The impact of spatial diversification on earthworm communities in agroecosystems

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Declaration of Authorship

I hereby certify that I have written this thesis independently and have not used any other sources or aids than those indicated by me. My own contribution is listed for every included publication. I have not previously submitted this thesis to any other doctorate procedure.

Berlin, 07. September 2023

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This thesis is a cumulative work based on the following manuscripts:

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Own contribution: I contributed to the conception of the study. I conceptualized the earthworm sampling and contributed to the field work. I conducted the earthworm taxonomy. I conducted the statistical analysis and visualization (figures and tables) of the earthworm and soil characteristics data. I contributed to the first draft of the paper and led the writing on the earthworms and soil characteristics parts of the paper and reviewed and edited it with regards to the suggestions made by the co-authors and journal reviewers.

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Contents

Summary

Soil organisms are organized in highly diverse communities that provide numerous ecosystem services and contribute decisively to the productivity and resilience of agricultural systems. Over the last decades, however, agricultural intensification has led to a loss of biodiversity, compromising the beneficial functions performed by soil communities. To counteract this decline, a number of agri-environmental schemes have been implemented to maintain and promote biodiversity. For example, spatial diversification (e.g., flower strips) can effectively promote aboveground biodiversity, whereas little is known on the impact of such measures on belowground communities. Earthworms are an integral component of soil communities as they perform key ecological functions. Earthworms are negatively affected by intensive agricultural management, especially intensive soil management. Consequently, the implementation of perennial structures into agricultural systems (e.g., perennial flower strips and tree rows through agroforestry) is expected to benefit earthworm communities. However, field-based studies validating this assumption, remain scarce. Here, we conducted two studies (Chapters 2 and 3) to evaluate the impact of flower strips and alley-cropping agroforestry on earthworm communities.

For that purpose, we sampled earthworms using chemical extraction with allyl isothiocyanate (AITC) under different flower strip mixtures (two annual and two perennial mixtures) and a grassy field margin vegetation at three different sites in Germany (Chapter 2). We found that perennial flower strip mixtures harbored greater earthworm population density and biomass than field margin vegetation, whereas population density and biomass were lower in annual flower strip mixtures as compared to the field margins and perennial flower strip mixtures. The absence of tillage in the field margins and the perennial flower strips as well as high plant diversity of the perennial flower strips are expected to cause the promotion of earthworms.

Similar effects of soil management were observed in an alley-cropping agroforestry system in Germany (Chapter 3). Here, we used AITC extraction to sample earthworms in the tree rows, at different distances from the trees into the crop row, and in an adjacent cropland monoculture without trees. We found increased earthworm population density and biomass as well as an altered community composition under the trees as compared to the crop row and the monoculture cropland. The absence of tillage under the trees was most likely the main beneficial factor influencing earthworm communities. In addition, increased above- and belowground litter input in close proximity to the trees might also have promoted earthworms, as some of the recorded positive effects also extended into the crop row. Despite our findings, several knowledge gaps regarding the impact of spatial

diversification measures on earthworms remain (e.g., influence of different flower strip mixtures, tree row orientation, and age of the perennial structures).

To fill these knowledge gaps, more field-based studies are required. However, commonly used methods for earthworm sampling and species determination are demanding and expensive. The standardized sampling method for earthworms requires hand sorting of the excavated topsoil and subsequent chemical extraction (e.g., with AITC) of the subsoil. Although this method offers high recovery rates of earthworms, hand sorting is labour-intensive, time-consuming, and destructive towards the sampling site. In Chapter 4 we, therefore, compared this standardized method to a method using only AITC extraction without hand sorting at eleven different sites in Germany. We found AITC extraction without hand sorting to be a viable alternative for investigations regarding anecic earthworms and overall species richness as well as for on-site comparisons of the whole community.

Following earthworm sampling, determination of the collected individuals on species level is a necessary step in order to draw conclusions regarding earthworm functions. Species determination is mostly carried out through morphological identification, which is time-consuming, requires taxonomical expertise, and is usually not suitable for the identification of juveniles and cryptic species. Molecular approaches such as DNA barcoding, however, are expensive and hence not commonly used. In Chapter 5, we investigated the potential of high-resolution melting (HRM) curve analysis as a cost-saving alternative to DNA barcoding. In our study, HRM curve analysis enabled the distinction between eight earthworm species commonly found in European agricultural soils. We were also able to distinguish different haplotypes of the earthworm species *Allolobophora chlorotica* using HRM curve analysis, which indicates the potential of the method to differentiate between cryptic species. Additionally, HRM curve analysis is suitable for the identification of juveniles and damaged individuals and could thus serve as a complementary tool to morphological identification.

Overall, the results presented in this thesis show that spatial diversification through perennial flower strips and agroforestry systems generally benefits earthworm communities. Furthermore, it can be concluded that for certain research questions, AITC extraction and HRM curve analysis are viable options to facilitate field-based earthworm research. By this, we hope that remaining knowledge gaps regarding the response of earthworm communities to agricultural management practices can be filled and thereby further practises that preserve the integrity of earthworm communities in agricultural soils can be identified and implemented into agri-environmental schemes.

Zusammenfassung

Bodenorganismen bilden hoch diverse Gemeinschaften, welche eine Vielzahl an Ökosystemleistungen erbringen, wodurch sie entscheidend zum Erhalt produktiver und resilienter landwirtschaftlicher Systeme beitragen. Über die letzten Jahrzehnte führte die landwirtschaftliche Intensivierung jedoch zu einem Verlust an Biodiversität, wodurch das Potential der Bodengemeinschaften zur Erbringung von Ökosystemleistungen eingeschränkt wird. Es wurden daher eine Reihe biodiversitätsfördernder Agrarumweltmaßnahmen eingeführt, um diesem Rückgang entgegenzuwirken. Beispielsweise können räumliche Diversifizierungsmaßnahmen (z.B. Blühstreifen) oberirdische Biodiversität nachweislich fördern. Zum Einfluss solcher Strukturen auf das Bodenleben ist jedoch wenig bekannt. Regenwürmer sind ein integraler Bestandteil von Bodengemeinschaften und erbringen diverse Ökosystemleistungen. Jedoch werden auch Regenwürmer durch intensives landwirtschaftliches Management, insbesondere intensive Bodenbearbeitung, negativ beeinträchtigt. Folglich ist zu erwarten, dass sich räumliche Dauerstrukturen (z.B. mehrjährige Blühstreifen und Baumreihen in Agroforstsystemen) positiv auf Regenwurmgemeinschaften auswirken. Feldbasierte Studien, die diese Annahme bestätigen könnten, sind jedoch rar. Daher führten wir zwei Studien zum Einfluss von Blühstreifen und Agroforstsystemen auf Regenwürmer durch (Kapitel 2 und 3).

Dafür haben wir an drei Standorten in Deutschland Regenwürmer mittels chemischer Extraktion mit Allylisothiocyanat (AITC) in Plots mit verschiedenen Blühstreifenmischungen (zwei einjährige und zwei mehrjährige Mischungen) und einer grasbasierten Feldrandvegetation erhoben (Kapitel 2). Es zeigte sich, dass mehrjährige Blühstreifen eine höhere Populationsdichte und Biomasse an Regenwürmern beherbergten als die Feldrandvegetation. In den einjährigen Blühstreifen hingegen waren Populationsdichte und Biomasse geringer als in der Feldrandvegetation und den mehrjährigen Blühstreifen. Wir vermuten, dass sowohl die pfluglose Bodenbearbeitung der Feldrandvegetation und der mehrjährigen Blühstreifen als auch die hohe Pflanzendiversität in den mehrjährigen Blühstreifen ausschlaggebend für die Förderung der Regenwurmgemeinschaften waren.

Ähnliche Effekte der Bodenbearbeitung konnten wir in einem Agroforstsystem in Deutschland zeigen (Kapitel 3). Hier verwendeten wir AITC-Extraktion, um Regenwürmer in den Baumreihen, in der Getreidereihe an unterschiedlichen Distanzen zu den Baumreihen und in einer nahegelegenen Getreidereinkultur zu erheben. Verglichen mit der Getreidereihe und der Getreidereinkultur zeigten Baumreihen dabei höhere Populationsdichten und Biomasse sowie eine veränderte Artzusammensetzung der

Regenwurmgemeinschaften. Hauptgrund für diese Förderung der Regenwurmgemeinschaften unter den Bäumen war vermutlich, dass in den Baumreihen keinerlei Bodenbearbeitung stattfindet. Einige der positiven Effekte waren außerdem bis in die Getreidereihe hinein messbar, was darauf hindeutet, dass erhöhter ober- und unterirdischer Streueintrag durch die Bäume ebenfalls zu der Förderung beigetragen hat. Trotz unserer Ergebnisse bleiben diverse Wissenslücken zum Einfluss räumlicher Strukturen auf Regenwurmgemeinschaften bestehen (z.B. Einfluss verschiedener Blühstreifenmischungen, Ausrichtung der Baumreihen und Alter der mehrjährigen Strukturen).

Mehr feldbasierte Regenwurmstudien sind notwendig, um diese Wissenslücken zu schließen. Übliche Methoden für Regenwurmerhebung und Artbestimmung sind jedoch aufwändig und kostenintensiv. Die standardisierte Erhebungsmethode für Regenwürmer sieht vor, dass ausgehobener Oberboden händisch auf Regenwürmer durchsucht wird und anschließend im Unterboden verbliebene Würmer mittels chemischer Extraktion ausgetrieben werden (z.B. mit AITC). Diese Form der Erhebung hat zwar eine hohe Effizienz, dafür ist sie arbeitsintensiv und zeitaufwändig und darüber hinaus destruktiv für die Probenahmestelle. In Kapitel 4 haben wir daher an elf verschiedenen Standorten in Deutschland die standardisierte Methode mit einer Methode verglichen, bei welcher nur AITC-Extraktion ohne Handsortierung verwendet wird. Wir konnten zeigen, dass AITC-Extraktion ohne Handsortierung eine praktikable Alternative ist, wenn nur anektische Regenwürmer oder die Gesamtartenvielfalt auf unterschiedlichen Flächen verglichen werden sollen. Die Methode ist außerdem geeignet für Vergleiche der gesamten Regenwurmgemeinschaft innerhalb einer Fläche.

Damit im Feld erhobene Regenwurmdaten mit Bezug auf die Funktionen von Regenwürmern ausgewertet werden können, ist es notwendig alle Individuen auf Artniveau zu bestimmen. Die Artbestimmung bei Regenwürmern erfolgt in der Regel morphologisch. Morphologische Bestimmung ist jedoch zeitaufwändig, benötigt taxonomische Expertise und ist ungeeignet für die Bestimmung von juvenilen Tieren und Kryptospezies. Molekulare Ansätze wie DNA-Barcoding sind hingegen kostenintensiv und finden in der Praxis bislang kaum Anwendung. In Kapitel 5 untersuchten wir daher das Potential hochauflösender Schmelzkurven (HRM) Analyse als kostenschonende Alternative zu DNA-Barcoding. In unserer Studie ermöglichte HRM-Analyse die Unterscheidung von acht Regenwurmarten, welche in europäischen Ackerböden typisch sind. Mittels HRM-Analyse waren wir auch in der Lage verschiedene Haplotypen der Regenwurmart *Allolobophora chlorotica* voneinander zu unterscheiden, was darauf hindeutet, dass die Methode verwendet

werden könnte, um zwischen Kryptospezies zu differenzieren. HRM-Analyse ist außerdem in der Lage juvenile und beschädigte Individuen auf Artniveau zu bestimmen und wäre daher eine wertvolle Ergänzung zur morphologischen Bestimmung.

Zusammengefasst zeigen die in dieser These präsentierten Ergebnisse, dass räumliche Diversifizierung durch mehrjährige Blühstreifen und Agroforstsysteme für Regenwürmer förderlich ist. Außerdem konnte gezeigt werden, dass für bestimmte Fragestellungen AITC-Extraktion und HRM-Analyse praktikable Optionen sind, um feldbasierte Regenwurmforschung zu vereinfachen. Wir erhoffen uns, dass dadurch bestehende Wissenslücken über den Einfluss landwirtschaftlicher Maßnahmen auf Regenwurmgemeinschaften geschlossen werden können und weitere für Regenwürmer förderliche Maßnahmen identifiziert und in Förderprogramme implementiert werden.

1 Introduction

Most terrestrial biodiversity is located in soils (Anthony *et al.* 2023) and covers prokaryotic (i.e., bacteria and archaea) and eukaryotic life forms (e.g., collembolans, earthworms, fungi, isopods and nematodes) (e.g., Bardgett & van der Putten 2014). These organisms form diverse and complex belowground communities which provide several beneficial soil functions. For example, the cycling of nutrients in soil is mainly mediated by soil microorganisms (Van Der Heijden *et al.* 2008), plant health is, *inter alia*, promoted by earthworms (e.g., Plaas *et al.* 2019), and soil microstructure is strongly influenced by collembolans (e.g., Rusek 1998). Consequently, a taxonomically and functionally diverse soil biome is key to soil health, which in turn is crucial for the provision of food, feed, fibre and fuel as well as other ecosystem services provided by soils, such as climate regulation and erosion control (Kibblewhite *et al.* 2008). Soil health is also directly linked to human health (e.g., Brevik *et al.* 2020) and thus, the protection and restoration of soils is a main objective of a number of political strategies (e.g., EU Soil Strategy for 2030, EU Biodiversity Strategy for 2030, and 2030 Agenda for Sustainable Development by the United Nations). Among soil organisms, earthworms are an important bioindicator for soil health (Paoletti 1999), contribute to a number of ecological functions (e.g., water infiltration, nutrient cycling, and soil formation), and make up a large share of the biomass of the soil macrofauna (Fragoso & Lavelle 1992).

1.1 Functions of earthworms in agricultural soils

Ecological groups of earthworms

Based on behavioural and morphological traits, lumbricid earthworms are commonly classified into three distinct ecological groups (i.e., anecic, endogeic and epigeic, see Figure 1) introduced by Bouché in 1972. To which degree different earthworm species provide certain functions is depending on, *inter alia,* the ecological group they belong to, as the three groups vary widely in earthworm size, lifestyle and behaviour (e.g., Bouché 1977).

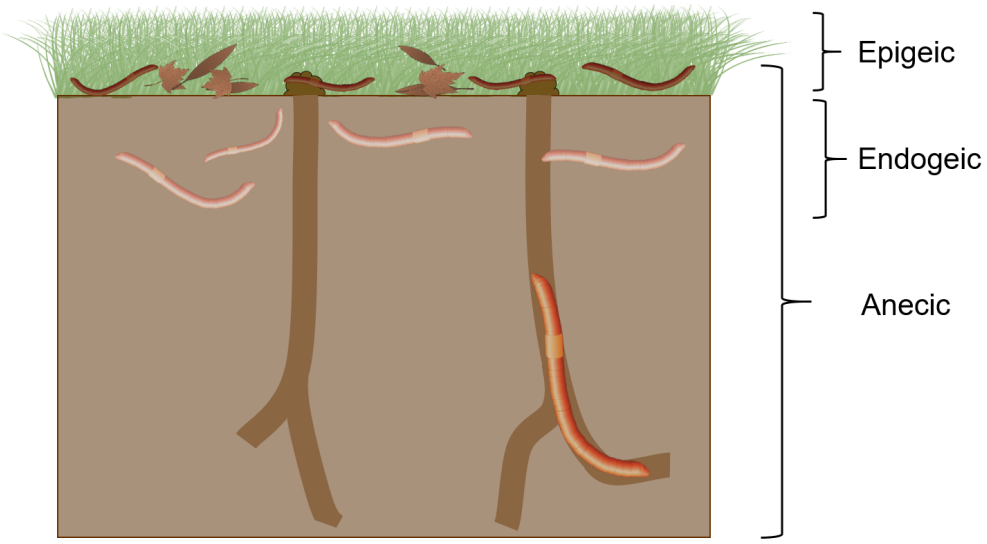

Figure 1: Schematic representation of the three common ecological groups of earthworms (i.e., epigeic, endogeic and anecic) and their occurrence in different soil layers. Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library)

Anecic earthworms are generally characterized by their relatively large size and heavy pigmentation which is restricted to the dorsal end of the body (Edwards and Bohlen 1996). They create up to 6 m long vertical, permanent burrows reaching from the soil surface into the subsoil (Bouché 1977). They are mainly detritivorous feeding on organic material such as leaf litter or straw, which they find on the soil surface and then pull into their burrows (Bouché 1977) where it is decomposed by microorganisms, such as fungi, which are also a feeding source for anecic earthworms (e.g., Curry & Schmidt 2007). Anecic species prefer soils with low sand content, as high sand content might destabilize their burrows (Römbke *et al.* 2005) and require habitats with consistent input of organic material on the surface. Consequently, they suffer from tillage events and intensive soil management, as it destroys their burrows and leaves the soil surface bare, which deprives them of their feeding source (Chan 2001). Important anecic species common to German croplands are *Lumbricus terrestris* and *Aporrectodea longa* (Jänsch *et al.* 2013).

Endogeic species vary in size but are generally characterized by a lack of pigmentation, as they live in the mineral topsoil (Edwards and Bohlen 1996, Bouché 1977). They are geophagus and feed mainly on mineral soil enriched with organic material by burrowing horizontally through the topsoil (Bouché 1977). Their habitat requirements are species-specific and they are found in a variety of habitats (e.g., Jänsch *et al.* 2013). In German agricultural soils, *Allolobophora chlorotica*,

Aporrectodea caliginosa, *Aporrectodea rosea* and *Octolasion tyrtaeum* are the most important representatives of this group (Jänsch *et al.* 2013).

Epigeic earthworms are generally rather small (1 to 3 cm, Bouché 1977) and fully pigmented, as their habitat is the vegetation layer and the O horizon on top of the mineral soil layers (Edwards and Bohlen 1996, Bouché 1977). They show little to no burrowing activity in the soil and feed almost exclusively on organic material (Edwards and Bohlen 1996). Since they do not colonize the soil, they require habitats that constantly contain accumulations of organic material on the soil surface, such as decomposing plant material or manure (Bouché 1977). Consequently, they are rarely found in cropland soils, as those are often associated with periods in which the soil lays bare (e.g., after tillage events) and thus cannot provide habitat or feeding source for epigeic earthworm species. In German croplands, only *Lumbricus rubellus* and *Lumbricus castaneus* are commonly found (Jänsch *et al.* 2013), as they show some intermediate behaviour between ecological groups.

Based on these different characteristics, it is not surprising that earthworm species of different ecological groups provide different ecological functions (e.g., litter incorporation by anecic species). It should be noted however, that a classification based on these three groups is not always enough to predict a species' ability to provide a certain ecological function (e.g., Capowiez *et al.* 2014), as some species might have intermediate positions between groups and would thus require a classification based on seven groups (Bouché 1972, Bottinelli *et al.* 2020, Bottinelli & Capowiez 2021).

Soil structure

In their role as ecosystem engineers (Jones *et al.* 1994), earthworms provide a number of ecological functions, such as aggregate formation, increased water infiltration and greater gas exchange by changing the soil structure (Lee & Forster 1991).

Already in 1975, Ehlers reported a positive connection between water infiltration rate and earthworm density, due to their ability to increase the number of macropores in the soil (Ehlers 1975). Bouché and Al-Addan made similar observations in 1997, reporting a positive correlation between water infiltration rate and earthworm biomass (Bouché & Al-Addan 1997). Capowiez *et al.* (2009) found that large macropores created by earthworms increased water infiltration in a reduced tillage system and that earthworms can restore macropores and thus water infiltration rate after a compaction event (Capowiez *et al.* 2012). In the same study however, it was pointed out that a complete restoration of macropores after compaction takes up to two years, indicating that earthworm activity takes considerable amounts of time to

changes soil structure (Capowiez *et al.* 2012). The ability to increase water infiltration rates varies between earthworm ecological groups (Ernst *et al.* 2009a). For example, Ernst *et al.* (2009a) found that endogeic earthworm species increase water infiltration stronger than anecics due to their higher burrowing activity. Contrasting to that, Capowiez and co-authors (2014) reported that anecic earthworms generally create longer burrows that unlike burrows of endogeic species are not refilled by soil and that endogeic species hence have only a small impact on water infiltration compared to anecics (Capowiez *et al.* 2021). The authors also point out that some species have intermediate positions between ecological groups and thus, belonging to an ecological group might not be suited to predict a species capability to increase water infiltration (Capowiez *et al.* 2014).

Earthworms can also increase the porosity of soils by forming water-stable aggregates, whereby bacteria in the earthworm gut and fungi hyphae in the soil drive the stabilization of the aggregates as long as sufficient organic material is available in the soil (Swaby 1950). However, others found that aggregates created by earthworms are generally less stable than natural aggregates but enriched with organic carbon (Zhang & Schrader 1993, Schrader & Zhang 1997). In 2010, Bottinelli *et al.* found similar results on the stability of earthworm casts, reporting a high turnover rate for fresh casts and potentially even a decline in soil porosity, as aggregates produced by earthworms easily dissolve under rainfall events and thus, the soil is more prone to compaction. Unlike Schrader and Zhang (1993), the authors also found a reduced amount of organic C in the casts of *M. posthuma* as compared to the bulk soil (Bottinelli *et al.* 2010). These contrasting results may be explained with the different earthworm species investigated, as Schrader and Zhang (1993) found that earthworm species with contrasting feeding behaviour (i.e., *L. terrestris* and *A. caliginosa*) showed varying concentrations of organic carbon in their casts, which might consequently also lead to different stabilization rates of the casts (Zhang & Schrader 1993, Schrader & Zhang 1997). Similar results were described by Ketterings *et al.* (1997), who reported an increase of water-stable aggregates enriched with C and N in plots with increased earthworm populations (Ketterings *et al.* 1997). In a mesocosms experiment, Coq *et al.* (2007) also found an increase of water-stable macroaggregates in treatments with earthworms and slightly higher C concentrations in earthworm casts as in the bulk soil (Coq *et al.* 2007). In the same study, the authors also described higher mineralization of carbon in the earthworm casts and lower total C concentrations in mesocosms containing earthworms than in those without earthworms (Coq *et al.* 2007) which hints towards the importance of earthworm-derived aggregates for soil fertility.

Soil fertility

Soil fertility is another main aspect influenced by earthworms and over the last decades, a vast number of research has been committed towards this topic. It has been reviewed multiple times, that earthworms can enhance soil fertility formation mainly by increasing the mineralization of organic matter, thus enhancing nutrient availability, and by incorporating C into soil aggregate fractions, facilitating C sequestration (e.g., Bhadauria 2010, Blouin *et al.* 2013, Ahmed & Al-Mutairi 2022). Earthworms also enhance soil and humus formation by accelerating both mineral weathering and decomposition of organic material (Carpenter *et al.* 2007, Blouin *et al.* 2013).

Already in 1992, Tomati and Galli reported more available nutrients and polysaccharides in the casts of *A. caliginosa* and *E. fetida* as compared to the corresponding control soils. In 2003, Chaoui *et al.* were able to show that earthworm casts contained higher amounts of nutrients compared to soil and reduced salinity stress for plants compared to compost and NPK fertilizers (Chaoui *et al.* 2003). A number of studies were able to show that earthworms enhance nutrient mineralization by stimulating microbial communities in their guts (e.g., Edwards & Fletcher 1988, Fujii 2012, Liu *et al.* 2017). Additionally, earthworms facilitate decomposition of organic material by incorporating it into the soil (e.g., Fahey *et al.* 2013) and enhancing soil aeration through their burrowing activity (e.g., Edwards 2004), both of which helps in providing more favourable conditions for microbial degradation of organic material.

Contrary to their ability to increase nutrient availability, earthworms are also known to help sequester C by producing clay-humus complexes and incorporating them into micro aggregates (e.g., Bossuyt *et al.* 2004, Ferlian *et al.* 2014). Many different factors influence this trade-off between mineralization and fixation, such as earthworm species (e.g., Hale *et al.* 2005), composition of organic material (e.g., Ernst 2009b), interactions with plants (e.g., van Groeningen *et al.* 2019), and land use (e.g., Pulleman 2005). In a long-term mesocosms experiment, Lubbers *et al.* (2017) were able to show that earthworms stabilize C in aggregates while simultaneously increase C mineralization and CO_2 emissions.

Certain earthworm species are also used in the production of fertile vermicompost from biological waste (Edwards *et al.* 2010). Vermicompost contains high amounts of plant-available nutrients (e.g., Lim *et al.* 2015), lower salinity levels as compared to regular compost (Lazcano *et al.* 2008), and has even been shown to suppress phytopathogens (e.g., Sahni *et al.* 2008). Consequently, the application of

vermicompost as an organic fertilizer is gaining more popularity, highlighting yet another way by which earthworms can enhance soil fertility.

Phytopathogens and soil suppressiveness

Natural control of soil-borne phytopathogens is another main function earthworms can provide in agricultural soils through a number of direct and indirect mechanisms (e.g., Brown 1995). Pathogenic fungi are among the most important soil-borne phytopathogens, and a vast number of studies investigated the mechanisms by which earthworms and vermicompost can inhibit fungal pathogens, subsequently enhancing soil suppressiveness (e.g., Schrader *et al.* 2013, Gudeta *et al.* 2022).

One potential mechanism by which earthworms reduce plant pathogenic fungi is direct consumption, as fungi are generally assumed to be a major food source for earthworms (Curry & Schmidt 2007) and many fungi species are negatively affected by earthworm gut fluids (Byzov *et al.* 2007). In the same study, the authors were also able to show that the soil microbial community was altered by passing through the gut of different earthworm species (i.e., *Aporrectodea caliginosa*, *Lumbricus terrestris* and *Eisenia fetida*), selecting for certain bacteria and fungi species as well as rising the bacteria-to-fungi ratio in the earthworm casts (Byzov *et al.* 2007). Some earthworm species, such as *Lumbricus terrestris*, have even been shown to have a feeding preference towards straw infected with pathogenic fungi (Moody *et al.* 1995) and certain pathogenic *Fusarium* and *Rhizoctonia* strains (Bonkowski *et al.* 2000). Similar results were obtained by Oldenburg and co-authors (2008), who reported faster incorporation rates by *Lumbricus terrestris* for straw infected with *Fusarium culmorum* than for non-infected straw. In accordance with that, in a microcosm experiment, Schrader *et al.* (2009) were able to show that earthworms (i.e., *Lumbricus terrestris)* feed on straw infected with *Fusarium culmorum*, thereby accelerating its degradation and also reducing the concentration of the mycotoxin deoxynivalenol (DON) in soil. This ability of *Lumbricus terrestris* to suppress *Fusarium culmorum* in the soil under field conditions was later confirmed in a mesocosm experiment by Meyer-Wolfarth *et al.* (2017). Euteneuer *et al.* (2019) found similar results on the consumption of the pathogenic fungi *Sclerotinia sclerotiorum* by *Lumbricus terrestris* (Euteneuer *et al.* 2019).

Even though digestion and earthworm metabolism are one potential pathway by which earthworms reduce fungal pathogens in soils, it cannot serve as a sole explanation, as some fungi species are known to still be viable after digestion (Byzov et a. 2007). Already in 1994, Toyota and Kimura found that earthworms of the genus *Pheretima* reduce the abundance of plant pathogenic *Fusarium oxysporum* in a microcosm experiment, even though the spores of the fungi were still viable after gut

passage. Additionally, earthworms are known to suppress pathogenic fungi by polysaccharides (Wang *et al.* 2007) and coelomic fluid (Plavšin *et al.* 2017). Additionally, earthworms can also indirectly inhibit pathogens by incorporating infected straw into the soil and thereby enhancing its decomposition (e.g., Wolfarth *et al.* 2011a), increasing the number of microbial antagonists (e.g., Hume *et al.* 2015) and improving nutrient availability for plants and thereby promoting plant health (e.g., Plaas *et al.* 2019).

It should be noted that not all earthworm species have the same antifungal potential and that the pathways by which they suppress pathogenic fungi depend on their lifestyle, feeding habits and ecological group. Wolfarth *et al.* (2011 a,b) have shown that *Lumbricus terrestris* significantly reduced soil surface cover by incorporating infected wheat straw, reduced biomass of the plant pathogen *Fusarium culmorum* and enhanced degradation of DON. Whereas in the same study, the contribution of the endogeic species *Aporrectodea caliginosa* towards pathogen suppression was rather minor and restricted to fungal material already belowground (Wolfarth *et al.* 2011 a,b). However, in a pot experiment with wheat infected with take-all disease Hume *et al.* (2015) found that *Aporrectodea caliginosa* did not improve decomposition of infected straw but still reduced the severity of the disease, which they attributed mainly to enhancement of microbial antagonists and improved nutrient availability. Plaas and co-authors (2019) concluded that deep burrowing, detritivorous species such as *Lumbricus terrestris* mainly suppress fungi by digestion and incorporation of infected straw, while geophagus earthworm species enhance soil suppressiveness rather indirectly by improving plant health. Bongiorno and co-authors (2019) found a positive correlation between soil suppressiveness and earthworm density in soils from 10 long-term field experiments in Europe using a bioassay with *Pythium ultimum* - *Lepidium sativum*, demonstrating the important role earthworms play in preserving soil health.

Soil greenhouse gas emissions

Despite all their useful ecological functions, it should also be noted that it is currently discussed whether earthworm activity can have negative implications for greenhouse gas (GHG) emissions from soil (carbon dioxide (CO_2), nitrous oxide (N_2O) and methane). In a meta-analysis of 57 studies, Lubbers *et al.* (2013a) found that the presence of earthworms generally increases the emission of CO_2 and N_2O from soil. Earthworms can increase GHG emissions through the promotion of denitrifying bacteria in their gut (Horn *et al.* 2003) as well as by increasing decomposition rates of organic material in the soil and on the soil surface (Lubbers *et al.* 2015). To what extend earthworms increase GHG emissions however, is still not clear, as their effect

on GHG emissions is strongly influenced by, *inter alia*, soil moisture content (Bertora *et al.* 2007), weather conditions (Lubbers *et al.* 2013b), and ecological group of earthworms (Giannopoulos *et al.* 2010). Their influence on GHG emissions has also been shown to change over time (Lubbers *et al.* 2013a) and earthworms might potentially even lead to decreased emission rates of certain GHGs after a longer period of time (Bertrora *et al.* 2007).

1.2 Earthworms in sustainable agricultural systems

All the ecological functions provided by earthworms underline their importance in agricultural soils. Consequently, maintaining healthy earthworm communities in agricultural landscapes is a crucial objective to maintain soil health and thus the productivity of sustainable agricultural systems. It has long been known that land use strongly influences earthworm communities (Fragoso *et al.* 1999), and that agricultural intensification can lead to a loss of earthworm diversity and biomass (Decaëns *et al.* 2002). Identifying management practices that promote earthworms and reduce the negative effect of agricultural land use is therefore in the focus of current earthworm research.

Crop rotation and tillage are among the most studied management aspects known to have an impact on earthworm communities. In a review article from 2001, Chan described a strong influence of tillage on the abundance and diversity of earthworms which was dependent on, *inter alia*, ecological group of earthworms, soil characteristics and type of tillage (e.g., tillage depth). Especially deep burrowing anecic species of earthworms are generally negatively influenced by soil tillage (Chan 2001). In 2022, Torppa and Taylor found higher densities of anecic earthworms under no tillage as compared to conventional tillage but only a small effect of crop rotation that was only visible under conventional tillage. In the same study, the authors reported increased densities of endogeic earthworms under a wide crop rotation (i.e., winter wheat – peas – oilseed rape) as compared to a narrow crop rotation (i.e., winter wheat – spring barley) independent of the tillage regime (Torrpa & Taylor 2022). Contrasting results were obtained by Capowiez *et al.* (2009) who were able to show that the effect of three different cropping systems on earthworm abundance was more pronounced under reduced tillage as compared to conventional tillage. Similarly, Rodriguez and co-authors (2020) described a promotion of earthworm densities and biomass through diversification and intensification of crop rotations under no-till management. Generally, however, earthworms seem to mostly benefit from reduced tillage and diversified crop rotations.

Consequently, it can be expected that spatial diversification (e.g., by the introduction of non-tilled field margins) should have positive impacts on earthworm

communities. Unsurprisingly, Smith *et al.* (2007) found that the establishment of field margins in a winter wheat field increased earthworm density and diversity but the effect was strongly influenced by management of the margins, especially when it involved soil disturbance. In a large study comparing earthworm communities under 50 field margins and adjacent crop fields in Finland, the authors reported higher earthworm densities as well as a shift towards anecic and epigeic species and increased species richness in the margins (Nieminen *et al.* 2011). Similar results were obtained by Crittenden *et al.* in 2015, who also found higher earthworm densities in the field margins as compared to the adjacent arable fields. In 2017, Frazão *et al.* were also able to show that the positive effect of field margins on earthworm communities was influenced by management of the margins (i.e., mulching and margin age) as well as the surrounding landscape (i.e., area of arable land).

While the influence of field margins on earthworm communities is relatively well described, only a small number of studies has investigated the influence of flower strip establishment on earthworms. The introduction of flower strips is a popular way to increase spatial diversity, promote pollinator services and increase natural pest control (e.g., Geppert *et al.* 2020, Tschumi *et al.* 2015, Albrecht *et al.* 2020). Since soil management in perennial flower strips is similar to field margins, positive effects on earthworm communities can be expected. So far however, only the study by Kohli *et al.* (1999) investigated earthworms under flower strips and indicated a potential positive effect of flower strips on earthworms. More studies are needed to investigate the influence of different flower strip mixtures, management, spacing, and placement as well as to describe whether positive effects on earthworm communities can expand into the adjacent crop fields.

Since reduced soil disturbance has been established as a major promoting driver for earthworm communities, the introduction of even more permanent spatial structures such as tree rows is of great interest for earthworm research. The introduction of trees into arable fields, also known as agroforestry, is a common practice in the tropics and is gaining increasing popularity in the temperate zone, as agroforestry systems bring numerous benefits compared to cropland monocultures (Veldkamp *et al.* 2023). The main advantages of such systems are reduced soil erosion (e.g., van Ramshorst *et al.* 2022) as well as improved nutrient cycling due to the trees (e.g., Allen *et al.* 2004). Some first studies, however, also indicate potential positive effects on earthworm communities (e.g., Price and Gordon 1998, Cardinael *et al.* 2019, D'Hervilly *et al.* 2022). In 2019, Cardinael *et al.* sampled earthworms in 13 agroforestry systems in France and found higher earthworm densities under the trees as compared to the cropland control plots without trees. In the same study, the

authors found no difference in earthworm density between sampling points at 2.5 m distance from the trees and in the middle of the crop row within the agroforestry system (Cardinael *et al.* 2019). In 2021, D'Hervilly *et al.* found more earthworms at 1 m distance from the trees as compared to 2.5 m in an alley-cropping agroforestry system in France and also reported a shift towards anecic species under the trees. Similar results were obtained by the same authors in 2020 and 2022, where they also reported more anecic earthworms under the trees as compared to the crop rows (D'Hervilly *et al.* 2020, D'Hervilly *et al.* 2022). However, all the studies described above measured at maximum two different distances from the trees, which does not allow to draw conclusions on how far earthworms are being promoted into the crop row. Little information is also known on the effect of different tree species and was so far only investigated by Price and Gordon (1998), who found an increase of earthworm densities under poplar but a decrease under ash compared to adjacent crop fields (Price & Gordon 1998).

1.3 Methods to investigate earthworm communities in the field

Studying earthworms directly in the field is a main prerequisite to understand the influence of agricultural management on earthworm communities and to identify sustainable management systems that promote earthworms and their functions. For that purpose, a variety of methods have been developed over time to investigate earthworm communities in the field. Most of these methods focus on extracting and counting the earthworms from the soil, while a few methods are based on earthworm activity rather than population density.

Manual hand sorting of excavated soil monoliths is one of the most common methods to collect earthworms from the soil (Phillips *et al.* 2021). For this method, soil monoliths that are usually 20 to 30 cm deep and cover a surface of 1/16 m² to 1 m² are excavated at the sampling site and subsequently sorted through by hand either on site or at a nearby location or laboratory. Hand sorting works well for adult endogeic species, as they live in the topsoil (e.g., Raw 1959, Pelosi *et al.* 2009). The method however, is not suitable for adult anecic species, as they generally burrow deeper than 30 cm, allowing them to escape into the subsoil (e.g., Callaham & Hendrix 1997, Chan & Munro 2001) and excavating down to the end of their burrows is usually not feasible. Hand sorting also underestimates very small juveniles and motionless, inactive earthworms, as they are harder to spot (e.g., Čoja *et al.* 2008). The method is also rather time consuming and labour intensive, especially for studies with a large number of sampling points, as the soil needs to be sorted through manually (e.g., Callaham & Hendrix 1997). Time and efficiency of the method also depend on the

experience of the persons performing the sorting (Bartlett *et al.* 2010). The method is also destructive towards the sampling site, as excavating and sorting through the soil destroys the soil structure (Čoja *et al.* 2008).

The main disadvantage of hand sorting remains its inefficiency for anecic species, which led to the development of alternative methods utilizing chemicals or irritating solutions to extract earthworms from deeper soil layers. For this method, water-based solutions with irritating substances are poured on a defined area of the soil surface and surfacing earthworms are collected either by hand or with tweezers. In the past, formaldehyde was commonly used but because of its toxicity for the handlers and the environment a number of alternatives have since been explored (e.g., Gunn 1992, Zaborski 2003, Steffen *et al.* 2013). Potential alternatives are, *inter alia*, hot mustard, chili powder, onion extracts or allyl isothiocyanate (AITC), with hot mustard and AITC being the most common (Pelosi *et al.* 2009). Chemical extraction is less time-consuming and requires fewer people compared to hand sorting. Depending on the stability and toxicity of the expulsion agent, it is also not destructive towards the sampling site and thus allows other parameters to be investigated in the same plot (Čoja *et al.* 2008). This method does, however, require large amounts of water to be carried to the sampling locations (Iannone *et al.* 2012), which can be quite tedious in remote locations. In the case of the irritating AITC, chemical extraction also needs to be handled carefully by experienced personnel (Valckx *et al.* 2011), which usually excludes its application in citizen science projects, where hot mustard would be more suitable (Iannone *et al.* 2012). Using chemical extraction works well for anecic species but underestimates endogeics because of their horizontal burrowing behaviour (e.g., Chan & Munro 2001, Bartlett *et al.* 2006, Gutiérrez-López *et al.* 2016). It is also not suitable for inactive earthworms that are in diapause because they do not react fast enough to the expulsion agent (Eisenhauer *et al.* 2008).

Consequently, combinations of chemical extraction and hand sorting are suggested when it comes to surveying the entire earthworm community (e.g., Zaborski *et al.* 2008, Bartlett *et al.* 2010). Two approaches are possible when combining both methods: First soil monoliths are excavated and sorted through by hand, afterwards the expulsion solution is poured into the remaining pit to extract earthworms in deeper soil layers. Alternatively, the expulsion solution is poured directly to the soil surface and the drenched soil is sorted through afterwards. The latter method is less common as it is making hand sorting more difficult and depending on the expulsion agent more dangerous (Andriuzzi *et al.* 2017). A combination of hand sorting and chemical extraction has a high efficiency for all three ecological groups and is recommended for earthworm sampling in an International Standard guideline

(ISO 23611-1:2018(E)). It does however, still underestimate very small juveniles and inactive earthworms and depends on the experience of the persons performing the hand sorting. It also requires large amount of water, experts handling the expulsion solution, and remains destructive towards the sampling site. It also requires even more time, personnel, and consequently money than hand sorting alone, which often makes it unfeasible for large study designs or monitoring projects (Iannone *et al.* 2012).

Another way to actively drive earthworms out of the soil is by using electricity. In 1986, Thielemann developed the electrical octet method, which uses electric voltage to force earthworms to the soil surface (Thielemann 1986). The method is non-destructive and non-toxic, which makes it especially attractive for surveys in urban areas, such as city parks where excavating the soil or application of chemical solvents are not possible (Pelosi *et al.* 2020). Unlike chemical extraction, electrical extraction has been shown to work better for juvenile worms, as large adult ones are more likely to be damaged by the electricity (Čoja *et al.* 2008). Additionally, the method requires large and expensive technical equipment to be carried to the study site (Čoja *et al.* 2008), experienced handling, and does not work well when soil moisture content is low (Eisenhauer *et al.* 2008).

Earthworms can also be extracted utilizing heat, for example using Kempson extraction. This method requires soil to be taken from the sampling site and brought to the laboratory, where it is placed in a Kempson extractor. In short, lamps hanging over the soil emit light and heat, which drives the earthworms deeper into the soil where they ultimately fall through a mesh bottom into a collection tray. This method shows the highest efficiency for juvenile earthworms (e.g., Čoja *et al.* 2008) but is extremely effortful, as all the soil needs to be transported back to the laboratory. Consequently, it is only applicable for small study designs and study sites that do not contain anecic species.

All the methods described above lead to earthworms being removed from a defined area of soil. Afterwards, earthworms are usually preserved in for example ethanol, or stored in water or soil until species, biomass and density are determined. Another approach to investigate earthworm communities is to monitor their activity. Methods for that include counting the number of casts on the surface or determine the rate by which straw or leaf litter is incorporated into the soil (Raw 1959). These methods are usually only applicable for estimating the activity of anecic earthworms and only during active periods.

1.4 Methods of earthworm species identification

Since earthworm functions differ strongly between ecological groups and species of earthworms, the identification of earthworms to species level is a necessary requirement for most monitoring or research questions that involve the collection of earthworms in the field.

Identification of earthworm species is commonly based on morphological traits (e.g., prostomium, clitellum, segments, and setae) with the help of local identification keys (e.g., Graff 1953, Bouché 1972, Sims & Gerard 1985, Krück 2018)). This can either be done with preserved dead worms (e.g., in ethanol) or living worms. Aside from ethical reasons, the latter also allows behavioural traits to be considered for the identification (Thielemann 1986) and does not require preservatives, which reduces health risks for the taxonomists, thus enabling its use in citizen science (e.g., Stroud 2019). However, morphological identification is time-consuming, often not possible for juvenile earthworms (Richard *et al.* 2010), and in most cases requires taxonomic expertise, which is increasingly hard to find (Decaëns *et al.* 2013). Additionally, not all species can be distinguished based on morphology due to the existence of cryptic species (King *et al.* 2008).

Consequently, molecular approaches to identify earthworms (e.g., DNA barcoding) are of increasing interest (Decaëns *et al.* 2013). Huang *et al.* (2007) were among the first to propose DNA barcoding of cytochrome c oxidase I (COI) genes as a complementary method to identify earthworms next to morphological identification (Huang *et al.* 2007). This technique was first described by Herbert *et al.* in 2003 and has since then been successfully applied to a number of taxa. Chang *et al.* (2009) however, pointed out that the use of the COI region on earthworms is limited due to intraspecific divergences of some earthworm species.

In general, existing molecular identification methods are associated with much higher costs as compared to morphological methods (i.e., for DNA extraction and sequencing), require specialised equipment and trained personnel, which makes them unfeasible for large studies and citizen science projects. However, molecular approaches offer the ability to identify cryptic species, damaged worms and juveniles, which would not be possible using morphological identification (e.g., Richard *et al.* 2010, Decaëns *et al.* 2013).

In 2010, Bartlett *et al.* reviewed that morphological identification based on identification keys is still the most common method for earthworm species identification, stressing the need to further develop alternative methods in order to overcome the limitations of species identification based on morphological traits. Nevertheless, morphological determination remains the most common technique in

earthworm studies today (e.g., Ashwood *et al.* 2019, Billaud *et al.* 2020) since molecular methods are still too expensive and other alternatives such as micro-computed tomography (Fernández *et al.* 2014), mid-infrared spectroscopy (Pham *et al.* 2021) and machine-learning approaches (Andleeb *et al.* 2021) are not yet established.

1.5 Aims

Earthworms provide a number of ecological functions (e.g., Blouin *et al.* 2013, Bertrand *et al.* 2015) but their abundance and diversity in arable lands are declining (Barnes *et al.* 2023), thus threatening crucial soil functions in agricultural systems. The influence of climate change and agricultural management on earthworm communities remains still largely unknown and more data is needed to identify and develop sustainable agricultural systems that can maintain healthy earthworm communities. Gaining more data under field conditions is crucial but laborious as the most common methods to sample and determine earthworms are time-consuming, labour-intensive, and expensive (e.g., Čoja *et al.* 2008). This limits the implementation of large-scale field studies or monitoring programs of earthworms.

Therefore, the main aims of this work were to i) investigate earthworms in sustainable agricultural systems with a focus on the implementation of perennial spatial structures (i.e., perennial flower strips and alley-cropping agroforestry systems) and ii) to compare and establish methods to investigate earthworms in the field and determine them at species level.

2 Earthworm and Soil Microbial Communities in Flower Strips

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RESEARCH ARTICLE

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').

Keywords Flower strips · Earthworms · Soil microbiome · Soil archaea · Soil bacteria · Soil fungi · Soil-N-cycling genes · Arbuscular mycorrhizal fungi (AMF)

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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 Pfleger (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 flowers 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 (Luvisoil 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

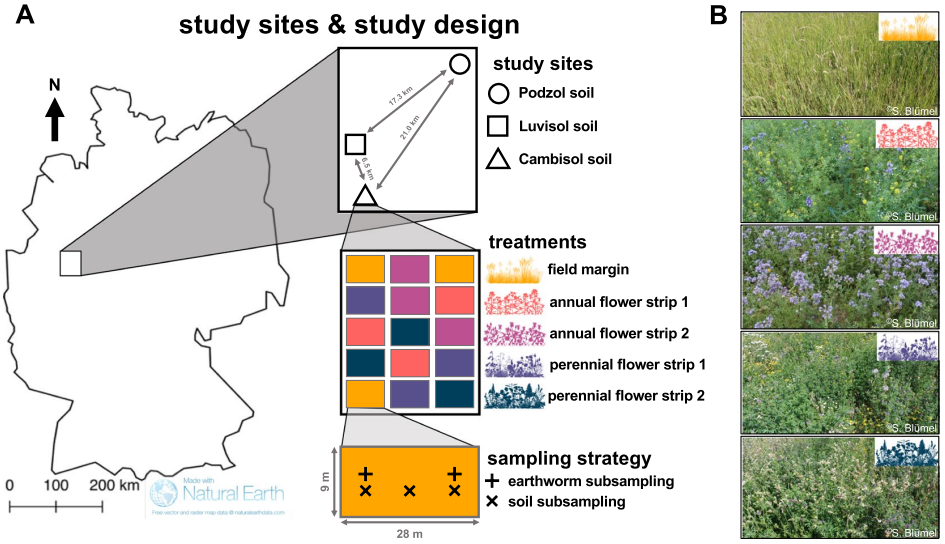

Fig. 1 Study sites and study design. Study sites and study design (**A**) and photos of the flower strips taken in July 2022 at the study site on the Cambisol soil (**B**). Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library)

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 cm^3 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_{CaCl2}), 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 (⌀ 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 H_2O 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 (VDLUFA 1991a) and Mg_{CaCl2} as per (VDLUFA 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 H_2O (ddH_2O) 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 Peqstar 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 CTAAKCC-3′ (Klindworth et al. 2013) and ITS1-F_KYO2 (5’-TAGAGGAAGTAAAAGTCGTAA-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 ddH_2O 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 ddH_2O for a negative control. The mastermix comprised ddH_2O, buffer (10 mM Tris–HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 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 $MgCaCl_2$) 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_{CaCl2}.

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 ± 6.1%), *Proteobacteria* (16.4 ± 1.8%), and *Acidobacteriota* (12.5 ± 1.8%). The dominating bacterial classes were *Actinobacteria* (20.1 ± 6.6%), *Alphaproteobacteria* (11.3 ± 1.1%), and *Planctomycetes* (7.9 ± 2.3%) (Fig. 3A). The fungal community was dominated by *Ascomycota* (65.4 ± 14.0%), *Mortierellomycota* (12.3 ± 9.5%), and *Basidiomycota* (12.0 ± 8.2%) on

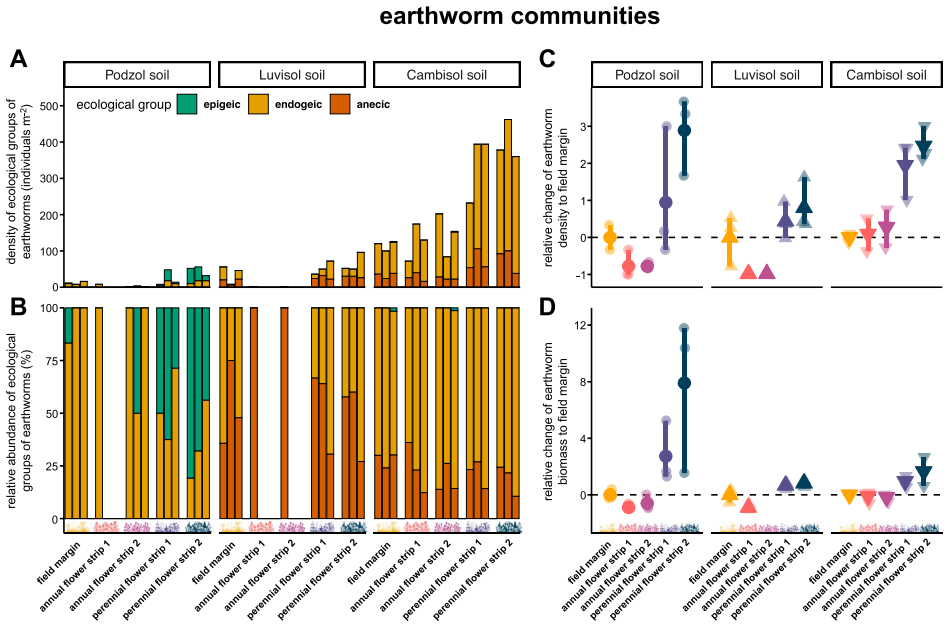

Fig. 2 Earthworm communities. Population densities of ecological groups of earthworms (**A**) and their relative abundance within the earthworm communities (**B**). Bars represent individual replicate plots ($n=3$). Relative change of earthworm density (**C**) and biomass (**D**) in response to flower 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 ± 16.0%), *Dothideomycetes* (17.2 ± 11. 6%), and *Mortierellomycetes* (12.2 ± 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

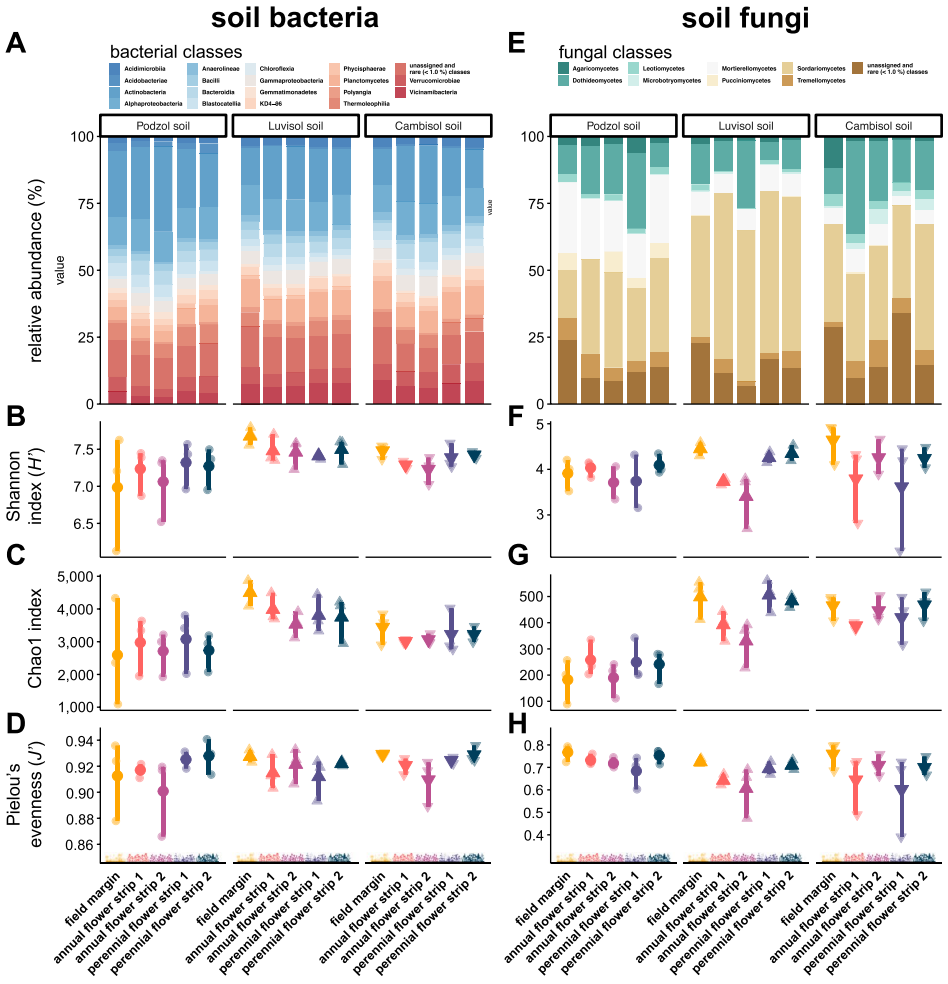

Fig. 3 Community composition and alpha diversity of soil bacteria and fungi. Mean relative abundance of bacterial (**A**) and fungal classes (**E**) per plant mixture and soil type. Alpha diversity indices of bacterial (**B**, **C**, **D**) and fungal communities (**F**, **G**, **H**). Non-transparent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

($p \leq 0.0011$; t-ratio = 5.9 to 10.3). Likewise, in the Luvisol soil, relative abundances of *Abditibacteriota* 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).

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

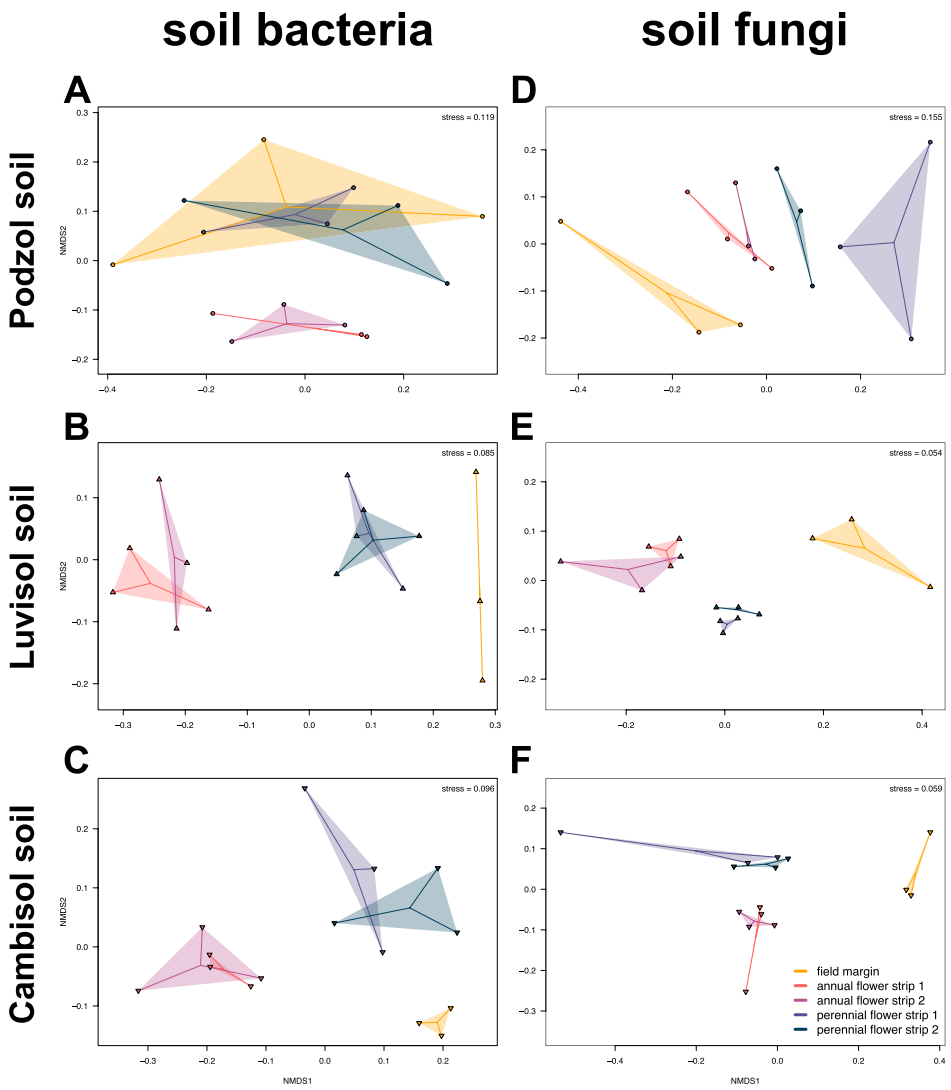

Fig. 4 Non-metric multidimensional scaling (NMDS) of Bray–Curtis dissimilarities of soil bacterial and fungal communities. NMDS plots of bacterial (**A**, **B**, **C**) and fungal communities (**D**, **E**, **F**) within each soil type. Dots and triangles represent individual data points (i.e. replicate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

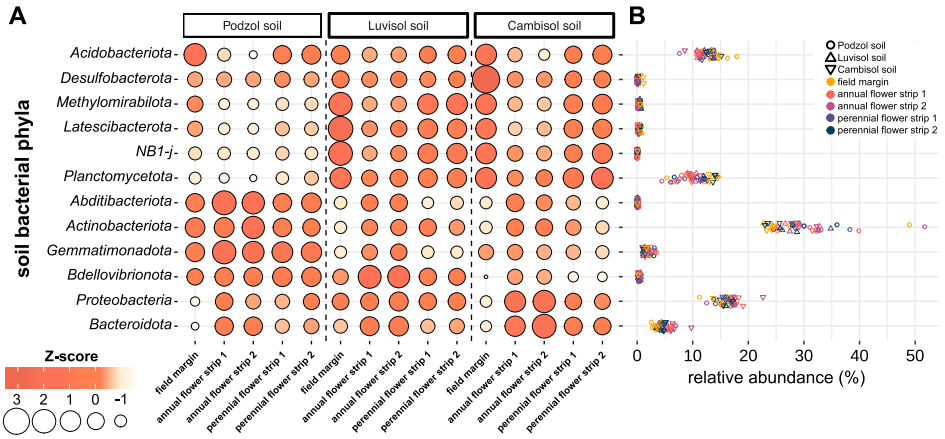

Fig. 5 Selected soil bacterial phyla in flower strips. Z-score normalized relative abundance (**A**) and relative abundance (**B**) of bacterial phyla in three different soil types. Colored dots and triangles represent individual data points (i.e. replicate plots) (**B**)

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; Capowiez 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,

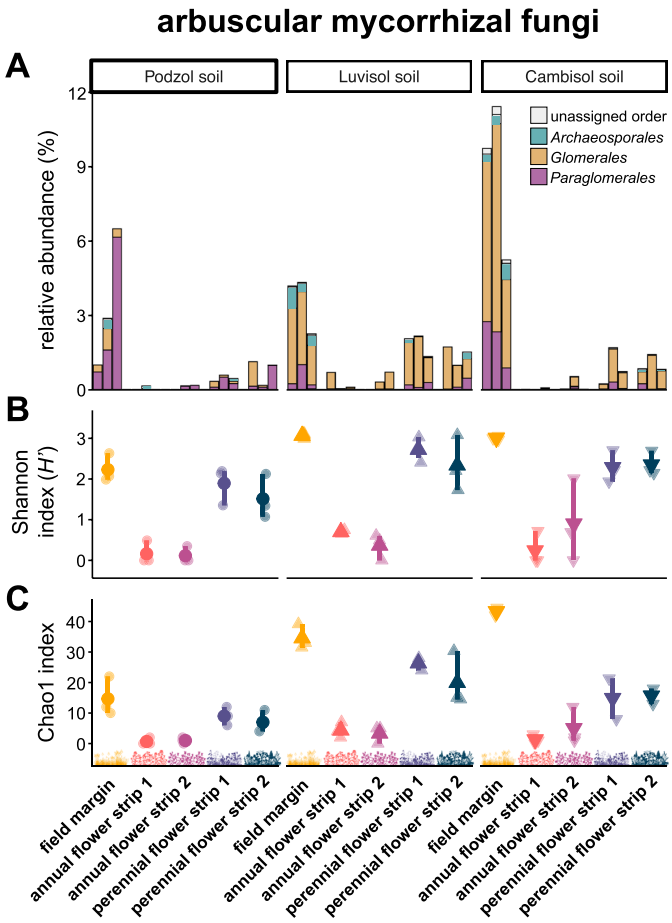

Fig. 6 Arbuscular mycorrhizal fungi (AMF – *Glomeromycota*) in flower strips. Relative abundance of AMF orders in three different soil types (**A**). Bars represent individual replicate plots ($n = 3$). Shannon (H') (**B**) and Chao1 index (**C**) of AMF. Non-transparent dots and triangles represent 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. Degrune 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 functionally 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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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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3 Tree-Distance and Tree-Species Effects on Soil Biota in a Temperate Agroforestry System

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RESEARCH ARTICLE

Tree-distance and tree-species effects on soil biota in a temperate agroforestry system

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Abstract
Aims Cropland agroforestry systems are land-use systems with numerous environmental advantages over monoculture croplands including promotion of soil life. This study aimed to investigate tree-species and tree-distance effects on soil biota in a temperate agroforestry system.
Methods Our study was conducted at a paired alley-cropping and monoculture cropland system. The tree rows of the agroforestry system comprised of blocks of poplar Fritzi Pauley, poplar Max 1 or black locust. Within the agroforestry system, soil microbial and earthworm communities were collected along transects spanning from the center of the tree rows into the crop rows. Archaea, bacteria, and fungi were quantified using real-time PCR. The community composition of fungi and earthworms was deciphered using amplicon sequencing and morphological identification, respectively.
Results Tree rows promoted the abundance of bacteria and earthworms, which we attribute mainly to tree litter input and the absence of tillage. Fungal community composition was altered by the tree rows, resulting in an increased proportion of ectomycorrhizal fungi in the tree-row associated mycobiome. The proportion of *Blumeria graminis,* the causal agent of powdery mildew, increased with increasing distance from the trees. We suggest that enhanced microbial antagonism, increased earthworm densities and/or altered microclimate contributed to the suppression of *B. graminis* in vicinity of the trees. Tree-species effect had a minor influence on the abundance and composition of soil communities at our study site.
Conclusions In comparison to monoculture cropland, agroforestry benefits the abundance, diversity, and function of soil biota and may enhance soil suppressiveness.

Keywords Temperate agroforestry · Alley cropping · Earthworms · Soil microorganisms · Soil mycobiome · Soil suppressiveness

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Introduction

Agroforestry systems are land-use systems that combine trees with crops and/or livestock. In the temperate zone, alley-cropping agroforestry systems that alternate rows of trees with rows of crops are gaining popularity. Tree rows of these systems usually consist of either fast-growing trees (e.g. poplar or willow) for biomass production or quality hardwoods such as cherry or walnut trees. The environmental benefits of temperate agroforestry practice are well recognized and include, *inter alia*, increased carbon sequestration (e.g. Mayer et al. 2022), reduced nutrient leaching (e.g. Allen et al. 2004; Wang et al. 2011), reduced soil erosion, and promotion of biodiversity (e.g. Varah et al. 2013, 2020). Certain advantages of alley-cropping systems over monoculture croplands, such as the complementary use of resources, are due to interspecific interactions between the trees and the crops (Jose et al. 2004). For example, deep-rooting trees are able to take up nutrients leached below the rooting zone of the crops ('safety-net'-function of the trees; Allen et al. 2004) and make them available to crops via litter fall. This process was recently dubbed as 'nutrient pumping' (Isaac and Borden 2019) and is supported by findings of increased soil nutrient availability in vicinity of the tree rows of temperate agroforestry systems (Pardon et al. 2017).

Soil biota are known to predominantly benefit from temperate agroforestry practice (Beule et al. 2022a; Marsden et al. 2020). Several studies on soil microorganisms in alley-cropping systems revealed that the tree rows promote microbial population size and that this effect is not only limited to the tree rows but extends gradually into the crop rows (i.e. stronger promotion in vicinity of the trees) (e.g. Beule et al. 2022b; Guillot et al. 2021). Furthermore, soil microbiome studies reported that the composition of bacterial and fungal communities strongly differs between the tree and crop rows (e.g. Beule et al. 2021; Beule & Karlovsky 2021a). Based on these studies, it was recently reviewed that alley-cropping systems increase microbial diversity in soil through increased beta diversity rather than alpha diversity (Beule et al. 2022a). Recent articles showed that soil fungal communities strongly respond to temperate agroforestry practices. For example, one study found that Basidiomycota in soil were up to 330 times more abundant in poplar tree rows of alley-cropping systems as compared to adjacent cropland monocultures (Beule et al. 2021). The same study used amplicon sequencing to investigate the composition of the soil fungal community and found that certain ectomycorrhizal fungi (*Cortinarius*, *Geopora*, and *Inocybe*) were particularly promoted by poplar trees, which holds great potential to improve nutrient acquisition in agroforestry systems (Beule et al. 2021). In the present study, we aimed to explore the impact of different tree species on soil fungal communities in agroforestry systems.

In 1999, Seiter and co-workers (1999) observed that most of the tree leaf litter in an alder–maize alley-cropping system was pulled into the burrows by *Lumbricus terrestris* but did not provide data to substantiate their field observation. Few months earlier, Price and Gordon (1998) published an article demonstrating tree-species specific promotion of earthworms through tree rows in an alley-cropping system. Since then, greater densities of earthworms in tree rows of temperate agroforestry systems as compared to bordering crop rows or adjacent monoculture croplands have been reported (Cardinael et al. 2019; D'Hervilly et al. 2022). Yet, studies investigating earthworm communities in temperate agroforestry systems along fine spatial gradients from the tree rows into the crop rows comprising more than two distances from the tree rows are scarce. Furthermore, most temperate agroforestry systems feature either a single tree species or an intermixture of tree species (e.g. Cardinael et al. 2019), thus disabling direct comparisons among tree species within the same system. To our best knowledge, the investigation by Price and Gordon (1998) is the only study to assess earthworm communities under different tree species within the same temperate agroforestry system. However, the authors did not report species identities or ecological groups of collected earthworms. Recent studies indicated differences in the response of different ecological groups of earthworms to agroforestry practice (Cardinael et al. 2019; D'Hervilly et al. 2022), highlighting the importance of investigating them.

In the present study, we chose two poplar species, as poplar trees are the most commonly planted short-rotation trees in modern alley-cropping agroforestry systems in the temperate zone. Although not commonly planted in temperate agroforestry systems, we further chose black locust as it is a fast-growing, nitrogen-fixing tree species. We expect differences

in overall soil biota communities under different tree species because root architecture, litter quality, and soil-nutrient cycling are tree species-specific (e.g. Das and Chaturvedi 2008, Aponte et al. 2013, Borden et al. 2017).

This study aimed to investigate the impact of three different tree species (two poplar species and black locust) on representatives of the soil biota community (archaea, bacteria, fungi, and earthworms) in a temperate alley-cropping agroforestry system. Furthermore, tree-distance effects on soil biota were tested by sampling multiple locations along transects spanning from the tree row into the crop row of the agroforestry system. We hypothesized that tree rows promote the abundance and alter the community composition of soil biota. We further hypothesized that these changes are dependent on the distance to the trees (tree-distance effect) as well as the tree species (tree-species effect).

Materials and Methods

Study site and sampling design

Our study site was located on a Gleyic Cambisol soil (IUSS Working Group WRB 2015) near Forst, Brandenburg, Germany (51°47′11″N, 14°38′05″E; m.a.m.s.l.: 67 m; mean annual temperature: 9.6 ± 0.2 °C; mean annual precipitation: 568 ± 21 mm; Fig. 1A), which is located in the glacially influenced region of the North German Plain. General biochemical and physical soil properties of the site were recently characterized by Schmidt et al. (2021). According to aerial images, the study site was under agricultural use for at least 50 years prior to conversion of cropland monoculture to alley-cropping agroforestry. At the study site, a conventionally managed alley-cropping agroforestry system was spatially paired with a conventional monoculture cropland system that served as a reference land use. The agroforestry system was established in 2010 and was 12 years old during sampling. The system consisted of 12 m-wide rows of trees (north–south orientation) that were alternated with 48 m-wide rows of arable crops (Fig. 1B). Tree rows were hand planted and consisted of three different tree species. The tree rows comprised four individual rows of trees (Fig. 1 C, D, E). The tree rows that defined the crop row consisted of three different tree species that were planted in alternating segments at a length of approx. 165 m (Fig. 1B). The different tree species were i) *Populus trichocarpa* cv. Fritzi Pauley (referred to as 'poplar Fritzi Pauley'; Fig. 1C), ii) *P. nigra* × *P. maximowiczii*

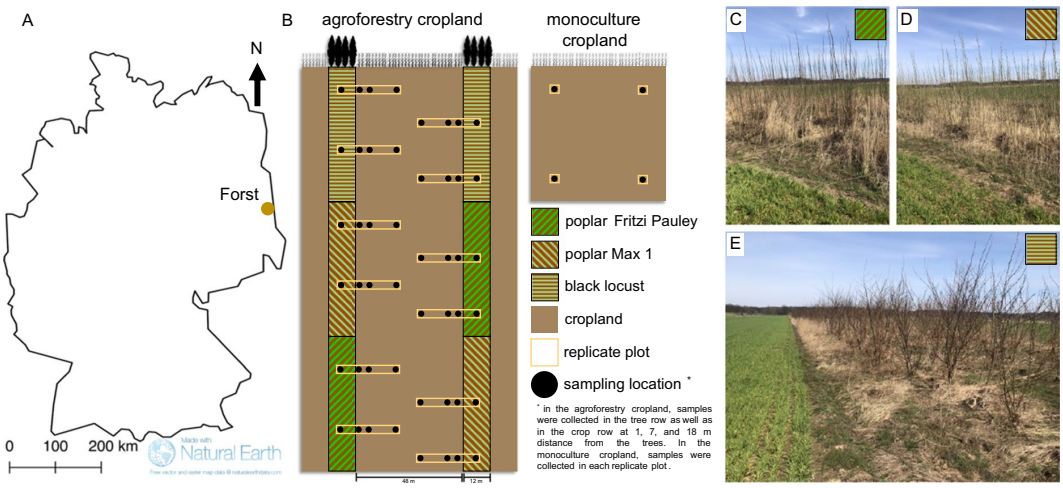

Fig. 1 Study site and study design. Location of the study site near Forst (federal state of Brandenburg), Germany (**A**), study design (**B**), and photos of the three different tree species cultivated at the site (poplar Fritzi Pauley (**C**), poplar Max 1 (**D**), and black locust (**E**)) taken in April 2022. Photo credit: Lukas Beule

cv. Max 1 (referred to as 'poplar Max 1'; Fig. 1D), and iii) black locust (*Robinia pseudoacacia*) (referred to as 'black locust'; Fig. 1E). The aboveground biomass of the trees was harvested using a forage harvester in February 2015, March 2018, and February 2021. Tree harvests were conducted when the soil was frozen to avoid soil compaction by the machinery.

The management of the crop rows was identical to that of the monoculture cropland. The crop rotation was maize (*Zea mays*) – summer barley (*Hordeum vulgare*) – summer oat (*Avena strigosa*) – winter wheat (*Triticum aestivum*) – winter barley (*H. vulgare*) (2018 – 2019 – 2020 – 2021 – 2022). In March 2022, the crop row of the agroforestry system and the monoculture cropland received 40 – 0 – 0 kg N – P – K ha^{-1} in the form of mineral fertilizer. Maize and small-grain cereal crops were harvested with a standard 24-m wide combine harvester. Crop rows and monoculture cropland were conventionally ploughed at a depth of 25 cm.

It is well established that trees in alley-cropping systems introduce spatial heterogeneity (e.g. Guillot et al. 2021; Beule et al. 2020). Thus, samples in the agroforestry system were collected along four transects (replicate plots) per tree species spanning from the tree row into the crop row. Samples were collected in the center of the tree row as well as at 1 m, 7 m, and 18 m distance from the trees within the crop row (3 tree species × 4 transects × 4 sampling locations within each transect = 48 samples; Fig. 1B). In the adjacent monoculture cropland, samples were collected in the center of each replicate plot (4 samples; Fig. 1B).

Soil and earthworm sampling

Soil samples for the determination of the microbial community and general soil properties (soil pH, soil organic carbon (SOC), total N, extractable nutrients) in the upper 5-cm topsoil were collected on 23 February 2022 (prior to fertilization). Three soil samples with a volume of 250 cm^3 were collected at each sampling location using stainless steel cylinders (5 cm height), transferred to a sterile polyethylene bag, and thoroughly homogenized. An aliquot of approx. 50 g fresh soil was transferred into a sterile 50-ml Falcon tube and frozen at -20 °C in the field. Upon arrival at the laboratory, samples were stored at -20 °C until freeze-drying for 48 h. The freeze-dried material was finely ground using a vortexer (Beule et al. 2019a) and stored at -20 °C until DNA extraction. The remaining fresh soil in the polyethylene bag was used for determination of the general soil properties. Since earthworm activity at our site is generally low in February, earthworm communities were sampled on 11 and 12 April 2022 as described below (see Extraction of earthworms). Soil samples (250 cm^3) for the determination on soil bulk density and water-filled pore space (WFPS) were collected on all sampling dates and sampling locations (see above).

Extraction of earthworms

Earthworms were directly extracted from soil by applying 5 L of 0.01% (w/v) allyl isothiocyanate (AITC) solution on an area of a quarter square meter as described by Zaborski (2003). The extractant was prepared in the field by adding 500 mg AITC (predissolved in 50 ml isopropanol (w/v)) to 4.95 L tap water immediately before extraction. To ensure that the extractant was applied only to a surface of 0.25 m^2, it was poured into a 50 × 50 cm open metal frame which was embedded approx. 5 cm deep into the soil (Figure S1 A). To allow better monitoring of extracted worms, plant material within the frame was carefully removed prior to the application of AITC. Following the AITC application, the soil surface within the frame was continuously monitored for at least 30 min and any surfacing earthworms were collected using tweezers, thoroughly washed, and stored in tap water (Figure S1 B). Upon arrival to the laboratory, earthworms were stored at 5 °C in the dark until morphological identification. Morphological identification and recording of the fresh weight (including gut content) of each individual earthworm were carried out within 48 h post sampling and earthworms were subsequently released. Furthermore, earthworms were classified into three ecological groups: anecic, endogeic, and epigeic species, which were introduced by Bouché in 1972. Earthworm data have been deposited at the BonaRes repository (https://doi.org/10.20387/bonares-y3je-zz30).

Soil DNA extraction

DNA from soil was extracted using a CTAB-based protocol with phenol and chloroform/isoamyl alcohol extraction (Beule et al. 2019a). Briefly, 50 mg

finely ground soil was suspended in a mixture containing 1 ml CTAB, 1 µL 2-mercaptoethanol, and 2 µL pronase E (30 mg/ml). The mixture was incubated at 42 °C and subsequently at 65 °C for 10 min each and 900 µL phenol were added. The mixture was shaken, centrifuged at 4,600×*g* at room temperature for 10 min, and 800 µL of the supernatant were transferred into a new 2 mL tube. 800 µL chloroform/isoamyl alcohol (24:1 (v/v)) were added, the mixture was shaken, incubated on ice for 10 min, and centrifuged at 4,600×*g* at room temperature for 10 min. Following centrifugation, 700 µL of the supernatant were transferred into a new 2 mL tube, 700 µL chloroform/isoamyl alcohol were added, the mixture was shaken, incubated on ice for 10 min, and centrifuged at 4,600×*g* for 10 min at room temperature. DNA was precipitated by transferring 600 µL of the supernatant into a new 1.5 mL tube containing 200 µL PEG 6,000 (30% (w/v)) and 100 µL 5 M NaCl. The mixture was incubated at room temperature for 20 min and centrifuged at 20,240×*g* at room temperature for 15 min to pellet the DNA. DNA pellets were washed with 70% (v/v) EtOH twice and dried using a vacuum centrifuge. Dried pellets were re-dissolved in 50 µL 1×TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA); adjusted to pH 8.0 with HCl) and incubated at 42 °C for 2 h to facilitate re-dissolving. Extracted DNA was inspected using gel electrophoresis on 1% (w/v) agarose gels stained with SYBR Green Solution I (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time PCR

Real-time PCR assays for the quantification of total bacteria, total fungi, Basidiomycota, Ascomycota, Acidobacteria, Actinobacteria, Alpha-, Beta-, Gammaproteobacteria, Bacteriodetes, and Firmicutes were performed in a Peqstar 96Q thermocycler (PEQLAB, Erlangen, Germany). The composition of the mastermix and the thermocycling conditions correspond to those described by Beule et al. (2020). Total archaea were amplified using the primer pair ARC787F and ARC1059R (Yu et al. 2005). The reaction mixture comprised of 3 µL mastermix (double distilled H_2O, buffer (10 mM Tris–HCl, 50 mM KCl, 2.0 mM $MgCl_2$, pH 8.3 at 25 °C); 100 µM of each deoxynucleoside triphosphate (New England Biolabs, Beverly, Massachusetts, USA); 0.3 µM of each primer; 0.1×SYBR Green I solution (Thermo Fisher Scientific, Waltham, MA, USA); 1 µg μL^{-1} bovine serum albumin; 0.03 u μL^{-1} Hot Start *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts, USA)) and 1 µL of template DNA or double distilled H_2O for a negative control. Thermocycling conditions were as follows. Initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation (95 °C for 20 s), annealing (62 °C for 30 s), and elongation (68 °C for 20 s). Final elongation was performed at 68 °C for 5 min. Following amplification, melting curves were generated by step-wise heating of the PCR product from 65 to 95 °C by 0.5 °C per step under continuous fluorescence measurement. DNA extracts were tested for PCR inhibitors according to Guerra et al. (2020) and diluted 1:50 (v/v) in double distilled H_2O prior to PCR to overcome PCR inhibition. Real-time PCR data have been deposited at the BonaRes repository (https://doi.org/10.20387/bonares-y3je-zz30).

Library preparation and amplicon sequencing of soil fungi

Sequencing libraries were prepared by amplifying the fungal ITS1 region using the primer pair ITS1-F_KYO2 (5'-TAGAGGAAGTAAAAGTCGTAA-3') (Toju et al. 2012) / ITS86R (5'-TTCAAAGAT TCGATGATTCA-3') (Vancov & Keen 2009). PCR reactions were carried out in 96-well plates in an Eppendorf Mastercycler EP Gradient S thermocycler (Eppendorf, Hamburg, Germany) in 25 µL reaction volume within one PCR run using the same mastermix for all libraries. The reaction volume comprised 18.75 µL mastermix and 6.25 µL template DNA diluted 1:50 (v/v) in double distilled H_2O or double distilled H_2O for negative a control. The mastermix contained double distilled H_2O, buffer (10 mM Tris–HCl, 50 mM KCl, 2.0 mM $MgCl_2$, pH 8.3 at 25 °C), 100 µM of each deoxynucleoside triphosphate (New England Biolabs, Beverly, Massachusetts, USA), 0.5 µM of each primer (ITS1-F_KYO2 / ITS86R), 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 adapters (5'-GACGTG TGCTCTTCCGATCT-3' for the forward primer and 5'-ACACGACGCTCTTCCGATCT-3' for the reverse

primer) at the 5`-end. The thermocycling conditions were as per (Beule and Karlovsky 2021a, b): initial denaturation (95 °C for 2 min), 3 touch-up cycles of denaturation (95 °C for 20 s), annealing (50 °C for 30 s), and elongation (68 °C for 60 s) followed by 25 cycles of denaturation (95 °C for 20 s), annealing (58 °C for 30 s), and elongation (68 °C for 60 s), and final elongation (68 °C for 5 min). Following thermocycling, successfulness of the amplification was confirmed by visualizing 2 µL of the PCR product on 1.7% (w/v) agarose gels stained with SYBR Green I solution (Thermo Fisher Scientific GmbH, Dreieich, Germany). Gel electrophoresis was performed at 4.6 V cm^{-2} for 60 min. Libraries were shipped to the facilities of LGC Genomics (Berlin, Germany) for a second amplification step using standard i7- and i5-sequencing adapters and Illumina sequencing. The second PCR was performed in 20 µL reaction volume containing 1 × MyTaq buffer (Bioline GmbH, Luckenwalde, Germany), 15 pmol of each forward and reverse i7- and i5- sequencing adapters, 2 µl of BioStabII PCR Enhancer (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and 0.075 u $µL^{-1}$ MyTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany).The thermocycling conditions were as follows: initial denaturation (96 °C for 1 min), 3 touch-up cycles of denaturation (96 °C for 15 s), annealing (50 °C for 30 s), and elongation (68 °C for 90 s) followed by 7 cycles of denaturation (96 °C for 15 s), annealing (58 °C for 30 s), and elongation (68 °C for 90 s), and final elongation (70 °C for 2 min). DNA quantity was assessed on agarose gels and indexed sequencing libraries were pooled. The multiplexed libraries were sequenced using on an Illumina MiSeq using V3 chemistry (2 × 300 bp) (Illumina, Inc., San Diego, CA, USA) at LGC Genomics, Berlin, Germany. Amplicon sequencing data have been deposited at NCBI's Short Read Archive (PRJNA885015).

Processing of amplicon sequencing data

Raw paired-end sequencing data were demultiplexed using Illumina's bcl2fast v. 2.20 (Illumina, San Diego, CA, USA) and sorted by their barcodes. Barcodes with more than two mismatches as well as one-sided and conflicting barcode pairs were excluded. Furthermore, Illumina sequencing adapters and primers (allowing three mismatches per primer) were clipped, whereas reads shorter than 100 bp were excluded. Afterwards, reads were processed in QIIME 2 version 2022.2 (Bolyen et al. 2019). Sequence quality was evaluated utilizing the 'q2-demux' plugin. Using DADA2 (Callahan et al. 2016), forward and reverse reads were truncated to 220 and 180 bp, respectively, quality filtered (allowing two expected errors per read), and merged. Moreover, chimeras and singletons were removed. Afterwards, reads were clustered into exact amplicon sequence variants (ASVs). ASVs were taxonomically classified against the UNITE database version 8.3 QIIME developer release (Abarenkov et al. 2021) using a scikit-learn Naive Bayes machine-learning classifier ('q2-fit-classifier-naive-bayes' and 'q2- classify-sklearn' plugin) in the 'balanced' configuration ([6,6]; 0.96) as suggested by Bokulich et al. (2018). After non-fungal ASVs were removed, 3,945,026 sequence counts remained. Sequence counts were normalized to 41,564 counts per sample using scaling with ranked subsampling (SRS) (Beule and Karlovsky 2020) in the R environment v. 4.2.1 (R Core Team 2013) utilizing the 'SRS' R package version 0.2.3 (Heidrich et al. 2021). SRS curves were generated using the same R package. The normalized dataset contained 2,949 fungal ASVs.

Soil properties

Soil pH was determined from sieved (< 2 mm) and air-dried soil in deionized H_2O at a ratio of 1:2.5 (soil/water (w/v)). Soil bulk density was determined from 250-cm^3 soil cores dried at 105 °C for 24 h. WFPS was determined from the same sample assuming a particle density of 2.65 g cm^{-3}. Double lactate-extractable phosphorus (P_{DL}) and potassium (K_{DL}) were determined from sieved (< 2 mm) and air-dried soil as per (VDLUFA 1991a). Calcium chloride-extractable magnesium (Mg_{CaCl2}) was determined as described previously (VDLUFA 1991b). Carbonates in sieved (< 2 mm) and air-dried soil were removed using acid fumigation as per Harris et al. (2001) and concentrations of SOC and total nitrogen were determined on a CNS elemental analyzer (Vario EL Cube, Elementar, Germany). Soil properties data have been deposited at the BonaRes repository (https://doi.org/10.20387/bonares-y3je-zz30).

Statistical analysis

For each tree species, differences in soil properties, microbial abundance, and earthworm parameters among the different sampling locations within the agroforestry system (i.e. tree row, 1 m, 7 m, and 18 m into the crop row) and the monoculture cropland were tested using one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD). Relationships between parameters (i.e. soil properties, microbial abundance, and earthworm parameters) were explored using Spearman's rank correlation test. For the soil fungal community, alpha (Shannon diversity, Chao1 index, and Pielou's evenness) and beta diversity (Bray–Curtis dissimilarity index) were computed using 'vegan' R package v. 2.6.2 (Oksanen et al. 2013). Differences in fungal community composition among the different sampling locations within the agroforestry system and the monoculture cropland were identified using permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis dissimilarities using the 'vegan' R package. Multivariate homogeneity of dispersions was tested using the same R package. Furthermore, differential abundance analysis using data collapsed at genus level was performed using the 'metacoder' R package v. 0.3.5 (Foster et al. 2017). The same package was utilized to generate heat trees. For each tree species, differences in alpha diversity and taxonomic groups of fungi among the different sampling locations within the agroforestry system and the monoculture cropland were tested using one-way ANOVA followed by Tukey HSD test or Kruskal–Wallis test followed by Dunn's test. All data were tested for normality of residuals (Shapiro–Wilk test) and homogeneity of variance (Levene's test). Statistical significance was considered at $p < 0.05$. All tests were performed in R version 4.1.2.

Results

Mean earthworm density in the tree rows of the agroforestry system was 2.9 to 12.3 times greater than in the crop rows and the monoculture cropland (Fig. 2A) ($p \leq 0.0216$). Likewise, mean earthworm biomass was 5.7 to 34.8 times greater in the tree rows than in the crop rows and the monoculture cropland (Fig. 2B) ($p \leq 0.0014$). A gradual decline in earthworm density from the trees into the crop rows and monoculture cropland was evident for all three tree species (Fig. 2A). For example, earthworm density was

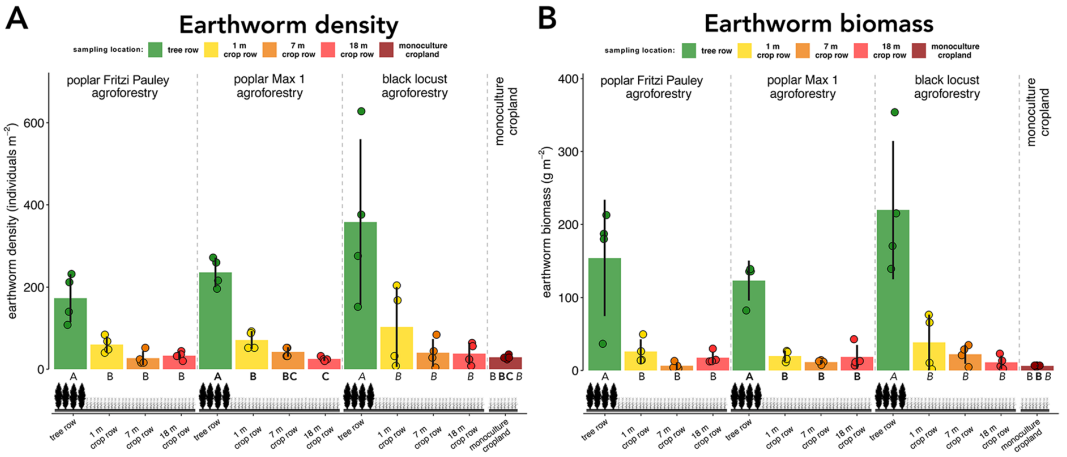

Fig. 2 Earthworm density (**A**) and biomass (**B**) in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation ($n = 4$). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences ($p < 0.05$)

greater at 1 m than at 18 m distance from the trees and monoculture cropland under poplar Max 1 cultivation (Fig. 2A) ($p = 0.0344$). The gradual decline in population density was mainly driven by endogeic (Figure S2 A) rather than anecic species (Figure S2 C). Across sampling locations, population density and biomass of earthworms were strongly positively correlated ($r = 0.84$; $p < 0.0001$). In total, five different earthworm species of three different ecological groups were found at our study site: *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea rosea* (endogeic species), *Lumbricus rubellus* (epigeic species), and *Lumbricus terrestris* (anecic species). The monoculture cropland was dominated by endogeic species with no anecic and epigeic species present (Fig. 3). Anecic species were always present in the tree rows of all three tree species ranging from 13.8 to 44.9% of the total community and were

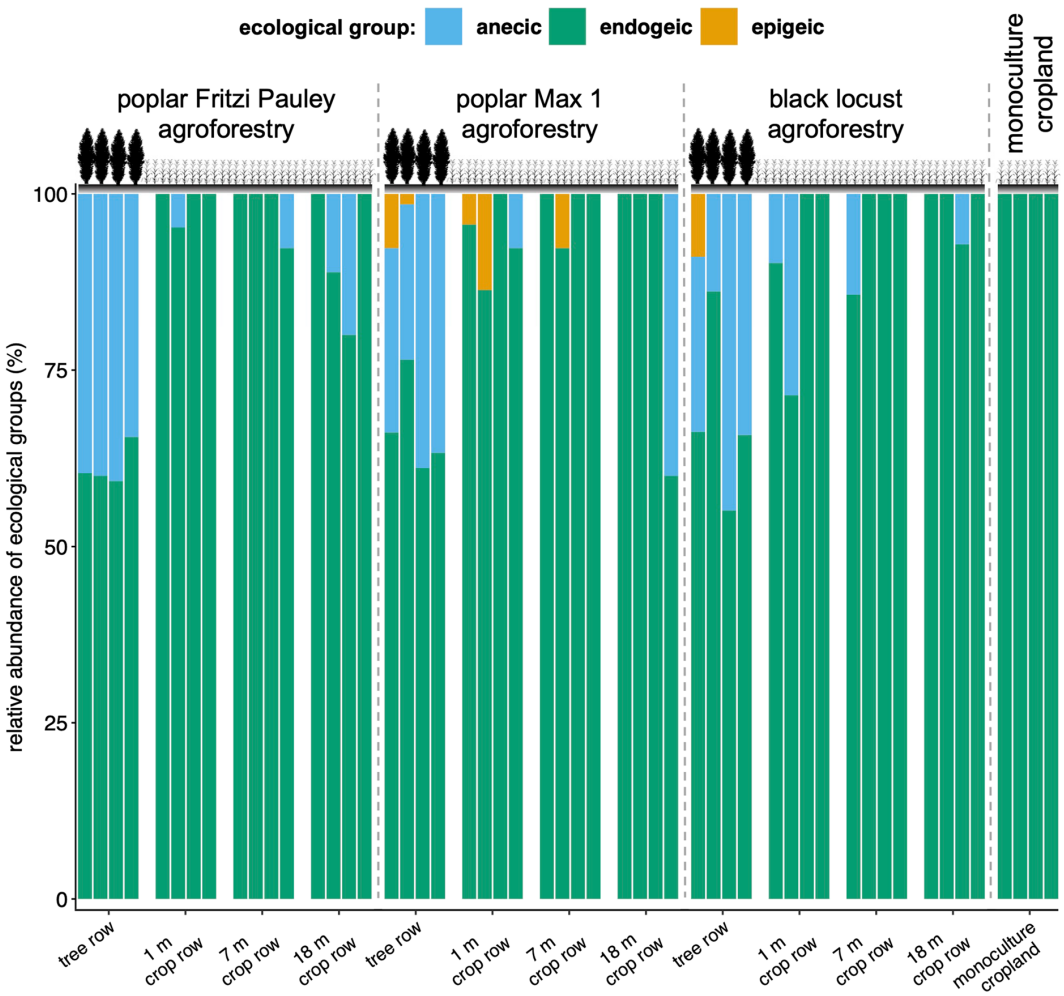

Fig. 3 Relative abundance of ecological groups of earthworms in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars represent individual samples ($n = 4$)

occasionally recovered in the crop rows (Fig. 3). In the poplar Max 1 system, the epigeic species (i.e. *L. rubellus*) was found in half of all replicate plots in the tree row and at 1 m distance from the trees as well as in a quarter of all the replicate plots at 7 m distance (Fig. 3). Epigeic earthworms were only detected in one replicate plot of the tree rows in the black locust system (Fig. 3).

We quantified 12 groups of soil microorganisms (total bacteria, total fungi, total archaea, Basidiomycota, Ascomycota, Acidobacteria, Actinobacteria, Alpha-, Beta-, Gammaproteobacteria, Bacteriodetes and Firmicutes) and found only positive or no effects of agroforestry on microbial abundance. Total soil bacterial abundance was greater in the tree rows than at 18 m distance from the trees within the crop rows (Fig. 4A) ($p \leq 0.0378$). In the poplar Max 1 system, gene abundance of bacteria as well as Actinobacteria was greater under the trees as compared to 7 and 18 m into the crop row and the monoculture cropland (Fig. 4A, C) ($p \leq 0.0288$). In the same system, greater abundance of Acidobacteria and Bacteroidetes was detected in the tree row as compared to the crop row and the monoculture cropland (Fig. 4B, S3 A) ($p \leq 0.0290$) and Firmicutes were more abundant under the trees than at 18 m distance into the crop row and the monoculture cropland (Figure S3 B) ($p \leq 0.0092$). The abundance of Basidiomycota in the poplar Max 1 system was greater in the tree row than in the crop row at 7 and 18 m distance and in the monoculture cropland (Figure S4 C) ($p \leq 0.0313$). Gene abundances of total soil fungi, Ascomycota, total archaea, Alpha-, Beta-, and Gammaproteobacteria did not differ among sampling locations (Figure S4 A-B, S5, S6).

The soil fungal community was dominated by Ascomycota ($51.4 \pm 16.7\%$), Basidiomycota ($32.4 \pm 22.4\%$), and Mortierellomycota ($5.8 \pm 4.0\%$). The dominant fungal classes were Sordariomycetes ($28.7 \pm 14.4\%$), Agaricomycetes ($27.4 \pm 22.8\%$), Dothideomycetes ($6.9 \pm 5.3\%$), and Mortierellomycetes ($5.8 \pm 4.0\%$) (Fig. 5A). Acremonium ($8.6 \pm 6.2\%$), Mortierella ($4.9 \pm 3.4\%$), Coprinellus ($4.5 \pm 8.9\%$), Laccaria ($3.2 \pm 9.8\%$), and Agrocybe ($2.9 \pm 10.7\%$) were the most abundant genera in the dataset. Alpha diversity (Shannon diversity, Chao1 index, and Pielou's evenness) did not differ among sampling locations (i.e. tree row, 1, 7, 18 m crop rows, and monoculture cropland) within each agroforestry system (i.e. poplar Fritzi Pauley, poplar Max 1, and black locust agroforestry system) (Table S1). Sampling location significantly affected fungal community composition (Table S2) ($p = 0.0001$) and was driven by the differences between the tree row and the arable land (crop row and monoculture cropland) (Fig. 5B). Multivariate homogeneity of dispersions was given under all PERMANOVA test conditions performed ($p \geq 0.32$). Differential abundance analysis was visualized by heat trees (Figure S 7) and a total of 15 genera and species were identified whose relative abundance was either positively or negatively affected by agroforestry (Fig. 6). Relative abundance

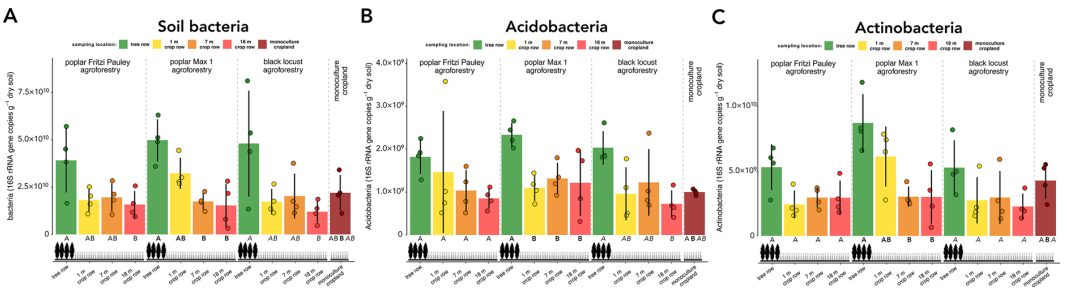

Fig. 4. 16S rRNA gene abundance of total bacteria (**A**), Acidobacteria (**B**), and Actinobacteria (**C**) in soil of a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation ($n = 4$). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences ($p < 0.05$)

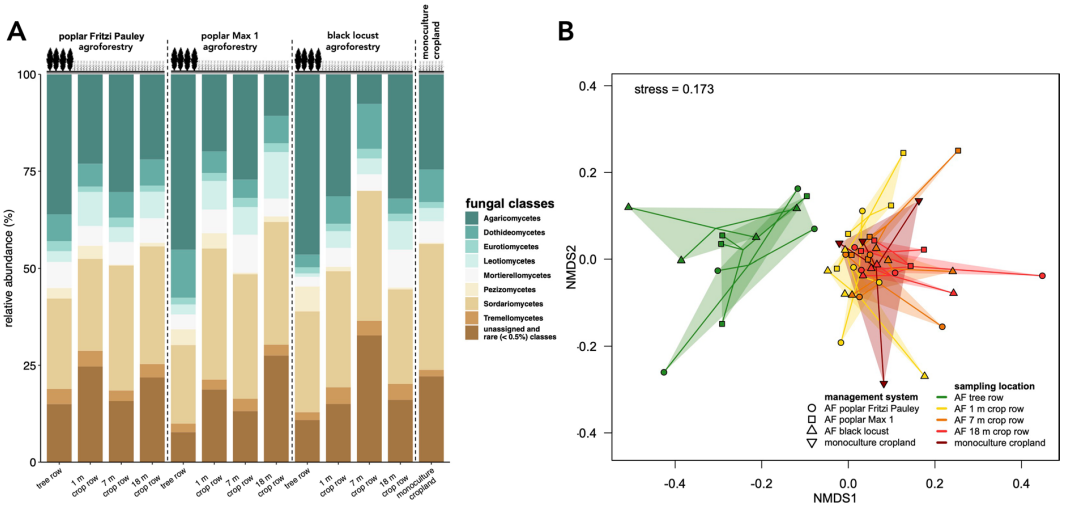

Fig. 5 Soil fungal community composition in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, soil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row ($n=4$). Relative abundances of dominant ($\geq 0.5\%$ mean relative abundance) fungal classes (**A**) are shown. Beta diversity is shown by non-metric multidimensional scaling (NMDS) of Bray–Curtis dissimilarities (**B**). Circles, squares, and triangles represent individual data points. AF = agroforestry

of *Gamsia, Ilyonectria, Laccaria*, and *Preussia* spp. were generally enhanced by the tree rows, whereas the increased relative abundance of *Inocybe* spp. was specific for poplar Max 1 tree rows (Fig. 6). Relative abundance of *Protomyces* spp., *Sporobolomyces* spp., and *Blumeria graminis* showed a positive trend with increasing distance from the trees. Among these, *Blumeria graminis* was absent in the tree rows. Relative abundance of *Mycosphaerella tassiana* as well as *Dioszegia*, *Itersonilia*, *Lectera*, *Leucosporidium*, *Microdochium*, and *Neosetophoma* spp. was overall higher in the crop row or monoculture cropland compared to the tree rows; however, no trends regarding sampling distance were observed (Fig. 6).

In the black locust system, SOC and total N concentrations in topsoil (upper 0–5 cm soil) were 54 to 97% greater in the tree rows than in the crop row and monoculture cropland (Fig. 7) ($p \leq 0{,}0004$). Likewise, in the poplar Max 1 system, SOC concentrations in topsoil were greater in the tree rows than in the crop row and monoculture cropland (Fig. 7A) ($p \leq 0.0376$). Tree rows of poplar Fritzi Pauley showed greater SOC concentrations than the crop row at 18 m distance (Fig. 7A) ($p = 0.0093$) but similar total N concentrations among all sampling locations (Fig. 7B). Soil bulk density showed slight differences across sampling locations, however, no distinct spatial pattern among sampling locations was detected (Table S3). In the black locust system, Mg_{CaCl2} was greater under the trees than in the crop row ($p = 0.0001$ – 0.0004) (Table S3). Similarly, Mg_{CaCl2} in the system with poplar Fritzi Pauley was greater in the tree row than in the crop row and monoculture cropland ($p = 0.0006$ – 0.0086) (Table S3). In both, the poplar Max 1 and the black locust system, K_{DL} increased in the tree row as compared to the crop row and monoculture cropland ($p < 0.0001$ – 0.0073) (Table S3). Tree rows of poplar Max 1 showed greater P_{DL} as compared to monoculture cropland ($p = 0.0106$). Earthworm density and biomass were positively correlated to SOC and total N concentrations, K_{DL}, Mg_{CaCl2}, and WFPS ($r = 0.35$ – 0.56; $p < 0.0001$ – 0.011) but not P_{DL} ($r = 0.04$ – 0.14; $p = 0.32$ – 0.77) (Figure S8). Positive relationships were found between soil bacteria, Bacteroidetes, Acidobacteria, and Actinobacteria and SOC and total N concentrations, K_{DL}, Mg_{CaCl2}, and P_{DL} ($r = 0.28$ – 0.54; $p < 0.0001$ – 0.041) (Figure S9).

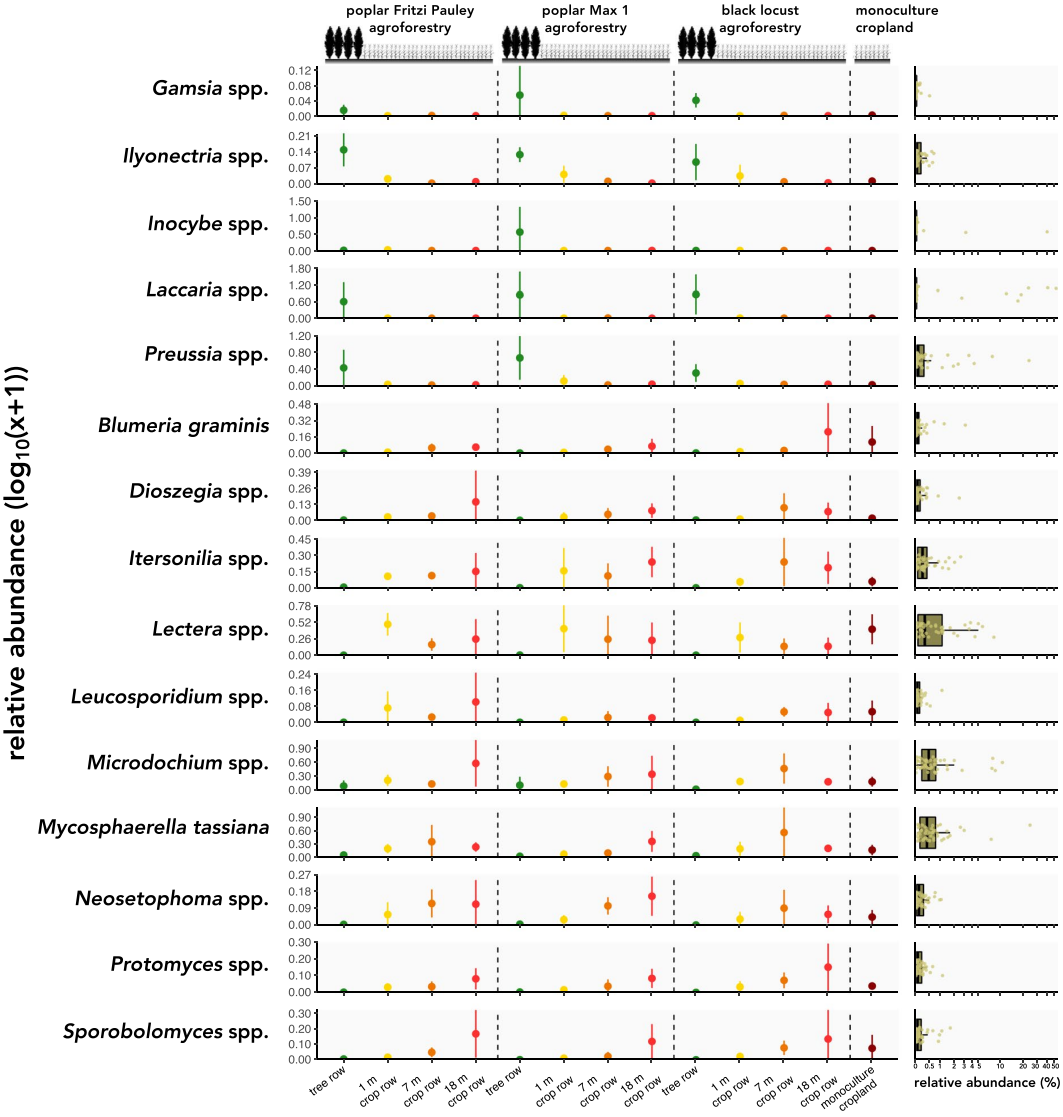

Fig. 6 Differentially abundant genera and species in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, soil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row ($n = 4$)

Discussion

Improved soil fertility through agroforestry

In agreement with previous findings (e.g. Pardon et al. 2017), soil fertility (i.e. SOC, total N, and extractable nutrients (K_{DL} and Mg_{CaCl2})) increased under the trees (Fig. 7, Table S3). This increase was likely due to the input of above- and belowground tree litter-derived nutrients, which declines with increasing distance

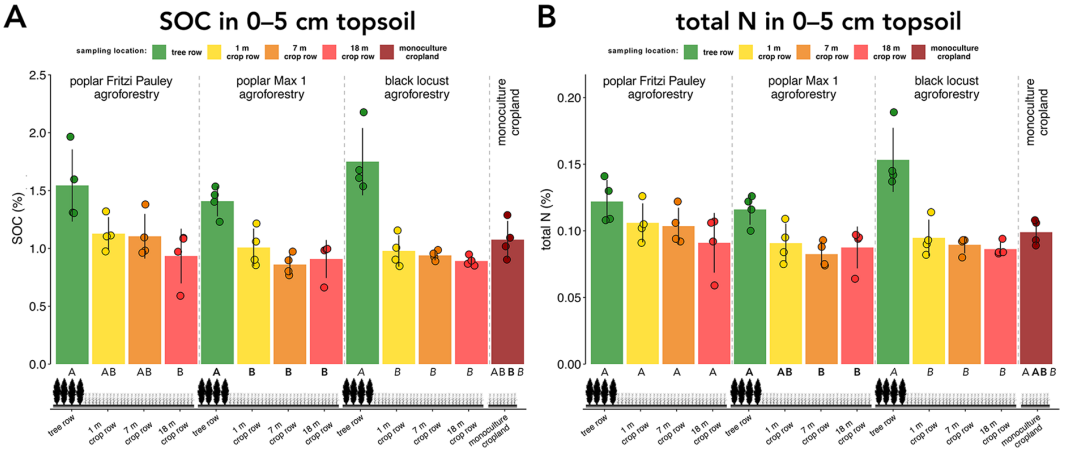

Fig. 7 Soil organic carbon (SOC) (A) and total N concentrations (B) in 0–5 cm topsoil in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation ($n=4$). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences ($p<0.05$)

from the trees (Schmidt et al. 2021). Although tree-derived nutrient inputs can reach several meters into the crop row (Schmidt et al. 2021), increased soil fertility did not gradually extend into the crop rows which we attribute to the relatively young age (12 years) of our system (*cf.* Pardon et al. 2017). We expect that as our system ages and more tree litter is deposited, the increase in soil fertility under the trees will increasingly extend into the crop rows.

Agroforestry promotes earthworm communities

In the present study, earthworms were sampled using AITC extraction without hand sorting. Compared to hand sorting combined with AITC extraction (e.g. as per ISO (2018) 23611–1), using exclusively AITC extraction for earthworm sampling may result in reduced efficacy for juveniles and endogeic species whereas adult anecic earthworms are well recovered (e.g.Pelosi et al. 2009; Čoja et al. 2008). Hand sorting, however, is rarely feasible for large-scale studies as it requires a substantial amount of labour and may prolong the sampling campaign (Iannone et al. 2012). Furthermore, if additional on-site data needs to be collected, chemical extraction is preferred over hand sorting since it does not physically disturb the soil (Iannone et al. 2012; Lees et al. 2016; Tóth et al. 2020).

Unlike Price and Gordon (1998), we did not detect tree-species effects on earthworm abundance, diversity or community composition at our study site. In line with previous studies (Cardinael et al. 2019; D'Hervilly et al. 2022), population size of earthworms was greater in the tree rows as compared to the crop rows (Fig. 2A). In agreement with the results of Cardinael et al. (2019), the increase in density was evident for all three ecological groups (Figure S2). Furthermore, we were able to demonstrate that earthworm abundance decreases with increasing distance from the trees (Fig. 2A). This gradual decline was driven mainly by endogeic species (*cf.* Figure S2 A, C, E), which almost exclusively colonize the topsoil. A recent study conducted in two similarly managed alley-cropping agroforestry systems of similar age in Germany found that tree roots in the topsoil of the tilled crop rows are scarce (Schmidt et al. 2021). Consequently, endogeic species in the crop row likely benefited from tree litter input in vicinity of the trees rather than tree root litter. To our best knowledge, the present study is the first to investigate earthworm communities at more than two distances from the tree rows into the crop rows. Although a greater number

of sampling locations is demanding, we believe that such study designs are essential to improve the characterization of spatial gradients within agroforestry systems, and thus, the extent by which the tree rows influence the crop rows and vice versa.

Earthworm communities in the tree rows were characterized by a shift towards anecic earthworms (Fig. 3), which is in agreement with previous findings (D'Hervilly et al. 2020, 2021). In contrast to the gradual decrease of endogeic earthworms with increasing distance from the trees, density of anecic earthworms did not decline gradually but rapidly dropped which is most likely due to the tillage in the crop rows. It is well established that anecic earthworms benefit from reduced tillage (Chan 2001; Ernst & Emmerling 2009). Unsurprisingly, the absence of tillage under the trees has recently been identified as a main driver for the abundance increase of anecic earthworms in the tree rows of agroforestry systems (Cardinael et al. 2019). Since anecic earthworms feed on the soil surface, litter fall and understory vegetation should also be considered as promoting factors for deep burrowing species. Additionally, Gilbert et al. (2014) were able to show that saprophagous earthworms, including *Lumbricus terrestris*, benefit from tree fine roots and fine root associated-mycorrhizae as a feeding source. In order to disentangle the impact of soil management (i.e. tillage) and food resource availability (i.e. litter, fine roots, mycorrhizae, and understory vegetation) on the abundance of anecic earthworms, studies in no-till agroforestry systems are required.

The sporadic detection of epigeic individuals identified as *L. rubellus* under as well as in close proximity to the trees (Figure S2 E, F) is in accordance with the results of D'Hervilly et al. (2020). We argue that this is likely due to tree litter input, as those species require a permanent surface cover with organic material and are therefore mostly absent in arable croplands. We speculate that depending on the size of the agroforestry system, the colonization of tree rows by epigeic earthworms requires several years to reach a spatially homogeneous equilibrium state.

Effects of agroforestry on soil microbial population size

The population size of total bacteria, Acidobacteria, and Actinobacteria was greater in the vicinity of trees in all agroforestry systems (Fig. 4), which is in line with results of recent studies (Beule et al. 2020; Guillot et al. 2021). The positive effect of trees in agroforestry systems on the abundance of soil microorganisms is well-described (Banerjee et al. 2016; Beuschel et al. 2019; Beule et al. 2020; Guillot et al. 2021; Luo et al. 2022) and is likely due to differences in soil management (i.e. absence of tillage in the tree rows versus tilled crop rows) and vegetation cover (i.e. woody perennials versus annual crops) (Beule et al. 2022a). Furthermore, some studies were able to demonstrate that microbial abundance increases with decreasing distance from the trees (e.g. Guillot et al. 2019, Beule et al. 2020; D'Hervilly et al. 2021; Luo et al. 2022). At our study site, no such trends were observed except for total bacteria (Fig. 4A), Actinobacteria (Fig. 4C), Firmicutes (Figure SI2 B), and Bacteriodetes (Figure SI 3C) in the poplar Max 1 system. In contrast to previous studies where trees were either not harvested at all (Guillot et al. 2019, 2021; D'Hervilly et al. 2021) or harvested four to five years (Beule et al. 2020; Luo et al. 2022) prior to soil sampling, the aboveground biomass of the trees at our study site was harvested one year prior to soil sampling. Therefore, we suggest that tree harvesting temporally alters soil microbial communities throughout agroforestry systems. We relate these alterations to changes in substrate input due to reduced tree litter input and/or altered tree root functioning (e.g. dying off of roots, changes in quantity and quality of root exudation). Furthermore, this hypothesis may explain why in contrast to previous studies (Beuschel et al. 2019; Beule et al. 2020; Guillot et al. 2021), soil fungal abundance at our study site was not promoted by agroforestry (Figure SI 3). Further studies exploring temporal dynamics along tree rotation cycles are required to investigate this hypothesis.

Agroforestry alters the soil mycobiome and putative phytopathogen abundance

Our finding that agroforestry does not affect alpha (Table S1) but beta diversity of soil fungi (Table S2, Fig. 5B) agrees with previous studies on the soil mycobiome of agroforestry systems (Beule et al. 2021, Beule & Karlovsky 2021b). Differences in fungal community composition were mainly driven by the tree rows (Fig. 5B) which are known to exert strong influence on the soil fungal community even few months after tree planting (Beule & Karlovsky

2021b). Across all three tree species, the tree-row associated mycobiome was characterized by the promotion of genera harbouring ectomycorrhizal fungi (EMF) such as *Laccaria* and *Preussia* spp. (Fig. 6). Notably, affiliates of the genus *Inocybe*, harbouring EMF that can associate with poplar (e.g. Long et al. 2016), were strongly promoted under poplar Max 1 (Fig. 6) which indicates a tree species-specific tree-EMF interaction. Colonization of roots by mycorrhiza is often advantageous for plant growth as it improves nutrient and water acquisition. Under field conditions, poplar and black locust trees can associate with both arbuscular (AMF) as well as ectomycorrhizal fungi (e.g. Khasa et al. 2002; Bratek et al. 1996). For poplar trees, colonization rates of AMF and EMF have been shown to be poplar genotype-specific (Khasa et al. 2002), whereas black locust genotype specificity of mycorrhizal associations has not been investigated yet. Our findings on EMF highlight the importance of investigating several tree species (e.g. hardwood and fast-growing tree species) at the same study site in order to disentangle tree-species from study-site effects.

Concerns regarding an increased risk of crop diseases is among the main impediments of farmers to implement temperate agroforestry. In our study, a gradual decrease in relative abundance of *Blumeria graminis* (formerly known as *Erysiphe graminis*) with decreasing distance from tree rows was observed (Fig. 6). *B. graminis* is the causal agent of powdery mildew, one of the most common cereal diseases worldwide that can reduce grain quality and cause significant yield losses in temperate zones (e.g. Bélanger et al. 2002; Dreiseitl 2011; Dean et al. 2012). The ability of *B. graminis* to cause disease is host specific and thus *forma specialis* dependent (Wyand and Brown 2003). Sequencing of short-read amplicons (2 × 300 bp) did not enable identification of *B. graminis* on *forma specialis* level, which is not surprising considering the limited taxonomic resolution of short-read amplicon sequencing (Heidrich & Beule 2022). Furthermore, *B. graminis* can overwinter in form of cleistothecia on plant residues (Zhang et al. 2005). Thus, it was not possible to relate *B. graminis* to any of the host crops that were grown in the crop rotation at our study site. Hence, we refer to *B. graminis* as a putative pathogen. Still, our results provide the first evidence of a positive effect of tree rows in temperate agroforestry systems on the reduction of the putative pathogen *B. graminis*. As microbial antagonists of *B. graminis* have been isolated in Germany (Köhl et al. 2019), antagonistic interactions in soil and on aboveground crop parts may have contributed to the suppression of *B. graminis* at our study site. In addition to microbial antagonism, the ability of earthworms to suppress soil-born fungal phytopathogens is well recognized (e.g. Plaas et al. 2019; Stephens et al. 1994). Thus, the gradual increase in endogeic earthworm abundance with decreasing distance from the trees may have enhanced biological control within the crop rows and thereby contributed to the suppression of *B. graminis*.

In addition to potential biological control, tree rows in agroforestry systems increase structural diversity and act as physical barriers which is expected to lower the spread of crop diseases through the dilution of the host crop (host dilution effect) (Beule et al. 2019b). In 2019, Kanzler et al. reported reduced wind speed and evaporation rates at our study site under the agroforestry as compared to the monoculture cropland. Furthermore, reductions of wind speed and evaporation were dependent on the distance from the trees (Kanzler et al. 2019). Such microclimatic alterations are known to affect the epidemiology of plant diseases (e.g. Aust & von Hoyningen-Huene 1986, Waggoner 1965). Overall, the enhanced control of *B. graminis* in the agroforestry as compared to the monoculture cropland system cannot be attributed directly to one of the factors listed above. We rather expect that the control of *B. graminis* was due to a combination of factors that cannot be disentangled in our experimental setting.

As of writing, the study conducted by Beule et al. (2019b) is the only study that investigated the effect of temperate agroforestry on crop health. Their results revealed that colonization of oilseed rape plants with *Verticillium longisporum* and wheat grain with *Fusarium tricinctum* was lower in temperate agroforestry systems compared to monoculture cropland systems. Colonization of wheat and barley grain and oilseed rape plants with other major fungal pathogens did not differ between agroforestry systems and monoculture systems (Beule et al. 2019b). Furthermore, the authors observed a relationship between abundance of a phytopathogen and distance to the trees only for the phytopathogen *Leptosphaeria biglobosa* in oilseed rape plants. Considering our findings on *B. graminis,* we hypothesize that suppression of fungal phtyopathogens within agroforestry

systems is limited to certain pathogens and is a function of the distance to the trees (i.e. suppressiveness increases as the distance to the trees decreases). Studies on diseases and disease related factors in temperate agroforestry systems are scarce. We suggest that future research should investigate crop diseases in agroforestry systems of different spatial designs (e.g. wide versus narrow crop allays) as well as management practices. New findings could help farmers to optimize the design of future agroforestry systems in order to maximize the beneficial effects of these systems on disease control.

Conclusion

The integration of tree rows into arable land (agroforestry) increased the abundance of soil bacteria and earthworms (anecic, endogeic, and epigeic species) as compared to monoculture cropland. We attribute this mainly to the absence of tillage and the input of large amounts of tree litter under the trees. Community composition of soil fungi was altered by the tree rows, resulting in a tree-row associated mycobiome, which was particularly characterized by an increased proportion of EMF. The tree-row associated mycobiome not just enhances overall fungal diversity of agroforestry systems but is also expected to alter soil functions such as nutrient cycling. As the distance from the trees decreased, the proportion of *Blumeria graminis,* the causal agent of powdery mildew, decreased. We suggest that enhanced microbial antagonism, increased earthworm densities and/or altered microclimatic conditions contributed to the suppression of *B. graminis* within the agroforestry system. Whereas distinct tree-distance effects were observed, tree-species effects were identified as a minor driver of the abundance and composition of soil communities at our study site. Overall, agroforestry benefits the abundance, diversity, and function of soil biota and may enhance soil suppressiveness. Future research should investigate crop diseases in agroforestry systems of different spatial designs and management practices in order to maximize the beneficial effects of these systems on disease control.

Author contributions AV and LB contributed to the conception and design of the study. AV and LB performed the field work. AV, ZB, and LB performed the laboratory work. AV, ZB, and LB performed the statistical analysis. AV, ZB, and LB wrote the first draft of the manuscript. NH, BH, and VEMR contributed resources and critically revised the manuscript. All authors read and approved the manuscript.

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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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4 Comparison of two earthworm extraction methods in different arable soils

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In preparation

Abstract

Background

Investigating earthworm communities in the field is a major prerequisite to understand their role in agriculture and their response to agricultural management. Several methods for earthworm extraction are available that have different advantages and disadvantages. Currently, the most common method used is a combination of hand sorting the topsoil and subsequent chemical extraction of earthworms from deeper soil layers. However, hand sorting is time-consuming and labour-intensive, which impedes its implementation in large-scale studies. The aim of this study was to investigate the efficiency of allyl isothiocyanate (AITC) extraction without hand sorting in different arable soils and evaluate its potential as a fast and low-cost alternative to the combination of hand sorting and chemical extraction.

Methods

For that purpose, we collected earthworms at eleven agricultural sites across Germany using i) hand sorting of the topsoil and subsequent AITC extraction or ii) direct AITC extraction without preliminary hand sorting and compared biomass, population density, and species composition. We further investigated the influence of different soil characteristics (i.e., soil pH, soil texture, total C, total N, soil organic matter, and effective cation exchange capacity) on the efficiency of AITC extraction without hand sorting.

Results

AITC extraction without hand sorting underestimated total earthworm population density and biomass and showed especially low efficiency for the extraction of endogeic earthworms. Species richness as well as population density and biomass of anecic earthworms however, did not differ between the two methods, while the extraction without hand sorting required substantial less working hours. Soil

properties and development stage of earthworms showed no effect on the extraction efficiency of AITC without hand sorting.

Conclusions

AITC extraction without hand sorting recovers anecic earthworms and species richness with the same efficiency as the combination method and is suitable for comparisons of anecic as well as endogeic earthworms within one study site. Due to its low costs, it represents a powerful tool for large-scale studies and monitoring.

Introduction

Soil biodiversity and its ecosystem services in agroecosystems are threatened by intensive land use, one-sided crop rotations, intensive tillage and climate change. Measures to reverse the process of biodiversity loss are urgent. Earthworms are a key component of soil communities in agricultural soils and provide a number of ecological functions, such as improved water infiltration by the creation of burrows, or decomposition of organic material to reduce phytopathogenic pressure on crops (e.g., Ehlers 1975, Tomati & Galli 1995, Cortez & Bouché 1998). In agricultural systems, earthworm communities are strongly influenced by management practices and land-use changes (e.g., Frazao *et al.* 2017, Spurgeon *et al.* 2013). While the influence of ploughing on earthworms is well described (Chan 2001), the effect of other agricultural management practices on the abundance, diversity, and activity of earthworm communities is still mostly unknown. In order to fill these knowledge gaps and provide advice for farmers and policy makers, more earthworm studies in agricultural fields under different management practices and land-use systems are needed (e.g., Barnes *et al.* 2023, Blouin *et al.* 2013). However, realisation of such studies is often hindered due to the high costs associated with earthworm sampling (Bartlett *et al.* 2010).

In the scientific literature of the last decades a number of different earthworm sampling methods are described, which vary strongly in efficiency and sampling effort. Commonly used methods include hand sorting, chemical extraction (primarily with formaldehyde or allyl isothiocyanate (AITC), or rarely hot mustard), electrical octet method, or a combination of different methods (e.g., Valckx *et al.* 2011). The efficiency of those methods differs; especially between the three ecological groups of earthworms (i.e., endogeic, epigeic and anecic as introduced by Bouché in 1972). While hand sorting is considered to work better for endogeic and epigeic earthworms (e.g., Pelosi *et al.* 2009), chemical extraction and electrical octet method are generally regarded to work better for deep-burrowing anecic earthworms (e.g., Chan & Munro 2001, Pelosi *et al.* 2020). Consequently, a combination of hand sorting and chemical

extraction is often recommended for earthworm sampling, as it works well for all ecological groups and has been standardized (ISO 23611-1:2018(E)). For large-scale study designs however, this method is often not feasible, because it is time-consuming and personnel-intensive.

In order to resolve this problem, multiple research groups investigated the efficiency (i.e., relative number of earthworms recovered compared to a combination of hand sorting and chemical extraction) of less cost intensive methods, to evaluate their limitations and possibilities for different research or monitoring questions. While the electrical octet method introduced by Thielemann (1986) seems to be a good alternative to hand sorting in urban areas (Pelosi *et al.* 2020), its efficiency in other land-use systems fluctuates. For example, Čoja *et al.* 2008 reported a higher efficiency of the electrical octet method than extraction with AITC, while Eisenhauer *et al.* 2008 found mustard extraction to be more effective than the octet method. Another problematic factor regarding the electrical octet method is that it is time-consuming and requires complex equipment (e.g., Čoja *et al.* 2008). Therefore, the sampling costs remain too high for many study designs, which are also not feasible for hand sorting. Consequently, chemical extraction of earthworms seems to be a more promising alternative for extensive samplings, as it is significantly less time- and labour-consuming than hand sorting (e.g., Bartlett *et al.* 2010). The most common chemical expellants for extracting earthworms are formaldehyde, allyl isothiocyanate, and hot mustard solution, whereby the use of formaldehyde is no longer recommended, as it is a carcinogen and has been shown to have the same or even lower efficiency than mustard and AITC (e.g., Gunn 1992, Pelosi *et al.* 2009, Valckx *et al.* 2011). A study conducted at 22 agricultural fields in France found no difference in extraction efficiency between pure AITC and hot mustard but found varying contents of AITC in the hot mustard depending on the storage time of the mustard, which impedes the reproducibility of this method compared to using pure AITC (Pelosi *et al.* 2014).

So far, only a limited number of studies compared the efficiency of AITC with and without hand sorting. Zaborski (2003) found similar numbers of earthworms with AITC and hand sorting on a soybean field in Illinois (USA) but did not include a combination of both methods to the comparison. Čoja *et al.* (2008) compared, *inter alia*, the efficiency of AITC with a combination of hand sorting and subsequent formaldehyde extraction and reported generally more earthworms with the combination method, even though the difference remained insignificant. Another study performed on two agricultural fields in France found that AITC extraction underestimates earthworm density compared to a combination with hand sorting,

especially for endogeic species (Pelosi *et al.* 2009). Similar results were reported by two studies investigating the efficiency of hot mustard solution instead of pure AITC (e.g., Bartlett *et al.* 2006, Lawrence & Bowers 2002). Unfortunately, most comparative studies investigating AITC extraction efficiency have been limited to a small number of study sites (Čoja *et al.* 2008). Therefore, the effect soil type has on the efficiency of a method is often not sufficiently regarded (Čoja *et al.* 2008). The study of Gutierrez-Lopez and co-authors (2016) is one of the few studies comparing AITC extraction with and without hand sorting on a large number of sampling locations distributed across different soils and habitat types in Central-Western Spain and is thus limited to Mediterranean soils and earthworm communities. In the same study, the authors reported an influence of soil characteristics on the efficiency of AITC extraction for certain earthworm species of the study region (Gutierrez-Lopez *et al.* 2016).

In this study, we aimed to investigate the AITC extraction efficiency for German earthworm communities in agricultural soils by comparing AITC extraction with and without hand sorting at eleven different sites across Germany. We hypothesize that the extraction efficiency of AITC will depend on I) the ecological groups and II) developmental stage (juvenil or adult) of extracted earthworms, as well as III) the soil characteristics, especially soil texture, of the study site.

Material and methods

Site description

Earthworms were sampled at eleven different cropland sites across Germany (Figure 1 B). Sampling was done at four sites near Straubing (i.e., Deggendorf, Landau a. d. Isar, Puchhausen, Straubing) in April 2022, one site near Alt Madlitz in April 2022, four sites near Cologne (i.e., Euskirchen, Mechernich-Wachendorf, Rommerskirchen-Vanikum, Wülfrath) in April 2021, one site near Otterndorf in October 2021, and one site near Prenzlau in October 2021. Detailed study site descriptions (i.e., geographic location and soil characteristics) are shown in Table S1. At each site, earthworms and soil parameters were sampled along a transect spanning through the field with at least 20 m distance from the field edge. The two different sampling techniques for earthworms were used alternately along the transect. Distance between sampling plots varied depending on the field size with a minimum of 10 m between sampling plots on the smallest field (Figure 1 A).

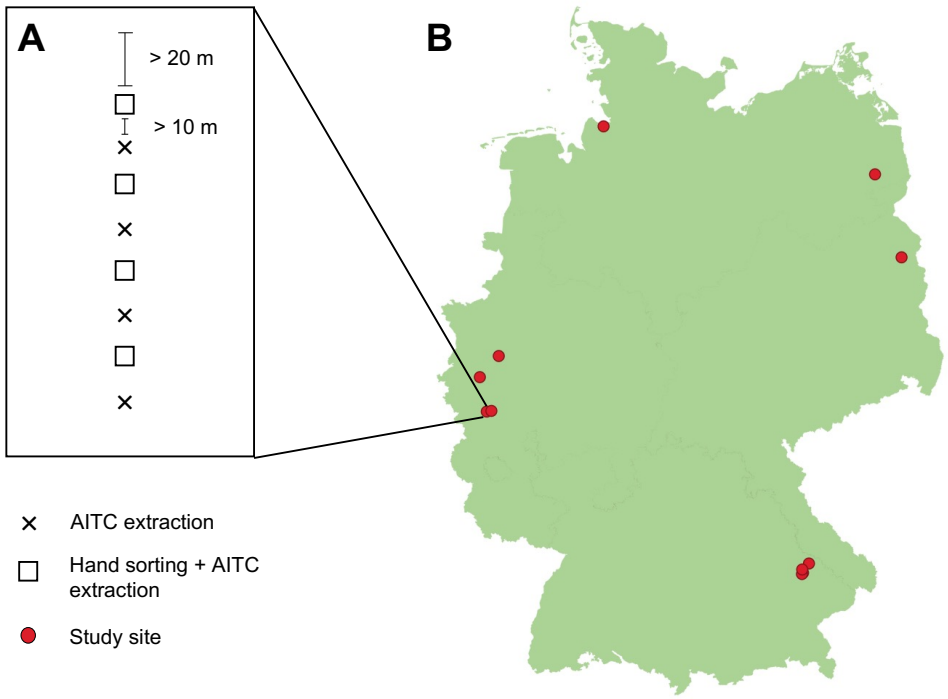

Figure 1. Study design. Schematic illustration of the study design (**A**) and location of the eleven study sites (**B**). Map created with QGIS version 3.4.4-Madeira.

Earthworm sampling

Earthworm sampling was done from 2021 to 2022 and was limited to April and October when earthworm activity peaks. Earthworms were sampled using two different methods: one method combining hand-sorting of excavated soil and subsequent extraction of remaining anecic earthworms with AITC and one method using only AITC extraction without excavating the soil. Independent of the used method, all collected worms were stored in cold water or soil and morphologically determined to species level. Afterwards, the fresh weight (including gut content) and developmental stage of the earthworms was determined before they were released alive.

Hand sorting and AITC extraction of earthworms

The combination of hand sorting and AITC extraction followed ISO 23611-1:2018(E) with slight modifications. Briefly summarized, the upper 20 cm of soil within an area of a squared 1/8 m^2 were excavated into a separate container, and searched for earthworms by hand. In order to collect earthworms that were deeper in the soil, 2.5 l (20 l per m²) of a 0.01 % (w/v) AITC solution (0.1 g AITC l^{-1}) were slowly poured into the hole, while a metal frame guides the infiltration of solution. Afterwards, the hole was carefully observed for 30 minutes, and surfacing earthworms were collected using tweezers.

AITC extraction of earthworms without hand sorting

For the AITC extraction of earthworms without hand sorting, adapted from Zaborski (2003) and Vaupel *et al.* (2023), the sampling site was prepared by pressing a squared 1/8 m^2 or 1/4 m^2 metal frame a few centimetres into the soil. Plants within the metal frame were removed using garden shears. Afterwards, depending on the frame size, 2.5 l or 5 l (20 l per m²) of a 0.01 % (w/v) AITC solution were poured slowly into the metal frame, allowing it to seep into the ground. Directly after pouring, the soil within the frame was carefully observed for 30 min, and earthworms surfacing were collected using tweezers.

Soil sampling and soil characteristics

Soil characteristics (i.e., soil pH, soil texture, total C, total N, soil organic matter (SOM), and effective cation exchange capacity (CEC_{eff})) were determined from homogenized, air-dried and sieved (< 2 mm) excavated topsoil (0 – 20 cm) which was sorted for earthworms during hand sorting. Soil pH was determined at a 1:2.5 soil-to-water ratio (w/v). Total C and total N were determined using a CNS elemental analyzer (Vario EL Cube, Elementar, Germany) according to DIN ISO 10694,1996 and DIN ISO 13878,1998. Soil organic matter content was calculated from weight difference after ashing at 550 °C according to DIN 19684-3 (2000). CEC_{eff} was determined using coupled optical plasma emission spectroscopy (ICP-OES, iCAP 7000 series, Thermo Fischer Scientific, USA) and $BaCl_2$ solution according to DIN ISO 11260 (1997).

Statistical analysis

Statistical analysis was performed with R version 4.1.0. Shapiro–Wilk and Levene's test were performed to check the assumptions for one-way ANOVA (i.e., normality of residuals and homogeneity of variance) and all data were subsequently root-transformed to fulfil the criteria. To explore differences between the two sampling methods linear mixed models were fitted by maximizing the restricted log-likelihood with the 'lmerTest' R package v 3.1-3 and the 'lme4' R package v 1.1-27.1 and then tested with ANOVA. Within the model, study site was always regarded as a random effect and sampling method as a fixed effect. Correlation coefficients between extraction efficiency and soil properties were calculated with Spearman's rank correlation test. For all tests, statistical significance was assumed for p-values < 0.05.

Results

In total, we detected six different species of earthworms across the study sites: endogeic species: *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea rosea, Octolasion tyrtaeum*, anecic species: *Lumbricus terrestris*, and

epigeic species: *Lumbricus castaneus.* While *Aporrectodea caliginosa* and *Lumbricus terrestris* were present at all eleven sites, *Octolasion tyrtaeum* and *Lumbricus castaneus* were only detected sporadically at two and one site, respectively. The total species number per site did not differ between the two methods ($p = 0.2211$), which found 3.6 ± 1.0 (hand sorting + AITC) and 3.0 ± 1.0 (AITC) species per site. Total earthworm population density ranged from 5 to 267 individuals per m² and was on average 2.4 times larger using hand sorting and AITC than using only AITC ($p < 0.0001$, Figure 2A). This results in an average extraction efficiency of 39 ± 20 % using only AITC compared to a combination of hand sorting and AITC. Developmental stage of earthworms did not improve the extraction efficiency of AITC, as adults were extracted with an efficiency of 33 ± 31 % ($p = 0.0001$, Figure S1A) and juveniles with an efficiency of 41 ± 18 % ($p < 0.001$, Figure S1B) compared to the combination of hand sorting and AITC. Similarly, total biomass was 1.9 times larger for earthworms sampled with hand sorting and AITC as compared to the AITC extraction without hand sorting ($p = 0.0007$, Figure 2B).

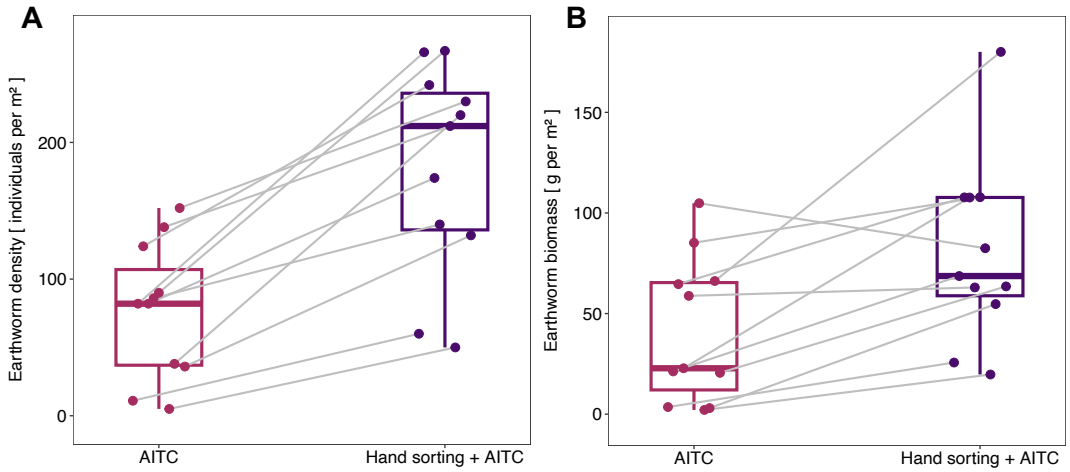

Figure 2. Earthworm population density and biomass. Population density (**A**) and biomass (**B**) of all earthworms. Individual points represent the mean (4 or 8 replicates per site) of each study site (n = 11). Grey lines connect data points sampled at the same site with different methods (i.e., AITC extraction and the combination of hand sorting + AITC extraction).

Regarding the different ecological groups of earthworms, population density and biomass of anecic earthworms (i.e., *Lumbricus terrestris*) did not differ between the two sampling methods ($p = 0.5858$, Figure 3A and $p = 0.0943$, Figure 3B respectively). The population density and biomass of endogeic species were significantly lower using AITC without hand sorting compared to the combination method ($p < 0.0001$, Figure 3C, D). For epigeic species, no comparison was possible,

since only two individuals of the epigeic earthworm species *Lumbricus castaneus* were found at a single sampling point.

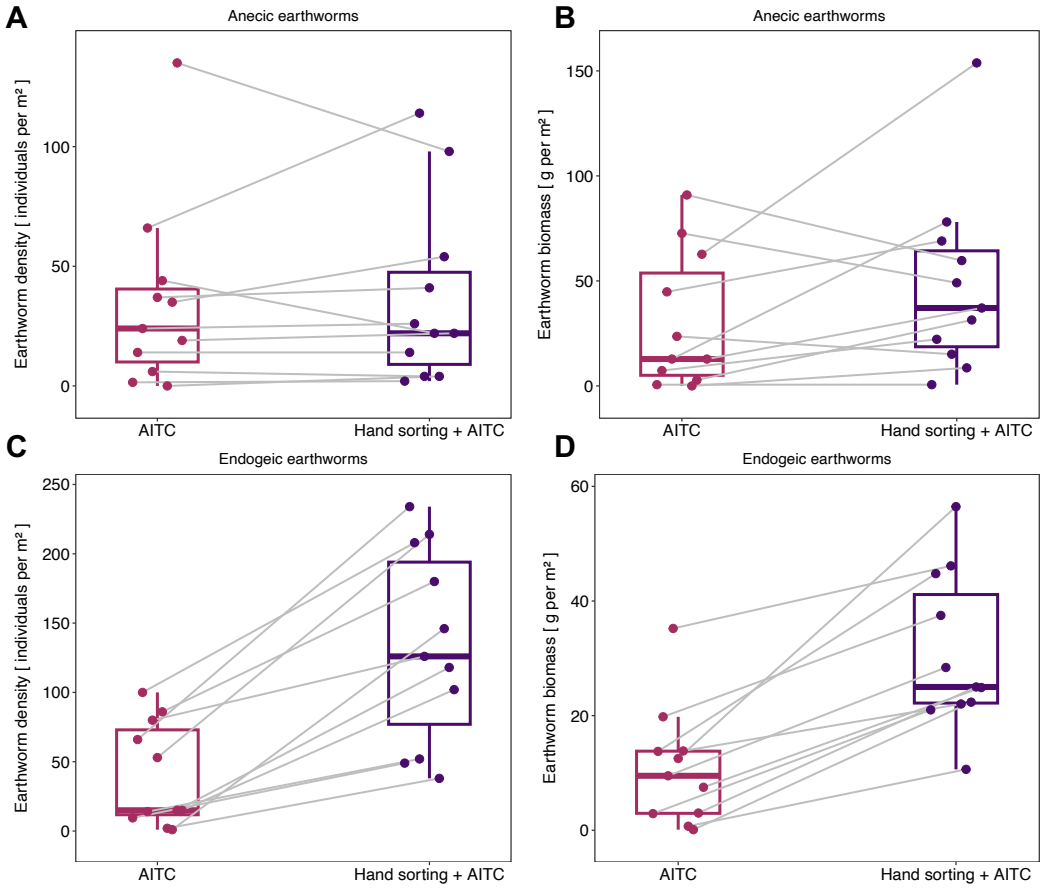

Figure 3. Population density and biomass of ecological earthworm groups. Density (**A**, **C**) and biomass (**B**, **D**) of anecic (**A**, **B**) and endogeic (**C**, **D**) earthworms. Individual points represent the mean (4 or 8 replicates per site) of an individual site (n=11). Grey lines connect data points sampled at the same site with different methods (i.e., AITC extraction and the combination of hand sorting + AITC extraction).

No relationships were found between soil texture (i.e., sand, silt and clay content) and total extraction efficiency (r = -0.1727 – 0.3909, p = 0.2345 – 0.6115, Figure S2 A, B, C). All other investigated soil properties (i.e., soil pH, C/N ratio, CEC_{eff} and SOM) also showed no correlation with extraction efficiency (r = -0.1549 – 0.3000, p = 0.3701 – 0.6493, Figure S3 A, B, C, D).

Discussion

Earthworm diversity

In accordance with the results of previous studies (Valckx *et al.* 2011, Gutierrez-Lopez *et al.* 2016), we found no significant differences in the number of

species recovered by the two different methods. German agricultural fields are known to harbour a low diversity of earthworms (Jänsch *et al.* 2013), as only few species are adapted to such intensively used systems. Consequently, it is not surprising that our study found no significant difference in species number, neither between study sites nor between sampling methods. In areas with higher earthworm diversity however, the AITC extraction without hand sorting could potentially recover a lower number of species, as less abundant species are more likely to be overlooked due to the lower efficiency of the method. For agricultural sites however, our results indicate that AITC extraction seems to be sufficient to determine earthworm diversity. Additionally, it should be noted that even hand sorting cannot always recover all present species. At an experimental meadow in Austria for example, Čoja *et al.* (2008) found that none of the five sampling methods tested in their study (i.e., AITC extraction, formalin extraction, Kempson extraction, electrical octet method, and hand sorting) was able to recover all species present at the study site.

Earthworm density and biomass

In agreement with previous studies investigating chemical extraction (Bartlett et al. 2006, Pelosi *et al.* 2009 and Gutierrez-Lopez *et al.* 2016), using only AITC for earthworm sampling resulted in significantly lower earthworm population density and biomass as compared to using hand sorting in combination with AITC. Possible reasons for this include earthworms horizontally escaping from the sampling point, inactive earthworms not reacting to the AITC or especially smaller earthworms not being able to reach the surface. For anecic earthworm species, these limitations seem to be less relevant, as their vertical burrows allow them to easily escape to the surface, while simultaneously preventing them to escape horizontally (Lawrence & Bowers 2002). In consonance with this, anecic earthworm species in our study (i.e., *Lumbricus terrestris*) where equally well recovered with both methods (Figure 3A). The population density of endogeic species, however, was significantly lower using only chemical extraction (Figure 3C), which is in accordance with previous studies (Bartlett *et al.* 2006, Pelosi *et al.* 2009, and Gutiérrez-López *et al.* 2016).

Correlation with soil properties

Contrary to the results of Gutierrez-Lopez *et al.* (2016), we were not able to detect an effect of soil properties on the extraction efficiency (Figures S2, S3). Similar results were reported by Lawrence and Bowers (2002), who also found no relationship between the efficiency of hot mustard extraction and a variety of soil properties, including soil texture. Nonetheless, extraction efficiency of our study still varied strongly between study sites, ranging from 10 to 66 %. As was discussed above, differences in community composition are one explanation for this variation in

efficiency. Aside from that, different activity levels of the respective earthworm communities might also have caused this variation, since earthworms are more likely to be found in deeper soil layers during diapause (e.g., Jiménez & Decaëns 2000, Lagerlöf *et al.* 2002), which would decrease their ability to escape to the surface independent of the soil type.

Advantages and limitations

During our study, sampling using only AITC took approximately 250 % less working hours as the combined sampling with hand sorting. This reduction in sampling time offers a huge advantage for large scale study designs or monitoring projects, which require a vast sample size and would not be feasible using hand sorting. Our results indicate that using only AITC extraction without hand sorting is sufficient for investigating the population density of anecic species (e.g., *Lumbricus terrestris*), and to capture the species richness. The method is also appropriate for onsite comparisons, for example for investigating spatial structural elements (Vaupel *et al.* 2023). It is, however, not suitable for assessing the whole population of a study site or for research questions specifically targeting endogeic species or the community composition, as chemical extraction is biased towards anecic species (e.g., Chan & Munro 2001, Bartlett *et al.* 2006 and Pelosi *et al.* 2020).

Conclusion

In our study, we were able to show that AITC extraction without hand sorting can be a powerful alternative tool for investigating earthworms in German agricultural landscapes. The greatest potential of the method lays in its fast and low-cost application, which allows for large-scale studies that would not be feasible for hand sorting. Though not suitable for estimating the whole earthworm community or studies focussing on endogeic species, the method provides reliable data for anecic earthworms and species richness. AITC extraction would also be applicable for onsite comparisons investigating for example spatial structures or different management strategies. However, further research is required to identify environmental influences on the methods effectiveness, investigate its effect on epigeic species and improve the efficiency for endogeic earthworms.

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5 High-resolution melting (HRM) curve analysis as a potential tool for the identification of earthworm species and haplotypes

High-resolution melting (HRM) curve analysis as a potential tool for the identification of earthworm species and haplotypes

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ABSTRACT

Background. Earthworm communities are an important component of soil biodiversity and contribute to a number of ecosystem functions such as soil-nutrient cycling. Taxonomic identification is an essential requirement to assess earthworm biodiversity and functionality. Although morphological identification of species is labour-intensive, it is the most commonly used method due to a lack of cost-efficient alternatives. Molecular approaches to identify earthworms at species and haplotype level such as DNA barcoding are gaining popularity in science but are rarely applied in practice. In contrast to barcoding, the differentiation of PCR products based on their thermal denaturation properties using high-resolution melting (HRM) curve analysis is a fast and cost-efficient molecular closed-tube, post-PCR tool that allows identification of taxa.

Methods. We developed a HRM curve assay to identify eight earthworm species common to agricultural soils in Central Europe (*Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Apo. limicola*, *Apo. longa*, *Apo. rosea*, *Lumbricus castaneus*, *L. rubellus*, and *L. terrestris*). For this, a new primer pair targeting a 158-bp long subregion of the cytochrome c oxidase I (COI) gene was designed. Our HRM assay was further tested for the differentiation of COI haplotypes using 28 individuals of the earthworm species *Allo. chlorotica*. Furthermore, we developed a novel extraction method for DNA from earthworm tissue that is fast and requires minimal consumables and laboratory equipment.

Results. The developed HRM curve assay allowed identifying all eight earthworm species. Performing the assay on 28 individuals of the earthworm species *Allo. chlorotica* enabled the distinction among different COI haplotypes. Furthermore, we successfully developed a rapid, robust, scalable, and inexpensive method for the extraction of earthworm DNA from fresh or frozen tissue.

Conclusions. HRM curve analysis of COI genes has the potential to identify earthworm species and haplotypes and could complement morphological identification, especially for juvenile or damaged individuals. Our rapid and inexpensive DNA extraction method from earthworm tissue helps to reduce the costs of molecular analyses and thereby promote their application in practice.

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Keywords High-resolution melting (HRM) curve analysis, Earthworm identification, Earthworm taxonomy, COI, Real-time PCR, Species, Haplotypes

INTRODUCTION

Soil meso- and macrofauna is a substantial part of soil biodiversity and contributes to key ecosystem functions such as nutrient cycling (*e.g.*, *Reichle, 1977*) and digestion of pathogens (*e.g.*, *Friberg, Lagerlöf & Rämert, 2005*; *Sofo, Mininni & Ricciuti, 2020*). Earthworms are important members of the soil faunal community and involved in multiple beneficial ecosystem functions. For example, earthworms contribute to the decomposition of organic material (*e.g.*, *Cortez, 1998*) and interact with beneficial as well as phytopathogenic soil microorganisms (*e.g.*, *Doube et al., 1994*). Earthworms can also serve as an indicator for soil health (*Linden et al., 1994*). Abundance, biomass and species diversity are common measures to characterize earthworm populations and their potential functions in the soil ecosystem. According to their ecology, earthworms can also be classified into three different major ecological groups (*i.e.*, anecic, endogeic and epigeic species). Recently, however, the suitability of these widely accepted three groups to reflect the functions of earthworms was questioned. *Bottinelli & Capowiez (2021)* suggested that the seven categories proposed by *Bouché (1972)* should be used instead. In addition to functional classification, taxonomic identification is an essential prerequisite for the assessment of earthworm biodiversity.

Identification of species by their morphology using regional identification keys (*e.g.*, *Graff, 1953*; *Stöp-Bowitz, 1969*; *Bouché, 1972*; *Ljungström, 1970*; *Sims & Gerard, 1985*) is still the most common technique applied in current earthworm surveys (*e.g.*, *Pérès et al., 2011*; *Tsiafouli et al., 2015*; *Ashwood et al., 2019*). The main advantages of this method are its low costs and few required equipment. Additionally, morphological identification of worms can be done non-invasive (*i.e.*, individuals can be released following examination), provided the worms are not stored in preservative agents (*e.g.*, ethanol). Depending on the identification key as well as the required accuracy, even citizen science approaches are possible, enabling larger surveys at low costs. Such approaches, however, are often limited to identification at genus level or ecological/morphological groups (*e.g.*, *Stroud, 2019*; *Billaud, Vermeersch & Porcher, 2021*). Therefore, they are only suitable for certain research questions. Even if morphological identification is carried out by trained specialists, identification of juveniles or damaged tissue is often not possible, consequently leading to their exclusion in surveys (*Richard et al., 2010*).

DNA barcoding of taxonomically informative loci such as cytochrome c oxidase I (COI) genes is a popular molecular approach for the identification of metazoa (*Hebert et al., 2003*). COI is the dominating marker gene for metazoa and efforts were undertaken to build databases for COI sequencing data. For example, in 2005, The Barcode of Life Data Systems (BOLD) was launched to acquire, store, and analyze COI gene sequence data (*Ratnasingham & Hebert, 2007*). The potential of DNA barcoding for the identification of earthworms has been pointed out over a decade ago (*Huang et al., 2007*), but is still rarely used in practice (*e.g.*, *Rutgers et al., 2016*). *Richard et al. (2010)* highlighted that DNA

barcoding enables identification of earthworms at all life stages, including juveniles, which has the potential to re-integrate juvenile individuals in earthworm studies and reduce possible identification bias.

High-resolution melting (HRM) curve analysis is a rapid and cost-effective tool, apart from the cost-intensive basic equipment, that allows differentiation among PCR products that differ by as little as one base pair. In this post-PCR technique, PCR products are differentiated by their thermal denaturation properties; their so-called "melting behaviour". For this, the PCR product is heated stepwise (commonly 0.1 or 0.2 ° C per step) and the dissociation of the double-stranded DNA is quantified after every step using a fluorescent DNA-intercalating dye. Amplification and subsequent HRM curve analysis are commonly performed in a real-time PCR thermocycler. The main fields of application are genotyping and the detection of mutations. Analysis of HRM curves is successfully used in molecular diagnostics to distinguish clinically relevant bacteria (*e.g.*, *Naze et al., 2015*), fungi (*e.g.*, *Fidler et al., 2016*), and viruses (*e.g.*, *Lin et al., 2008*). Furthermore, HRM curve analysis can serve as an alternative tool to DNA barcoding for the taxonomic identification and differentiation of species (*e.g.*, *Ngui, Lim & Chua, 2012*). The analysis of HRM curves can also be used to identify invertebrates such as mosquitos (*Ajamma et al., 2016*), oysters (*Wang et al., 2014*), and nematodes (*Skorpikova et al., 2020*) as well as vertebrates (*Ouso et al., 2020*). Recently, the potential of HRM curve analysis for the differentiation of cryptic earthworm species was shown by *Baudrin et al. (2020)*, who performed DNA barcoding and HRM analysis on a subregion of the 16S rRNA gene of the earthworm species *Allolobophora chlorotica*.

This work aimed to (i) demonstrate the potential of HRM curve analysis of the COI gene for the distinction of earthworm species and haplotypes common to agricultural soils in Central Europe and (ii) develop a rapid and inexpensive extraction method for DNA from earthworm tissue to reduce the costs for molecular analyses.

MATERIALS & METHODS

Reference earthworm material

Earthworm species common to agricultural soils in Central Europe were identified utilizing the Edaphobase database (*Burkhardt et al., 2014*). Eight agriculturally relevant species were selected (*Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Apo. limicola*, *Apo. longa*, *Apo. rosea*, *Lumbricus castaneus*, *L. rubellus*, and *L. terrestris*) covering 87.6% of all database entries. Morphologically identified reference material of all eight species sampled in agricultural soils in Germany was kindly provided by Dr. Stefanie Krück. Total DNA was extracted using Qiagen's DNeasy® Blood & Tissue extraction kit (Qiagen N.V., Hilden, Germany) according to the manufacturers' instructions. The extracts were checked on 1.7% (w/v) agarose gels stained with SYBR Green I. Prior to amplification, extracts were diluted 1:50 (v/v) in double-distilled H2O (ddH_2O). For the identification of different haplotypes, DNA from 28 morphological identified individuals of *Allo. chlorotica* collected in October 2021 at an agricultural field near Otterndorf, Germany (53°48′32.69″N, 8°54′2.45″E) was extracted using a cetyltrimethylammonium bromide (CTAB)-based protocol as per

Table 1 Primer used for high-resolution melting (HRM) curve analysis.

Primer name	Primer sequence (5′–3′)	Source
EW_COI_F1	CATG CATT YGTD ATAA TYTT CTT	This study
EW_COI_F2	GTVT TYAT YGGN GGNT TYGG AAA	This study
EW_COI_F3	ATRG TDGG DGCH GGWA TRAG	This study
EW_COI_R1	CCDG THCC DGCN CCYT TTTC	This study
EW_COI_R2	AGAA TNAG NGAD GGRG GNAR NA	This study
EW_COI_R3	GADG CWCC HGCY ARRT GDAR DGA	This study
16S-Ac-F1	CTAAATTCTGACCCTTATTC	*King et al. (2010)*
WORM-16S-R1	CCTAAGCCAACATCGAGGTG	*King et al. (2010)*
COI-Al-F2	TGGCTTCTACCTCTAATACT	*King et al. (2010)*
COI-Al-R2	ATGAAGGGAGAAGATGGCCA	*King et al. (2010)*

Brandfass & Karlovsky (2008). Extracts were checked on 1.7% agarose gels and diluted 1:50 prior to PCR as described above.

Rapid extraction method of DNA from earthworm tissue

We developed a rapid, robust, and inexpensive extraction method to extract amplifiable DNA from earthworm tissue. The protocol was tested using earthworm tissue of *Allo. chlorotica, Apo. caliginosa*, and *L. terrestris*. Fresh or frozen earthworm tissue (approx. 2× 5 mm tissue) was placed in 1.5 mL tubes containing 50 µL ddH_2O and two spatula tips of glass beads (Ø250–300 µm). The tissue was ground within the tube for 10 s using a sterile plastic micropestle and incubated at 70 °C for 10 min. Following incubation, the tube was centrifuged at 10,000× g for 1 min and the supernatant was transferred into a new 1.5 mL tube. The supernatant was diluted 1:10 (v/v) in ddH_2O prior to PCR.

Primer design for HRM curve analysis

We designed three forward and three reverse primers (Table 1) targeting different subregions of the COI gene. Reference sequences of all eight earthworm species were obtained from NCBI's GenBank (accession numbers are given in Fig. S1) and aligned in MEGA version 11.0.10 (*Kumar, Stecher & Tamura, 2016*) using ClustalW (*Thompson, Higgins & Gibson, 1994*). Primers were designed manually by selecting suitable primer binding sites (Fig. S1 for primer pair EW_COI_F2 and EW_COI_R1, Fig. S2 for all primer) within the COI genes under consideration of potential dimerization, melting temperature, and degeneracy of the designed primers as well as sequence heterogeneity among species.

Development of HRM curve assay

Morphologically identified reference DNA material of all eight earthworm species was amplified using nine different primer combinations (Table 2). Amplifications were carried out in a CFX 384 Thermocycler (Biorad, Rüdigheim, Germany) in 384-well microplates in 4 µL reaction volumes consisting of ddH_2O; buffer (10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, pH 8.3 at 25 °C); 125 µM of each deoxynucleoside triphosphate (Bioline, Luckenwalde, Germany); 0.3 µM of each EW_COI primer (Table 1); 1 µg μL^{-1} bovine serum albumin; 0.5 µM EvaGreen® solution (Jena Bioscience, Jena, Germany); 0.03 u μL^{-1}

Table 2 Primer combinations tested in this study and their PCR product length.

Primer combination	PCR product length (bp)
EW_COI_F1 × EW_COI_R1	194
EW_COI_F1 × EW_COI_R2	287
EW_COI_F1 × EW_COI_R3	152
EW_COI_F2 × EW_COI_R1	158
EW_COI_F2 × EW_COI_R2	251
EW_COI_F2 × EW_COI_R3	116
EW_COI_F3 × EW_COI_R1	293
EW_COI_F3 × EW_COI_R2	386
EW_COI_F3 × EW_COI_R3	251

Hot Start Taq DNA Polymerase (New England Biolabs, Beverly, Massachusetts, USA) and 1 µL template DNA or ddH_2O for negative controls. Thermocycling conditions for all nine primer combinations were as follows: Initial denaturation for 120 s at 95 °C followed by 40 cycles of denaturation for 20 s at 95 °C, annealing for 30 s at 55 °C, and elongation for 30 s at 68 °C. Final elongation was performed for 5 min at 68 °C. Following amplification, PCR products were heated to 95 °C for 60 s, cooled to 65 °C for 60 s, followed by a stepwise temperature increase from 65 °C to 95 °C by 0.1 °C per step with continuous fluorescence measurement to generate HRM curves.

Since a clear distinction between *Apo. caliginosa* and *Apo. longa* was not achieved by the optimized HRM curve assay (see *HRM curve analysis of earthworms*), we expanded a previously described multiplex PCR assay (*King et al., 2010*) for these two species by the generation of melting curves. Briefly, a 116 bp subregion of the 16S rRNA gene of *Apo. caliginosa* was amplified using the primer pair 16S-Ac-F1 and WORM-16S-R1 or a 213 bp subregion of the COI gene of *Apo. longa* using the primer pair COI-Al-F2 and COI-Al-R2 (Table 1). The composition of the reaction volume as well as the thermocycling conditions were identical to those described above except that four primers were used.

Furthermore, we aimed to test the suitability of our optimized HRM curve assay for the identification of different COI haplotypes. For this, HRM curve analysis was performed on different COI haplotypes across 28 individuals of *Allo. chlorotica* using the primer pair EW_COI_F2 and EW_COI_R1. The composition of the reaction volume as well as the thermocycling conditions were identical to those described for the HRM curve analysis of all eight earthworm species above. To confirm the findings from HRM curve analysis of potentially different haplotypes, COI genes of all 28 individuals of *Allo. chlorotica* were sequenced as described below (see *Sanger sequencing of the COI region of Allolobophora chlorotica*).

Sanger sequencing of the COI region of Allolobophora chlorotica

The COI gene of 28 individuals of *Allo. chlorotica* was amplified using the primer set LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) (*Folmer et al., 1994*). Amplifications were carried out in an

Eppendorf Mastercycler EP Gradient S thermocycler (Eppendorf, Hamburg, Germany) in 200 µL PCR tubes in 15 µL reaction volumes consisting of ddH_2O; buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100, pH 8.8 at 25 °C); 125 µM of each deoxynucleoside triphosphate (Bioline, Luckenwalde, Germany); 0.3 µM of each primer (LCO 1490 and HCO2198); 1 µg μL^{-1} bovine serum albumin; 0.03 u μL^{-1} Taq DNA Polymerase (New England Biolabs, Beverly, MA, USA) and 1 µL template DNA or ddH_2O for negative controls. Thermocycling conditions were as follows. Initial denaturation for 120 s at 95 °C followed by 40 cycles of denaturation for 20 s at 95 °C, annealing for 30 s at 55 °C, and elongation for 60 s at 68 °C. Final elongation was performed for 5 min at 68 °C. Following amplification, PCR products were loaded on 1.7% agarose gels, bands of the expected product size were excised from the gel, and extracted utilizing the FastGene Gel/PCR Extraction Kit (Nippon Genetics Europe GmbH, Düren, Germany) according to the manufacturer's instructions. Extracts were diluted 1:50 (v/v) in ddH_2O and re-amplified as described above. Re-amplified PCR products were purified using isopropanol as described previously (*Beule et al., 2017*), quantified using a spectrophotometer (BioPhotometer plus, Eppendorf, Hamburg, Germany), and subjected to Sanger sequencing at the facilities of LGC Genomics (Berlin, Germany). The quality of the obtained sequences was checked manually and sequences were aligned in MEGA version 11.0.10 (*Kumar, Stecher & Tamura, 2016*) using ClustalW (*Thompson, Higgins & Gibson, 1994*). A phylogenetic tree was constructed using maximum likelihood analysis. All sequences were deposited at NCBI's GenBank (accession numbers ON242065 to ON242092).

HRM data processing and taxonomic assignment

HRM data was processed as described by *Schiwek et al. (2020)*. Briefly, relative fluorescence unit (RFU) data was obtained from the CFX Maestro™ Software (Bio-Rad CFX Maestro 1.1 version 4.1.2433.1219, Biorad, Rüdigheim, Germany) and the negative first derivate was calculated to obtain melting curves. Difference curves were generated by subtracting the melting curve data of each reference earthworm from the mean melting curve data of all reference earthworms. All raw fluorescence and negative derivative of fluorescence data can be found in File S1.

RESULTS

Rapid and inexpensive extraction of earthworm DNA

We developed a rapid extraction method to recover earthworm DNA from fresh or frozen tissue in less than 15 min. The method is easy, robust, and scalable. Furthermore, the method requires only minimal laboratory equipment (incubation at 70 °C and centrifugation) and comes at extremely low costs. As expected, extracted DNA was fragmented but sufficient amounts of amplifiable DNA were obtained (Fig. S3).

HRM curve analysis of earthworms

Of our nine primer combinations, only the combination EW_COI_F2 and EW_COI_R1 (see Fig. S1 for alignment) yielded successful amplification of all eight species and enabled

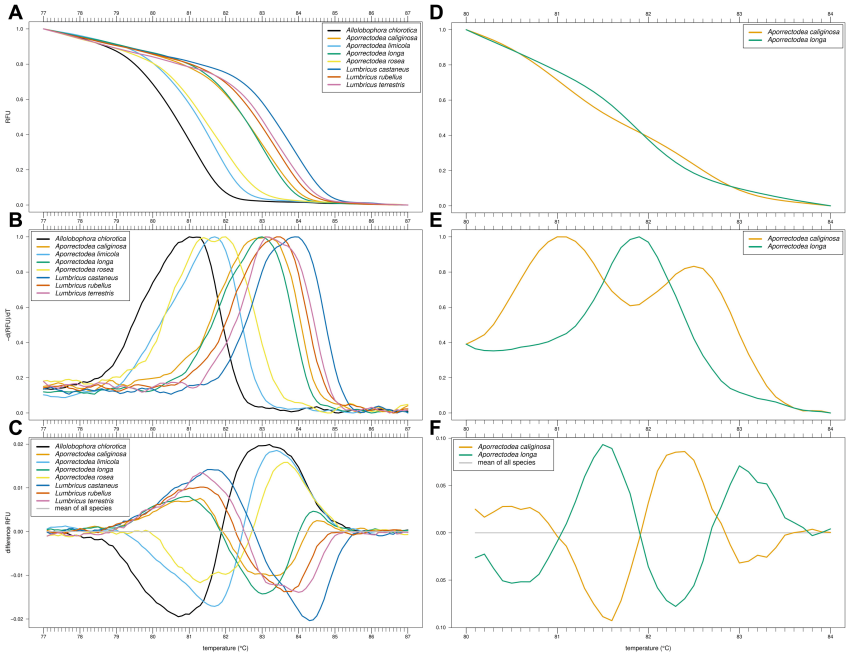

Figure 1 High-resolution melting (HRM) curve assay for the identification of earthworm species. Denaturation curves of eight earthworm species (A) and *Aporrectodea. caliginosa* and *Apo. longa* (D). Melting curves of eight earthworm species (B) and *Apo. caliginosa* and *Apo. longa* (E). Difference curves of eight earthworm species (C) and *Apo. caliginosa* and *Apo. longa* (F). PCRs were carried out using the primer pair EW_COI_F2 and EW_COI_R1. Differences curves were generated by subtracting the data of each curve from the mean of all curves. All curves represent means of five technical replicates.

Full-size DOI: 10.7717/peerj.13661/fig-1

the distinction of *Allo. chlorotica*, *Apo. limicola*, *Apo. rosea*, *Lumbricus castaneus*, *L. rubellus*, and *L. terrestris* (Figs. 1A–1C). The subsequent multiplex real-time PCR assay with HRM curve analysis to distinguish *Allo. caliginosa* from *Allo. longa* was done using different markers (see *Development of HRM curve assay*) (Figs. 1D–1F).

Sanger sequencing of the COI region of 28 individuals of *Allo. chlorotica* revealed eight different COI sequence variants (COI haplotypes) among individuals within the COI subregion amplified by the primer pair EW_COI_F2 and EW_COI_R1 (Fig. 2A). HRM curve analysis of the 28 individuals of *Allo. chlorotica* using the primer pair EW_COI_F2 and EW_COI_R1 mirrored the genetic distances among COI haplotypes (cf. Figs. 2A–2C). We were able to successfully distinguish several different COI haplotypes within *Allo. chlorotica* (Figs. 2B, 2C); however, the samples clustered into three groups of COI haplotypes. Group 1 of COI haplotypes comprised haplotype 1, group 2 comprised haplotypes 2, 3, 6, and 7, and group 3 comprised haplotypes 4, 5 and 8. None of the COI haplotypes of *Allo. chlorotica* reported in this work were identical with any previously reported COI haplotype.

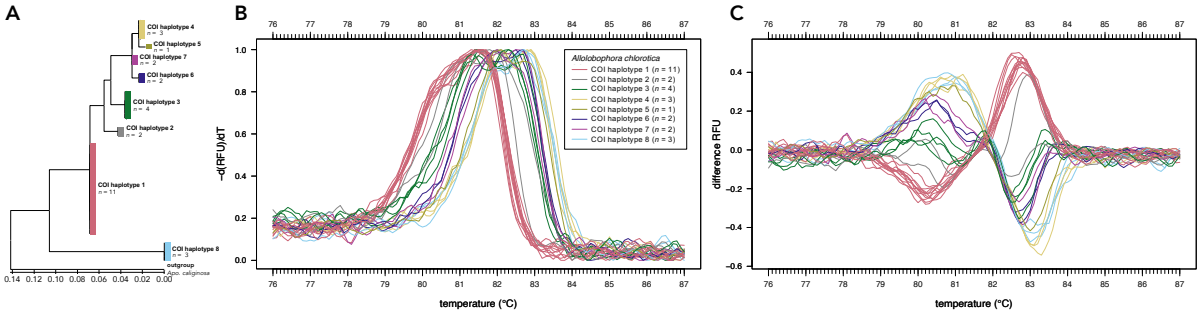

Figure 2 **High-resolution melting (HRM) curve assay for the distinction of earthworm COI haplotypes of *Allolobophora chlorotica*.** Phylogenetic tree of 28 individuals of *Allo. chlorotica* (A). The tree was constructed from the COI subregion amplified by the primer pair EW_COI_F2 and EW_COI_R1. The tree was constructed using maximum likelihood analysis. Melting curves (B) and difference curves (C) of 28 individuals of *Allo. chlorotica*. PCRs were carried out using the primer pair EW_COI_F2 and EW_COI_R1. Differences curves were generated by subtracting the data of each curve from the mean of all curves.

Full-size DOI: 10.7717/peerj.13661/fig-2

DISCUSSION

Identification of soil fauna is an essential part of belowground biodiversity research and new primer sets targeting soil fauna are continuously being developed (*e.g.*, *Capra et al., 2016*). Most studies that used amplicon sequencing for metabarcoding of invertebrate communities worked with communities captured in traps and just few studies aimed to sequence soil fauna directly from soil samples (*e.g.*, *Watts et al., 2019*). Although direct amplicon sequencing from soil offers high taxonomic resolution as well as the ability to include invertebrates irrespective of their life stage and locomotion, such approaches have certain disadvantages. For example, amplicon sequencing data is compositional and does not reveal the absolute population size (*Gloor et al., 2017*) and thereby also ignores population dynamics (*e.g.*, *Beule, Arndt & Karlovsky, 2021*). Therefore, it was suggested to always accompany amplicon sequencing by absolute quantification approaches (*Beule, Arndt & Karlovsky, 2021*). Additionally, tremendous differences in the biomass of organisms lead to distinct differences in the DNA content per organism. Consequently, the number of sequences per individual is expected to increase with increasing biomass. Thus, relative abundances of taxa obtained from metabarcoding of complex communities reflect proportions of the biomass rather than population size. Earthworms are among the largest soil invertebrates and are frequently investigated in soil biodiversity surveys. For the sampling of earthworms, individuals are usually either extracted manually by hand-sorting or by using a combination of an expellant (*e.g.*, allyl isothiocyanate or formaldehyde) combined with hand-sorting. Such sampling and identification of individuals allows for the determination of population size and dynamics.

New tools for the identification of earthworms are continuously emerging. For example, in 2014, *Fernández et al. (2014)* showed that earthworms specimens can be identified taxonomically using micro-computed tomography. In 2021, the use of mid-infrared spectroscopy was proposed as a tool for earthworm identification (*Pham et al., 2021*). The

same year, *Andleeb et al. (2021)* developed a machine-learning model for the identification of earthworms based on digital images. Currently, these techniques are rather experimental and have not established themselves in practice yet. A more widely established method besides traditional morphological identification is DNA barcoding of taxonomically informative loci such as COI.

Molecular tools such as DNA barcoding are associated with higher costs as morphological identification, partly due to the required basic equipment and the extraction of DNA that is commonly done using commercial extraction kits. Here, we introduce a simple, rapid, robust, inexpensive, and scalable homemade extraction method that enables the extraction of amplifiable DNA from earthworm tissue in less than 15 min. We believe that such techniques could help to promote the use of molecular techniques for earthworm taxonomy.

For large-scale use, HRM curve analysis is faster and less expensive compared to barcoding approaches as it is a closed-tube, post-PCR method. Still, investment costs for a real-time PCR thermocycler capable of generating HRM curves (*i.e.*, step-wise heating of PCR products at 0.1 to 0.2 °C per step under continuous fluorescence measurement) as well as costs for commercial software for the generation of HRM curves (unless raw fluorescence data is processed outside of a commercial software) remain a major hurdle for many laboratories. In the present study, we were able to distinguish eight earthworm species common to agricultural soils in Central Europe by using HRM curve analysis of a subregion of the COI gene (Fig. 1). However, as for barcoding approaches, suitable marker genes must be selected carefully. For example, large intraspecific variations in the COI gene of earthworms may limit its usage as a marker for DNA barcoding at species level (*e.g.*, *Chang, Rougerie & Chen, 2009*). Furthermore, since *Apo. caliginosa* and *Apo. longa* could not be clearly distinguished in the HRM curve assay alone, a subsequent multiplex PCR assay expanded by the generation of HRM curves must be performed to identify all eight species. Although our assay is an important first step towards HRM curve-based identification of earthworms, more work is required to overcome such limitations.

Sequencing of the COI gene of 28 individuals of *Allo. chlorotica* revealed eight different COI haplotypes within a short subregion (158 bp) of COI (Fig. 2A). Some of these COI haplotypes were distinguishable by using HRM curve analysis (Figs. 2B, 2C), highlighting its potential for intraspecies differentiation. However, we also found that several COI haplotypes (*i.e.*, those within COI haplotype group 2 and 3) are undistinguishable using our HRM curve assay, limiting haplotype typing. Although sequencing remains necessary in our case to uncover the full diversity of haplotypes, HRM curve-based differentiation of haplotypes can serve as a first indication of haplotype diversity. Similar findings among individuals of *Allo. chlorotica* were obtained by *Baudrin et al. (2020)* for a subregion of the 16S rRNA gene. Besides its potential to assess intraspecific variability, intraspecific marker gene variations may impede robust identification at species level using HRM curve analysis, particularly if the variation of melting profiles induced by intraspecific marker gene variation is large. Until suitable marker genes or marker gene subregions are found, we suggest that HRM curve analysis of earthworms could support morphological investigations, especially for juveniles and damaged specimens that cannot be identified

morphologically. If combined, morphological identification and HRM curve analysis may be a suitable alternative to DNA barcoding.

CONCLUSION

HRM curve analysis of COI genes has the potential to simultaneously identify earthworm species and assess intraspecific variations. For robust species identification, we encourage researchers to search for marker genes or marker gene subregions that are suitable for species differentiation by HRM curve analysis and have no to minimal intraspecific variation. Morphological identification remains the most common method to identify earthworm species; therefore, we suggest that HRM curve analysis can be used to confirm morphological identification, especially for juveniles and damaged tissue. Molecular analyses have several advantages over morphological identification but are cost and labour intensive. Our rapid and inexpensive DNA extraction method from earthworm tissue helps to reduce the costs of molecular analyses and thereby promote their application in practice.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Anna Vaupel conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Bernd Hommel conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Lukas Beule conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

All sequences are available at NCBI's GenBank: ON242065 to ON242092.

Data Availability

The following information was supplied regarding data availability:

All raw fluorescence and negative derivative of fluorescence data are available in the Supplementary File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.13661#supplemental-information.

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6 Discussion

6.1 Diversification measures in agriculture

The recent loss of biodiversity and associated ecosystem services, driven by agricultural intensification over the last decades, is threatening the resilience and functionality of agricultural systems (e.g., Tilman *et al.* 2002, Tscharntke *et al.* 2012, Hufnagel *et al.* 2020). For example, in 2015 Chagnon *et al.* reviewed that the systemic use of insecticides causes a decline in the diversity of many important taxonomical groups, such as earthworms and pollinators, and negatively affects ecosystem services, such as decomposition and nutrient cycling. Likewise, in a large-scale pan-European study, Emmerson *et al.* (2016) found that agricultural intensification has negative implications for the diversity of birds, carabid beetles and arable plants, all of which provide important ecosystem services. Additionally, Watson *et al.* (2021) were able to show that the loss of ecosystem services caused by agriculture usually does not follow a linear decline but is rather characterized by tipping points. Correspondingly, a recent study that investigated the resilience of 30 cropping systems with varying degrees of intensification in France found systems with a high capacity for providing ecosystem services to be more stable than intensified systems (Dardonville *et al.* 2022). Adding to that, Oliver *et al.* (2015) summed up the importance of biodiversity and ecosystem services to maintain the resilience of ecosystems in the long-term. Moreover, functional diversity has been described to decline even stronger under agricultural intensification than biodiversity (Flynn *et al.* 2009). Similar results were obtained by Tarifa *et al.* (2021), who investigated the effect of agricultural intensification on the diversity of herb cover under Mediterranean olive groves. The authors found that functional and taxonomic diversity decline under agricultural intensification and described a functional filtering driven by increasing landscape simplification (Tarifa *et al.* 2021).

The agriculturally driven loss of habitat, biodiversity and ecosystem services has been estimated to increase even further in the future as the global demand for food increases (Tilman *et al.* 2001). Adaptation of agricultural systems towards biodiversity conservation has thereby been argued to be a necessary step in maintaining agricultural productivity for future generations (Tscharntke *et al.* 2012). Diversification of agricultural systems is one potential way to counteract the negative effects of agricultural intensification (Hufnagel *et al.* 2020). Diversification measures are generally classified into temporal and spatial diversification measures. In a recent review article, Hufnagel and colleagues (2020) summarized temporal and spatial crop diversification measures.

Temporal diversification measures include, *inter alia*, diversification of crop rotations, double cropping, and the introduction of catch crops (Hufnagel *et al.* 2020). In a long-term field experiment, Peralta *et al.* (2018) were able to show that a diverse crop rotation increases the abundance of disease suppressive functional genes in soil as compared to less diverse crop rotations. Additionally, diversification of crop rotations have been shown to increase SOC and total nitrogen, thus enhancing soil fertility (Tiemann *et al.* 2015). Correspondingly, Zhang *et al.* (2021) reviewed that diversified crop rotations have a positive impact on C cycling and SOC formation. Similar positive effects on C cycling were observed for the introduction of catch crops into the crop rotation. For example, Gentsch *et al.* (2020) described an increased net uptake of atmospheric C and microbial biomass under catch crops as compared to a fallow. In the same study, the authors also found that the positive effect on C uptake increased with increasing catch crop diversity (Gentsch *et al.* 2020). Moreover, catch crops can reduce mineral nitrogen pools in deeper soil layers, consequently reducing nutrient leaching (e.g., Heuermann *et al.* 2019). Furthermore, catch crops can have positive effects on plant health, as has been shown by Mielniczuk *et al.* (2020). The authors found that catch crops (i.e., white mustard and lacy phacelia) grown prior to oat reduced the abundance of total soil fungi, while increasing the abundance of antagonistic fungi and improved oat plant health as compared to no catch crops (Mielniczuk *et al.* 2020).

In contrast to temporal diversification measures, spatial diversification measures are characterized by the simultaneous growth of different plants and include, *inter alia*, alley cropping, intercropping, mixed cropping, or the introduction of buffer and flower strips (Hufnagel *et al.* 2020). For example, in a long-term field experiment, Hauggaard-Nielsen *et al.* (2008) found that intercropping of legumes and barley led to a more efficient use of resources, higher weed suppression, disease reduction and higher N_2-fixation as compared to the same crops being grown separately. Additionally, in a large field experiment investigating the effect of different intercropping systems, Li *et al.* (2009) observed higher yields, increased land equivalent ratio and disease reduction in the intercropped fields as compared to monocultures. The authors argued that even though intercropping in their study required more labour and fertilizer, its potential to increase food production by utilizing the same amount of land could be of great benefit especially in developing countries with increasing food demand (Li *et al.* 2009). In a review article published in 2014, it was hypothesized that enhanced nutrient mobilization by simultaneous growth of two or more crops is a main reason for the overyielding frequently observed in intercropping systems (Li *et al.* 2014). Intercropping also leads to increased microbial

abundance, activity and functions as compared to monocultures (Lian *et al.* 2019). Similar effects can be observed in alley-cropping agroforestry systems, as a specialized form of intercropping which combines rows of trees with rows of crops (Beule *et al.* 2022a). Spatial diversification can also help to reduce pest induced plant damage by increasing the abundance of natural enemies, as was shown for flower strips (Tschumi *et al.* 2015). It should be noted however, that under unfavourable management, the implementation of such spatial measures can also have negative implications for biodiversity. For example, Ganser *et al.* (2019) found that ploughing of annual flower strips has negative effects on overwintering arthropods and concluded that annual flower strips could therefore act as an ecological trap.

Overall, however, diversification of farming systems and the related alteration of agricultural practices, such as reduced soil management, has been reviewed to strengthen the resilience towards climate change and hence increase the sustainability of agricultural systems (Mijatović *et al.* 2012). Consequently, agricultural systems that include biodiversity management strategies have been shown to increase ecosystem services while maintaining yields (Garbach *et al.* 2016).

6.1.1 Earthworm communities under flower strips

Establishment of semi-natural vegetation strips, such as field margins, buffer strips or flower strips, is a common spatial diversification measure in agricultural management with numerous environmental benefits. For example, flower strips have been shown to increase pollination services, natural pest control and insect diversity (e.g., Ouvrard *et al.* 2018, Tschumi *et al.* 2016, Haaland *et al.* 2011). While much is known about the positive effects of flower strips aboveground, their influence belowground is less well described and so far, the study of Kohli *et al.* 1999 is the only one investigating earthworms under strips of wild flowers. Grassy field margins, however, have been shown to increase species diversity and abundance of earthworms and to alter the community composition of earthworms as compared to adjacent croplands (e.g., Smith *et al.* 2008, Crittenden *et al.* 2015, Nieminen *et al.* 2011). These positive effects, however, are strongly influenced by the management of the margins (Frazão *et al.* 2017) and Smith *et al.* (2008) found soil management to be a key element in the potential of field margins to benefit earthworms. Given the well-established negative impact of tillage on many earthworm species (e.g., Chan 2001), these results are unsurprising and in accordance with the results reported in Chapter 2 of this thesis. At two of our three study sites, we found untilled field margin vegetations to harbor a higher abundance of earthworms compared to annual flower strip plots that were reestablished every year. Perennial flower strip mixtures however, showed higher density and biomass of earthworms than annual flower strip mixtures

at all our study sites. This indicates that similar to grassy field margins (Smith *et al.* 2008), soil management of flower strips is of great importance when it comes to the promotion of earthworms. At two of our three study sites, we were also able to show higher density of earthworms under the perennial flower strip vegetation as compared to the field margin vegetation. These findings indicate that under certain management, flower strips can be even more effective in promoting earthworm communities than grassy vegetation strips. Since soil management was identical in both treatments, the higher plant diversity in flower strips likely caused the additional promotion of earthworms, which is in accordance with previous studies where higher plant diversity (and consequently higher plant and fine root biomass) in grasslands was found to increase earthworm abundance and biomass (Spehn *et al.* 2000, Zaller & Arnone 1999). In addition to higher above- and belowground plant biomass, increased nutritional value of more diverse plant mixtures might also be a promoting factor for earthworm communities (e.g., Milcu *et al.* 2006, Curry & Schmidt 2007).

Consequently, our results indicate that in addition to the numerous aboveground benefits, incorporation of flower strips can also positively influence belowground biota, which further confirms their potential in agri-environmental schemes (AES). However, management of flower strips should also be taken into consideration, as we were able to show that perennial flower strips are of much greater value for earthworm communities than annual flower strips. In order to fully unlock the potential of flower strips in AES, more research addressing the different aspects of flower strip management is required. For example, the influence of differently diverse plant mixtures should be investigated, as our results indicated that plant species richness has an influence on earthworms. In addition to the species richness, plant composition of similarly diverse flower strip mixtures should also be investigated, as different earthworm species are known to have feeding preferences towards different plant species (e.g., Neilson & Boag 2003, Curry & Schmidt 2007). Furthermore, Frazão *et al.* (2017) found mulching as well as the surrounding landscape to be important factors influencing earthworm communities in herbaceous/grassy field margins and similar effects can be expected in flower strips but remain yet to be investigated. In the same study, the authors also reported an influence of field margin age on earthworm abundance (Frazão *et al.* 2017). In our study, already one and a half years after establishment, non-tilled field margin and perennial flower strip mixtures showed higher earthworm density and biomass as compared to the tilled annual flower strip mixtures. These results indicate the promotional effect of flower strips after a short period of time but potential long-term positive or negative effects of ageing flower strips on earthworm communities are still

unknown. Moreover, Nieminen *et al.* (2011) found decreasing earthworm densities in arable fields with increasing distance from adjacent field margins. Similar results were reported in Chapter 3 of this thesis for earthworm communities in crop rows alternated with rows of trees. Both studies indicate a potential spillover effect of perennial structural elements into adjacent crop fields, but studies investigating earthworms at increasing distances from flower strips are still missing. Such a potential spillover could also impact the design of future flower strip establishments. As of writing, most flower strips are established at the field edge whereas spatial designs that incorporate them into the field (e.g., in a linear design like the tree rows of alley-cropping agroforestry systems), might be even more beneficial.

6.1.2 Earthworm communities in agroforestry

Another, even more permanent form of spatial diversification is the integration of trees into arable land (agroforestry). Agroforestry systems include, *inter alia*, shelterbelts, silvopastures, and alley-cropping systems, the latter being one of the most popular types of modern agroforestry in the temperate zone with numerous ecological advantages over cropland monoculture (e.g., Quinkenstein et al. 2009, Veldkamp *et al.* 2023). Here, rows of trees are planted alternatingly with rows of crops, which reduces soil erosion (e.g., van Ramshorst *et al.* 2022), improves nutrient cycling (e.g., Wolz *et al.* 2018) and creates additional habitat for above- and belowground biota (e.g., Varah *et al.* 2013, Beule *et al.* 2022b).

Earthworm communities also benefit from alley-cropping agroforestry. A number of studies reported higher numbers of earthworms under the tree rows as compared to the crop rows or cropland monocultures (serving as a reference land use) (Cardinael *et al.* 2019, D'Hervilly *et al.* 2020, D'Hervilly *et al.* 2021). The results presented in Chapter 3 confirm these findings, as we found 2.9 to 12.3 times more earthworms in the tree rows as compared to the crop rows and the cropland monoculture. The magnitude of this increase is even higher as compared to previous results reported by Cardinael *et al.* (2019) who found on average 2.05 to 2.5 times more earthworms in the tree rows as compared to the crop rows and cropland without trees. In our study, anecic earthworms were especially promoted under the trees, which caused a shift in earthworm community composition. This observation is in accordance with the findings of D'Hervilly *et al.* (2020, 2021) who also reported a community shift towards anecic species in the tree rows as compared to the crop rows of three alley-cropping agroforestry sites in France. Since anecic species create permanent vertical burrows and feed on the soil surface, their promotion under the trees was most likely caused by the absence of tillage in the tree rows as well as increased feeding resources on the soil surface due to tree leaf litter input. While the

promotion of anecic earthworms in our study was limited to the tree row, endogeic earthworms in the crop row showed a gradual decline with increasing distance from the trees, which was most likely caused by tree leaves being incorporated into the soil through tillage. These results indicate a potential spill-over of positive effects on earthworms from the tree rows into the crop row. In order to confirm this finding, more studies investigating earthworms along fine spatial gradients in agroforestry systems are needed. As of writing, our study is the only one where earthworms were sampled at more than two distances from the trees in an alley-cropping agroforestry system. At our study site, epigeic earthworms were only found sporadically and in close distance to or in the tree rows, which is in accordance with results from D'Hervilly *et al.* (2020). Cardinael *et al.* (2019) also reported higher densities of epigeic earthworms under the trees, indicating a promoting influence of agroforestry on these species. This is unsurprising, as epigeic earthworms are particularly dependent on a permanent layer of organic material on the soil surface, which can be found under the trees. In general, earthworms are promoted by the implementation of agroforestry systems, mainly due to reduced soil disturbance in the tree rows as well as increased above- and belowground litter input (i.e., leaves and fine roots) in the tree and crop rows of the system.

Nonetheless, a number of questions remain unanswered regarding the influence of agroforestry practice on earthworm communities, which, if answered, could help to improve the design of future systems and maximize their ecological benefits. For example, as age is an important factor regarding the beneficial effects of field margins, even bigger age effects can be expected in agroforestry systems due to the long lifetime of these systems. Additionally, while we were not able to detect an effect of different tree species (i.e., two poplar clones and black locust) in our study, Price and Gordon (1998) found tree species (i.e., ash, poplar and maple) to have an impact on earthworm communities at an agroforestry system in Ontario, Canada. More studies on agroforestry systems with different tree species and understory vegetation are needed to elucidate the influence of tree row composition on earthworm communities. Moreover, orientation of the tree rows can be expected to have an influence on earthworms, as East-West oriented tree rows will provide more shade and are expected to retain more soil moisture than rows planted in a North-South orientation. In short rotation agroforestry systems that are harvested every two to five years, harvest of trees leads to a decline in above- and belowground biomass, as roots die off and regrowing trees produce less leaf litter in the first years post-harvest. Consequently, tree harvest intervals can be expected to affect earthworm communities, but studies investigating earthworm over an entire tree rotation cycle

would be required to confirm this. Similar to flower strips and field margins (Frazão *et al.* 2017), the surrounding landscape of agroforestry systems should also be considered in future study designs. This is of particular interest when it comes to species diversity and the recolonization of epigeic earthworms into arable land, as those species are often missing in tilled cropland monocultures (Stroud 2019).

6.2 Earthworm research in agricultural systems

All knowledge gaps described above are stressing the need for field-based earthworm research. However, common methods for extraction and species determination of earthworms are time-consuming and expensive (e.g., Iannone et al 2012). Additionally, answering many of those complex research questions would require spatially (large number of sampling points or/and study sites) or temporally (repeated samplings or long-term monitoring) large study designs. Such large-scale designs are associated with even more extended sampling durations and higher costs as well as an increased number of collected earthworms that need to be processed (i.e., determination of biomass, development stage and species), which further increases cost and time frame of a given research project. Consequently, studies investigating earthworm communities in the field are scarce as compared to micro- and mesocosm experiments, which however, cannot depict the complexity of natural systems and are thus not suitable for a number of research questions. Rapid and low-cost alternatives for earthworm extraction and species determination are therefore urgently needed.

6.2.1 Extracting earthworms in the field

There are various methods to extract earthworms from soils (e.g., hand sorting, chemical extraction and electrical octet method). Hand sorting with chemical extraction is a standardized method that is regarded to work well for the quantitative and qualitative assessment of the whole earthworm community (ISO 23611-1:2018(E)). However, hand sorting is time-consuming (e.g., Callaham & Hendrix 1997, Iannone et al 2012), which is why it is often not feasible for large-scale studies or monitoring programs. Thus, alternative approaches such as chemical extraction without hand sorting receive increased attention.

As common expellants are either highly toxic (formaldehyde) or not reproducible (commercial hot mustard), the use of AITC as an alternative expellant for chemical extraction is gaining popularity (e.g., Pelosi *et al.* 2009, Pelosi *et al.* 2014). However, most method comparisons either use mustard instead of AITC (e.g., Lawrence & Bowers 2002), do not include hand sorting (e.g., Pelosi *et al.* 2014), or have a small number of study sites (e.g., Čoja *et al.* 2008). As of writing, our study

(Chapter 4) is the first to investigate AITC extraction efficiency compared to a combination of hand sorting and AITC extraction over a wide range of study sites in Germany. We found that the use of AITC extraction without hand sorting underestimates total earthworm density and biomass, which is in accordance with previous results for AITC and other chemical expellants (e.g., Bartlett *et al.* 2006, Pelosi *et al.* 2009, Gutiérrez-López *et al.* 2016). This is unsurprising, as some earthworms can be expected to escape horizontally, which limits the methods efficiency, especially for endogeic species. Our results on different ecological groups of earthworms showed that the discrepancy between total earthworm densities between the two methods was mainly explained by endogeic earthworms. In contrast, recovery of anecic earthworms did not differ between the two methods, which is probably due to the fact that anecic earthworms create vertical burrows with openings at the soil surface (Bouché 1977). Consequently, if only anecic species are of interest, AITC extraction without hand sorting would be a viable tool for quantitative comparisons among study sites. The same holds true for research questions focussing on species diversity, such as biodiversity monitoring, as we found no difference in species richness between the two methods. Unlike Gutiérrez-López *et al.* (2016) but in accordance with Lawrence & Bowers (2002), we did not find a correlation between chemical extraction efficiency and any of the tested soil properties (i.e., soil pH, soil texture, total C, total N, soil organic matter, and effective cation exchange capacity). Studies along even finer gradients of different soil properties would be required to fully understand if and if so, how chemical extraction efficiency is influenced by soil properties. Future investigations should also include different land-use systems such as grass- and woodlands and record other potentially important environmental factors. For example, soil moisture and soil temperature are known to influence earthworm activity levels and should hence be monitored not only during but also in the weeks prior to sampling. Additionally, more data on the extraction efficiency of epigeic earthworms is required, as they were not present in sufficient quantities at our study sites. A first indication for the extraction efficiency of epigeics was given by Lawrence and Bowers (2002) who reported 100 % extraction efficiency for the epigeic earthworm *Dendrobaena octaedra* using hot mustard extraction. However, their study design included only two study sites (Lawrence and Bowers 2002). Until all those uncertainties are conquered, hand sorting remains necessary for quantitative investigations of the whole earthworm community across multiple study sites. However, for on-site comparisons, AITC extraction without hand sorting can be a suitable alternative even for quantitative assessments of the whole community, as demonstrated in Chapter 2 and 3 of this thesis.

Overall, it can be concluded that for certain research questions, AITC extraction without hand sorting is a rapid and cost-saving alternative to the standardized combination of chemical extraction with hand sorting. The method provides reliable information on the abundance of anecic earthworms and overall species richness and is also applicable for on-site comparisons of the whole community. The method is especially valuable for large-scale studies and allows for non-destructive sampling, which can be of importance at certain sampling sites. Finally, method selection should always be tailored to the research question and available resources as all existing methods for earthworm samplings are associated with specific limitations and disadvantages (Čoja *et al.* 2008).

6.2.2 Species determination

Most applied earthworm research in agriculture aims to evaluate management practices that maintain or promote earthworm ecological functions. However, as earthworm functions differ among species (Capowiez *et al.* 2014), determination to species level is a key prerequisite for a meaningful interpretation of earthworm data in such studies. Identification based on morphological keys (e.g., Graff 1953, Bouché 1972, Sims & Gerard 1985, Krück 2018) is time-consuming, requires taxonomical expertise (Decaëns *et al.* 2013), and does not work well for juveniles (e.g., Richard *et al.* 2010), damaged worms, and cryptic species (King *et al.* 2008) but is still the most commonly used method. Alternatively, molecular techniques, such as DNA barcoding, have been successfully tested for earthworms (Huang *et al.* 2007) but are rarely used due to its high costs.

High resolution melting (HRM) curve analysis is a post-PCR technique, which is widely used in clinical diagnostics (e.g., Tindall *et al.* 2009) and for species identification of different invertebrates (e.g., Ajamma *et al.* 2016, Wang *et al.* 2014, Skorpikova *et al.* 2020). In our study (Chapter 5), we were able to distinguish eight earthworm species commonly found in agricultural soils using HRM curve analysis of a subregion of the COI gene. Furthermore, Baudrin *et al.* (2020) indicated the potential application of HRM analysis for the identification of cryptic species within the earthworm species *Allolobophora chlorotica*. In accordance with this, HRM curve analysis in our study enabled the differentiation between different haplotypes of *Allolobophora chlorotica*, underlining the potential application of HRM for the differentiation between cryptic species. In addition to that, the method is faster and less expensive compared to DNA barcoding and unlike morphological identification, it requires only a small amount of earthworm tissue, which makes it suitable for the identification of juveniles and damaged individuals. Currently, juvenile individuals are

often excluded from interpretation, as they usually cannot be determined morphologically (Richard *et al.* 2010). Here, HRM could offer a rapid low-cost solution, especially when used in combination with morphological identification.

HRM curve analysis has the potential to become a fast and inexpensive alternative for earthworm identification, especially in cases where morphological identification reaches its limits (e.g., juveniles, damaged worms and cryptic species). However, more species need to be tested and successfully differentiated to make the method widely applicable. Additionally, other methodologies such as micro-computed tomography (Fernández *et al.* 2014), mid-infrared spectroscopy (Pham *et al.* 2021) and machine-learning models based on digital images (Andleeb *et al.* 2021) should also be explored to further increase the array of potential identification methods and offer suitable alternatives to morphological identification for all sorts of research questions.

7 Conclusion

The implementation of spatial diversification measures into agricultural systems provides new habitats in and around agricultural fields that can maintain and promote above- and belowground biodiversity. In this thesis, spatial diversification through perennial flower strips and agroforestry promoted the abundance and biomass of earthworm communities and also led to a community shift towards anecic and epigeic species. These combined effects can be expected to result in an increased potential of earthworms to provide ecological functions within these systems. However, more research on these and other agri-environmental schemes and their management is required to further understand and improve their impact on earthworm communities (e.g., spillover effects from flower strips into arable land, tree species effects, and landscape structure).

In order to gain a deeper understanding of the impact of spatial diversification measures on earthworms, more field-based studies are necessary. Common methods for earthworm sampling and species determination are impractical for large-scale studies or monitoring, which is why earthworms are often excluded from such projects (*cf.* Rutgers *et al.* 2016 and references there within). In our study, AITC extraction without hand sorting recovered anecic earthworm species and overall species richness equally well as the combination of AITC extraction and hand sorting. The method also allows for on-site comparisons of the whole community and is non-destructive towards the sampling site while being less expensive and less time-consuming than hand sorting. Therefore, AITC extraction without hand sorting offers a reliable alternative, especially for large-scale studies and monitoring where hand sorting is not feasible. Regarding species determination, using HRM curve analysis allowed for the differentiation of eight common earthworm species in our study and is of high value for juvenile and damaged individuals. Unlike morphological identification, HRM can potentially be used to distinguish cryptic species as we were able to differentiate between 8 different haplotypes of *Allolobophora chlorotica* in our study. The method is also less expensive and faster than DNA barcoding and could be especially valuable when being used in addition to morphological determination. Overall, we suggest that both methods (AITC without hand sorting and HRM curve analysis), either alone or combined, have the potential to facilitate field-based earthworm research and enable the incorporation of earthworms in monitoring projects and large-scale research studies. By this, we hope that remaining knowledge gaps regarding the response of earthworm communities to agricultural management

practices can be filled and thereby further practises that preserve the integrity of earthworm communities in agricultural soils can be identified and implemented into agri-environmental schemes.

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Appendix

A1 Earthworm and Soil Microbial Communities in Flower Strips

File SI 1 Composition of the plant mixtures in 2022 (2 years post sowing). "x" indicates the presence of the plant species. Plant data are provided by Blümel *et al.* (unpublished).

File SI 1. Composition of the plant mixtures in 2022 (2 years post sowing). "x" indicates the presence of the plant species. Plant data are provided by Blümel *et al.* (unpublished).

plant mixture:	field margin		
soil type:	Podzol	Luvisol	Cambisol
Festuca pratensis			x
Dactylis glomerata	x	x	x
Lolium perenne	x	x	x
Phleum pratense	x	x	x

plant mixture:	annual flower strip 1		
soil type:	Podzol	Luvisol	Cambisol
Trifolium alexandrinum		x	x
Fagopyrum esculentum	x	x	x
Trifolium incarnatum	x	x	x
Camelina sativa	x	x	
Raphanus sativus	x	x	x
Trifolium resupinatum	x	x	x
Phacelia tanacetifolia	x	x	x
Ornithopus sativus	x	x	
Helianthus annuus	x	x	x
Sinapis alba	x	x	x
Melilotus albus	x	x	x

plant mixture:	annual flower strip 2		
soil type:	Podzol	Luvisol	Cambisol
Phacelia tanacetifolia	x	x	x
Centaurea cyanus	x	x	x
Calendula officinalis	x	x	x
Papaver rhoeas	x	x	x
Anethum graveolens	x	x	
Nigella sativa			
Carthamus tinctorius	x	x	x
Helianthus annuus	x	x	x
Coriandrum sativum	x	x	x
Ornithopus sativus	x	x	
Melilotus albus		x	x
Trifolium incarnatum	x	x	x
Trifolium resupinatum	x	x	x

plant mixture:	perennial flower		
soil type:	Podzol	Luvisol	Cambisol
Phacelia tanacetifolia			x
Centaurea cyanus			
Calendula officinalis			x
Papaver rhoeas	x		
Anethum graveolens			x
Nigella sativa			
Carthamus tinctorius			
Helianthus annuus			
Coriandrum sativum			
Ornithopus sativus			
Melilotus albus			
Inkarnatklee			
Perserklee		x	x
Achillea millefolium	x	x	x
Carum carvi			
Cichorium intybus	x	x	x
Daucus carota	x	x	
Foeniculum vulgare	x	x	x
Leucanthemum vulgare	x	x	
Lotus corniculatus	x	x	x
Trifolium repens			
Medicago sativa	x	x	x
Onobrychis	x	x	x
Plantago lanceolata	x	x	x
Sanguisorba minor	x	x	x
Anthemis tinctoria	x	x	
Pastinaca sativa	x	x	x
Salvia officinalis	x		x
Melilotus officinalis		x	x
Trifolium hybridum	x	x	x

plant mixture:	perennial flower		
soil type:	Podzol	Luvisol	Cambisol
Achillea millefolium	x	x	x
Agrimonia eupatoria			x
Anthemis tinctoria	x	x	x
Anthriscus sylvestris			
Artemisia vulg./cam pestris	x	x	x
Barbarea vulgaris			
Carum carvi			
Centaurea scabiosa	x	x	
Cerastium holosteoides			
Cichorium intybus	x	x	x
Clinopodium vulgare	x	x	x
Crepis biennis	x	x	x
Daucus carota	x	x	
Dipsacus fullonum	x	x	x
Echium vulgare	x	x	x
Galium album	x	x	x
Galium verum	x		x
Heracleum sphondylium			
Hypericum perforatum			
Leucanthemum incultanum	x	x	x
Malva moschata	x	x	x
Malva sylvestris	x	x	x
Oenothera biennis	x	x	x
Origanum vulgare	x		
Pastinaca sativa			
Plantago lanceolata	x	x	x
Prunella vulgaris	x	x	x
Reseda luteola	x	x	
Salvia pratensis	x		x
Sanguisorba minor	x		x
Silene dioica	x	x	x
Silene latifolia subsp. alba	x	x	x
Silene vulgaris	x	x	x
Lychnis flos-cuculi		x	x
Tanacetum vulgare	x	x	x
Verbascum lychnitis, nigrum, thapsus			
Anethum graveolens			
Borago officinalis			x
Camelina sativa			
Carthamus tinctorius			
Coriandrum sativum			
Fagopyrum esculentum			
Foeniculum vulgare	x	x	
Guizotia abyssinica			
Helianthus annuus			
Linum usitatissimum			
Medicago lupulina			
Medicago sativa	x	x	x
Petroselinum crispum			
Phacelia tanacetifolia			
Trifolium pratense	x	x	x

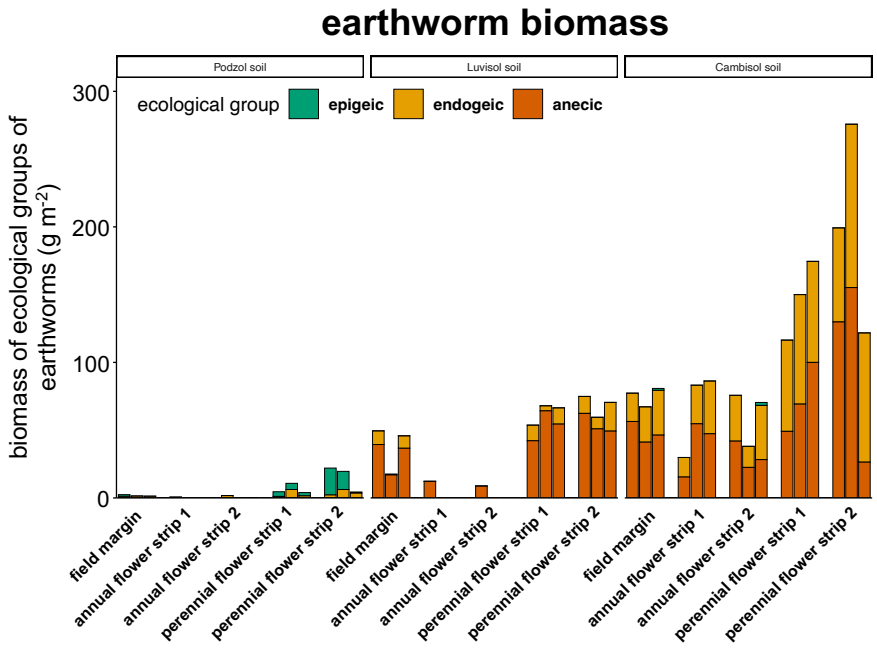

Figure SI 1 Earthworm biomass. Biomass (g m^{-2}) of ecological groups of earthworms. Bars represent individual replicate plots (n = 3).

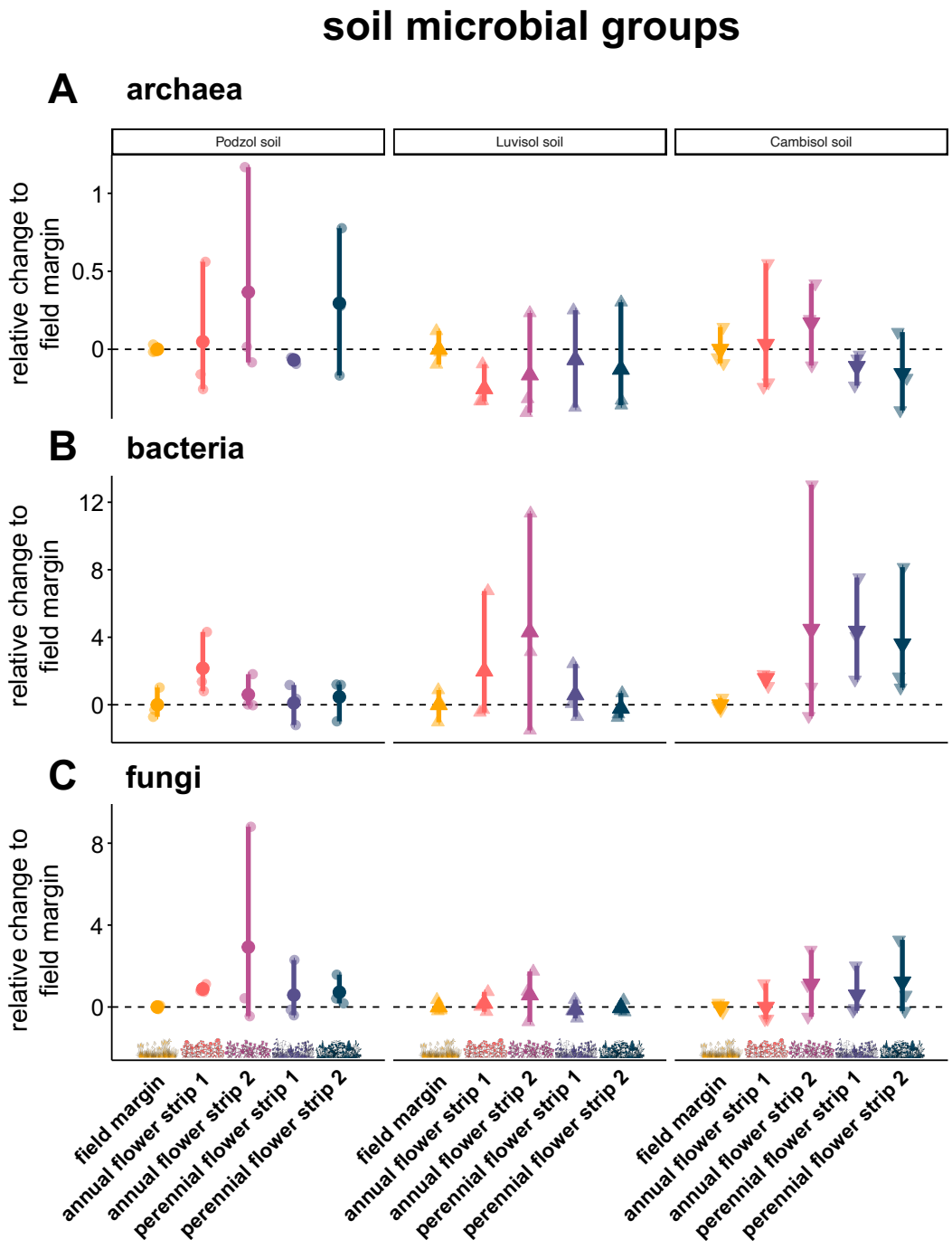

Figure SI 2. Relative change of (A) soil archaea, (B) bacteria, and (C) fungi in response to flower strips. Non-transparent dots and triangles represent means and vertical bars range from the minimum to the maximum value (*n* = 3). Transparent dots and triangles represent individual data points (i.e. replicate plots). Archaea, bacteria, and fungi were quantified by using real-time PCR (see Quantification of soil microbial groups using real-time PCR for details). See Statistical analysis for details regarding the calculation of the relative change. Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library).

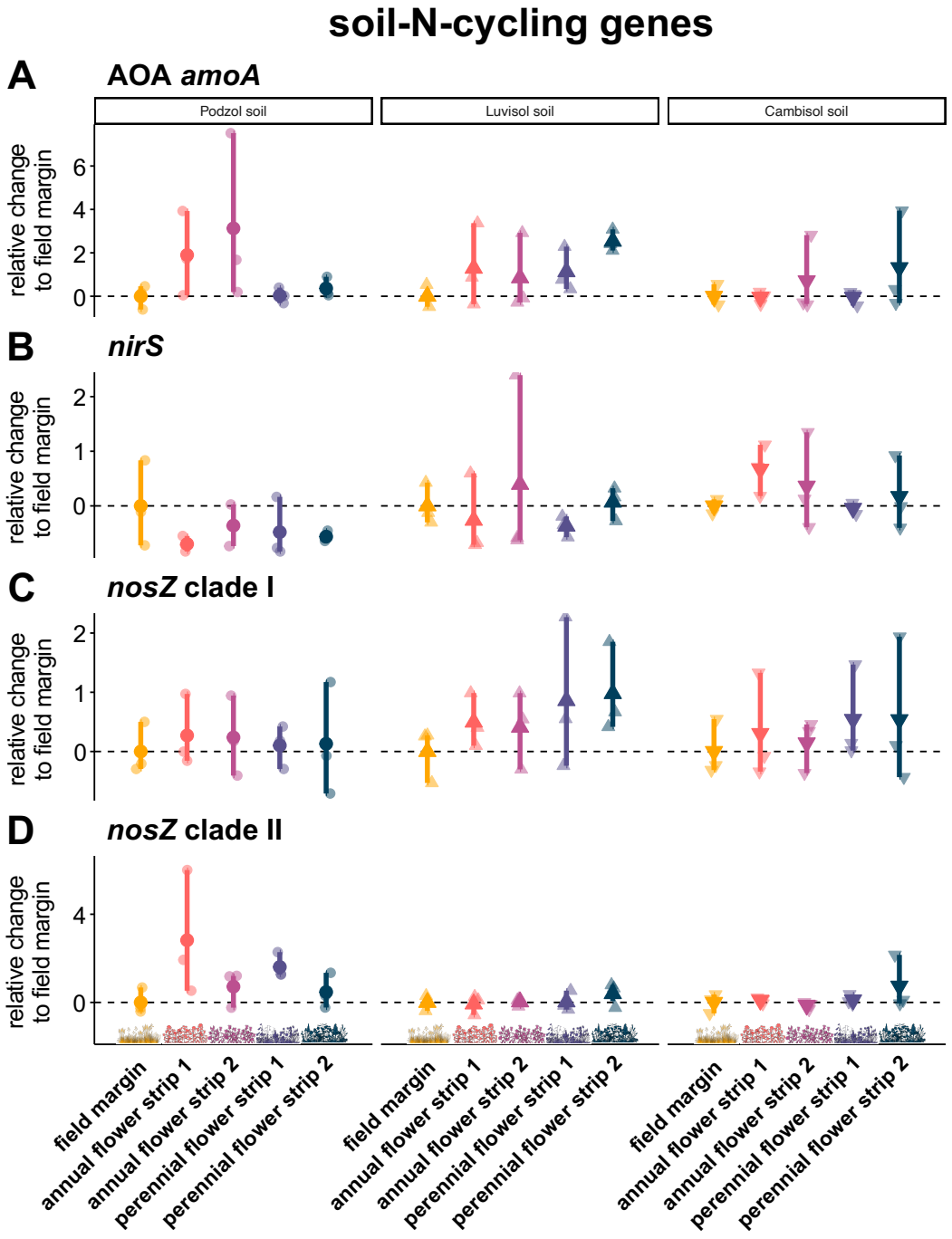

Figure SI 3. Relative change of ammonia-oxidizing archaea (AOA) *amoA* (A), *nirS* (B), *nosZ* clade I (C), and *nosZ* clade II genes (D) in response to flower strips. Non-transparent dots and triangles represent means and vertical bars range from the minimum to the maximum value (n = 3). Transparent dots and triangles represent individual data points (i.e. replicate plots). AOA *amoA*, *nirS*, and *nosZ* clade I and II genes were quantified by using real-time PCR (see Quantification of soil microbial groups using real-time PCR for details). See Statistical analysis for details regarding the calculation of the relative change. Images are courtesy of the Integration and Application Network (ian.umces.edu/media-library).

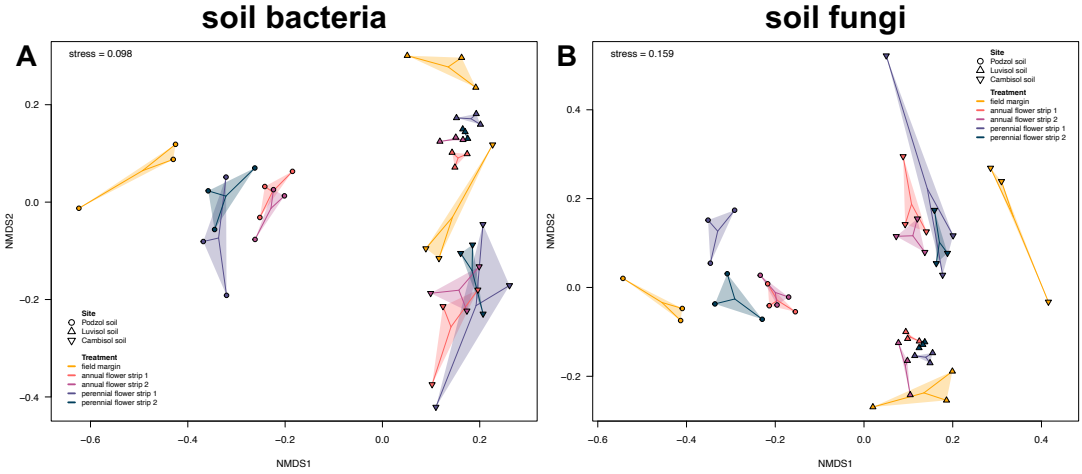

Figure SI 5. Non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities of soil bacterial (A) and fungal (B) communities. Dots and triangles represent individual data points (i.e. replicate plots) (*n* = 3) which are connected with the centroid of their respective plant mixture.

Table SI 1. Study site description and general soil properties. [a] data for all sites was obtained from a weather station near Werl (Germany) of the German Meteorological Service (station ID 3031 for Lippetal and 5480 for Merklingsen and Ense); [b] determined from soil samples collected at 0 – 30 cm soil depth; [c] determined from soil samples collected at 0 – 5 cm soil depth.

Study site	Lippetal	Merklingsen	Ense
Location	(51°39'47.7"N, 8°12'22.8"E)	(51°33'59.9"N, 8°00'29.8"E)	(51°30'34.3"N, 8°01'33.0"E)
Soil type	Gleyic Podzol	Gleyic Luvisol	Stagnic Cambisol
Elevation (m.a.m.s.l.)	72	99	223
Annual mean precipitation (mm)[a]	765.9		777.0
Annual mean temperature (°C)[a]	10.3		10.5
Soil pH (1:2.5 in H_2O)[b]	6.3 ± 0.2	7.4 ± 0.1	7.7 ± 0.1
Soil bulk density (g cm^{-3})[c]	1.19 ± 0.07	0.99 ± 0.06	1.18 ± 0.15
Soil organic C (%)[b]	1.89 ± 1.00	2.01 ± 0.18	1.68 ± 0.11
Total N (%)[b]	0.15 ± 0.07	0.21 ± 0.02	0.19 ± 0.01
C/N ratio	12.93 ± 1.16	9.27 ± 0.46	9.00 ± 0.00
Double lactate-extractable P (mg kg^{-1})	85.4 ± 18.2	76.1 ± 29.0	269.4 ± 40.9
Double lactate-extractable K (mg kg^{-1})	47.4 ± 16.0	257.7 ± 88.1	208.3 ± 26.1
Calcium chloride-extractable magnesium Mg (mg kg^{-1})	32.2 ± 6.9	58.0 ± 6.3	56.8 ± 3.5
Soil texture (sand – silt – clay in %)[b]	95 – 0 – 5	3 – 74 – 23	7 – 56 – 37

Effective cation exchange capacity ($mmol_c\ kg^{-1}$)[b]	4.2	15.0	17.9
Base saturation (%)[b]	64.1	80.2	84.7

Table SI 2. Composition of the plant mixtures at sowing.

Field margin	Annual flower strip 1	Annual flower strip 2	Perennial flower strip 1	Perennial flower strip 2
Festuca pratensis	*Trifolium alexandrinum*	*Phacelia tanacetifolia*	*Phacelia tanacetifolia*	*Achillea millefolium*
Dactylis glomerata	*Fagopyrum esculentum*	*Centaurea cyanus*	*Centaurea cyanus*	*Agrimonia eupatoria*
Lolium perenne	*Trifolium incarnatum*	*Calendua officinalis*	*Calendua officinalis*	*Anthemis tinctoria*
Phleum pratense	*Camelina sativa*	*Papaver rhoeas*	*Papaver rhoeas*	*Anthriscus sylvestris*
	Raphanus sativus	*Anethum graveolens*	*Anethum graveolens*	*Artemisia vulg./campestris*
	Trifolium resupinatum	*Nigella sativa*	*Nigella sativa*	*Barbarea vulgaris*
	Phacelia tanacetifolia	*Carthamus tinctorius*	*Carthamus tinctorius*	*Carum carvi*
	Ornithopus sativus	*Helianthus annuus*	*Helianthus annuus*	*Centaurea scabiosa*
	Helianthus annuus	*Coriandrum sativum*	*Coriandrum sativum*	*Cerastium holosteoides*
	Sinapis alba	*Ornithopus sativus*	*Ornithopus sativus*	*Cichorium intybus*
	Meliotus albus	*Melilotus albus*	*Melilotus albus*	*Clinopodium vulgare*
		Trifolium incarnatum	*Trifolium incarnatum*	*Crepis biennis*
		Trifolium resupinatum	*Trifolium resupinatum*	*Daucus carota*
			Achillea millefolium	*Dipsacus fullonum*
			Carum carvi	*Echium vulgare*
			Cichorium intybus	*Galium album*
			Daucus carota	*Galium verum*
			Foeniculum vulgare	*Heracleum sphondylium*
			Leucanthemum vulgare	*Hypericum perforatum*
			Lotus corniculatus	*Leucanthemum ircutianum*
			Trifolium repens	*Malva moschata*
			Medicago sativa	*Malva sylvestris*
			Melilotus officinalis	*Oenothera biennis*
			Onobrychis	*Origanum vulgare*
			Plantago lanceolata	*Pastinaca sativa*
			Sanguisorba minor	*Plantago lanceolata*
			Anthemis tinctoria	*Prunella vulgaris*
			Pastinaca sativa	*Reseda luteola*
			Salvia officinalis	*Salvia pratensis*
			Trifolium hybridum	*Sanguisorba minor*
				Silene dioica
				Silene latifolia subsp. alba
				Silene vulgaris
				Lychnis flos-cuculi
				Tanacetum vulgare
				Verbascum lychnitis, nigrum, thapsus
				Anethum graveolens

	Borago officinalis
	Camelina sativa
	Carthamus tinctorius
	Coriandrum sativum
	Fagopyrum esculentum
	Foeniculum vulgare
	Guizotia abyssinica
	Helianthus annuus
	Linum usitatissimum
	Medicago lupulina
	Medicago sativa
	Petroselinum crispum
	Phacelia tanacetifolia
	Trifolium pratense

Table SI 3. Mean ± standard deviation of the relative abundance of soil bacterial phyla (*n* = 3). Different uppercase letters of the same font indicate statistically significant differences ($p < 0.05$).

relative abundance of soil bacterial phyla	Podzol soil					Luvisol soil					Cambisol soil				
	field margin	annual flower strip 1	annual flower strip 2	perennial flower strip 1	perennial flower strip 2	field margin	annual flower strip 1	annual flower strip 2	perennial flower strip 1	perennial flower strip 2	field margin	annual flower strip 1	annual flower strip 2	perennial flower strip 1	perennial flower strip 2
***Acidobacteriota* (%)**	15.3 ± 3.24 A	11.0 ± 0.40 AB	9.83 ± 1.94 B	13.1 ± 0.84^{AB}	13.5 ± 0.53 ^{A}	13.3 ± 0.01 **A**	11.6 ± 1.12 **A**	11.8 ± 1.31 **A**	12.5 ± 0.91$^{\mathbf{A}}$	12.9 ± 0.80$^{\mathbf{A}}$	14.6 ± 0.07 *A*	11.5 ± 0.67 *AB*	10.5 ± 1.67 *B*	12.6 ± 1.10^{AB}	13.5 ± 1.44^{AB}
***Desulfobacterota* (%)**	0.03 ± 0.05 A	0.02 ± 0.02 A	0.03 ± 0.02 A	0.06 ± 0.01^{A}	0.02 ± 0.02^{A}	0.14 ± 0.02 **A**	0.06 ± 0.03 **B**	0.09 ± 0.02 **AB**	0.07 ± 0.02$^{\mathbf{B}}$	0.10 ± 0.02$^{\mathbf{AB}}$	0.95 ± 0.29 *A*	0.06 ± 0.06 *B*	0.05 ± 0.05 *B*	0.12 ± 0.06^{B}	0.12 ± 0.08^{B}
***Methylomirabilota* (%)**	0.27 ± 0.14 A	0.11 ± 0.05 A	0.11 ± 0.01 A	0.16 ± 0.07^{A}	0.17 ± 0.05^{A}	0.68 ± 0.03 **A**	0.23 ± 0.11 **B**	0.25 ± 0.01 **B**	0.49 ± 0.02$^{\mathbf{A}}$	0.53 ± 0.15$^{\mathbf{A}}$	0.52 ± 0.03 *A*	0.22 ± 0.02 *BC*	0.19 ± 0.08 *C*	0.41 ± 0.05^{AB}	0.45 ± 0.15^{A}
***Latescibacterota* (%)**	0.20 ± 0.16 A	0.07 ± 0.02 A	0.06 ± 0.03 A	0.15 ± 0.15^{A}	0.14 ± 0.06^{A}	0.73 ± 0.10 **A**	0.22 ± 0.06 **C**	0.25 ± 0.02 **C**	0.41 ± 0.003$^{\mathbf{B}}$	0.40 ± 0.07$^{\mathbf{B}}$	0.46 ± 0.08 *A*	0.14 ± 0.04 *C*	0.16 ± 0.05 *BC*	0.36 ± 0.10^{AB} *C*	0.38 ± 0.15^{AB}

***NB1-j* (%)**	0.003 ± 0.006 A	0.002 ± 0.003 A	0.000 ± 0.000 A	0.005 ± 0.009 A	0.002 ± 0.003 A	0.089 ± 0.023 A	0.014 ± 0.013 B	0.021 ± 0.010 B	0.049 ± 0.003 B	0.054 ± 0.024 B	0.059 ± 0.015 A	0.019 ± 0.003 A	0.014 ± 0.013 A	0.033 ± 0.013 A	0.052 ± 0.039 A
***Planctomycetota* (%)**	7.81 ± 2.16 A	7.17 ± 0.78 A	6.47 ± 1.75 A	8.08 ± 1.86 A	8.69 ± 1.75 A	13.4 ± 1.21 A	9.85 ± 0.78 C	10.7 ± 0.61 BC	11.7 ± 0.81 C	12.0 ± 0.16 AB	13.4 ± 0.89 A	9.73 ± 0.28 B	10.2 ± 1.05 B	12.7 ± 1.40 A	13.9 ± 0.21 A
***Abditibacteriota* (%)**	0.15 ± 0.03 B	0.31 ± 0.03 A	0.26 ± 0.02 AB	0.15 ± 0.08 B	0.18 ± 0.04 B	0.03 ± 0.01 C	0.11 ± 0.02 AB	0.12 ± 0.05 A	0.01 ± 0.01 C	0.03 ± 0.03 BC	0.02 ± 0.02 A	0.13 ± 0.06 A	0.09 ± 0.03 A	0.06 ± 0.09 A	0.02 ± 0.01 A
***Actinobacteriota* (%)**	33.9 ± 13.1 A	34.4 ± 4.78 A	39.2 ± 10.9 A	32.1 ± 5.45 A	30.2 ± 5.70 A	23.8 ± 0.90 A	28.3 ± 3.97 A	28.3 ± 3.89 A	30.1 ± 4.41 A	27.5 ± 1.57 A	24.1 ± 0.68 B	29.7 ± 1.54 A	29.7 ± 2.35 A	27.5 ± 1.68 AB	24.8 ± 2.72 AB
***Gemmatimonadota* (%)**	2.12 ± 0.29 A	3.05 ± 0.50 A	2.65 ± 0.54 A	2.46 ± 0.57 A	2.38 ± 0.58 A	1.13 ± 0.14 C	1.59 ± 0.16 AB	1.68 ± 0.31 A	1.18 ± 0.09 BC	1.15 ± 0.11 BC	1.52 ± 0.21 A	1.55 ± 0.13 A	1.44 ± 0.22 A	1.41 ± 0.43 A	1.25 ± 0.11 A
***Bdellovibrionota* (%)**	0.37 ± 0.08 A	0.46 ± 0.10 A	0.46 ± 0.15 A	0.50 ± 0.09 A	0.49 ± 0.03 A	0.34 ± 0.07 B	0.68 ± 0.04 A	0.62 ± 0.06 A	0.41 ± 0.05 B	0.39 ± 0.03 B	0.12 ± 0.02 B	0.33 ± 0.02 A	0.31 ± 0.08 A	0.19 ± 0.02 B	0.18 ± 0.01 A
***Proteobacteria* (%)**	13.9 ± 2.35 A	16.6 ± 1.25 A	15.6 ± 2.65 A	15.4 ± 1.20 A	16.0 ± 1.94 A	16.2 ± 0.28 A	16.8 ± 0.71 A	17.3 ± 0.24 A	16.5 ± 0.79 A	16.6 ± 0.68 A	14.4 ± 0.66 B	18.4 ± 0.64 A	19.1 ± 3.06 A	16.1 ± 0.88 AB	15.9 ± 1.16 AB
***Bacteroidota* (%)**	2.83 ± 0.35 B	5.30 ± 0.83 A	5.34 ± 1.48 A	4.00 ± 0.40 AB	4.27 ± 0.49 AB	3.95 ± 0.39 C	5.49 ± 0.20 AB	5.75 ± 0.51 A	4.02 ± 0.34 C	4.24 ± 0.73 BC	3.32 ± 0.34 C	6.07 ± 0.35 AB	7.61 ± 1.87 A	5.19 ± 0.86 AB	4.91 ± 0.15 BC

A2 Tree-Distance and Tree-Species Effects on Soil Biota in a Temperate Agroforestry System

Figure S1. Allyl isothiocyanate (AITC) extraction of earthworms in the field. Application of 5 L of 0.01 % (w/v) AITC solution to a quarter square meter of soil framed by an open metal frame (**A**). Surfacing earthworms within the open metal frame following AITC application (**B**). Photo credit: Anna Vaupel

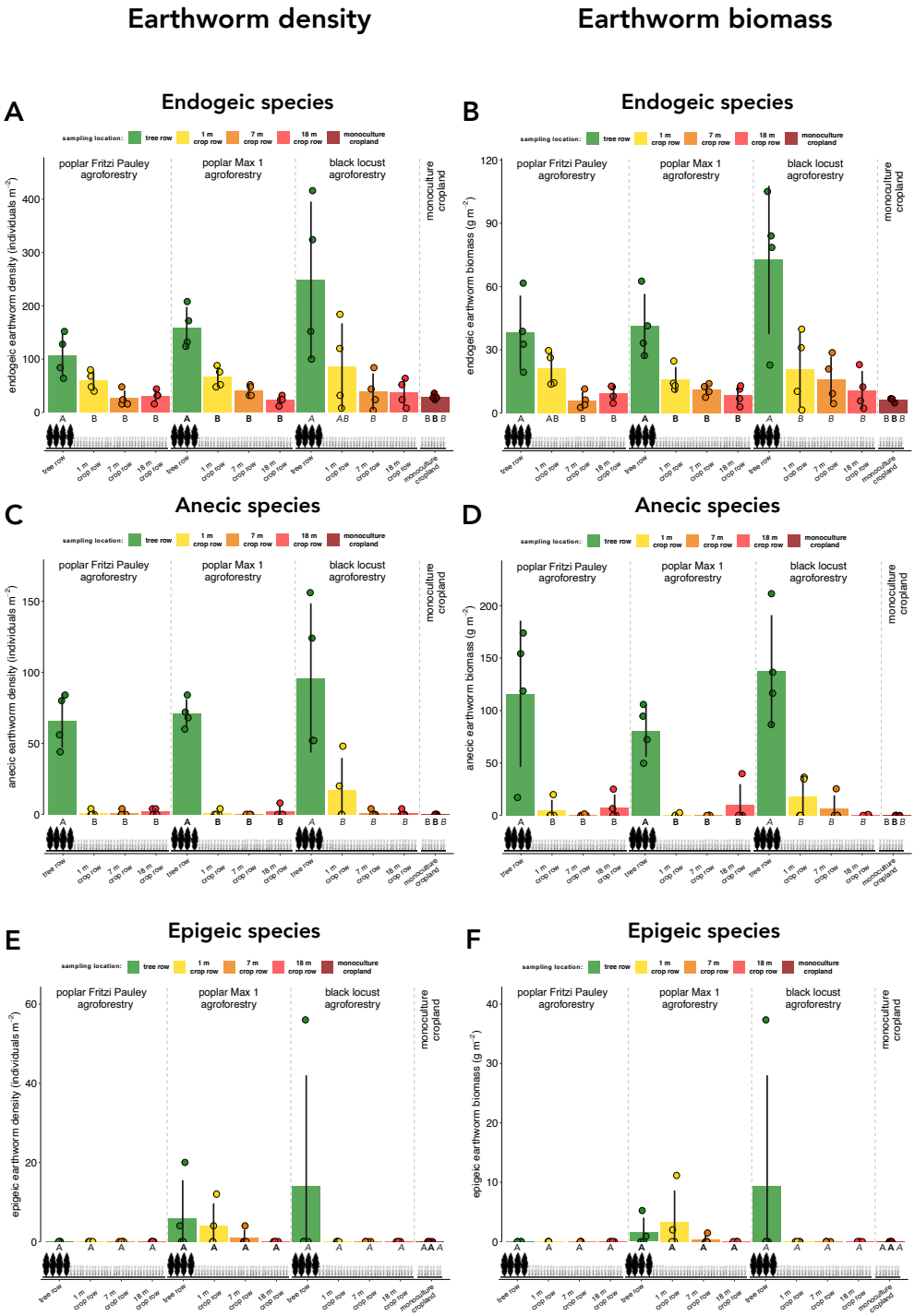

Figure S2. Density and biomass of endogeic (A, B), anecic (C, D), and epigeic (E, F) earthworms in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation (n = 4). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences ($p < 0.05$)

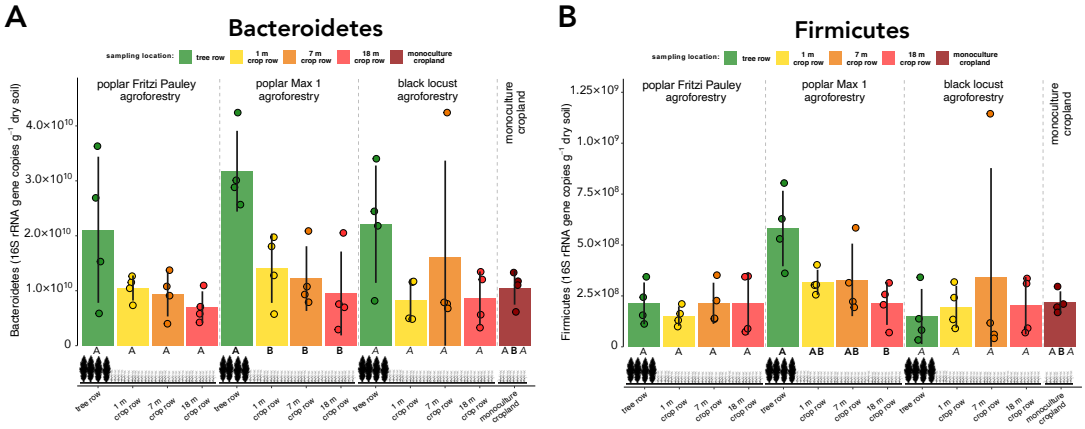

Figure S3. 16S rRNA gene abundance of Bacteroidetes (A) and Firmicutes (B) in soil of a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation (n = 4). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences (p < 0.05)

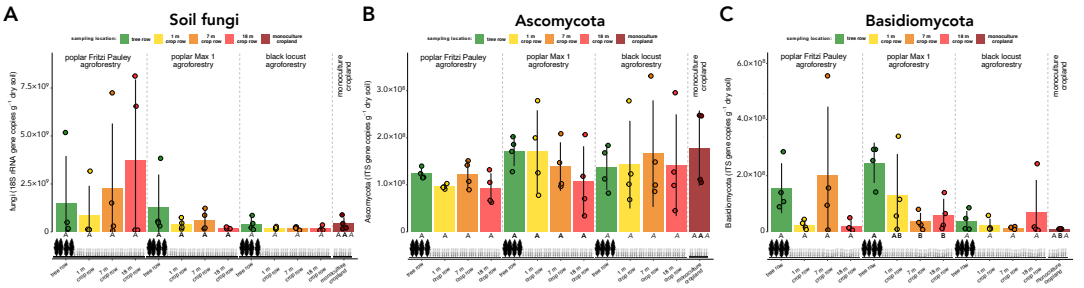

Figure S4. 18S rRNA gene abundance of total fungi (A) and ITS gene abundance of Ascomycota (B), and Basidiomycota (C) in soil of a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation (n = 4). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences (p < 0.05)

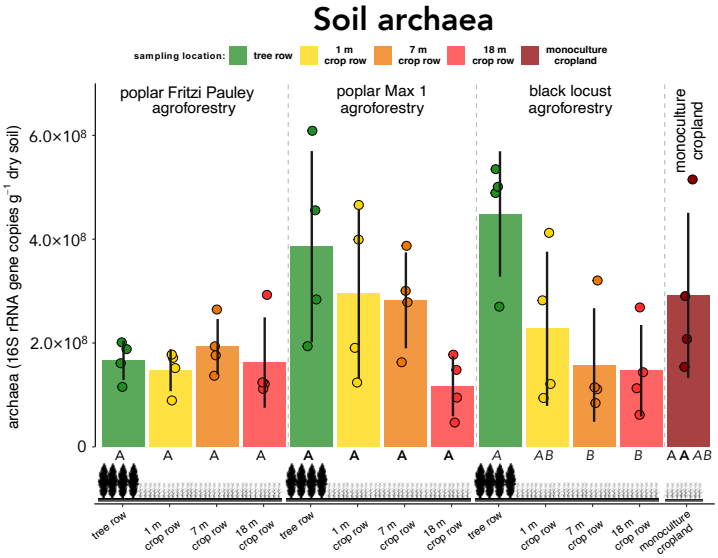

Figure S5. 16S rRNA gene abundance of total archaea in soil of a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation (n = 4). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences (p < 0.05)

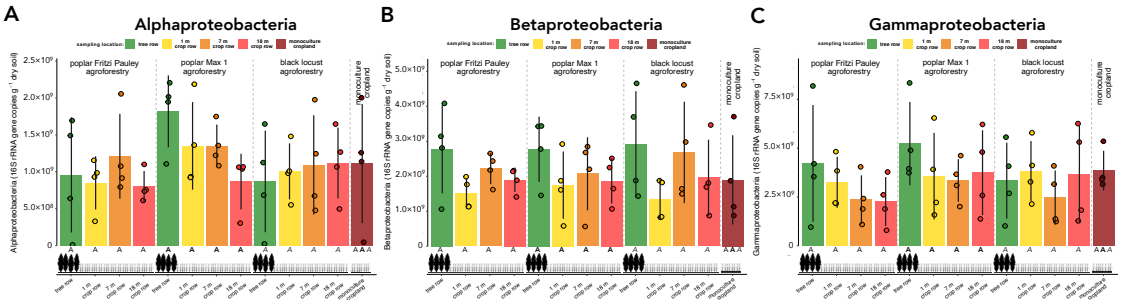

Figure S6. 16S rRNA gene abundance of Alpha- (A), Beta- (B), and Gammaproteobacteria (C) in soil of a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row. Bars with error bars represent the mean and its standard deviation (n = 4). Dots represent individual data points. Different uppercase letters of the same font indicate statistically significant differences (p < 0.05)

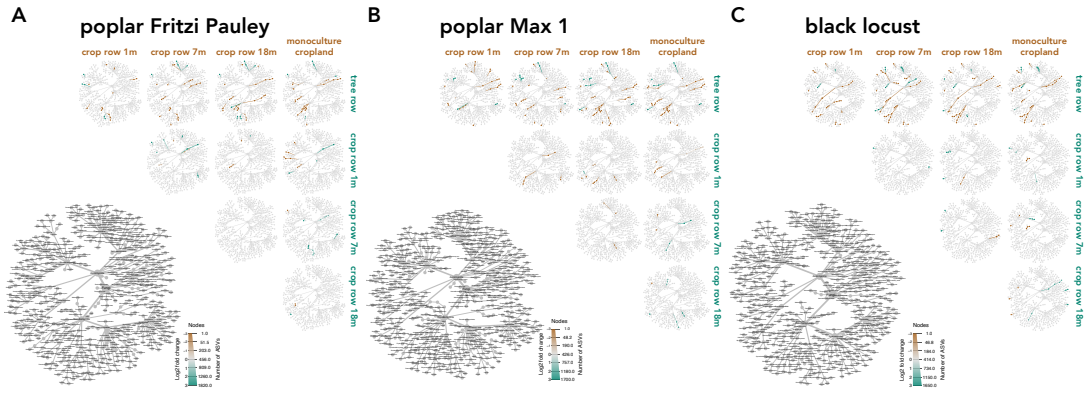

Figure S7. Heat trees visualizing results of differential abundance analysis of soil fungal groups in a paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley (**A**), poplar Max 1 (**B**), and black locust (**C**) are different tree species within the agroforestry system. Within the agroforestry system, soil samples were collected in the tree row as well as at 1 m, 7 m, and 18 m distance from the tree row within the crop row (n = 4). Heat trees were collapsed at genus level. For each panel, a key containing taxonomic labels is provided in the lower left. Within the pairwise heat trees on the upper right, turquoise and brown nodes represent statistically significant (p < 0.05) differences between two sampling locations. Turquoise and brown nodes indicate greater abundance of a taxonomic group in the sampling locations listed in rows and columns, respectively. Different node sizes indicate the number of amplicon sequence variants (ASVs) detected per taxonomic group

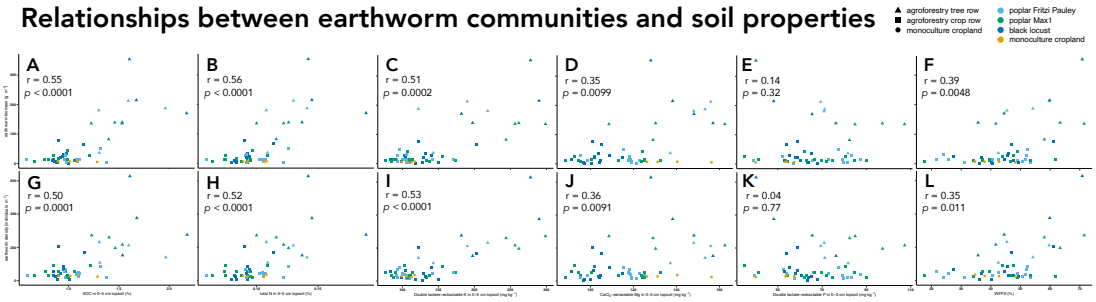

Figure S8. Relationships between earthworm biomass (A-F), earthworm density (G-L) and soil properties in soil of paired temperate alley-cropping agroforestry and monoculture cropland system in Germany. Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, soil samples were collected in the tree row, at three different distances from the tree row within the crop row as well as in a monoculture cropland (n = 4). Relationships were investigated with Spearman's rank correlation test (r = Spearman´s rank correlation coefficient).

Different colors represent different tree species, whereas different shapes represent different sampling locations.

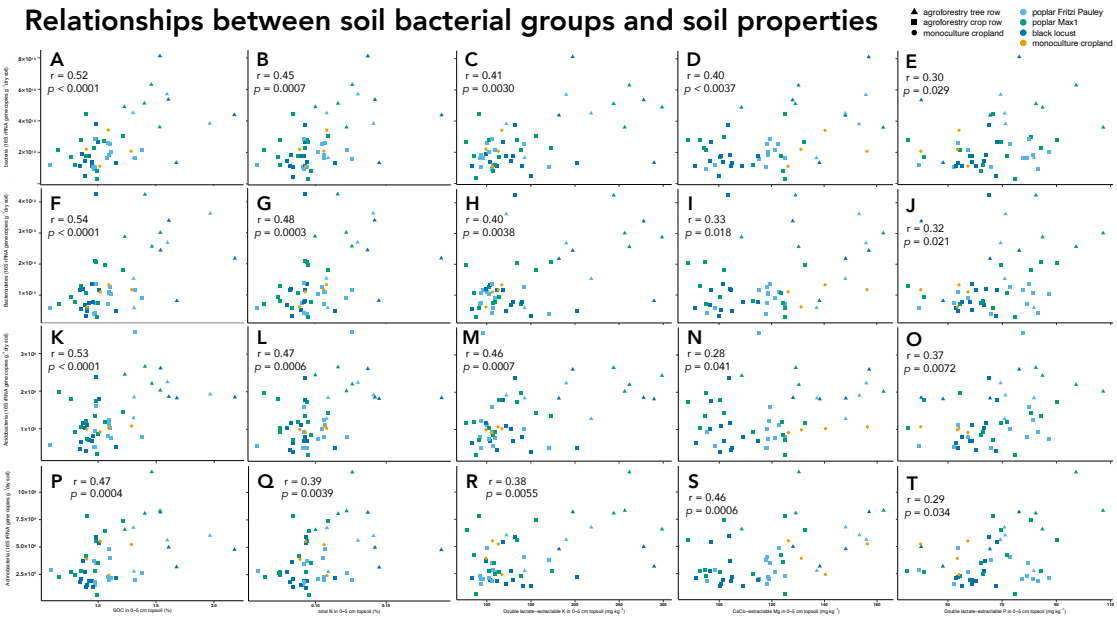

Figure S9. **Relationships between soil bacteria (A-E), Bacteroidetes (F-J), Acidobacteria (K-O), and Actinobacteria (P-T) and soil properties in soil of paired temperate alley-cropping agroforestry and monoculture cropland system in Germany.** Poplar Fritzi Pauley, poplar Max 1, and black locust are different tree species within the agroforestry system. Within the agroforestry system, topsoil samples were collected in the tree row, at three different distances from the tree row within the crop row as well as in a monoculture cropland (n = 4). Relationships were investigated with Spearman's rank correlation test (r = Spearman´s rank correlation coefficient). Different colors represent different tree species, whereas different shapes represent different sampling locations

A3 Comparison of two earthworm extraction methods in different arable soils

Table S1: Study site locations and soil characteristics. Soil properties were determined from four ([a]or eight) biological replicates per site, except for soil texture and CECeff, which were determined from composite samples.

Study Site	Location	Soil pH	Soil texture (sand – silt – clay in %)	C/N ratio	Soil organic matter (%)	CEC_{eff} (cmol kg^{-1})
Alt Madlitz[a]	52°22'25.0" (N), 14°16'53.5" (E)	6.0 ± 0.1	68 – 26 – 6	10.27 ± 0.87	1.7 ± 0.2	4.14
Deggendorf	48°47'21.8" (N), 12°28'57.2 (E)	6.1 ± 0.1	7 – 69 – 24	9.09 ± 0.16	5.6 ± 0.7	14.50
Euskirchen	50°38'39.8" (N), 6°51'17.2" (E)	6.2 ± 0.1	15 – 68 – 16	9.03 ± 0.14	4.5 ± 0.2	12.89
Landau a. d. Isar	48°44'6.4" (N), 12°28'41.7" (E)	6.4 ± 0.1	9 – 63 – 28	8.86 ± 0.16	4.7 ± 0.2	16.03
Mechernich-Wachendorf	50°38'11.4" (N), 6°46'14.6" (E)	6.4 ± 0.0	20 – 58 – 22	8.98 ± 0.32	5.1 ± 0.3	15.27
Otterndorf	53°47'50.0" (N), 8°53'12.6" (E)	6.7 ± 0.0	33 – 57 – 10	9.67 ± 0.20	3.4 ± 0.2	11.50
Prenzlau[a]	53°16'50.7" (N), 13°48'2.8" (E)	6.7 ± 0.1	61 – 29 – 10	10.04 ± 0.50	3.4 ± 0.4	8.34
Puchhausen	48°44'59.4 (N), 12°29'51.3 (E)	6.6 ± 0.0	15 – 56 – 30	9.88 ± 0.48	6.1 ± 0.2	19.54
Rommerskirchen-Vanikum	51°1'48.1" (N) 6°38'44.8" (E)	6.1 ± 0.1	2 – 80 – 18	10.85 ± 0.49	4.0 ± 0.1	13.18
Straubing	48°51'37.8 (N), 12°36'16.4" (E)	6.3 ± 0.1	5 – 72 – 23	8.62 ± 0.23	4.2 ± 0.3	13.66
Wülfrath	51°16'11.7" (N), 6°59'18.4" (E)	6.3 ± 0.1	2 – 81 – 17	9.61 ± 0.27	4.1 ± 0.3	10.43

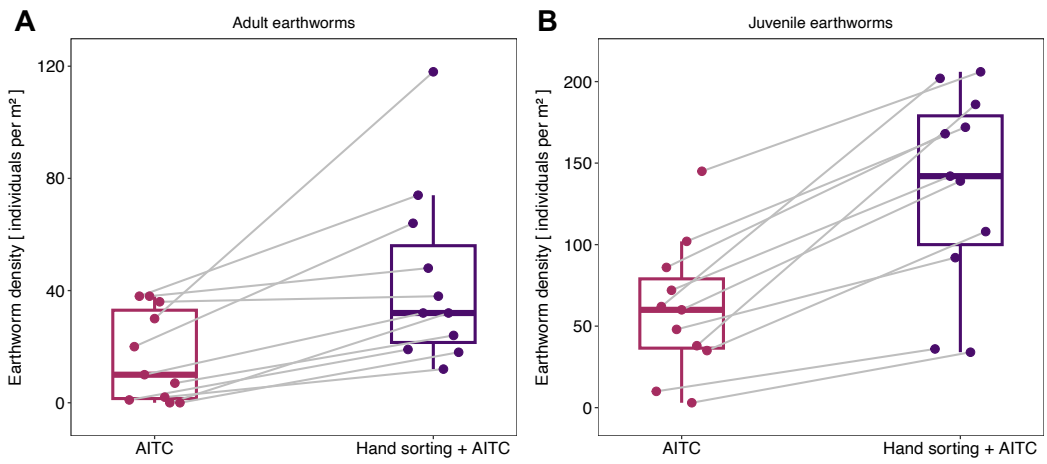

Figure S1. Earthworm population density of different age groups. Population density of adult (**A**) and juvenile (**B**) earthworms. Individual points represent the mean (4 or 8 replicates per site) of each study site (n = 11). Grey lines connect data points sampled at the same site with different methods (i.e., AITC extraction and the combination of hand sorting + AITC extraction).

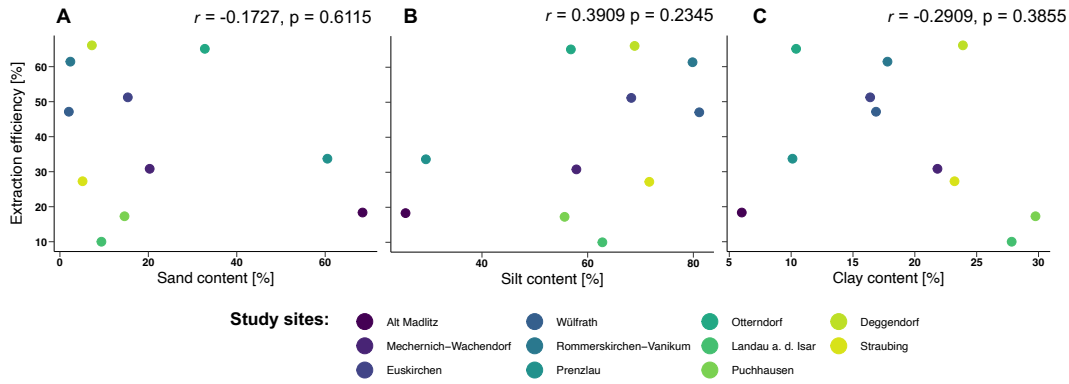

Figure S2. Correlation between soil texture and extraction efficiency. Spearman's rank correlation (*r* = Spearman's rank correlation coefficient, p = p-value) between sand (**A**), silt (**B**) and clay (**C**) content and total earthworm extraction efficiency. Individual points represent the mean (4 or 8 replicates per site) of an individual site (n=11).

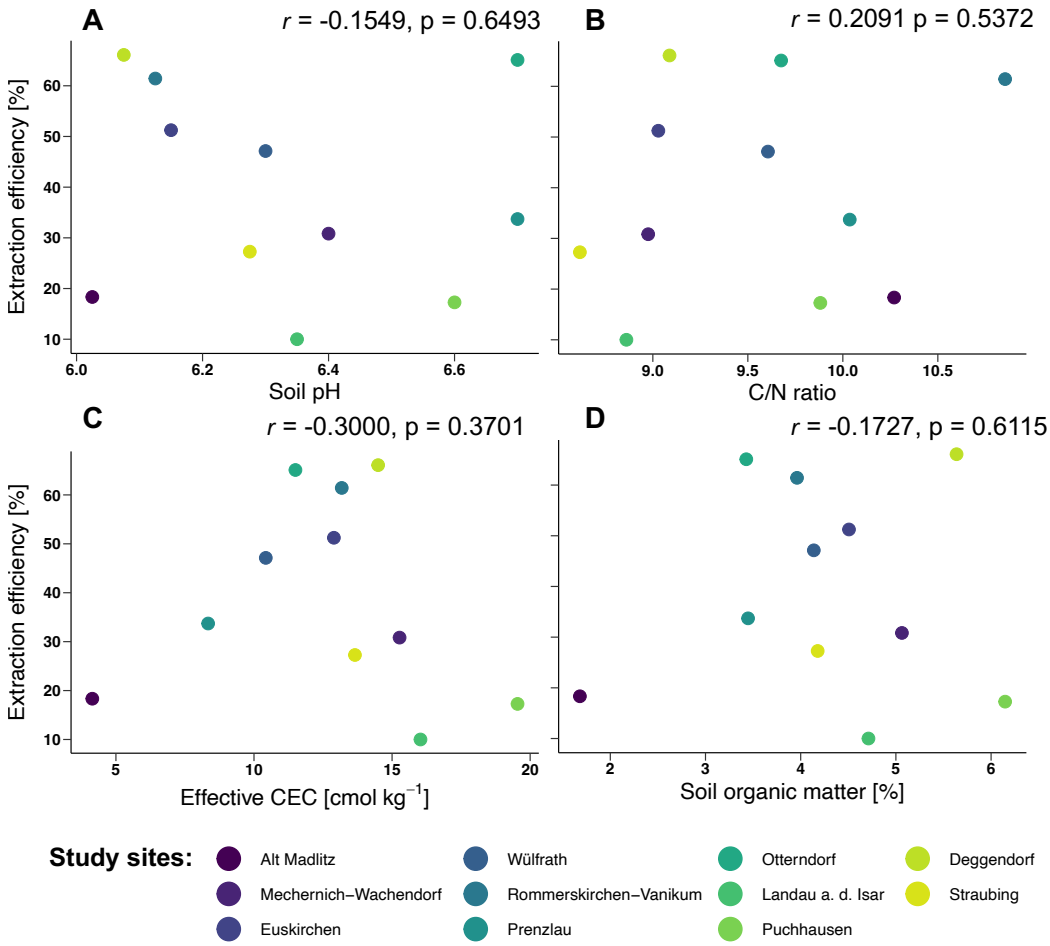

Figure S3. Correlation between soil properties and extraction efficiency. Spearman's rank correlation (*r* = Spearman's rank correlation coefficient, p = p-value) between soil pH (**A**), C/N ratio (**B**), CEC_{eff} (**C**), and SOM (**D**) and total earthworm extraction efficiency. Individual data points represent the mean (4 or 8 replicates per site) of each study site (n=11).

A4 High-resolution melting (HRM) curve analysis as a potential tool for the identification of earthworm species and haplotypes

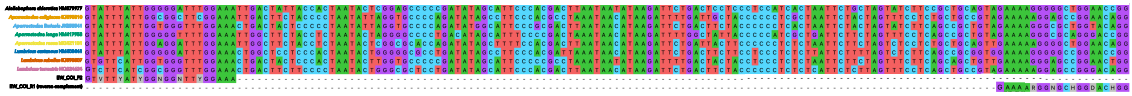

Figure S1. Sequence alignment of the newly developed COI primer pair EW_COI_F2 and EW_COI_R1 used for identification of eight earthworm species by high-resolution melting curve analysis Reference sequences were obtained from NCBI's GenBank (accession numbers are given in the figure) and aligned in MEGA version 11.0.10 (Kumar, Stecher & Tamura, 2016) using ClustalW (Thompson, Higgins & Gibson, 1994). For the reverse primer EW_COI_R1, the reverse complement is shown. DOI: https://doi.org/10.7717/peerj.13661/supp-1

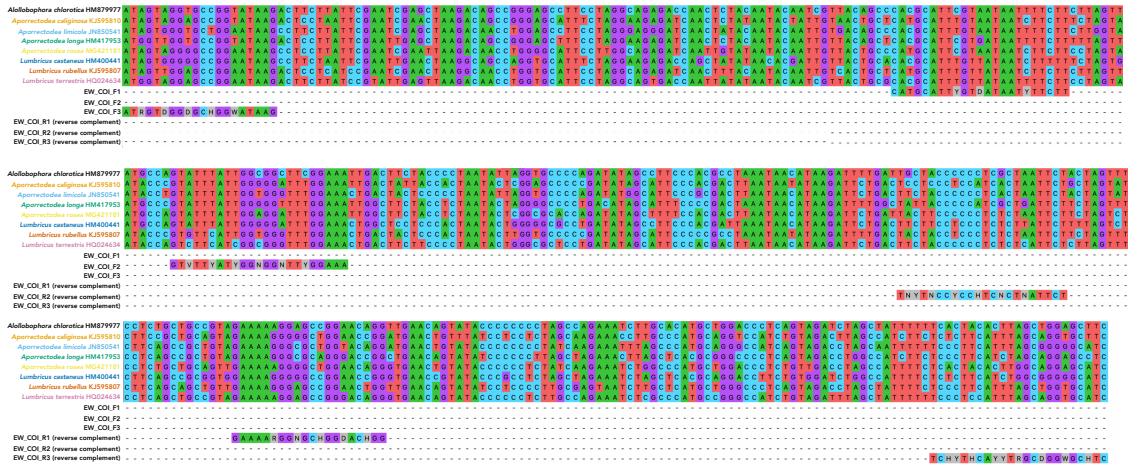

Figure S2. Sequence alignment of the three newly developed COI primer pairs used for identification of eight earthworm species by high-resolution melting curve analysis Reference sequences were obtained from NCBI's GenBank (accession numbers are given in the figure) and aligned in MEGA version 11.0.10 (Kumar, Stecher & Tamura, 2016) using ClustalW (Thompson, Higgins & Gibson, 1994). For reverse primers, the reverse complements are shown. DOI: https://doi.org/10.7717/peerj.13661/supp-2

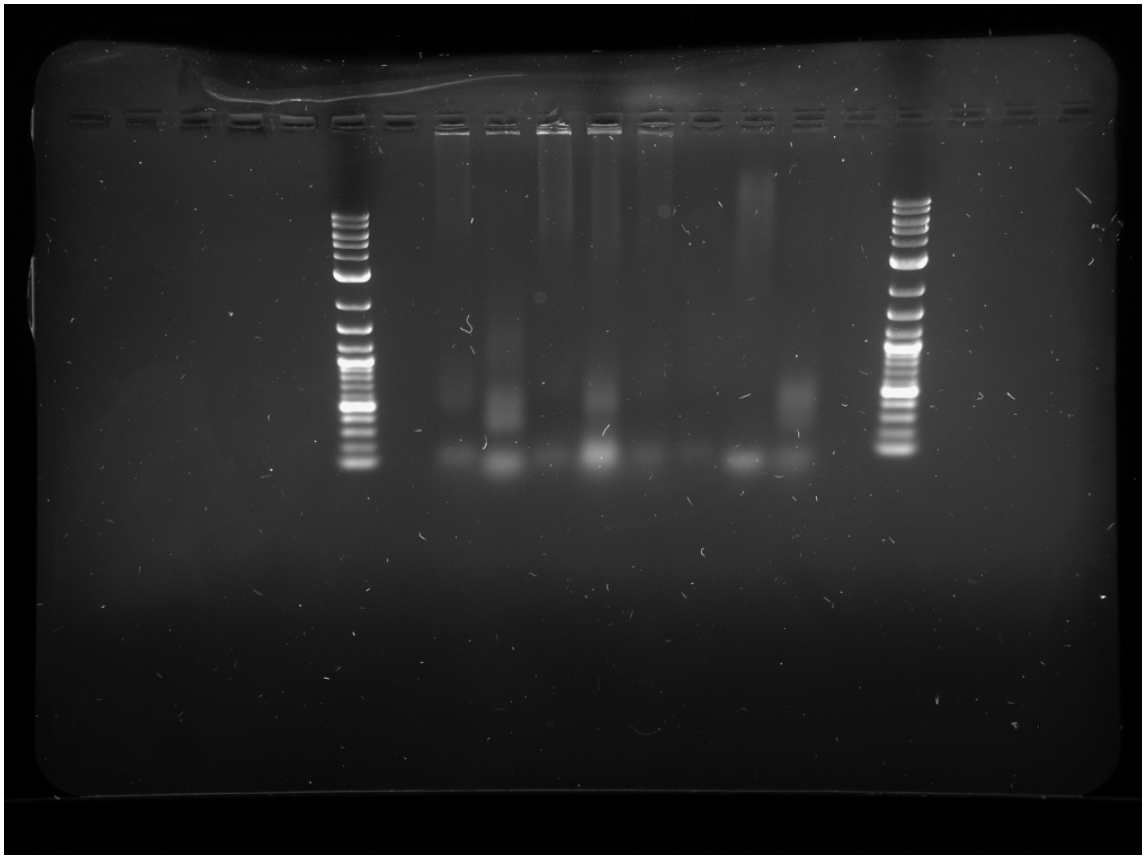

Figure S3. Agarose gel (1% (w/v)) showing earthworm DNA extracts obtained by using the developed rapid and inexpensive extraction protocol Lanes 8 to 15: 3 µL of DNA extract of Allolobophora chlorotica, Aporrectodea caliginosa, Apo. limicola, Apo. longa, Apo. rosea, Lumbricus castaneus, L. rubellus, and L. terrestris, respectively. Lanes 6 and 17: 1 µL of 1 kb Plus DNA Ladder (New England Biolabs, Beverly, Massachusetts, USA). The agarose gel was stained using PicoGreen (Thermo Fisher Scientific, Waltham, MA, USA) and run at 4.6 V/cm for 60 min. DOI: https://doi.org/10.7717/peerj.13661/supp-3

Supplementary file 4. Raw fluorescence and negative derivative of fluorescence data DOI: https://doi.org/10.7717/peerj.13661/supp-4