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Original Article

Soil bacteriobiome under wheat fertilization in a long-term multicrop rotation experiment in Kyrgyzstan

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Abstract

Crop rotation, one of the most fundamental agronomic practices, has been widely used to avoid drastically compromising soil quality. From the longest in Central Asia multicrop rotation experiment we collected Calcisol samples with the aim to assess bacteriobiome structure and diversity using 16S rRNA gene metabarcoding; in this pilot study we used the plots cropped for winter wheat under different fertilization treatments (no fertilizers, NPK and NPK+manure) and collected soil samples three months after wheat harvest to allow the effects of soil disturbance and post-harvest phytomass residues input in soil to subside. In this first survey all major dominant phyla, namely *Pseudomonadota*, *Acidobacteriota*, *Actinomycetota*, *Bacillota* and *Bacteioidota*), together accounted for 85%, each having the same abundance under different fertilization. Overall, the long-term fertilization under multicrop rotation was not found to have a notable effect on soil bacteriobiome as only minor or rare taxa had changes in their abundance that were very small in size and hence hardly ecologically and agronomically significant. Soil bacteriobiome α-biodiversity indices were not affected by fertilization as well: the repetitive management practices might have increased the homogeneity of ecological niches for bacteria, thus equalizing biodiversity. Such bacterial genera as *Sphingomonas*, *Stenotrophobacter* and *Pseudarthrobacter*, as the most responsive to changes in soil environment under different treatments and being the drivers of β-biodiversity, warrant further research attention as related to the arable Calcisols functioning.

Keywords: 16S rRNA genes, Illumina Miseq, Metabarcoding, Crop rotation, Calcisol

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Introduction

Crop rotation, one of the most fundamental and predominant agronomic practices, has been a widely used technique aimed at sustaining crop yields without drastically compromising soil quality. Although by now there is better understanding of the mechanisms how crop diversity increases productivity, the role of soil and plant microbiome remains much less clear, despite the universal consensus that the plant–soil–microbiome interactions determine the benefits of crop diversification for plant growth and development (Jing et al., 2022). As plant exudates shape the rhizosphere microbiome, different crops in the same soil can differ in their rhizosphere microbiota diversity (Hu et al., 2018), leaving a certain legacy to affect the subsequent crop (Benitez et al., 2021).

Truly long-term field experiments, embracing sometimes more than a century (Perryman et al., 2018; Górska et al., 2022), with fertilization and crop rotation are very rare. Such experiments "take years to mature, are vulnerable to loss, and have yet to be comprehensively inventoried or networked" (Richter et al., 2007) and represent globally unique objects for research. The available field experiments can and should be used for addressing a range of contemporary issues, providing important information to soil management, including how the latter can establish greater control over nutrient cycling and microorganisms-soil environment interactions. In Central Asia the longest (58 years) multicrop rotation and fertilization field trial has been maintained by the Kyrgyz National Agrarian University (KNAU, Bishkek, Kyrgyz Republic) since 1967. Alfalfa, winter wheat, spring wheat, sugar beet and corn have been the main crops, with some of them used twice or thrice (alfalfa) in the 9-year field succession. The crops were grown under the mineral or mineral and manure fertilizers application. Fertilization, affecting various aspects of plant–soil–microbiome interactions, modifies the effect of crop diversification, but the degree and mechanisms of such modification have not been studied in detail. Therefore, there is limited information regarding the importance of fertilization in shaping soil microbiome over crop rotation stages. The Sustainability Report 2022 of the International Fertilizer Association (IFA, 2023) states that "Mineral fertilizers feed about 50% of the global population every day, and with continuously rising population numbers, the role of fertilizers will be even more indispensable to ensure food availability and affordability in the coming decades".

So the main objective of this study was to investigate the soil microbiome in the KNAU experiment under different fertilization treatments to find the effect of almost six decades of application on the soil bacteriobiome. For this study we used some of the plots from this field experiment, namely winter wheat without fertilizers and fertilized with NPK or NPK combined with manure, to reveal Calcisol bacteriobiome composition and structure using 16S rRNA gene metabarcoding and chart future avenues for research within the framework of this experiment.

Material and Methods

Experimental site and conditions

The study was performed using some plots of the field trial started in 1967 at the experiment station of the then Kyrgyz Agricultural Institute and currently Kyrgyz National Agrarian University named after Skryabin (KNAU, Bishkek, Kyrgyz Republic). This KNAU field trial is the longest existing one in Central Asia, having been established as the basic trial in the geographical network of field trials with mineral fertilizers under the supervision of the All-Union Institute of Fertilizers and Agricultural Soil Science named after D.N. Pryanishnikov (Kuznetsov et al., 2003b).

The experimental field is located in the Sukuluk administrative district of the Chuisky region of Kyrgyzstan (42.96 N, 74.38 E). The relief of the experimental area is flat, with a slight (0.2°) incline northward. The ground water can be found at 1.3–1.5 m. According to the geomorphology and the Soil Classification adopted in Kyrgyzstan, the soil of the experiment is classified as a serozem meadow soil (or gray-meadow soil) of semi closed inter-mountain depressions (500–1000 m above sea level). The topsoil is loam. According to the World Reference Base for Soil Resources (IUSS, 2022), the soil is classified as Gleyic Calcisol (Siltic, Aric).

The climate of the Chuisky Valley, where the field trial is carried out, is characterized by hot summers, moderately cool winters and large deficit of precipitation. Annual precipitation, as averaged over 1981-2010, is 451 mm, and with mean daily maximal temperatures from May till October ranging 35-40 °C (WMO, 2025).

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Experimental setup

The field trial was started in 1967. Since 1968 regular measurements of crop yields, as well as occasional measurements of soil and plant properties, have been conducted. The total experiment area comprises 5 ha, occupied by 56 plots, corresponding to 14 treatment variants with four replicates, the plots separated by protection strips of 1.5 m wide. Although the crop rotation sequence was somewhat modified, since 1995 it has been as following: 1) spring barley with alfalfa, 2) 2nd year alfalfa, 3) 3rd year alfalfa, 4) first winter wheat, 5) sugar beet, 6) spring wheat, 7) first corn, 8) second winter wheat and 9) second corn.

Furrow irrigation system has been maintained since the start of the experiment. Mineral fertilizers were applied in the form of ammonium nitrate (34.0% N, 120 kg N ha^{-1}), superphosphate (19.5% P_2O_5, 78.6 kg P ha^{-1}) and potassium chloride (48-52% K_2O, 49.8 kg K ha^{-1}). Cattle manure was applied at the rate of 30 t/ha for both corn fields, i.e. manure application, closest to the soil sampling for the study, was four years prior. As for pesticide application, winter wheat was treated with insecticide Mospilan Art (Nippon Soda Co, Tokyo, Japan) and herbicides Estor (Syngenta, Basel, Switzerland) and Ovsjugen (Shelkovo Agrochem, Shelkovo, Russia).

In the year of soil sampling for this study (2022) the unfertilized winter wheat yielded 3.6 t/ha, whereas wheat under NPK and NPK plus manure yielded 5.7 and 5.2 t/ha, respectively. Despite the long term of the experiment, most of its published results pertained to the agronomic efficacy of mineral and organic fertilizers and the fertilizers effect on the crop yields (Akhmatbekov et al., 2023; Kuznetsov et al., 2003b; Mambetov et al., 2016), whereas the effect of the long-term fertilization on soil chemical properties received less attention.

Soil sampling and chemical analyses

To study the effect of fertilizers, we investigated a subset of the treatments from the experiment, namely the plots cropped for winter wheat (the 4th crop in the rotation) in the year of sampling: the control no-fertilization treatment (hereinafter designated as No), treatment with mineral NPK fertilizers (NPK) and NPK combined with manure (NPK+M) treatment. Winter wheat was chosen as the major staple grain crop, both in Kyrgyzstan and Russia.

Soil sampling took place in late October 2022, i.e. three months after harvesting wheat, to allow the effect of disturbance and stress on soil microbiome to subside, as recommended by Nannipieri et al. (2019). Samples were collected from the 0–20 cm layer from each plot: six separate subsamples were collected with the help of a soil corer and bulked together into a composite sample. Five such soil samples, i.e. individual soil replicates, were collected for each of the treatment, chosen for the study, i.e. in total 15 composite soil samples. After removing roots and plant fragments, soil was passed through a 2 mm sieve and portions were taken for drying and, storage at +4°C and for the DNA extraction. Soil properties were determined using standard methods (Carter and Gregorich, 2008). Briefly, the amount of organic matter in the soil was measured based on mass loss upon combustion at 550 °C for 12 hours. Soil total carbon and nitrogen contents were estimated using an elemental analyzer (CHNS/O 2400 Serie II, Perkin Elmer, Waltham, MA, USA). Nitrate content was determined potentiometrically in 0.015 M K_2SO_4 solution (soil:solution ratio 1:5 w/v); available soil P was extracted by 0.1 M $(NH_4)_2C_2H_4O(COO)_2$ solution (pH=5.7; 1:20 w/v) and determined calorimetrically. Exchangeable cations (Ca, Mg, Na, K) and cation exchange capacity (calculated as a sum of the cations) were measured by extraction in 1 M CH_3COONH_4 solution (pH=7.0; 1:10 w/v) and estimated by atomic absorption spectrometer with flame atomization (Kvant-2A, Russia). Soil pH was measured by equilibrating 10 g of field-moist soil with 25 ml of deionized water. All analyses were performed in triplicates, and the data expressed on the oven (105 °C)-dry basis (Table 1).

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Table-1. Soil properties of Calcisol (0–20 cm) in the studied fields (mean ± standard deviation).

Soil property	Wheat		
	No	NPK+M	NPK
Sand, %	16.4 ± 0.6 b	15.4 ± 0.5 a	15.2 ± 1.0 a
Silt, %	63.8 ±1.6 a	70.1 ±1.5 b	69.3 ± 1.1 b
Clay, %	19.8 ± 1.0 b	14.5 ± 1.1 a	15.5 ± 1.7 a
Total C, %	1.61 ± 0.05 a	1.85 ± 0.08 c	1.69 ± 0.05 b
Total N, %	0.12 ± 0.01 a	0.14 ± 0.01 b	0.13 ± 0.01 a
C/N ratio	15.6 ± 1.0	16.0 ± 0.9	15.2 ± 1.7
SOM, mg g^{-1} soil	30.3 ± 0.8	34.2 ± 3.2	30.6 ± 1.5
pH_{water}	8.04 ±0.03 b	8.01 ± 0.06 ab	7.95 ± 0.06 a
EC, µS	430 ± 39 a	449 ± 45 ab	535 ± 35 b
Nitrates, mg N/kg	74 ± 8 a	66 ±10 a	96 ± 9 b
Available P, mg P_2O_5/kg	25 ± 2 a	45 ± 4 b	153 ± 24 c
Exchangeable cations, cmol(+)/kg	22.7 ± 0.3 a	23.3 ± 0.5 b	22.4 ± 0.4 a

NPK+M stands for NPK+Manure fertilization. Values in rows followed by the different letters differ significantly (P≤0.05, Fisher's test). SOM stands for soil organic matter.

16S metabarcoding

The DNA extraction and libraries preparation were performed exactly as we described earlier (Naumova et al., 2024). Briefly, V3-V4 region of 16S rRNA genes were amplified with the primer pair 343F and 806R. Sequencing was also performed by the Miseq sequencer at the Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia). The raw reads were submitted to the NCBI SRA under bioproject accession number PRJNA1271229.

Bioinformatic analysis

The bioinformatic analysis was carried out as previously described (Naumova et al., 2024), analyzing raw sequences with UPARSE pipeline (Edgar, 2013), using Usearch v.11.0.667, the UPARSE-OTU algorithm, SINTAX (Edgar, 2013) for the taxonomic attribution, referenced with the 16S RDP training set v.19 (Wang et al., 2007).

The operational taxonomic units (OTUs) datasets were analyzed by individual rarefaction (graphs are not shown) with the help of the PAST software (Hammer et al., 2001): the numbers of OTUs detected, which plateaued as the number of sequences increased, indicated that sampling was nearly saturated for all samples, making it sufficient to compare biodiversity in the non-rarefied datasets in order to preserve as much information as possible (Willis, 2019).

Statistical analysis

Statistical analyses (descriptive statistics, ANOVA, PCA, PCoA, SIMPER, ANOSIM and PLS) were performed by using Statistica v.13.3 (TIBCO Software Inc., Palo Alto, CA, USA) and PAST v. 4.16 (Hammer et al., 2001) software packages. Analysis of variance (ANOVA) was performed to find differences in taxa relative abundance. Principal components analysis (PCA) provided hypothetical (new) variables (principal components) as linear combinations of the original variables accounting for as much as possible of the variance in the multivariate datasets; these new variables were also analyzed by ANOVA to find the variables that can contribute to the differences between treatments. Non-parametric analysis of similarity (ANOSIM) was carried out to compare fertilization treatments, based on Bray-Curtis distance measure converted to ranks. To assess which taxa are primarily responsible for the observed difference between the fertilization treatments, a similarity percentage (SIMPER) was evaluated, its significance of the difference being assessed by ANOSIM. Tests' statistics were considered statistically significant at the

$p \leq 0.05$ level. Two-block Partial Least Squares (PLS) was used as an ordination method with the objective of maximizing covariance between two sets (blocks) of variates (in this study soil properties as one block and taxa relative abundance or α-biodiversity indices as another block) for different fertilization treatments. OTUs-based α- and β-biodiversity indices were calculated using PAST software. As it was recently verified that sequencing depth had no impact on the total number of amplicon sequence variants and singletons (Cassol et al., 2025), and aiming to use as much information as possible (Willis, 2019), α-biodiversity indices were calculated on the non-rarefied datasets.

Results

General taxonomic diversity of the soil bacteriobiome

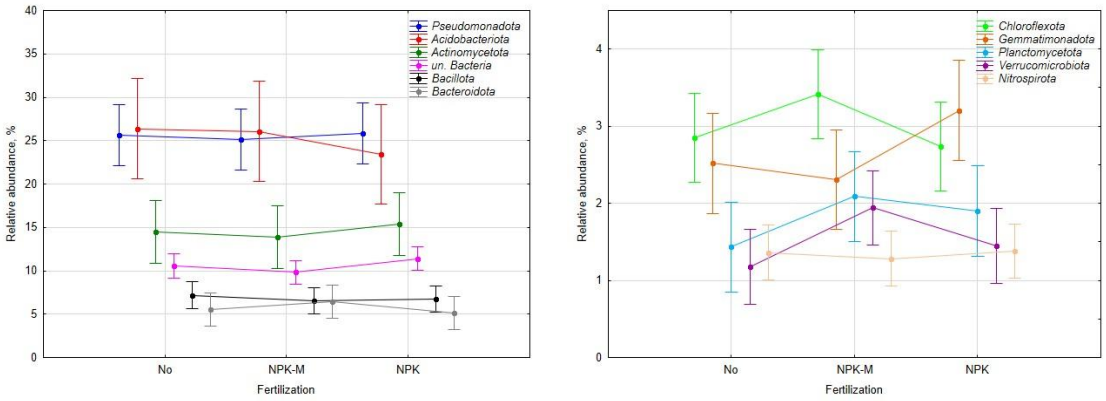
We identified 2108 OTU-level clusters, belonging to 21 phyla, 76 classes, 136 orders, 237 families and 420 genera. Of the total number of OTUs 24% belonged to *Pseudomonadota*, whereas *Actinomycetota* and *Acidobacteriota* contributed 14% and 12%, respectively. These three phyla together accounted for half of the OTUs in the dataset of the study. Notably, almost 15% of the OTUs were identified as *Bacteria* only, without attributing the cluster to lower hierarchical levels.

Soil bacteriobiome taxonomic composition as related to fertilization

As for the relative abundance of sequence reads, *Pseudomonadota* was on par with *Acidobacteriota* (Figure 1a) contributing 25.5 ± 3.3% (mean ± s.d.) and 25.3 ± 5.6, respectively, as averaged over all treatments. Notably, all major dominants, i.e. phyla with ≥ 5% of the relative abundance, showed very similar pattern under different fertilization without any statistically significant differences.

The minor phyla dominants, i.e. the ones contributing from 1 to 5% into the total number of sequence reads, showed more pronounced variation in their relative abundance between the treatments (Figure 1b). Statistically significant difference was found for the *Verrucomicrobiota* phylum as its relative abundance in the NPK+manure treatment was higher as compared with the NPK only.

Among the dominant genera, only *Rubrobacter* showed differential abundance, being twice as low in the NPK field as compared with the control and NPK+manure fields (Table 2).



(a) **(b)**

Figure-1. Relative abundance of the major (with ≥ 5% of the relative abundance, (a) and minor (1– 5%), (b) dominant bacterial phyla in arable Calcisol under different fertilization (mean ± confidence interval).

Table-2. The relative abundance of the dominant bacterial genera in the arable Calcisol fields under different fertilization (mean ± standard deviation) and scores of some principal components extracted from the entire data set of the genera (percentage in brackets gives the contribution of the principal component in the total data variance).

Genus	No	NPK+manure	NPK
un. *Acidobacteria*_Gp6	11.1 ± 1.0	12.6 ± 1.7	10.0 ± 2.0
un. *Acidobacteria*_Gp4	6.1 ± 0.6	4.8 ± 0.9	4.3 ± 0.7
Sphingomonas	3.6 ± 0.5	4.1 ± 0.7	3.7 ± 0.5
Niallia	2.4 ± 0.2	1.9 ± 0.1	2.0 ± 0.3
Skermanella	2.1 ± 0.4	2.4 ± 0.4	2.4 ± 0.4
Pseudarthrobacter	2.0 ± 0.8	2.0 ± 0.3	1.7 ± 0.3
un. *Acidobacteria*_Gp16	1.7 ±0.1	1.7 ± 0.2	2.2 ± 0.2
Stenotrophobacter	1.7 ± 0.1	2.1 ± 0.4	1.7 ± 0.3
un. *Acidobacteria*_Gp3	1.6 ± 0.0	1.5 ± 0.2	1.6 ± 0.2
Nitrospira	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.2
Rubrobacter	1.3 ± 0.1 b	1.4 ± 0.2 b	0.7 ± 0.1 a
Neobacillus	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Gaiella	1.2 ± 0.1	1.1 ± 0.2	1.2 ± 0.1
Microvirga	1.1 ± 0.2	1.3 ± 0.2	1.1 ± 0.1
Tepidisphaera	1.0 ± 0.5	1.6 ± 0.2	1.4 ± 0.2
Actinoplanes	0.1 ± 0.0	0.1 ± 0.0	1.4 ± 1.3
PC1(60%)	0.1 ± 0.7	0.2 ± 1.1	-0.3 ± 1.2
PC2 (14%)	0.2 ± 1.2 ab	-0.7 ± 0.8 a	0.5 ± 0.6 b
PC3 (13%)	-0.44 ± 0.45	-0.05 ± 0.56	0.49 ±1.56
PC4 (4%)	-0.8 ± 0.8 a	0.3 ± 1.1 ab	0.5 ± 0.7 b

"un." stands for unclassified. Different letters in rows indicate that the values are different (P≤0.05, Fisher's LSD test); the absence of letters denote the absence of statistically significant differences. PC stands for a principal component extracted from the entire data set of the genera; percentage in brackets gives the contribution of the principal component in the total variance of the data.

Overall, 59 genera showed relative abundance as differential to fertilization; however, with exception of *Rubrobacter*, most of them contributed much lower than 1% in the total number of sequence reads, i.e. they were rare genera. Some of the principal components, extracted from the entire dataset of the genera relative abundance, showed statistically significant changes due to fertilization (Table 2), grasping the variance between the NPK+M and NPK fields (PC2) and the No and NPK fields (PC4). Such genus-level clusters as un. *Acidobacteria*_Gp4 and Gp6, as well as *Pseudarthrobacter* and *Sphingomonas* contributed the main (≥ 0.05 each) share in the PC2; however, the latter was mostly defined by the unidentified *Bacteria* (0.43). As for the PC4, it was mostly defined by un. *Acidobacteria*_Gp4 genus-level cluster, as well as *Pseudarthrobacter*, *Actinoplanes*, un. *Chitinophagaceae* and un. *Gemmatimonadaceae*.

The bacteriobiome α-biodiversity indices were similar in the studied fields (Table 3).

As for the β-biodiversity, samples from different fields were not separated distinctly on the graphs (Figure 2, a and b).

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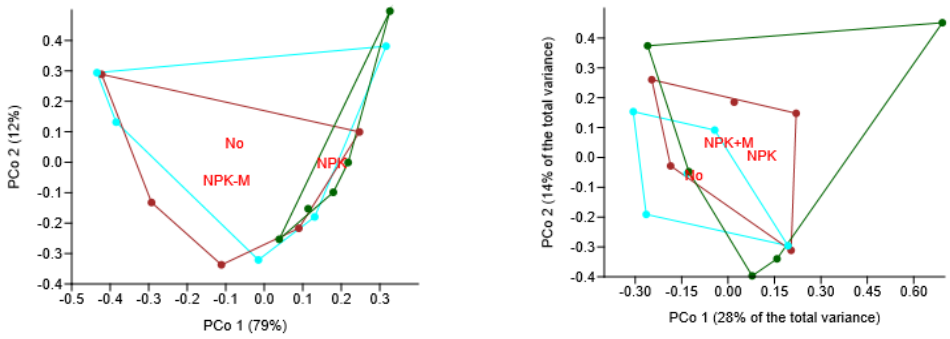
Table-3. Bacteriobiome α-biodiversity indices of Calcisol under different fertilization (calculated on the OTU's basis; mean ± standard deviation).

Index	No	NPK+M	NPK
OTU richness	1411 ± 310	1397 ± 232	1599 ±78
Chao-1	1634 ± 246	1662 ± 153	1789 ± 43
Simpson (S)	0.994 ± 0.001	0.994 ± 0.001	0.994 ± 0.000
Shannon's	6.1 ± 0.1	6.1 ± 0.0	6.1 ± 0.1
Evenness	0.34 ± 0.06	0.33 ± 0.05	0.28 ± 0.04
Equitability	0.85 ± 0.02	0.85 ± 0.02	0.82 ± 0.02
Berger-Parker	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.02
Dominance (1-S)	0.006 ± 0.001	0.006 ± 0.000	0.006 ± 0.002

The absence of letters after numbers in rows denote the absence of statistically significant differences.



a b

Figure-2. Principal coordinates analysis of the soil bacteriobiome composition (Bray-Curtis dissimilarity distance) under different fertilization: location of samples in the plane of the first two coordinates (phylum level, a, and OTU level, b). Symbols: turquoise dots denote no fertilization, brown dots denote NPK+manure, and dark green dots denote NPK fertilization.

Analysis of similarity percentage (SIMPER), used for assessing which OTUs were mainly responsible for some shifts between fertilization treatments (Figure 2b), showed that four OTUs (*Sphingomonas* sp., *Pseudarthrobacter* sp., un. *Acidobacteria*_Gp4 and *Skermanella* sp.) contributed ≥1% in shifting between No and NPK+manure, with average dissimilarity in this case being 30%. Six OTUs (the same four mentioned above plus *Niallia* sp. and *Actinoplanes* sp.) contributed ≥1% in shifting between No and NPK, overall average dissimilarity being 33%. And, except for *Niallia* sp., the other five of those OTUs contributed ≥1% in shifting between NPK and NPK+manure), the average dissimilarity being the same as between No and NPK. Analysis of similarities (ANOSIM), based on the Bray-Curtis dissimilarity distance and comparing distances between groups with distances within groups, provided nearly statistically significant p-value of 0.066 for the distance between No and NPK bacteriobiome.

Soil bacteriobiome structure and biodiversity as related to soil properties

To access the relationship between soil properties and bacteriobiome taxonomic structure we performed two-block partial least squares (PLS) analysis to find latent variables (called axes in PAST) maximizing covariance between original variables in the two blocks (soil properties, Block1, and bacterial taxa abundance, Block2). Location of samples in the

Block1-Block2 plane of the Axis 1 scores showed clear separation of NPK-fertilized samples from No and NPK+manure ones, both on the basis of phyla and genera abundance as Block 2 variables (Figure 3a and b). Samples location and variables in the plane of the first two axes, together accounting for 70% of the total covariance, showed that some phyla have predilection for nitrates, available phosphorus and electric conductivity, whereas some major (*Pseudomonadota*, *Bacteroidota*) and minor (*Planctomycetota* and *Verrucomicrobiota*) dominant phyla tended to group near soil organic matter, total C and N, being in the same semiplane with exchangeable cations, cation exchange capacity, silt and NPK+manure fertilized samples (Figure 3a). The *Bacillota* phylum, one of the major dominants, with relative abundance averaging over all samples at 6.8%, as well as *Nitrospirota* (1.3%), are located near soil total C/N ratio. The squared covariance, i.e. a measure of the overall squared covariance between the soil and bacteriobiome blocks of variables, in percent relative to the maximum possible (all correlations equal to 1) accounted for 6.1%. As for the same analysis, but with the genus-level abundance in Block 2 instead of the phylum-level one, several genus-level clusters showed positive correlations with some soil properties (Figure 3d).

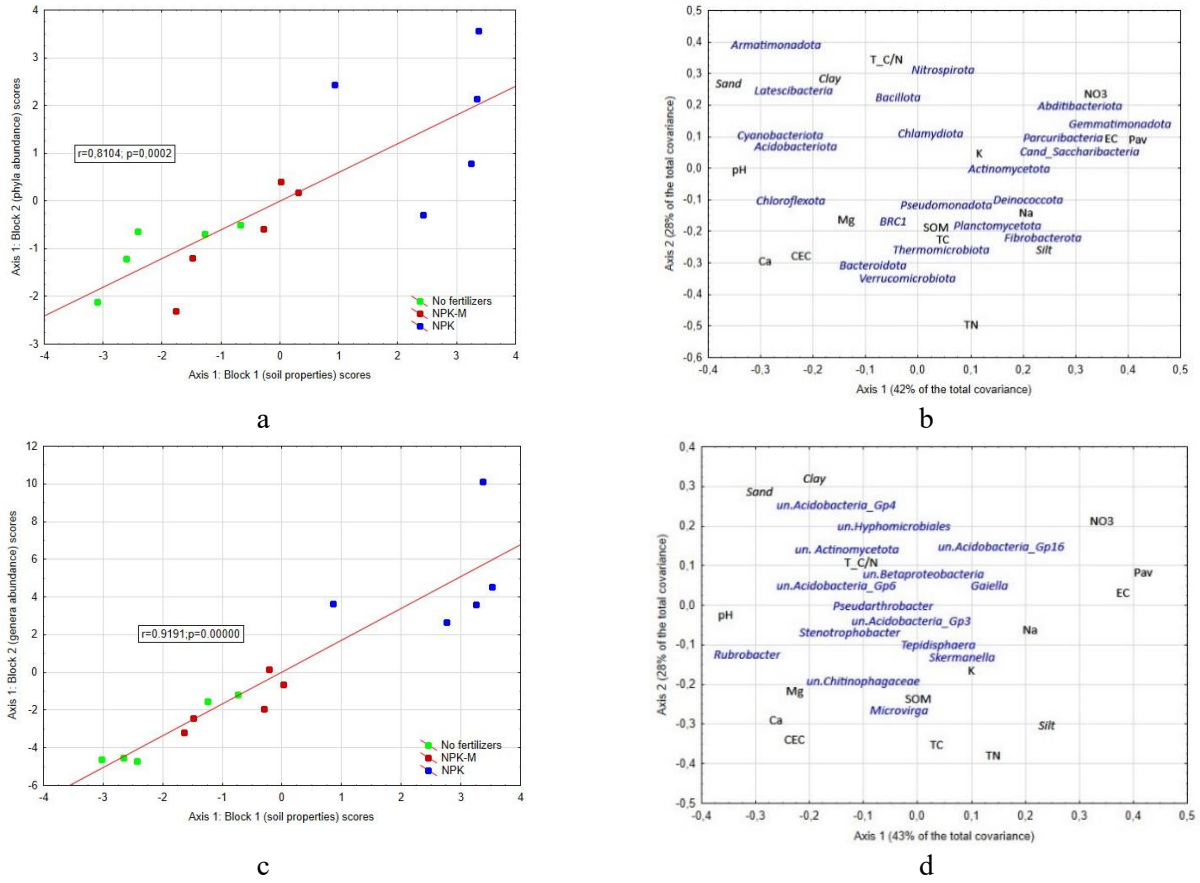


Figure-3. Two-blocks PLS analysis: soil samples score in the Block 1–Block 2 plane of Axis 1 (a, c), and variable loadings (b, d) in the plane of Axis 1–Axis 2: bacterial phyla (a, b) and genera (c, d). Abbreviations: EC – electrical conductivity, CEC – cation exchange capacity, Pav – available phosphorus, SOM – soil organic matter, TC – soil total carbon, TN – soil total nitrogen, T_C/N – the ratio of soil total C to total N, NO_3 – soil nitrate content; Ca, Mg, Na and K – soil exchangeable cations.

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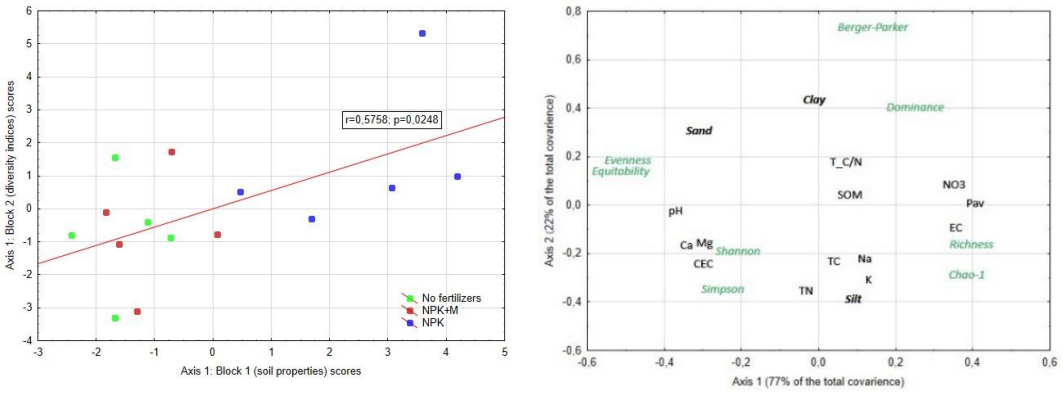
Location of soil samples in the Block1 (soil properties)–Block2 (α-biodiversity indices) plane showed clear separation of the NPK-fertilized plots from No and NPK-M samples (Figure 4a), the squared covariance percentage being 6.5. The bacteriobiome OTUs richness and Chao-1 followed closely soil nitrate, available phosphorus and electrical conductivity (Figure 4, b). Both Shannon and Simpson indices were closely related to soil exchangeable Ca and Mg content and cation exchange capacity. Shannon and richness indices were located at the opposite poles of PLS-Axis 1, accounting for 77% of the total covariance in soil properties and α-biodiversity data (Figure 4d), whereas dominance indices (Berger-Parker and Simpson's Dominance) were located at the positive pole of Axis 2, i.e. opposite to Shannon, Simpson, and richness (both observed and potential, i.e. Chao1).



a b

Figure-4. Two-blocks PLS analysis of the soil properties and α-biodiversity indices: soil samples score in the Block 1–Block 2 plane of Axis 1 (a) and variable loadings in the plane of Axis 1–Axis 2 (b). Abbreviations: EC – electrical conductivity, CEC – cation exchange capacity, Pav – available phosphorus, SOM – soil organic matter, TC – soil total carbon, TN – soil total nitrogen, T_C/N – the ratio of soil total C to total N, NO3 – soil nitrate content; Ca, Mg, Na and K – soil exchangeable cations.

Discussion

To our knowledge, there are no reports about Calcisol bacteriobiome taxonomic composition (as assessed by 16S metabarcoding), and here we provide its first inventory. At the phylum level it followed the global pattern of the composition and structure (Delgado-Baquerizo et al., 2018), being similar to the bacteriobiomes reported for other long-term crop rotation experiments, with *Pseudomonadota*, *Acidobacteriota* and *Actinomycetota* dominating significantly. For example, in a more than 100-years-old field crop rotation experiment on Luvisol, *Pseudomonadota* (former *Proteobacteria*) accounted for 27-29% of the total number of reads, *Actinomycetota* and *Acidobacteriota* accounting for 12-26 and 14-17%, respectively (Perryman et al., 2018). The long-term nine-crop rotation experiment with fertilization that we studied has been under consistent management for more than five decades, i.e. enough time for the soil bacteriobiome response to establish and become fully evident. The finding that bacteriobiome of the studied arable Calcisol was dominated a limited number of taxa strongly implies their prevailing role in soil ecosystem processes in this experimental environment.

The effect of fertilizers on soil bacteriobiome

In this study the application of fertilizers for many years under multicrop rotation was not found to have a notable effect on soil bacteriobiome structure at different taxonomic levels as changes in the relative abundance a) concerned only minor or rare taxa, and b) were very small in size and hence hardly ecologically and agronomically significant individually. For instance, the *Rubrobacter* genus, one of the minor dominants in the study, had 2-fold decreased abundance in the NPK field as compared with the NPK+manure and non-fertilized fields (Table 2), but its abundance, as averaged over all fertilization treatments, was only 1.1±0.4%. The representatives of the genus, mainly moderately thermophilic

chemoorganoheterotrophs, are found in diverse habitats, including those with rather harsh environmental conditions, like volcanic soil (Norman et al., 2017), hyperarid soil of Atacama Desert (Demergasso et al., 2023); some of the *Rubrobacter* representatives are multi-extremophilic (Kouřilová et al., 2021). Thus, the presence of the genus in the Calcisol in the area with high temperatures, high solar radiation and precipitation deficit is hardly surprising. However, the decreased abundance of the genus in association with NPK application is difficult to explain on the basis of the currently available information about the genus ecophysiology. Notably, the principal component 4, extracted from the entire set of the genera (i.e. not only the dominants) and grasping the changes between the non-fertilized and NPK fields, accounted for only four per cents of the original data variance. Such small effect of mineral fertilizers in our study of the long-term experiment is in agreement with some short-term studies: for instance, crop rotation stage was shown to exert a greater effect on soil microbiome than fertilization (Xie et al., 2022). In a long-term wheat-corn rotation experiment on an alluvial soil in China it was found that combined NPK+manure fertilization altered bacterial community composition, whereas addition of NPK only had little effect (Li et al., 2017a). Our study showed opposite results in a way that only rare taxa were affected, and NPK+manure was closer to the treatment without fertilization than NPK. Yet in another study the bacterial community was shown to be mainly driven by fertilization, in this case especially by chemical fertilizers (Liao et al., 2018). Such inconsistent conclusions are abundant, and their detailed discussing is beyond the framework of this article; suffice it to reiterate the plethora of differences in the designs of reported studies.

Although in our study several dozens of the genera had differential abundance as related to fertilization, like *Rubrobacter*, they hardly may be of any agronomical relevance, both individually (showing changes of fractions of a per cent of the genus relative abundance) and combinationally (as major contributors in the PC4). The principal component 2 is apparently responsible for the difference between the NPK and NPK+manure treatments, accounting for 14 per cents of the original data variance. As opposite to the mineral fertilization, manure is applied twice: before corn in the seventh and the ninth year of rotation. So the soil of NPK+manure winter wheat field was sampled four years after manure was applied, and despite the propensity of manure and its composts to modify soil microbiome structure for about several months (Naumova et al., 2024), the effect could have subsided since the soil acts as an effective buffer against exogenous microbiota addition. However, in our study the difference in bacteriobiome structure between NPK and NPK+manure fields, albeit small, was revealed four years after manure application, i.e. somewhat unexpectedly long time. Since the main bacterial constituents of cattle manure are representatives of the *Bacillota* phylum (Zalewska et al., 2024), many of which are spore-producers, their spores may persist in soil for a long time, being indicative of the addition and bringing about shifts in bacteriobiome composition. And repeated manure application, albeit not annual, also sustained the effect.

Bacteriobiome α- and β-biodiversity

Our finding that soil bacteriobiome α-biodiversity indices were not affected by long-term fertilization, both NPK and NPK combined with manure, agrees with results from the studies where some agronomic aspects were similar to ours: for example, the long-term fertilization (NPK or NPK combined with manure) of soil cropped for wheat was also found to have no impact on soil bacteriobiome α-biodiversity indices (Li et al., 2017b). This finding that despite different fertilization α-biodiversity indices did not differ was somewhat unexpected. We are inclined to believe that such result might be due to two reasons. Firstly, as indicated above, soil was sampled three months after harvesting, during which time the decomposition of fresh plant residues and their more easily available compounds were mostly completed, and the effect on bacteriobiome had subsided. The latter was the main reason we collected samples long after harvest to incur minimal disturbance and stress, as recommended by Nannipieri et al. (2019), caused by harvesting *per se*. Secondly, in part the result might be due to the inability of the indices to "provide a consistent view of community change", as stated in some other reports (Neal et al., 2021). The latter also concluded that "… estimates of biodiversity do not capture important facets of community adaptation to stresses adequately". As fertilizers, including nitrogen, are believed to be stressors for soil biodiversity in agroecosystems (Romero et al., 2023), we assume that in our study consistent management practices (fertilization and crop rotation) over more than half a century, have ceased to be stressors, and soil bacteriobiome had become rather stabilized in its

composition. This conclusion is confirmed indirectly by such α-biodiversity indices as high equitability and low dominance. The absence of fertilization effect on bacterial α-biodiversity was also reported for agronomical designs, more divergent from ours (Wu et al., 2023) as compared with the work by Li et al. (2017b).

The positive correlation of the observed and potential (Chao-1) OTUs richness with electrical conductivity, nitrates and available phosphorus content may indicate bacteria direct requirement for total solids dissolved in soil solution as nutrients and hence better soil environment for maintaining more species. For instance, the bacterial community was reported to be closely related to the shifts in soil phosphorus in a fertilization experiment before (Liao et al., 2018). Also, these indices, i.e. observed and potential OTUs richness, may be affected indirectly, that is via stimulated plant production (including exudation, root litter, etc.). The close association of Shannon and Simpson indices with soil cation exchange capacity, an important soil property, characterizing soil fertility and plant nutrient availability, suggests that even a very small CEC gradient between our samples may tune bacteriobiome major α-biodiversity indices.

The set of OTUs, contributing the most in the dissimilarity distance (β-biodiversity) between the fertilization treatments, i.e. *Sphingomonas* sp., *Skermanella* sp., *Pseudarthrobacter* sp., *Niallia* sp., *Actinoplanes* sp. and some *Acidobacteria*_Gp4 OTU-level cluster, are regarded as generally beneficial bacteria. *Sphingomonas* bacteria are common in diverse terrestrial environments, able to decompose recalcitrant natural compounds and regarded as generally beneficial bacteria (Asaf et al., 2020; Stolz, 2009); the same goes for *Skermanella*, which can fix C and N (Zhang et al., 2023), and *Pseudarthrobacter*, possessing plant-growth promoting and metal resistance properties (Park et al., 2025).

Bacterial taxa abundance and biodiversity as related to soil properties

The finding that one of the major dominants, namely the *Pseudomonadota* (25% on average) phylum, together with some other prevailing phyla, such as *Planctomycetota*, *Thermomicrobiota* and BRC1, correlated positively with soil organic matter and total C content three month after harvesting (and hence wheat post-harvest residue input into soil) strongly suggest that the phyla were mainly involved in soil organic matter transformation, as the decomposition of plant deadmass had been mostly completed, and low-molecular mass substrates, available for bacterial utilization due to hydrolysis of plant polymers, had been depleted by the time of soil sampling for the study. The dominants *Bacillota* (6.8%) and *Nitrospirota* (1.3%) correlated positively with soil total C/N ratio, and could be participating in recalcitrant plant matter decomposition, utilizing low molecular mass substrates after fungi.

The positive correlation of the *Acidobacteriota* phylum, accounting for a significant (25%) fraction of soil bacterial community in our study, with soil pH was rather unexpected within a very small between-treatments pH changes, and the phylum's tendency to prefer acidic environment (Kalam et al., 2020). Some genus-level *Acidobacteria*_Gp6 cluster positively correlated with soil total C/N ratio: *Acidobacteria*l genomes possess genes encoding enzymes for the degradation of complex carbohydrate polymers, such as xylan, cellulose, hemicelluloses, pectin, starch, and chitin (Ward et al., 2009), which, except for chitin, contribute to increasing the C/N ratio of organic matter present in soil.

Sphingomonas sp., *Skermanella* sp. and *Pseudarthrobacter* sp. appear to have a propensity for higher SOM and total soil C (Figure 3d), and hence, most likely, for the amount of organic input with post-harvest plant material, as the latter usually correlates with crop yields.

As for the α-biodiversity indices, the finding that in our experiment with a very small, if not tiny, gradient of soil properties along the fertilization treatments (Table 1), Shannon and Simpson indices positively correlated with CEC, soil exchangeable Mg and Ca content and, to a lesser degree, with pH, is in line with other reports (Fierer and Jackson, 2006), albeit on a continental scale. Notably though, that in the latter study Shannon and richness indices were correlated, and in our case they were not, being at the opposite poles of PLS-Axis 1, accounting for 77% of the total covariance in soil properties and α-biodiversity data (Figure 4). Noteworthy is the revealed correlation between the observed and potential OTUs' richness and such soil properties as electrical conductivity, nitrates and available P content: this relationship may imply the importance of ions' content in the soil solution for shaping bacteriobiome in this hot and alkaline soil environment. The absence of the fertilization effect on the α-diversity indices, despite its effect on the wheat yield, makes us agree with Osburn et al. (2023), who after comprehensive

consideration of all potential bacterial drivers of the soil processes concluded that "bacterial α-diversity *per se* was never among the most important predictors of ecosystem functions".

As for the β-biodiversity, i.e. a species variation among different sites, in case of the studied fields is most likely determined by species nestedness, reflecting the diversity of niches available across different environmental condition (Wang et al., 2021). With differences in plant and organic matter input among different fertilization treatments the combination of niches available in each case may be somewhat different, resulting in 30% dissimilarity (according to SIMPER results).

General considerations

It is very challenging to compare our results with results from other crop rotation and fertilization experiments, as so many factors, from environmental (weather conditions, soil, photosynthetically active radiation) to those related to an experimental design (crops, their number and sequence in rotation, the duration of the experiment), let alone the factors related to soil sampling and analytical methodologies (from the primers to bioinformatic pipelines, etc.), contextually shape soil microbiome. As noted above, some researchers found practically no alteration of bacterial diversity under fertilization with NPK only, whereas NPK in combination with manure changed bacterial community composition. (Li et al., 2017a). The discrepancy with our results here in that soil bacteriobiome under NPK+manure was closer to the no-fertilization treatment than to the NPK one most likely results from many differences between the studies, starting from the soil type, crop (corn *vs.* wheat), duration of time elapsed after harvest (<1 month *vs.* 3 months), the number of crops in rotation (2 *vs*.9), fertilization rate, the longevity duration of the experiment (24 *vs*. 54 years), etc. Our results concerning soil bacteriobiome comply with those of Struijk et al. (2023) that at all rotational stages soil bacterial community structure, albeit estimated by different technique (phospholipid fatty acid analysis), was similar. These are just two telling examples of many, illustrating the equivocal nature of results obtained for soil bacteriobiome diversity in long-term field experiments.

It is universally accepted that soil chemical properties influence the composition, diversity and activity of microbial communities, although some studies did not reveal any significant correlation between soil chemical properties and microbial abundance that could explain trends in soil quality or land-use, specifically for agricultural soils reporting no significant relationship between any chemical property and the abundance of dominant prokaryotic microbes (Yoon et al., 2024). The latter, however, in their turn, change the properties of their immediate environment (Philippot et al., 2024). Thus, strictly speaking, all statistical analyses, based on correlation/covariation, contribute mostly to giving an idea where and when to find bigger numbers of specimen or percentages and speculate about the reasons/mechanisms, rather than reveal cause-effect relationship, needed for developing novel agronomical technologies involving beneficial modification of soil agromicrobiome.

It is worth emphasizing that the results obtained in this study, i.e. the absence of between-treatments differences in the major taxa relative abundance of sequence reads and α-biodiversity indices, by no means should be perceived as if reflecting no between-treatments difference in the soil bacterial biomass and/or activity as well. On the contrary, as soil microbial biomass usually positively and strongly correlates with soil organic matter content and phytomass input into soil (Fierer et al., 2009) and increases after organic matter interventions, it implies that the fertilized wheat plots had bigger microbial, including bacterial, biomass than the non-fertilized one, as fertilization led to higher soil TC content (by 5-15%) and wheat yields (by 40-60%).

Conclusions

This is the first study to explore bacteriobiome diversity in the Calcisol in winter wheat-cropped plots of the long-term multicrop rotation experiment with different fertilization (no, NPK and NPK combined with manure). In the studied treatments we found very similar relative abundance of the dominant bacterial taxa, with some changes occurring only at the level of minor and rare taxa. Besides that, the number of dominant bacterial taxa in the study was few at all hierarchical levels. This was accompanied by similarity in the bacteriobiome α-biodiversity indices. All these together indicate that under repetitive management practices the physicochemical properties of the soil environment could have been affected in a way increasing the homogeneity of ecological niches for bacteria to thrive in, hence narrowing the "opportunity space" (Hu et al., 2018; Neal et al., 2021)

and equalizing biodiversity. Such bacteria as *Sphingomonas*, *Stenotrophobacter*, *Pseudarthrobacter*, *Acidobacteria*_Gp4, *Gemmatimonadaceae*, *Betaproteobacteria* and *Chitinophagaceae*, as apparently the most responsive to changes in soil environment under different treatments, warrant further research attention as putatively important actors in the functioning of arable Calcisols. We conclude that long-term multicrop rotation induces taxonomic homogenization of soil bacteria.

Several months that elapsed between harvesting and soil sampling for this study might have also contributed to the absence of major shifts in soil bacteriobiome due to different fertilization: it emphasizes the need to focus research on the temporal aspects of soil microbiome variability, both seasonal and long-term.

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Data Availability Statement

The read data reported in this study were submitted to the NCBI Short Read Archive under the study accession PRJNA1271229 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1271229).

Use of Generative AI Tools Statement

None of the AI tools have been used in the study.

Contribution of Authors

Akhmatbekov M, Barsukov P & Mambetov K: Conceptualized the idea of the study, designed methodology, planned and supervised research experiments and analyses.

Kabilov M: Developed software for the study.

Baturina O, Rusalimova O, Sydykov A, Mamytkanov S, & Dzhainakova G: Conducted experiments, performed chemical analyses, collected and curated the data.

Naumova N & Sydykov A: Validated, visualized and interpreted data and wrote the first draft of manuscript.

Akhmatbekov M & Kabilov M: Supervised resources and funds, reviewed and edited the final draft of the manuscript.

All authors read and approved the final draft of the manuscript.

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