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Article

Multivariate Analyses of Chemical and Microbiological Soil Properties and CO₂ Emission Under Long-Term Fertilizer Experiment and Four-Crop Rotation on Luvic Chernozem

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Abstract: A key objective of contemporary agriculture is to restore biodiversity, preserve ecosystem health, reduce the effects of climate change, and produce safe and healthy foods. Maintaining high soil fertility while reducing greenhouse gas emissions requires a precise assessment of how fertilization and crop rotation affect the carbon and nitrogen cycles in agroecosystems. In the context of a long-term fertilizer experiment on Luvic Chernozem, a monitoring of chemical and microbiological soil properties and CO₂ emission was carried out. The fertilization treatments that have been continuously applied for 64 years under four-crop (wheat, barley, corn, and beans) rotation are N, P, K, NP, NK, PK, NPK, and 0. The highest organic carbon content was observed in the treatments NPK (1.42%) and NP (1.43%), and the lowest in the control (1.05%). The constant application of nitrogen as single fertilizer led to lower SOC values (1.22%). On average, CO₂ emissions were the most intensive in NP (66.34 kg ha⁻¹ day⁻¹), NPK (67.82 kg ha⁻¹ day⁻¹) and PK (60.16 kg ha⁻¹ day⁻¹) treatments. The nitrogen fertilizer application most significantly alters soil properties including pH, EC, and SOC, which alters the environment for soil microbial development and influences the number and composition of soil microbial communities.

Keywords: long-term fertilizer experiment; fertilization; soil organic carbon; soil microbiota; CO₂ emissions

1. Introduction

The primary agricultural technique that significantly increases crop yields is mineral fertilization. The biological, chemical, and physical processes of the soil are also impacted by altering the nutrient regime and the environment for plant growth [1–5]. To obtain information on the types and amounts of fertilizers to increase agricultural yields and plant nutrition, numerous long-term fertilizer experiments were set up [6]. These studies offer a chance to look into how long-term application of mineral fertilizers and their combinations affect the soil characteristics. Mineral fertilization effects on soil quality must be investigated over an extended period of time to understand how it impacts soil parameters that are slowly shifting. Additionally, they allow to develop the theoretical framework for the sustainable crop yields and the preservation of a healthy soil environment [7].

The cycles of water, carbon and biogenic elements are the basis of life. Agriculture manipulates and interferes with parts of these cycles. The link between the carbon cycle and climate change is now well established. Land use is responsible for about a third of the increase in atmospheric carbon dioxide over the past 150 years, mainly due to the loss of soil organic matter [8]. The main goals of modern sustainable agriculture are mitigating climate change, avoiding long-term environmental

damage and meeting the growing need for food [9]. Fertilization is the most effective management practice for ensuring global agricultural production since the discovery of the role of nutrients in crop growth and development. However, excessive or irrational mineral fertilization can lead to a decrease in soil fertility and an increase in nutrient loss [10]. This leads to many interrelated negative effects on the environment and human health, such as increased greenhouse gas emissions, pollution of surface and groundwater, and food [11]. Nitrogen is a key element for plant growth and yield formation and is a limiting factor in the productivity of ecosystems [12]. Nitrogen fertilization strongly affects biomass production, microbial activity, decomposition of accumulated biomass and hence the dynamics of soil organic matter [13]. The effect of nitrogen addition, through mineral fertilization, leads to higher yields, a higher amount of plant residues and a higher intensity of their decomposition, which leads to the release of CO₂ [14]. Cumulative CO₂ fluxes also increase with higher nitrogen rates [15] and with long-term application of combined NP and NPK [16]. Thus, the impact of fertilization practices on soil C and N cycling in agroecosystems needs to be accurately assessed to achieve the goals of maintaining high soil fertility and, in the meantime, mitigating greenhouse gas emissions [17].

In the current climate change context, another key goal of agriculture is optimizing fertilization management in terms of greenhouse gas emission reduction, improvement of soil carbon sequestration and to ensure food security, it is essential to understand how fertilizing strategies impact the dynamics of soil organic carbon (SOC) [16]. A growing number of researchers suggest that agricultural soils have significant potential to mitigate climate change by carbon sequestration [18–21]. Agricultural lands have the potential to sequester approximately 5500–6000 Mg CO₂-eq. yr⁻¹ to 2030 [22]. The accumulation of soil organic matter and the release of greenhouse gas emissions in arable land can be managed by various agricultural practices [23,24], including applied fertilization [15]. Long-term field experiments provide an opportunity to study the processes in the soil that occur during the application of mineral fertilizers in relation with the solution of these global problems.

Ecosystem functions are result of the interactions between plants, soil and microorganisms, and any change in soil properties influences the microbial structure, which in turn influence ecological processes [25]. Fertilization rates and crop rotations can affect soil pH, nutrients and organic matter through their effects on microbial processes [26]. Fertilization can indirectly affect soil microorganisms by changing soil properties or directly through applied nutrients [27]. Soil microbiota plays an essential role in the cycling of soil carbon, nitrogen, phosphorus and sulphur and is important component in maintaining soil quality and fertility [28,29]. Changes in soil microbial communities also affect the productivity and functioning of agroecosystems.

The 64-year fertilization experiment under four crop rotation in the Institute of Agriculture and Seed Science (IASS) “Obraztsov Chiflik” Rousse is one of the few long-term field experiment in Bulgaria which has been still maintained till now. On this experiment, we performed monitoring to determine the interaction between soil microbiota, mineral fertilization, and soil chemical properties.

2. Materials and Methods

2.1. Site Description

A long-term fertilization experiment was carried out in the experimental field of IASS Obraztsov Chiflik, region Ruse, (43.807, 26.041). The area is located in the Danube plain (Figure 1) and bare the traits of a moderate continental climate.

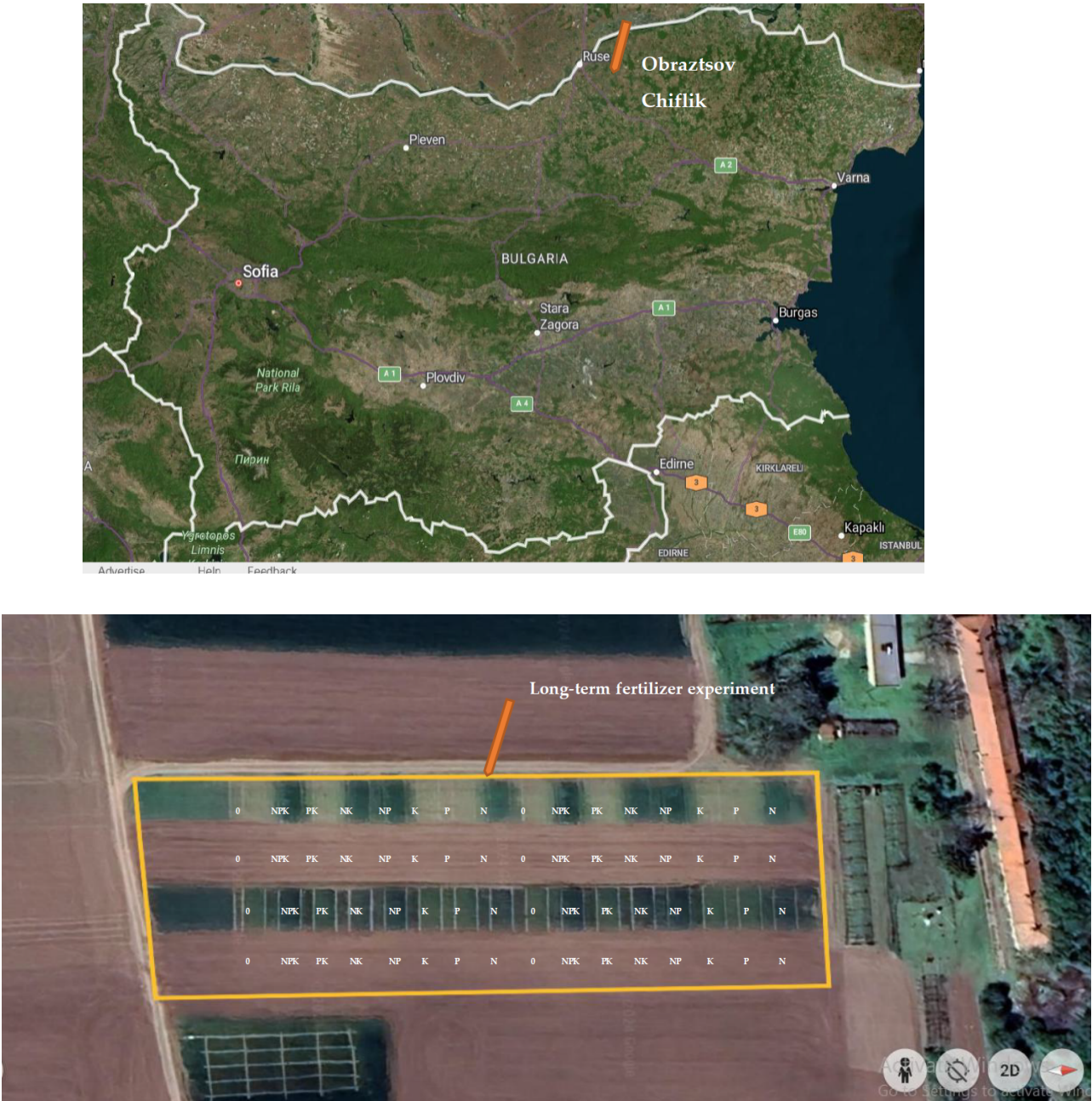


Figure 1. Location of the site.

The soil type is Luvic Chernozem (IUSS Working Group WRB, 2022) with low organic carbon content and moderately acidic soil reaction (pH in H₂O 5,5– 6,0) in the 0-40 cm layer. The texture is classified as Silty Clay Loam (SiCL) with 30% clay content (Table 1).

Table 1. Textural fractions in the humus horizons of Luvic Chernozem under grass, IZ "Obratzov Chiflik".

Soil layer	Fractions according to FAO (2006), %			USDA	Texture class
cm	Sand 2-0.063 mm	Silt 0.063-0.002 mm	Clay <0.002 mm	Sand 2-0.05 mm	
0-5	5,7	65,5	28,8	8,1	SiCL
10-15	4,0	65,6	30,3	7,5	SiCL
15-20	4,4	65,1	30,4	7,5	SiCL

2.2. Experiment Design

The experiment was established in its current format in 1958. It is located on area of 1 ha. The size of the trial plots is 100 m², and the harvest plots - 60 m². The applied methods of fertilization had been carried out the same places for 64 years on four agricultural crops - wheat (variety "Danube"), beans (variety "Obratzov chiflik 12"), barley (variety "Agate") and maize (hybrid "Ruse 464") (Figure 2). In the rotation, wheat replaces beans, maize replaces wheat, beans - barley, barley- maize.

m	a	i	z	e				m	a	i	z	e			
b	a	r	l	e	y			b	a	r	l	e	y		
b	e	a	n	s				b	e	a	n	s			
w	h	e	a	t				w	h	e	a	t			
N	P	K	NP	NK	PK	NPK	O	N	P	K	NP	NK	PK	NPK	O

Figure 2. Experimental design 2021-2022.

Seven treatments have been tested with individual (single) and combined application of the three macroelements N, P, K. Nitrogen rates for wheat and maize were N - 150 kg ha⁻¹; for beans N - 50 kg ha⁻¹ and for barley N - 100 kg ha⁻¹. Phosphorus -120 kg ha⁻¹ and potassium – 70 kg ha⁻¹ were applied for all crops. For comparison, the unfertilized control N0P0K0 is maintained. Phosphorus and potassium fertilizers are applied once, before the main tillage, and nitrogen fertilizers - once after the sprouting of the crops, as an early spring feeding.

2.3. Chemical and Microbiological Analyses

Soil samples for chemical and microbiological analyses were taken once in two periods - May and October, in 2023, from all treatments and two replicates of the experiment, for all crops, at depth 0-20 cm.

The ammonium and nitrate nitrogen were determined according to Kjeldal and acetate-lactate method for the determination of available phosphorus and potassium forms was used. The microbial biomass carbon (Cmic) measurements were determined by the substrate-induced respiration method (SIR). Total organic carbon content (SOC,%) was determined by wet digestion by the modified Tjurin's method [dichromate digestion at 125° C, 45 min] [30]. The soil pH and electrical conductivity (EC) of the samples were measured in water suspension 1:2.5 by a combined pH-EC meter. Ammonium and nitrate nitrogen were determined according to the method of Kjeldal. The acetate-lactate method was used for determination of the plant-available forms of phosphorus and potassium by simultaneous extraction. The potassium was determined directly by a flame photometer. The phosphorus was determined by a spectrophotometer after a colour reaction (according to Murphy – Riley, 1962).

The substrate-induced respiration method (SIR) was used for measuring the microbial biomass (Cmic) according to the Anderson-Domsch method [31]. The number of microorganisms was determined by the plate counts technique on selective agar media. Ammonifying bacteria were cultivated on Nutrient broth agar, actinomycetes – on starch-ammonium agar medium, microscopic fungi – on Czapek’s agar medium, nitrifying bacteria on Winogradski medium, nitrogen fixing bacteria on Ashby medium [32].

Thre greenhouse gas emissions were measured during the crop growing seasons in the three year period 2021-2023. CO₂ fluxes were measured on site using infrared gas analysers - the calibrated tester "ALMEMO" with a CO₂ sensor. Plastic chambers were used to collect the emissions.

The air CO₂ concentrations were measured at a height of 1 m above the plots. After placing the chambers, they were closed and an incubation period of 30 min was maintained, after which the

accumulated CO₂ in the closed chambers was measured. Then the leakage of the respective gas (kg ha⁻¹ day⁻¹) was calculated, according to [33]:

$$E\text{ (CO}_2\text{)} = (M \times P \times V \times (C_2 - C_1)) / (R \times T \times A \times (t_2 - t_1)) \tag{1}$$

where: E (CO₂) - CO₂ release from the soil (kg ha⁻¹ h); then it can be converted to a day. M-molar mass of CO₂ (kg mol⁻¹); P-air pressure (Pa); V-chamber volume (m³); C₁ - initial concentration of CO₂ (μmol mol⁻¹); C₂-concentration of CO₂ after incubation time (μmol mol⁻¹); R-gas constant (J mol⁻¹ K⁻¹); T-air temperature (K); A-chamber surface area (m²); t₂-t₁-incubation period.

2.4. Statistical Analyses

Descriptive statistical analyses, ANOVA, correlation, principal component and regression analysis of the obtained data were carried out using STATGRAPHICS Plus.

3. Results

3.1. Soil Chemical Properties and Organic Matter Content

According to the applied treatments, all of the soil chemical indicators showed statistically significant differences (Table 2). The cultivated crop also affected some of the parameters, including pH, EC, available forms of phosphorus, and mineral N. Because two of the crops in the rotation were winter cereals, one was a spring cereal (maize), and the other was a spring leguminous crop (beans), the agricultural technologies differed greatly. This was reflected in the nitrogen fertilizer dosages, fertilization schedules, and crop growth stages, where nutrient uptake varies.

As anticipated, the long-term nitrogen single fertilization increased EC and decreased pH. On average across all plots, the electrical conductivity was the highest in all variations with nitrogen fertilization (N, NPK, NK). In the zero variations, it was the lowest. Since the pH was greater in the K, P, and zero treatments, the variants with nitrogen fertilizers application showed the greatest pH drop. In general, a decrease in pH was observed in variants N, NP (5.44), NK (5.30), NPK (5.42). The average pH of the zero treatment was 5.82.

On average, soil organic carbon was most significantly increased by the NP and NPK treatments. Variants NK (1.25%), PK (1.18%), and N (1.22%) exhibited lower soil organic matter as a result of long-term, consistent mineral fertilization. The highest increase in soil organic carbon was result of fertilization with both nitrogen and phosphorus - in the NPK treatment, this was 1.42%, and in NP, it was 1.43%. The zero treatment had the lowest average organic carbon content (1.05%) (Table 2). Microbial biomass carbon was stimulated most strongly by single and combined nitrogen and potassium fertilization. The microbial biomass carbon increased in the variants (NK, K, N).

Table 2. Analysis of variance by controlling factors (treatment, season, crop) on mineral N (N-NO₃, N-NH₄) (mg kg⁻¹), available P₂O₅ (mg 100g⁻¹), available K₂O (mg 100g⁻¹), EC (μS cm⁻¹), pH, SOC (%), Cmic (mg kg⁻¹).

Treatment	N (N-NO ₃ , N-NH ₄)		P ₂ O ₅		K ₂ O		EC		pH		SOC		Cmic	
N	121.74 d		15.97 a		27.13 a		90.43 c		5.44 ab		1.22 bc		300.9 bc	
P	28.88 ab		35.21 c		34.09 b		29.13 a		5.90 d		1.34 cd		274.3 abc	
K	28.81 ab		21.02 ab		42.1 c		29.92 ab		5.89 cd		1.24 bc		304.0 bc	
NP	83.00 bcd		27.58 bc		29.74 ab		46.79 ab		5.44 ab		1.43 d		245.2 ab	
NK	90.06 cd		16.39 a		43.95 c		56.39 abc		5.30 a		1.25 bc		343.8 c	
PK	36.74 abc		31.83 c		46.17 c		39.01 ab		5.66 bc		1.18 ab		303.3 bc	
NPK	121.29 d		37.53c		45.83 c		70.54 bc		5.42 a		1.42 d		263.8 abc	
0	21.92 a		17.11 a		31.23 ab		25.10 a		5.82 cd		1.05 a		209.1 a	
ANOVA	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
factors	ratio	value	ratio	value	ratio	value	ratio	value	ratio	value	ratio	value	ratio	value
Treatment	4.36	0.00007	5.77	0.0000	11.66	0.0000	2.54	0.025	8.02	0.000	5.94	0.0000	2.11	0.059

Season	NS	NS	NS	NS	NS	NS	7.43	0.009	19.47	0.0001	NS	NS	22.40	0.000
Crop	6.86	0.0006	9.66	0.0000	2.23	0.095	3.51	0.021	6.39	0.0009	NS	NS	NS	NS

A principal component analysis (PCA) was performed to establish the main relationships between the available forms of macroelements, physicochemical properties, organic carbon and Cmic (Table 3). The model describes 69.71% of the variability of the data. Component 1 reflects 42% of the variation in the indicators. On the vector of principal component 1 (PC1) increases the values of mineral nitrogen, which leads to higher EC and a lower pH and a very slight increase in SOC. The enhanced SOC was the strongest in the second component (PC2), and the available forms of phosphorus and potassium also arose with PC2. pH decreased in all three components. Cmic was growing in the first component. In the diagram (Figure 3), the parameters considered to be positively correlated with each other when they are at an acute angle, when their vectors form a right angle, they are not correlated, and when they are at an obtuse angle they are negatively correlated. From the PCA figure (Figure 3), a positive relationship can be established between mineral nitrogen and soil organic matter, as well as with microbial biomass carbon.

Table 3. Variable loadings of the first three components of principal components analysis. Soil mineral N (N-NO₃, N-NH₄) (mg kg⁻¹), P- available P₂O₅ (mg 100g⁻¹), K- available K₂O (mg 100g⁻¹), EC (μS cm⁻¹), pH, SOC (%), Cmic (mg kg⁻¹).

	Component 1	Component 2	Component 3
Eigenvalue	2.23	1.35	1.27
Percent of variance	31.83	19.26	18.20
N	0.632	0.072	-0.028
P	-0.070	0.402	-0.522
K	-0.126	0.581	0.230
pH	-0.423	-0.416	-0.321
EC	0.611	-0.118	-0.045
SOC	0.087	0.261	-0.710
Cmic	0.145	-0.490	-0.255

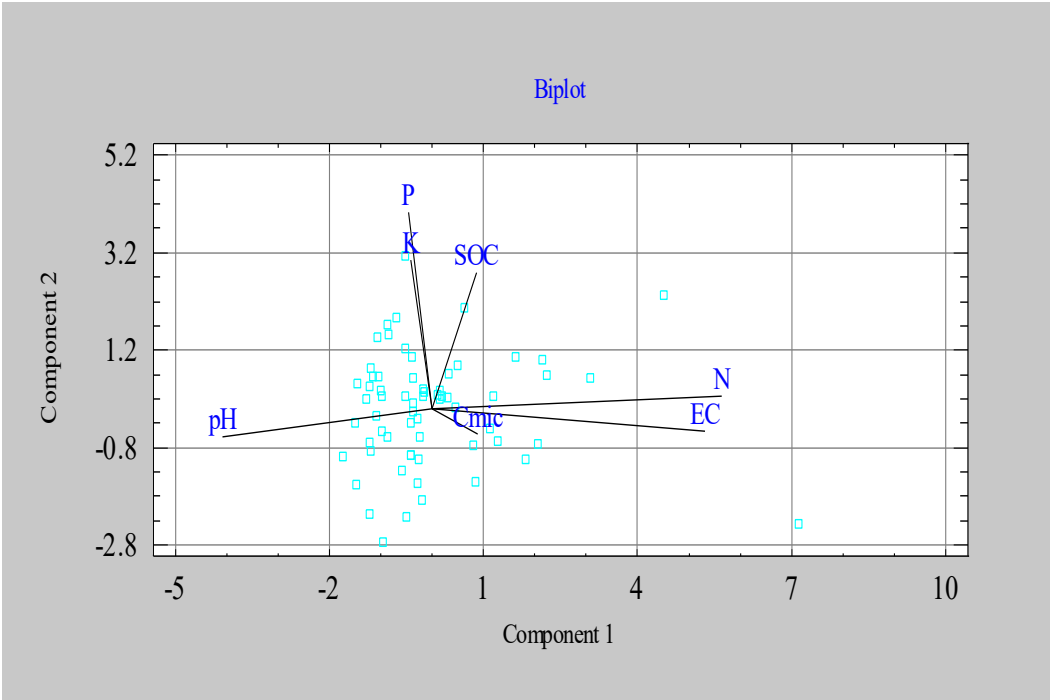


Figure 3. Biplot of the first two components in the analysis carried out on a dataset including: soil mineral N (N-NO₃, N-NH₄) (mg kg⁻¹), P- available P₂O₅ (mg 100g⁻¹), K- available K₂O (mg 100g⁻¹), EC (μS cm⁻¹), pH, SOC (%), Cmic (mg kg⁻¹).

Available forms of phosphorus and potassium according to PCA have a stronger impact on SOC content, while mineral nitrogen has a stronger positive relationship with microbial carbon. However, organic matter was improved most strongly in plots with combined nitrogen and phosphorus fertilization (Table 2), but nitrogen effect in treatments N, with separate nitrogen fertilization was low.

3.2. Effects of Mineral Fertilization on Soil CO₂ Emissions

The most intensive CO₂ emissions were recorded in the NP and NPK variants (Figure 4). Also in these treatments, the highest accumulation of organic carbon was established (Table 2). Combined nitrogen and phosphorus fertilization, stimulated organic matter accumulation, which also stimulates soil respiration. Correlation coefficient between SOC and CO₂ flows were positive and significant ($p < 0.05$) in bean, barley and wheat plots. Soil CO₂ fluxes were the most intensive in wheat plots (66.38 kg ha⁻¹day⁻¹) and lowest in beans plots (33.22 kg ha⁻¹day⁻¹). Despite the low amount of SOC, recorded in the zero variants, CO₂ emissions in the absence of fertilization were not the most strongly reduced. CO₂ fluxes in N, P, K treatments were lower compared to the zero control.

A principal component analysis (PCA) was also conducted (Figure 5). The PCA was applied to determine the relationship between agrochemical indicators, soil organic matter and greenhouse gas emissions. The model describes 85.86% of the variability of original data. The data were loaded into two principal components (PC1 and PC2), which explain most of the variations between the indicators by variants of fertilization. A positive relationship was found between the content of available forms of phosphorus and potassium, mineral nitrogen, soil organic carbon and CO₂ emissions. The relation between mineral nitrogen and CO₂ was lower than impact of phosphorus and potassium. Cmic showed negative relation with all parameters in these analyses. There was negative correlation between Cmic and CO₂ in wheat ($r = -0.44$, $p = 0.09$) and maize ($r = -0.64$, $p = 0.007$). In beans and barley these coefficients were also negative ($r = -0.12$; $r = -0.61$), but not significant.

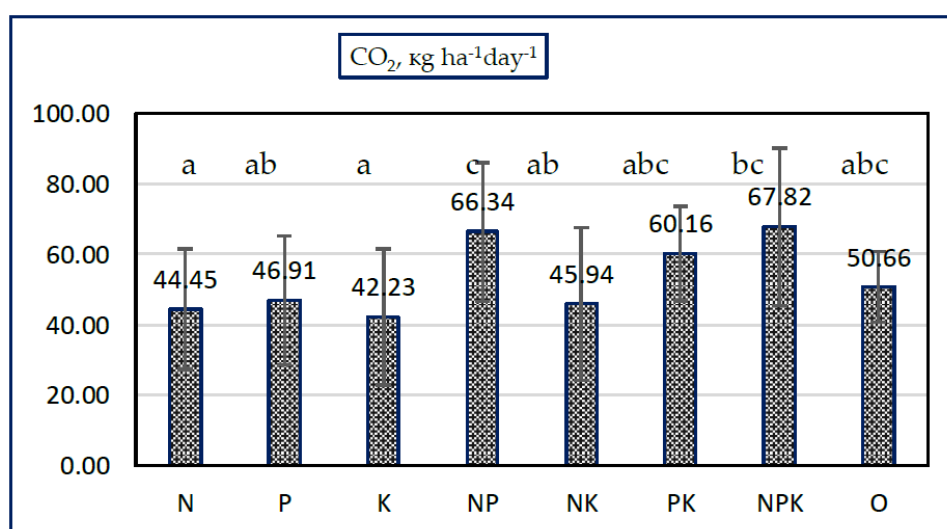


Figure 4. Soil CO₂ emissions, kg ha⁻¹day⁻¹. Vertical bars denote standard deviation. ANOVA: $p = 0.0578$.

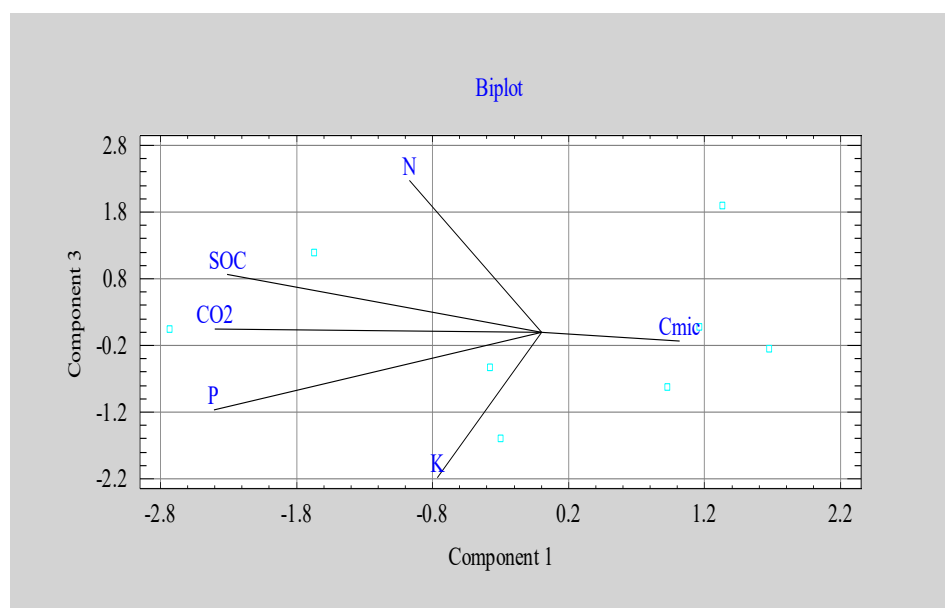


Figure 5. Biplot of the first two components in the analysis carried out on a dataset including: CO₂ emissions (kg ha⁻¹day⁻¹), mineral N (N-NO₃, N-NH₄) (mg kg⁻¹), P- available P₂O₅ (mg 100g⁻¹), K- available K₂O (mg 100g⁻¹), Cmic (mg kg⁻¹).

Table 4. Variable loadings of the first three components of principal components analysis. CO₂ emissions (kg ha⁻¹day⁻¹), mineral N (N-NO₃, N-NH₄) (mg kg⁻¹), P- available P₂O₅ (mg 100g⁻¹), K- available K₂O (mg 100g⁻¹), Cmic (mg kg⁻¹).

	Component 1	Component 2	Component 3
Eigenvalue	2.47	1.46	1.22
Percent of variance	41.18	24.36	20.35
N	-0.220	0.450	0.655
P	-0.545	-0.065	-0.334
K	-0.173	0.498	-0.629
CO ₂	-0.544	-0.064	0.012
Cmic	0.230	0.729	-0.039
SOC	-0.524	0.101	0.250

3.3. Effects of Long-Term Mineral Fertilization on Soil Microbiota.

Microorganisms are highly sensitive and their number strongly depends on many factors and the variations in their quantity are very strong. Of all the factors studied, the season has the strongest influence on the number of the studied groups of microorganisms, followed by the growing crop. The influence of the treatments is statistically significant when combining all factors.

In general, microscopic fungi and actinomycetes correlated in number ($r=+0.34$, $p=0.006$), while nitrogen cycle bacteria correlated positively with each other. The increased number of microscopic fungi was observed in treatments with high EC and low pH (N, NP, NK, NPK), but there was not established positive correlation between N and number of fungi. The number of bacteria utilizing mineral nitrogen was highest in variants with arising amounts of nitrifying and nitrogen-fixing bacteria in NP, NK, NPK treatments (Table 5). The numbers of nitrogen-fixing and nitrifying bacteria were also high in the N treatments, which was not expected, but can be explained by improved soil quality and conditions due to the enhanced amount of organic matter. The number of these three groups of microorganisms, nitrogen-fixing ($r=+0.53$, $p=0.03$), nitrifying ($r=+0.51$, $p=0.04$) and assimilating mineral nitrogen ($r=+0.14$), had a positive correlation with the available phosphorus forms in beans plots and negative correlation with phosphorus in all cereal's plots. These three groups of microorganisms had positive correlation with nitrogen in all crops, most strongly in barley (NFIX, $r=+0.51$, $p=0.03$; NITR, $r=+0.62$, $p=0.04$; NUTIL, $r=+0.53$, $p=+0.05$). Nitrogen had a suppressive

effect only in maize and wheat in the N variant, while in beans and barley no such effect was observed, maybe due to the lower doses of nitrogen applied.

Table 5. Analysis of variance by controlling factors on ammonifying bacteria (CFU x 10⁶ g⁻¹), nitrogen utilizing bacteria (CFU x 10⁶ g⁻¹), nitrogen fixing bacteria, (CFU x 10⁶ g⁻¹), nitrifying bacteria (CFU x 10⁶ g⁻¹), actinomycetes (CFU x 10⁶ g⁻¹), microscopic fungi CFU x 10⁶ g⁻¹).

Treatment	Ammonifying bacteria		Nitrogen fixing bacteria		Nitrogen utilizing bacteria		Nitrifying bacteria		Actinomycetes		Microscopic fungi	
N	29.32b	±27.3	10.35ab	±15.1	11.43abc	±26.5	3.40a	±5.9	0.51ab	±0.3	0.061abc	±0.03
P	23.42a	±13.9	13.31ab	±19.4	12.53ab	±14.7	12.54abc	±16.7	0.44a	±0.4	0.043a	±0.06
K	20.29a	±20.1	12.14ab	±19.7	15.12ab	±19.6	23.32c	±26.6	0.55ab	±0.5	0.046ab	±0.05
NP	19.04a	±22.6	14.64ab	±16.7	24.24bcd	±29.2	10.23ab	±14.1	0.40a	±0.3	0.069c	±0.07
NK	25.94a	±32.6	17.61a	±44.1	34.16cd	±72.7	15.47bc	±39.3	0.48ab	±0.5	0.061bc	±0.06
PK	21.59a	±32.3	5.57	±8.2	9.27ab	±21.2	3.28a	±5.6	0.66b	±0.5	0.047ab	±0.05
NPK	22.46a	±27.8	14.35a	±31.9	19.78a	±46.0	16.68a	±41.3	0.41a	±0.3	0.050ab	±0.04
0	23.90b	±32.3	5.42ab	±4.4	22.63d	±33.1	4.14bc	±5.0	0.32a	±0.3	0.044ab	±0.04
ANOVA	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
factors	<i>ratio</i>	<i>value</i>	<i>ratio</i>	<i>value</i>	<i>ratio</i>	<i>value</i>	<i>ratio</i>	<i>value</i>	<i>ratio</i>	<i>value</i>	<i>ratio</i>	<i>value</i>
Treatment	1.45	0.186	1.25	0.277	3.65	0.001	3.25	0.003	1.79	0.092	2.32	0.0276
Season	67.76	0.000	41.01	0.000	73.28	0.000	49.96	0.000	35.96	0.000	265.27	0.000
Crop	4.77	0.003	2.42	0.068	2.34	0.074	2.97	0.033	5.02	0.002	13.61	0.000

The ratio of bacteria under different fertilization types shows that in the zero control, ammonifying and mineral nitrogen-utilizing bacteria predominated. The trend was similar for the N and PK variants (Figure 6). This indicated enhanced mineralization and a much lower amount of nitrogen-fixing and nitrifying bacteria. The largest share of NFIX and NUTIL were recorded in the K, NPK treatments.

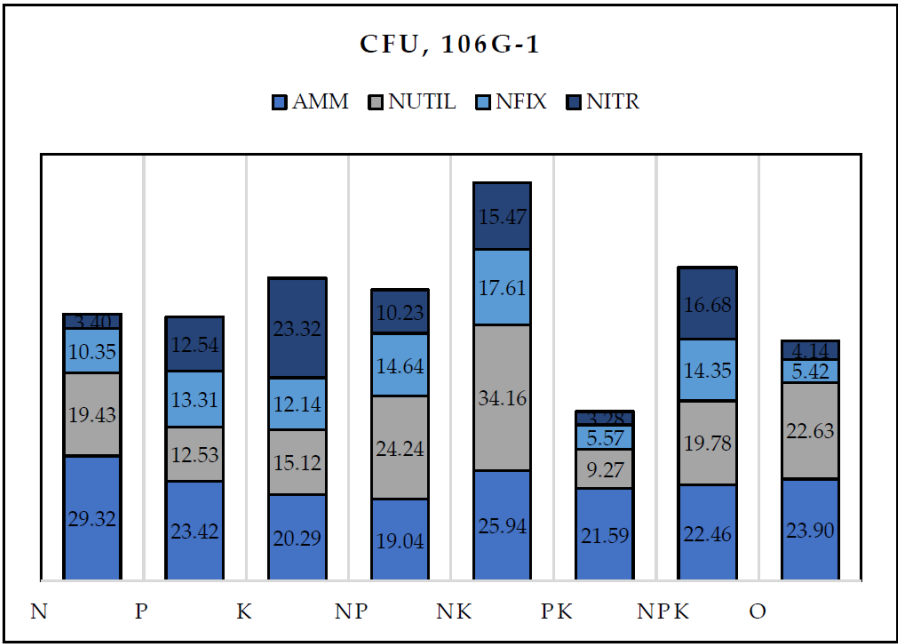


Figure 6. Number of AMM- ammonifying bacteria (CFU x 10⁶ g⁻¹), NUTIL- nitrogen utilizing bacteria (CFU x 10⁶ g⁻¹), NFIX-nitrogen fixing bacteria, (CFU x 10⁶ g⁻¹), NITR-nitrifying bacteria (CFU x 10⁶ g⁻¹).

PCA had been performed on mineral nitrogen content, available forms of P₂O₅, K₂O, main groups soil microorganisms to establish the connections between nutrients content and soil

microbiota. The model describes 68.04% of the variability of the data (Table 6). The data were loaded into three principal components (PC1, PC2 and PC3), that explain most of the variations between variables. On PC1 and PC2, the population of ammonifying, nitrifying, nitrogen-fixing and mineral nitrogen-utilizing bacteria increased, with the first component showing a slight increase in nitrogen, and the second component showing an increase in phosphorus and potassium.

Table 6. Variable loadings of the first *three* components of principal components analysis of dataset including: soil mineral N (N-NO₃, N-NH₄) (mg kg⁻¹), P- available P₂O₅ (mg 100g⁻¹), K- available K₂O (mg 100g⁻¹), AMM- ammonifying bacteria (CFU x 10⁶ g⁻¹), NUTIL- nitrogen utilizing bacteria (CFU x 10⁶ g⁻¹), NFIX-nitrogen fixing bacteria, (CFU x 10⁶ g⁻¹), NITR-nitrifying bacteria (CFU x 10⁶ g⁻¹), ACT-actinomycetes (CFU x 10⁶ g⁻¹), FUNGI-microscopic fungi CFU x 10⁶ g⁻¹).

	Component 1	Component 2	Component 3
Eigenvalue	5.54	1.53	1.06
Percent of variance	39.29	17.00	11.75
N	0.078	-0.244	0.724
P	-0.107	0.533	0.377
K	0.033	0.505	0.402
ACT	-0.181	0.504	-0.326
FUNGI	-0.271	0.249	-0.179
AMM	0.433	0.012	-0.090
NITR	0.464	0.223	0.005
NFIX	0.476	0.180	-0.059
NUTIL	0.496	0.049	-0.146

The model describes positive relation between nitrogen and nitrifying, nitrogen fixing, ammonifying and nitrogen utilizing bacteria (Figure 7). A positive effect of K was established on NFIX and NUTIL. Both actinomycetes and fungi were affected by available phosphorus and potassium.

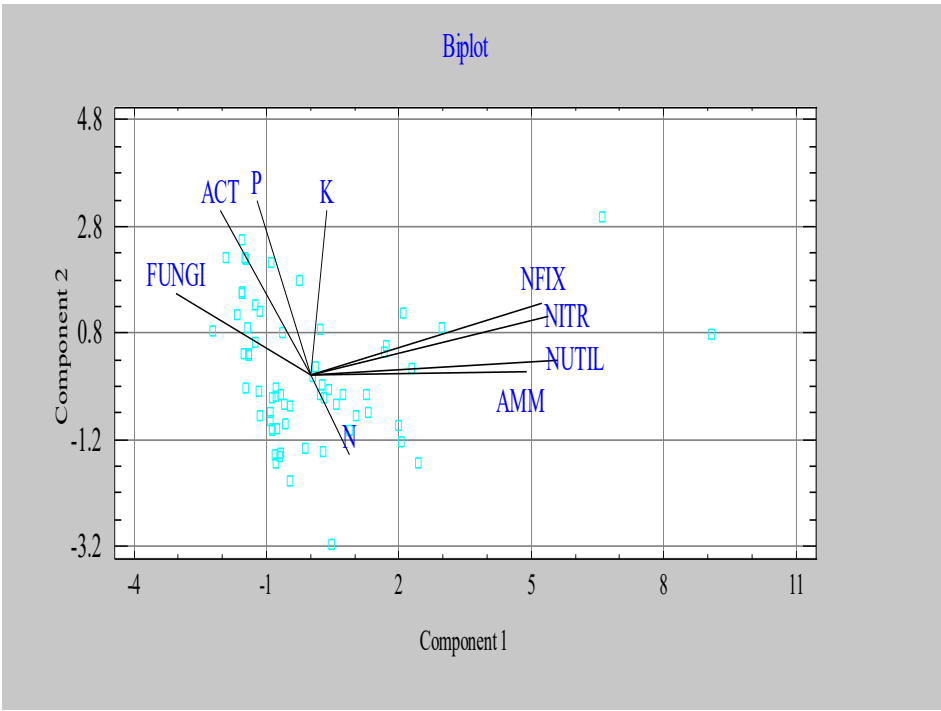


Figure 7. Biplot of the first two components in the analysis carried out on a dataset including: N (N-NO₃, N-NH₄) (mg kg⁻¹), P- available P₂O₅ (mg 100g⁻¹), K- available K₂O (mg 100g⁻¹), AMM- ammonifying bacteria (CFU x 10⁶ g⁻¹), NUTIL- nitrogen utilizing bacteria (CFU x 10⁶ g⁻¹), NFIX-nitrogen fixing bacteria, (CFU x 10⁶ g⁻¹), NITR-nitrifying bacteria (CFU x 10⁶ g⁻¹), ACT-actinomycetes (CFU x 10⁶ g⁻¹), FUNGI-microscopic fungi CFU x 10⁶ g⁻¹).

A PCA was performed on CO₂ emissions and numbers of main groups of microorganisms (Table 7, Figure 8). The model describes 72.20% of data. CO₂ increases on two vectors (PC1 and PC2), with the second component increasing CO₂ emissions and all groups of microorganisms. The model shows a stronger relationship of actinomycetes and microscopic fungi with CO₂ emissions and a weaker relationship with NUTIL, NFIX, AMM, NITR.

Table 7. Variable loadings of the first three components of principal components analysis of dataset including: CO₂ emissions (kg ha⁻¹day⁻¹), AMM- ammonifying bacteria (CFU x 10⁶ g⁻¹), NUTIL- nitrogen utilizing bacteria (CFU x 10⁶ g⁻¹), NFIX-nitrogen fixing bacteria, (CFU x 10⁶ g⁻¹), NITR-nitrifying bacteria (CFU x 10⁶ g⁻¹), ACT-actinomycetes (CFU x 10⁶ g⁻¹), FUNGI-microscopic fungi CFU x 10⁶ g⁻¹).

	Component 1	Component 2
Eigenvalue	3.77	1.29
Percent of variance	53.81	18.40
CO2	0.307	0.498
NFIX	-0.450	0.285
NITR	-0.441	0.255
NUTIL	-0.470	0.220
ACT	0.161	0.384
AMM	-0.415	0.137
FUNGI	0.298	0.626

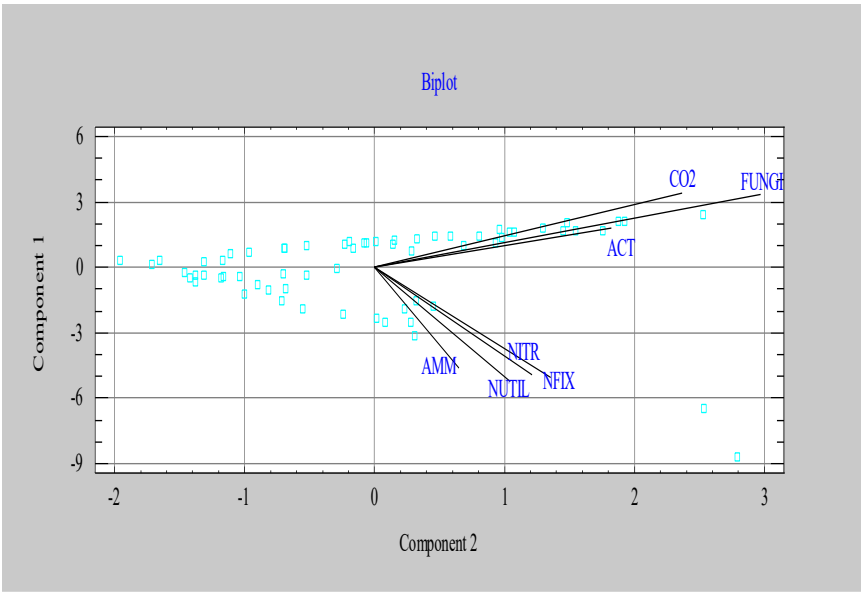


Figure 8. Biplot of the first two components in the analysis carried out on a dataset including: CO₂ emissions (kg ha⁻¹day⁻¹)AMM- ammonifying bacteria (CFU x 10⁶ g⁻¹), NUTIL- nitrogen utilizing bacteria (CFU x 10⁶ g⁻¹), NFIX- nitrogen fixing bacteria, (CFU x 10⁶ g⁻¹), NITR-nitrifying bacteria (CFU x 10⁶ g⁻¹), ACT-actinomycetes (CFU x 10⁶ g⁻¹), FUNGI-microscopic fungi CFU x 10⁶ g⁻¹).

4. Discussion

There are different views on the impact of mineral nitrogen fertilizers on organic carbon sequestration [34]. Some have suggested that nitrogen fertilizers reduce SOC stocks because they accelerate the mineralization of organic matter [35,36], and other reports suggest that nitrogen fertilizers increase SOC stocks [26,37]. In this study, long-term application of mineral nitrogen as a single fertilizer resulted in much lower SOC levels than single phosphorus fertilizer and combined fertilization. SOC stocks were highest with combined NP and NPK fertilization.

A detailed analyses of 84 permanent fertilizer experiments, showed that soils, fertilized with NPK, N, P, and K, have increased SOC with 10, 5, 5, and 2%, respectively, and these changes

depended on cropping system, duration of experiment, geographic region, and degree of fertilization [38].

It was found that while phosphorus had no significant impact on net primary productivity, nitrogen boosted it by 10.16%. Fertilization with both N and P also enhanced net photosynthetic production by 21.97%. As a result, the soils containing plant leftovers had a larger carbon input. According to the findings the simultaneous addition of N and P stimulates carbon sequestration capacity of N-limited soils [39].

The structure, composition and activity of soil microflora are very sensitive indicators of changes in soil conditions. Soil microorganisms are directly related to greenhouse gas emissions and the formation and stabilization of soil organic matter [40–42]. Soil microbial community diversity and distribution responses to fertilization have been widely reported [43,44]. It was established that the most significant effect on bacterial communities had pH and available phosphorus content, while on fungal diversity the strongest impact had mineral nitrogen, pH, SOC mineral nitrogen [44]. The increased number of microscopic fungi was observed in treatments with high EC and low pH (N, NP, NK, NPK), but there was not established positive correlation between N and number of fungi. In our study Cmic had a positive correlation with the amount of actinomycetes, fungi and different groups of bacteria, depending on the crop. In research of [45], long-term N fertilization declined Cmic and dehydrogenase activity, while combined NP, NPK fertilization increased these parameters. In current research microbial biomass carbon was stimulated most strongly by single and combined nitrogen and potassium fertilization (N, K, NK).

Higher nitrogen application rates resulted with higher GHG emissions [15,46,47], and cumulative CO₂ fluxes under long-term NP and NPK treatments were higher than under zero and NK fertilization [16,38]. The CO₂ emissions in our experiment were higher in the NP and NPK treatments, in contrast to the control and the K, N, and P fertilization treatments. Increased biomass accumulation and increase of C sequestration resulted by combined N and P fertilization intensify microbiological activities and CO₂ emissions.

5. Conclusions

The strongest influence on the formation of soil organic matter is the content of mineral nitrogen, but in combination with phosphorus (NP) and with phosphorus and potassium (NPK). Combined nitrogen and phosphorus fertilization lead to an increase in soil carbon sequestration by 37% compared to the unfertilized control and by 17% compared to single nitrogen fertilization and 8% compared to single phosphorus fertilization on Luvic Chernozem. The nitrogen fertilizer application most significantly alters soil properties including pH, EC, and SOC, which alters the environment for soil microbial development and influences the number and composition of soil microbial communities. The enhancement of total organic carbon leads to a higher intensity of soil respiration and more intense CO₂ emissions in NP and NPK treatments.

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