IMPACT OF LONG-TERM FLAX MONOCULTURE VERSUS CROP ROTATION ON THE STRUCTURAL DIVERSITY OF PROKARYOTIC COMMUNITIES IN SOD-PODZOLIC SOIL

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Abstract. Modern molecular-biological methods, such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis and real-time PCR, open up new possibilities for understanding the phylogenetic and functional diversity of microbial communities in agricultural soils. Studying the structure and diversity of the soil microbiome is crucial for assessing the state of agroecosystems and developing sustainable farming practices. This article presents the results of a long-term study on the comparative analysis of prokaryotic genetic diversity in sod-podzolic soil under long-term flax cultivation (*Linum usitatissimum* L.) in conditions of monoculture and crop rotation. It was found that continuous flax cultivation, as well as keeping the soil under bare fallow, lead to a significant depletion of the genetic resources of the soil microbiome and a fundamental change in its qualitative composition compared to crop rotation. The use of T-RFLP and real-time PCR methods allowed for a more comprehensive and rapid characterization and comparison of prokaryotic community structure, identification of dominant groups, and assessment of the impact of various agricultural practices. Understanding the real taxonomic diversity of the soil microbiome is necessary for an objective evaluation of the impact of agrotechnologies on soil biogeochemical processes and interactions in the soil-microorganism-plant system.

Keywords: *Linum usitatissimum*; sod-podzolic soil; microbial community; T-RFLP; real-time PCR; crop rotation.

INTRODUCTION

The productivity of agricultural crops is significantly influenced by various abiotic stresses caused by the intensive use of pesticides, climate change, and environmental pollution, as well as biotic stresses from pathogenic organisms, phytophagous insects [6, 21]. The determining component in yield formation is the biological component of the soil (microbiocenosis), and the interaction within the «soil – microorganisms – plant» system includes the functioning and homeostasis of agroecosystems. In this regard, studies on the formation of the microbiome in the plant rhizosphere significantly deepen our knowledge and help identify mechanisms influencing the soil microbiome [2, 28]. Nevertheless, studying and managing soil microbiomes remains a complex task, as most soil microorganisms have not yet been isolated, and the molecular details underlying their functions are far from being fully understood [15]. A review and synthesis of recent literature indicates a growing trend in publications focused on researching significant representatives of the soil microbiome (prokaryotes, micromycetes, archaea) that influence soil activity and plant growth through the decomposition of organic matter, solubilization of nitrogen, phosphorus, and potassium elements, symbiotic relationships, production of plant growth hormones, inhibition of pathogens, and induction of plant resistance [37]. The interaction between microorganisms and plants has a noticeable influence on initial soil formation, especially in arid and semi-arid regions, over a short period of time under changing climate conditions. Furthermore, soil and climate characteristics are crucial for both the current structure of the soil microbial community and interactions, and for the future development of the soil and the response of microorganisms [4, 30]. It has been established that the formation of different functional pools of soil organic matter is linked to various properties of micromycete organisms, and that so-called «multifunctional» species with intermediate investments in a key trait group (namely, carbon efficiency, 16S rRNA gene copy numbers of proteins and phenolic compounds) contribute to soil organic matter formation, functional complexity, and stability. The research results highlight the importance of synergy among microbial traits for the formation of functionally complex soil organic matter [38].

The level of photosynthesis is linked to the microflora that transforms organic substances, and along with induced changes in the structure of the microbial complex, the nutrient trophic pathways and the products of synthesis and degradation by rhizomicroorganisms change, which is a key issue in the formation of the rhizosphere and the functioning of all its components. Rhizosphere functioning is known to significantly influence plant development and soil quality indicators, as microbial metabolism in this environment determines the ontogenesis of the host plant, for example, adaptation to stress conditions (deficiency of moisture and mineral resources, presence of plant pathogens, etc.) [17].

Over the past decade, modern fields such as genomics and metagenomics, with powerful instrumental methodologies, have enabled revolutionary research to describe and understand the formation of soil biota diversity, particularly the functional role of microorganisms important for agriculture. [13, 32].

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Thus, the soil microbiome plays a key role in the functioning of agroecosystems, determining nutrient cycling, soil fertility, and plant health. Soil microbial diversity is a sensitive indicator of its state and reacts quickly to anthropogenic impacts, including various farming systems and agricultural practices.

Traditional cultivation methods allow the characterization of only a small fraction of soil microorganisms («the great plate count anomaly»), which limits the understanding of the true structure and functions of microbial communities. Nucleic acid-based methods (DNA and RNA analysis), such as 16S rRNA gene sequencing, metagenomic sequencing, Terminal Restriction Fragment Length Polymorphism (t-RFLP), and quantitative PCR (qPCR), provide a more complete picture of the taxonomic and functional diversity of microbial communities, regardless of their ability to be cultured [3, 8, 20]. Studies using these approaches have shown the vast genetic diversity of soil bacteria and have revealed the influence of various factors, such as soil type, vegetation, and agricultural practices, on the structure of microbial communities. In particular, the works of Patyka M.V. and co-authors using T-RFLP and pyrosequencing [22, 23] demonstrated the capabilities of these methods for assessing microbial diversity in various soil types of Ukraine, including podzolic soils and chernozems, and identifying the influence of farming systems.

Fiber flax (*Linum usitatissimum* L.) is an important agricultural crop, however, its long-term continuous cultivation often leads to soil fatigue, reduced yield, and disease development. It is assumed that one of the reasons for this phenomenon may be the negative impact of flax monoculture on the soil microbiome, including a decrease in its diversity and the accumulation of phytopathogenic microorganisms. Despite the relevance of the problem, the specific effects of long-term flax cultivation on the genetic diversity of the prokaryotic complex in sod-podzolic soils have been insufficiently studied.

We hypothesized that long-term continuous flax cultivation creates a specific selective pressure that reduces the structural diversity of the prokaryotic community and favors specific dominant taxa compared to a diverse crop rotation system, which maintains a more balanced and resilient microbiome.

The aim of the work is to assess the impact of long-term flax cultivation under continuous cropping and crop rotation on the structure and genetic diversity of the prokaryotic complex in sod-podzolic soil using T-RFLP and real-time PCR methods.

MATERIALS AND METHODS

Site Description and Sampling. The study analyzes archived soil samples collected in 2013–2014 from the stationary plots of the Classical Long-term Field Experiment, established in 1912 (coordinates: 55°50′N, 37°33′E). The site represents the typical conditions of the Central Non-Chernozem zone. The soil is classified as sod-podzolic (Albeluvisol according to WRB) with a light loamy texture, pH KCl of 4.5–5.0, and soil organic carbon content of 1.0–1.2%. Immediately after sampling, the soil was transported to the laboratory and stored at -80°C to preserve DNA integrity prior to the molecular analysis. Sampling was performed from the arable horizon (0–15 cm) with 10 replicates.

Experimental Design and Treatments. The long-term experiment compares two cropping systems: (1) Continuous flax monoculture, where flax has been cultivated on the same plots annually since 1912; and (2) Crop rotation, consisting of a 6-course rotation: 1. Bare fallow, 2. Winter rye, 3. Potato, 4. Oats with clover, 5. Clover, 6. Flax. Consequently, in the crop rotation system, flax was sown after clover (predecessor).

The fertilizer treatments included:

- Mineral fertilizers (NPK): Applied annually at rates of N45 P60 K60 kg ha.
- Farmyard Manure: Applied at an annual equivalent rate of 10 t ha.
- Control: No fertilizers applied.

For this study, the following specific variants were analyzed:

1. Continuous Flax (Control, no fertilizers);
2. Continuous Flax + NPK;
3. Continuous Flax + Manure;
4. Flax in Crop Rotation + NPK;
5. Bare Fallow (from the crop rotation system). The area of each experimental plot was 100 m².

Classical microbiological analyses. The counts of the main groups of microorganisms (bacteria, streptomycetes, micromycetes) were determined by plating on appropriate selective nutrient media (Winogradsky's starch-glucose agar medium) [11, 12]. Phenotypic diversity was assessed based on the morphological and cultural characteristics of colonies [16, 24, 36].

DNA extraction and purification. Total DNA was extracted from soil samples (0.5-1.0 g of soil) using a modified CTAB method based on the protocol by Doyle and Doyle, Torsvik et al. [7, 35]. Typical modifications for soil samples include:

1. Mechanical cell disruption (using a bead homogenizer or grinding in liquid nitrogen).
2. Lysis in CTAB buffer (2.0% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl), often with the addition of reagents to remove inhibitors, such as polyvinylpyrrolidone (PVP) and β-mercaptoethanol, at elevated temperature (65^0C).
3. Purification from proteins and polysaccharides by extraction with a chloroform:isoamyl alcohol mixture (24:1).
4. DNA precipitation from the aqueous phase with cold isopropanol (usually 0.6-0.7 volumes) or ethanol.
5. Washing the DNA pellet with 70.0% ethanol to remove salts.

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6. Dissolving the purified DNA in sterile buffer (TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA) or deionized water. Additionally, treatment with RNase A to remove RNA. The quality (purity) and quantity of the isolated DNA were assessed spectrophotometrically (by the ratios A260/A280 and A260/A230) and by electrophoresis on a 1.0% agarose gel. Purification of DNA from humic acids, if necessary, was carried out according to Moreira [18].

T-RFLP Analysis The 16S rRNA gene fragments were amplified using the universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') labeled with 6-FAM (fluorescein) at the 5'-end and 1492R (5'-GGYTACCTTGTTACGACTT-3'). PCR was performed in a 50 µl reaction mixture containing: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, 1 U of Taq DNA polymerase, and 50 ng of template DNA. The thermal cycling conditions were: 94°C for 5 min (initial denaturation), followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s, with a final extension at 72°C for 10 min.

Purified PCR products were subjected to digestion with restriction endonucleases HhaI and MspI (Thermo Fisher Scientific) in separate reactions to increase resolution. The digestion was carried out at 37°C for 3 hours. The restriction fragments were separated by capillary electrophoresis using an ABI 3130xl Genetic Analyzer (Applied Biosystems) with a GeneScan LIZ 600 internal size standard.

Quantitative Real-time PCR (qPCR). The abundance of the bacterial 16S rRNA gene was quantified using real-time PCR. The reaction mixture (25 µl) contained 1× SYBR Green Master Mix, 0.4 µM of each primer (Eub338F and Eub518R), and 10 ng of template DNA. Amplification was performed using an iCycler iQ5 (Bio-Rad) with the following thermal profile: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Standard curves were generated using serial 10-fold dilutions of plasmid DNA containing the cloned 16S rRNA gene of *Escherichia coli*. Data are expressed as log10 copy numbers per gram of dry soil. Melting curve analysis was performed to confirm the specificity of the amplification products [9].

Calculation of ecological diversity indices. Based on the T-RFLP data matrix (relative intensities of T-RFLPs, pi, where pi is the proportion of the intensity of the i-th T-RFLP out of the total intensity in the profile), indices were calculated for assessing the α-diversity of prokaryotic communities:

– Shannon index (H′): Assesses diversity, taking into account both the number of species (T-RFLPs) and the evenness of their representation.

$$H' = -\sum_{i=1}^{S} p_i \ln(p_i)$$

where S is the total number of T-RFLPs in the profile, pi is the relative intensity of the i-th T-RF. Higher values of H′ indicate greater diversity.

– Simpson's dominance index (D): Shows the probability that two randomly selected individuals (in this case, T-RFLPs) belong to the same type.

$$D = \sum_{i=1}^{S} p_i^2$$

Higher values of D indicate greater dominance of one or more T-RFLPs and, accordingly, lower diversity. Derivative indices are often used:

– Simpson's diversity index: 1−D. Values range from 0 to 1, where 1 represents maximum diversity.
– Inverse Simpson index: 1/D. The value equals 1 at minimum diversity and increases with increasing diversity.

Statistical analysis. Comparison of diversity indices and community structure using similarity analysis [19, 25] Odum between experimental variants was performed using appropriate statistical tests.

RESULTS

Microorganism abundance and phenotypic diversity: According to counts on nutrient media, bacteria numerically dominated in all experimental variants, their number exceeding the abundance of streptomycetes and micromycetes by 2-3 orders of magnitude. The application of both mineral (NPK) and organic (manure) fertilizers led to an increase in the abundance of all studied groups of microorganisms, especially in the variant with continuous manure application (Table 1). Analysis of phenotypic diversity revealed changes in the dominant morphotypes of bacteria and micromycetes depending on the experimental variant.

Genetic diversity of prokaryotes (T-RFLP): Comparative analysis of prokaryotic community T-RFLP profiles showed significant differences between experimental variants both in terms of the number of detected T-RFLPs (operational taxonomic units) and the structure of their relative abundance (Fig. 1).

The highest species richness of prokaryotes (T-RFLP quantity) was observed in the variant with flax

Table 1. Abundance of microorganisms in sod-podzolic soil under flax crops, *Linum usitatissimum* L. (autumn, after harvest)

Experimental variant	Bacteria, CFU 106/g soil	Actinobacteria, CFU 103/g soil	Micromycetes, CFU 103/g soil
	Permanent crops		
Without fertilizers (control)	23.3	1.0	10.2
NPK	34.0	7.5	17.3
Manure	32.0	17.1	22.0
	Crop rotation		
Flax + NPK	22.8	17.0	30.7
Pure fallow	15.6	17.1	42.2

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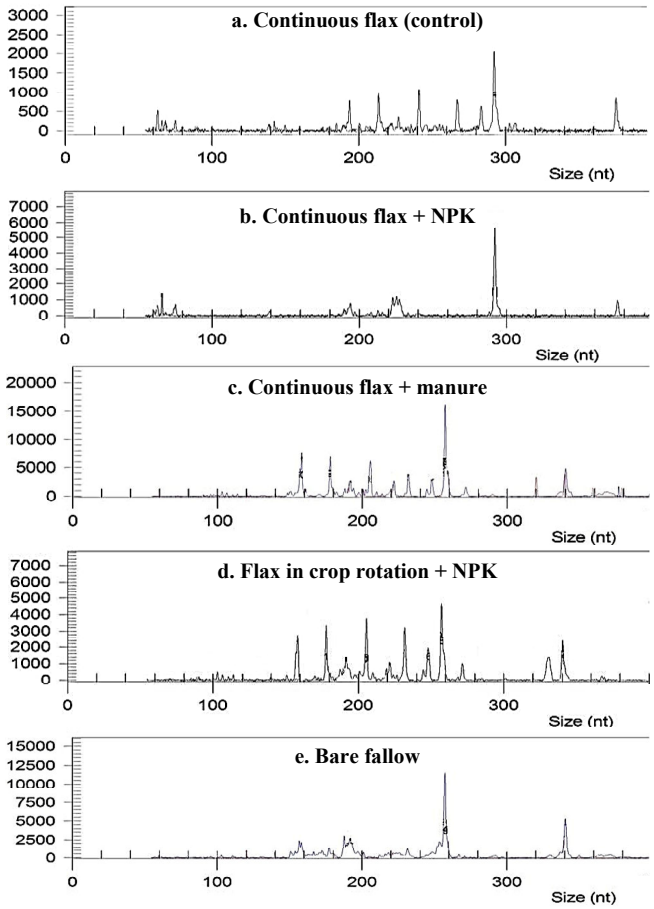

Figure 1. T-RFLP profiles of prokaryotic communities in sod-podzolic soil under different usage variants (Primer Eu3, endonucleases HhaI+MspI). Axes: X – Fragment size (nt), Y – Relative fluorescence intensity. (a – Continuous flax (control); b – Continuous flax + NPK; c – Continuous flax + manure; d – Flax in crop rotation + NPK; e – Bare fallow). Note: The Y-axis scale (Relative fluorescence intensity) is automatically adjusted for each panel to visualize low-abundance peaks effectively.

cultivation in crop rotation against the background of NPK, as well as with permanent manure application. The minimum amount of T-RFLP was recorded in variants with permanent flax cultivation (especially against the background of NPK) and in bare fallow.

Analysis of ecological indices confirmed these observations (Table 2). The highest genetic diversity according to the Shannon index (H=4.01) and the lowest dominance according to the Simpson index (D=0.063) were noted in the «flax in crop rotation + NPK» variant. Continuous flax cultivation without fertilizers and with NPK, as well as bare fallow, were characterized by significantly lower Shannon index values (2.37-2.90) and higher dominance (0.21-0.23). Manure application under continuous flax cultivation contributed to maintaining a relatively high level of diversity (H=3.68) and low dominance (D=0.12), bringing the indicators closer to the crop rotation variant.

Analysis of the similarity of prokaryotic community structure revealed significant differences between variants. The community similarity index of continuous flax cultivation (without fertilizers and with NPK) relative to crop rotation was extremely low (0.059-0.074). At the same time, the community of the variant with manure application had significantly higher similarity to crop rotation (0.667), although even in this case, only about 67% of the taxonomic units were common. This indicates the formation of specific microbial complexes under different farming systems.

Total number of prokaryotes (qPCR). Real-time PCR data showed that the total prokaryotic 16S rRNA gene copy numbers (estimated by the number of 16S

Table 2. Ecological indices of diversity of the prokaryotic complex of sod-podzolic soil (according to T-RFLP)

Indicators	The permanent culture of flax	Pure fallow	Flax in crop rotation
	Fertilizer-free	NPK	Manure
Diversity (Shannon's H)	2.37	2.90	3.68
Dominance (C Simpson))	0.210	0.230	0.120
Similarity* (relative to crop rotation+NPK)	0.074	0.059	0.667

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* Factor pairwise similarity is calculated according to the formula (Odum, 1975)

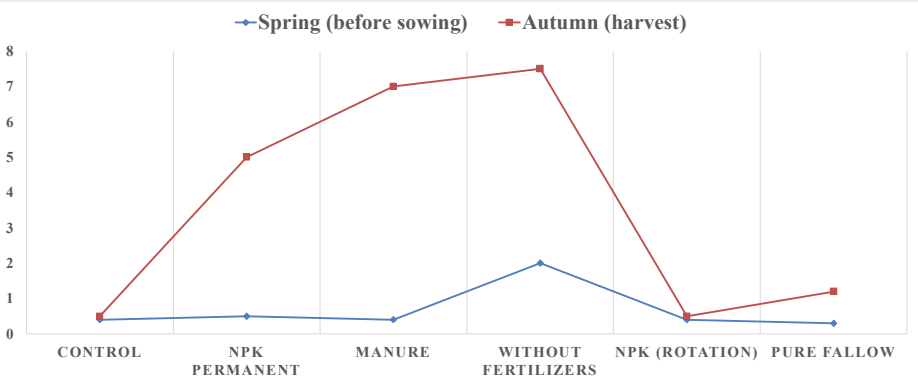

Figure 2. Abundance of 16S rRNA gene copies in sod-podzolic soil under different cropping systems. Y-axis: Log10 16S rRNA gene copy numbers g dry soil. Data are presented as mean values (n=3). Standard deviations were within 5–10% of the mean.

rRNA gene copies) was lowest in variants with continuous flax cultivation without fertilizers and in bare fallow. Application of fertilizers (both NPK and manure) and flax cultivation in crop rotation led to an increase in the total prokaryotic abundance by more than an order of magnitude (Fig. 2). These results are generally consistent with data from classical microbiological counts. The qPCR method allows for the estimation of total prokaryotic 16S rRNA gene copy numbers, including unculturable forms.

DISCUSSION

The results obtained convincingly demonstrate the significant impact of long-term agricultural use, in particular the system of flax cultivation (monoculture vs. crop rotation) and the use of fertilizers, on the structure and genetic diversity of the prokaryotic complex of sod-podzolic soil.

The key finding of the study is the negative impact of continuous flax cultivation on the soil microbiome. As shown by the T-RFLP data and ecological index calculations, flax monoculture, especially without fertilizers or with only mineral fertilizers (NPK), leads to a significant decrease in prokaryotic genetic diversity (decrease in the Shannon index) and an increase in the dominance of certain taxa (increase in the Simpson index) compared to crop rotation. This is consistent with general understanding of the impact of monocultures on soil microbial communities, where selection of a specific group of microorganisms adapted to the root exudates and plant residues of the given crop occurs, leading to the impoverishment of the total taxonomic composition [23, 26, 29]. Similar effects of diversity reduction under monoculture were observed for other crops, for example, wheat on chernozems [10, 14, 27]. Furthermore, the decrease in total prokaryotic 16S rRNA gene copy numbers, revealed by the qPCR method in continuous flax variants, may indicate a general suppression of microbial activity.

Bare fallow also showed a low level of genetic diversity and total prokaryotic abundance. This is to be expected, as the lack of vegetation cover and fresh organic matter intake limits resources for most soil microorganisms [1, 31, 34, 40].

On contrast, growing flax in crop rotation contributed to maintaining high genetic diversity and a more even structure of the prokaryotic community. Alternating crops provides the input of diverse plant residues and root exudates, creating conditions for the development of a wider range of microbial groups.

It is interesting to note the positive impact of organic fertilizer (manure) even under continuous flax cultivation. Manure application not only increased the total abundance of microorganisms but also maintained genetic diversity at a level close to crop rotation and significantly increased the similarity of the microbial community to the crop rotation variant. This emphasizes the importance of organic matter input for maintaining the health and biodiversity of the soil microbiome in agroecosystems.

The observed impoverishment and change in the structure of the microbial complex under continuous flax cultivation is likely one of the key reasons for the so-called «flax fatigue» of the soil. Reduced diversity can lead to a disruption of soil suppressiveness and the accumulation of specific phytopathogens, as well as an imbalance in the performance of important biogeochemical functions by microorganisms.

The molecular methods used (T-RFLP and qPCR) confirmed their effectiveness for the comparative characterization of microbial communities under different variants of agricultural impact. The T-RFLP method, as previously shown [22, 23, 25] by Patyka M.V. and co-authors for podzolic soils, allows for quickly obtaining a «phylogenetic fingerprint» of the community and assessing its diversity and structure, identifying dominant groups and differences between samples. qPCR provides a quantitative estimation of total prokaryotic 16S rRNA gene copy numbers. Joint use of these methods with classical approaches gives a more complete picture of the state of the soil microbiome. For a deeper understanding of the taxonomic composition and functional potential of communities, the use of next-generation sequencing (NGS) methods is promising, such as 16S rRNA gene

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amplicon sequencing or metagenomic sequencing [5, 33, 39, 41].

Thus, this study emphasizes the critical importance of crop rotation and organic fertilizer application for maintaining the genetic diversity and functional stability of the soil microbiome in agroecosystems. Flax monoculture leads to the degradation of the microbial community, which may be related to reduced agroecosystem productivity. The obtained data can be used for developing recommendations on optimizing farming systems with the aim of preserving soil biodiversity and increasing the sustainability of agricultural production.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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