



Crop diversification improves the diversity and network structure of the prokaryotic soil microbiome at conventional nitrogen fertilization

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Abstract

Background Sustainable agriculture seeks to optimize the application of nitrogen (N) fertilizers to reduce adverse economic and ecological effects. Crop diversification has been proposed to increase the efficiency of N fertilization. An open question is how the soil microbiome responds to these beneficial practices.

Methods In a field study we investigated the effects of mineral N fertilizer with a 0-control, a conventional amount of 150 kg N ha⁻¹ and an excessive application of 250 kg N ha⁻¹ on the soil microbiome within a diversified cropping system with oil radish

and undersown ryegrass over a period of 2.5 years and a non-diversified control, both in rotation of potato, winter rye and maize.

Results N-fertilizations and crop rotations altered the pH, but differences were less pronounced with the diversified system. Compared to the crop species and season, N fertilization and crop diversification had less influence on the abundance of soil bacteria, archaea and fungi. The crop diversification showed a much stronger effect on archaeal than on bacterial or fungal abundances, while the microbial carbon use efficiency correlated strongly with bacterial abundance. At the end of the growing seasons, crop diversification increased prokaryotic richness and Shannon diversity in response to N addition, with a greater increase in the conventional N. At conventional N supply, prokaryotic co-occurrence networks revealed a much denser and complex structure in the diversified system.

Conclusions The diversified cropping system under conventional N application rates showed positive effects on the prokaryotic soil microbiome by increasing their richness, Shannon diversity, and promoting a more elaborated network structure.

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Introduction

Nitrogen (N) is a major growth limiting factor in cropping systems and has therefore traditionally been applied in agricultural systems in plentiful amounts as organic or mineral N-fertilizers (Foyer et al. 2016; Vitousek et al. 2002). Such surplus N applications however are not only economically wasteful but may also trigger environmental problems. More than half of applied N fertilizer can be lost from agricultural fields due to nitrate leaching and nitrogenous oxide gas emission, both potentially also resulting from microbial N transformations (Erisman et al. 2008; Foyer et al. 2016; Francioli et al. 2016; Hartmann and Six 2022; He et al. 2020).

Nitrate leaching from agricultural soils can be greatly affected by the employed cropping system (Beaudoin et al. 2005; Hall et al. 2001; Smith et al. 2013; Tonitto et al. 2006; Toth and Fox 1998). Inclusion of cover crops into the crop rotation as a diversification strategy has been shown to improve N use efficiencies of plants (Hauggaard-Nielsen et al. 2012; Kankanen and Eriksson 2007; Thorup-Kristensen et al. 2012). Introduction of undersown ryegrass in cereal/wheat production systems reduced N leaching by 50%, as shown by a meta-analysis of 35 studies in Nordic countries (Valkama et al. 2015). While such cases have demonstrated the usefulness of diversification in increasing N-use efficiency of crops, there is still limited understanding of the molecular mechanisms underlying the role of diversified cropping systems in limiting N losses.

It is well known that the soil microbiome carries out all crucial steps in the biogeochemical cycling of N, including nitrification, denitrification, N fixation, or the nitrite-dependent oxidation of ammonium (Kuypers et al. 2018). A diversified cropping system could prevent the N losses by supporting the ecosystem function of the soil microbial community in soil biogeochemical cycling, *e.g.* via building up more biomass and stabilizing a more diversified microbiome, since the diversity of the microbial community correlates with its capacity to transform N (Delgado-Baquerizo et al. 2016). In agricultural fields, diversified cropping systems, like cover crops were shown to improve soil structure by the formation of aggregates, which provide diverse microhabitats, likely to promote a more diverse microbiome (Qi et al. 2022). It also could increase the soil microbial biomass carbon

(C) and N by at least 40% as revealed from 81 different studies (Muhammad et al. 2021). Furthermore, an analysis of 60 studies found that soil microbial abundance and activity increased with cover crops by up to 27%, (Kim et al. 2020). In tropical cropping systems with maize it was demonstrated that the presence of cover crops affected the abundance of microbial N-cycling genes, and thereby possibly increase N-fertilization efficiency (Momesso et al. 2022; Rocha et al. 2020), and this may also apply to other climatic conditions where crops are cultivated. Given the complex composition and high diversity of the microbiome, including bacteria, archaea and fungi, with their specific and highly heterogeneous properties, different soil microbial taxa may not equally respond to crop diversification and change of N supply *e.g.*, cover crops favored fungi more than bacteria, and ammonia-oxidizing archaea showed a stronger correlation with cover crop biomass than ammonia-oxidizing bacteria (Momesso et al. 2022; Muhammad et al. 2021). While these studies demonstrate that microbial groups and functional potentials can differentially be affected by specific cropping systems, it is still difficult to see a more general response pattern of the soil microbiome including those mediating N transformation processes.

Microbial carbon use efficiency (CUE) is a physiological parameter that indicates how energy rich carbon resources are utilized for microbial growth *versus* immediate loss by maintenance respiration (Geyer et al. 2019). Its usefulness to describe the response of the soil microbiome to N fertilization was already explored but the influence of cropping systems is still poorly understood. N fertilization could reduce microbial C uptake with lower microbial respiration and lower microbial growth rate, potentially by inhibiting the degradation of aromatic compounds (Spohn et al. 2016). However, studies with grassland microbiomes found CUE to be robust towards changes imposed by N and phosphate fertilization (Widdig et al. 2020). Such contrasting results of CUE could be an outcome of both direct and indirect effects on the microhabitat conditions *e.g.*, in agricultural systems rhizodeposition is an important factor that can influence microbial habitats (Denef et al. 2009). Microorganisms tended to use more root-derived C and presented higher microbial CUE in the rhizosphere with abundant N fertilization, potentially owing to the low energy costs for cell uptake under the condition of

high C supply, which was associated with higher N fertilizations (Bicharanloo et al. 2020). In fact, most studies on CUE in cropping systems suffer from the fact that only single sampling events were analyzed and such snapshots may not sufficiently consider the impact of seasonal changes and crop rotations over years. Both have a tremendous effect on the living conditions of soil microorganisms (Liu et al. 2022; Simon et al. 2020). Thus, we still lack a more systematic understanding of the impacts of mineral N fertilization and crop diversification on soil microbial CUE, specific microbial functional groups and the temporal resilience of compositional changes of the soil microbiome.

The objective of this study was to elucidate the effects of different mineral N fertilization rates and the influence of crop diversification on soil physicochemical parameters, soil microbial CUE and the diversity of the soil microbiome, with emphasis to their prokaryotic community members. From an agricultural field experiment located in Lower Saxony, Germany, we collected soil samples over a period of 2.5 years before and after the cropping season from two cropping systems (winter cover crop and undersown ryegrass vs. non-diversified) amended with an excessive amount of 250 kg N ha⁻¹ (250N), a conventional amount of 150 N and a control without any additional N supply. The dynamics of physicochemical soil parameters (pH, C_{org} and N_{total}) were followed along with a cropping regime including a succession of potato, winter rye and maize. The determination of soil microbial CUE was conducted on the soil samples collected from the first three sampling events, while the prokaryotic community dynamics were investigated in those soil samples from the first four sampling events. We hypothesized that conventional 150 N fertilization combined with crop diversification will increase the soil microbial diversity and microbial CUE by providing plentiful C and N sources. In contrast, we suspected that the excessive N fertilization would decrease microbial diversity as well as their CUE by accelerating soil acidification and thereby neutralize the positive effect of crop diversification seen at lower N supplies. Finally, because of the different microhabitat conditions and nutrient supplies in spring and autumn, we assumed that soil microbiomes as analyzed before and after the cropping season would show seasonal differences.

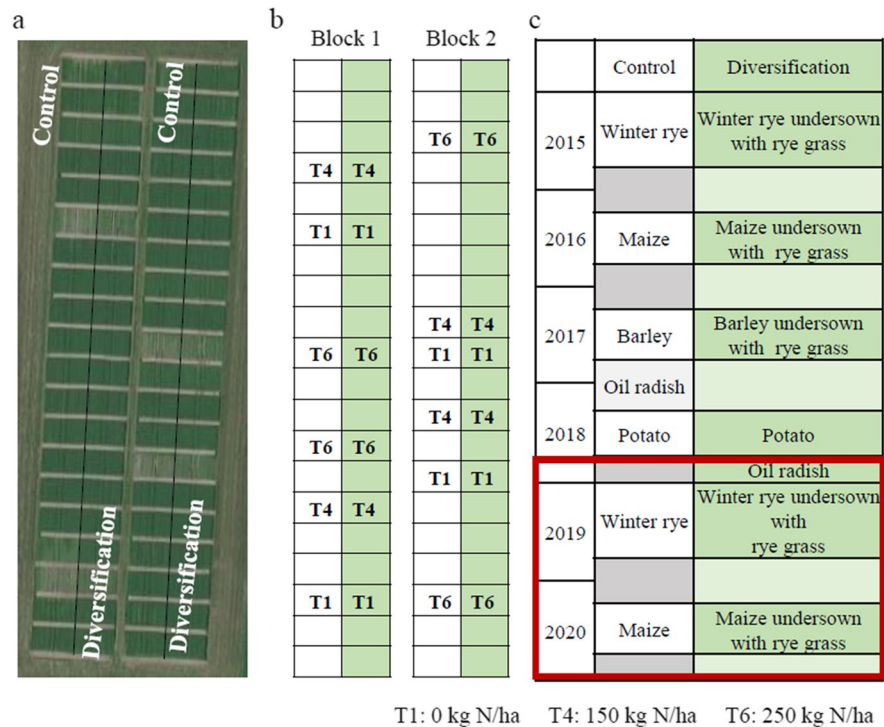
Materials and methods

Experimental site and soil sampling

The soil was collected from an ongoing crop diversification and N fertilization trial initiated in 2014, located at Hamerstorf, Germany (52°54'22" N, 10°27'6" E, 50 m a.s.l.). This field site is maintained as part of a field-experimental-system under the technical authority of the Chamber of Agriculture Lower Saxony (Landwirtschaftskammer Niedersachsen), and it is financed by the water-treatment charge of Lower Saxony. The average annual precipitations of the region were 406 mm, 628 mm and 542 mm, while the mean annual temperatures were 10.4 °C, 10.4 °C and 10.6 °C from 2018 to 2020, respectively. The soil type was classified as silty sand (Su2) with 83% sand, 14% silt, and 3% clay, respectively.

This experimental field simulated a model for a water protection area to analyze how risks of N leaching into ground and surface water can be reduced by crop diversification and N fertilizer management. There were two cropping trials in a split-block design, including a control system and crop diversification system, both cultivated with potato (*Solanum tuberosum*), winter rye (*Secale cereale*), and maize (*Zea mays*) from 2018 to 2020, respectively (Fig. 1). The diversified cropping system included winter cover crops (oil radish) between winter rye (2019) and potato (2018) cultivations, and undersown ryegrass with cultivation of winter rye and maize. In addition to the crop diversification, different amounts of mineral N fertilizer treatments were implemented in each cropping trial. The mineral N fertilizer consisted of half ammonium and half nitrate nitrogen. Irrespective of the treatments, above ground crop residues were removed after harvesting. This study selected three N fertilization treatments including T1 (without N fertilization, abbreviated here as 0N), T4 (amended with a conventional amount of 150 kg N ha⁻¹; 150N) and T6 (amended with an excessive amount of 250 kg N ha⁻¹; 250N). Prior to this study during the growing season of 2018 and adjusted for the N demand of potato, the conventional amount of N fertilizer was 160 kg N ha⁻¹ in T4 treatment, and 240 kg N ha⁻¹ in T6 treatment, respectively. Each treatment included four replicate plots ordered in a randomized block design (9 m×12 m), which were all sampled. The

Fig. 1 **a** Aerial view of the Hamerstorf field site indicating the field blocks with control and diversified cropping systems (image generated by Google Earth on June 2, 2017); **b** Schematic view of the field plots with their respective N applications; **c** Crop rotations of the two cropping systems (control and crop diversification) since 2015. The red lined frame indicates the time period of sampling for this study



fertilizers were applied at the beginning and the middle of the respective crop growth cycles.

Soil samples were collected in spring (S) at the onset and in autumn (A) at the end of the growing season. The exact sampling dates mainly depended on the weather condition and local agronomic plan, thus it varied between years. The sampling date in autumn 2018 was October 25 (abbreviated here as 2018A), in spring 2019 March 25 (2019S) and in autumn October 24 (2019A), in spring 2020 May 7 (2020S) and in autumn October 15 (2020A). Soil samples were collected by shovel and sampled from four spatially distinct spots in the center area of each plot from a depth of 1 to 10 cm after gently removing the upper-most surface soil. Soils from these spots were well mixed to generate a composite sample of each plot. These soil samples were kept at ambient temperature for a maximum of 3 h and then processed in the laboratory, where the soil was first air-dried and then sieved to 2 mm in order to remove residual straw and plant roots. Soil pH was measured in 0.01 M calcium chloride with a pH meter (HI221 Microprocessor, Hanna Instruments Germany). C_{org} and N_{total} were analyzed by dry combustion in an elemental analyzer (Leco TruMac, St. Joseph, MI). Soil pH, C_{org} and N_{total}

were measured for all soil samples collected in this study, and so was the determination of the abundance of bacteria, archaea and fungi by quantitative PCR (qPCR). Prokaryotic diversity and community composition were characterized from samples of the first four sampling events (2018A to 2020S), thus with annual replication for each season, and soil microbial carbon use efficiency (CUE) was determined from samples of the first three sampling events. Analyses of these parameters were always based on all four independent replicates of each treatment.

DNA extraction, quantification and sequencing

Microbial DNA was extracted from 500 mg of soil with the FastDNA®SPIN Kit (MP Biomedicals, Eschwege, Germany) following the manufacturer's protocol. The DNA samples were stored at -20°C for gene quantification and -80°C for 16S rRNA sequencing. The abundance of bacteria, archaea and fungi was quantified with the qPCR method by using a Biorad CFX96 Realtime PCR cycler with C1000 Touch (Biorad, Feldkirchen, Germany). TaqMan assays were used to estimate bacterial 16S rRNA genes using the primer pair BAC338F/BAC805R

with probe BAC516F (Yu et al. 2005). The archaeal gene copy numbers were quantified with primers and probe: ARC787F, ARC1059R and ARC915F (Yu et al. 2005). The qPCR reactions for bacteria and archaea 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. The qPCR cycling conditions for bacterial and archaeal gene quantification consisted of an initial 10 min at 95 °C followed by 39 cycles of denaturing at 95 °C for 15 s and annealing/polymerase extension at 60 °C for 1 min. The abundance of fungi was estimated by quantifying a fragment of the ITS1 region by qPCR, using the primer pair NSII/ 58A2R, in 20 μL reactions: 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 (Martin and Rygielwicz 2005). Its cycling condition consisted of an initial 10 min at 95 °C, followed by 39 cycles of 95 °C for 15 s, 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 with culture strains obtained from the DSMZ, Braunschweig, Germany, *i.e.* *Bacillus subtilis*, *Methanobacterium oryzae* (DSM 11,106) and *Fusarium culmorum* (DSM 62,191), respectively. Amplification efficiencies were 95% (bacteria), 96% (archaea) and 93% (fungi); correlation coefficients were above 0.998 for bacteria, 0.996 for archaea, and 0.995 for fungi.

For 16S rRNA sequencing, 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 \times 300 bp) for the 16S rRNA gene PCR amplicons was performed on the Illumina MiSeq platform by LGC Genomic GmbH, Berlin, Germany. All the samples were sequenced together in the same run and all the sequencing data were analyzed together with the QIIME2 platform (Bolyen et al. 2019). Considering the blind ligation of Illumina adaptor and amplicon fragments, raw sequence reads were firstly reoriented into respective forward and reverse read files by using an in-house Python

script (github.com/DamienFinn/MiSeq_read_reorientation). The Cutadapt paired-end method was used to demultiplex the reoriented sequence reads on the QIIME2 platform (Martin 2011). Later, the forward and reverse reads were merged by Vsearch join-pairs function (Rognes et al. 2016). After the merge step, sequence reads were truncated at positions 280 and 40 by DADA2 denoise-single function (Callahan et al. 2016). In this study, amplicon sequence variants (ASV) were adopted and assigned with the Silva 138 database (Quast et al. 2013; Yilmaz et al. 2014). Eukaryote-associated ASV (mitochondria and chloroplasts) were removed. The DNA sequence reads have been deposited in the European Nucleotide Archive database <https://www.ebi.ac.uk/ena/browser/> Project Accession number PRJEB47841).

Determination of soil microbial growth, respiration and carbon use efficiency (CUE)

Soil microbial CUE was determined with the ^{18}O -labeling method. This method quantifies microbial growth via the incorporation of ^{18}O from H_2^{18}O into microbial DNA as previously described in detail (Poeplau et al. 2019; Schroeder et al. 2021). Briefly, 20 g to 25 g fresh weight of soil from each sample was adjusted to 45% (wt/wt) water-holding capacity, then preincubated for 7 d at 15 °C in the dark. After the preincubation, two aliquots of approximately 400 mg fresh weight of soil were collected into a 2 mL incubation microtube and put into a 20 mL air-tight gas vial later sealed with a rubber and aluminum cap for the assessment of ^{18}O -incorporation into newly formed DNA, where one aliquot represented the natural abundance reference and the other was labelled. The soil water content in the two incubation microtubes per sample was adjusted to 60% water holding capacity with ddH_2O in the unlabeled incubation microtube and with H_2^{18}O (97% atom percent w/w) in labelled incubation microtube with the syringe. Specifically, the ^{18}O content in the final soil water was maintained at 20% (wt/wt). In order to allow the determination of microbial respiration rates from the increase in CO_2 concentration, the starting conditions of the labeled samples were adjusted to 349 ppm CO_2 and 1.3 bar. Both unlabeled and labeled incubation microtubes were kept at 15 °C for 24 h in the dark. Headspace gas samples were extracted only from the labelled incubation microtubes using a gas-tight syringe for CO_2

concentration measurement to determine the respiration rate, while the soil samples from both unlabeled and labeled incubation microtubes were immediately frozen with liquid N₂ and stored at -80 °C for DNA extraction. The CO₂ concentration was measured by gas chromatography with an electron capture detector (Agilent 7890A GC, Agilent Technologies). The amount of respired CO₂ (microbial respiration, ng C g⁻¹ dry weight soil h⁻¹) was calculated as described before (Schroeder et al. 2021). DNA was extracted by FastDNA®-SPIN Kit (MP Biomedicals, Eschwege, Germany) and eluted in 100 µL DNase-free water. Later the DNA concentration was quantified by QuantiT PicoGreen dsDNA Kit (Invitrogen, Carlsbad, CA, USA). And 60 µL DNA from both unlabeled and labeled samples were dried at 60 °C in silver capsules. The abundance of ¹⁸O was measured by a high-temperature conversion/elemental analyzer (TC/EA) (Thermo Fisher Scientific) coupled with a Delta V Plus isotope ratio mass spectrometer via a ConFloIV interface (Thermo Fisher Scientific). The total DNA produced in 24 h (DNA_{produced}, µg) was calculated as described in detail elsewhere (Poelau et al. 2019; Schroeder et al. 2021).

To convert newly formed DNA into C directed to microbial biomass during the 24-h incubation, a conversion factor is needed (Spohn et al. 2016; Schroeder et al. 2021). Therefore, microbial biomass was measured on 7 g dry mass aliquots of soil using the chloroform-fumigation extraction method (Vance et al. 1987). The fumigation process lasted 24 h at room temperature in a dark environment. Fumigated and non-fumigated samples were extracted with 0.5 M K₂SO₄ solution in a 1:4 dry weight/volume ratio. The C_{org} was measured in a DIMATOC 2000 (Dimatec, Essen, Germany). The efficiency of microbial C extraction was assumed as 0.45 (Joergensen 1996). Based on the result of microbial biomass C content (µg C g⁻¹ dw soil) and DNA concentration (µg g⁻¹ dw soil) derived from PicoGreen screening, the conversion factor f_{DNA} was determined by their ratio for each sample. This conversion factor f_{DNA} was used to calculate the amount of microbial biomass C production (microbial growth, ng C g⁻¹ dw soil h⁻¹) during 24 h incubation based on the DNA_{produced} (µg) derived from ¹⁸O labelling for each sample. The CUE was indicated by the ratio of microbial biomass C production (microbial growth) and total C uptake, which was the sum of microbial respiration and microbial growth.

Data analysis

Rarefaction analysis was performed to estimate the sequence coverage with the vegan package (v.2.5–7) in R (v.4.0.3) (Revelle 2022). Sequences per sample were rarefied to 6,311 to normalize sequencing depth for the following analyses. The Shannon index, richness and evenness were calculated in R using the vegan package. Fisher's Least Significant Difference (LSD) post hoc test was performed with the agricolae package (v. 1.3–3) in R (Mendiburu 2021), and the significant differences were considered as $p < 0.05$. The fit of LMEs was calculated with restricted maximum likelihood with the lmerTest package (v. 3.1–3) in R (Kuznetsova et al. 2017). Spearman's correlation coefficient was calculated with the package stats (v. 4.1.1). Non-metric multidimensional scaling (NMDS) and Analysis of similarities (ANOSIM) tests were conducted with the vegan package (v.2.5–7). Statistically significant differences in taxonomic ranks (family and ASV) between different treatments were calculated using the 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 psych package (v.2.1.9), funrar package (v.1.4.1) and igraph package (v.1.2.6) in R (Csardi and Nepusz 2006; Grenié et al. 2017; Revelle 2022; Wickham et al. 2021). For generating correlation networks only ASV with an average read of above one in all samples were considered. More stringent conditions with higher average reads of above 3, 5 and 7 were also analyzed. The correlation matrix was generated by the psych package, and correlation coefficients ($r > 0.7$ or $r < -0.7$) and FDR (false discovery rate) corrected p -values less than 0.05 were used to form the networks. The network images were created with igraph package, as well as the topological features. The microbial functional profile was predicted using the Functional Annotation of Prokaryotic Taxa (FAPROTAX, v.1.2.4) (Louca et al. 2016). FAPROTAX is a database which can map taxonomic annotation profiles of ASV to their potential functions. It is a software for converting ASV into putative functional profiles. It distinguishes over 80 different functions and considers over 7,500 functional annotations obtained from over 4,600 taxa.

Results

Effect of N application rates and crop diversification on physicochemical soil parameters

Both mineral N fertilization and cropping regimes significantly affected the soil pH ($p < 0.05$; Table 1). The acidification effect of N addition was significant ($p < 0.01$) at all five sampling events, and, for the non-diversified cropping system, the shift was related to the amount of N added. In the winter rye season, the crop growth increased the soil pH by 0.7 in 150N and 0.8 in 250N on average while maize cultivation caused a decline in soil pH value by 0.6 in 150N and 0.9 in 250N on average ($p < 0.05$). In contrast, for the diversified system, there was no clear relationship with the amount of N added. The pH shift after winter rye crop was only around 0.1 on average with both 150N and 250N, respectively. Similarly, maize cultivation caused a relatively minor decline in soil pH around 0.4, irrespective of the amount of N supply.

Neither mineral N fertilization nor crop diversification affected the soil C_{org} ($p > 0.05$). In contrast, cultivation of winter rye appeared to increase C_{org} , especially in diversified cropping systems, while maize cultivation caused a slight reduction, which was on average higher with 2.56% in non-diversified cropping systems than in diversified cropping systems with 1.86% ($p < 0.05$). Irrespective of diversification or N supply, C_{org} showed a clear trend to decrease over the winter season. Overall, the 2.5 years of cultivation caused a minor loss of soil C_{org} .

For N_{total} , crop cultivation of both winter rye and maize resulted in a decrease during the growing season, but this loss was restored over winter ($p < 0.05$). Crop diversification showed no significant effect on soil N_{total} ($p > 0.05$). Mineral N fertilization affected the N_{total} in 2019A and 2020S ($p < 0.05$), but not at other sampling dates. N_{total} reached a peak in 150N in 2019A and for the 250N treatment in 2020S, regardless of crop diversification.

The total soil C:N ratio was neither affected by N fertilization nor crop diversification ($p > 0.05$), except in the samples taken in 2019A, *i.e.* after cultivation of winter rye. Overall, the C:N ratio increased during crop growth and declined over winter ($p < 0.05$). Due to the split block design of the non-diversified and diversified cropping systems, the block effect was also considered, and there were no significant effects

on physicochemical soil parameters detectable, with the one exception of N_{total} in the samples taken in 2020A, *i.e.* after maize with or without undersown ryegrass.

Effect of the N fertilization and crop diversification on soil microbial abundance

The mineral N fertilization affected fungal abundances consistently across all sampling dates ($p < 0.05$; Fig. 2; Table S1-2), while the effects on the abundance of bacteria and archaea were inconsistent. Crop diversification surprisingly showed a stronger influence on archaeal abundance than on bacteria and fungi. At three of the five sampling events, the archaeal gene copy numbers were different between the control and diversified cropping systems except for 2020S and 2020A ($p < 0.05$). In contrast, crop diversification affected the bacterial and fungal abundance only in 2019S ($p < 0.05$). The increased abundance of all three microbial groups in 2019S can be linked to a better microbial survival during winter in the presence of oil radish in the diversified system as compared to bulk soil in the non-diversified control. For bacterial abundance, significant differences were seen in only 3 of 15 cases ($p < 0.05$; Fig. 2), but interestingly these always indicated a positive effect by crop diversification; for fungi, five cases were significantly different, and four of them also indicated positive effects. In contrast, the archaea were the most responsive group to diversification in terms of abundance, and showed a negative response to diversification in seven out of eight cases.

Overall, the abundance of soil bacteria, archaea and fungi appeared to be more strongly influenced by crop species and season than by N fertilization and crop diversification, respectively. During the winter rye season, the soil bacterial, archaeal and fungal abundances increased, but they decreased after the growth of maize ($p < 0.05$) (2020A). There was no significant block effect on soil microbial abundance.

The changes in bacterial ($p < 0.05$), archaeal ($p < 0.001$) and fungal ($p < 0.01$) abundances were highly associated with fluctuations of the pH, according to Spearman's correlation coefficient. Positive correlations occurred in the range between pH 4.4 to 5.8. For C_{org} and N_{total} , there were positive correlations with bacterial ($p < 0.05$) and fungal ($p < 0.05$) abundance, but not archaeal abundances ($p > 0.05$).

Table 1 Physicochemical soil parameters of different treatments (sampling events, cropping system, fertilization). A indicates autumn, S indicates spring sampling events

	Sampling event	Block	Crop diversi- fication	Nitrogen fertilization	Crop * Nitrogen	Control			Diversification			
						ON			ON			
						150N	250N	150N	250N	150N	250N	
pH	2018A	Potato	ns	**	ns	5.6±0.1	5.6±0.1	5.6±0.0	5.6±0.1	5.5±0.1	5.6±0.1	5.6±0.1
	2019S	Winter rye	ns	***	ns	5.4±0.1	4.6±0.1	5.5±0.1	4.7±0.1	4.7±0.1	4.5±0.1	4.5±0.1
	2019A		ns	***	*	5.5±0.2	5.3±0.2	5.3±0.1	4.8±0.1	4.8±0.1	4.6±0.2	4.6±0.2
	2020S	Maize	ns	**	ns	5.5±0.2	5.5±0.3	5.3±0.1	5.1±0.3	5.1±0.3	4.8±0.3	4.8±0.3
	2020A		ns	***	ns	5.6±0.1	4.9±0.1	5.4±0.1	4.7±0.2	4.7±0.2	4.4±0.1	4.4±0.1
C _{org}	2018A	Potato	ns	ns	ns	8.43±0.36	8.39±0.40	8.22±0.74	8.35±0.32	8.35±0.32	8.42±0.51	8.42±0.51
	2019S	Winter rye	ns	ns	ns	8.41±0.35	7.91±0.81	7.75±1.04	7.68±0.56	7.68±0.56	8.48±0.77	8.48±0.77
	2019A		ns	ns	ns	8.04±0.42	8.66±0.25	8.17±0.66	7.87±1.12	8.31±0.39	8.49±0.68	8.49±0.68
	2020S	Maize	ns	ns	ns	7.73±0.57	7.79±0.49	8.12±0.46	7.66±0.89	7.87±0.24	8.08±0.57	8.08±0.57
	2020A		ns	ns	ns	7.53±1.06	7.45±0.55	8.06±0.96	7.58±0.67	7.66±0.48	7.93±0.49	7.93±0.49
N _{total}	2018A	Potato	ns	ns	ns	0.60±0.12	0.56±0.10	0.52±0.07	0.57±0.07	0.57±0.07	0.63±0.08	0.63±0.08
	2019S	Winter rye	ns	ns	ns	0.60±0.08	0.59±0.08	0.72±0.14	0.62±0.11	0.60±0.06	0.62±0.05	0.62±0.05
	2019A		ns	**	ns	0.47±0.04	0.60±0.03	0.52±0.08	0.51±0.04	0.60±0.01	0.55±0.08	0.55±0.08
	2020S	Maize	ns	*	ns	0.65±0.04	0.68±0.03	0.77±0.04	0.66±0.05	0.67±0.06	0.70±0.08	0.70±0.08
	2020A		**	ns	ns	0.52±0.04	0.55±0.14	0.62±0.12	0.49±0.07	0.52±0.07	0.49±0.10	0.49±0.10
C:N ratio	2018A	Potato	ns	ns	ns	15±3	15±2	16±1	15±1	15±1	14±1	14±1
	2019S	Winter rye	ns	ns	*	14±1	14±1	13±1	13±1	13±1	14±0	14±0
	2019A		ns	*	ns	17±2	15±1	15±1	14±2	14±2	16±1	16±1
	2020S	Maize	ns	ns	ns	12±1	11±1	12±1	12±1	12±1	12±1	12±1
	2020A		ns	ns	ns	15±1	14±3	16±3	15±2	15±2	16±3	16±3

Different letters indicate statistical differences among different sampling campaign, $p < 0.05$; Differences of values between sampling events are indicated with a to d, starting with the highest values; The significance levels are indicated as follows: ***0.001 **0.01 *0.05, ns indicates not significant

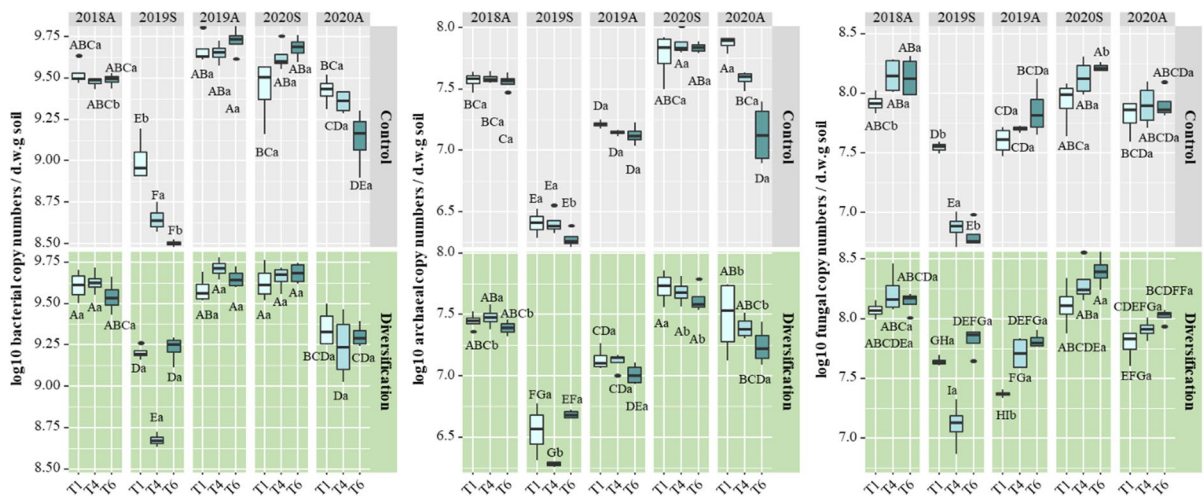


Fig. 2 Effect of inorganic N fertilization and crop diversification on the abundances of bacteria, archaea and fungi at the experimental field site, as assessed by qPCR from directly extracted soil DNA. Capital letters and lower-case letters indi-

cate significant differences among different N fertilizations (T1: 0 kg N/ha; T4: 150 kg N/ha; T6: 250 kg N/ha; $n=4$) and between two cropping systems (non-diversified control vs. crop diversification)

Furthermore, there was a negative correlation between abundances of both archaea and fungi, with the C:N ratio ($p < 0.05$).

Microbial carbon use efficiency

Neither mineral N fertilization nor crop diversification affected the soil microbial CUE in any of the three sampling events (2018A, 2019S, 2019A) analyzed ($p > 0.05$; Fig. 3; Table S3-4). However, crop diversification reduced microbial respiration and growth in the 2019A, and this was most pronounced with the 250N supply. At the beginning of the winter rye growing season (2019S), the N fertilization reduced microbial respiration, but only in the diversified cropping system ($p < 0.05$). After the winter rye growing season (2019A), N fertilization promoted microbial growth in the non-diversified cropping system ($p < 0.05$). This promotion was stronger with 250N than with 150N. Notably, CUE varied with crop species and season.

Across the three sampling events, microbial CUE was found to be driven by alternations of microbial growth rather than the changes of microbial respiration rates (Fig. 4). Strong negative correlations were found between microbial CUE value and soil N_{total} ($p < 0.01$), while the C:N ratio correlated positively with the CUE values ($p < 0.05$). In addition to the

correlation with soil chemical parameters, the abundance of bacteria but not of archaea and fungi correlated positively with microbial CUE, respiration and growth ($p < 0.001$), respectively.

Composition and diversity of the prokaryotic communities

In total, 3.63 million high-quality 16S rRNA gene amplicon sequences were obtained from the 96 samples included in this study. These grouped into 31,733 ASV. Sequences assigned to bacteria and archaea were 96.2% and 3.8%, respectively (Fig. S1). The most abundant bacterial phyla were *Proteobacteria* (relative abundance 29.2%), *Actinobacteriota* (21.8%), *Acidobacteriota* (11.8%), *Bacteroidota* (10.4%), *Firmicutes* (7.05%), *Chloroflexi* (5.19%), *Verrucomicrobiota* (4.50%), *Planctomycetota* (3.47%), *Gemmatimonadota* (3.12%), *Myxococcota* (2.34%) and *Patescibacteria* (1.12%). The dominant archaea were *Crenarchaeota* (99.0%).

Correlation analyses were conducted to evaluate the response of specific bacterial phyla to the different treatments and field conditions. *Bacteroidota* showed a strong positive correlation with C:N ratio ($p < 0.001$; Table S5). Furthermore, the relative abundance of *Planctomycetota* correlated positively with microbial respiration ($p < 0.01$) and microbial

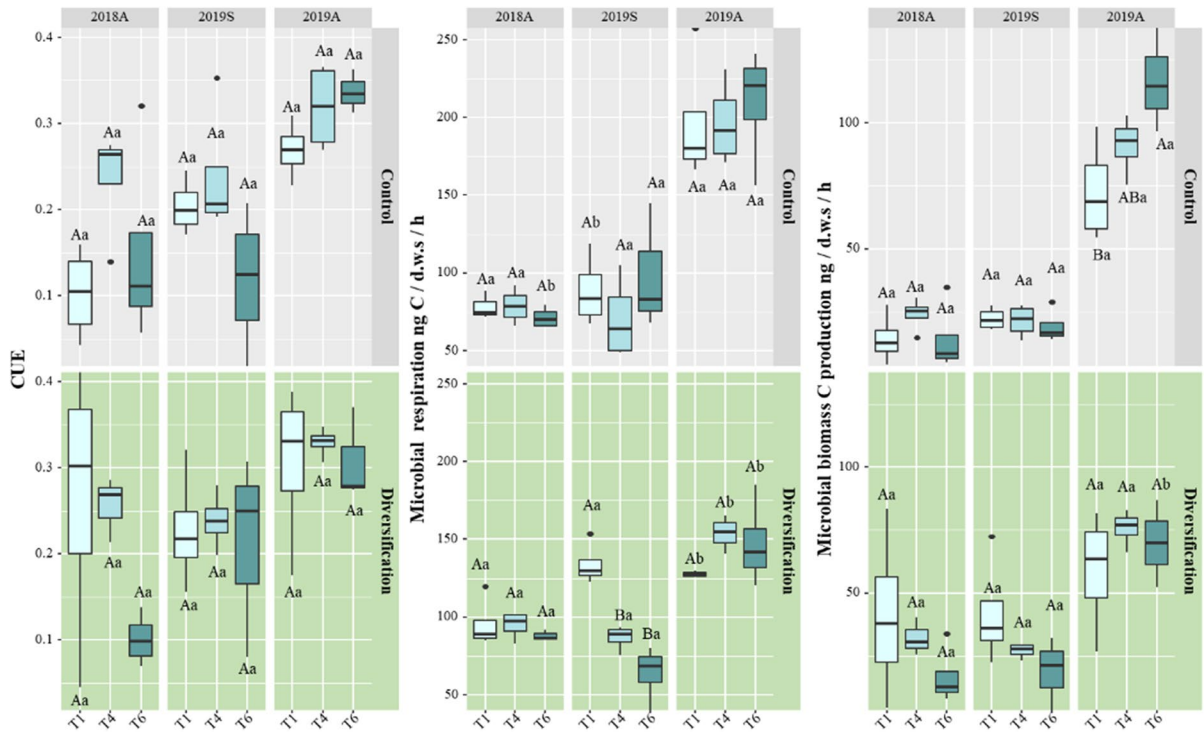


Fig. 3 Effect of N fertilizations and crop diversification on soil microbial carbon use efficiency (CUE), microbial respiration and growth. Capital letters and lower-case letters indicate significant difference among different N fertilizations (T1: 0 kg N/ha; T4: 150 kg N/ha; T6: 250 kg N/ha; n=4) and between

two cropping systems (non-diversified control vs. crop diversification) according to LSD test, respectively. Three values are missing in microbial respiration and four in microbial growth due to technical problems

growth ($p < 0.05$), respectively. In contrast, the relative abundance of *Firmicutes* correlated negatively with microbial growth and CUE ($p < 0.05$). *Myxococcota* correlated negatively with soil pH ($p < 0.01$), and positively with microbial CUE ($p < 0.05$), microbial respiration and microbial growth ($p < 0.01$). In addition, the relative abundance of both *Proteobacteria* and *Bacteroidota* correlated negatively with microbial respiration ($p < 0.05$). On the other hand, microbial respiration correlated positively with the relative abundance of *Gemmatimonadota* ($p < 0.05$). Overall, the phylum-specific correlations suggest different adaptations of the soil microbiome to changing soil conditions as represented by the selected parameters.

Neither the level of N fertilization nor the crop diversification changed the community evenness ($p > 0.05$; Fig. S2). For the non-diversified cropping system, N fertilizations had no effect on the ASV richness and Shannon diversity, the latter indicated by the Shannon index ($p > 0.05$). In contrast, crop

diversification increased both richness and Shannon diversity in the 150N treatment by the end of crop growing seasons ($p < 0.05$).

Effect of the N fertilization and crop diversification on the prokaryotic community and network structure

Non-metric multidimensional scaling (NMDS) plots were constructed to compare the overall differences in soil prokaryotic communities between different treatments (Fig. 5; Table S6). ANOSIM revealed that the community structures differed ($p < 0.001$) among different sampling events accompanied by distinct crop species and growth stages. The mineral N fertilization showed a significant impact on the prokaryotic community ($p < 0.05$) with the one exception of samples taken in spring 2020. Crop diversification significantly changed the community structure at the end of the growing season ($p < 0.05$) but not at the beginning ($p > 0.05$).

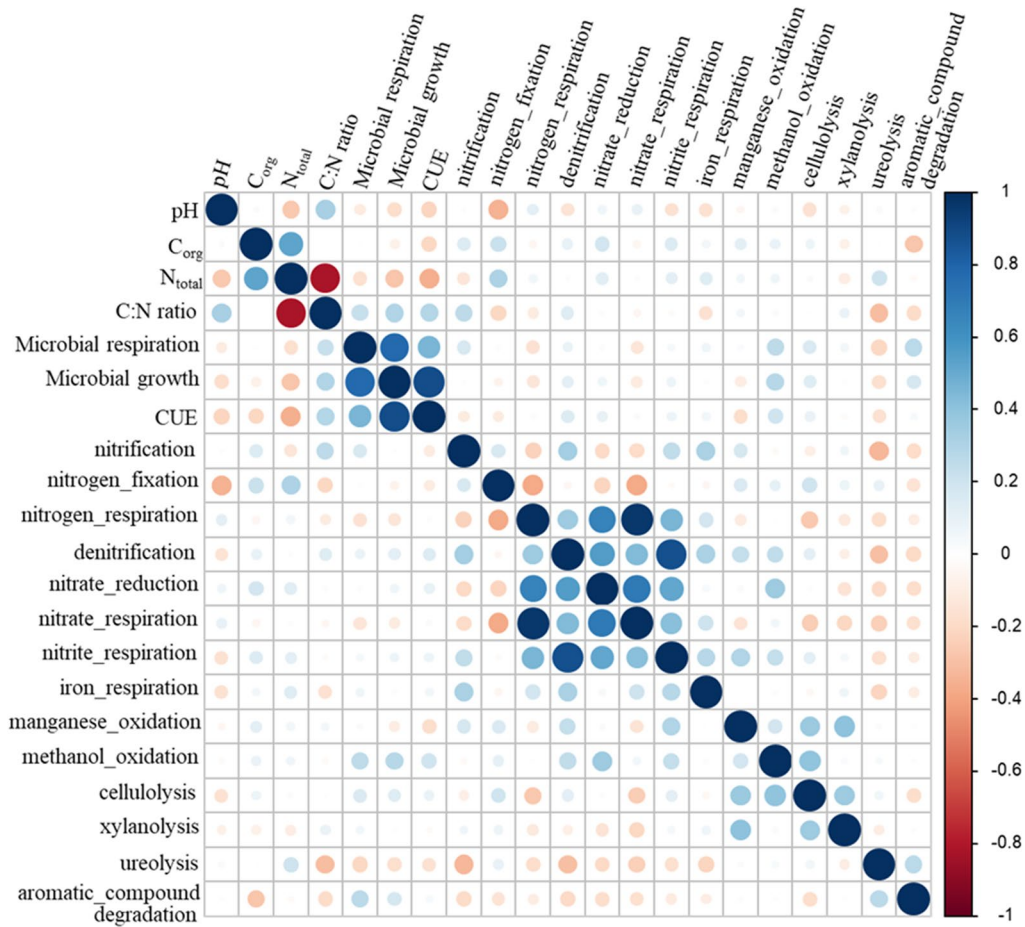


Fig. 4 Spearman’s rank correlation matrix of physicochemical soil parameters, parameters of soil microbial metabolism and the relative abundance of microbial functional groups. Strong correlations are indicated by large circles; weak correlations are indicated by smaller circles. Positive correlations are dis-

played in blue and negative correlations in red. Both circle size and colour intensity are proportional to the spearman correlation coefficients. The correlation analysis was based on 72 replicates (three N treatment with four replicates in two cropping systems and all three sampling events combined)

The identification of responsive prokaryotic taxa was performed at the family and ASV level (Fig. S3 and S4), but by means of pairwise comparison among three mineral N treatments and two cropping systems in four sampling events, no significantly responding ASV or family were detected.

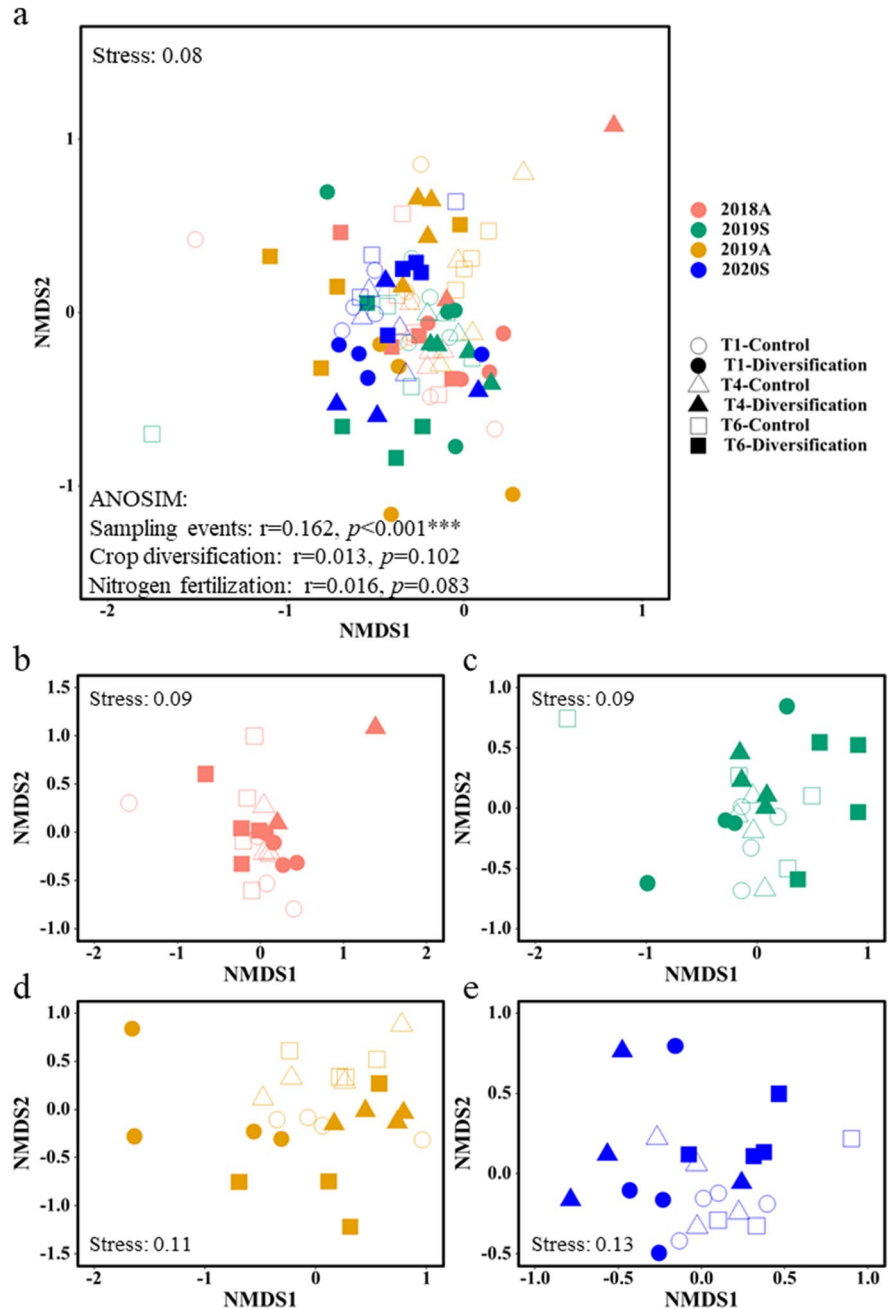
Correlation networks of co-occurring ASV were used to explore assemblage patterns under different conditions of N fertilization and crops (Fig. 6 and Fig. S5; Table 2). The network constructed with data from all four sampling times revealed a striking difference between the non-diversified and the diversified cropping systems at the conventional 150N supply:

Networks of the diversified system were characterized by a higher number of nodes, edges, and other descriptors demonstrating a much more elaborated structure.

Effect of the N fertilization and crop diversification on suspected microbial functional groups

FAPROTAX analysis allowed to assign 17.8% (5,640 out of 31,733) of all ASV to at least one potential ecological type. Overall, those assigned ASV encompassed a total of 63 different ecological types, with chemo-heterotrophy and aerobic chemo-heterotrophy being the dominant functions. A total of 14 ecological

Fig. 5 Non-metric multidimensional scaling (NMDS) plots for prokaryotic communities across four sampling events (**a**) and individual sampling events (**b–e**). Bray–Curtis distance matrix was used to generate the NMDS plots and stress values are presented



types which represented functional groups mainly contributing to the C and N cycles were selected for further analyses, as these functions were considered as being most relevant for the arable land use in this study (Fig. 4 and Fig. S6). Except for nitrification, N-transforming functional groups involved in nitrogen respiration, denitrification, nitrate reduction,

nitrate respiration and nitrite respiration, responded in a similar way. Crop diversification showed strong effects on increasing the relative abundance of taxa associated with nitrification, while decreasing the proportion of nitrogen and nitrate respiration, nitrate reduction and aromatic compound degradation functional groups in the excessive 250N treatment. This

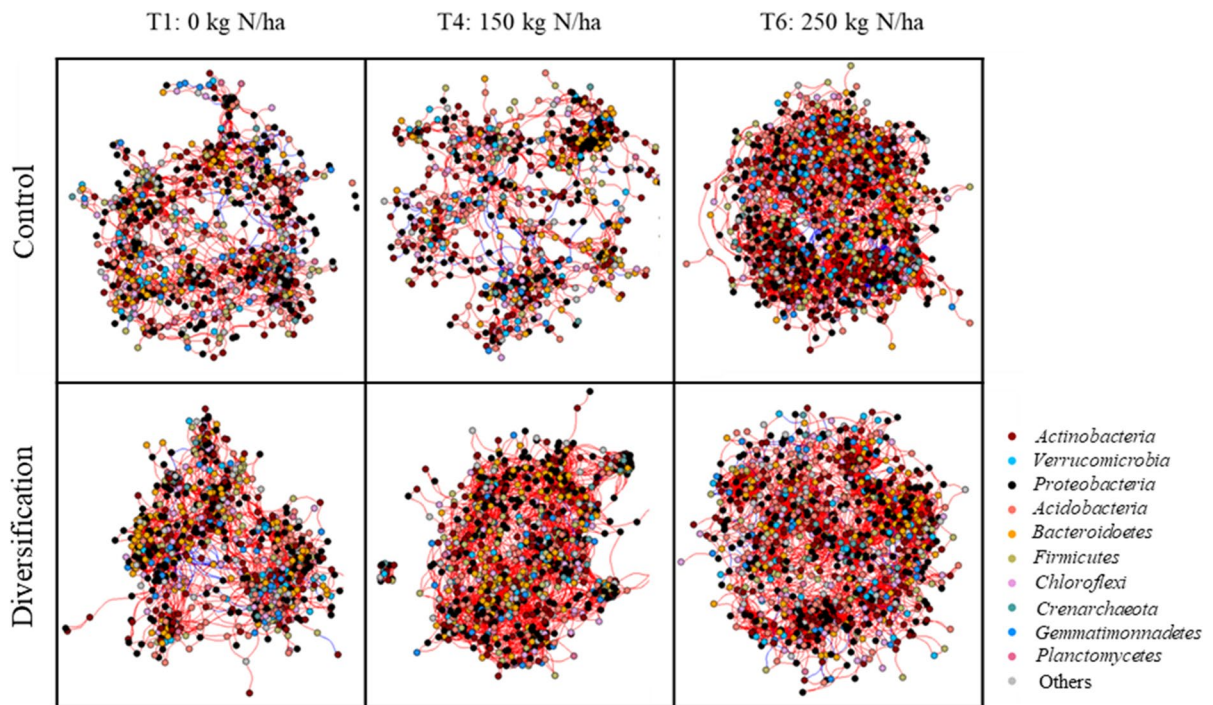


Fig. 6 Co-occurrence networks of amplicon sequence variants (ASV) representing the prokaryotic communities across four sampling events in different treatments based on average ASV numbers of above 1 in all samples. Connections indicate strong ($r > 0.7$ or $r < -0.7$) and significant (corrected p -value < 0.05) correlations. The positive and negative correlations between

nodes are displayed with red and blue lines, respectively. Networks were constructed based on 16 replicates (each treatment, with four replicates and all four sampling events combined). For network variants based on higher average ASV see Supplemental material (Fig. S5)

Table 2 The topological features of the co-occurrence networks

Crop	Nitrogen fertilization	Nodes	Edges	Average degree	Edge density	Clustering coefficient	Betweenness centralization	Degree centralization
Control	T1: 0N	845	6,046	7.155	0.017	0.813	0.070	0.059
	T4: 150N	694	3,418	4.925	0.014	0.583	0.129	0.038
	T6: 250N	1,118	15,177	13.575	0.024	0.702	0.045	0.087
Diversification	T1: 0N	957	9,570	10.000	0.021	0.718	0.052	0.047
	T4: 150N	1,041	12,607	12.110	0.023	0.830	0.078	0.063
	T6: 250N	973	8,686	8.927	0.018	0.652	0.035	0.041

effect was most pronounced with samples from 2019A, *i.e.*, after winter rye with or without under-sown ryegrass. The relative abundance of ASV associated with N fixation correlated negatively with soil pH ($p < 0.05$) but positively with C_{org} ($p < 0.05$) and N_{total} ($p < 0.05$). ASV associated with methanol oxidation and aromatic compound degradation correlated positively with the microbial CUE, growth and respiration, while the ureolysis showed a negative relationship with those parameters.

Discussion

The cultivation of crops on arable land has immediate consequences for the living conditions of the soil microbiome. Soil tillage, the addition of fertilizers and the sequences of different crops with their particular root architectures and organic exudations all together can change soil microhabitats and, thus, are likely of inducing adaptation processes emerging in structurally and functionally different microbiomes.

Both soil pH and organic nutrients have been shown to act as major selective factors for shaping the soil microbiome (Fierer and Jackson 2006; Szoboszlay et al. 2017). In our study, along with a sequence of cultivating potato, winter rye and maize, we observed shifts of the soil pH in a range between 4.4 and 5.8. The application of mineral N fertilizer caused soil acidification, which is also known from other studies (Schroder et al. 2011; Snapp and Surapur 2018; Yang et al. 2018). Thus, as a consequence of mineral fertilization, the microbiome does not only receive an additional source of N, but also faces a decrease in pH. While additional N will change the soil C:N ratio, which could promote growth of N-limited microorganisms, the decline in pH may also result in mobilization of otherwise soil-adsorbed nutrients, due to weakening anionic bonds with clay minerals by a decline of bivalent cations *i.e.* Ca^{2+} or Mg^{2+} (Goulding 2016). In fact, a positive growth effect of additional N was seen for fungal abundance, but not for bacteria or archaea. To merely explain this effect by the additional N would be surprising, considering that fungi have a higher C:N ratio (Six et al. 2006) and therefore lower N requirement than bacteria (Holland and Coleman 1987). But it was already demonstrated that the availability of N would not always suffice to explain differential growth between fungi and bacteria (Rousk and Baath 2007). Possibly, the decrease in pH mobilized C adsorbed at mineral surfaces, which would preferentially be metabolized by fungi and thus support their growth rather than growth of bacteria.

A lack of consistent N fertilizer impact on bacterial growth was also indicated by the fact that there were no clear N-induced compositional shifts in the prokaryotic community structure (which was represented to 96% by bacteria). Furthermore, neither microbial growth, as measured by incorporation of ^{18}O from H_2^{18}O into DNA, nor microbial respiration or the CUE were significantly and continually affected by the N additions. Apparently, these results contradicted the conclusion on N-stimulated fungal growth obtained with qPCR. The different techniques and their respective sensitivities used in this study remain to be determined, for characterizing properties of the soil microbiome under different environmental conditions. Even the compositional analyses of the prokaryotic microbiome determined in this study by massively parallel 16S rRNA gene amplicon sequencing failed to detect tangible effects of the N fertilizations on the community

structure, thus supporting results obtained with respiration, microbial growth and CUE. Especially considering the two-step PCR for sequencing preparation was used in this study, the response of low-abundant microorganisms to N fertilization and crop diversification might be underestimated (Finn et al. 2022).

Possibly the failure to detect a fertilizer effect on the prokaryotic community structure was influenced by the fact that we sampled bulk soil rather than rhizosphere, since we wanted to consider the impact on the whole soil community and not only on a specific compartment. The sampling of bulk soil rather than rhizosphere in this study probably captured more 16S rRNA gene signals from inactive or even dead bacterial cells, thus masking the indirect effects triggered by root exudation. Furthermore, it should be noted that the experimental design of this agricultural field study only allowed four replicates for each treatment, which probably limited our capacity to detect less pronounced but significant effects, given the typical intrinsic environmental variation under field conditions.

The cropping system analyzed in this study included a regionally common rotation with potato, winter rye and maize. Comparisons between spring and autumn samples indicated that the cultivation of winter rye increased soil pH while maize decreased it. Compared to the non-diversified control, the diversification included undersown ryegrass in both winter rye and maize season, and in addition during the winter season, oilseed radish. The diversification was suspected to have a positive effect by translocation of more photosynthetically fixed organic C into soil due to higher root density and rhizodeposition. This striking effect was clearly seen in this study after the winter season in which the diversified system, with its oilseed radish, supported a relatively higher gene copy numbers than in the non-diversified control, thereby indicating a higher microbial population during winter. Since the soil microbiome can also provide nutrients, *e.g.* in the subsequent growing season, the maintenance of a higher biomass during winter should be ecologically and economically beneficial. Similar beneficial effects of the over-winter stabilization of the soil microbiome under diversified systems were observed in a long-term field study, where in addition to cover crops, crop residues were returned to soil (Liu et al. 2022).

With cultivation of winter rye, we observed an increase in soil C_{org} while the cultivation of maize

caused a decline. Such variation can be explained by the specific root architectures of the respective crops, their capacity to assimilate soil nutrients and specific agricultural management practices, including time of fertilization and soil tillage. Interestingly, the shifts of C_{org} in both directions were less pronounced with the diversified cropping system with its undersown ryegrass. These additional plants apparently increased the higher buffering capacity in soils, resulting in less variable soil microhabitat conditions. Due to the stabilized microhabitat conditions along with additional rhizodepositions of the ryegrass we expected that diversified systems support the presence of more complex and less changeable microbiomes.

In the non-diversified system, N fertilization had no effect on the prokaryotic richness and diversity, but in the diversified systems both parameters increased. This beneficial effect can be explained by the additional rhizodepositions which provide most likely other C sources than the main crops, and generating more diverse soil microhabitats, thus promoting additional bacterial taxa (ASV) to grow as compared to the non-diversified system. However, despite the clear indication by the diversity and richness indices, we were unable in this study to identify specific taxa or phylogenetic groups specifically promoted by the diversified systems. A statistically robust identification of indicator taxa probably would have required a higher number of replicates and/or annual replications, considering that field studies have a higher variation than simple systems, *i.e.* microcosms.

While we could not unravel soil bacterial indicators for diversification, ASV association networks provided a clear indication that the prokaryotic communities were more elaborated and complex after addition of conventional amounts of N fertilizer in soils of diversified cropping systems than in non-diversified controls. This difference can be explained by growth of a more diverse prokaryotic community which takes advantage of a higher diversity of rhizodepositions in soils of the diversified cropping system. With the excessive N-supply such benefits of diversified cropping systems on the prokaryotic association networks, however, disappeared, perhaps because higher N supplies would activate the decomposition of additional organic C from soil. Interestingly, at this excessive N supply, the functional prediction model that we used in this study, *i.e.* the FAPROTAX tool, indicated at one sampling event, *i.e.* after cultivation of rye, strong differences caused by

the diversification (undersown ryegrass), a shift in the N cycle, by increasing the relative abundance of nitrifiers in comparison to denitrifiers. Although this predictive tool certainly cannot replace whole metagenome analyses, this model was useful in other studies to estimate microbial metabolic phenotypes and it showed a relatively good consistency with metagenomics results (Louca et al. 2016; Sansupa et al. 2021; Wei et al. 2020). While it would be too speculative to explain a potential promotion of nitrifiers by the diversified cropping system in this study, the data indicate that most likely functional changes in the microbiome occur when non-diversified cropping systems are replaced by diversified.

In contrast to the indicated changes of the prokaryotic community in response to crop diversification, CUE, as determined in this study, was not affected. The values varied between crops and seasons, but not with diversification. This is in accordance to conclusions obtained analyzing factors driving the abundance of bacteria, archaea and fungi. The strong correlations seen between bacterial abundance and CUE suggest that the value was mainly driven by bacteria, even though fungi are also known to strongly contribute to respiration, *i.e.* one of the major factors determining the CUE. At the end of one growing season (2019A), crop diversification decreased respiration, most pronounced with the highest N supply, but this effect was not tangible at other sampling occasions. Thus, we could not confirm in this study that diversification promoted CUE and thereby the stabilization of a microbiome in soil. Considering that the diversification treatments at the field site were started only three years before the onset of this study, the time period for selecting characteristic soil microbiomes may not have been long enough.

Conclusion

The diversified cropping system showed positive effects on the prokaryotic soil microbiome at conventional N application rates, specifically as a higher richness, diversity, and a more elaborated network structure, rather than by promoting growth of specific indicator taxa. The positive effects of the diversified systems can be attributed to the higher buffering of soil pH and a suspected higher amount and diversity of exudates due to the additional soil input of photosynthetically fixed organic C by higher root density and rhizodeposition. Thereby the results of this study underline the beneficial effects of

crop diversification on the soil microbiomes and their ecosystem.

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Author contributions Christoph C. Tebbe conceptualized the experimental question and defined the objectives and analytical tools. Hauke Ahnemann carried out the field experiments. Soil sampling, lab work, data collection and analysis were performed by Bei Liu. Julia Schroeder and Christopher Poeplau guided the work of soil microbial carbon use efficiency determination. Christoph C. Tebbe supervised the project. The first draft of the manuscript was written by Bei Liu and Christoph C Tebbe, and all authors commented on previous versions of the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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