Under temperate climate, the conversion of grassland to arable land affects soil nutrient stocks and bacteria in a short term

Michael M. Obermeier a, Friederike Gnädinger a, Abilash C. Durai Raj a, Wolfgang A. Obermeier b, Christoph A.O. Schmid a, Helga Balàzs a and Peter Schröder a,\*

a Helmholtz Zentrum München GmbH, Research Unit for Comparative Microbiome Analysis, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

b Ludwig-Maximilians-Universität München, Research and Teaching Unit for Physical Geography and Land Use Systems, Luisenstraße 37, 80333 München, Germany

**\*** Correspondence: peter.schroeder@helmholtz-muenchen.de; Tel.: +49-089-3187-4056

**Abstract**

Projected population growth and climate change will make it inevitable to convert neglected and marginal land into productive arable land. We investigate the influence of agricultural management practices on nutrient stocks and soil functions during the conversion of former extensively used grassland to arable land. Effects of grassland removal, tillage, intercropping with faba bean (*Vicia faba*) and its later incorporation were studied with respect to soil properties and bacterial community structure. Therefore, composite samples were collected with a core sampler from the topsoil (0 – 20 cm) in (a) the initial grassland, (b) the transitional phase during the vegetation period of *V. faba*, (c) after ploughing the legume in, and (d) untreated controls. In all samples, nitrate-N, ammonium-N, dissolved organic carbon (DOC) and total nitrogen bound (TNb) were analyzed and comparisons of the bacterial community structure after 16S-amplicon sequencing were performed to assess soil functions. Mineralization after grassland conversion followed by the biological nitrogen fixation of broad beans enhanced the nitrate-N content in bulk soil from 4 to almost 50 µg N g-1 *dw*. Bacterial community structure on phylum level in bulk soil was dominated by *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, and *Bacteroidetes* and remained almost stable. However, alpha and beta-diversity analysis revealed a change of the bacterial composition at the final state of the conversion. This change was primarily driven by increasing abundances of the genera *Massilia* and *Lysobacter*, both members of the *Proteobacteria*,after the decay of the leguminous plant residues. Furthermore, increasing abundances of the family *Gaiellaceae* and its genus *Gaiella* fostered this change and wererelated to the decreasing carbon to nitrogen ratio. In short, gentle management strategies could replace the input of mineral fertilizer with the aim to contribute to future sustainable and intensified production even on converted grassland.

**Keywords:** Bacterial community structure, sustainable agriculture, nitrogen fixation, turnover processes, incorporation of plant residues

1. Introduction

Projections show that feeding a world population of 9.1 billion people will require 70% increase in global food production by 2050 (FAO, 2009). In particular, increased plant production will be necessary to ensure food and feed supply, and to respond to the need for biomass as renewable energy and industrial feedstock application. In order to meet these challenges, we must improve biomass production and utilization to satisfy the social, economic, and environmental demand of the growing population (Schröder et al., 2018). Including neglected and upgrading marginal sites will be unavoidable and the gentle management of such sites indispensable to maintain or even improve soil quality, functionality, and health and thus to contribute to a more sustainable agriculture (Lal, 2016, Schröder et al., 2019).

In contrast, mineral fertilizers have been used since decades to increase plant production in conventional farming with diverse effects on fertility and physical properties of soils (Aggelides and Londra, 2000, Ahmed et al., 2017). To mitigate resulting negative effects, techniques have to be developed or rediscovered to replace or at least minimize the input of inorganic fertilizer to commonly used agricultural farmlands and also to lower the release of nitrate-N into groundwater (EC, 2000). Therefore, undersowing of leguminous species (Schröder et al., 2008), intercropping of faba bean (Fan et al., 2006), incorporation of leguminous plant residues (Ordóñez-Fernández et al., 2018) as well as organic amendments have been proven to be beneficial and also to enhance crop yield (Diacono and Montemurro, 2011, Scotti et al., 2015, Lori et al., 2018).

In addition, it will be inevitable to convert neglected and marginal land into productive arable land. Even the conversion of poor grassland into economically attractive cropland may be considered if it can be reached in a sustainable manner. To do this, nutrient pools need to be stabilized and the prevailing bacterial community structures and activities have to be maintained or even enhanced. It will be essential to face possible negative effects following grassland break-up like increased nitrogen losses, due to nitrate leaching and nitrous oxide emissions, following the mineralization of soil organic nitrogen and the decomposition of grass residues (Buchen et al., 2017). To compensate these effects, intercropping of faba bean seems to be promising since it not only facilitates atmospheric nitrogen fixation and thus improves soil fertility (Stagnari et al., 2017) but also reduces nitrate leaching if it is used as cover crop (Plaza-Bonilla et al., 2015). However, little is known about the influence of intercropping legumes and their later incorporation during grassland conversion on bacterial community structure.

Several studies revealed the importance of bacterial community structure on ecosystem services such as nutrient cycling in soil (Barrios, 2007, Zhong et al., 2010, Kaiser et al., 2016). It is crucial to know that climate (Sheik et al., 2011) and different land use intensities (Estendorfer et al., 2017) can change bacterial communities. Since the intervention in ecosystems (e.g. the conversion of grassland to arable land) can also disturb and change bacterial diversity and composition (Gatica and Cytryn, 2013, Carbonetto et al., 2014, Hartmann et al., 2015) such conversion must be well planned and monitored.

Being part of an interdisciplinary project, we hypothesize that neglected land can be re-activated as high-value cropland without losses in nutrient pools or decreases in ecosystem services. To test this, the grassland of a small-scale dairy farm in southern Bavaria was converted into cropland and broad bean was used as intercrop and later incorporated into the soil. We aimed to assess the early consequences of such land use change on nutrient availability and bacterial community structure and thus to contribute to a more sustainable and intensified agriculture. The focus was hereby on studying the changes induced by mineralization processes during grassland conversion, the nitrogen fixation of *V. faba*, and degradation processes after incorporating its residues into the soil. Finally, it was intended to provide enough nitrate-N for further cropping of arable plants and thus to replace the additional input of mineral fertilizer.

2. Materials and Methods

2.1. Site Description

The study site is based at Martlhof, a traditional small-scale dairy farm, raising sheep and pigs on pasture, on former extensively used grassland, in Ostin am Tegernsee (Bavaria, Germany, 47° 44' 37.30" N and 11° 45' 38.32" E). The field trial (1 ha) is located 784 meters above sea level with a gently sloping relief. Climatic conditions are in the transition zone of the warm-temperate climate of Western Europe and the colder continental climate of Eastern Europe. A mean annual precipitation of 991 mm, a mean annual temperature of 7.5 °C, and a mean annual sunshine duration of 1571 h characterize the climate in this region. The site’s bedrock is calcareous, the colluvial topsoil contains 28.2 % sand, 43.1 % silt, and 28.8 % clay. Its texture has been classified as clayey loam with an average pH ranging from 5.2-5.6.

2.2. Experimental Layout and Agricultural Management Practices

To analyze the effects of grassland transformation on bacterial community structure, a short-term field trial was started in May 2016. Therefore, the experimental field (32 x 32 m) as a whole was subdivided into six subplots of 10.7 x 14 m (see Supplementary Figure S1). The six subplots (I, II, III, IV, V, and VI) were separated from the beginning of the experiment and complemented with four untreated grassland controls of 8 x 4 m size each. Randomized sampling was performed in the center of each plot to avoid transition effects between the subplots (composite of 12 subsamples). A phytosociological survey of the grassland was performed according to Ellenberg (1992). The Ellenberg indicator values (e.g. individuality, sociability, temperature, nitrogen) are given in Supplementary Table S1. The grass scar was mechanically mulched and the residual green cover was incorporated into the soil. Following the milling of the top soil (12 cm) broad beans (*Vicia faba* L.) were sown (200 seeds/m²) as cover crop. This was done to homogenize the field area, to facilitate biological fixation of nitrogen, and at the same time to avoid weed invasion and leaching of nutrients. In April 2017, the leguminous plant residues were incorporated and in May the top soil was tilled to a depth of 18 cm using a three-furrow turning plough. After milling with a harrow, the field reached its final state of transition.

2.3. Soil and Plant Sampling Procedure

Soil sampling to analyze soil properties and bacterial community structure up to a depth of 20 cm was performed using a core sampler. Bacterial analysis was done for a total sample number of 20. Therefore, the 12 subsamples of each plot were pooled and homogenized. The samples were sieved (2 mm), frozen on dry ice and subsequently stored at -80 °C for later bacterial analysis. The soil samples used for analysis of nitrate, ammonia, total nitrogen bound, dissolved organic carbon and pH were stored at 4 °C. Moisture and temperature were measured on the field using a time domain reflectometer UMP-1 BTim (Umwelt-Geräte-Technik GmbH, Müncheberg, Germany). Sampling was performed at four different sampling dates. Sampling 1 (IG) in July 2016 describes the initial status of the grassland (Supplementary Figure S1). Sampling 2 (TP) was performed in November 2016 during the vegetative period of *V. faba* and describes the transitional phase. Sampling 3 (FS) was conducted in June 2017, describing the final state of the conversion to arable plots after incorporation of the leguminous plant residues. Additionally, Sampling 4 (CG) was accomplished in August 2017 and describes the status of the grassland without any management practices and acts as control, directly adjacent to the converted plots. Additionally, in TP, six plant samples of *V. faba* were taken from each plot to analyze the content of pigments (Chl *a*, Chl *b* and total carotenoids) as well as the plant fresh weight and height.

2.4. Nutrient stocks (DOC, TNb, Nitrate-N and Ammonium-N) and pH

Dissolved organic carbon (DOC) and total nitrogen bound (TNb) in bulk soil were extracted from 5 g of field fresh samples using 20 mL of 0.01 M CaCl2. After shaking the samples for 45 min on a horizontal shaker the samples were filtered through a Whatman folded filter (type 595, diameter 110 mm, GE Healthcare, Buckinghamshire, United Kingdom). TNb and DOC were measured on a DIMATOC®2000 (DIMATEC, Langenhagen, Germany). Concentrations of nitrate (NO3--N) and ammonium (NH4+-N) were analyzed photometrically by continuous flow measurements using an autoanalyzer (CFA-SAN Plus, Skalar Analytik, Erkelenz, Germany). To determine the gravimetric water content, subsamples of the bulk soil were dried for 24 h at 105 °C. Soil pH measurements followed the guidelines of the OECD (ISO, 10390) adding 25 mL of 0.01 M CaCl2 to 5 g of bulk soil samples.

2.5. Pigment analysis

Chlorophylls (Chl *a* and Chl *b*) and total carotenoids of *V. faba* plants were analyzed following the protocol of Lichtenthaler and Buschman (2001), slightly modified by Obermeier et al. (2015) (see legend of Supplementary Table S2).

2.6. Nucleic acid Extraction

DNA was extracted from 0.5 g of bulk soil (-80 °C) using the Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, United States) according the manufacturer’s instructions. Negative controls were included using empty extraction tubes. DNA concentrations were measured in duplicates using Quant-iTPico™ Green® ds DNA assay Kit (Thermo Fisher Scientific, Waltham, United States) following the manufacturer’s protocol. Measurements were performed at 520 nm using a SpectraMax Gemini EM Fluorescence Plate Reader Spectrometer (Molecular Devices, Ismaning, Germany). Non-target controls were used to correct for background fluorescence. All DNA extracts were stored at -80°C for further usage.

2.7. 16S Library Preparation and Illumina Sequencing

Polymerase chain reaction (PCR) of the 16S rRNA region was performed on 1 ng of DNA extracts in triplicates using primer S-D-Bact-0008-a-S-16 (5′-AGAGTTTGATCMTGGC-3′) and primer S-D-Bact-0343-a-A-15 (5'-CTGCTGCCTYCCGTA-3') to amplify the V1-V2 region (Klindworth et al., 2013). PCR conditions were the following: denaturation at 98 °C for 30 s, followed by 28 cycles each at 98 °C for 10 s (denaturation), 60 °C for 30 s (annealing) and 72 °C for 30 s (elongation), followed by 72°C for 5 min (final elongation). A non-target control (NTC) and a positive control with the target gene were also performed following the same PCR conditions. The reaction mix contained 12.5 µL of NEBNext High-Fidelity Master Mix (New England Biolabs, Ipswich, United States), 5 pmol of each primer, 10.5 µl of DEPC water, 2.5 µL of 3 % bovine serum albumin (BSA) and 1 ng of DNA extract. The quality of the PCR amplicons was checked on 1 % agarose gels. Triplicate DNA reactions were pooled and purified using Agencourt®AMPure®XP kit (Beckman Coulter Inc., Webster, United States) according to the manufacturer’s instructions (with the modification of using 78 µL beads for 60 µL of sample volume). DNA quantification and quality controls were performed using the DNF-473 standard sensitivity Kit (1 bp – 6000 bp) on a Fragment Analyzer device (Advanced Analytical Technologies GmbH, Heidelberg, Germany).

The Nextera XT Index Kit v2 (Illumina Inc., San Diego, United States) was used for indexing 10 ng of the 16S rRNA gene amplicons, according to the manufacturer’s protocol. The PCR comprised initial denaturation with 98 °C for 30 s, followed by 8 cycles each at 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, ending with a final elongation at 72 °C for 5 min. The indexed PCR products were purified, and quality as well as quantity, were checked as described above. Next-generation sequencing was performed on 10 pM of indexed DNA, using the Illumina MiSeq platform (Illumina Inc., San Diego, United States).

2.8. Sequencing Data Analysis

To remove primers and adapters, the raw data from Illumina Sequencing was processed using the software AdapterRemoval (V. 2.1.7) (Lindgreen, 2012) separately for reverse and forward reads. For further processing, the R package DADA2 (V. 1.8.0) was used (Callahan et al., 2016). After checking read quality plots, quality filtering and trimming of forward reads was performed at 10 and 200 bp. For the reverse reads, trimming was done at 60 and 180 bp only. Remaining PhiX contaminations were removed during filtering. Subsequently, the samples were dereplicated and denoised before forward and reverse reads were merged. Thereafter, an ASV table was constructed and chimeras removed. Finally, taxonomic annotations of ASVs against the SILVA database version 128 (Quast et al., 2013) were performed.

Sequence data were imported to R (V. 3.5.1) (R Core Team, 2018) using the phyloseq package (V. 1.25.2) (McMurdie and Holmes, 2013), plotted using the ggplot2 package (V. 3.0.0) (Wickham, 2016) and statistically analysed using the package agricolae (V. 1.2.8) (De Mendiburu, 2014). After filtering ASVs that were not assigned to bacteria (NA and eukaryota), chloroplasts and mitochondria, ASVs present in negative controls and ASVs that were present in only a single sample were removed. For the filtered data, a phylogenetic tree was calculated using the software RaxML-NG (V. 0.6.0) (Kozlov et al., 2018). Alpha diversity indices were plotted using the plot\_richness function of the phyloseq package. Non-metric multidimensional scaling (NMDS) was performed on genus level to visualize the dissimilarities between the sampling points based on Bray-Curtis distances. NMDS was done for a reduction to two dimensions with a maximum of 500 tries using the vegan package (V. 2.4-6) (Oksanen et al., 2018). Ninety-five percent confidence ellipses were plotted for each sampling time. Tukey’s post-hoc test based on Bray-Curtis dissimilarities in conjunction with a one-way ANOVA on relative abundances of phyla, orders, families, and genera was run to indicate which sampling time point differs significantly from others (p < 0.05). Relative abundances and standard deviation on phylum, order, family, and genus level are shown to indicate effects of the management practices as well as the homogeneity within the subplots of the experimental layout. Further, a one-way ANOVA (p < 0.05) in conjunction with Tukey’s post-hoc test was performed to analyze soil and plant data using basic R functions (R Core Team, 2018). The nucleotide sequence data are available in the NCBI Sequence Read Archive (SRA) (Leinonen et al., 2011) under the BioProject accession number PRJNA471669 (https://www.ncbi.nlm.nih.gov/sra/PRJNA471669).

3. Results

3.1. Climate, initial situation and soil properties

From January 2016 to October 2017, a typical temperature and precipitation pattern for the sub-continental climate prevailed at the experimental site (Supplementary Figure S2). In the transitional phase (TP), four strong rain events (> 30 mm/day) could be observed during the vegetation period of *V. faba*. Air temperatures at the sampling dates were 19.7 °C (IG – initial grassland), 5.0 °C (TP – transitional phase), 13.7 °C (FS – final state), and 26.3 °C (CG – control grassland).

***Insert Table 1***

Soil temperature and moisture were homogeneously distributed within the field plots at the different sampling dates (see Table 1). Furthermore, soil pH-values were homogeneous within the field plots and remained constant with only slight variations during the complete sampling period (5.4 ± 0.2). The basic inventory of the initial and the control situation of the grassland following the guidelines of Ellenberg values (Ellenberg, 1992) identified it as grassland of moderate quality, slightly moist without indications for salt stress (Supplementary Table S1).

3.2. Nutrient stocks (DOC, TNb, Nitrate-N and Ammonium-N)

A strong two-fold increase of DOC in bulk soil was observed from 15.3 ± 7.7 µg g-1 *dw* in IG to 30.9 ± 12.1 µg g-1 *dw* in TP (Table 2). Subsequently, DOC decreased to 12.5 ± 3.4 µg g-1 *dw* in FS. With 20.5 ± 5.8 µg g-1 *dw*, the DOC content in the control grassland (CG) was not significantly different from the initial situation (IG).

***Insert Table 2***

TNb increased three-fold from 5.2 ± 1.6 µg g-1 *dw* in IG to 15.2 ± 5.4 µg g-1 *dw* in TP and finally reached its maximum of 45.3 ± 5.0 µg g-1 *dw* at FS. In contrast, the contents of TNb in the control grassland (CG) showed no significant changes and remained with 8.1 ± 1.2 µg g-1 *dw* on the low level of the initial grassland (IG). A decrease of the DOC/TNb ratio from 3.0 in IG to 2.0 in TP and 0.3 in FS was observed during the transformation. The control grassland (CG) had DOC/TNb ratios similar to the initial grassland situation (IG).

***Insert Fig. 1 a and 1 b***

Contents of nitrate-N exhibited similar trends compared to TNb (Fig. 1a). With 16.9 ± 5.9 µg nitrate-N g-1 *dw* a strong increase was already observed at TP to values three times higher than in IG (4.2 ± 1.1 µg nitrate-N g-1 *dw*). Subsequently, another three-fold increase (compared to TP) to 49.6 ± 5.5 µg nitrate-N g-1 *dw* in FS followed. Again, CG and IG had similar nitrate content (6.1 ± 1.9 µg nitrate-N g-1 *dw*).

Similar to TNb and nitrate-N, an increasing trend for ammonium-N was observed throughout the experiment (Fig. 1b). However, this increase was not significant and much less pronounced than the strong increase of nitrate-N and TNb. The ammonium-N content in the initial bulk soil doubled from 0.14 ± 0.07 µg ammonium-N g-1 *dw* (IG) to 0.28 ± 0.12 µg ammonium-N g-1 *dw* (TP) and finally reached 0.51 ± 0.22 µg ammonium-N g-1 *dw* (FS). Ammonium-N contents in the initial and final grassland (IG and CG) had the lowest values.

Different from the strong increase of nitrate-N and TNb the DOC content outlined its maximum in the transitional phase causing a decrease of the DOC/TNb ratio during the conversion. The nutrient stocks within the initial and the control grassland remained constant.

3.3. Plant performance (TP – transitional phase)

Performance of intercropped *V. faba* after 70 days of vegetation showed a rather homogeneous distribution pattern of biomass development and pigments within the field experiment (Supplementary Table S2). The average plant height of *V. faba* plants was 51.6 ± 7.2 cm containing 1.05 ± 0.14 mg g-1 *fw* chlorophylls (*a*+*b*) and 0.26 ± 0.01 mg g-1 *fw* total carotenoids (*x*+*c*).

3.4. Bacterial community structure

3.4.1. Sequencing data

A total of 5.75 million raw reads were obtained from the sequencing platform of which 4.95 million raw reads (86.2 % of total raw reads) remained after filtering and trimming. 4.60 million (80.1 % of total raw reads) remained after denoising forward and reverse reads. After merging the reads 3.60 million (62.5 % of total raw reads) and removal of chimeras 3.12 million (54.2 % of total raw reads) reads remained. After removing ASVs not assigned to bacteria (NA and eukaryota), as well as those assigned to chloroplasts and mitochondria 3.11 million reads (54.0 % of total raw reads) were remaining. 2.99 million reads (52.1 % of total raw reads) were remaining after removing the negative controls and 2.80 million reads (48.7 % of total raw reads) after filtering ASVs that were present in only one of the samples. The final ASV table contained on average 140,100 reads per sample with a minimum of 69,378 and a maximum of 320,927 reads counting for a sum of 8690 taxa. In total 27 phyla, 73 classes, 125 orders, 183 families, 314 genera and 24 species were unique.

***Insert Fig. 2***

The bacterial richness was not significantly different for the four sampling dates (Supplementary Figure S3). Simpson’s index of diversity (0.9990 ± 0.0003) indicated a highly diverse bacterial community structure on genus level throughout the entire experiment (Fig. 2). Although the diversity of FS was also very high (0.9986 ± 0.0002), it decreased significantly (F = 5.745 and *p* < 0.007) compared to the other sampling times (Supplementary Figure S3).

***Insert Fig. 3***

Non-metric multidimensional scaling of beta-diversity revealed a good representation (stress-value of 0.108) of the sampling dates within two dimensions (Fig. 3). High similarity of the bacterial community structure could be seen for the initial grassland (IG) and the transitional phase (TP). Almost all of the individual samples clustered in the 95 % confidence intervals of these sampling dates. The control grassland (CG) was most similar to the initial grassland (IG) and only slightly separated from the other sampling dates. Finally, FS clearly separated from the other sampling dates indicating a shift of the bacterial composition at the end of the experiment.

3.4.2. Soil bacterial communities

The most abundant phyla in our dataset were *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, and *Bacteroidetes* in decreasing order (Fig. 4). Members of these phyla accounted for 87.0 ± 6.9 % of the total bacterial community structure. With an averaged relative abundance of 32.9 ± 2.0 %, *Proteobacteria* was the predominant phylum during the entire experiment. However, also *Actinobacteria* (25.5 ± 1.9 %), *Acidobacteria* (17.2 ± 1.9 %), *Chloroflexi* (6.2 ± 0.4 %), and *Bacteroidetes* (5.0 ± 0.8 %) showed high abundances at the four sampling dates. *Proteobacteria* was the only of these phyla that changed significantly during the experiment having comparable values in IG, TP, and CG but higher abundances at the final state of the conversion (FS).

***Insert Fig. 4***

At the final state (FS) the higher abundance of the phylum *Proteobacteria* was correlated to the strong increase of members of the order *Betaproteobacteriales* (see Supplementary Table S3). Within samplings IG, TP, and CG averaged abundances of 6.6 ± 0.6 % were observed, which more than doubled to 13.9 ± 2.3 % at FS. Remarkably, the family *Burkholderiaceae* (*Betaproteobacteriales*) contributed strongly to this increase (Fig. 5). Starting with comparable abundances of 1.4 ± 0.3 % (IG) and 1.7 ± 0.4 % (TP) this family finally reached abundances of 10.4 ± 2.5 % (FS), representing the most abundant family within the whole experiment. With an averaged abundance of 4.6 ± 1.7 % at FS, the genus *Massilia* (*Burkholderiaceae*) strongly contributed to this trend exhibiting the highest abundance of all genera within the entire experiment (see Supplementary Table S4). Interestingly, *Massilia* was not significantly present at the other sampling dates (IG, TP, and CG).

***Insert Fig. 5***

On order level members of *Rhizobiales* (*Proteobacteria*) were predominant during the experiment (see Supplementary Table S3). However, their abundance decreased significantly from 10.1 ± 1.0 % (IG) to 7.8 ± 0.9 (TP) but finally increased again to 9.9 ± 0.3 % (FS). With abundances of 6.8 ± 0.7 % (IG) and 7.8 ± 0.3 % (CG), the family *Xanthobacteraceae* was the most abundant family in the grassland plots contributing also to the high occurrence of *Rhizobiales* (Fig. 5). However, their abundance significantly decreased to 5.1 ± 0.7 % (TP) but later reached the final state of 6.4 ± 2.8 % (FS). *Pseudolabrys* was the most abundant genus within the family *Xanthobacteraceae* outlining highest abundances of 2.0 ± 0.3 % in the grassland plots (Supplementary Table S4). Abundances during growth of *V. faba* and its incorporation were significantly lower (1.5 ± 0.2 % (TP) and 1.6 ± 0.1 % (FS)).

*Myxococcales* were observed as third most abundant order of the phylum *Proteobacteria*. Members of this order exhibited similar abundances of 4.6 ± 0.4 % (IG, TP, and CG) which significantly decreased to 3.0 ± 0.3 % at FS. Members of the genus *Haliangium* had the strongest influence on the decrease of *Myxococcales* at FS (see Supplementary Table S3). Similar trends were found for the fourth most abundant family, the *Nitrosomonadaceae* (*Proteobacteria*). This family outlined comparable values of 3.2 ± 0.2 % within IG, TP, and CG but decreased significantly to 2.1 ± 0.1 % at FS (Fig. 5).

Members of the order *Gaiellales*, which belong to the phylum *Actinobacteria*, showed similar abundances of 7.0 ± 0.6 % (IG and TP) at the beginning of the experiment (see Supplementary Table S3). Interestingly, their abundances increased significantly to 9.5 ± 1.7 % at FS. The most abundant genus, the *Gaiella*, outlined similar values of 1.8 ± 0.3 % (IG) and 1.9 ± 0.5 (TP) at the beginning of the experiment. However, a significant increase after incorporation of the leguminous plant residues to 2.9 ± 0.9 % (FS) was observed (see Supplementary Table S4). Similar trends on genus level were observed for *Lysobacter* (*Proteobacteria*), which was almost not present within IG, TP, and CG but significantly showed up in FS outlining abundances of 1.0 ± 0.3 % (see Supplementary Table S4).

The most pronounced increase was observed for members of the order *Betaproteobacteriales*, its family *Burkholderiaceae* and therein its genus *Massilia* following the cultivation of *V. faba* and its subsequent incorporation (FS). However, members of the order *Rhizobiales* and its family *Xanthobacteraceae* were found to be predominant during the entire experiment.

4. Discussion

The present study shows the successful transformation of a former marginal grassland (IG) to arable land (FS) via a transitional nitrogen fixing phase (TP).

Already in the beginning of the experiment after ploughing and milling the initial grassland and during growth of *V. faba* (TP) a strong enrichment of nitrate-N and TNb was observed. Mineralization processes following the incorporation of the residual green of the initial grassland (Chen et al., 2014) dominated this increase in the transitional phase. The subsequent further increase at the final state of the conversion was dominated by nitrogen fixation of the legume (Fan et al., 2006) and the later incorporation of the leguminous plant residues (Ordóñez-Fernández et al., 2018). In total, the combined effects led finally to the high amount of 50 µg nitrate-N g-1 *dw* (150 kg N/ha) which is already sufficient for future crop cultivation.

Unlike the strong increase of nitrate-N and TNb, the carbon content (DOC) outlined its maximum in the transitional phase (TP) after incorporation of the grass residues and later decreased after incorporation of the leguminous plant residues (FS). The later decrease of DOC might indicate the utilization of soil organic carbon for bacterial immobilization of nitrogen after incorporation of the leguminous crop residues (Reichel et al., 2018).

The increasing amounts of TNb, as well as the decrease of DOC at the final state of the experiment, explained the decreasing trend of the DOC/TNb ratio. This ratio was with 3.0 highest in the initial grassland and only slightly lower in the control grassland. Interestingly, during the conversion the ratio decreased during growth of *V. faba* (TP) and reached its minimum after incorporation of the leguminous plant residues (FS) into the soil.

Soil properties of the initial and control grassland were comparable with respect to concentrations of nitrate-N, ammonium-N, DOC, TNb and its ratio DOC/TNb. It may hence be concluded that the strong increase of nitrate-N and the variations in DOC and the ratio DOC/TNb mainly depended on farm management and not on seasonal effects.

Plant performance and health of *V. faba* (Supplementary Table S2) observed on this field followed a homogenous pattern that is suitable for subsequent bacterial analysis. The prevailing pH was 5.4 ± 0.2 and thus optimal for root nodule formation (around 60 per plant) in *V. faba* (Torabian et al., 2019). The constant pH is one benefit of incorporating leguminous plant residues to enrich nutrient stocks within soil instead of using e.g. ammonium-based fertilizers since the increase of the net H+ concentration after application of ammonium-based fertilizers leads to acidification of agricultural soils with negative effects on plants and organisms (Crews and Peoples, 2004). Different studies indicate pH as important factor for shaping bacterial community composition in grassland and agricultural soils (Kaiser et al., 2016, Wu et al., 2017). However, in our study effects of pH on bacterial community structure could be excluded because the pH remained constant independent of conversion state and spatial distribution within the field (see Table 1).

Simpson’s index of diversity showed an extremely diverse bacterial community structure within the entire field experiment. The initial grassland (IG), the transitional phase (TP), and the control grassland (CG) had highest diversity during the conversion. In the final state (FS) diversity was significantly lower indicating a slight shift of the bacterial composition to more dominant species when plant cover was lacking.

This trend was supported by a strong shift of beta-diversity toward the final state of the experiment. Furthermore, the NMDS analysis revealed high similarity for the initial grassland (IG) and the transitional phase (TP) indicating a quite stable bacterial community structure in the beginning of the experiment. However, although the control grassland (CG) outlined an overlap with the initial grassland (IG) and the transitional phase (TP), it slightly changed. Since soil nutrient stocks (nitrate-N, ammonium-N, DOC, and TNb) and environmental factors (soil temperature, soil moisture, and soil pH) of the initial and the control grassland remained quite stable this bacterial shift leads to the assumption that seasonal effects influenced the bacterial community structure in our grassland. Still, the control grassland differed significantly from the final state of the conversion (FS), indicating that the effects of the different conversion steps were much stronger than the seasonal effects. Drenovsky et al. (2010) and Xue et al. (2018) suggested accordingly that effects of precipitation and elevation have a weaker influence on shaping bacterial communities than soil properties and agricultural practices.

Phylogenetic lineage analyses based on 16S rRNA gene sequences showed highest abundances on phylum level for *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, and *Bacteroidetes*. Similar observations for the dominant phyla in temperate grasslands were observed by Kaiser et al. (2016) and Delgado-Baquerizo et al. (2018). *Proteobacteria* were the predominant phylum during this study, significantly increasing at the final state after incorporation of the leguminous plant residues (FS). *Proteobacteria* are good indicators of crop residue degradability (Pascault et al., 2010) which explains their higher occurrence during decomposition of the incorporated legumes. Interestingly, no further significant changes on phylum level could be observed. It may hence be concluded, that grassland conversion and management practices did only slightly influence the bacterial community structure on phylum level. However, the previously described increase was mainly caused by increasing abundances of members of the order *Betaproteobacteriales* after the incorporation of the leguminous plant residues (FS). This was mainly driven by the strong increase in abundance of the family *Burkholderiaceae* and its genus *Massilia*. Recent studies describe *Massilia*, formerly aligned to the family *Oxalobacteraceae*, as rhizosphere associated (Ofek et al., 2012) and plant-growth promoting rhizobacteria in leguminous plants (Xiao et al., 2017). Their high occurrence in bulk soil after the incorporation of leguminous plant residues can be explained by bacterial decomposition of plant material and its release to the soil. Pascault et al. (2010) similarly reported strong increases of *Massilia* for early stages of decomposition of plant material. Other studies showed that several isolates of *Massilia* are able to reduce nitrate (Zhang et al., 2006, Bailey et al., 2014) which furthermore explains their occurrence in line with the highest nitrate-N content in bulk soil at the final state.

Similarly, increasing abundances of *Lysobacter* (*Proteobacteria*) after incorporation of *V. faba* may be explained by degradation processes of *V. faba* residues. Similar effects were also observed in tillage-residue management studies of Chávez-Romero et al. (2016). Interestingly, members of the genus *Lysobacter* have been described as promising candidates for biological control of plant diseases (Hayward et al., 2010), and increased abundances of *Lysobacter* might indicate improved soil quality (Wang et al., 2017).

Furthermore, members of the family *Gaiellaceae* strongly increased toward the final state of the conversion. The family *Gaiellaceae* was found to be a good indicator of the carbon to nitrogen ratio (Hermans et al., 2017). The postulated negative correlation of its abundance to the DOC/TNb ratio could be supported by our findings, which showed an increasing amount of *Gaiellaceae* after incorporation of *V. faba* plant residues in line with the lowest DOC/TNb ratio.

Interestingly, members of the family *Nitrosomonadaceae* finally developed their lowest abundances after incorporation of the plant residues. Similar results for decreasing abundances of the ammonia-oxidizing family after incorporation of some cover crops and organic fertilizer application have been reported (Fernandez et al., 2016). Similarly, members of the order *Myxococcales* outlined their lowest abundance in the final state. Herzog et al. (2015) showed lowered abundance of *Myxococcales* in line with lower carbon to nitrogen ratios. The most abundant phylotype within this order could be assigned to the genus *Haliangium*. High abundances of *Haliangium* in german grassland soils have also been shown by Kaiser et al. (2016).

All grassland plots were dominated by members of the order *Rhizobiales*. Kaiser et al. (2016) showed similar results for temperate grassland soils. Decreasing abundances in the transitional phase during growth of *V. faba* were linked to lower plant density and species diversity following the removal of the grass scar. Members of the family *Xanthobacteraceae* were most responsible for this decrease during *V. faba* growth and have been predominant in the grassland plots. *Pseudolabrys*, *Afipia*,and *Bradyrhizobium*, the three most abundant genera aligned to this family, have been identified to be diminished in the transitional phase. The subsequent increase at the final state may be due to the release of some members of *Rhizobiales* after the decomposition of the leguminous plant material. Denison and Kiers (2011) have reported similar effects on bulk soil for rhizobia (e.g. *Bradyrhizobium*) escaping from senescing nodules.

**5. Conclusion**

Overall, this study elucidates responses of soil bacteria after converting a temperate grassland to agricultural land via a transitional nitrogen-fixing phase. Results revealed a quite stable bacterial composition on phylum level, which was dominated by *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, and *Bacteroidetes*. Bacterial richness did not change during this short-term field trial. However, Simpson’s index of diversity revealed a highly diverse bacterial community structure, which slightly decreased after the conversion. This change at the final state was supported by our beta-diversity analysis indicating changes due to the management practices. The study also revealed slight seasonal variations within the grassland plots. However, the change in the bacterial community structure was much more pronounced after converting the initial grassland to its final agricultural state. Strongest increase was observed for the family *Burkholderiaceae*, its genus *Massilia* as well as the genus *Lysobacter* after incorporation and decomposition of *V. faba* plants. The increase of the family *Gaiellaceae*, its genus *Gaiella* as well as the decrease of members of the order *Myxococcales* was linked to the decrease of the carbon to nitrogen ratio during the conversion. Furthermore, changes appearing already in the transitional phase were mainly induced by decreasing abundances of *Rhizobiales,* especially of its family *Xanthobacteraceae* caused by the lower plant diversity. The strongly enriched nitrate-N, the lowered DOC/TNb ratio and effects occurring from decomposition processes were the main drivers of the community changes. Mineralization processes after grassland conversion, the nitrogen fixation of *V. faba* and its subsequent incorporation contributed to the strong mobilization of the nitrate-N pool in the final plots, ideal for further cropping of arable plants.

**Abbrevations**

*ASV* Amplicon sequence variant

*bp* Base pairs

*BSA* Bovine serum albumin

*C* Carbon

*CG* Control grassland

*Chl* Chlorophyll

*DNA* Deoxyribonucleic acid

*DOC* Dissolved organic carbon

*dw* Dry weight

*FS* Final state

*fw* Fresh weight

*FACCE-JPI* Joint Programming Initiative on Agriculture, Food Security and Climate Change

*IG* Initial grassland

*INTENSE* Intensify production, transform biomass to energy and novel goods and protect soils in Europe

*N* Nitrogen

*NA* Not assigned

*NMDS* Non-metric multidimensional scaling

*spp.*  Species pluralis

*PCR* Polymerase chain reaction

*rRNA*  Ribosomal ribonucleic acid

*TNb* Total nitrogen bound

*TP* Transitional phase

**Funding information**

This work was supported and financed by the Joint Programming Initiative on Agriculture, Food security and Climate Change (FACCE-JPI) of the European Research Area (ERA-NET), in the frame of the INTENSE project.

**Data availability**

The nucleotide sequence data reported are available in the SRA database (NCBI) under the BioProject ID PRJNA471669.

**Acknowledgments**

The authors would like to thank the team of the Research Unit for Comparative Microbiome Analysis (COMI) of the Helmholtz Zentrum München for its support within this project. In addition, we like to thank Viviane Radl and Andrés Sauvêtre for revising the manuscript, Silvia Gschwendtner and Johan S. Sáenz for their support with the sequence data analysis as well as Georg Gerl and Christoph Poschenrieder for the management of the experimental site.

**Conflicts of Interest**

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

**References**

Aggelides, S.M., Londra, P.A., 2000. Effects of compost produced from town wastes and sewage sludge on the physical properties of a loamy and a clay soil. Bioresource Technol., 71:253-259.

Ahmed, M., Rauf, M., Mukhtar, Z., Saeed, N.A., 2017. Excessive use of nitrogenous fertilizers: an unawareness causing serious threats to environment and human health. Environ. Sci. Pollut. Res., 24:26983-26987.

Bailey, A.C., Kellom, M., Poret-Peterson, A.T., Noonan, K., Hartnett, H.E., Raymond, J., 2014. Draft genome sequence of *Massilia* sp. strain BSC265, isolated from biological soil crust of Moab, Utah. GenomeAnnounc., 2 e01199-14.

Barrios, E., 2007. Soil biota, ecosystem services and land productivity. Ecol. Econ., 64:269–285.

Buchen, C., Well, R., Helfrich, M., Fuß, R., Kayser, M., Gensior, A., Benke M., Flessa, H., 2017. Soil mineral N dynamics and N2O emissions following grassland renewal. Agri. Ecosyst. Environ., 246, 325-342.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data.Nat. Methods, 13:581-583.

Carbonetto, B., Rascovan, N., Álvarez, R., Mentaberry, A., Vázquez, M.P., 2014. Structure, composition and metagenomic profile of soil microbiomes associated to agricultural land use and tillage systems in argentine pampas*.* PLoS One, 9 e99949.

Chávez-Romero, Y., Navarro-Noya, Y.E., Reynoso-Martínez, S.C., Sarria-Guzmán, Y., Govaerts, B., Verhulst, N., Dendooven, L., Luna-Guido, M., 2016. 16S metagenomics reveals changes in the soil bacterial community driven by soil organic C, N-fertilizer and tillage-crop residue management. Soil Tillage Res., 159:1–8.

Chen, B., Liu, E., Tian, Q., Yan, C., Zhang, Y., 2014. Soil nitrogen dynamics and crop residues. A review. Agron. Sustain. Dev., 34:429-442.

Crews, T.E., Peoples, M.B., 2004. Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agriculture, ecosystems & environment, 102(3), pp.279-297.

De Mendiburu, F., 2014. Agricolae: statistical procedures for agricultural research. R package version 1.

Denison, R.F., Kiers, E.T., 2011. Life histories of symbiotic rhizobia and mycorrhizal fungi. Curr. Biol., 21:775-785.

Delgado-Baquerizo, M., Oliverio, A.M., Brewer, T.E., Benavent-González, A., Eldridge, D.J., Bardgett, R.D., Maestre, F.T., Singh, B.K., Fierer, N., 2018. A global atlas of the dominant bacteria found in soil. Science, 359:320–325.

Diacono, M., Montemurro, F., 2011. Long-Term Effects of Organic Amendments on Soil Fertility, in: Lichtfouse, E., Hamelin, M., Navarrete, M., Debaeke, P. (Eds.) Sustainable Agriculture, Volume 2. Springer, Dordrecht.

Drenovsky, R.E., Steenwerth, K.L., Jackson, L.E., Scow, K.M., 2010. Land use and climatic factors structure regional patterns in soil microbial communities. Global Ecol. Biogeogr., 19:27-39.

EC, 2000. Establishing a framework for community action in the field of water policy*.* Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000. Off. J. Eur. Union,(Brussels, Belgium):1-77.

Ellenberg, H., 1992. Zeigerwerte der Gefäßpflanzen (ohne *Rubus*). Scr. Geobot., 18:9–166.

Estendorfer, J., Stempfhuber, B., Haury, P., Vestergaard, G., Rillig, M.C., Joshi, J., Schröder, P., Schloter, M., 2017. The influence of land use intensity on the plant-associated microbiome of *Dactylis glomerata* L. Front. Plant. Sci., 8:930.

Fan, F., Zhang, F., Song, Y., Sun, J., Bao, X., Guo, T., Li, L., 2006. Nitrogen fixation of faba bean (*Vicia faba* L.) interacting with a non-legume in two contrasting intercropping systems. Plant Soil, 283:275–286.

FAO, 2009. How to feed the world 2050, High level expert forum. FAO, Rome, 2009.

Fernandez, A.L., Sheaffer, C.C., Wyse, D.L., Staley, C., Gould, T.J., Sadowsky, M.J., 2016. Structure of bacterial communities in soil following cover crop and organic fertilizer incorporation. Appl. Microbiol. Biot., 100:9331-9341.

Gatica, J., Cytryn, E., 2013. Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. Environ. Sci. Pollut. Res., 20:3529–3538.

Hartmann, M., Frey, B., Mayer, J., Mäder, P., Widmer, F., 2015. Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 9:1177–1194.

Hayward, A.C., Fegan, N., Fegan, M., Stirling, G.R., 2010. *Stenotrophomonas* and *Lysobacter*: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. J. Appl. Microbiol., 108:756–770.

Hermans, S.M., Buckley, H.L., Case, B.S., Curran-Cournane, F., Taylor, M., Lear, G., 2017. Bacteria as emerging indicators of soil condition. Appl. Environ. Microbiol., 83 e02826-16.

Herzog, S., Wemheuer, F., Wemheuer, B., Daniel, R., 2015. Effects of fertilization and sampling time on composition and diversity of entire and active bacterial communities in german grassland soils. PloS One, 10 e0145575.

Kaiser, K., Wemheuer, B., Korolkow, V., Wemheuer, F., Nacke, H., Schöning, I., Schrumpf, M., Daniel, R., 2016. Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests. Sci. Rep., 6 33696.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res., 41:e1.

Kozlov, A., Darriba, D., Flouri, T., Morel, B., Stamatakis, A., 2018. RAxML-NG: A fast, scalable, and user-friendly tool for maximum likelihood phylogenetic inference. bioRxiv. 447110.

Lal, R., 2016. Soil health and carbon management. Food Energy Secur.,5:212–222.

Leinonen, R., Sugawara, H., Shumway, M., International Nucleotide Sequence Database Collaboration, 2010. The sequence read archive. Nucleic Acids Res., 39, D19-D21.

Lichtenthaler, H., Buschman, C., 2001. Chlorophylls and carotenoids : measurement and characterization by UV-VIS spectroscopy, in: Wrolstad, R.E., Acree, T.E., Decker, E.A., Penner, M.H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Smith, D., Sporns, P. (Eds.) Handbook of Food Analytical Chemistry. Wiley, New York, pp 1–8.

Lindgreen, S., 2012.AdapterRemoval: Easy cleaning of next generation sequencing reads. BMC Res. Notes, 5:337.

Lori, M., Symanczik, S., Mäder, P., Efosa, N., Jaenicke, S., Buegger, F., Tresch, S., Goesmann, A., Gattinger, A., 2018. Distinct nitrogen provisioning from organic amendments in soil as influenced by farming system and water regime. Front. Environ. Sci., 1-14.

McMurdie, P.J., Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One, 8 e61217.

Obermeier, M., Schröder, C.A., Helmreich, B., Schröder, P., 2015. The enzymatic and antioxidative stress response of *Lemna minor* to copper and a chloroacetamide herbicide. Environ Sci. Pollut. Res., 22:18495–18507.

Ofek, M., Hadar, Y., Minz, D., 2012. Ecology of root colonizing *Massilia* (*Oxalobacteraceae*). PloS One, 7 e40117.

ISO 10390, 2005. Soil quality – Determination of pH; Technical Committee ISO/TC 190, Soil quality. Subcommittee SC3. Chemical methods and soil characteristics. OECD Publishing Paris, France:1-7.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R., O’Hara, R.B., Simspon, G.L., Solymos, P. et al., 2018. Vegan: community ecology package. [accessed 2018 Feb 21]. https://cran.r-project.org.

Ordóñez-Fernández, R., Repullo-Ruibérriz de Torres, M.A., Márquez-García, J., Moreno-García, M., Carbonell-Bojollo, R.M., 2018. Legumes used as cover crops to reduce fertilisation problems improving soil nitrate in an organic orchard. Eur. J. Agron., 95:1–13.

Pascault, N., Cécillon, L., Mathieu, O., Hénault, C., Sarr, A., Lévêque, J., Farcy, P., Ranjard, L., Maron, P.A., 2010. In situ dynamics of microbial communities during decomposition of wheat, rape, and alfalfa residues. Microbial Ecol., 60:816-828.

Plaza-Bonilla, D., Nolot, J.M., Raffaillac, D., Justes, E., 2015. Cover crops mitigate nitrate leaching in cropping systems including grain legumes: field evidence and model simulations. Agric. Ecosyst. Environ., 212, 1-12.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res., 41:590-596.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [accessed 2018 Mar 23]. https://www.R-project.org.

Reichel, R., Wei, J., Islam, M.S., Schmid, C., Wissel, H., Schröder, P., Schloter, M., Brüggemann, N., 2018. Potential of wheat straw, spruce sawdust, and lignin as high organic carbon soil amendments to improve agricultural nitrogen retention capacity: an incubation study. Front. Plant Sci., 9, 900.

Schröder, P., Huber, P., Reents, H.R., Munch, J.C., Pfadenhauer, J., 2008. Outline of the Scheyern project, in: Schröder, P., Pfadenhauer, J., Munch, J.C. (Eds.), Perspectives for Agroecosystem Management - Balancing Environmental and Socio-Economic Demands. Elsevier, Amsterdam, pp 3-16.

Schröder, P., Beckers, B., Daniels, S., Gnädinger, F., Maestri, E., Marmiroli, N., Mench, M., Millan, R., Obermeier, M.M., Oustriere, N. et al., 2018. Intensify production, transform biomass to energy and novel goods and protect soils in Europe—A vision how to mobilize marginal lands. Sci. Total Environ., 616–617:1101–1123.

Schröder, P., Sauvêtre, A., Gnädinger, F., Pesaresi, P., Chmeliková, L., Doğan, N., Gerl, G., Gökçe, A., Hamel, C., Millan, R., et al., 2019. Discussion paper: Sustainable increase of crop production through improved technical strategies, breeding and adapted management–A European perspective. Sci. Total Environ., 678, 146-161.

Scotti, R., Bonanomi, G., Scelza, R., Zoina, A., Rao, M.A., 2015. Organic amendments as sustainable tool to recovery fertility in intensive agricultural systems. J. Soil Sci. Plant Nut., 15:333-352.

Sheik, C.S., Beasley, W.H., Elshahed, M.S., Zhou, X., Luo, Y., Krumholz, L.R., 2011. Effect of warming and drought on grassland microbial communities. ISME J., 5:1692–1700.

Stagnari, F., Maggio, A., Galieni, A., Pisante, M., 2017. Multiple benefits of legumes for agriculture sustainability: an overview. Chem. Biol. Technol. Agric., 4(1), 2.

Torabian, S., Farhangi-Abriz, S., Denton, M.D., 2019. Do tillage systems influence nitrogen fixation in legumes? A review. Soil Tillage Res., 185, 113-121.

Wang, R., Zhang, H., Sun, L., Qi, G., Chen, S., Zhao, X., 2017. Microbial community composition is related to soil biological and chemical properties and bacterial wilt outbreak. Sci. Rep., 7:343.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer, New York, 2016.

Wu, Y., Zeng, J., Zhu, Q., Zhang, Z., Lin, X., 2017. pH is the primary determinant of the bacterial community structure in agricultural soils impacted by polycyclic aromatic hydrocarbon pollution. Sci. Rep.,7:40093.

Xiao, X., Fan, M., Wang, E., Chen, W., Wie, G., 2017. Interactions of plant growth-promoting rhizobacteria and soil factors in two leguminous plants. Appl. Microbiol. Biotechnol., 101:8485-8497.

Xue, P.P., Carrillo, Y., Pino, V., Minasny, B., McBratney, A.B., 2018. Soil properties drive microbial community structure in a large scale transect in South Eastern Australia. Sci. Rep., 8:11725.

Zhang, Y.Q., Li, W.J., Zhang, K.Y., Tian, X.P., Jiang, Y., Xu, L.H., Jiang, C.L., Lai, R., 2006. *Massilia dura* sp. nov., *Massilia albidiflava* sp. nov., *Massilia plicata* sp. nov. and *Massilia lutea* sp. nov., isolated from soils in China. Int. J. Syst. Evol. Microbiol., 56:459-463.

Zhong, W., Gu, T., Wang, W., Zhang, B., Lin, X., Huang, Q., Shen, W., 2010. The effects of mineral fertilizer and organic manure on soil microbial community and diversity. Plant Soil, 326:511–522.