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Organic Amendments in a Long-term Field Trial—Consequences for the Bulk Soil Bacterial Community as Revealed by Network Analysis

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Abstract

This study intended to elucidate the long-term effects of organic soil amendments on bacterial co-occurrence in bulk soil with and without addition of mineral fertiliser. Previous research mostly neglected the bacterial co-occurrence structure and focussed mainly on the parameters species diversity and abundance changes of species. Here we present a systematic comparison of two frequently used soil amendments, manure and straw, with regard to their impact on bacterial co-occurrence in a long-term field trial in Speyer, Germany. The approach involved 16S amplicon sequencing in combination with a bacterial network analysis, comparing the different fertiliser regimes. The results show an increase of bacterial diversity as well as an accumulation of bacteria of the order Bacillales in plots fertilised with manure compared to a control treatment. In the straw-amended plots neither an increase in diversity was found nor were indicative species detectable. Furthermore, network analysis revealed a clear impact of mineral fertiliser addition on bacterial co-occurrence structure. Most importantly, both organic amendments increased network complexity irrespective of mineral fertilisation regime. At the same time, the effects of manure and straw exhibited differences that might be explained by differences in their nutritional/chemical contents. It is concluded that bacterial interactions are a crucial parameter for the assessment of amendment effects regarding soil health and sustainability.

Keywords Microbial interactions · Organic amendments · Manure · Straw · 16S amplicon sequencing · Long-term experiment

Introduction

Following the demand of an increasing world population for food, agriculture had to face the challenge of maximising yields under the constraints of reduced availability of sites with high soil quality, during the last centuries. With the implementation of the Haber-Bosch process into agroindustry, synthetic fertilisers became the tool of choice to reach this goal. Initially, the awareness of potential environmental damages associated with the excessive use of mineral fertilisers in practice was neglected [1, 2]. However, today, we are aware that abandoning traditional soil amendments like manure, compost and plant residues is detrimental for soil as well as for plant health and finally soil fertility [3–5]. Much research has been done to elucidate the effects of mineral fertilisers on soil organisms [6–8]. A frequent result of these studies is a decreasing diversity mainly of soil microbes, commonly linked to decreased soil fertility. In contrast, the application of organic soil amendments resulted in positive effects and increased bacterial diversity as well as plant health [9–11]. In this respect, manure has been identified as a strong mitigator of detrimental effects from mineral fertilisation, while plant residues seem to be much less effective [12, 13]. Despite that, manure application on arable land might be critical due to an enrichment of antibiotic resistance genes [14–17].

In the long term, it has been considered that organic amendments also influence soil quality positively [18]. Thus, recent approaches in agriculture do not only rely on a combined use of organic and mineral fertilisers [19, 20], but also include the application of additional single organic amendments every 3–5 years with the aim to restore soil quality [21]. To address potential effects of such approaches, however, long term trials are needed, as time spans needed to improve soil quality are

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long. Furthermore, time-series are needed as especially α-diversity is known to change over the course of the year [22]. Thus, data on the influence of additional single organic amendment applications on the soil microbial community, which is one of the major drivers for soil quality, is rare.

Most amendments introduce complex, often polymeric, organic substances into the soil that are degraded and metabolised by specialised microorganisms. Lignin, cellulose and hemicellulose constitute major portions of the material introduced for amendment [23]. Given the complexity of these compounds, their biological degradation requires several specialised enzymes [24–26]. It has been observed that for an efficient degradation of cellulose, more than one bacterial species is necessary [27, 28]. Stable cellulose-degrading communities even harbour species that do not themselves degrade cellulose, but facilitate degradation by maintaining favourable oxygen and pH conditions [27, 29]. It is therefore conceivable, that when assessing the fertility of amended soils, bacterial diversity is only one side of the coin, while an investigation on how organisms interact is the second.

In this study, we present results obtained from a long-term experimental field study in Speyer, Germany, where additional single organic amendment applications were implemented into different mineral fertiliser regimes. Organic materials based on both horse manure and plant residues were used and effects were compared to controls where only mineral fertiliser was applied. We used a molecular barcoding approach to assess bacterial diversity patterns based on DNA directly extracted from bulk soil, amplification of the 16S rRNA gene by PCR and high-throughput sequencing of the obtained amplicons. We analysed samples at different time points throughout the vegetation period. Besides α- and ß-diversity, we assessed novel co-occurrence networks, and we investigated the stimulation of such co-occurrence patterns by complex organic materials. Furthermore, the difference in co-occurrence patterns between plots with differing organic amendments (horse manure vs. plant residues) was elucidated.

Materials and Methods

Experimental Layout

The present study was conducted at the International Organic Nitrogen Fertilisation Trial (IOSDV), Rinkenbergerhof, Speyer, Germany [30, 31]. The plots for this long-term trial were established in 1983. Each plot has a size of 7.5 × 6 m. The trial investigates two factors, namely, the use of different organic soil amendments and increasing mineral N application rates. As for the organic amendments, horse manure and plant residues are applied, along with untreated control plots. Previously stored horse manure is applied every third year in autumn after winter barley had been grown (cf. crop rotation in next section) at a concentration of 300 dt FM ha^{-1}. Plant residues are cereal straw or sugar beet leaves that are leftovers from each harvest (2015: 10.2 dt DM ha^{-1} barley straw) as well as cut cover crops. Hence, the main difference between the amendment managements is the source of the amendment material, i.e. animal manure vs. vegetable biomass. As for mineral N application rates, these are applied in five levels reaching from 0 to 240 kg ha^{-1} (200 for winter barley). Each combination of organic amendment and mineral N application rate is replicated three times in the field. A complete field plan is available in Online Resource 1.

Agricultural Management of Field Sites

The applied crop rotation includes sugar beet, winter wheat and winter barley. Additionally, *Raphanus sativus* var. *oleiformis* is used as a cover crop every third year after winter barley in the plant residue treatment only and mulched before tilling. All plots received a basal dressing with a total of 35 kg P ha^{-1}, 232 kg K ha^{-1}, 66 kg Mg ha^{-1} and 1.6 kg Mn ha^{-1} in spring (P, K, Mg) or autumn (Mn). During the sampling campaigns for this study, mineral N was applied twice in May '16. Tillage (plough) has been done equally on each plot up to a depth of 30 cm in November.

Site Characteristics

The soil has been characterised as a Cambisol with an average pH of 6–6.5 containing 8.9% clay, 48.3% silt and 42.8% sand. Total N ranged from 0.06 to 0.1%, and total C from 0.5 to 1.0%. In non-amended control plots, the agricultural practices that were described above led to a reduction of organic carbon contents from originally 0.83% C to 0.60–0.70% C in the 0–30 cm soil layer. Plots amended with either horse manure (0.74–0.85% C) or plant residues (0.74–0.82% C), however, were close to the original value. Similarly, the organic N content of originally 0.07% was found reduced to 0.053–0.065% N in the control plots, while almost unchanged in the horse manure (0.066–0.077% N) and plant residue (0.068–0.076% N) treatments. The climate at the experimental farm is characterised by a mean annual temperature of 10.0 °C, a mean annual precipitation of 593 mm and an annual sunshine duration of 1441 h.

Soil Sampling Procedure

In the frame of this study, plots from two levels of mineral N fertilisation were sampled, i.e. 'without' (0 kg N ha^{-1}) and 'with' (120 + 60 kg N ha^{-1} to sugar beet, 90 + 50 + 40 kg N ha^{-1} to winter wheat and 80 + 30 + 40 kg N ha^{-1} to winter barley). Since the selected plots included all organic amendment variants, this corresponds to a total of 18 plots sampled in four temporal replicates. As the study aimed at long-term changes in bacterial communities, sampling campaigns were performed either prior to or well after management activities, to avoid effects caused by heavy disturbances. They took place in September '15 (fallow ground with volunteer crops/cover crop; before manure application), February '16 (fallow ground with volunteer crops/cover crop), June '16 (sugar beet) and November '16 (sugar beet). Thus, we covered the complete vegetation period of sugar beet. Sampling was performed with a core sampler up to a depth of 30 cm. From each plot, 12 soil cores were taken and thoroughly mixed by hand. A sub-sample of 100 g was taken for bacterial community analysis, sieved to 2 mm, and frozen on dry ice in the field. The remaining soil material was stored at 4 °C and subsequently used for the analysis of soil chemical properties (ammonia, nitrate and dissolved organic carbon) according to VDLUFA [32]. Samples from replicated plots were treated as true replicates.

16S Library Preparation

Five hundred milligrammes of soil were extracted using the NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. A negative control was done using empty extraction tubes for every sampling date. The DNA extract was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, US-MA). The V1-V2 region of the 16S rRNA gene was amplified using the primers S-D-Bact-0008-a-S-16 (5′-AGAGTTTGATCMTGGC-3′) and S-D-Bact-0343-a-A-15 (5'-CTGCTGCCTYCCGTA-3') described by [33]. PCRs were carried out in triplicate using the NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs, Ipswich, US-MA) with 10 ng input DNA. The PCR program included an initial denaturation step of 5 min at 98 °C followed by 25 cycles—denaturation 10 s 98 °C; annealing 30 s 60 °C; elongation 30 s 72 °C—and a final elongation time of 5 min at 72 °C. PCR amplicons were checked on 1% agarose gels. For each sample, three independent PCR reactions were performed and pooled. Purification of PCR products was performed using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) and analysed on a 2100 Bioanalyzer Instrument (Agilent Technologies, Inc., Santa Clara, US-CA). Ten nanogrammes of amplified DNA per sample were indexed using the Nextera XT Index Kit v2 (Illumina Inc., San Diego, US-CA) for multiplexed short-read sequencing. The PCR program used included an initial denaturation step of 30 s at 98 °C followed by 8 cycles—denaturation 10 s 98 °C; annealing 30 s 55 °C; elongation 30 s 72 °C—and a final elongation time of 5 min at 72 °C. The resulting amplicons were checked as described above, diluted to 4 nM and pooled equimolar for sequencing on a MiSeq System (Illumina Inc., San Diego, US-CA) using the MiSeq Reagent Kit v3 (600 cycle) for paired end sequencing following the manufacturer's instructions. A final amount of 11 pM DNA was loaded. As a positive control, PhiX (Illumina Inc., San Diego, US-CA) was used as a spike-in.

Processing of the Sequencing Data

De-multiplexed raw data from the MiSeq system was subjected to primer and adapter removal using the software AdapterRemoval V. 2.1.7 [34]. Subsequently, reads associated to PhiX were excluded and forward and reverse reads were merged using DeconSeq V. 0.4.3 [35]. For further data processing, the QIIME pipeline V. 1.9.1 was used [36]. Prior to 97% OTU clustering, the reads were filtered at a Phred quality score of 3; for downstream analyses, only merged reads within a size range of 300 to 400 bp were processed further. For taxonomic assignments, the RDP classifier V. 2.2 [37] retrained with the GreenGenes database V. 13.8 [38] was used. Rare sequences with a relative abundance below 0.005% were filtered out. Finally, OTUs present in either negative extraction or negative PCR controls (0.3% of reads) as well as chloroplast sequences were filtered out from the data set.

Statistical Data Analysis

Sequence data were imported to R V. 3.3.2 [39] using the phyloseq package V. 1.19.1 [40]. Plotting was carried out using the package ggplot2 [41]. Shannon diversity was computed from unfiltered OTU tables as recommended in the phyloseq documentation. Species evenness was computed as Pielou's evenness index. Soil chemical parameters, soil DNA content, alpha diversity and taxa abundances were compared between treatments using a Bayesian model equivalent to a linear mixed model with time as a random factor. Long-term average crop yield data were supplied by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Germany. Yields between 2005 and 2016, i.e. four complete crop rotations, were compared using a Bayesian two-way ANOVA-like procedure [42]. For our dataset, we considered Bayesian approaches more suitable than frequentist methods, as these are more flexibly adjustable to the data structure while still producing valid results even at low numbers of replicates. One experimental plot (treatment combination: mineral fertilised, without organic amendment) proved to be an extreme outlier in crop yield, DNA content, alpha diversity and community composition and was excluded from further analyses. For the production of a representative sample of the posterior distribution, Markov Chain Monte Carlo (MCMC) sampling was performed using the software packages JAGS V. 3.2.0 [43] and Stan V. 2.10.0 [44] as well as utility functions provided by Kruschke [42]. Four independent chains were sampled for 2,000,000 steps after a burn-in and an adaption period of 2000 and 1000 steps, respectively. Thinning was set to every 10th step. Chain convergence was checked using trace plots to ensure that all chains came to the same result and none of them got stuck in a local maximum. Autocorrelation within the MCMC chain was assessed by the effective sample size (ESS) aiming at a lower limit of 10,000 for the relevant parameters, thereby ensuring that a truly representative sample of the posterior distribution was produced. In the following, we will use the common notation of Bayesian statistics and use the term credibility rather than significance despite their similarity in interpretation. Ninety-five percent highest density intervals (HDI), rather than Bayesian *p* values or Bayes factors, served for checking the credibility of observed differences, as they provide more information about the posterior distribution and are generally more robust than Bayesian *p* values or Bayes factors (cf. [42]). A 95% HDI for a mean difference was produced by calculating the difference at every step of the MCMC chain, thus generating a posterior distribution of the mean difference. The 95% HDI then is the interval that contains the values with the 95% highest posterior probabilities. Finally, an observed difference was considered credible, when its 95% HDI did not include zero, i.e. a difference of zero had low posterior probability.

Ordination Analysis

Beta diversity analysis was performed based on generalised UniFrac distances [45] of 97% OTUs using partial constrained analysis of principal coordinates (CAP) as implemented in the capscale function from the vegan package V. 2.4–1 [46]. In order to assess exclusively the influence of fertiliser regimes on beta diversity, the soil parameters water content, pH, contents of ammonium, nitrate, mineral sulphur, phosphorus, potassium and magnesium, and the sampling time were partialled out in CAP. All of these proved to be significant drivers of microbiome structure in an earlier analysis step on a 5% significance level. Subsequently, a permutation test ($n = 999$) was carried out to assess significance of the factors mineral and organic fertilisation using the date of the sampling as strata for permutations to correct for repeated measurements. The ordination was plotted as a split plot with 95% confidence ellipses for the organic fertilisation treatments in the sample plot. OTUs were plotted using their annotated taxonomical order, reducing overlap for readability.

Identification of Main Responders

OTUs indicative of each organic amendment treatment were identified by means of Bayesian predictive modelling. For this purpose, the BioMiCo algorithm [47] was employed with a delay of 5000 steps. Two thirds of all samples were used for training and one third for testing the model. Multiple runs were conducted in order to ensure that local maxima were avoided. OTU assemblages indicative of a treatment were defined as those that were present in the top 95% posterior distribution of a single treatment only. The 20 OTUs with highest posterior probability in each assemblage were chosen and their absolute abundances plotted in a heatmap.

Computation of Co-occurrence Networks

Bacterial co-occurrence analysis was computed in the MetaMIS software V. 1.02 with default settings [48]. One network per treatment combination was calculated in order to compare treatment effects. The input data for the visual comparison were mean abundances over the three field replicates per time point collapsed to taxonomic families. For a statistical evaluation of network, topology networks were computed for each plot and the resulting consensus networks exported to Gephi V. 0.9.1 [49]. Average degree, average clustering coefficient and average path length were computed and compared statistically using a Bayesian two-way ANOVA-like procedure.

Data Availability The nucleotide sequence data reported are available in the SRA databases under the BioProject ID PRJNA388309.

Results

Crop Yields

Yield increases due to organic amendments depend on the planted crop as well as soil characteristics [50]. A more detailed analysis of crop yields is presented in Online Resource 1. Dry mass yields increased credibly for all crops when mineral N was supplied to plots (sugar beet 114.2 dt ha^{-1}, winter wheat 49.3 dt ha^{-1}, winter barley 42.4 dt ha^{-1}) as compared to non-N-fertilised control plots (s.b. 61.2 dt ha^{-1}, w.w. 20.1 dt ha^{-1}, w.b. 16.4 dt ha^{-1}). The organic amendments exerted highest influence on the sugar beet yields. However, their effect is most pronounced in the non-N-fertilised plots, where addition of any amendment credibly increased yield (95% HDI [12.2, 56.8]) compared to control conditions. When combined with mineral N, the overall amendment effects are diminished (HDI [− 8.2, 42.2]) and only plant residues increase sugar beet dry mass yield credibly compared to the control (HDI [4.4, 60.2]).

Abiotic Soil Parameters

Gravimetric water content of the soil differed only by sampling date, but not by treatment. Potassium contents in soil were credibly higher in plots fertilised with either organic amendment compared to the control (HDIs: manure [− 56.6; − 15.2], plant residues [− 55.3; − 15.7]), while the manure and plant residue treatments were at the same level (HDI [− 16.7; 16.9]). All other analytes did not show differences due to organic amendments, but only to mineral N additions. Results of the soil chemical analysis are summarised in tabular form in Online Resource 2. Compared to the control treatment without organic amendments, soil DNA was found to be elevated in both the manure and the plant residue treatment (95% HDI none vs. manure [− 3.1; − 1.6]; none vs. residues [− 3.3; − 1.8]; Online Resource 1). No credible difference was found between applied amendments (HDI [− 0.9; 0.5]). Treatments without mineral fertilisation showed an evident, yet non-credible increase in soil DNA content when compared to fertilised plots (HDI [− 0.01; 0.96]).

Sequencing Data

A total of 7.2 million reads were obtained from sequencing of which on average 96.0% were retained after decontamination and merging of reads, 88.4% after removal of low-quality reads and application of length filters, 66.5% after OTU calling and taxonomic assignments and 49.8% (3.6 million) after application of abundance filters. The final OTU table contained on average 50,223 reads per sample with a minimum of 24,452 and a maximum of 84,406 reads. This resulted in overall 3282 OTUs (97% identity). Taking account of the recent discussion in scientific literature concerning normalisation methods for unequal read counts [51, 52], alpha and beta diversity analyses were performed on non-normalised, rarefied and relative abundance data. We found no evidence for library size artefacts. Following the ideas of McMurdie & Holmes [51], we therefore present results based on the full, non-rarefied data. All samples show a sufficient coverage of the bacterial community as confirmed by rarefaction curves (Online Resource 1).

Bacterial α-Diversity

Bacterial α-diversity, presented as the Shannon index, exhibited similar seasonal trends in all treatment combinations with the lowest diversity observed in June (Fig. 1a). Diversities were credibly higher in the treatment with manure when compared to the control treatment (HDI [− 0.17; − 0.01]). Plant residues did not change α-diversity compared to the control (HDI [− 0.1; 0.02]) and showed a tendency for lower values than in manure fertilised plots (HDI [− 0.01, 0.12]). Bacterial α-diversity was increased in plots fertilised with mineral N when compared to control plots without mineral N fertilisation (HDI [− 0.13; − 0.01]). Species evenness did not differ between organic fertilisation regimes. However, mineral N addition resulted in bacterial communities being slightly, but credibly, more even when compared to communities from plots without mineral fertiliser addition (HDI [− 0.012; − 0.003], Fig. 1b).

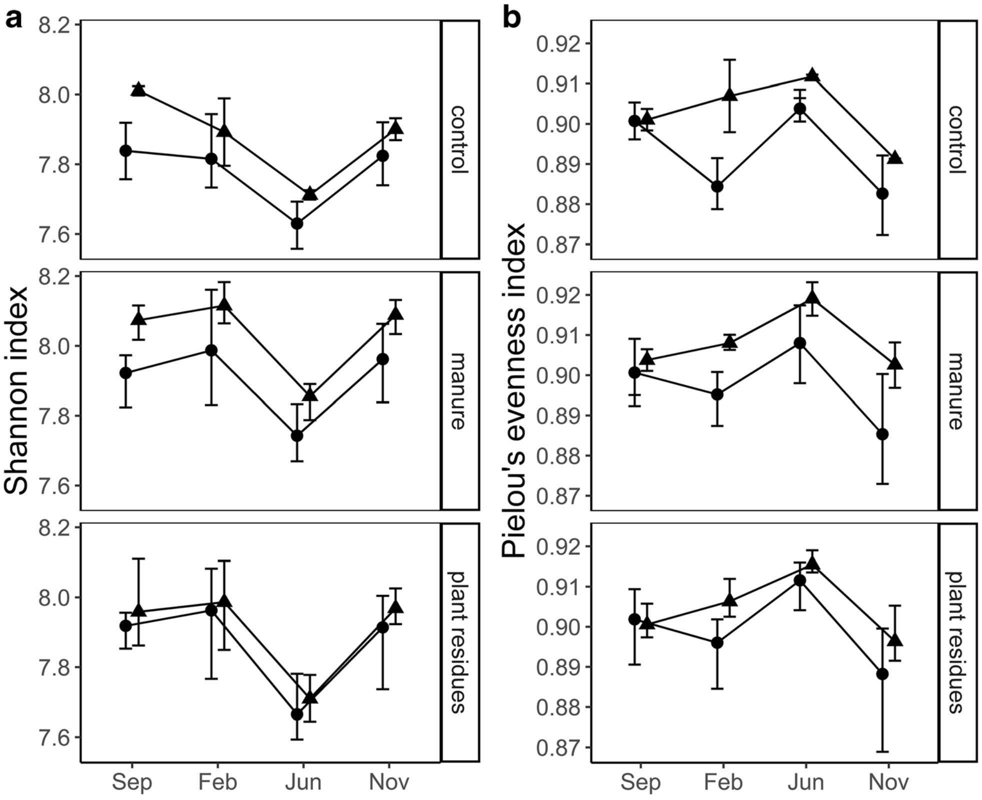


Fig. 1 Alpha diversity (**a**) and species evenness (**b**). The values presented are mean values, error bars show 95% confidence intervals. $n = 3$ (2 for no organic, with mineral fertiliser). Circles represent plots without, triangles plots with addition of mineral N. For results of statistical analyses, see text

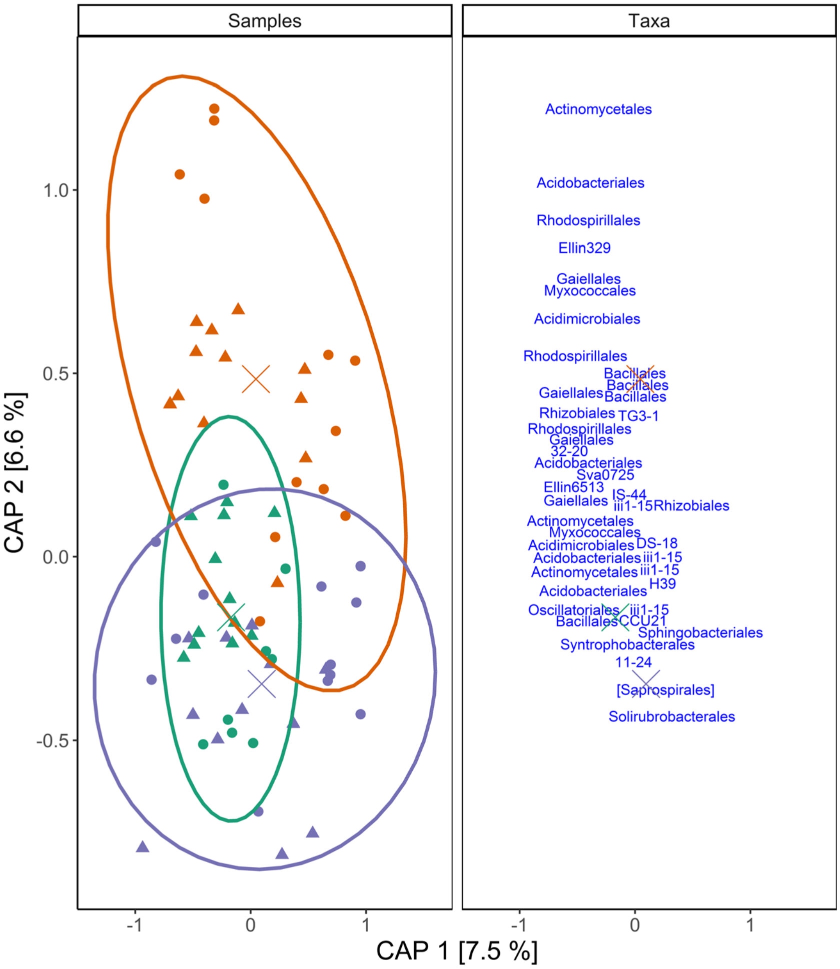


Fig. 2 Partial CAP on generalised UniFrac distances. Sample scores are plotted on the left hand side, while taxa scores are plotted on the right hand side with reduced overlap to enhance readability. Centroids, i.e. the average cases per amendment group, are presented by "X" and can be used as a reference to find the most typical species in the taxa plot. Circles show samples from plots with, triangles from plots without mineral N fertilisation. Colours refer to soil amendment treatments with green, orange and purple representing control, manure and plant residue, respectively. For analysis details, see text

Bacterial β-Diversity Analysed by Ordination

In a first analysis step, β-diversity was assessed with time and fertiliser regimes as predictors of community composition using partial CAP. The sampling date as well as fertilisation regimes proved to be significant determinants of community structure in a permutation test (date: $F(3,53) = 3.65$, $p < 0.001$; mineral: $F(1,53) = 5.21$, $p < 0.001$; organic: $F(2,53) = 4.16$, $p < 0.001$). The ordination plot indicated differentiation between the sampling

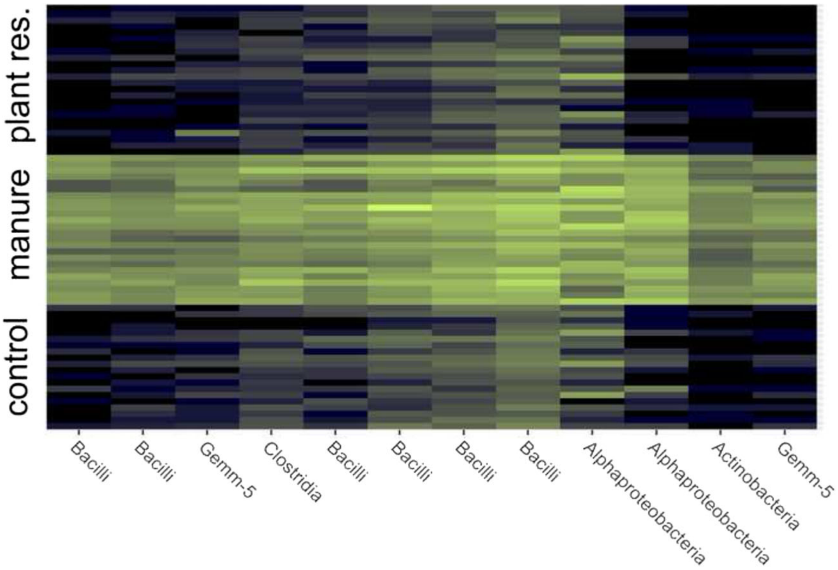


Fig. 3 Heatmap of identified main responders to horse manure amendment. Shading is based on total abundance with black representing absence, and brighter shades of green representing an increasing abundance of the respective OTU in a sample. The x-axis shows the assigned taxonomic class of each OTU

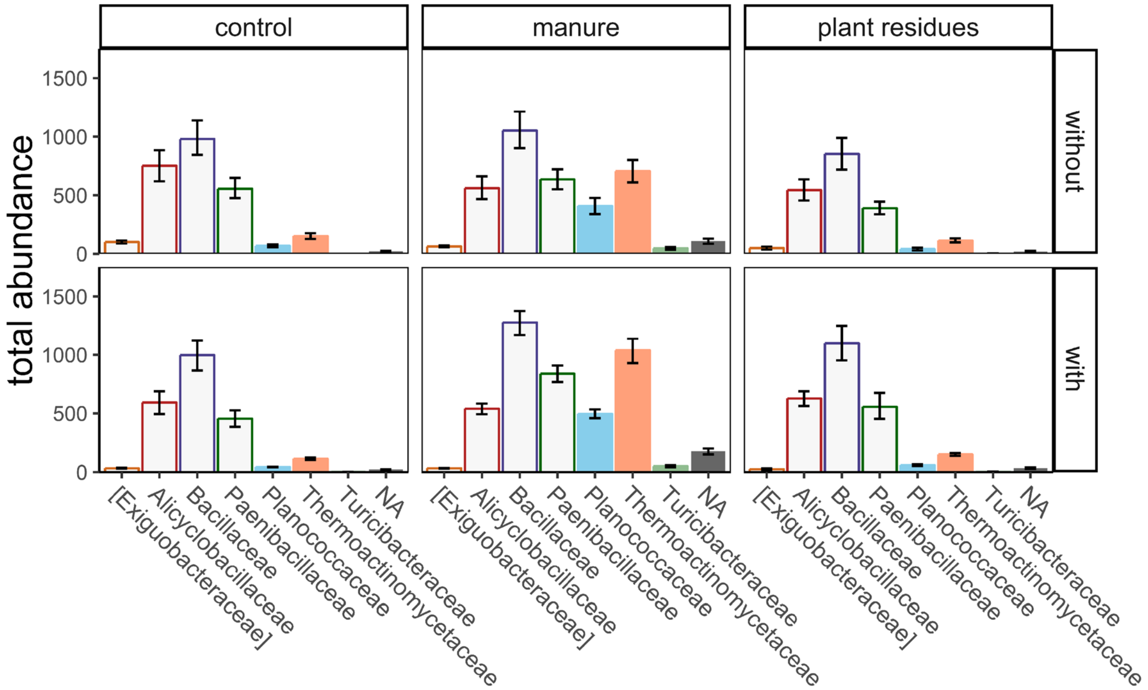


Fig. 4 Total abundance of families within the class Bacilli. The values are mean total abundances over the four sampling campaigns, error bars show 95% confidence intervals. Bars without fill represent families that do not change credibly with soil amendment treatment, while filled bars represent families that are credibly enriched in the horse manure amended plots

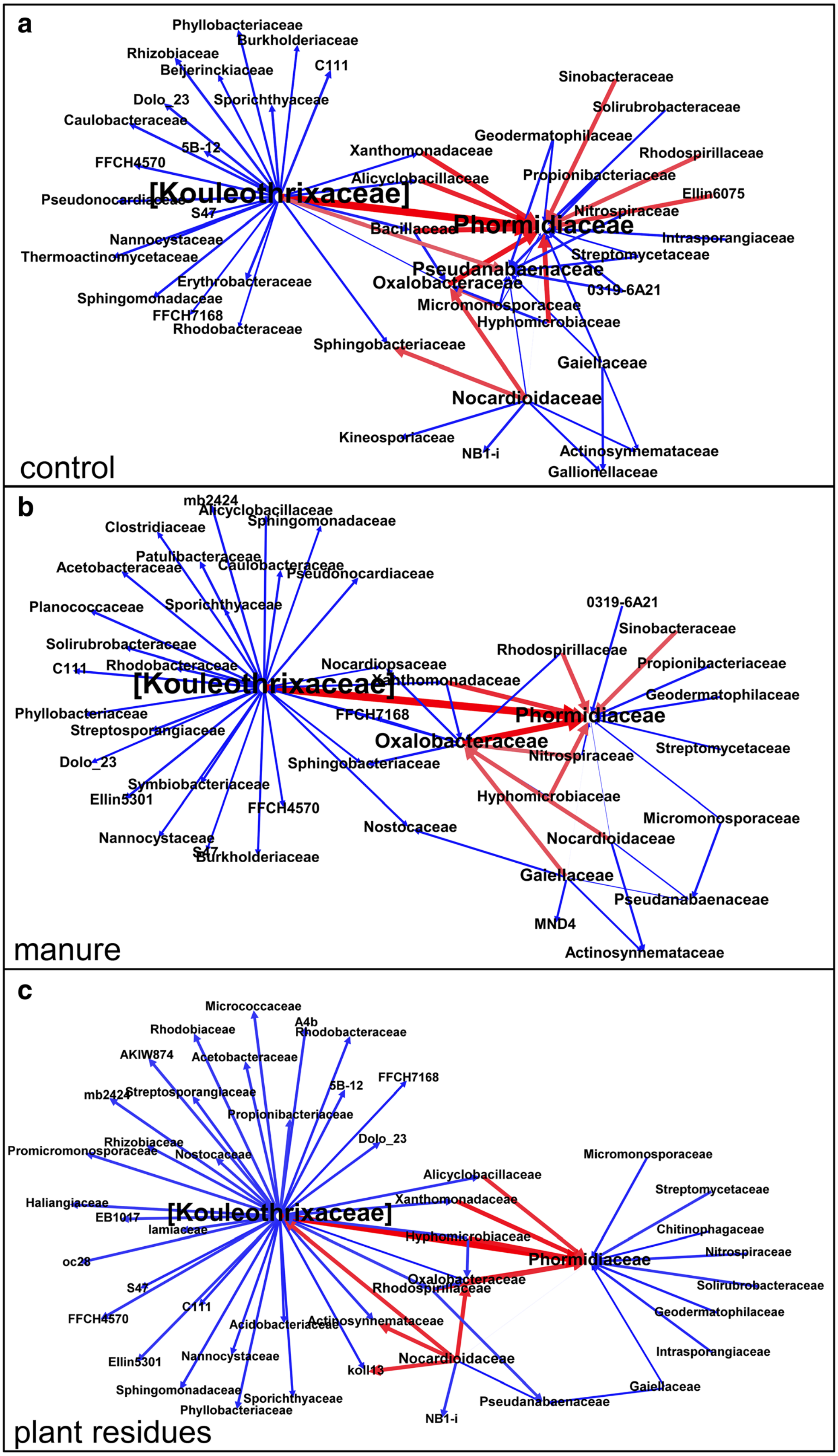
campaigns in September and February from those in June and November, possibly due to a change from fallow soil to sugar beet cultivation (not shown). In a second step, the sampling date was partialled out in order to further focus the analysis on the influence of the fertilisation regime on bacterial community structure. This revealed a clear separation of plots amended with manure from those without organic amendments and amended with plant residues (Fig. 2). Both mineral N and organic amendments were significant factors of influence in the permutation test (mineral: $F(1,53) = 4.64$, $p < 0.001$; organic: $F(2,53) = 4.11$, $p < 0.001$). Variance was divided by CAP into 7.2% constrained variance, 62.6% conditional variance and 30.2% unconstrained variance. From the taxa plot of the ordination, the order Bacillales appears to be typical and indicative part of the bacterial community on the manure-amended plots as it appears close to the centroid of the manure plots.

Identification of Main Responders

A more detailed exploration of the taxa that are typical for the different variants of organic fertilisation was done using the BioMiCo algorithm. Prediction accuracy of affiliation to the three organic amendment groups in several runs of the analysis was consistently around 50% and hence higher than the 33% expected from random chance. Using the posterior probabilities for both OTU assemblages as well as OTUs within assemblages, a subset of potential responders for the different fertiliser variants was chosen. Figure 3 shows the absolute abundances of these responding bacterial groups as a heatmap. Visual interpretation of the graph reveals several species that appear to be more abundant in the plots fertilised with manure relative to the other treatments. The taxonomic identities of these OTUs harbour a striking proportion of members of the class Bacilli. To further support this observation, all taxonomic families within Bacilli were statistically compared between

Table 1 Summary of statistical analysis of taxonomical families reacting to horse amendment with horse manure on field sites. The presented values are the most probably difference between the named treatment groups. Values in brackets give the lower and upper limit of the 95% HDI. Credible differences between treatment groups are marked with *

	Comparison		
Family	Manure vs. control	Manure vs. plant residues	Plant residues vs. control
Planococcaceae	126 [109; 144]*	133 [114; 149]*	− 6 [− 15; 3]
Thermoactinomycetaceae	194 [166; 228]*	205 [177; 238]*	− 11 [− 25; 5]
Turicibacteraceae	15 [14; 16]*	15 [13; 17]*	0.3 [− 0.2; 0.8]
not assigned	31 [25; 38]*	31 [24; 38]*	0.4 [− 3.3; 4.0]


a
Phyllobacteriaceae
Burkholderiaceae
Rhizobiaceae
Beijerinckiaceae
C111
Dolo_23
Sporichthyaceae
Caulobacteraceae
5B-12
FFCH4570
Pseudonocardiaceae
[Kouleothrixaceae]
S47
Nannocystaceae
Thermoactinomycetaceae
Erythrobacteraceae
Sphingomonadaceae
FFCH7168
Rhodobacteraceae
Sinobacteraceae
Solirubrobacteraceae
Geodermatophilaceae
Xanthomonadaceae
Rhodospirillaceae
Alicyclobacillaceae
Propionibacteriaceae
Ellin6075
Nitrospiraceae
Bacillaceae
Phormidiaceae
Intrasporangiaceae
Streptomycetaceae
Pseudanabaenaceae
Oxalobacteraceae
0319-6A21
Micromonosporaceae
Hyphomicrobiaceae
Sphingobacteriaceae
Gaiellaceae
Nocardioidaceae
Kineosporiaceae
NB1-i
Actinosynnemataceae
Gallionellaceae
control
b
mb2424
Alicyclobacillaceae
Sphingomonadaceae
Clostridiaceae
Patulibacteraceae
Acetobacteraceae
Caulobacteraceae
Pseudonocardiaceae
Planococcaceae
Sporichthyaceae
0319-6A21
Sinobacteraceae
Solirubrobacteraceae
Rhodospirillaceae
C111
Rhodobacteraceae
Nocardiopsaceae
Propionibacteriaceae
[Kouleothrixaceae]
Xanthomonadaceae
Geodermatophilaceae
Phyllobacteriaceae
FFCH7168
Phormidiaceae
Streptosporangiaceae
Oxalobacteraceae
Streptomycetaceae
Nitrospiraceae
Dolo_23
Sphingobacteriaceae
Symbiobacteriaceae
Hyphomicrobiaceae
Ellin5301
FFCH4570
Micromonosporaceae
Nostocaceae
Nocardioidaceae
Nannocystaceae
S47
Burkholderiaceae
Gaiellaceae
Pseudanabaenaceae
MND4
Actinosynnemataceae
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c
Micrococcaceae
Rhodobiaceae
A4b
Rhodobacteraceae
AKIW874
Acetobacteraceae
FFCH7168
5B-12
mb2424
Streptosporangiaceae
Propionibacteriaceae
Dolo_23
Rhizobiaceae
Promicromonosporaceae
Nostocaceae
Micromonosporaceae
Alicyclobacillaceae
Streptomycetaceae
Haliangiaceae
Xanthomonadaceae
EB1017
[Kouleothrixaceae]
lamiaceae
Chitinophagaceae
Hyphomicrobiaceae
Nitrospiraceae
oc28
Phormidiaceae
Solirubrobacteraceae
Oxalobacteraceae
S47
Rhodospirillaceae
Geodermatophilaceae
C111
FFCH4570
Acidobacteriaceae
Actinosynnemataceae
Intrasporangiaceae
Nannocystaceae
Ellin5301
koll13
Nocardioidaceae
Gaiellaceae
Sphingomonadaceae
Sporichthyaceae
Pseudanabaenaceae
Phyllobacteriaceae
NB1-i
plant residues

Fig. 5 MetaMIS consensus networks of plots *without* addition of mineral N. Sub-plots represent the bacterial co-occurrence in control (**a**), manure (**b**) and plant residue (**c**) treatment. Red edges represent positive, blue edges represent negative correlation. Edge thickness corresponds to estimated interaction strength. The font size depicts the node degree, i.e. how many connections the corresponding family possesses in the network

treatments (Fig. 4). The families Planococcaceae, Thermoactinomycetaceae and Turicibacteraceae as well as those Bacillales not assigned to any family proved to be credibly more abundant in the plots fertilised with manure in comparison to both the control and the plant residue treatment (cf. Table 1).

Bacterial Co-occurrence Patterns

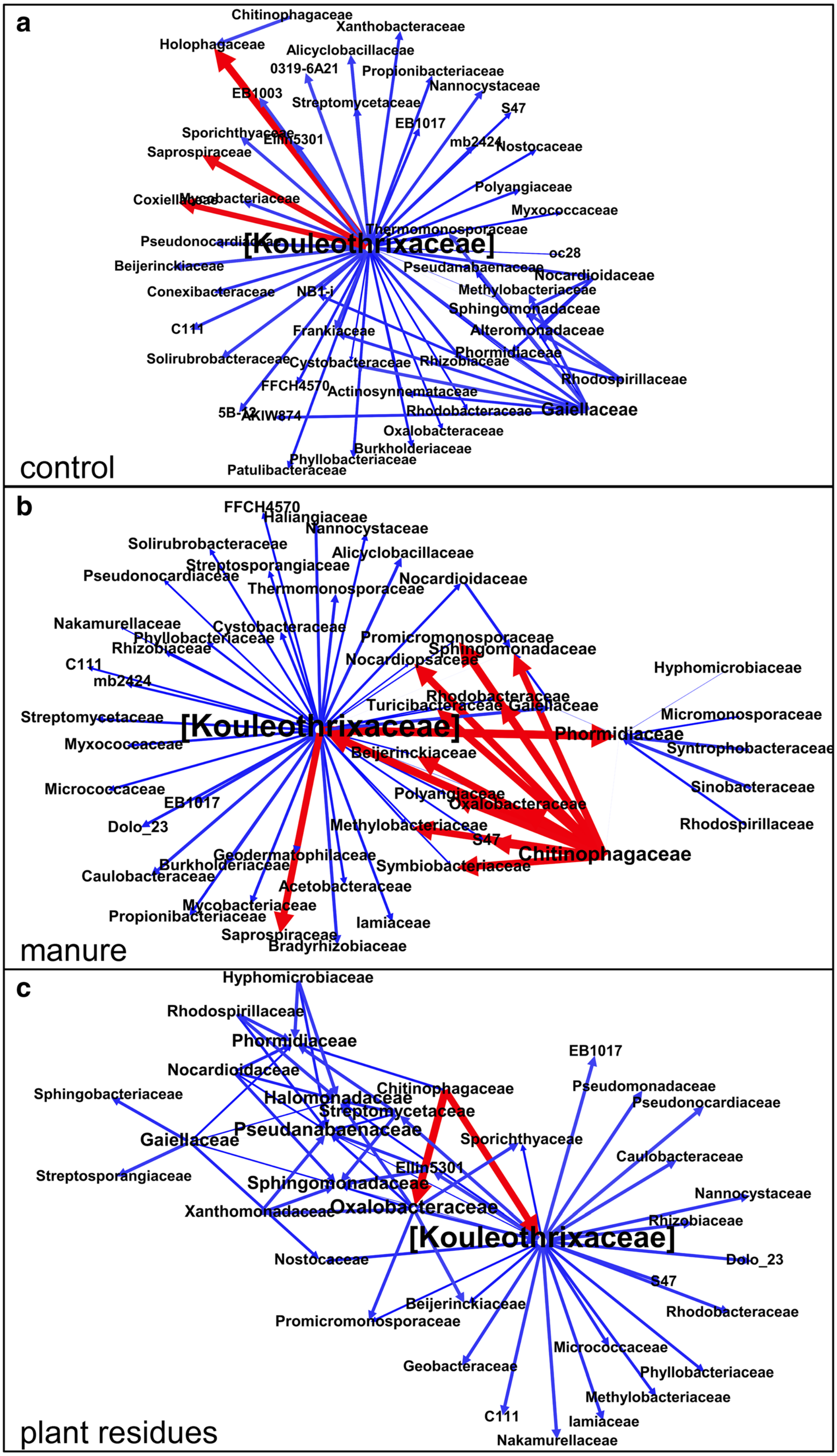
In order to compare the co-occurrence pattern of bacterial communities, MetaMIS consensus networks were computed and general network structure was compared qualitatively between treatments. Predicted community compositions by the underlying model exhibited low Bray-Curtis dissimilarities of around 0.1, showing the accuracy of the approach. The resulting networks showed a similar structure for the plots to which no mineral fertilisation was applied (Fig. 5a–c). Two evident hub taxa, namely, Kouleothrixaceae (phylum Chloroflexi) and Phormidiaceae (Cyanobacteria), connected by a strong positive edge originating from the former are present in each of the networks. Proportions of positive interactions are 21.0, 16.1 and 16.4% in control, manure and plant residues treatments, respectively, as indicated by red edges.

Network structure changed profoundly when mineral fertiliser had been added to the plots. Both the non-amended control plots and the plant residue treatments lacked the prominent positive network edge between Kouleothrixaceae and Phormidiaceae (Fig. 6a, c) and the portion of positive correlations dropped to 4.8 and 3.2%, respectively. However, from the plots fertilised with both mineral fertiliser and manure, the resulting network resembled more the ones without additional mineral fertilisation (Fig. 6b). The positive correlation highlighted above was present (represented by the red edge) as well as a higher overall portion of 22.2% positive interactions. However, most of these positive interactions were originating from only one family, i.e. Chitinophagaceae, while in plots that did not receive mineral N, they were spread out on several nodes in the network. The statistical evaluation of the network topology showed a credible increase in average path length for plots amended with organic fertilisers as compared to non-amended plots (HDI [− 0.593; − 0.115], Online Resource 1) indicating a more complex network structure. The type of organic amendment did not credibly influence average path length of co-occurrence networks (HDI [− 0.152; 0.408]). Neither average degree nor the average clustering coefficient changed credibly due to fertilisation regimes (not shown).

Discussion

Bacilli as Main Responders to the Amendment with Organic Fertilisers

General effects of fertilising practices on the soil microbiome are very comparable in studies from different parts of the world analysing different soil types [53–57]. Several authors state that mineral fertilisation practice is a major determinant of the bacterial community structure [54, 58–60]. Indeed, also in this study, the presence or absence of mineral fertilisation could be identified as a strong driver influencing the structure of the bacterial community. Possible explanations for this are the acidification of soil by NPK additions or the higher total nitrogen content [61, 62]. Another observation with empirical evidence from agriculture is the obvious mitigating effect of organic amendments like manure and straw on soil degradation by mineral fertilisers [55, 63]. Most commonly, higher bacterial diversity is found in soils amended with manure, but not straw, and this increase is then interpreted as one factor influencing soil fertility [60, 64, 65]. For the IOSDV trial, our study in fact demonstrates that manure exerts such an effect on bacterial diversity [66]. Accordingly, either of the organic fertilisers actually increases crop yield on these field sites. However, it should be noted that relating yield increases to bacterial community changes is problematic, because fertilisation affects both plant yield and the bacterial community. This is why the effects of the community on the crop yield cannot be easily distinguished from fertiliser effects and, hence, cannot be evaluated separately. To assess the effects of the bacterial community on yield, a future experimental approach would need to manipulate the bacterial community while keeping all other factors equal. However, this can likely be done only in a controlled pot experiment. In spite of the positive effects on overall diversity, abundance changes of soil-dwelling bacteria are usually relatively small and only connected to few species when compared to the more profound changes associated with NPK fertilisation [57]. Species that are often found to be typical for changes in fertiliser regimes, especially when contrasting pure mineral with organic amended soils, belong to the taxa Actinomycetales and Bacilli [57, 59, 64, 67]. In this study, recent bioinformatical tools were used to identify typical taxa within the different amendment treatments. The most evident changes were recorded in the class Bacilli, specifically in the order Bacillales, where three families with credible changes due to manure addition to field sites were identified. It is hypothesised that these do not stem from the manure itself, as most species present in horse manure belong to the orders Clostridiales and Lactobacillales [68, 69]. Similarly, Chu et al. [63] described a specific 16S PCR band stemming from a *Bacillus* sp. in manure-amended soil that was not present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Chitinophagaceae
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EB1017
Sporichthyaceae
Ellin5301
mb2424
Nostocaceae
Saprospiraceae
Polyangiaceae
Coxiellaceae
Mycobacteriaceae
Myxococcaceae
Thermomonosporaceae
Pseudonocardiaceae
[Kouleothrixaceae]
oc28
Beijerinckiaceae
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Nocardioidaceae
Methylobacteriaceae
Conexibacteraceae
NB1-j
Sphingomonadaceae
C111
Frankiaceae
Alteromonadaceae
Phormidiaceae
Solirubrobacteraceae
Cystobacteraceae
Rhizobiaceae
Rhodospirillaceae
FFCH4570
Actinosynnemataceae
5B-12
AKIW874
Rhodobacteraceae
Gaiellaceae
Oxalobacteraceae
Burkholderiaceae
Phyllobacteriaceae
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Micrococcaceae
Sinobacteraceae
Polyangiaceae
EB1017
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Chitinophagaceae
Geodermatophilaceae
Burkholderiaceae
Symbiobacteriaceae
Caulobacteraceae
Acetobacteraceae
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Rhodobacteraceae
Promicromonosporaceae
Micrococcaceae
Geobacteraceae
Phyllobacteriaceae
Methylobacteriaceae
C111
Iamiaceae
Nakamurellaceae
plant residues

Fig. 6 MetaMIS consensus networks of plots *with* addition of mineral N. Sub-plots represent the bacterial co-occurrence in control (**a**), manure (**b**) and plant residue (**c**) treatment. Red edges represent positive, blue edges represent negative correlation. Edge thickness corresponds to estimated interaction strength. The font size depicts the node degree, i.e. how many connections the corresponding family possesses in the network

in the original amendment material. Bacteria from the Bacillus and Paenibacillus groups were found to play roles in denitrification, nitrogen fixation, cellulose, hemicellulose and pectin degradation as well as proteolysis [70–74]. What is more, most of the members are saprophytes [75, 76]. It seems therefore conclusive to find Bacillales species in the plots amended with manure that contains cellulose, hemicellulose as well as proteins. However, for the families found to be responders to the manure treatment in this study, knowledge is scarce. All of them, Planococcaceae, Thermoactinomycetaceae as well as Turicibacteraceae, seem to not be plant-associated as they were found to negatively correlate with living willow plant biomass [77]. This might hint to a similar, saprophytic life style as in other Bacillales. For the plant residue treatment in this experiment on the other side, no evidence was found for associated higher taxa. A possible explanation might be the chemical composition of the amendments, which does not drastically differ from, e.g. rotting roots that are present in all plots after harvest. Microbes specialised on the decomposition of plant material dwell in all treatments and can therefore not be effectively used to distinguish the treatments in the present study. In addition to that it is conceivable that the treatments might prove to differ in their microbial community structure when taking other organisms, e.g. fungi and archaea, into account.

Bacterial Co-occurrence

Using network statistics, general aspects of network topology can be compared and hence the influence of external factors on the interactive structure of bacterial networks can be estimated [78]. However, our results only consider the bacterial community, while other organisms, e.g. fungi and archaea, might play crucial roles in microbial interactions as well [79–83]. Nevertheless, we are confident that the immense abundance and well-known ecological importance of soil-dwelling bacteria enable us to depict a representative part of the community. In this study, the hypothesis that application of organic fertilisers will lead to a more complex co-occurrence pattern of the soil bacterial community was addressed. Indeed, it could be demonstrated that addition of organic amendments increases network complexity in soil. While earlier studies mainly focused on more general parameters like abundance, diversity or evenness [84, 85], a change in co-occurrence as shown by this study directly enhances our understanding of the underlying mechanisms involved in the mitigation of detrimental NPK effects. Interestingly, an increased complexity of interactions caused by straw was also found. Without taking co-occurrence aspects into account, the influence of straw on the bacterial community might easily be underestimated. Indeed, earlier studies found little effects of straw on the bacterial community, even though empirically positive effects on crop yield are known [12, 13, 66]. Another reason for studies to find only weak effects on the bacterial community might be short run times of the corresponding experiments as compared to the long-term experiment investigated in our study. Concerning the most evident taxa in the co-occurrence networks, an exhaustive discussion is difficult due to the scarcity of information about those found in literature. However, as both Phormidiaceae (Cyanobacteria) and Kouleothrixaceae (Chloroflexi) belong to taxa that harbour photoautotrophic organisms, their relation might actually be limited to the very surface of the plots, e.g. in biological soil crusts [86]. These crusts could in turn be influenced by the increased plant cover in mineral-fertilised plots, thereby disrupting the relationship found in unfertilised plots with higher bare soil coverage. Other taxa with high connectivity in the networks described here are more likely to be present in deeper soil layers. The family Gaiellaceae with the only known member *Gaiella occulta* is able to reduce nitrate to nitrite and might therefore be part of the nitrogen cycle in soil [87]. Accordingly, it appears with higher connectivity in networks derived from the plots fertilised with mineral N, although this is reversed in the manure treatment. An evident difference to other treatments is the hub taxon Chitinophagaceae in the mineral-fertilised, manure amended plots, exhibiting exclusively positive interactions to other nodes. This might relate to the chitin and especially cellulose hydrolysing capability of *Chitinophagus* species [88, 89], presumably making metabolites of this pathway available to other organisms. Regarding the hypothesis that different soil amendments will affect co-occurrence networks differently, a qualitative comparison lets us conclude that the respective networks differ, especially in mineral fertilised soils. This might be explained by the chemical differences of the organic material applied to the plots, whose decomposition involves other bacteria that will consequently have other interaction structures.

Conclusions

Former comparisons of soil amendments that did not take bacterial co-occurrence into account, might have underestimated the effect of straw on bacterial life in soil. Here, both manure and straw had a clear influence on the co-occurrence pattern of bacteria. While restricted to the bacterial community, these results clearly show the benefits and additional value of studying not only general parameters of microbial communities exposed to organic fertilisation, but also taking the interactive structure of the organisms into account. Future efforts should consider other organisms, expanded and denser time-series and experiments on smaller scale to enhance the resolution of the inferred interactions. Agricultural long-term experiments are an essential prerequisite for these investigations. While 16S sequencing analyses can guide to recognise key species in the centre of the observed changes, we need to find out about the specific functions these species are involved in. However, a possible step forward in understanding the effects of organic fertilisers on the community structure and function in soil requires the employment of novel methods. Future investigations should therefore comprise metagenomic and transcriptomic analyses of the communities as well as characterisations of single species with respect to their metabolism and life traits. Only then the ecology of the observed changes in interaction structures can be evaluated and explained.

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