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Changes in *Eutric Cambisol* due to long-term mineral fertilisation: A case study in Serbia

Nikola Koković,[1] Goran Jačimović,[2] Biljana Sikirić,[1] Vladimir Čirić,[2] Vladan Ugrenović,[1] Aigul Zhapparova,[3] Elmira Saljnikov[1,4]

[1]Institute of Soil Science, Belgrade, Serbia; [2]University of Novi Sad, Faculty of Agriculture, Novi Sad, Serbia; [3]Kazakh National Agrarian Research University, Republic of Kazakhstan; [4]Mitscherlich Academy for Soil Fertility (MITAK) GmbH, Paulinenaue, Germany

Highlights

- *Due to a long-term mineral fertilisation Eutric Cambisol transformed into Dystric Cambisol.*
- *A sharp drop in soil pH did not affect the yield of crops.*
- *The total number of microflora correlated with the amount of fungi in the soil.*
- *Degradation of soil fertility parameters persists under mineral fertilisation.*

Abstract

The paper presents the results of a 50-year fertilisation experiment on *Eutric Cambisol* with increasing doses of nitrogen and constant doses of phosphorus and potassium. The changes in main parameters of soil fertility were compared with the initial level at the beginning of the experiment in 1963 (baseline), as well as with the adjacent natural meadow soil. The long-term application of mineral fertilisers without replenishment of calcium resulted in strong acidification, an increase in clay content, an increase in the number of fungi, and a decrease in the number of ammonifiers and oligonitrophiles. In the fertilised treatments, an increase in the content of plant-available phosphorus and potassium and a significant decrease in the plant available boron and zinc with an increase in the N dose were observed. Long-term addition of high doses of N harmed the total number of microflora, actinomycetes, ammonifiers, and oligonitrophiles compared to untreated meadow soil. Significant changes in soil's physical and chemical properties resulted in the transformation of *Eutric Cambisol* into *Dystric Cambisol.* Inappropriate agricultural practices, such as adding only mineral fertilisers, can lead to significant soil degradation even on the sites with favourable bioclimatic conditions for cropping. The results showed that causal chains between microbiological and chemical parameters need to be better researched and understood in the future. This and other long-term experimental results should be used to calibrate agro-ecosystem models in Serbia.

Correspondence: Elmira Saljnikov, Institute of Soil Science, Department of Pedology, Teodora Drajzera 7, 11000 Belgrade, Serbia.
E-mail: soils.saljnikov@gmail.com

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Introduction

Agricultural intensification, one of the key strategies for enhanced food production, is dependent on increased flows of plant nutrients to crops to secure higher yields. However, if a balanced supply of nutrients does not support agricultural intensification, it will lead to land degradation and jeopardize the sustainability of agriculture (FAO, 2006). Therefore, in the European Commission's new 'A Farm to Fork' strategy 'For a fair, healthy and environmentally friendly food system' COM (2020) 381 and Zero Pollution action plan, which comprehensively respond to the challenges of sustainable food systems, measures are prescribed to reduce nutrient losses in the soil by at least 50%, and the use of fertilisers by at least 20% by 2030, while ensuring that there is no reduction in soil fertility (European Commission, 2019).

However, the ever-growing population of the Earth requires increased food production while the area of agricultural land is decreasing (Saljnikov *et al*., 2022). This means that higher yields should be obtained per unit area, which is achieved using higher fertilizer doses in most cases. This leads to intensive exploitation of arable land and its degradation. To meet the goals of environmentally friendly crop management, Sustainable Soil Management (FAO, 2017), Land Degradation and Restoration

Assessment (IPBES, 2018) as well as the target 15.3 of the Sustainable Development Goals (UNDP, 2015), clearly defined further strategies that emphasise the importance of soil health in future agricultural policies, environmental protection, and climate change are required (Akhtar-Schuster *et al*., 2017; Montanarella and Panagos, 2021).

Long-term application of mineral N can lead to unfavourable changes in physical, chemical, and biological properties of soil and cause or contribute to soil degradation processes (Koković *et al*., 2018; Gupta *et al*., 2019; Sainju *et al*., 2019; Danilov *et al*., 2020; Jones *et al.,* 2020; Zhang, 2020). This ultimately affects the adequate ecological functioning of the soil and its sustainability (Zhou *et al*., 2020; Craig *et al*., 2021). Huang *et al*. (2019) showed that long-term fertilisation altered the microbial community but failed to restore SOC stocks to the level of natural meadow soils of the Tibetan Plateau. Dal Ferro *et al*. (2020), studying the changes in organic carbon content in different types of soils, concluded that the long-term establishment of permanent grassland along with the application of manure or crop residues with minimal tillage is possible at great depths in natural conditions on sandy and loamy soils with low content of organic carbon. Jakšić *et al*. (2020), in a study to the present work, investigated acid soils in Serbia and suggested that continuous application of inorganic fertiliser without organic amendments had led to a decrease of SOC in topsoil. The latter found high rates of organic C reduction in topsoil in the early stage of cultivation. However, during permanent cultivation, deep tillage showed the potential to preserve SOC in the deeper soil layer and prevent carbon loss from the topsoil.

Oversaturation with mineral nitrogen may cause a toxic effect on plants, inhibiting their growth and development (Li *et al*., 2020) and contaminating groundwater (Yang *et al*., 2017). In addition, only 30-50% of the applied fertiliser N is absorbed by the growing crop in the year of application, depending on the crop species and cultivar (Raza *et al*., 2020), while the rest of the added N can be lost via leaching, volatilisation, and erosion.

Soil microorganisms play an essential role in ecosystem function and are considered a reliable indicator of soil quality and fertility. Soil microorganisms can be limited by carbon or phosphorus, while net primary production in terrestrial ecosystems is generally limited by nitrogen availability. Excess N supply can sometimes inhibit soil microorganisms, indicating that microbes are not always nitrogen-restricted (Treseder, 2008).

The effect of chemical fertilisers on soil quality is not immediately apparent due to soil buffering ability. However, over time, the buffering capacity may deteriorate due to a nutrient imbalance (Jiang *et al.,* 2018). Therefore, the critical challenge is to meet crop nutrient requirements while minimising nutrient losses to maintain a sustainable environment and economic benefits for farmers. In this regard, long-term field experiments provide reliable data to monitor the effect of the continuous addition of mineral fertilisers on soil quality and plant nutrition (Wen *et al.,* 2020; Körschens, 2021).

Eutric Cambisol (WRB, 2015) is the most representative soil in agricultural landscapes in central Serbia (about 650,000 ha). It is developed mainly on loose carbonates and has a loamy texture. The upper horizon is well supplied with organic matter (>3%) and characterised by a stable structure and slightly acidic to neutral reaction. However, human activity has significantly altered the chemical properties of the studied agricultural soil. For example, the processes of lessivage and podzolisation led to a deterioration of this soil's physical and chemical properties (the topsoil layer was acidified, and the content of organic matter dropped to ≤2%).

Moreover, these processes are amplified by irregular cultivation, imbalanced and improper fertilisation. In Serbia, to develop an appropriate fertilisation scheme and monitor the effects of different combinations and doses of mineral fertiliser, a multi-variable field experiment was set up in 1963 at the Soil Research Institute. Since then, the original experimental design has been maintained, and soil and plant quality changes have been systematically monitored. The objective of this study was to evaluate the changes in selected physical, chemical, and biological properties of the soil after 50 years of continuous NPK fertilisation on a two-year crop rotation (wheat/corn) under increasing doses of nitrogen and constant doses of phosphorus and potassium. All the results obtained were compared with the initial state (the beginning of the field experiment in 1963) and the adjacent meadow soil.

Materials and methods

Study site and experimental design

The soil was sampled from the Institute of Soil Science's long-term experimental station 'Mladenovac' located 55 km from Belgrade (44°24'58'' N and 20°10'34'' E) in Serbia. The elevation is 161 m above sea level with mean annual precipitation and temperature of about 700 mm and 11°C (Table 1). Calcium ammonium

Table 1. Average monthly air temperature and precipitation during the study years (2013-2014) and the 50-year average (1961-2011).

Years	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Aver.
						Mean air temperature (°)							
2013	2.5	3.5	5.5	12.8	17.6	20.1	22.4	23.5	15.7	12.0	8.4	1.9	12.2
2014	3.9	6.2	9.1	12.3	15.5	19.3	21.8	21.0	16.9	12.6	8.3	2.8	12.5
50-year average (1961-2011)	0.2	2.5	6.7	11.6	16.5	19.6	21.3	21.2	17.1	12.0	6.6	1.6	11.4
Years						Precipitation, mm							Sum
2013	69.2	68.6	94.6	27.0	89.1	42.0	30	36.0	48.7	50.7	45.1	11.1	561.4
2014	31.9	13.8	51.7	108	241.2	73.0	151	90.0	73.1	59.7	16.5	82.1	991.9
50-year average (1961-2011)	44.0	45.3	48.0	53.5	64.0	91.2	60.7	55.1	56.0	50.7	52.5	56.5	677.4

nitrate (CAN), monocalcium phosphate (superphosphate fertiliser, $Ca(H_2PO_4)_2$), and potassium chloride (KCl) were applied in the period from 1963 to 1973. Since then, urea, monoammonium phosphate fertiliser (MAP), and KCl have been used according to the described rates and timing below. The cultivated crops were winter wheat (*Triticum aestivum*) and corn (*Zea maize* L.) with harvest residues left on the field. After the wheat harvest in the first ten days of July, mouldboard ploughing was done in September to a depth of 25 cm. At the end of March, NPK fertiliser was applied, followed by disking and fine pre-sow field processing. Corn was sown in early April. After harvesting corn in the first ten days of September, heavy disking to 20 cm depth was performed. Then, the whole amount of phosphorus and potassium and 1/3 part of nitrogen fertilisers were added, followed by heavy disking. After that, in October, wheat was sown. In the third ten days of February, the remaining amount of N fertiliser was added. The crops were grown in rainfed conditions.

The experiment was arranged in a randomised block design, with each treatment replicated in four blocks of a total of 60 plots (6×10 m), including a control treatment that did not receive any fertiliser. In addition, a composite soil sample was taken from the natural meadow site with the same soil type located near the experimental field in the year the experiment was established in 1963 and the year of study in 2013. Among large numbers of the fertilisation treatments, the following treatments were selected for this study: i) control (without fertilisation); ii) N1P2K2 (N1, 60/90/80 kg ha^{-1} per year); iii) N2P2K2 (N2, 90/90/80 kg ha^{-1} per year); iv) N3P2K2 (N3, 120/90/80 kg ha^{-1} per year); v) N4P2K2 (N4, 150/90/80 kg ha^{-1} per year); vi) natural meadow.

Soil sampling

For chemical and physical analyses, soil samples were taken from the surface 0-25 cm plough layer in autumn 2013 and for microbiological analysis in autumn 2013 and spring 2014. Five subsamples were taken from each replicates and then mixed to make a composite sample for each replicate. All the collected soil samples were air-dried and sieved through a 2.0 mm mesh sieve. Samples for microbiological analyses were taken twice (spring and autumn) from the plough soil layer (0-10 cm) using the method of scattered sampling (Vojinovic *et al.,* 1966). In 1963, soil samples were taken from the field before establishing the experiment and subjected to primary soil agrochemical analyses (pH, organic carbon, mineral nitrogen, available forms of phosphorus and potassium).

Analytical methods

Particle size distribution was determined with a combined sieving and pipette method-modified International 'B' method. Soil pH was determined with a glass electrode pH meter in 1 mol L^{-1} KCl (pH KCl; in ratio 1:2.5 (w/v)) and in distilled water (pH in H_2O with ratio 1:20 (w/v)).

Hydrolytic acidity (Hy) and the sum of exchangeable basis (S) were determined by the Kappen method (Kappen, 1929). Cation exchange capacity (CEC) was determined using the tube leaching method with 1 mol L^{-1} ammonium acetate. The method consisted of the following steps: i) soil extraction with sodium-acetate to replace exchangeable cations with Na^+; ii) removing excess sodium with alcohol; iii) exchange of Na^+ ions with $NH4^+$; iv) determination of Na^+ concentration in the resulting solution by atomic emission spectrometry (Burt, 2014).

Soil total carbon (TC) and nitrogen (TN) were measured on an elemental CNS analyser (Vario model EL III-ELEMENTAL Analysis systems GmbH, Hanau, Germany) by dry combustion at 1150°C. The content of inorganic carbon ($CaCO_3$) was determined volumetrically with a Scheibler calcimeter. The organic carbon (OC) was calculated by subtracting the $CaCO_3$ carbon from the amount of total carbon content. The Al-method (Egnér *et al*., 1960) determined available P and K in the soil, where 0.1 mol L^{-1} ammonium lactate (pH=3.7) was used as an extract. After extraction, potassium was determined by an FP 6440 FP flame emission photometry (Nanbei Instrument Ltd., China), and phosphorus by UV-160A spectrophotometer (Shimadzu, Japan) after colour development with ammonium molybdate and $SnCl_2$. Exchangeable Al (mg 100 g^{-1} soil) was determined by the titration method of Sokolov: extraction with 1 mol L^{-1} KCl (1:2.5) after shaking for 1 h and titration with 0.01 mol L^{-1} NaOH (Jakovljević *et al.,* 1985). Soil Ca and Mg were extracted by ammonium acetate and determined with a SensAA dual atomic absorption spectrophotometer (Dandenong, Australia). Particle size distribution was determined by the pipette method (ISO 11277:1998); bulk density (BD) was measured by drying the cores at 105°C to a constant weight (ISO 11272:1993).

Microelements were determined on an iCAP 6300 ICP optical emission spectrometer (Thermo Electron Corporation, Cambridge, UK), after digestion with concentrated HNO_3 for the extraction of hot acid-extractable forms and with diethylenetriaminepentaacetic acid for the (DTPA) extractable elements (Soltanpour *et al.,* 1996). Merck standards were used for determinations on ICP and SensAA Dual.

The basic parameters for assessing soil biogenity during long-term nitrogen fertilisation were: total microflora, the total number of fungi, actinomycetes, ammonifiers, *Azotobacter,* and ologonitrophiles. The number of microorganisms was determined by a dilution method on the appropriate nutrient medium using decimal dilutions (10^{-1}-10^{-8}) and expressed by colony forming units (CFU) or most probable number (MPN) technique (Williams and Busta, 2003). The total number of microflora was determined using the plating method on the agarised soil extract, fungi on the Chapek medium, and actinomycetes on the synthetic agar with saccharose, according to Krasilnikov (Govedarica, 1996). The number of ammonifiers was determined on a liquid medium with asparagines and *Azotobacter* sp. with mannitol as the source of nitrogen and oligonitrophiles on the medium, according to Fyodorov (Stajković-Srbinović *et al.,* 2018).

Statistics

The results were statistically processed using a one-way blocked ANOVA: SPSS version 16 software and COSTAT. Significant differences between physical and chemical parameters and different doses of N fertiliser were assessed using the t-test (95%) for Tuckey HSD. In addition, the differences between the applied fertiliser treatment and the total number of microorganisms were tested using the Tukey HSD, $P<0.05$ probability level.

Results and discussion

Particle size distribution

The results of particle size distribution are presented in Table 2. Because the initial (50 years ago) data of particle size distribution (PSD) was missing, we compared PSD data from the fertilisation treatments with the control treatments and the adjacent native meadow. The content of sand particles, both >0.2 mm and 0.2-0.02 mm, did not show significant differences between all studied treatments, while the content of silt and clay fractions differed significantly ($P<0.05$). Higher doses of N resulted in a decrease in silt content, and an increase in the content of clay fractions. The 90 kg N ha^{-1} significantly differed from the lowest dose and the control. While the doses of 90, 120, and 180 N did not significantly differ.

A small but statistically significant ($P<0.05$) increase in the fraction of clay in fertilised treatments can be explained, firstly, by significant acidification of fertilised soils that might result in a non-reversible dissolution of clay mineral and a reduction in cation exchange capacity, accompanied by structural changes (Goulding, 2016; Wei *et al*., 2020). The decrease in the content of the silt fraction was probably due to the partial dissolution of the allophone minerals. Okada *et al*. (2005) established that the Si/Al ratios in soil samples were reduced due to the loading of orthophosphate due to the partial replacement of SiO_4 with PO_4 tetrahedrons. However, the partial dissolution of allophane compensated for this effect due to the loading of polyphosphate. Another factor might be associated with the change in the distribution of clay and silt fractions.

Soil pH

The main agrochemical properties of the soil from the study are presented in Table 3, including the data on the initial soil characteristic at the start of the experiment in 1963 (Ivović *et al.,* 1979). A comparison of pH of samples analysed in 2013 with baseline pH values analysed in 1963 showed that the soil acidity changed significantly over 50-years of using mineral N or no fertilisation when growing wheat and corn. A clear downward trend in soil pH was observed with an increase in N application rates between the control and fertilized plots (Table 3). A comparison of the soil pH between the adjacent natural landscape and the experimental plots showed that the meadow soils retained the acidity observed in 1963.

Soil pH is one of the main factors affecting solubility and phytoavailability of many macro- and microelements. In our study, long-term use of mineral N led to a significant change in the acidity: from weakly to very acidic soil (Table 3). It is well known that most inorganic fertilisers containing N, unless specifically treated, tend to acidify soil due to the presence of NH_4^+ ions or its production in the soil (*e.g.*, Sainju *et al*., 2019; Raza *et al*., 2020). Sainju *et al*. (2015) found in a similar study a decrease in soil pH from an initial 6.75 to 6.15 after 30-years of N-fertilisation in soil grown continuously with spring wheat. Although soils with a higher buffer capacity are resistant to acidification, the studied *Eutric Cambisol* lost its basic cations due to the prolonged addition of mineral N (Table 4). Saljnikov *et al*. (2010), studying the same experiment, found that 40 years of phosphorus (MAP) and K (KC1) fertilisation resulted in an increase in soil acidity and a decrease in soil base cations due to leaching. In our study, the dose of applied N was decisive for the degree of soil acidification. Ghimire *et al*. (2017) found that surface soil pH dropped from 5.70 in control conditions to 5.0 after 70 years of N-application

Table 2. Particle size distribution in the *Eutric Cambisol* soil (0-25 cm) as affected by increasing doses of nitrogen fertiliser applied over 50 years.

Treatment	Fertiliser ($kg\ ha^{-1}$)	Sand >0.2 (mm)	Sand 0.2-0.02	Silt (%) 0.02-0.002	Clay <0.002	Total Sand >0.2-0.02	Silt + Clay <0.002-0.02	Texture class
Control	0.00	0.98±0.10	26.03±0.34	42.03^a±0.41	30.98^a±0.10	27.00±0.39	73.01	CL
N1	$N_{60}P80K_{80}$	0.98±0.09	26.20±0.63	41.75^b±0.15	31.08^b±0.59	27.18±0.68	72.83	CL
N2	$N_{90}P80K_{80}$	1.00±0.06	26.30±0.78	41.45^c±0.31	31.25^c±0.58	27.30±0.77	72.70	CL
N3	$N_{120}P80K_{80}$	0.95±0.03	25.95±0.45	41.40^c±0.28	31.70^c±0.21	26.90±0.45	73.10	CL
N4	$N_{150}P80K_{80}$	0.89±0.06	25.99±0.21	41.18^c±0.18	31.95^c±0.08	26.88±0.26	73.13	CL
t-test		*NS*	*NS*	**	**			
Meadow	0.00	0.8	25.9	42.1	31.4	26.7	73.5	CL

*Significantly different at $P<0.05$ (t-test for Tuckey HSD); NS, not significant; CL, clay loam.

Table 3. Agrochemical properties in the *Eutric Cambisol* soil (0-25 cm) as affected by increasing doses of nitrogen fertiliser applied over 50 years.

Treatment	Fertiliser ($kg\ ha^{-1}$)	pH H_2O	pH KCl	OC (%)	TN	C/N	Avail. P ($mg\ 100\ g^{-1}$)	Avail. K
Initial state (1963)								
	0.00	6.20	5.20	1.51	0.120	12.60	1.80	13.60
Autumn 2013								
Control	0.00	5.35^a±0.04	4.56^a±0.02	0.92 ±0.02	0.104^a±0.01	8.44	6.90^a±0.57	21.44^a±0.71
N1	$N_{60}P80K_{80}$	5.00^b±0.13	4.08^b±0.06	0.98^b±0.02	0.119^b±0.00	8.23	20.60^b±0.91	26.12^{bc}±0.77
N2	$N_{90}P80K_{80}$	4.75^c±0.06	3.91^c±0.08	1.08^c±0.03	0.125^{bc}±0.01	8.64	21.51^b±0.59	27.80^b±0.90
N3	$N_{120}P80K_{80}$	4.60^{cd}±0.05	3.74^d±0.05	1.13^c±0.03	0.126^c±0.00	8.97	21.08^b±1.55	26.53^{bc}±0.82
N4	$N_{150}P80K_{80}$	4.48^c±0.02	3.63^d±0.07	1.14^c±0.02	0.127^c±0.01	8.98	19.50^b±1.28	25.55^c±0.84
t-test		**	**	**	**		**	**
Meadow	0.00	6.01	5.13	2.87	0.270	10.77	9.27	29.07

*Significantly different at $P<0.05$ (for Tuckey HSD); OC, organic carbon; TN, total nitrogen; Avail., available; $^{a-c}$different letters within columns mean statistically significant difference.

at a dose of 135-180 kg N ha^{-1}. This study confirmed that the continuous addition of mineral N results in a constant gradual decrease in pH, accompanied by the loss of basic cations (Ca^{2+}, K^{+}, Mg^{2+}) due to leaching (Pavlova *et al*., 2019). This, in turn, leads to a decrease in base saturation and an increase in Al^{3+} saturation, and thus the availability of nutrients is reduced (Goulding, 2016).

Organic carbon and total nitrogen content

Soils from all treatments showed a significantly lower organic carbon content than the unmanaged meadow soil (Table 3). Among fertilised treatments, there were statistically significant differences between the treatment N1treatment (60 kg N ha^{-1}) and the other treatments N2, N3, and N4 (90, 120, and 150 kg N ha^{-1}). However, there was no significant difference in the organic carbon (OC) content between N2, N3, and N4 treatments. The content of OC decreased significantly over the 50-year cultivation of wheat and corn on fertilised plots compared with the initial values in 1963.

As expected, the total nitrogen content (TN) showed a tendency to increase following the applied N doses. The changes in N content in the fertilised treatments were significant (Table 3) compared to the control ($P<0.05$). In the treatments with the doses of N 60 and 90 kg, the soil nitrogen content (0.119% and 0.125%, respectively) was close to the initial content (0.120%). A decrease in OC and increase in total N content in the fertilised treatments resulted in a narrower C/N ratio than the initial C-to-N ratio of 12.6. The natural meadow showed higher content of OC and total N than those on the soils from fertilised and control treatments and soil samples from natural landscapes.

The increase in organic carbon content at higher doses of N ranged from 6% to 17% and was in line with the applied N doses. However, 50 years of fertilisation were not sufficient to maintain the organic carbon content at the baseline level regardless of the amount of fertiliser applied. The initial content of soil OC was 1.51%, as reported by Ivović *et al*. (1979). Thirty-six years after the soil was brought to cultivation, Kresović (1999) established that the content of OC in the same experimental field decreased by 45.03%, 35.76%, 30.46%, 28.48%, and 27.81% in the N1 to N4 fertilised treatments, respectively. The lowest content of organic C in the control was associated with the lowest crop yields (Table 5), which produced both the lowest above- and below-ground biomass that returned the least organic residues to soil (Kresović, 1999; Li *et al*., 2020; Ghimire *et al*., 2022). Berti *et al*. (2016), in studying the effect of organic C amendments on humification, found that carbon sources from the roots of crops showed a 1.9 times higher humification coefficient compared to the above-ground plant residues implying greater accumulation of stable soil organic matter under the treatments with greater root biomass. Berti *et al*. (2016) proposed that the quality of C input is fundamental to assessing the dynamics of SOM in different cropping systems. In our study, the biomass of roots and stubble left in the fields contributed to the greater organic carbon accumulation in the fertilised plots.

Typically, total soil N increases with high doses of N applied over a more extended period (Ghimire *et al*., 2017). However, high doses of added N do not always lead to increased crop yields (Guo *et al.,* 2019; Pasley *et al*., 2019) or efficient use of added N. Moreover, because crops utilise the required amount of N, excess N either volatilises or leaches into deep soil, contaminating groundwater sources (Sainju, 2019). In our study, higher doses of N maintained some organic carbon and total nitrogen replenishment with a higher yield (Table 5). However, the mineral fertilisation alone was insufficient to maintain the baseline soil organic carbon, regardless of the amount applied. Table 5 presents yield characteristics of wheat harvested in July 2013 before sampling in autumn 2013 and corn in 2014, sown after sampling in April 2014. The multi-year average yield from 1963 to 2014 is also shown. Generally, added mineral nitrogen significantly positively affected the yield of both crops in line with the dose of added N. The exceptions were the last two treatments with the highest doses of N (N3 and N4), which did not differ significantly.

In addition to mineral fertiliser, high yields of both crops, especially corn in 2014, were also influenced by the favourable distribution of precipitation in the vegetation period. A comparison of the yield data with the soil chemical characteristics (Table 3 and Table 5) indicates that a sharp drop in soil pH did not affect the yield of the studied crops. This phenomenon can be explained because the pronounced acidity in the fertilised plots was not accompanied by toxic concentrations of exchangeable aluminium (Table 6). The studied *Cambisol*, despite over 50 years of acidification, maintained a concentration of Al below the toxic limit of 10 mg $100g^{-1}$ (Sikirić *et al*., 2009), which is closely related to the composition of the parent rock (Mc Lean and Brown, 1984; Kovačević *et al*., 2003). In addition, higher crop yield under higher nitrogen doses contributed to the higher crop residues and added to general soil fertility (Vojnov *et al*., 2020; Koković *et al*., 2021; Šeremešić and Ćirić, 2022).

There is controversial information in the literature on the effect of nitrogen fertilisation on the accumulation and/or loss of organic carbon in the soil. Some authors argue that nitrogen fertilisation increases OC content, given that crop residues are returned to the soil (Alvarez, 2005; Kong *et al*., 2008), while others found that nitrogen fertilisation led to the loss of organic carbon (Khan *et al*., 2007). Although these differences are influenced by the type of soil and crops grown, climatic conditions, and soil management (Sainju *et al*., 2015), the discrepancy between the results when studying the effect of long-term use of nitrogen fertilisers on SOM content indicates that soil organic matter is a dynamic parameter that is sensitive to both individual factors (a dose of fertiliser) and a combination of factors (a dose of fertiliser and the changes in soil physical and chemical parameters).

Available phosphorus, potassium, macro-and microelements

The content of available phosphorus (P_2O_5) was the lowest in the control (6.9 mg 100 g^{-1}), while the content of P_2O_5 in fertilised treatments was uniform and ranged from 19.50-21.51 mg 100 g^{-1} (Table 3). However, compared with the initial phosphorus content in 1963, its concentration in the control was approximately 3.8 times higher, and in the fertilized treatments 10 times higher. The available potassium (K_2O) content was also uniform among fertilised treatments and slightly higher than in the control and almost doubled the initial state of 1963. The content of available P in the natural meadow was very similar to the control plot (9.27 and 6.90 mg per 100 g of soil, respectively). In contrast to phosphorus, potassium content in the natural meadow was higher (29.07 mg 100 g^{-1}) than in other treatments. All samples tested contained high amounts of Mg and sufficient Ca (Table 4) with a favourable Ca/Mg ratio (Mengel *et al*., 2001). The highest Mg and Ca content was in the control and significantly decreased with increasing doses of N ($P<0.01$). Concentrations of exchangeable Fe and Mn increased, while the content of Zn, Cu, and B decreased.

The total phosphorus content in soils is generally low in the upper horizon and is less mobile in soil than nitrogen and potassium (Weil and Brady, 2017). Continuous addition of NPK resulted in a tripling in the concentration of P_2O_5 compared to the control and almost ten times compared to the initial baseline values in 1963

(Table 3). Since phosphorus added to the acidic soil is prone to fixation with sesquioxides of aluminium and iron, a massive reserve of phosphorus accumulated in the soil over 50 years (Čakmak *et al*., 2010a). Among the fertilised treatments, the phosphorus concentration was distributed relatively evenly, given that the dose of applied phosphorus was the same. A slight decrease in P_2O_5 was observed with an increased dose of N, which is explained by the removal with the harvest. Pasley *et al*. (2019) reported that nitrogen fertiliser increased plant uptake of P by 280%, indicating the potential for mitigating nutrient deficiencies other than nitrogen. In contrast, meadow vegetation forms higher phosphorus levels in the soil due to a biochemical cycle that returns organic phosphorus with rich herbal vegetation (FAO, 2006).

In contrast to phosphorus, potassium concentrations in the fertilised treatments were only slightly higher than in the control. Higher doses of N promoted potassium uptake by wheat (Guo *et al*., 2019). Soluble potassium is less prone to binding with aluminium and iron oxides (Chatterjee *et al*., 2014), reflected in the higher content of soluble potassium in natural landscapes in our study. On the other hand, although the potassium added with fertilisers is available biologically for uptake, plants cannot use significant amounts of potassium in a single growing season (Rehm and Schmitt, 2002), so excess amounts are prone to leaching (Gupta *et al*., 2019; Zhang, 2020).

The content of potassium was the highest in the natural meadow. Generally, soils contain large reserves of K, but only a small fraction is available for plant uptake (Rehm and Schmitt, 2002). Almost all of the potassium contained in the soil is included in the structural component of soil minerals, implying that potassium supply depends upon the type of soil and ultimately dictates the amount of potassium to be applied through fertilisation (Madaras and Koubová, 2015).

The studied soil showed high availability of Fe and Mn associated with the natural richness of *Eutric Cambisol* in these microelements, and an increase in their solubility in an acidic environment (Sikirić *et al*., 2015). This is closely correlated with the values obtained from the N3 and N4 treatments, where the lowest soil pH and highest Fe and Mn concentrations were observed. On the other hand, low B and Zn content is associated with naturally low micronutrient content in this soil and leaching from acidic soil (Mahedi *et al*., 2019). In addition, soil CEC exerts a significant impact on the adsorption of microelements, especially Zn (Belanović *et al*., 2012). As expected, meadow soil contained favourable contents of all macro- and microelements, whose levels were higher than in the experimental field since there was no removal of nutrients by crops, with all the extracted nutrients returned to the soil.

Soil adsorption complex

Long-term agricultural production markedly increased the hydrolytic acidity (Hy) of the studied Cambisol compared to the initial values, which increased linearly with an increase in the dose of N (Table 6), where significant changes were observed between the control and fertilised treatments. The most significant increase in hydrolytic acidity and the most significant decrease in total exchangeable bases (S) and base saturation (BS) were obtained in the treatment with the highest dose of N (N4 treatment). The BS and S parameters changes were significant between the treatments, resulting in a significant loss of soil bases under higher doses of N. The treatments with the highest doses of N (N3 and N4) had a BS below 50%. The least content of exchangeable Al was found in the control and increased significantly with increasing N dose. Although an intense soil acidification process was noted, the exchangeable Al content in fertilised treatments was below the toxic limit (Mrvić *et al*., 2007).

Contrary to expectations and soil acidification due to the long-

Table 4. Content of micro- and macronutrients in the *Eutric Cambisol* soil (0-25 cm) as affected by increasing doses of nitrogen fertiliser applied over 50 years (1963-2014).

Treatment	Fertiliser ($kg\ ha^{-1}$)	Ca ($mg\ 100\ g^{-1}$)	Mg	Ca/Mg	Fe	Mn	Zn ($mg\ kg^{-1}$)	Cu	B
Control	0.00	$318^a \pm 1.1$	$58^a \pm 1.4$	3.30	$89.16^a \pm 1.9$	$74.70^a \pm 1.0$	$0.98^a \pm 0.07$	$3.46^a \pm 1^{-2}$	$0.40^a \pm 1^{-2}$
N1	$N_{60}P_{80}K_{80}$	$310^b \pm 0.8$	$57^a \pm 0.8$	3.30	$120.11^b \pm 1.4$	$75.93^{ab} \pm 1.4$	$0.95^{ab} \pm 0.02$	$3.41^a \pm 1^{-2}$	$0.35^a \pm 1^{-2}$
N2	$N_{90}P_{80}K_{80}$	$311^b \pm 0.8$	$58^a \pm 1.4$	3.20	$124.95^c \pm 1.8$	$78.10^b \pm 1.0$	$0.88^{cb} \pm 0.05$	$3.4^a \pm 1^{-2}$	$0.34^a \pm 1^{-2}$
N3	$N_{120}P_{80}K_{80}$	$308^c \pm 0.8$	$55^b \pm 0.8$	3.40	$125.36^c \pm 0.5$	$88.80^c \pm 1.0$	$0.78^d \pm 0.01$	$3.37^c \pm 1^{-2}$	$0.25^c \pm 4^{-2}$
N4	$N_{150}P_{80}K_{80}$	$305^d \pm 0.7$	$54^b \pm 1.1$	3.40	$136.05^d \pm 1.7$	$98.00^d \pm 0.8$	$0.79^d \pm 0.02$	$3.35^c \pm 1^{-2}$	$0.25^c \pm 2^{-2}$
t-test		*	*		*	*	*	*	*
Meadow	0.00	490	70	4.20	121.92	74.61	3.21	3.09	0.84

*Significant at $P<0.05$; [a-d]different letters within columns signify statistically significant difference (for Tuckey HSD test).

Table 5. Yield of crops as affected by increasing doses of nitrogen fertiliser applied over 50 years.

Fig	Fertiliser $kg\ ha^{-1}$	Wheat yield (2013) $kg\ ha^{-1}$	Index %	Corn yield (2014) $kg\ ha^{-1}$	Index %	Wheat yield (1963-2014) $kg\ ha^{-1}\ year^{-1}$	Corn yield (1963-2014)
Control	0.00	3192.6^a	100.00	8003.0^a	100.00	2896.4	4120.3
N1	$N_{60}P_{80}K_{80}$	4168.3^b	130.56	9806.1^b	122.53	3854.3	6816.1
N2	$N_{90}P_{80}K_{80}$	5011.3^c	156.97	11845.7^c	148.02	4952.9	8145.7
N3	$N_{120}P_{80}K_{80}$	5979.2^d	187.29	12885.3^d	161.01	5679.2	9485.3
N4	$N_{150}P_{80}K_{80}$	6065.1^d	189.98	13045.4^d	163.01	5947.1	10133.8

[a-d]Different letters within columns mean statistically significant difference at $P<0.05$ (for Tuckey HSD test).

term application of mineral nitrogen, no decrease in cation exchange capacity (CEC) was found (Table 6). This may be due to an increase in the proportion of clay in the treatments with high doses of nitrogen fertilisers that resulted in a sharp reduction of soil pH (Čakmak *et al*., 2010a; Belanović *et al*., 2012; Wei *et al*., 2020; Niu *et al*., 2021). All study treatments showed an increase in CEC compared to the baseline CEC (1963), consistent with an increasing proportion of clay. The highest CEC values in the meadow soil were explained by the higher organic matter content (Yan *et al*., 2007). Significant reductions in cation exchange (S) and saturation (BS) capacities, along with other changes in soil properties, resulted in the original *Eutric Cambisol* soil being converted to *Dystric Cambisol* (Čakmak *et al*., 2010b). The loss of bases is a consequence of prolonged use of physiologically acidic fertilisers that do not contain calcium, associated with removal of nutrients by harvest and losses via leaching. Our results confirm that the higher the yield, the greater the amount of bases harvested, according to the dose of N added, leading to imbalanced nutrient inputs and outputs (Čakmak *et al*., 2010b).

Microbiological characteristics

The results of the microbiological analyses are shown in Table 7. Since microbiological properties were not studied until 2013, a comparison with the initial state was not conducted. Therefore, for comparison and explanation of the current state of soil biogenity, soils were selected from control and meadow samples. The total number of examined microbiological groups generally showed a higher content in autumn than in spring.

One-way analysis of variance showed a significant effect of the long-term application of different doses of N on the number of all studied microbiological groups. In both sampling periods, all fer-

Table 6. Soil adsorption complex and content of exchangeable Al in the *Eutric Cambisol* soil (0-25 cm) as affected by increasing doses of nitrogen fertiliser applied over 50 years (1963-2014).

Treatment	Fertiliser (kg ha^{-1})	Hy	S (cmol kg^{-1})	CEC	BS (%)	Exch. Al (mg 100 g^{-1})
Initial state (1963)						
	0.00	6.10	17.90	24.00	74.30	-
Current state (Autumn 2013)						
Control	0.00	11.38^{a}±0.54	16.00^{a}±0.13	27.38^{a}±0.61	58.45^{a}±1.38	1.00^{a}±0.11
N1	$N_{60}P_{80}K_{80}$	11.70^{a}±0.35	15.20^{b}±0.27	26.90^{a}±0.58	56.51^{b}±0.42	1.30^{b}±0.10
N2	$N_{90}P_{80}K_{80}$	13.98^{b}±0.33	14.80^{b}±0.14	28.78^{b}±0.42	51.43^{c}±0.56	1.50^{b}±0.18
N3	N_{120}P80K_{80}	14.63^{bc}±0.15	14.00^{c}±0.21	28.63^{b}±0.34	48.91^{d}±0.91	1.90^{d}±0.17
N4	$N_{150}P_{80}K_{80}$	14.95^{cd}±0.13	12.40^{d}±0.29	27.35^{a}±0.30	45.34^{e}±0.61	2.60^{e}±0.12
t-test		*	*	*	*	*
Meadow	0.00	9.26	22.40	31.66	70.75	0.10

*Significant at P<0.05; Hy, hydrolytic acidity; BS, base saturation; S, total exchangeable bases; CEC, cation exchange capacity; Exch., exchangeable; $^{a-e}$different letters within columns mean statistically significant difference at P<0.05(for Tuckey HSD test).

Table 7. Microbiological properties of the *Eutric Cambisol* soil (0-10 cm) as affected by increasing doses of nitrogen fertiliser applied over 50 years (1963-2014).

Treatment	Fertiliser (kg ha^{-1}) ×10^{4} CFU	Total number of microflora	Actinomycetes ×10^{4} CFU	Fungi ×10^{4} CFU	Ammonifiers ×10^{5} MPN	Azotobacter MPN	Oligonitrophiles ×10^{5} CFU
Autumn 2013							
Control	0.00	561.75±25.50	0.58±0.11^{a}	13.67±1.334^{a}	42.00±2.00^{a}	35.00±10.00^{a}	24.90±2.72^{ac}
N1	$N_{60}P_{80}K_{80}$	1124.33±34.66	2.14±0.03^{b}	24.00±4.00^{b}	25.00±3.00^{b}	95.00±5.00^{b}	36.67±2.41^{b}
N2	N_{90}P8$_{80}K_{80}$	1127.97±58.60^{bc}	2.42±0.07^{ce}	32.67±3.67^{c}	12.00±2.00^{dc}	92.00±3.00^{b}	30.09±2.97^{bc}
N3	$N_{120}P_{80}K_{80}$	1375.20±11.20^{c}	2.25±0.10^{be}	34.33±3.00^{c}	15.00±1.00^{c}	75.00±20.00^{c}	24.25±2.50^{ac}
N4	$N_{150}P_{80}K_{80}$	1268.50±63.00^{bc}	1.50±0.05^{d}	38.33±2.33^{c}	10.00±2.00^{d}	56.00±2.00^{b}	18.83±1.67^{a}
t-test		***	***	***	***	***	***
Meadow	0.00	1609.67	3.33	13.33	35	25	26.33
Spring 2014							
Control	0.00	479.50±29.50^{a}	0.57±0.09^{a}	13.33±2.00^{a}	40.00±3.00^{a}	27.00±10.00^{a}	22.10±2.72^{ac}
N1	$N_{60}P_{80}K_{80}$	892.33±36.33^{b}	1.58±0.2^{b}	22.67±2.33^{b}	25.00±2.00^{b}	95.0±6.00^{b}	33.07±2.41^{b}
N2	N_{90}P8$_{80}K_{80}$	1066.25±73.96^{c}	1.98±0.15^{c}	28.67±1.67^{c}	10.00±1.00^{cd}	87.00±3.00^{b}	26.19±2.97^{a}
N3	$N_{120}P_{80}K_{80}$	1275.2±80.2^{c}	1.73±0.12^{c}	28.00±1.67^{c}	15.00±2.00^{c}	65.00±10.00^{c}	21.35±2.50^{ac}
N4	$N_{150}P_{80}K_{80}$	1210.50±83.5^{c}	1.41±0.15^{b}	33.00±2.33^{d}	9.00±2.00^{d}	55.00±1.00^{db}	16.25±1.25^{c}
t-test		***	***	***	***	***	***
Meadow	0.00	1410	4.3	32.67	27	45	31

One-way ANOVA shows mean values ±SD for individual treatment; $^{a-d}$values followed by the same letter in a column are not significantly different (Tukey HSD, P<0.05); CFU, colony-forming units; MPN, most probable number; CFU g^{-1} of dry soil or MPN g^{-1} of dry soil.

tilised treatments positively affected the total number of the following groups: microflora, actinomycetes, fungi, and *Azotobacter* compared to the control. The number of ammonifiers and oligonitrophiles increased at the lowest N (N1 treatment) compared to the control but decreased with an increasing dose of applied N (N2, N3, and N4 treatments). In addition, *Azotobacter* showed an increase in number in all fertilised treatments compared to the control. The most significant increase in the total number of microflora was in the treatment N3, while fungi and actinomycetes were most abundant in the treatment N4 and N2 in both periods (Table 7).

Quantity and diversity of soil microbiological communities greatly depend on soil pH, availability and nature of organic substrate, land use, and soil type (Zhou *et al.,* 2020; Craig *et al*., 2021). In our study, a decrease in the number of all tested microorganisms, except for fungi and oligonitrophiles, was observed in the treatment receiving the highest dose of N (150 kg ha^{-1}) with the most significant decrease in soil pH (Table 7). Stanojkovic-Sebic *et al*. (2012) investigated the effects of different amounts of NPK fertilisers on the total number of fungi, *Azotobacter*, ammonifiers, and oligonitrofilrs in Cambisol. In her study, the amount of fertiliser used stimulated the growth of fungi, and there were no significant differences between N doses in the range of 90 to 150 kg ha^{-1} and the number of fungi. A high dose of added NPK inhibited the growth of *Azotobacter*. The same trends of change in the number of these two groups of microorganisms were confirmed in our study. Most oligonitrophic bacteria can fix N_2, and their presence in the N-poor soils is more significant than in other soils. In our study, the use of high doses of N inhibited the growth of oligonitrophiles and ammonifiers in the *Eutric Cambisol*. Similar results were obtained in other studies using different amounts of NPK fertilisers (Pešaković *et al*., 2006; Stanojković-Sebić *et al*., 2012).

Conclusions

Based on the study of the effect of long-term application of increasing doses of nitrogen fertiliser on changing the properties of *Eutric Cambisol*, the following conclusions can be drawn:

- Over a 50-year period, mineral fertilisation resulted in strong acidification, and at higher doses, a loss of base saturation (<50%) accompanied by an increase in clay content, hydrolytic acidity, Fe, Mn, and exchangeable Al consistent with increasing doses of N.
- In all treatments, including control, the content of organic carbon was lower than the baseline.
- The observed changes in soil's physical and chemical properties resulted in the original *Eutric Cambisol* soil transforming into *Dystric Cambisol* soil.
- The increase in the total number of microflora with an increase in the dose of applied mineral N correlated with an increase in the amount of fungi in the soil. Long-term application of mineral fertilisers negatively affected the total number of microflora, actinomycetes, ammonifiers, and ologonitrophiles compared with the natural meadow soil.
- Although the addition of high doses of mineral N contributed to higher crop yields, it could not prevent progressive degradation of soil quality parameters.

This and future field experiments should be used to calibrate agro-ecosystem models. In addition, our data show that causal chains between microbiological and chemical parameters need to be researched more thoroughly and better understood.

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