 2021) was used to normalize the bacterial and fungal ASV table to 19,219 and 18,318 sequence counts per sample, respectively. The normalized data sets contained 44,009 bacterial and 3,648 fungal ASVs.

Statistical analysis

To test the effect of plant mixtures (i.e. field margin and different flower strips) on soil properties (soil pH, bulk density, SOC, total N, P_{DL}, K_{DL}, and MgCaCl₂) per site (i.e. soil type), we used one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) test. To test the effect of plant mixtures on the abundance of soil communities per site, we calculated the relative change of earthworm density and biomass as well as the abundance of archaea, bacteria, fungi, and N-cycling genes in response to the flower strips as follows:

$$\text{relative change} = \frac{a - b}{b}$$

where *a* is the observed response and *b* is the average response of field margin per site. Differences in the relative change of earthworm density and biomass as well as absolute abundance of archaea, bacteria, fungi, and N-cycling genes per site were determined using one-way ANOVA followed by Tukey HSD test.

We determined alpha diversity indices covering entropy (Shannon index (*H'*)), species richness (Chao1 index) and evenness (Pielou's evenness (*J'*)) of bacterial and fungal communities using the 'vegan' R-package (version 2.5–7) (Oksanen et al. 2019). We then tested the effect of plant mixtures on alpha diversity indices per site using one-way ANOVA followed by Tukey HSD test. Differences in relative abundance of microbial taxa obtained from amplicon sequencing among treatments per site were determined from log(*x* + 1)-transformed data and tested using one-way

ANOVA followed by Tukey HSD test. Prior to running one-way ANOVA tests, all data were manually inspected for homoscedasticity and normal distribution of the residuals and tested using Levene's and Shapiro–Wilk test, respectively. Relationships among different parameters were tested using Spearman rank correlations.

Compositional differences of bacterial and fungal amplicon sequencing data were calculated using pairwise Bray–Curtis dissimilarities and visualized using non-metric multidimensional scaling (NMDS) as implemented in the 'vegan' R-package. To test the effects of site and plant mixture on the bacterial and fungal community composition, we performed permutational multivariate analysis of variance (PERMANOVA) on Bray–Curtis dissimilarities using 999 permutations [adonis2(dissimilarity matrix ~ soil type + plant mixture + soil type:plant mixture, nperm=999)]. Additionally, we tested the effect of plant mixture on the bacterial and fungal community composition per site by running subsets of the datasets per site [adonis2(dissimilarity matrix ~ plant mixture, nperm=999)]. Complementary to each PERMANOVA model, we assessed the dispersion of samples in each group using multivariate homogeneity of group dispersions.

All statistical analyses were performed in R (version 4.1.2) (R Core Team 2017). For all statistical tests, statistical significance was considered at $p < 0.05$.

Results

General soil properties

Within each soil type, soil properties remained unaffected by the recent introduction of flower strips. Flower strips did not affect soil pH, bulk density, SOC, total N, P_{DL} , K_{DL} , and Mg_{CaCl_2} .

Earthworm communities

Earthworm density and biomass were strongly correlated with each other ($r=0.95$; $p<0.0001$) and increased from the Podzol to the Luvisol to the Cambisol soil (Fig. 2 A, Figure SI 1). The classification of the species into ecological groups (i.e. anecic, endogeic, and epigeic) revealed that earthworm

community composition was site-specific. In the Podzol soil, anecic earthworms were absent and epigeic earthworms accounted for a large share of the community. In contrast, epigeic earthworms were not present in the Luvisol soil. The Cambisol soil harbored all three ecological groups (Fig. 2 B).

Perennial flower strips strongly promoted earthworm population density and biomass across soils (Fig. 2A, C, D). In contrast, annual flower strips showed consistently lower density and biomass than the field margin in the Podzol and Luvisol soil (Fig. 2A, C, D). In these two soils, earthworms were almost absent under the annual flower strips (Fig. 2A). In the Podzol soil, the perennial flower strip 2 increased earthworm density and biomass by a factor of 3.7 to 17.5 compared to the field margin and the annual flower strips ($p \leq 0.031$; t -ratio = -4.7 to -3.9), which was mainly driven by the increased occurrence of epigeic earthworms in the perennial flower strip 2. Earthworm density in the flower strips in the Luvisol soil did not differ statistically significant from the field margin. However, earthworm densities were 79 to 99 times larger in perennial than in annual flower strips ($p \leq 0.036$; t -ratio = -4.4 to -3.5). In the same soil, earthworm biomass was 15.4 to 23.3 times larger in perennial flower strips and 9.2 to 12.8 times larger in the field margin ($p \leq 0.025$; t -ratio = -8.0 to 4.2) as compared to annual flower strips. The Cambisol soil was the only soil in which annual flower strips showed earthworm densities and biomass similar to those in the field margin. In this soil, perennial flower strips increased earthworm density by 171 to 247% as compared to the annual flower strips and field margin ($p \leq 0.018$; t -ratio = -5.8 to -3.9).

Soil microbiome

Gene copy numbers of archaea, bacteria, fungi, and functional groups involved in soil N-cycling were not affected by flower strips (Figure SI 2, Figure SI 3). Across soils, soil bacterial communities were dominated by the phyla of *Actinobacteriota* ($29.4 \pm 6.1\%$), *Proteobacteria* ($16.4 \pm 1.8\%$), and *Acidobacteriota* ($12.5 \pm 1.8\%$). The dominating bacterial classes were *Actinobacteria* ($20.1 \pm 6.6\%$), *Alphaproteobacteria* ($11.3 \pm 1.1\%$), and *Planctomycetes* ($7.9 \pm 2.3\%$) (Fig. 3A). The fungal community was dominated by *Ascomycota* ($65.4 \pm 14.0\%$), *Mortierellomycota* ($12.3 \pm 9.5\%$), and *Basidiomycota* ($12.0 \pm 8.2\%$) on

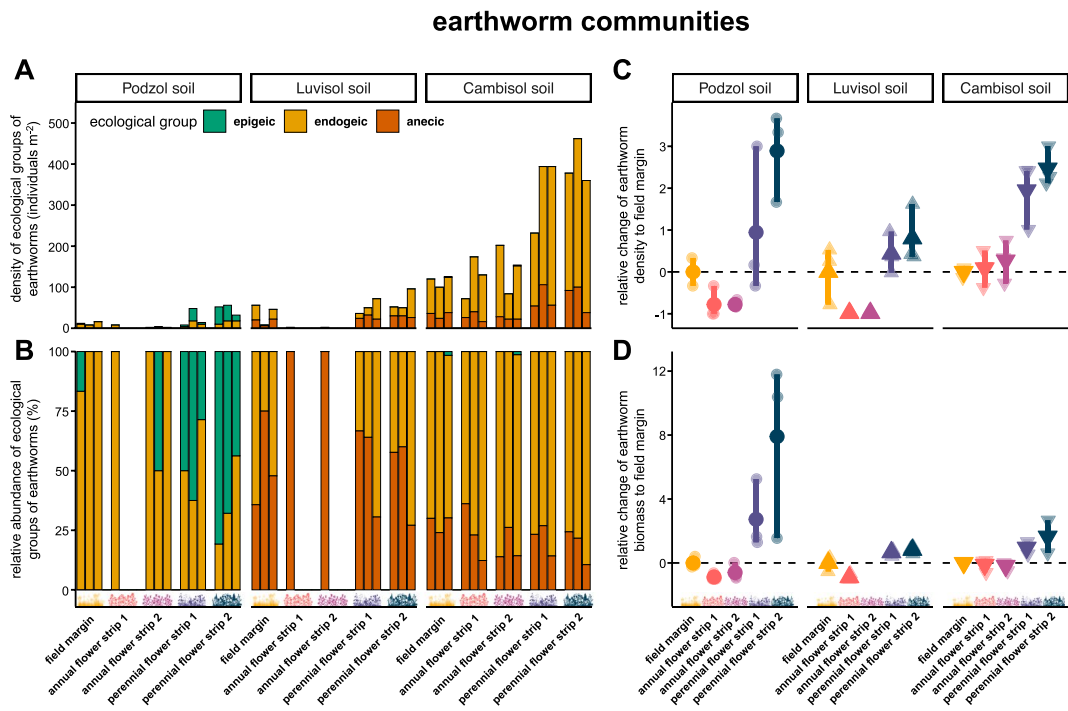


Fig. 2 Earthworm communities. Population densities of ecological groups of earthworms (A) and their relative abundance within the earthworm communities (B). Bars represent individual replicate plots ($n=3$). Relative change of earthworm density (C) and biomass (D) in response to flower strips. Non-

transparent dots and triangles represent means and vertical bars range from the minimum to the maximum value. Transparent dots and triangles represent individual data points (i.e. replicate plots). Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library)

phylum level and *Sordariomycetes* ($41.5 \pm 16.0\%$), *Dothideomycetes* ($17.2 \pm 11.6\%$), and *Mortierellomycetes* ($12.2 \pm 8.1\%$) on class level (Fig. 3E). Alpha diversity indices (Shannon index (H'), Chao1 index, and Pielou's evenness (J')) were not affected by flower strips (Fig. 3B, C, D, F, G, H), except fungal Shannon diversity in the Luvisol soil which was higher in the perennial flower strips and the field margin compared to the annual flower strip 2 ($p=0.036$; t -ratio = -3.9 to 4.3) (Fig. 3F).

Soil type (i.e. Podzol, Luvisol, and Cambisol soil) and plant mixture (i.e. field margin and different flower strips) affected community composition of both bacteria and fungi (Table 1, Figure SI 4). For both communities, the effect of soil type on community composition was stronger than the effect of plant mixture (Table 1). Plant mixture effects per site were visualized using NMDS (Fig. 4). In the Luvisol and Cambisol soil the field margin, the annual flower strips, and the perennial flower strips each formed a distinct cluster in the NMDS for both bacteria and

fungi (Fig. 4B, C, E, F). In the Podzol soil, two clusters emerged comprising the non-tilled plant mixtures (i.e. the field margin and the perennial flower strips) and the tilled plant mixtures (i.e. the annual flower strips) for bacterial communities (Fig. 4A).

The relative abundance of several bacterial phyla was affected by the plant mixtures (Fig. 5; see Table SI 3 for p-values) and reflected the clustering in the NMDS. For example, relative abundance of *Desulfobacterota* in the Cambisol soil were greater in the field margin than in the flower strips ($p \leq 0.0001$; t -ratio = 7.6 to 8.5). In the same soil, the field margin increased the relative abundance of *Methylomirabilota* and *Latescibacterota* as compared to the annual flower strips ($p \leq 0.015$; t -ratio = 4.0 to 5.0). In contrast, compared to the field margin, annual flower strips promoted the relative abundance of *Actinobacteria*, *Bdellovibrionota*, and *Proteobacteria* in the Cambisol soil ($p \leq 0.037$; t -ratio = -6.7 to -3.6). In the Luvisol soil, *Latescibacterota* showed greater relative abundance in the field margin than in the flower strips

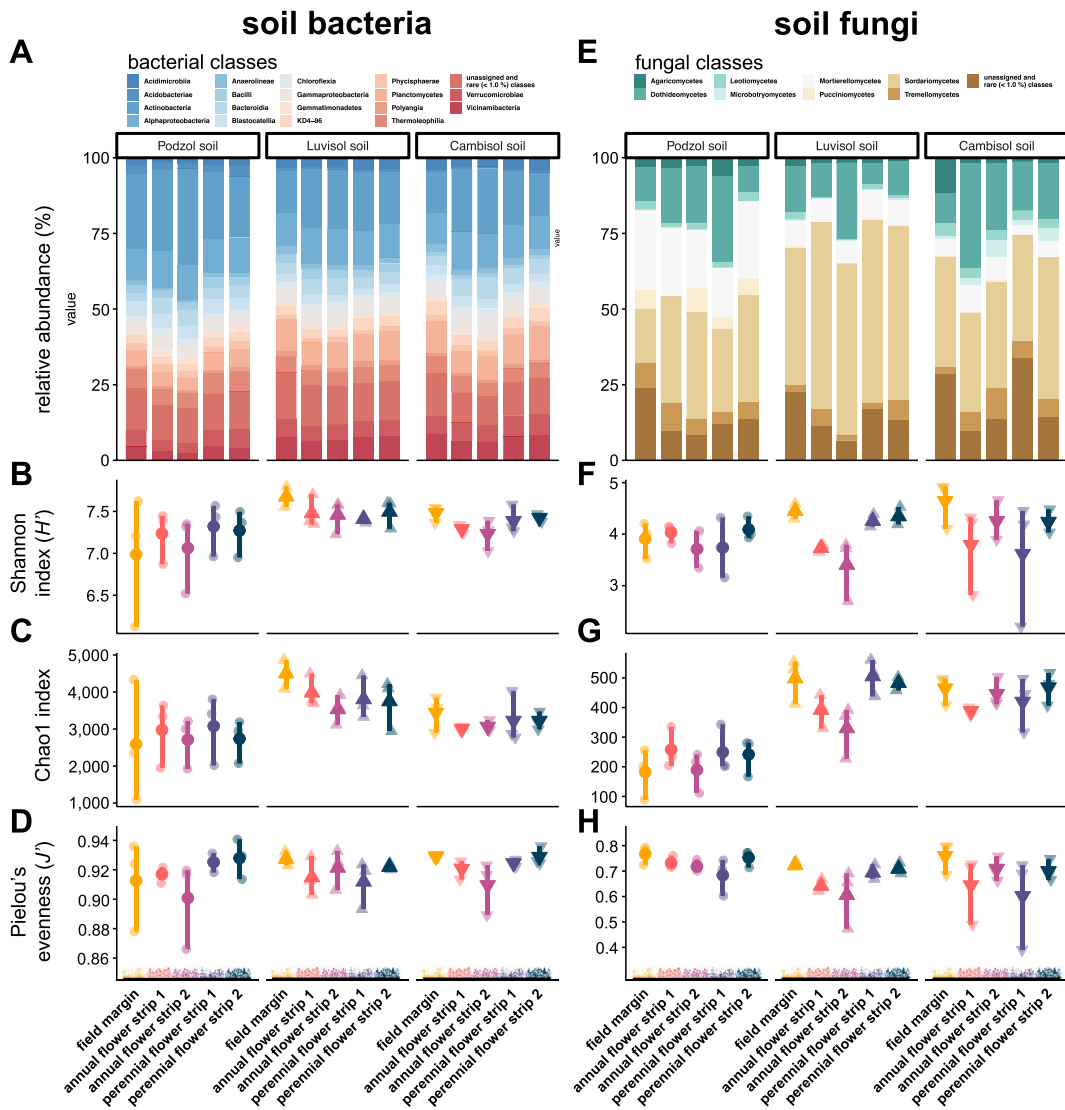


Fig. 3 Community composition and alpha diversity of soil bacteria and fungi. Mean relative abundance of bacterial (A) and fungal classes (E) per plant mixture and soil type. Alpha diversity indices of bacterial (B, C, D) and fungal communities (F, G, H). Non-transparent dots and triangles represent means

($p \leq 0.0011$; t -ratio=5.9 to 10.3). Likewise, in the Luvisol soil, relative abundances of *Auditibacteriota* and *Gemmatimonadota* were greater in the annual flower strips than in the field margin ($p \leq 0.043$; t -ratio=-4.0 to -3.4). In all soil types, relative abundance of *Bacteroidota* were greater in the annual flower strips than in the field margin ($p \leq 0.021$; t -ratio=-6.9 to -3.9).

and vertical bars range from the minimum to the maximum value ($n=3$). Transparent dots and triangles represent individual data points (i.e. replicate plots). Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library)

Within the fungal community, the abundance and diversity of affiliates of the monophyletic phylum *Glomeromycota* (containing all AMF), were altered by the plant mixtures (Fig. 6). Patterns of relative abundance of AMF were not consistent across soil types, except that the relative abundance of AMF was greater in the field margin mixture as compared to the annual flower strip mixtures ($p \leq 0.007$;

Table 1 Permutational multivariate analysis of variance (PERMANOVA) results for soil bacterial and fungal community composition. PERMANOVA was performed with 999 permutations using ASV count data. df=degrees of freedom; sum Sq=sum of squares; R^2 =coefficient of determination; F =pseudo-F ratio. ^a three soil types (Podzol, Luvisol, and Cambisol soil). ^b Five plant mixtures (field margin, annual flower strip 1, annual flower strip 2, perennial flower strip 1, perennial flower strip 2)

| Source of variance | df | sum Sq | R^2 | F | p -value |
|--|----|--------|-------|-------|------------|
| Soil bacteria | | | | | |
| Soil type ^a | 2 | 5.68 | 0.49 | 23.11 | 0.001 |
| Plant mixture ^b | 4 | 1.00 | 0.08 | 2.02 | 0.006 |
| Soil type ^a ×plant mixture ^b | 8 | 1.29 | 0.11 | 1.31 | 0.084 |
| Residuals | 30 | 3.69 | 0.32 | | |
| Total | 44 | 11.66 | 1.00 | | |
| Soil fungi | | | | | |
| Soil type ^a | 2 | 4.29 | 0.36 | 15.05 | 0.001 |
| Plant mixture ^b | 4 | 1.85 | 0.15 | 3.26 | 0.001 |
| Soil type ^a ×plant mixture ^b | 8 | 1.63 | 0.14 | 1.43 | 0.012 |
| Residuals | 30 | 4.27 | 0.35 | | |
| Total | 44 | 12.04 | 1.00 | | |

t -ratio=1.9 to 2.9). Furthermore, in the Cambisol soil, relative abundance of AMF in the perennial flower strips was lower than in the field margin ($p \leq 0.0003$; t -ratio=2.3 to 2.4).

Across sites and plant mixtures, 249 ASVs were assigned to AMF, covering three orders, namely *Archaeosporales*, *Glomerales*, and *Paraglomerales* (Fig. 6A). Relative abundance of *Archaeosporales* in the Luvisol and the Cambisol soil was greater in the field margin as compared to the annual and perennial flower strips ($p \leq 0.016$; t -ratio=0.4 to 0.7). Furthermore, in the Luvisol soil, relative abundance of *Glomerales* was greater in the field margin and perennial flower strip 1 than in the annual flower strips ($p \leq 0.043$; t -ratio=1.3 to 1.7). Relative abundance of *Glomerales* in the Cambisol soil was greater in the field margin compared to the annual and perennial flower strips ($p \leq 0.0008$; t -ratio=2.0 to 2.6). In the Podzol soil, plant mixtures only affected the community share of *Paraglomerales* which was greater in the field margin compared to the annual flower strips ($p \leq 0.036$; t -ratio=2.1). In the Cambisol soil, relative abundance of *Paraglomerales* was lower in the annual and perennial flower

strips as compared to the field margin ($p \leq 0.0007$; t -ratio=1.4 to 1.5). In contrast, community share of *Paraglomerales* did not differ among plant mixtures in the Luvisol soil.

Alpha diversity (Shannon index (H') and Chao1 index) of AMF differed significantly among the plant mixtures (Fig. 6B, C). In each soil type, alpha diversity of AMF was greater in the field margin as compared to the annual flower strips ($p \leq 0.0053$; t -ratio=3.2 to 4.2 and $p \leq 0.0066$; t -ratio=23.7 to 53.5 for Shannon index and Chao1, respectively). Furthermore, alpha diversity of AMF did not differ between field margin and the perennial flower strips in the Podzol and Luvisol soil. In the Cambisol soil, however, Chao1 index was greater in the field margin than in the perennial flower strips ($p \leq 0.0001$; t -ratio=39.2 to 40.0). According to Shannon index, alpha diversity of AMF was greater in the perennial flower strips as compared to the annual flower strip 1 in all soil types ($p \leq 0.012$; t -ratio=2.4 to 3.6).

Discussion

Earthworm communities

We collected earthworms using AITC extraction without hand-sorting (Vaupel et al. 2023). While using exclusively AITC extraction works well for adult anecic earthworms, endogeic species and juveniles may be recovered with reduced efficacy without additional hand-sorting (Čoja et al. 2008; Pelosi et al. 2009). Chemical extraction is a non-destructive sampling technique that is often preferred over hand-sorting when additional data needs to be collected at the sampling location (e.g. Lees et al. 2016; Tóth et al. 2020).

In the present study, croplands or fallow were sown with a mixture of four grasses commonly found in field margins, annual flower strip, and perennial flower strip mixtures. In the Podzol and Luvisol soil, annual flower strips showed the lowest earthworm density and biomass (Fig. 2A, Figure SI 1), which we attribute to their annual re-establishment that included tillage (grubber and rotary harrow). Although all plots in the Luvisol soil had to be re-established one and a half years prior to earthworm sampling due to high weed pressure, differences among tillage regimes were already apparent following just one

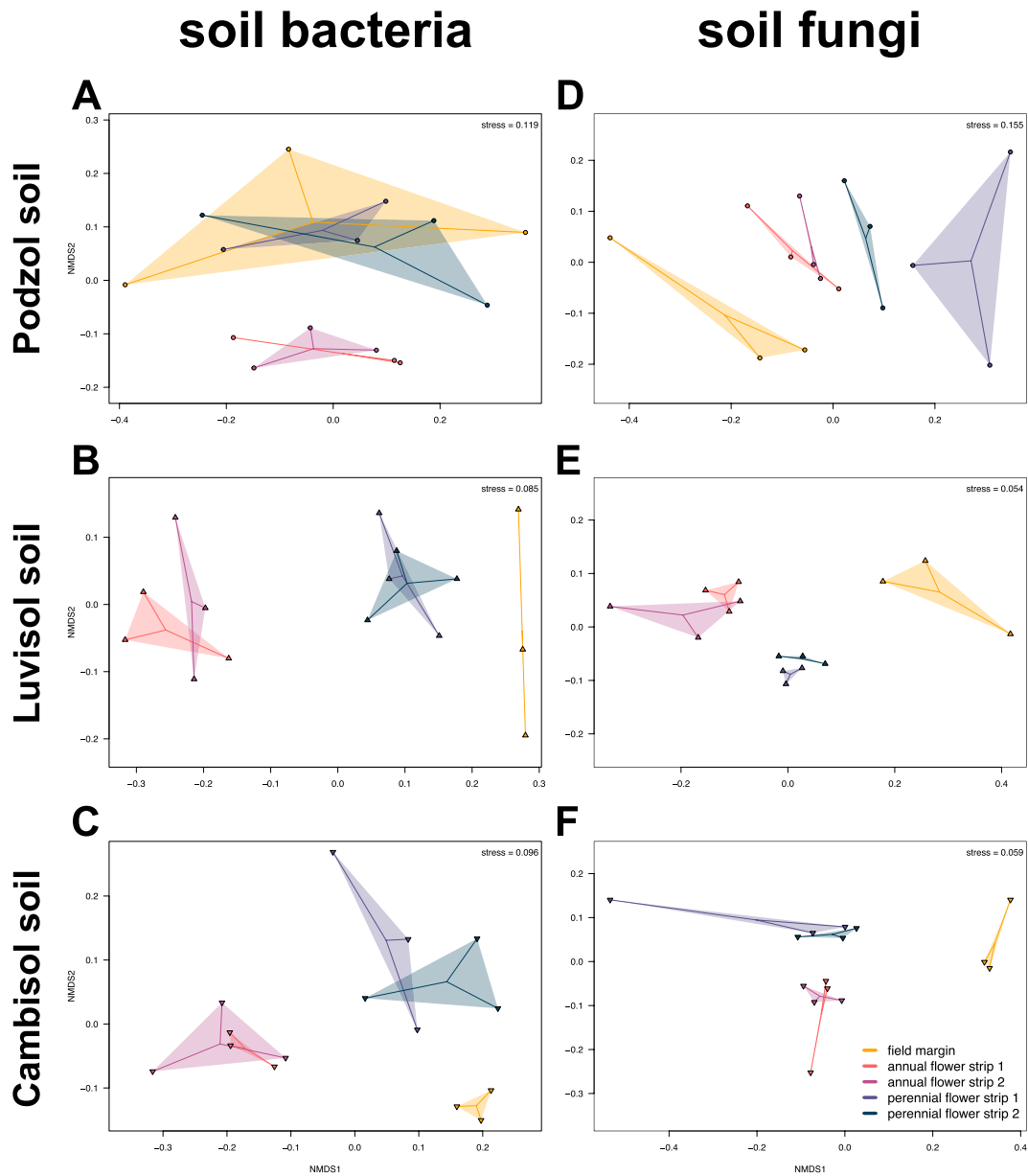


Fig. 4 Non-metric multidimensional scaling (NMDS) of Bray–Curtis dissimilarities of soil bacterial and fungal communities. NMDS plots of bacterial (A, B, C) and fungal communities (D, E, F) within each soil type. Dots and triangles

represent individual data points (i.e. replicate plots) ($n=3$) which are connected with the centroid of their respective plant mixture

tillage operation (Fig. 2A, Figure SI 1). Such rapid recovery of earthworm populations following tillage agrees with previous studies showing population recoveries within few months post tillage (Marinissen 1992; Boström 1995). Tillage is well-known to affect density, biomass, and community composition

of earthworms (Chan 2001; Ernst and Emmerling 2009). While density of anecic species generally decreases under tillage due to physical damage and the removal of plant litter from the soil surface (e.g. Ernst and Emmerling 2009), responses of endogeic species to tillage are rather inconsistent. While some

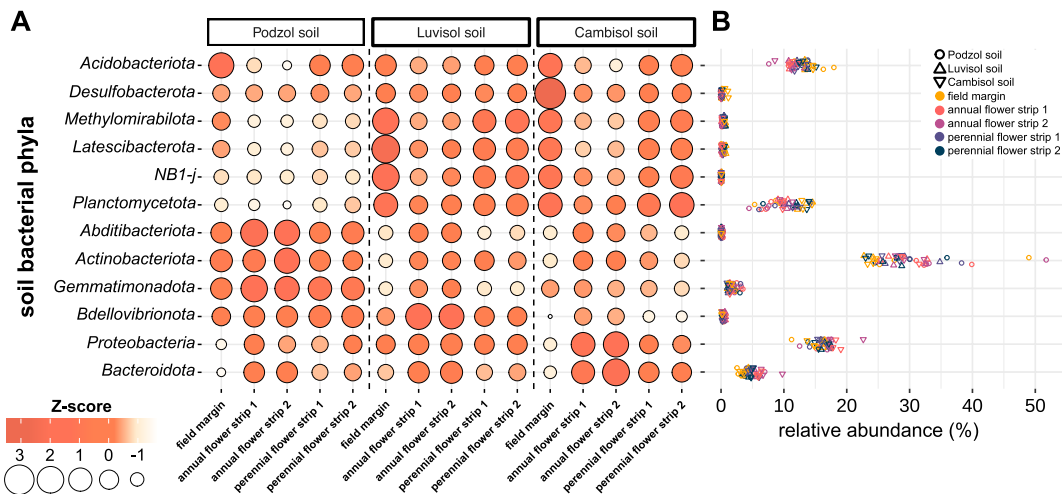


Fig. 5 Selected soil bacterial phyla in flower strips. Z-score normalized relative abundance (**A**) and relative abundance (**B**) of bacterial phyla in three different soil types. Colored dots and triangles represent individual data points (i.e. replicate plots) (**B**)

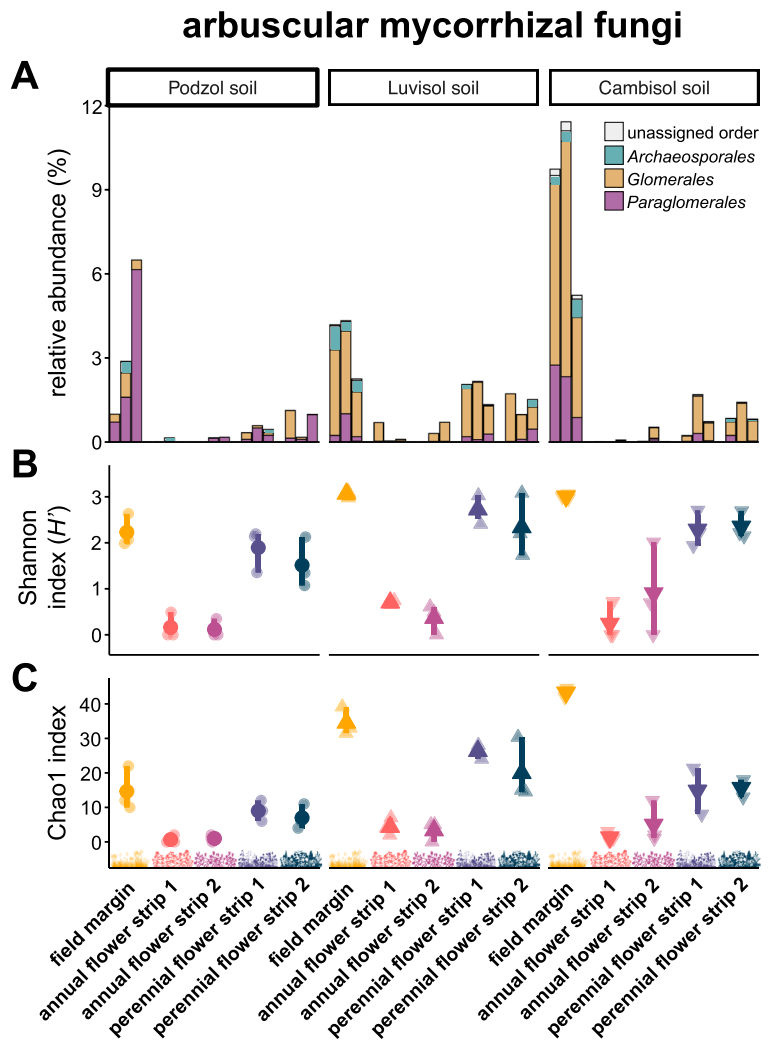
studies showed that the density of endogeic species is either unaffected (e.g. Pelosi et al. 2014; Torppa and Taylor 2022) or greater in ploughed soils as compared to soils under reduced or no tillage (e.g. Ernst and Emmerling 2009; Capowicz et al. 2009), due to the incorporation of plant residues that serve as a food resource, other studies found a negative impact of tillage on endogeic earthworm density (e.g. Edwards and Lofty 1982; Simonsen et al. 2010). In view of these inconsistent results, Briones and Schmidt (2017) recently conducted a global meta-analysis on the effects of tillage on earthworm abundance and biomass. Their results revealed that the population densities of all three ecological groups benefit from reduced tillage and that epigeic and anecic species benefit more than endogeic (Briones and Schmidt 2017). Their results agree with our findings of a decline in all three ecological groups of earthworms (epigeic, endogeic, and anecic) under the tilled annual flower strips as compared to the non-tilled field margin and perennial flower strips (Fig. 2A).

Although differences in tillage regimes can explain the low earthworm densities in the annual flower strips, they do not explain the increased population densities in the non-tilled perennial flower strips as compared to the non-tilled field margin (Fig. 2A, C, D). The impacts of plant richness and biomass on earthworm communities have frequently been studied in grasslands. While some studies revealed a positive impact of plant richness and biomass on earthworm

density and biomass (Zaller and Arnone 1999; Spehn et al. 2000; Eisenhauer et al. 2013), other studies were not able to confirm this (Wardle et al. 1999; Hedlund et al. 2003). These discrepancies among studies may be related to, *inter alia*, interactions with other soil biota (Milcu et al. 2006) and plant community composition (Gastine et al. 2003; Milcu et al. 2006, 2008; Eisenhauer et al. 2009). In our study, perennial flower strip mixtures showed higher plant species richness of sown plants than field margin mixtures (File SI 1), possibly contributing to the higher earthworm density and biomass observed in all three soil types (Fig. 2A, C, D, Figure SI 1). Although plant biomass was not determined in our study, previous studies showed that plant biomass production (and consequently plant litter production) generally increases with plant richness (e.g. Cardinale et al. 2007). Thus, we suggest that compared to the field margin, earthworm communities in the perennial flower strips benefited from higher quantities of above- and belowground plant litter (i.e. food resources). We further suggest that perennial flower strips not just increase the quantity of food input but also alter its quality which may be even more important for soil decomposer communities (e.g. Milcu et al. 2006; Eisenhauer and Reich 2012).

The spatial design of our study may have also affected the community dynamics of earthworms. At each study site, earthworm communities are likely to respond to a change in management through differential recruitment from the local species pool. However,

Fig. 6 Arbuscular mycorrhizal fungi (AMF – *Glomeromycota*) in flower strips. Relative abundance of AMF orders in three different soil types (A). Bars represent individual replicate plots ($n=3$). Shannon (H') (B) and Chao1 index (C) of AMF. Non-transparent dots and triangles represent means and vertical bars range from the minimum to the maximum value ($n=3$). Transparent dots and triangles represent individual data points (i.e. replicate plots). Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library)



although our plots were fully randomized, our study design did not restrict influxes of earthworms from spatially close soil environments. In this context, habitat connectivity represents an important driver of earthworm migration (Palm et al. 2013).

Soil microbiome

Plant mixture (i.e. field margin and different flower strips) was identified as a strong driver of bacterial and fungal community composition (Table 1, Fig. 4). Dissimilarities in community composition of bacteria and fungi between the annual flower strips and the other plant mixtures in each soil type (Fig. 4) may be related to tillage during the re-establishment of the annual flower strips. There is compelling evidence

of not only changes in microbial population size (Mathew et al. 2012) but also in community composition of bacteria and fungi in response to tillage intensity (e.g. Degruene et al. 2016; Smith et al. 2016; Yin et al. 2017; Frøslev et al. 2022). For example, a global meta-analysis revealed that conservation tillage benefits bacterial and fungal biomass in soil (Chen et al. 2020). Another meta-analysis from the same year showed that the absence of tillage increased the relative abundance of *Acidobacteria* but decreased the relative abundance of *Actionobacteria* (Li et al. 2020). These results agree with our findings on these two phyla (Fig. 5, Table SI 3). Furthermore, a recent study was able to show that tillage also changes the vertical distribution of bacterial and fungal communities in soil (Sun et al. 2018). In light of the strong

impact of tillage on soil structure (Pagliai et al. 2004) and the subsequent consequences for soil as a biological habitat (Young and Ritz 2000), it is conclusive that tillage can affect the composition of soil microbiomes.

Besides differences in soil management, differences in plant species composition as well as richness of the plant mixtures (field margin < annual flower strips < perennial flower strips; see File SI 1) likely contributed to the observed changes in community composition. Considering the plant richness, this assumption is supported by the differences in community composition between the non-tilled field margin and the non-tilled perennial flower strips. Although not determined in our study, differences in plant biomass among treatments may also have affected community composition. There are numerous interactions between plants and soil microorganisms that shape the soil microbiome. For example, plant root exudates shape the soil microbiome (especially in the rhizosphere) by recruiting plant-beneficial microorganisms (Vives-Peris et al. 2020). The quantity and quality of root exudates depend on abiotic and biotic stressors but also plant species and age (Badri and Vivanco 2009). Thus, it is reasonable to assume that microbial community composition was driven by the variation in the root exudation due to differences in plant species composition of the different plant mixtures. Indeed, a recent microcosm experiment proposed root exudates as an important link between plant richness and the soil microbiome (Steinauer et al. 2016). Furthermore, differences in plant species composition are expected to result in differences in the quantity and quality of above- (leaves, stalks) and belowground (roots) plant litter among plant mixtures which have been identified as a driver of soil microbial communities (e.g. Allison et al. 2005) and could thus have contributed to the observed community shifts.

The soil bacterial community composition was strongly affected by the plant mixture at phylum level (Fig. 5). There are several studies showing that microbial community composition drives ecosystem functions (Wagg et al. 2014), whereas the concept of functional redundancy within microbial systems (Louca et al. 2018) challenges this relationship. Currently, there is no consensus on the relationship between microbial composition and microbiome functionality as some recent studies from distinct environments demonstrated (Fierer et al. 2013; Galand et al. 2018).

Another issue connected to this is that linking microbial identities to functional potentials of the microbiome remains challenging (Fierer 2017). Although there are several tools to predict functional potential profiles from the taxonomical profiles of microbiome data sets (Djemiel et al. 2022), we decided to not use these tools because microbiome data generated from short-read amplicons may not be suitable to accurately predict microbiome functions (Heidrich and Beule 2022). Instead, we suggest that future studies should measure actual microbial processes in flower strips and link these with microbiome data in order to test whether flower strips alter the functionality of the soil microbiome. The plant mixtures (Figure SI 3) did not alter functional genes involved in soil-N cycling. Abundances of soil-N-cycling genes have shown pronounced temporal dynamics, which are likely to be determined by plant growth stages (Regan et al. 2017). Since our study comprised of a single sampling point in time, we recommend that future studies should quantify N-cycling microorganisms repeatedly across the vegetation period to capture temporal dynamics of these communities. Finally, we argue that complementing microbiome data obtained from amplicon sequencing with absolute quantification of functional groups of microorganisms is a step towards understanding microbial functions in environmental systems.

In contrast to the differences in beta diversity (i.e. compositional dissimilarities among plant mixtures) discussed above, overall alpha diversity of bacteria and fungi remained mostly unaffected by the plant mixtures (Fig. 3). These results agree with the findings of Prober et al. (2015) who found that plant richness in grasslands is a predictor of beta but not alpha diversity. Alpha diversity of AMF, however, was affected by the plant mixtures (Fig. 6B, C). In addition to the diversity of AMF, plant mixtures also affected the relative abundance of AMF (Fig. 6A). The greater community share and diversity of AMF in the non-tilled (field margin and perennial flower strips) than in the tilled (annual flower strips) plant mixtures (Fig. 6) agrees with previous studies that showed that reduced tillage favors AMF (e.g. Säle et al. 2015; Bowles et al. 2017). Recently, Holden et al. (2019) compared AMF communities in field margins to those in arable land and found that field margins alter AMF community composition and increase AMF diversity as compared to arable land. Few years earlier, Verbruggen et al.

(2012) proposed that AMF colonization could take place via different nearby landscape elements such as field margins. Although neighboring croplands were not investigated in this study, we hypothesize that perennial flower strips serve as a reservoir for AMF and enhance AMF colonization of neighboring crops.

Conclusion

Grassy field margins, annual and perennial flower strips harbor distinct earthworm and soil microbial communities. Compared to field margins, earthworm density and biomass declined or remained unaffected in annual flower strips but increased in perennial flower strips. Soil type was the strongest predictor of bacterial and fungal community composition. However, plant mixture (i.e. field margin, annual and perennial flower strips) affected microbiome assembly within each soil type. Although overall alpha diversity of bacteria and fungi remained mostly unaffected by the plant mixtures, AMF showed greater diversity and community share in non-tilled (i.e. field margin and perennial flower strips) as compared to tilled plant mixtures (i.e. annual flower strips). We attribute the observed changes in soil biota mainly to differences in tillage and plant richness. Overall, our data suggests that perennial flower strips serve as refugia for soil biota in agricultural landscapes. Thus, future studies should compare the population size, diversity, and functionality of soil biota in flower strips to those in adjacent agricultural fields in order to assess the belowground benefits of flower strips. Furthermore, we suggest to investigate whether beneficial effects on belowground biota are restricted to the perennial flower strips or extend into adjacent agricultural fields ('spillover') as they do for certain aboveground biota. We hope that our work provides a starting point for research on the biodiversity and function of belowground communities in flower strips.

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Author contributions ZB, AV, SB, VHK, and LB contributed to the conception and design of the study. ZB, AV, and LB performed the field work. ZB, AV, and LB performed the laboratory work. ZB, AV, and LB performed the statistical analysis. ZB, AV, SB, and LB wrote the first draft of the manuscript.

NH, BH, and VHK contributed resources and critically revised the manuscript. All authors read and approved the manuscript.

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Data Availability We uploaded our data to different repositories (NCBI and BonaRes Data Centre) and indicated this in the manuscript.

Declarations

Conflict of Interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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