UNIWERSYTET PRZYRODNICZY w LUBLINIE

Wydział Agrobioinżynierii
Dyscyplina naukowa Rolnictwo i Ogrodnictwo

mgr inż. Edyta Kwiatkowska

Rozprawa doktorska

Parametry aktywności bakterii i grzybów glebowych oraz fitotoksyczność gleby, jako wskaźniki oceny skutków oddziaływania na środowisko odpadów pochodzących z rolnictwa i przemysłu chemicznego

Parameters of soil bacterial and fungal activity and soil phytotoxicity as indicators for assessing the environmental impact of waste from agriculture and chemical industry

Rozprawa doktorska wykonana w
Katedrze Mikrobiologii Środowiskowej

Promotor: dr hab. Jolanta Joniec, profesor uczelni

Lublin 2024

Pragnę złożyć najserdeczniejsze podziękowania
Pani dr hab. Jolancie Joniec, prof. uczelni
za wiarę we mnie,
za cenne wskazówki i rady w trakcie tworzenia tej rozprawy oraz ogólnej współpracy,
za cierpliwość, życzliwość, wyrozumiałość, poświęcony czas oraz ogromne wsparcie,
a także za zaufanie,
Dziękuję!

Chciałabym również podziękować
Panu Prof. dr hab. Cezaremu Kwiatkowskiemu
za udostępnienie modelu doświadczalnego
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Spis treści

1. Wykaz publikacji naukowych stanowiących przedmiot rozprawy doktorskiej

PUBLIKACJA P.1

Joniec J., **Kwiatkowska E.**, Kwiatkowski C.A. 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. *Agriculture*, **2022**, 12, 1190. https://doi.org/10.3390/agriculture12081190

MEiN 100 pkt; IF = 3,6

Indywidualny wkład pracy w publikację: udział w opracowaniu koncepcji badań, udział w zaplanowaniu badań, przeprowadzenie wszystkich badań, opracowanie wyników i analiza statystyczna, udział w redagowaniu manuskryptu, udział w procesie publikacyjnym oraz w poprawie manuskryptu po recenzjach, jako autor korespondencyjny.

PUBLIKACJA P.2

Kwiatkowska E., Joniec J. Effects of agricultural management of spent mushroom waste on phytotoxicity and microbiological transformations of C, P, and S in soil and their consequences for the greenhouse effect. *Int. J. Environ. Res. Public Health*, **2022**, 19, 12915. https://doi.org/10.3390/ijerph191912915

MEiN 140 pkt; IF = 0,0

Indywidualny wkład pracy w publikację: *udział w opracowaniu koncepcji badań, udział w zaplanowaniu badań, przeprowadzenie wszystkich badań, opracowanie wyników i analiza statystyczna, udział w redagowaniu manuskryptu, udział w poprawie manuskryptu po recenzjach.*

PUBLIKACJA P.3

Kwiatkowska E., Joniec J., Kwiatkowski C.A, Kowalczyk K., Nowak M., Leśniowska-Nowak J. 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. *Int. Agrophys.*, **2024,** 38, 139-154. https://doi.org/10.31545/intagr/184175

MEiN 100 pkt; IF = 2,2

Indywidualny wkład pracy w publikację: *udział w opracowaniu koncepcji badań, udział w zaplanowaniu badań, przeprowadzenie wszystkich badań, opracowanie wyników i analiza statystyczna, udział w redagowaniu manuskryptu, udział w poprawie manuskryptu po recenzjach.*

PUBLIKACJA P.4

Kwiatkowska E., Joniec J., Kwiatkowski C.A. Involvement of soil microorganisms in C, N and P transformations and phytotoxicity in soil from post-industrial areas treated with chemical industry waste. *Minerals*, **2023,** 13, 12. https://doi.org/10.3390/min13010012

MEiN 100 pkt; IF = 2,5

Indywidualny wkład pracy w publikację: *udział w opracowaniu koncepcji badań, udział w zaplanowaniu badań, udział w pobieraniu materiału glebowego, przeprowadzenie wszystkich badań, opracowanie wyników i analiza statystyczna, udział w redagowaniu manuskryptu, udział w poprawie manuskryptu po recenzjach.*

Dane naukometryczne prac wchodzących w skład rozprawy:

Liczba punktów **MEiN** [według punktacji obowiązującej w roku wydania pracy]:	**440**
Impact Factor [zgodnie z rokiem opublikowania]:	**8,3**
Liczba cytowań wg Web of Science /bez autocytowań	**7/4**
Liczba cytowań wg Scopus /bez autocytowań	**8/5**

2. Streszczenie

Degradacja gleb jest zjawiskiem nieuniknionym i stanowiącym realne zagrożenie dla ograniczenia jej użyteczności. Głównymi czynnikami przyczyniającymi się do pogłębiania tego zjawiska oraz nasilających się w związku z tym zmian klimatu są obecnie intensywne rolnictwo oraz przemysł. Wczesna ocena degradacji środowiska glebowego może zapobiec jej dalszemu postępowaniu. Dlatego też istnieje silna potrzeba poszukiwania nowych oraz weryfikacji przydatności już używanych wskaźników do monitorowania stanu środowiska glebowego. Analiza możliwości wykorzystania parametrów mikrobiologicznych może odegrać istotną rolę zarówno w opracowaniu zrównoważonego zarządzania ekosystemami, jak i w polityce ochrony środowiska glebowego, uwzględniającej postępowanie z różnymi odpadami.

W związku z tym głównym celem niniejszej rozprawy doktorskiej była próba weryfikacji przydatności wskaźników mikrobiologicznych do monitorowania stanu środowiska glebowego, poddanego działaniu różnych odpadów, pochodzących z działalności zarówno rolniczej, jak i przemysłowej. Wykorzystano do tego celu takie parametry jak: ogólną liczebność bakterii oligotroficznych, kopiotroficznych oraz grzybów strzępkowych, liczebność bakterii i grzybów celulolitycznych oraz proteolitycznych, względną zawartość DNA, stężenie dsDNA, nasilenie procesów amonifikacji i nitryfikacji, aktywność oddechową, aktywność enzymów glebowych (proteazy, ureazy, dehydrogenaz, fosfatazy kwaśnej i zasadowej, arylosulfatazy, β-glukozydazy, aktywność hydrolityczną diooctanu fluoresceiny (FDA)), a także wskaźniki fitotoksyczności gleby. Badane parametry analizowano na tle właściwości fizycznych, chemicznych i fizykochemicznych, oraz warunków środowiskowych, takich jak opady atmosferyczne i temperatura.

Badania zostały oparte na dwóch modelach doświadczalnych. Materiał glebowy, w pierwszym modelu, stanowiła gleba pochodząca z trzyletniego doświadczenia polowego, w którym poszczególne poletka zostały nawiezione podłożem popieczarkowym oddzielnie lub łączenie z nawożeniem mineralnym NPK w dwóch dawkach lub obornikiem. Drugi model badawczy zlokalizowany był na glebie pochodzącej z terenu poprzemysłowego, narażonej na oddziaływanie płynnego odpadu z przemysłu chemicznego. Materiał glebowy pobierano w trzech punktach zlokalizowanych w różnej odległości od zbiornika z odpadem.

Badania dotyczące oddziaływania podłoża popieczarkowego wykazały, że odpad ten wpłynął na ogół pozytywnie na liczebność badanych bakterii i grzybów, a także na

względną zawartość DNA oraz na większość badanych parametrów związanych z przemianami mikrobiologicznymi węgla i azotu w glebie. Jednak wpływ ten wraz z upływem czasu osłabł. Natomiast w przypadku aktywności enzymów związanych z przemianami fosforu i siarki oraz zawartości dsDNA oddziaływanie zastosowanego podłoża popieczarkowego było z reguły negatywne. Należy zaznaczyć, że efekt zmniejszył się w kolejnych latach. Wyniki dotyczące wpływu odpadu i obornika na nasilenie procesów oddychania i nitryfikacji w glebie wykazały, że odpad popieczarkowy bardziej niż obornik przyczynił się do nasilenia emisji CO_2 z gleby. Natomiast stymulujący wpływ obornika na proces nitryfikacji, którego produkty mogą być transformowane do N_2O, utrzymywał się znacznie dłużej niż odpadu popieczarkowego.

Badania dotyczące oceny degradacji gleby ciekłym odpadem wykazały istotne zmiany poziomu aktywności wszystkich zastosowanych parametrów w poszczególnych punktach poboru prób. Najbardziej czułymi okazały się: ogólna liczebności bakterii i grzybów, aktywności fosfatazy kwaśnej i zasadowej oraz aktywność hydrolityczna fluoresceiny. Najniższymi wartościami omawianych aktywności oraz najwyższą fitotoksycznością charakteryzowała się gleba zlokalizowana najbliżej zbiornika z odpadem. W tym punkcie odnotowano również nasilenie emisji CO_2 z gleby.

Uzyskane wyniki wskazują, że analizowane w niniejszych badaniach parametry jakości gleby, użyte na tle właściwości chemicznych, fizycznych i fizykochemicznych, są czułymi wskaźnikami zmian zachodzących w glebie poddanej oddziaływaniu różnej antropopresji. Przedstawione badania sugerują również, że do monitorowania zmian zachodzących w glebie nawiezionej odpadem popieczarkowym wskazane jest łączne stosowanie różnych metod badawczych, zarówno klasycznych, jak i nowoczesnych. Użyte techniki okazały się dobrym narzędziem do oceny skuteczności zastosowanych zabiegów nawozowych oraz ryzyka związanego z powstawaniem gazów cieplarnianych w glebie poddanej oddziaływaniu różnych odpadów. Poniższe badania mogą być pomocne przy ograniczeniu negatywnych skutków rolniczej działalności człowieka, jak i ocenie stopnia degradacji środowiska glebowego spowodowanej oddziaływaniem zbiorników z ciekłymi odpadami, a także przy ocenie skuteczności ich zabezpieczeń.

Słowa kluczowe: wskaźniki biologiczne, podłoże popieczarkowe, degradacja, odpady, bakterie i grzyby glebowe, gazy cieplarniane, fitotoksyczność

Summary

Soil degradation is an unavoidable phenomenon and poses a real threat by limiting soil usability. Currently, intensive agriculture and industry are the primary factors contributing to the exacerbation of this phenomenon and the resulting increasing climate changes. Early assessment of soil degradation can prevent its further progression. Consequently, there is a pressing need to explore new indicators and reassess the effectiveness of existing ones for monitoring the condition of the soil environment. The analysis of the potential utilization of microbiological parameters can play a significant role both in developing sustainable ecosystem management and in environmental soil protection policies that would include the management of various types of waste.

Therefore, the main objective of this dissertation was to verify the usefulness of microbiological indicators for monitoring the condition of the soil environment subjected to the influence of various types of waste originating from both agricultural and industrial activities. For this purpose, parameters were utilized such as the total abundance of oligotrophic and copiotrophic bacteria, filamentous fungi, the abundance of cellulolytic and proteolytic bacteria and fungi, relative DNA content, dsDNA concentration, intensity of ammonification and nitrification processes, respiratory activity, soil enzyme activity (proteases, ureases, dehydrogenases, acid and alkaline phosphatases, arylsulfatases, β-glucosidases, fluorescein diacetate hydrolytic activity - FDA), as well as soil phytotoxicity indicators. The analyzed parameters were examined considering the physical, chemical, and physicochemical properties, as well as environmental conditions such as atmospheric precipitation and temperature.

The research was based on two experimental models. Soil material in the first experimental model consisted of soil from a three-year field experiment, where individual plots were fertilized with ash substrate separately or in combination with two doses of NPK mineral fertilization, or with manure. The second research model was situated on soil originating from an industrial area, exposed to the influence of liquid waste from the chemical industry. Soil samples were collected at three points located at varying distances from the waste reservoir.

The research on the influence of spent mushroom substrate indicated that this waste generally had a positive impact on the abundance of the studied bacteria and fungi, as well as on the relative DNA content, and most of the analyzed parameters related to microbial transformations of carbon and nitrogen in the soil. However, this impact gradually

diminished over time. Regarding the activity of enzymes associated with phosphorus and sulfur transformations, as well as the dsDNA content, the influence of the applied spent mushroom substrate was generally negative. It should be noted that this effect decreased in subsequent years. The results regarding the influence of waste and manure on the intensity of soil respiration and nitrification processes indicated that spent mushroom substrate contributed more than manure to the intensification of CO_2 emissions from the soil. However, the stimulating effect of manure on the nitrification process, whose products can be transformed into N_2O, persisted significantly longer than that of spent mushroom substrate.

The research concerning the assessment of soil degradation by liquid waste showed significant changes in the level of activity of all applied parameters at individual sampling points. The most sensitive parameters were found to be: total abundance of bacteria and fungi, activity of acid and alkaline phosphatase and fluorescein hydrolase. The soil located closest to the waste reservoir exhibited the lowest values of the discussed activities and the highest phytotoxicity. At this site, an intensification of soil CO_2 emissions was also recorded.

The results obtained in this study suggest that the analyzed soil quality parameters, when considered alongside the chemical, physical, and physicochemical properties, can serve as sensitive indicators of changes occurring in soil affected by different human pressures. The present research also suggests that for monitoring changes occurring in soil fertilized with spent mushroom substrate, it is advisable to employ a combination of various research methods, both classical and modern. The techniques used proved to be effective tools for assessing the effectiveness of the applied fertilization treatments and the risk associated with greenhouse gas emissions in soil subjected to the influence of various wastes. The studies presented here can be helpful in mitigating the negative effects of human agricultural activities and assessing the degree of soil environmental degradation caused by the influence of liquid waste reservoirs, as well as in evaluating the effectiveness of their protection measures.

Keywords: biological indicators, spent mushroom substrate, degradation, waste, soil bacteria and fungi, greenhouse gases, phytotoxicity.

3. Wstęp

Gleba to jeden z najważniejszych zasobów naturalnych Ziemi. Jest podstawą systemów produkcji żywności, uprawy roślin na paszę, błonnik oraz paliwo, a także odgrywa ważną rolę w zwalczaniu i łagodzeniu zmian klimatycznych [Trasar-Cepeda i in., 2016; Lehmann i in., 2020]. Równocześnie środowisko glebowe jest niezwykle wrażliwe i wystawione na szereg zagrożeń, zarówno ze względu na szybko postępujące zmiany klimatyczne, jak i intensywną działalność człowieka [Borrelli i in., 2020; Kuzyakov i Zamanian, 2019]. Wszelkie procesy i działania powodujące pogorszenie właściwości fizycznych, chemicznych i biologicznych pedosfery określane są mianem degradacji gleby.

Obecnie w dobie szybko postępującej cywilizacji, degradacja gleb jest jednym z najpoważniejszych problemów społeczno-ekonomicznych i środowiskowych zagrażających przetrwaniu i dobrobytowi ludzkości [Santorufo i in., 2021]. Według FAO 33 % gleb na Ziemi jest już zdegradowanych, a ponad 90% może jej ulec do 2050 r. Największy odsetek terenów zagrożonych degradacją lub już zniszczonych występuje w Europie (15,2 %), Afryce (10,7 %) oraz w Azji (10,4 %) [FAO i ITPS, 2015; IPBES, 2018].

Przyczyny degradacji gleby są złożone i mają różnorodny charakter. Najczęściej definiowana jest ona na podstawie trzech, ściśle ze sobą powiązanych, aspektów: fizycznego, chemicznego oraz biologicznego. Degradacja fizyczna dotyczy m.in. erozji wodnej i osuwisk. Obejmuje przemieszczenie i/lub zmianę położenia cząstek gleby bez zmiany ich składu chemicznego. Degradacja chemiczna wiąże się głównie z wysoką koncentracją soli w roztworach glebowych, z naruszeniem równowagi jonowej gleby, zakwaszeniem jej albo nadmierną alkalizacją. Degradacja biologiczna dotyczy w szczególności spadku ilości i jakości materii organicznej gleby, a także obniżenia bioróżnorodności organizmów glebowych, zarówno makrofauny, jak i mikroflory [Keesstra in., 2018; Saljnikov i in., 2022].

Postępujący deficyt materii organicznej, która jest jednym z podstawowych wskaźników jakości gleby, zależnym od różnych biotycznych i abiotycznych cech ekosystemu, jest jednym z problemów związanych z degradacją gleby [Rutkowska i Pikuła, 2013]. Przy aktualnie pogłębiających się zmianach warunków klimatycznych, a co za tym idzie także glebowych, zawartość materii organicznej nabiera coraz większego znaczenia nie tylko dla prawidłowego funkcjonowania ekosystemów, ale także dla rozwoju społeczno-gospodarczego wielu regionów świata [Komatsuzaki i Ohta, 2007]. Deficytem

materii organicznej charakteryzują się przede wszystkim gleby lekkie (piaszczyste), ze względu na słabo rozwiniętą strukturę agregatową, małą zdolność do retencji wody, niski poziom składników odżywczych oraz słabą zdolność do ich przechowywania i wymiany [Peake i in., 2014; Osman, 2018; Yost i Hartemink, 2019; Zhou i in., 2019; Usowicz i Lipiec, 2021]. Szacuje się, że ten rodzaj gleb zajmuje na świecie ok. 900 ml ha [Yost i Hartemink, 2019]. Zjawisko to wymusza poszukiwanie sposobów poprawienia ich jakości i produktywności. Jednym z nich jest wprowadzanie do gleb coraz większych ilości nawozów naturalnych i organicznych [Zhou i in., 2019; Frąc i in., 2021; Kwiatkowski i Harasim, 2021; Lipiec i in., 2021].

Duży potencjał nawozowy wykazują zwłaszcza odpady rolnicze, powstające na obszarach wiejskich w wyniku przetwórstwa płodów rolnych i działalności rolniczej. Jednym z takich odpadów o charakterze organicznym jest podłoże po uprawie pieczarki (*Agaricus bisporus* L.) [Hanafi i in., 2018]. Według the Food and Agriculture Organization Corporate Statistical Database, wielkość światowej produkcji grzybów i trufli w 2020 roku wyniosła 42 792 893 ton, podczas gdy na przykład w 2000 r. zaledwie 8 781 004 ton, czyli 20 % całkowitej aktualnej produkcji. Na świecie głównym producentem grzybów i trufli są zdecydowanie Chiny (40 004 574 ton w 2020 r.), natomiast w Europie (1 270 241 ton w 2020 r.) przodują głównie Holandia (260 000 ton – 2020 r.), Polska (182 900 ton – 2020 r.) oraz Hiszpania (166 010 ton – 2020 r.) [FAOSTAT, 2022]. Tak intensywna światowa produkcja skutkuje powstawaniem ogromnych ilości zużytego podłoża grzybowego, które szacuje się na około 60 mln ton rocznie [Leong i in., 2022]. Efektywne wykorzystanie i utylizacja tak dużej ilości wytworzonego corocznie materiału jest dużym wyzwaniem dla współczesnej gospodarki.

Ze względu na skład (głównie wysoką zawartość materii organicznej) źle składowany odpad popieczarkowy może stanowić zagrożenia dla środowiska, poprzez rozwój mikroflory patogennej i rozprzestrzenianie się chorób grzybowych, niekontrolowaną biodegradację odpadów przez mikroorganizmy i w konsekwencji emisję gazów cieplarnianych do atmosfery oraz wymywanie związków biogennych do wód powierzchniowych i gruntowych [Rinker, 2017; Leong i in., 2022].

Z powodu rosnących obaw o środowisko, niezbędna jest odpowiednia utylizacja i postępowanie z nadmiernie gromadzonym podłożem popieczarkowym. Aktualne badania jednoznacznie wskazują, że rolnicze wykorzystanie jest najlepszym sposobem jego recyklingu, w związku z dużymi walorami nawozowymi [Owaid i in., 2017; Kwiatkowski i Harasim, 2021; Velusami i in., 2021; Prasad i in., 2022]. Podłoże to jest cennym źródłem

substancji organicznej oraz składników pokarmowych łatwo dostępnych dla roślin [Owaid i in., 2017; Zhou i in., 2019; Frąc i in., 2021]. Wprowadzone do gleby poprawia szereg jej właściwości w szczególności: strukturę, odczyn, a także pojemność wodną [Malińska i in., 2018; Lipiec i in., 2021]. Odpad ten wykorzystywany jest również m.in.: w bioremediacji, do uprawy roślin w uprawach szklarniowych i polowych, w produkcji preparatów promujących wzrost roślin, w szkółkach i kształtowaniu krajobrazu [Paula, 2017; Rinker, 2017; Corral-Bobadilla i in., 2019; Zied i in., 2020; Kwiatkowski i Harasim, 2021]. Te sposoby zagospodarowania podłoża popieczarkowego pozwalają również rozwiązać pośrednio problem innych odpadów, tj. tych, które zostały wcześniej użyte do jego skomponowania. Do przygotowania podłoża do uprawy pieczarek stosuje się różne składniki, takie jak: słoma, obornik drobiowy, rzadziej obornik koński, substancje odżywcze oraz strukturotwórcze – mocznik, węglany, włókno kokosowe i odtłuszczoną śrutę sojową. Jako przykrycie wykorzystuje się torf niski lub przejściowy, niezamulony lub lekko zamulony, z różnym udziałem torfu wysokiego i dodatków alkalizujących – dolomitu, wapna [Becher, 2013]. Ponadto zużyte podłoże popieczarkowe może być poddawane kompostowaniu także z użyciem np. gnojowicy czy osadów ściekowych, co dodatkowo pozwala na recykling kolejnych odpadów [Grimm i Wösten, 2018; Meng i in., 2018]. Biorąc pod uwagę dużą różnorodność i zmienność poszczególnych podłoży popieczarkowych zalecane jest badanie ich składu oraz ewentualne zbilansowanie składników poprzez uzupełnienie nawożeniem mineralnym. Wprowadzenie odpadu do gleby m.in. w celach nawozowych wpisuje się również w ideę gospodarki o obiegu zamkniętym. Idea ta polega na odpowiednim doborze nie tylko działań związanych z poszczególnymi etapami produkcji, ale również ponownym wykorzystaniem odpadów, które powstają w wyniku tej działalności [Zied i in., 2020].

Ściśle z degradacją fizyczną i biologiczną związana jest również degradacja chemiczna, która według Richmonda [2015] jest, zaraz po erozji, jej najbardziej rozpowszechnioną formą. Degradacja chemiczna będąca skutkiem antropopresji może być związana z niektórymi praktykami agrotechnicznymi (np. nadmierne stosowanie nawozów mineralnych, herbicydów, insektycydów), ale przede wszystkim z intensywnym rozwojem przemysłu, zwłaszcza rolno-spożywczego, papierniczego i celulozowego [Richmond, 2015; Gaur i in., 2020; Srivastava i in., 2023]. Jak podają Gaur i in. [2020] przemysł celulozowo-papierniczy należy do najbardziej zanieczyszczających gałęzi przemysłu na świecie, generując niebezpieczne ścieki i odpady na dużą skalę. Niekontrolowane, źle zlokalizowane i niewłaściwie zaprojektowane składowiska różnych rodzajów odpadów,

takich jak stałe i ciekłe odpady komunalne lub przemysłowe, uważane są za poważne potencjalne źródła zanieczyszczeń ekosystemów lądowych i wodnych [Gaur i in., 2020; Mester i in., 2022; Srivastava i in., 2023]. Na stopień zanieczyszczenia mają wpływ, takie czynniki jak: ilość i skład odcieków, czas eksploatacji obiektu, rodzaj gleby, poziom wód gruntowych, odległość od gruntów rolnych lub środowiska wodnego [Mester i in., 2022]. Mogą mieć one charakter zanieczyszczenia rozproszonego, jak i punktowego, który ma wpływ na biotyczne i abiotyczne funkcje gleby, jakość upraw oraz zdrowie zwierząt i ludzi [Richmond, 2015; Keesstra in., 2018]. Nagromadzenie w środowisku glebowym różnych substancji toksycznych prowadzi między innymi do naruszenia równowagi jonowej gleby, a także jej zakwaszenia lub nadmiernej alkalizacji [Keesstra in., 2018; Saljnikov i in., 2022]. Odczyn gleby determinuje losy substancji w środowisku glebowym, wpływa na liczne biologiczne, chemiczne i fizyczne właściwości gleby oraz procesy, które wpływają na aktywność mikroorganizmów, wzrost roślin i plon biomasy [Neina, 2019]. Niektóre mikroelementy są bardziej dostępne w warunkach kwaśnych, podczas gdy inne w warunkach zasadowych. Rozwój silnie kwaśnych gleb (poniżej 5,5 pH) może skutkować słabym wzrostem roślin. Natomiast gleby alkaliczne charakteryzują się zmniejszoną dostępnością fosforu i mikroskładników, co też wpływa negatywnie na rośliny [Jiang in., 2017]. W związku z tym, iż chemiczne i biologiczne procesy degradacji wchodzą ze sobą w interakcje, wymagają one dokładnego i stałego monitorowania.

Degradacja gleby jest zjawiskiem nieuniknionym, stanowiącym realne zagrożenie dla realizacji wizji 17 Celów Zrównoważonego Rozwoju Organizacji Narodów Zjednoczonych [Keesstra in., 2018]. Dlatego należy dążyć do łagodzenia jej skutków przy jednoczesnym utrzymaniu wydajności rolnictwa i równowagi społeczno-ekologicznej. Intensywny rozwój i chemizacja gospodarki wymuszają szukanie nowych naturalnych alternatyw dla poprawy jakości stanu gleb, bez szkodliwego ingerowania w ekosystemy.

Przy wyborze sposobu zagospodarowania odpadów organicznych, w tym generowanych w rolnictwie, należy wziąć pod uwagę możliwość emisji gazów cieplarnianych w wyniku przemian węglowej i azotowej materii. Rolnictwo jest podstawowym czynnikiem przyczyniającym się do ich emisji, którą szacuje się na 10 % do 20% całkowitej antropogenicznej emisji gazów cieplarnianych [Allen i in., 2020]. Zarówno nawozy, jaki i odpady, zwłaszcza organiczne, zawierają duże ilości węgla organicznego, którego zasoby na gruntach rolnych odgrywają kluczową rolę w zrównoważonym rolnictwie. To właśnie materia organiczna, której głównym źródłem może być podłoże popieczarkowe, wpływa na tempo mineralizacji i gromadzenia się bądź

emisji węgla z gleby oraz na złożone interakcje między glebowymi procesami biologicznymi i fizyko-chemicznymi a warunkami środowiskowymi [Rahman, 2013]. Sekwestracja glebowa węgla, czyli zwiększanie w glebie ilości tego pierwiastka, zmagazynowanego jako materia organiczna, może poprawić jakość gleby i zmniejszyć udział rolnictwa w emisji CO_2 [Minasny i in., 2017; Navarro-Pedreño i in., 2021]. Istotne jest, aby z wniesieniem materii organicznej do gleby wraz z odpadami, jednocześnie analizować wpływ tej aplikacji na procesy glebowe i aktywność drobnoustrojów. Biorąc pod uwagę, że około 90 % CO_2 emitowanego z gleby jest pochodzenia mikrobiologicznego, jest to główny strumień w ramach globalnego obiegu węgla, który emituje do atmosfery około 10 razy więcej CO_2 rocznie niż spalanie paliw kopalnych [Bond-Lamberty i Thomson, 2010; Le Que´re´i in., 2013]. Dokładniejsze zrozumienie poszczególnych procesów mikrobiologicznych związanych z przemianami tlenków azotu w glebie, pozwoli na stosowanie lepszych praktyk zarządzania środowiskiem glebowym, mających na celu zwiększenie efektywności wykorzystania tego biogenu i równoczesne ograniczenie emisji gazów cieplarnianych. Według IPCC [2013] gleby rolnicze są głównymi antropogenicznymi źródłami gazów cieplarnianych i odpowiadają za około 60 % emisji CH_4, 15 % CO_2 i 61 % emisji N_2O. Wykorzystanie odpadów organicznych w rolnictwie prowadzi do poprawy jakości gleb, ale może także prowadzić do zanieczyszczenia atmosfery poprzez zwiększenie emisji gazów cieplarnianych z gleby [Jezierska-Tys i Frąc, 2007]. W związku z postępującymi zmianami klimatycznymi i szybko rosnącym zaludnieniem Ziemi, utrzymanie jakości gleby na wysokim poziomie, szczególnie na obszarach rolniczych, uznawane jest za jeden z najbardziej krytycznych wyzwań dla społeczeństwa XXI wieku [Santorufo i in., 2021].

Rosnące obecnie zainteresowanie zrównoważonym rozwojem oraz chęć oceny wpływu użytkowania gruntów i praktyk zarządzania nimi powoduje, że jednym z najważniejszych celów współczesnej nauki, zajmującej się środowiskiem glebowym, jest zrozumienie znaczenia jakości gleby i jej ocena [Santorufo i in., 2021]. Pomocne, w ocenie stanu środowiska glebowego poddanego różnego rodzaju presji człowieka, są różne parametry mikrobiologiczne, biochemiczne oraz enzymatyczne. Mogą być one wykorzystywane zarówno w przypadku pozytywnych aspektów (np. analizy: skuteczności rekultywacji, wzrostu żyzności gleby, ograniczenia emisji gazów cieplarnianych), jak i negatywnych (np. ocena stopnia zdegradowania środowiska glebowego czy nasilenia emisji gazów cieplarnianych).

Drobnoustroje glebowe to fundament wielu różnych funkcji w ekosystemach, a ich liczebność i bioróżnorodność są wrażliwe na zmiany środowiska glebowego, dlatego też uznawane są one za wczesne wskaźniki zmian jego jakości [Nosrati i Collins, 2019; Ananyeva i in., 2021; Qiu i in., 2021; Mencel i in., 2022; Naylor i in., 2022; Shah i in., 2022]. Jak podają Chen i in. [2020] jeden gram gleby zawiera do 1 miliarda bakterii i 10 milionów strzępek grzybów. Ogromne bogactwo biologiczne gleby stanowi podstawę jej funkcjonowania, a co za tym idzie pośredniczy w zapewnieniu dobrej jakości żywności, łagodzeniu zmian klimatu, a także magazynowaniu i oczyszczaniu wody oraz zapobieganiu erozji [Wall i in., 2015; Yang i in., 2018; Chen i in., 2020; Fan i in., 2023]. Drobnoustroje są ściśle związane z rozkładem materii organicznej, uwalnianiem składników mineralnych, obiegiem składników odżywczych, czy sekwestracją węgla, przez co determinują stabilność i odporność ekosystemów [Ananyeva i in., 2021; Mencel i in., 2022; Naylor i in., 2022]. Skład i liczebność mikrobioty glebowej zależy od wielu różnych czynników, m.in.: właściwości fizykochemicznych gleby, jej rodzaju, zawartości składników odżywczych i materii organicznej, warunków klimatycznych, szaty roślinnej oraz sposobu jej użytkowania [Geisen i in., 2019; Chen i in., 2020; Mencel i in., 2022]. Wszelkie zmiany w mikrobiocie glebowej mają istotny wpływ na obieg składników odżywczych, węgla, azotu, a także na emisję gazów cieplarnianych [Muhammad i in., 2022; You i in., 2022]. Ze względu na znaczenie różnorodności mikrobiologicznej gleby dla wielofunkcyjności ekosystemów zasadne wydaje się uwzględnianie jej analizy przy badaniu wszelkich mechanizmów odpowiedzi środowiska glebowego na zmiany klimatyczne, jak też na różnorodną działalność człowieka. Oba te czynniki znacząco wpływają na właściwości fizyczne, jaki i chemiczne gleby, a to z kolei przekłada się na aktywność, liczebność i bioróżnorodność drobnoustrojów glebowych [Borrelli i in., 2020; Kuzyakov i in., 2020]. Ocena ilości oraz bioróżnorodności drobnoustrojów w glebie jest niezbędna dla lepszego zrozumienia dynamiki ich populacji, a także prowadzonych przez nie procesów biochemicznych. Skład ilościowy i jakościowy mikroorganizmów glebowych jest uważany za czuły wskaźnik jakości gleby, ponieważ jest to żywy składnik środowiska glebowego, który szybko reaguje na czynniki antropogeniczne [Hermans i in., 2020; Frąc i in., 2021; Jezierska-Tys i in., 2021; Joniec i in., 2021; Wyszkowska i in., 2023].

Ważnym, obok liczebności i różnorodności, wskaźnikiem aktywności biologicznej gleby jest intensywność procesów biochemicznych mierzona zawartością produktów działalności mikroorganizmów glebowych, np. jonów $N\text{-}NO_3$, $N\text{-}NH_4$, czy CO_2.

Aktywność oddechowa uznawana jest za dobry wyznacznik zmian zachodzących w środowisku glebowym [Joniec i in., 2015; Gyawali i in., 2019]. Większość CO_2 emitowanego z gleby jest to końcowy produkt mineralizacji oraz utleniania substancji organicznych przez drobnoustroje bytujące w glebie, ale także efekt procesów oddechowych roślin oraz rozkładu związków organicznych, wnoszonych do gleby wraz z korzeniami [Kuzyakov, 2006]. Dlatego też zmiany aktywności procesów oddechowych mogą wskazywać na zaburzenia ekologiczne oraz na duży udział mikroorganizmów w metabolizmie gleby i globalnym ociepleniu. Z tego względu aktywność oddechowa została uznana przez wielu innych autorów za dobry wyznacznik szybkości rozkładu materii organicznej lub mikrobiologicznej biomasy [Álvarez-Martín i in., 2016; Paula i in., 2017; Joniec i in., 2019; Elsakhawy i El-Rahem, 2020; Joniec i in., 2021].

Mikroorganizmy glebowe uczestniczą nie tylko w przemianach węgla, ale biorą także udział w cyklu biogeochemicznym, kolejnego ważnego biogenu, jakim jest azot [Barabasz i in., 2002; Barton i McLean, 2019]. Jest to jeden z najważniejszych pierwiastków w przyrodzie o kluczowym znaczeniu dla przetrwania wszystkich żywych organizmów. Na jego obieg składa się szereg różnych procesów, między innymi amonifikacja, denitryfikacja czy nitryfikacja. Procesy te tworzą tak zwany cykl azotowy, odpowiadający za większość przemian tego pierwiastka oraz odgrywają istotną rolę w jego losie w ekosystemach Ziemi. Amonifikacja to proces wytwarzania amoniaku z rozkładu azotu organicznego, a nitryfikacja polega na utlenianiu amoniaku do azotynów NO_2^-, a następnie do azotanów NO_3^- [Prangnell i in., 2019]. Zdaniem Sierra i in. [2012] nagromadzanie się mineralnych form azotu w wyniku mineralizacji odpadowej materii organicznej może być zjawiskiem niekorzystnym dla środowiska. Jest to związane z podatnością mineralnej formy azotu na ługowanie, co w konsekwencji grozi zanieczyszczeniem wód i stratami tego pierwiastka z gleby. Z kolei nitryfikacja, jak i denitryfikacja są istotnym źródłem N_2O w glebach rolniczych, który jest jednym z głównych gazów cieplarnianych, o ok. 320 razy wyższym potencjale tworzenia efektu cieplarnianego niż CO_2 [Lai i in., 2019; Yoon i in., 2019]. Ze względu na rolę jaką pełnią, powyższe parametry, powinny być one często wykorzystywane jako wskaźnik aktywności biologicznej gleby, a także do określania wpływu różnych czynników na stan biologiczny środowiska glebowego.

Istotnym narzędziem, w monitorowaniu zmian zachodzących w środowisku glebowym, jest również aktywność enzymatyczna, która jest ściśle związana z mikrobiomem glebowym. Enzymy glebowe jako naturalne katalizatory wielu procesów

zachodzących w środowisku glebowym, odgrywają ważną rolę w rozkładzie materii organicznej i obiegu składników odżywczych, a tym samym odzwierciedlają trendy i charakter cykli biogeochemicznych [Gianfreda i Rao, 2014; Utobo i Tewari, 2015]. Aktywność enzymatyczna wykazuje dużą czułość i wrażliwość na zmiany środowiskowe. Szybka reakcja tego parametru, wywołana różnymi praktykami powoduje, że aktywność enzymatyczna uznawana jest za istotny wskaźnik wykorzystywany w ocenie jakości gleb i odpowiedzi drobnoustrojów na zmiany klimatyczne [Lee i in., 2020; Song i in., 2021; Fanin i in., 2022; Mencel i in., 2022]. Jak donoszą Alkorta i in. [2003] enzymy mogą reagować na różnego rodzaju zmiany znacznie wcześniej niż inne parametry gleby. Co więcej, aktywność enzymatyczna wykazuje często ścisłe korelacje z krytycznymi parametrami jakości gleby, takimi jak: materia organiczna, właściwości fizyko-chemiczne gleby czy biomasa i aktywność mikrobiologiczna [Song i in., 2017; Furtak i Gałązka, 2019; Joniec i in., 2022; Kwiatkowska i Joniec, 2022]. Ponadto techniki oznaczania enzymów są dość tanie, proste i dają wysoką powtarzalność wyników [Utobo i Tewari, 2015].

Ze względu na ważną rolę jaką pełnią mikroorganizmy glebowe w kształtowaniu zdrowotności gleb i kondycji roślin, właściwe jest łączenie badań ich aktywności oraz liczebności z badaniami fitotoksyczności danego środowiska. Powszechnie wiadomo, że wpływ rożnego rodzaju odpadów na środowisko glebowe jest zróżnicowany. W glebach zdegradowanych chemicznie występują duże ilości związków toksycznych zakłócających aktywność procesów życiowych gleby, w tym m.in. dostępność, pobieranie i mobilność składników odżywczych [Richmond, 2015]. Z kolei odpady organiczne, takie jak podłoże popieczarkowe, na ogół pozytywnie wpływają na właściwości gleby [Malińska i in., 2018; Lipiec i in., 2021]. Wprowadzenie do środowiska glebowego odpadowej materii organicznej, niesie jednak ze sobą pewne ryzyko zaburzenia warunków życia roślin. Dlatego istotne jest monitorowanie skutków oddziaływania różnych odpadów na parametry związane ze wzrostem oraz rozwojem roślin. W celu kontrolowania środowiska glebowego pod wspomnianym kątem zaleca się stosowanie biotestów, przykładem takich analiz jest fitotest z udziałem *Lepidium sativum* L. [Kucaj i in., 2019; Szymanski i Dobrucka, 2022]. Parametry fitotoksyczne są często wykorzystywane do określania wpływu różnych związków chemicznych, w tym pochodzenia odpadowego, na kiełkowanie i wzrost roślin [Alvarenga i in., 2015; Pampuro i in., 2017; Manas i de las Heras, 2018; Clasen i de Moura Lisbôa, 2019; Joniec i in., 2019; Seneviratne i in., 2019; Godlewska i in., 2022].

Wskaźniki mikrobiologiczne, biochemiczne oraz enzymatyczne gleby, a także fitotoksyczność mają potencjał szybkiego reagowania na zmiany środowiskowe. Dlatego też mogą służyć do oceny skutków oddziaływania odpadów pochodzenia rolniczego i przemysłowego [Joniec i in., 2022; Kwiatkowska i Joniec, 2022; Kwiatkowska i in., 2023; Kwiatkowska i in., 2024].

4. Cele i hipotezy badawcze

Intensywne rolnictwo oraz przemysł są obecnie jednymi z głównych czynników degradacji gleb oraz nasilających się w związku z tym zmian klimatu. Dlatego też istnieje silna potrzeba poszukiwania nowych oraz weryfikacji przydatności już używanych parametrów do monitorowania stanu środowiska glebowego. Analiza możliwości wykorzystania parametrów mikrobiologicznych może odegrać kluczową rolę, zarówno w opracowaniu zrównoważonego zarządzania ekosystemami, jak i w polityce ochrony środowiska glebowego, uwzględniającej postępowanie z różnymi odpadami. W związku z tym celem nadrzędnym niniejszej rozprawy doktorskiej była próba weryfikacji przydatności wskaźników do monitorowania stanu środowiska glebowego poddanego działaniu różnych odpadów pochodzących z działalności rolniczej, jak i przemysłowej.

W ramach głównego celu wyodrębniono następujące cele szczegółowe:

- zbadanie i porównanie wpływu odpadu popieczarkowego oraz obornika, na wskaźniki jakości gleby, jakimi są: liczebność i różnorodność mikroorganizmów glebowych, aktywność biochemiczna oraz enzymatyczna związana z mikrobiologicznymi przemianami N, C, P, S, a także na fitotoksyczność gleby;
- ocenę liczebności mikroorganizmów glebowych i ich aktywności biochemicznej, a także enzymatycznej oraz analizę fitotoksyczności gleby z terenów poprzemysłowych, poddanej odziaływaniu odpadu z przemysłu chemicznego;
- zbadanie wpływu mikroorganizmów glebowych na powstawanie gazów cieplarnianych w glebie z dodatkiem różnych odpadów.

Cel nadrzędny oraz cele szczegółowe badań zostały zrealizowane w oparciu o następujące hipotezy badawcze:

- odpad popieczarkowy stosowany do celów nawozowych pozytywnie wpływa na wskaźniki jakości gleby, w tym na bioróżnorodność i aktywność drobnoustrojów glebowych oraz nie wywołuje fitotoksycznego działania na początkowe etapy wzrostu roślin;
- odpad popieczarkowy jest dobrą alternatywą nawożenia dla obornika i może być stosowany co roku;
- analizowanie populacji mikroorganizmów glebowych z wykorzystaniem jednocześnie odpowiednio dobranych klasycznych i nowoczesnych wskaźników pozwala na uzyskanie pełniejszego obrazu stanu gleb nawożonych odpadem popieczarkowym;

- podłoże popieczarkowe, w przeciwieństwie do obornika, nie przyczynia się do nasilenia się efektu cieplarnianego;
- użyte w badaniach wskaźniki aktywności drobnoustrojów glebowych i fitotoksyczności są odpowiednie do monitorowania stanu gleb zdegradowanych chemicznie na skutek silnej alkalizacji;
- alkalizacja środowiska glebowego powoduje zmiany w aktywności drobnoustrojów nie tylko w górnej, ale również w dolnej warstwie gleby, oraz że zmiany te utrzymują się nawet w dużej odległości od emitera zanieczyszczenia.

5. Materiał i metody

5.1. Model badawczy I (publikacje: P.1, P.2, P.3)

Pierwszy eksperyment polowy został założony na terenie Gospodarstwa Doświadczalnego w Czesławicach (Polska, województwo lubelskie, 51°18'23''N, 22°16'02''E) należącego do Uniwersytetu Przyrodniczego w Lublinie. Doświadczenie prowadzono w układzie bloków losowych, w trzech powtórzeniach, a powierzchnia pojedynczego poletka wynosiła 3 m^2 (1,5 m x 2,0 m). Poszczególne poletka oddzielono od siebie ścieżkami o szerokości 1m. Doświadczenie zlokalizowano na glebie płowej wytworzonej z lessu, należącej do II klasy bonitacyjnej [PSSS, 2009; WRB, 2015]. Skład uziarnienia gleby był następujący: frakcja 1,0 – 0,1 mm - piasek średni (4 %), frakcja 0,1 – 0,02 mm - piasek drobny - pył gruby (52 %), frakcja 0,02 – 0,002 mm - pył drobny (35 %), frakcja < 0,002 mm - ił koloidalny (9 %).

Podłoże popieczarkowe i obornik bydlęcy stosowano przez trzy lata (jesienią) w jednorazowej dawce 20 t ha^{-1}. Podłoże popieczarkowe, użyte w doświadczeniu, zostało skomponowane na bazie: słomy zbożowej (pszenicy ozimej), torfu i obornika kurzego. W przypadku dwóch obiektów z tym podłożem, stosowano również uzupełniające nawożenie mineralne azotem (N), fosforem (P) i potasem (K). Było to spowodowane wyjściową zasobnością gleby w przyswajalne składniki pokarmowe, a także z hipotetycznie przyjętym szybkim uwalnianiem się z tego odpadu składników pokarmowych, a co za tym idzie z krótkotrwałym działaniem nawozowym samego podłoża popieczarkowego (bez nawożenia NPK). Dlatego azot wprowadzono w formie saletry amonowej, w dawkach N1 – 50 i N2 – 100 kg ha^{-1}, fosfor w postaci superfosfatu potrójnego granulowanego w dawkach P1 – 30 i P2 – 60 kg ha^{-1} oraz potas, jako siarczan potasowy w dawkach K1 – 70 i K2 – 140 kg ha^{-1}. Rośliną testową była życica wielokwiatowa (*Lolium multiflorum* Lam.) – odmiana tetraploidalna Turtetra (Kroto), wysiewana każdego roku w drugiej dekadzie kwietnia w ilości 30 kg ha^{-1}, w rozstawie rzędów 25 cm, na głębokość 1 cm. Obiekt kontrolny stanowiła gleba bez nawożenia. Charakterystykę gleby, podłoża popieczarkowego oraz obornika przedstawiono w Tabeli 1.

Tabela 1. Wybrane właściwości gleby oraz zastosowanych odpadów [Joniec i in., 2022].

Właściwości	Jednostka	Gleba	Podłoże popieczarkowe	Obornik
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		1660	15800	2240
K	mg kg^{-1}	2350	6330	11100
Mg		1390	1240	1550
Zn			86,0	
Cu			16,6	
Ni			2,81	
Cr	mg kg^{-1}	n.o.	1,84	n.o.
Cd			0,055	
Pb			0,956	
Hg			0,07	

Skróty: TOC – węgiel organiczny ogółem, TN – azot ogólny, TP – potas ogólny, n.o. – nieoznaczone

Fotografia 1. Uprawa pieczarki.

Prace badawcze prowadzono w latach 2018 – 2020. Materiał glebowy pobierano za pomocą świdra żłobiącego, z warstwy 0 – 25 cm, z wytypowanych losowo 10 miejsc w obrębie każdego poletka badawczego w dwóch terminach, tj. wiosną (czerwiec) i jesienią (wrzesień). Pobrane próbki przesiano przez sito o średnicy 2 mm i przechowywano w plastikowych workach w temperaturze 4°C, z wyjątkiem gleby do analiz DNA, którą przechowywano w temperaturze -80°C.

Schemat doświadczenia:

1. gleba bez nawożenia – obiekt kontrolny (C)
2. gleba + podłoże popieczarkowe (SMS)
3. gleba + podłoże popieczarkowe + N1P1K1 (SMS+N1P1K1)
4. gleba + podłoże popieczarkowe + N2P2K2 (SMS+N2P2K2)
5. gleba + obornik bydlęcy (M).

5.2. Model badawczy II (publikacja: P.4)

Materiał glebowy pochodził z terenu poprzemysłowego zlokalizowanego w województwie mazowieckim (51°28′54″N, 21°27′01″E). Próbki gleby pobierano z trzech miejsc (S1, S2, S3) znajdujących się w różnych odległościach od zbiornika z płynnym odpadem. Punkt S1 był oddalony o 5,88 m, punkt S2 o 22,70 m, a punkt S3 o 50,08 m. Punkty poboru nie przebiegały wzdłuż jednej linii, co umożliwiło zbadanie czy ewentualne zanieczyszczenie gleby rozprzestrzenia się w środowisku w jednym kierunku czy rotacyjnie. Ciecz znajdowała się w zamkniętych cysternach, umieszczonych nad betonowym zbiornikiem, który pierwotnie miał być wtórnym zabezpieczeniem przed ewentualnym przesączaniem się cieczy do gleby. Ciekły odpad to pozostałość po działalności przemysłu chemicznego związanej m.in. z produkcją celulozy i klejów. Zbiorniki ustawiono w latach siedemdziesiątych XX wieku. Charakterystyka ciekłego odpadu została zamieszczona w Tabeli 2.

Tabela 2. Charakterystyka odpadu, [Kwiatkowska i in., 2023].

	pH 1 mol KCl	**Ca** mg kg^{-1}	**K** mg kg^{-1}	**Na** mg kg^{-1}
odpad	≈ 14	37,6	328	87000

Fotografia 2. Cysterny z płynnym odpadem, umieszczone nad betonowym zbiornikiem.

Próbki gleby do analiz pobierano latem (lipiec) i jesienią (wrzesień) 2022 roku z głębokości 0 – 20 cm i 20 – 40 cm. Materiał pobierano losowo z 4 miejsc w obrębie każdego z trzech punktów poboru tj.: S1, S2 i S3 oddzielnie dla poszczególnych warstw. Pobraną glebę przesiano przez sito o średnicy 2 mm i przechowywano w plastikowych workach w temperaturze 4°C.

5.3. Metody badawcze

5.3.1. Liczebność mikroorganizmów

W materiale glebowym metodą płytkową, zgodnie z procedurą opisaną przez Foght i Aislabie [2005], oznaczono liczebność: bakterii oligotroficznych na podłożu z wyciągiem glebowym i K_2HPO_4, bakterii kopiotroficznych na podłożu Bunta i Roviry [1955], grzybów strzępkowych na podłożu Martina [1950], grzybów celulolitycznych na agarze mineralnym przykrytym krążkiem bibuły Whatmana, a także bakterii oraz grzybów rozkładających białko na podłożu Fraziera [Rodina, 1968]. W przypadku grzybów do podłoża dodano antybiotyki [Martin, 1950; Gil i in., 2009]. Wyniki ww. analiz podano w postaci jednostek tworzących kolonie (jtk). Ponadto oznaczano liczebność bakterii celulolitycznych za pomocą metody najbardziej prawdopodobnej liczby NPL [Foght i Aislabie, 2005]. W przypadku tych bakterii zastosowano pożywkę płynną wg Pochon i Tardieux [1962], a wyniki przedstawiono jako najbardziej prawdopodobną liczbę (NPL) odczytaną z tabel McCrady'ego. Hodowle bakterii prowadzono w temperaturze 28°C przez 4 dni, z wyjątkiem bakterii celulolitycznych gdzie inkubacja trwała 14 dni. Natomiast grzyby inkubowano w temperaturze 25°C przez 3 dni (grzyby strzępkowe i proteolityczne) i 14 dni grzyby celulolityczne.

5.3.2. Analizy molekularne

Całkowite genomowe DNA zostało wyekstrahowane z analizowanych próbek gleby przy użyciu zestawu Soil DNA Purification Kit (EurX) zgodnie z protokołem producenta. Dla każdej próbki użyto 100 mg gleby. Integralność uzyskanych próbek DNA określono za pomocą elektroforezy w 1,5 % żelu agarozowym barwionym bromkiem etydyny. Czystość próbek określono spektrofotometrycznie przy użyciu aparatu NanoDrop 2000 (Thermo Scientific) obliczając stosunki A260/A280 i A260/A230. Stężenie analizowanych próbek DNA określono za pomocą oceny fluorometrycznej przy użyciu zestawu odczynników dsDNA Quantitation BR (Thermo Fisher Scientific) zgodnie z instrukcjami producenta. W celu oznaczenia ilościowego 4 µl wyekstrahowanej próbki genomowego DNA mieszano z 196 µl roztworu roboczego Qubit, worteksowano przez 5 sekund i inkubowano

w temperaturze pokojowej przez 2 minuty. Przygotowane próbki były następnie mierzone fluorometrycznie przy użyciu fluorometru Qubit 2.0 (Thermo Fisher Scientific).

Analizy ilościowe materiału genetycznego bakterii i grzybów w badanych próbkach gleby przeprowadzono przy użyciu techniki ilościowego PCR (qPCR). Jako matrycę do każdej reakcji użyto 80 ng całkowitego genomowego DNA. Amplifikacja specyficznych sekwencyjnie fragmentów genu 16S rRNA i 18S rRNA została wykorzystana do ilościowego oznaczenia zawartości odpowiednio bakteryjnego i grzybowego DNA w próbce. Do amplifikacji użyto dwóch zestawów starterów specyficznych dla sekwencji: 515F (5'-GTGYCAGCMGCCGCGTAA-3') i 806R (5'-GGACTACNVGGTWTCTAAT-3') [Apprill i in., 2015; Parada i in., 2015] dla genu 16S rRNA oraz FungiQuant-F (5'-GGRAAACTCACCAGGTCCAG-3') i FungiQuant-R (5'-GSWCTATCCCCAKCACGA-3') [Liu i in., 2012] dla genu 18S rRNA. Do analizy użyto SYBR Select Master Mix (Thermo Fisher Scientific) zgodnie z protokołem producenta. Wszystkie analizy przeprowadzono przy użyciu aparatu QuantStudio 3 (Thermo Fisher Scientific) wraz z pakietem oprogramowania Thermo Fisher Connect. Każdą próbkę analizowano w trzech powtórzeniach. Do analizy danych zastosowano względny model kwantyfikacji, w którym ilość amplikonu w próbce kontrolnej ustawiono jako 1, a zawartość amplikonów we wszystkich innych próbkach przedstawiono jako zmianę w porównaniu z próbką kontrolną. Specyficzność reakcji amplifikacji potwierdzono dla każdej próbki za pomocą analizy krzywej topnienia.

5.3.3. Aktywność biochemiczna

Nasilenie amonifikacji oznaczano w 25 g gleby z dodatkiem 0,1 % asparaginy jako substratu, następnie po 3 dniach, stężenie jonów amonowych określono metodą Nesslera [Nowosielski, 1974] i wyrażono w mg $N\text{-}NH_4$ kg^{-1} s.m. gleby 3 d^{-1}. Nasilenie nitryfikacji oznaczono w próbkach gleby o masie 25 g, stosując jako substrat 0,1 % $(NH_4)H_2PO_4$. Jony azotanowe oznaczano, po 7 dniach, metodą brucynową [Nowosielski, 1974], a ich zawartość wyrażono w mg $N\text{-}NO_3$ kg^{-1} s.m. gleby 7 d^{-1}.

Aktywność oddechową oznaczano, metodą Rühlinga i Tylera [1973] w 20 g próbkach gleby z dodatkiem 1 % glukozy jako substratem i wyrażono w mg $C\text{-}CO_2$ kg^{-1} s.m. gleby 24 h^{-1}.

5.3.4. Aktywność enzymatyczna

Aktywność proteazy oznaczano metodą Ladda i Butlera [1972] w 2 g gleby z dodatkiem substratu, którym był kazeinian sodu i wyrażono jako mg tyrozyny kg^{-1} s.m.

gleby h^{-1}. Aktywność ureazy oznaczano metodą Zantua i Bremnera [1975], w 10 g gleby z dodatkiem mocznika jako substratu i wyrażono jako mg $N\text{-}NH_4$ kg^{-1} s.m. gleby 18 h^{-1}. Aktywność dehydrogenaz oznaczano metodą Thalmanna [1968] z użyciem chlorku 2,3,5-trifenylotetrazoliowego (TTC) jako substratu i wyrażona jako mg TPF kg^{-1} s.m. gleby d^{-1}. Do oznaczenia aktywności fosfatazy kwaśnej i zasadowej, wykorzystano metodę Tabatabai i Bremnera [1969]. Aktywność obu tych enzymów oznaczano w 1 g gleby, stosując jako substrat p-nitrofenylofosforan. W przypadku fosfatazy kwaśnej inkubację prowadzono w buforze uniwersalnym o pH = 6,5, natomiast w przypadku fosfatazy zasadowej w buforze uniwersalnym o pH = 11. Aktywność arylosulfatazy oznaczano metodą Tabatabai i Bremnera [1970] w 1 g gleby z 4-nitrofenylosiarczanem potasu (PNS) jako substratem. Aktywność β-glukozydazy oznaczono w 1 g gleby, stosując jako substrat p-nitrofenylo-β-D-glukozyd (PNG) [Eivazi i Tabatabai, 1988]. Aktywność zarówno fosfataz, arylosulfatazy, jak i β-glukozydazy, została wyrażona jako mg PNP kg^{-1} s.m. gleby h^{-1}. Aktywność hydrolityczną dioctanu fluoresceiny (FDA) oznaczono metodą Schnurera i Rosswalla [1982] w 1 g gleby z dodatkiem FDA jako substratu i wyrażono jako mg fluoresceiny kg^{-1} s.m. gleby h^{-1}.

5.3.5. Fitotoksyczność

W ramach oceny fitotoksyczności gleby wykonano dwa fitotesty przy użyciu pieprzycy siewnej (*Lepidium sativum* L.), jako rośliny testowej.

Test Masciandaro i in. [1997] miał na celu określenie wpływu całokształtu warunków powstałych w glebie na rozwój *L. sativum,* po zastosowaniu badanych wariantów nawożenia organicznego. W tym celu na 50-cio gramowe naważki świeżej gleby, umieszczone na płytkach Petriego wysiano po 100 nasion *L. sativum.* Inkubację prowadzono przez 4 dni w temperaturze 22°C utrzymując stały poziom wilgotności gleby (60 % c.p.w.). Po upływie wyznaczonego czasu zliczono liczbę wykiełkowanych nasion oraz oznaczono ich ciężar. W oparciu o te parametry obliczono indeks wzrostu (IW %) zgodnie ze wzorem Masciandaro i in. [1997]:

$$IW\ \% = P\ (^{T}/_{C})$$

P – % wykiełkowanych nasion w glebie poddanej różnym zabiegom, w stosunku do wartości tego parametru w glebie kontrolnej; T – średni ciężar świeżych kiełków *L. sativum* wyrosłych w glebie poddanej różnym zabiegom; C – średni ciężar kiełków *L. sativum* w glebie kontrolnej.

Drugi fitotest umożliwił przeanalizowanie wpływu substancji potencjalnie toksycznych rozpuszczonych w roztworze glebowym na kiełkowanie i przyrost korzenia *L. sativum,* po 2 i 4 dniach. W tym celu, na płytkach Petriego umieszczano 20-sto gramowe naważki świeżej gleby, w sześciu powtórzeniach, które przykryto sterylnymi krążkami bibuły. Następnie na 3 płytki wyłożono po 90 nasion *L. sativum*, a na pozostałe 3 płytki po 10 nasion. Inkubację prowadzono w temp. 22°C. Po dwóch dniach zliczono liczbę wykiełkowanych nasion na wszystkich płytkach. Zmierzono również długość korzeni kiełków po 2 oraz 4 dniach, na płytkach, na których znajdowało się po 10 nasion.

5.3.6. Analizy chemiczne, fizyczne i fizykochemiczne

Uzupełnieniem analiz mikrobiologicznych, biochemicznych, enzymatycznych oraz fitotoksyczności były analizy chemiczne (wykonane w Centralnym Laboratorium Badawczym Uniwersytetu Przyrodniczego w Lublinie) i fizykochemiczne. Poniższe metody zastosowano zarówno dla próbek gleby, jak i badanych odpadów w poszczególnych modelach badawczych. Do oznaczenia odczynu wykorzystano metodę elektrometryczną z roztworem KCl. Wilgotność oznaczano metodą wagową. Pomiar węgla organicznego wykonano metodą spektrometrii IR, azot całkowity oznaczano metodą Kjeldahla, a fosfor całkowity metodą spektrofotometrii. Z kolei wapń, potas, magnez oraz sód oznaczano metodą płomieniowej absorpcyjnej spektrometrii atomowej (FAAS). Metale ciężkie oznaczano metodą spektroskopii absorpcji atomowej (AAS).

5.3.7. Analizy statystyczne

Wszystkie analizy przeprowadzono w trzech równoległych powtórzeniach i przedstawiono, jako średnią arytmetyczną z tych powtórzeń. Wyniki poddano analizie statystycznej przy użyciu oprogramowania STATISTICA wersja 13.0 (TIBCO Software Inc., Palo Alto, CA, USA) z modelami ANOVA i wielokrotnymi t *Tukeya*-testy na poziomie istotności $\alpha = 0{,}05$. Wyniki przedstawiono na wykresach słupkowych z zaznaczonym odchyleniem standardowym. Zależności między analizowanymi parametrami mikrobiologicznymi, biochemicznymi, enzymatycznymi, fitotoksycznością i parametrami fizykochemicznymi, chemicznymi oraz warunkami środowiskowymi analizowano za pomocą analizy składowych głównych (metoda PCA) oraz korelacji Persona na trzech poziomach istotności: $p < 0.001$, $p < 0.01$, $p < 0.05$. Wyniki analiz korelacji przedstawiono w postaci mapy cieplnej, gdzie dla poszczególnych przypadków przyjęto skale kolorów od ciemnozielonej (niższe wartości) do ciemnoczerwonej (wyższe wartości), z odpowiednimi kolorami przejściowymi pomiędzy tymi skrajnościami. Analiza

skupień posłużyła do zidentyfikowania grup obiektów wykazujących podobieństwo pod względem: liczebności drobnoustrojów oraz aktywności enzymatycznej i biochemicznej. Aglomerację właściwości oceniano metodą analizy skupień Warda z odległością euklidesową.

6. Wyniki i dyskusja

6.1. Wyniki uzyskane w publikacji P.1

Joniec J., **Kwiatkowska E.**, Kwiatkowski C.A. 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. *Agriculture,* **2022**, 12, 1190. https://doi.org/10.3390/agriculture12081190

W publikacji **P.1** przedstawiono wyniki badań dotyczących oceny potencjału podłoża popieczarkowego do poprawy wskaźników jakości gleby, takich jak liczebność oraz aktywność mikroorganizmów, związanych z przemianami azotu. W badaniach tych dokonano również oceny wpływu zastosowanych sposobów nawożenia (podłoże popieczarkowe i obornik) na zwiększanie się efektu cieplarnianego. Materiał glebowy pochodził z trzyletniego doświadczenia polowego, zlokalizowanego na glebie płowej, gdzie poszczególne poletka nawożono podłożem popieczarkowym lub podłożem wraz z uzupełniającym nawożeniem mineralnym, a także w wariancie z samym obornikiem (model badawczy I). W niniejszych badaniach stwierdzono, że zastosowane podłoże popieczarkowe oraz obornik miały istotny wpływ na mikrobiologiczne przemiany azotu. Odpady te, z różnym natężeniem pobudzały lub hamowały poszczególne etapy obiegu tego biogenu. Nasilenie zaburzenia homeostazy środowiska glebowego mogło mieć również negatywny wpływ na jakość powietrza.

Wyniki przedstawione w **P.1** wykazały, że zastosowanie odpadu popieczarkowego spowodowało na ogół pozytywne zmiany zarówno w liczebności bakterii, jaki i grzybów proteolitycznych. Nasilenie tych zmian różniło się w zależności od czasu, jak i zastosowanego sposobu nawożenia. Najsilniejsze pobudzenie rozwoju tych grup drobnoustrojów odnotowano wiosną w I roku trwania doświadczenia, w przypadku bakterii w wariancie z samym podłożem popieczarkowym, a grzybów w wariancie z niższą dawką nawożenia mineralnego. Stymulacja bakterii i grzybów proteolitycznych została zapewne spowodowana dostarczeniem dodatkowych składników pokarmowych, w postaci podłoża popieczarkowego. Można przyjąć, że to właśnie odpad, zastosowany także łącznie z NPK, jest głównym aktywatorem tych dwóch grup drobnoustrojów. W pozostałych latach, tj. w II i III roku, pozytywny wpływ podłoża popieczarkowego znacznie osłabł zarówno w przypadku bakterii, jak i grzybów, ale nadal utrzymywał się w obiektach z dodatkiem nawożenia mineralnego. Jak wynika z danych literaturowych niskie dawki

tego nawożenia mają pozytywny wpływ na właściwości mikrobiologiczne oraz agrochemiczne gleby, gdyż przyspieszają tempo rozkładu i zwiększają ilość glebowej materii organicznej [Sivojiene i in., 2021; Chen i in., 2022]. Z kolei sezonowe zmiany liczebności bakterii i grzybów były prawdopodobnie spowodowane wahaniami temperatury i wilgotności w warunkach polowych. O zależności rozwoju drobnoustrojów glebowych od warunków atmosferycznych donoszą również Li i in. [2018], Fan i in. [2021] oraz Sivojiene i in. [2021].

Analiza aktywności proteazy w ciągu 3-lat trwania doświadczenia wykazała istotne zmiany pod wpływem zastosowanego nawożenia odpadem popieczarkowym w różnych wariantach. Efekt ten miał zróżnicowany charakter i był widoczny z różnym nasileniem w zależności od rodzaju użytego nawożenia oraz czasu jego oddziaływania. Na aktywność proteazy najsilniej zadziałało zastosowanie podłoża popieczarkowego w połączeniu z niższą dawką nawożenia mineralnego, ale tylko w II roku trwania doświadczenia. W pozostałych terminach i latach zastosowanie odpadu popieczarkowego w poszczególnych wariantach nie wywarło istotnego wpływu na aktywność proteazy lub spowodowało jej inhibicję. Być może jest to związane tym, że produkcja proteaz zewnątrzkomórkowych może być hamowana przez łatwo dostępny węgiel [Vranova, 2013]. Sposób użytkowania i glebowa materia organiczna wpływają na cykl azotu poprzez modyfikacje w składzie zbiorowisk drobnoustrojów zaangażowanych w ten cykl, szczególnie drobnoustrojów proteolitycznych [Lori i in., 2020]. Również poszczególne grupy drobnoustrojów mogą kodować bardziej lub mniej wydajne proteazy. Odnotowane zmiany w aktywności proteaz mogą być też spowodowane warunkami klimatycznymi, tj. wilgotnością i temperaturą, ponieważ ekspresja genów jest regulowana przez wiele czynników środowiskowych m.in. przez C, P, Ca, pH czy wilgotność [Vranova, 2013].

W przypadku aktywności ureazy zastosowanie odpadu popieczarkowego spowodowało pobudzenie tego parametru w większej liczbie obiektów i terminów niż w przypadku proteazy. Najsilniej, w ciągu całego okresu badań, uwidoczniło się ono w I roku stosowania nawożenia, zwłaszcza z samym podłożem popieczarkowym. Również zastosowanie odpadu łącznie z niższą dawką nawożenia mineralnego okazało się korzystne, natomiast w przypadku obiektu z wyższą dawką nawożenia mineralnego odnotowano spadek tego parametru. W II i III roku tendencja co do aktywności ureazy była podobna, wszystkie zastosowane warianty z odpadem popieczarkowym wpływały na ogół stymulująco na badany parametr. Dlatego też możemy przypuszczać, że podobnie jak w przypadku liczebności mikrobiologicznych, produkty transformacji popieczarkowej

materii organicznej oraz zmiany jakie wywołały one w środowisku glebowym przyczyniły się w prezentowanych badaniach do stymulacji aktywności omawianego enzymu. Pobudzenie aktywności ureazy pod wpływem odpadu popieczarkowego odnotowali w swoich badaniach także Kuziemska i in. [2020] oraz Ma i in. [2021]. Na uwagę zasługuje fakt, że aktywność tego enzymu, oprócz obiektów z samym podłożem popieczarkowym, była na ogół istotnie wyższa w obiektach z obornikiem w ciągu całego okresu badań. Obserwacja ta może wskazywać na większe prawdopodobieństwo zachodzenia niekorzystnego zjawiska, jakim jest strata azotu z gleby. Dzieje się to na skutek uchodzenie do atmosfery gazowych produktów reakcji katalizowanych przez ureazę tj. amoniaku, właśnie w obiektach z obornikiem [Grzyb i in., 2021; Klimczyk i in., 2021].

Analiza danych, dotyczących aktywności biochemicznej, wykazała, że aplikacja odpadu popieczarkowego w różnych kombinacjach, w ciągu całego okresu badań, wpłynęła na ogół w małym stopniu na proces amonifikacji. Należy odnotować, że zastosowanie podłoża popieczarkowego łącznie z uzupełniającym nawożeniem mineralnym, w obu wariantach, spowodowało jedyną wyraźną stymulację tego parametru, wiosną w II roku trwania doświadczenia. Na podstawie uzyskanych wyników można stwierdzić, że zanik wraz z upływem czasu stymulacji procesu amonifikacji w obiektach z odpadem popieczarkowym jest zjawiskiem pozytywnym. Nagromadzanie się mineralnych form azotu w wyniku mineralizacji odpadowej materii organicznej może być niekorzystne dla środowiska [Sierra i in., 2012]. Jest to związane z podatnością mineralnej formy azotu na ługowanie, co w konsekwencji grozi zanieczyszczeniem wód i stratami tego pierwiastka z gleby.

Podobnie jak w przypadku amonifikacji, najkorzystniejsze odziaływanie nawożenia na nitryfikację, odnotowano we wszystkich wariantach z podłożem popieczarkowym wiosną w II roku eksperymentu. Wyraźną stymulację stwierdzono również jesienią, ale tylko w obiekcie z nawożeniem wyższa dawką nawozu mineralnego. W przypadku aktywności nitryfikacji długość stosowania nawożenia wpłynęła niekorzystnie na ten parametr, ale z punktu widzenia ochrony środowiska jest to zjawisko pozytywne. Z produktów nitryfikacji, w procesach oddechowych, korzystają drobnoustroje odpowiadające za procesy denitryfikacji. Efektem tej redukcji jest m.in. N_2O zaliczany do gazów cieplarnianych [Barton i McLean, 2019]. Tak więc zarówno nitryfikacja, jak i denitryfikacja są istotnym źródłem N_2O w glebach rolniczych, a więc im niższa aktywność tych procesów tym mniejsza emisja tego gazu do atmosfery [Lai i in., 2019; Yoon i in., 2019]. Jednocześnie należy podkreślić, że w obiekcie z obornikiem nasilenie

procesu nitryfikacji było na ogół silniejsze i podlegało stymulacji w ciągu całego okresu badań. Obserwacja ta potwierdza hipotezę, że nawożenie odpadem popieczarkowym niesie ze sobą mniejsze ryzyko pogłębiania efektu cieplarnianego niż nawożenie obornikiem.

Obornik, w przeciwieństwie do podłoża popieczarkowego, nie wpłynął tak wyraźnie na rozwój bakterii i grzybów proteolitycznych. Mogło być to spowodowane tym, że podłoże popieczarkowe charakteryzuje się co prawda zróżnicowaną, ale wyższą zawartością materii organicznej w porównaniu z obornikiem bydlęcym lub świńskim [Atiyeh i in., 2000; Becher i in., 2021]. Jeśli chodzi o aktywność enzymatyczną, zastosowanie obornika wywarło na ogół pozytywny wpływ na aktywność ureazy. Najkorzystniejszy był on wiosną w I roku, ale z biegiem czasu osłabł. Zastosowanie obornika, podobnie jak podłoża popieczarkowego, spowodowało zróżnicowany wpływ na aktywność proteazy. Najwyższe wartości w przypadku tego wariantu nawożenia odnotowano wiosną zarówno w I, jaki i II roku trwania doświadczenia. W pozostałych latach i sezonach uwidocznił się na ogół brak wpływu obornika lub jego hamujący wpływ na ten parametr. Proces amonifikacji, w kombinacji z obornikiem, kształtował się w ciągu 3 lat badań na poziomie zbliżonym do kontroli. Jedynie jesienią w I roku w glebie wzbogaconej obornikiem odnotowano niewielką istotną stymulację tej aktywności. Natomiast jak wspomniano wcześniej obornik, w porównaniu z podłożem popieczarkowym, wpłynął silniej na nitryfikację, powodując jej wyraźne nasilenie utrzymujące się we wszystkich latach badań.

6.2. Wyniki uzyskane w publikacji P.2

Kwiatkowska E., Joniec J. Effects of agricultural management of spent mushroom waste on phytotoxicity and microbiological transformations of C, P, and S in soil and their consequences for the greenhouse effect. *Int. J. Environ. Res. Public Health,* **2022**, 19, 12915. https://doi.org/10.3390/ijerph191912915

W publikacji **P.2** przedstawiono wyniki badań dotyczących kontynuacji oceny wpływu podłoża popieczarkowego oraz obornika na wskaźniki jakości gleby, takich jak aktywność biochemiczna oraz enzymatyczna, związanych z mikrobiologicznymi przemianami tym razem C, P i S oraz z fitotoksycznością gleby. Zweryfikowano również tezy zakładające: że podłoże popieczarkowe może stać się nawozową alternatywą dla obornika, stosowaną corocznie oraz, że rolnicze zagospodarowanie podłoża popieczarkowego nie przyczynia się do zwiększenia się efektu cieplarnianego. Do tego

celu wykorzystano ten sam model badawczy jak w publikacji **P.1.** Uzyskane wyniki wykazały istotne zmiany parametrów związanych z przemianami mikrobiologicznymi C, P i S w glebie pod wpływem podłoża popieczarkowego. Analizowane parametry fitotoksyczności gleby, również podlegały istotnym zmianom, zarówno pod wpływem obornika, jak i odpadu popieczarkowego.

Zastosowanie podłoża po uprawie pieczarek spowodowało istotne pobudzenie parametrów związanych z przemianami mikrobiologicznymi węgla w glebie, tj. oddychania i aktywności dehydrogenaz. Nasilenie aktywności tych parametrów, a w szczególności procesu oddychania, mierzonego ilością wydzielanego CO_2, utrzymywało się z różnym nasileniem przez cały okres badań w obiektach, gdzie odpad zastosowano łącznie nawożeniem mineralnym. W przypadku oddychania najwyższe ilości wydzielanego CO_2 odnotowano w III roku badań. Z kolei odnośnie dehydrogenaz najwyższe wartości odnotowano w I roku. Wzrost, chodź z różnym nasileniem, aktywności oddechowej, jaki dehydrogenaz, w glebie był zapewne spowodowany wkładem materii organicznej wraz z podłożem popieczarkowym oraz obornikiem, stanowiącymi źródło substratów oddechowych dla mikroorganizmów glebowych. O stymulacji procesów oddechowych poprzez dodatek materii organicznej do gleby donoszą również inni autorzy [Owaid i in., 2017; Zhou i in., 2019; Frąc i in., 2021; Hernandez i in., 2021]. Natomiast odnotowany, okresowo w II roku, spadek w przypadku aktywności dehydrogenaz był prawdopodobnie wywołany rozłożeniem łatwiej dostępnych substancji pokarmowych. Powyższe obserwacje świadczą o tym, że odpadowa materia została włączona w mikrobiologiczne procesy związane z obiegiem węgla. Ważną rolę, w przypadku tych parametrów, odegrało podobnie jak przy liczebnościach drobnoustrojów proteolitycznych przedstawionych w **P.1**, zastosowanie nawożenia mineralnego, które przyczyniło się do stymulacji rozkładu materii organicznej gleby [Kátai i in., 2020; Sivojiene i in., 2021; Chen i in., 2022]. Dynamika zmian aktywności omawianych parametrów mogła być spowodowana również warunkami środowiskowymi, o czym świadczą odnotowane istotne korelacje zarówno oddychania, jaki i dehydrogenaz, z opadami i temperaturą. Zwłaszcza dehydrogenazy wykazują dużą wrażliwość na zmiany związane z porami roku, ponieważ pozostają w ścisłym związku z dynamiką aktywności mikroorganizmów [Wolińska i Stępniewska, 2012].

Wpływ obornika zarówno na proces oddychania, jaki i aktywność dehydrogenaz, uwidocznił się zdecydowanie słabiej niż w przypadku odpadu po uprawie pieczarek.

Pobudzenie aktywności oddechowej pod wpływem tego nawożenia odnotowano tylko jesienią w I-szym roku. W kolejnych latach odnotowano zanik stymulacji a nawet spadek aktywności oddechowej. Natomiast w przypadku dehydrogenaz istotny wpływ obornika odnotowano jedynie jesienią w II roku w postaci pobudzenia tego parametru.

Uzyskane wyniki wykazały, że nawozowe zastosowanie odpadu popieczarkowego przyczyniło się do wzrostu ilości wydzielanego CO_2. Efekt ten nasilił się wraz z upływem czasu. Niestety obserwacje te nie potwierdzają, postawionej hipotezy, że taki sposób gospodarowania odpadami nie przyczynia się do nasilenia efektu cieplarnianego poprzez zwiększenie emisji CO_2 z gleby. W tym kontekście obornik okazał się bezpieczniejszym nawozem, ponieważ nie spowodował istotnego utrzymującego się wzrostu emisji CO_2.

Aktywność enzymów odpowiedzialnych za przemiany fosforu i siarki tj. fosfatazy i arylosulfatazy, podlegały hamowaniu pod wpływem podłoża popieczarkowego. Należy zaznaczyć, że negatywny wpływ odpadu z czasem osłabł, a nawet zanikł, ale utrzymywał się w przypadku arylosulfatazy nawet w III roku w obiektach z odpadem i nawożeniem mineralnym w niższej dawce. Dlatego też możemy przypuszczać, że materia organiczna wprowadzona w postaci podłoża popieczarkowego nie odegrała kluczowej roli w przypadku tych enzymów. Jak wykazali inni autorzy, aktywność fosfatazy może być hamowana przez obecność w glebie fosforu mineralnego [Perez-de-Mora i in., 2012; Dotaniya i in., 2019; Manzoor i in., 2022]. Również w niniejszych badaniach odegrał on istotną rolę o czym świadczą odnotowane istotne korelacje między aktywnością fosfatazy a zawartością przyswajalnego fosforu mineralnego. Kolejnym parametrem, który mógł mieć wpływ na aktywność omawianych enzymów jest azot. Analiza skupień wykazała istotne korelacje tego czynnika zarówno z fosfatazą kwaśną, jak i arylosulfatazą. Prawdopodobnie to właśnie dodanie azotu, w postaci nawożenia mineralnego, zwiększyło dostępność siarki w glebie, a to przełożyło się na zmniejszenie aktywności arylosulfatazy. Do podobnych wniosków doszli m.in. Mori i in. [2020], z kolei Sawicka i in. [2020] odnotowali istotny wpływ nawożenia mineralnego na aktywność fosfatazy kwaśnej. Innymi czynnikami, które mogły przyczynić się do zmian w aktywnościach omawianych parametrów są zarówno pH, jaki warunki środowiskowe, a potwierdzeniem tych obserwacji są odnotowane istotne korelacje między tymi czynnikami a omawianymi enzymami.

Wpływ obornika, na aktywność fosfatazy kwaśnej i arylosulfatazy nie był ukierunkowany. W I roku odnotowano w tym obiekcie spadek aktywności fosfatazy w obu terminach. Natomiast w kolejnych latach istotny wpływ odnotowano jedynie w II roku

jesienią w postaci stymulacji. Zastosowanie obornika wywołało istotne zmiany w aktywności arylosulfatazy, ale tylko w I roku badań. Odnotowano spadek tego parametru enzymatycznego w obu terminach.

Ocenę fitotoksyczności gleby przeprowadzono na podstawie dwóch fitotestów. Pierwszy test, miał na celu określenie wpływu całokształtu warunków powstałych w glebie, po zastosowaniu badanych wariantów nawożenia organicznego, na rozwój *L. sativum,* tj. na kiełkowanie nasion i masę kiełków. Z kolei drugi test umożliwił przeanalizowanie wpływu substancji potencjalnie toksycznych, rozpuszczonych w roztworze glebowym, na początkowe etapy rozwoju rośliny testowej, tj. na kiełkowanie i przyrost korzenia *L. sativum* L.*,* po 2 i 4 dniach. Wyniki dotyczące wpływu odpadu popieczarkowego, zarówno na masę kiełków, jaki i ilość wykiełkowanych nasion oraz na przyrost korzenia *L. sativum* L., wykazały, że miał on zróżnicowany charakter w zależności od czasu. Pozytywny wpływ odnotowano w przypadku indeksu wzrostu, w całym okresie badań, podczas gdy w przypadku przyrostu długości korzenia i kiełkowania, jedynie w III roku badań. W początkowym latach przyrost korzenia, szczególnie mierzony po 4 dniach, był mniejszy w obiektach z odpadem. Podobne obserwacje dotyczą wpływu obornika. Uzyskane wyniki badań dotyczące parametru długości korzenia *L. sativum,* mierzonego zarówno po 2, jak i 4 dniach, wskazują, że parametr ten jest najbardziej czuły na ewentualne szkodliwe związki, występujące lub powstałe w wyniku przemian wprowadzonej wraz z podłożem popieczarkowym i obornikiem materii organicznej, w roztworze glebowym. Do podobnych wniosków w swoich badaniach doszli również Godlewska i in. [2022]. Wpływ badanych materiałów nawozowych na ten parametr był dość zróżnicowany w zależności od kombinacji i czasu trwania doświadczenia. Spadek toksyczności w tym przypadku mógł być prawdopodobnie związany ze zmniejszonym działaniem czynnika toksycznego w wyniku jego degradacji lub wypłukania. Na analizowane parametry związane z fitotoksycznością wpływ może mieć również skład podłoża popieczarkowego, bowiem jak donoszą Catal and Peksen [2020] amoniak, sole, różne metale ciężkie, związki organiczne o małej masie cząsteczkowej występujące w zawartości tych odpadów mogą również uniemożliwiać kiełkowanie nasion i rozwój korzeni. Uzyskane wyniki wykazały, że całokształt zaistniałych, w analizowanej glebie po dodaniu podłoża popieczarkowego, warunków fizykochemicznych i chemicznych wpływa korzystnie na początkowy rozwój rośliny, wyrażony indeksem wzrostu. Niekorzystne oddziaływanie odpadu popieczarkowego na badane parametry fitotoksyczności uwidoczniło się najsilniej w roztworach glebowych, co

wskazywałoby, że poprawa ww. warunków glebowych niwelowała negatywny wpływ związków zawartych w roztworze glebowym. Ale z drugiej strony jak donoszą Canellas i Olivare [2014] rośliny uprawiane w optymalnych warunkach żywieniowych przeznaczają mniej energii na rosnące korzenie. Wyniki badań nad wpływem podłoża popieczarkowego na fitotoksyczność potwierdziły brak negatywnego wpływu na indeks wzrostu, czyli parametr związany z kiełkowaniem i masą kiełków. Natomiast biorąc pod uwagę przyrost korzenia, którego hamowanie odnotowano w I i II roku badań należy stwierdzić, że fitotoksyczność gleby uległa okresowemu pogorszeniu.

6.3. Wyniki uzyskane w publikacji P.3

Kwiatkowska E., Joniec J., Kwiatkowski C.A., Kowalczyk K., Nowak M., Leśniowska-Nowak J. 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. *Int. Agrophys.*, **2024**, 38, 139-154. https://doi.org/10.31545/intagr/184175

Badania przedstawione w publikacji **P.3** stanowią kontynuację oceny wpływu podłoża popieczarkowego oraz obornika na środowisko glebowe. Są również próbą weryfikacji przydatności i doboru odpowiednich wskaźników biologicznych (klasycznych i nowoczesnych) do monitorowania stanu środowiska glebowego i oceny skuteczności zastosowanych zabiegów nawozowych. Do tego celu wykorzystano liczebność drobnoustrojów, względną zawartość DNA, stężenie dsDNA oraz aktywność enzymatyczną. Badania wykonano na tym samym modelu badawczym, co badania opisane w publikacjach **P.1** i **P.2**. Na podstawie uzyskanych wyników stwierdzono, że zastosowanie podłoża popieczarkowego miało zróżnicowany wpływ na analizowane grupy drobnoustrojów. W przypadku względnej zawartości DNA, zastosowanie odpadu zarówno oddzielnie, jak i łącznie z obiema dawkami NPK, okazało się korzystne, szczególnie w przypadku grzybów. Najistotniejsze zmiany, analizowanych parametrów, również aktywności enzymatycznej, odnotowano głównie w pierwszych latach badań. Ponadto uzyskane wyniki wskazują, że łączne stosowanie odpowiednio dobranych klasycznych i nowoczesnych wskaźników pozwala na uzyskanie lepszego obrazu stanu gleb nawożonych m.in. odpadem popieczarkowym.

Wyniki uzyskane w publikacji **P.3** wykazały, że aplikacja zarówno podłoża popieczarkowego, jaki i obornika, wywołała istotne zmiany w liczebnościach

poszczególnych grup bakterii i grzybów, oznaczonych metodą płytkową. Liczebność bakterii kopiotroficznych oraz grzybów strzępkowych, a także drobnoustrojów celulolitycznych podlegała istotnym zmianom w ciągu całego okresu badań. Najwyraźniej odpad popieczarkowy wpłynął na te grupy drobnoustrojów w I roku trwania doświadczenia, gdzie odnotowano największą stymulację ich rozwoju. Najkorzystniejsze dla analizowanych parametrów mikrobiologicznych, w tym okresie, okazało się zastosowanie podłoża popieczarkowego łącznie z nawożeniem mineralnym, w przypadku obu grup bakterii oraz grzybów celulolitycznych z niższą, a grzybów strzępkowych z wyższą dawką NPK. Podobne obserwacje, dotyczące pobudzenia rozwoju bakterii i grzybów, odnotowano również w stosunku do liczebności drobnoustrojów proteolitycznych w publikacji **P.1**. Zastosowanie materiałów organicznych, w postaci podłoża popieczarkowego spowodowało zapewne początkową stymulację liczebności analizowanych grup drobnoustrojów. Odpad ten jest bogaty w materie organiczną oraz w różnego rodzaju makro i mikroelementy [Becher i in., 2021; Velusami i in., 2021]. W przypadku drobnoustrojów celulolitycznych kluczową rolę odegrała zapewne celuloza, która jest elementarnym składnikiem zarówno podłoża popieczarkowego, jak i obornika [Leong i in., 2022]. W kolejnych latach, tj. II i III roku badań, oddziaływanie odpadu popieczarkowego na rozwój omawianych parametrów mikrobiologicznych znacznie osłabło, a nawet znikło. Za spowolnienie mineralizacji materii organicznej w tym przypadku mógł być odpowiedzialny m.in. węglan wapnia, który jest jednym z podstawowych komponentów podłoża popieczarkowego [Becher i in., 2021], a jak sugerują Medina i in. [2012] organiczne cząsteczki węgla są lepiej chronione przed zniszczeniem przez aktywność mikrobiologiczną w glebach wapiennych. Reakcja mikroorganizmów glebowych, na stosowanie różnego rodzaju nawożenia, jest również dość mocno zależna od warunków klimatycznych, m.in. od wahań temperatury i wilgotności w warunkach polowych, a to z kolei przekłada się na różną wrażliwość drobnoustrojów na te czynniki. Zdaniem Li i in. [2022], bakterie wykazują większa wrażliwość na zmiany, np. opadów niż grzyby.

Zastosowanie obornika spowodowało podobne zmiany w liczebnościach omawianych grup drobnoustrojów, jak aplikacja podłoża popieczarkowego. Również w przypadku tego odpadu najwyraźniejszą stymulację rozwoju poszczególnych grup bakterii i grzybów odnotowano w I roku badań. W kolejnych latach efekt ten osłabł, a nawet w III roku odnotowano spadek liczby analizowanych grup drobnoustrojów w stosunku do obiektu kontrolnego.

W przypadku kolejnego wskaźnika, jakim jest stężenia dsDNA, zaaplikowanie podłoża popieczarkowego, szczególnie łącznie z nawożeniem NPK, spowodowało spadek tego parametru. Efekt ten wystąpił w I i II roku trwania doświadczenia. Pozytywny wpływ odpadu popieczarkowego na omawiany wskaźnik biologiczny odnotowano jedynie wiosną w II roku badań, w obiekcie z samym odpadem. W ostatnim roku trwania doświadczenia nie odnotowano istotnych zmian w stężeniu dsDNA w poszczególnych wariantach. Metody oparte na ekstrakcji dsDNA z gleby posiadają wiele zalet, ale niosą ze sobą również pewne wątpliwości, które mogą rzutować na otrzymane wyniki [Li i in., 2021; Roumani i in., 2023]. Ponadto na ilość i jakość wyizolowanego dsDNA mają wpływ także różne czynniki takie jak np.: rodzaj gleby, liczba mikroorganizmów, rodzaj uprawy, klimat i itp. [Wolińska i in., 2013, Rincon-Florez i in., 2013; Semenov, 2021; Wydro, 2022].

W przypadku względnej zawartości DNA, zarówno bakteryjnego, jak i grzybowego, odnotowano jego wzrost pod wpływem podłoża popieczarkowego zastosowanego zarówno oddzielnie, jak i łącznie z obiema dawkami nawożenia mineralnego. Jednak w przeciwieństwie do liczebności wcześniej omawianych grup drobnoustrojów efekt ten utrzymywał się dłużej, tj. w przypadku bakterii w II i III roku, a w przypadku grzybów przez wszystkie lata trwania doświadczenia. Należy zaznaczyć, że bardziej korzystne okazało się zastosowanie odpadu łącznie z nawożeniem mineralnym niż samego podłoża popieczarkowego. Najkorzystniejsze odnośnie tego parametru, jeśli chodzi o grzyby, okazało się zastosowanie odpadu łącznie z nawożeniem NPK, szczególnie w niższej dawce. O stymulacji rozwoju grzybów, oznaczonych metodami molekularnymi w glebie, pod wpływem podłoża popieczarkowego, donoszą m.in. Frąc i in. [2021].

Oddziaływanie obornika na stężenie dsDNA nie było ukierunkowane w poszczególnych latach i terminach. Jesienią w I roku odnotowano spadek tego parametru, z kolei wiosną w II roku oraz jesienią w III roku odnotowano jego wzrost. W pozostałych terminach zmiany nie były istotne. Z kolei w przypadku względnej zawrtości DNA, wprowadzenie do gleby obornika spowodowało spadek względnej zawartości bakteryjnego DNA oraz wzrost tego parametru w przypadku grzybów. Efekt ten utrzymywał się przez cały okres badań.

Warte podkreślenia są różnice uzyskane między stężeniem dsDNA i względną ilością DNA, zarówno bakteryjnego, jaki i grzybowego, a w wynikami oznaczonymi metodą płytkową dla liczebności mikroorganizmów. W przypadku bakterii różnice te wynikają zapewne z ograniczenia zdolności wzrostu niektórych grup tych drobnoustrojów na podłożach sztucznych [Rincon-Florez i in., 2013; Wydro, 2022]. Z kolei odnośnie

wyników uzyskanych dla grzybów należy zauważyć, że ich rozwojowi towarzyszył wzrost względnej ilości DNA, co świadczy o zgodności wyników otrzymanych metodami konwencjonalnymi i nowoczesnymi. Obserwacje te mogą wskazywać na konieczność łączenia obu tych technik w przyszłości.

Aktywność enzymatyczna, podobnie jak liczebność poszczególnych grup drobnoustrojów, wykazała istotne różnice w poszczególnych obiektach z zastosowanym nawożeniem. Zmiany te nie były jednak tak ukierunkowane jak w przypadku liczebności mikroorganizmów i wystąpiły tylko w I i II roku badań. W przypadku zarówno aktywności β-glukozydazy, jaki i aktywności hydrolitycznej fluoresceiny początkowo najkorzystniejsze okazało się zastosowanie samego odpadu popieczarkowego, a następnie odpadu z niższą dawką NPK. Dlatego można przypuszczać, że to właśnie produkty transformacji popieczarkowej materii organicznej przyczyniły się w pierwszych latach badań, do stymulacji omawianych enzymów. Wrażliwość tych parametrów, na zmiany właściwości gleby, wynika prawdopodobnie z ich silnego związku z zawartością i jakością materii organicznej [Gajda i in., 2016; Adetunji i in., 2017; Song i in., 2017]. W kolejnych latach jednak odnotowano spadek aktywności tych enzymów w kombinacji z samym odpadem. Odwrotna tendencja wystąpiła w przypadku stosowania odpadu łącznie z wyższą dawką NPK, gdzie odnotowano spadek aktywności obu enzymów w I roku badań. Należy jednak zaznaczyć, że zarówno stymulacja, jak i inhibicja, aktywności enzymatycznych na ogół zanikła w III roku badań. Ważną rolę w aktywności badanych enzymów, odegrał zapewne odczyn gleby. O zmianach odczynu gleby pod wpływem nawożenia mineralnego donoszą m.in. Ge i in. [2018] oraz Souza i in. [2023]. Z kolei Adetunji i in. [2017], jak i Dotaniya i in. [2019] podają, że β-glukozydaza, ze względu na swoją wrażliwość na zmiany pH, może służyć za jeden z lepszych wskaźników jakości gleby. Hydrolizę dioctanu fluoresceiny (FDA) przeprowadza wiele różnych enzymów [Dzionek i in., 2018; Patle i in., 2018], dlatego jej podatność na wahania odczynu gleby może być jeszcze większa. W przypadku kombinacji z NPK wahania aktywności β-glukozydazy i FDA, mogą wynikać także z dodatkowego źródła azotu i fosforu, w postaci nawożenia mineralnego. O stymulacji aktywności β-glukozydazy pod wpływem azotu donoszą m.in. Geisseler i Scow [2014]. Z kolei Davies i in. [2022] podają, że azot nie miał większego wpływu na aktywność omawianych enzymów, ale zwracają uwagę, że istotną rolę w ich aktywności mogły odegrać zmiany sezonowe. Również w poniższych badaniach czynniki klimatyczne, takie jak opad atmosferyczny i temperatura, miały istotny wpływ na aktywność analizowanych parametrów enzymatycznych.

Wpływ obornika na aktywność analizowanych enzymów był dość zróżnicowany. W przypadku aktywności β-glukozydazy, nawiezienie gleby tym odpadem spowodowało spadek jej aktywności w pierwszym roku badań. Natomiast w II roku odnotowano stymulację tego parametru. Aktywność hydrolityczna fluoresceiny pod wpływem tego nawożenia podlegała stymulacji zarówno w I, jak i II roku trwania eksperymentu. Spadek poziomu tego parametru odnotowano jedynie jesienią w I roku badań.

6.4. Wyniki uzyskane w publikacji P.4

Kwiatkowska E., Joniec J., Kwiatkowski C.A. Involvement of soil microorganisms in C, N and P transformations and phytotoxicity in soil from post-industrial areas treated with chemical industry waste. *Minerals,* **2023,** 13, 12. https://doi.org/10.3390/min13010012

W publikacji **P.4** przedstawiono wyniki dotyczące oceny przydatności wskaźników mikrobiologicznych oraz fitotoksyczności do monitorowania stanu gleb poddanych odziaływaniu odpadu z przemysłu chemicznego. Materiał glebowy pochodził z terenu poprzemysłowego (model badawczy II). Próbki gleby pobrano, z górnej i dolnej warstwy (0-20 i 20-40 cm), z trzech miejsc zlokalizowanych w różnej odległościach, tj. punkt S1 - 5,88 m, S2 - 22,70 m, S3 - 50,08 m, od zbiornika z płynnym odpadem poprodukcyjnym. Odpad ten pochodził z przemysłu chemicznego związanego, m.in. z produkcją klejów i celulozy. Powyższe badania wykazały, że wskaźniki mikrobiologiczne, biochemiczne oraz enzymatyczne gleby, a także wskaźniki fitotoksyczności mają potencjał szybkiego reagowania na zmiany środowiskowe. Dlatego też mogą służyć do oceny skutków oddziaływania różnych odpadów na gleby użytkowane zarówno agrotechnicznie, jak i poddane działalności przemysłowej. Ponadto odnotowane silne zmiany w aktywności populacji bakterii i grzybów w glebie zlokalizowanej najbliżej zbiornika z odpadem, gdzie odczyn był najwyższy (pH 10) sugerują, że mogło tu mieć miejsce wyselekcjonowanie się drobnoustrojów odpornych na wysokie pH. W związku z czym, obserwacje te wskazują na potrzebę kontynuowania badań pod kątem tym razem bioróżnorodności mikrobioty i mykobioty zasiedlających to miejsce.

Wyniki przedstawione w publikacji **P.4** dowodzą, że zmiany właściwości chemicznych gleby, mają istotny wpływ na analizowane parametry mikrobiologiczne w poszczególnych punktach poboru prób. Dane zebrane zarówno dla bakterii oligotroficznych, jaki i grzybów strzępkowych, wykazały podobną tendencję odnośnie liczebności obu tych grup drobnoustrojów. Najmniejszą liczebność drobnoustrojów

glebowych odnotowano w punkcie S1, tj. zlokalizowanym najbliżej zbiornika z odpadem, gdzie gleba charakteryzowała się zasadowym odczynem, co spowodowane było przenikaniem cieczy ze zbiornika do gleby. Efekt ten widoczny był w obu warstwach gleby i utrzymywał się przez cały okres badań. W miarę oddalania się od źródła zanieczyszczenia poziom omawianego parametru podlegał istotnemu wzrostowi, co zapewne było związanez poprawą warunków chemicznych gleby (spadek pH). Prezentowane wyniki są dowodem na dużą wrażliwość drobnoustrojów glebowych na stresujący czynnik, którym w tym przypadku był odczyn gleby. Potwierdzają to istotne różnice w liczebności między poszczególnymi analizowanymi punkami na stosunkowo małej odległości. Należy zauważyć, że w przypadku obu badanych grup drobnoustrojów, na ogół wyższe liczebności odnotowano w górnej warstwie gleby. Zdaniem Naylor i in. [2022] powierzchniowe warstwy gleby posiadają większą porowatość i intensywniejszy przypływ świeżych substratów oraz składników odżywczych, co przekłada się na stosunkowo wyższą aktywność drobnoustrojów. Ponadto minerały takie jak sód są bardziej podatne na wypłukiwanie, dlatego ich stężenie zwykle zwiększa się wraz z głębokością. To z kolei może przekładać się na pogorszenie warunków fizykochemicznych gleby, z którymi tak silnie związane są mikroorganizmy glebowe [Hermans i in., 2020; Shi i in., 2021]. Również zapewne zmiany sezonowe miały istotny wpływ na ten analizowany parametr, a potwierdzają to odnotowane korelacje.

Istotne różnice odnotowano także w przypadku nasilenia się procesów oddechowych. Najwyższe wartości parametr ten osiągnął latem w puntach S1 i S2, a najniższe w S3, czyli miejscu najbardziej oddalonym od zbiornika. Odmiennie kształtowało się nasilenie procesu oddychania jesienią, wraz z oddalaniem się od zbiornika z odpadem parametr ten istotnie wzrastał, zwłaszcza warstwie 0 – 20 cm. Uzyskane wyniki wskazują na zwiększone wydzielanie CO_2 z gleby w punktach zlokalizowanych bliżej zbiornika z odpadem. Obserwacje te wskazują, że z gleby zdegradowanej emitowana jest zwiększona ilość gazu cieplarnianego, co może przyczyniać się do pogłębiania się efektu cieplarnianego [Lal, 2020]. Dlatego też aktywność oddechowa może być dobrą miarą czynników stresowych, ponieważ po pierwsze odzwierciedla wydajność drobnoustrojów, a po drugie w warunkach stresowych wytwarzane są większe ilości CO_2 [Gonzalez-Quinones i in., 2011]. W analizowanych warunkach nasilenie procesu oddechowego było spowodowane przedostającym się ze zbiornika do gleby odpadem, który nie tylko spowodował wzrost odczynu gleby, ale również przyczynił się do zmian w jej strukturze. Jak donoszą Mavi i Marschnera [2017] zwiększenie nasycenia sodem w glebie powoduje rozproszenie

materii organicznej i cząstek ilastych, a tym samym niszczy agregaty i strukturę gleby. Co z kolei prawdopodobnie przyczynia się do uwalniania materii organicznej w nich zgromadzonej.

Na aktywność badanych enzymów, tak jak w przypadku wcześniej analizowanych parametrów, najistotniejszy wpływ miały zapewne zmiany w odczynie gleby. Świadczą o tym odnotowane najniższe wartości wszystkich analizowanych aktywności enzymatycznych, w punkcie położonym najbliżej zbiornika z odpadem. Potwierdzają to wyniki m.in. analizy składowych głównych (PCA), gdzie odnotowano ujemne korelacje odczynu gleby z analizowanymi enzymami. Dlatego też mógł być to jeden z czynników ograniczających aktywność enzymów glebowych zwłaszcza w punkcie położonym najbliżej zbiornika z odpadem. Na podkreślnie zasługuje także fakt, że aktywnosć fosfatzy zasadowej kształtowała się na znacznie wyższym poziomie niż fosfatazy kwaśnej. Jak pokazują niniejsze badania enzymy glebowe mają duży potencjał szybkiego reagowania na zmiany środowiskowe i dlatego mogą służyć jako wskaźniki zdrowia i jakości środowiska glebowego.

Przeprowadzone badania wskazują, że to przedostający się ze zbiornika do środowiska odpad chemiczny był głównym czynnikiem ograniczającym wzrost roślin w prezentowanych badaniach. Świadczy o tym zahamowanie kiełkowania rośliny testowej *L. sativum* w glebie pobranej najbliżej zbiornika z odpadem. Było to zapewne spowodowane mocną alkalizacją środowiska glebowego. Na uwagę zasługuje również fakt, że w miarę oddalania się od źródła zanieczyszczenia poziom odczynu gleby spadł, a to z kolei przełożyło się na poprawę badanych parametrów fitotoksycznych. Odczyn gleby był również czynnikiem ograniczającym zarówno kiełkowanie, jak i długość przyrostu korzenia *L. sativum*. Stymulacja parametrów związanych z fitotoksycznością jesienią była zapewne spowodowana lepszą dostępnością podstawowego składnika pokarmowego, ważnego z punktu widzenia żywienia roślin, jakim jest azot.

7. Podsumowanie i wnioski

Wyniki przedstawione w niniejszej rozprawie dowodzą, że wskaźniki jakości gleby takie jak: liczebność i różnorodność mikroorganizmów glebowych, nasilenie procesów biochemicznych, aktywność enzymatyczna, a także wskaźniki fitotoksyczności gleby, użyte na tle właściwości chemicznych, fizycznych i fizykochemicznych są czułymi parametrami zmian zachodzących w glebie poddanej oddziaływaniu różnych odpadów, pochodzących zarówno z działalności rolniczej, jak i przemysłowej. Wykorzystane metody okazały się dobrym narzędziem do oceny skuteczności zastosowanych zabiegów nawozowych, a także ryzyka związanego z powstawaniem gazów cieplarnianych w glebie, poddanej różnej antropopresji. Poniższe badania dostarczają wskazówek, które mogą być pomocne przy ograniczeniu negatywnych skutków rolniczej działalności człowieka, jak i ocenie stopnia degradacji środowiska glebowego spowodowanego oddziaływaniem zbiorników z ciekłymi odpadami, a także przy ocenie skuteczności ich zabezpieczeń.

Ze względu na obszerny materiał badawczy poniżej zamieszczono uogólnione wnioski. Bardziej szczegółowe wnioski, nawiązujące do poruszanych, w prezentowanych badaniach aspektów, zostały zawarte w publikacjach wchodzących w skład rozprawy.

1. Zastosowanie podłoża popieczarkowego spowodowało na ogół pobudzenie większości badanych parametrów związanych z przemianami mikrobiologicznymi C i N w glebie, ale wraz z upływem czasu efekt ten ulegał osłabieniu. Jedynie aktywność oddechowa podlegała nasileniu utrzymującemu się, z różną intensywnością, przez cały okres badań.

2. Aktywność enzymów odpowiedzialnych za przemiany P i S, tj. fosfatazy kwaśnej i arylosulfatazy, podlegały hamowaniu pod wpływem podłoża popieczarkowego. Należy zaznaczyć, że negatywny wpływ odpadu z czasem osłabł, a nawet zanikł, ale utrzymywał się w przypadku arylosulfatazy również w III roku w obiektach z odpadem i nawożeniem mineralnym w niżej dawce.

3. Przeprowadzone badania wykazały, że korzystny wpływ nawożenia odpadem popieczarkowym na aktywność drobnoustrojów ma charakter krótkoterminowy i dotyczy pierwszych dwóch lat stosowania.

4. Wyniki badań nad wpływem zużytego podłoża popieczarkowego na

fitotoksyczność potwierdziły brak negatywnego wpływu na indeks wzrostu. Natomiast biorąc pod uwagę przyrost korzenia, którego hamowanie odnotowano w I i II roku badań należy stwierdzić, że fitotoksyczność gleby uległa okresowemu pogorszeniu.

5. Najkorzystniejsze dla analizowanych parametrów mikrobiologicznych okazało się zastosowanie podłoża popieczarkowego łącznie z niższą dawką nawożenia mineralnego.

6. Przedstawione badania wskazują również, że do monitorowania zmian zachodzących w glebie nawiezionej odpadem popieczarkowym wskazane jest łączne stosowanie różnych metod badawczych, zarówno klasycznych, jak i nowoczesnych.

7. Odpad popieczarkowy w mniejszym stopniu niż obornik przyczynia się do wzrostu ilości produktów nitryfikacji, które następnie mogą potencjalnie prowadzić do powstania gazu cieplarnianego, tj. N_2O i tym samym przyczyniać się do wzrostu efektu cieplarnianego.

8. Nawozowe zastosowanie odpadu popieczarkowego przyczyniło się do wzrostu ilości wydzielanego CO_2, którego ilość wzrastała wraz z upływem czasu. Obserwacje te wskazują, że taki sposób gospodarowania tym odpadem może przyczyniać się do nasilenia efektu cieplarnianego poprzez zwiększenie emisji CO_2 z gleby.

9. Wpływ obornika na emisję gazów cieplarnianych nie był jednoznaczny. W przypadku CO_2, nie spowodował on istotnego utrzymującego się wzrostu jego emisji. Natomiast stymulujący wpływ obornika na proces nitryfikacji, którego produkty mogą być transformowane do N_2O, utrzymywał się znacznie dłużej niż odpadu popieczarkowego.

10. Zastosowane parametry aktywności drobnoustrojów oraz wskaźniki fitotoksyczności są czułymi markerami zmian spowodowanych oddziaływaniem ciekłego odpadu na glebę. Spośród analizowanych parametrów, najczulszymi w ocenie zmian środowiska glebowego, pod wpływem silnej alkalizacji, okazały się: ogólna liczebność bakterii i grzybów, aktywności fosfatazy kwaśnej

i zasadowej oraz aktywność hydrolityczna fluoresceiny.

11. Wyniki dotyczące zanieczyszczenia gleby ciekłym odpadem wykazały, że jego negatywne oddziaływanie na populacje mikroorganizmów glebowych nie ogranicza się jedynie do górnej warstwy gleby (0 - 20 cm), ale jest również wyraźnie widoczne w jej dolnej warstwie, tj. 20 – 40 cm. Natomiast nie odnotowano negatywnych zmian w dalszej odległości od zbiornika.

12. Uzyskane wyniki wskazują, że z gleby zdegradowanej emitowana jest zwiększona ilość CO_2, co może przyczyniać się do pogłębiania się efektu cieplarnianego. Aktywność oddechowa jest dobrą miarą czynników stresowych, ponieważ po pierwsze odzwierciedla wydajność drobnoustrojów, a po drugie w warunkach stresowych wytwarzane są większe ilości CO_2.

8. Bibliografia

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9. Publikacje wchodzące w skład rozprawy doktorskiej

9.1. Publikacja P.1 - *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.*

Joniec J., **Kwiatkowska E**., Kwiatkowski C.A.
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.
Agriculture, **2022**, 12, 1190.
https://doi.org/10.3390/agriculture12081190

agriculture

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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

Citation: Joniec, J.; Kwiatkowska, E.; Kwiatkowski, C.A. 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. *Agriculture* **2022**, *12*, 1190. https://doi.org/10.3390/agriculture12081190

Academic Editors: Sylwia Andruszczak and Monika Skowrońska

Received: 11 July 2022
Accepted: 8 August 2022
Published: 10 August 2022

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%).

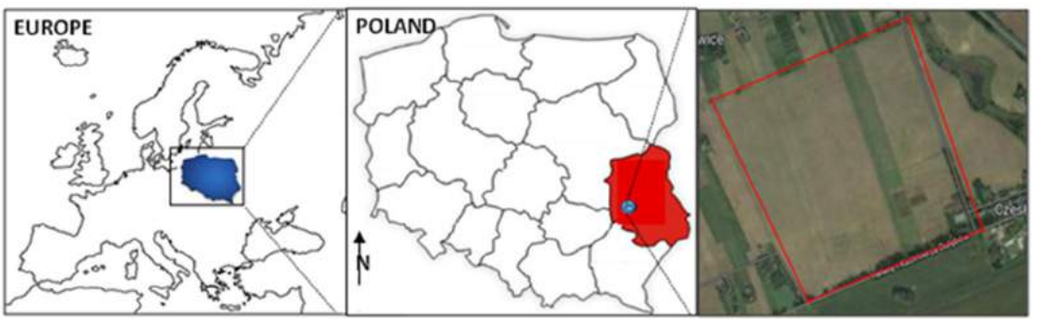

Figure 1. Location of the research area against the background of Europe, Poland and Lublin Region; red lines mark the area of Experimental Farm in Czesławice.

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.

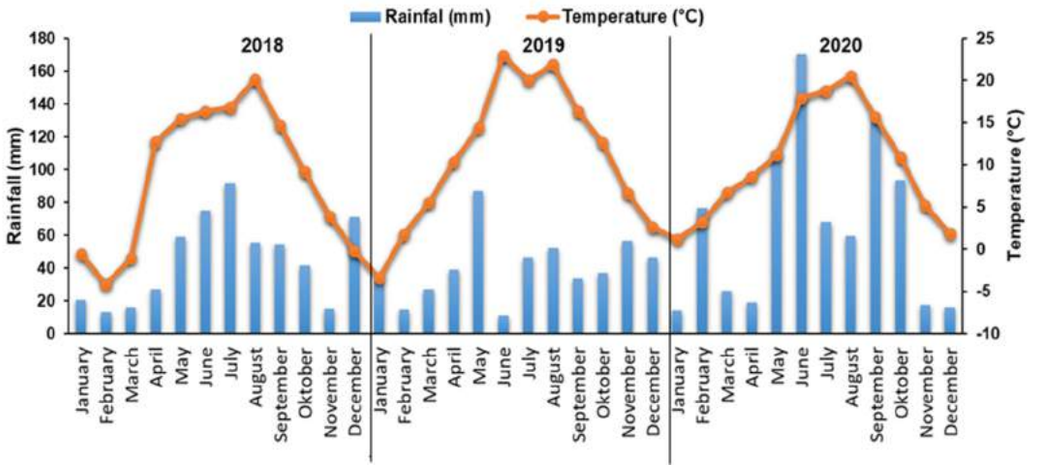

Figure 2. Mean monthly temperatures and rainfall in the experimental site during the experimental period.

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.

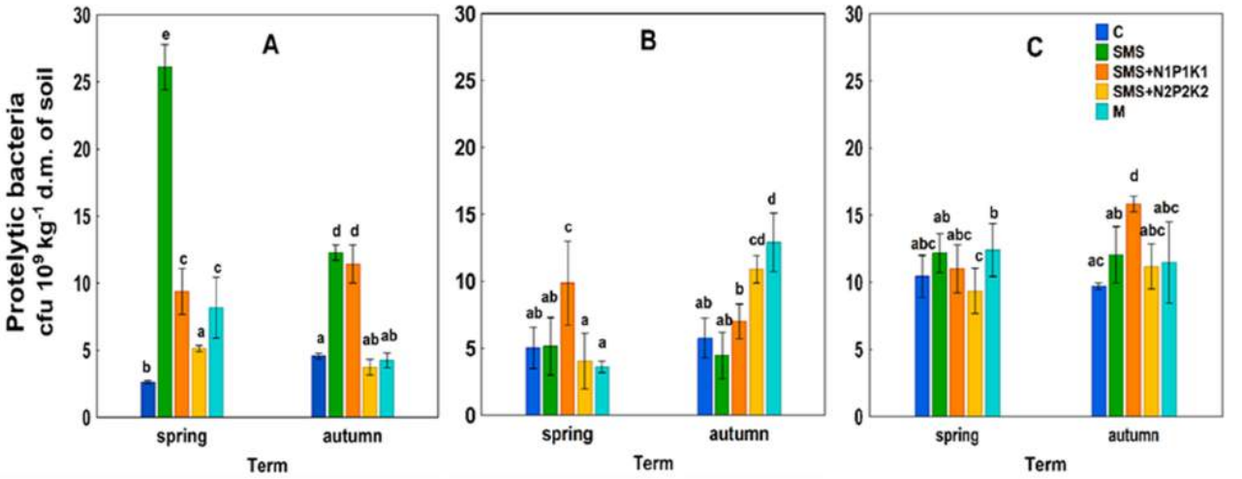

Figure 3. Number of proteolytic bacteria in the soil. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure; (**A**)—1st year; (**B**)—2nd 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.

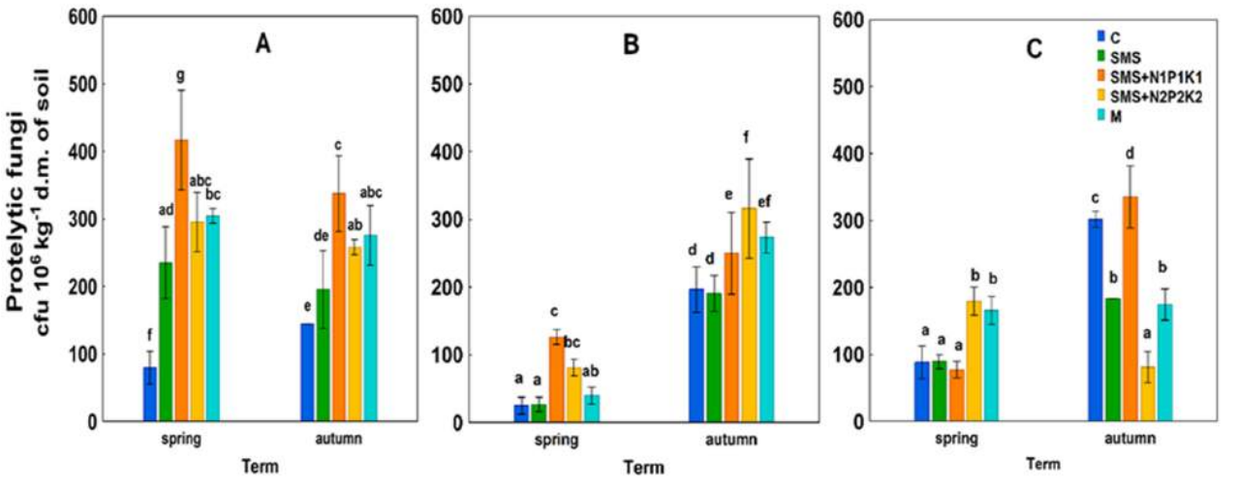

Figure 4. Number of proteolytic fungi in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization 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).

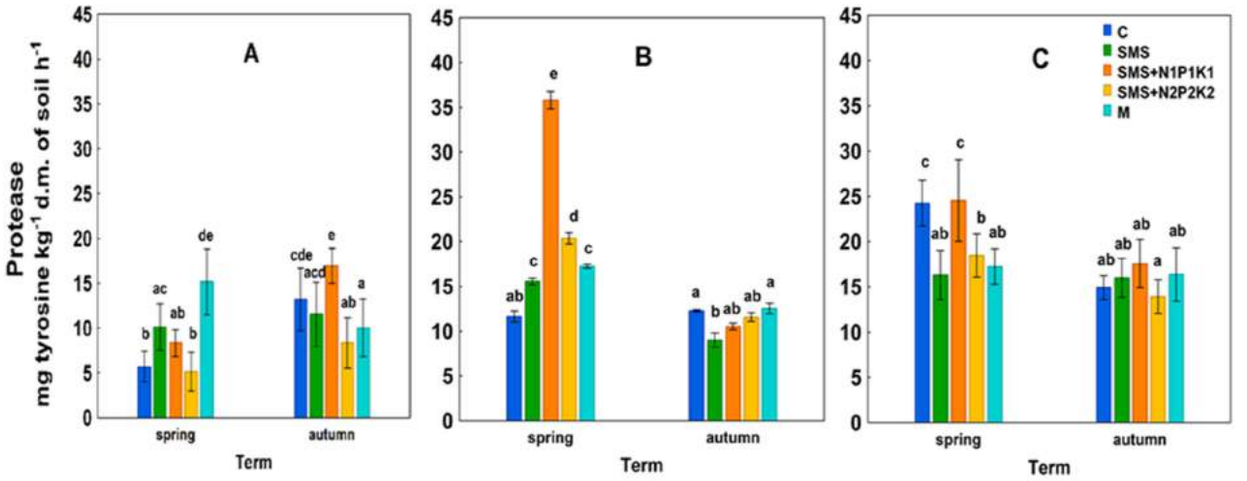

Figure 5. Activity of protease in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; 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.

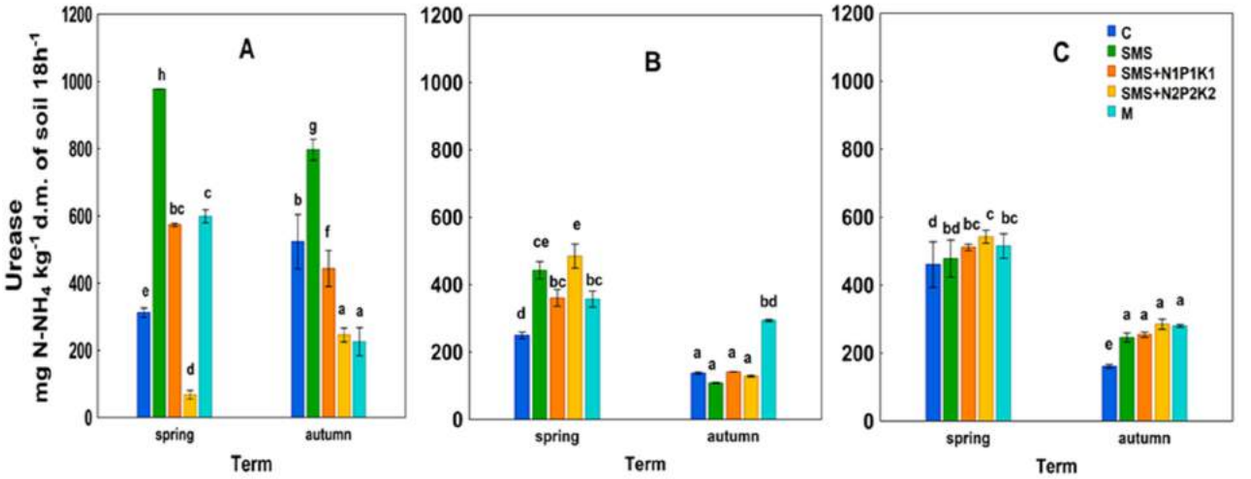

Figure 6. Activity of urease in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; 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.

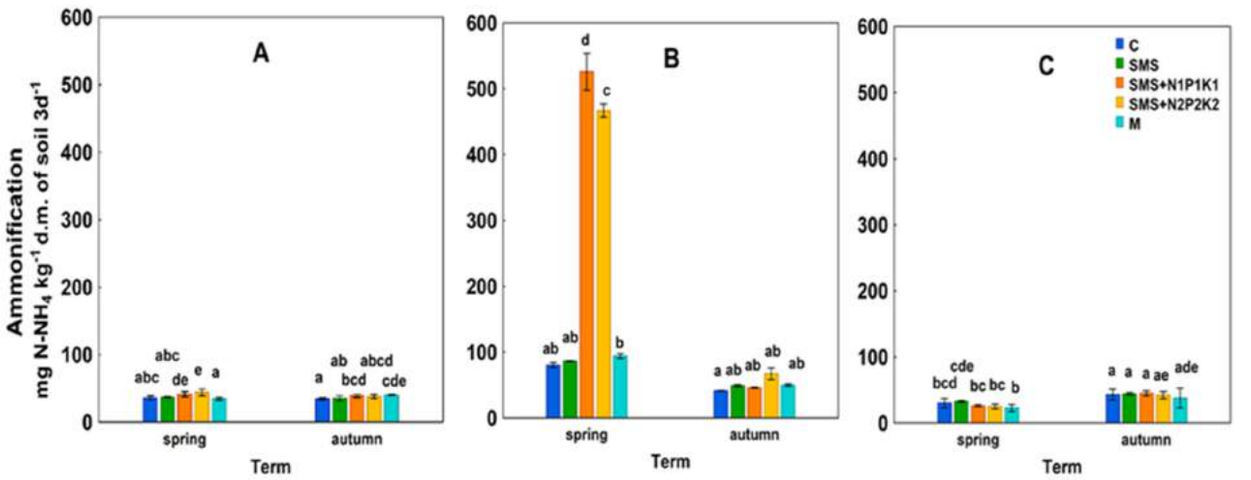

Figure 7. Ammonification in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; 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.

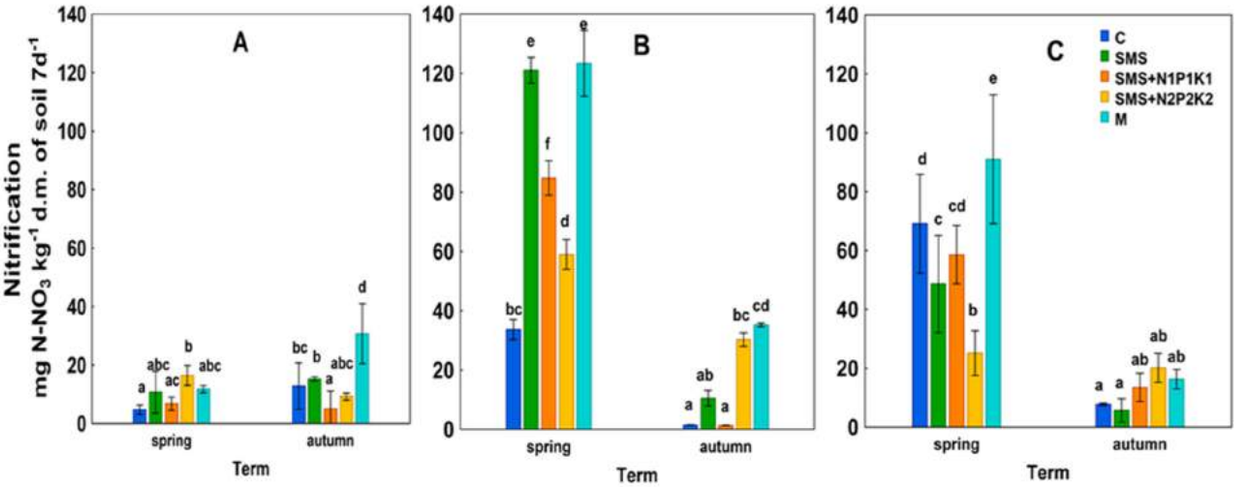

Figure 8. Nitrification in the control soil and soil under different treatment strategies. Legend: C—control; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; 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].

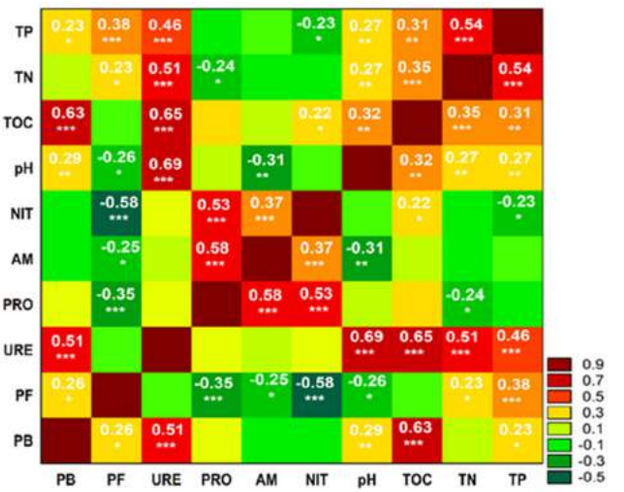

Figure 9. Heatmap displaying the Pearson's correlation coefficients between soil physico-chemical, chemical, and microbial properties. BP—proteolytic bacteria, PF—proteolytic fungi, URE—urease, PRO—protease, AM—ammonification, NIT—nitrification, TOC—total organic carbon, TN—total nitrogen, TP—total potassium. 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).

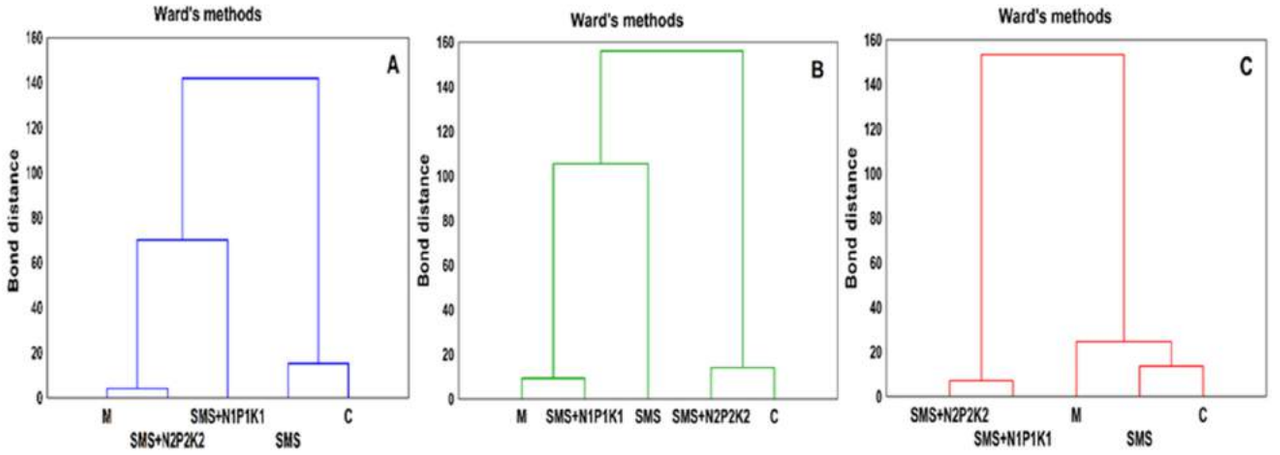

Figure 10. Tree diagram—Ward's dendogram for (**A**) microbial counts; (**B**) enzymatic activity; and (**C**) biochemical activity. C—control soil; SMS—soil + spent mushroom substrate, SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate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

Author Contributions: Conceptualization, J.J. and E.K.; methodology, J.J.; software, J.J. and E.K.; validation, J.J. and E.K.; formal analysis, J.J. and E.K.; investigation, J.J.; resources, J.J. and C.A.K.; data curation, J.J.; writing—original draft preparation, E.K.; writing—review and editing, J.J. and E.K.; visualization, J.J. and E.K.; supervision, J.J.; project administration, J.J. and C.A.K.; funding acquisition, J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the University of Life Sciences in Lublin.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Additional information is provided by the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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9.2. Publikacja P.2 - *Effects of agricultural management of spent mushroom waste on phytotoxicity and microbiological transformations of C, P, and S in soil and their consequences for the greenhouse effect.*

Kwiatkowska E., Joniec J.
Effects of agricultural management of spent mushroom waste on phytotoxicity and microbiological transformations of C, P, and S in soil and their consequences for the greenhouse effect.
Int. J. Environ. Res. Public Health, **2022,** 19, 12915.
https://doi.org/10.3390/ijerph191912915

International Journal of
Environmental Research and Public Health

MDPI

Article

Effects of Agricultural Management of Spent Mushroom Waste on Phytotoxicity and Microbiological Transformations of C, P, and S in Soil and Their Consequences for the Greenhouse Effect

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Abstract: The huge volumes of currently generated agricultural waste pose a challenge to the economy of the 21st century. One of the directions for their reuse may be as fertilizer. Spent mushroom substrate (SMS) could become an alternative to manure (M). A three-year field experiment was carried out, in which the purpose was to test and compare the effect of SMS alone, as well as in multiple variants with mineral fertilization, and in manure with a variety of soil quality indices—such as enzymatic activity, soil phytotoxicity, and greenhouse gas emissions, i.e., CO_2. The use of SMS resulted in significant stimulation of respiratory and dehydrogenase activity. Inhibition of acid phosphatase and arylsulfatase activity via SMS was recorded. SMS showed varying effects on soil phytotoxicity, dependent on time. A positive effect was noted for the growth index (GI), while inhibition of root growth was observed in the first two years of the experiment. The effect of M on soil respiratory and dehydrogenase activity was significantly weaker compared to SMS. Therefore, M is a safer fertilizer as it does not cause a significant persistent increase in CO_2 emissions. Changes in the phytotoxicity parameters of the soil fertilized with manure, however, showed a similar trend as in the soil fertilized with SMS.

Keywords: spent mushroom substrate; manure; phytotoxicity; soil respiration; greenhouse effect; dehydrogenases; enzymatic activity; *Lepidium sativum* L.; waste; soil microorganisms

Citation: Kwiatkowska, E.; Joniec, J. Effects of Agricultural Management of Spent Mushroom Waste on Phytotoxicity and Microbiological Transformations of C, P, and S in Soil and Their Consequences for the Greenhouse Effect. *Int. J. Environ. Res. Public Health* **2022**, *19*, 12915. https://doi.org/10.3390/ijerph191912915

Academic Editor: Andrzej Klasa

Received: 15 September 2022
Accepted: 6 October 2022
Published: 9 October 2022

1. Introduction

The intensification of human economic and livelihood activities is associated with the generation of huge amounts of various types of waste [1]. Therefore, the modern economy is increasingly open to production based on technologies that allow the integration of the broadly understood waste back into the production cycle. Among the many directions for their reuse, fertilizer application is particularly important. Agricultural wastes generated in rural areas as a result of crop processing and agricultural activities show a particularly high fertilizing potential. One such waste of organic origin is spent mushroom substrate (SMS) (*Agaricus bisporus* L.) [2].

According to the Food and Agriculture Organization Corporate Statistical Database, the global production of mushrooms and truffles in 2020 was 42,792,893 tons compared, for example, to only 8,781,004 tons in 2000, i.e., 20% of the total current production. Globally, China is, by far, the main producer of mushrooms and truffles (40,004,574 tons in 2020), while Europe (1,270,241 tons in 2020) is led mainly by the Netherlands (260,000 tons in 2020), Poland (182,900 tons in 2020), and Spain (166,010 tons in 2020) [3]. Such intensive global production results in the generation of large quantities of spent mushroom substrate, estimated at approximately 60 million tons per year [4,5]. The efficient use and disposal of such a large volume of annually generated material is, therefore, a major challenge for the modern economy.

Due to its composition (mainly high organic matter content), poorly stored spent mushroom substrate can pose environmental hazards through the development of pathogenic

microflora and the spread of fungal diseases, uncontrolled waste biodegradation by microorganisms, and the consequent emission of greenhouse gases into the atmosphere; this is as well as the leaching of nutrients into surface and groundwater [5,6].

Due to growing environmental concerns, the proper disposal and handling of excess SMS accumulation are essential. Current research has clearly indicated that, due to its high fertilizing value, agricultural application is the most efficient method for SMS recycling [7–10]. SMS is a valuable source of organic matter and nutrients that is readily available to plants [8,11,12]. It is important to note that the composition of SMS varies greatly depending on location, type of mushroom grown, and other factors [5]. It also improves a number of soil properties, including structure, pH, and water-holding capacity [13,14]. Additionally, this method of management indirectly solves the problem of other wastes, i.e., those previously used to compose mushroom substrate, e.g., straw; poultry and cattle manure, waste gypsum from electrostatic precipitators, phosphogypsum and $CaCO_3$ [2,5]. In addition, spent mushroom substrate can be composted with the addition of other wastes, i.e., liquid manure or sewage sludge, which also allows for the recycling of these additional wastes [15,16]. Considering the wide variety and variability of individual spent mushroom substrates, it is advisable to study their composition and possibly balance the components by supplementing them with mineral fertilization.

It is important to carry out soil toxicity tests due to the possibility of toxic compound formation, which arises as a result of the microbiological transformation of waste organic matter. In order to monitor the soil environment in this respect, it is recommended to use biotests, e.g., a phytotest using *Lepidium sativum* L. [17,18]. *L. sativum* L. has been repeatedly used as a bio-indicator to determine the effects of various chemical compounds, including those of waste origin, on plant germination and growth [19–23].

When selecting the method of managing organic waste, including waste generated in agriculture, one should take into account the possibility of greenhouse gas (GHG) emissions as a result of the transformation of carbon and nitrogen matter. Agriculture is the main sector contributing to their emissions, estimated at between 10% and 20% of the total anthropogenic GHG emissions [24]. Both fertilizers and waste, especially organic waste, contain large amounts of organic carbon, whose resources in farmland play a key role in sustainable agriculture. It is the organic matter, the main source of which can be SMS, that influences the rate of mineralization, accumulation, or emission of carbon from the soil and the complex interactions between biological and physico-chemical soil processes and environmental conditions [25]. Soil carbon sequestration, i.e. increasing the amount of this element in the soil, stored as organic matter, can improve soil quality and reduce the contribution of agriculture to CO_2 emissions [26,27]. However, simply adding organic matter to the soil will not solve the problem. In addition, it is also important to assess the impact of this application on soil processes and microbial activity. As approximately 90% of CO_2 emitted from the soil is of microbial origin, it is, therefore, the main component in the global carbon cycle, emitting about ten times more CO_2 per year into the atmosphere than burning fossil fuels [28,29]. Therefore, changes in the activity of respiratory processes may indicate ecological disturbances and also a large contribution of microorganisms to soil metabolism and global warming. An indirect indicator of the total number and activity of microorganisms in the soil is respiratory activity, which can be a marker of changes occurring in this environment [30]. CO_2 emitted from the soil is the final product of mineralization and the oxidation of organic substances by soil microorganisms, but also the result of plant respiratory processes and the decomposition of organic compounds brought into the soil with roots [31]. Therefore, respiratory activity has been recognized by many other authors as a good determinant of the rate of organic matter decomposition or microbial biomass [21,32–35].

Soil enzymes also play a significant role in ecosystem processes, participating in multiple reactions that are an integral part of various biogeochemical cycles [36]. They regulate, among others, the decomposition of organic matter and determine the availability of nutrients in the soil; therefore, they are critical for the carbon cycle in ecosystems [37,38].

Given that microorganisms contribute significantly to organic matter cycling and long-term soil carbon stabilization, it is thus necessary to monitor the impact of climate change (this includes, in particular, the greenhouse effect), on microbial communities and soil carbon cycling rates. Enzyme activity reflects the metabolic requirements of the microbial community and may therefore be an important indicator of microbial function in response to climate change [39,40]. Both acid phosphatase and arylsulfatase have been used multiple times to assess the condition of soil environments, including those fertilized with various types of organic waste [21,34,41–43].

A number of agricultural wastes, including spent mushroom substrate, have significant fertilizing potential. A multi-year field study was conducted as part of a series [44] to investigate and compare the effects of spent mushroom waste and manure on soil quality indicators, such as biochemical and enzymatic activity related to microbial transformations of C, P, and S, as well as soil phytotoxicity. Pertaining to this research, the authors posed the following hypotheses: (1) spent mushroom waste is a good fertilizer alternative to manure and can be applied annually; (2) spent mushroom waste has no phytotoxic effects on the initial stages of plant growth, i.e., germination, root growth, and sprout weight; (3) agricultural management of SMS does not contribute to the greenhouse effect. In view of the above assumptions, the authors assumed that the obtained results would allow for better management of agricultural waste, including spent mushroom waste, in a manner that ensures an increase in soil fertility in accordance with the principle of sustainable development. The presented research may be helpful in achieving the United Nations' Sustainable Development Goals (SDGs), such as climate action, responsible consumption and production, and the elimination of poverty and hunger [45,46].

2. Materials and Methods

2.1. Site and Experimental Setup and Soil Sampling

The experiment with the use of spent mushroom substrate and manure was carried out at the Czesławice Experimental Station (Lublin region, Poland, 51°18′26″ N, 22°16′1″ E) (Figure 1) in a randomized block design.

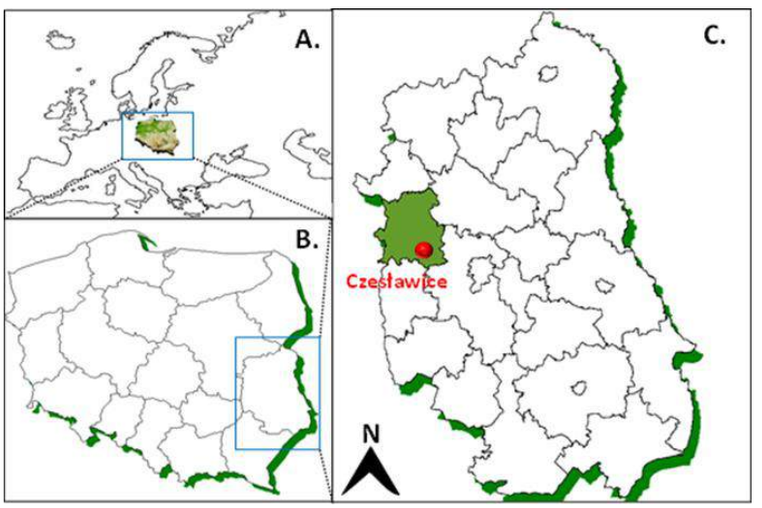

Figure 1. Location of the research area: (**A**) location of Poland against the background of Europe; (**B**) location of the Lublin region in Poland; and (**C**) location of the "Czesławice" farm in the Lublin region.

Experimental plots were located on a lessive soil belonging to the second quality class [47,48]. 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%); and fraction <0.002 mm—colloidal clay (9%). The plots were established in triplicate (the area of a single plot was 3 m^2) and fertilized for three years (in the fall) with

single doses (20 t ha^{-1}) of spent mushroom medium SMS (moisture 67%) and composted cattle manure M (moisture 77%). The spent mushroom medium was composed on the basis of winter wheat straw, peat, and chicken manure. It did not contain any mineral additives, as it was intended for organic farming. Supplemental mineral fertilization with nitrogen (N), phosphorus (P), and potassium (K) was also applied to the sites with this substrate. This was due to the initial abundance of assimilable nutrients in the soil and from the hypothesized rapid release of nutrients from this waste, and thus the short-term fertilizing effect of the spent mushroom substrate alone (without NPK fertilization). Therefore, nitrogen was introduced in the form of ammonium nitrate at doses of N1—50 kg ha^{-1} and N2—100 kg ha^{-1}, phosphorus in the form of granular triple superphosphate at doses of P1—30 kg ha^{-1} and P2—60 kg ha^{-1}, and potassium as potassium sulfate at K1—70 kg ha^{-1} and K2—140 kg ha^{-1}. Soil without fertilizer constituted the control object. Italian ryegrass (*Lolium multiflorum* Lam.) was used as the test plant. The characteristics of the spent mushroom substrate and manure are presented in Table 1.

Table 1. Properties of soil and wastes [44].

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}	No.	86.0	No.
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, and TP—total potassium.

Experimental scheme:

1. Soil without fertilizer (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).

Research was carried out from 2018 to 2020. Soil material was collected with a gouging drill, from the 0–25 cm layer, from ten randomly selected sites within each test plot at two time points, i.e., in the spring (June) and fall (September). The average soil sample from each plot (about 4 kg) consisted of a mixture of 10 soil cores, each 4 cm in diameter. The collected samples were sifted through a 2 mm sieve and stored in plastic bags at 4 °C.

2.2. Meteorological Conditions

Weather conditions were recorded by the Meteorological Station in Czesławice, located ~800 m from the field experiment. The total precipitation in 2018, 2019, and 2020 was 539.3, 481.8, and 799.7 mm, respectively, while the average annual air temperature was 8.6, 11.0, and 10.1 °C, respectively. Analyzing the weather conditions in the months of soil sampling—i.e., June and September—it was found that monthly precipitation varied considerably and amounted to 74.8 and 54.7 in 2018, 11.2 and 33.5 in 2019, and 170.3 and 128.5 in

2020. The highest temperature during the entire study period was observed in June 2019 (22.9 °C), while values of 16.3, 17.9, 14.7, 16.3, and 15.6 were recorded at the remaining time points—June 2018 and 2020, and September 2018, 2019, and 2020, respectively (Figure 2).

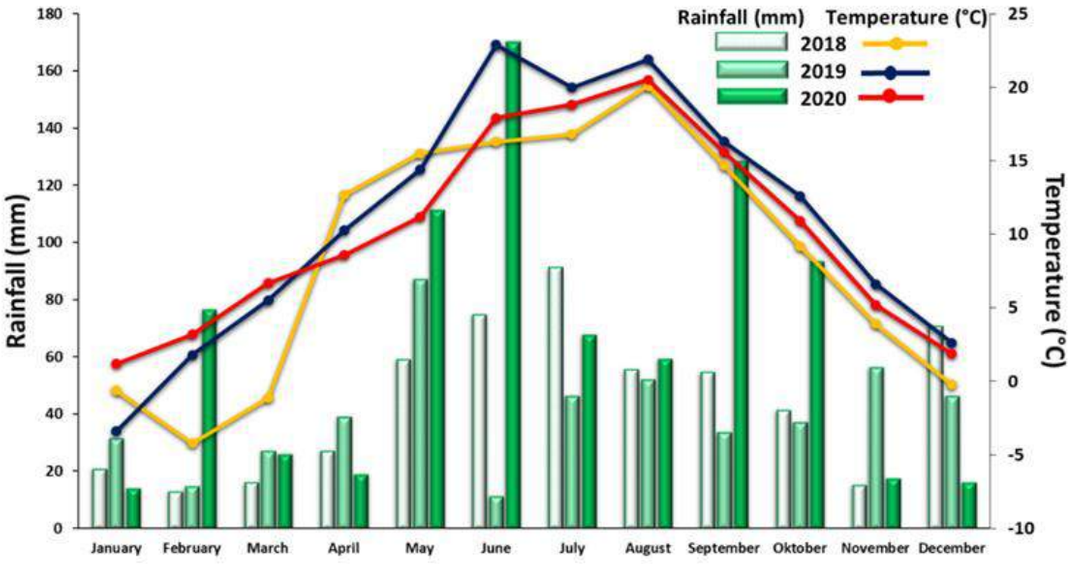

Figure 2. Average monthly temperatures and monthly rainfall totals in the experimental area during the research period.

2.3. Biochemical and Enzymatic Analyses

Respiratory activity was determined using the method of Rühling and Tyler [49]. Soil samples (20 g) with 1% glucose addition were incubated for 24 h in the presence of 0.2 M NaOH solution. After incubation, the excess unbound sodium hydroxide was titrated with 0.1 M HCl in the presence of $BaCl_2$ and phenolphthalein.

Thalmann's [50] method was used to determine dehydrogenase activity. Further, soil samples (5 g) with 2,3,5–triphenyltetrazolium chloride addition as the substrate were incubated in 0.1 M tris(hydroxymethyl)aminomethane buffer (Tris–HCl pH 7.4) for 48 h at 30 °C. Enzymatic activity was determined colorimetrically (λ = 485 nm) by measuring the extinction of the TPF (triphenylformazan) produced.

The method of Tabatabai and Bremner [51] was used to determine acid phosphatase activity. Soil samples (1 g) with p-nitrophenyl disodium phosphate (PNPNa) as a substrate were incubated for one hour at 37 °C in a modified universal buffer (pH 6.5). For arylsulfatase, soil samples (1 g) were incubated for 1 h at 37 °C in the presence of p-nitrophenol sulfate (PNS) in a modified universal buffer (pH 5.8) [52]. The activity of both enzymes was determined spectrophotometrically at 400 nm and expressed as para-nitrophenol-mg PNP kg^{-1} dm soil h^{-1}.

All analyses were carried out in triplicate, and activities were calculated based on dry soil weight.

2.4. Phytotoxicity

As part of the soil phytotoxicity evaluation, two phytotests were performed using garden cress (*Lepidium sativum*) as a test plant.

The test of Masciandaro et al. [53] was used for the purposes of determining the effect of the overall conditions in the soil on the development of *L. sativum*, following the application of the tested variants of organic fertilization. For this purpose, 100 seeds of *L. sativum* were sown (in triplicate) on 50-gram weights of fresh soil placed in Petri dishes (moisture content—60%WHC). Incubation was carried out for four days at 22 °C, maintaining a constant moisture level. Subsequently, the number of germinated seeds was

counted and their weight was determined. The growth index (GI) was calculated based on these parameters, according to the formula of Masciandaro et al. [53]:

$$GI\% = P\left(\frac{T}{C}\right)$$

P—mean % of germinated seeds in the reclaimed soil relative to the value for the control soil; *T*—mean weight of fresh *L. sativum* sprouts in the reclaimed soil; *C*—mean weight of fresh *L. sativum* sprouts in the control soil.

The second test analyzed the effect of potentially toxic substances dissolved in soil solution on the sprouting and growth of *L. sativum* roots after 2 and 4 days. For this purpose, fresh soil weights (20 g) (moisture content—60%WHC) were placed on Petri dishes covered with sterile disks of blotting paper in six replicates. Following this, 90 *L. sativum* seeds were placed on 3 plates, and 10 seeds on the remaining 3 plates. Incubation was carried out at 22 °C. The number of germinated seeds on all plates was counted after two days. The length of sprout roots was also measured after two and four days on plates containing ten seeds each.

2.5. Chemical Analyses

Chemical analyses complemented biochemical, enzymatic, and phytotoxicity tests (Tables 1 and 2). The pH was determined from the soil extract in KCl (10 g of soil in 25 mL of KCl) using an electrometric method. Organic carbon (TOC) was determined by IR spectrometry. The Kjeldahl method was used to determine total nitrogen (TN), and total phosphorus (TP) was determined by spectrophotometry. Flame atomic absorption spectrometry (FAAS) was used to determine calcium, potassium, and magnesium. All of the above methods were applied to soil, as well as spent mushroom substrate and manure samples. In addition, heavy metals in the spent mushroom substrate were determined using atomic absorption spectroscopy (AAS).

Table 2. Selected physico-chemical and chemical properties of the soil [44].

	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

Table 2. *Cont.*

	Year	Season	C	SMS	SMS + N1P1K1	SMS + N2P2K2	M
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

Abbreviations: TOC—total organic carbon, TN—total nitrogen, and 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, and M—soil + manure.

2.6. Statistical Analysis

Descriptive statistics involved calculating the arithmetic means of three replicates obtained for a given sample, along with the standard deviation. The results were presented in the form of bar graphs. Statistical evaluation of result variability was carried out using a two-factor analysis of variance, where each year was analyzed separately. The basic ANOVA assumptions, including normality of the dataset and homogeneity of variance, were checked with the Shapiro–Wilk and Levene tests. The significance of differences between means was verified using Tukey's post hoc test. Significance was assumed at α=0.05. The relationships between the analyzed biochemical, enzymatic, phytotoxic, and physicochemical parameters and environmental conditions were analyzed via principal component analysis (PCA). These relationships were also analyzed at the level of experimental combinations using Pearson correlations at three levels of significance: $p < 0.001$, $p < 0.01$, and $p < 0.05$; in addition, the results were presented as heat maps. All statistical analyses were performed using the Statistica 13.1 package (TIBCO Software Inc.; Palo Alto, CA, USA).

3. Results

The data presented in Figure 3 and Table 3 show that the application of spent mushroom substrate and manure significantly affected soil respiration. During the three years of the experiment, this activity was stimulated in the spent mushroom substrate sites (SMS, SMS + N1P1K1, and SMS + N2P2K2), and its intensity varied from site to site and changed with time. Respiration reached the highest values in the third year in the sites with NPK fertilization—i.e., SMS + N1P1K1 and SMS + N2P2K2 (234.08–240.72 mg)—while the lowest values were recorded in the second year in the site with spent mushroom substrate alone, i.e., SMS (34.78 mg). The introduction of waste into the soil separately and in combination with NPK at both doses resulted in the stimulation of respiration, which over time was limited to the sites where SMS was introduced together with NPK (SMS + N1P1K1 and SMS + N2P2K2). The highest stimulation of this parameter was recorded at these sites in the spring of the first year and in the spring and fall of the third year. In contrast, the impact of SMS alone was not as directional over time. In the second year, a decrease and then a subsequent increase in respiration were recorded under its influence, and in the third year, its effect disappeared.

The effect of manure on respiration was significantly less apparent than that of the spent mushroom substrate. Stimulation of this process was recorded in the manure sites only in the first year and occurred more strongly in the fall, where its value was 98.74 mg compared to 68.95 mg in the control (C). In subsequent years, there was a loss of stimulation and even a decrease in respiratory activity.

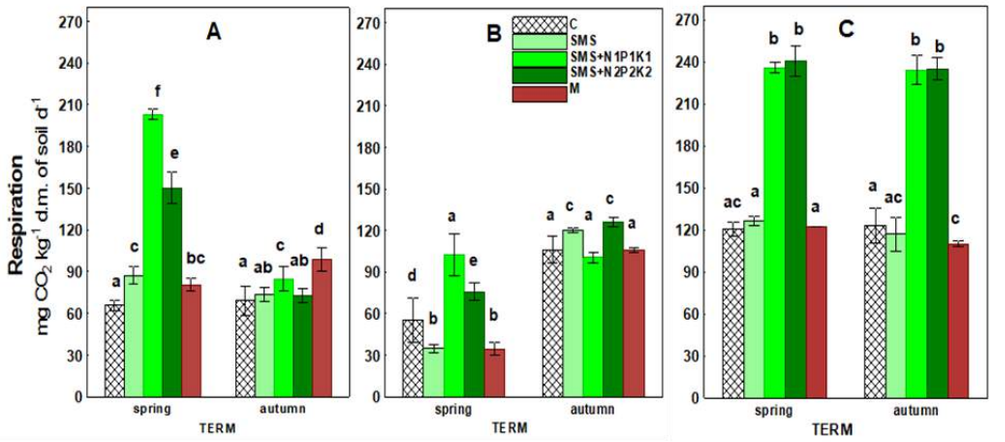

Figure 3. Respiratory activity in control soil and soil under different treatment strategies. (**A**) 1st year; (**B**) 2nd year; and (**C**) 3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; and M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

Table 3. Biochemical, enzymatic activity and soil phytotoxicity parameters (annual averages).

Years	Experimental Treatments	RES	DEH	AcP	ARS	GI	GERM	RL2	RL4
2018	C	67.28 a	4.89 abc	36.60 b	63.95 i	100.00 ab	99.33 g	1.99 f	3.74 g
	SMS	80.58 cd	11.60 i	38.58 b	60.43 h	92.86 a	99.00 g	1.70 de	2.92 de
	SMS + N1P1K1	143.84 h	9.23 gh	34.25 b	27.44 c	93.75 a	99.17 g	1.62 de	2.83 cde
	SMS + N2P2K2	111.40 f	3.77 a	24.35 a	23.58 a	176.86 e	99.00 g	1.57 d	2.98 def
	M	89.65 d	4.88 ab	23.60 a	31.28 d	145.86 d	98.50 g	1.50 cd	1.99 ab
2019	C	80.75 cd	5.31 bc	48.76 c	42.58 ef	100.00 ab	88.00 de	1.18 ab	3.13 efg
	SMS	77.76 bc	3.94 a	48.93 c	45.51 g	220.27 f	88.42 ef	1.30 bc	2.50 bcd
	SMS + N1P1K1	101.64 e	4.64 ab	43.92 c	26.50 bc	144.20 d	87.00 cde	1.15 ab	2.09 ab
	SMS + N2P2K2	100.92 e	4.37 ab	57.06 de	24.65 ab	116.94 abc	90.58 f	1.00 a	1.65 a
	M	70.38 ab	6.01 cd	55.59 d	40.23 e	181.52 e	88.83 ef	1.30 bc	2.24 abc
2020	C	121.85 g	6.72 de	61.98 ef	46.72 g	100.00 ab	85.00 bc	1.59 d	2.55 bcde
	SMS	121.63 g	7.80 ef	62.14 ef	42.06 ef	134.31 cd	83.50 b	1.52 cd	2.55 bcde
	SMS + N1P1K1	234.88 i	9.66 h	67.60 g	41.06 e	145.80 d	85.50 bc	1.87 ef	3.55 fg
	SMS + N2P2K2	237.80 i	8.77 fgh	65.00 fg	44.56 fg	92.83 a	85.75 bcd	1.84 ef	3.03 def
	M	116.22 fg	8.25 fg	64.69 fg	46.56 g	122.13 bcd	80.25 a	1.99 f	3.04 def

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; RES—respiration of soil (mg CO_2 kg^{-1} d.m. of soil d^{-1}); DEH—dehydrogenases (mg TPF kg^{-1} d.m. of soil d^{-1}); AcP—acid phosphatase (mg PNP kg^{-1} d.m. of soil h^{-1}); ARS—arylsulfatase (mg PNP kg^{-1} d.m. of soil h^{-1}); GI—growth index *L. sativum* (%); GERM—germination of *L. sativum* (the number of seeds germinated); RL2—root length of *L. sativum* after two days (cm); and RL4—root length of *L. sativum* after four days (cm). Different letters indicate significant differences at $p < 0.05$.

Figure 4 and Table 3 show data concerning dehydrogenase activity. The introduction of a spent mushroom substrate in various combinations and manure into the soil caused significant changes in the activity of these enzymes. As with respiration, this parameter was also generally subject to stimulation under the influence of the spent mushroom substrate. This effect also lasted the longest at sites where spent mushroom substrate was introduced in combination with NPK (SMS + N1P1K1, SMS + N2P2K2). The dynamics of changes over time were similar to that recorded for respiration. The highest stimulation was recorded in the first year of the study, where the value of enzymatic activity in the spring at the SMS only site was 16.37 mg, in the fall 6.84 mg, while only 8.41 mg and 1.38 mg in the control

(C), respectively. The positive effect of SMS disappeared in the second year of the study, and there was even a decrease recorded in dehydrogenase activity in the spring. However, in the third year of SMS application, its stimulating effect occurred again in the sites with mineral fertilization (SMS + N1P1K1 and SMS + N2P2K2).

Manure, as in the case of respiration, had a significantly weaker effect on dehydrogenase activity than the spent mushroom substrate. Its significant impact was recorded only in autumn in the second year in the form of an increase in this parameter. The enzyme activity value in the manure site (M) was 5.20 mg at this time point, while in control (C), it was 3.18 mg.

Figure 5 and Table 3 present data regarding acid phosphatase activity. The results showed that the impact of SMS was not directional and exhibited varying intensity throughout the study period. During the first two years of the experiment, it caused a decrease, an increase, or no significant effect on the discussed enzymatic activity at individual time points, depending on the variant in which the spent mushroom substrate was applied. The use of SMS alone had an effect only in the first time point in the form of phosphatase activity stimulation. The introduction of SMS in combination with NPK fertilization (SMS + N1P1K1 and SMS + N2P2K2), on the other hand, caused a decrease in activity at this time point. This effect apparently occurred in the fall in the facility with a higher NPK dose (SMS + N2P2K2). The activity at this site was 27.99 mg, while it was 41.00 mg in control (C). The negative effect of SMS at this site disappeared over time, and stimulation of phosphatase activity was already observed in the second year. The value of this parameter at this site (SMS+N2P2K2) was 66.36 mg, while it was 56.76 mg in the control (C). The impact of the waste at all sites completely disappeared in the third year of the study.

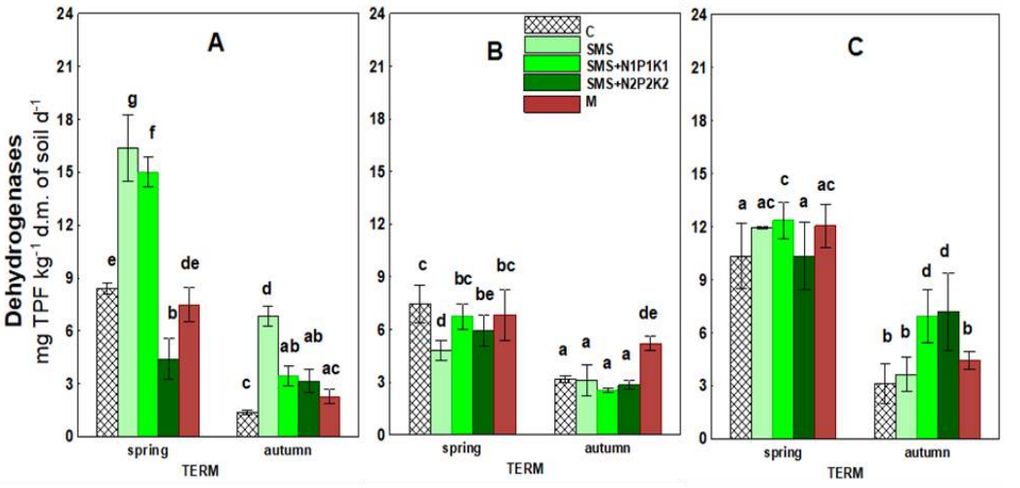

Figure 4. Activity of dehydrogenases in control soil and soil under different treatment strategies. (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

The effect of manure was also not directional. In the first year, there was a decrease in phosphatase activity at this site (M) and at both time points. In the following years, a significant impact was recorded only in the second year in the fall in the form of stimulation. It should be noted that phosphatase activity reached the highest value of 67.24 mg in this time point compared to 56.76 mg in the control.

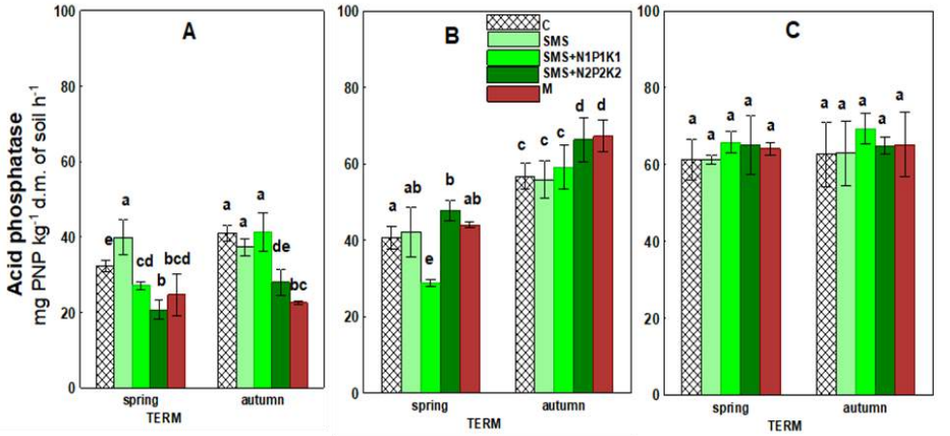

Figure 5. Acid phosphatase activity in control soil and soil under different treatment strategies. (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

The effect of spent mushroom substrate and manure on arylsulfatase activity, as opposed to phosphatase, was directional (Figure 6, Table 3). The activity of arylsulfatase was subject to significant inhibition persisting in the spent mushroom substrate with varying intensity throughout the study period. In the first year, spent mushroom substrate, introduced both separately and together with both NPK doses (SMS, SMS + N1P1K1, and SMS + N2P2K2), caused a decrease in arylsulfatase activity. It should be noted that inhibition was the strongest during this year, and the value of this parameter in the SMS and N1P1K1 site was only 7.38 mg, while in the control (C) it was 56.56 mg. The negative effect of waste also persisted in the second year in the sites with NPK fertilization (SMS + N1P1K1 and SMS + N2P2K2) and in the third year in the site with SMS and N1P1K1. The only positive effect that was noted for spent mushroom substrate (SMS) was in the fall in the second year of the study.

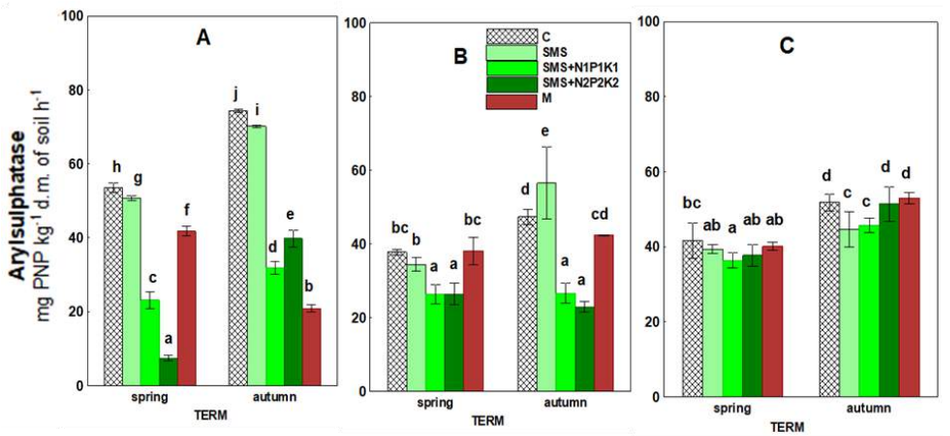

Figure 6. Arylsulfatase activity in control soil and soil under different treatment strategies. (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

The application of manure induced significant changes in arylsulfatase activity, but only in the first year of the study. There was a decrease in this parameter in both spring and fall. In the fall, the activity of this enzyme was the lowest of all the time points for this site (M) and was 20.81 mg compared to 74.33 mg in the control.

Figure 7 and Table 3 present the growth index (GI) data of the test plant. Data analysis showed that the introduction of SMS and manure into the soil resulted in an increase in this parameter. Initially, this effect became apparent only in sites where SMS was applied in combination with N2P2K2 fertilization.

In time, i.e., in the second year, the stimulation of this parameter intensified and, in the fall, it was already visible in all sites with waste. The highest stimulation that was recorded was for SMS alone, and the values recorded were 121.67% and 118.17%. In the third year of the study, the recorded stimulation weakened and was evident in fewer sites.

The effect of manure on the GI persisted over three years in the form of stimulation of this parameter. This effect was strongest in the second year and amounted to 96% and 66%. It was weaker in the third year and finally disappeared.

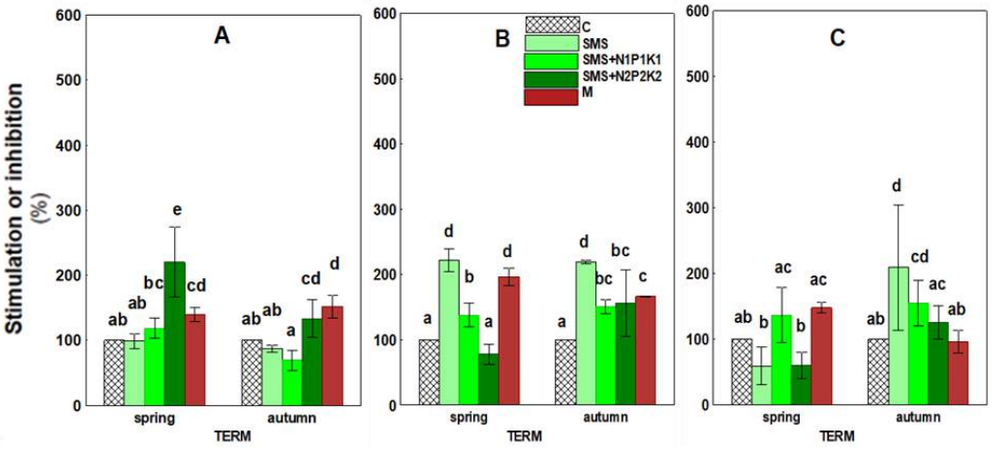

Figure 7. Growth index *Lepidium sativum* in soil under different treatment strategies. (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

The results concerning seed germination of the test plant are shown in Figure 8 and Table 3. The application of spent mushroom substrate in different variants had no significant effect on this parameter during the first two years of the experiment. In the third year, the germination process differed between individual time points. In the spring, germination was inhibited in all sites with SMS. Inhibition became most apparent when spent mushroom waste was applied alone. The number of germinated seeds in this site was 79 compared to 93 in the control. In autumn, however, the process of seed germination was stimulated. The strongest increase in the number of germinated seeds was also observed in the site with waste alone and with waste applied in combination with N1P1K1, which amounted to 88 and 86 seeds, respectively, compared to 77 seeds in the control.

The effect of manure also occurred only with time and was not uniform. In the second year of the study, there was a significant stimulation of seed germination in the fall, with the number of seeds reaching 85 compared to 82 in the control. In contrast, the negative effect of manure on this process became apparent in the third year at both time points. There was also a decrease in the number of germinated seeds (from 94 and 77 in the control soil to 89 and 72 in the soil with manure).

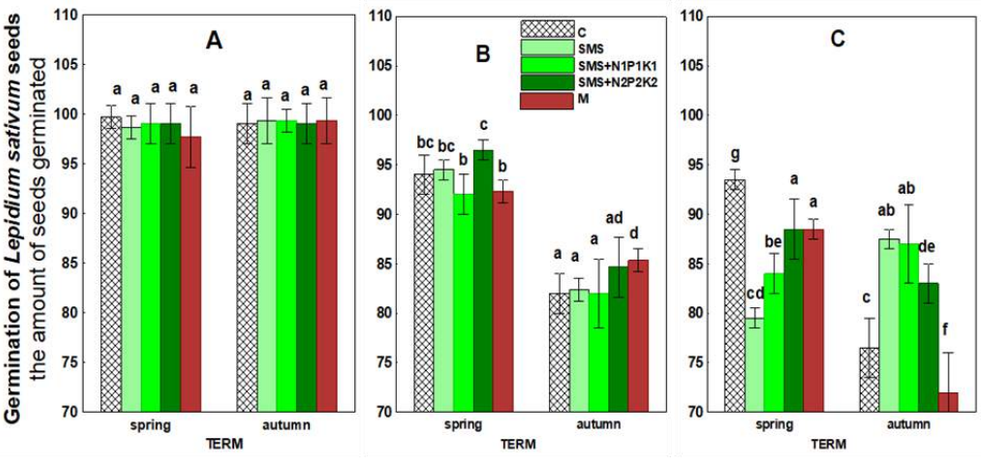

Figure 8. *Lepidium sativum* seed germination in the control soil and soil under different treatment strategies. (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

The data shown in Figures 9 and 10, as well as Table 3, refer to the increase in root length of the test plant measured after 2 and 4 days, respectively. The results showed that the applied waste and manure significantly affected the growth of the roots of the test plant seedlings. These changes developed with varying intensity over the three years of the study. However, in the case of root growth measured after four days, these alterations occurred in a greater number of sites.

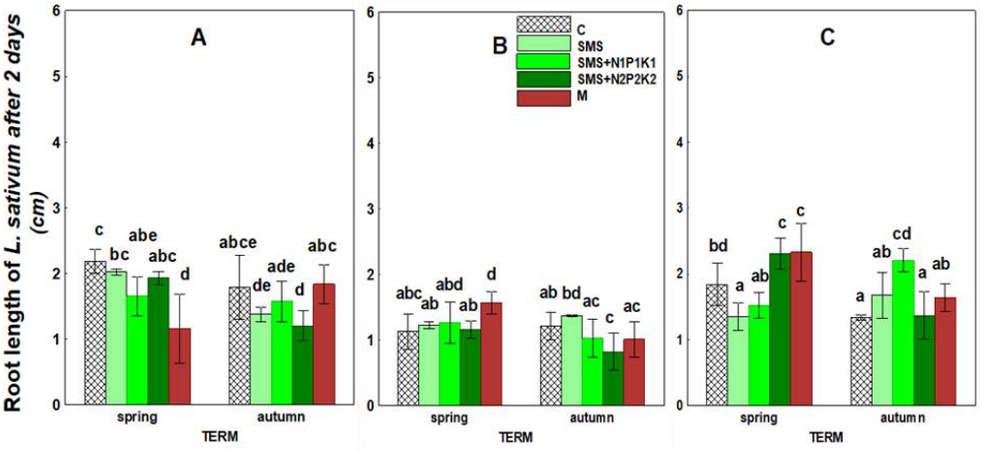

Figure 9. Increase in root length of *Lepidium sativum* in control soil and soil in different treatment strategies after two days. (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

In the first year, there was a decrease in root growth (when compared to the control), both after two and four days. After two days, the lowest root growth that was recorded in the fall was at the site with SMS and N2P2K2, and after four days in the spring it was at the site with SMS and N1P1K1. In the second year of the study, the negative impact of

SMS on root growth after two days almost disappeared and persisted only in the site with N2P2K2. However, the inhibition was still present when the measurement was taken after four days. It was particularly pronounced in the spring because it occurred in all sites with SMS, while in the autumn it was present only in the site with SMS and N2P2K2. In the third year of the study, the negative effect of spent mushroom substrate on root growth after 2 and 4 days disappeared and occurred only in a single site with SMS alone. In the other combinations and time points, the parameters studied were subject to stimulation.

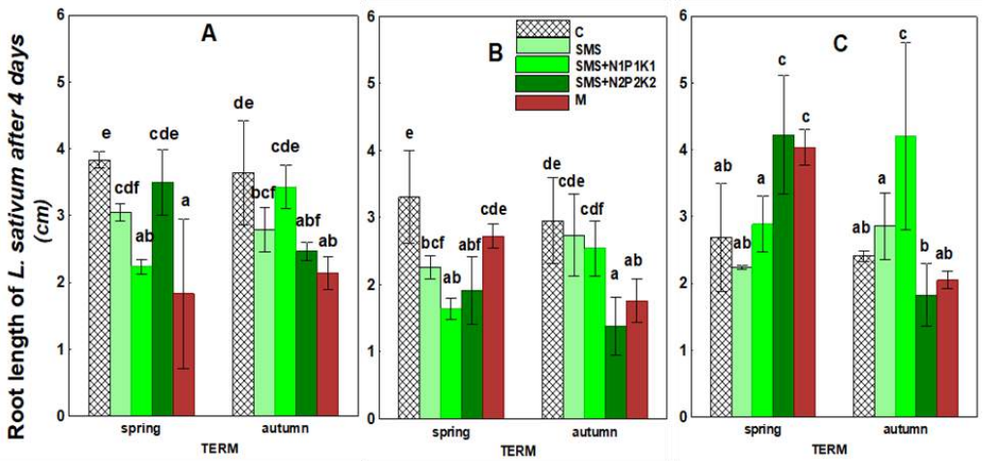

Figure 10. Increase in root length of *Lepidium sativum* in control soil and soil in different treatment strategies after four days. (**A**)—1st year; (**B**)—2nd year; (**C**)—3rd year. C—control soil; SMS—soil + spent mushroom substrate; SMS + N1P1K1—soil + spent mushroom substrate + mineral fertilization N1P1K1; SMS + N2P2K2—soil + spent mushroom substrate + mineral fertilization N2P2K2; M—soil + manure. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$, and each year was analyzed independent of each other.

The effect of manure on root length growth after two and four days also showed some dynamics. Initially, inhibition of these parameters relative to controls was observed in the first two years, particularly when measured after four days. In the third year, as in the case of the spent mushroom substrate, the parameter was stimulated in the spring (after 2 and 4 days), and no effect was noted in the fall.

4. Discussion

Soil respiration is a particularly important parameter in assessing the condition and quality of the soil, and thus the fertilizer value of various types of organic waste, since it reflects the full range of its biological activity. CO_2 released in this process is derived mainly from the decomposition of organic matter by soil microorganisms (SOM) [31]. The rate of organic carbon mineralization depends on, among others, temperature, humidity, salinity, pH, and soil aeration, as these factors are closely related to the living conditions of soil microorganisms [54]. However, as the available literature shows, carbon mineralization is primarily related to organic matter [55]. Therefore, the stimulation—albeit with varying degrees of intensity—of respiratory activity observed in the current study was likely due to the input of organic matter along with spent mushroom substrate and manure, i.e., sources of respiratory substrates for soil microorganisms. Stimulation of respiratory processes by the addition of organic matter to the soil has also been reported by other authors [8,11,12,56]. The initial increase in respiratory activity in soil may have been due to the decomposition of readily available compounds brought in with spent mushroom substrate. On the other hand, the decrease in the activity of the analyzed parameter (which is noted later) could be the result of the depletion of these compounds or the induction phase of microbial enzymes. Additionally, it could also be the result of uptake and storage of carbon by microorganisms,

without its utilization for cell structure repair or growth [57]. However, in the third year of the present study, significant stimulation of respiratory activity in all variants was observed, including the control soil, which could suggest that environmental conditions could have affected this analyzed parameter. This was confirmed by a cluster analysis which showed a positive correlation between respiration and precipitation and a negative correlation with temperature (Figure 11).

With regard to temperature, negative correlations were recorded only in the variants with SMS alone and manure (Figure 12). Perhaps the key role in the other variants was played by the applied supplemental mineral fertilization, which helped stimulate the decomposition of soil organic matter. The available literature shows that low doses of this type of fertilization have a beneficial effect on the microbiological and agrochemical properties of the soil, as they accelerate decomposition and increase the amount of soil organic matter [58–60]. Hernandez et al. [56] point out that combined organic and mineral fertilization is a good substitute for mineral nitrogen fertilization. For precipitation, positive correlations in all variants were recorded and, in combinations with different mineral fertilization variants, they were at a significance level of $p < 0.001$, similar to manure, while in the variant with SMS alone, they were at a fairly high level of $p < 0.01$ (Figure 12).

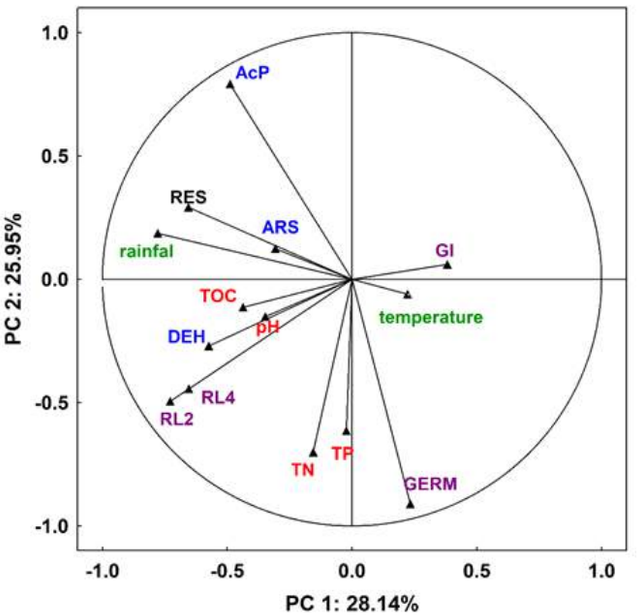

Figure 11. Principal component analysis (PCA) for the results of analyzed parameters in the soil-loading plot. RES—respiration of soil, DEH—dehydrogenases, ARS—arylsulfatase, AcP—acid phosphatase, GI—growth index of *L. sativum*, GERM—germination of *L. sativum*, RL2—root length of *L. sativum* after two days, RL4—root length of *L. sativum* after four days, TOC—total organic carbon, TN—total nitrogen, and TP—total potassium.

Reports of other authors have confirmed the current observations, as they have also recognized the relationship of soil fertilization with spent mushroom substrate and weather conditions [61]. This may be due to the ability of the spent mushroom substrate to retain water in the soil which, in turn, results in the better reaction of crops to periodic drought conditions. This is a compelling argument that can give SMS an advantage over other fertilizers in terms of its impact on yield and crop quality. Other physicochemical and chemical parameters did not play a significant role in the respiratory activity analyzed in the current study. Positive correlations were found with pH only in the case of variants with mineral fertilization at a significance level of $p < 0.001$ (SMMS+N1P1K1) and with TOC at $p < 0.05$ (SMS+N2P2K2) (Figure 13). The effects of organic waste on respiratory activity were also analyzed with

varying results, in studies, among others, by Joniec [21], Álvarez-Martín et al. [32], Elsakhawy and El-Rahem [33], Joniec et al. [34], and Paula et al. [35].

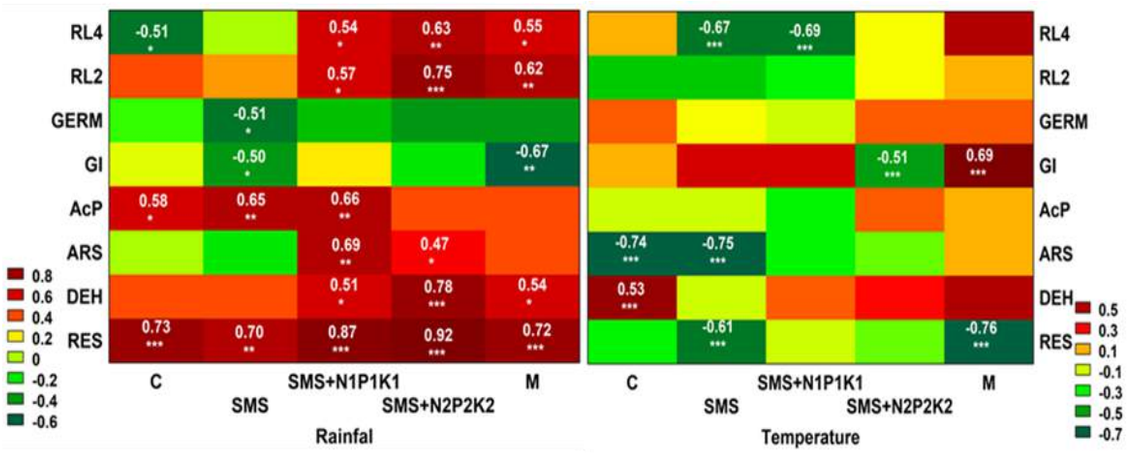

Figure 12. Heat map displaying the Pearson correlation coefficients between environmental factors (rainfall and temperature); biochemical and enzymatic activity; and phytotoxic parameters; as well as physicochemical and chemical properties at the combination level. Significance noted at * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$, respectively. RL4—root length of *L. sativum* after four days, RL2—root length of *L. sativum* after two days, GERM—germination of *L. sativum*, GI—growth index of *L. sativum*, AcP—acid phosphatase, ARS—arylsulfatase, DEH—dehydrogenases, RES—respiration of soil, 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.

Microbial respiration is also related to the activity of dehydrogenases, whose determination allows controlling changes in the population of soil microorganisms, which is an important parameter of soil quality. Soil enzymes are biological catalysts for many biochemical processes in the soil environment, including those related to the emission of greenhouse gases CO_2 and N_2O [62]. They are also suitable markers of soil fertility as they are involved in the cycle of the most important nutrients [63,64]. It is well known that dehydrogenase activity in soil depends on organic carbon content. Therefore, as in the case of biochemical activity, it can be assumed that these were the transformation products of spent mushroom substrate organic matter and the changes they induced in the soil environment that contributed to the stimulation of dehydrogenase activity in the current study. This was confirmed by a cluster analysis that showed positive correlations of the analyzed enzyme with TOC, TN, TP, and pH (Figure 11). At the same time, it should be noted that the correlations with TOC of the variants with SMS were at the significance level of $p < 0.001$, and for manure only at $p < 0.05$ (Figure 13). As in the case of respiration, the initial increase in dehydrogenase activity was probably caused by the introduction of readily degradable nutrients into the soil along with SMS, which resulted in improved conditions for many microbial groups, and this translated into the stimulation of dehydrogenases. The improvement of these conditions was also evidenced by the recorded significant correlations of the enzymes with pH in all variants with SMS at $p < 0.01$ (SMS + N1P1K1) and $p < 0.05$ (SMS and SMS + N2P2K2). For manure, the recorded significance of the results was also at the level of $p < 0.05$ (Figure 13). In contrast, the later decrease in the activity of these enzymes was probably caused by the breakdown of more readily available nutrients. The dynamics of changes in the activity of dehydrogenases, similarly to biochemical activity, could also have been caused by environmental conditions, as evidenced by positive correlations with precipitation and temperature (Figures 11 and 12). Dehydrogenases are fairly sensitive to changes associated with seasons because they are closely associated with the dynamics of microbial activity [65]. The effect of spent mushroom substrate medium on dehydro-

genase activity was studied by, among others, Meng et al. [16], Álvarez-Martín et al. [32], Elsakhawy and El-Rahem [33], and Gong et al. [66].

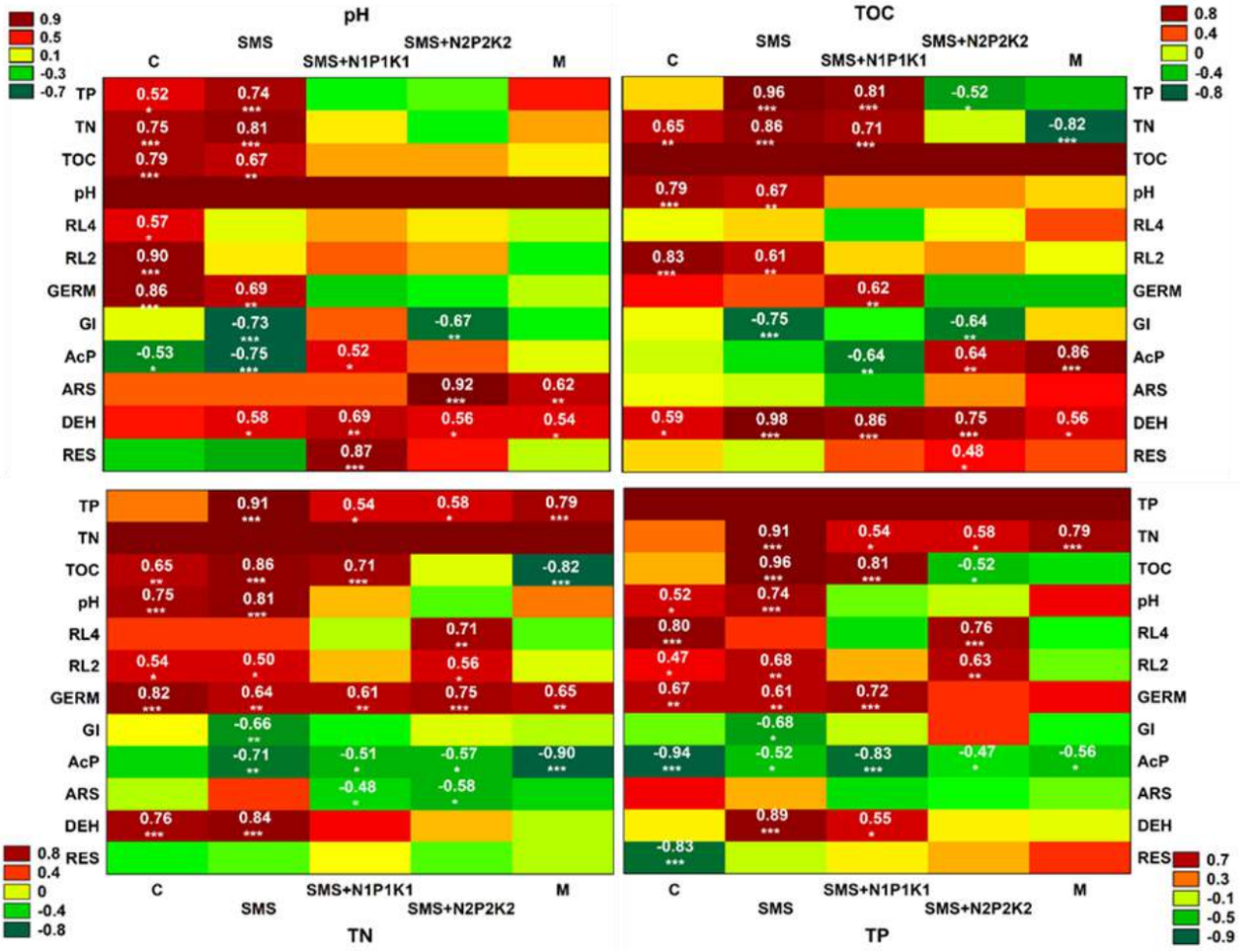

Figure 13. Heat map displaying the Pearson correlation coefficients between chemical and physicochemical properties; biochemical and enzymatic activity; and phytotoxic parameters at the combination level. Significance noted at * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$, respectively. TOC—total organic carbon, TN—total nitrogen, TP—total potassium, RL4—root length of *L. sativum* after four days, RL2—root length of *L. sativum* after two days, GERM—germination of *L. sativum*, GI—growth index of *L. sativum*, AcP—acid phosphatase, ARS—arylsulfatase, DEH—dehydrogenases, RES—respiration of soil, 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.

Other enzymes involved in the circulation of major nutrients are acid phosphatase and arylsulfatase, which are associated with phosphorus and sulfur metabolism. They catalyze transformations of various substrates, releasing available inorganic forms of phosphate and sulfate, which serve as key energy sources for plants and soil organisms. They are also sensitive indicators of agriculture-induced changes in soil properties due to their strong association with soil organic matter content and quality [67,68]. Cluster analysis showed a positive correlation of acid phosphatase and arylsulfatase with TOC, but in the combined variants, significant results were recorded only for phosphatase (Figures 11 and 13). In addition, the activity of the discussed enzymes was generally inhibited by the wastes applied, although their negative effect weakened over time and even disappeared; however, for arylsulfatase, it persisted even in the third year (SMS+N1P1K1).

Therefore, we could surmise that organic matter introduced in the form of spent mushroom substrate and manure did not play a key role in the activity of these enzymes. Hence, it disproves the first hypothesis regarding the fertilizing qualities of the spent mushroom substrate. As demonstrated by other authors, phosphatase activity may inhibit the presence of mineral phosphorus in the soil [68–70]. It was likely that this factor also played a key role in the present study, as evidenced by the recorded negative correlations between phosphatase activity and the content of bioavailable mineral phosphorus (Figures 11 and 13). TN was another parameter that could influence the activity of the discussed enzymes. Cluster analysis showed negative correlations of this factor with both acid phosphatase and arylsulfatase (Figure 11). For the first enzyme, negative correlations with TN were recorded in all variants with the spent mushroom substrate, while with SMS alone, they were at $p < 0.01$, and with mineral fertilization they were at $p < 0.05$ (Figure 13). For arylsulfatase, negative correlations at the $p < 0.05$ level were recorded only in combinations with mineral fertilization (Figure 13). Likely it was the addition of nitrogen in the form of mineral fertilization that increased the availability of sulfur in the soil, and this translated into a decrease in the activity of arylsulfatase. Similar conclusions were reached, among others, by Mori et al. [71], while Sawicka et al. [72] noted a significant effect of mineral fertilization on the activity of acid phosphatase. Another possible cause for alterations in the activity of the analyzed hydrolases was the change in the soil pH. This assumption was confirmed by the observed positive correlations between the analyzed enzymes, but it was much stronger in the case of arylsulfatase (Figures 11 and 13); these differences were probably due to the various sensitivity of these enzymes to this same chemical parameter [68]. The activity of these enzymes could also be influenced by environmental conditions, as evidenced by positive correlations with rainfall and negative correlations with temperature (Figures 11 and 12). Both hydrolases are frequently utilized to assess the condition of soil environments, including those fertilized with various types of organic waste [21,34,41–43,73].

Spent mushroom substrate introduced into soil generally has a positive effect on the physical, chemical, and microbiological properties of the soil environment. However, the introduction of waste organic matter into the soil also carries a certain risk of disturbing the living conditions of plants. Therefore, it is important to monitor the effects of unconventional organic fertilizers, such as spent mushroom substrate, on parameters related to plant growth and development. The conducted research shows that the organic matter and mineral compounds that were introduced with spent mushroom substrate and manure contributed to the stimulation of *L. sativum* growth in the initial period. This was likely caused by better availability of valuable nutrients, important from the point of view of plant nutrition, and a better aggregate structure of the soil. On the other hand, the decrease in the activity of this parameter observed later could be related to the activation of previously unavailable pollutants as a result of organic matter mineralization. Joniec et al. [21] reached similar conclusions in their study. Transformations of organic carbon in the soil influenced not only GI, but also the germination of *L. sativum*, as evidenced by the observed negative correlations with soil respiration and in the case of GI with dehydrogenase (Figure 11). In turn, with regard to the increment in the root length, cluster analysis showed positive correlations of this parameter with both biochemical and enzymatic activity, but only after two days (Figure 11). The ecotoxicological parameters related to plant growth were also likely influenced by the transformation of other nutrients, which was confirmed by the reported negative correlations of GI with arylsulfatase and in the germination with acid phosphatase (Figure 11). Chemical parameters such as TN and TP also played an important role, especially with respect to germination and root growth. This was confirmed by significantly positive correlations of germination with TN for all the experimental variants at the significance level of $p < 0.001$ and $p < 0.01$ and for the root growth (after two days) at the level of $p < 0.05$ (Figure 13). TP also played an important role in these parameters, as demonstrated by correlations at the level of $p < 0.001$ and $p < 0.01$ for virtually all combinations (Figure 13). The influence of these elements on the growth of *L. sativum* was

also reported by Mohamed et al. [74]. The results of the current study concerning *L. sativum* root length, measured after two and four days, indicated that this parameter was most sensitive to potentially harmful compounds occurring or resulting from changes in organic matter introduced with SMS and M in soil solution. Similar conclusions were also reached by Godlewska et al. [20]. The influence of the tested fertilizing materials on this parameter was fairly varied and depended on the combination and duration of the experiment. The decrease in toxicity, in this case, could probably be related to a reduced effect of the toxic agent as a result of its degradation or leaching. The analyzed parameters related to phytotoxicity may also be influenced by SMS composition because, as reported by Catal and Peksen [73], ammonia, salts, various heavy metals, or low molecular weight organic compounds present in SMS may also prevent seed germination and root development. The data presented in Table 1 indicate that a certain pool of heavy metals was brought in together with the spent mushroom substrate each year in the discussed experiment. The obtained results showed that the overall physicochemical and chemical conditions in the analyzed soil after the addition of spent mushroom substrate positively influenced the initial development of the plants. The adverse effects of SMS on the studied parameters were most apparent in soil solutions, which would indicate that improving the aforementioned soil conditions eliminated the negative impact of compounds present in the soil solution. On the other hand, Canellas and Olivare [75] reported that plants grown under optimal nutritional conditions spent less energy on growing roots. The results obtained in this study, therefore, are difficult to relate to the data of other authors due to the scarcity of reports concerning the effect of spent mushroom substrate on such parameters as the growth index, germination, and root length increment in L. *sativum* [73], strictly speaking.

5. Conclusions

The use of spent mushroom substrate significantly increased the parameters related to microbiological soil carbon transformations, i.e., respiration and dehydrogenase activity. The intensity of the respiration process, measured by the amount of CO_2 and the activity of dehydrogenases, was maintained with varying intensity throughout the research period in sites where waste was applied jointly with mineral fertilization. For respiration, the highest CO_2 release was recorded in the third year of the study. These observations indicate that waste matter has been incorporated into the microbial processes involved in the carbon cycle. This study partially confirms that spent mushroom substrate is a good fertilizer for increasing soil microbial activity and that it can be applied every year. On the other hand, the activity of enzymes responsible for phosphorus and sulfur metabolism, i.e., phosphatase and arylsulfatase, was inhibited by a spent mushroom substrate. It should be noted that the negative effect of waste weakened and even disappeared over time, but it persisted in the case of arylsulfatase also in the third year in the sites with waste and N1P1K1 fertilization. Therefore, the hypothesis concerning the fertilization values of a spent mushroom substrate, which can be applied every year, was rejected, in part, concerning its influence on the transformation of phosphorus, especially sulfur.

The obtained results showed that the fertilizing application of spent mushroom substrate contributed to an increase in the amount of released CO_2, which increased over time. Unfortunately, these observations do not confirm that such a method of waste management does not contribute to the exacerbation of the greenhouse effect by increasing CO_2 emissions from the soil. In this context, manure proved to be a safer fertilizer as it did not cause a significant persistent increase in CO_2 emissions.

The results of the effect of spent mushroom substrate on parameters related to the initial stage of the test plant development showed that its nature varied depending on the time period. A positive effect was noted for GI and root growth, but only in the third year of the study. In the initial year, root growth was lower in the sites with a spent mushroom substrate. Similar observations apply to the impact of manure. The results of the research on the impact of a spent mushroom substrate on phytotoxicity confirmed that the growth index was not negatively affected. On the other hand, considering the root growth, whose

inhibition was recorded in the first and second years of the study, it should be concluded that soil phytotoxicity deteriorated periodically.

It should be emphasized that one of the goals of sustainable development is to preserve or increase soil fertility, while also reducing GHG emissions from the agricultural sector. The obtained results may be helpful in making decisions on the fertilization of mushroom waste in light of the principles of sustainable development.

Author Contributions: Conceptualization, J.J. and E.K.; methodology, J.J.; software, E.K.; validation, J.J. and E.K.; formal analysis, J.J. and E.K.; investigation, J.J. and E.K.; resources, J.J.; data curation, E.K.; writing—original draft preparation, E.K.; writing—review and editing, J.J. and E.K.; visualization, E.K.; supervision, J.J.; project administration, J.J.; funding acquisition, J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the University of Life Sciences in Lublin (RKM/S/21/2020).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Additional information can be provided by the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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9.3 Publikacja P.3 - *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.*

Kwiatkowska E., Joniec J., Kwiatkowski C.A, Kowalczyk K., Nowak M., Leśniowska-Nowak J.
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.
Int. Agrophys., **2024**, 38, 139-154.
https://doi.org/10.31545/intagr/184175

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Int. Agrophys., 2024, 38, 139-154
doi: 10.31545/intagr/184175

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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Received December 28, 2023; accepted February 16, 2024

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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**This work was financially supported by the University of Life Sciences in Lublin (RKM/S/21/2020).

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

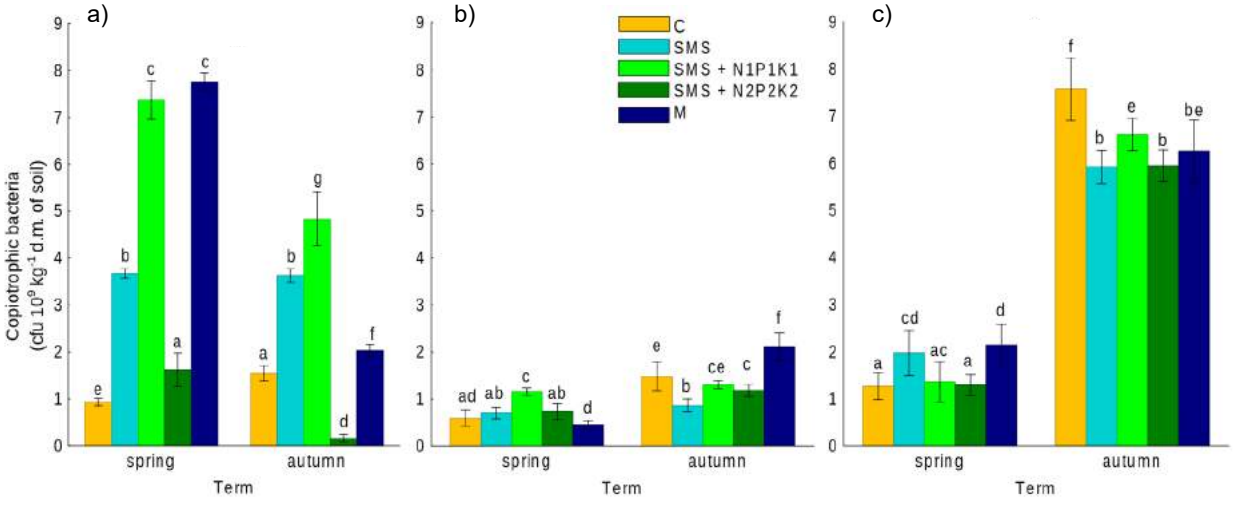

Fig. 1. Number of copiotrophic bacteria in the control soil and soil under different treatment strategies: a) 1st year, b) 2nd year, c) 3rd year. The vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p<0.05$, each year was analyzed 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 $p<0.05$.

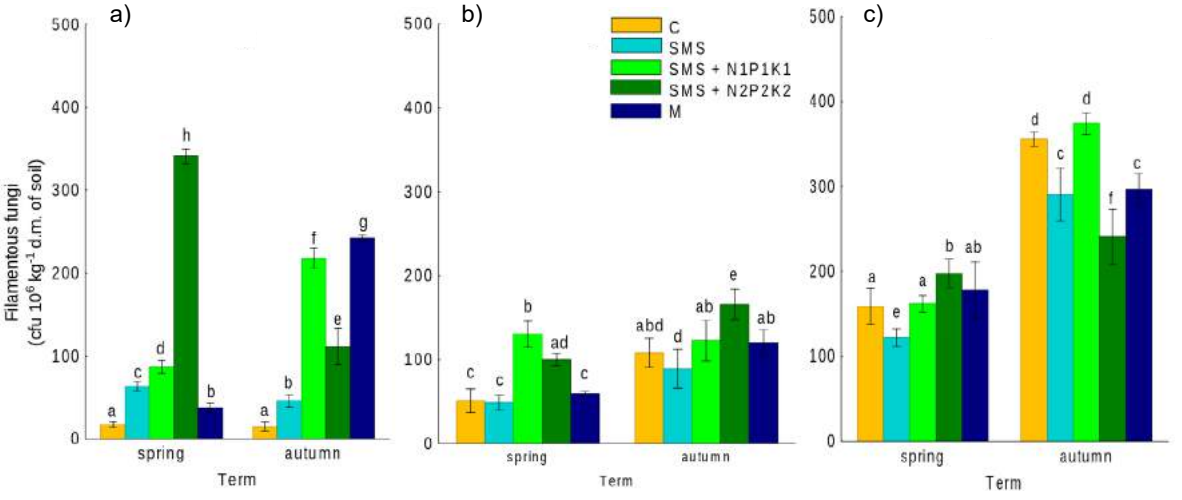

Fig. 2. Number of filamentous fungi in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

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).

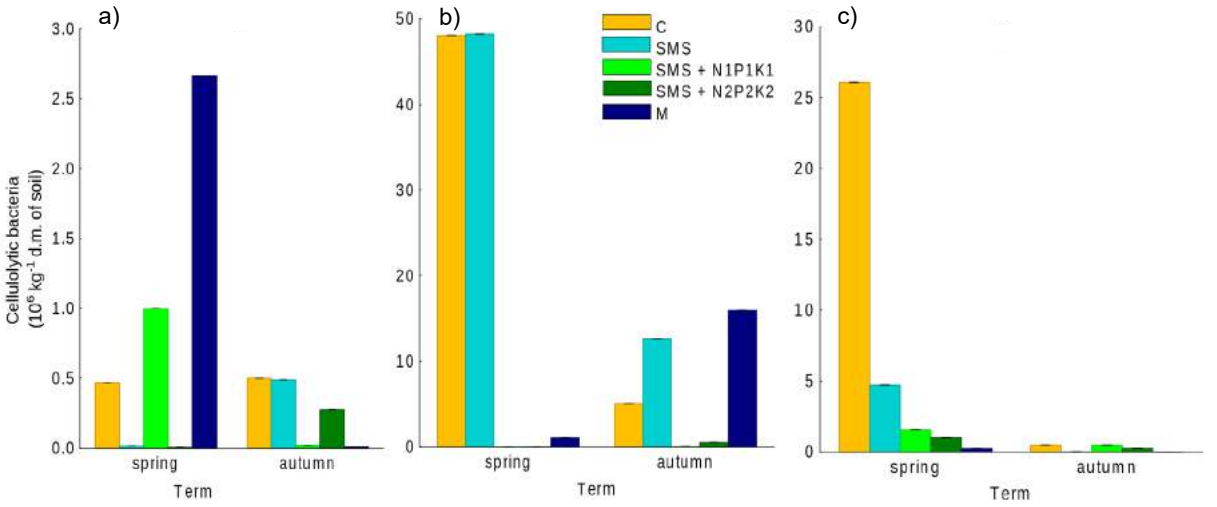

Fig. 3. Number of cellulolytic bacteria in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

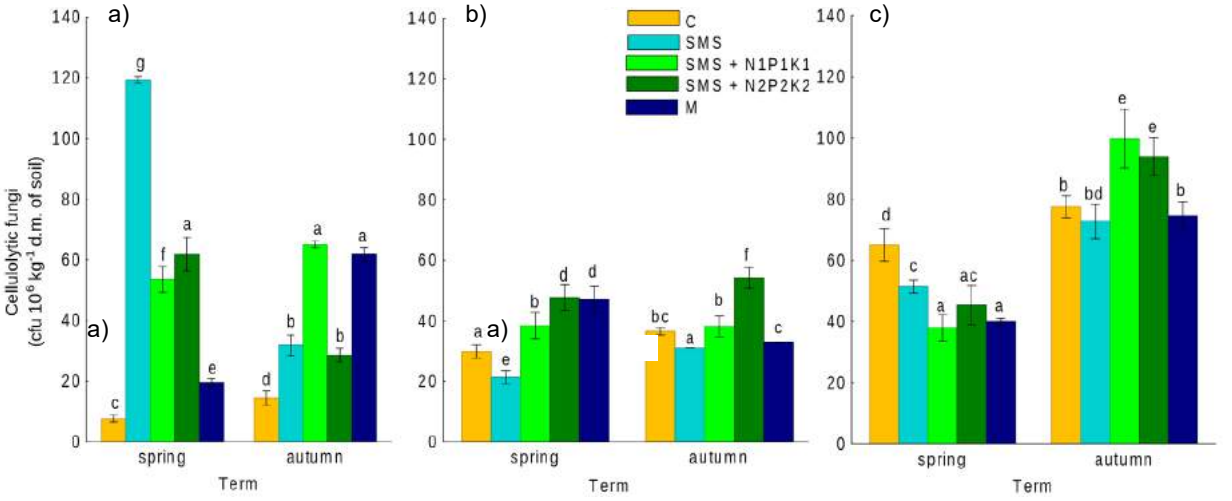

Fig. 4. Number of cellulolytic fungi in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

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

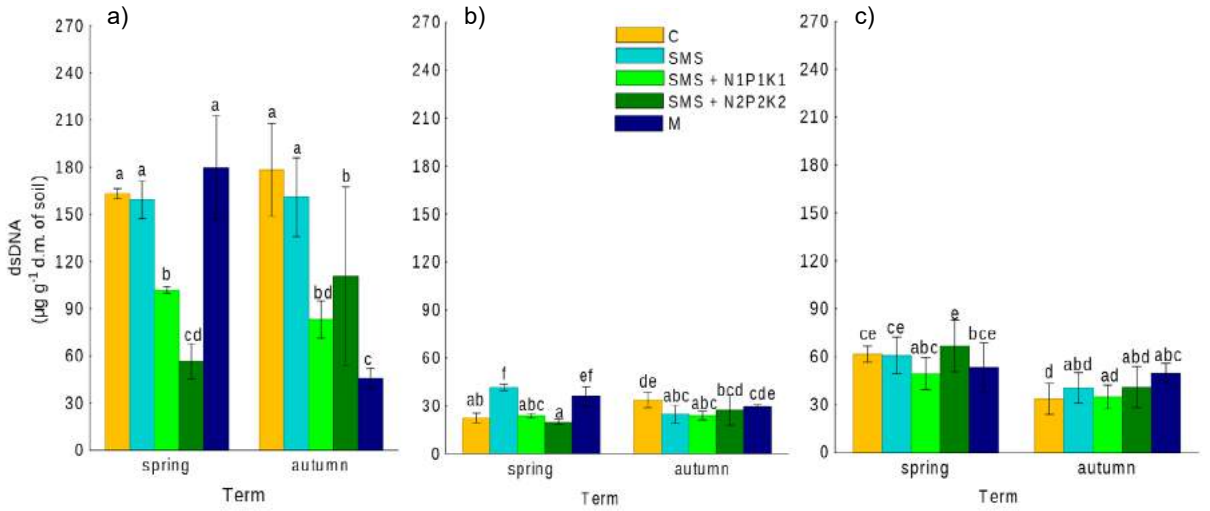

Fig. 5. dsDNA concentration in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

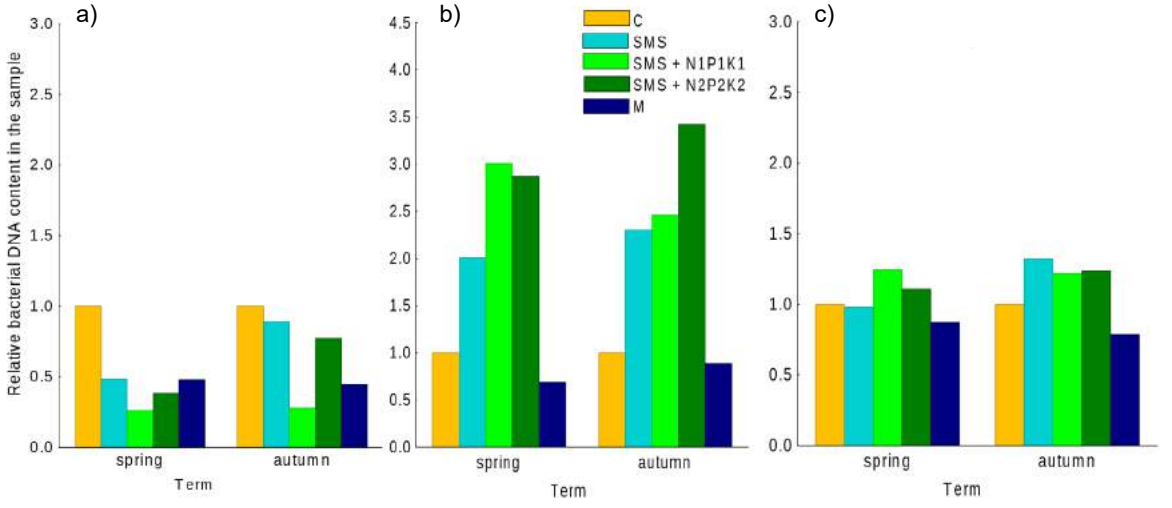

Fig. 6. Relative bacterial DNA content in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

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.

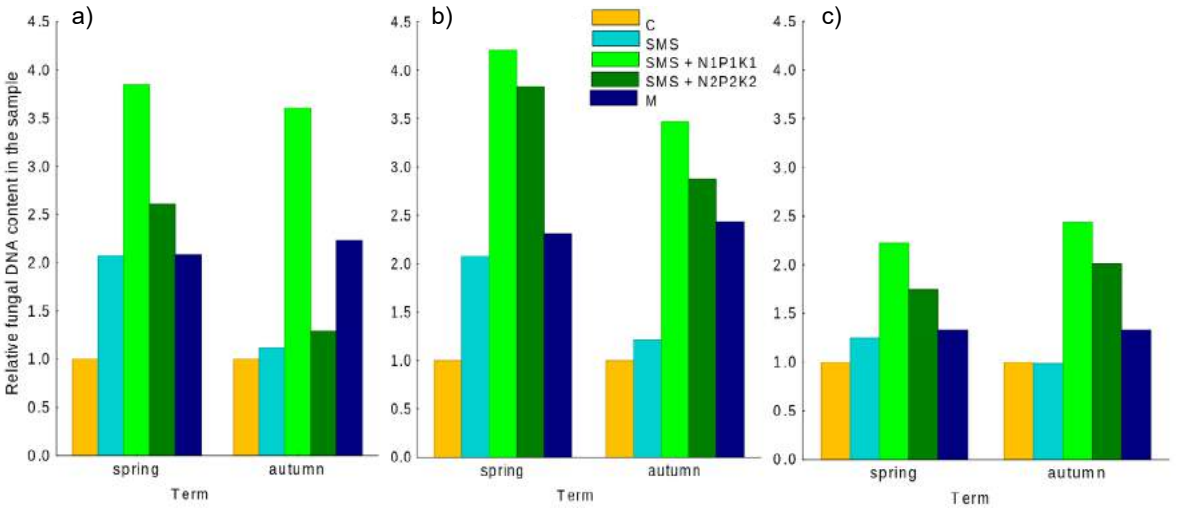

Fig. 7. Relative fungal DNA content in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

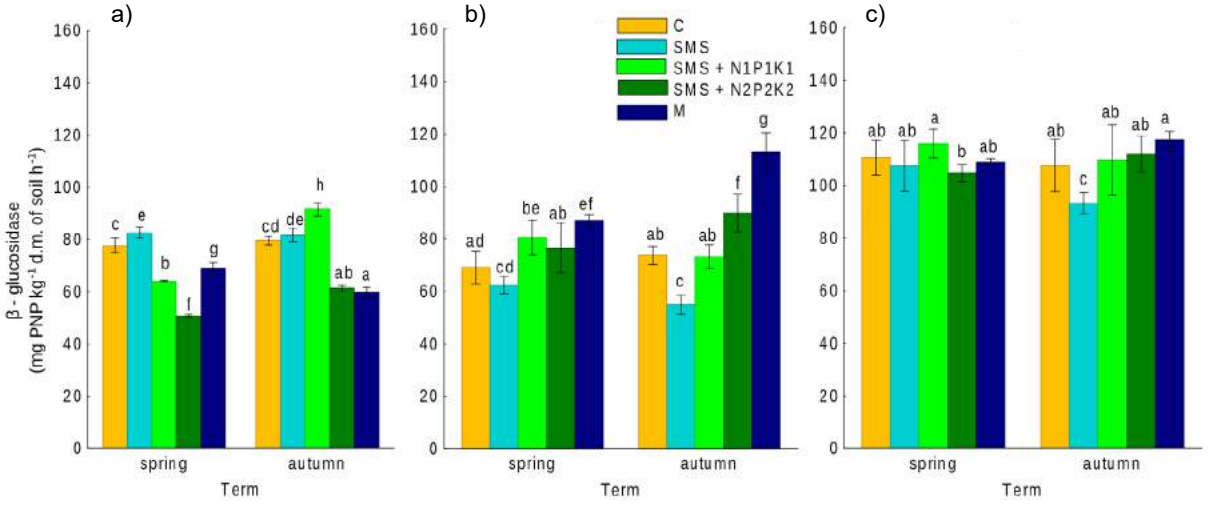

Fig. 8. Activity of β-glucosidase in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

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

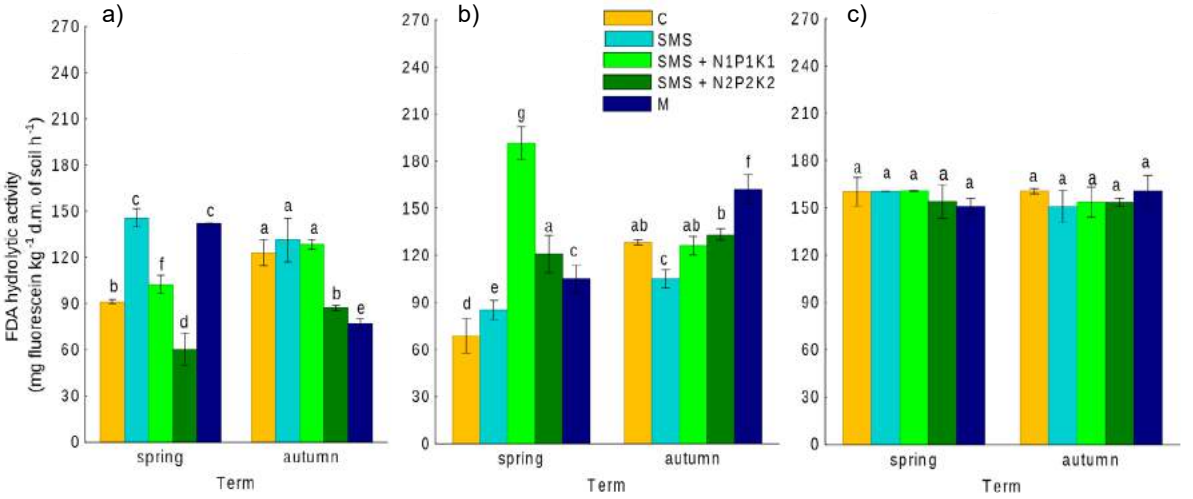

Fig. 9. FDA hydrolytic activity in the control soil and soil under different treatment strategies. Explanations as in Fig. 1.

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).

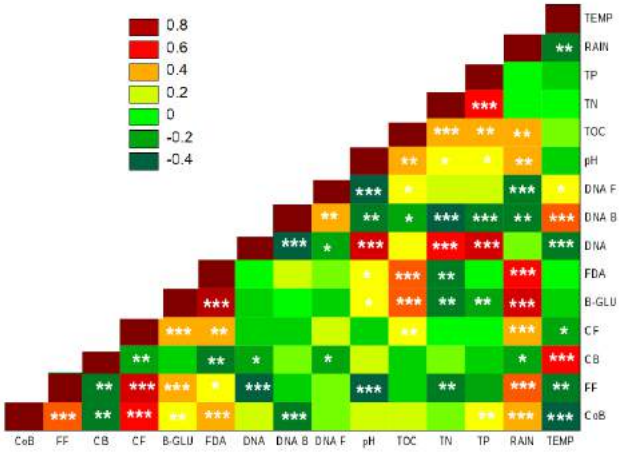

Fig. 10. Heatmap displaying the Pearson's correlation coefficients between soil physico-chemical, chemical, environmental factors and microbial, enzymatic activity. Signifcant at * $p<0.05$; ** $p<0.01$; *** $p<0.001$, respectively. CoB – copiotrophic bacteria, FF – filamentous fungi, CB – cellulolytic 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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9.4. Publikacja P.4 - *Involvement of soil microorganisms in C, N and P transformations and phytotoxicity in soil from post-industrial areas treated with chemical industry waste.*

Kwiatkowska E., Joniec J., Kwiatkowski C.A.
Involvement of soil microorganisms in C, N and P transformations and phytotoxicity in soil from post-industrial areas treated with chemical industry waste.
Minerals, **2023,** 13, 12.
https://doi.org/10.3390/min13010012

minerals

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Involvement of Soil Microorganisms in C, N and P Transformations and Phytotoxicity in Soil from Post-Industrial Areas Treated with Chemical Industry Waste

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Abstract: Soil degradation is an unavoidable phenomenon that poses a real threat, as it limits soil utility and reduces its resources. Early assessment of soil degradation can prevent its further deterioration. Various parameters of soil microbial activity may be helpful in this evaluation. Therefore, the purpose of the study was to assess the usefulness of microbiological (total abundance of oligotrophic bacteria and filamentous fungi), biochemical (soil respiration) and enzymatic (dehydrogenase, protease, acid and alkaline phosphatase activity and fluorescein hydrolytic activity) indicators, as well as phytotoxicity, in monitoring the condition of chemically degraded soils due to severe alkalization. The experimental material was soil collected in three sites located at different distances from the reservoir with liquid post-production waste. The analyzed indicators were correlated with the physical and chemical properties of the soil in three variants at the level of sampling sites, soil profile and seasonal variability. All analyzed parameters showed significant changes in the level of their activity at individual sampling sites. The location closest to the waste reservoir was characterized by the lowest values of the discussed activities and the highest phytotoxicity. Individual activities also showed changes depending on the season and soil layer. Considering the usefulness in monitoring changes in soils exposed to chemical degradation, total bacterial and fungal counts, as well as acid and alkaline phosphatase activities and fluorescein hydrolytic activity proved to be the most sensitive indicators.

Keywords: soil bacteria and fungi; chemical degradation; waste; phytotoxicity; enzymatic activity; soil respiration; microbial indicators; the reaction of microorganisms to stress

Citation: Kwiatkowska, E.; Joniec, J.; Kwiatkowski, C.A. Involvement of Soil Microorganisms in C, N and P Transformations and Phytotoxicity in Soil from Post-Industrial Areas Treated with Chemical Industry Waste. *Minerals* **2023**, *13*, 12. https://doi.org/10.3390/min13010012

Academic Editors: Maxim Muravyov and Anna Panyushkina

Received: 26 November 2022
Revised: 18 December 2022
Accepted: 19 December 2022
Published: 22 December 2022

1. Introduction

In addition to water and air, soil quality has a huge impact on the natural environment. It is defined as "the ability of the soil to function within the boundaries of the ecosystem and land use to maintain biological productivity, environmental quality, as well as plant and animal health" [1]. However, the soil is at the same time extremely vulnerable and exposed to a number of hazards due to both rapidly advancing climate change and intensive human activity [2,3]. All processes and activities causing deterioration of the chemical, physical and biological properties of the pedosphere are referred to as soil degradation. It leads to reduced soil productivity and thus to a decrease in other ecosystem functions [4,5].

Soil degradation is a worldwide phenomenon. According to the FAO, 33% of the Earth's soils are already degraded, and more than 90% could be degraded by 2050. Every 5 s, soil the size of a football field is being degraded. In contrast, it can take up to 1000 years to produce just 2–3 cm of soil. The highest percentage of areas at risk of degradation or already destroyed are located in Europe (15.2%), Africa (10.7%) and Asia (10.4%) [6,7].

The causes of soil degradation are complex and diverse. They range from biophysical, i.e., land use, cropping system, agricultural practices, deforestation, to socio-economic

(institutions, markets, poverty) and political (politics, political instability, conflicts) [8]. Soil degradation is most often characterized in terms of three closely related aspects: physical, biological and chemical. Physical degradation includes water erosion and landslides. It involves the displacement and/or repositioning of soil particles without changing their chemical composition. Biological degradation concerns, among others, a decrease in the quantity and quality of soil organic matter (SOM), as well as reduced biodiversity of soil organisms, both macrofauna and microflora [9,10].

Chemical degradation is closely related to the first two types. According to Richmond [11], this type of degradation is the most common form, second to erosion. It is a common form of both diffuse and point pollution that affect biotic and abiotic soil functions, crop quality, and animal and human health [9,11]. It is mainly associated with pollutants, e.g., heavy metals, toxic organic compounds, municipal or industrial waste, spills of toxic substances, but also excessive use of organic fertilizers, herbicides and insecticides. This, in turn, leads to high salt concentrations in soil solutions, disruption of the soil ionic balance, as well as its acidification or excessive alkalization [9,10]. Among the many aspects associated with this type of degradation, acidification or alkalization of the soil environment is one of the most important. Soil pH determines the fate of substances in the soil environment, influences countless biological, chemical and physical properties of the soil, and processes that affect microbial activity, plant growth and biomass yield [12]. Some micronutrients are more available in acidic conditions, while others in alkaline environments. Development of strongly acidic soils (below 5.5 pH) can result in poor plant growth. Alkaline soils, on the other hand, are characterized by reduced availability of phosphorus and micronutrients, which also negatively affect plants [13]. Soils with extremely alkaline pH (>9) are likely to have high sodium levels.

Soil degradation is an inevitable phenomenon that poses a real threat to the implementation of the vision of the 17 United Nations' Sustainable Development Goals [9]. Therefore, efforts should be made to mitigate hazards to soil function caused by degradation and thus to agricultural productivity and socio-ecological sustainability. Early assessment of soil degradation can help identify various socio-economic and biophysical causes and prevent its further deterioration [14]. Various parameters of soil microbial activity are helpful in assessing the condition of the soil environment subjected to various types of human pressure. The most commonly used indicators are the number and diversity of microorganisms and biochemical and enzymatic activity [15–19].

The soil microbiome is an essential component of the soil ecosystem, responsible for most biological activity in the pedosphere. Microorganisms are closely linked to organic matter decomposition, mineral release, nutrient cycling or carbon sequestration, thereby determining the stability and resistance of ecosystems [20–22]. The composition and diversity of microbial communities depends on various factors, such as changes in soil acidity [23,24] or depth of the soil profile [22]. According to Shi et al. [24] and Wang et al. [25], soil pH is an important selector of the biodiversity of soil bacterial and fungal populations. In the case of the soil profile, it is entirely colonized by microorganisms, but their composition and diversity vary between individual soil layers [22]. The sensitivity of heterotrophic soil microorganisms to changes in the properties of the soil environment meant that the number of bacteria and fungi was repeatedly used to monitor changes in soils subjected to various anthropopressures [18,19,26]. An important indicator of soil biological activity, besides abundance, is the intensity of biochemical processes and the content of products of soil microorganism activity, such as $N\text{-}NO_3$, $N\text{-}NH_4$ or CO_2. Respiratory activity is considered a good indicator of changes occurring in the soil environment [27,28]. Since about 90% of CO_2 emitted from the soil is of microbial origin, the remainder is the effect of plant respiratory processes and decomposition of organic compounds, brought into the soil with the roots [29].

Enzyme activity is closely related to the soil microbiome and thus is considered a good indicator of soil quality due to these relationships, ease of measurement, and rapid reflection of changes caused by soil use [30,31]. Enzymes are involved in many biogeochem-

ical cycles (in the carbon cycle—dehydrogenase, nitrogen cycle—proteases, phosphorus cycle—phosphatases) [32]. In addition, hydrolysis of fluorescein diacetate (FDA) is used to measure the total microbial activity in the soil. Fluorescein diacetate hydrolysis assessment is proposed as a prospective method for determining total microbial activity, as it covers several classes of enzymes including lipases, esterases and proteases. The spectrophotometric determination of fluorescein diacetate (FDA) hydrolysis has been shown to be a simple, sensitive and rapid method for determining soil microbial activity [33].

Monitoring these properties seems justified, because soils with greater microbial diversity and biochemical and enzymatic activity are characterized by higher resistance to environmental changes [30,32]. Although soils undergoing severe degradation are generally characterized by lower diversity and activity, they also constitute locations where populations of exthermophilic microorganisms can emerge [34]. Microorganisms obtained from such environments can subsequently be used in the composition of biopreparations applied in bioremediation [35,36]. Therefore, studies were conducted to assess the abundance, as well as biochemical and enzymatic activity of soil bacteria and fungi in soil from post-industrial areas subjected to strong alkalization. Due to the important role played by soil microorganisms in the health condition of soils and plants, research on the activity and abundance of the microbiome and mycobiome was combined with studies on the phytotoxicity of this environment, determining the impact of existing conditions on the development of plants at the initial stage, i.e., germination and seedling root growth.

The authors formulated two research hypotheses: the first assumed that the analyzed indicators of soil microbial activity and phytotoxicity would be suitable for monitoring the condition of chemically degraded soils due to strong alkalization; the second assumed that the alkalization of the environment would cause changes in the activity of microorganisms not only in the upper layer, but also in the lower one, and they would persist in the soil even at a great distance from the pollution emitter.

2. Materials and Methods

2.1. Description of the Research Area

The soil material was derived from a post-industrial area located in the Mazowieckie region in central-eastern Poland (51°28′54″ N, 21°27′01″ E). The climate of this region is transitional between maritime and continental. The average annual air temperature is 10–11 °C; the sum of average annual precipitation varies between 650 and 750 mm. Soil samples were collected from three locations at different distances from the reservoir with liquid post-production waste, i.e., sodium hydroxide (Scheme 1).

Scheme 1. Sampling location.

Site S1 was 5.88 m away from the liquid tailings tanks, site S2 was 22.7 m away, and site S3 was 50.08 m away. The sampling sites did not run along a single line, which helped to analyze whether possible soil contamination spread in the environment in one direction or evenly in all directions. The liquid was contained in sealed tanks placed in a concrete

reservoir, which was originally intended as a secondary protection against possible leaching of the liquid into the soil. The liquid waste was a remnant from chemical industry activity related, among others, to the production of cellulose and adhesives. The tanks were set up in the 1970s. The characteristics of the liquid waste are listed in Table 1.

Table 1. Waste characteristics.

	pH 1 mol KCl	Ca mg kg^{-1}	K mg kg^{-1}	Na mg kg^{-1}
waste	14	37.6	328	87,000

2.2. Sampling Description

Soil samples for analysis were collected in the summer (2 July) and autumn (27 September) of 2022 from depths of 0–20 and 20–40 cm. The material was randomly collected from 4 locations within each of the three collection sites, i.e., S1, S2 and S3, separately for individual layers. Soil samples were collected using a cylindrical sampler with a diameter of 4 cm and transferred to plastic bags. The collected material was then sieved through a 2 mm mesh to remove any roots, gravel and other fragments. The samples were stored in plastic containers at +4 °C.

2.3. Characteristics of Soil Chemical Properties

The chemical and physical properties of the soil (Table 2) were analyzed as a supplement to the microbiological, biochemical, enzymatic and phytotoxicity tests. The pH of the soil extract in KCl (10 g of soil in 25 mL of KCl) was determined electrometrically. Soil moisture was determined using the gravimetric method. IR spectrometry was used to determine organic carbon (TOC). Total nitrogen (TN) was determined by the Kjeldahl method.

Table 2. Soil characteristics.

Sampling Location	Depth cm	pH 1 mol KCl		M %		TOC g kg^{-1}		TN g kg^{-1}	
		s	a	s	a	s	a	s	a
S1	0–20	9.6	9.5	10.98	6.59	13.03	11.60	0.30	0.40
	20–40	8.9	9.2	11.96	5.36	11.90	9.00	0.10	0.20
S2	0–20	7.9	8.1	3.39	8.96	10.69	21.00	0.60	0.50
	20–40	8.0	8.0	4.73	5.16	18.21	15.10	0.90	0.10
S3	0–20	7.9	7.9	5.25	9.77	8.69	13.00	0.40	0.60
	20–40	7.6	7.4	6.44	8.55	10.11	11.60	0.70	0.60

Abbreviations: S1—site 5.88 m away, S2—22.7 m away, S3—50.08 m away. M—soil moisture, TOC—total organic carbon, TN—total nitrogen, s—summer, a—autumn.

2.4. Microbiological Analyses

According to the procedure described by Foght and Aislabie [37], the count of oligotrophic bacteria and filamentous fungi was determined using the plate method. Bacterial counts were determined on soil extract medium and K_2HPO_4, while fungal counts were determined on Martin's medium with antibiotics [38]. Cultures were carried out for bacteria at 28 °C for 4 days, and for fungi at 25 °C for 3 days. The analyses were performed in triplicate, and the results are given as colony-forming units (CFU) per gram of dry matter.

2.5. Biochemical Analyses

The method of Rühling and Tyler [39] was used to determine soil respiratory activity. In the presence of 0.2 M NaOH solution, 20 g of soil sample with 1% glucose was incubated for 24 h. After incubation, excess unbound sodium hydroxide was titrated with 0.1 M HCl in the presence of $BaCl_2$ and phenolphthalein.

2.6. Enzymatic Analyses

Dehydrogenase activity was determined by the method of Thalmanna [40]. Soil samples (5 g) were incubated for 48 h at 30 °C in the presence of 0.1 M tris(hydroxymethyl) aminomethane buffer (Tris-HCl pH 7.4). 2,3,5-triphenyltetrazolium chloride was used as a substrate. Enzyme activity was determined colorimetrically (λ = 485 nm) by measuring the extinction of the produced TPF (triphenylformazan). Protease activity was determined according to the method of Landd and Butler [41]. Soil samples (2 g) were incubated in 0.2 M tris(hydroxymethyl)aminomethane buffer (Tris-HCl pH 8.0) for 1 h at 50 °C. Sodium caseinate solution (5 mL) was used as a substrate. The level of released tyrosine was measured spectrophotometrically at 578 nm. The method of Tabatabai and Bremner [42] was used to determine acid and alkaline phosphatase activity. The activity of both these enzymes was determined in soil samples (1 g) incubated for 1 h at 37 °C. Disodium 4-nitrophenyl phosphate disodium salt hexahydrate (PNPNa) was used as a substrate. For acid phosphatase, incubation was conducted in universal buffer at pH = 6.5, while for alkaline phosphatase at pH = 11. The activity of both enzymes was determined spectrophotometrically at 400 nm and expressed as mg PNP kg^{-1} d.m. soil h^{-1}.

Fluorescein diacetate (FDA) hydrolysis level was determined using the method of Schnurer and Rosswall [43]. Soil samples (1 g) were incubated for 2 h at 25 °C. Incubation was carried out in the presence of fluorescein diacetate substrate and 60 mM sodium phosphate buffer (pH 7.6). Enzyme activity was determined spectrophotometrically at 490 nm and expressed as mg fluorescein kg^{-1} d.m. soil h^{-1}.

2.7. Soil Phytotoxicity

Soil phytotoxicity was assessed using a phytotest, which enabled the analysis of the effect of potentially toxic substances dissolved in the soil solution on germination and root growth of *Lepidium sativum* L. after 2 and 4 days. This test consisted of placing 20 g weighed amounts of fresh soil from the 0–20 cm layer in Petri dishes in 6 replicates for each combination. Subsequently, the soil was soaked with distilled water exceeding their total water capacity by 2 mL and thoroughly mixed. On the second day, after equilibrium in the soil solution was established, the soil was covered with a filter paper disc. The next step was placing 90 seeds of *L. sativum* on 3 plates and 10 seeds on the remaining 3 plates (100 seeds in total) and incubation at 22 °C. The number of germinated seeds on all plates was counted after two days. After 2 and 4 days, the length of sprout roots was also measured on plates with 10 seeds each.

2.8. Statistical Analysis

The results were presented in the form of arithmetic mean values from three replicates obtained for a given sample together with the standard deviation. Statistical analysis of the results of microbiological, biochemical, enzymatic and phytotoxicity analyses was performed using the STATISTICA 13.3 program (TIBCO Software Inc., Palo Alto, CA, USA). Data were analyzed using a three-way analysis of variance (ANOVA) to compare means. The post hoc analysis used Tukey's honestly significant difference (HSD) test at the significance level of $p < 0.05$. Pearson's correlation analysis was also conducted at three levels of significance: $p < 0.001$, $p < 0.01$, $p < 0.05$. The relationships between microbiological, biochemical, enzymatic, phytotoxicity and physical and chemical parameters were also analyzed in three variants, at the level of: sampling sites ("combinations"), soil profile and seasonal variation. Color scales ranging from dark green (lower values) to dark red (higher values) were adopted for each case, with corresponding transition colors between these extremes. Principal component analysis (PCA) was also performed for all analyzed parameters.

3. Results

The results presented in Figure 1A,B refer to the abundance of oligotrophic bacteria and filamentous fungi. Data concerning bacteria (Figure 1A) showed that their numbers in

both soil layers (0–20 and 20–40 cm) differed significantly between individual sampling sites (S1, S2 and S3) throughout the study period. The smallest number was recorded at site S1, i.e., closest to the reservoir with liquid waste (0.04–0.06 cfu 10^9 kg^{-1}). The level of this parameter increased significantly with the distance from the source of pollution in both soil layers in summer and autumn. The highest number was recorded in autumn (2.98 and 3.04 cfu). At that time, the number of bacteria was significantly higher in the upper soil layer than in the lower layer.

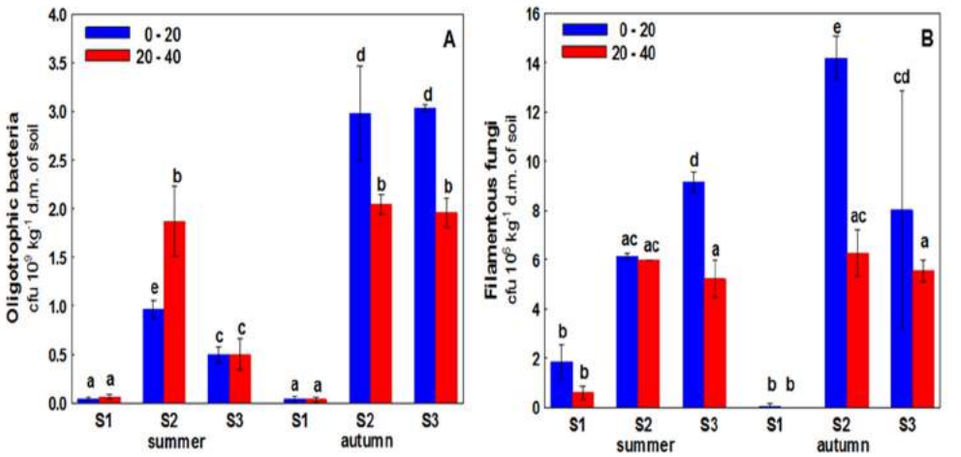

Figure 1. Number of selected groups of bacteria and fungi in soil from 0–20 and 20–40 cm depths: (**A**) oligotrophic bacteria; (**B**) filamentous fungi. Legend: S1, S2, S3—sampling sites located S1—5.88 m, S2—22.7 and S3—50.08 m from the reservoir with liquid post-production waste. Vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$.

The results obtained for filamentous fungi (Figure 1B) showed a similar development tendency of this group of microorganisms as for bacteria. The lowest abundance of fungi was recorded in the soil at site S1. This effect was visible in both soil layers and persisted throughout the study period. The level of this parameter at S1 ranged from 0, i.e., no filamentous fungi in the lower soil layer in autumn, to 1.85 cfu 10^6 kg^{-1} in the upper layer in summer. In the remaining sampling sites, i.e., S2 and S3, the development of fungi was significantly higher than in S1. The highest values were recorded in the upper soil layer, i.e., 0–20 cm (9.16–14.20 cfu), which was particularly pronounced in autumn. At that time, the number of fungi was significantly smaller in the lower soil layer.

Figure 2A presents the soil respiration data. Significant differences were noted in the intensity of this process at individual time points. In the summer, the highest values of this parameter were recorded at sites S1 and S2, (upper layer—133.68 and 146.21 mg kg^{-1}; lower layer—112.51 and 127.20 mg kg^{-1}). Significantly lower values were recorded at site S3, i.e., the farthest from the reservoir. Respiration in the analyzed layers at this location reached 76.23 and 73.90 mg, respectively. The intensity of the respiration process was different in autumn. Respiration in the upper soil layer (0–20 cm) was lowest at S1 and amounted only to 65.48 mg. It remained at a similar level in the lower soil layer at all sampling sites (S1, S2 and S3). On the other hand, in the 0–20 cm layer, respiration increased significantly with the distance from the waste reservoir. The highest value was recorded at site S2 (171.00 mg).

The results concerning the activity of dehydrogenases presented in Figure 2B showed that similarly to the number of bacteria and fungi, the lowest values for the upper layer were recorded for this parameter at site S1. This phenomenon was observed in the entire study period (0.28 and 0.44 mg kg^{-1}). The activity of dehydrogenases significantly increased with the distance from the reservoir with liquid waste, reaching the highest value in summer at S2 (2.93 mg) and in autumn at S3 (1.81 mg). The opposite tendency was observed in

the lower soil layer (20–40 cm) in summer compared to the upper soil layer (0–20 cm). The enzyme activity was the lowest at the most distant site, i.e., S3, and amounted to only 0.67 mg, while it was significantly higher at S1 and S2—2.15 and 1.93 mg, respectively. This parameter in autumn in the lower soil layer was at a similar level at all sampling sites, i.e., S1, S2 and S3 (0.35–0.52 mg).

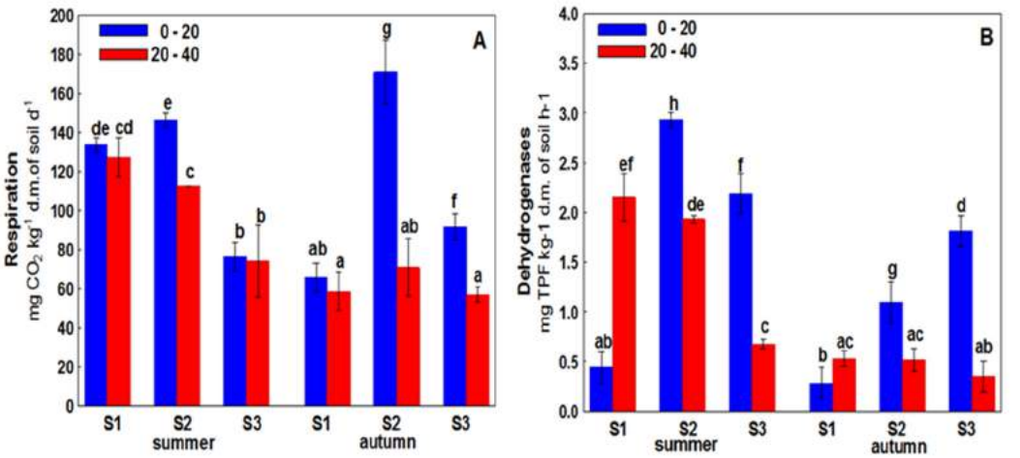

Figure 2. Biochemical and enzymatic activity in soil from 0–20 and 20–40 cm depths: (**A**) respiration; (**B**) dehydrogenases activity. Legend: S1, S2, S3—sampling sites located S1—5.88, S2—22.7 and S3—50.08 m from the reservoir with liquid post-production waste. Vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$.

Figure 3A,B show the results for acid and alkaline phosphatase activity. Acid phosphatase activity (Figure 3A) reached the lowest values in both soil layers, at the site closest to the waste tank, i.e., S1 (1.07–3.12 mg kg^{-1}). At the remaining sites, i.e., S2 and S3, the discussed activity was significantly higher (10.58–28.52 mg kg^{-1}). This effect persisted throughout the study period and was most pronounced in the upper soil layer in autumn. Acid phosphatase activity was significantly lower in the lower soil layer (20–40 cm).

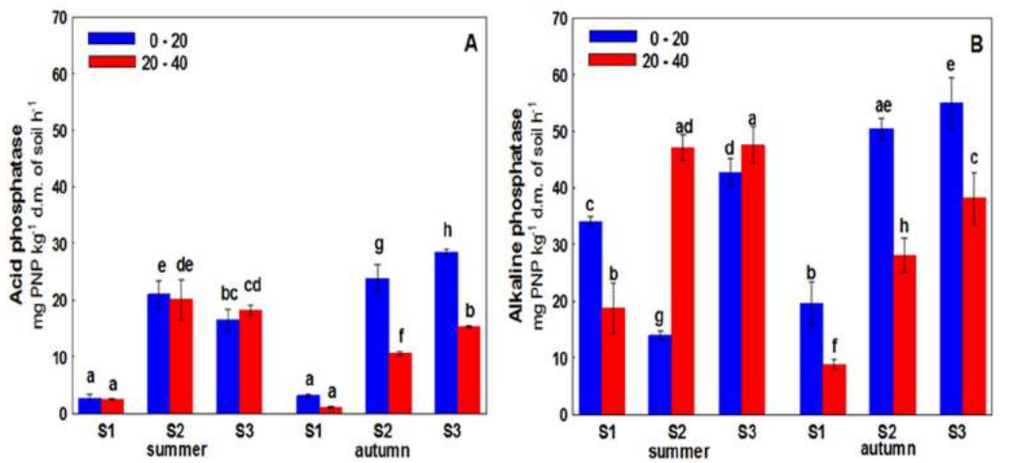

Figure 3. Enzymatic actvity in soil from 0–20 and 20–40 cm depths: (**A**) acid phosphatase; (**B**) alkaline phosphatase. Legend: S1, S2, S3—sampling sites located S1—5.88, S2—22.7 and S3—50.08 m from the reservoir with liquid post-production waste. Vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$.

Data on alkaline phosphatase activity (Figure 3B) showed that the activity of this enzyme reached higher values compared to acid phosphatase. For alkaline phosphatase, different trends were noted between individual time points and soil layers. In summer, the

lowest values of this parameter in the 0–20 cm layer were reached at S2 (13.97 mg kg^{-1}). In the remaining sites, i.e., S1 and S3, it was significantly higher, reaching the highest level at S3 (42.76 mg kg^{-1}). The lowest value in the lower soil layer was recorded at S1 (18.74 mg kg^{-1}). At the other sites, i.e., S2 and S3, it was significantly higher (47.13–47.53 mg kg^{-1}). In autumn, the activity of alkaline phosphatase in both soil layers showed a similar tendency as acid phosphatase. It had the lowest values at S1 (19.59 mg and 8.76 mg kg^{-1}). At other sites, it was significantly higher (54.95 mg and 50.51 mg kg^{-1}). The values recorded in both time points for the upper layer were generally significantly higher than for the lower layer.

Figure 4A presents the results for protease activity. The data were significantly different in individual dates. In summer, the lowest values in both soil layers were recorded at the site most distant from the liquid reservoir, i.e., S3 (8.07 and 10.21 mg kg^{-1}). However, at S1 and S2, the proteolytic activity was significantly higher, reaching the highest values at S2 (19.95 and 18.65 mg kg^{-1}). In contrast to S3, there were no significant differences between the layers at S1 and S2. In autumn, a reverse trend was observed, i.e., the lowest values of the enzyme activity were observed at S1 and S2 (1.26-6.23 mg) and significantly higher at S3 (9.08 and 14.29 mg kg^{-1}). In autumn, the values at most sites were significantly higher in the upper layer than in the lower one.

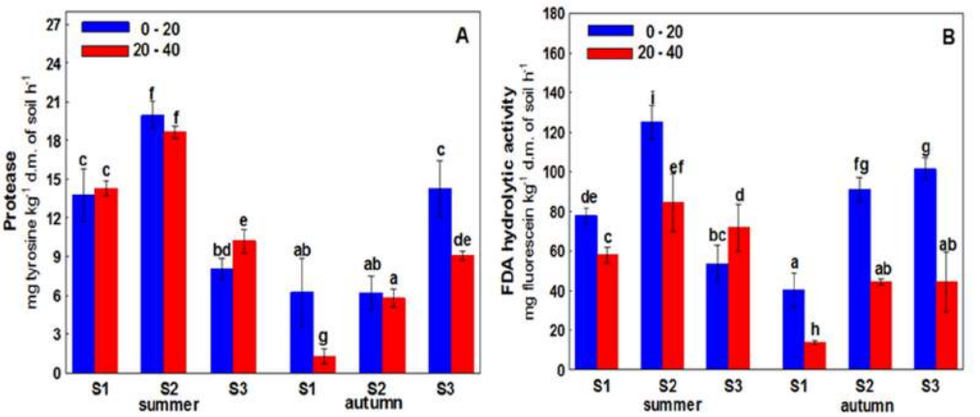

Figure 4. Enzymatic activity in soil from 0–20 and 20–40 cm depths continued: (**A**) protease; (**B**) FDA hydrolytic activity. Legend: S1, S2, S3—sampling sites located S1—5.88, S2—22.7 and S3—50.08 m from the reservoir with liquid post-production waste. Vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$.

The hydrolytic activity of fluorescein (Figure 4B), similarly as proteolytic activity, greatly varied in individual experimental time points in both soil layers. In summer, the lowest values in the 0–20 cm layer were recorded at S3 (53.50 mg kg^{-1}). In the remaining sites, i.e., S1 and S3, this activity was significantly higher, reaching the highest level at S2 (124.96 mg kg^{-1}). In the 20–40 cm layer, the studied activity was lowest at S1 (57.94 mg kg^{-1}), while at the other sites, it was significantly higher reaching the highest value similarly to the upper layer at S2 (84.55 mg kg^{-1}). In autumn, fluorescein hydrolytic activity was the lowest in both soil layers at the site closest to the reservoir, i.e., S1, reaching 40.35 and 13.67 mg kg^{-1}, respectively. At the other sampling sites, i.e., S2 and S3, the values were significantly higher, especially at S3 (101.32 mg kg^{-1}). The hydrolytic activity of fluorescein throughout the study period was significantly lower in the lower soil layer than in the upper one.

The results concerning seed germination and root growth of *L. sativum* seedlings (Figure 5A–C) indicated that the distance from the liquid waste reservoir had a very significant effect on the phytotoxicity of the top soil layer (0–20 cm). Seed germination data (Figure 5A) demonstrated that at S1, i.e., closest to the reservoir, seeds did not germinate either in summer or in autumn. Therefore, no data were obtained for root length increment in the soil from this site (Figure 5B,C). At the other sites, i.e., S2 and S3, the number of

germinated seeds was generally similar (97 seeds) in both time points. The number of germinated seeds was slightly but significantly higher only in autumn at S2 and amounted to 99 seeds.

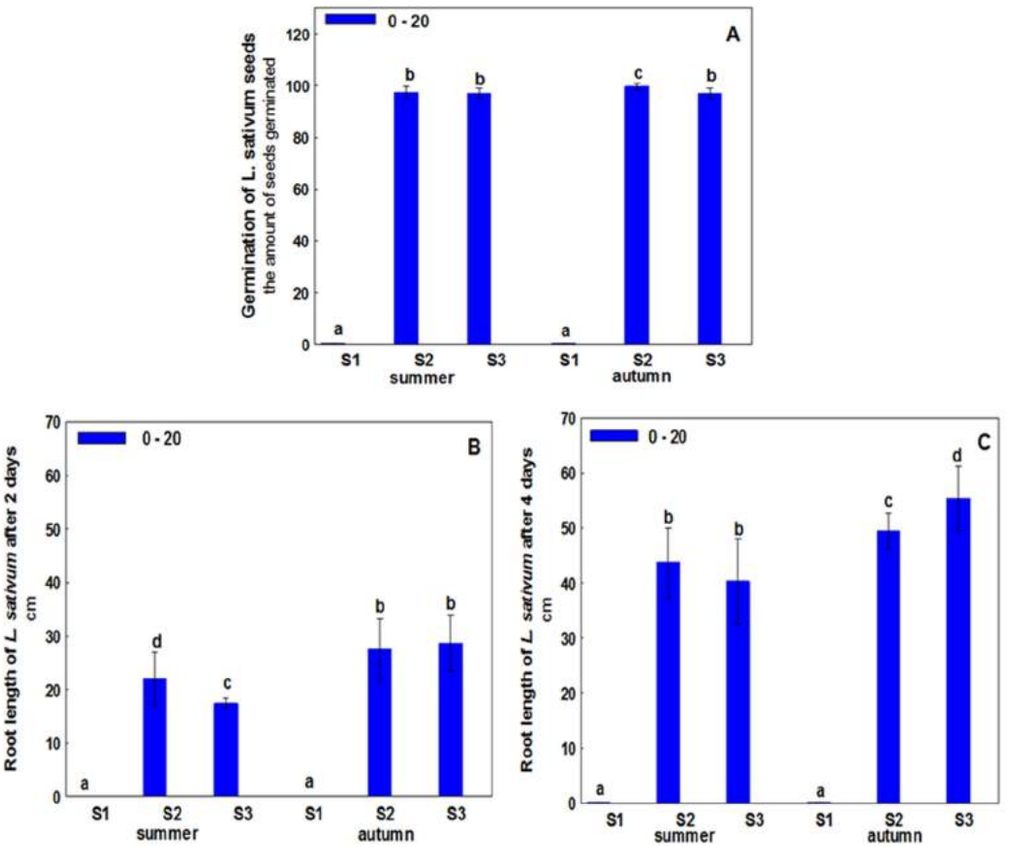

Figure 5. Soil phytotoxicity indicators from the 0–20 cm layer: (**A**) *L. sativum* seed germination; (**B**) seedling root length after 2 days; (**C**) seedling root length after 4 days. S1, S2, S3 —sampling sites located S1—5.88, S2—22.7 and S3—50.08 m from the reservoir with liquid post-production waste. Vertical lines indicate the standard deviation. Different letters above the columns indicate significant differences at $p < 0.05$.

The results concerning the root length measured after 2 and 4 days (Figure 5B,C) showed that the increments in autumn at both sites, i.e., S2 and S3, were significantly higher than in summer. Root length measured after 2 days (Figure 5B) in autumn did not differ significantly between individual points and amounted to 27.57 and 28.70. In contrast, root length in summer was significantly greater at S2 (22.03). Different observations were made for root measurements after 4 days (Figure 5C), where in summer, there were no significant differences between S2 and S3 (40.37–43.75). In contrast, such differences occurred in autumn. Significantly, the highest value was obtained for the farthest site, i.e., S3 (55.37 cm).

4. Discussion

Soil microorganisms are the foundation of many different ecosystem functions [20–22], and their abundance, richness and composition are sensitive to changes in the soil environment [44,45]; thus, they are considered early indicators of changes in its quality [46]. All changes in the soil microbiota have a significant impact on the cycle of nutrients, carbon, nitrogen, as well as greenhouse gas emissions [47,48]. Considering the importance of soil microbial diversity for the multifunctionality of ecosystems, it seems justified to include its analysis when studying all mechanism of the soil environment's response to climate change, as well as to various human activities. Both of these factors significantly affect the physical and chemical properties

of the soil [2,3], which in turn translate into the activity, abundance and biodiversity of soil microorganisms, which have been confirmed in the present study. Significant changes in the number of both bacteria and fungi recorded between individual sampling sites proved that changes in the chemical properties of the soil had the main impact on this parameter. This was confirmed by principal component analysis, which showed a negative correlation of both oligotrophic bacteria and filamentous fungi with soil pH (Figure 6). In addition, the smallest number of soil microorganisms was recorded in S1, characterized by a strongly alkaline pH (>9.0). Along with increasing distance from the source of pollution, the value of this parameter also grew significantly, which was probably related to the improvement in soil chemical conditions (decrease in pH).

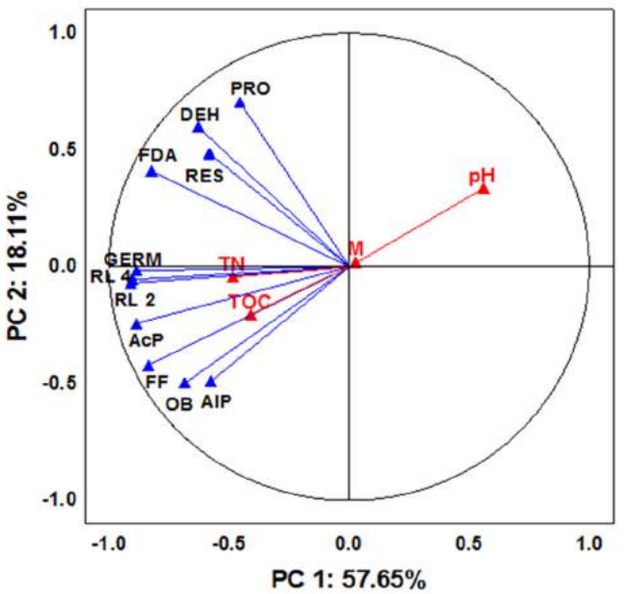

Figure 6. Principal component analysis (PCA) for the results of analyzed parameters in the soil. OB—oligotrophic bacteria, FF—filamentous fungi, RES—respiration of soil, DEH—dehydrogenases, PRO—protease, FDA—fluorescein diacetate hydrolysis activity, AcP—acid phosphatase, AlP—alkaline phosphatase, GERM—germination of *L. sativum*, RL2—root length of *L. sativum* after two days, RL4—root length of *L. sativum* after four days, M—moisture, TOC—total organic carbon, TN—total nitrogen.

This was confirmed by the correlation results at the "combination" level where significant positive correlations were recorded at S2 and S3 (Figure 7). The presented results may also be evidence of the high sensitivity of soil microorganisms to the stress factor, i.e., soil pH, due to the rather significant differences in the abundance between the individual analyzed sites at a relatively short distance.

Confirmation of the negative impact of soil pH on both groups of microorganisms was also shown for the correlation results at the level of the soil profile, where significant negative correlations for both bacteria and fungi were recorded (Figure 8). Generally, higher numbers were observed in the upper soil layer for both tested groups of microorganisms. Perhaps this was due to the fact that soil surface layers were porous and characterized by a more frequent occurrence of dry–wet cycles. This, in turn, caused an influx of fresh substrates and nutrients, which translated into relatively higher microbial activity [22]. Moreover, as reported by Naylor et al. [22], minerals such as sodium were more susceptible to leaching; thus, their concentration tended to increase with depth. This, in turn, could translate into deterioration of soil physicochemical conditions, with which soil microorganisms were strongly associated [23,24].

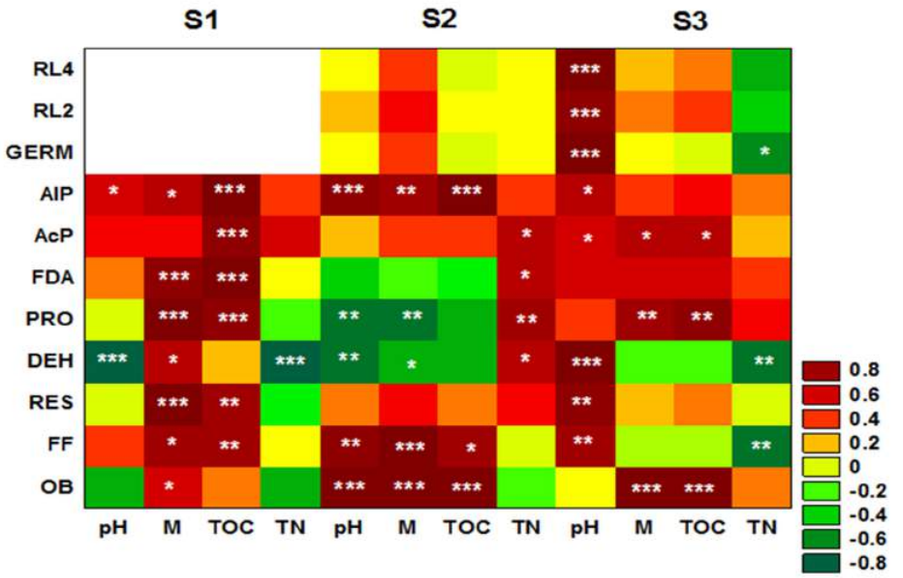

Figure 7. Heat map displaying the Pearson correlation coefficients between chemical and physico-chemical properties; microbial, biochemical and enzymatic activity; and phytotoxic parameters at the "combination" level. Significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, respectively. S1, S2, S3—sampling sites located S1—5.88, S2—22.7 and S3—50.08 m from the reservoir with liquid post-production waste. OB—oligotrophic bacteria, FF—filamentous fungi, RES—respiration of soil, DEH—dehydrogenases, PRO—protease, FDA—fluorescein diacetate hydrolysis activity, AcP—acid phosphatase, AlP—alkaline phosphatase, GERM—germination of *L. sativum*, RL2—root length of *L. sativum* after two days, RL4—root length of *L. sativum* after four days, M—moisture, TOC—total organic carbon, TN—total nitrogen.

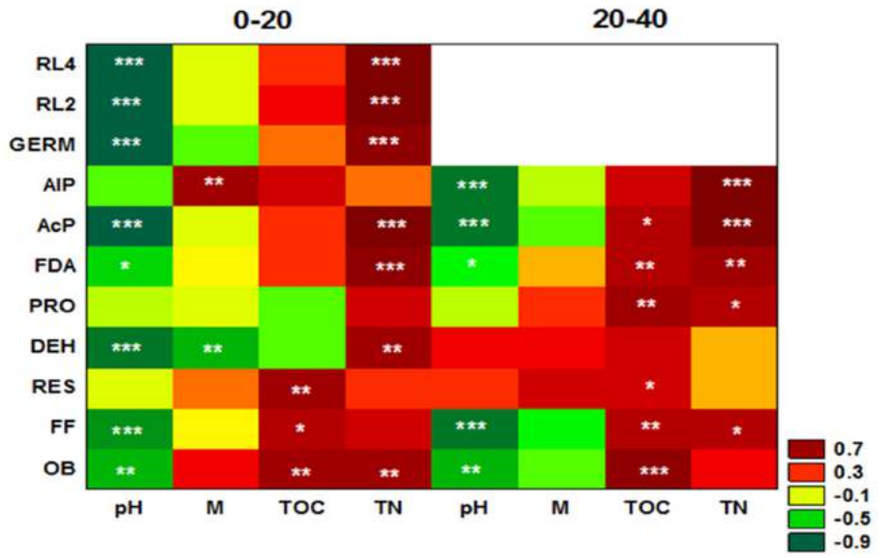

Figure 8. Heat map displaying the Pearson correlation coefficients between chemical and physico-chemical properties; microbial, biochemical and enzymatic activity; and phytotoxic parameters at the level of the soil profile. Significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, respectively. Legend: 0–20, 20–40—soil profiles in cm. OB—oligotrophic bacteria, FF—filamentous fungi, RES—respiration of soil, DEH—dehydrogenases, PRO—protease, FDA—fluorescein diacetate hydrolysis activity, AcP—acid phosphatase, AlP—alkaline phosphatase, GERM—germination of *L. sativum*, RL2—root length of *L. sativum* after two days, RL4—root length of *L. sativum* after four days, M—moisture, TOC—total organic carbon, TN—total nitrogen.

For both bacteria and fungi, the highest numbers were recorded in autumn. A similar trend was observed by Fan et al. [49]. This proved that the obtained microbial counts were probably also affected by seasonal changes. They were shown to be related to climatic fluctuations, including humidity and temperature in field conditions, which according to Li et al. [50], were key indicators affecting the soil microbiome. More pronounced changes in the case of bacteria were probably due to their greater sensitivity to unfavorable conditions compared to fungi, which showed greater resistance [51].

Soil respiratory activity, i.e., CO_2 emissions from the soil surface, can also be a good indicator of ecological disturbance of the soil environment. CO_2 can have different sources [30]; therefore, its generation stream is of global importance. It is a powerful regulator of the greenhouse effect and the global climate because it affects the global carbon cycle [52,53]. According to Grzyb et al. [54] and Kwiatkowska et al. [55], carbon mineralization was primarily related to organic matter. In turn, Bao et al. [56] and Hou et al. [57] proved that climatic factors, such as temperature and humidity, mainly influenced soil respiratory activity. It is likely that soil water content may have been one of the factors that contributed to the stimulation, albeit with varying degrees of intensity, of respiratory activity between the different sampling points in the current study. This was partially confirmed by the correlation results at the "combination" level, where significant positive correlations were recorded at S1 between carbon content and moisture content (Figure 7). Quemada and Menacho [58] already reported that soil respiration was strongly influenced by water content and temperature. We also obtained the highest values of this parameter in summer, when high temperatures and water content probably contributed to the stimulation of respiration [58,59]. Sodium hydroxide was also a factor that could have affected the respiratory activity of the soil. According to Wong et al. [60], soil organic carbon could be rapidly lost under sodium conditions. This is due to the dispersion of soil aggregates and thus the release of organic matter accumulated in them. This may have stimulated, in our study, the activity of microorganisms in relation to carbon mineralization, by decomposing not only easily degradable carbon compounds, but also those that are hard to access. These observations were confirmed by significant positive correlations of respiratory activity with TOC at the "combination" level in sites located closest to the waste reservoir (Figure 7). We also recorded positive correlations of these parameters at the level of soil profiles: 0–20 and 20–40 cm (Figure 8). We recorded the highest significant positive correlation of this indicator with TOC in autumn. Perhaps some additional source of fresh organic matter also contributed to this effect, e.g., in the form of plant residues during the growing season. On the other hand, the soil microbiota utilizes only part of the carbon contained in the substrates for growth and maintenance of microbial structures, while the rest is released into the atmosphere in the form of CO_2 [61]. Respiratory activity can be a good measure of stress factors because, firstly, it reflects the efficiency of microorganisms, and secondly, higher amounts of CO_2 were shown to be produced under stress conditions [61]. This was also confirmed by the present results, indicating an increased CO_2 emission from the soil at the site with most unfavorable conditions, i.e., S1. These observations additionally indicated that an increased amount of greenhouse gas was emitted from degraded soil, which could contribute to the worsening of the greenhouse effect [62].

In addition to biological and biochemical activity, soil enzymes are other rapid and sensitive "receptors" of environmental and anthropogenic stress factors. They are similarly closely associated with the physical and chemical properties of the soil and climatic conditions [31,49,63]. The latter reports were also confirmed by our research, in which both soil moisture and pH, as well as seasonal changes, were probably the main factors that contributed to the fluctuations in the activity of the enzymes studied. This was supported by the results of principal component analysis, where we noted negative correlations of soil pH with all analyzed enzymes (Figure 6). Therefore, it could be one of the factors limiting the activity of soil enzymes, especially at the site closest to the waste reservoir. This applied, among others, to the activity of dehydrogenase, acid phosphatase and fluorescein hydrolytic activity (FDA). These observations were also confirmed by significant

negative correlations between dehydrogenase and soil pH in sites located closest to the waste reservoir (Figure 7). The dependence of the activity of soil enzymes on soil pH has been repeatedly analyzed in various conditions [18,64,65]. However, in the case of acid phosphatase activity and FDA, negative correlations with soil pH were recorded at the level of both soil profiles (Figure 8). With respect to acid phosphatase, we also noted negative correlations with soil pH at the level of seasonal changes (Figure 9). The situation was different for alkaline phosphatase, where the tested conditions, especially soil pH, had a stimulating effect on this enzyme. This was confirmed by positive correlations with soil pH at all sampling sites (Figure 7). The varying effects of soil pH on the enzymatic activity could be due to the high complexity of the role of this parameter. It affects, among others, the process of decomposition and mineralization of soil organic molecules, dispersion and aggregation of soil colloids, the number and activity of microorganisms, and redox reactions, which in turn translated into the activity of soil enzymes [66]. As with respiratory activity, varying enzyme activities were probably also influenced by sodium hydroxide, which not only increased soil pH, but also affected soil structure. These observations were confirmed by significant positive correlations with TOC at the site closest to the reservoir for all analyzed enzymes, except for dehydrogenase. As reported by Mavi and Marschnera [67], increasing sodium saturation in the soil caused the dispersion of organic matter and clay particles, thereby damaging aggregates and soil structure, which probably contributed to the release of organic matter accumulated in them. Positive correlations with TOC were also recorded for protease, FDA and acid phosphatase for the 20–40 cm profile (Figure 8). This was probably also related to the presence of sodium, whose concentration increased with depth [22]. The positive correlations with TOC of the tested parameters in autumn (Figure 9) were associated, as in the case of microorganisms and respiratory activity, with the supply of an additional source of organic matter, such as plant residues during the growing season. Nitrogen was another biogen that also significantly affected the activity of enzymes. This was confirmed by both principal component analysis (Figure 6) and the significant positive correlations recorded for all TN levels with the enzymes studied (Figures 7–9). The important role of nitrogen in shaping soil enzyme activity has also been demonstrated by other authors [68,69]. The activity of the analyzed enzymes was also affected by climatic conditions, especially moisture. We recorded significant positive correlations of moisture content with all analyzed enzymes (Figure 9). The present study has shown that soil enzymes have great potential to respond rapidly to environmental changes, and can therefore serve as indicators of the health and quality of the soil environment.

Chemical degradation typically negatively affects the physical, chemical and microbiological properties of the soil environment, which also carries the risk of disturbing the living conditions of plants [11]. Therefore, it is important to monitor the effects of different types of harmful substances on parameters related to plant growth and development. Phytotoxic parameters are often used to assess the effects of various substances on the soil environment [17,70–72]. The conducted research indicated that sodium hydroxide was the main factor limiting plant growth in the current study. This was evidenced by germination inhibition of the test plant *L. sativum* in the soil collected closest to the waste reservoir. This was probably due to the strong alkalization of the soil environment. These observations were confirmed by the recorded significant negative correlations of soil pH with all parameters related to phytotoxicity (Figure 6). As the distance from the source of contamination increased, the soil pH decreased, which in turn translated into an improvement in phytotoxic parameters. This was supported by the results of correlations at the "combination" level between the discussed parameters, where significant positive correlations were observed at site S3, located farthest from the waste reservoir (Figure 7). Soil pH was also a limiting factor for both germination and root length increments of *L. sativum* at the soil profile level (Figure 8). The stimulation of parameters related to phytotoxicity in autumn was, to some extent, caused by better availability of the basic nutrient, important in terms of plant nutrition, i.e., TN (Table 2). This was confirmed by the recorded significant positive correlations between these parameters (Figure 9).

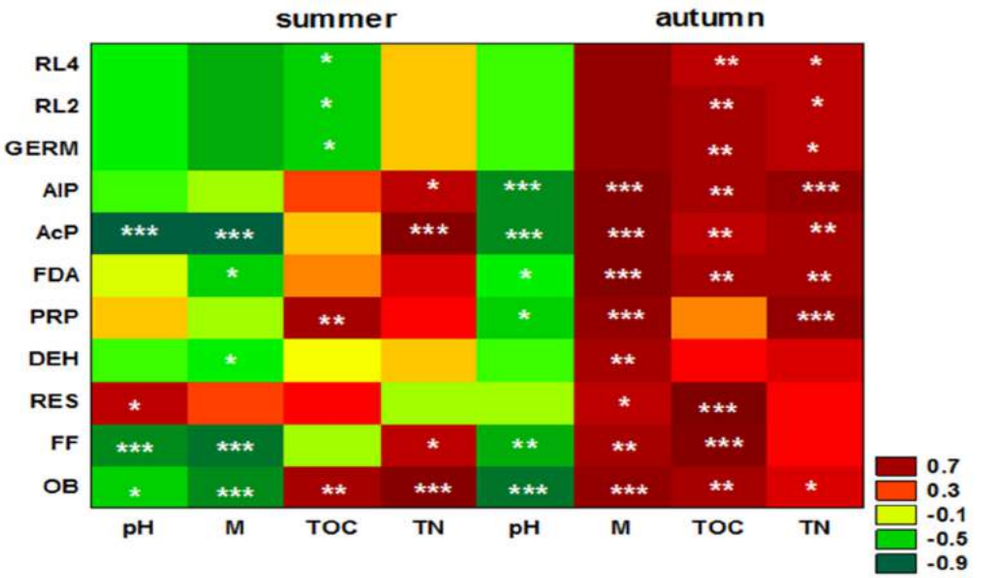

Figure 9. Heat map displaying the Pearson correlation coefficients between chemical and physico-chemical properties; microbial, biochemical and enzymatic activity; and phytotoxic parameters at the level of the seasonal variability. Significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, respectively. VII, IX—months of sampling, OB—oligotrophic bacteria, FF—filamentous fungi, RES—respiration of soil, DEH—dehydrogenases, PRO—protease, FDA—fluorescein diacetate hydrolysis activity, AcP—acid phosphatase, AlP—alkaline phosphatase, GERM—germination of *L. sativum*, RL2—root length of *L. sativum* after two days, RL4—root length of *L. sativum* after four days, M—moisture, TOC—total organic carbon, TN—total nitrogen.

The present study has shown that soil microbiological, biochemical and enzymatic indicators, as well as phytotoxicity, have the potential to respond quickly to environmental changes. Therefore, they can be used to assess the effects of the impact of various wastes on soils used for various purposes, e.g., arable soils and soils of post-industrial areas [55,73].

5. Conclusions

All the analyzed parameters of the activity of soil microorganisms and phytotoxicity showed significant changes in the level at individual sampling sites. The soil located closest to the liquid waste reservoir had the lowest values of the microbiological, biochemical and enzymatic activities, while phytotoxicity had the highest. Individual activities showed changes depending on the season and soil layer. Bacterial and fungal counts and acid phosphatase activity remained at the lowest levels at S1 in spring and autumn in both soil layers (0–40 cm). For the other activities, i.e., respiration, protease, alkaline phosphatase and fluorescein hydrolytic activity, the effect was more pronounced in both layers only in autumn. The phytotoxicity results showed that conditions near the emitter of pollution were unfavorable for seed germination. The above observations support the hypothesis that the applied microbial activity parameters are sensitive indicators of soil changes caused by liquid waste. Considering the duration of these changes and the profile level at which they persisted, it should be pointed out that the total bacterial and fungal counts, and the activities of acid phosphatase, alkaline phosphatase and fluorescein hydrolytic activity were the most useful in monitoring the condition of soils exposed to degradation caused by increased pH. The results also partially confirmed the second hypothesis that soil contamination with waste would affect soil microbial populations in the deeper soil layer, i.e., 20–40 cm. In contrast, it does not result in negative changes further away from the reservoir.

Such strong changes in the activity of bacterial and fungal populations in the soil located closest to the waste reservoir, where the pH was the highest (pH 9), suggested that

this could be a site of selection of microorganisms resistant to high pH. These observations suggest the need to continue research into the biodiversity of microbiota and mycobiota inhabiting this site.

The present study provides guidelines that can be helpful in assessing the degree of soil environment degradation caused by liquid waste reservoirs and in evaluating the effectiveness of safeguards for such reservoirs.

Author Contributions: Conceptualization, J.J. and E.K.; methodology, J.J.; software, E.K. and C.A.K.; validation, J.J., E.K. and C.A.K.; formal analysis, J.J. and E.K.; investigation, J.J. and E.K.; resources, J.J. and C.A.K.; data curation, E.K.; writing—original draft preparation, E.K.; writing—review and editing, J.J. and E.K.; visualization, E.K.; supervision, J.J.; project administration, J.J.; funding acquisition, J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the University of Life Sciences in Lublin.

Data Availability Statement: Additional information is provided by the corresponding author.

Acknowledgments: The authors thank Michał Bańko for inspiration for research, for help in collecting soil material and in documentation.

Conflicts of Interest: The authors declare no conflict of interest.

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10. Oświadczenia o współautorstwie

Lublin, 4.03.2024r.

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w niżej wymienionych pracach:

Joniec J., Kwiatkowska E., Kwiatkowski C.A. 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. *Agriculture,* **2022**, 12, 1190. https://doi.org/10.3390/agriculture12081190

mój udział polegał na:

- opracowaniu koncepcji badań
- zaplanowaniu badań
- przeprowadzeniu wszystkich badań
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- koordynowaniu, jako autor korespondencyjny, procesu publikacyjnego, w tym poprawy manuskryptu po recenzjach

Kwiatkowska E., Joniec J. Effects of agricultural management of spent mushroom waste on phytotoxicity and microbiological transformations of C, P, and S in soil and their consequences for the greenhouse effect. *Int. J. Environ. Res. Public Health,* **2022**, 19, 12915. https://doi.org/10.3390/ijerph191912915

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- opracowaniu koncepcji badań
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- redagowaniu manuskryptu
- poprawie manuskryptu po uwagach recenzentów

Kwiatkowska E., Joniec J., Kwiatkowski C.A, Kowalczyk K., Nowak M., Leśniowska-Nowak J. 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. *Int. Agrophys.*, **2024**, 38, 139-154. https://doi.org/10.31545/intagr/184175

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- poprawie manuskryptu po uwagach recenzentów

Kwiatkowska E., Joniec J., Kwiatkowski C.A. Involvement of soil microorganisms in C, N and P transformations and phytotoxicity in soil from post-industrial areas treated with chemical industry waste. *Minerals*, **2023**, 13, 12. https://doi.org/10.3390/min13010012

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- pobieraniu materiału glebowego
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- opracowaniu wyników i analizie statystycznej
- redagowaniu manuskryptu
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Kwiatkowska Edyta

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Podpis

Lublin, 4.03.2024r.

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w niżej wymienionych pracach:

Joniec J., Kwiatkowska E., Kwiatkowski C.A. 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. *Agriculture,* **2022,** 12, 1190. https://doi.org/10.3390/agriculture12081190

mój udział polegał na:

- opracowaniu koncepcji badań
- zaplanowaniu badań
- tworzeniu manuskryptu

Kwiatkowska E., Joniec J. Effects of agricultural management of spent mushroom waste on phytotoxicity and microbiological transformations of C, P, and S in soil and their consequences for the greenhouse effect. *Int. J. Environ. Res. Public Health,* **2022,** 19, 12915. https://doi.org/10.3390/ijerph191912915

mój udział polegał na:

- opracowaniu koncepcji badań
- zaplanowaniu badań
- tworzeniu manuskryptu
- pełnieniu funkcji autora korespondencyjnego

Kwiatkowska E., Joniec J., Kwiatkowski C.A, Kowalczyk K., Nowak M., Leśniowska-Nowak J. 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. *Int. Agrophys.*, **2024**, 38, 139-154. https://doi.org/10.31545/intagr/184175

mój udział polegał na:

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- zaplanowaniu badań
- tworzeniu manuskryptu
- pełnieniu funkcji autora korespondencyjnego

Kwiatkowska E., Joniec J., Kwiatkowski C.A. Involvement of soil microorganisms in C, N and P transformations and phytotoxicity in soil from post-industrial areas treated with chemical industry waste. *Minerals*, **2023**, 13, 12. https://doi.org/10.3390/min13010012

mój udział polegał na:

- opracowaniu koncepcji badań
- zaplanowaniu badań
- tworzeniu manuskryptu
- pełnieniu funkcji autora korespondencyjnego

Jolanta Joniec

Podpis

Lublin, 4.03.2024r.

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w niżej wymienionych pracach:

Joniec J., Kwiatkowska E., Kwiatkowski C.A. 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. *Agriculture,* **2022,** 12, 1190. https://doi.org/10.3390/agriculture12081190

mój udział polegał na:

- założeniu i prowadzeniu doświadczenia polowego
- poborze materiału glebowego
- poprawie manuskryptu po recenzjach

Kwiatkowska E., Joniec J., Kwiatkowski C.A, Kowalczyk K., Nowak M., Leśniowska-Nowak J. 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. *Int. Agrophys*., **2024**, 38, 139-154. https://doi.org/10.31545/intagr/184175

mój udział polegał na:

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- poborze materiału glebowego
- poprawie manuskryptu po recenzjach

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mój udział polegał na:

- przeprowadzeniu badań i opracowaniu wyników

Cezary Kwiatkowski
Podpis

Lublin, 4.03.2024r.

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy

Kwiatkowska E., Joniec J., Kwiatkowski C.A, Kowalczyk K., Nowak M., Leśniowska-Nowak J. 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. *Int. Agrophys.*, **2024**, 38, 139-154. https://doi.org/10.31545/intagr/184175

mój udział polegał na:

- przeprowadzeniu badań molekularnych i interpretacji wyników

Podpis

Lublin, 4.03.2024r.

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:

Kwiatkowska E., Joniec J., Kwiatkowski C.A, Kowalczyk K., Nowak M., Leśniowska-Nowak J. 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. *Int. Agrophys.*, **2024**, 38, 139-154. https://doi.org/10.31545/intagr/184175

mój udział polegał na:

- przeprowadzeniu badań molekularnych i przeliczeniu wyników

Podpis

Lublin, 4.03.2024r.

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:

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mój udział polegał na:

- przeprowadzeniu badań molekularnych i przeliczeniu wyników

Podpis

11. Wykaz dorobku naukowego i dane bibliometryczne

05.03.2024, Lublin

Biblioteka Główna UP w Lublinie
Baza Publikacji Pracowników Uniwersytetu Przyrodniczego

Raport autora mgr inż. Kwiatkowska Edyta za lata 2012 – 2024

1. Publikacje w czasopismach naukowych

1.1. Publikacje w czasopiśmie naukowym posiadającym Impact Factor IF

Lp	Opis bibliograficzny	IF	Pkt. MNiSW
1.	**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.** [AUT.] EDYTA KWIATKOWSKA, [AUT. KORESP.] JOLANTA JONIEC, [AUT.] CEZARY KWIATKOWSKI, KRZYSZTOF KOWALCZYK, MICHAŁ NOWAK, JUSTYNA LEŚNIOWSKA-NOWAK. *Int. Agrophys.* 2024 Vol. 38 Iss. 2 s. 139-154, il., bibliogr., sum. DOI: 10.31545/intagr/184175	2,200	100,00
2.	**Involvement of soil microorganisms in C, N and P transformations and phytotoxicity in soil from post-industrial areas treated with chemical industry waste.** [AUT.] EDYTA KWIATKOWSKA, [AUT. KORESP.] JOLANTA JONIEC, [AUT.] CEZARY KWIATKOWSKI. *Minerals* 2023 Vol. 13 Iss. 1 Article number 12, il., bibliogr., sum. DOI: 10.3390/min13010012	2,500	100,00
3.	**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.** [AUT.] JOLANTA JONIEC, [AUT. KORESP.] EDYTA KWIATKOWSKA, [AUT.] CEZARY KWIATKOWSKI. *Agriculture* 2022 Vol. 12 Iss. 8 Article number 1190, il., bibliogr., sum. DOI: 10.3390/agriculture12081190	3,600	100,00
4.	**Microbiological nitrogen transformations in soil treated with pesticides and their impact on soil greenhouse gas emissions.** [AUT.] STEFANIA JEZIERSKA-TYS, [AUT. KORESP.] JOLANTA JONIEC, [AUT.] JOANNA BEDNARZ, EDYTA KWIATKOWSKA. *Agriculture* 2021 Vol. 11 Iss. 8 Article number 787, il., bibliogr., sum. DOI: 10.3390/agriculture11080787	3,408	100,00
5.	**Reaction of microorganisms to long-term waste**	2,818	100,00

	reclamation of soil degraded by the sulfur mining industry. [AUT. KORESP.] JOLANTA JONIEC, [AUT.] GRAŻYNA ŻUKOWSKA, MARTA BIK-MAŁODZIŃSKA, EDYTA KWIATKOWSKA, KAMILA ROJEK. *Minerals* 2021 Vol. 11 Iss. 11 Article number 1226, il., bibliogr., sum. DOI: 10.3390/min11111226		
6.	Effect of reclamation treatments on microbial activity and phytotoxicity of soil degraded by the sulphur mining industry. [AUT. KORESP.] JOLANTA JONIEC, [AUT.] PATRYK OLESZCZUK, STEFANIA JEZIERSKA-TYS, EDYTA KWIATKOWSKA. *Environ. Pollut.* 2019 Vol. 252 Part B s. 1429-1438, il., bibliogr., sum. DOI: 10.1016/j.envpol.2019.06.066	6,793	100,00
7.	Fungal frequency and diversity in the nests of wetland birds from Poland: relationships between birds, nest properties and inhabiting fungi. [AUT. KORESP.] TERESA KORNIŁŁOWICZ-KOWALSKA, [AUT.] IGNACY KITOWSKI, JUSTYNA BOHACZ, EDYTA KWIATKOWSKA. *Avian Biol. Res.* 2018 Vol. 11 No. 4 s. 245-262, il., bibliogr., sum. DOI: 10.3184/175815618X15360537405342	0,853	25,00
8.	Changes in the spore numbers of am fungi and in am colonisation of roots of clovers and grasses on a peat-muck soil with respect to mineral fertilisation. [AUT.] TERESA KORNIŁŁOWICZ-KOWALSKA, BERNADETA WOJDYŁO-KOTWICA, EDYTA KWIATKOWSKA. *Pak. J. Bot.* 2016 Nr Vol 48(2) 729-738, il., bibliogr., sum.	0,690	20,00
9.	Application of biological indicators for estimation of remediation of soil degraded by sulphur industry. [AUT.] JOLANTA JONIEC, JADWIGA FURCZAK, EDYTA KWIATKOWSKA. *Ecol. Chem. Eng., S* 2015 vol. 22 Nr 2 269-283, il., bibliogr., sum. DOI: 10.1515/eces-2015-0016	0,552	15,00
10.	Microbiological activity of soil amended with granulated fertilizer from sewage sludge. [AUT.] JOLANTA JONIEC, EDYTA KWIATKOWSKA. *J. Elem.* 2014 Vol. 19 No. 1 143-154, il., bibliogr., streszcz., sum. DOI: 10.5601/jelem.2014.19.1.586	0,690	15,00
	Suma:	24,104	675,00

1.2 Publikacja w czasopiśmie naukowym nieposiadającym IF

Lp	Opis bibliograficzny	Pkt. MNiSW
1.	Effects of agricultural management of spent mushroom waste on phytotoxicity and microbiological transformations of C, P,and S in soil and their consequences for the greenhouse effect. [AUT.] EDYTA KWIATKOWSKA, [AUT. KORESP.] JOLANTA JONIEC. *Int. J. Environ. Res. Public Health* 2022 Vol. 19 Issue 19 Article number 12915,	140,00

il., bibliogr., sum. DOI: 10.3390/ijerph191912915

Suma: 140.00

2. Inne

2.3. Materiały konferencyjne

Lp	Opis bibliograficzny
1.	**Udział mikroorganizmów glebowych w przemianach C, N i P w glebie z terenów poprzemysłowych, poddanej działaniu odpaduz przemysłu chemicznego.** [AUT.] EDYTA KWIATKOWSKA, JONIEC JOLANTA, CEZARY KWIATKOWSKI. W: 55 Jubileuszowa Konferencja Mikrobiologiczna „Mikrobiologia w badaniach środowiskowych – rys historyczny i perspektywy na przyszłość". Puławy, 14-15 września 2023 roku s. 73. [Puławy] 2023, Instytut Uprawy Nawożenia i Gleboznawstwa - Państwowy Instytut Badawczy, 978-83-7562-400-7.
2.	**Wpływ kilkuletniego nawożenia odpadem popieczarkowym na populacje bakterii i grzybów glebowych.** [AUT.] EDYTA KWIATKOWSKA, JOLANTA JONIEC, KOWALCZYK KRZYSZTOF, MICHAŁ NOWAK, JUSTYNA LEŚNIOWSKA-NOWAK, CEZARY KWIATKOWSKI. W: VII ogólnopolskie Sympozjum Mikrobiologiczne. „Metagenomy różnych środowisk". Materiały konferencyjne. Lublin, 20-21 czerwca 2023 s. 132. [Lublin] 2023, Instytut Agrofizyki im. Bohdana Dobrzańskiego Polskiej Akademii Nauk, 978-83-89969-79-8.
3.	**Reakcja populacji mikroorganizmów glebowych na nawiezienie gleby odpadem popieczarkowym.** [AUT.] JOLANTA JONIEC, MICHAŁ NOWAK, KRZYSZTOF KOWALCZYK, EDYTA KWIATKOWSKA, CEZARY KWIATKOWSKI. W: VI Ogólnopolskie Sympozjum Mikrobiologiczne: "Metagenomy Różnych Środowisk" : Puławy, 23-24 czerwca 2022 : Materiały Konferencyjne s. 83. [b.m.] 2022, Instytut Uprawy Nawożenia i Gleboznawstwa - Państwowy Instytut Badawczy, 978-83-7562-382-6.
4.	**Badania wstępne nad wpływem podłoża popieczarkowego na wybrane parametry aktywności bakterii i grzybów glebowych.** [AUT.] JOLANTA JONIEC, CEZARY KWIATKOWSKI, EDYTA KWIATKOWSKA. W: V Ogólnopolskie Sympozjum Mikrobiologiczne „Metageny różnych środowisk". Abstrakty. Warszawa, 17-18 czerwca 2021 roku s. 79. Warszawa 2021, [b.w].
5.	**Ocena rocznego oddziaływania na środowisko glebowe podłoża popieczarkowego, przy użyciu wybranych parametrów aktywności enzymatycznej.** [AUT.] JOLANTA JONIEC, EDYTA KWIATKOWSKA, CEZARY KWIATKOWSKI. W: 54. Konferencja Mikrobiologiczna „Mikroorganizmy różnych środowisk", 20-21 września 2021, Lublin 2021. Abstrakty s. 83. Lublin 2021, Uniwersytet Marii Curie-Skłodowskiej w Lublinie.
6.	**Phytotoxicity and respiratory activity of soil in the first year of fertilization with spent mushroom substrate.** [AUT.] JONIEC, J., KWIATKOWSKA, E., KWIATKOWSKI, C.. W: 13th International Conference on Agrophysics: Agriculture in changing climate - BOOK OF ABSTRACTS. 15-16 november, Lublin, Poland s. 121. [b.m.] 2021, Polska Akademia Nauk, 978-83-89969-72-9.
7.	**Aktywność enzymatyczna jako wskaźnik zmian zachodzących w glebie poddanej wieloletniej rekultywacji z wykorzystaniem odpadów organicznych i mineralnych.**

[AUT.] JOLANTA JONIEC, STEFANIA JEZIERSKA-TYS, EDYTA KWIATKOWSKA, MARTA BIK-MAŁODZIŃSKA. W: "Nauka dla zrównoważonego rozwoju i biogospodarki"- Międzynarodowa Konferencja Naukowa połączona z Jubileuszem 75-lecia Wydziału Agrobioinżynierii, Lublin, 12 -13.06.2019 r s.73, bibliogr. Lublin 2019, [b.w].

8. **Ocena skuteczności wieloletniej rekultywacji gleby na podstawie parametrów aktywności drobnoustrojów glebowych przeprowadzających przemiany azotu i fosforu.** [AUT. KORESP.] JOLANTA JONIEC, [AUT.] STEFANIA JEZIERSKA-TYS, EDYTA KWIATKOWSKA, MARTA BIK-MAŁODZIŃSKA. W: "Mikroorganizmy w zrównoważonym rolnictwie, ochronie środowiska i procesach biotechnologicznych" pod redakcją Doroty Swędrzyńskiej i Agnieszki Mocek-Płóciniak-53 Ogólnopolska Konferencja Mikrobiologiczna, Poznań, 8-11.09.2019 s. 55. Poznań 2019, Uniwersytet Przyrodniczy w Poznaniu.

9. **Wykorzystanie aktywności drobnoustrojów w monitorowaniu stanu gleby zdegradowanej, poddanej wieloletniej rekultywacji odpadami.** [AUT.] JOLANTA JONIEC, STEFANIA JEZIERSKA-TYS, EDYTA KWIATKOWSKA, MARTA BIK-MAŁODZIŃSKA. W: Bioróżnorodność funkcjonalna gleb Polski : Konferencja Naukowa. Puławy, 18-19 października 2018 : Materiały konferencyjne s. 60. Puławy 2018, [Dział Upowszechniania i Wydawnictw], 978-83-7562-293-5.

10. **Badania nad następczym wpływem różnych odpadów na liczebność i aktywność drobnoustrojów związanych z przemianami węgla, w glebie zdegradowanej przez przemysł wydobywczy siarki.** [AUT.] JOLANTA JONIEC, EDYTA KWIATKOWSKA. W: Konferencja naukowa : Bioróżnorodność środowiska, znaczenie, problemy, wyzwania : Puławy, 30-31 maja 2017 r. Materiały konferencyjne s. 105. Puławy 2017, IUNG - PIB, 978-7562-258-4.

11. **Następcze oddziaływanie różnych odpadów na aktywność mikroorganizmów, związaną z przemianami N, S i P, w glebie zdegradowanej poddanej 3 letniej rekultywacji.** [AUT.] JOLANTA JONIEC, EDYTA KWIATKOWSKA. W: Mikrobiologia środowiskowa szansą bezpiecznego życia i postępu biotechnologicznego : 51. Ogólnopolska Konferencja Mikrobiologiczna : Materiały Konferencyjne, 5-8 września 2017, Toruń - Ciechocinek. Redakcja Dominika Thiem, Patrycja Golińska s. 55-56. [b.m.] 2017, [b.w.].

12. **Oddziaływanie różnych odpadów na aktywność enzymatyczną związaną z przemianami C,N,P w glebie zdegradowanej, poddanej rocznej rekultywacji (Impact of different wastes on enzymatic activity related to C,N, and P transformations in degraded soil subjected to 1-year reclamation).** [AUT.] JOLANTA JONIEC, EDYTA KWIATKOWSKA. W: Konferencja Międzynarodowa "Biogospodarka w Rolnictwie, Puławy, 21-22 czerwca 2016 = International Conference "Bioeconomy in agriculture", Puławy, 21-22 June 2016 s.83-86. Puławy, IUNG - PIB, 2016, 978-83-7562-219-5.

13. **Kształtowanie się liczebności wybranych grup mikroorganizmów oraz aktywności biochemicznej związanej z przemianami C i N w glebie zdegradowanej poddanej rocznej rekultywacji różnymi odpadami.** [AUT.] JOLANTA JONIEC, JADWIGA FURCZAK, EDYTA KWIATKOWSKA. W: Odpady organiczne - problemy i sposoby zagospodarowania. Ogólnopolska Konferencja Naukowa, Falenty, 20-21 września 2012 roku s. 39. [Falenty] 2012, [b.w.], 2012.

Suma:

UNIWERSYTET PRZYRODNICZY w Lublinie

BIBLIOTEKA GŁÓWNA UNIWERSYTETU PRZYRODNICZEGO W LUBLINIE

Sumaryczny IF-24,104
Sumaryczna liczba punktów MNiSW- 815,00

Szczegółowe wyjaśnienia:

- Wskaźnik Impact Factor został podany na podstawie bazy Journal Citation Reports (JCR) dla roku wydania publikacji z wyjątkiem publikacji z lat 2023 i 2024 - gdzie jego wartość została podana na podstawie ostatniej edycji JCR ed. 2022
- Punktacja została podana na podstawie Wykazu Czasopism Punktowanych MNiSW/MEiN obowiązujących dla roku wydania publikacji z wyjątkiem:
• publikacji z 2017 i 2018 r., którym punkty zostały przypisane na podstawie "Wykazu czasopism naukowych zawierający historię czasopisma z publikowanych wykazów za lata 2013-2016";
• publikacji z roku 2019-2022, którym punkty zostały przypisane na podstawie: „Komunikatu Ministra Edukacji i Nauki z dnia 21 grudnia 2021 r. o zmianie i sprostowaniu komunikatu w sprawie wykazu czasopism naukowych i recenzowanych materiałów z konferencji międzynarodowych".

Wykaz sporządziła:

Anna Starek

Lublin, 5.03.2024

Zestawienie wyników cytowań sporządzone na dzień 5.03.2024 r.

mgr inż. Edyta Kwiatkowska

Według bazy Web of Science Core Collection – Basic Search	
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Liczba cytowań opublikowanych prac	46
Liczba cytowań opublikowanych prac bez autocytowań	32
Liczba artykułów cytujących	38
Liczba artykułów cytujących bez autocytowań	32
Średnia liczba cytowań na pozycję	4,6
Indeks Hirsha	4
Według bazy Web of Science Core Collection – Author Search	
Liczba prac indeksowanych w bazie	10
Liczba cytowań opublikowanych prac	49
Liczba cytowań opublikowanych prac bez autocytowań	35
Indeks Hirsha	4