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Assessment of the impact of spent mushroom substrate on biodiversity and activity of soil bacterial and fungal populations based on classical and modern soil condition indicators**

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A b s t r a c t. In the present study, biological indicators were used to assess the impact of applying spent mushroom substrate and manure on the soil environment. The use of spent mushroom substrate had a varied effect on the microorganisms. Stimulation was recorded in the abundance of copiotrophic bacteria and fungi, but only in the first year of the study. In the case of cellulolytic bacteria, this effect was visible only in single plots. Similar observations were also noted regarding the relative DNA content (in relation to the control), which increased for both bacteria and fungi after applying spent mushroom substrate. In the soil fertilized with spent mushroom substrate, a decrease in DNA concentration was observed, but only in the first and second year. For enzymatic activity, the use of spent mushroom substrate alone proved to be more favorable, but this effect was again observed only in the first year of the study. The application of manure caused similar changes as observed with the use of spent mushroom substrate. These observations indicate a similar impact of spent mushroom substrate and manure on the parameters tested. The research presented suggests the use of both classical methods and methods based on the analysis of DNA extracted from soil to study the impact of spent mushroom substrate on the activity of soil microbial populations.

K e y w o r d s: biological indicators, soil enzymes, spent mushroom substrate, bacteria and fungi, biodiversity, DNA

1. INTRODUCTION

The soil environment is a rich and complex ecosystem characterized by immense biodiversity. There are 10,000 different species of organisms per 1 m^2 of soil, among which bacteria are the most numerous and diverse (Orgiazzi *et al*., 2016; Delgado-Baquerizo *et al*., 2018). As reported by Chen *et al*. (2020), one gram of soil contains up to 1 billion bacteria and 10 million fungal hyphae. The composition and abundance of soil microbiota depend on various factors, including the physicochemical properties of the soil, its type, nutrient and organic matter content, climatic conditions, vegetation cover, and land use practices (Geisen *et al*., 2019; Chen *et al*., 2020; Mencel *et al*., 2022).

The immense biological richness of the soil serves as the foundation for its functioning, and consequently, it plays a crucial role in providing food of good quality, mitigating climate change through carbon sequestration, as well as accumulating and purifying water and preventing erosion (Wall *et al*., 2015; Yang *et al*., 2018; Chen *et al*., 2020; Fan *et al*., 2023). Soil biodiversity is of great importance to life. Despite this, it is threatened and destroyed by various human activities worldwide (Yang *et al*., 2018; Geisen *et al*., 2019). Therefore, monitoring of soil quality, and consequently, finding appropriate and sensitive indicators, is of crucial importance for a better and more accurate understanding of the impact of land management on the soil ecosystem. Currently, determining the state of the soil environment is based mainly on physical, chemical and hydrological indicators, but the biological functions of the soil and its biodiversity are also increasingly

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appreciated. As reported by Costantini and Mocali (2022), the European Commission has recommended the inclusion of soil biodiversity as one of the six indicators of soil health. Moreover, the Food and Agriculture Organization (FAO) has identified soil biological activity as one of the indicators that should be applied (FAO, 2020).

Assessing the quantity and quality of microorganisms in the soil is essential to better understand the dynamics of their populations and the related biochemical and enzymatic processes. The quantitative and qualitative composition of soil microorganisms is considered a sensitive indicator of soil quality because it represents a living component of the soil environment that responds rapidly to anthropogenic factors (Hermans *et al*., 2020; Frąc *et al*., 2021; Jezierska-Tys *et al*., 2021b; Joniec *et al*., 2021; Wyszkowska *et al*., 2023).

Many different methods are used to quantify the abundance of soil microorganisms, but finding the most optimal one is still a matter of debate. Determining the population of microorganisms using the plate count method and expressing it as colony-forming units (CFU), while providing valuable information about viable cells, is considered by some to not fully reflect the actual state of soil microorganism populations (Wydro, 2022). Currently, intensive development of molecular techniques such as PCR, sequencing and metagenomics is observed. Methods based on DNA extraction from soil have many advantages and seem to be more reliable, but they also raise some concerns (Rincon-Florez *et al*., 2013; Sidstedt *et al*., 2020; Semenov, 2021; Wydro, 2022). One of the risks is that DNA isolated from the soil environment may originate from sources other than bacterial cells, such as plant residues, fungi, algae, or protozoa (Taylor *et al*., 2002). Moreover, these techniques do not allow distinction DNA of living bacteria from DNA of dead cells (Li *et al*., 2021; Roumani *et al*., 2023). In addition, soil microbiologists still have doubts concerning the weight of the soil sample that should be collected for DNA analysis to ensure the most reliable results (Semenov, 2021). Soil is also a complex matrix, characterized by a diverse and variable composition, presence of inhibitors, and a large amount of organic substances that can inhibit DNA polymerase activity and affect hybridization protocols (Sidstedt *et al*., 2020; Wydro, 2022). Therefore, aspects such as the complexity of analysis, research experience and facilities, as well as associated costs, are not without significance when selecting an appropriate molecular method (Rincon-Florez *et al*., 2013). It is widely believed that molecular techniques provide a more accurate picture of microbial communities, as the ability of microorganisms to grow on artificial media is limited (Rincon-Florez *et al*., 2013; Wydro, 2022). However, as reported by Bonnet *et al*. (2019) and Rodrigues *et al*. (2022), after a period of stagnation in the development of plate count techniques, this field is currently experiencing a resurgence. At present, emerging new culture media and cultivation conditions increasingly resemble the natural environment of microorganisms. Culture media remain an important tool for isolating microorganisms, despite being abandoned by a significant number of researchers (Bonnet *et al*., 2019). The choice of one technique over another is individual and depends on the researcher's hypothesis and resources. Therefore, combining different methods increases the possibility of obtaining better results and more information (Rincon-Florez *et al*., 2013). The studies conducted by Joniec (2019) and Wolińska *et al*. (2013) demonstrate the usefulness of the combined application of these parameters as indicators of the activity of living microorganisms in the soil. The positive correlations observed by the authors between DNA concentration and microbial abundance, respiratory activity, and dehydrogenase activity indicate the dominance of intracellular DNA in the soil. As research shows, combining both techniques for determining the quantitative and qualitative composition of soil microorganisms is still quite common (Joniec, 2019; Li *et al*., 2021; Chaudhary *et al*., 2022; Pu *et al*., 2022; Wyszkowska *et al*., 2023).

Enzymatic activity is also an important tool in tracking changes in the soil environment. Soil enzymes are responsible for many processes occurring in the soil environment and therefore play a crucial role in the decomposition of organic matter and nutrient cycling, thus reflecting trends and the character of biogeochemical cycles (Gianfreda and Rao, 2014; Utobo and Tewari, 2015). This parameter exhibits high sensitivity and responsiveness to environmental changes. This rapid reaction, induced by various agricultural practices, makes enzymatic activity an effective means of assessing soil quality and a significant indicator of microbial response to climate changes (Lee *et al*., 2020; Song *et al*., 2021; Fanin *et al*., 2022; Mencel *et al*., 2022). As reported by Alkorta *et al*. (2003), enzymes can respond to various types of changes much earlier (within months to 1 to 2 years) than other soil properties. Furthermore, enzymatic activity often exhibits strong correlations with critical soil quality parameters, such as organic matter, physico-chemical properties of the soil, biomass, and microbial activity (Song *et al*., 2017; Furtak and Gałązka, 2019; Joniec *et al*., 2022; Kwiatkowska and Joniec, 2022). In addition, assays determining enzymatic activity are relatively inexpensive, simple and provide high reproducibility of results (Utobo and Tewari, 2015). Both β-glucosidase and fluorescein diacetate hydrolysis (FDA) have been widely used for assessing the condition of the soil environment (Kracmarova *et al*., 2020; Joniec *et al*., 2021; Song *et al*., 2021; Chaudhary *et al*., 2022; Davies *et al*., 2022; Wyszkowska *et al*., 2022, Kwiatkowska *et al*., 2023). The cited authors confirmed the sensitivity of enzymatic activity to various factors such as fertilization, waste management or environmental conditions.

The analysis of microbiological parameters is crucial for the development of sustainable ecosystem management and soil environmental policies. Monitoring not only the immediate responses of microorganisms but also seasonal changes in their populations caused by various human activities, can help achieve the goals of sustainable ecosystem management and environmental protection. This allows for the assessment of soil environmental balance over an extended period. This knowledge can also help mitigate the negative impact of various agricultural practices on climate change (Jezierska *et*

al., 2021a; Lynch *et al*., 2021; Holka *et al*., 2022). Therefore, in this study, microbiological and enzymatic activity parameters, along with DNA analysis, were used to assess the impact of the application of spent mushroom substrate (SMS) and manure (M) on the soil environment. An attempt was also made to verify the usefulness of these indicators for monitoring the condition of the soil environment and evaluating the effectiveness of the applied fertilization practices. These studies are part of a comprehensive research project, lasting several years, aimed at assessing the trend, intensity and persistence of changes in soil microbial activity (Joniec *et al*., 2022; Kwiatkowska and Joniec, 2022). The research will improve existing knowledge regarding the selection of appropriate microbiological indicators for soil monitoring in the coming years. Pertaining to this assumptions, the authors have formulated the following hypotheses: (I) the application of spent mushroom substrate for fertilization positively influences soil microbial biodiversity and activity; (II) analyzing soil microbial populations using a combination of appropriately selected classical and modern indicators allows for a more comprehensive assessment of soil health.

2. MATERIALS AND METHODS

2.1. Study sites

The experiment was located at the Experimental Farm in Czesławice (Poland, Lubelskie Region, 51°18'23"N, 22°16'02"E) of the University of Life Sciences in Lublin. The experiment was set up using a randomized block design with three replications, where individual plots measuring 1.5 m × 2.0 m were fertilized with spent mushroom substrate or manure (Table 1). Spent mushroom substrate and cattle manure were applied for three years in a single dose of 20 t ha^{-1} in autumn (before autumn ploughing was carried out to cover the fertilizers with the soil – the first 10 days of October). They were applied separately or in combination with supplementary NPK fertilization at two different doses

Table 1. Properties of soil und wastes (Joniec *et al*., 2022)

Property	Unit	Soil	Spent mushroom substrate	Manure
pH_{KCl}	1 mol KCl	7.0	6.6	7.3
TOC	g kg^{-1}	14.98	105.0	135.8
TN	g kg^{-1}	1.51	6.50	9.47
TP	g kg^{-1}	0.19	0.25	0.25
Ca	mg kg^{-1}	1660	15800	2240
K		2350	6330	11100
Mg		1390	1240	1550
Zn	mg kg^{-1}	n.o.	86.0	n.o.
Cu			16.6	
Ni			2.81	
Cr			1.84	
Cd			0.055	
Pb			0.956	
Hg			0.07	

TOC – total organic carbon, TN – total nitrogen, TP – total potassium.

Table 2. Selected, physico-chemical and chemical properties of the soil (Joniec *et al*., 2022)

	Year	Season	C	SMS	SMS+ N1P1K1	SMS+ N2P2K2	M
pH 1 mol KCl	2018	spring	7.03	7.20	6.41	5.16	7.47
		autumn	6.86	7.60	5.98	6.60	5.44
	2019	spring	6.42	6.75	5.88	5.84	6.20
		autumn	6.34	6.04	6.18	5.53	6.24
	2020	spring	6.87	6.85	6.68	6.79	6.56
		autumn	6.25	6.13	6.33	6.64	6.50
TOC g kg^{-1}	2018	spring	14.98	19.50	17.21	12.83	13.45
		autumn	13.59	14.39	14.34	11.46	12.16
	2019	spring	12.19	12.99	14.75	15.60	14.89
		autumn	12.02	10.63	13.25	13.28	18.18
	2020	spring	15.62	16.30	14.90	15.33	17.75
		autumn	13.34	12.54	13.85	14.91	14.78
TN g kg^{-1}	2018	spring	1.51	1.82	2.13	1.46	1.36
		autumn	1.37	1.44	1.39	1.18	1.28
	2019	spring	1.50	1.10	1.00	1.30	1.10
		autumn	0.96	0.97	1.30	0.84	1.00
	2020	spring	1.70	1.20	0.98	1.40	1.10
		autumn	0.97	0.80	1.20	0.55	1.10
TP g kg^{-1}	2018	spring	0.19	0.21	0.21	0.17	0.22
		autumn	0.16	0.16	0.14	0.15	0.18
	2019	spring	0.15	0.13	0.19	0.10	0.10
		autumn	0.11	0.10	0.11	0.13	0.15
	2020	spring	0.10	0.15	0.12	0.16	0.15
		autumn	0.12	0.13	0.14	0.11	0.14

TOC – total organic carbon, TN – total nitrogen, TP – total potassium. C – control soil; SMS – soil + spent mushroom substrate, SMS+N1P1K1 – soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS+N2P2K2 – soil + spent mushroom substrate + mineral fertilization N2P2K2; M – soil + manure.

(N1P1K1 and N2P2K2). Nitrogen fertilization was applied in doses of N1-50 and N2-100 kg ha^{-1} in the form of ammonium nitrate, phosphorus P1-30 and P2-60 kg ha^{-1} in the form of granulated triple superphosphate, and potassium K1-70 and K2-140 kg ha^{-1} in the form of potassium sulfate. Italian ryegrass (*Lolium multiflorum* Lam.), a tetraploid variety of Turtetra (Kroto), was used as the test plant, and was sown each year in the second decade of April in the amount of 30 kg ha^{-1}, with a row spacing of 25 cm, at a depth of 1 cm. A three-year field experiment was established on luvisol soil formed from loess, belonging to the 2nd valuation class (PSSS, 2009; WRB, 2022). Soil grain size composition was as follows: fraction 1.0-0.1 mm – medium sand (4%), fraction 0.1-0.02 mm – fine sand-coarse dust (52%), fraction 0.02-0.002 mm – fine dust (35%), fraction <0.002 mm – colloidal clay (9%).

Experimental scheme:

1. Soil without fertilization (control object) (C),
2. Soil + spent mushroom substrate (SMS),
3. Soil + spent mushroom substrate + N1P1K1 (SMS+N1P1K1),
4. Soil + spent mushroom substrate + N2P2K2 (SMS+N2P2K2),
5. Soil + cattle manure (M).

2.2. Soil sampling

Soil samples were collected from the 0-25 cm layer over a period of 3 years, twice during each growing season, *i.e.*, in spring (June) and autumn (September), randomly from 10 locations within each research plot. Soil material from individual plots was a mixture of 10 soil cores with a diameter of 4 cm each. All samples were sieved through a 2 mm mesh before analysis. The samples were stored in plastic bags at 4°C, except for the soil samples for DNA analysis, which were stored at –80°C.

Selected soil properties (pH, TOC, TN, TP) were determined on the same dates as other microbiological activities are listed in Table 2 (Joniec *et al*., 2022).

2.3. Meteorological conditions

The total precipitation during the field experiment, *i.e.*, from 2018 to 2020, varied and amounted to 539.3, 481.8, and 799.7 mm, respectively. The average annual air temperature was 8.6, 11.0, and 10.1°C for the same respective years. The meteorological conditions during the months of soil sample collection in June and September were as follows: monthly precipitation and average monthly temperature were 74.8 mm and 16.3°C, and 54.7 mm and 14.7°C, respectively, in 2018; 11.2 mm and 22.9°C, and 33.5 mm and 16.3°C, respectively, in 2019; 170.3 mm and 17.9°C, and 128.5 mm and 15.6°C, respectively, in 2020. Detailed meteorological data have been published in previous studies (Joniec *et al*., 2022; Kwiatkowska and Joniec, 2022).

2.4. Microbiological analyses

The abundance of individual groups of microorganisms in the soil material was determined using the plate count method (Foght and Aislabie, 2005) on the following media: copiotrophic bacteria – Bunt and Rovira medium (1955), filamentous fungi – Martin medium (1950), and cellulolytic fungi – mineral agar covered with a Whatman filter paper disk. For the fungal analysis, antibiotics (streptomycin, chloramphenicol) were added to the medium (Martin, 1950; Gil *et al.,* 2009). The results of the aforementioned analyses are expressed as colony-forming units (CFU). Additionally, the abundance of cellulolytic bacteria was determined using the most probable number (MPN) method, as described by Foght and Aislabie (2005). For these bacteria, a liquid medium described by Pochon and Tardieux (1962) was used, and the results are presented as the most probable number (MPN) read from the McCrady tables. Bacteria were cultured at 28°C for 4 days (copiotrophic bacteria) and 14 days (cellulolytic bacteria), while fungi were cultured at 25°C for 3 days (filamentous fungi) and 14 days (cellulolytic fungi).

2.5. Molecular analyses

Total genomic DNA was extracted from analyzed soil samples using Soil DNA Purification Kit (EurX) according to the manufacturer's protocol. For each sample, 100 mg of fresh soil has been used. The integrity of the obtained DNA samples was determined through of electrophoresis in 1.5% agarose gel stained with ethidium bromide. The purity of samples was determined spectrophotometrically using a NanoDrop 2000 (Thermo Scientific) by calculating A260/A280 and A260/A230 ratios. The concentration of analyzed DNA samples was determined using of fluorometric assessment using a dsDNA Quantitation BR reagent kit (Thermo Fisher Scientific) according to the manufacturer's instructions. For quantitation 4 µl of extracted genomic DNA sample was mixed with 196 µl of Qubit working solution, vortexed for 5 s, and incubated at room temperature for 2 min. The prepared samples were then measured fluorometrically using the Qubit 2.0 fluorometer (Thermo Fisher Scientific).

Quantitative analyses of bacterial and fungal genetic material in examined soil samples were performed using the quantitative PCR (qPCR) technique. As a template 80 ng of total genomic DNA has been used for each reaction. The amplification of the sequence-specific fragments of the 16S rRNA gene and 18S rRNA gene was used for the quantification of bacterial and fungal DNA content in the sample, respectively. For amplification two sets of sequence-specific primers were used: 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill *et al*., 2015; Parada *et al*., 2015) for 16S rRNA gene, and FungiQuant-F (5'-GGRAAACTCACCAGGTCCAG-3') and FungiQuant-R (5'-GSWCTATCCCCAKCACGA-3') (Liu *et al*., 2012) for 18S rRNA gene. For analysis, SYBR Select Master Mix (Thermo Fisher Scientific) has been used according to the manufacturer's protocol. All analyses were performed using QuantStudio 3 (Thermo Fisher Scientific) apparatus together with the Thermo Fisher Connect software suite. Each sample was analyzed in three replications. For data analysis, a relative quantification model has been used, where the number of amplicon in control sample was set as 1, and the content of amplicons in all other samples was presented as a change compared to the control sample. The specificity of the amplification reaction was confirmed for each sample by means of melt curve analysis.

2.6. Enzymatic analyses

The activity of β-glucosidase was determined in 1 g soil samples, incubated in a modified universal buffer with a pH of 6.0 for 1 h at 37°C, using p-nitrophenyl-β-D-glucopyranoside (PNG) as the substrate (Eivazi and Tabatabai, 1988). The activity of this enzyme was determined spectrophotometrically at 400 nm and expressed as mg PNP kg^{-1} d.m. soil h^{-1}.

The level of hydrolysis of fluorescein diacetate (FDA) was determined using the method described by Schnurer and Rosswall (1982) in 1 g soil samples with FDA addition as the substrate. Incubation was conducted in the presence of 60 mM sodium phosphate buffer (pH = 7.6) for

2 h at a temperature of 25°C. The activity of this enzyme was determined spectrophotometrically at 490 nm and expressed as mg of fluorescein per kg^{-1} soil d.m. h^{-1}.

2.7. Statistical analysis

Statistical analyses were carried out using the Statistica 13.1 software package (TIBCO Software Inc.; Palo Alto, CA, USA). The results were statistically analyzed using analysis of variance (ANOVA) and Tukey's test at a significance level of $\alpha = 0.05$. Each year was analyzed separately. Additionally, Pearson's correlation analysis was used to determine the relationships between microbiological and enzymatic parameters, and the physical, chemical, and environmental conditions, at three levels of significance: $p<0.001$, $p<0.01$, $p<0.05$. The results were presented in the form of a heat map.

3. RESULTS

The data presented in Figs 1-4 and Table 3 revealed significant changes in the abundance of individual bacterial and fungal groups as a result of the applied fertilization.

The abundance of bacteria with high nutritional requirements fluctuated significantly throughout the study period (Fig. 1, Table 3). The most noticeable impact of the spent mushroom substrate occurred in the first year of the study, where a clear stimulation of their development was observed. The highest number of these bacteria was found in the treatment where spent mushroom substrate was applied together with mineral fertilizer at a lower dose (SMS+N1P1K1). The combined application of spent mushroom substrate with a higher dose of mineral fertilizer (SMS+N2P2K2) proved to be unfavorable for the growth of these bacteria. This led to a decline in their development in the autumn compared to the unfertilized control treatment (C). In the second and third year of the study, the impact of spent mushroom substrate on the growth of copiotrophic bacteria weakened and even disappeared. The positive impact of the spent mushroom substrate on copiotrophic bacteria persisted only in specific treatments during the spring season: in the second year, this effect was observed in the treatment where the spent mushroom substrate was combined with a lower dose of NPK fertilizer (SMS+N1P1K1) and in the third year, in the treatment with spent mushroom substrate (SMS) alone. In the autumn of the second year of the study, a significant decrease in the number of copiotrophic bacteria was observed in the treatments with the addition of waste alone (SMS), and in the third year in all treatments with waste (SMS, SMS+N1P1K1, SMS+N2P2K2).

Fertilization of the soil with manure (M) also increased the development of bacteria with high nutritional requirements, which was particularly evident in the first year of the study. In subsequent years, this effect weakened and was only observed in single seasons. In the autumn of the third year of the study, manure caused a decrease in the growth of these microorganisms compared to the control treatment (C).

Similar changes over the 3 years of the study were observed in the population of filamentous fungi under the influence of the spent mushroom substrate (Fig. 2, Table 3). The effect of spent mushroom substrate on this parameter was most evident in the first year of the study. In both spring and autumn, fungal growth was found to be stimulated in all treatments with the addition of spent mushroom substrate (SMS, SMS+N1P1K1, SMS+N2P2K2). The highest number of filamentous fungi was recorded in the treatment with spent mushroom substrate combined with a higher dose of mineral fertilizer (SMS+N2P2K2), followed by the

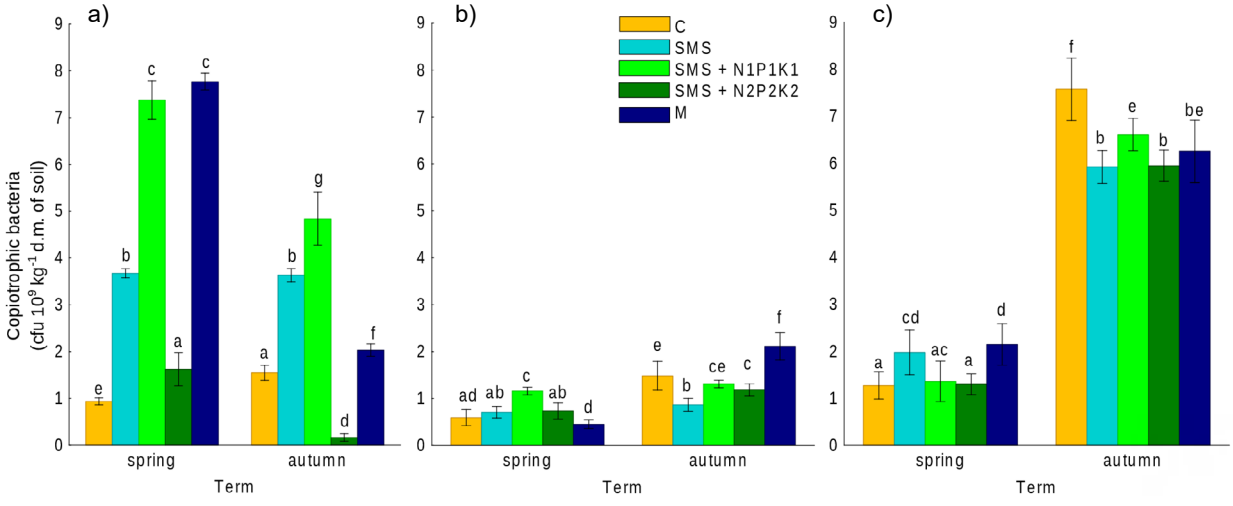

Fig. 1. Number of copiotrophic bacteria in the control soil and soil under different treatment strategies: a) 1st year, b) 2nd year, c) 3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p<0.05$, each year was analyzed independent of each other. C – control soil; SMS – soil and spent mushroom substrate, SMS+N1P1K1 – soil, spent mushroom substrate and mineral fertilization N1P1K1; SMS+N2P2K2 – soil, spent mushroom substrate and mineral fertilization N2P2K2; M – soil and manure.

Table 3. Microbiological and enzymatic activity in soil (annual averages)

Years	Experimental treatments	CopB	FF	CB	CF	dsDNA	B-GLU	FDA
	C	1.24 b	16.11 a	0.48	11.06 a	170.81 g	78.63 d	107.21 c
	SMS	3.65 c	54.77 b	0.25	75.60 j	160.22 g	82.25 d	138.58 e
2018	SMS+N1P1K1	6.11 g	152.48 f	0.51	59.34 gh	92.49 ef	77.94 cd	115.46 c
	SMS+N2P2K2	0.90 a	226.28 h	0.14	45.18 e	83.60 e	56.12 a	73.91 a
	M	4.90 f	139.75 ef	1.34	40.70 d	112.77 f	64.37 b	109.55 c
	C	1.04 ab	79.53 cd	26.56	33.20 c	28.15 ab	71.46 c	98.33 b
	SMS	0.79 a	68.93 bc	30.42	26.17 b	33.26 abcd	58.68 ab	95.08 b
2019	SMS + N1P1K1	1.24 b	126.60 e	0.03	38.28 d	23.93 a	76.89 cd	158.93 f
	SMS + N2P2K2	0.96 a	132.95 e	0.26	50.95 f	23.61 a	83.24 d	127.01 d
	M	1.28 b	90.03 d	8.48	40.02 d	32.83 abc	100.11 e	133.60 de
	C	4.42 e	257.18 i	13.29	71.30 i	47.63 bcd	109.10 f	160.47 f
	SMS	3.95 d	206.29 g	2.38	62.10 h	50.76 cd	100.37 e	155.63 f
2020	SMS+N1P1K1	3.99 d	267.96 i	1.04	68.87 i	42.15 abcd	112.76 f	157.27 f
	SMS+N2P2K2	3.62 c	219.07 gh	0.64	69.66 i	53.94 d	108.39 f	153.82 f
	M	4.20 de	237.00 h	0.14	57.24 g	51.51 cd	113.14 f	155.81 f

C – control soil; SMS – soil and spent mushroom substrate, SMS+N1P1K1 – soil, spent mushroom substrate and mineral fertilization N1P1K1; SMS+N2P2K2 – soil, spent mushroom substrate and mineral fertilization N2P2K2; M – soil and manure. CopB – copiotrophic bacteria (cfu 109 kg^{-1} d.m. of soil), FF – filamentous fungi (cfu 106 kg^{-1} d.m. of soil), CB – cellulolytic bacteria (106 kg^{-1} d.m. of soil), CF – cellulolytic fungi (cfu 106 kg^{-1} d.m. of soil), dsDNA – DNA concentration (μg g^{-1} d.m. of soil), B-GLU – β-glucosidase (mg PNP kg^{-1} d.m. of soil h^{-1}, FDA – FDA hydrolytic activity (mg fluorescein kg^{-1} d.m. of soil h^{-1}). Different letters indicate significant differences at $p<0.05$.

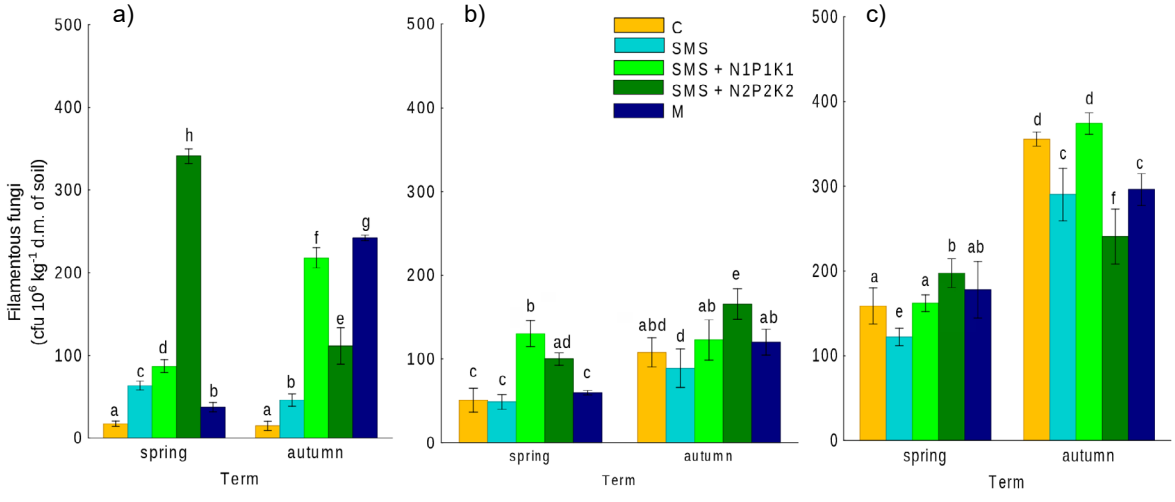

Fig. 2. Number of filamentous fungi in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

treatment with spent mushroom substrate combined with a lower dose of mineral fertilizer (SMS+N1P1K1). The least favorable results were observed when only spent mushroom substrate (SMS) without any additional mineral fertilizer was applied. In the second year of the study, the stimulating effect of spent mushroom substrate noticeably declined and was visible mainly in the treatments where mineral fertilization was applied (SMS+N1P1K1; SMS+N2P2K2). In the third year of the study, the positive impact of spent mushroom substrate persisted only in the spring and was observed in the treatment with a higher dose of mineral fertilization (SMS+N2P2K2). The addition of spent mushroom substrate alone (SMS) inhibited the growth of filamentous fungi throughout the year. A similar unfavorable effect was observed in the autumn in the treatment with higher mineral fertilizer application (SMS+N2P2K2).

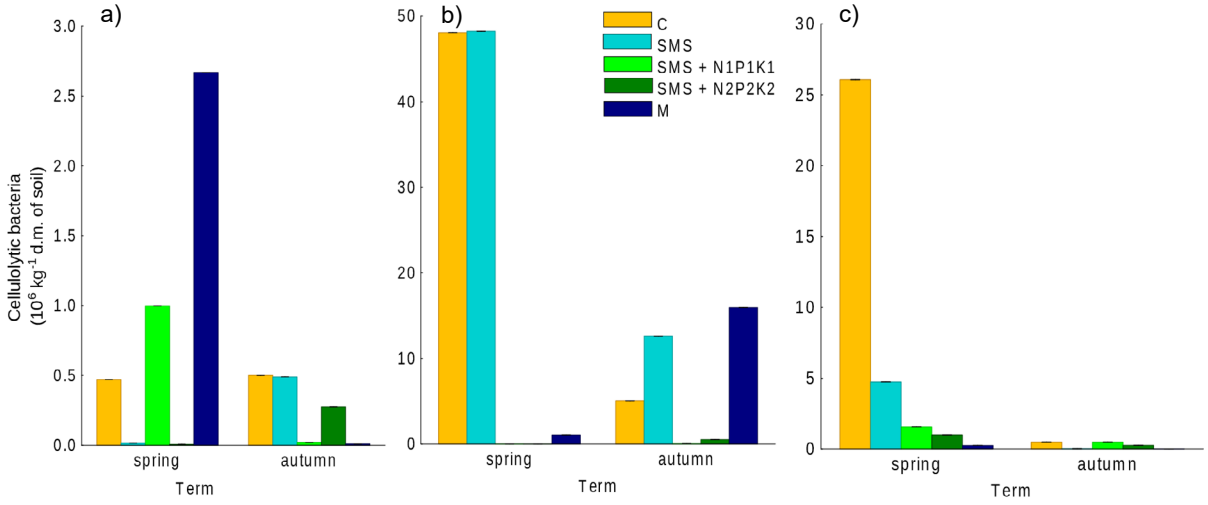

Fig. 3. Number of cellulolytic bacteria in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

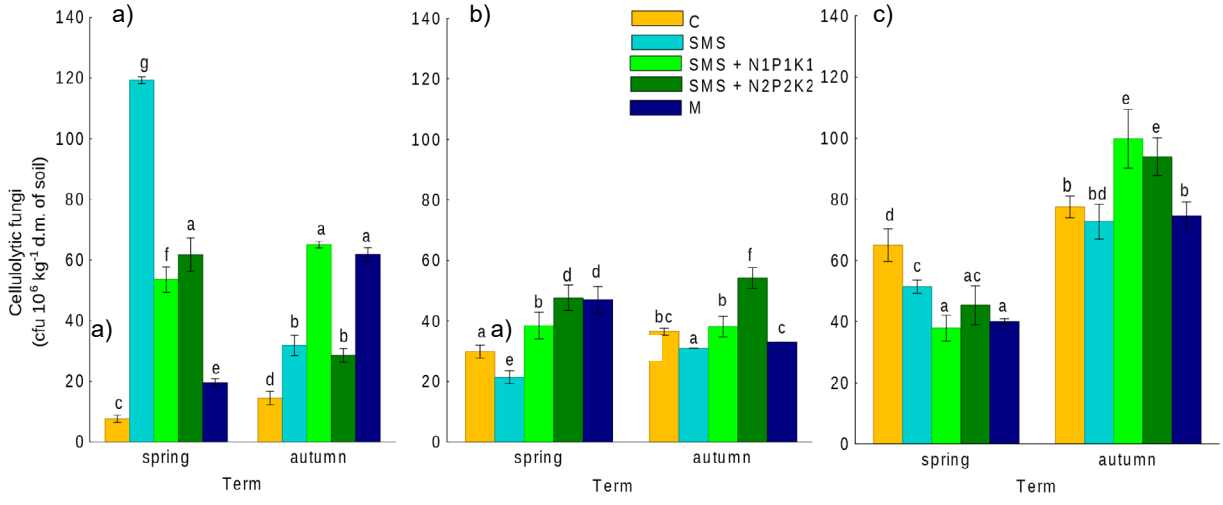

Fig. 4. Number of cellulolytic fungi in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

The addition of manure (M) also stimulated the growth of filamentous fungi, but this effect was observed only in the first year of the study. In subsequent years, the beneficial effect of manure disappeared. In the third year in autumn, the addition of manure resulted in a significant decrease in the abundance of filamentous fungi compared to the control treatment (C).

The data presented in Fig. 3 and Table 3 indicated that fertilization of the soil with various variants of spent mushroom substrate resulted in changes in the abundance of cellulolytic bacteria. The introduction of spent mushroom substrate into the soil, combined with mineral fertilization in both lower and higher doses, resulted in a decrease in the development of cellulolytic bacteria, which persisted during all years of the study. A beneficial effect of spent mushroom substrate, applied together with a lower dose of mineral fertilization (SMS+N1P1K1), on the development of cellulolytic bacteria was observed only in the first season of the study. The effect of spent mushroom substrate (SMS) alone on the analyzed parameter was not consistent. During the first and second year in this plot, there were either decreases, increases, or no significant differences in the abundance of these bacteria compared to the control object (C). However, in the third year of the study, a decrease in this parameter was observed under the influence of spent mushroom substrate alone at both time points.

Manure application (M) during the first and second year resulted in either a decrease or an increase in the number of cellulolytic bacteria. In the third year, the addition of manure, similar to SMS alone, led to a decrease in the development of this group of bacteria, which persisted throughout the year.

The results presented in Fig. 4 and Table 3 showed that, similarly to the total number of copiotrophic bacteria and filamentous fungi, the development of cellulolytic fungi was most significantly stimulated in the first year of the study. The most favorable for the development of this group of fungi was the use of SMS alone, followed by the addition of spent mushroom substrate together with a lower dose of mineral fertilization (SMS+N1P1K1). In the following years of

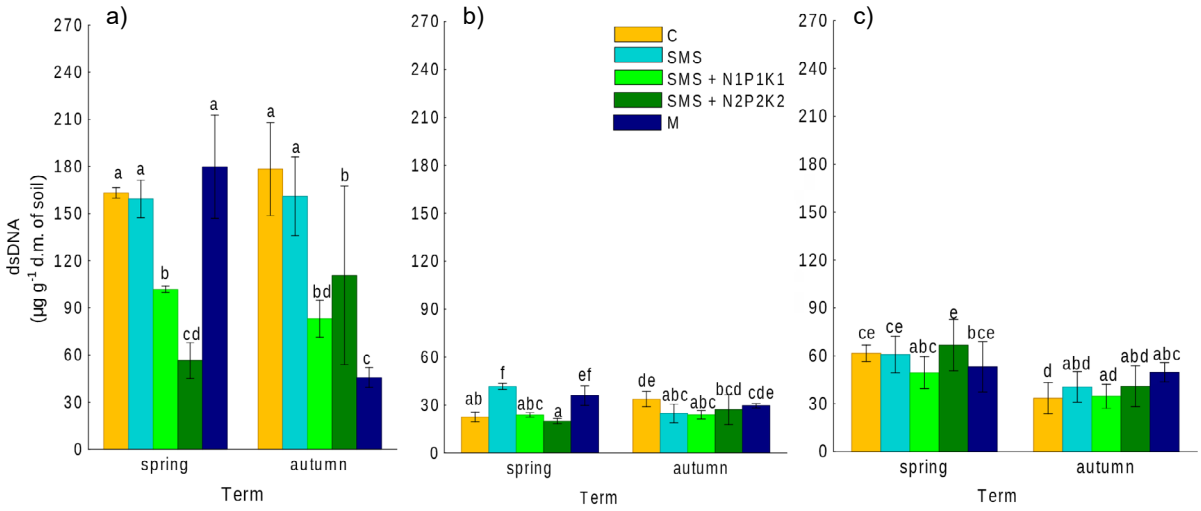

Fig. 5. dsDNA concentration in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

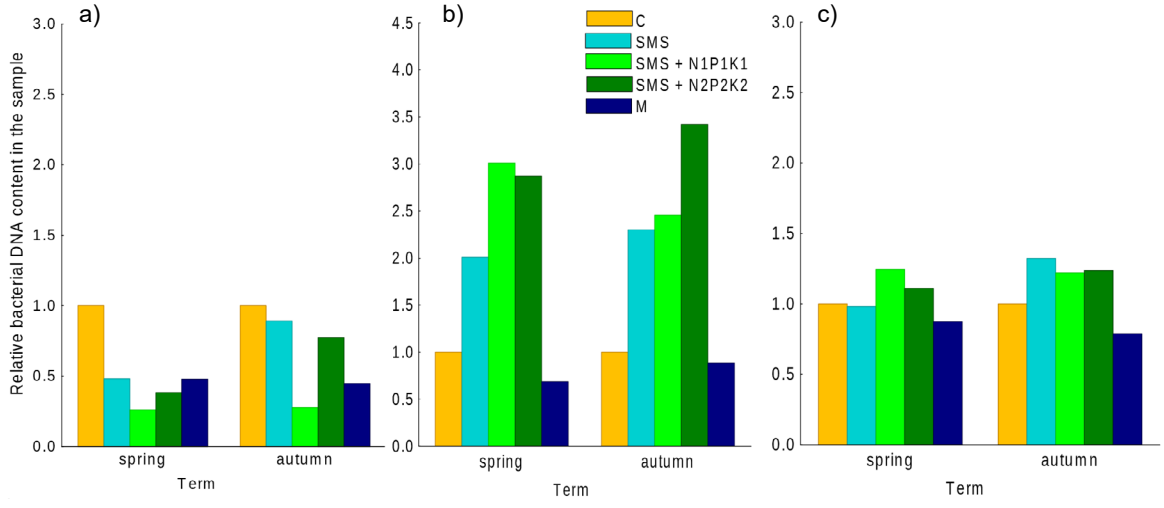

Fig. 6. Relative bacterial DNA content in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

the study, the positive effect of SMS application alone disappeared. Throughout the entire second year and in the spring of the third year, a decrease in the development of these fungi was observed in the plot with only spent mushroom substrate (SMS). The positive effect of waste applied together with mineral fertilization (SMS+N1P1K1, SMS+N2P2K2) persisted in almost all time points in the second and third year. Only in the spring of the third year, a decrease in the development of cellulolytic fungi was observed in these treatments compared to the control treatment (C).

Manure also caused an increase in the number of cellulolytic fungi, which was evident in the autumn of the first year and in the spring of the second year (M). In the remaining time points and years, this effect did not occur, and in the spring of the third year, there was even a reduction in the number of these fungi compared to the control treatment (C).

The concentration of dsDNA in the soil enriched with spent mushroom substrate underwent statistically significant changes in the first and second year of the study (Fig. 5, Table 3). In the first year, a decrease in dsDNA concentration was recorded after the application of spent mushroom substrate together with NPK in both doses (SMS+N1P1K1, SMS+N2P2K2). The lowest dsDNA concentration was observed in the spring. In the following year of the study, the level of this parameter was lower in all treatments compared to the previous year. The adverse effect of the spent mushroom substrate applied together with NPK, but only with a lower dose of NPK (SMS+N1P1K1), was visible in this year, but only in the autumn. In this period, the addition of spent mushroom substrate alone (SMS) also resulted in reduced dsDNA concentration. A positive effect of spent mushroom substrate on the analyzed parameter was only observed in the spring of the second year of the study, in the plot with spent mushroom substrate alone (SMS). In the third year of the experiment, no significant changes in dsDNA concentration were observed in individual treatment variants with SMS.

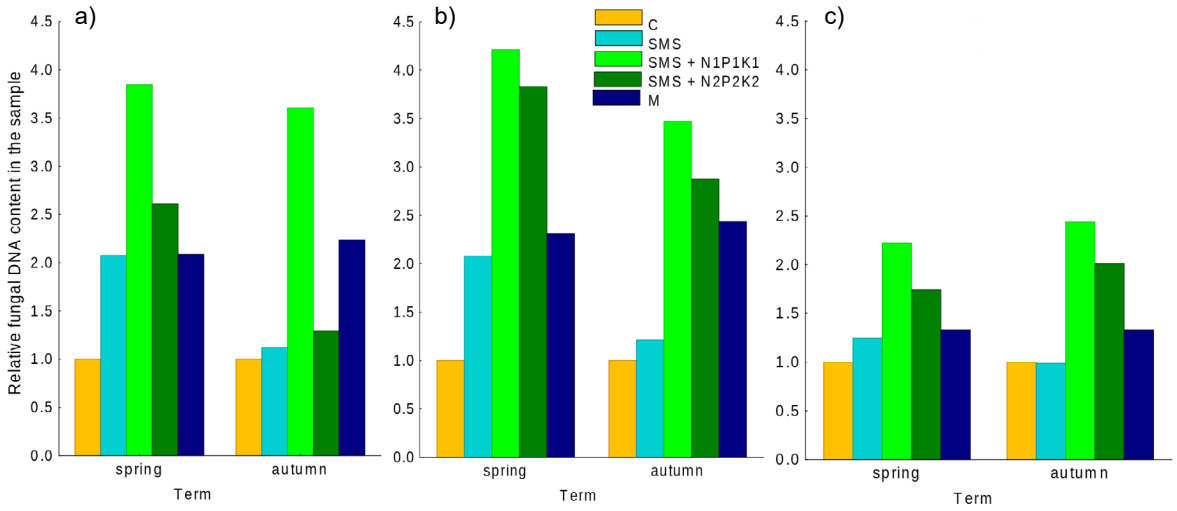

Fig. 7. Relative fungal DNA content in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

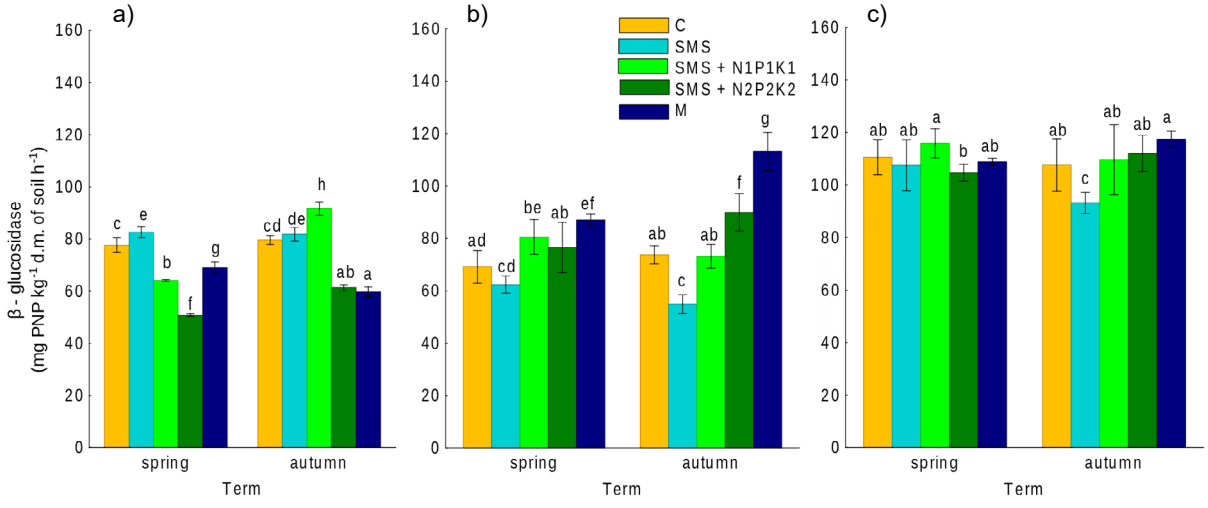

Fig. 8. Activity of β-glucosidase in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

The effect of manure (M) on dsDNA concentrations was not consistent across the years and time points (Fig. 5, Table 1). In the first year, a decrease in its content was observed in the autumn. However, an increase was observed in the spring of the second year and in the autumn of the third year. In the remaining time points, the changes were not significant.

The relative content of both bacterial and fungal DNA was subject to changes due to the applied spent mushroom substrate (Figs 6 and 7). Concerning bacteria, this parameter in the first year of the study was lower in all plots with the spent mushroom substrate (SMS, SMS+N1P1K1, SMS+N2P2K2) compared to the control soil (C). For bacteria in the following years, *i.e.*, second and third, and for fungi in all years, stimulation of this parameter was observed under the influence of the spent mushroom substrate introduced into the soil both separately and in combination with NPK fertilization (SMS, SMS+N1P1K1, SMS+N2P2K2). It should be noted that the use of spent mushroom substrate together with mineral fertilization proved to be more beneficial than using SMS alone. At all time points and years, the relative content of fungal DNA was highest in the plot with a lower NPK dose (SMS+N1P1K1).

The addition of manure (M) to the soil resulted in a decrease in the relative content of bacterial DNA and an increase in this parameter for fungi (Figs 6 and 7). This effect persisted throughout the entire study period.

Similarly to microbial counts, enzymatic activity also showed significant differences among individual fertilization treatments (Figs 8 and 9, Table 3). However, these changes were not as consistent as those observed for the microbial counts, and they occurred only in the first and second year of the study.

The activity of β-glucosidase showed significant fluctuations, with different patterns observed in each treatment, sampling period, and year. In the first year of the study, a positive effect of spent mushroom substrate on the enzymatic parameter tested was observed in the spring in the

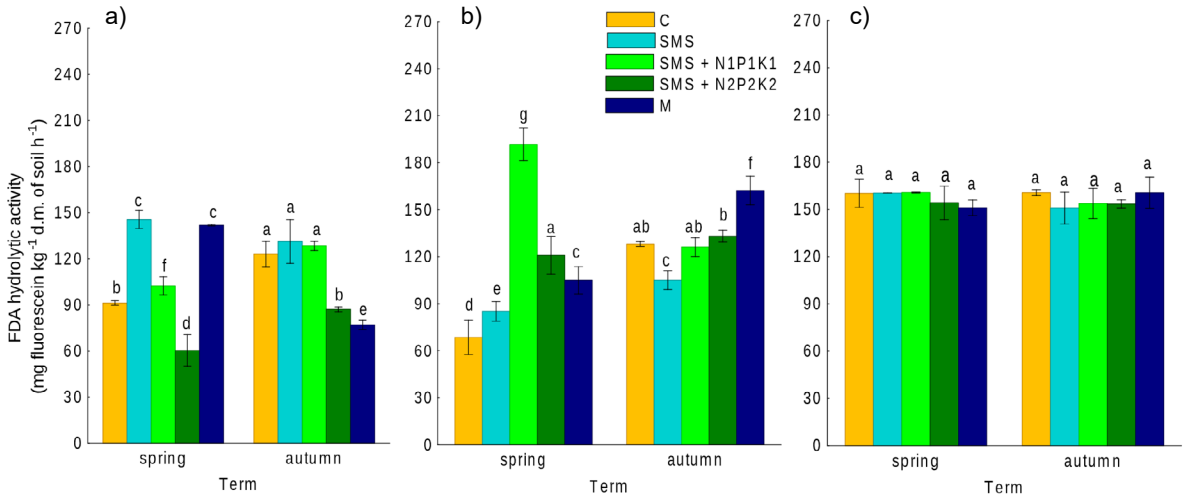

Fig. 9. FDA hydrolytic activity in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

treatment with waste alone (SMS) and in the treatment with waste applied together with a lower dose of mineral fertilizer (SMS+N1P1K1). In the other treatments with mineral fertilization, a decrease in this enzymatic activity was observed compared to the control treatment (C). The application of spent mushroom substrate together with a higher dose of mineral fertilizer (SMS + N2P2K2) proved most unfavorable. In the second year of the study, the negative impact of the waste declined and was only noticeable in the autumn in the plot with waste alone (SMS). A stimulation of β-glucosidase activity was observed in individual plots with mineral fertilization (SMS+N1P1K1; SMS+N2P2K2).

Fertilization of the soil with manure (M) resulted in a decrease in β-glucosidase activity in the first year. However, in the second year of the study, manure application stimulated this parameter.

The hydrolytic activity of fluorescein also showed significant changes in the first and second year of the study. These changes varied in individual seasons. An increase in this enzymatic parameter was observed in the spring in almost all treatments with spent mushroom substrate in the first year (SMS, SMS+N1P1K1) and in all treatments in the second year (SMS, SMS+N1P1K1, SMS+N2P2K2). In the first year of the study, the highest activity was observed in the plot with spent mushroom substrate (SMS) alone, while in the second year, it was in the plot with spent mushroom substrate applied together with a lower dose of mineral fertilizer (SMS+N1P1K1). Initially, the application of spent mushroom substrate together with a higher dose of mineral fertilizer (SMS+N2P2K2) exerted a negative effect on this enzymatic activity, resulting in a decrease in its level in the first period of the study. A decrease in hydrolase activity was also observed in individual plots in the autumn of both the first and second year of the study (SMS; SMS+N2P2K2).

In contrast to the application of spent mushroom substrate (SMS), the use of manure (M) resulted in an increase in fluorescein hydrolase activity in both the first and second year, which persisted in almost all periods of the study. A decrease in the level of this parameter was only observed in the autumn of the first year of the study.

4. DISCUSSION

The application of organic materials in the form of spent mushroom substrate and manure initially stimulated the abundance of the analyzed bacterial and fungal groups (except cellulolytic bacteria). The available literature indicates that spent mushroom substrate is a waste material rich in organic matter and various macro- and micronutrients (Becher *et al*., 2021; Velusami *et al*., 2021). Furthermore, Lipiec *et al*. (2021) reported that the application of spent mushroom substrate, especially in the long term, increased the organic matter content in the soil. The addition of this waste in the present study also likely contributed to the increase in organic carbon content in the soil (Joniec *et al*., 2022). However, it should be noted that its concentration showed only minor fluctuations over time, which was consistent with the observations of Medina *et al*. (2012). The latter authors also observed an increase in the organic matter content in the soil after adding the spent mushroom substrate. At the same time, this parameter showed minor alterations over time, which the authors attributed to the stability of the organic matter originating from the waste material. Moreover, as reported by Powlson *et al*. (1987), microbial biomass responded to management practices much more rapidly than the total organic carbon content in the soil. This suggests that the soil microbiome may be influenced by agricultural practices, impacting soil quality long before the effects are detectable through measurements of total organic carbon in the soil. This can also be confirmed by the lack of significant positive correlations between TOC and the studied groups of microorganisms (Fig. 10). The observed decrease in the abundance of the analyzed parameters in later periods could have resulted from the depletion of readily available compounds, leaving only those more resistant to microbial degradation. The key role here

was played by cellulose, which is the basic component found both in the spent mushroom substrate and manure (Leong *et al*., 2022). Confirmation of these observations comes from the significant positive correlations of cellulolytic fungi with TOC ($p<0.01$) (Fig. 10).

The slow mineralization of organic matter could also have been influenced by the root exudates from the developed plant biomass during the experiment. Reports from Wen *et al*. (2022) and Lei *et al*. (2023) highlighted the varied impact of root exudates on the mineralization of organic matter. Root exudates may disturb the homeostasis of the microbial C:N ratio. This, in consequence, may lead to inhibition of SOM decomposition by microorganisms that are responsible for these processes (Sun *et al*., 2021). Calcium carbonate may also be responsible for the deceleration of organic matter mineralization. This compound is one of the fundamental components of the spent mushroom substrate (Becher *et al*., 2021). Medina *et al*. (2012) suggested that organic carbon molecules are better protected from degradation by microbial activity in calcareous soils. In our research, mineral fertilization combined with SMS generally had a positive impact on the abundance of microorganisms. This was confirmed by the longest-lasting stimulatory effect observed in the plots with a lower NPK dosage for copiotrophic bacteria and a higher NPK dosage for filamentous fungi. The favorable conditions for the development of fungi in these combinations were likely due to a decrease in soil pH. This was confirmed by significant negative correlations between pH and the studied fungi ($p< 0.001$) (Fig. 10). Mineral fertilization contributed to a decrease in pH, which was particularly visible in combinations with its higher dose (Table 2). Other researchers also reported a decrease in soil pH as a result of mineral fertilization in their studies (Ge *et al*., 2018; Souza *et al*., 2023).

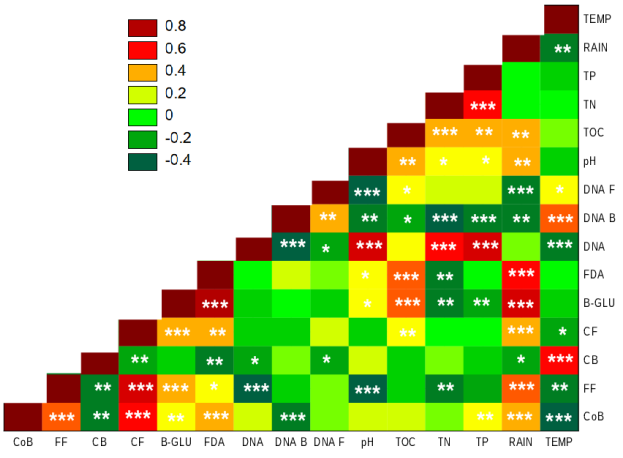

Fig. 10. Heatmap displaying the Pearson's correlation coefficients between soil physico-chemical, chemical, environmental factors and microbial, enzymatic activity. Signifcant at * $p<0.05$; ** $p<0.01$; *** $p<0.001$, respectively. CoB – copiotrophic bacteria, FF – filamentous fungi, CB – cellulolytic bacteria, CF– cellulolytic fungi, B-GLU – β-glucosidase, FDA – fluorescein diacetate hydrolysis activity; DNA – dsDNA concentration; TOC – total organic carbon, TN – total nitrogen, TP – total potassium; RAIN – rainfall, TEMP – temperature.

To assess the stability of agroecosystems subject to various agricultural practices, including fertilization, it is also necessary to track seasonal changes in the soil microbiome (Lacerda-Júnior *et al*., 2019). These changes are mainly due to fluctuations in temperature and humidity in field conditions. According to Li *et al*. (2022), bacteria show greater sensitivity to changes in rainfall compared to fungi. As our research shows, the response of soil microorganisms to the application of various types of fertilizers is also strongly dependent on climatic conditions. These observations were confirmed by significant correlations of all tested groups of bacteria and fungi with precipitation and temperature (Fig. 10). The analysis of the obtained correlations showed positive relationships between the studied groups of fungi and copiotrophic bacteria with precipitation, and negative relationships with temperature. In the case of cellulolytic bacteria, opposite relationships were observed. Positive correlations of bacteria and fungi with precipitation were at the highest level of significance in all cases, *i.e.* $p<0.001$. The relationship with the highest level of significance in the case of temperature occurred for both groups of bacteria. Changes in soil microorganisms under the influence of climatic conditions have been the subject of extensive research for many years (Šťovíček *et al*., 2017; Koyama *et al*., 2018; Li *et al*., 2022; Yu *et al*., 2022).

It is worth noting that significant correlations were also observed between almost all analyzed groups of microorganisms (Fig. 10). They may indicate a strong cooperation among microorganisms in the transformation of organic matter. Similar conclusions were also drawn by other authors who observed similar relationships between microorganisms under the influence of organic corrections (Luo *et al*., 2022).

The differences observed between the results obtained for dsDNA concentration and relative DNA abundance, both for bacterial and fungal communities, and the results acquired using the plate count method, are noteworthy. These observations may indicate the need to combine both of these techniques in the future. Regarding bacteria, these differences in the results could be due to the limited growth capacity of some groups of these microorganisms on artificial substrates (Rincon-Florez *et al*., 2013; Wydro, 2022). Concerning the result of fungal analysis, it is important to note that their growth and development correlated with an increase in relative DNA abundance, indicating the consistency between the results obtained using conventional and modern methods. Stimulation of fungal development in soil after spent mushroom substrate introduction, as assessed by molecular methods, has been reported *e.g.*, by Frąc *et al*. (2021). In the present study, the total pool of dsDNA, was significantly correlated with pH (Fig. 10). This is likely associated with changes in the soil environment resulting from the addition of exogenous organic matter and NPK fertilization, as mentioned earlier by the authors. The total pool of

dsDNA was also significantly positively correlated with the phosphorus and nitrogen content in the soil (Fig. 10). This is likely associated with NPK mineral fertilization, which can alter the composition and proportion of bacterial communities carrying genes encoding enzymes responsible for the transformations of these elements (Ye *et al*., 2020; Lang *et al*., 2021; Sieradzki *et al*., 2023). N and P are also the major building blocks of nucleic acids, which could further impact the observed correlations (Silberbach *et al*., 2005; Malhotra *et al*., 2018). Other authors also reported positive correlations between dsDNA concentration and the abundance of soil microorganisms (Wolińska, 2013; Joniec, 2019). In the current study, negative correlations were observed with copiotrophic and cellulolytic bacteria, as well as with filamentous fungi. Methods based on soil DNA extraction have many advantages, but they also bring certain concerns, such as distinguishing DNA from living and dead cells (Li *et al*., 2021; Roumani *et al*., 2023). The quantity and quality of isolated DNA depend on various factors, including soil type, soil conditions, microbial population, crop type, climate, and others (Wolińska *et al*., 2013; Rincon-Florez *et al*., 2013; Semenov, 2021; Wydro, 2022).

Soil enzymes are important parameters that allow monitoring changes in the soil environment, especially caused by human activities. Their sensitivity to changes in soil properties primarily results from their strong association with the content and quality of organic matter (Gajda *et al*., 2016; Adetunji *et al*., 2017; Song *et al*., 2017). Therefore, it can be assumed that the transformation products of the organic matter from the spent mushroom substrate and manure contributed to the stimulation of both β-glucosidase and fluorescein diacetate hydrolysis (FDA) activities in the initial years of our experiment. These observations were confirmed by the reported strong positive correlations ($p<0.001$) of TOC with the analyzed enzymes (Fig. 10). The soil pH played an important role in the activity of the enzymes studied by us. This was indicated by the observed positive correlations between β glucosidase and FDA activities and pH (Fig. 10). According to both Adetunji *et al*. (2017) and Dotaniya *et al*. (2019), β-glucosidase, due to its sensitivity to pH changes, can serve as one of the better indicators of soil quality. The hydrolysis of fluorescein diacetate (FDA) is carried out by many different enzymes (Dzionek *et al*., 2018; Patle *et al*., 2018), which can make it even more susceptible to fluctuations in soil pH. With respect to NPK combinations, the authors observed fluctuations in the activity of β-glucosidase and FDA hydrolysis. These variations could be attributed to the additional nitrogen and phosphorus source provided by mineral fertilization. The reported significant negative correlations of β glucosidase with TN and TP ($p<0.01$) and FDA with TN ($p<0.01$) can be considered as confirmation of these observations (Fig. 10). Nitrogen-induced stimulation of β glucosidase activity has been reported, among others, by Geisseler and Scow (2014). In contrast, Davies *et al*. (2022) reported that nitrogen had negligible effect on the activity of these enzymes, but noted that seasonal changes may have played a role in their activity. In our study, climatic factors such as precipitation also affected the activity of the enzyme parameters analyzed. This was evidenced by the recorded significant positive correlations of FDA and β-GLU with precipitation (Fig. 10). Noteworthy are the numerous positive correlations between the tested groups of microorganisms and the enzymes analyzed (Fig. 10). This could indicate their microbial origin, which is in line with the reports of Dotaniya *et al*. (2019) and Furtak and Gałązka (2019). Additionally, Furtak and Gałązka (2019) have pointed out that fungi are the main producers of β-glucosidase. The positive correlations ($p<0.001$) observed between FF and CF with β-glucosidase in our research may support this finding.

It is worth noting that the changes in microbial and enzymatic parameters persisted with varying intensities throughout the entire study period. The continuous occurrence of these changes may suggest that the new equilibrium in the soil fertilized with spent mushroom substrate has not yet been established during these 3 years.

5. CONCLUSIONS

The application of spent mushroom substrate has led to significant changes in the development of the analyzed bacterial and fungal groups. However, the beneficial impact of spent mushroom substrate became evident primarily in the initial period of the study, specifically in the first year.

In subsequent years of the study, the beneficial effects of spent mushroom substrate disappeared and even contributed to a decline in the growth of these microorganisms. In general, application of the waste in combination with mineral fertilization proved to be more favorable for the development of microbial groups than using spent mushroom substrate alone.

Regarding another indicator, namely the relative DNA content, an increase was observed under the influence of spent mushroom substrate. However, in contrast to the aforementioned population changes, this effect on the relative DNA content persisted for a longer period. The most beneficial approach was the combination of spent mushroom substrate with NPK fertilization, particularly with a lower NPK dose. It should be noted that in the soil treated with spent mushroom substrate, especially in combination with NPK fertilization, a decrease in the concentration of dsDNA was observed. However, this effect occurred only in the first and second year.

Initially, the use of spent mushroom substrate alone proved to be more favorable in terms of enzymatic activity. However, in the following year, it led to a decrease in enzymatic activity. The opposite trend occurred when spent mushroom substrate was applied in combination with mineral fertilization. It should be noted that both the stimulation and inhibition of enzymatic activity ceased in the third year of the study. The effect of different spent mushroom substrate fertilization treatments on enzymatic activity was not

as directional or consistent as observed for the previously discussed parameters. Furthermore, unlike the growth of bacteria and fungi, and the relative DNA content, the impact of spent mushroom substrate on enzymatic activity was observed only during the first two years of fertilizer application. This suggests that these parameters are more sensitive indicators of soil condition under these specific conditions compared to enzymatic activities.

The application of manure resulted in similar changes as the application of spent mushroom substrate. These observations indicate that fertilizing with spent mushroom substrate has a similar effect on the development and enzymatic activity of soil bacteria and fungi as traditional manure fertilization.

The observed inhibition of the development of the studied microbial groups in the third year of the study suggests that fertilization with spent mushroom substrate may exert only short-term beneficial effects, specifically for the first 1-2 years.

Changes in the analyzed indicators of microbiological activity, persisting with varying intensity, suggest that it is advisable to combine various research methods, *i.e.* classical and modern techniques, to monitor the alterations occurring in the soil fertilized with spent mushroom substrate.

As a continuation of the presented research, the authors plan to deepen this topic with a genetic analysis of bacterial and fungal communities in the soil with the addition of spent mushroom substrate.

Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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