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Assessment of the Effects of Soil Fertilization with Spent Mushroom Substrate in the Context of Microbial Nitrogen Transformations and the Potential Risk of Exacerbating the Greenhouse Effect

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Abstract: The intensification of agriculture leads to worrying changes in agro-ecosystems. Research has been conducted to bridge the gap between the desire to maintain ecological balance and harmful interference with ecosystems. Spent mushroom substrate (SMS) can become the basis of a farming system that improves soil quality. The aim of the study was to assess the potential of SMS in improving the following soil quality indicators: abundance and activity of microorganisms, and to assess the impact of SMS and manure (M) on the increase in the greenhouse effect. The plots were fertilized with SMS, M, and SMS in combination with NPK mineral fertilization. The application of SMS had a varied but generally positive effect on the parameters studied, particularly on the number of proteolytic microorganisms, urease activity but also ammonification and nitrification. In contrast, inhibition of protease activity was observed. The stimulation of most of the indicators was recorded in the first and second years, followed by a weakening of their effect. M also positively influenced the tested parameters, especially nitrification, where this effect lasted longer than for SMS. This indicates that the application of manure contributes more to the formation of products from which denitrification can potentially generate greenhouse gases.

Keywords: spent mushroom substrate (SMS); manure; soil enzyme activity; nitrification; ammonification; proteolytic bacteria and fungi; soil; the greenhouse effect

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1. Introduction

Soil is one of the most important natural resources of the Earth, non-renewable on the human time scale. It is the basis of human food production systems, crop cultivation for fodder, fiber, and fuel, and plays an important role in controlling and mitigating climate change [1,2]. Currently, in the age of rapidly advancing civilization, soil degradation is one of the most serious socio-economic and environmental problems that threaten the survival and well-being of mankind. Due to the progressive climate change and the rapidly growing population of the Earth, maintaining the quality of the soil at a high level, especially in agricultural areas, is considered one of the most critical challenges for society in the 21st century [3].

One of the problems related to soil degradation is the progressive deficit of organic matter (OM), which is one of the basic indicators of soil quality, dependent on various biotic and abiotic characteristics of the ecosystem [4]. With the currently worsening changes in climatic conditions, and thus also soil conditions, OM content is becoming increasingly important, not only for the proper functioning of ecosystems, but also for the socio-economic development of many regions of the world [5]. The deficit of organic matter is mainly observed in light (sandy) soils, due to the poorly developed aggregate structure, low water retention capacity, low nutrient levels, and poor

nutrient retention and exchange capacity [6–10]. It is estimated that this type of soil covers about 900 million ha in the world [9]. This phenomenon necessitates searching for methods to improve their quality and productivity. One of these is the introduction of increasing amounts of natural and organic fertilizers into soils [11]. Until recently, manure was the basic natural fertilizer that maintained an appropriate level of organic carbon and humus [12]. Currently, various types of waste are used to improve the fertility of soils, including spent mushroom substrate [10,13–19]. According to Gapiński [20], 1 m^3 of the substrate contains the amount of nutrients present in 2–3 m^3 of fresh cattle manure. Considering the deficit of organic matter in soils and the high fertilization value of mushroom waste, which is considered a source of humus formation, it seems reasonable to use this waste for soil fertilization [21,22]. Spent mushroom substrate has a high content of organic and mineral matter, thus it is rich in macro- and micro-nutrients, and above all in easily assimilable nitrogen [10,13–19]. After introducing this waste into the soil, it improves a number of its properties, including structure, pH, and water-holding capacity [12,20,23]. This waste is also used, among other areas, in bioremediation, plant cultivation in greenhouse and field crops, as a general supplement/fertilizer for soil, in the production of plant growth—promoting formulations, as well as nurseries and landscaping [24–28]. For the preparation of the mushroom growing substrate, various components are used, such as: straw, poultry manure, less often horse manure, nutrients, and structure-forming substances—urea, carbonates, coconut fiber, defatted soybean meal. Low or transitional peat, not silted or slightly silted, with a different proportion of high peat and alkalizing additives—dolomite, defecation lime is used as a cover [29]. It should be noted that in *Agaricus bisporus L.* cultivation, each 1 kg of fresh mushrooms generates 3.24 kg of fresh SMS [30]. Global mushroom and truffles production exceeded 41,736,063 tons in 2019 [31]. Meanwhile, the storage of spent mushroom substrate may have a negative impact on the environment due the weathering and leaching processes, contributing to the deterioration of air, soil, and water condition [27]. Introduction of waste into the soil, e.g., for fertilization purposes, also fits in with the idea of a circular economy. This idea is based on the appropriate selection of not only activities related to individual production stages, but also the reuse of waste generated as a result of this activity [28].

The growing interest of mankind in sustainable development and the desire to assess the impact of land use and management practices makes soil quality assessment one of the most important goals of modern science [3]. The microbiological, biochemical, and enzymatic properties of soils are considered useful indicators of soil quality, as these factors are sensitive to both environmental stress and anthropogenic changes [32,33].

Soil microorganisms transform biogens, including nitrogen, thereby supporting biogeochemical cycles [34]. Therefore, the intensity of biochemical processes and the content of products of soil microbial activity, e.g., CO_2, N-NO_3, N-NH_4, and N_2O ions, can be considered, in addition to the abundance, important soil biological activity indicators, reflecting its fertility [35]. The nitrogen cycle consists of several key processes, including ammonification, nitrification, or enzymatic processes of decomposition of organic nitrogen compounds for which specialized microorganisms are responsible [36]. Controlling the course of these processes (including nitrification, ammonification, urea hydrolysis) is also supported by the fact that the resulting gaseous products may contribute to the exacerbation of the greenhouse effect [37,38]. It is estimated that human activities related to agriculture emit about 60% CH_4, 15% CO_2 and 61% N_2O [39,40].

Soil enzymes are natural catalysts for many processes in the soil environment, including: processes of decomposition and formation of soil humus, organic matter decomposition, molecular nitrogen fixation, release of mineral nutrients, as well as their delivery to plants and circulation of elements [41,42]. They react fairly quickly and sensitively to both environmental and anthropogenic factors compared to other soil properties [43]. Of particular importance for soil transformations are hydrolases, especially proteases and ureases, which are involved in the soil nitrogen cycle [44]. These enzymes can be useful in developing and applying strategies of effective nitrogen management [45].

Better understanding of soil enzyme function and activity, as well as learning about soil biochemical properties, can lead to improved soil management and quality. Intensive

development and chemicalization of the economy are forcing the search for new natural alternatives to improve the quality of soils, without harmful interference with ecosystems. Therefore, the present study was conducted to examine and compare the influence of spent mushroom substrate and manure on the activity of microorganisms associated with the nitrogen cycle, i.e., ammonification, nitrification, protease and urease activity, and the number of proteolytic microorganisms. The authors made two hypotheses. One of them assumed that the use of spent mushroom substrate would improve soil quality indicators. The second hypothesis was that spent mushroom substrate, unlike manure, did not contribute to the increase in the greenhouse effect. Consequently, the authors assumed that the obtained results would be a guideline for sustainable soil management based on the condition of the soil microbiome and enzymatic activity, and spent mushroom substrate would find practical application in modern agriculture and become an alternative to manure.

2. Materials and Methods

2.1. Research Area and Characteristics of Experimental Plots

The field experiment was established at the Experimental Farm in Czesławice (Poland, Lublin Region, 51°18′′23′ N, 22°16′′02′ E) belonging to the University of Life Sciences in Lublin (Figure 1). The experiment was carried out in a random block design, in three replications, and the area of a single plot was 3 m^2 (1.5 m × 2.0 m). The individual plots were separated by 1 m wide paths. The experiment was located on loess soil, 2nd soil quality class [46,47]. 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%).

Figure 1. Location of the research area against the background of Europe, Poland and Lublin Region; red lines mark the area of Experimental Farm in Czesławice.

Spent mushroom substrate and cattle manure were applied for three years in a single dose of 20 t ha^{-1} in autumn. Supplementary mineral fertilization with nitrogen (N), phosphorus (P), and potassium (K) in the plots with spent mushroom substrate was applied in each year of the study in spring at two levels (N1P1K1 and N2P2K2) after the beginning of crop vegetation. 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 K-70 and K2-140 kg ha^{-1} in the form of potassium sulfate. The adopted doses and levels of supplemental NPK mineral fertilization were based on the initial abundance of bioavailable nutrients in the soil and the hypothesized rapid release of nutrients from spent mushroom substrate and, consequently, the short-term fertilization effect of spent mushroom substrate alone (without NPK fertilization). The control plot was soil without fertilization.

The substrate used in the experiment was composed of cereal straw (winter wheat), peat, and chicken manure. It should be noted that the substrate did not contain any mineral additives because it was intended for ecological cultivation. Characteristics of spent mushroom substrate and manure are presented in Table 1.

Table 1. Properties of soil und wastes.

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	15,800	2240
K		2350	6330	11,100
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	

Abbreviations: TOC—total organic carbon, TN—total nitrogen, TP—total potassium.

Experiment scheme:

- Soil without fertilizing, control (C);
- Soil + spent mushroom substrate (SMS);
- Soil + spent mushroom substrate + N1P1K1 (SMS + N1P1K1);
- Soil + spent mushroom substrate + N2P2K2 (SMS + N2P2K2);
- Soil + manure (M).

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.

2.2. Meteorological Conditions

The course of meteorological conditions during the experiment is shown in Figure 2. They were obtained from the Meteorological Station in Czesławice, located approximately 800 m from the field experiment. The presented data show that in the first two research years, i.e., 2018 and 2019, the annual sums of precipitation were similar and amounted to 539.3 mm and 481.8 mm, respectively. The year 2020, on the other hand, differed significantly from the first two years, as it had an annual rainfall of 799.7 mm. The highest monthly rainfall over the 3-year experimental period was recorded in the sampling months, i.e., June and September 2020, at 170.3 and 128.5 mm, respectively, and the lowest in June 2019 at 11.2 mm.

The average annual temperature in the initial year of the experiment, i.e., 2018, was 8.6 °C. It was significantly lower than the annual averages in 2019–2020, which were similar and amounted to 11.0 and 10.1 °C. Analyzing the weather conditions in the months of soil sampling, the highest temperature was recorded in June 2019 (22.9 °C), while the temperatures in the other periods (June 2018, September 2018, September 2019, June 2020, September 2020) were similar and amounted to 16.3, 14.7, 16.3, 17.9, and 15.6 °C, respectively.

2.3. Soil Sampling

The soil material was collected for a period of 3 years, twice during each growing season, i.e., in spring (June) and autumn (September). Topsoil samples (0–25 cm) were taken from 10 randomly selected sites from each plot using a gouging drill. Average soil sample from each plot consisted of a mixture of 10 soil cores, 4 cm diameter each. The samples were placed in plastic containers and stored at 4 °C to reduce any changes in microbial populations. Before the analyses, the soil samples were sieved through a sieve

with a 2 mm diameter. Microbiological, biochemical, and enzymatic tests in the collected soil material were performed within two weeks.

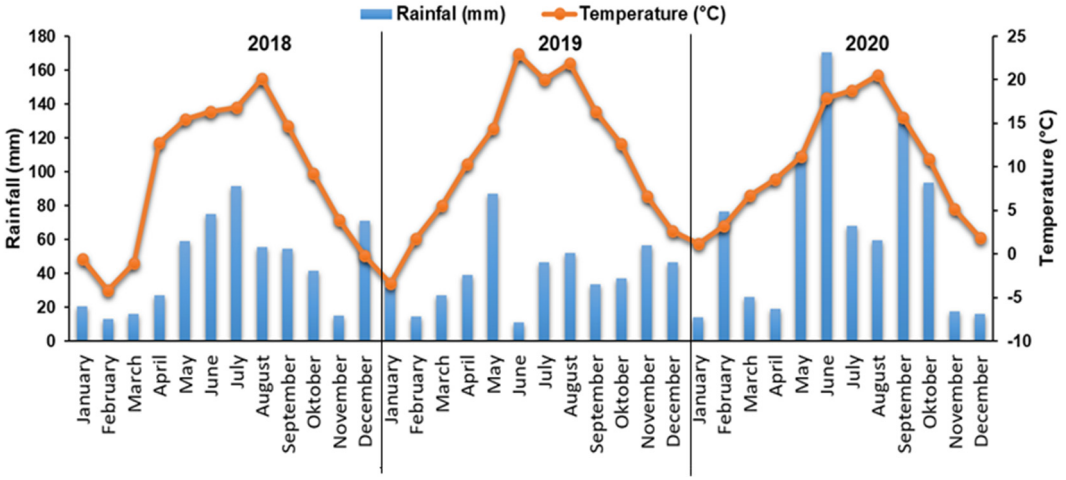

Figure 2. Mean monthly temperatures and rainfall in the experimental site during the experimental period.

2.4. Chemical Analyses

The microbiological and enzymatic analyses were supplemented with chemical determinations. The methods below were used for both soil samples, spent mushroom waste and manure (Table 1), and for soil samples at individual test time points (Table 2). The pH was measured by electrometry from soil extract in KCl (10 g of soil in 25 mL of KCl). Total N was measured by the Kjeldahl method, total organic carbon (TOC) by IR spectrometry, and total phosphorus using spectrophotometry. Calcium, potassium, and magnesium were determined by flame atomic absorption spectrometry (FAAS). Heavy metals were determined only for spent mushroom substrate by atomic absorption spectroscopy (AAS).

Table 2. Selected, physico-chemical and chemical properties of the soil.

	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

Table 2. *Cont.*

	Year	Season	C	SMS	SMS + N1P1K1	SMS + N2P2K2	M
TP g kg^{-1}	2020	spring	1.70	1.20	0.98	1.40	1.10
		autumn	0.97	0.80	1.20	0.55	1.10
	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

Abbreviations: 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.

2.5. Microbiological Analyses

The number of bacteria and protein-decomposing fungi was determined in the soil material, using the plate method on the Frazier gelatin substrate, following the procedure described by Foght and Aislabie [48,49]. For fungi, antibiotics were added to the medium in the amounts recommended by Martin [50]. Cultures were carried out for bacteria at 28 °C for 4 days, and for fungi at 25 °C for 3 days. After incubation, plate surfaces were poured over with a thin layer of Frazier's reagent (water solution of HCl and $HgCl_2$—Chempur, Piekary Śląskie, Poland) with protein denaturing properties, manifested by a milky color of the medium. Both in the case of bacteria and fungi, only colonies surrounded by a transparent zone were counted, which indicated proteolytic abilities. The results are expressed in colony forming units (cfu) per gram dry weight.

2.6. Enzymatic Analyses

Protease activity was determined in 2 g soil samples incubated in 0.1 M tris (hydroxymethyl) aminomethane buffer (Tris-HCl pH 8.0—Sigma-Aldrich, Wien, Austria) for 1 h at 50 °C using sodium caseinate solution—Sigma-Aldrich, Wien, Austria, (5 mL) as a substrate [51]. The level of released tyrosine was measured spectrophotometrically at 578 nm. Urease activity was determined by the method of Zantua and Bremner [52] in 10 g soil samples using urea solution—Chempur, Piekary Śląskie, Poland, as a substrate and incubating for 18 h at 37 °C. Ammonium ion concentration was measured spectrophotometrically at a wavelength of 410 nm. A UV 1800 spectrophotometer (Rayleigh, Beijing, China) was used to measure the enzyme activity.

2.7. Biochemical Analyses

Ammonification activity was determined in 25 g soil samples with 0.1% asparagine—Sigma-Aldrich, Wien, Austria. Ammonium ions were extracted, after 3 days of incubation, with 2 M KCl—Chempur, Piekary Śląskie, Poland, (stirred for 20 min) and their content was determined using the Nessler method [53]. Intensification of the nitrification process was determined in 25 g soil samples using 0.1% ammonium phosphate as a substrate—Chempur, Piekary Śląskie, Poland. After 7 days of incubation, nitrate ions were extracted with 2 M KCl (stirred for 20 min) and their levels were measured using the brucine method [53]. A UV 1800 spectrophotometer (Rayleigh, Beijing, China) was used to measure the biochemical activity.

2.8. Statistical Analysis

All analyses were performed in three parallel repetitions and presented as a mean of these repetitions. The results were statistically analyzed using STATISTICA version 13.0 software (TIBCO Software Inc., Palo Alto, CA, USA) with ANOVA models and mul-

tiple Tukey's *t*-tests at the significance level of $\alpha = 0.05$. In order to check whether the assumptions of ANOVA, including normality of the dataset and homogeneity of variance were met, the Shapiro–Wilk and Levene tests were used, respectively, and showed that indeed these criteria were fulfilled. The results are presented in graphs with standard deviation indicated. The results were additionally correlated with the obtained chemical parameters and presented in the form of a heat map. Cluster analysis was used to identify groups of objects showing similarity in terms of: microbial abundance and enzymatic and biochemical activity. Agglomeration of properties was assessed using Ward's cluster analysis method with Euclidean distance.

3. Results

3.1. Abundance of Microorganisms

The results presented in Figure 3A–C and Table 3 showed that the application of spent mushroom substrate generally resulted in positive changes in proteolytic bacteria abundance. The severity of these changes varied with time, as well as with the method of fertilization.

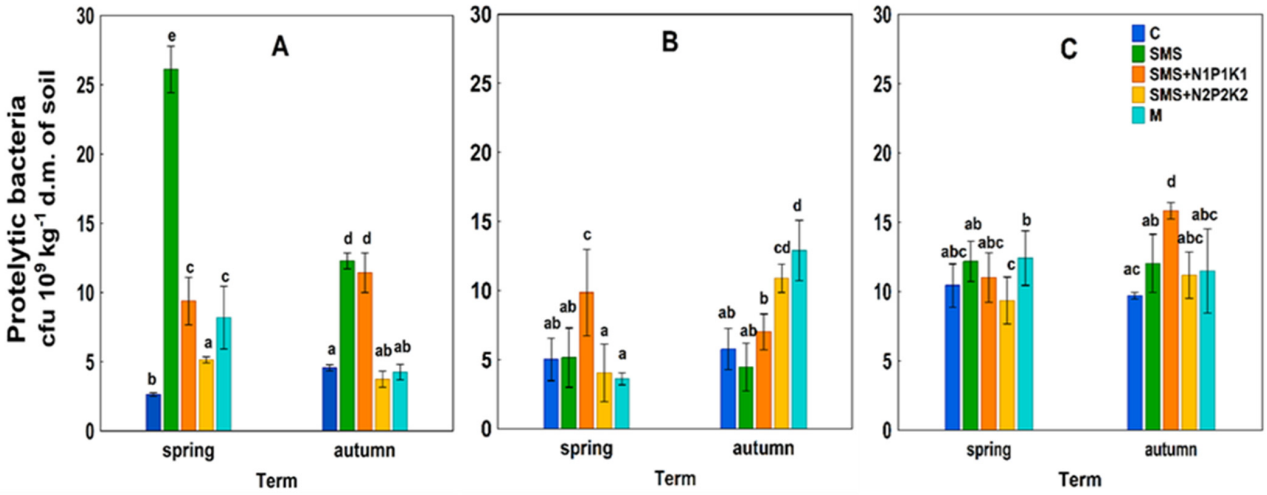

Figure 3. Number of proteolytic bacteria in the soil. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure; (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences, each year was analyzed independently of each other.

The strongest stimulation of this group of microorganisms was recorded in spring in the first year of the experiment in combination only with spent mushroom substrate (SMS) where its abundance was 26.1 cfu 10^9 kg^{-1}, compared to only 2.6 cfu in control (Figure 3A). Stimulation of the development of proteolytic bacteria was also recorded in the remaining plots with SMS both in the spring (SMS + N1P1K1, SMS + N2P2K2) and autumn (SMS, SMS + N1P1K1), but at a significantly lower level. In the second year of the experiment, the positive effect of SMS weakened (Figure 3B) and became apparent only in the spring in the plot with a lower dose mineral fertilization (SMS + N1P1K1) and in the autumn in the plot with mineral fertilization at a higher dose (SMS + N2P2K2). Bacterial counts in these plots were 9.8 and 10.9 cfu, respectively, compared to 5.1 and 5.7 cfu in the control plots. In the last year of the experiment (Figure 3C), the impact of SMS was visible only in the autumn in the plot where it was applied in combination with a lower dose of mineral fertilization (SMS + N1P1K1). The abundance of proteolytic bacteria was 15.8 cfu at this plot. In the remaining plots with the substrate (SMS, SMS + N2P2K2), the abundance was at a level

similar to that in control (C). The application of manure throughout the study period in most time points did not significantly affect the growth of the tested group of bacteria. Its stimulating effect on the growth of these bacteria was observed only in the spring of the first year (Figure 3A) and in autumn in the second year (Figure 3B). In these plots (M), 8.2 and 12.9 cfu were recorded at individual time points, respectively.

Table 3. Microbiological, enzymatic, and biochemical activity in soil (Annual averages).

Year	Experimental Treatments	PB	PF	URE	PRO	AM	NIT
2018	C	3.60 a	112.31 a	417.75 g	9.45 b	35.29 a	8.79 ab
	SMS	19.20 j	215.39 f	887.20 i	10.84 bc	36.32 a	12.99 abc
	SMS + N1P1K1	10.40 gh	377.14 h	508.27 h	12.63 cd	40.37 a	5.95 a
	SMS + N2P2K2	4.44 ab	276.58 g	156.63 a	6.76 a	41.25 ab	12.83 abc
	M	6.22 bc	290.16 g	412.21 g	12.60 cd	37.45 a	21.22 bcd
2019	C	5.39 ab	110.68 a	193.39 a	11.96 bc	60.98 bc	17.59 abcd
	SMS	4.81 ab	108.78 a	275.76 bcd	12.29 cd	67.76 c	65.77 h
	SMS + N1P1K1	8.43 def	188.25 def	251.21 b	23.17 g	285.94 d	43.05 fg
	SMS + N2P2K2	7.48 cd	198.65 ef	306.82 cd	15.97 e	267.04 d	44.65 fg
	M	8.25 de	156.62 bcd	325.29 de	14.90 de	72.04 c	79.29 i
2020	C	10.07 efg	194.96 ef	311.10 cd	19.58 f	36.72 a	38.41 ef
	SMS	12.11 hi	136.28 abc	362.48 ef	16.17 e	38.82 a	27.15 de
	SMS + N1P1K1	13.42 i	206.27 ef	382.99 fg	21.05 fg	35.89 a	36.04 ef
	SMS + N2P2K2	10.26 fgh	130.27 ab	413.97 g	16.21 e	33.85 a	22.68 cd
	M	11.95 ghi	170.38 cde	397.90 fg	16.82 e	30.58 a	53.64 gh

Abbreviations: 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. PB—proteolytic bacteria (cfu 10^9 kg^{-1} d.m. of soil), PF—proteolytic fungi (cfu 10^6 kg^{-1} d.m. of soil), URE—urease (mg N-NH_4 kg^{-1} d.m. of soil 18 h^{-1}), PRO—protease (mg tyrosine kg^{-1} d.m. of soil h^{-1}), AM—ammonification (mg N-NH_4 kg^{-1} d.m. of soil 3 d^{-1}), NIT—nitrification (mg N-NO_3 kg^{-1} d.m. of soil 7 d^{-1}). Different letters indicate significant differences at $p < 0.05$.

The influence of the spent mushroom substrate and manure on the development of proteolytic fungi was generally positive, and its intensity varied significantly over the three years (Figure 4 A–C and Table 3). Regarding the fungal abundance, the highest values were also recorded in the first year in spring, but with the application of spent mushroom substrate in combination with a lower dose of mineral fertilization (SMS + N1P1K1). Fungal abundance was 416.71 cfu 10^6 kg^{-1} in this facility, compared to 79.95 cfu in the control soil (C) (Figure 4A). This year, the number of mushrooms was also increased in the remaining plots with SMS but to a lower extent. In the second year (Figure 4B), proteolytic fungi were favorably affected by the application of spent mushroom substrate, but in combination with two variants of mineral fertilization (SMS + N1P1K1, SMS + N2P2K2). Fungal abundance in spring was lower than in autumn and was 24.34 in the control (C) plot and 126.39 and 81.15 cfu, respectively, in the plots where stimulation was recorded. In contrast, in autumn, fungal growth in the control plot (C) was 196.43 cfu, and 250.1 and 316.15 cfu, respectively, in plots where microorganisms were stimulated. In the third year (Figure 4C), the beneficial effect became visible only in single sites with mineral fertilization, i.e., in spring with its higher dose addition (SMS + N2P2K2), and in autumn with a lower dose (SMS + N1P1K1). Fungal abundance at these plots was 179.32 and 335.26 cfu, respectively. It should be noted that a decrease in the number of these microorganisms was recorded in autumn in the plot with only SMS and SMS together with mineral fertilization at a higher dose (SMS + N2P2K2), which in these plots amounted to 183.2 and 81.2 cfu, respectively.

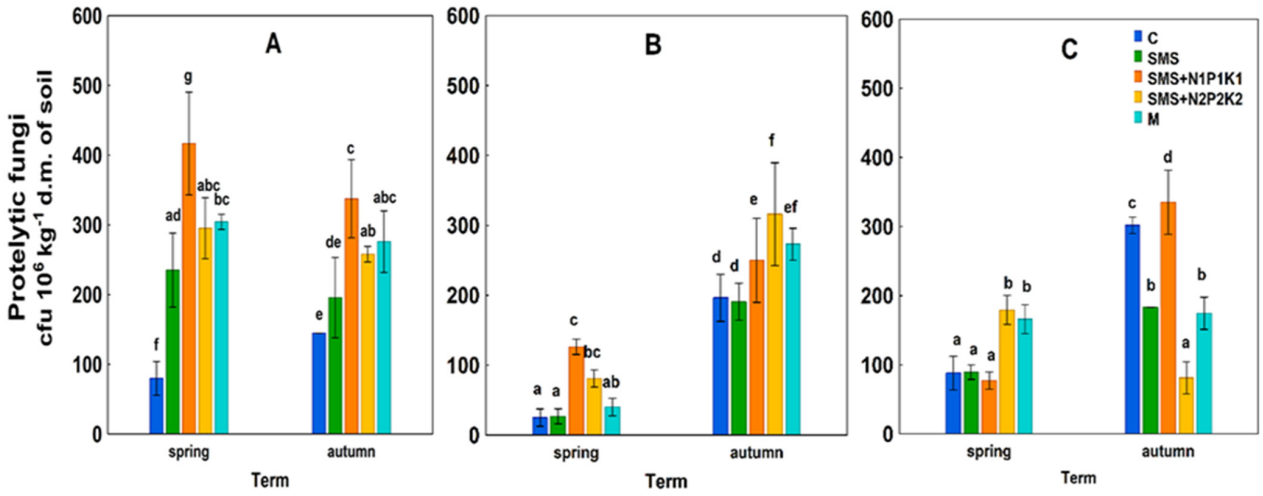

Figure 4. Number of proteolytic fungi in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure; (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences, each year was analyzed independently of each other.

Manure also generally exerted a stimulating effect on proteolytic fungi, but slightly lower than SMS applied in the different variants. In the first and second year, the values for this fertilization were the highest, generally ranging from 273.13 to 304.36 cfu. In addition, the lowest value, i.e., 40.11 cfu, was recorded in the spring of the second year. Fungal stimulation was still evident at this site in the spring of the third year, with a count of 166.15 cfu. Inhibition of the development of the analyzed microorganisms under the influence of manure was recorded only in the autumn in the third year of the study. Their number was 174.62 cfu, while in control it was 301.73.

3.2. Enzymatic Activity

The analysis of protease activity during the 3-year experiment showed significant changes under the influence of the applied fertilization with spent mushroom substrate in different variants and with manure (Figure 5A–C and Table 3). This effect varied and was observed with different intensity depending on the type of fertilizer and the time of its action. Protease activity throughout the experiment was most strongly affected by the application of SMS in combination with a lower dose of mineral fertilization (SMS + N1P1K1). The highest value for this enzyme was recorded in this plot in spring in the second year (35.80 mg kg^{-1}), while in the control plot, it was lower and amounted to 11.64 mg (Figure 5B). Stimulation of the discussed parameter, but weaker, was also noted in other plots in this period (SMS, SMS + N2P2K2) and in the first year in spring in the plot with SMS alone (Figure 5A). Protease activity in these objects was at the level of 15.56, 20.37, and 10.31 mg, respectively. In the remaining time points and years, the use of spent mushroom substrate in individual variants did not have a significant effect on protease activity or caused its inhibition. It should be noted that inhibition was observed at certain time points in plots with SMS or applied together with a higher fertilization dose (SMS + N2P2K2). The strongest decrease was recorded in the third year in spring, when protease activity in these plots was 16.32 and 18.48 mg, respectively, while in the control plot, it was 24.25 mg (Figure 5C).

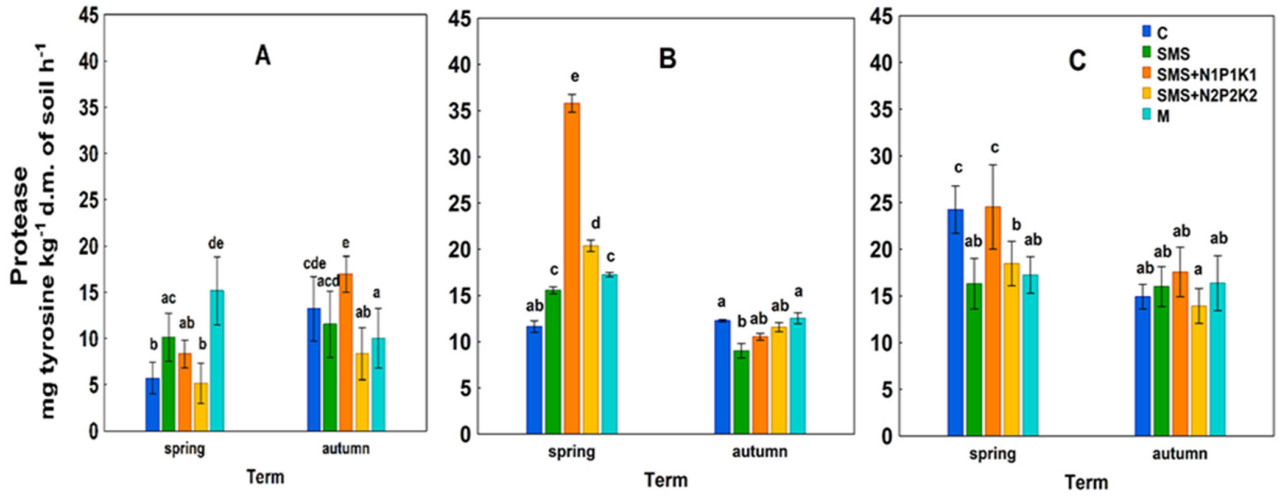

Figure 5. Activity of protease in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure; (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences, each year was analyzed independently of each other.

The application of manure also resulted in a differential effect on protease activity. The highest values of this fertilization variant (M) were recorded in spring, both in the 1st and 2nd year of the experiment, they were 15.15 and 17.27 mg, respectively, while only 5.72 and 11.64 mg in the control plots. In the remaining years and seasons, no effect of manure or an inhibitory effect on this parameter was visible.

With respect to urease activity, the use of SMS caused an increase in this parameter in a greater number of plots and time points compared to protease (Figure 6A–C and Table 3). It was most noticeable in the first year of fertilization, especially with SMS, and ranged from 797.10 to 977.30 mg kg^{-1}, while 312.07–523.42 mg in the control soil (C) (Figure 6A). Moreover, the use of SMS together with a lower dose of mineral fertilization (SMS + N1P1K1) turned out to be beneficial, and the activity in this plot was 573.51 mg. In the case of the object with a higher dose of mineral fertilization (SMS + N2P2K2), a decrease in this parameter was noted as compared to the control soil, where in the spring in the first year, it was the lowest in the entire research period (67.68 mg). In the second and third year, the tendency of urease activity was similar, all the applied SMS variants (SMS, SMS + N1P1K1, SMS + N2P2K2) had a stimulating effect on the tested parameter. It is noteworthy that the stimulating effect of SMS was more pronounced in the second year (Figure 6B). The activity in the plots with SMS ranged from 361.00 to 485 mg, while it was 249.52 mg in control. On the other hand, in the third year, the stimulation was weaker and the activity in the plots with SMS ranged from 478.26 to 342.61 mg, while this activity in control (C) was 460.87 mg (Figure 6C).

In general, the use of manure during the three years of the experiment, similarly to SMS, had a positive effect on urease activity. It exerted the most beneficial effect in the spring of the first year, when the activity of this enzyme was 598.83 mg. At other time points, its positive impact was weaker. The decrease in the discussed enzymatic parameter in the plot with manure (M) was recorded only in the autumn of the first year.

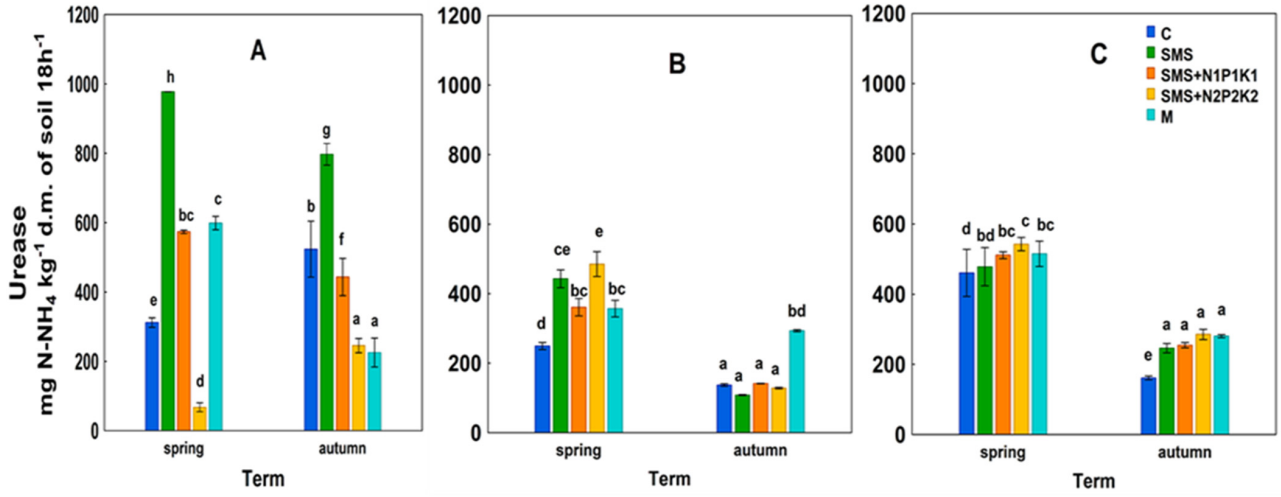

Figure 6. Activity of urease in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure; (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences, each year was analyzed independently of each other.

3.3. Biochemical Activity

Figure 7A–C and Table 3 show data on the effects of SMS and M in individual variants on the ammonification process. The analysis of the data showed that SMS application in various combinations generally had a small but nevertheless stimulating effect on this parameter throughout the study period.

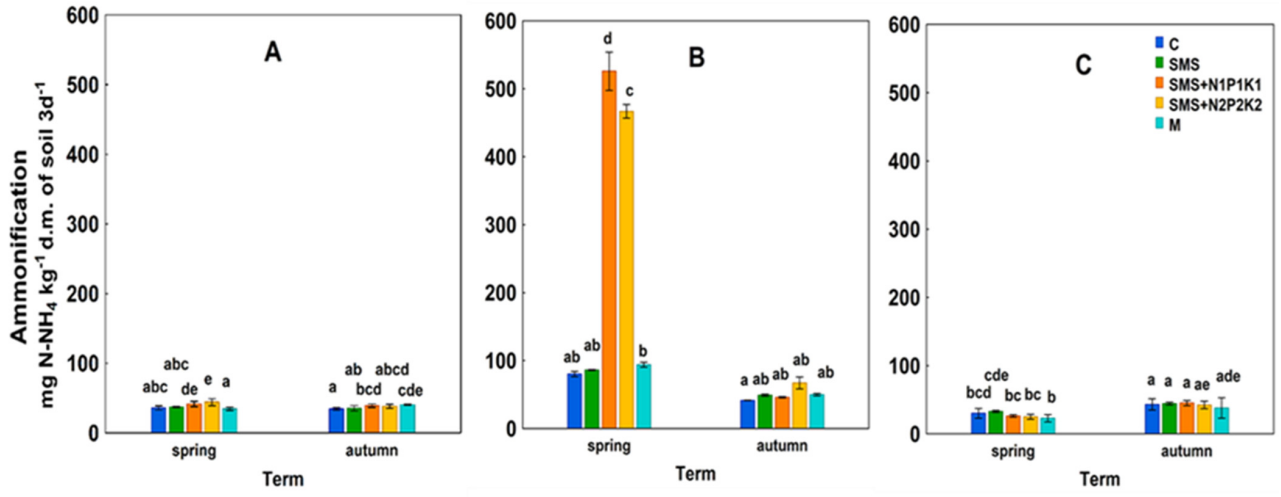

Figure 7. Ammonification in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure; (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences, each year was analyzed independently of each other.

It should be noted that only in the spring of the second year, the application of SMS in combination with supplemental mineral fertilization caused a clear stimulation of the

ammonification process in both variants (SMS + N1P1K1, SMS + N2P2K2), (Figure 7B); this activity was 525.91 mg kg^{-1} and 466.86 mg, respectively, and only 80.47 mg in the control (C) soil. Stimulation of ammonification was also recorded at these plots in the first year, but its level was significantly lower (Figure 7A).

The ammonification process in combination with manure was at a level similar to control during the 3 years of research (Figure 7A–C) Only in the autumn of the first year, a slight significant stimulation of this activity was recorded in the soil enriched with manure (M).

The course of nitrification in the analyzed seasons and years of research is presented in Figure 8A–C and Table 3. As in the case of ammonification, the most favorable effect of waste fertilization on nitrification was recorded in the spring of the second year.

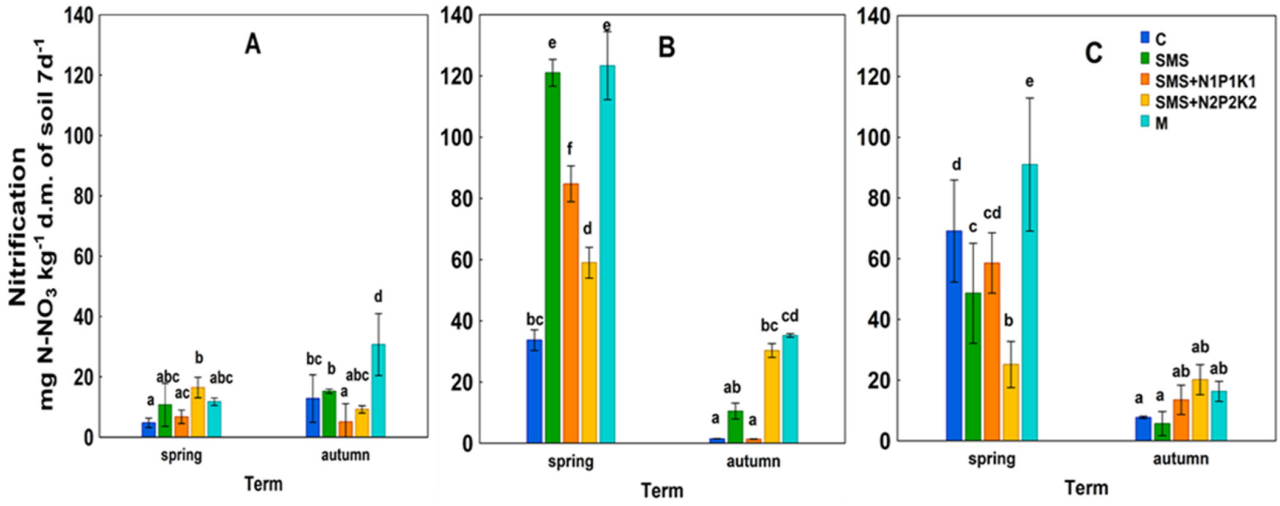

Figure 8. Nitrification in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure; (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences, each year was analyzed independently of each other.

The highest value was recorded in combination with SMS alone, i.e., 121.01 mg, followed by the plots with mineral fertilization (SMS + N1P1K1, SMS + N2P2K2), where the activity was 84.75 and 58.89 mg, respectively, and only 33.67 mg in the control (C) (Figure 8B). A clear stimulation was also recorded in the autumn, but only in the plot with fertilization with a higher dose of mineral fertilizer (SMS + N2P2K2), where the value of the tested parameter was 30.30 mg, while only 1.50 mg in control (C). With regard to nitrification activity, the duration of fertilization application had a negative effect on this biochemical activity. In spring, in the third year of application of the tested waste, inhibition was observed in all combinations with the waste (SMS—48.65 mg; SMS + N1P1K1—58.56 mg; SMS + N2P2K2—25.18 mg) in relation to the control plot (C—69.11 mg) (Figure 8C).

Manure, as compared to spent mushroom substrate, exerted a stronger and more significant effect on nitrification. In all years, stimulation of this process was recorded in the plot with manure (M), most clearly visible in the second year of the study both in the spring and autumn. The highest activity was recorded in the spring of the second year, and it was 123.34 mg.

4. Discussion

4.1. Abundance of Microorganisms

The amount of nitrogen available to plants in soil depends on the processes of nitrogen immobilization and mineralization. These processes are carried out by a variety of soil microbiota, which initiates and is responsible for virtually all processes occurring in the soil, but its activity, abundance, and biodiversity depend on many environmental factors, including, e.g., the availability of organic matter [54–56]. This is probably the main factor that positively affected the number of proteolytic microorganisms, both bacteria and fungi, in the present study. Stimulation of their development was probably caused by the supply of additional nutrients, whose main source in this study was SMS. This was confirmed by the significant positive correlations found between proteolytic bacteria and TOC (0.63) and TP (0.23), and between fungi and TP (0.38) and TN (0.23) (Figure 9). We could assume that it was SMS and SMS applied together with NPK, as the primary source of these components, was the main activator of these two groups of microorganisms. The available literature shows that SMS is an organic waste material rich in macro- and micronutrients, especially nitrogen, which are readily available to plants [57,58]. The positive effect of organic waste on microbial growth was also observed in a study by Frąc et al. [14], Joniec [59] and Joniec et al. [60].

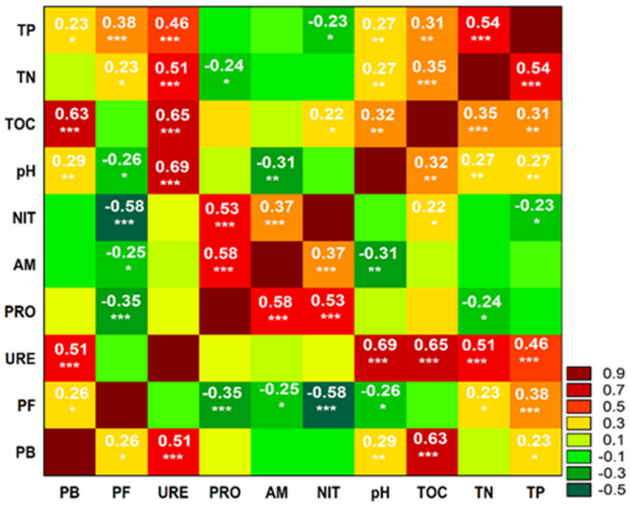

Figure 9. Heatmap displaying the Pearson's correlation coefficients between soil physico-chemical, chemical, and microbial properties. BP—proteolytic bacteria, PF—proteolytic fungi, URE—urease, PRO—protease, AM—ammonification, NIT—nitrification, TOC—total organic carbon, TN—total nitrogen, TP—total potassium. Significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, respectively.

The stimulation effect lasted longest in the plots with the addition of mineral fertilizer, low doses of which were shown to have a positive effect on the microbiological and agrochemical properties of the soil, as they accelerated the rate of decomposition and increased the amount of soil organic matter [55,61]. Currently, some authors have suggested that nutrients, such as nitrogen or phosphorus have a more restrictive effect on microorganisms compared to pH [56,62]. Our study partially confirmed this because it also showed significant correlations of microbial abundance with pH (Figure 9), but at a significance level of $p > 0.01$ for bacteria and at $p > 0.05$ regarding fungi. The positive correlation of fungi with phosphorus were at a higher level of significance ($p > 0.001$). Cluster analysis showed that the abundance of bacteria and fungi differed in the plots with waste applied together with NPK from the plot with waste alone and control. This confirmed previous observations regarding the significant influence of organic matter and p on these parameters (Figure 10A).

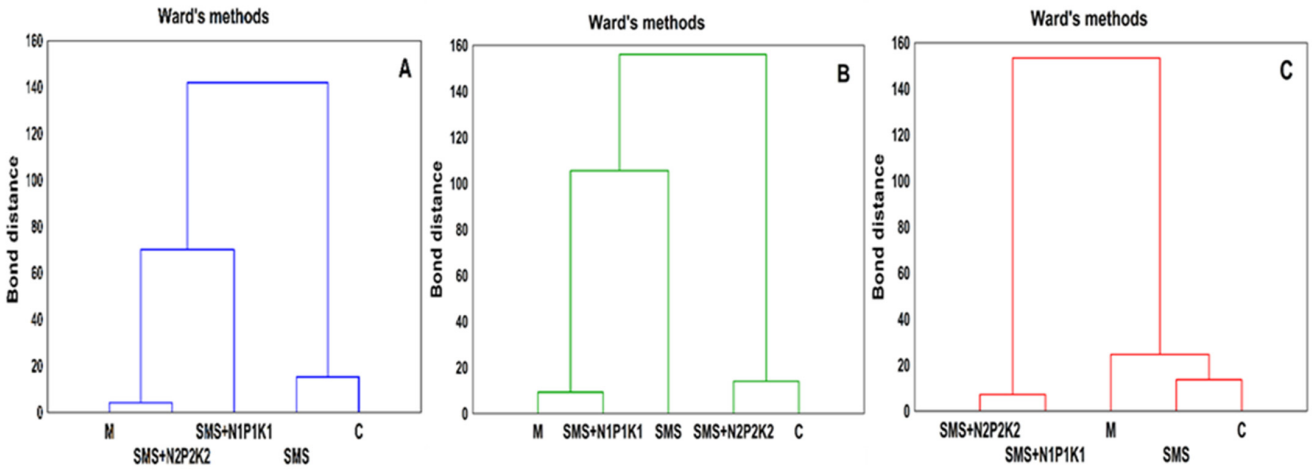

Figure 10. Tree diagram—Ward's dendogram for (**A**) microbial counts; (**B**) enzymatic activity; and (**C**) biochemical activity. C—control soil; SMS—soil + spent mushroom substrate, SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure.

The growth of bacteria and fungi, and consequently their activity, is also largely influenced by climatic conditions, including humidity and temperature [63]. Seasonal changes in the abundance of bacteria and fungi can be caused by fluctuations in temperature and humidity under field conditions (Figure 2). The stronger changes observed in bacteria were probably due to their higher sensitivity to unfavorable conditions compared to fungi, which showed greater resistance [63]. The increase in the number of fungi, persisting longer and in a higher number of plots with SMS alone, compared to bacterial counts, could suggest that fungi were better adapted to utilize this additional nutrient source. In contrast, Wang et al. [58] found that these were bacteria, compared to fungi, that could acclimate to new conditions resulting from the addition of spent mushroom substrate to the soil in a shorter period of time.

Manure, unlike SMS, did not significantly affect the development of the microbial groups throughout the experiment. This could be due to the fact that SMS was characterized by a diversified but higher content of organic matter compared to cattle or pig manure [57,64].

4.2. Enzymatic Activity

Further indicators of soil quality, i.e., soil enzymes are closely related to the soil microbiome. They can be of both plant and animal origin, but primarily their main source is microorganisms [65]. Their activity is strongly associated with the biomass and structure of microbial communities, substrate availability, the size of soil aggregates and environmental conditions. Literature data indicate that hydrolases are strongly related to the content of organic matter in soil, and thus directly involved in its mineralization [66]. Therefore, we can assume that similarly as for the microbiological parameter, transformation products of spent mushroom substrate and the changes they caused in the soil environment contributed to the stimulation of urease activity in our study. This was also confirmed by positive correlations of urease activity with TOC (0.65), TN (0.51), TP (0.46), and pH (0.69) (Figure 9). The strong correlation of urease with proteolytic bacteria (0.51) might also confirm that SMS, as the primary source of organic matter, was the main activator of this enzyme, but might also suggest that this enzyme was of microbial origin. Stimulation of urease activity under SMS was also observed by Kuziemska et al. [67] and Ma et al. [68]. It should be noted that urease activity, in contrast to the plots with SMS, was significantly higher in the plots with manure during the entire study period. This observation could indicate a higher probability of the adverse phenomenon of nitrogen loss from the soil through the release into the atmosphere of gaseous products of reactions catalyzed by urease, i.e., ammonia, precisely in the plots with manure.

The situation was opposite for protease, because according to our analysis, it was inhibited over time in individual SMS plots. Perhaps this was related to the fact that the production of extracellular proteases as a result of catabolic repression was inhibited by readily available carbon [65]. Land use and soil organic matter affect the N cycle through modifications in the composition of microbial communities involved in this cycle, especially proteolytic microorganisms [69]. In the present study, there was a negative correlation of protease activity with fungi (−0.35), suggesting that they were not the main producers of these enzymes under the conditions analyzed (Figure 9). Similar observations were noted by Graham et al. [70], who reported that bacteria rather than fungi were mainly responsible for the release of proteases. In addition, the lack of correlation of proteases with the abundance of proteolytic bacteria supports the thesis that abundance does not always translate into proteolytic activity [69]. This is because individual microorganisms may encode more or less efficient proteases. In addition, gene expression is regulated by many environmental factors including C, P, Ca, pH, or humidity [65]. Therefore, the recorded changes in protease activity may also be due to climatic conditions, i.e., humidity and temperature.

Cluster analysis showed that enzyme activity differed between the control plot and the plots with organic fertilization, i.e., SMS and M, as well as the plot with SMS in combination with NPK, but its lower dose. This indicated that the combination with N2P2K2 was the least favorable for these activities (Figure 10B).

4.3. Biochemical Activity

Nitrogen, as we have repeatedly pointed out, is one of the most important biogenic elements in nature with a key role in the survival of all living organisms. Its circulation in the environment consists of a number of different processes that are part of the so-called nitrogen cycle, responsible for most of the element's transformations and playing a key role in its fate in the Earth's ecosystems [34]. The nitrogen cycle is a whole cycle of individual and interdependent processes, such as ammonification and nitrification. Ammonification is the process of producing ammonia from the decomposition of organic nitrogen, while nitrification involves the oxidation of ammonia to nitrite NO_2^- and then to nitrate NO_3^+ [71]. According to Sierra et al. [72], the accumulation of mineral forms of nitrogen as a result of mineralization of waste organic matter can be an adverse environmental phenomenon. It is related to the leaching of the mineral form of nitrogen, which, in turn, poses a risk of water pollution and loss of this element from the soil. Therefore, the disappearance of ammonification stimulation in time in the plots with SMS indicated the lack of such risk. The mutual positive correlations (at the significance level of $p > 0.001$) between the activity of proteases, intensity of ammonification and nitrification processes recorded in this study indicated that the nitrogen cycle at these stages proceeded without interference (Figure 9).

The next stage of this cycle is denitrification. Both nitrification and denitrification are important sources of N_2O in agricultural soils [73,74]. Denitrification causes direct emissions of nitrous oxide (N_2O), one of the major greenhouse gases (GHGs), with about 320 times higher greenhouse-forming potential than CO_2 [73]. Denitrifiers are microorganisms that use nitrification products in their respiratory processes. The effect of this reduction, among others, is precisely N_2O, classified as a greenhouse gas [34]. Therefore, the disappearance of nitrification process intensification in the plots with SMS, or even its inhibition over time, was a favorable phenomenon. At the same time, it should be noted that the intensification of the nitrification process in the plot with manure was generally stronger and subject to stimulation throughout the study period. This observation supports the hypothesis that fertilizing with SMS carries a lower risk of exacerbating the greenhouse effect than fertilizing with manure. Cluster analysis showed that the process of ammonification and nitrification was different in the plots with waste applied separately and in combination with mineral fertilization, and yet different in the plot with manure (Figure 10C). This confirmed the observation that the addition of manure permanently

enhanced the process of nitrification, while the effect of spent mushroom substrate on this parameter in the other plots disappeared.

The observed changes in the intensity of the nitrification process from season to season may have been due to the influence of temperature and humidity. The dependence of nitrifiers, denitrifiers, and thus the impact of N_2O emissions on temperature conditions was previously reported by Lai et al. [74].

Better understanding of these individual microbial N-oxide reduction pathways in soil will allow for better management practices to increase N utilization efficiency and reduce greenhouse gas emissions, as agricultural soils are the main anthropogenic sources of greenhouse gases and are responsible for approximately 60% of CH_4, 15% of CO_2, and 61% of N_2O emissions [39]. The use of organic waste in agriculture leads to neutralization and improvement of soil quality, but can also lead to atmospheric pollution by increasing greenhouse gas emissions from the soil [75].

In summation, it should be noted that the used mushroom substrate and manure had a significant effect on microbiological nitrogen transformations. These wastes, with varying degrees of intensity, stimulated or inhibited individual stages of the circulation of this nutrient. The severity of disturbed soil environment homeostasis may also have negative effects on air quality.

5. Conclusions

The spent mushroom substrate caused an increase in the number of proteolytic bacteria and fungi at individual time points. It should be noted that this effect has weakened in time, and even disappeared in certain variants. It lasted longest in plots with waste applied in combination with mineral fertilization. The effect of waste on enzymatic activity was not as unidirectional as in the case of abundance and was subject to changes over the three years of the study. Urease activity was stimulated at most time points, mainly in the plot with waste alone, and then with mineral fertilization. This effect intensified over time. In contrast, protease activity was subject to inhibition with time in individual plots with SMS. Ammonification and nitrification processes were stimulated in the plots with SMS, but at three time points. With time, this effect weakened, and even a decrease in the intensity of nitrification was observed. Our research showed that SMS application resulted in an improvement of the analyzed microbiological, enzymatic, and biochemical parameters, which translated into a higher overall fertility and quality of the soil. Thus, the first hypothesis that the application of spent mushroom substrate would improve soil quality indicators was confirmed.

Manure also had a generally positive effect on the parameters studied. It should be noted that its stimulation of the nitrification process lasted longer than in the case of SMS. This confirmed the authors' second hypothesis, which assumed that spent mushroom substrate, to a lesser extent, contributed to the increase in the amount of nitrification products, which could then potentially lead to greenhouse gas formation, i.e., N_2O, thereby contributing to the increase in the greenhouse effect.

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