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Disentangling the impact of contrasting agricultural management practices on soil microbial communities – Importance of rare bacterial community members

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ABSTRACT

Agriculture has a strong effect on soil microbial communities, but it is still unclear how different management practices drive their diversity and composition. To disentangle the effects of temporally contrasting crop management practices on soil microbial abundance and prokaryotic diversity, we analysed samples from a long-term agricultural field experiment, in which plant residues were exported (RE) or returned to soil, i.e., restituted (RR), over a period of 60 years. For 2.5 years, we followed a cropping sequence of maize, winter wheat and barley, in which, as an additional treatment, wheat cultivation was diversified once with pea intercropping. Based on soil-extracted DNA, abundances of bacteria, archaea and fungi were analysed by domain-specific qPCR and the diversity and composition of the prokaryotic community by 16S rRNA gene amplicon sequencing. The abundance of bacteria and fungi, but not for archaea, increased with the long-term restitution, but this effect was only detectable in spring due to their stabilized abundance during winter. The long-term effect of crop restitution on bacterial diversity became tangible when rare and dominant community members were differentiated, with higher sensitivity shown for the rare. In contrast, the cropping sequence equally affected members of both groups. The short-term effect of crop diversification by intercropping was much stronger in the C-depleted RE soils, than in RR soils where the C-loss was compensated, indicating that crop residue restitution increased the environmental resilience of soil microbial communities. Finally, we could confirm that rare bacterial community members, suspected to represent more oligotrophs and synergistic bacteria, formed stronger network structures to each other than the dominant, suspected to be more copiotrophic and competitive. Therefore, our results emphasize the importance to consider the response of rare microbial community members when evaluating long-term effects of agricultural management on the soil microbiome.

1. Introduction

The management of arable soils introduces physical, chemical and biological factors which together have the potential to strongly modify soil properties and thereby the living conditions for the soil microbiome. This microbiome is typically composed of a highly diverse and complex assemblage of bacteria, archaea, fungi, protists and viruses (Fierer, 2017). Their diversity results from the coexistence of an uncountable number of microhabitats in immediate vicinity to each other (Young et al., 2008). These microhabitats, which emerge from aggregation and disintegration of mineral and organic soil building blocks (Tisdall and Oades, 1982; Jastrow, 1996), are probably the most important scale for controlling the biogeochemical processes mediated by the microbiome in soil (Lavelle et al., 2016; Rillig et al., 2017). Fine scale studies have revealed that different microhabitats harbour distinct microbial communities (Davinic et al., 2012; Wilpiszeski et al., 2019; Szoboszlay and Tebbe, 2021) which interact with each other while they provide important ecosystem services, i.e. improving the soil structure through decomposition and storage of organic material, or supporting crop growth by facilitating their nutrient supply in the rhizosphere (Schimel and Schaeffer, 2012).

Both soil tillage and the incorporation of plant residues have been shown to change the microbial community composition (Mikha and Rice, 2004; Kallenbach et al., 2015; Smith et al., 2016). In undisturbed

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soils, the formation and disintegration of soil aggregates is driven by organic substances released from plants or microbial cells (Totsche et al., 2018), but soil tillage can cause immediate disturbances and destructions of aggregates (Six et al., 1999). Microbial habitats are thereby modified or lost and the diversity and functionality of microbiomes may decline with the consequence of impairing multiple ecosystem functions (Wagg et al., 2014; Banerjee et al., 2019). To counteract such adverse processes and to enhance the nutritional status of soils, their restitution with plant residues or addition of organic fertilizers is proposed as a useful management option for protecting soils (Edmeades, 2003; Abiven et al., 2009). However, the recovery of soil structure and stable organic carbon is usually a slow process (Powlson et al., 2011; Dungait et al., 2012). The role of the soil microbiome in this process is also ambivalent, as on one side, microbial communities produce polysaccharides which can act as gluing agents for soil aggregate formation (Blanco-Canqui and Lal, 2004) and storage of carbon in their biomass, but on the other side, the microbiome mineralizes organic carbon and thereby contributes to greenhouse gas emission (Janzen, 2006; Powlson et al., 2011). The response of the microbiome and its individual constituents to organic soil restitutions is not well understood, which limits our capacity to favour beneficial microbial activities with targeted management options (Schimel and Schaeffer, 2012; Hartman et al., 2018).

The most important biological factor acting on the soil microbiome in arable lands is the crop with its developing and changing root system as it seasonally grows from seed to mature plants (Taschen et al., 2017; Li et al., 2021). As a chemical factor, mineral or organic fertilizers are applied to support plant growth by providing nitrogen and other plant nutrients. Typically, under field conditions, physical, chemical and biological factors act together, thus, making it difficult to evaluate their effects separately. Roots also modify soil conditions by exudation of energy rich carbon sources, thereby supporting microbial growth, which then results in a higher microbial cell density and activity compared to root-free soil (Berg and Smalla, 2009; Kuzyakov and Blagodatskaya, 2015). However, rhizosphere inhabiting microorganisms may also limit for their own benefit the access to nitrogen and other nutrients, change the soil pH and soil water availability - all factors which also change the microbial community structure and activity (Kuzyakov and Blagodatskaya, 2015).

In comparison to bare soil, crop roots were shown to stabilize microbial abundance, activity and diversity on agricultural fields, thus advocating for cover crops instead of keeping bare soil during winter seasons (Verzeaux et al., 2016; Kim et al., 2020). Since different crops have distinct root architectures and chemically characteristic exudates, microbial communities in rhizospheres are plant specific (Berg and Smalla, 2009; Berendsen et al., 2012), and consequently, total microbial diversity and activity is higher in rhizospheres, when different crops are cultivated at the same time, as done by intercropping (McDaniel et al., 2014; Granzow et al., 2017). Increased soil microbial diversity was shown to have beneficial effects, i.e., suppression of plant pathogens (Lupatini et al., 2017; Wu et al., 2020; Wang et al., 2021).

While ample field studies have already revealed that soil microbiomes respond to various agricultural management practices (Drenovsky et al., 2010; Shange et al., 2012; Ashworth et al., 2017; Li et al., 2021), we still lack a more systematic understanding about their impact on specific microbial groups or taxa and the time-scale and resilience of compositional changes of the microbiome. Some agricultural practices target immediate or short-term effects, i.e. the application of fertilizers or pesticides, while others are aiming at long-term effects, e.g., the improvement of soil structure and soil carbon by incorporation of crop residues. It is yet unclear whether such short-term and long-term objectives are equally reflected by changes in the soil microbiome. For example, additions of an organic fertilizer to sugarcane field only caused a transient change during a period of one year in the diversity of the soil microbiome, indicating a resilience of the microbiome (Lourenco et al., 2018). A 2-year nitrogen fertilization had no tangible effects on bacterial communities in a wheat maize cropping system (Zhao et al., 2019), but more than 30 years of nitrogen fertilizations in a wheat soybean crop rotation caused a significant decrease in bacterial diversity (Sun et al., 2015). In contrast, wheat straw incorporation over 30 years on the same fields and crop rotation had little effects on the bacterial community (Sun et al., 2015), but in other studies straw return increased soil bacterial diversity (Luo et al., 2020).

A direct comparison of results from different field studies is difficult because of the contrasting local conditions including soil types, cropping regimes and management intensities. Furthermore, there is strong variation caused by the different laboratory methods to characterize the abundance and diversity of soil microbial taxa. An increasing number of studies, all taking advantage of massively parallel PCR amplicon sequencing, have revealed contrasting patterns depending on the abundance of soil microbial community members to environmental changes (Jiao et al., 2019; Gao et al., 2020; Liang et al., 2020) and the specific importance of those members contributing to the "rare biosphere" (Pascoal et al., 2021). Contrasting patterns between dominant and rare community members can e.g. be explained by different prevalence of distinct life-styles, i.e. more copiotrophic for the dominant and more oligotrophic for the rare in the soil environment (Fierer et al., 2007; Sun et al., 2018). Thus, differentiating between both groups should have the potential to unravel effects on microbial communities that could be hidden when both groups are not distinguished from each other.

The objective of this study was to analyse how different crop management practices affect the soil microbiome, more specifically the abundance of bacteria, archaea and fungi and the diversity of bacteria. For the latter, we differentiated also between dominant and rare community members. An agricultural long-term field experiment in Gembloux, Belgium, allowed us to compare field plots on which plant residues were returned to soil (residues restitution; RR) or exported (RE) over a period of 60 years, thus producing a long-term effect on soil organic carbon and other parameters. Microbial community dynamics were followed on these plots along a 2.5-year cropping regime including a succession of maize, winter wheat and barley. As a short-term effect, we analysed a diversification by intercropping of wheat with pea. We asked (1) How strong and for how long is the abundance of bacteria, archaea and fungi and diversity of prokaryotes affected by the long-term soil restitution, by the cropping regime and by the short-term single intercropping event? (2) Are microbial responses to short-term effects, different between plots depending on their previous long-term management (RE vs. RR) and crop diversification? (3) Are there specific microbial taxa or subcommunities that respond to the short-term and long-term management practices?

We hypothesized that (1) the long-term crop residue restitution (RR) would increase the abundance and change the diversity of microbial groups, as more nutrients and organo-mineral microhabitats would become available, and that (2) cropping systems have a less dynamic microbial response on RR soils than on RE because the relative increase in nutrients provided by the respective crops was less. We also hypothesized that (3) the long-term soil restitution treatment selects of a specific community characterized by more complex relationships than those stimulated by the cropping regime or diversification, as the latter reflects only immediate nutritional effects, which would more likely select for competing microbial copiotrophs, while communities in the restitution treatment could evolve over a long time. The results of this study should be helpful to understand the importance of specific microbial community responses to agricultural management measures and thus contribute to the development of environmentally friendly sustainable cropping systems.

2. Materials and methods

2.1. Experimental sites and soil sampling

The soils of this study originated from the long-term field experiment

of the Walloon Agricultural Research Centre (CRA-W) in Gembloux, Belgium (50°33′28″ N, 4°43′39″ E, 170 m asl) (Buysse et al., 2013). This area is exposed to a temperate maritime climate with an average rainfall of 830 mm and annual temperature of 9.6 °C. The soil type was classified as Eutric Cambisol: the clay, silt and sand proportions are 17.3%, 80.5% and 2.19%, respectively. The experiment was initiated in 1959. During the first 16 years it included a four-year rotation (sugar beet, cereal, legume, cereal) which was followed by a three-year rotation (sugar beet, winter wheat or horse bean -winter barley or wheat) until 1990. From 1991 to 2017, rotations of sugar beet, winter wheat and barley in subsequent years were implemented (Fig. 1). In 2018, maize was cultivated, and, after harvesting, two different types of cultivation were initiated: winter wheat (*Triticum aestivum*; non-diversified cropping, ND), and winter wheat intercropping with pea (*Pisum sativum*; diversified cropping, DI). This cultivation divergence was only performed once, then barley was cultivated on all plots in 2020 (Fig. 1).

Since its beginning, in the year 1959, this long-term experiment aimed to investigate the impact of different crop residue restitutions and management of organic matter on soil properties (Buysse et al., 2013). There were six block-randomized treatments presented in a rectangular plot (10 m × 24 m) with 12 replicates of each in the field (Fig. 1). In this study, two treatments were selected to explore the effects on soil microbial communities. All treatments were managed in the same way. The plots were ploughed every year at a depth of ca. 25 cm. In the residue export treatment (RE), crop residues were removed and no organic matter was added to the soil. In the restitution treatment (RR), crop residues were incorporated into the soil while cover crops were grown during the winter right before the sugar beet crop season (Fig. 1). The cover crop was thus implemented into the restitution treatment every three years. Before 2017, the cover crop was usually restricted to vetch, mustard and phacelia in the pure crop. In 2017, the cover crop was a mix of cereal and legumes.

Soil samples for this study were collected after the growing season in 2018, 2019 and 2020, before the growing season in 2019 and 2020, thus for a period of 2.5 years. In each plot, soil samples were collected from four spatially distinct spots from a depth of 0–10 cm. These samples were mixed to create a composite sample. The soil samples were homogenized by 2-mm sieving after air-drying at room temperature, and stored at 4 °C until use. Soil pH was measured in 0.01M calcium chloride with pH meter (HI221 Microprocessor, Hanna Instruments, Vöhringen Germany). Carbon and nitrogen contents were analysed by dry combustion in an elemental analyser (Leco TruMac, St. Joseph, MI). The soil physicochemical parameters (soil pH, TC and TN) and microbial abundances (bacteria, archaea and fungi) were quantified in the samples from all five sampling events, while the diversity and composition of the prokaryotic community were characterized in the samples from the first four sampling events.

2.2. DNA extraction, quantification and sequencing

DNA was extracted from 500 mg of soil by FastDNA ®SPIN Kit (MP Biomedicals, Eschwege, Germany) following the manufacturer's procedures. The DNA samples were stored at −20 °C until quantification and −80 °C until sequencing. Quantitative real time PCR (qPCR) was used to estimate the abundance of bacteria, archaea and fungi using a Biorad CFX96 Real time PCR cycler with C1000 Touch (Biorad, Feldkirchen, Germany). TaqMan assays were to quantify bacterial 16S rRNA genes using the primer pair BAC338F/BAC805R with TaqMan BAC516F (Yu et al., 2005). The corresponding copy numbers for archaea were quantified with primers and probes: ARC787F, ARC1059R, ARC915F (Yu et al., 2005). All qPCR reactions were conducted in 20 μl reactions containing 10 μl SYBR (Maxima SYBR Green qPCR, Master Mix 2X no ROX, Thermo Fisher Scientific, Erlangen, Germany), 0.2 μl of each primer (50 μM), 0.08 μl of 50 μM TaqMan, 7.52 μl of DNA/RNAase-free water for qPCR (Thermo Fisher Scientific) and 2 μl of extracted soil DNA. QPCR cycling conditions consisted of initial 10 min at 95 °C followed by 39 cycles of denaturing at 95 °C for 15 s and annealing at 60 °C for 1 min. Fungal abundance was estimated by quantifying a fragment of the ITS1 region by qPCR, using the primer pair NSII/58A2R (Martin and Rygiewicz, 2005), in 20 μl reaction: 10 μl SYBR (Maxima SYBR Green qPCR, Master Mix 2 X no ROX, Thermo Fisher Scientific), 0.2 μl of each primer (50 μM), 7.6 μl of DNA/RNAase-free water for qPCR (Thermo Fisher Scientific) and 2 μl of extracted soil DNA. The cycling conditions consisted of initial 10 min at 95 °C, followed by 39 cycles of 95 °C for 15 s,

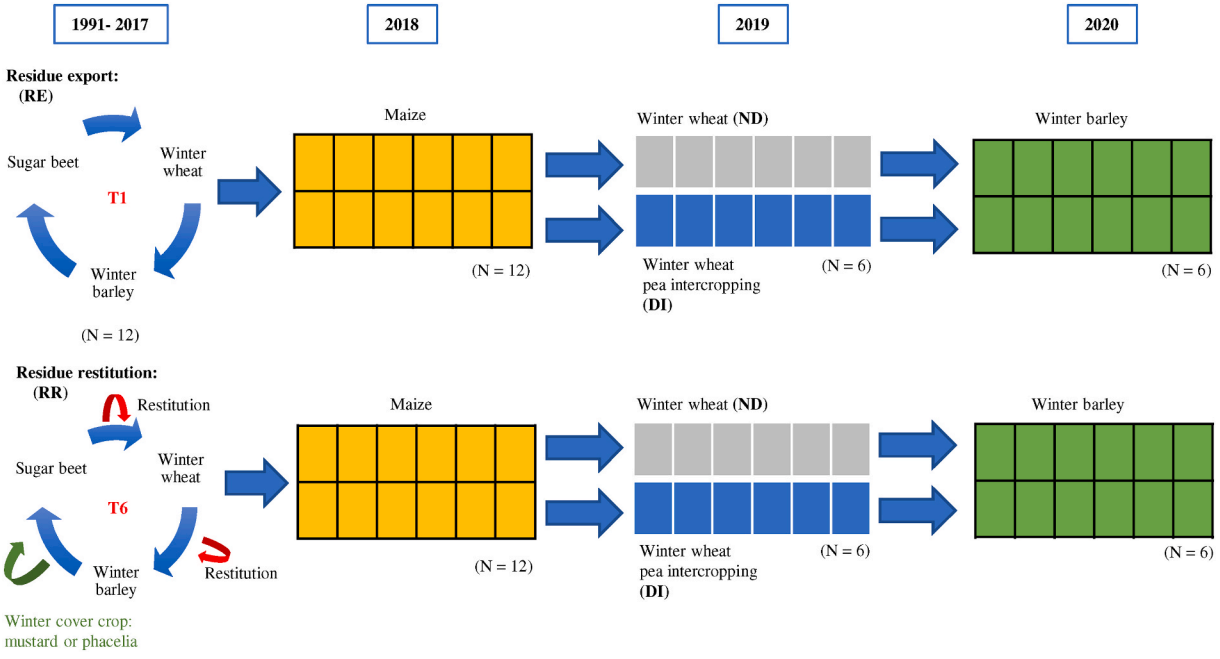

Fig. 1. Schematic view of the farming practices conducted at the field site before (1991–2017) and during sampling (2018–2020) for this study. RE, residue export; RR, residue returned to soil (restituted); ND, non-diversified (no intercropping); DI, diversified (intercropping in summer 2019); A indicates autumn; S indicates spring. The actual plots were located at the field site in a randomized design.

annealing at 52 °C for 30 s, extension at 72 °C for 30 s and 79 °C for 15 s. Standard curves for bacteria, archaea and fungi were prepared using type culture strains obtained from the DSZM, Braunschweig, Germany, i.e. *Bacillus subtilis*, *Methanobacterium oryzae* (DSM 11106) and *Fusarium culmorum* (DSM 62191), respectively. The determined amplification efficiencies were ca. 96%, and the correlation coefficients >0.99.

To determine the diversity of bacteria and archaea, the V4 region of the 16S rRNA gene was amplified with the 515F/806R primer set (Caporaso et al., 2011) and sequenced as described elsewhere (Herbold et al., 2015). Paired-end sequencing (2 × 300 bp) for the 16S PCR amplicons was performed on Illumina MiSeq platform by LGC Genomic GmbH, Berlin, Germany. All the samples from different treatments and sampling seasons were sequenced in the same run and the sequencing data were analysed together on QIIME2 platform (Bolyen et al., 2019). Raw sequence reads were reoriented into respective forward and reverse read files due to the blind ligation of Illumina adaptor and amplicon fragments with an in-house Python script available at: github.com/DamienFinn/MiSeq_read_reorientation. Reoriented reads were demultiplexed by cutadapt paired-end methods (Martin, 2011). Then the forward and reverse reads were merged using the Vsearch join-pairs function (Rognes et al., 2016). The merged reads were truncated at the position 280 and 40 with DADA2 denoise-single function (Callahan et al., 2016). With the respect to the higher resolution of sequences, amplicon sequence variants (ASV) were adopted and assigned with Silva 138 database (Quast et al., 2013; Yilmaz et al., 2014). Eukaryote-associated ASVs (mitochondria and chloroplasts) were removed from the final dataset. The raw DNA sequences have been deposited at the European Nucleotide Archive database (Project Accession number PRJEB47825).

2.3. Data analysis

Rarefaction was performed to estimate the sequence coverage with the Vegan package (v.2.5–7) in R (v.4.0.3) (Oksanen et al., 2020). Sequences per sample were rarefied to 9096 to normalize sequencing depth for the following analysis. Shannon index was calculated in R using the Vegan package. Statistically significant differences in physicochemical soil parameters, microbial abundance and Shannon index were examined using Fisher's Least Significant Difference (LSD) post hoc test with the agricolae packages (v. 1.3–3) in R, and the significant differences were considered as $p < 0.05$ (Mendiburu, 2021). Principal component analysis (PCA) plots based on Euclidean distance were created with the Vegan package in R. Permutational multivariate analysis of variance (adonis) was performed to determine the differences between different microbial communities with 999 permutations. Venn diagrams were generated with the VennDiagram package (v.1.6.20) in R (Chen, 2018). Statistically significant differences in taxonomic ranks (phylum, class, order, family, genus, ASV) between different treatments were calculated using Aldex2 package (v.1.22.0) in R, and the significant differences were considered as Benjamini-Hochberg-corrected P-values less than 0.05 (Fernandes et al., 2013, 2014). Network analysis was performed to explore the microbial co-occurrence patterns with the dplyr package (v.1.0.5), funrar package (v.1.4.1), WGCNA package (v.1.70–3) and igraph package (v.1.2.6) in R (Csardi and Nepusz, 2006; Langfelder and Horvath, 2008, 2012; Grenié et al., 2017; Wickham et al., 2021). The co-occurrence network was based on the correlation matrix constructed by WGCNA package. The correlation coefficients larger than 0.7 and FDR-corrected P-values less than 0.05 were used to form the networks. The nodes in those networks represented ASV, and the edges between these nodes represented the correlations between ASV. The network images were generated with igraph package, and the topological features in each network were calculated in the same package. The feature set included average degree and clustering coefficient, represented the number of adjacent edges and the probability that the adjacent nodes of a node are connected, respectively.

3. Results

3.1. Effect of the long-term and short-term treatments on physicochemical soil parameters

The pH value of plots from which plant residues were exported (RE) and those where they were left (RR) did not significantly differ at the end of the growing seasons 2018, 2019 and 2020, respectively (Fig. 2 A and B., Fig. S1). However, with the exception of the diversified system in 2020, the restitution with residues showed lower pH values compared to the residue export at the beginning of the growing seasons.

The restitution treatment (RR) over a period of 59–61 years resulted in 14.8% higher soil organic carbon (C_{org}) compared to control without restitution ($p < 0.001$; with one outlier at the onset of this study for RE plots; Fig. 2 C. andD. Fig. S1). In parallel, total soil nitrogen (N_{tot}) was also 11.5% higher in RR ($p < 0.001$; including the same outlier; Fig. 2 E. and F., Fig. S1). As detected after the growing season in 2019, the diversification by intercropping with pea increased C_{org} in both RE and RR plots. The average increase of C_{org} was 5.8% ($p < 0.05$) in RE plots and 3.8% with RR, the latter not significant though. Diversification triggered an increase of N_{tot} in the RE (18.3%, $p = 0.09$) but not in the RR plots. During the following winter and growing season with barley, the increases of C_{org} and N_{tot} of the diversified system from the RE plots levelled out.

3.2. Effect of the long-term and short-term treatments on soil microbial abundance

A comparison of the spring and autumn samples collected in 2019 and 2020 revealed that generally, but not always significant, the soil bacterial, archaeal and fungal abundance increased during the growing seasons (Fig. 3, Fig. S2). Soils from RR showed in comparison to RE higher bacterial and fungal abundances at the beginning of the growing seasons ($p < 0.05$) but not at the end ($p > 0.05$). For archaeal abundance, the residue restitution had no significant effect. The diversification by intercropping did not increase the bacterial, archaeal or fungal abundance, neither in the RE nor the RR plots ($p > 0.05$). The differences in bacterial abundances seen at the beginning of the growing season (2019 and 2020) between the RE and RR group were not significant with the diversified cropping system.

3.3. Composition and diversity of the prokaryotic communities

The 16S rRNA gene PCR amplicon sequencing was performed at four sampling events, i.e., at the end of growing seasons 2018 and 2019 and the beginning of growing seasons 2019 and 2020, respectively. In total, 96 samples provided 3.73 Million high-quality sequences grouped into 19,240 ASV, with a range of 9,096 to 100,145 sequences for the individual samples. Sequences assigned to bacteria and archaea were 92.7% and 7.3%, respectively. In total, the ASV could be assigned into 43 bacterial and 2 archaeal phyla.

The most abundant bacterial phyla were Proteobacteria (27.5%), Actinobacteria (20.9%), Acidobacteria (14.2%), Bacteroidetes (7.9%), Verrucomicrobia (6.18%), Chloroflexi (5.67%), Firmicutes (3.32%), Planctomycetes (2.78%), Myxococcota (2.57%), and Gemmatimonadetes (2.24%). Within Proteobacteria, Alpha- and Gammaproteobacteria showed by far the highest relative abundance. The dominant archaea were classified into Crenarchaeota (99.5%) and Thermoplasmatota (0.46%) (for more details, see Supplemental Material, Fig. S3, and Supplemental data file).

The restitution (RR compared to RE) had generally no tangible effect on the soil prokaryotic diversity, as indicated by the Shannon index ($p > 0.05$; Fig. S4). Equally, the crop diversification with pea intercropping apparently did not change the overall diversity. In contrast, a significant increase of diversity was detected from spring to autumn in 2019 ($p = 0.001$). This increase in prokaryotic diversity was stronger in the RE

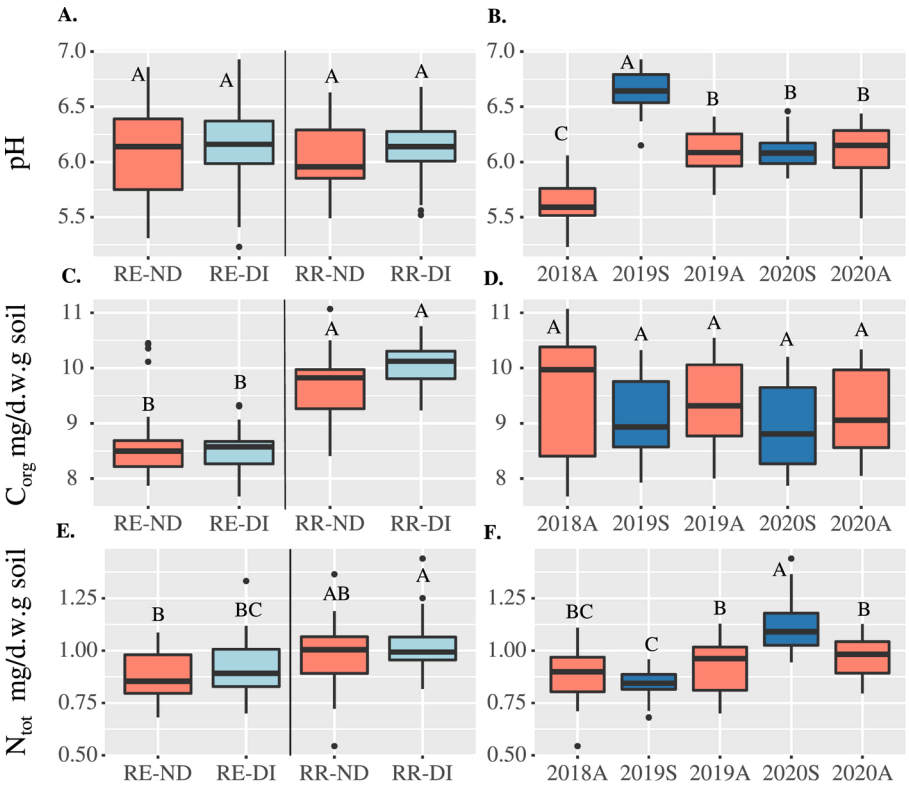

Fig. 2. Physicochemical soil parameters (soil pH, TC and TN) assessed from the field site soils. Effects of crop restitution and intercropping on soil pH (A.), TC (C.) and TN (E.), box plots represent the variation among the five sampling events (n = 30). Seasonal variation (B.-, D., F.) on soil parameters among different crop management practices (n = 24). Data for each of the crop management practices and sampling events during the course of this study are presented in detail in Fig. S1. Boxes not sharing the same letter are significantly different ($p < 0.05$; Fisher's Least Significant Difference (LSD) post hoc test). RE, residue export; RR, residue restitution; ND, non-diversified (no intercropping); DI, diversified (intercropping in summer 2019); A indicates samples taken in autumn; S indicates those collected spring.

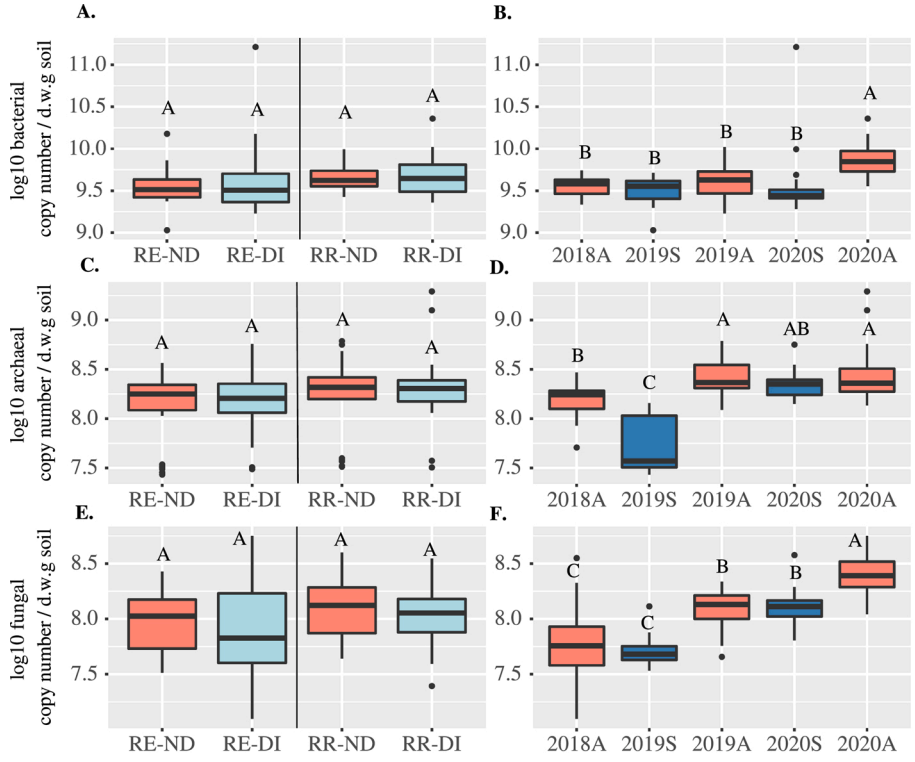

Fig. 3. Abundances of bacteria, archaea and fungi in the field site soils, as assessed by qPCR from directly extracted soil DNA. For details of the treatments and sampling events see legend of Fig. 2. Results from the five individual sampling events of this study and their statistical analyses are shown in more detail in the Supplemental Material (Fig. S2).

plots compared to RR plots. Despite the inclusion of pea, there was no clear stimulatory effect of this legume on the native soil inhabiting rhizobia. Their abundance in the field soil was generally low (<1% at the genus level) and not dynamic (Fig. S5).

3.4. *Effect of the long-term and short-term treatments on soil prokaryotic community structure*

The major impact on the prokaryotic community composition was caused by the respective crops, as indicated by comparing the four

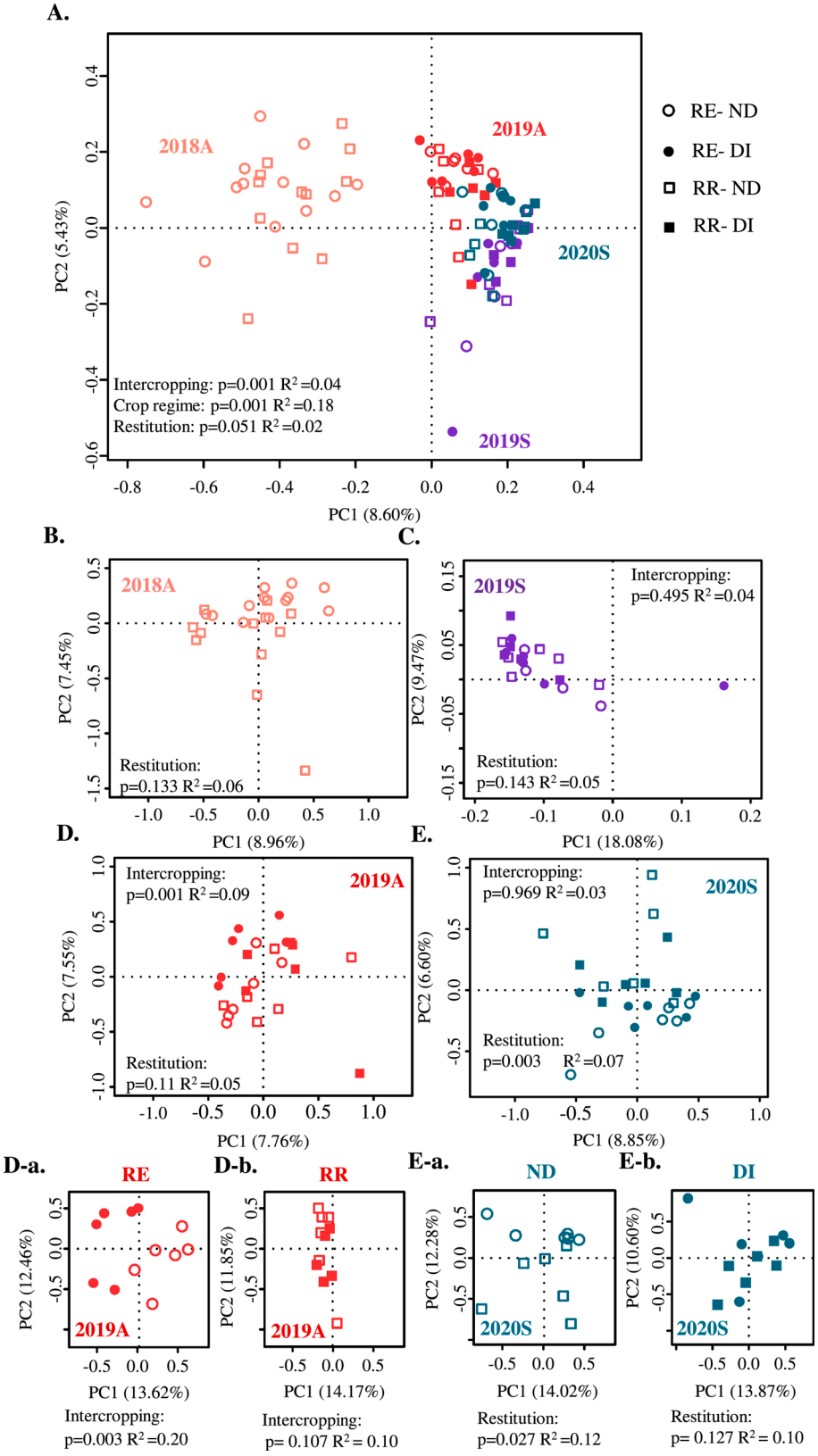

Fig. 4. Principle components analysis (PCA) of the soil prokaryotic community structure from four sampling events (A.), and individual sampling events from 2018A to 2020S (B. to E.). The effect of intercropping on the soil samples collected from residue export treatment (D-a.) and residue restitution treatment (D-b.) in 2019A. The effect of crop restitution on the soil samples collected from no intercropping system (E-a.) and intercropping (E-b.) in 2020S. Variation explained by axes one and two are shown. Permutational multivariate analysis of variance (adonis) was performed to determine the impact of crop diversification (single crop and intercropping) and restitution treatment (residue export treatment and residue restitution treatment). RE, residue export; RR, residue restitution; ND, non-diversified (no intercropping); DI, diversified (intercropping in summer 2019); A indicates autumn; S indicates spring.

sampling events (Fig. 4 A.). Surprisingly, the long-term residue restitution (RR) had no consistently significant impact on the overall prokaryotic community structure. No effect was detected at three of the four sampling events, between autumn 2018 to autumn 2019. (Fig. 4 B. to D.). Only the samples from the non-diversified treatment (no intercropping) collected in spring 2020 showed significantly distinct microbial community structures between residue export (RE) and restitution plots (RR) (Fig. 4 E. and E-a.), but the samples from the diversified cropping showed no such effect (Fig. 4 E-b.). Furthermore, PCA and the Adonis analysis revealed a significant effect of the diversified cropping at the end of the growing season in autumn 2019, but not subsequently in spring 2020 (Fig. 4 D. and E.). This crop diversification effect was only significant in the RE plots (Fig. 4 D-a. and D-b.), indicating that the incorporated crop material buffered the immediate impact of the crop diversification.

3.5. Identification of responsive prokaryotic taxa

Responsive taxa were identified by means of pairwise comparison among the different treatments and cropping regimes as implemented over the two years of this study. Different levels of different phylogenetic resolution were analysed separately.

No significantly responding tax or group were detectable in response to the residue restitution treatment or the diversification by intercropping (Figs. S6–S11). In fact, among the 29 comparisons made, only 5 revealed significantly responding units, and these were all linked to changes as caused by the cropping regimes during the 2.5 years of this study (Table 1). The most pronounced differences were found on field plots with residue export and the non-diversified system (RE-ND), between autumn 2018 and autumn 2019. A lower, but still well pronounced difference, was seen on the field plots with residue restitution between autumn 2018 and spring 2019, thus during the transition of maize to wheat.

For the phylogenetic ranks, the family level was found to be most significant in responding to the cropping regimes, followed by their immediate lower and higher neighbouring ranks, i.e., genus and order (Table 1). The least sensitive was the ASV level, most likely due to the low abundance values and higher fluctuation, affecting the significance testing. The responsive families came from diverse lineages, including as the most dominant Sphingomonadaceae, Chitinophagaceae, Xanthomonadaceae, and Nitrososphaeraceae (Crenarchaeota) (Tables S1–S4). The latter was not different between autumn (maize) and spring (wheat) but it strongly declined between autumn and autumn, indicating that maize was more favourable for this taxon than wheat. Its phylogenetic assignment suggests that it had a potential for oxidizing ammonium.

3.6. Response of dominant and rare taxa to the treatments

In order to analyse potentially different responses of high abundance and low abundance taxa to the environmental variables of this study (restitution, diversification by intercropping, cropping regime), we clustered ASVs according to their relative abundance and defined as dominant taxa those with larger than 0.1% in each sample, and those with less than 0.1% as rare taxa. The dominant taxa community covered 14 phyla, 26 class, 52 orders, 69 family and 76 genera on average, which was much lower than in the rare taxa community, the latter covering 38 phyla, 100 class, 212 orders, 302 family and 450 genera on average (Fig. S12 A.). Dominant taxa accounted for about half of the sequence reads (mean = 47.9%) with a significantly lower proportion of ASVs (mean = 5.7%) in each sample compared to the rare taxa community (mean = 52.1% and 94.3%, respectively; Fig. S12 B.). The most abundant phyla in dominant and rare taxa community were similar. However, oligotrophic-associated phyla like Acidobacteria, Chloroflexi, Planctomycetes, Myxococcota and Verrucomicrobia showed clearly higher abundance and more diverse family lineages in the rare taxa community compare to the dominant taxa community (Fig. S13). The proportion of consistently occurring ASV in the dominant taxa community (mean = 64.3%) was significantly larger than in the rare taxa community (mean = 29.4%), as indicated by Venn diagrams (Fig. S14). Thus, the variability between treatments and crop regimes was much higher for the rare than for the dominant community members.

The PCA plot based on dominant and rare taxa revealed a significant influence of the different crops and crop diversification for both groups, but for the long-term residue restitution, only the rare taxa responded significantly ($p = 0.02$) (Fig. S12 C.). The significant response of the rare taxa to restitution was confirmed for most of the sampling events, and thus not strongly affected by the cropping regime, while there was no consistently significant response of the dominant taxa (Fig. S15). An exception was autumn 2019, possibly linked to the interfering crop diversification effect. In fact, diversification by intercropping showed similar impacts on taxa forming the dominant and rare communities ($p = 0.001$; Fig. S12 C.). Interestingly, the PCA plots for each individual sampling event (Fig. S15) showed the short-term diversification (intercropping) treatment significantly affected the structure of both dominant and rare taxa communities ($p = 0.001$; $p = 0.004$), but only after the growing season and not at their beginning ($p = 0.482$; $p = 0.208$) in 2019. With winter barley growing in spring 2020, no significant impact on both dominant and rare communities were detectable in response to the previous intercropping.

The distinct response patterns of dominant and rare community members were further analysed with co-occurrence networks, for each group separately. Networks based on dominant taxa appeared less connected and complex than networks constructed with ASVs of rare abundance (Fig. 5 A. and B.). At first sight, this difference was owed to the higher numbers of ASVs from the rare compared to the dominant taxa group, but, when ASVs of the rare community were adjusted to the number of ASVs contributing to the dominant group by random selection, rare taxa communities still displayed a stronger network structure (Fig. 5 C.). In fact, the topological features retrieved from all networks showed that the rarefied dataset of 420 ASVs caused four times more

Table 1
Summary of the results of pairwise comparisons[a] for detecting prokaryotic taxa significantly responding on field plots with two soil conditions (crop residues exported; non-restituted vs. crop residues returned to soil; restituted) to two cropping systems (non-diversified vs. diversified) over a period of two years.[b].

Phylogenetic rank	RE-ND (maize A vs. wheat S)	RE-ND (maize A vs. wheat A)	RR-ND (maize A vs. wheat S)	RR-ND (maize A vs. wheat A)	RR-DI (wheat-pea; S vs. A)	Total number of responsive units
ASV	–	8	1	–	–	9
Genus	3	35	15	10	–	63
Family	11	39	17	11	4	82
Order	12	32	15	11	3	73
Class	10	27	14	5	2	58
Phylum	1	10	2	3	2	18

RE, residue export; ND, non-diversified (no intercropping); RR, residue restitution: DI, diversified (intercropping in summer 2019); A indicates autumn; S indicates spring.

[a] Total number of comparisons between treatments and crop regimes was 29.

[b] Details of all comparisons based on volcano plots are shown in the Supplemental Material (Figs. S6–S11).

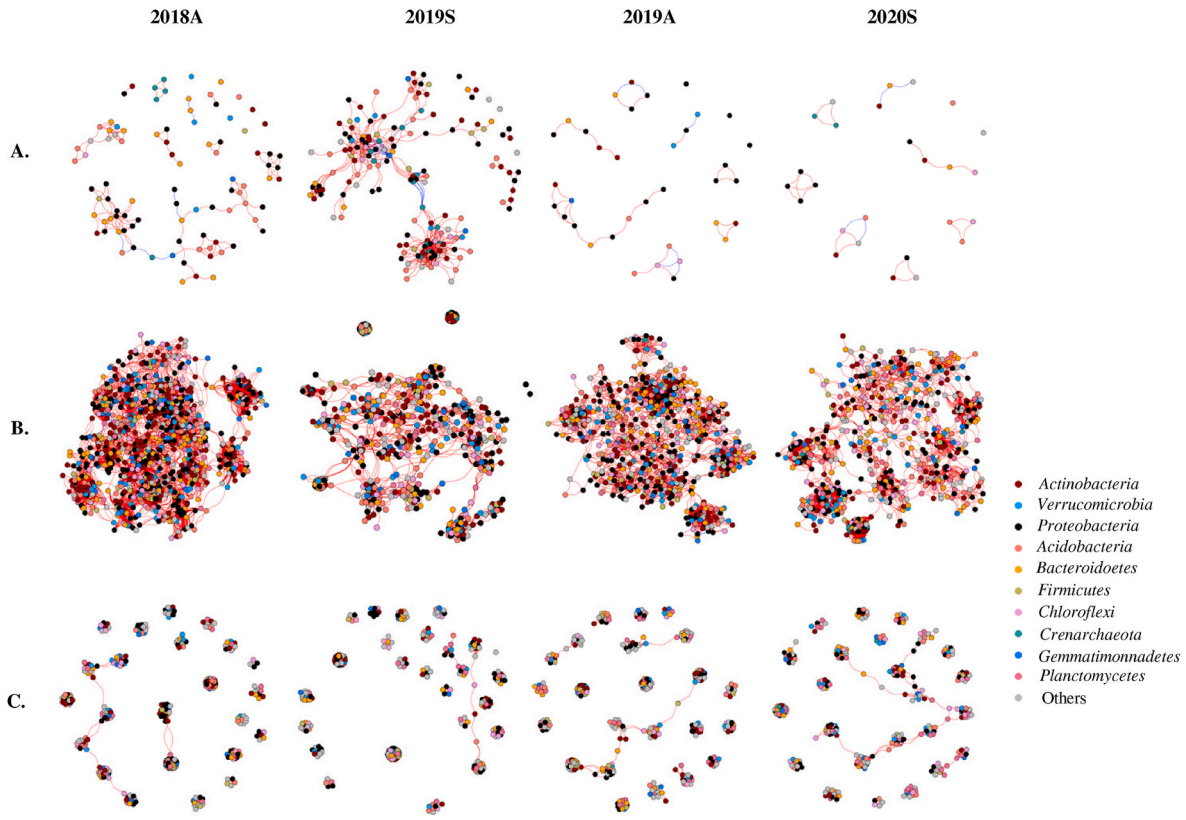

Fig. 5. Co-occurrence networks of amplicon sequence variants (ASV) representing the dominant taxa community (A), the rare taxa community (B) and randomly selected 420 ASV from the rare taxa community (C) to rarefy the dataset. Connections indicate strong ($r > 0.7$ or $r < -0.7$) and significant (FDR-corrected p-value < 0.05) correlations. The positive and negative correlations between nodes are displayed with red and blue lines, respectively. **A** indicates samples collected in autumn; **S** in spring. Networks were constructed based on 24 replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
The topological features of the co-occurrence networks. A indicates autumn; S indicates spring.

	Sampling date	ASV Number	Nodes	Edges	Average degree	Clustering coefficient
Dominant taxa	2018A	419	92	148	3.22	0.564
	2019S	421	170	682	8.02	0.569
	2019A	423	36	35	1.94	0.529
	2020S	418	26	25	1.92	0.843
Rare taxa	2018A	1554	1033	15,494	30.00	0.866
	2019S	1697	803	14,833	36.94	0.974
	2019A	1825	721	5878	16.31	0.773
	2020S	1707	578	4091	14.16	0.786
Random selected rare taxa	2018A	420	355	3027	17.05	0.976
	2019S	420	360	8332	46.29	1.000
	2019A	420	330	2293	13.90	0.969
	2020S	420	317	2150	13.56	0.957

nodes, 17 times more edges and a two-fold higher clustering coefficient, respectively (Table 2). This suggests that compared to the dominant taxa, rare community members were more linked to each other.

4. Discussion

The three variables included in this field study to evaluate responses of the soil microbial communities included a 60-year, annually repeated long-term plant residue restitution, a crop rotation including maize, winter wheat and barley, and a short-term diversification by inter-cropping winter wheat with pea. The residue restitution (RR) caused a significantly higher soil organic carbon and total soil N compared to the residue export (RE), corroborating results obtained from soil analyses at the same site ten years earlier (Buysse et al., 2013). Interestingly, these higher values only compensated the loss of SOC since 1959. The decline of SOC due to agricultural use, is a common phenomenon which can be accompanied by significant reduction of microbial abundance, diversity, and ecosystem functions (George et al., 2021). The compensation with added plant residues replenished the C-pool but basically replaced the more stable C-fractions with fresh material which was potentially more easily accessible as nutrients for plants and microbial metabolism. While these additions may restore soil fertility to a certain extent, they unlikely contribute much to preserving the soil structure considering that the stable SOC fractions dominantly occur in organo-mineral fractions and micro-aggregates ((Totsche et al., 2018; Lehmann et al., 2020).

In addition to the plant residues, the C pool in this study was further enhanced by intercropping of wheat with pea, probably because of more plant coverage and, thus, higher primary production compared to single

cropping wheat (Mao et al., 2012; Pelzer et al., 2012). Interestingly, soil from the residue exported plots (RE), which suffered from SOC losses over the years, presented a higher capacity to absorb additional C and N from intercropping than the RR soils. Probably the RR soils were more saturated with fresh plant residues already and, thus, the added C may have been less protected against microbial metabolism than in the C-deprived RE plots. The capacity of a soil to stabilize new C is related to the soil C-saturation deficit (Stewart et al., 2008), which was clearly higher in the RE compared to the RR plots. Furthermore, new inputs of nitrogen-rich organic material preferentially bind directly to soil mineral surfaces (Kopittke et al., 2018) which can initiate the process of rebuilding lost soil organic matter.

The abundance of soil bacteria, archaea and fungi, as indicated by their gene copy numbers, generally increased between spring and autumn, suggesting that primary production and warmer seasonal conditions supported their growth. Such a seasonal increase is not always observed on arable fields (Daniell et al., 2001; Chernov et al., 2015; Peltoniemi et al., 2021), but, considering that roots enhance microbial growth and abundance (Berg and Smalla, 2009; Kuzyakov and Blagodatskaya, 2015), these trends of increasing microbial abundance during crop cultivation were not surprising. Seasonal differences were also important for detecting an effect of the long-term restitution (RR), as it caused higher bacterial and fungal abundances only in the spring, but not in autumn samples. The plant residue additions apparently stabilized the bacterial and fungal community abundance over winter. Consequently, on the RE plots with less SOC, the seasonal growth of soil bacteria and fungi was more dynamic and sensitive to nutrients. The short-term stimulatory effect of crop residue restitution was not seen for archaea, suggesting they were less dependent on organic inputs and decomposition. The typically low relative abundance of archaea in the rhizosphere microbial communities of maize (Dohrmann et al., 2013; Szoboszlay et al., 2019) or other plants and in aerobically decomposing plant material (Su et al., 2017; Dong et al., 2021) supports this assumption.

Surprisingly, the additional input of C and N by intercropping did not further enhance the abundance of bacteria and fungi, neither on RE nor on RR plots. Apparently, the additional nutrients from intercropping were not as easily accessible as those released from plant residues during decomposition in the RR treatment. It can be suspected that the gains of C and N by intercropping originated mainly from an increase of root material, considering a higher resource efficiency and root density compared to single cultured crops (Li et al., 2006). Thus, the added C and N was first physically protected in plant roots and root nodules, and their microbial use would be delayed until decomposition of the plant material. Such a slow process of nutrient release may support oligotrophic microorganisms or maintenance of microbial biomass rather than boosting microbial copiotrophs (Fierer et al., 2007).

The prokaryotic diversity assessed in this study by means of 16S rRNA gene sequencing was mainly a bacterial diversity, considering that bacteria represented 93% of all quality filtered sequences. This proportion of bacteria and archaea was also reflected by the qPCR data of the respective groups in this study and is not unusual for arable soils from temperate climatic regions i.e. Europe (Szoboszlay et al., 2017). The overall bacterial diversity, as indicated by Shannon index, was not affected by the long-term restitution or the short-term intercropping. Pairwise comparisons to identify treatment specific microbial taxa failed to detect any, neither for long-term crop residue restitution nor for the single diversification event. In contrast, positive correlations were found between the seasonally increasing abundance of bacteria and their overall Shannon diversity. Apparently, the cultivation of wheat and wheat-pea intercropping supported the development of a bacterial community more diverse than at the onset of the season, irrespective of the long-term soil restitution. The seasonal shifts were accompanied by the occurrence of different bacterial taxa, probably because the different crops and associated diverse root exudates promoted the growth of distinct taxa in their rhizosphere (Berg and Smalla, 2009; Dennis et al., 2010). Statistical analyses of differences between the overall prokaryotic community structures confirmed that temporal succession was the major driver of the community compositions, and it is likely, that the respective crops cultivated during this succession with their characteristic root exudates were the main reason for these differences. Pairwise comparisons revealed a number of significantly shifting bacteria at different levels of taxonomic resolution. The highest number of responsive taxa were detected on field plots with residue export (RE) and the non-diversified system. This confirmed that the bacterial communities were more susceptible and stimulated by the short-term crop diversification in the soil that was assumingly more depleted with C and N. Thus, residue restitution increased the stability of the bacterial community not only during adverse winter conditions, but also when primary production supported their growth. Our data clearly confirm that residue restitution has positive effects on soils and their inhabiting microbiome (Hiel et al., 2018; Drost et al., 2020; Luo et al., 2020).

The distinction between dominant (abundant) and rare bacterial community members in our dataset revealed partially contrasting response patterns and it increased the sensitivity of detecting effects on microbiomes, as recently also reported from other studies (Jiang et al., 2019; Ji et al., 2020; Jiao and Lu, 2020). The shifts of the bacterial communities to the cropping regime and diversification were detectable for both groups, but the effect of the long-term restitution treatment was much more pronounced with the rare taxa. Other studies analyzing a residue restitution failed to detect effects on the composition of the soil bacterial communities, possibly because the inertia of the dominant taxa masked the pattern of the responsive rare community members (Essel et al., 2019). In our study, the residual plant material applied over 60-years likely resulted in the development of a bacterial community that was specifically adapted to the additional microhabitats introduced with the organic inputs. Considering their low abundance, it can be suspected that these bacteria would mainly be slow growing and adapted to living with more complex organic substrates. In contrast, both the rare and dominant community responded to the seasonal effects and the diversification by intercropping. The striking differences that we found between the less complex network structure of the dominant compared to the rare community suggests, that the long-term restitution treatment allowed their community members to develop more elaborated interactions. This could be a result of longer time the rare communities had to develop synergistic activities for extracting energy and carbon from the more complex organic substrates enriched during the conversion of fresh to more aged plant residues. In contrast, the more easily available nutrients and energy, as supplied by roots and their exudates during the growing season would promote competition between fast growing copiotrophs, resulting in a poorer network structure.

In summary, our study confirmed for bacteria and fungi, but not for archaea, our hypothesis that the long-term crop restitution increased the microbial abundance, but this effect was seasonally variable and despite 60-years of treatment only detectable in spring, due to a stabilized abundance during winter. The long-term restitution effect on bacterial diversity became more tangible with rare community members. In contrast, the cropping regime affected members of both dominant and rare soil bacteria. The short-term effect of crop diversification by intercropping was stronger in soils suffering from C-depletion than in soils where C-loss was compensated with plant residues, confirming our hypotheses that crop residue restitution would stabilize soil microbial communities. Finally, we could confirm that rare bacterial community members, suspected to represent more oligotrophs and synergistic bacteria, formed a stronger network to each other than the dominant, suspected to be more copiotrophic and competitive. Therefore, our results emphasize the importance to consider the response of rare microbial community members when evaluating long-term effects of agricultural management on the soil microbiome.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108573.

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