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Changes in Soil Labile Organic Matter as Affected by 50 Years of Fertilization with Increasing Amounts of Nitrogen

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Abstract: Microbially mediated soil organic matter is an extremely sensitive pool that indicates subtle changes in the quality parameters responsible for the soil's ecological and productive functions. Fifty years of mineral fertilization of a wheat-corn cropping system has a strong impact on soil quality parameters. The goal of the research was to study the dynamics and quality of soil biological parameters affected by increasing amounts of mineral nitrogen. Soil respiration, potentially mineralizable C and N, microbial biomass C and N and light-fraction OM on Cambisol were analyzed in the following treatments: (1) Control (without fertilization); (2) NPK (60/51/67); (3) NPK (90/51/67); (4) NPK (120/51/67); (5) NPK (150/51/67 kg ha^{-1}). The parameters studied were significantly affected by the long-term application of mineral fertilizer compared with both the control and the adjacent native soil. The highest amounts of nitrogen (N150) did not significantly differ from N120 and N90 for most of the parameters studied. Potentially mineralizable C represented the largest labile carbon pool, while microbial biomass N was the largest labile nitrogen pool. The mineralization rates for C and N were oppositely distributed over the seasons. The sensitivity index correlated with the amount of light-fraction OM. The results give a deeper insight into the behavior and distribution of different pools of labile SOM in the agro-landscapes and can serve as a reliable basis for further research focused on zero soil degradation.

Keywords: fertilization; *eutric cambisol*; microbial biomass C and N; light-fraction OM; potentially mineralizable C and N; sensitivity index

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1. Introduction

Anthropogenic impacts, including the intensive and long-term addition of mineral nitrogen, often adversely affect soil properties and ultimately the adequate ecological functioning of the soil and its sustainability. Huang et al. [1] showed that long-term fertilization altered the microbial community but failed to restore stocks of soil organic carbon (SOC) to the level of the natural meadow soils of the Tibetan Plateau. The key challenge is to meet crop nutrient requirements while minimizing nutrient losses to maintain a sustainable environment and economic benefits for farmers [2,3] at the same time as maintaining zero loss of soil fertility and carbon sequestration.

Microorganisms are the most sensitive part of the soil, influencing the ecological stability and biological productivity of cropland and grassland ecosystems [4,5] by participating in the biochemical transformation of mineral fertilizers and the synthesis of

biologically active substances and nitrogen fixation [6,7]. Microbial communities such as fungi and bacteria may not necessarily be limited by the same elements that limit the plant community. Soil microorganisms can be limited by carbon or phosphorus, while net primary production in terrestrial ecosystems is generally limited to nitrogen availability. Excess N may inhibit soil microorganisms' activity, indicating that microbes are not always nitrogen restricted [8,9].

Aboveground biomass production typically increases after nitrogen fertilization, while plant residues returning to the soil can increase the carbon source for soil microorganisms. On the other hand, the indirect effects of long-term N fertilization can cause significant changes in C availability and a dramatic loss of organic C [10].

It is well known that indicators of microbial activity such as respiration, microbial biomass C and N (MBC and MBN), and metabolic quotient qCO_2, as well as light-fraction OM, potentially mineralizable C and N (PMC and PMN) and their C/N ratios are sensitive indicators to detect subtle changes in soil fertility parameters [11] compared with soil organic C [12]. Woolf and Lehmann [13] showed that the microbially mediated organic carbon changes are consistent with the global distribution of SOC. The microbial metabolic quotient qCO_2, as an indicator of microbial carbon utilization efficiency, represents the changes in both biotic factors (TOC, TN, C/N ratio) and abiotic factors (pH, moisture, temperature) [14,15]. The changes in MBC/TOC and MBC/MBN ratios might show a shift in relative dominance between bacterial and fungal communities, and a higher MBC/MBN ratio could indicate greater fungal community dominance [14]. However, the nature of the changes due to the long-term addition of mineral fertilizer can take different directions and vary in intensity, with the above-mentioned parameters and indicators undergoing similar, different or opposite changes. The objective of this study was to reveal the effect that 50 years of different amounts of nitrogen fertilizer have had on biological indices of Cambisol with a bipolar crop rotation (wheat/corn) in two seasons before and after harvest. The main goal was to reveal whether and what amounts of nitrogen adversely change the biological properties of microbially mediated soil.

2. Materials and Methods

2.1. Study Site and Experimental Design

The *Eutric Cambisol* studied [16] is the most representative soil in agricultural landscapes in central Serbia (about 650,000 ha). Different combinations and amounts of mineral fertilizer were set up in a multi-variable field experiment in 1963 at the Institute of Soil Science's "Mladenovac" experimental station, located 55 km from Belgrade (44°24′58″ N and 20°10′34″ E) in Serbia (Figure 1). The elevation is 161 m above sea level with a mean annual precipitation and temperature of about 700 mm and 11 °C, respectively.

Calcium ammonium nitrate (CAN), monocalcium phosphate (superphosphate fertilizer, $Ca(H_2PO_4)_2$ and potassium chloride (KCl) were applied in the period from 1963 to 1973. Since then, urea, monoammonium phosphate fertilizer (MAP) and KCl have been used. The cultivated crops were winter wheat (*Triticum aestivum* L.) and corn (*Zea maize* L.). Selected experimental treatments and control plots were arranged in a randomized block design in four replications (a single plot size was 6 × 10 m). The wheat was harvested in July 2013, followed by soil sampling that August. In September, the soil was then tilled by conventional ploughing to a depth of 20–25 cm. In March 2014, the soil was fertilized and prepared for sowing corn in April. In addition, a composite soil sample was taken from the adjacent natural meadow to establish the magnitude of any changes due to the long-term application of mineral fertilizers.

The following treatments were selected for this study in autumn 2013 and spring 2014:

1. Control (without fertilization)
2. N60/P51/K67 kg ha^{-1}
3. N90/P51/K67 kg ha^{-1}
4. N120/P51/K67 kg ha^{-1}
5. N150/P51/K67 kg ha^{-1}

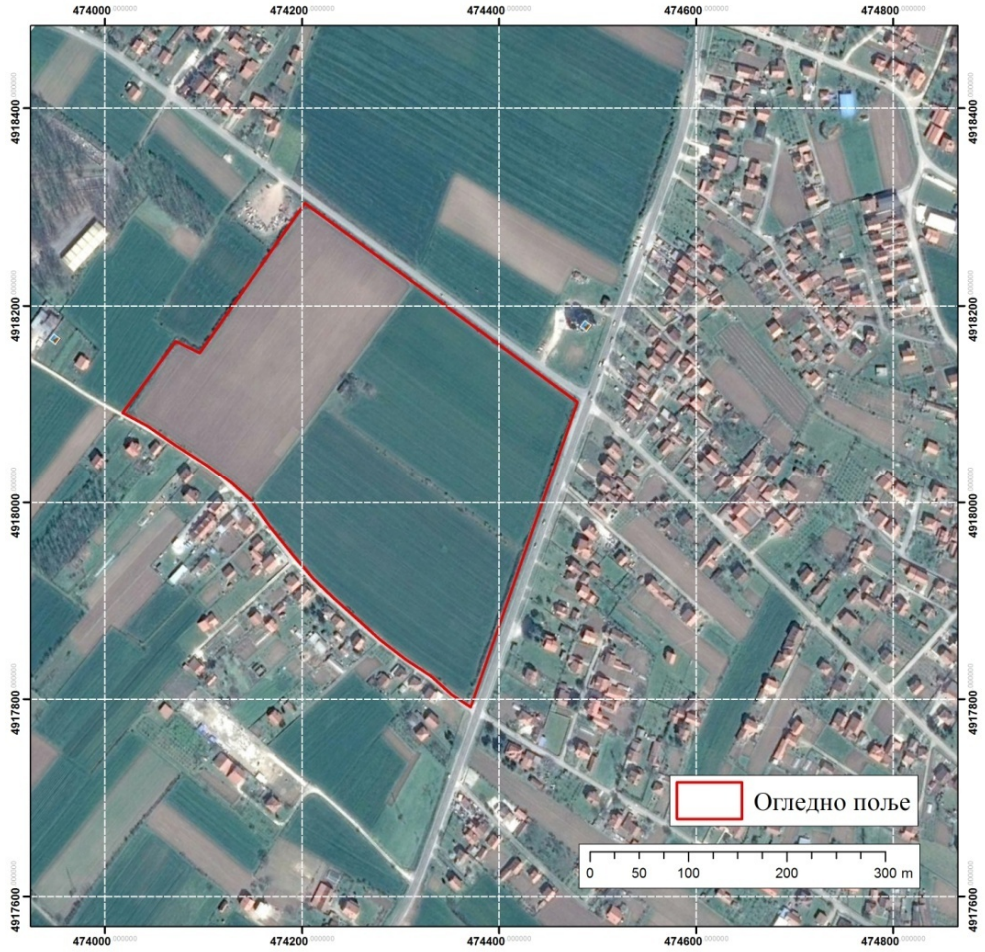

Figure 1. "Mladenovac" experimental field (in the red square) since 1963 (Institute of Soil Science, Serbia).

2.2. Soil Sampling and Analytical Methods

Soil Sampling

Soil samples were taken twice (spring and autumn) from the surface horizons at 0–10 cm for biological and microbiological analysis using the scattered sampling method. Five composite subsamples were taken from each replicate.

2.3. Analytical Methods

The soil total carbon (TC) and total nitrogen (TN) were measured on an elemental CNS analyzer (Vario model EL III -ELEMENTAL Analysis systems GmbH, Hanau, Germany) by the dry combustion of samples at 1150 °C [17]. The content of inorganic carbon ($CaCO_3$) was determined volumetrically with a Scheibler calcimeter, whereas the organic carbon (OC) was calculated by subtracting the mineral carbon from the total amount of carbon.

Labile Fractions of SOM

Soil respiration was measured as CO_2 evolved due to organic C mineralization under controlled laboratory conditions (temperature 28 °C and soil moisture 50% WHC) at the following intervals: 3, 9, 16, 30, 44, 62, and 83 days for each treatment in four replications. The carbon dioxide released was trapped by 0.2 mol L^{-1} NaOH, and the amount of residual free NaOH was determined by titration with 0.02 mol L^{-1} HCl. The amount of carbon dioxide released for each incubation period was calculated from the difference between the amount of NaOH taken for carbon dioxide fixation and that determined by titration with HCl.

The potentially mineralized carbon was calculated from the cumulative data on the sequential respiration during 83 days of incubation (for autumn 2013 and spring 2014), using Equation (1) of the first-order kinetic model:

$$C\mathit{min} = C_0(1 - \mathit{exp}\,(-\mathit{kt})) \quad (1)$$

where C*min* is the experimentally obtained value of mineralized C (mg kg^{-1}) during *t* (days), C_0 is the potentially mineralizable carbon (PMC) (mg kg^{-1}), and *k* is the nonlinear mineralization constant, i.e., the rate of mineralization (the amount that is mineralized per day) (d^{-1}) (SPSS Inc., Sigma Plot 2011). The microbial metabolic quotient (qCO_2) was calculated by dividing the soil respiration with the MBC [18].

Potentially mineralizable N (PMN). The amount of mineralized nitrogen was determined by aerobic incubation at an optimal temperature of 28 °C and a soil moisture of 50% WHC [17], followed by sequential measurement of the amount of mineralized N for each treatment in four replications. The amount of mineralized N was analyzed at intervals of 3, 9, 16, 30, 44, 62 and 83 days for each treatment in four replications. Mineral nitrogen was extracted with 0.5 M of K_2SO_4 at a ratio of 5:1 [19] and determined using the Kjeldahl distillation method.

The amount of potentially mineralizable nitrogen (PMN) was obtained after fitting the data to the first-order kinetic model using Sigma Plot software [20]. An initial amount of immobilized mineralized nitrogen was added to Equation (2) as a variable (Sigma Plot 2011):

$$N_{min} = N_{imb} + N_0(1 - \mathit{exp}(-\mathit{kt})) \quad (2)$$

where N_{min} is the experimentally obtained value of mineralized N (mg kg) during *t* (days), N_0 is the potentially mineralizable nitrogen (PMN) (mg kg^{-1}), *k* is the nonlinear mineralization constant, i.e., the rate of mineralization (amount of nitrogen mineralized per day), and N_{imb} is an amount of immobilized nitrogen.

Microbial biomass carbon and nitrogen (MBC and MBN). The microbial biomass carbon and nitrogen were determined using the fumigation-incubation method [19]. Soil samples were taken from each field treatment, in 4 replicates. In a laboratory, the soil moisture content was set to 50% WHC, followed by the fumigation of 17 g of soil with chloroform in a vacuum desiccator for 17 h. After defumigation, 3 g of fresh soil sample from the same treatment was added as an inoculant. Simultaneously, 20 g of non-fumigated soil was set as a control for a further 5-day incubation under controlled moisture and temperature conditions (28 °C) together with 0.2 mol L^{-1} NaOH. Microbial carbon was determined using the same alkali trap method followed by titration as described for soil respiration. Microbial biomass nitrogen (MBN) was extracted with 0.5 M of K_2SO_4 at a ratio of 5:1 [19] followed by determination using the Kjeldahl digestion method. From the difference between fumigated and non-fumigated soil, the amount of nitrogen and carbon in the living part of the organic matter of the soil was calculated as biomass $C = (C_f - C_{uf})/K_{ec}$ and biomass $N = (N_f - N_{uf})/K_{ec}$ [19].

Light fraction of organic matter. The light fraction of organic matter (LFOM) is organic residues with a recognizable cellular structure. The light fraction can originate from a variety of sources, but is usually dominated by pieces of plant debris and serves as an easily accessible source of energy and nutrients for soil organisms. The LFOM was isolated using the densitometry method with a NaI solution after adjusting its density to 1.8 g cm^{-3} [21]. Ten grams of air-dry soil were suspended in a 40-mL sodium iodide (NaI) solution. After centrifugation, the suspended material, or light fraction (LF), was transferred directly to the filtration unit. The LF was then washed three times with 10 mL $CaCl_2$, and three times with distilled water, followed by drying at 70 °C for 15 h, after which it was weighed. The residue was re-suspended, and the procedure was repeated to determine whether all the LF had been retrieved. The LF composite sample was finely ground and analyzed on a CNS analyzer for its C and N content (LFC and LFN, respectively).

2.4. Statistics

The results obtained were statistically processed using one-way ANOVA (SPSS version 16 software). Significant differences were assessed using the *t*-test (95%) proposed by Pearson to gain Fisher's LSD. The mineralization rates and the amount of potentially mineralizable C and N were obtained by fitting the data of mineralized C and N into a non-linear dynamic model (SYSTAT SigmaPlot 14 software). The Sensitivity Index (SI) of labile C and N was calculated as in Equation (3):

$$SI = (A - B)/(B*100) \quad (3)$$

where A is a C or N fraction in treatment and B is a C or N fraction in the control [22].

3. Results

3.1. Organic Carbon and Total Nitrogen Content

Soils from the fertilized treatments maintained a significantly lower content of organic carbon compared to the initial OC determined in 1963 (Table 1). Among the fertilized treatments, a statistically significant difference was observed only between N60 and other treatments. However, there was no significant difference in the OC content between the N90, N120 and N150 treatments. The total N content increased in line with the applied amounts of N, although the changes between fertilized treatments were insignificant (Table 1). Only the control treatment showed a statistically significant difference with each of the fertilized treatments ($p < 0.01$).

Table 1. Content of organic carbon, total nitrogen depending on the N added and compared with the initial state, and the aboveground wheat biomass in 2013.

Treatment	NPK Fertilizer	OC	TN	pH (KCl)	Clay, % <0.002	CEC, (cmol kg^{-1})	Wheat Grain Yield, 2013
	kg ha^{-1}	%					
Initial state, 1963	0	1.51	0.120	5.20	NA	24.00	
control	$N_{60}P_{51}K_{67}$	0.92b ± 0.02	0.104b ± 0.01	4.56a ± 0.02	30.98a ± 0.10	27.38a ± 0.61	3192.6a
N60	$N_{90}P_{51}K_{67}$	0.98b ± 0.02	0.119c ± 0.00	4.08b ± 0.06	31.08b ± 0.59	26.90a ± 0.58	4168.3b
N90	$N_{120}P_{51}K_{67}$	1.08c ± 0.03	0.125c ± 0.01	3.91c ± 0.08	31.25c ± 0.58	28.78b ± 0.42	5011.3c
N120	$N_{150}P_{51}K_{67}$	1.13c ± 0.03	0.126c ± 0.00	3.74d ± 0.05	31.70c ± 0.21	28.63b ± 0.34	5979.2d
N150	0	1.14c ± 0.02	0.127c ± 0.01	3.63d ± 0.07	31.95c ± 0.08	27.35a ± 0.30	6065.1d
t-test		**	**	**	**	**	

Note: ** Significantly different at $p < 0.01$; values followed by the same letter in a column are not significantly different; OC—organic carbon; TN—total nitrogen; NA—not available.

3.2. Grain and Biomass Yield of Wheat (2013)

The grain yield of wheat ("Euclide" variety) increased gradually from the control to the N150 treatment in line with the amounts of N added (Table 1). All differences were significant except between N120 and N150. If we consider the yield of wheat from the control treatment as 100%, then the increase in wheat yield was 30.56%, 56.97%, 87.29% and 89.98% for the N60, N90, N120 and N150 treatments, respectively.

3.3. Soil Mineral Nitrogen (N_{min})

The initial concentrations of mineral nitrogen are presented in Figure 2. In the fertilized treatments, the N_{min} concentrations gradually increased from the control to the N150 treatment. It is characteristic that the amount of N_{min} was higher in the autumn than in spring soil in fertilized plots. In addition, the differences were more pronounced with an increase in the amounts of added nitrogen.

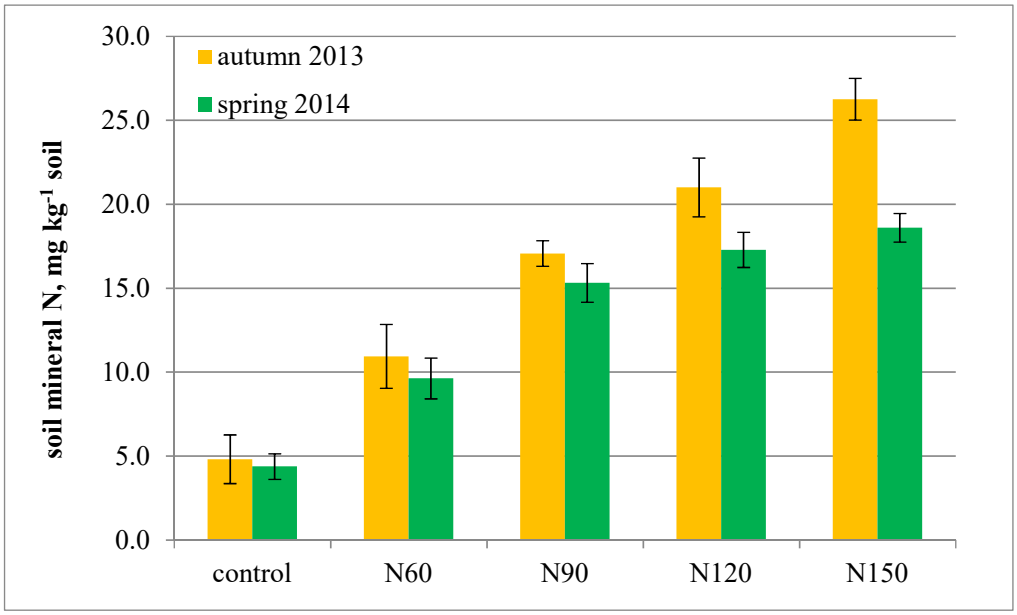

Figure 2. Soil mineral nitrogen in 0–10 cm in autumn 2013 and spring 2014 under increasing amounts of N fertilizer. N60—60 kg; N90—90 kg, N120—120 kg and N150—150 kg N ha^{-1}.

3.4. Microbial Biomass, Microbial Carbon and Nitrogen (MBC and MBN)

The data on the carbon and nitrogen of the biomass (MBC and MBN) are presented in Table 2.

Table 2. Microbial biomass C and N (MBC and MBN) in 0–10 cm of Cambisol under increasing amounts of N fertilizer in autumn 2013 and spring 2014.

Treatment	NPK Fertilizer	MBC	MBN	C/N	As a Part of SOM (%)	
		mg kg^{-1} Soil			As MBC	As MBN
			Autumn 2013			
control	0	188.31a	100.43a	1.9	1.75	8.51
N60	$N_{60}P_{51}K_{67}$	228.66b	109.85a	2.1	1.84	8.52
N90	$N_{90}P_{51}K_{67}$	295.91c	144.37b	2.0	2.29	10.46
N120	$N_{120}P_{51}K_{67}$	342.99d	150.65b	2.3	2.66	10.92
N150	$N_{150}P_{51}K_{67}$	352.14d	164.33b	2.1	2.69	11.03
			Spring 2014			
control	0	141.36g	59.27e	2.4	1.31	5.02
N60	$N_{60}P_{51}K_{67}$	141.72g	71.29f	2.0	1.14	5.53
N90	$N_{90}P_{51}K_{67}$	148.26g	84.34f	1.8	1.15	6.11
N120	$N_{120}P_{51}K_{67}$	154.32g	87.88f	1.8	1.20	6.37
N150	$N_{150}P_{51}K_{67}$	154.48g	96.12f	1.6	1.18	6.45
t-test		***	***			
			Autumn			
Meadow	0	926.19	689.57	1.3	2.89	23.30
			Spring			
Meadow	0	1442.70	353.04	4.1	4.51	11.93

Note: Values followed by the same letter in a column are not significantly different; *** Significant at $p < 0.001$.

Both parameters increased with an increase in the amount of fertilizer nitrogen added. The MBC and MBN showed different trends depending on the sampling season, with autumn soil showing significantly higher values than spring soil.

The MBC/MBN ratio in the autumn samples was generally evenly distributed and was around two, while the MBC/MBN ratio decreased in the spring samples, with an

increase in the amount of nitrogen added with the fertilizer. In both seasons, in the N120 and N150 treatments, the MBC and MBN were not statistically different. In the spring samples, MBC did not differ between treatments, including the control. For MBN, only the control was significantly different from the fertilized treatments. Only the autumn MBC showed a statistically significant difference between treatments.

The adjacent native meadow soils with identical pedology and topography was also analyzed in order to establish the magnitude of the changes in the soil due to 50 years of fertilization. Comparison of the absolute values of native soil and mean values from the fertilized soils showed that in autumn, the amount of MBC in the meadow soil exceeded N150 and the control treatments by 2.63 and 4.92 times, respectively. In the spring, this excess even doubled, from 9.34 to 10.21 times more than in N150 and the control treatments, respectively. The MBN values in meadow soils were 4.20–6.87 and 3.67–5.96 times higher in autumn and spring soils, respectively.

3.5. Labile Fractions of SOM

3.5.1. Potentially Mineralizable Carbon (PMC)

Fertilization resulted in a significant increase in PMC compared to the control (Table 3). Between the fertilized plots, PMC values increased in line with the addition of nitrogen. The highest PMC was in the N150 treatment in both sampling periods. Similar to the microbial biomass C, the N120 and N150 treatments also did not differ in the amount of potentially mineralizable C in the autumn. Differences in the amount of accumulated labile C (PMC) between the autumn and spring samples ranged from 1.1 to 1.3 times for each pair of treatments. The rate constant of carbon mineralization in autumn was significantly higher than in the spring samples (Table 3). Comparison of the absolute values of native soil and mean values from the fertilized soils showed that the meadow accumulated labile carbon 7 and 5.1 times more compared to the control and N150, for the autumn and spring samples, respectively. The microbial metabolic quotient (qCO_2) varied significantly and was higher in the spring than in autumn. The PMC-to-PMN ratio corresponded to the qCO_2 values.

3.5.2. Potentially Mineralizable Nitrogen (PMN)

The amounts of mineral N applied showed a great impact on the potentially mineralizable N (Table 3). As expected, the autumn samples accumulated significantly more labile organic N, which was 1.73, 1.85, 2.15, 2.36 and 2.18 times higher than in the spring samples for the control, N60, N90, N120 and N150 treatments, respectively.

The rate constant of mineralization showed that in the autumn, the soil OC mineralized at a higher speed than in the spring. The opposite trend was observed for nitrogen, where the rate of mineralization in spring was much higher than in autumn (Table 3).

Fitting the data on N mineralization to the first-order kinetic model showed that active immobilization of mineralized N (N_{imb}) occurred during the incubation (Table 3). For both seasons, the amount of immobilized N was linear with the amount of nitrogen added with fertilizer.

Comparison of absolute values of native soil and mean values from the fertilized soils in autumn showed that the amount of PMN in the meadow soil was 2.22 to 3.79 times higher than in N150 and the control treatment, respectively. A similar trend was observed for the spring samples, where the meadow soil accumulated 2.29–3.11 times more PMN than the fertilized soils.

Table 3. Potentially mineralizable C and N in 0–10 cm Cambisol under increasing amounts of N fertilizer in autumn 2013 and spring 2014.

Treatment	NPK Fertilizer	Respiration	PMC	Mineralization Rate Constant *k*	PMN	Mineralization Rate Constant *k*	N_{imb}	qCO_2	PMC/PMN	As a Part of SOM (%)	
	kg ha^{-1}	mg CO_2-C kg^{-1} Soil Day^{-1}	mg kg^{-1}		mg kg^{-1}		mg kg^{-1}			As PMC	As PMN
Autumn 2013											
control	0	9.725	913.91a	0.0100	51.40a	0.0057	6.63	3.664a	17.78	8.47	4.36
N60	$N_{60}P_{51}K_{67}$	10.727	1287.95b	0.0750	56.00a	0.0084	12.4	4.562b	23.00	10.34	4.34
N90	$N_{90}P_{51}K_{67}$	10.692	1893.50c	0.0050	69.40b	0.0082	19.5	5.694c	27.28	14.68	5.03
N120	$N_{120}P_{51}K_{67}$	11.979	1840.94c	0.0054	87.00c	0.0072	23.5	4.743b	21.16	14.27	6.30
N150	$N_{150}P_{51}K_{67}$	12.350	2054.64c	0.0052	88.20c	0.0249	23.7	5.331c	23.30	15.72	5.92
Spring 2014											
control	0	14.281	689.92f	0.0700	29.70f	0.0224	7.00	6.465d	23.23	6.39	2.52
N60	$N_{60}P_{51}K_{67}$	19.041	1043.20g	0.0048	30.30g	0.0396	8.5	9.088f	34.43	8.37	2.35
N90	$N_{90}P_{51}K_{67}$	19.589	1684.80h	0.0030	32.30g	0.0410	11.8	12.771g	52.16	13.06	2.34
N120	$N_{120}P_{51}K_{67}$	19.180	1626.63h	0.0032	36.90gh	0.0356	15.7	11.929g	44.08	12.61	2.67
N150	$N_{150}P_{51}K_{67}$	19.820	1877.45c	0.0030	40.40h	0.0303	18.3	13.30gh	46.47	14.36	2.71
t-test			***	***	**	***	***	**			
			$p < 0.001$	$p < 0.0001$	$p < 0.05$	$p < 0.0001$	$p < 0.0001$	$p < 0.05$			
Autumn											
Meadow	0	30.779	6942.22	0.0037	195.00	0.0055	10.48	6.223	35.60	21.69	6.59
Spring											
Meadow	0	27.433	5763.89	0.0024	92.50	0.0083	16.89	4.812	62.31	18.01	3.13

Note: Values followed by the same letter in a column are not significantly different (*t*-test, $p < 0.05$); *** significant at $p < 0.001$; for the first-order kinetic fitting $p < 0.0001$; ** significant at $p < 0.05$; qCO_2—metabolic quotient; N_{imb}—nitrogen immobilized.

3.5.3. Light-Fraction OM

The data on the light-fraction organic matter as dry matter (LFDM), LF carbon and LF nitrogen are shown in Table 4. All the LF parameters studied, including the proportion of LFDM, LFC and LFN in total OC, showed higher values in autumn than in spring for each pair of treatments, although the difference between some fertilized treatments was not statistically significant, as indicated by the same letters. A significant difference was observed only between the control and all other treatments.

Table 4. Light-fraction OM, C and N (LFC and LFN) in 0–10 cm Cambisol under increasing amounts of N fertilizer in autumn 2013 and spring 2014.

Treatment	NPK Fertilizer	LF, Dry Matter	LFC	LFN	C/N	% LF in A Total SOC	As A Part of SOM (%)	
	kg/ha	g/kg	mg/kg				LFC	LFN
			Autumn 2013					
control	0	1.606a	332.44a	17.39a	19	14.88	3.08	1.47
N60	$N_{60}P_{51}K_{67}$	2.79b	589.55b	30.77b	19	22.39	4.73	2.39
N90	$N_{90}P_{51}K_{67}$	2.93 b	590.69b	35.13b	17	22.71	4.58	2.55
N120	$N_{120}P_{51}K_{67}$	3.356c	650.16c	40.47c	16	26.02	5.04	2.93
N150	$N_{150}P_{51}K_{67}$	3.428c	680.49c	42.20c	16	26.23	6.73	3.66
			Spring 2014					
control	0	1.47a	303.48a	15.38a	20	13.62	2.81	1.30
N60	$N_{60}P_{51}K_{67}$	2.45b	496.20b	25.73ba	19	19.66	3.98	1.99
N90	$N_{90}P_{51}K_{67}$	2.51b	490.03b	26.66ba	18	19.46	3.80	1.93
N120	$N_{120}P_{51}K_{67}$	3.01bc	543.94b	32.69b	17	23.33	4.22	2.37
N150	$N_{150}P_{51}K_{67}$	3.02bc	585.85b	34.94b	17	23.11	4.48	2.35
t-test		***	***	***				
			Autumn					
Meadow	0	6.95	1692.88	98.52	17	21.71	5.29	3.33
			Spring					
Meadow	0	5.10	1266.3	65.89	19	15.93	3.96	2.23

Note: Values followed by the same letter in a column are not significantly different (Duncan test, $p < 0.05$); *** significant at $p < 0.001$.

4. Discussion

4.1. Organic Carbon, Total and Mineral Nitrogen

Many authors note that intensive cultivation, inadequate fertilization and poor land management are the main factors for the loss of OC [23–27], which causes the destruction of structural aggregates, increases aeration and promotes more intensive decomposition of organic matter.

In our study, the greatest loss of organic C observed in the control was associated with the lowest yield in the unfertilized plot (control), which produced the lowest post-harvest residues that failed to replenish SOM [28]. Nitrogen fertilization had less effect on total soil nitrogen than on organic carbon, but expectations were confirmed that the amount of soil nitrogen would increase linearly with amounts applied over a longer period [29]. Although higher amounts of N maintained the replenishment of a certain amount of organic carbon and total nitrogen sourced from crop residues [30], results from our long-term field experiment showed that mineral fertilization alone was not sufficient to maintain the baseline soil organic carbon, regardless of the amount applied.

Karbozova-Saljnikov et al. [20] found that nitrogen mineralization is more sensitive to recent inputs of post-harvest wheat residues, while carbon mineralization better reflects long-term inputs of post-harvest residue. This is strongly supported by the higher amounts of LF dry matter, LFC and LFN in autumn than in spring, which are directly influenced by wheat biomass and thus the organic substrate returned to the soil. Typically, in spring, higher moisture contributes to better SOM mineralization, suggesting that there is a greater accumulation of mineral N. Although nutrients are harvested from the soil in the autumn,

the results of this study indicate that a recent supply of fresh organic residues provided a sufficient organic substrate for microbial activity and the accumulation of mineral nitrogen in the soil, but not enough to maintain effective humification, i.e., carbon sequestration.

4.2. Microbial Biomass Carbon and Nitrogen (MBC and MBN)

Microbial biomass stores plant nutrients as biologically active compounds that are easily available for crops [20]. The autumn samples showed greater variability in MBC values between treatments than spring samples. The proportion of MBC in SOC ranged from 1.75% to 2.89% and 1.18% to 4.51% in the autumn and spring, respectively (Table 2) and was similar to the figures reported by [18] where the percentage of MBC in SOC amounted from 2.3 to 2.9 in various cropping systems. Microbial C values were within the range reported by [31], who found a range of MBC values from 245 to 3720 mg kg^{-1} in the 240 soil cores studied. The portion of MBN in SOM was significantly higher than MBC and ranged from 8.51% to 24.52% in autumn and from 5.02% to 11.93% in spring. This is probably associated with the fact that during the fumigation-incubation procedure, soil mineral N was immobilized by microorganisms, and an increase in microbial biomass resulted in a greater immobilization. In addition, an increased proportion of microbial biomass with the increasing amounts of N added can be associated with an increased biomass of fungi [32]. Prolonged application of nitrogen fertilizers negatively affected the total amount of microflora, actinomycetes, ammonifiers and oligonitrophiles, associated with negative changes in the soil physical and chemical properties, particularly with the largest decrease in soil pH in the N150 treatment [33,34]. Although the addition of high amounts of N inhibited the growth of oligonitrophiles and ammonifiers in the *Cambisol* studied, the total microbial population increased due to the increased population of bacteria. This meant that the MBC/MBN ratio was much narrower than usual (for bacteria it is around 5:1, and for fungi it is around 10:1 [35]). Generally, the greater amount of MBC and MBN in the autumn samples is associated with a greater number of microorganisms in this sampling season, an abundance of organic substrate and favorable moisture and temperature levels, which promoted the growth of microbial biomass compared with the spring samples [36].

Although statistical analysis could not be applied for the soils from native meadow, based on the absolute values we propose that the higher microbial biomass in the meadow samples was associated with a constant influx of fresh organic matter; filamentous fungi in particular are responsible for the decomposition of lignocellulosic materials—fallen leaves, branches and grass [36]. The slower decomposition of organic matter in the meadow soil was associated with the absence of agrotechnical disturbances contributing to the accumulation and stabilization of organic matter, which promoted C sequestration.

4.3. Soil Respiration, Potentially Mineralizable Carbon and Nitrogen (PMC and PMN) and Light-Fraction OM (LFDM, LFC and LFN)

Earlier studies on wheat-based cropping systems showed that carbon mineralization is not directly related to the amount of plant residues from the previous year, but is highly positively correlated with the perennial accumulation of organic substrate [30,37]. Soil respiration indicates the level of microbial activity and can be used to assess the circulation of nutrients in the soil and the ability of the soil to support plant growth. According to the USDA classification (Table 5), soil respiration was more affected by the sampling season than by the amount of organic substrate added as annual crop residues (Table 6).

Table 5. Classification of soils according to their level of respiration, mg CO_2-C kg soil per day (adapted from [38]; modified by Saljnikov E.).

Very Low Soil Activity	Moderately Low Soil Activity	Medium Soil Activity	Ideal Soil Activity	Unusually High Soil Activity
<12	12–20	20–40	40–80	>80

Table 6. Classes of microbial activity in the soils fertilized with increasing amounts of mineral nitrogen.

Treatment	CO_2-C/Day, mg kg^{-1}	Class of Soil Microbial Activity	CO_2-C/Day, mg kg^{-1}	Class of Soil Microbial Activity
	Autumn 2013		Spring 2014	
control	9.72a	Very low	14.28a	Moderately low
N60	10.73a	Very low	19.04b	Moderately low–Medium
N90	10.69a	Very low	19.59b	Moderately low–Medium
N120	11.98ab	Moderately low	19.18b	Moderately low–Medium
N150	12.35b	Moderately low	19.82b	Moderately low–Medium
Meadow	30.78	Medium	31.75	Medium

Values followed by the same letter in a column are not significantly different.

Lower respiration in the autumn compared with the spring samples may be due to a less favorable moisture content and an abundance of fresh straw residues with a wider C/N ratio. In contrast, in the spring, favorable humidity and partially decomposed crop residues with a narrower C/N ratio contributed to the microbial growth and activity. The medium class of respiration in the meadow soil is probably explained by the large number of roots in the upper soil layer [39,40], which consists of a high-molecular-weight organic substrate that is less available for soil biota compared to aboveground biomass.

The increase in PMC and PMN values with increasing amounts of added N can be explained by the fact that the samples taken in the autumn contained significantly more labile fractions of organic matter returned after harvest, which was confirmed by the yield of wheat and amount of light fraction (Tables 1 and 4).

The labile OM consists of amino acids, simple hydrocarbons, fractions of microorganisms and other simple organic components [41] that are rich in nitrogen compounds, primarily root secretions and total microorganisms. Liang et al. [42] found that in ecosystems of the temperate zone, 55% of SOC in soils under cropping are microbial necromass. In addition, higher amounts of fertilizer nitrogen resulted in a higher amount of light fraction accumulated with crop residues, which provided more mineral N released at a higher rate due to the favorable humidity and temperature provided in the laboratory incubation.

The correlation coefficients (Table 7) support the assumption that the labile N is closely related to the fresh organic substrate. PMN correlates more strongly with other parameters of labile and microbial carbon and nitrogen in both seasons than PMC. This is due to the mineralization of N from the light fraction, which changes over time due to the seasonal input of plant residues [23,43]. Namely, from the second half of October, when samples were taken, until early April, when repeated soil samples were taken, the light fraction underwent decomposition, as evidenced by its weight loss.

The LFC/LFN ratio was favorable for soil biota in both seasons, indicating the availability of nutrient and energy sources for growth. The proportion of LF in the total OC was high, ranging from 14.88–26.23% in the autumn, to 13.62–23.33% in the spring, in the fertilization treatments. Our results showed that higher crop yields build up a greater supply of labile organic substrate, which in general creates a greater possibility for carbon sequestration in the soil [44].

The fact that higher amounts of N applied resulted in a greater immobilization of N by soil microorganisms is associated with a higher yield and greater amount of crop residues added to the soil. More intensive immobilization of N in autumn than in spring was due to the priming effect: the addition of fresh wheat straw [45] in autumn resulted in a N-limit environment (the C/N ratio of straw is about 80), therefore soil microorganisms began to actively bind available mineral nitrogen.

Due to the high ability of PMN, MBC, MBN, LFC and LFN to provide nutrients [46], the yield correlated strongly with these parameters in autumn soils, except PMC. However, in spring, the most significant correlation with productivity was only observed for PMC.

This implies that the feedback of labile C more closely reflects the accumulation of organic matter over a longer period.

Table 7. Correlation between the parameters studied in Cambisols under long-term mineral fertilization in autumn 2013 and spring 2014.

	TN	OC	PMC	PMN	LFDM	LFC	LFN	MBC	MBN	Yield
Autumn 2013										
TN	1									
OC	0.996 **	1								
PMC	0.853 *	0.811 *	1							
PMN	0.978 **	0.959 **	0.926 **	1						
LFDM	0.986 **	0.990 **	0.783 *	0.953 **	1					
LFC	0.994 **	0.996 **	0.804 *	0.960 **	0.998 **	1				
LFN	0.994 **	0.992 **	0.831 *	0.974 **	0.997 **	0.998 **	1			
MBC	0.997 **	0.995 **	0.836 *	0.977 **	0.993 **	0.996 **	0.998 **	1		
MBN	0.999 **	0.994 **	0.866 *	0.980 **	0.979 **	0.988 **	0.989 **	0.995 **	1	
Yield	0.939 *	0.887 *	0.948 *	0.978 **	0.948 *	0.903 *	0.975 **	0.996 **	0.964 **	1
Spring 2014										
TN	1									
OC	0.996 **	1								
PMC	0.772 *	0.720	1							
PMN	0.991 **	0.978 **	0.840 *	1						
LFDM	0.964 **	0.975 **	0.614	0.928 **	1					
LFC	0.982 **	0.991 **	0.654	0.952 **	0.995 **	1				
LFN	0.982 **	0.986 **	0.677	0.956 **	0.996 **	0.997 **	1			
MBC	0.958 **	0.938 **	0.896 **	0.982 **	0.851 *	0.889 **	0.890 **	1		
MBN	0.964 **	0.941 **	0.908 **	0.988 **	0.867 *	0.900 **	0.905 **	0.996 **	1	
Yield	0.948 *	0.916 *	0.975 **	0.866	0.947 *	0.910 *	0.953 *	0.946 *	0.985 **	1

**. Correlation is significant at $p < 0.01$; *. Correlation is significant at $p < 0.05$.

4.4. Distribution of the Labile C and N

Figure 3 shows the distribution of labile C and N (MBC, PMC and LFC), where PMC has the largest share of labile OC, followed by LFC and MBC in both seasons. A different pattern was observed for the labile N fractions, where MBN was the largest fraction in both seasons, followed by PMN and LFN.

The largest pool of PMC among the three carbon fractions studied was due to MBC, and LFC fractions are readily available sources of nutrients and energy for soil microbes. It is generally agreed that the labile fractions of SOM mainly consist of plant debris [47], with microbial necromass occupying a significant proportion of SOM/SOC [42,48]. During mineralization in optimal moisture and temperature conditions, light-fraction OM and microbial necromass contributed to the amount of PMC [42]. In the case of labile N, the predominance of microbial N in both seasons can probably be explained by the active immobilization of mineralized N, which occurred during the incubation period, as confirmed by the values of N_{imb} in the dynamic regression models (Table 3). Moreover, LF can be a strong short-term sink for mineral N, incorporating up to 39% of ammonium and 17% of nitrate during the incubation period [49].

4.5. Sensitivity Index of Carbon and Nitrogen Fractions

In fertilized treatments, the sensitivity index (SI) was linear with the amounts of N added with fertilizer (Figure 4), and the lowest SI was observed for the control (no fertilization). In agricultural soil, fresh residues and organomineral complexes are repeatedly mixed. Therefore, due to mechanical breakdown and better aeration, fresh residues mineralize faster [50,51].

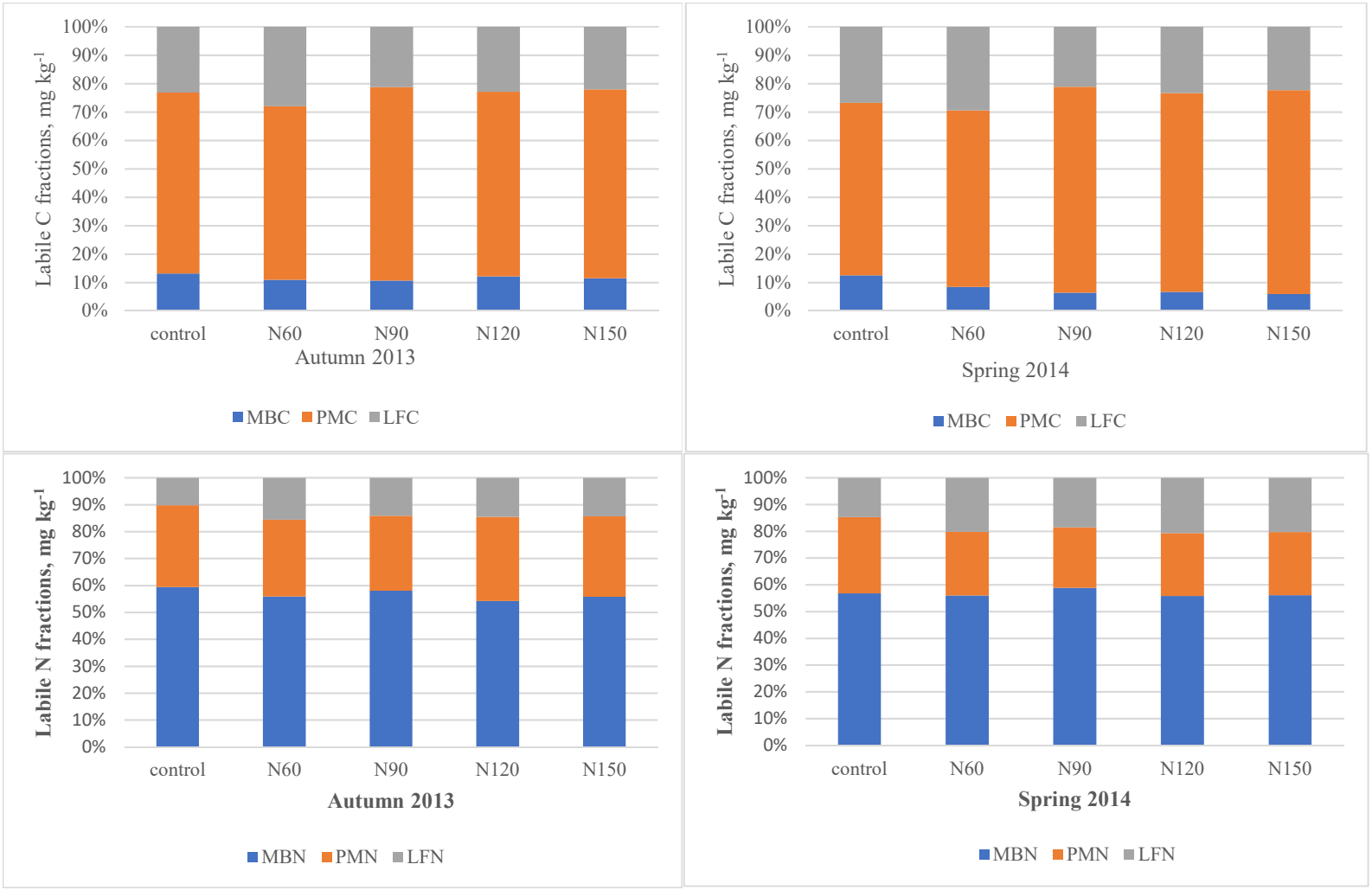

Figure 3. Distribution of labile fractions of carbon (MBC, PMC and LFC) and nitrogen (MBN, PMN and LFN) in the soil fertilized with increasing amounts of N, in Cambisol. MBC—microbial biomass carbon; MBN—microbial biomass nitrogen; PMC—potentially mineralizable carbon; PMN—potentially mineralizable nitrogen; LFC—light-fraction carbon; LFN—light-fraction nitrogen. Control—no fertilization; N60—60 kg N ha^{-1}; N90—90 kg N ha^{-1}; N120—120 kg N ha^{-1}; N150—150 kg N ha^{-1}.

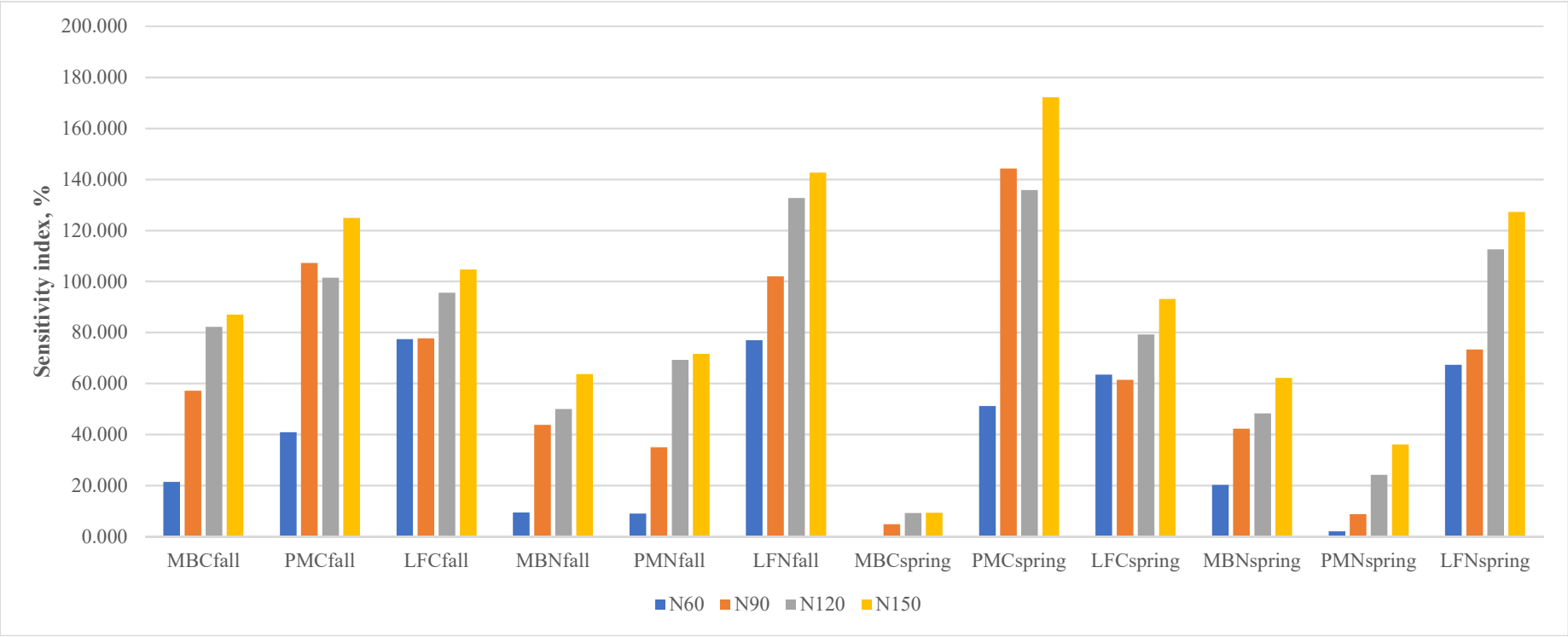

Figure 4. Sensitivity indices (SIs) of microbial C (MBC), potentially mineralizable C (PMC), light-fraction carbon (LFC), microbial N (MBN), potentially mineralizable N (PMN) and light-fraction nitrogen (LFN). N60—60 kg N ha^{-1}; N90—90 kg N ha^{-1}; N120—120 kg N ha^{-1}; and N150—150 kg N ha^{-1} in a wheat-corn bipolar cropping system on Cambisol.

The results of this study indicated that higher amounts of N fertilizer (N120 and N150) and associated recent inputs of fresh organic residues provide a sufficient organic substrate for microbial activity and the accumulation of mineral nitrogen in the soil, but are insufficient to maintain effective humification.

Long-term field experiments are the only true basis of reliable data for assessing organic matter depletion in agricultural systems and for setting realistic carbon sequestration targets based on specific landscapes [52]. The results showed that labile C more accurately reflects long-term organic matter accumulation, while labile N correlates better with fresh mulch application. The addition of only mineral fertilizers worsened the biological properties of Cambisol over 50 years (as compared with the adjacent native soils), therefore further research should focus on restoring lost soil fertility and maintaining zero soil degradation by optimizing the mineralization/accumulation balance of SOM.

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