



Digging deeper: microbial communities in subsoil are strongly promoted by trees in temperate agroforestry systems

Lukas Beule · Victor Guerra · Ena Lehtsaar · Anna Vaupel

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Abstract

Aims Temperate alley-cropping agroforestry systems maintain agricultural production while offering several environmental benefits. Central benefits of agroforestry systems such as the ‘safety-net’-role of the trees for leached nutrients are mainly due to processes occurring below the soil surface: the subsoil. Microorganisms in the subsoil may play a key role in the ‘safety-net’-function as they can improve the capturing and uptake of nutrients by the trees. Systematic investigations of microbial communities in temperate

agroforestry systems, however, are restricted to topsoil.

Methods We quantified bacteria, fungi, and functional groups of microorganisms in the topsoil and subsoil of two alley-cropping systems using real-time PCR. Topsoil and subsoil samples were collected in the tree rows and at multiple distances from the trees within the crop rows of the agroforestry systems as well as at an adjacent monoculture cropland.

Results Microbial population size decreased with soil depth likely due to limited resource availability in subsoil. Tree rows in agroforestry systems not only promote soil microbial populations in both the topsoil and subsoil but the promotion also extends gradually into the crop rows of the systems. The promotion of microorganisms through trees is stronger in subsoil than topsoil, pointing at more intense resource scarcity in the subsoil than topsoil.

Conclusions We propose that tree root-derived resources and root litter, which are scarce in agricultural subsoils, triggered the strong positive response of the subsoil community to the trees. Finally, we provide initial evidence that subsoil microorganisms contribute to the ‘safety-net’-role of the trees in agroforestry systems.

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Keywords Temperate agroforestry · Alley-cropping systems · Soil depth · Topsoil · Subsoil · Real-time PCR (qPCR) · Soil bacteria · Soil fungi · Nitrogen-cycling genes

Introduction

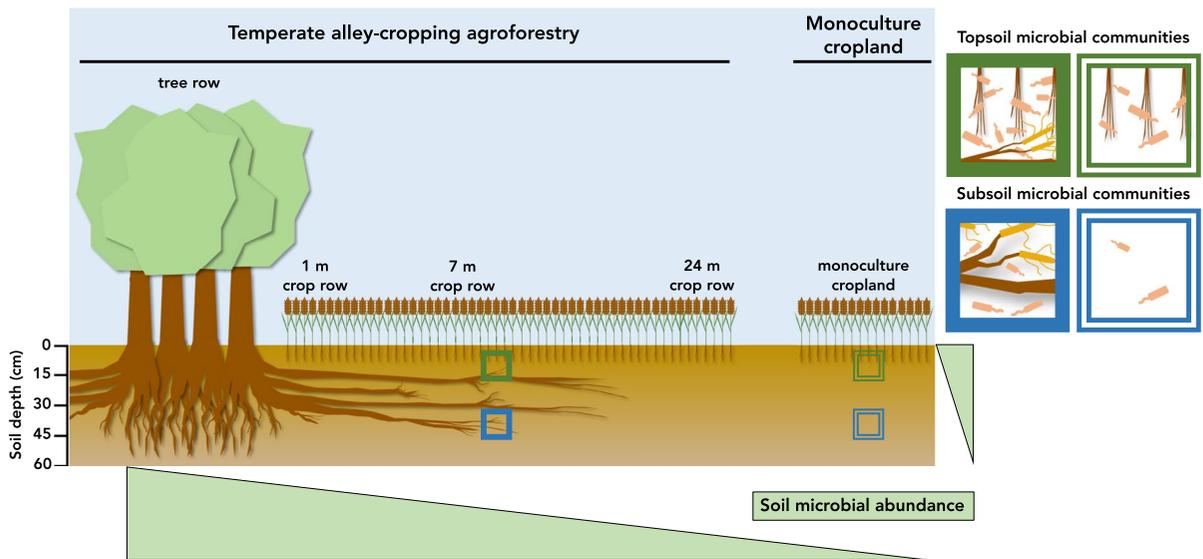
Temperate alley-cropping systems are agroforestry systems that alternate rows of trees with rows of crops and have multiple environmental benefits over monoculture croplands (e.g. Tsonkova et al. 2012). For example, deep-rooting trees in these systems can take up nutrients leached below the rooting zone of the crops and, thus, act as ‘safety-nets’ for leached nutrients (Rowe et al. 1999; Allen et al. 2004). Incorporation of recovered nutrients in the biomass of the trees and their release through tree litter has recently been described as ‘nutrient pumping’ (Isaac and Borden 2019) and can contribute to increased soil fertility close to the trees (Pardon et al. 2017). Furthermore, temperate alley-cropping systems promote soil biota such as communities of earthworms (e.g. Cardinael et al. 2019), springtails (e.g. Boinot et al. 2019), and microorganisms (e.g. Beule et al. 2020).

Soil microorganisms contribute to key soil functions such as nutrient cycling and soil health. For example, microorganisms involved in soil-N cycling enzymatically regulate nitrification and denitrification and thereby leaching (e.g., nitrate) and gaseous (e.g., nitrous oxide (N₂O)) N losses. In a recent review article, we found that soil microbial abundance, diversity, and functionality increase through temperate alley-cropping agroforestry and likely yield enhanced biological soil fertility (Beule et al. 2022). All of the studies reviewed, however, were limited to the upper 30 cm topsoil. In agricultural systems, topsoil is defined as the soil above the present or historical tillage depth (usually 20 to 30 cm) while subsoil is the soil below this depth. Although a recent study investigated microorganisms at varying soil depths of up to 40 cm depth (Wang et al. 2022), the authors ignored the spatial heterogeneity within agroforestry systems (Guillot et al. 2021) by randomly setting their plots within the studied systems. Although subsoil makes up the majority of agricultural soil, microorganisms in subsoils are highly understudied as compared to those in topsoils (e.g. Naylor et al. 2022), especially in temperate agroforestry systems. Furthermore, certain benefits of agroforestry systems, such as the ‘safety-net’-role of the trees, occur in the subsoil rather than topsoil. In this context, we recently hypothesized that tree root-associated microorganisms in subsoil, such as

mycorrhizae, help trees to capture and uptake nutrients more effectively and thereby contribute to their ‘safety-net’-role (Beule et al. 2022).

Microbial community composition is frequently reported to differ between topsoil and subsoil (Fierer et al. 2003b; Zhang et al. 2017; Sosa-Hernández et al. 2018a), which is not surprising considering that soil properties vary with depth. Furthermore, microbial population size usually declines with depth as overall resource availability decreases (Lavahun et al. 1996; Taylor et al. 2002; Kramer et al. 2013). Interestingly, certain enzyme activities do not decrease but increase with depth (Kramer et al. 2013). Recently, it was shown that although the active microbial biomass decreases with depth, subsoil communities can respond rapidly to added resources (Min et al. 2021), again underscoring resource scarcity in subsoils. Sampling of subsoil is demanding and requires special equipment. Additionally, the recovery of sufficient quantities of nucleic acids from subsoil poses a major hurdle for molecular investigations of microbial communities in subsoil. Besides low microbial population size in subsoil, high clay content in subsoils can hamper DNA recovery as nucleic acids can strongly bind to clay particles (Guerra et al. 2020). Despite all these challenges, the characterisation of subsoil microbial communities in agricultural systems is crucial to understand how these organisms contribute to soil functioning such as nutrient cycling.

This study aimed to recover microbial DNA from topsoil and subsoil of two temperate poplar-based alley-cropping systems and quantify soil bacteria and fungi as well as different functional groups of microorganisms (nitrifying, denitrifying, methanotrophic, and methanogenic microorganisms) at different soil depths using real-time PCR. Our study design included sampling at multiple soil depths (0–15, 15–30, 30–45, and 45–60 cm) in the tree rows as well as multiple distances to the tree rows within the crop rows of the agroforestry systems and at an adjacent reference land use (monoculture cropland). We hypothesized that i) microbial abundance decreases with soil depth, ii) tree rows promote microbial communities in both the topsoil and subsoil, and iii) the promotion of microorganisms through the tree rows is stronger in the subsoil than topsoil. We illustrated these hypotheses in Fig. 1.



Hypotheses:

- i) Microbial abundance decreases with soil depth
- ii) Tree rows promote microbial communities in both the topsoil and subsoil
- iii) The promotion of microorganisms through the tree rows is stronger in the subsoil than topsoil

Fig. 1 Schematic illustration of the hypotheses

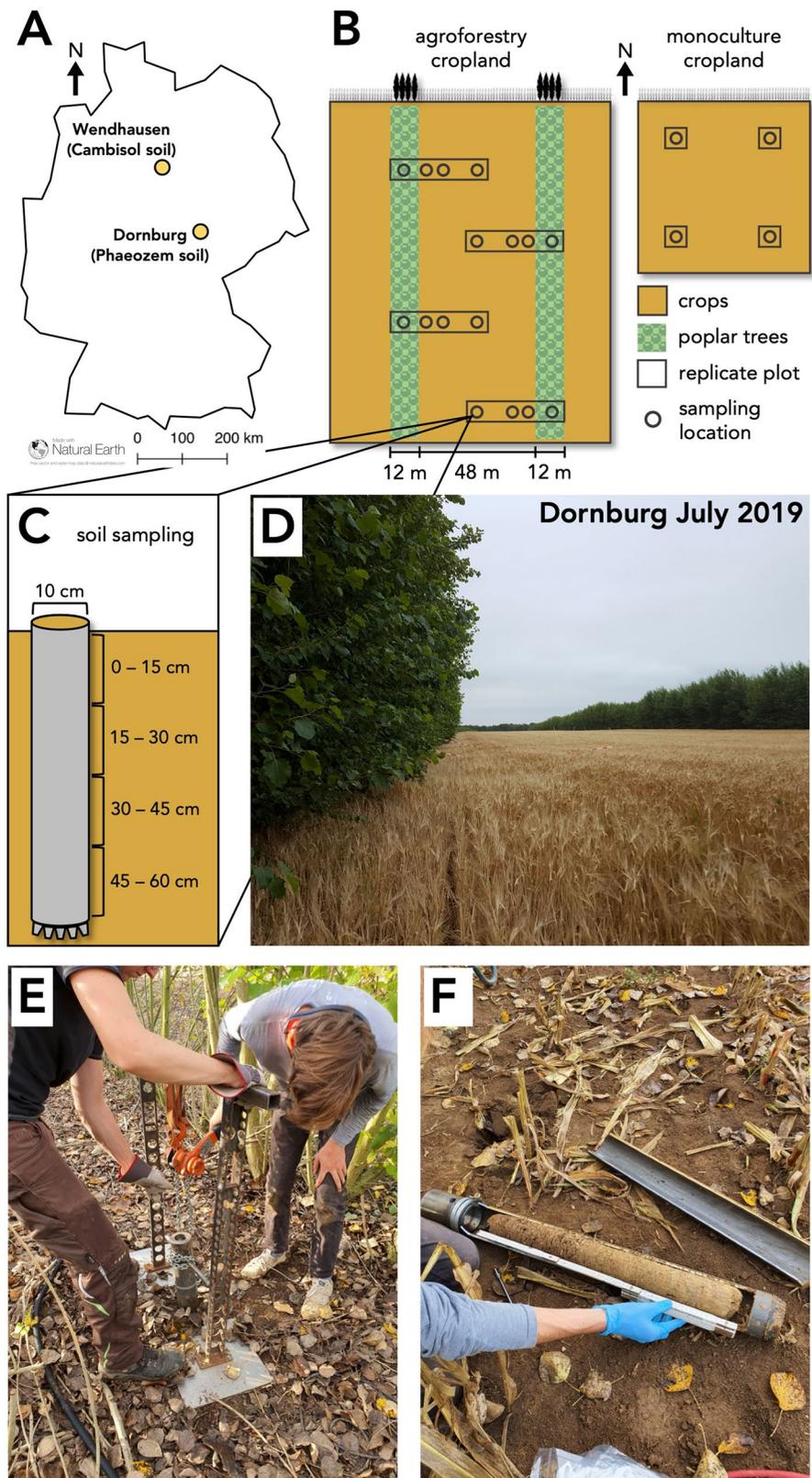
Materials and Methods

Study sites and sampling design

Our study was conducted in two conventionally managed temperate alley-cropping agroforestry systems with adjacent conventional monoculture cropland systems as a reference land use. The study sites were located in a Vertic Cambisol soil near Wendhausen, Lower Saxony, Germany (52°20′00″N, 10°37′55″E, 82 m above mean sea level, mean annual air temperature: 9.6 ± 0.2 °C, mean annual precipitation: 637 ± 23 mm) and in a Calcaric Phaeozem soil near Dornburg, Thuringia, Germany (51°00′40″N, 11°38′46″E, 289 m above mean sea level, mean annual air temperature: 9.9 ± 0.1 °C, mean annual precipitation: 608 ± 21 mm) (Fig. 2a). Hereafter, we refer to the study sites by their soil types (i.e., Cambisol and Phaeozem). General biochemical and physical properties of the two systems have been reported previously by Schmidt et al. (2021). The agroforestry system in the Cambisol was established 2008; the system in the Phaeozem in 2007. At both sites, the agroforestry system was established by converting

conventional monoculture cropland to an alley-cropping system through planting of 12-m wide rows of poplar trees (clone Max 1; *Populus nigra* × *P. maximowiczii*). The tree rows were planted from poplar cuttings using a dibble bar. To minimize shading of the neighbouring crops, tree rows were planted in North-South orientation. The above-ground biomass of the trees in the Cambisol was first harvested in January 2014 and in January 2015 in the Phaeozem. The tree rows were alternated with 48-m wide rows of crops that were managed identically to their corresponding monoculture cropland (i.e., identical crops, fertilization and soil management regime, and plant protection product application) (Fig. 2b). The tree rows did not receive fertilizer or plant protection products and were not tilled. The crop rotation in the Cambisol was winter oilseed rape—winter wheat—winter wheat—maize (2016—2017—2018—2019) and summer barley—winter oilseed rape—winter wheat—summer barley (2016—2017—2018—2019) and in the Phaeozem. In the year of sampling (2019), fertilization rates were 101—0—0 kg N—P—K ha⁻¹ in the Cambisol and 36—22—31 kg N—P—K ha⁻¹ in the Phaeozem.

Fig. 2 Study sites and study design of paired temperate alley-cropping agroforestry and monoculture cropland systems. Study sites were located near Wendhausen (Germany) in a Vertic Cambisol and Dornburg (Germany) in a Calcaric Phaeozem (a). Soil samples within the agroforestry systems were collected along transects that spanned from the centre of the tree row to the centre of the crop row (b). Samples were collected in the centre of the agroforestry tree rows as well as at 1 m, 7 m, and 24 m distance from the tree rows within the agroforestry crop rows. In the adjacent monoculture cropland, soil samples were collected in the centre of each replicate plot. At each sampling location (agroforestry tree row, 1 m, 7 m, 24 m agroforestry crop row, and monoculture cropland), soil samples were collected in topsoil (0–15 and 15–30 cm soil depth) and subsoil (30–45 and 45–60 cm soil depth) (c). Photos of the agroforestry site near Dornburg (d), the double-gear hand cable puller used to pull out the steel soil sampling cylinder (e), and an in-tact soil core retrieved using the steel soil sampling cylinder (f). Photos were provided by V. Guerra (d) and E. Lehtsaar (e, f)



Soil sampling and DNA extraction

Soil samples were collected after harvest and prior to tillage in July 2019 in the Calcaric Phaeozem and in October 2019 in the Vertic Cambisol. To cover the spatial heterogeneity within the agroforestry systems, soil samples were collected along four transects per site (Fig. 2b). Within each transect, soil samples were collected within the tree row as well as at 1 m, 7 m, and 24 m distance from the tree rows within the crop rows (Fig. 2b). In the reference land use (monoculture cropland), four soil samples were collected (Fig. 2b). At each sampling location (agroforestry tree row, 1 m, 7 m, 24 m distance from the tree row within the crop row, and monoculture cropland), a 60-cm soil core with 10 cm diameter was obtained using a steel soil sampling cylinder with a hardened steel cutting head (Fig. 2c, f). The cylinder was driven into the soil by an electric caulking hammer (Makita HM1400, Makita, Fischamend, Austria) and pulled out using a double-gear hand cable puller (Hebezone R5007, Hebezone GmbH, Hanau, Germany) (Fig. 2e). The outer few millimetres (approximately 5 mm) of the soil cores were carefully removed to avoid the transfer of topsoil into subsoil material during the sampling procedure. Soil cores were divided into topsoil (0–15 and 15–30 cm soil depth) and subsoil (30–45 and 45–60 cm soil depth) and soil of each depth was thoroughly homogenized in a sterile polyethylene bag. From the homogenized sample, approximately 50 g of fresh soil was transferred into a sterile 50-mL Falcon tube (SARSTEDT, Nümbrecht, Germany) and frozen at $-20\text{ }^{\circ}\text{C}$ in the field. Upon arrival at the laboratory, samples were freeze-dried for 72 h and finely ground using a swing mill (Retsch MM400, Retsch, Haan, Germany) at 25 Hz for 1 min.

DNA was extracted from 200 mg ground soil using an in-house subsoil DNA extraction protocol as described by Guerra and co-workers (2020). Briefly, 200 mg ground soil were suspended in 250 μL 1 M phosphate buffer and incubated at $65\text{ }^{\circ}\text{C}$ for 10 min with frequent inversion. Following incubation, the suspension was centrifuged, 90 μL of the supernatant was diluted 1:10 (v/v) in double distilled H_2O (dd H_2O) and 900 μL phenol was added. The mixture was shaken and centrifuged for 10 min. After centrifugation, 800 μL of the supernatant were extracted twice with chloroform/isoamyl alcohol (24:1 (v/v)) and precipitated using polyethylene glycol 6000 and

NaCl. Precipitated DNA was pelleted using centrifugation and obtained pellets were dried using vacuum centrifugation. Dried pellets were re-suspended in 50 μL 1 \times TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and incubated at $42\text{ }^{\circ}\text{C}$ for 2 h to facilitate resuspension. Quantity and quality of extracted DNA was assessed on 0.8% (w/v) agarose gels. Gel electrophoresis was carried out at 4.6 V cm^{-1} for 60 min. DNA extracts were tested for PCR inhibitors using a DNA amplification inhibition test as described previously (Guerra et al. 2020).

Real-time PCR assays

We quantified total bacteria, total fungi, Asco- and Basidiomycota, six functional genes involved in N cycling (ammonia-oxidizing archaea (AOA) *amoA* and ammonia-oxidizing bacteria (AOB) *amoA* for the oxidation of ammonia, *nirK* and *nirS* for the reduction of nitrite, and *nosZ* clade I and II the reduction of N_2O) as well as two genes involved in CH_4 cycling (*pmoA* for methanotrophic and *mcrA* for methanogenic microorganisms) in all soil samples using real-time PCR (qPCR). Amplifications were carried out in a CFX384 Thermocycler (Bio-Rad, Rüdigenheim, Germany) in 384-well microplates. Primer, master-mix composition, and thermocycling conditions for the quantification of bacteria, fungi, Ascomycota, and Basidiomycota correspond to those described by Beule et al. (2020). N-cycling genes (AOA *amoA*, AOB *amoA*, *nirK*, *nirS*, *nosZ* clade I and II) were amplified as described by Beule et al. (2019). Genes involved in CH_4 cycling (*pmoA* and *mcrA*) were amplified in 4 μL reaction volume consisting of dd H_2O , reaction buffer (10 mM Tris-HCl, 50 mM KCL, 1.5 mM MgCl_2 , pH 8.3), 100 μM of each deoxyribonucleoside triphosphate (Bioline, Luckenwalde, Germany), 0.5 μM of each primer (A189f (Holmes et al. 1995)/mb661 (Costello and Lidstrom 1999) for *pmoA*; mlas-mod-F (Angel et al. 2011)/mcrA-rev (Steinberg and Regan 2008) for *mcrA*), 1 mg ml^{-1} bovine serum albumin, 0.1 \times SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 0.03 μL^{-1} Hot Start *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts, USA), and 1 μL template DNA solution or dd H_2O for negative controls. The thermocycling conditions for *pmoA* and *mcrA* were as follows: initial denaturation ($95\text{ }^{\circ}\text{C}$ for 2 min), 40 cycles of denaturation ($95\text{ }^{\circ}\text{C}$ for 20 sec), annealing

(57 °C for 30 sec) and elongation (68 °C for 45 sec), and final elongation (68 °C for 5 min). For all qPCR assays, melting curves were obtained by heating the samples to 95 °C for 60 sec and cooling to 55 °C for 60 sec followed by a step-wise temperature increase from 55 °C to 95 °C by 0.5 °C per step with continuous fluorescence measurement.

Soil properties

Soil pH was determined by suspending freeze-dried soil in deionized H₂O (1:4 (w/v)). Carbonates were removed prior to the determination of soil organic C (SOC) and total N using acid fumigation (Harris et al. 2001). Following acid fumigation, samples were dried at 60 °C and finely ground using a swing mill (Retsch MM400, Retsch, Haan, Germany) at 25 Hz for 1 min. SOC and total N were determined using a multi N/C 2100S Analyzer (Analytik Jena, Jena, Germany). All values are reported in Table S1.

Statistical analysis

All data were checked for normal distribution of the residuals using Shapiro-Wilk test and for homoscedasticity using Levene's test. In addition to these tests, data were manually inspected for normal distribution of the residuals and homoscedasticity. Differences in microbial abundance among sampling locations (i.e., tree row, 1 m, 7 m, 24 m crop row, and monoculture cropland) within each soil depth as well as among soil depths within each sampling location were tested by performing one-way ANOVA with Tukey's HSD on logarithmically transformed data. Within each soil depth, relative change of microbial population size in response to temperate alley-cropping agroforestry was calculated as follows.

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

where *a* is one of the sampling locations (tree row, 1 m, 7 m, 24 m crop row or monoculture cropland) within each transect of the agroforestry system or the monoculture cropland and *b* is the mean of the monoculture cropland per site. Differences in soil properties among sampling locations within each soil depth as well as among soil depths within each sampling location per site were tested by using one-way ANOVA with Tukey's HSD test or Kruskal–Wallis

test with Dunn's test. Relationships between soil properties (i.e., soil pH, SOC, total N, and C/N ratio) and microbial population sizes (i.e., bacteria, fungi, and functional groups of microorganisms) were analysed using Spearman's rank correlation test. For all tests, statistical significance was considered at $p \leq 0.05$. All statistical analysis was performed in R version 4.0.4 (R Core Team 2017). All data generated in this study were deposited at the Bonares repository (<https://doi.org/10.20387/bonares-sqrj-4643>).

Results

Microbial population size, SOC, and total N decrease with soil depth

The abundance of all successfully quantified taxonomical (bacteria, fungi, Ascomycota, and Basidiomycota) and functional groups of microorganisms (nitrifier, denitrifier, and methanotrophs) decreased with soil depth (Fig. 3, SI 1 B, D, SI 2 B, D, SI 3, B, D, see Table S2 for statistical analysis). Similarly, SOC and total N decreased with soil depth (Table S1). In both soils, soil pH increased with increased soil depth (Table S1) due to the calcareous parent material. Within each soil and soil depth, either no or only minor differences in soil pH, SOC, and total N were found among sampling locations (i.e., agroforestry tree row, 1 m, 7 m, 24 m agroforestry crop row, and monoculture cropland) within each soil type (Table S1).

Populations of soil bacteria and fungi

Although soil bacterial abundance within each soil type did not differ among the different sampling locations within each depth, we observed a noticeable trend of decreasing mean bacterial abundance with increasing distance from the trees in the subsoil (i.e., 30–45 and 45–60 cm soil depth) in the Cambisol soil (Fig. 4a, b). Furthermore, in the Cambisol soil, the relative change of mean bacterial abundance through the conversion of monoculture cropland to agroforestry was greater at 30–45 than at 45–60 cm soil depth (Fig. 4a, b).

Overall, tree rows promoted soil fungal abundance in both soil types. In the Cambisol soil, fungal population size in the subsoil of the tree row

was greater than at 7 and 24 m into the crop row and monoculture cropland ($p \leq 0.0048$) (Fig. 4c). Fungal abundance gradually increased with decreasing distance from the trees and was 12.3 and 4.5 times greater in the tree row than in the monoculture cropland at 30–45 and 45–60 cm soil depth, respectively (Fig. 4c). In the topsoil at 0–15 cm soil depth, fungal abundance was greater in the tree row than at 7 and 24 m into the crop row ($p \leq 0.0355$) (Fig. 4c). In the Phaeozem soil, fungal abundance at 15–30 cm soil depth increased with decreasing distance to the trees and was greater in the tree row as compared to 7 and 24 m into the crop row and monoculture cropland ($p \leq 0.0108$) (Fig. 4d). In the subsoil (i.e., below 30 cm soil depth), the tree row harboured greater fungal population size than the monoculture cropland ($p \leq 0.0134$) (Fig. 4d). Compared to the monoculture cropland, the tree row had 1.1, 3.6, and 2.0 times greater fungal abundance at 15–30, 30–45, and 45–60 cm soil depth, respectively (Fig. 4d).

The ratio of fungi to bacteria decreased with soil depth (Fig. SI 4 B, D). In the subsoil (i.e., below 30 cm soil depth) of the Cambisol soil, the fungi-to-bacteria ratio was greater in the tree row and at 1 m into the crop row than at 24 m into the crop row and in the monoculture cropland ($p \leq 0.0253$) (Fig. SI 4 A). Compared to the monoculture cropland, the fungi-to-bacteria ratio in the subsoil at the tree row and at 1 m into the crop row were 3.0 to 9.5 and 1.4 to 5.6 times greater, respectively (Fig. SI 4 A). In the upper 15-cm topsoil of the Cambisol soil, the ratio of fungi to bacteria was greater under the trees than at 7 and 24 m into the crop row ($p \leq 0.0309$) (Fig. SI 4 A). In the Phaeozem soil at 15–30 cm soil depth, the fungi-to-bacteria ratio was lower in the monoculture cropland than in the tree row and at 1 m into the crop row ($p \leq 0.0310$) (Fig. SI 4 C).

Abundance of Ascomycota was not altered through the introduction of trees rows in arable land (Fig. 5a, b). Among all taxonomical and functional groups of microorganisms, Basidiomycota showed the strongest increase in response to the tree rows. At all soil depths in the Cambisol soil, abundance of Basidiomycota was greater in the tree row than at 7 and 24 m into the crop row and monoculture cropland ($p \leq 0.0050$) (Fig. 5c). In the Cambisol soil, Basidiomycota in the tree row were 30.7 to 48.5 and 231.9 to 198.6 times more abundant than in the monoculture cropland in the topsoil

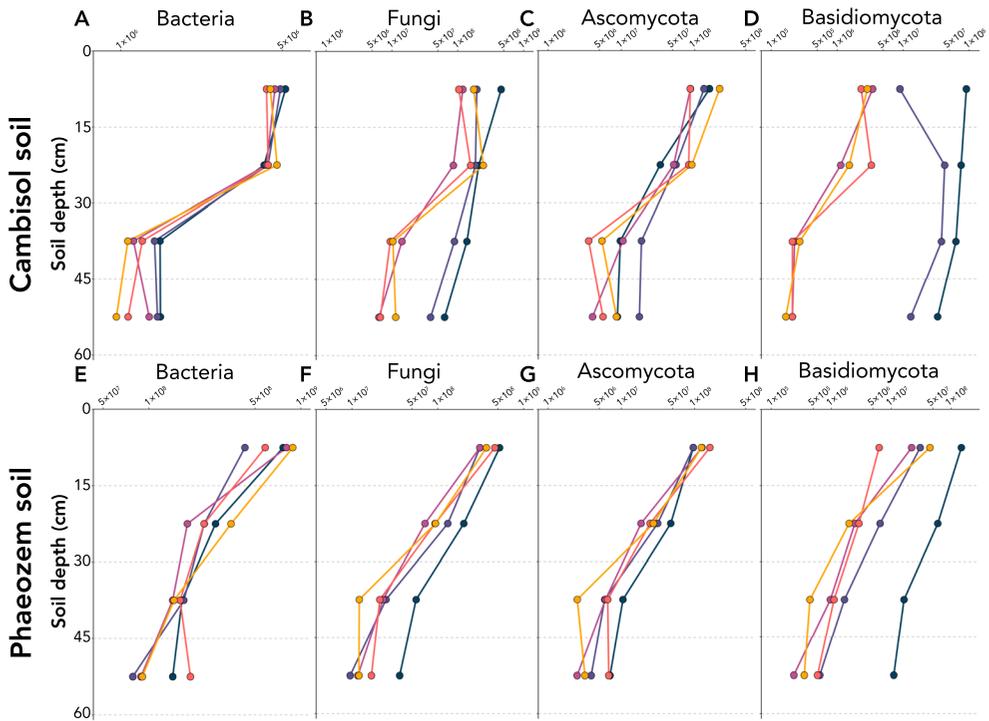
and subsoil, respectively (Fig. 5c). Similarly, the tree row in the Phaeozem increased the population size of Basidiomycota as compared to 7 and 24 m into the crop row and monoculture cropland at the three lowest soil depths (i.e., 15–30, 30–45, and 45–60 cm soil depth) ($p \leq 0.0097$) (Fig. 5d). In the same soil type, the population size of Basidiomycota in the tree row was 29.1, 36.0, and 30.1 times greater than in the monoculture cropland at 15–30, 30–45, and 45–60 cm soil depth, respectively (Fig. 5d).

Functional groups of soil microorganisms

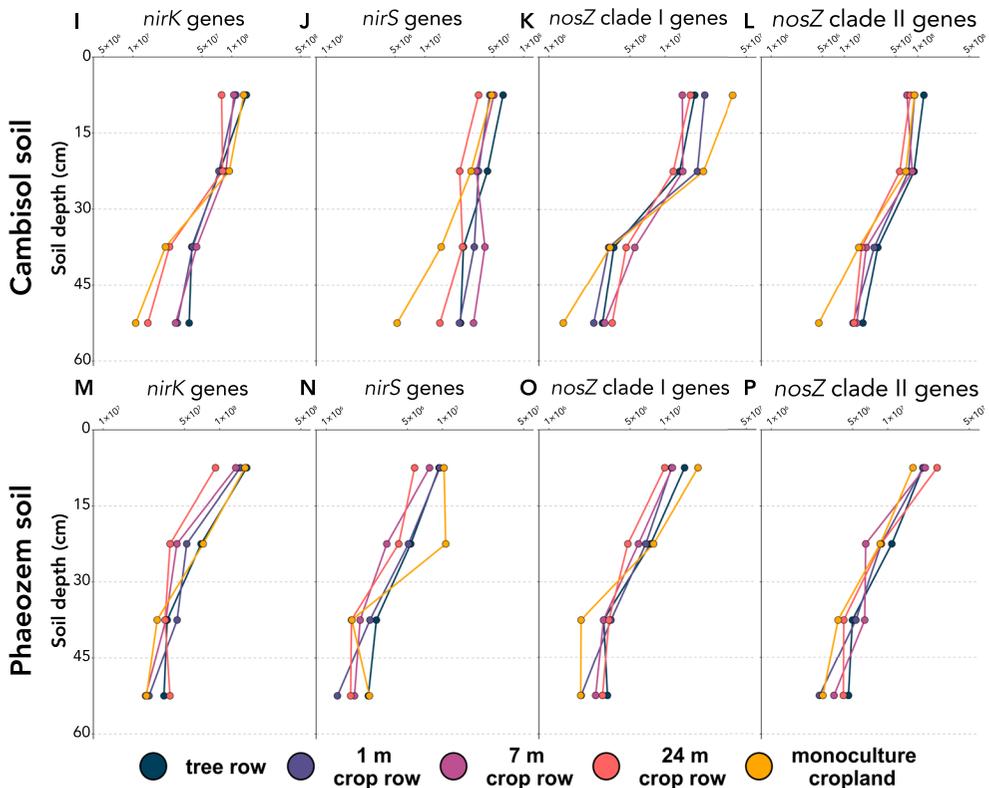
While methanogenic microorganisms were never detected above the limit of quantification, methanotrophic microorganisms were detected in all samples. Although the mean abundance of methanotrophic microorganisms in the subsoil of the Cambisol soil decreased gradually with increasing distance from the trees, only the tree row and 1 m into the crop row at 45–60 cm depth differed from the monoculture cropland ($p \leq 0.0082$) (Fig. SI 1 A, B). No differences among sampling locations within each depth were found in the Phaeozem soil (Fig. SI 1 C, D). Likewise, in the same soil type, no differences in AOA abundance among sampling locations within each depth were found (Fig. SI 2 C, D). In contrast, in the subsoil (i.e., below 30 cm soil depth) of the Cambisol soil, all sampling locations within the agroforestry system (i.e., tree row, 1 m, 7 m, and 24 m into the crop row) harboured 4.2 to 8.7 times more AOA than the monoculture cropland ($p \leq 0.0017$) (Fig. SI 2 A, B). While AOB were always detected in the topsoil, they were only occasionally detected in the subsoil. In the topsoil of the Cambisol soil, AOB abundance did not differ among sampling locations (Fig. SI 3 A, B). In the Phaeozem, the mean population size of AOB was consistently lower at the different sampling locations within the agroforestry system as compared to the monoculture cropland (Fig. SI 3 C, D).

In both soil types, the population size of *nirK*-type denitrifiers did not differ among sampling location within each depth (Fig. 6a, b); however, in the subsoil (i.e., below 30 cm depth) of the Cambisol soil, we observed that the mean *nirK* gene abundance decreased with increasing distance from the tree row (Fig. 6a). Abundances of *nirS*-type denitrifiers at 45–60 cm soil depth in the Cambisol soil were greater in the tree row, at 1, and 7 m distance into the crop row than in the monoculture cropland (p

Taxonomical groups



Denitrification genes



● tree row ● 1 m crop row ● 7 m crop row ● 24 m crop row ● monoculture cropland

◀**Fig. 3** Soil microbial abundance decreases with soil depth. Dots represent mean gene copy numbers g^{-1} dry soil of bacteria (a, e), fungi (b, f), Ascomycota (c, g), Basidiomycota (d, h), and denitrification genes (*nirK* (i, m), *nirS* (j, n), *nosZ* clade I (k, o) and II (l, p)) ($n = 4$). Soil samples were collected in topsoil (0–15 cm and 15–30 cm soil depth) and subsoil (30–45 cm and 45–60 cm soil depth) of two paired agroforestry and monoculture cropland systems (in a Cambisol (a, b, c, d, i, j, k, l) and Phaeozem soil (e, f, g, h, m, n, o, p)) in Germany. Tree row, 1 m, 7 m, and 24 m crop row are sampling locations within the agroforestry systems. Taxonomical groups and denitrification genes were quantified by using real-time PCR (see *Real-time PCR assays* for details)

≤ 0.0337) (Fig. 6c). Although not statistically significant, mean *nirS* gene abundance in the Phaeozem soil at 30–45 cm soil depth decreased gradually with increasing distance from the trees (Fig. 6d). In the same soil

type at 15–30 cm soil depth, *nirS*-type denitrifiers were less abundant at 7 and 24 m into the crop row as compared to the monoculture cropland ($p \leq 0.0194$) (Fig. 6d). Population sizes of *nosZ* clade I and II-denitrifiers were not affected by the conversion of monoculture cropland to agroforestry (Fig. SI 5); however, greater mean *nosZ* clade I and II gene abundances in the Cambisol at 45–60 cm soil depth were observed in the agroforestry system than in the monoculture cropland (Fig. SI 5 A, C).

Relationships between soil microorganisms and soil properties

Population sizes of all microorganisms were positively related to SOC and total N ($r = 0.41$ to 0.90 ; $p \leq 0.0001$) (Fig. SI 6). Bacteria, Ascomycota,

Fig. 4 Relative change of the abundance of soil bacteria (a, b) and fungi (c, d) in response to temperate alley-cropping agroforestry. Soil samples were collected in topsoil (0–15 cm and 15–30 cm soil depth) and subsoil (30–45 cm and 45–60 cm soil depth) of two paired agroforestry and monoculture cropland systems (in a Cambisol (a, c) and Phaeozem soil (b, d)) in Germany. Tree row, 1 m, 7 m, and 24 m crop row are sampling locations within the agroforestry systems. Relative change within each soil depth was calculated as $[(\text{sampling location} - \text{mean(monoculture cropland)}) / \text{mean(monoculture cropland)}]$ (see *Statistical analysis* for details). Dots with vertical bars represent means and standard error ($n = 4$), respectively. Bacteria and fungi were quantified by using real-time PCR for bacterial 16S rRNA and fungal 18S rRNA genes, respectively (see *Real-time PCR assays* for details)

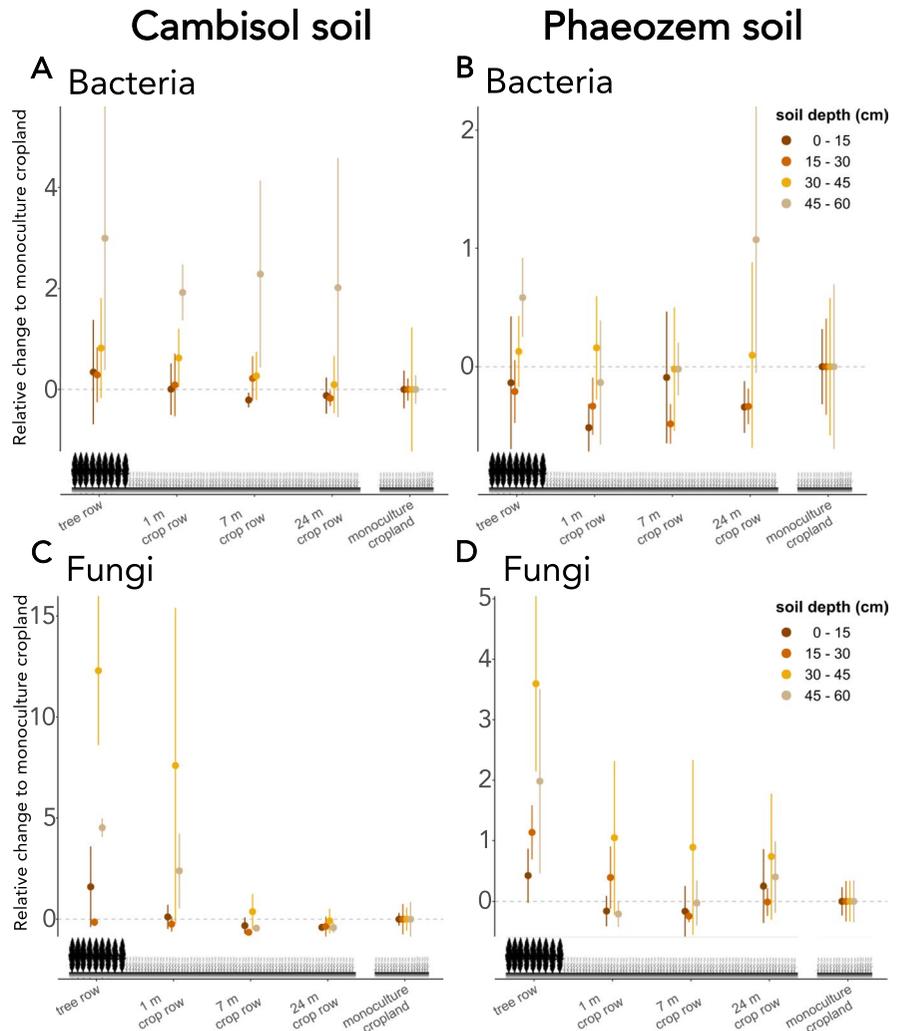
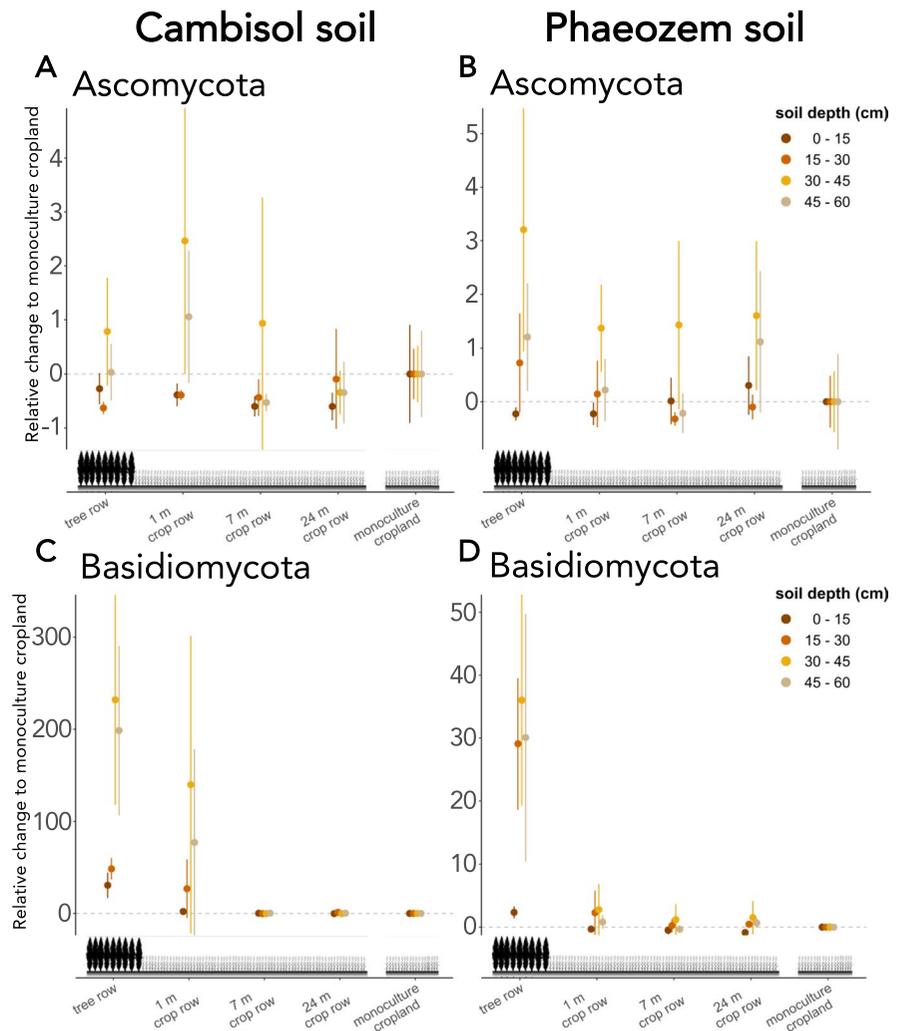


Fig. 5 Relative change of the abundance of Ascomycota (a, b) and Basidiomycota (c, d) in soil in response to temperate alley-cropping agroforestry. Soil samples were collected in topsoil (0–15 cm and 15–30 cm soil depth) and subsoil (30–45 cm and 45–60 cm soil depth) of two paired agroforestry and monoculture cropland systems (in a Cambisol (a, c) and Phaeozem soil (b, d)) in Germany. Tree row, 1 m, 7 m, and 24 m crop row are sampling locations within the agroforestry systems. Relative change within each soil depth was calculated as $[(\text{sampling location} - \text{mean}(\text{monoculture cropland})) / \text{mean}(\text{monoculture cropland})]$ (see *Statistical analysis* for details). Dots with vertical bars represent means and standard error ($n = 4$), respectively. Ascomycota and Basidiomycota were quantified by using real-time PCR for ITS genes (see *Real-time PCR assays* for details)



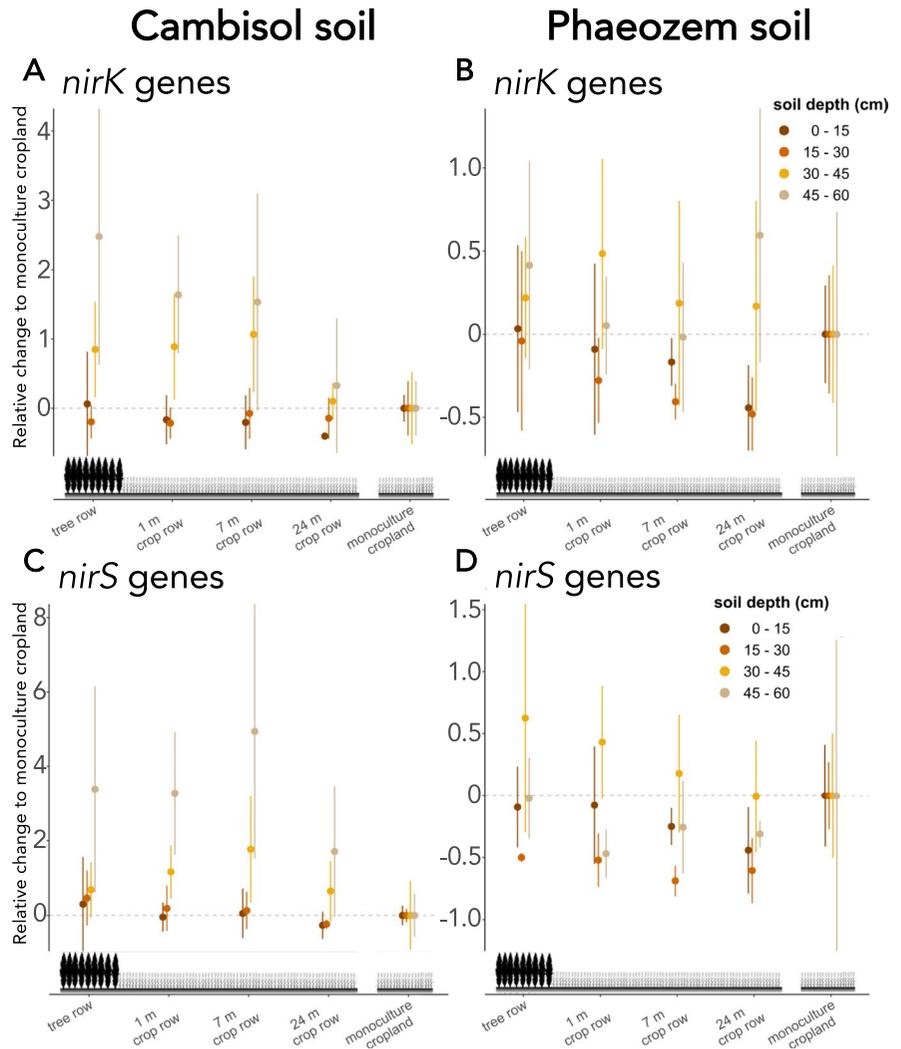
methanotrophs, nitrifiers, and all denitrifiers except *nirK*-carrying microorganisms were positively correlated with the C/N ratio ($r = 0.19$ to 0.54 ; $p \leq 0.014$) (Fig. SI 6). All fungal groups (i.e., total fungi, Ascomycota, Basidiomycota) as well as *nirK*- and *nosZ* clade I-type denitrifiers were negatively associated with soil pH ($r = -0.23$ to -0.40 ; $p \leq 0.0037$) (Fig. SI 6). The ratio of fungi to bacteria was negatively correlated with soil pH, SOC, and C/N ratio ($r = 0.19$ to 0.54 ; $p \leq 0.0010$) (Fig. SI 6).

Discussion

Subsoil accounts for the majority of agricultural soil, however, microbial communities in subsoils are far

less studied compared to those in topsoils (Naylor et al. 2022). Besides greater sampling efforts associated with subsoil collection, molecular investigations of subsoil communities are often facing challenges recovering DNA from subsoil (e.g. Hurt et al. 2014). Here, we successfully recovered microbial DNA from two clayey subsoils using a phosphate lysis buffer-protocol optimized for subsoils (Guerra et al. 2020). Furthermore, we were able to quantify bacteria, fungi, methanotrophs, nitrifiers, and denitrifiers from the soil DNA extracts using qPCR. Our results revealed that several effects were restricted either to topsoil or subsoil or even to specific soil depths within topsoil (e.g., 0–15 or 15–30 cm) or subsoil (e.g., 30–45 or 45–60 cm). Therefore, we argue that sampling the topsoil alone does not reflect the vertical complexity

Fig. 6 Relative change of the abundance of *nirK*-type (a, b) and *nirS*-type denitrifiers (c, d) in soil in response to temperate alley-cropping agroforestry. Soil samples were collected in topsoil (0–15 cm and 15–30 cm soil depth) and subsoil (30–45 cm and 45–60 cm soil depth) of two paired agroforestry and monoculture cropland systems (in a Cambisol (a, c) and Phaeozem soil (b, d)) in Germany. Tree row, 1 m, 7 m, and 24 m crop row are sampling locations within the agroforestry systems. Relative change within each soil depth was calculated as [(sampling location – mean(monoculture cropland))/mean(monoculture cropland)] (see *Statistical analysis* for details). Dots with vertical bars represent means and standard error ($n = 4$), respectively. *nirK* and *nirS* genes were quantified by using real-time PCR (see *Real-time PCR assays* for details)



of soils as well as their inhabitants. This becomes particularly important in complex three-dimensional systems such as agroforestry systems (Beule et al. 2022).

Soil microbial population size of bacteria, fungi, and functional groups decreased with soil depth (Fig. 3, SI 1 B, D, SI 2 B, D, SI 3, B, D), confirming our first hypothesis (Fig. 1). A decrease of microbial population size with soil depth is commonly observed in agricultural soils (e.g. Hao et al. 2021) and is likely due to limited available resources for microorganisms in deeper soil layers (Fierer et al. 2003b). Our results reveal that SOC and total N decreased with increased soil depth (Table S1) and were positively correlated with all microbial groups (Fig. SI 6). SOC and total N are indicators of soil fertility

and their decrease with depth suggests limited available resources in subsoil, which may explain the observed decrease of microbial population size with depth. In addition to resource availability, other soil biochemical and physical soil properties that change with depth and are known to impact the soil microbiome such as soil bulk density (e.g. Ye et al. 2019) and oxygen diffusion rates (e.g. Lemon and Erickson 1952), likely contributed to the decrease in microbial abundance but were not assessed in this study. It is worth mentioning though that several specialized microorganisms are well adapted to subsoils and thrive in these environments. For example, arbuscular mycorrhizal fungal communities in topsoil and subsoil of agricultural soil not just differ from each other (Sosa-Hernández et al. 2018a) but evidence emerged

that some of these fungi are specialized to subsoils (Sosa-Hernández et al. 2018b). Similar findings exist for prokaryotes. For example, in 2019, Brewer and co-workers reported that affiliates of the candidate phylum *Dormibacteraeota* thrive in subsoil due adaptation to resource scarcity. Overall, successful colonization of subsoil requires special adaptation but several microorganisms succeed in colonizing this resource-limited environment.

The promotion of microbial abundance through tree rows within each soil depth (Figs. 4, 5, 6, SI 1 A, C, SI 2 A, C, SI 3, A, C, SI 5) agrees with previous studies in temperate alley-cropping agroforestry systems (Beuschel et al. 2019; Beule et al. 2020, 2021; Beule and Karlovsky 2021; Guillot et al. 2021). In contrast to previous studies, however, we were able to show that the promotion of microorganisms is not limited to the topsoil but extends into the subsoil, thereby confirming our second hypothesis (Fig. 1). In addition, we were able to show that microbial abundances decrease gradually with increasing distance from the tree rows into the crop rows in both topsoil and subsoil, which may be linked to spatial gradients of above- and belowground tree litter input (Oelbermann et al. 2004; Schmidt et al. 2021) and, consequently, soil fertility (Pardon et al. 2017) within agroforestry systems. Consistent with our previous findings (Beule et al. 2020; Luo et al. 2022), the promotion of microbial abundance and its gradual decrease into the crop rows also included functional populations of microorganisms involved in denitrification, especially nitrite reducers (i.e., *nirK*- and *nirS*-carrying microorganisms) (Fig. 6). The introduction of tree rows into arable land through agroforestry is well known to alter microbial functions in soil. For example, tree rows increase the catabolic potential (Udawatta et al. 2008, 2009; Beuschel et al. 2019) as well as the substrate-use efficiency (Mungai et al. 2005; Beuschel et al. 2019; Guillot et al. 2021) of the microbial community and reduce non-CO₂ greenhouse gas emissions (CH₄ and N₂O) from soil (Amadi et al. 2016, 2017; Luo et al. 2022). The denitrifiers quantified in our study are involved in both the emission and uptake of N₂O from soil. Although these organisms are involved in denitrification, the quantification of their population size measured by gene abundance may not reflect N₂O fluxes (e.g., Dandie et al. 2008). Recently, Luo et al. (2022) determined gross N₂O emission and uptake at our study sites

using ¹⁵N₂O pool dilution technique and correlated these fluxes with abundances of denitrification genes. The authors found that gross fluxes of N₂O were rather driven by substrate availability than denitrification gene abundance. Therefore, they concluded that denitrification gene abundance represents the genetic potential for denitrification rather than a predictor for actual process rates (Luo et al. 2022).

We consistently found that if microorganisms were promoted by the tree rows, the promotion was stronger in subsoil than topsoil, confirming our third hypothesis (Fig. 1). In 2006, Mulia and Dupraz reported popular roots in the subsoil below the rooting zone of cash crops, supporting the hypothesis of a ‘safety-net’-role of tree roots for leached nutrients in the subsoil (Rowe et al. 1999; Allen et al. 2004). Although tree root distribution patterns were not assessed in this study, Schmidt et al. (2021) recently measured tree root mass at our study sites and reported that tree roots in both topsoil and subsoil extend gradually into the crop rows as illustrated in Fig. 1. Roots form microhabitats in soil (e.g., the rhizosphere) that are considered hotspots for plant-microbe interactions and microbial activity (e.g. Pathan et al. 2020). Furthermore, plants exert strong selection on the rhizosphere microbiome and promote specific microbial taxa through root exudates (Hartmann et al. 2009). Recently, Min et al. (2021) demonstrated that although subsoil communities inhabit resource-limited environments as compared to those in the topsoil, they can respond rapidly to added resources. Furthermore, Fierer et al. (2003a) showed that when resources (i.e., nutrients) are added to subsoil communities, their relative respiration rate increases with depth due to increasing resource limitation. Thus, rhizodeposits (i.e., exudates, fine root litter, lysates, etc.) of tree roots in the subsoil (resource inputs) likely promoted microbial populations. We assume that the positive response of the microbial populations to the tree rows in the subsoil was stronger than in topsoil due to more intense resource scarcity in the subsoil.

In agreement with previous studies (Turner et al. 2017; Hsiao et al. 2018), the ratio of fungi to bacteria decreased within increasing soil depth (Fig. SI 4 B, D). Sradnick et al. (2014) suggested that this decrease is due to a lack of fresh organic matter in subsoil to which fungi are more sensitive than bacteria. Interestingly, we found that the fungi-to-bacteria ratio in the subsoil increases with decreasing distance to the trees (Fig. SI 4 A, C). Following the argumentation of Sradnick et al.

(2014), we suggest that this increase may be due to increased input of tree root litter into the subsoil in the vicinity of the trees which supports saprophytic fungi. Additionally, fungi may directly interact with tree roots in the subsoil through mycorrhization. Poplar trees can be colonized to varying degrees by arbuscular mycorrhizal as well as ectomycorrhizal fungi (Khasa et al. 2002). In 2021, Carteron et al. reported a strong shift from saprotrophic to mycorrhizal fungi with soil depth in temperate forests. In line with this, we recently formulated the hypothesis that tree-root associated microorganisms such as mycorrhizae contribute to the ‘safety-net’-function by increasing nutrient capturing and uptake by the trees in the subsoil (Beule et al. 2022). Since the phylum Basidiomycota harbours most ectomycorrhizal fungi, the tremendous increase of Basidiomycota in the subsoil beneath or adjacent to (1 m) the tree row (Fig. 5c, d) may be due to an increase of ectomycorrhizal fungi. Although this hypothesis was not tested here, our results provide initial evidence that may support the hypothesis that ectomycorrhizal fungi in the subsoil contribute to the ‘safety-net’-role of trees in agroforestry systems. We suggest that future studies in temperate agroforestry systems should decipher the subsoil microbiome and its functions to better understand if and to which degree microorganisms in subsoil contribute to the enhanced soil functions of these systems.

Conclusion

Microbial population size in agricultural soils decreases with soil depth, likely due to limited available resources for microorganisms in deeper soil layers. Tree rows in temperate alley-cropping agroforestry systems increase soil microbial abundance in both topsoil and subsoil. The promotion of microorganisms through the trees extends gradually into the crop rows and is stronger in subsoil than topsoil. This demonstrates that the soil microbiome of the crop rows also benefits from the introduction of tree rows in arable land through agroforestry. We suggest that the strong positive response of the subsoil community to the tree rows is due to tree root-derived resources (e.g. exudates) and root litter that are otherwise scarce in agricultural subsoils. Furthermore, we provide initial evidence that microbial communities in the subsoils of agroforestry systems contribute to the ‘safety-net’-role of the trees.

If confirmed, the ‘safety-net’-role of deep tree roots should be extended by their associated microbial partners. Finally, we argue that the insights obtained from sampling multiple soil depths including subsoil are worth the efforts associated with soil sampling and extraction of nucleic acids.

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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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