UNIWERSYTET RZESZOWSKI
Kolegium Nauk Przyrodniczych

mgr inż. Paulina Książek-Trela

ROZPRAWA DOKTORSKA

Wpływ komercyjnych preparatów mikrobiologicznych na degradację herbicydów i wielopierścieniowych węglowodorów aromatycznych

Praca wykonana pod kierunkiem:
Promotora dr hab. inż. Ewy Szpyrki, prof. UR
Promotora pomocniczego dr Leszka Potockiego
w Instytucie Biotechnologii, Katedrze Biotechnologii
Dziedzina nauki ścisłe i przyrodnicze
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Składam serdeczne podziękowania:

Pani Promotor dr hab. Ewie Szpyrce, prof. UR

za opiekę naukową i merytoryczną, stworzenie warunków umożliwiających rozwój naukowy, wszelką pomoc, nieustającą życzliwość oraz wyrozumiałość, wsparcie, cierpliwość i poświęcony czas.

Panu Promotorowi pomocniczemu dr Leszkowi Potockiemu

za pomoc i cenne wskazówki podczas realizacji niniejszej pracy oraz za okazaną życzliwość.

Mężowi

za wsparcie, nieustającą wiarę we mnie i moje możliwości oraz pomoc w zachowaniu równowagi między pracą i życiem prywatnym.

Rodzicom i Rodzinie

za trud włożony w moje wychowanie, wsparcie i wiarę we mnie i moje życiowe decyzje.

Spis treści

Wykaz stosowanych skrótów

DHA – aktywność dehydrogenazy (ang. *Dehydrogenase Activity*)

DT50 – czas zanikania 50% substancji czynnej

DT90 – czas zanikania 90% substancji czynnej

EM – efektywne mikroorganizmy (ang. *Effective Microorganisms*)

IPM – Integrowana Ochrona Roślin (ang. *Integrated Pest Management*)

MSM – podłoże mineralne (ang. *Mineral Salt Medium*)

ORP – potencjał oksydo-redukcyjny (ang. *Oxidative Redox Potential*)

s.m. – sucha masa

$\mathbf{t_{1/2}}$ – czas połowicznego zanikania

TTC – 2,3,5-chlorek trifenylotetrazoliowy

TPF – 1,3,5-formazan trifenylotetrazoliowy

WWA – wielopierścieniowe węglowodory aromatyczne

Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Podstawę niniejszej dysertacji stanowi artykuł przeglądowy oraz trzy oryginalne artykuły opublikowane w czasopismach naukowych:

Publikacja 1. **Książek-Trela P.**, Szpyrka E. 2022. The effect of natural and biological pesticides on degradation of synthetic pesticides. Plant Protection Science 58(4), 273–291. DOI: 10.17221/152/2021-PPS.

IF_{2022} – 1,3; $MEiN_{2022}$ – 100 pkt; cytowania: 11

Publikacja 2. Szpyrka E., **Książek-Trela P.**, Bielak E., Słowik-Borowiec M. 2024. The influence of commercial yeast preparations on the degradation of herbicide mixtures in the soil and the effect on the shell pea (*Pisum sativum* L.) cultivation. Journal of Soil Science and Plant Nutrition. DOI:10.1007/s42729-024-01671-7.

IF_{2022} – 3,9; $MEiN_{2024}$ – 100 pkt; **cytowania: 0**

Publikacja 3. **Książek-Trela P.**, Bielak E., Wężka D., Szpyrka E. 2022. Effect of three commercial formulations containing effective microorganisms (EM) on diflufenican and flurochloridone degradation in soil. Molecules 27(14), 4541. DOI: 10.3390/molecules27144541.

IF_{2022} – 4,6; $MEiN_{2022}$ – 140 pkt; **cytowania: 4**

Publikacja 4. **Książek-Trela P.**, Figura D., Wężka D., Szpyrka E. 2024. Degradation of a mixture of 13 polycyclic aromatic hydrocarbons (PAHs) by commercial effective microorganisms. Open Life Sciences 19(1), 20220831. DOI: 10.1515/biol-2022-0831.

IF_{2022} – 2,2; $MEiN_{2024}$ – 40 pkt; **cytowania: 0**

Sumaryczny Impact Factor: **12**

Sumaryczna liczba punktów wg MEiN: **380**

Streszczenie

Występowanie w środowisku wielopierścieniowych węglowodorów aromatycznych (WWA) i pozostałości środków ochrony roślin jest ściśle związane z działalnością człowieka. Zanieczyszczenia te stanowią poważne zagrożenie dla bezpieczeństwa i zdrowia ludzi oraz zwierząt, ze względu na swoje toksyczne właściwości i trwałość. Biodegradacja jest naturalnym procesem usuwania zanieczyszczeń ze środowiska, w którym toksyczne związki są przekształcane do mniej szkodliwych form.

Głównym celem badań była ocena wpływu komercyjnych preparatów mikrobiologicznych stosowanych w rolnictwie na degradację herbicydów i WWA w glebie. Dodatkowo analizowano wpływ tych preparatów na parametry fizyko-chemiczne gleby (pH i potencjał oksydo-redukcyjny) oraz aktywność dehydrogenazy.

Na podstawie przeglądu literatury pod kątem preparatów mikrobiologicznych zalecanych w Integrowanej Ochronie Roślin stwierdzono, że najważniejsze i najbardziej perspektywiczne są bakterie z rodzajów *Bacillus* spp. i *Pseudmonas* spp., grzyby z rodzaju *Trichoderma* spp. oraz drożdże *Saccharomyces cerevisiae* ze względu na ich skuteczność w degradacji pestycydów oraz dużą liczbę dostępnych na rynku preparatów komercyjnych zawierających wspomniane mikroorganizmy.

Do badań wykorzystano dostępne na rynku preparaty mikrobiologiczne, zawierające bakterie i/lub drożdże, stosowane do rewitalizacji, poprawy składu, kondycji i aktywności mikrobiologicznej gleby. Niektóre z nich wspomagają także naturalną odporność roślin i chronią przed chorobami. Z przeprowadzonych badań wynika, że preparaty bakteryjne oraz drożdżowe mają wpływ na degradację badanych WWA i herbicydów: chlomazonu, fluazyfopu-P-butylu, flurochloridonu, metrybuzyny, pendimetaliny, propyzamidu, z wyjątkiem diflufenikanu. Preparaty zawierające w swoim składzie drożdże pozytywnie wpływają zarówno na remediację gleby, jak i kiełkowanie nasion i wzrost roślin grochu zwyczajnego.

Wyniki niniejszych badań jednoznacznie potwierdzają, że preparaty mikrobiologiczne mogą być zarówno uzupełnieniem, jak i alternatywą dla chemicznych środków stosowanych w rolnictwie. Ich stosowanie powinno być rekomendowane ze względu na poprawę jakości i bezpieczeństwa środowiska.

Summary

The presence of polycyclic aromatic hydrocarbons (PAHs) and residues of plant protection products in the environment is closely associated with man's activities. These contaminations pose a serious threat to safety and health of humans and animals, due to their toxic properties and persistence. Biodegradation is a natural process for removing contaminations from the environment, during which toxic compounds are transformed into their less harmful forms.

The primary aim of the study was to evaluate the influence of commercially available microbiological formulations used in the agriculture on herbicides and PAHs degradation in the soil. Additionally, the influence of those formulations on physical and chemical parameters (the pH and the oxidoreduction potential) of the soil, as well as dehydrogenase activity were analysed.

On the basis of the literature review focusing on microbiological formulations recommended in the Integrated Plant Protection it was found that bacteria belonging to *Bacillus* spp. and *Pseudmonas* spp., *Trichoderma* spp. fungi, and *Saccharomyces cerevisiae* yeasts were the most important and the most promising, due to their effectiveness in pesticide degradation and a large number of commercial formulations containing those microorganisms available in the market.

The study analysed commercially available microbiological formulations containing bacteria and/or yeasts and used for revitalisation and for improving composition, condition and microbiological activity of the soil. Some of them also support natural immunity of the plants and protect them against diseases. The conducted studies show that bacterial and yeast formulations influence degradation of studied PAHs and herbicides: clomazone, fluazifop-P-butyl, flurochloridone, metribuzin, pendimethalin, and propyzamide, except for diflufenican. The formulations containing yeasts positively influence the soil remediation, as well as seed germination and plant growth in the cultivated pea.

The results of this study clearly confirm that microbiological formulations can be either a supplement or an alternative to chemical agents used in the agriculture. Their use should be recommended due to improved quality and safety of the environment.

1. Wstęp

Obecność zanieczyszczeń w wodzie, glebie, powietrzu i żywności stanowi poważne zagrożenie dla zdrowia i bezpieczeństwa ludzi. Występowanie w środowisku wielopierścieniowych węglowodorów aromatycznych (WWA) i pozostałości pestycydów, jest ściśle związane z działalnością człowieka. Obecność pierwszej grupy związków wynika przede wszystkim z niepełnego spalania paliw, natomiast drugiej ze stosowania środków ochrony roślin w rolnictwie, ogrodnictwie i leśnictwie (Abdel-Shafy i Mansour, 2016; Freedman, 2018).

Herbicydy

Intensyfikacja produkcji rolnej spowodowana jest głównie wzrostem liczby ludności, zmianami klimatycznymi oraz występowaniem chwastów, szkodników i chorób roślin, które powodują znaczne straty ekonomiczne na etapie ich uprawy, transportu i przechowywania (Morales-Cedeño i in., 2021; Saldaña-Mendoza i in., 2023). Nadmierne i/lub nieprawidłowe stosowanie środków ochrony roślin, może powodować negatywny wpływ na środowisko naturalne, np. ograniczać bioróżnorodność, powodować pojawianie się organizmów odpornych na ich działanie oraz występowanie ich pozostałości w płodach rolnych (Pathak i in., 2022).

Chociaż niektóre pestycydy zostały zakazane i wycofane ze sprzedaży w Unii Europejskiej (UE), są one nadal w użyciu w krajach rozwijających się (Rani i in., 2021). Niewielka ilość środków ochrony roślin (<0,1%) wykazuje toksyczność wobec patogenów roślin, natomiast ich pozostałości mogą długo utrzymywać się w środowisku, zanieczyszczając je i wywołując negatywne skutki (Stolte i in., 2016; Ukalska-Jaruga i in., 2020).

Herbicydy (łac. *herb* zioło, chwast, *caedeo* – zabijać) to chemiczne środki stosowane w rolnictwie, ogrodnictwie i sadownictwie w ochronie roślin przed chwastami, w celu poprawy ilości i jakości plonów (Giglio i Vommaro, 2022).

W 2020 roku na całym świecie sprzedano 2 661 124 ton środków ochrony roślin. Największą sprzedaż odnotowano w Stanach Zjednoczonych – 407 779 ton, następnie w Brazylii – 377 176 ton i Chinach – 262 700 ton (Our World in Data). Herbicydy stanowiły 34% całkowitej sprzedaży tych preparatów (Eurostat, 2020). Również w Polsce, według danych

Głównego Urzędu Statystycznego, największy udział w sprzedaży środków ochrony roślin miały herbicydy. W 2021 roku ich sprzedaż wyniosła blisko 45 ton. Polska zajmowała 4 miejsce wśród krajów UE pod względem ilości sprzedanych herbicydów (GUS, 2022).

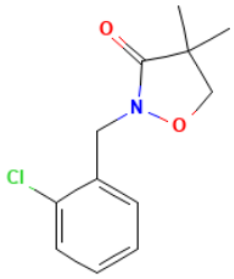
Wśród środków ochrony roślin, herbicydy stanowią grupę związków charakteryzujących się największą trwałością w środowisku. Podczas jednej uprawy stosuje się kilka zabiegów preparatami chemicznymi o podobnym działaniu, ale zawierającymi inną substancję czynną. Wiele herbicydów stanowi trwałe zanieczyszczenia środowiska, charakteryzujące się długotrwałą degradacją, wynoszącą od kilku dni do nawet kilku lat, m.in. diflufenikan (maksymalne wartości DT50 do 621 dni, DT90 do 1900 dni), glifosat (DT50 do 80 dni, DT90 do 1600 dni), napropamid (DT50 do 400 dni, DT90 do 1000 dni), chlorotoluron (DT50 do 140 dni, DT90 do 796 dni) oraz chlomazon (DT50 do 195 dni, DT90 do 645 dni) (PPDB, 2024).

W 2015 r. w ramach programów badawczych finansowanych przez UE naukowcy zbadali 317 próbek gleby pobranych z nieużytków rolnych. Badania obejmowały 76 substancji czynnych środków ochrony roślin spośród ponad 400 dopuszczonych do stosowania w UE. Pozostałości pestycydów wykryto w 83% próbek gleby, z których 25% zawierało jedną pozostałość, a w 58% wykryto obecność mieszaniny substancji czynnych badanych związków (Silva i in., 2019). Trwałe środki ochrony roślin występujące w glebie mogą być pobierane przez rośliny uprawne, co skutkuje występowaniem pozostałości tych zanieczyszczeń w żywności (Schleiffer i Speiser, 2022).

We wszystkich państwach UE najwyższe dopuszczalne poziomy pozostałości (NDP) pestycydów w żywności i paszy regulowane są Rozporządzeniem (WE) nr 396/2005 Parlamentu Europejskiego i Rady (Rozporządzenie 2005). W raporcie sporządzonym przez Europejski Urząd ds. Bezpieczeństwa Żywności (ang. *The European Food Safety Authority*, EFSA) w 2020 r. 5,9% próbek zawierało pozostałości pestycydów powyżej ustalonych NDP, natomiast rok później 3,9% (EFSA, 2020; EFSA, 2021).

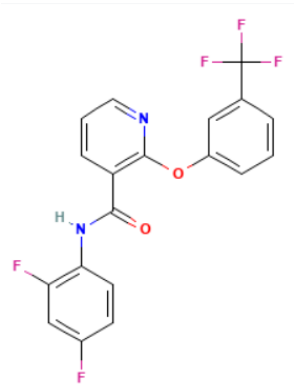
Badaniami przedstawionymi w niniejszej rozprawie doktorskiej objęto siedem herbicydów. Poniżej przedstawiono ich charakterystykę, z uwzględnieniem nazw IUPAC oraz wzorów strukturalnych, mechanizmu działania według klasyfikacji Globalnego Komitetu ds. Działań w zakresie Odporności na Herbicydy (ang. *Herbicide Resistance Action Committee,* HRAC), trwałości i mobilności w glebie, zastosowania w zwalczaniu chwastów oraz ochrony roślin uprawnych.

Chlomazon (nazwa IUPAC 2-[(2-chlorophenyl)methyl]-4,4-dimethyl-1,2-oxazolidin-3-one) to nietrwały, średnio mobilny w glebie, rozpuszczalny w wodzie herbicyd z grupy izoksazolidionów o działaniu selektywnym, stosowany doglebowo (Rysunek 1). Według klasyfikacji HRAC należy do grupy F4 – inhibitorów biosyntezy karotenoidów (HRAC, 2024). Stosowany do zwalczania chwastów szerokolistnych i traw w różnych uprawach (PPDB, 2024). W Polsce zarejestrowany jest do ochrony: rzepaku ozimego i jarego, ziemniaków, grochu, fasoli, marchwi, ogórków, kukurydzy oraz soi (MRiRW, 2023).



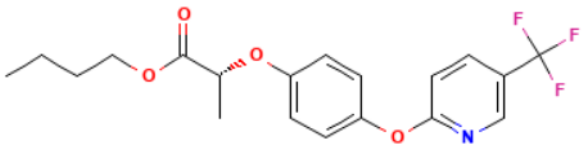
Rysunek 1. Struktura chemiczna chlomazonu (PubChem).

Diflufenikan (nazwa IUPAC N-(2,4-difluorophenyl)-2-[3-(trifluoromethyl)phenoxy] pyridine-3-carboxamide) to średnio trwały, niemobilny w glebie, nierozpuszczalny w wodzie herbicyd pirydynokarboksyamidowy, o działaniu selektywnym (Rysunek 2). Według klasyfikacji HRAC należy do grupy F1 – inhibitorów biosyntezy karotenoidów (inhibitor funkcjonowania desaturazy fytonowej) (HRAC, 2024). Stosowany jest do zwalczania traw i chwastów szerokolistnych (PPDB, 2024). W Polsce zarejestrowany jest do ochrony zbóż ozimych: pszenicy, jęczmienia, żyta i pszenżyta, zbóż jarych: pszenicy i jęczmienia, ziemniaków oraz sadów jabłoniowych (MRiRW, 2023).



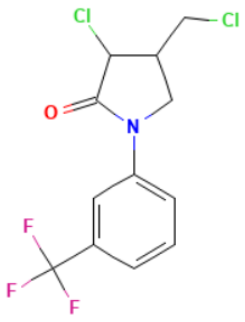
Rysunek 2. Struktura chemiczna diflufenikanu (PubChem).

Fluazyfop-P-butylu (nazwa IUPAC butyl (2R)-2-[4-[5-(trifluoromethyl)pyridin-2-yl]oxyphenoxy]propanoate) to nietrwały i lekko mobilny w glebie, nierozpuszczalny w wodzie herbicyd aryloksyfenoksypropionianowy, o działaniu selektywnym, wchłaniany przez powierzchnię liści (Rysunek 3). Według klasyfikacji HRAC należy do grupy A1 – inhibitorów biosyntezy lipidów (inhibitor funkcjonowania karboksylazy acetylo-CoA) (HRAC, 2024). Stosowany do zwalczania chwastów trawiastych, głównie w uprawach roślin szerokolistnych (PPDB, 2024). W Polsce preparaty zawierające fluazyfop-P-butylu zarejestrowane są do ochrony: rzepaku ozimego, ziemniaków, fasoli szparagowej, truskawek, buraków cukrowych oraz kapusty białej (MRiRW, 2023).



Rysunek 3. Struktura chemiczna fluazyfopu-P-butylu (PubChem).

Flurochloridon (nazwa IUPAC 3-chloro-4-(chloromethyl)-1-[3-(trifluoromethyl)phenyl] pyrrolidin-2-one) to średnio trwały i lekko mobilny w glebie, nierozpuszczalny w wodzie herbicyd pirolidynowy, o działaniu selektywnym, wchłaniany przez korzenie i łodygę (Rysunek 4). Według klasyfikacji HRAC należy do grupy F1 – inhibitorów biosyntezy karotenoidów (inhibitor funkcjonowania desaturazy fytonowej) (HRAC, 2024). Stosowany jest do zwalczania różnych chwastów w uprawach baldaszkowatych, zbożach i ziemniakach (PPDB, 2024). W Polsce zarejestrowany jest do ochrony: pszenicy ozimej i pszenżyta ozimego, żyta, ziemniaków, marchwi, pietruszki, selera oraz pasternaku (MRiRW, 2023).

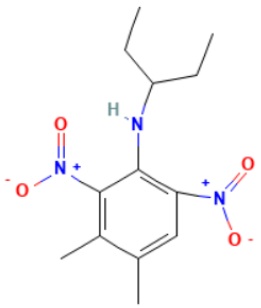


Rysunek 4. Struktura chemiczna flurochloridonu (PubChem).

Metrybuzyna (nazwa IUPAC 4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one) to nietrwały, mobilny w glebie, rozpuszczalny w wodzie herbicyd triazynowy, o działaniu selektywnym (Rysunek 5). Według klasyfikacji HRAC należy do grupy C1 – inhibitorów fotosyntezy na poziomie fotosystemu II (HRAC, 2024). Stosowany do zwalczania chwastów w zbożach i wielu innych uprawach (PPDB, 2024). W Polsce preparaty zawierające metrybuzynę zarejestrowane są do ochrony zbóż ozimych: jęczmienia, pszenicy, pszenżyta i żyta, a także: soi, ziemniaków, pomidorów, bakłażanów oraz marchwi (MRiRW, 2023).

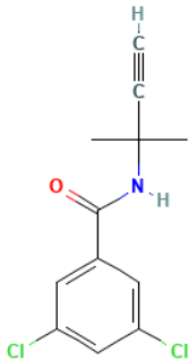
Rysunek 5. Struktura chemiczna metrybuzyny (PubChem).

Pendimetalina (nazwa IUPAC 3,4-dimethyl-2,6-dinitro-N-pentan-3-ylaniline) to trwały, niemobilny w glebie herbicyd dinitroanilinowy, o działaniu selektywnym, wchłaniany przez korzenie i liście (Rysunek 6). Według klasyfikacji HRAC należy do grupy K1 – inhibitorów mitozy (HRAC, 2024). Stosowany do zwalczania większości jednorocznych traw i pospolitych chwastów w zbożach, owocach i warzywach (PPDB, 2024). W Polsce preparaty zawierające pendimetalinę zarejestrowane są do ochrony zbóż ozimych: pszenicy, jęczmienia, żyta i pszenżyta oraz: kukurydzy, plantacji traw nasiennych, ziemniaków, marchwi, kapusty białej i czerwonej, kalafiora, cebuli, grochu oraz truskawek (MRiRW, 2023).



Rysunek 6. Struktura chemiczna pendimetaliny (PubChem).

Propyzamid (nazwa IUPAC 3,5-dichloro-N-(2-methylbut-3-yn-2-yl)benzamide) to średnio trwały, lekko mobilny w glebie, nierozpuszczalny w wodzie herbicyd amidowy, o działaniu selektywnym i układowym (Rysunek 7). Według klasyfikacji HRAC należy do grupy K1 – inhibitorów mitozy (HRAC, 2024). Stosowany do zwalczania chwastów jednorocznych i wieloletnich (PPDB, 2024). W Polsce zarejestrowany jest do ochrony rzepaku ozimego, sałaty oraz jabłoni (MRiRW, 2023).



Rysunek 7. Struktura chemiczna propyzamidu (PubChem).

Wielopierścieniowe węglowodory aromatyczne

WWA to grupa związków organicznych o płaskiej strukturze, które zawierają co najmniej dwa połączone pierścienie aromatyczne. Tworzą grupę składającą się z kilkuset związków pokrewnych chemicznie o odmiennej toksyczności (Zelinkova i Wenzl, 2015). Związki te zaliczane są do trwałych substancji zanieczyszczających środowisko i często występują w postaci mieszanin (Ekanem i in., 2019). Substancje te powstają w wyniku naturalnych procesów zachodzących w środowisku takich jak pożary lasów czy erupcje wulkanów oraz w wyniku działalności człowieka jako produkt niepełnego spalania paliw kopalnych w celu wytwarzania ciepła i energii (Han i in., 2019; Faboya i in., 2020). WWA są wszechobecne w środowisku, występują w glebie, wodzie, atmosferze i powietrzu (Zelinkova i Wenzl, 2015; Adeniji i in., 2019). Charakteryzują się działaniem rakotwórczym, toksycznym i mutagennym, są także bardzo silnymi środkami immunosupresyjnymi (Di Duca i in., 2023; Maciejczyk i in., 2023). Mechanizm toksyczności WWA polega na zaburzaniu funkcji błon komórkowych i uszkadzaniu układów enzymatycznych (Patel i in., 2020).

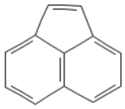
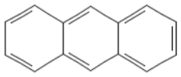
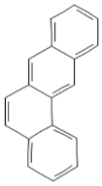
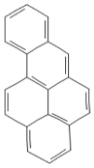
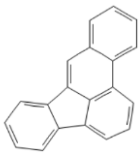
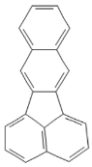
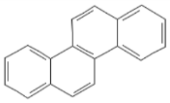
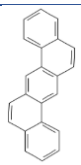
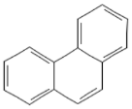
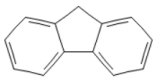
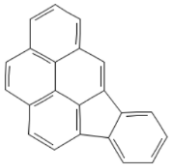
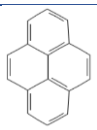
Większość WWA w glebie wiąże się z jej cząsteczkami. Proces ten, znany jako sorpcja, zależy od właściwości gleby, takich jak: skład mineralny, zawartość materii organicznej, wartość pH i przewodność (Han i in., 2019). Mobilność WWA w glebie również zależy od tych czynników. WWA o niskiej masie cząsteczkowej są bardziej mobilne i łatwiej dostępne dla mikroorganizmów glebowych. Z kolei WWA o dużej masie cząsteczkowej są mniej mobilne, ale bardziej niebezpieczne ze względu na trwałość, a także słabą rozpuszczalność w wodzie (Hou i in., 2018; Patel i in., 2020).

WWA obecne w glebie mogą być pobierane przez rośliny uprawne i stanowić zagrożenie dla zdrowia konsumentów (Guo i in., 2023). W celu ochrony ich zdrowia ustalono najwyższe dopuszczalne poziomy dla tych substancji określone w Rozporządzeniach Komisji (UE) nr 835/2011 oraz nr 2023/915 (Rozporządzenie 2011, Rozporządzenie 2023).

Według badań przeprowadzonych przez Instytut Uprawy Nawożenia i Gleboznawstwa z 2020 r. podczas Monitoringu Chemizmu Gleb Ornych Polski, średnia zawartość sumy 13 WWA w glebach rolniczo użytkowanych wynosiła 470,8 µg/kg (IUNG). Z kolei na podstawie badań wykonanych na próbkach gleby pobranych w sąsiedztwie szlaków komunikacyjnych oraz w miejscach objętych ruchem turystycznym i transportowym stwierdzono podwyższone lub przekraczające dopuszczalne limity stężenia badanych WWA (wynoszące 2,979 mg/kg) (Kicińska i Dmytrowski, 2023).

Poniżej w Tabeli 1. przedstawiono nazwy i wzory strukturalne WWA badanych w niniejszej pracy.

Tabela 1. Wzory strukturalne badanych WWA (PubChem).

WWA	Wzór strukturalny	WWA	Wzór strukturalny
acenaftylen		antracen	
benzo[a]antracen		benzo[a]piren	
benzo[b]fluoranten		benzo[k]fluoranten	
benzo[ghi]perylen		chryzen	
dibenzo[a,h]antracen		fenantren	
fluoren		indeno[1,2,3-cd]piren	
piren			

Biodegradacja trwałych zanieczyszczeń środowiska

Biodegradacja jest jednym z głównych sposobów usuwania pestycydów, WWA i innych trwałych zanieczyszczeń ze środowiska (Ruomeng i in., 2023; Thacharodi i in., 2023). Proces ten polega na przekształcaniu ksenobiotyków w mniej toksyczne i bardziej przyjazne dla środowiska substancje (Bala i in., 2022). Mikroorganizmy odpowiedzialne za rozkład zanieczyszczeń mogą występować naturalnie w środowisku lub być celowo do niego

wprowadzane. W ciągu ostatnich dziesięcioleci nastąpił ogromny postęp w badaniach nad biodegradacją pestycydów i WWA (Ghosal i in., 2016). Biodegradacja trwałych zanieczyszczeń i bioremediacja gleby należą do najbardziej ekonomicznych i przyjaznych dla środowiska innowacji biotechnologicznych (Edbeib, 2020; Kirkinci i in., 2021; Raffa i Chiampo, 2021; Aliyu i in., 2022; Bala i in., 2022). Mikroorganizmy mogą wykorzystywać zanieczyszczenia organiczne obecne w glebie, takie jak pestycydy czy WWA, jako źródło energii i węgla w procesach metabolicznych (Kebede i in., 2021; Raffa i Chiampo, 2021).

Zanikanie zanieczyszczeń w glebie zależy od wielu czynników, takich jak: rodzaj gleby, zawartość substancji mineralnych i organicznych, pH, wilgotność, temperatura, obecność mikroorganizmów oraz budowa chemiczna i stężenie substancji, które będą ulegały rozkładowi (Fernandes i in., 2020; Kim i in., 2023; Koh i Khor, 2023).

Pestycydy i WWA mogą być usuwane ze środowiska poprzez: biodegradację, degradację fotochemiczną, wymywanie, bioakumulację lub adsorpcję. Poszczególne procesy wpływają na zanieczyszczenia w różny sposób, ponieważ każdy związek ma unikalną strukturę i inne właściwości chemiczne, biologiczne i fizyczne (Abdel-Shafy i Mansour, 2016; Kuppusamy i in., 2017). Preferowanym i głównym sposobem rozkładu herbicydów i WWA jest biodegradacja, ponieważ umożliwia całkowite usunięcie tych związków (Dhar i in., 2022). Mikroflora występująca w danym środowisku musi przystosować się do degradacji zanieczyszczeń. Mikroorganizmy zazwyczaj nie są w stanie bezpośrednio rozkładać tych substancji, dlatego potrzebują czasu, aby rozwinąć zdolność do wytwarzania niezbędnych enzymów. Czas ten zależy od rodzaju drobnoustrojów i właściwości zanieczyszczeń. Pojedynczy mikroorganizm nie jest w stanie rozłożyć wszystkich ksenobiotyków. Z tego powodu biodegradacja jest procesem wieloetapowym, zachodzącym przy udziale dużej liczby mikroorganizmów działających synergistycznie. Na poszczególnych etapach biodegradacji różne mikroorganizmy wykorzystują swoje specyficzne enzymy do rozkładu zanieczyszczeń na coraz mniejsze i prostsze związki (Wu i in., 2023).

W procesie biodegradacji pestycydów kluczową rolę odgrywają bakterie i grzyby, które wykorzystują te substancje jako składniki odżywcze i źródło energii (Vanitha i in., 2023). Mikroorganizmami wykazującymi największą aktywność w degradacji pestycydów są bakterie z rodzaju *Bacillus, Pseudomonas, Flavobacterium, Micrococcus, Acinetobacter, Arthrobacter, Aerobacter, Alcaligenes, Burkholderia, Sphingomonas, Clostridium, Flavobacterium*

i *Actinomycetes* oraz grzyby z rodzajów *Fusarium, Aspergilus, Penicillium, Lentinulaedodes, Lecanicillium* i *Oxysporum* (Wołejko i in., 2016; Huang i in., 2018; Szpyrka i in., 2020; Kumari i in., 2022). Istnieje również kilka doniesień na temat wykorzystania drożdży do degradacji pestycydów. Dotyczy to przede wszystkim gatunku *S. cerevisiae*, który może rozkładać m.in.: azoksystrobinę, piraklostrobinę, iprodion, boskalid (Wołejko i in., 2016), glifosat (Low i in., 2005), tiuram (Kitagawa i in., 2002), atrazynę, terbutylazynę (Hack i in., 1997) i pirymifos metylowy (Đorđević i Đurović-Pejčev, 2016). Należy jednak zaznaczyć, że badania nad wykorzystaniem drożdży do biodegradacji pestycydów są wciąż na wczesnym etapie, a ich udział w tym procesie jest słabo poznany.

Podobnie jak w przypadku pestycydów, w procesie degradacji WWA również bakterie odgrywają kluczową rolę (Khan i in., 2021; Varjani i Upasani, 2021). Wiele badań nad biodegradacją WWA koncentruje się na bakteriach z rodzajów *Bacillus, Pseudomonas, Gordonia, Micrococcus, Rhodococcus, Arthrobacter, Mycobacterium, Flavobacterium, Corynebacterium, Klebsiella, Alcaligenes* i *Nocardia*. Oprócz bakterii, w biodegradacji WWA uczestniczą również grzyby z rodzajów: *Penicillium, Aspergillus, Trichoderma, Candida* i *Fusarium* (Ekhaise i Nkwelle, 2011; Hadibarata i in., 2017; Kuang i in., 2018; Feng i in., 2020; Achife i in., 2021). Choć istnieje wiele doniesień na temat degradacji WWA przez bakterie i grzyby, to publikacji opisujących skuteczną degradację WWA przy użyciu drożdży jest stosunkowo niewiele. Wpływ drożdży na degradację ropy naftowej lub WWA z miejsc skażonych analizowano tylko w kilku badaniach, które dotyczyły gatunków: *S. cerevisiae, Yarrowia lipolytica, Hanseniaspora valbyensis, H. opuntiae* i *Debaryomyces hansenii* (Ferreira i in., 2012; Abioye i in., 2013; Mandal i in., 2016; Mandal i in., 2018).

W bioremediacji trwałych zanieczyszczeń istotną rolę odgrywają enzymy pochodzące z bakterii, grzybów i drożdży. Biodegradacja za pomocą enzymów jest wydajna i selektywna, ze względu na większą szybkość reakcji oraz zdolność do katalizowania reakcji w szerokim zakresie temperatur i wartości pH (Patel i in., 2020). Dotychczas zidentyfikowano różne enzymy biorące udział w biodegradacji pestycydów m.in. hydrolazy, esterazy, peroksydazy, dehalogenazy, lakazy i oksydazy (Baćmaga i in., 2015; Lushchak i in., 2018; Raffa i Chiampo, 2021; Chen i in., 2022; Ghosh i Sarkar, 2023; Guerrero Ramírez i in., 2023). Do enzymów odpowiedzialnych za degradację WWA należą m.in. oksygenazy, dehydrogenazy, peroksydazy, katalazy, lakazy i oksydazy (Kadri i in., 2017; Elufisan i in., 2020; Fallahi i in., 2023).

Integrowana Ochrona Roślin

Od 1 stycznia 2014 roku wszyscy profesjonalni użytkownicy środków ochrony roślin zobowiązani są do stosowania zasad Integrowanej Ochrony Roślin (ang. *Integrated Pest Management,* IPM), zgodnie z postanowieniami art. 14 Dyrektywy 2009/128/WE oraz Rozporządzenia (WE) nr 1107/2009. Celem tych regulacji jest ograniczenie stosowania chemicznych środków ochrony roślin do niezbędnego minimum (Dyrektywa 2009; Rozporządzenie 2009).

IPM to metoda ochrony roślin przed organizmami szkodliwymi, w której pierwszeństwo ma stosowanie metod niechemicznych: biologicznych, fizycznych, agrotechnicznych, mechanicznych, hodowlanych i innych niechemicznych, zapewniających ochronę przed szkodnikami. Jednocześnie wykorzystuje zgromadzoną wiedzę na temat biologii i szkodliwego działania tych organizmów, a także antagonistycznych mikroorganizmów, aby w bezpieczny sposób eliminować ze środowiska organizmy niepożądane (Deguine i in., 2021; MRiRW 2022).

Biologiczna ochrona roślin to metoda zwalczania szkodników oraz zapobiegania i zwalczania patogenów roślin uprawnych z wykorzystaniem: mikroorganizmów (bakterii, grzybów, drożdży, pierwotniaków i wirusów) będących konkurentami agrofagów, organizmów pożytecznych (roztoczy i owadów drapieżnych), związków nieorganicznych i naturalnych substancji pochodzenia roślinnego i zwierzęcego, a także substancji semiochemicznych (Kempka, 2014; He i in., 2021; Książek-Trela i Szpyrka, 2022). Stosowanie środków kontroli biologicznej stanowi przyjazną dla środowiska strategię ochrony roślin, która nie wpływa negatywnie na zdrowie konsumentów, ani pożyteczne mikroorganizmy (Bolívar-Anillo i in., 2020; Fira i in., 2018; Moraes Bazioli i in., 2019).

Biopestycydy są obiecującą alternatywą dla ochrony upraw, bezpieczną dla zdrowia ludzi i zwierząt (Nathiely Ramírez-Guzmán i in., 2018; Saldaña-Mendoza, Chavez-González i in., 2023). Stanowią około 5% całego rynku środków ochrony roślin. Liczba biopestycydów zarejestrowanych w UE jest mniejsza niż w innych krajach, takich jak Chiny, Indie czy Stany Zjednoczone, co wynika ze skomplikowanych procesów rejestracyjnych (Damalas i Koutroubas, 2018). Biopestycydy są korzystne dla wzrostu roślin, mogą działać jako: insektycydy, fungicydy, bakteriocydy, nicieniocydy, moluskocydy, induktory odporności, regulatory wzrostu, repelenty

czy atraktanty, a jednocześnie mogą degradować zanieczyszczenia środowiska oraz wpływać na rewitalizację i poprawę jakości gleb (Książek-Trela i Szpyrka, 2022).

W biologicznej ochronie roślin stosowane są naturalne i biologiczne środki ochrony roślin. Preparaty mikrobiologiczne mogą być stosowane również jako produkty nawozowe, preparaty poprawiające właściwości gleby, a także jako środki wspomagające uprawę roślin (Ustawa 2007).

Efektywne mikroorganizmy

Efektywne mikroorganizmy (EM) to kompozycja oddziałujących na siebie mikroorganizmów pochodzenia naturalnego, bezpiecznych dla środowiska. Wykorzystuje się je do produkcji preparatów biologicznych, nawozów naturalnych i organicznych oraz środków wspomagających uprawę roślin czy poprawiających właściwości gleb. EM poprawiają strukturę i żyzność gleby, a także różnorodność biologiczną poprzez zwiększenie aktywności mikroorganizmów glebowych. Ułatwiają pobieranie składników odżywczych, wpływając tym samym na wzrost i rozwój roślin. Mogą przyczyniać się do zwiększenia plonów i poprawy ich jakości, a także przyspieszać proces kompostowania oraz rozkład materii organicznej z resztek pożniwnych, odpadów organicznych i pozostałości pestycydów (Jusoh i in., 2013; Olle i Williams, 2013; Joshi i in., 2019; Książek-Trela i in., 2022; Younas i in., 2022).

Obecność EM w glebie przyczynia się także do zmniejszenia konieczności stosowania chemicznych środków ochrony roślin. Może to być obserwowane w przypadku zastępowania pestycydów mikroorganizmami w procesie biologicznej ochrony roślin w rolnictwie naturalnym i ekologicznym (Hernández-Rosas i in., 2020). Do eliminacji lub zwalczania szkodników i patogenów służą naturalne strategie działania pożytecznych mikroorganizmów, które m.in. inicjują naturalne mechanizmy odporności roślin, działają antagonistycznie lub konkurują o przestrzeń i składniki odżywcze (BPDB, 2024; Köhl i in., 2019; Książek-Trela i Szpyrka, 2022; Nayak i in., 2022).

2. Hipoteza badawcza i cele badań

Hipoteza badawcza niniejszej rozprawy doktorskiej zakłada, że komercyjne preparaty mikrobiologiczne, a w szczególności mieszaniny mikroorganizmów, mogą znacząco przyśpieszać rozkład herbicydów i WWA w glebie.

Głównym celem badań było określenie wpływu wybranych komercyjnych preparatów mikrobiologicznych na degradację herbicydów i WWA w glebie. Dodatkowym celem badań była analiza wpływu preparatów mikrobiologicznych na parametry fizyko-chemiczne gleby – pH i potencjał oksydo-redukcyjny (ORP) oraz aktywność dehydrogenazy (DHA).

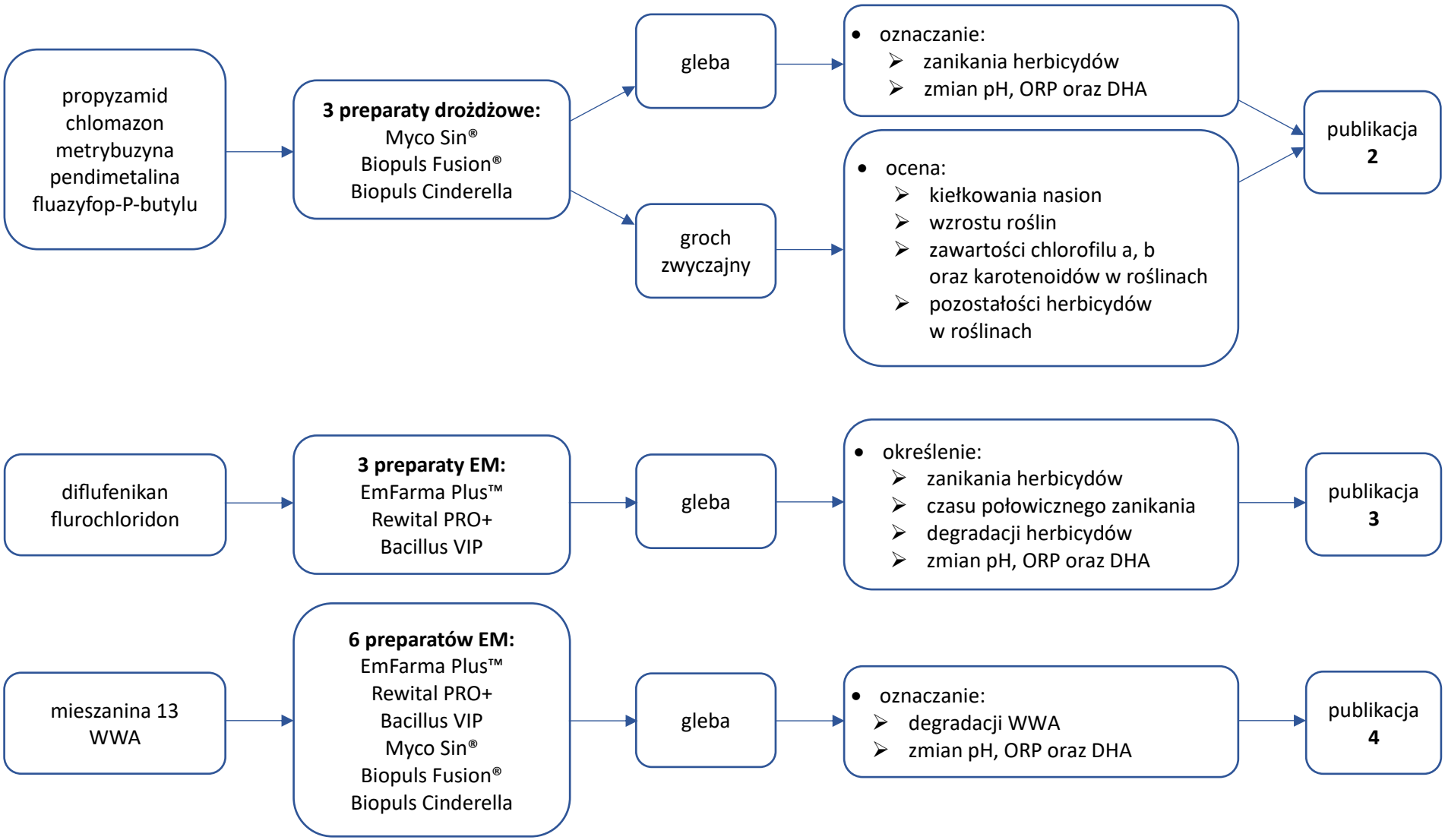
Cele szczegółowe zostały przedstawione w omówieniu każdej publikacji.

3. Omówienie publikacji

Publikacja 1. **Książek-Trela P.**, Szpyrka E. 2022. The effect of natural and biological pesticides on degradation of synthetic pesticides. Plant Protection Science 58(4), 273–291. DOI: 10.17221/152/2021-PPS.

Na podstawie danych zawartych w Unijnej bazie danych dotyczących pestycydów (ang. *EU Pesticide Database*) przeprowadzono przegląd substancji czynnych środków ochrony roślin. W dniu 2 lutego 2022 roku zatwierdzonych do stosowania było 512 substancji czynnych, z czego 97 stanowiły mikroorganizmy (EU Pesticide Database, 2024). Sporządzono spis naturalnych i biologicznych substancji czynnych, z podziałem na bakterie, grzyby, drożdże, wirusy, związki nieorganiczne i inne. Wyszczególniono również ich rodzaje oraz mechanizmy działania w biologicznej kontroli (Tabela 1 w publikacji 1). Z przedstawionych danych wynika, że do najczęściej wykorzystywanych mechanizmów działania substancji czynnych należą: antybioza, rywalizacja o przestrzeń i składniki odżywcze, stymulacja naturalnych mechanizmów odporności roślin, wydzielanie enzymów, antybiotyków lub toksyn killerowych (BPDB, 2024; Ciesielska i in., 2011). Następnie dokonano przeglądu literatury pod kątem zastosowania poszczególnych mikroorganizmów i substancji naturalnych do degradacji pestycydów. Zebrane dane zestawiono w postaci tabelarycznej, podając maksymalną procentową degradację oraz czas trwania doświadczeń (Tabela 2 w publikacji 1). Podsumowano zebrane dane, które przedstawiono w postaci wykresu (Rysunek 1 w publikacji 1) podając dla każdego mikroorganizmu liczbę badanych substancji czynnych oraz maksymalną degradację wyrażoną w procentach. Stwierdzono, że najważniejszymi i najbardziej perspektywicznymi w IPM są bakterie z rodzajów *Bacillus* spp. i *Pseudmonas* spp., grzyby z rodzaju *Trichoderma* spp. oraz drożdże *S. cerevisiae* ze względu na ich skuteczność w degradacji pestycydów oraz dużą liczbę dostępnych na rynku preparatów komercyjnych zawierających te mikroorganizmy. Stosowanie pestycydów biologicznych zalecanych w IPM może znacząco poprawić jakość gleby, środowiska oraz bezpieczeństwo i zdrowie ludzi.

Szczegółowy zakres zrealizowanych badań laboratoryjnych (publikacje 2-4) przedstawiono na Rysunku 8.



Rysunek 8. Zakres badań zrealizowanych w ramach pracy doktorskiej.

Publikacja 2. Szpyrka E., **Książek-Trela P**., Bielak E., Słowik-Borowiec M. 2024. The influence of commercial yeast preparations on the degradation of herbicide mixtures in the soil and the effect on the shell pea (*Pisum sativum* L.) cultivation. Journal of Soil Science and Plant Nutrition. DOI:10.1007/s42729-024-01671-7.

Cele badań

Celem badań była analiza wpływu preparatów komercyjnych zawierających różne gatunki drożdży na degradację mieszaniny herbicydów: propyzamidu, chlomazonu, metrybuzyny, pendimetaliny i fluazyfopu-P-butylu w glebie. Kolejnym celem była ocena wpływu preparatów drożdżowych na kiełkowanie nasion, wzrost roślin, zawartość chlorofilu a i b oraz karotenoidów grochu zwyczajnego (*Pisum sativum* L.), uprawianego w glebie zanieczyszczonej mieszaniną herbicydów. Dodatkowo określono zmiany parametrów fizyko-chemicznych – pH i ORP oraz DHA w próbkach gleby.

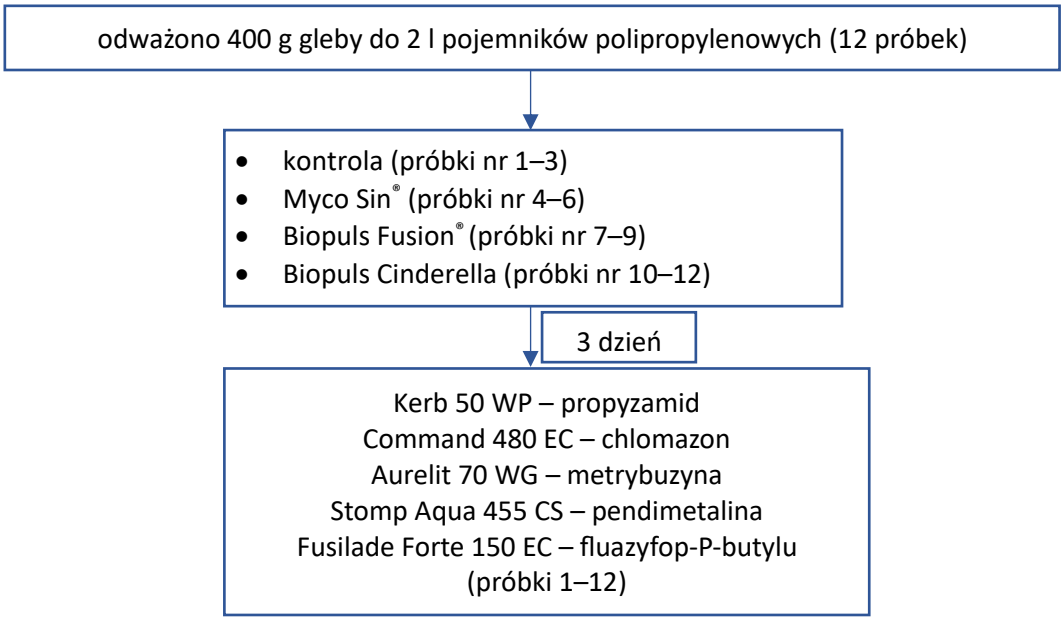
Materiały i metody

We wszystkich doświadczeniach (publikacje 2-4) wykorzystano glebę uniwersalną, zalecaną pod uprawę ogrodniczą (PPUH Zielona Oaza I, Brzozów, Polska). Parametry gleby przedstawiono w Tabeli 2.

Tabela 2. Charakterystyka gleby.

Typ	ogrodnicza
Skład	torf wysoki i niski, piasek, kora sosnowa, perlit, dolomit oraz nawozy mineralne
Stężenie soli	0,5 – 1,0 g KCl/dm^3
pH	6,0 – 7,3

Doświadczenie przeprowadzono zgodnie ze schematem przedstawionym na Rysunku 9.



Rysunek 9. Schemat doświadczenia z mieszaniną herbicydów i preparatami mikrobiologicznymi.

Poniżej w Tabeli 3. przedstawiono charakterystykę chemicznych środków ochrony roślin zastosowanych w przeprowadzonych badaniach (publikacja 2), natomiast w Tabeli 4. zaprezentowano opis preparatów mikrobiologicznych zastosowanych w badaniach (publikacje 2 i 4).

Tabela 3. Chemiczne środki ochrony roślin zastosowane w doświadczeniu (Etykieta Command 480 EC; Etykieta Fusilade Forte 150 EC; Etykieta Aurelit 70 WG; Etykieta Stomp Aqua 455 CS; Etykieta Kerb 50 WP).

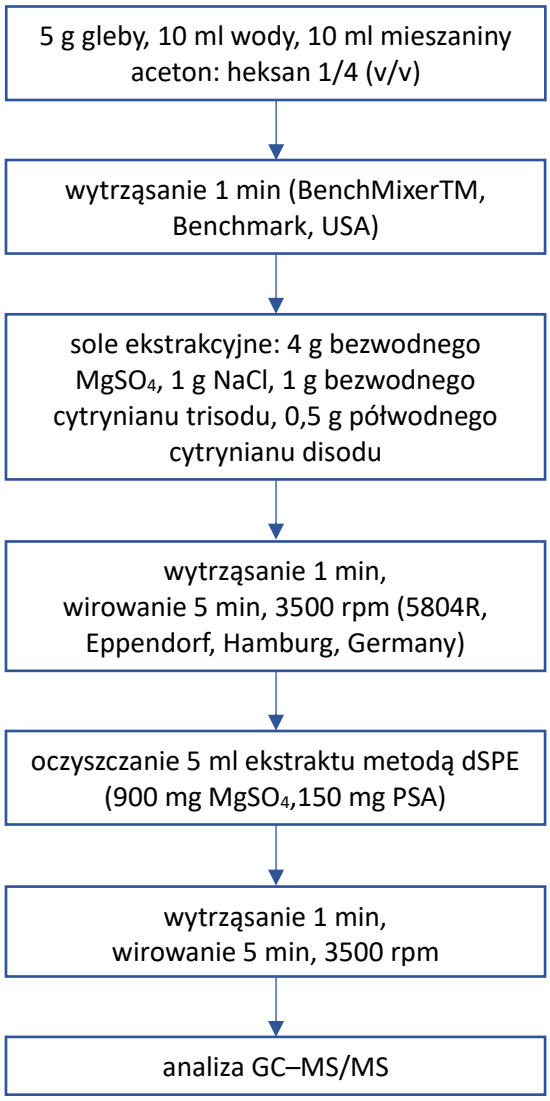
Nazwa preparatu	Substancja czynna	Zawartość substancji czynnej	Zastosowanie	Pobieranie
Command 480 EC (FMC Chemical, Królestwo Belgii)	chlomazon	480 g/l	zwalczanie jednorocznych chwastów dwuliściennych i jednoliściennych	korzenie
Fusilade Forte 150 EC (Syngenta, Polska)	fluazyfop-P-butylu	150 g/l	zwalczanie perzu właściwego oraz jednorocznych chwastów jednoliściennych	liście
Aurelit 70 WG (ADAMA Deutschland GmbH, Republika Rederalna Niemiec)	metrybuzyna	700 g/kg	zwalczanie chwastów dwuliściennych i niektórych jednoliściennych w uprawie ziemniaka i pomidora	korzenie i liście
Stomp Aqua 455 CS (BASF SE, Republika Federalna Niemiec)	pendimetalina	455 g/l	zwalczanie jednorocznych chwastów dwuliściennych i jednoliściennych	korzenie i części nadziemne
Kerb 50 WP (Dow AgroSciences, Polska)	propyzamid	500 g/kg	zwalczanie chwastów jednoliściennych i dwuliściennych w rzepaku ozimym	korzenie

Tabela 4. Preparaty mikrobiologiczne zastosowane w badaniach (Etykieta Biopuls Cinderella; Etykieta BioPuls Fusion®; Etykieta Myco Sin®).

Nazwa preparatu	Rodzaj preparatu	Skład preparatu	Zastosowanie
Myco Sin® (Biocont, Poland)	preparat zwiększający odporność roślin oraz rewitalizujący glebę	nieaktywne, mielone, suszone drożdże – *Saccharomyces cerevisiae,* suchy ekstrakt skrzypu (*Equisetum arvense L.*) oraz siarczan glinu tetradekahydrat	zwiększanie odporności roślin, ułatwienie pobierania składników odżywczych, zwiększanie aktywności biologicznej gleby, wzmacnianie roślin
Biopuls Fusion® (Microlife, Poland)	preparat rewitalizujący glebę	drożdże *Yarrowia lipolytica*	poprawianie struktury i zwiększanie materii organicznej gleby, ulepszanie mrozoodporności gleby i reakcji na suszę, stanowienie źródła łatwo dostępnych składników odżywczych, zwiększanie siły wschodu nasion i rozwoju sadzonek, wzmacnianie systemu korzeniowego roślin, ograniczanie i osłabianie patogennych grzybów, pobudzanie wzrostu pożytecznej mikroflory
Biopuls Cinderella (Microlife, Poland)	preparat rewitalizujący glebę, fungicyd	drożdże *Debaryomyces hansenii* i ich metabolity oraz bakterie z rodzaju *Bacillus*	ograniczanie szkodliwych grzybów, wspieranie mechanizmów odpornościowych w roślinach, wspomaganie roślin w biologicznej kontroli chorób grzybowych i bakteryjnych, ograniczanie strat w czasie przechowywania zebranych płodów, stanowienie źródła mikroelementów, wspomaganie rozwoju owoców i warzyw oraz jakości i masy plonów

Przygotowanie próbek gleby do analiz pozostałości herbicydów.

Pozostałości herbicydów w glebie (publikacje 2 i 3) analizowano z zastosowaniem zmodyfikowanej metody znormalizowanej PN–EN 15662:2018-6 „Żywność pochodzenia roślinnego – Multimetoda do oznaczania pozostałości pestycydów z zastosowaniem analizy opartej na GC i LC po ekstrakcji/podziale acetonitrylem i oczyszczaniu metodą dyspersyjnej SPE – Metoda modułowa QuEChERS" (PN-EN 15662:2018-06). Metoda ta została zmodyfikowana w zakresie rozpuszczalnika wykorzystywanego do ekstrakcji (Rysunek 10). Jestem współautorką opublikowanego artykułu naukowego, w którym przedstawiono walidację tej metody (Słowik-Borowiec i in., 2022).



Rysunek 10. Metoda QuEChERS zastosowana w badaniach.

Analiza GC–MS/MS (publikacje 2-4).

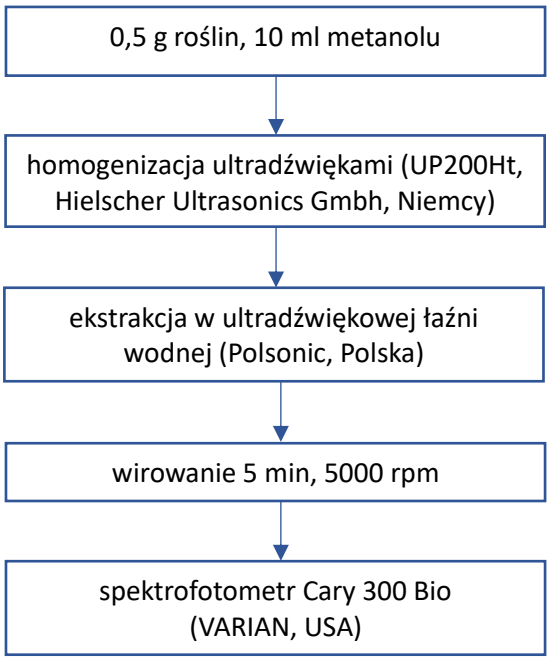
Ekstrakty analizowano techniką chromatografii gazowej sprzężonej ze spektrometrią mas (GC–MS/MS QqQ) (model aparatu 7890A, model detektora mas 7000 Agilent Technologies, Palo Alto, CA, USA). Warunki pracy GC–MS/MS, czasy retencji i analizowane reakcje wielokrotne dla badanych związków zostały zawarte w publikacji Słowik-Borowiec i in. (2022).

Ocena wpływu preparatów drożdżowych na kiełkowanie nasion, wzrost roślin oraz zawartość barwników w grochu zwyczajnym uprawianym w glebie zanieczyszczonej mieszaniną herbicydów.

Do badań wykorzystano ten sam schemat doświadczenia co w degradacji herbicydów (Rysunek 9). W 7 dniu obliczono współczynnik kiełkowania nasion, według wzoru podanego poniżej (Ling i in., 2014).

$$\text{współczynniki kiełkowania } (\%) = \frac{\text{liczba wykiełkowanych nasion}}{\text{liczba nasion}} \cdot 100\%$$

W 16 dniu zbadano zawartość chlorofilu a i b oraz karotenoidów zgodnie z metodyką opisaną przez Wellburn (1994) (Rysunek 11).



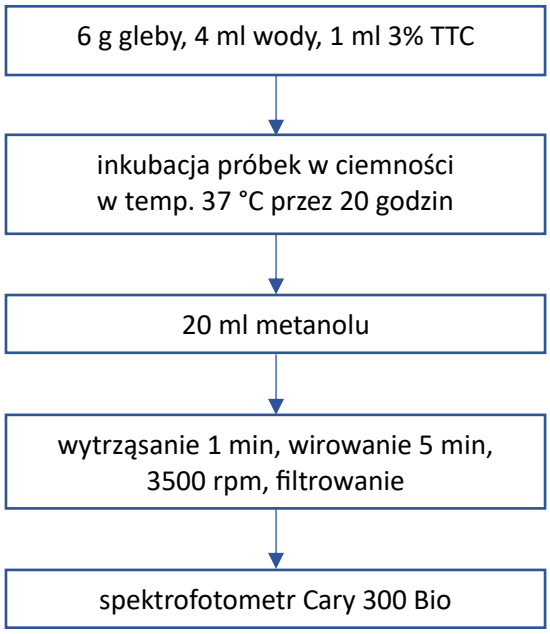
Rysunek 11. Procedura oznaczania barwników.

Badanie pH oraz ORP (publikacje 2-4).

W każdym dniu poboru próbek badano pH oraz ORP za pomocą miernika elektrycznego ADWA AD14 (Adwa, Węgry).

Oznaczanie DHA w próbkach gleby (publikacje 2-4).

DHA w próbkach gleby oznaczano z zastosowaniem metodyki opisanej przez Casida i in. (1964), Wołejko i in. (2017) oraz Tabatabai (2018) przedstawionej na Rysunku 12. Wyniki analiz wyrażono w mikromolach 1,3,5-formazanu trifenylotetrazoliowego (TPF) wytworzonego przez 1 gram suchej masy (s.m.) gleby w ciągu 20 godzin (Książek-Trela i in., 2022).



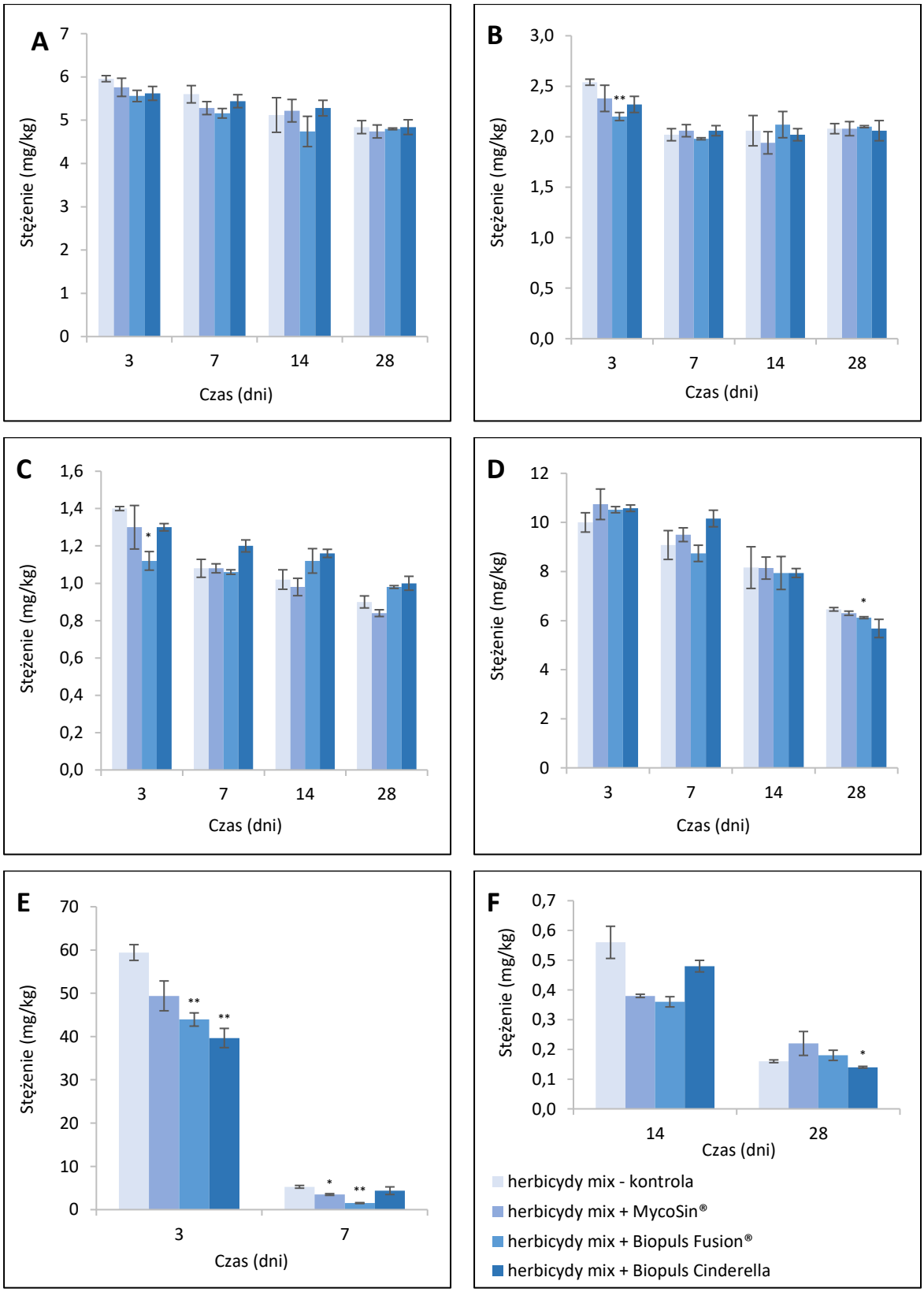
Rysunek 12. Procedura oznaczania DHA.

Analiza statystyczna wyników (publikacje 2-4).

Różnice istotne statystycznie dla każdego dnia pobierania próbek wyznaczono testem t-Studenta (program Excel Microsoft 365). Wartości *p*, istotne statystycznie, przedstawiono jako $p < 0,05$ (*), $p < 0,01$ (**) i $p < 0,001$ (***).

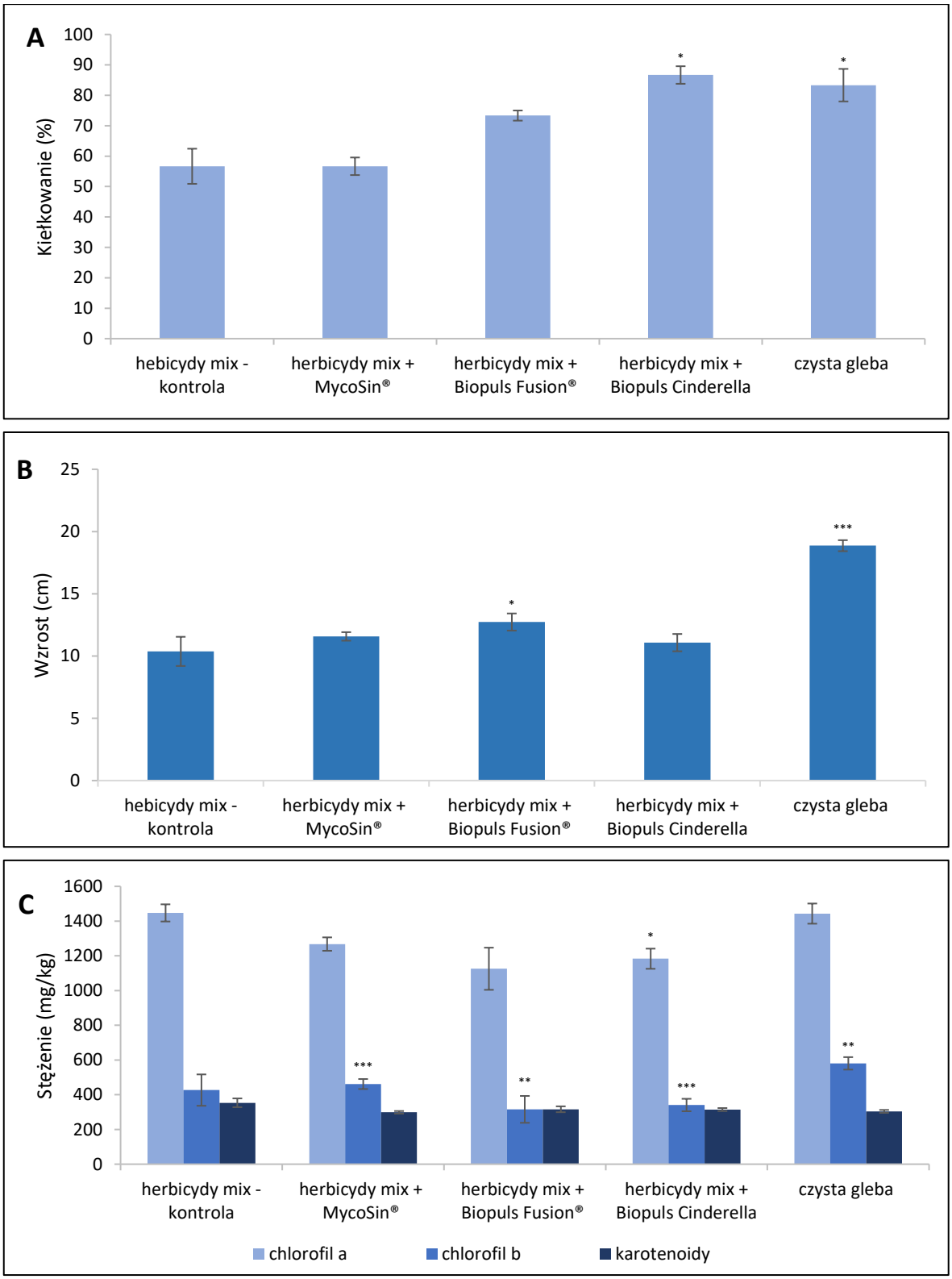
Omówienie wyników

Na Rysunku 13. przedstawiono kinetykę zanikania badanych herbicydów w próbkach gleby. Po zastosowaniu preparatu Biopuls Fusion® zawierającego *Y. lipolytica* uzyskano najwyższą różnicę w degradacji dla wszystkich badanych herbicydów w porównaniu do próbek kontrolnych. Maksymalna różnica dla pendimetaliny wynosiła 5,3% (dzień 28), chlomazonu – 7,9% (dzień 7), propyzamidu – 13,4% (dzień 3), metrybuzyny – 20,0% (dzień 3) oraz fluazyfopu-P-butylu – 71,2% (dzień 7).



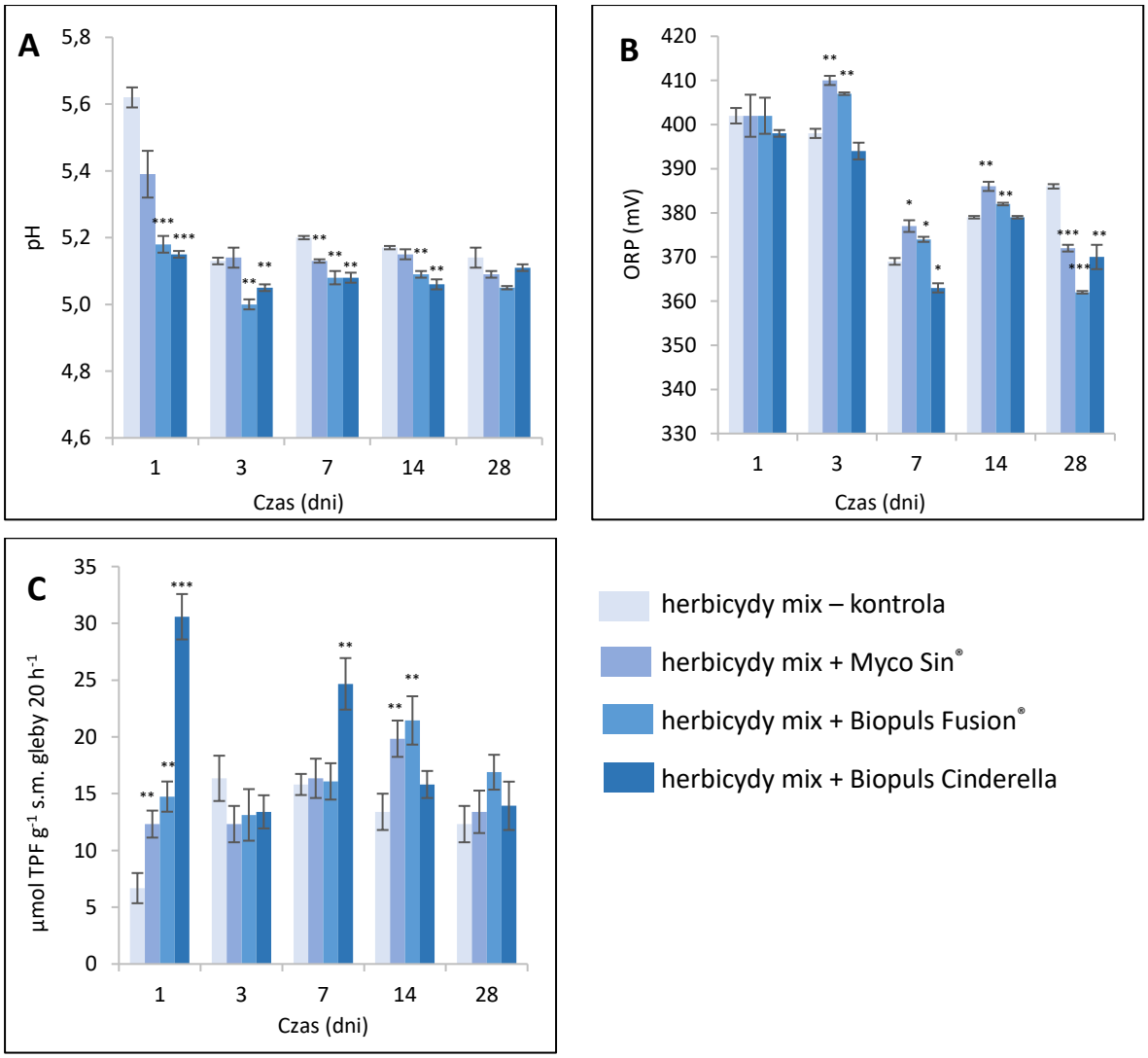
Rysunek 13. Zanikanie badanych herbicydów w czasie: chlomazonu – A, propyzamidu – B, metrybuzyny – C, pendimetaliny – D, fluazyfopu-P-butylu – E oraz F. Istotne statystycznie wartości *p* przedstawiono na rycinach jako $p < 0,05$ (*) oraz $p < 0,01$ (**).

Na Rysunku 14. przedstawiono wpływ preparatów drożdżowych oraz mieszaniny herbicydów na kiełkowanie nasion, wzrost roślin oraz zawartość barwników w grochu zwyczajnym. Mieszanina herbicydów znacznie ograniczała kiełkowanie nasion. Współczynnik kiełkowania był najwyższy w glebie z preparatami Biopuls Fusion® i Biopuls Cinderella oraz w glebie czystej. Największą i istotnie statystyczną wartość stwierdzono dla próbki z preparatem Biopuls Cinderella (Rysunek 14A). Wzrost roślin mierzono w 16 dniu badań. Mieszanina herbicydów znacząco ograniczyła wzrost grochu zwyczajnego. W glebie z mieszaniną herbicydów i preparatem Biopuls Fusion® zawierającym *Y. lipolytica* rośliny charakteryzowały się najszybszym wzrostem (Rysunek 14B). Zastosowanie preparatów drożdżowych wpłynęło na zawartość barwników w roślinach grochu, zwłaszcza chlorofilu b. Na ten parametr mógł mieć wpływ szybszy wzrost roślin uprawianych na glebie z dodatkiem preparatów drożdżowych (Rysunek 14C). Po 16 dniach oznaczono także pozostałości herbicydów w roślinach, aby ocenić pobieranie tych zanieczyszczeń z gleby przez roślinę. Wszystkie próbki zawierały metrybuzynę (średnio na poziomie 0,1 mg/kg) i chlomazon (0,02 mg/kg). Rośliny grochu zwyczajnego nie zawierały pozostałości propyzamidu i fluazyfopu-P-butylu powyżej granicy oznaczalności (LOQ) wynoszącej 0,01 mg/kg.



Rysunek 14. Wpływ mieszaniny herbicydów (kontrola) oraz mieszaniny herbicydów i preparatów mikrobiologicznych na: kiełkowanie nasion – A, wzrost roślin – B oraz zawartość chlorofilu a i b oraz karotenoidów – C. Istotne statystycznie wartości *p* przedstawiono na rycinach jako $p < 0,05$ (*), $p < 0,01$ (**) oraz $p < 0,001$ (***).

Podczas trwania doświadczenia, najwyższe wartości pH odnotowano dla próbek kontrolnych, zatem preparaty mikrobiologiczne obniżały pH gleby (Rysunek 15A). Po zastosowaniu mieszaniny herbicydów nastąpił wzrost ORP dla próbek z preparatem Myco Sin® i Biopuls Fusion®. W kolejnych dniach parametr ten zmniejszał się we wszystkich próbkach (Rysunek 15B). Po aplikacji preparatów drożdżowych DHA gleby była najwyższa w próbkach z preparatem Biopuls Cinderella. Po zastosowaniu mieszaniny herbicydów wartość tego parametru uległa obniżeniu. W kolejnych dniach pobierania próbek stężenie DHA było wyższe w glebie zawierającej drożdże w porównaniu do próbek kontrolnych (Rysunek 15C).



Rysunek 15. Parametry próbek gleby: pH – A, ORP – B, DHA – C, po zabiegu preparatami mikrobiologicznymi w 1 dniu oraz po aplikacji mieszaniny herbicydów w 3, 7, 14 i 28 dniu. Istotne statystycznie wartości *p* przedstawiono na rycinach jako $p < 0,05$ (*), $p < 0,01$ (**) oraz $p < 0,001$ (***).

Dyskusja

Dostępne na rynku preparaty mikrobiologiczne zawierają różne gatunki drożdży, m.in. *S. cerevisiae* wykorzystywane do ochrony roślin, czy *Y. lipolytica* i *D. hansenii* stosowane do rewitalizacji gleby. W niniejszych badaniach wykorzystano trzy preparaty drożdżowe do analizy ich potencjału degradacji mieszaniny herbicydów w glebie oraz ich wpływu na uprawę grochu zwyczajnego (*Pisum sativum* L.). W literaturze niewiele jest informacji na temat wpływu preparatów drożdżowych na degradację herbicydów.

Drożdże *S. cerevisiae* mają szerokie zastosowanie w rolnictwie jako bionawozy czy biofungicydy. Szczep *S. cerevisiae* LAS02 został zatwierdzony w UE jako substancja czynna niskiego ryzyka (EU Pesticide Database, 2024). Mechanizm jego działania związany jest ze zwiększaniem rozpuszczalności fosforu, utlenianiem siarki i produkcją fitohormonu – kwasu indolo-3-octowego (IAA). Natomiast jako biofungicyd szczep ten konkuruje z grzybami chorobotwórczymi o składniki odżywcze i przestrzeń kolonizacyjną (Hernández-Fernández i in., 2021). W sadzie jabłoniowym badano wpływ biofungicydu Myco Sin® zawierającego *S. cerevisiae* na degradację fluksapyroksadu. Stopień degradacji tego fungicydu po zastosowaniu preparatu drożdżowego wyniósł 59,9% dla odmiany jabłek Gala i 43,8% dla odmiany Idared (Podbielska i in., 2020). Ponadto stwierdzono, że *S. cerevisiae* jest w stanie degradować także inne pestycydy, w tym iprodion, boskalid, azoksystrobinę i piraklostrobinę występujące w warzywach liściastych (Wołejko i in., 2016). W badaniach przeprowadzonych w ramach niniejszej pracy doktorskiej preparat Myco Sin®, zawierający *S. cerevisiae*, rozłożył 33,7% fluazyfopu-P-butylu w ciągu 7 dni badań. W przypadku tego preparatu drożdżowego nie stwierdzono wpływu na kiełkowanie i wzrost grochu zwyczajnego. Myco Sin® zwiększył zawartość chlorofilu b w tej roślinie. W innych badaniach zastosowanie *S. cerevisiae* w uprawie ryżu znacząco zwiększyło poziom barwników fotosyntetycznych w roślinach i pobudziło ich wzrost (Verma i in., 2019), natomiast zastosowanie trzech izolatów – *D. hansenii* Dh-67, *Lachancea thermotolerans* Lt-69 i *S. cerevisiae* Sc-6 w sadzonkach kukurydzy sprzyjało rozwojowi siewek (wzrost s. m. i zawartości chlorofilu na poziomie 10%) (Fernandez-San Millan i in., 2020).

Drożdże *Y. lipolytica* mogą być stosowane jako bionawóz (zwiększający rozpuszczalność fosforu) i jako środek owadobójczy (Hernández-Fernández i in., 2021), a także jako środek

bioremediacyjny do usuwania m.in. pestycydów (Wierzchowska i in., 2022). W badaniach zrealizowanych w ramach niniejszej pracy doktorskiej, preparat Biopuls Fusion®, zawierający drożdże *Y. lipolytica*, okazał się najskuteczniejszy w degradacji herbicydów. Wzrost degradacji w odniesieniu do kontroli wyniósł dla propyzamidu 13,4% w 3 dniu, metrybuzyny 20% w 3 dniu, natomiast dla pendimetaliny 5,3% w 28 dniu i fluazyfopu-P-butylu osiągając maksymalny poziom 71,2% w 7 dniu. Preparat ten sprzyjał wzrostowi grochu zwyczajnego (rośliny były wyższe o 2,3 cm w stosunku do próbek kontrolnych). Gul Jan i in. (2019) oraz Verma i in. (2019) analizowali wpływ *Y. lipolytica* na wzrost kukurydzy i wykazali, że drożdże te sprzyjały wzrostowi roślin kukurydzy poprzez regulowanie ich metabolizmu i wydzielanie hormonów roślinnych tj. kwasu abscysynowego i IAA.

Drożdże *D. hansenii* wykazują wysoką odporność na ekstremalne warunki środowiskowe, takie jak niskie pH i wysokie ciśnienie osmotyczne. Są powszechnie stosowane w przemyśle spożywczym, ale w ostatnich latach znalazły także zastosowanie w rolnictwie. Wspomagają rośliny w walce z grzybami chorobotwórczymi w uprawach owoców i warzyw poprzez wydzielanie toksyn killerowych (Etykieta BioPuls Cinderella). W badaniach przeprowadzonych w ramach niniejszej pracy doktorskiej preparat Biopuls Cinderella zawierający *D. hansenii* wykazał zdolność do rozkładu fluazyfopu-P-butylu w glebie. W 3 dniu doświadczenia stopień degradacji tego herbicydu wyniósł 33,3% w porównaniu do próbek kontrolnych. W przypadku pozostałych badanych herbicydów nie stwierdzono statystycznie istotnych różnic w ich degradacji w glebie zawierającej preparat Biopuls Cinderella w porównaniu do gleby kontrolnej. Preparat Biopuls Cinderella wykazał natomiast istotny wpływ na kiełkowanie grochu zwyczajnego. Współczynnik kiełkowania w glebie z tym preparatem był wyższy o 30% niż w glebie zanieczyszczonej mieszaniną herbicydów, a także o 3,4% wyższy niż w glebie czystej. Badania Fernandez-San Millan i in. (2020) potwierdzają pozytywny wpływ *D. hansenii* na rośliny. Szczep Dh-67 sprzyjał wzrostowi i zawartości chlorofilu w kukurydzy.

Wyniki badań wskazują na pozytywny wpływ preparatów drożdżowych zarówno na remediację gleby, jak i wzrost roślin. Stosowanie tych preparatów może stanowić uzupełnienie do ochrony roślin chemicznymi preparatami i powinno być zalecane w celu poprawy jakości i bezpieczeństwa środowiska.

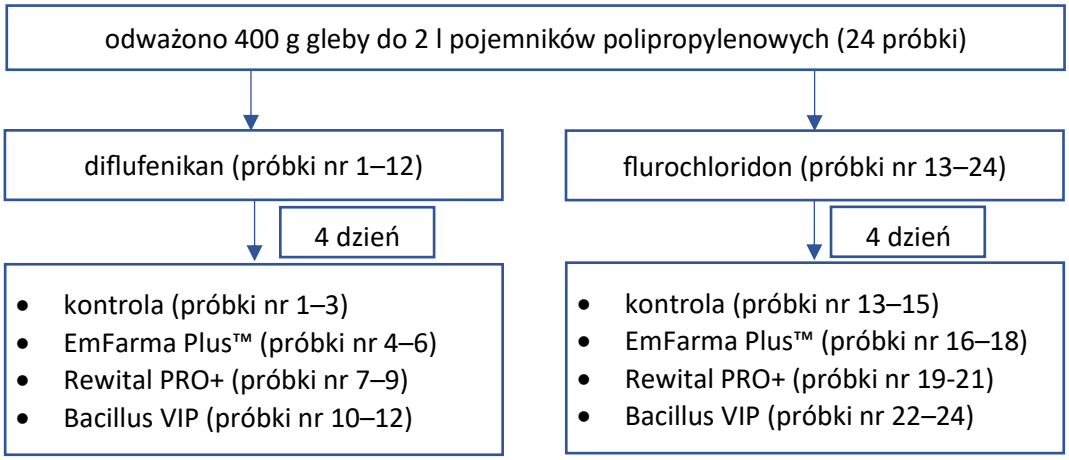
Publikacja 3. **Książek-Trela P.**, Bielak E., Wężka D., Szpyrka E. 2022. Effect of three commercial formulations containing effective microorganisms (EM) on diflufenican and flurochloridone degradation in soil. Molecules 27(14), 4541. DOI: 10.3390/molecules27144541.

Cele badań

Celem badań była ocena wpływu trzech komercyjnych preparatów mikrobiologicznych zawierających konsorcja bakterii i drożdży na degradację diflufenikanu i flurochloridonu w glebie w warunkach laboratoryjnych oraz obliczenie czasu zanikania substancji czynnych tych herbicydów w glebie. Dodatkowo analizowano zmiany pH, ORP oraz DHA w próbkach gleby.

Materiały i metody

Doświadczenie przeprowadzono zgodnie ze schematem przedstawionym poniżej (Rysunek 16).



Rysunek 16. Schemat doświadczenia z herbicydami i preparatami mikrobiologicznymi.

Poniżej przedstawiono charakterystykę chemicznych środków ochrony roślin (Tabela 5) (publikacje 3 i 4) oraz preparatów mikrobiologicznych zastosowanych w przeprowadzonych badaniach (Tabela 6) (publikacje 3 i 4).

Tabela 5. Chemiczne środki ochrony roślin zastosowane w badaniach (Etykieta Legato 500 SC; Etykieta Racer 250 EC).

Nazwa preparatu	Substancja czynna	Zawartość substancji czynnej	Zastosowanie	Pobieranie
Legato 500 SC (ADAMA, Polska)	diflufenikan	500 g/l	zwalczanie chwastów w zbożach przed lub wkrótce po ich wystąpieniu	liście i częściowo przez korzenie
Racer 250 EC (ADAMA Agan Ltd, Państwo Izrael)	flurochloridon	250 g/l	zwalczenie jedno- i dwuliściennych chwastów jednorocznych w zbożach ozimych i warzywach	korzenie i liścienie

Tabela 6. Preparaty mikrobiologiczne (Etykieta EmFarma Plus™; Etykieta Rewital PRO+; Etykieta Bacillus VIP Mikroorganizmy Probiotyczne).

Nazwa preparatu	Rodzaj preparatu	Skład preparatu	Zastosowanie
EmFarma Plus™ (ProBiotics, Poland)	preparat rewitalizujący glebę	bakterie z rodzajów: *Bifidobacterium, Lactococcus, Lactobacillus, Bacillus, Rhodopseudomonas* oraz *Streptococcus,* drożdże *Saccharomyces cerevisiae,* ekologiczna melasa z trzciny cukrowej, rewitalizowana woda, sól kamienna, kompleks minerałów	optymalizacja rozkładu materii organicznej, kondycjonowanie gleby, zwiększanie zawartości próchnicy i wody, intensyfikacja procesów mikrobiologicznych, poprawianie procesu kompostowania, przyspieszanie rozkładu pozostałości pestycydów w glebie
Rewital PRO+ (BIOGEN, Poland)	preparat rewitalizujący glebę	bakterie z rodzajów: *Streptomyces, Bacillus, Pseudomonas, Cellulomonas, Rhodococcus, Pseudonocardia, Arthrobacter* oraz *Paenibacillus,* pożywka startowa	przywracanie równowagi mikrobiologicznej gleby, poprawianie żyzności gleby oraz wartości biologicznej produktów roślinnych, przyspieszanie rozkładu resztek pożniwnych roślin, obornika i innych naturalnych nawozów organicznych, uwalnianie trudno dostępnych dla roślin składników pokarmowych, redukowanie chorobotwórczych dla roślin bakterii, wirusów i grzybów, poprawianie zdrowotności roślin uprawnych
Bacillus VIP Mikroorganizmy Probiotyczne (AGROBIOS, Poland)	preparat zwiększający żyzność gleby, wzrost i odporność roślin	8 szczepów bakterii z rodzaju *Bacillus*: *B. amyloliquefaciens, B. coagulans, B. laterosporus, B. licheniformis, B. mucilaginosus, B. megaterium, B. polymyxa* oraz *B. pumilus,* ekologiczna melasa trzcinowa, rewitalizowana woda	wytwarzanie substancji wzrostowych (aminokwasy, enzymy, fitohormony, stymulatory), przyspieszanie wzrostu kiełkowania roślin, rozkład związków fosforowych do dostępnego fosforu roślinnego, zwiększanie biodostępności azotu, potasu i żelaza, stymulacja wzrostu korzeni, zabezpieczanie roślin, w tym korzeni przed patogenami, promowanie wzrostu roślin i kwitnienia, zwiększanie odporności roślin na długotrwałe warunki stresowe

Określenie kinetyki zanikania substancji czynnych badanych herbicydów oraz czasu połowicznego zaniku.

Określono kinetykę zanikania substancji czynnych diflufenikanu i flurochloridonu, poprzez wykreślenie zależności stężenia od czasu. Zależność tę opisano równaniami wykładniczymi dla reakcji pierwszego rzędu, dla których wartość współczynnika determinacji (R^2) była najbliższa jedności.

$$R_t = R_0 \cdot e^{-kt}$$

gdzie:

R_t — stężenie substancji w danym czasie (t) (mg/kg),

R_0 — początkowe stężenie substancji (mg/kg),

k — stała szybkości reakcji.

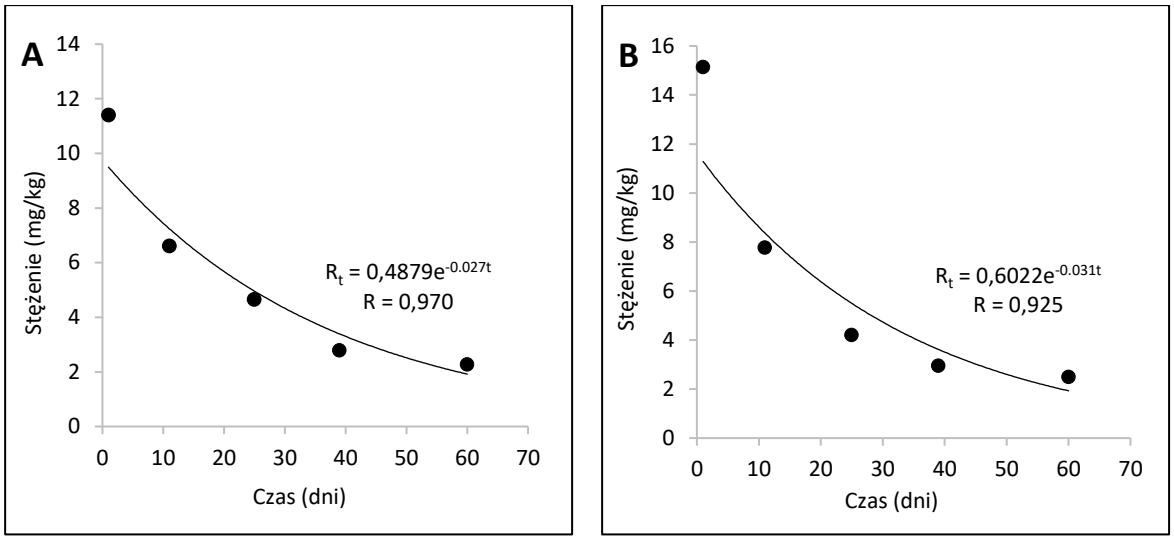
Na podstawie wyznaczonych równań obliczono czasy połowicznego zanikania badanych herbicydów w glebie (ang. *half-life*) (Szpyrka i in., 2020).

$$t_{1/2} = \frac{\ln(2)}{k}$$

Pozostałości herbicydów w próbkach gleby oznaczano za pomocą metody QuEChERS (Rysunek 10). Zmiany pH, ORP i DHA analizowano zgodnie z metodyką przedstawioną w publikacji 2.

Omówienie wyników

Na Rysunku 17. przedstawiono zanikanie diflufenikanu i flurochloridonu w glebie w czasie wraz z równaniami kinetycznymi i współczynnikami korelacji. Obliczono czas połowicznego zanikania ($t_{½}$), który w przypadku diflufenikanu wyniósł 25,7 dni, natomiast dla flurochloridonu 22,4 dni.



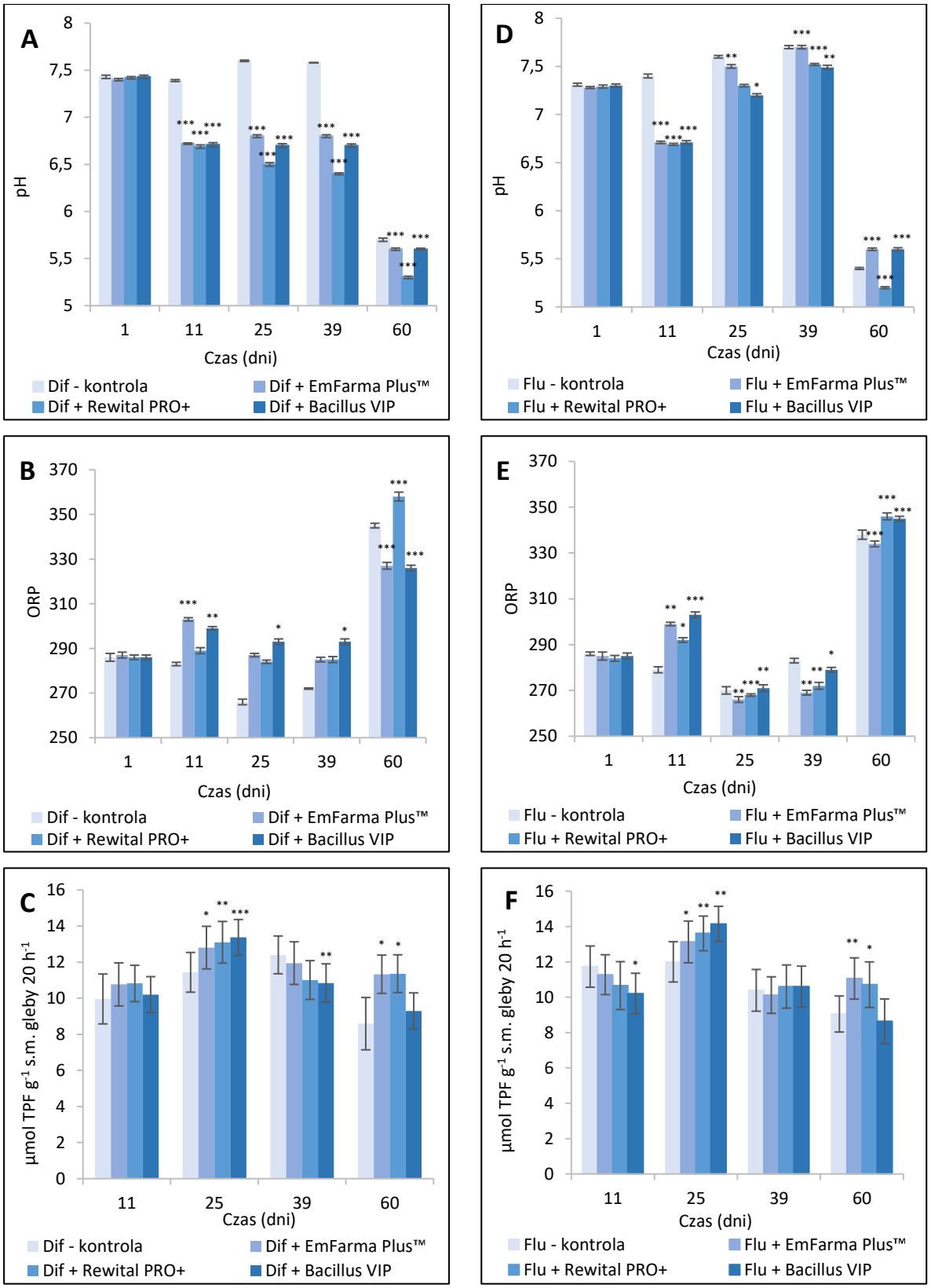
Rysunek 17. Zanikanie diflufenikanu – A i flurochloridonu – B w glebie w czasie.

Preparaty EM miały istotny wpływ na degradację diflufenikanu w glebie, ale efekt ten był odwrotny do oczekiwanego. Oznacza to, że preparaty biologiczne spowolniły rozkład tego herbicydu. Największe zahamowanie degradacji (36,5%) odnotowano po zastosowaniu preparatu EmFarma Plus™. Prawdopodobną przyczyną był znaczny spadek pH gleby po zastosowaniu preparatów EM, maksymalnie aż o 2,3 jednostki. W przypadku flurochloridonu najwyższą degradację (31,2%) uzyskano po aplikacji preparatu Bacillus VIP. Istotne statystycznie różnice zaobserwowano dla preparatów EmFarma Plus™ i Rewital PRO+ w przypadku diflufenikanu oraz Rewital PRO+ i Bacillus VIP w przypadku flurochloridonu (Tabela 7).

Tabela 7. Wpływ preparatów mikrobiologicznych na degradację diflufenikanu i flurochloridonu („-" oznacza, że szybkość degradacji zmniejszyła się). Istotne statystycznie wartości *p* przedstawiono jako $p < 0,05$ (*) i $p < 0,01$ (**).

Liczba dni po zabiegu preparatem EM	Degradacja (%)					
	diflufenikan			flurochloridon		
	EmFarma Plus™	Rewital PRO+	Bacillus VIP	EmFarma Plus™	Rewital PRO+	Bacillus VIP
11	-36,5**	-35,5	-32,6	18,3	7,1	22,0
25	7,2	1,4	7,2	4,9	27,7	29,8
39	3,5	-25,0*	-38,0	4,4	17,9	17,8
60	2,4	-9,1	-10,0	19,3	30,5*	31,2*

Po dodaniu preparatów EM do próbek gleby zawierających diflufenikan i flurochloridon, pH wszystkich badanych próbek było niższe w porównaniu do próbek kontrolnych. W 60 dniu we wszystkich próbkach zaobserwowano obniżenie wartości pH (Rysunek 18A). We wszystkich próbkach zawierających diflufenikan i preparaty EM, wartości ORP były wyższe w porównaniu z próbkami kontrolnymi, z wyjątkiem 60 dnia. W próbkach zawierających flurochloridon ORP był wyższy w porównaniu z próbami kontrolnymi w 11 dniu, natomiast w 25 i 39 dniu, utrzymywał się na podobnym poziomie jak w próbach kontrolnych. W 60 dniu nastąpił znaczny wzrost ORP we wszystkich badanych próbkach (Rysunek 18B). W 4 dniu doświadczenia, po dodaniu preparatów EM, początkowa wartość DHA w próbkach kontrolnych z diflufenikanem wynosiła 10,3 µM TPF/g s.m. gleby w ciągu 20 godzin, natomiast w próbkach z flurochloridonem 6,2 µM TPF/g s.m. gleby w ciągu 20 godzin. Najwyższą DHA zaobserwowano w próbkach z diflufenikanem i flurochloridonem oraz preparatami EM w 25 dniu, wartości te były wyższe niż w próbach kontrolnych (Rysunek 18C).



Rysunek 18. Parametry: pH, ORP, DHA w próbkach gleby z diflufenikanem (A–C) i flurochloridonem (D–F) w 1 dniu doświadczenia (kontrola) – przed aplikacją preparatów mikrobiologicznych oraz po ich aplikacji w 11, 25, 39 i 60 dniu. Istotne statystycznie wartości *p* przedstawiono jako $p < 0,05$ (*), $p < 0,01$ (**) i $p < 0,001$ (***).

Dyskusja

Według danych dostępnych w Bazie danych właściwości pestycydów (ang. *the Pesticide Properties Database*, PPDB), czas połowicznego zanikania dla diflufenikanu wynosi 41,4-318 dni w warunkach laboratoryjnych oraz 224-621 dni w badaniach polowych (PPDB, 2024). Inne doniesienia literaturowe wskazują, że $t_{1/2}$ diflufenikanu wynosił 101 dni w glebie uprawnej oraz 65 dni w glebie w sadzie gruszowym (Rouchaud i in., 1994; Rouchaud i in., 2000). W badaniach przeprowadzonych w ramach niniejszej pracy doktorskiej, diflufenikan zanikał szybciej. Czas połowicznego zanikania wynosił 25,7 dni, co może być spowodowane prowadzeniem doświadczenia w innych warunkach niż polowe (temperatura: 22°C, wilgotność: 67-74%).

W przypadku flurochloridonu czas połowicznego zanikania mieści się w zakresie 22,8-180 dni w warunkach laboratoryjnych oraz 11-65 dni w badaniach polowych (PPDB, 2024). Rouchaud i in. (1997) obliczyli $t_{1/2}$ flurochloridonu w warunkach polowych, który wynosił 41 dni w pierwszym roku prowadzenia doświadczenia oraz 50 dni po powtórzeniu zabiegu w kolejnym roku. Li i in. (2021) oznaczali $t_{1/2}$ flurochloridonu w glebie, wykazując, że czas ten mieścił się w zakresie 19,3-27,7 dni. Sharipov i in. (2021) prowadzili badania nad zanikaniem tego herbicydu w zależności od pH i składu gleby i wskazali, że $t_{1/2}$ flurochloridonu wynosił 38-51 dni. W badaniach przeprowadzonych w ramach niniejszej pracy doktorskiej czas połowicznego zanikania flurochloridonu wynosił 22,4 dni, co jest skorelowane z danymi uzyskanymi przez innych badaczy.

Wyniki przeprowadzonego doświadczenia w trakcie realizacji pracy doktorskiej wskazują, że preparaty EM miały istotny wpływ na degradację diflufenikanu w glebie, ale efekt ten okazał się odwrotny od oczekiwanego. Preparaty EM zahamowały degradację badanego herbicydu, prawdopodobnie ze względu na znaczny spadek pH gleby po ich zastosowaniu. Według Houot i in. (2000) gleby o pH poniżej 6,5 charakteryzują, się wolniejszą degradacją mikrobiologiczną. Istnieją doniesienia literaturowe na temat wpływu nawozów organicznych stosowanych doglebowo oraz mikroorganizmów na degradację diflufenikanu. Rouchaud i in. (1994) obliczyli czas połowicznego zanikania diflufenikanu, który wynosił 101 dni w próbkach kontrolnych oraz od 116 do 215 dni po zastosowaniu nawozów organicznych, które zwiększają trwałość herbicydu. Pinto i in. (2020) zbadali wpływ grzybów *Fusarium oxysporum* PP0030,

Paecilomyces variotii PP0040 oraz *Trichoderma viride* PP0050 na rozkład pestycydów, w tym diflufenikanu. Maksymalną degradację uzyskano po zastosowaniu *F. oxysporum*, wynoszącą 74,7% po 120 dniach prowadzonego doświadczenia.

Wyniki uzyskane w ramach niniejszej pracy doktorskiej wskazują, że najwyższą degradację flurochloridonu uzyskano po zastosowaniu preparatu Bacillus VIP. Preparat ten zawiera osiem szczepów z rodzaju *Bacillus*, które prawdopodobnie stymulują mikroflorę glebową i tym samym przyspieszają rozkład tego herbicydu. Istnieje tylko jedno doniesienie literaturowe dotyczące wpływu nawozów organicznych na degradację flurochloridonu. Rouchaud i in. (1997) zbadali rozkład flurochloridonu w warunkach polowych. Czas połowicznego zanikania flurochloridonu wynosił od 48 do 74 dni po zabiegach nawozami organicznymi oraz 41 dni w próbkach kontrolnych.

Otrzymane wyniki badań, opisane w publikacji 3 potwierdzają potrzebę prowadzenia dalszych badań nad degradacją diflufenikanu oraz poszukiwania mikroorganizmów zdolnych do przyspieszenia rozkładu tej substancji.

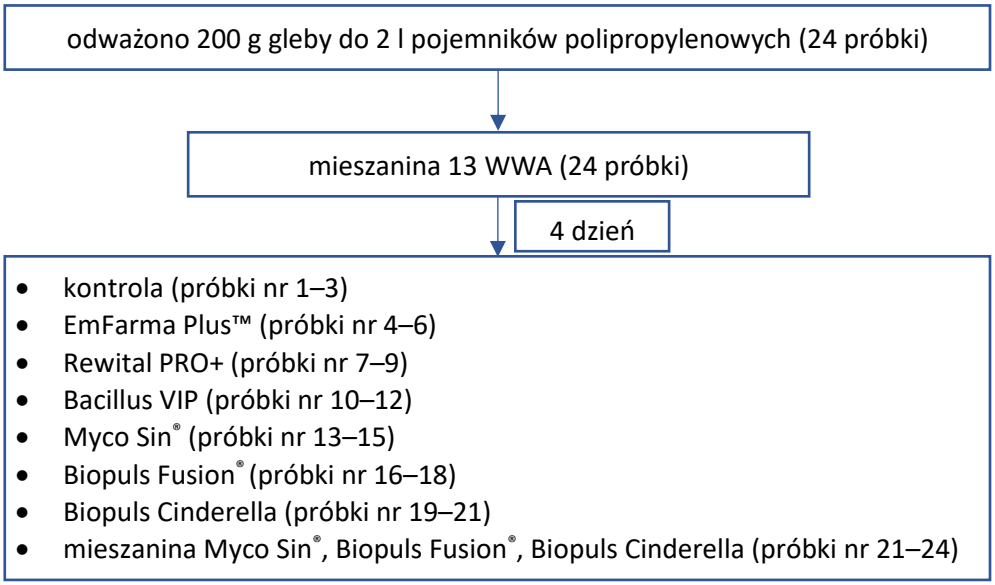
Publikacja 4. **Książek-Trela P.**, Figura D., Wężka D., Szpyrka E. 2024. Degradation of a mixture of 13 polycyclic aromatic hydrocarbons (PAHs) by commercial effective microorganisms. Open Life Sciences 19(1), 20220831. DOI: 10.1515/biol-2022-0831.

Cele badań

Celem badań była ocena wpływu sześciu komercyjnych preparatów mikrobiologicznych oraz mieszaniny preparatów drożdżowych na degradację 13 WWA: acenaftylenu, antracenu, benzo[a]antracenu, benzo[a]pirenu, benzo[b]fluorantenu, benzo[k]fluorantenu, benzo[ghi]perylenu, chryzenu, dibenzo[a,h]antracenu, fenantrenu, fluorenu, indeno[1,2,3-cd]pirenu oraz pirenu, w glebie w warunkach laboratoryjnych. Dodatkowo określono zmiany pH, ORP oraz DHA w próbkach gleby.

Materiały i metody

W dniu rozpoczęcia doświadczenia do wszystkich próbek gleby dodano wodny roztwór wzorca standardowego (EPA 525 PAH MIX B, Sigma-Aldrich, USA) zawierający mieszaninę 13 WWA. W 4 dniu do gleby dodano preparaty EM (Tabela 4 i 6). Kontrolę stanowiły próbki z mieszaniną WWA (Rysunek 19). Próbki gleby do analizy WWA przygotowano według metody QuEChERS (Rysunek 10) (Słowik-Borowiec i in., 2022). Zmiany pH, ORP i DHA analizowano zgodnie z metodyką przedstawioną wcześniej w opisie publikacji 2.



Rysunek 19. Schemat doświadczenia z WWA i preparatami mikrobiologicznymi.

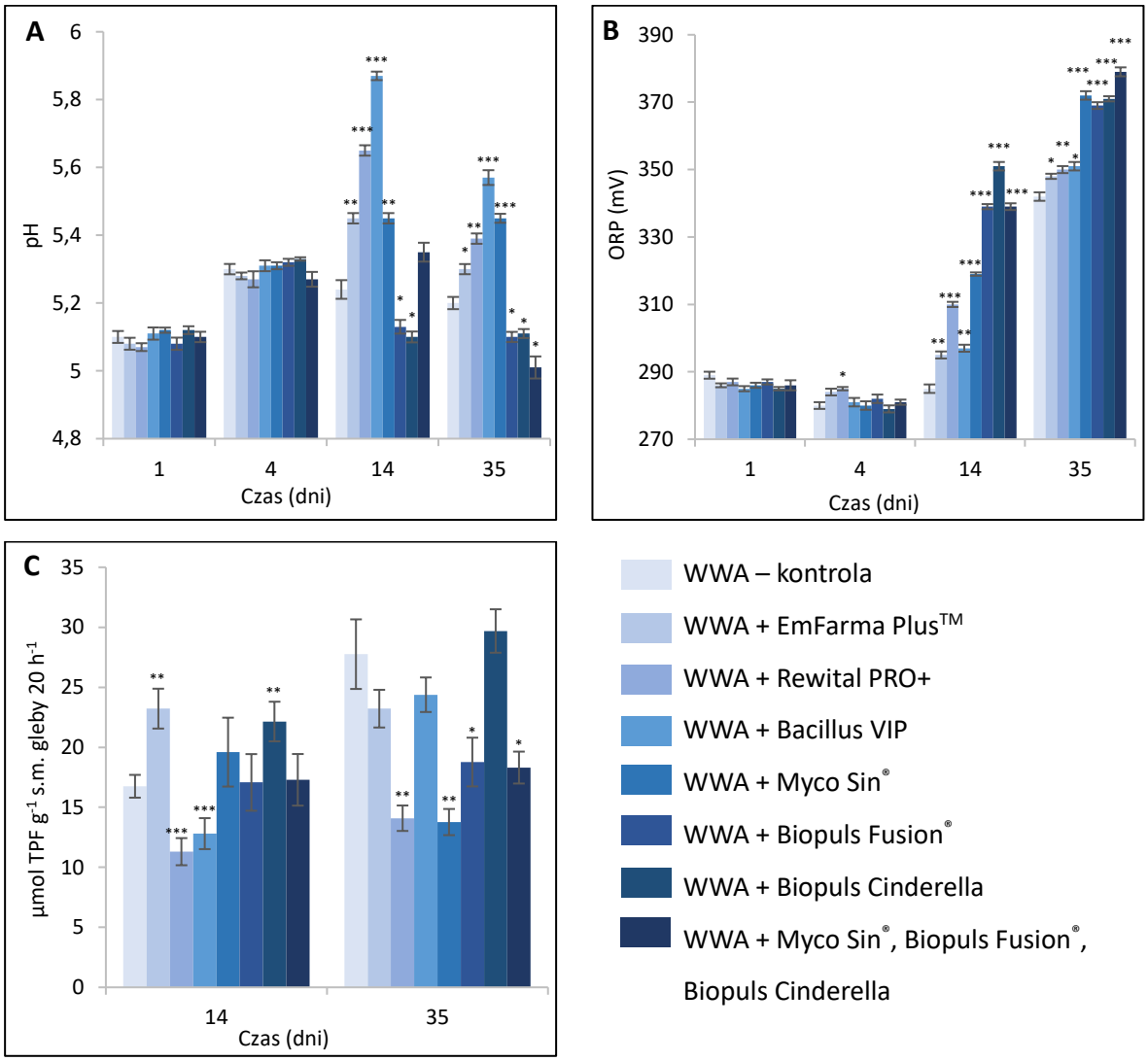
Omówienie wyników

W Tabeli 8. przedstawiono degradację badanych WWA po zastosowaniu preparatów EM w 35 dniu doświadczenia w porównaniu z próbkami kontrolnymi. Wartości maksymalne i minimalne degradacji dla próbek oznaczono odpowiednio kolorem zielonym i czerwonym. Najwyższą degradację, wynoszącą 95,5% uzyskano po zastosowaniu preparatu Bacillus VIP. Najbardziej skuteczny był preparat EmFarma Plus™, dla którego uzyskano najwyższą degradację w przypadku 8 badanych WWA. Najniższe wartości degradacji odnotowano po zastosowaniu preparatów Biopuls Fusion®, Biopuls Cinderella oraz mieszaniny preparatów drożdżowych.

Tabela 8. Degradacja WWA po zastosowaniu preparatów mikrobiologicznych.

Nazwa badanego WWA	Degradacja (%)							
	Kontrola	EmFarma Plus™	Rewital PRO+	Bacillus VIP	Myco Sin®	Biopuls Fusion®	Biopuls Cinderella	Myco Sin® Biopuls Fusion® Biopuls Cinderella
acenaftylen	82,6	91,9	92,7	91,8	91,3	90,7	89,2	87,5
antracen	90.3	94,5	93,9	95,1	92,2	92,3	91,4	92,9
benzo[a]antracen	85,2	90,7	90,4	90,8	88,9	89,6	90,3	88,2
benzo[a]piren	77,5	87,3	85,1	83,6	85,2	85,3	82,0	86,2
benzo[b]fluoranten	79,0	88,4	86,4	84,9	86,6	86,2	78,5	83,8
benzo[k]fluoranten	75,1	86,8	85,1	83,3	84,9	84,5	75,5	81,5
benzo[ghi]perylen	79,8	86,3	82,7	82,9	81,4	79,3	79,3	76,6
chryzen	75,9	85,5	80,8	82,0	81,7	80,3	80,8	82,6
dibenzo[a,h]antracen	80,5	87,9	85,4	82,0	85,6	83,2	77,5	84,0
fenantren	85,3	90,8	87,8	88,6	87,8	83,6	83,1	83,8
fluoren	92,6	94,6	93,5	95,5	93,3	92,0	91,8	91,8
indeno[1,2,3-cd] piren	70,0	85,1	83,7	85,6	85,3	87,0	84,4	83,3
piren	84,2	91,8	90,1	90,7	89,3	88,9	85,9	87,3

Na podstawie wyników badań oraz danych literaturowych, stwierdzono, że pH gleby miało wpływ na degradację WWA. W 14 i 35 dniu badań w próbkach z preparatem Bacillus VIP odnotowano najwyższe wartości pH gleby, co korelowało z maksymalną degradacją WWA. W próbkach gleby z preparatami drożdżowymi, gdzie degradacja była najniższa, pH było bardziej kwasowe, co sprzyjało trwałości tych związków (Rysunek 20A). W 14 dniu doświadczenia ORP wzrósł we wszystkich badanych próbkach w porównaniu z próbkami kontrolnymi. W 35 dniu parametr ten ponownie wzrósł osiągając najwyższą wartość w próbkach zawierających mieszaninę preparatów drożdżowych. W ostatnim dniu eksperymentu ORP wzrósł o prawie 100 mV w porównaniu z dniem 1 doświadczenia (Rysunek 20B). Początkowa wartość DHA w próbkach kontrolnych wynosiła 26,5 µM TPF/g s.m. gleby w ciągu 20 godzin. Po dodaniu WWA wartość ta znacznie spadła i w dniu 14 dla próbek kontrolnych wynosiła 16,8 µM TPF/g s.m. gleby w ciągu 20 godzin. W 14 dniu, zawartość DHA w próbkach z preparatami EM była wyższa niż w próbkach kontrolnych, z wyjątkiem preparatów Rewital PRO+ oraz Bacillus VIP. W przypadku preparatu Bacillus VIP, który wykazał najwyższe wyniki biodegradacji WWA, poziom DHA wzrósł dwukrotnie w 35 dniu w porównaniu do 14 dnia eksperymentu (Rysunek 20C).



Rysunek 20. Parametry próbek gleby: pH – A, ORP – B, DHA – C, z mieszaniną WWA (kontrola) w 1 i 4 dniu, przed aplikacją preparatów mikrobiologicznych oraz po ich aplikacji w 14 i 35 dniu eksperymentu. Istotne statystycznie wartości *p* przedstawiono jako $p < 0,05$ (*), $p < 0,01$ (**) oraz $p < 0,001$ (***).

Dyskusja

Przedmiotem badań przeprowadzonych w ramach niniejszej pracy doktorskiej były preparaty komercyjne zawierające bakterie oraz drożdże i ich mieszanina. Największy potencjał w degradacji badanych WWA miały bakterie, podczas gdy drożdże okazały się mniej efektywne. Najbardziej skuteczny był preparat EmFarma Plus™, dla którego uzyskano najwyższą degradację w przypadku 8 badanych WWA.

Barnes i in. (2023) zbadali wpływ 10 izolatów grzybowych: *Penicillium citrinum, Acremonium sclerotigenum, Aspergillus polyporicola, A. versicolor, Fusarium equiseti, Fusarium* sp.*, Aspergillus* sp.*, A. favus* i *A. sydowii* na degradację 8 WWA (acenaftylen, naftalen, acenaften, fluoren, fenantren, piren, benzo[a]antracen i indeno[1,2,3-c,d]piren) występujących w ropie naftowej. Po 23 dniach doświadczenia wszystkie badane WWA zostały zdegradowane w 100% po zastosowaniu przynajmniej jednego izolatu. Najbardziej efektywne w degradacji WWA okazały się *A. polyporicola, A. versicolor* i *F. equiseti*.

Biodegradację acenaftylenu badano za pomocą grzyba z gatunku *Pleurotus ostreatus* (Rocha i in., 2014). W przypadku antracenu zastosowano 39 szczepów grzybów z rodzaju *Micromycetes* oraz 2 izolaty *Fusiarium solani*. Degradację tej substancji analizowano także po aplikacji *Pseudomonas mosselii* i *P. mendocina* (Smułek i in., 2020). Rozkład benzo[a]antracenu badano z wykorzystaniem grzybów *F. solani* (Wu i in., 2010) oraz *P. ostreatus* (Rocha i in., 2014), benzo[a]pirenu za pomocą bakterii *Bacillus flexus* i *Paenibacillus* sp. (Kotoky i Pandey, 2020) oraz grzybów *Trichoderma* sp., *Fusarium* sp. i *Aspergillus niger* (Wang i in., 2008). Kumari i in. (2018) ocenili wpływ *Ochrobactrum anthropi, Stenotrophomonas maltophilia, Pseudomonas mendocina, P. aeruginosa* i *Microbacterium esteraromaticum* na degradację benzo[b]fluorantenu. Treviño-Trejo i in. (2021) udowodnili wpływ bakterii z rodzajów *Bacillus, Gordonia, Pseudomonas, Rhodococcus, Ochrobactrum* i *Amycolatopsis* na degradację tej substancji. W przypadku rozkładu benzo[k]fluorantenu wykorzystano *Stenotrophomonas maltophilia* (Arulazhagan i in., 2017) oraz *Sphingobium* sp. (Maeda i in., 2014). Wpływ 8 gatunków grzybów*: Agrocybe praecox, A. dura, Kuehneromyces mutabilis, Hypholoma capnoides, Stropharia rugosoannulata, S. coronilla, S. cubensis* i *S. hornemannii* na rozkład dibenzo[a,h]antracenu został udowodniony przez Steffen i in. (2007). W przypadku benzo[ghi]perylenu istnieje doniesienie dotyczące zastosowania *Bacillus licheniformis*, *B. subtilis* i *Pseudomonas aeruginosa* do jego degradacji (Amodu i in., 2019) oraz *Acinetobacter baumannii* i *Pseudomonas taiwanensis* na rozkład indeno[1,2,3-cd]pirenu (Huang, 2016). Grzyby *Mucor irregularis* szczep bpo1 (Bankole i in., 2021) i bakterie *Sphingobacterium* sp. (Nam i in., 2011) znalazły zastosowanie w degradacji fluorenu. Istnieją doniesienia odnośnie wykorzystania bakterii *B. licheniformis*, *B. subtilis* i *P. aeruginosa* (Amodu i in., 2019), *Pseudomonas citronellolis* i *Stenotrophomonas maltophilia* (Nzila i in., 2018) oraz grzybów *Fusarium* sp. (Li i in., 2005) i *Trichoderma* sp. (Hadibarata i in., 2007) w rozkładzie fenantrenu.

W przypadku badań na temat degradacji chryzenu wykorzystano grzyby P*olysporus* sp. (Hadibarata i Tachibana, 2009; Hadibarata i in., 2009), bakterie *Bacillus cereus* i *Pseudomonas putida* (Tuhuloula i in., 2017) oraz *Rhodococcus* sp., *Bacillus* sp. i *Burkholderia* sp. (Vaidya i in., 2018). Bakterie *Coriolopsis byrsina* (Agrawal i Shahi, 2017) oraz *Achromobacter xylosoxidans* (Nzila i in., 2018), a także grzyby *Aspergillus niger, Trichoderma* sp. i *Fusarium* sp. (Wang i in., 2008), były przedmiotem badań nad rozkładem pirenu.

W porównaniu do grzybów i bakterii, drożdże są zdecydowanie słabiej reprezentowane w badaniach nad degradacją WWA. Istnieją jednak doniesienia o ich potencjale w tym zakresie. Udowodniono, że *S. cerevisiae* wpływają na degradację ropy naftowej lub WWA z miejsc skażonych tymi substancjami (Abioye i in., 2013). Drożdże *Y. lipolytica* są stosowane jako środek bioremediacyjny do usuwania substratów hydrofobowych, takich jak ropa naftowa i WWA (Wierzchowska i in., 2022). *D. hansenii* może rozkładać aminy biogenne, benzo(a)piren i n-dodekan (Bäumlisberger i in., 2015; Padilla-Garfias i in., 2022; Azimian, 2023). Szczep drożdży *Candida* sp. S1 został wykorzystany w badaniach nad degradacją pirenu (Hadibarata i in., 2017). Natomiast Mandal i in. (2016 i 2018) przeprowadzili badania nad rozkładem benzo[ghi]perylenu i benzo[a]pirenu przez trzy izolaty *Rhodotorula* sp. NS01, *D. hansenii* NS03 i *Hanseniaspora valbyensis* NS04.

Wymienione powyżej doniesienia literaturowe stanowią silny argument za wykorzystaniem mikroorganizmów w remediacji miejsc zanieczyszczonych przez WWA, a także potrzebę prowadzenia dalszych badań w tym zakresie. Wydaje się, że szczegółowe poznanie mechanizmów biodegradacji, wpływu czynników środowiskowych i optymalnych strategii bioremediacji jest kluczowe dla efektywnego oczyszczania skażonych środowisk.

4. Podsumowanie

Realizacja niniejszych badań miała na celu określenie wpływu komercyjnych preparatów zawierających bakterie i drożdże na degradację herbicydów i WWA w glebie.

Uzyskane wyniki badań zaprezentowane w oryginalnych pracach stanowiących przedmiot rozprawy doktorskiej dowiodły, że badane mikroorganizmy wywierają wpływ na degradację trwałych zanieczyszczeń. Oznacza to, że mogą one zostać wykorzystane w bioremediacji gleb zanieczyszczonych herbicydami i WWA. Ważnym zagadnieniem, na które położono nacisk było badanie wpływu mieszanin bakterii i drożdży na degradację zanieczyszczeń, w celu stworzenia warunków najbardziej zbliżonych do tych panujących w środowisku naturalnym. Zastosowane w badaniach preparaty posiadają duże znaczenie w rewitalizacji, poprawie składu, kondycji i aktywności mikrobiologicznej gleb. Dodatkowo, niektóre z nich wspomagają także naturalną odporność roślin. Na podstawie przeprowadzonych badań, można jednoznacznie stwierdzić, że preparaty bakteryjne oraz drożdżowe mają istotny wpływ na degradację siedmiu badanych herbicydów, z wyjątkiem diflufenikanu. W przypadku WWA preparaty drożdżowe okazały się mniej skuteczne. Środki zawierające w swoim składzie drożdże pozytywnie wpływają zarówno na rekultywację gleby, jak i kiełkowanie nasion i wzrost roślin grochu zwyczajnego. Wyniki niniejszych badań wskazują, że stosowanie tego typu preparatów stanowi uzupełnienie do ochrony roślin preparatami chemicznymi i powinno być rekomendowane w celu poprawy jakości i bezpieczeństwa środowiska.

W przeprowadzonych doświadczeniach badano także zmiany pH, ORP oraz DHA jako znaczące czynniki wpływające na degradację zanieczyszczeń w glebie. Dehydrogenaza jest enzymem występującym we wszystkich żywych komórkach drobnoustrojów (Kaczyńska i in., 2015; Curyło i Telesiński, 2020; Gospodarek i in., 2021). Oznaczanie jej aktywności uważa się za jeden z najbardziej miarodajnych, kluczowych i czułych bioindykatorów związanych z żyznością gleby (Wolińska i Stępniewska, 2012), a także szybką i stosunkowo prostą metodę określenia ogólnej aktywności mikroorganizmów glebowych (Bueis i in., 2018; Małachowska-Jutsz i Matyja, 2019; Meena i Rao, 2021). Pestycydy, WWA i inne trwałe zanieczyszczenia gleby hamują DHA (Wyszkowska i Kucharski, 2004; Wolińska i Stępniewska, 2012; Järvan i in., 2014). Z badań przeprowadzonych przez Wołejko i in. (2017) i Książek-Trela i in. (2022) wynika

natomiast, że pestycydy mogą w pierwszym okresie stymulować aktywność mikroorganizmów, co oznacza wzrost DHA w glebie. Należy jednak zaznaczyć, że stymulacja DHA przez pestycydy jest zjawiskiem krótkotrwałym. Długotrwałe narażenie mikroorganizmów na pestycydy hamuje ich aktywność, co w konsekwencji prowadzi do spadku DHA. Dodatkowo kilka czynników środowiskowych, takich jak: wilgotność, temperatura, pH, ORP, zawartość materii organicznej, zanieczyszczenie WWA lub pestycydami oraz nawożenie gleby, może znacząco wpływać na DHA w glebie (Wolińska i Stępniewska, 2012).

ORP jest ważnym czynnikiem środowiskowym, który odzwierciedla tendencję środowiska do przyjmowania lub dostarczania elektronów w środowisku (Behl i in., 2023). Odgrywa kluczową rolę w regulacji aktywności drobnoustrojów i wpływa na aktywność enzymatyczną gleby, zwłaszcza DHA (Song i in., 2008). Zazwyczaj aktywność enzymów glebowych, w tym DHA, zwiększa się wraz ze wzrostem pH gleby (Błońska, 2010; Moeskops i in., 2010). Wolińska i Stępniewska (2012) wykazały, że niskie pH gleby powoduje silną inhibicję DHA w porównaniu z zasadowym. Poziomy zanieczyszczeń są często powiązane z pH zanieczyszczonych miejsc, ponieważ mikroorganizmy mogą nie być zdolne do przekształcenia pestycydów oraz WWA w warunkach kwaśnych lub zasadowych. Ekstremalne wartości pH, które można zaobserwować w niektórych glebach, negatywnie wpływają na zdolność populacji drobnoustrojów do degradacji zanieczyszczeń (Houot 2000, Pawar 2015).

Wyniki niniejszych badań jednoznacznie potwierdzają, że preparaty mikrobiologiczne mogą być zarówno uzupełnieniem, jak i alternatywą dla chemicznych środków ochrony roślin. Ich stosowanie powinno być rekomendowane ze względu na poprawę jakości i bezpieczeństwa środowiska. Otrzymane wyniki potwierdzają konieczność prowadzenia dalszych badań nad degradacją trwałych zanieczyszczeń środowiska.

5. Spis rysunków

6. Spis tabel

7. Literatura

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The effect of natural and biological pesticides on the degradation of synthetic pesticides

Paulina Książek-Trela, Ewa Szpyrka**

Department of Biotechnology, Institute of Biology and Biotechnology, College of Natural Sciences, University of Rzeszow, Poland

**Corresponding authors: pksiazek@ur.edu.pl; eszpyrka@ur.edu.pl*

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Abstract: Chemical plant protection methods have been used for decades. For some time now, society has paid attention to the hazards to human health resulting from the excessive use of chemical protection products. The presence of plant protection agent residues in crops causes changes in the natural environment, including biodiversity loss and the appearance of organisms harmful to plants, resistant to plant protection agents. To protect the health of humans, animals, and the environment, the principles of integrated plant protection have been introduced, giving priority to biological plant protection methods, for example, the use of biological active substances containing microorganisms (bacteria, yeasts, fungi) and natural substances. Microorganisms, as well as other natural substances, can accelerate the degradation of chemical plant protection products present in the environment and agricultural products. This review paper focuses on the effect of natural and biological pesticides on the degradation of synthetic pesticides. The most important and most perspective in integrated pest management (IPM) systems are *Bacillus* spp. and *Trichoderma* spp. because their effectiveness in pesticide degradation and the large number of commercial preparations containing these microorganisms available on the market. The application of biological pesticides recommended in IPM systems could significantly improve the quality of the soil, environment, and human health.

Keywords: pesticide; biocontrol; biodegradation; biopesticides; natural active substances

From January 1, 2014, all professional users of plant protection products (PPP) must apply the principles of the integrated plant protection scheme, according to provisions of Article 14 of Directive 2009/128/EC and of Regulation (EC) No. 1107/2009. The use of chemical plant protection products (PPP) is limited to a necessary minimum, to prevent economic losses in the yield [Council Directive 2009; Comission Regulation (EC) No. 1107/2009].

The integrated pest management scheme means the careful consideration of all available pest control techniques and the integration of measures that discourage the development of pest populations and keep pesticides to acceptable levels (Matyjaszczyk 2015).

Integrated plant protection is a method for protecting plants against harmful organisms, giving priority to the use of non-chemical methods: biological, physical and other non-chemical methods ensuring protection against pests. At the same time, it uses the accumulated knowledge on biology and the harmful effects of those organisms, as well as on antagonistic microorganisms, to safely remove pests from the environment (Korniłowicz-Kowalska 2000; Jensen 2016; Ministry of Agriculture and Rural Development 2022). Non-chemical plant protection methods include crop rotation, reasonable levels of fertilising, liming, drainage, irrigation, and the use of resistant or highly tolerant varieties (Gacek 2016).

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Biological plant protection is a method for pest control, and for the prevention of and fighting crop diseases using natural agents and beneficial organisms. Microorganisms that are agrophage competitors or pathogens are used, such as bacteria, fungi, protozoa, beneficial microorganisms (mites and predatory insects), viruses (used as ingredients of insecticide formulations), natural substances of plant (extracts) and animal origin, as well as semiochemicals (Wolny 2003; Kempka 2014).

By using biopesticides for plant protection, the development of pest resistance to chemical PPPs is prevented, and pesticide residue in the food and environment are maintained at a minimum level, improving the safety of humans and animals (Dominik & Schonthaler 2012).

Biopesticides account for about 5% of the total crop protection market. The number of biopesticides registered in the European Union (EU) is lower than that in countries, like China, India, or the United States, which results from the complex registration processes (Damalas & Koutroubas 2018).

This review paper focuses on the effect of natural and biological pesticides on the degradation of synthetic pesticides.

Table 1 presents a review of microorganisms and natural active substances approved in the European Union (as of February 2022), allocated to the relevant groups: bacteria, fungi, yeast, viruses, inorganic compounds, and other. The mechanisms of action used by the biocontrol agents, microorganisms and active substances to protect plants against pests, are also described.

Biodegradation of pesticides. Biodegradation is a process by which a pesticide is transformed into benign substances that are environmentally compatible. The degradation of pesticides can occur in plants, in the soil, and in water. Microorganisms degrading pesticides may be naturally present and/or intentionally introduced into the environment. The most common type of degradation is carried out in the soil by microorganisms, especially fungi, bacteria and yeasts, which use pesticides as a food and a source of energy. Organisms with the highest pesticide degradation activity are bacteria belonging to the *Arthrobacter, Bacillus, Corynebacterium, Flavobacterium*, *Pseudomonas* and *Rhodococcus* genera, fungi from the *Penicillium, Aspergillus, Fusarium* and *Trichoderma* genera, and the yeast species *Saccharomyces cerevisiae* (Różański 1992; Wróblewska-Krepsztul et al. 2017).

The microbiological degradation of pesticides can occur as a result of:

- enzymatic reactions – microorganisms use pesticides as a source of carbon, nitrogen and energy (fast decomposition), or these reactions are random transformations using extracellular enzymes (slow decomposition). The degradation occurs, for example, during oxidation, reduction, and hydrolysis reactions, transforming organic chemical compounds into small non-toxic molecules, i.e., carbon dioxide, nitrates, phosphates, ammonia, and water (Maloney 2001; Prabha et al. 2017; Huang et al. 2018);
- non-enzymatic reactions – changes in the environmental pH by microorganism metabolites or through the production of compounds interacting with pesticides (Różański 1992; Frąc 2019);
- cometabolism – independent cooperation of organisms in the form of substrate modification through oxidation and/or reduction, without using the product as a source of carbon and energy by that organism. The resultant product becomes available to another organism, which benefits from its decomposition. Following a change in the structure of molecules, a potentially toxic substance is removed from the environment or its toxicity is reduced. One of formulations metabolised this way is dichlorodiphenyltrichloroethane (DDT) (You et al. 1996; Baczyński et al. 2008).

The microbiological degradation of pesticides is influenced by factors including the number and type of microorganisms, metabolic activity of a given microorganism, microorganism resistance to environmental conditions, concentration and chemical structure of the processed pesticide, and environmental factors, like the pH, temperature, humidity, light, salinity, nutrients availability, and oxygen and carbon dioxide levels (Aislabie & Lloyd-Jones 1995; Huang et al. 2018). Table 2 presents a review of the literature on synthetic pesticide degradation by microorganisms and natural active substances used for plant protection, allocated to the relevant group: bacteria, fungi, yeast and inorganic compounds.

OVERVIEW OF PUBLISHED RESULTS

Microorganisms present in biopesticides are responsible for the degradation of the synthetic pesticide residue found in the environment. The literature

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Table 1. A review of natural and biological active substances used in plant protection used in the European Union*

Type	Active substance	Agent type	Mode of action**
Bacteria	*Bacillus amyloliquefaciens*	fungicide	antibiosis, effects of lytic enzymes, effective colonization of plant surfaces, competing for nutrients and induction of natural resistance mechanisms in plants
	Bacillus firmus	nematicide	production of enzymes and phytohormone, degradation of root exudates
	Bacillus pumilus	fungicide	preventing the germination of fungal spores on plants by forming a physical barrier between the spores and the leaf surface
	Bacillus subtilis	fungicide	production of substances disrupting functions of fungal cell membrane, competing for living space and nutrients, and induction of natural defence mechanisms in plants
	Bacillus thuringiensis	insecticide	gastric effect on larvae – in contact with digestive juices of a host it releases D-endotoxin that paralyses muscles of the digestive system, stopping larval trophic activity
	Pasteuria nishizawae	nematicide, insecticide	penetration of nematode epidermis, leads to the pest death
	Pseudomonas spp.	fungicide	induction of the resistance – production of siderophores leading to a restriction in plant root pathogen growth
	Spinosad – a chemical compound found in *Saccharopolyspora spinosa*	insecticide	stimulation of the insect's nervous system leading to involuntary muscle contractions and paralysis in the pest
	Streptomyces spp.	fungicide, bacteriocide	production of substances inhibiting pathogens growth: antibiotics, toxins, biosurfactants
Fungi	*Akantomyces muscarius* (formerly *Lecanicillium muscarium*)	insecticide	penetration and multiplication into the insect, causing death of the pest
	Ampelomyces quisqualis	fungicide	penetration and attack of the pathogens, degrading the cytoplasm leading to fungal death
	Aureobasidium pullulans	fungicide	microbial antagonist of fungal pathogens, production of antifungal enzymes (chitinase, glucanase), competing for nutrients, and induction of natural resistance of a host
	Beauveria bassiana	insecticide	mycelium mechanical penetration into the insect, causing loss of water and nutrients from the pest body
	Clonostachys rosea	fungicide, insecticide	hyperparasitism, secretion of enzymes
	Coniothyrium minitans	fungicide	mechanical penetration of the colonized fungus by dissolving its plasma walls, thus creating sources of infection
	Gliocladium catenulatum	fungicide	secretion of chemical substances inhibiting growth of other fungi, competing for space and nutrients
	Isaria fumosorosea	insecticide	hyperparasitism – production of enzymes disrupting insect growth and mechanical penetration of the insect body
	Metarhizium anisopliae	insecticide	attack on haemolymph by penetrating insect's epidermis resulting in loss of nutrients, physical disability and infection of the pest organs
	Phlebiopsis gigantea	fungicide	creating a natural coating on the cut or injured part of plant, preventing the invasion of pests
	Paecilomyces fumosoroseus	insecticide	spores act on a contact and germination on cuticle of insects
	Pythium oligandrum	fungicide	microbial antagonist of fungal pathogens, increase of plant resistance
	Purpureocillium lilacinum	insecticide, nematicide	secretion of the enzyme – chitinase, penetration the nematode egg shell
	Trichoderma afroharzianum	fungicide	inhibition of growth of pathogenic fungi by competing for nutrients and invasion sites, secretion of antifungal substances

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Table 1 to be continued

Type	Active substance	Agent type	Mode of action**
Fungi	*Trichoderma asperellum*	fungicide	competing for space and nutrients, mycoparasitism, production of enzymes able to degrade fungal cell walls, production of other antifungal substances and stimulation of the plant immune system
	Trichoderma atroviride	fungicide	competing for nutrients and space
	Trichoderma gamsi	fungicide	competing for space or nutrients, mycoparasitism, and secretion of enzymes able to degrade cell walls of parasites, production of antifungal substances, solubilization of inorganic components, and stimulation of the plant immune system
	Trichoderma harzianum	fungicide	parasitism, production of antibiotic substances (particularly, at low pH), competing for nutrients, secretion of enzymes dissolving the cell wall and enabling penetration into the host mycelium
	Verticillium albo-atrum	fungicide	induction of natural mechanisns of plant resistance
Yeasts	*Candida oleophila*	fungicide	competing for space and nutrients
	Cerevisane – a component of *Saccharomyces cerevisiae* cell wall	fungicide	induction of natural mechanisms of plant resistance
	Metschnikowia fructicola	fungicide	competiting for space and nutrients, secretion of chitinolytic enzymes
Viruses	*Cydia pomonella* granulosis virus	insecticide	insect tissues infected by the virus – reduced resistance to bacterial and fungal diseases
	Helicoverpa armigera nucleopolyhedrovirus	insecticide	on ingestion the virus invades the insects body, multiply leading to death
	Pepino mosaic virus	resistance inducer	virus inoculation for cross protection
	Spodoptera littoralis nucleopolyhedrovirus	insecticide	following ingestion the virus multiplies inside the insects body leading to death
	Granulovirus infecting the summer fruit tortrix (*Adoxophyes orana)*	insecticide	insect tissues infected by the virus – reduced resistance to bacterial and fungal diseases
Inorganic compounds	iron(III) phosphate	molluscicide	reduction of mucus secretion by snails; impact on calcium metabolism in slugs and snails intestine, so they stop feeding
	copper in form of copper oxychloride, tribasic copper(II) sulfate, copper(II) hydroxide	fungicide, bacteriocide	replacement of certain cations in the chitin wall (hydrogen, calcium, magnesium) with copper ions, contamination of structural and enzymatic proteins of the cell membrane – blocking of spore germination
	potassium hydrogen carbonate	fungicide, insecticide	changing conditions of the pest development by increasing the leaf surface alkalinity and the osmotic pressure on the plant surface
	sulphur	fungicide	penetrating into fungal cells through a cell membrane disrupting the osmoregulation process leading to fungal death, disrupted energy production in fungal cells
	azadirachtin A	insecticide	disrupting insect development at the pre-imago stage, inhibition of production and secretion of ecdysone, the main insect moulting hormone, repelling effect
	COS-OGA – chitooligosaccharides obtained by depolymerisation of natural chitosan and oligogalacturonides derived from natural pectins	immunity stimulator	induction of natural mechanisms of plant resistance
	green mint oil extract	growth regulator	disrupts potato sprouting by inhibiting cell growth
	ethylene	growth regulator	regulation of plant growth, development and death processes, stimulating fruit ripening
	fatty acids	insecticide, acaricide	mechanical penetration of the insect body disrupting osmoregulation and gas exchange

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Table 1 to be continued

Type	Active substance	Agent type	Mode of action**
Inorganic compounds	laminarin	immunity stimulator	stimulation of natural mechanisms of plant resistance
	paraffin oil	insecticide, acaricide	mechanical penetration of the insect body through stigmas resulting in their blocking, and in consequence, the insect death through asphyxiation, repelling effect on numerous phytophages
	orange oil	insecticide, fungicide	drying of cell walls in mycelium and spores
	rape seed oil	insecticide	forming a biofilm on the pest body surface, resulting in asphyxiation of insects and mites
	quartz sand	repellent	mechanical action, disruption by destroying insect mouthparts, and cuticle
	pyrethrins	insecticide	attacking insect nervous system, loss of movement coordination – paralysis
	sheep fat	repellent	repelling wild game with scent and flavour
	aliphatic acetate compounds	attractant	a pheromone attracting males of controlled insects

*EU Pesticides Database – https://ec.europa.eu/food/plants/pesticides/eu-pesticides-database_en

**BPDB (2022); Ciesielska et al. (2011)

review concerning the decomposition of pesticide residue indicates great interest in the use of biological methods for the degradation of active substances by bacteria, fungi, and yeasts.

Bacteria. In the case of bacteria, the biodegradation of pesticides is most frequently observed in bacteria from the *Bacillus* spp. genus. Their biodegradation ability results from the production of extracellular enzymes that act on a broad array of organic compounds (Bass & Field 2011). Enzymes participating in the biodegradation of pesticides by *Bacillus* spp. include: laccase, esterase (Gangola et al. 2018), hydrolase (Narayanan et al. 2020), carboxylesterases (Khan et al. 2016), and organophosphate hydrolase (Acharya et al. 2015). The largest number of studies focused on *Bacillus subtilis*.

The studies were concerned with the degradation of pesticides by four *B. subtilis* strains: DR-39, CS-126, TL-171 and TS-204 isolated from grapevines or the grape rhizosphere, analysed in a liquid culture, on grape berries and in vineyard soil. Each one of the four *B. subtilis* strains enhanced the degradation of profenofos in all three matrices. The results indicate that all four *B. subtilis* strains were able to degrade profenofos even when other carbon sources were available in the medium, at a level of 90% (TS-204, TL-171, CS-126) or 79% (DR-39), as compared to 52% degradation observed in the uninoculated control. The kinetics of the *in vitro* profenofos degradation showed that the half-life decreased from 12.90 days in the uninoculated spiked control to < 4.03 days in the presence of the *B. subtilis* strains (Salunkhe et al. 2013).

The *B. subtilis* KPA-1 strain was studied for the degradation of monocrotophos. It was demonstrated that the biodegradation of that pesticide is performed by the enzyme, phosphorus-organic hydrolase, resulting in 94.2% degradation of the monocrotophos in the soil in aerobic conditions (Acharya et al. 2015).

The process of quinalphos degradation by *B. subtilis* was optimised by Gangireddygari et al. (2017a). The maximum degradation of quinalphos (78.7%) was observed at a pH of 7.5 and an optimum temperature of 35–37 °C. Additional carbon (glucose) and nitrogen (yeast extract) sources marginally improved the rate of the quinalphos degradation (Gangireddygari et al. 2017a).

Other studies concerned with the *B. subtilis* 1D strain impact on the cypermethrin degradation. Under optimised growth conditions, the bacteria achieved cypermethrin degradation at a level of 95% after 15 days. The end products of the cypermethrin biodegradation under aerobic conditions were cyclododecylamine, phenol, 3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropane carboxylate, 1-decanol, chloroacetic acid, acetic acid, cyclopentan palmitoleic acid, and decanoic acid. This bacterium uses the enzyme lactase in the metabolic pathway of the cypermethrin degradation. The strain utilises cypermethrin as the sole source of carbon for its growth, which suggests

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Table 2. Review of the literature on synthetic pesticide degradation by natural and biological active substances

Type	Active substance	Degradation, references
Bacteria	*Bacillus amyloliquefaciens*	acibenzolar-*S*-methyl – 5.4–5.7 times faster degradation; metribuzin – 8–18%; napropamide – 9–11%; propamocarb hydrochloride – 15–36%; thiamethoxam – 11–22% (72 h) (Myresiotis et al. 2012)
		cypermethrin – 45% (5 days) (Lee et al. 2016)
		phoxim – 96.1% (48 h) (Meng et al. 2019a)
		chlorpyrifos – 18.7%; dichlorvos – 53%; dipterex – 5.5%; phoxim – 68.3%; triazophos – 96.3% (1 h) (Meng et al. 2019b)
		carbendazim – 41.8% (6 days) (Li et al. 2019)
	Bacillus firmus	fipronil – 100% (Mandal et al. 2014)
	Bacillus pumilus	acibenzolar-*S*-methyl – 5.4–5.7 times faster degradation; metribuzin – 8–18%; napropamide – 9–11%; propamocarb hydrochloride – 15–36%; thiamethoxam – 11–22% (72 h) (Myresiotis et al. 2012)
		chlorpyrifos and its metabolite product TCP – 90% (8 days) (Anwar et al. 2009)
	Bacillus subtilis	profenofos – 79–90% (Salunkhe et al. 2013)
		monocrotophos – 94.2% (Acharya et al. 2015)
		quinalphos – 78.7% (2 days) (Gangireddygari et al. 2017a)
		dimethomorph – 83.3–85.7% (15 days); 85.8–90.9% (30 days); carbendazim – 78.8–84.2% (15 days); 84.1–88.1% (30 days); thiophanate methyl – 88.9–94% (15 days); 98.3–98.9% (30 days); hexaconazole – 66.2–67.8% (15 days); 79.4–84.1% (30 days); tetraconazole – 79.3–83.9% (15 days); 94.6–95.2% (30 days); myclobutanil – 76.6–79.6% (15 days); 95.7–99.8% (30 days); buprofezin – 64.7–81.7% (15 days); 74.5–86.7% (30 days); benzoate emamectrin – 75.9–84% (15 days); 100% (30 days) (Suryawanshi et al. 2018)
		penthiopyrad – 5% (14 days) (Podbielska et al. 2020)
		acibenzolar-*S*-methyl – 5.4–5.7 times faster degradation; metribuzin – 8–18%; napropamide – 9–11%; propamocarb hydrochloride – 15–36%; thiamethoxam – 11–22% (72 h) (Myresiotis et al. 2012)
		cypermethrin – 95% (15 days) (Gangola et al. 2018)
		boscalid – 52%; pyraclostrobin – 41% (Podbielska et al. 2018)
	Bacillus thuringiensis	fipronil and its metabolites (Mandal et al. 2013)
		malathion – 50% (3 days) (Kamal et al. 2008)
		quinalphos (Gangireddygari et al. 2017b)
	Pseudomonas spp.	aldrin, chlorpyrifos, coumaphos, DDT, diazinon, endosulfan, endrin, hexachlorocyclohexane, parathion-methyl, monocrotophos, parathion (Huang et al. 2018)
		chlorpyrifos – 87% (20 days); 92% (30 days); atrazine, parathion, carbofuran (Gilani et al. 2016)
		endosulfan – 100% (12 days) (Jesitha et al. 2015)
		aldrin – 43% (12 days) (Doolotkeldieva et al. 2018)
		pentachlorophenol – 70%; chlorpyrifos – 75% (Arunachalam & Velmurugan 2010)
		quinalphos – 90.4% (Pawar & Mali 2014)
		permethrin, cypermethrin – 90% (15 days) (Mendoza et al. 2011)
		endosulfan – 50% (3 days) (Shivaramaiah & Kennedy 2006)
		diazinon – 83.6% (14 days) (Essa et al. 2016)
	Streptomyces spp.	diuron – 95% (5 days); 100% (10 days) (Castillo et al. 2006); cypermethrin – 100% (24–30 h) (Lin et al. 2011); β-cypermethrin – 23–37.5%; 73.1% (24 h) (Chen et al. 2012); lindane – 50–80% (20 h) (Benimeli et al. 2006); chlordane – 99.8% (24 h) (Cuozzo et al. 2012); chlorpyrifos – 90% (24 h) (Briceno et al. 2012); carbofuran – 66.5–95.3% (10 days) (Jayabarath et al. 2010); alachlor – 60–75% (14 days); 50% (7 days) (Sette et al. 2004; Sette et al. 2005); aldrin – 90% (72 h) (Benimeli et al. 2003); methoxychlor – 40–76% (28 days) (Bourguignon et al. 2014); diazinon – 10.8–31.7%; 13–61.7% (Briceno et al. 2016)

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Table 2 to be continued

Type	Active substance	Degradation, references
Fungi	*Aureobasidium pullulans*	chlorobenzoate herbicides (Rajendran & Gunasekaran 2006)
	Metarhizium anisopliae	diazinon – 68.2–85.6%; profenofos – 54.7–63.7%; malathion – 83.8–91% (20 days) (Abd El-Ghany & Masmali 2016)
		chlorpyrifos, cypermethrin > 80% (21 days) (Ong et al. 2019)
	Purpureocillium lilacinum	glyphosate – 80% (7 days) (Spinelli et al. 2021)
	Trichoderma atroviride	dichlorvos – 56.7% (24 h) (Sun et al. 2001)
		dichlorvos – 96% (Tang et al. 2009)
		carbendazim – 21% (5 days) (Sharma et al. 2016)
	Trichoderma harzianum	carbendazim – 85% (5 days) (Sharma et al. 2016)
		chlorpyrifos, ethion – 70–80% (21 days) (Harish et al. 2013)
		chlorpyrifos – 97.9% (10 days) (Jayaraman et al. 2012)
		DDT – 83%; dieldrin – 74%; endosulfan – 61%; pentachloronitrobenzene (PCNB) – 52%; pentachlorophenol (PCP) – 12% (Katayama & Matsumura 1993)
		chlorfenvinphos – 60% (165 days) (Oliveira et al. 2015)
		clomazone – 24.2%; fluazifop-*P*-butyl – 24.8%; metribuzin – 23.5% (Szpyrka et al. 2020)
		carbofuran – 81.5% (14 days) (Afify et al. 2012)
		bromoxynil – 45.6–60.3% (3 days) (Askar et al. 2007)
		oxamyl – 72.5% (10 days) (Afify et al. 2013)
		pentachlorophenol – 100% (7 days) (Vacondio et al. 2015)
		penthiopyrad – 34.2% (3 days); 56.9% (14 days) (Podbielska et al. 2020)
		boscalid – 52%; pyraclostrobin – 41% (Podbielska et al. 2018)
		diazinon – 84.8–91.8%; profenofos – 71.1–84.0%; malathion – 90.6–91.8% (20 days) (Abd El-Ghany & Masmali 2016)
Yeasts	Cerevisane – a component of *Saccharomyces cerevisiae* cell wall	azoxystrobin, pyraclostrobin, iprodione, boscalid (Wołejko et al. 2016)
		glyphosate – 21% (24 h) (Low et al. 2005)
		thiram (Kitagawa et al. 2002)
		atrazine, terbuthylazine (Hack et al. 1997)
		pirimiphos-methyl – 48.8% (72 h) (Đorđević & Đurović-Pejčev 2016)
Inorganic compounds	copper	cypermethrin $t_{1/2}$ 115.5 min → $t_{1/2}$ 9.0 min; cyhalothrin $t_{1/2}$ 173.3 min → $t_{1/2}$ 115.5 min (Liu et al. 2007)
		lambda-cyhalothrin $t_{1/2}$ 182.4 → $t_{1/2}$ 59.2 (Rafiq et al. 2012)
		chlorpyrifos, chlorpyrifos-methyl (Blanchet & St-George 1982)

the adaptation of *B. subtilis* to the oligotrophic environment (Gangola et al. 2018).

The disappearance of boscalid and pyraclostrobin residue in apple fruit was investigated using an organic fertiliser enriched with strains of the antagonistic microorganisms *Bacillus* spp., *Trichoderma* spp. and mycorrhizal fungi *Glomus* spp. The residue levels of boscalid and pyraclostrobin were reduced by 52% and 41%, respectively, versus the control. This organic fertiliser simulates plant growth and development, improves the soil fertility, and promotes the healthy growth of roots and the above ground parts of plants. In orchards, it increases the plants natural resistance, improves the quality of the fruit during storage, and reduces the levels of chemical plant protection product residue (Podbielska et al. 2018).

Suryawanshi et al. (2018), in 2017–2018, conducted extensive research on the influence of the *B. subtilis* DR-39 formulation on enhancing the pesticide degradation in grapes, for dimethomorph, carbendazim, thiophanate methyl, hexaconazole, tetraconazole, myclobutanil, buprofezin, and emamectin benzoate. They demonstrated that the degradation was similar throughout those years; however, a faster degradation rate was observed during the initial

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period of 15 days, versus the period of 15–30 days. The application of *B. subtilis* DR-39 at a dose of 2.5 g/L reduced the calculated half-life of the pesticides by 1–3 days, except for buprofezin and hexaconazole, where it was reduced by 5 and 6.5 days, respectively, in 2016–2017, and for hexaconazole, with a reduction by 6 days in 2017–2018. Studies show that *B. subtilis* DR-39 applications in vineyards can be utilised for the faster degradation of multi-class pesticide residue (Suryawanshi et al. 2018).

The interactions of *B. subtilis* GB03, *B. subtilis* FZB24, *B. amyloliquefaciens* IN937a and *B. pumilus* SE34 with acibenzolar-*S*-methyl, metribuzin, napropamide, propamocarb hydrochloride and thiamethoxam at two concentrations in a liquid culture and in soil microcosm were studied. The 72-h studies showed that the degradation of acibenzolar-*S*-methyl at low (1.0 mg/kg) and high (10.0 mg/kg) concentrations was 5.4 and 5.7 faster, respectively, versus the control. The interactions between those species also influenced the degradation of metribuzin (8–18%), napropamide (9–11%), propamocarb hydrochloride (15–36%), and thiamethoxam (11–22%) (Myresiotis et al. 2012).

The bacterial strain *B. pumilus* C2A1 isolated from the soil can degrade chlorpyrifos and its hydrolysis metabolite 3,5,6-trichloro-2-pyridinol (TCP). The research was conducted under different culture conditions: pH, inoculum density, presence of added carbon/nutrient sources and pesticide concentrations. The pesticide was utilised by the microorganisms as the sole source of carbon and energy. The maximum pesticide degradation was observed at a high pH (8.5) and a high inoculum density. The strain C2A1 showed 90% degradation of TCP (300 mg/L) within 8 days of incubation (Anwar et al. 2009).

Furthermore, the influence of *B. amyloliquefaciens* on the degradation of other pesticides was studied. Lee et al. (2016) demonstrated the cypermethrin degradation at a level of 45% in 5 days, using the *B. amyloliquefaciens* APO1 strain in the mineral medium. Furthermore, when 2% glucose was added to the medium, the rate of cypermethrin degradation by the AP01 strain increased to about 60%. Therefore, AP01 may serve as a promising strain in the bioremediation of soils polluted with cypermethrin (Lee et al. 2016).

Meng et al. (2019a) demonstrated the phoxim degradation at a level of 96.1% using the *B. amyloliquefaciens* YP6 strain. The bacterium uses phoxim as the sole source of phosphorus. The optimum biodegradation conditions were 40 °C, a pH of 7.20, and an inoculum size of 4.17% (v/v) (Meng et al. 2019a).

To expand the range of biodegrading enzymes, alkaline phosphatase (AP3): from *B. amyloliquefaciens* YP6 was characterised and used to test its effectiveness in the degradation of five phosphorus-organic pesticides. AP3 reached its optimal activity at 40 °C and a pH of 10.3. The study was conducted for 1 h, revealing the biodegradation of 96.3% of triazophos, 68.3% of phoxim, 53% of dichlorvos, 18.7% of chlorpyrifos and 5.5% of dipterex (Meng et al. 2019b).

Another enzyme that can biodegrade pesticides is hydrolase HY-1 from *B. amyloliquefaciens.* Studies were performed in food, and it was demonstrated that this enzyme degraded 41.8% of carbendazim from the cucumber surface during a 6-day study (Li et al. 2019).

B. thuringiensis is another bacterium from the *Bacillus* spp. genus, responsible for the degradation of pesticides. The *B. thuringiensis* isolate (MOS-5) was evaluated during the incubation time of 30 days. The removal of a considerable amount of malathion after three days of incubation was observed. For instance, in an inoculated salt media, more than 50% of malathion was degraded to other compounds. After one week of incubation, the residual malathion levels decreased to 26.5% and reached 0.68% after 30 days of incubation. MOS-5 was able to utilise malathion as the sole carbon and energy source and to degrade it cometabolically (Kamal et al. 2008).

B. thuringiensis was used in studies on quinalphos degradation. It was studied whether an additional source of carbon and nitrogen, the inoculum density, pesticide concentration, pH and the temperature influenced the level of the pesticide degradation. The maximum degradation of quinalphos was observed with an inoculum of 1.0 optical density (OD), an optimum pH (6.5–7.5), and an optimum temperature of 35–37 °C (Gangireddygari et al. 2017b).

B. firmus can degrade fipronil under laboratory conditions. Soil samples were fortified with fipronil at concentrations of 0.50–1.50 mg/kg and inoculated with *B. firmus* cells. The study was conducted for 56 days. Pesticide residue was not detected after 35 days at lower doses of fipronil (0.50, 0.75, 100 mg/kg), and at higher concentrations (1.25 and

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1.50 mg/kg), and the pesticide had completely degraded after 42 days (Mandal et al. 2014).

Several reports are available concerning the influence of bacteria from the *Pseudomonas* spp. genus on pesticide residue. *Pseudomonas* spp. is a diversified genus with a great deal of catabolic pathways and enzymes involved in pesticide degradation. In the case of chlorpyrifos, *Pseudomonas* was found to have a high degradation potential due to the widespread environmental adaptability, and it was followed by *Agrobacterium* and *Bacillus* (Abo-Amer 2012; Gilani et al. 2016).

Enzymes participating in the pesticide biodegradation by bacteria from the *Pseudomonas* genus include: oxidoreductases (Gox), cytochrome P450, dioxygenases (TOD), phosphotriesterases, and carboxylesterases (Ortiz-Hernandez et al. 2013; Saafan et al. 2016).

Pseudomonas ATCC 700113 degraded chlorpyrifos by reductive dechlorination to 3,5,6-trichloro-2-pyridinol (TCP) and then to carbon dioxide, water, chloride and ammonium (Feng et al. 1998). The chlorpyrifos degradation was noted at a level of 87% after 20 days and of 92% after 30 days (Gilani et al. 2016). *Pseudomonas* exhibits maximum degradation at a pH of 8 because at a high pH, enzymes have optimum activity and are able to degrade chlorpyrifos (Swetha & Phale 2005). *Pseudomonas* is adapted the best to low pesticide concentrations. The degradation of chlorpyrifos decreases with an increase in its concentration because the high concentrations affect the microorganisms involved in the degradation. Li et al. (2007) reported that when the concentration exceeds 200 mg/L, bacteria grow slowly and also stop degrading the intermediate TCP, while they function normally at low concentrations (Li et al. 2007).

P. fluorescens was studied for the degradation of endosulfan. It was demonstrated that this bacterium uses the pesticide as the sole source of carbon and energy. Endosulfan was completely degraded after 12 days. The formed metabolites implied that this pesticide is decomposed by hydrolysis (Jesitha et al. 2015).

In the studies on aldrin degradation by *P. fluorescens*, it was demonstrated that this microorganism degrades the pesticide using cytochrome P450 hydroxylase. The studied bacterium degraded 43.2% of aldrin during the 12-day study (Doolotkeldieva et al. 2018).

The level of the quinalphos degradation by *Pseudomonas* species isolated from the grape rhizosphere soils was determined. A total number of 14 *Pseudomonas* strains were isolated and screened for their tolerance to four concentrations of quinalphos (5 mg/L, 10 mg/L, 15 mg/L and 20 mg/L). The results indicated that only one strain could degrade the highest concentration of quinalphos at a level of 15 mg/L and 20 mg/L. The results showed that the isolated strain could degrade quinalphos at a level of up to 90.4% in the presence of glucose, and of up to 38.2% in the absence of glucose. This finding may be related to the role of glucose as an inducer of organism growth (Pawar & Mali 2014).

P. aeruginosa was isolated from agricultural drainage ditches (Fayoum, Egypt). A pure culture of *P. aeruginosa* was grown in a minimal medium supplemented with diazinon as the sole carbon source. The temperature and pH influence on the bacterial growth and the rate of diazinon degradation were investigated. The maximum diazinon degradation capability (83.6%) was achieved at a pH value of 7.0 and a temperature of 30 °C within 14 days (Essa et al. 2016).

P. putida and *P. mendocina* strains have great capacity for biodegrading permethrin and cypermethrin pesticides. The bioremediation of up to 90% can be achieved with the help of these bacterial strains within a period of 15 day (Mendoza et al. 2011).

Pseudomonas bacteria can degrade endosulfan. Endosulfan is metabolised into endosulfan sulfate, which is the only product of the endosulfan metabolism by bacteria. It resulted in 50% degradation of endosulfan within three days (Shivaramaiah & Kennedy 2006).

There are several reports of the effect of bacteria from the *Streptomyces* spp. genus on pesticide residue. *Streptomyces* spp. are able to remove organic and inorganic pollutants, such as: hydrocarbons, pesticides, aliphatic and aromatic compounds, making them good tools for the bioremediation process (Sambasiva Rao et al. 2012). Microorganisms represent an efficient source of oxidoreductases and hydrolytic enzymes. Amylases, protease, cellulase, xylanase, esterase, nitrile hydratase, laccase, dehydrogenase are some enzymes that could be involved in the pesticide degradation (Kariga & Rao 2011). *Streptomyces* spp. have the ability to grow and degrade several chemical families of pesticides, including: organochlorines, organophosphates, pyrethroids, ureas and chloroacetanilides (Briceno et al. 2013; Alvarez et al. 2017).

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Sette et al. (2004) and Sette et al. (2005) isolated 53 *Streptomyces* strains from alachlor contaminated soils. Sixteen strains were able to grow at a high pesticide concentration (720 mg/L) and six strains were able to degrade over 50% (72 mg/L) of alachlor from a mineral salt medium after 7 days (Sette et al. 2004; Sette et al. 2005).

Castillo et al. (2006) studied the biodegradation of diuron by 17 strains of *Streptomyces* spp. isolated from soils. All the strains were able to degrade the pesticide, but to different levels. Twelve strains degraded the herbicide by up to 50% and four of them by up to 70%. The study was conducted in a medium with 4 mg/L of diuron. *S. albidoflavus* A7-9 was the most efficient microorganism in the degradation of diuron, achieving 95% degradation after five days and no herbicide residue after 10 days (Castillo et al. 2006).

Lin et al. (2011) isolated *Streptomyces* sp. HU-S-01 from wastewater sludge and studied the degradation of cypermethrin by this microorganism. At pesticide concentrations lower than 50 mg/L, the strain completely degraded cypermethrin within 30 hours. At higher concentrations – 150–250 mg/L, the degradation of the pesticide was incomplete even after 48 h of incubation. The optimum degradation condition was at a temperature of 26–28 °C and a pH of 7.5. The influence of *Streptomyces* sp. HU-S-01 on the degradation of the major metabolite, 3-phenoxybenzoic acid (3-PBA), of cypermethrin hydrolysis in soil and water was also investigated. HU-S-01 completely degraded 3-PBA at a concentration 50 mg/L, but the degradation rate of the metabolite was slower than cypermethrin. Complete degradation occurred after 96 hours (Lin et al. 2011).

The degradation of chlorpyrifos (CP) by *Streptomyces* spp. isolated from agricultural soil was investigated. Two strains were selected because of their tolerance to 50 mg/L of CP. *Streptomyces* sp. AC5 and *Streptomyces* sp. AC7 were cultivated in a medium with CP at concentrations of 25 mg/L and 50 mg/L for 72 hours. Both strains were able to degrade CP with about 90% degradation after 24 hours. *Streptomyces* sp. AC5 degrade CP into 3,5,6-trichloro-2-pyridinol (TCP) reaching the maximum metabolite concentration of 0.46 mg/L, while the concentration of TCP was 1.31–4.32 mg/L for *Streptomyces* sp. AC7 (Briceno et al. 2012).

Another study was concerned with the degradation of carbofuran by three isolates: *S. alanonosinicus*, *S. album* and *S. atratus*, which were able to resist the pesticide and showed growth on a medium with a pesticide concentration of 20 µg/L. After 10 days, 65.5%, 64.9% and 34.8% of carbofuran was degraded by *S. alanonosinicus*, *S. atratus* and *S. album*, respectively (Jayabarath et al. 2010).

Bourguignon et al. (2014) isolated *Streptomyces* strains from pesticide-contaminated sediments. The microorganisms were able to grow in the presence of 1.66 mg/L of methoxychlor (MTX). The MTX degradation by *Streptomyces* sp. A14 was at level of 40% and 76% for the pesticide concentrations of 8.33 and 16.6 mg/kg (28 days), respectively. *Streptomyces* sp. A14 showed the best growth in the presence of MTX in the culture medium at 30 °C and a pH of 7 (Bourguignon et al. 2014).

Fungi. Fungi generally biotransform pesticides and other xenobiotics by introducing minor structural changes to the molecule, rendering it nontoxic. The biotransformed pesticide is released into the environment, where it is susceptible to further degradation by bacteria (Diez 2010).

Fungi tolerate high levels of pollutants better than bacteria (Evans & Hedger 2001). With characteristics such as specific biodegradation and/or growth morphology, fungi degrade the pollutants more effectively (Mollea et al. 2005).

Three possible mechanisms for pesticides degradation by fungi have been proposed: (1) the pesticide is used as a source of carbon or nitrogen by capture or extracellular decomposition of a compound; (2) cometabolism effected by enzymes secreted by the fungi, in which the pesticide is not used by a given fungus as a source of carbon and energy; (3) a detoxication mechanism in fungi exposed to toxic compounds (Ellegaard-Jensen 2012).

The fungi use enzymes such as extracellular oxidases and peroxidases, including laccases, manganese peroxidases, aryl alcohol oxidases, and lignin peroxidase, for the biodegradation of pesticides (Paszczyński & Crawford 2000; Novotny et al. 2004).

Fungi from the *Trichoderma* spp. genus are the most popular biocontrol agents (BCAs) known worldwide for their great ability to combat different soil and foliar diseases of agricultural crops. *Trichoderma* spp. are used in biopesticides because of their ability to destroy other fungi, induce resistance to plant pathogens, improve plant growth, solubilise plant nutrients, and bioremediate heavy metals and environmental pollutants, such as pesticides (Szpyrka et al. 2020). For the biodegradation

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of pesticides, *Trichoderma* spp. use mechanisms such as nutrient competition, antibiosis, the activity of cell wall-lytic enzymes, the induction of systemic resistance, and increased plant nutrient availability (Ene & Alexandru 2008). In studies on biodegradation of pesticides, the researchers' attention has been drawn to a species of fungi, *Trichoderma harzianum*.

Another study was concerned with the impact of *T. harzianum* and *Rhizopus nodosus* isolated from the contaminated soil on the degradation of two phosphorus-organic pesticides, chloropyrifos and ethion. The fungi were able to degrade 70–80% of the pesticides within 21 days of the incubation period. Furthermore, the degradation efficiency increased by 10–20% with the supplementation of 0.1% dextrose to the mineral media (Harish et al. 2013).

The *T. harzianum* influence on the decomposition of five pesticides: DDT, dieldrin, endosulfan, pentachloronitrobenzene (PCNB), and pentachlorophenol, was demonstrated, with a degradation level of 83%, 74%, 61%, 52%, and 12%, respectively. The discussed publication focused, in particular, on endosulfan degradation. The initial metabolic product of the endosulfan decomposition was endosulfan sulfate, and the oxidative system is the major enzyme system in *T. harzianum* responsible for the endosulfan degradation (Katayama & Matsumura 1993).

Another study was concerned with the degradation of several pesticides exposed to a mixture of fungi: *Penicillium citrinum, Aspergillus fumigatus, A. terreus* and *T. harzianum*, found in the aquatic environment. The analyses were conducted for 165 days and demonstrated that those microorganisms are able to degrade 60% of chlorfenvinphos, versus the control (Oliveira et al. 2015).

The influence of the commercially available biological fungicide containing *T. harzianum* Rifai T-22 on the dispersion and degradation kinetics of five herbicides: clomazone, fluazifop-*P*-butyl, metribuzin, pendimethalin and propyzamide, was studied in two types of soil. The study results showed that *T. harzianum* T-22 influences the degradation of pesticides and the dispersion kinetics of non-persistent herbicides: clomazone, fluazifop-*P*-butyl, and metribuzin. In the soil with a higher content of nitrogen, phosphorus and organic matter, the degradation increased by 24.2%, 24.8% and 23.5% for clomazone, fluazifop-*P*-butyl, and metribuzin, respectively. In the soil with a lower organic content, the degradation was at a low level, at 16.1%, 17.7% and 16.3% for clomazone, fluazifop-*P*-butyl, and metribuzin, respectively. The results obtained for more persistent pesticides: propyzamide and pendimethalin were not advantageous (Szpyrka et al. 2020).

Another area of investigation was the effect of gamma radiation on the carbofuran degradation by *T. harzianum*. The analysed pesticide was transformed into 3-ketocarbofuran. Fungus samples were collected and exposed to different doses of gamma radiation. Fungi from the 7-day-old culture were irradiated with doses of 0.0, 0.02, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, and 5.0 kGy. It was demonstrated that the radiation influenced the level of the pesticide degradation. During exposure to the gamma radiation, the carbofuran degradation by *T. harzianum* was at a level of 76.5% and 85% in sterile and non-sterile soils, respectively, during 14 days of incubation (Afify et al. 2012). These results were compared to earlier studies, where *T. harzianum* was not exposed to radiation, and the degradation was at a level of 75% and 81.5% in sterile and non-sterile soils, respectively, during 14 days of incubation (Afify & Shousha 1988).

Similar studies were also conducted on the influence of gamma radiation on the oxamyl decomposition. The results indicated that *Trichoderma* spp. used oxamyl as source of carbon and nitrogen, and had enzyme(s) acting on the amide and ester bond in the oxamyl structure. The oxamyl degradation by *T. harzianum* strain reached 72.5% within 10 days of incubation (Afify et al. 2013).

Furthermore, the *T. harzianum* influence on the bromoxynil decomposition was determined. The pesticide levels exposed to that fungus dropped to 45.61, 21.25, 20.63, 10.49, and 1.56 ppm after 3, 7, 14, 21, and 28 days of incubation, respectively (with an initial level of 100 ppm). Less than 2% of the initial concentration was detected after 28 days (Askar et al. 2007).

Vacondio et al. (2015) studied the degradation of pentachlorophenol (PCP) and its metabolite, 2,3,4,6-TeCA, by *T. harzianum* CBMAI 1677. After seven days, these compounds were completely decomposed. Enzymes, such as phenoloxidases and cellobiose dehydrogenase, operating simultaneously with ligninolytic enzymes degrade PCP (Vacondio et al. 2015).

The influence of *T. harzianum* on the degradation of phosophorus-organic pesticides: diazinon, profenofos and malathion, was studied for different pes-

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ticides concentrations, incubation periods and temperatures. *T. harzianum* degraded 91.8% (10 mg), 98.2% (20 mg) and 84.8% (40 mg) of diazinon within 20 days. Profenofos was degraded by 78.3%, 84.0% and 71.1% at concentrations of 10 mg, 20 mg, and 40 mg, respectively, after 20 days. In the case of malathion, 90.6%, 91.8% and 90.6% of its initial concentration were degraded within 20 days for the initial concentrations of 10 mg, 20 mg, and 40 mg, respectively. The optimal temperature for the pesticides degradation by *T. harzianum* is 35 °C. *T. harzianum* proved to be more effective in the degradation of those pesticides than *Metarhizium anisopliae* (Abd El-Ghany & Masmali 2016).

Furthermore, the influence of *T. harzianum, T. viride* and *T. atroviride* on the degradation of carbendazim in three concentrations of 100, 150 and 200 ppm was studied. After five days of incubation, the percentage biodegradation of carbendazim was 85% for *T. harzianum*, 47% for *T. viride*, and 21% for *T. atroviride* (Sharma et al. 2016).

The *T. atroviride* T23 strain was used to degrade dichlorvos. The pesticide was degraded in 56.7% to the following compounds: dichloroacetic acid, 2,2-dichloroethanol and phosphoric acid. The enzyme responsible for 2,2-dichlorovinyl dimethyl phosphate (DDVP) degradation was organophosphorus hydrolase. The highest activity of this enzyme was achieved at 35 °C, and the optimal pH was 8.5. The results showed that organophosphorus hydrolase provided a clue for the comprehensive understanding the mechanism underlying the organophosphorus pesticide degradation by filamentous fungi (Sun et al. 2001).

Other studies involved the application of *T. atroviride* transformants with the enzyme hygromycin B phosphotransferase to analyse the dichlorvos degradation. The transformants were characterised by an increased ability to degrade dichlorvos. Of 247 transformants, 76% showed improved a dichlorvos degradation ability when compared to the parent strain T23. Among them, eight transformants exhibited a 30% higher degradation rate than the parent isolate. The highest dichlorvos degradation rate achieved by the transformants was up to 96% (Tang et al. 2009).

Studies were also conducted on the influence of a fungus, *Metarhizium anisopliae*, on the degradation of pesticides, due to its ability to promote biological control in the environment, especially in crops. In was found that *M. anisopliae* shows high resistance to herbicides, acaricides and insecticides in the environment, so this fungus can be used together with chemical plant protection products to protect plants, and for further studies on the degradation of pesticides in the environment (Mochi et al. 2005).

The influence of *M. anisopliae* on the degradation of phosophorus-organic pesticides: diazinon, profenofos and malathion, was assessed for different pesticides concentrations, incubation periods and temperatures. The study results showed that profenofos, diazinon and malathion degradation increased with an increase in the incubation time, but, at the same time, decreased with an increase in the initial concentrations of those insecticides. *M. anisopliae* degraded 85.6% (10 mg), 77.2% (20 mg) and 68.2% (40 mg) of diazinon within 20 days. Profenofos was degraded at 54.7%, 62.4% and 63.7% at concentrations of 10 mg, 20 mg, and 40 mg, respectively, after 20 days. In the case of malathion, 89.2%, 91.0% and 83.8% of its initial concentration were degraded within 20 days for initial concentrations of 10 mg, 20 mg, and 40 mg, respectively. The optimal temperature for the pesticides degradation by *M. anisopliae* is 30 °C (Abd El-Ghany & Masmali 2016).

On the basis of the above studies, Ong et al. (2019) proposed the use of *M. anisopliae* for the biodegradation of two pesticides: cypermethrin and chlorpyrifos in the soil. The study was conducted for 21 days, and demonstrated that the degradation of those two insecticides exceeded 80% and was higher versus the control (47–61%). The chlorpyrifos and cypermethrin residues in *M. anisopliae* treated soils amounted to 19.39 ± 0.10 ppm and 19.68 ± 0.36 ppm, respectively, and were significantly lower than in the control (residue levels of 262.6 ± 7.6 ppm for chlorpyrifos and of 194.4 ± 4.3 ppm for cypermethrin, at $P < 0.05$) (Ong et al. 2019).

The literature review shows that only one research paper on the effect of a yeast-like fungus *Aureobasidium pullulans* on the degradation of pesticides has been published so far. *A. pullulans* is an effective agent for the biological control of storage diseases in many species of fruit, including apples (Castoria et al. 2001; Schena et al. 2003). Thus, an interest in further studies on the degradation of pesticides by this fungus may be stimulated (Vero et al. 2009). Rajendran and Gunasekaran (2006) found that *A. pullulans* degrades chlorobenzoic herbicides (Rajendran & Gunasekaran 2006).

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Yeast. Yeasts of the *Saccharomyces cerevisiae* species, whose cell wall is an active substance of biological plant protection agents, were also studied. *S. cerevisiae* is particularly active in the quick conversion of sugars into alcohol and carbon dioxide, thus contributing to the limited availability of nutrients for other organisms inhabiting the plant organs. Additionally, it is capable of producing so-called toxin killers which, as protein complexes, exhibit very strong inhibitory properties against the pathogens present in the same environment (Wołejko et al. 2016).

It was demonstrated that *S. cerevisiae* is responsible for the glyphosate degradation during the fermentation cycle of the breadmaking process, where 21% of the pesticide was degraded within 1 h (Low et al. 2005).

Other studies on *S. cerevisiae* concerned the degradation behaviour of pirimiphos methyl in wheat during fermentation. Yeast fermentation was especially effective for the reduction of pirimiphos methyl applied at 5 mg/kg (the maximum residue limit), causing a maximum dissipation of 48.8% (72 h). The pesticide reduction rate decreased with an increase in the fortification rate. Thus, in samples fortified with 25 and 75 mg/kg of the yeast, a reduction of up to 27.1%, and 23.7% was observed, respectively (Đorđević & Đurović-Pejčev 2016).

It was proven that the yeast *S. cerevisiae* is responsible for the biodegradation of*:* azoxystrobin, pyraclostrobin, iprodione, boscalid (Wołejko et al. 2016), thiram (Kitagawa et al. 2002), atrazine, and terbuthylazine (Hack et al. 1997).

Other. Following the literature review, it was found that studies on the influence of pesticide degradation were only conducted for copper among the inorganic and other substances used in biological and natural preparations.

Liu et al. (2007) analysed the effect of copper on cypermethrin and cyhalothrin in the soil and on their photodegradation in the aquatic system. In the soil, the degradation of pesticides was not accelerated. For the photodegradation, the half-life decreased from $t_{1/2}$ 173.3 min to $t_{1/2}$ 115.5 min for cyhalothrin, and from $t_{1/2}$ 115.5 min to $t_{1/2}$ 99 min for cypermethrin. The results suggested that copper influenced the degradation of the pesticides in the soil by affecting the activity of the microorganisms. However, it had a catalyst effect on the photodegradation in the water system. The difference was also observed for the efficiency of the degradation of the pyrethroid isomers in the soil. Copper could obviously accelerate the degradation of certain isomers (Liu et al. 2007; Wang et al. 2007).

The influence of copper on the photodegradation of two pesticides, imidacloprid and lambda-cyhalothrin, commonly used in cotton crops, was also investigated. For this purpose, different concentrations of pesticides were irradiated in a UV photoreactor with a wavelength of > 300 nm at different time intervals, i.e., 0–960 minutes. The Cu effect on photodegradation was studied, by adding Cu to the pesticide solution. After irradiation, the remaining concentrations of the pesticides were determined. The study showed that the photodegradation rate of lambda-cyhalothrin increased in the presence of Cu. This was confirmed by the reduction of the lambda-cyhalothrin half-time $t_{1/2}$, from 182.36 min to 59.2 min in the presence of Cu. On the contrary, the imidacloprid photodegradation is delayed after the addition of Cu. This was probably caused by the pesticide stabilisation through complexation with metal ions (Rafiq et al. 2012).

Furthermore, the influence of copper on chlorpyriphos and chlorpyriphos-methyl in buffered water solutions was demonstrated. As the concentration of copper(II) increased, the rate of hydrolysis increased until the concentration of copper(II) reached about 1.0×10^{-2} M. At this point, the rate of hydrolysis became independent of the copper(II) concentration. During the study on the pesticide degradation by copper, the presence of copper-pesticide complexes, an intermediate product in the hydrolysis reaction, was observed. The copper(II) ion forms a six-membered ring complex with nitrogen in the ring structure and sulfur of phosphorothioate moiety from the chlorpyrifos and chlorpyrifos–methyl (Blanchet & St-George 1982).

In Figure 1, the summary of the information included in Table 2 can be seen. All the microorganisms presented in this study, which have the potential of pesticide degradation are shown with the number of pesticides tested and their maximum degradation.

CONCLUSION

The use of natural and biological agents in environmental protection is associated with a number

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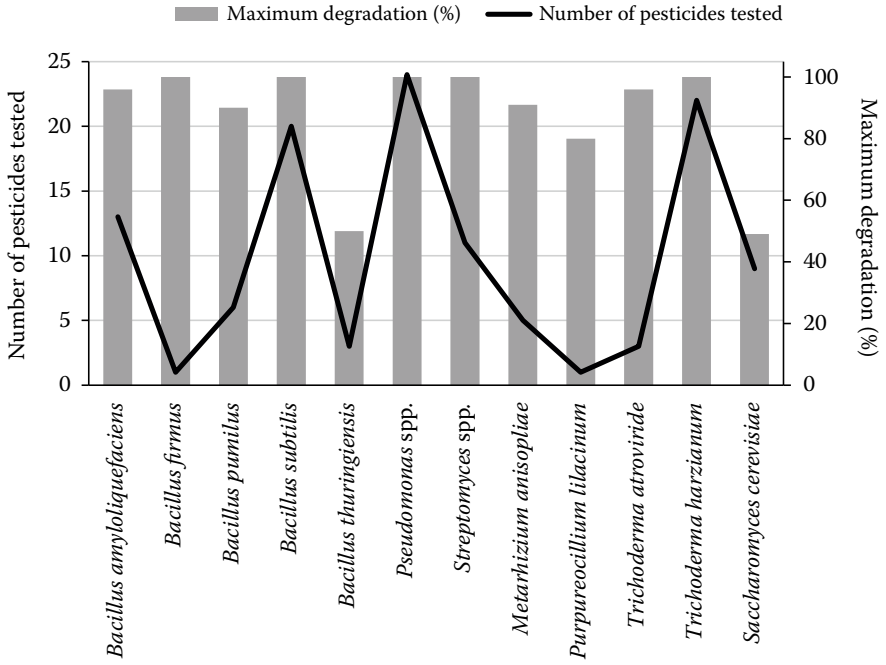

Figure 1. Number of pesticides tested and their maximum degradation (%) for the particular microorganisms

of advantages, including the prevention of pest resistance developing to chemical PPPs, an increase in the yield, an increase in the biodiversity, and the faster decomposition of toxic pollutants. The effects of these agents result, among other things, from the phenomenon of antibiosis, competing for space and nutrients, antagonism, and stimulation of host's natural resistance mechanisms. Biopesticides containing, e.g., microorganisms, are responsible for the degradation of pesticide residue in the environment. The most common mechanism underlying the biodegradation conducted by microorganisms is the decomposition of substances by enzymes secreted outside the cell, which degrade chemical compounds to their less toxic or non-toxic derivatives. The review of the literature on the biodegradation of pesticides by microorganisms implies a significant interest in this subject. The largest body of literature data concerns bacteria from the *Bacillus* spp. and *Pseudomonas* spp. genera, fungi from the *Trichoderma* spp. genus and the yeast *S. cerevisiae*. The most important and most perspective bacteria and fungi used in integrated pest management are *Bacillus* spp. and *Trichoderma* spp. because their effectiveness in pesticide degradation and the large number of commercial preparations containing these microorganisms available on the market. Application of biological pesticides recommended in integrated pest management programmes could significantly improve the quality of the soil, environment, and human health.

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Jednostka **Instytut Biotechnologii**

Promotor **dr hab. inż. Ewa Szpyrka, prof. UR**

Promotor pomocniczy **dr Leszek Potocki**

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ORIGINAL PAPER

The Influence of Commercial Yeast Preparations on the Degradation of Herbicide Mixtures in the Soil and the Effect on the Shell Pea (*Pisum sativum* L.) Cultivation

Ewa Szpyrka[1] · Paulina Książek-Trela[1] · Ewelina Bielak[1] · Magdalena Słowik-Borowiec[1]

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Abstract
Today, increasingly more attention is paid to the use of biological preparations that have an advantageous effect on the soil condition and boost plant health. The study analyzes the influence of three agrochemical preparations containing three different yeast species, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Debaryomyces hansenii*, on the degradation of a herbicide mixture in the soil. The second aim of the study was to evaluate the influence of yeast preparations on the shell pea (*Pisum sativum* L.) cultivated on a soil contaminated with a herbicide mixture. Experiments were performed in controlled laboratory conditions. The parameters analyzed in the soil samples included the pH, the oxidation–reduction potential, humidity, the dehydrogenase activity of microorganisms (DHA), and pesticide residues. In the second experiment, the analyses focused on germination of shell pea seeds, plant growth, chlorophyll and carotenoid content, and pesticide residues in plants. All yeast preparations enhanced the degradation of fluazifop-P-butyl. The preparation with *Y. lipolytica* had the greatest impact on the herbicides degradation, even on the one as stable as pendimethalin. Maximum degradation rates were 71.2% for fluazifop-P-butyl, 13.4% for propyzamide, 20% for metribuzin, and 5.3% for pendimethalin. No statistically important values were provided for clomazone. Additionally, the preparation with *Y. lipolytica* promoted the growth of shell pea (22%, versus the control), while the preparation with *D. hansenii* increased the seed germination capacity by 30%. Biological preparations containing yeasts can be a good means to reduce soil contamination by pesticides, to improve the health and quality of plants, and to enhance the safety of environment.

Keywords Herbicide · Degradation · Yeast · Effective microorganisms · Shell pea · *Pisum sativum* L

1 Introduction

The use of pesticides is an integral part of the intensification of crop production. In 2015, under programs funded by the European Union, scientists examined 317 soil samples taken from EU farmlands. The scope of the research covered only 76 active substances out of over 400 approved for use in the EU (Silva et al. 2019; EU Pesticides Database 2023). Eighty-three percent of the soil samples were contaminated with pesticide residues, where 25% of them contained one residue and 58% contained a mixture of studied compounds. Soil contaminants can be hazardous to soil organisms and succeeding crops, and can be taken up by cultivated plants and then consumed by people (Silva et al. 2019). Pesticides are present in vegetables that were grown in the ground. On average, in 1% of these vegetables, the pesticide concentration exceeds the maximum residue limits (MRLs) set by Regulation 396/2005/EU and can cause the acute dietary risk (Carrasco Cabrera and Medina Pastor 2022). The disappearance of pesticides in the soil depends on a number of factors, such as the soil type, its mineral and organic matter content, pH, moisture, temperature, presence of microorganisms, and the chemical structure of active substances.

The soil is a complex structure of minerals, organic matter, water, and air. It is an environment for a wide range of soil organisms and microorganisms like bacteria, fungi, archaea, viruses, and protist, which play a crucial role in soil processes. The microorganisms are an essential component

✉ Ewa Szpyrka
eszpyrka@ur.edu.pl

✉ Paulina Książek-Trela
pksiazek@ur.edu.pl

[1] Institute of Biotechnology, University of Rzeszów, 1 Pigoń St., 35-310 Rzeszów, Poland

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in cycles of carbon and other nutrients because they decompose and convert organic matter. These processes are dependent on various soil parameters like the temperature, the pH, or the soil moisture content (Joos and De Tender 2022). Some of microorganisms can use organic pollutants present in the soil, such as pesticides or polycyclic aromatic hydrocarbons (PAHs), as a source of carbon, and could be advantageous for soil remediation (Raffa and Chiampo 2021). Beneficial microorganisms, especially those promoting plant growth, increase the farm productivity and improve protection of the environment. They are harmless to the environment and non-toxic to other organisms, and recommended as a promising alternative to conventional fertilizers and pesticides. The introduction of beneficial microbes promotes the adaptability of plants to adverse biotic and abiotic stress by enhancing soil fertility, water uptake, and biocontrol of pests and diseases, as well as by inducing their systemic resistance. On the other hand, such microorganisms can improve the plant quality by removing soil pollutants, reducing the absorption and accumulation of harmful substances, and regulating the synthesis of secondary metabolites (Wang et al. 2022; Koskey et al. 2021).

Biological preparations are recommended in the plant protection schemes, and have a priority over chemical substances. A wide range of different effective microorganisms are proposed for the biological plant protection. They are beneficial for plant growth and can act as insecticides, nematicides, fungicides, bacteriocides, molluscicides, and resistance inducers, and at the same time, these microorganisms, especially bacteria and fungi, can degrade pesticides and enhance soil quality (Książek-Trela and Szpyrka 2022).

Yeasts are microorganisms which are readily and widely used as biofertilizers, biostimulants, and biopesticides. They can also be used in soil remediation, as biodegraders. The yeast effects on plants and their pathogens are not as comprehensively described as those of other microorganism, such as bacteria or filamentous fungi. They have a positive influence on plants, by reducing the development and impact of pathogens. The biocontrol of plant pathogens by yeasts involves multiple mechanisms, such as production of volatile organic compounds and toxins, competition for space and nutrients, production of lytic enzymes, induction of plant immunity, and mycoparasitism. Commercially available yeast-based bioproducts for the control of plant diseases contain *Aureobasidium pullulans*, *Candida oleophila*, *Metschnikowia fructicola*, and Cerevisane, a component of *Saccharomyces cerevisiae* cell wall. The yeasts are also used as a component of biofertilizers and preparations improving soil quality (Hernández-Fernández et al. 2021; Kowalska et al. 2022; Książek-Trela and Szpyrka 2022).

Some authors studied the effect of yeasts on the pesticide degradation. They used mainly *S. cerevisiae* to degrade azoxystrobin, pyraclostrobin, iprodione, boscalid (Wołejko et al. 2016), glyphosate (Low et al. 2005), thiram (Kitagawa et al. 2002), atrazine, terbuthylazine (Hack et al. 1997), and pirimiphos-methyl (Đorđević and Đurović-Pejčev 2016), *Rhodotorula glutinis* and *Rhodotorula rubra* to degrade chlorpyrifos; *Rhodotorula mucilaginosa* to degrade neonicotinoid insecticides (acetamiprid and thiacloprid); *Solicoccozyma terricola* to degrade glyphosate herbicide; *Clavispora lusitaniae* to degrade dinitroaniline herbicide; and *Pichia kudriavzevii* to degrade the s-triazine group of herbicides (Hernández-Fernández et al. 2021).

This study proposes a hypothesis that yeast preparations will promote plant growth and health, and simultaneously will be beneficial in remediation of the soil contaminated with herbicides.

The aim of this study was to analyze the influence of three agrochemical preparations containing different yeast species, *S. cerevisiae*, *Yarrowia lipolytica*, and *Debaryomyces hansenii*, on the degradation of a mixture of herbicides, containing propyzamide, clomazone, metribuzin, pendimethalin, and fluazifop-P-butyl, in the soil. The second aim of this study was to evaluate the influence of yeast preparations on the shell pea (*Pisum sativum* L.) cultivated on the soil contaminated with the herbicide mixture. The analyses focused on the influence of this preparation on shell pea germination, plant growth, and chlorophylls and carotenoids content.

2 Materials and Methods

2.1 Experimental Design

2.1.1 Chemicals

Acetone, n-hexane, and methanol of LC–MS grade were purchased from Honeywell Specialty Chemicals Seelze GmbH, Germany. QuEChERS Extraction Kits for extraction and cleanup were bought from Agilent, USA. Analytical standards of herbicides and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich, USA. 1,3,5-Triphenyl tetrazolium formazan (TPF) was bought from Tokyo Chemical Industry, Japan, and triphenyl phosphate (TPP) was bought from Supelco Inc., Bellefonte, PA., USA.

2.1.2 Soil Sample Preparation

The experiments were conducted in the soil of one type, i.e., horticultural (PPUH Zielona Oaza I, Brzozów, Poland). The soil parameters are presented in Table 1.

Four hundred grams of the soil was weighted into 2-L propylene transparent containers and 100 mL of the biological preparations dissolved in distilled water was separately added. The biological preparations were prepared at concentrations recommended in their labels (Table 2). The

Table 1 Soil characteristic

Type	Composition	Salt concentration	pH
Horticultural	High and low moor peat, sand, pine bark, perlite, dolomite, and mineral fertilizers	0.5–1.0 g of KCl/L	5.11 ± 0.01

control sample was prepared by adding 100 mL of water. After 3 days, 80 mL of the herbicide mixture (0.1 mL of Command 480 EC, 0.025 g of Aurelit 70 WG, 0.125 mL of Stomp Aqua 455 CS, 17 mL of Fusilade Forte 150 EC, and 0.05 g of Kerb 50 WP dissolved in 1 L of distilled water) was added to the soil samples. The control sample was a sample with no yeasts added, containing only the pesticide mixture. Each sample was prepared in triplicate. The temperature was maintained at a level of 21 ± 1 °C and the soil water content was in the range of 70–72%.

The soil samples for the DHA analysis were taken on days 1, 3, 7, 14, and 28. The herbicide mixture was applied on the third day of the experiment, and the soil samples for the pesticide analysis were taken on days 3 (12 h after the pesticide application), 7, 14, and 28.

2.2 Specific Analyses

2.2.1 Soil Samples Analyses

In each sample, the pH and the redox potential (the oxidation–reduction potential, ORP) were measured by AD14 Waterproof pH-ORP-TEMP Testers (Adwa, Hungary). The water content was measured by a weighing method after drying at 105 °C (S–40, Alpina, Poland) (PN-ISO 11465:1999). These parameters were measured before the experiment (day 0), and then on each sampling day.

The herbicide concentrations in the soil samples were determined using the standardized method, PN-EN 15662:2018 Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method, with modifications (Słowik-Borowiec et al. 2022). QuEChERS Extraction Kits (Agilent, USA) were used for the extraction and cleanup of samples. In brief, 5 g of the soil was weighted, hydrated with 10 mL of distilled water, and extracted with the mixture of acetone and hexane (1:4 v/v) and extraction salts (magnesium sulfate; sodium chloride; sodium citrate; and disodium citrate sesquihydrate). Sample extracts were cleaned up by the dispersive solid phase extraction (dSPE), with primary and secondary amines, PSA, and magnesium sulfate used as sorbents. A 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with a mass detector equipped with three quadrupoles, model 7000 (GC–MS/MS QqQ), was used to qualify and quantify the herbicide concentrations in the soil samples. A capillary column HP-5 MS Ultra Inert/30 m × 0.25 mm I.D. × 0.25-µm was used for analyte separation, with helium (5.0 purity) as a carrier gas, supplied at the flow rate of 2.1 mL/min. The samples were analyzed in the dynamic multi-reaction mode (dMRM). The following acquisition parameters were used: the electron ionization mode (EI − 70 eV); the sample injection mode, splitless; the volume, 2 µL; temperatures of the injector and the transfer line, 280 °C; the ion source, 300 °C; the quadrupoles, 180 °C; and the oven, 70 (2-min hold) to 150 °C at a rate of 25 °C/min, increased to 200 °C at 3 °C/ min, increased to 280 °C at 8 °C/min (Słowik-Borowiec et al. 2022). The total analysis run time was 31.87 min. The Mass Hunter, version B.06.00 software, was used for data acquisition and processing.

Certified analytical standards of herbicides were used for calibration of GC–MS/MS. TPP was used as the internal standard (ISTD) for the analysis. The retention times and ion transitions for the active substances and TPP are provided in Supplementary Material, Table S1.

The validation study was performed according to the guidelines for the analytical quality control and method validation procedures for the pesticide residues analysis (SANTE/12682/2019, 2019), now superseded by (SANTE 11312/2021, 2021). The validation procedure was described in our earlier report (Słowik-Borowiec et al. 2022). The summary of validation parameters (average recovery, average precision, linearity) for herbicides tested is provided in Supplementary Material, Table S2. The limit of quantification (LOQ) for all herbicides was 0.01 mg/kg.

DHA was determined in the soil samples according to (Casida et al. 1964; Tabatabai 2018; Wołejko et al. 2017). In brief, 6 g of the soil samples was weighted into a 50-mL propylene tube, and then 4 mL of distilled water and 1 mL of 3% aqueous solution of TTC were added. The samples were incubated at 37 °C for 20 h, protected from light. TPF was extracted by 20 mL of methanol, and the absorption was measured at 485 nm with a spectrophotometer Cary 300 Bio (VARIAN, USA). The unit used for DHA measurements was micromole TPF g^{-1} DM soil 20 h^{-1}, where DM represents the dry mass of the soil (Książek-Trela et al. 2022).

2.2.2 Analysis of the Shell Pea (*Pisum sativum* L.) Seed Germination, Plant Growth, and Pigment Content

The soil was prepared in the same manner as for the dissipation experiment. The yeast preparations were prepared

Table 2 Biological preparation used in experiments (Myco-Sin Label 2023; BioPuls Fusion Label 2023; BioPuls Cinderella Label 2023)

Biological preparation name (a name of the manufacturer, a country of origin)	Yeast	Other preparation ingredients	Recommended concentration	Biological action
Preparation 1 (Myco-Sin, Biocont, Kraków, Poland)	Inactive, ground, dried yeast — *Saccharomyces cerevisiae*	Aluminum sulfate tetradecahydrate, dry horsetail extract (*Equisetum arvense* L.)	10 g/1 L	It improves the uptake of nutrients, increases the biological soil activity, and induces resistance to pathogens
Preparation 2 (BioPuls Fusion, Microlife, Poznań, Poland)	*Yarrowia lipolytica*	Beneficial native soil bacteria, microorganic metabolites: enzymes, phytohormones, amino acids, vitamins, natural nutrients: dextrins, lignins, humic and fulvic acids and microelements in a form available to plants	20 mL/1 L	It supplies the soil with beneficial microorganisms, and improves soil structure, mechanics, and fertility. It is intended to be used in the cultivation of vegetables, fruit, and ornamental plants
Preparation 3 (BioPuls Cinderella, Microlife, Poznań, Poland)	live cells of *Debaryomyces hansenii* and its metabolites	Bacteria from *Bacillus* genus; the preparation provides 1,200,000 units of killer toxin activity per hectare of crops	2 g/1 L	It supports plants in fighting pathogenic fungi in fruit and vegetable crops by secreting killer toxins

according to recommendations in their labels (Table 2), and added to the soil samples. On day 3, the herbicide mixture was added to the soil. The soil was placed in plastic pots in three replicates, and 30 shell pea seeds were sown. Plants sown in the soil with the herbicide mixture and with no yeasts added were used as the control samples. Additionally, the plants were sown in the clean soil. The pots were kept at a temperature of 22/20 °C, photoperiod of 14/10, under the light of the intensity of 4000 Lux. The germination rates were determined by counting the germinated seeds on day 7. The germination rate (%) = (the number of germinated seeds/the total seed number) × 100% was calculated (Ling et al. 2014). After 16 days, when the plants reached the stage of 4 internodes (34 BBCH scale), above-ground parts were cut, and their content of chlorophyll a and b and carotenoids was analyzed. 0.5 g of the plant was homogenized with 10 mL of methanol in an ultrasonic mill (UP200Ht, Hielscher Ultrasonics Gmbh, Teltow, Germany) and extracted in an ultrasonic water bath (Polsonic, Poland) at 35 °C for five minutes (Zulqarnain et al. 2021). The extracts were centrifuged at 5000 rpm for 5 min (5804R, Eppendorf, Hamburg, Germany). Then, the sample content of chlorophyll a and b and carotenoids was analyzed using the spectrophotometer Cary 300 (VARIAN, USA) (Wellburn 1994).

After 16 days, the herbicide residues in the plants were also determined, to assess the uptake of these contaminants from the soil. The same QuEChERS procedure was used as for the soil samples, but the sample weight was 10 g, and sorbent kits for the plant material with a high chlorophyll content (150 mg of PSA, 45 mg of GCB and 900 mg of $MgSO_4$) were used for extracts cleanup (Słowik-Borowiec et al. 2022).

2.3 Statistical Analysis

The Student *t*-test (Excel Microsoft 365) was used to determine statistically significant differences between the samples, with and without the biological preparation added, for each sampling day.

3 Results

3.1 Soil Samples

pH values and ORP in the soil are presented in Fig. 1. Theses parameters were measured before the experiment (day 0), and then on each sampling day.

Before the experiment, the pH of soil was equal to 5.11 ± 0.01. On day 1, the pH increased to 5.62 ± 0.06 in the soil to which only water was added, and to 5.15 ± 0.02–5.39 ± 0.14 in the soil containing the biological preparation. On day 3, the herbicide mixture was

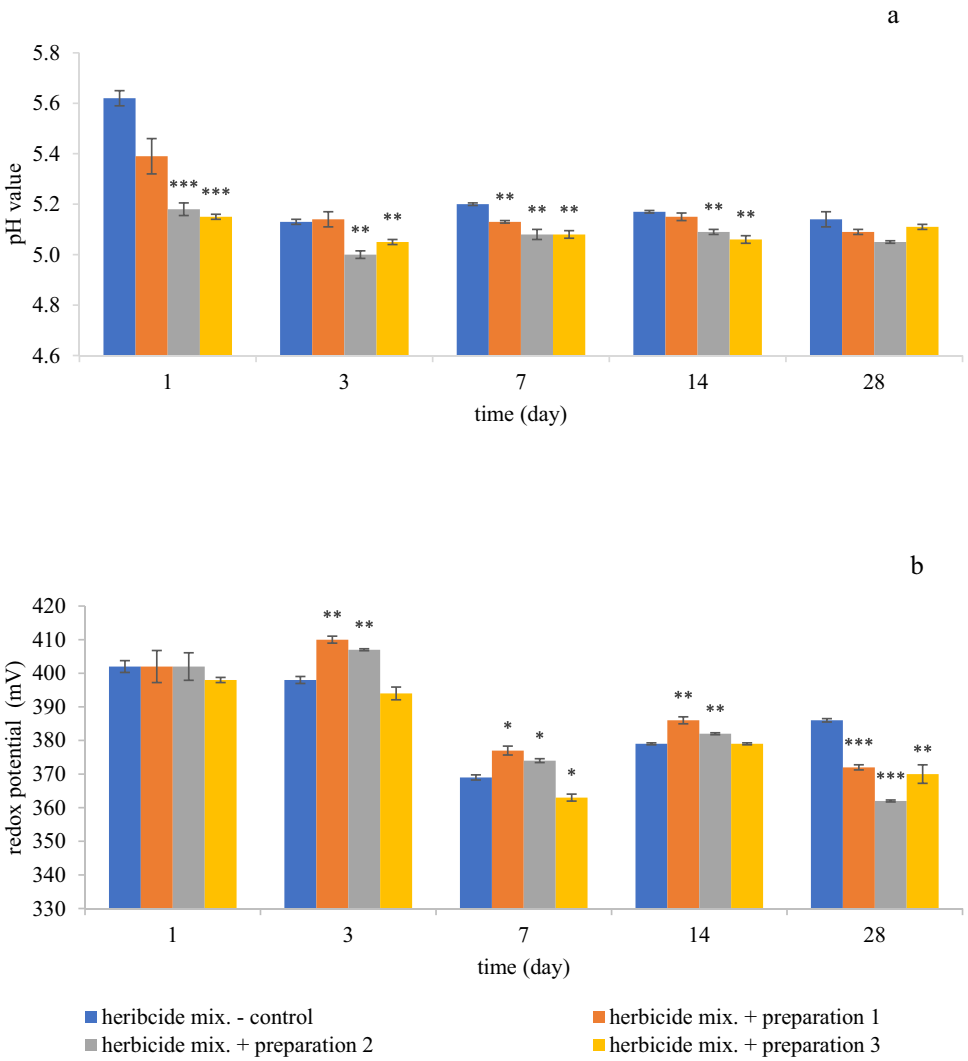

Fig. 1 Parameters of soil samples treated with the herbicide mixture (control) and with the herbicide mixture and yeast preparations: **a** pH, **b** redox potential. Statistically significant p values are shown in figures as $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***)

applied, and the pH values of the soil samples decreased to the range of 5.00 ± 0.03–5.14 ± 0.06. On successive sampling days, the pH values were stable in the range of 5.05 ± 0.01–5.20 ± 0.01. The highest pH values were recorded for the control samples during the entire experiment.

Before the experiment, ORP of the soil was 403 ± 2. On day 1, the ORP values in all samples were very similar and in the range of 398 ± 1–402 ± 9 mV. On day 3, after the herbicide mixture was applied, ORP of the soil sample was in the range of 394 ± 4–410 ± 2 mV. The highest value of this parameter was noticed for the sample with preparation 1 containing *S. cerevisiae*. On the successive sampling days, this parameter decreased in all samples and was in the range of 362 ± 1–386 ± 1 mV.

Dissipation of herbicides in the soil is presented in Fig. 2.

On day 3 of the experiment, the clomazone concentration was the highest in the control samples and equal to 5.96 ± 0.50 mg/kg (Fig. 2a). In the samples with the biological preparation, the clomazone concentrations were lower in all samples, except for the samples with preparations 1 and 3 on Day 14. The biggest difference was found after application of preparation 2 with *Y. lipolytica* (7.9% on day 7 of the experiment, versus the control samples with the pesticide mixture). During the experiment, no statistically significant differences were found for this herbicide.

For propyzamide, the highest concentration of 2.54 ± 0.03 mg/kg was found in the control samples (Fig. 2b). On day 3, the propyzamide concentrations in all samples with the yeast were lower than in the control samples, with the biggest difference found for preparation 2 with *Y. lipolytica* (13.4%), which was statistically significant.

The highest concentration of metribuzin, of 1.40 ± 0.01 mg/kg, was determined in the control samples after pesticide application (Fig. 2c). On day 3, the metribuzin concentrations in all samples with the yeast were lower than in the control samples, with the biggest difference found for

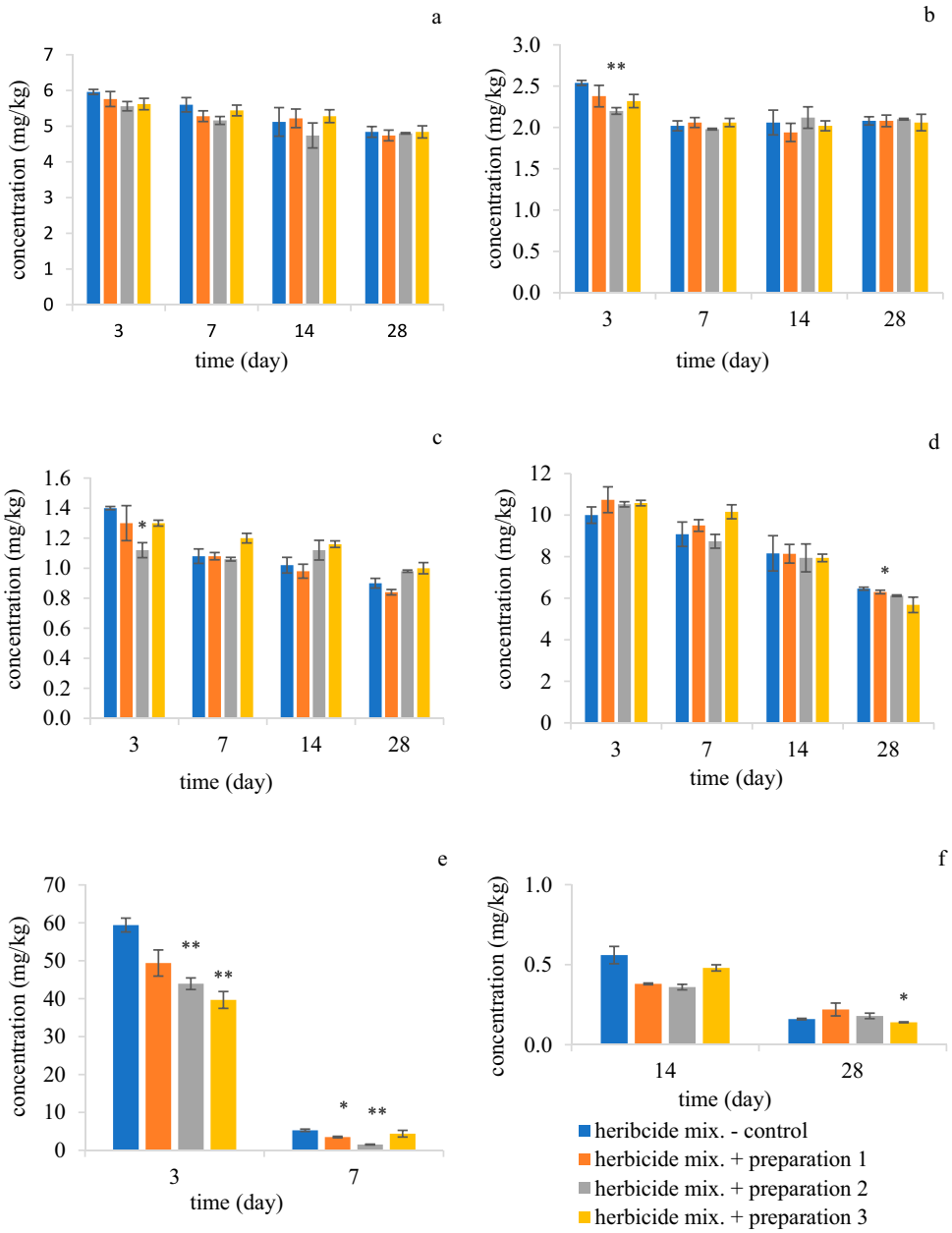

Fig. 2 Dissipation of herbicides in the soil: **a** clomazone, **b** propyzamide, **c** metribuzin, **d** pendimethalin, **e** and **f** fluazifop-P-butyl. Statistically significant *p* values are shown in figures as $p < 0.05$ (*) and $p < 0.01$ (**)

preparation 2 with *Y. lipolytica* (20.0%), which was statistically significant.

The highest pendimethalin concentration was 10.74 ± 0.62 mg/kg in the samples with the pesticide mixture and preparation 1 on day 3 of the experiment (Fig. 2d). The statistically significant difference was found for the samples with preparation 2 on day 28 (5.3% lower than in the control samples).

The highest fluazifop-P-butyl concentration was 59.42 ± 1.82 mg/kg for the control samples on day 3 (Fig. 2e and f). The biggest difference was found for the samples with preparation 2 with *Y. lipolytica* (71.2% on day 7), and it was statistically significant. For all yeast preparations, statistically significant differences were found especially on days 3 and 7 of the experiment.

After application of the yeast preparations into the soil, DHA increased significantly, but then on day 3, following the herbicide mixture application, this parameter decreased (Fig. 3). On successive sampling days, DHA was higher in the soil with the yeasts.

3.2 Shell Pea (*Pisum sativum* L.)

The soil for the plant experiment was prepared in the same manner as for the dissipation experiment. The yeast preparations were prepared according to recommendation in

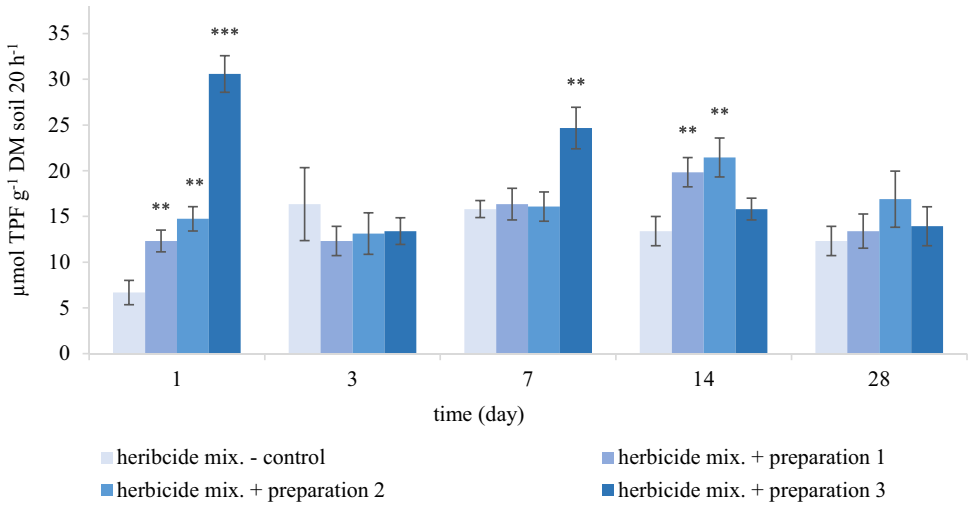

Fig. 3 DHA in the soil samples treated with the herbicide mixtures (control) and with the herbicide mixtures and the yeasts preparation. Statistically significant p values are shown in figures as $p < 0.01$ (**) and $p < 0.001$ (***)

their labels (Table 2) and added to the soil samples, and then the herbicide mixtures were applied on day 3. The plants sown in the soil with the herbicide mixture and with no yeasts added were used as the control samples. Additionally, the plants were sown in the clean soil. The herbicide mixture significantly decreased the germination of seeds. On day 7, the seed germination rate was on the level of 56.7 ± 11.5% in the control samples (Fig. 4a), while in the clean soil, it reached 83.3 ± 10.7%. The germination rate was higher in the soil with preparations 2 and 3, and in the clean soil. The highest and statistically significantly different value was determined for the sample with preparation 3 (86.7 ± 5.8%).

The plant growth was measured after 16 days. The results are presented in Fig. 4b. The herbicide mixture significantly reduced the plant growth; the average value in the control samples was 10.4 ± 2.4 cm, while in the clean soil, it reached 18.9 ± 0.8 cm. In the soil with the pesticide mixture and the biological preparations, the plants were higher, and reached 11.6 ± 0.6 cm for preparation 1, 12.7 ± 1.4 cm for preparation 2 (and this value was statistically significant), and 11.1 ± 1.4 cm for preparation 3.

The yeast preparation influence on the pigments content in the shell pea, especially on chlorophyll b, is shown in Fig. 4c. This parameter could have been influenced by the faster growth of plants cultivated on the soil with the yeast preparations added.

After 16 days, the herbicide residues in plants were also determined, to assess the uptake of these contaminants from the soil. All samples contained metribuzin (at the level of 0.10 ± 0.08 mg/kg on average) and clomazone (at the level of 0.02 ± 0.004 mg/kg). Pendimethalin residues were determined only in the control samples (at the level of 0.07 ± 0.01 mg/kg). The shell pea plants did not contain residues of propyzamide and fluazifop-P-butyl above the LOQ of 0.01 mg/kg.

4 Discussion

Intensive agricultural production, involving the use of pesticides, can lead to environmental pollution. Persistent substances, like some herbicides, can accumulate in the soil and then be taken up by plants, which are later consumed by humans. Therefore, increasingly often farmers consciously use biological plant protection products and biofertilizers. In addition to bacteria and fungi, such preparations can contain yeasts. Yeasts are microorganisms which are readily and widely used as biofertilizers, biostimulants, and biopesticides. Yeasts can act on plants directly, by providing plants with soluble nutrients (nitrogen, phosphorus, potassium, iron, and zinc), and producing organic acids and phytohormones. They can also act indirectly through their high antifungal and lower insecticidal and herbicidal activity. The biocontrol of plant pathogens by yeasts involves multiple mechanisms, such as production of volatile organic compounds and toxins, competition for space and nutrients, production of lytic enzymes, induction of plant immunity, and mycoparasitism (Hernández-Fernández et al. 2021; Kowalska et al. 2022). Yeasts can also increase plants' resistance to abiotic stresses such as drought, a reduced availability of minerals, and salinity. Yeast preparations can contribute to the promotion of plant growth and remediation of soils contaminated with heavy metal and pesticides (Abdel-Kareem et al. 2021; Hernández-Fernández et al. 2021). Commercially available plant protection products contain inter alia *S. cerevisiae*, while formulations for soil revitalization contain *Y. lipolytica* and *D. hansenii*. Those three yeast preparations were applied in our study, to analyze their potential in degradation of a mixture of herbicides, containing propyzamide, clomazone, metribuzin, pendimethalin, and fluazifop-P-butyl, in the soil, and to evaluate their influence on the shell pea (*Pisum sativum* L.) cultivation on the soil contaminated with the said herbicide mixture. In the literature,

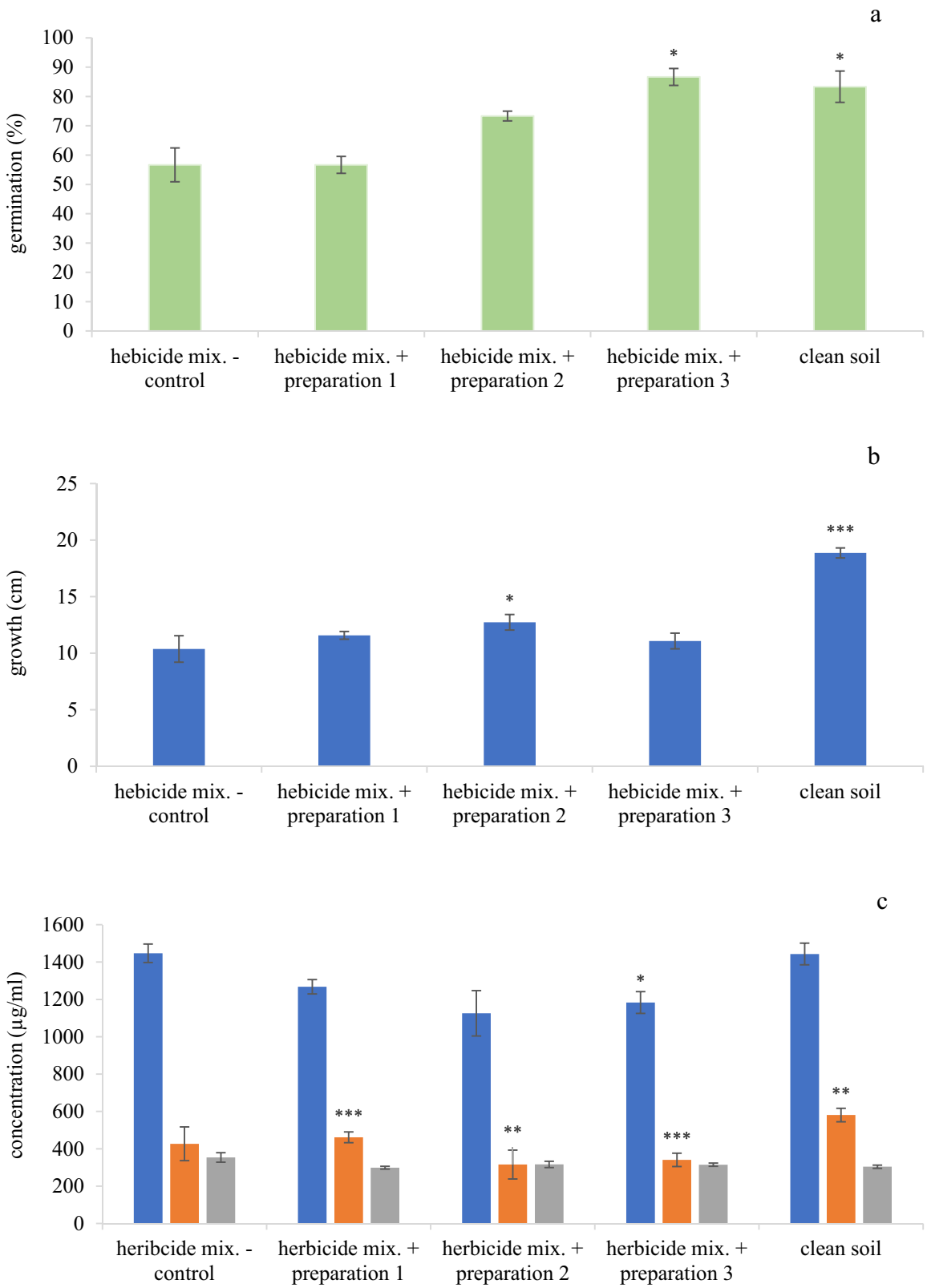

Fig. 4 The influence of the pesticide mixture (control) and the pesticide mixture with the biological preparation on **a** seeds germination, **b** growth, and **c** pigment contents of the shell pea

the information about the effect of yeast preparations on the herbicide degradation is very scarce. The novelty of this article also lies in the application of yeast preparations which are new on the market.

S. cerevisiae is very popular as a biofertilizer and a biofungicide. *S. cerevisiae* strain LAS02 was approved in the European Union as a low-risk active substance (EU Pesticides Database 2023). Its mechanism of action is associated with phosphorus solubilization, sulfur oxidation, and production of a phytohormone—indole-3-acetic acid (IAA). As a biofungicide, it competes with pathogenic fungi for nutrients and space (Hernández-Fernández et al. 2021). The *S. cerevisiae* cell wall is recommended for protection of plants against powdery mildew and grey mold (Kowalska et al. 2022). Biofungicide Myco-Sin, containing *S. cerevisiae*, was tested in an apple orchard. Degradation levels of the tested active substance, fluxapyroxad, in apples after application of the yeast preparation were 59.9% for Gala and 43.8% for Idared (Podbielska et al. 2020). In studies conducted in an aqueous solution, *S. cerevisiae* was able to degrade 96% of diazinon (Ehrampoush et al. 2017). The influence of *S. cerevisiae* on the pesticides degradation during fermentation was also studied. This yeast was able to decompose 21% of glyphosate (Low et al. 2005), and 48.8% of pirimiphos methyl (Đorđević and Đurović-Pejčev 2016). Furthermore, it was found that *S. cerevisiae* is also able to degrade other pesticides, including iprodione, boscalid, azoxystrobin, and pyraclostrobin in leafy vegetables (Wołejko et al. 2016). In our study, preparation 1, containing *S. cerevisiae*, was able to degrade fluazifop-P-butyl in the soil (33.7% on Day 7). For other pesticides, no statistically significant differences were found. No effect on shell pea germination and growth was established. Preparation 1 increased the chlorophyll b content in this plant. In the research conducted by (Fernandez-San Millan et al. 2020) in maize seedlings grown in the soil in a phytotron, three isolates (*D. hansenii* Dh-67, *Lachancea thermotolerans* Lt-69, and *S. cerevisiae* Sc-6) promoted the seedling development (an increase in dry weight and chlorophyll content at a level of 10%). In the research conducted by Verma et al. (2019) in rice, the application of *S. cerevisiae* significantly increased the levels of photosynthetic pigments in the rice plants and promoted plant growth.

Y. lipolytica can be used as a biofertilizer (it enhances P solubilization) and insecticide (Hernández-Fernández et al. 2021). It is well known as bioremediation agent for removal of hydrophobic substrates such as crude oil, pesticides, and polycyclic aromatic hydrocarbons (Wierzchowska et al. 2022). Under abiotic stress, this yeast can promote growth of maize plants through controlled metabolism and hormonal secretions (Gul Jan et al. 2019). In our study, preparation 2, containing *Y. lipolytica*, was the most effective in the pesticide degradation. It degraded propyzamide (13.4% on day 3), metribuzin (20% on day 3), pendimethalin (5.3% on day 28), and fluazifop-P-butyl (reaching a maximum level of 71.2% on day 7). The results are significant, because pendimethalin is a stable herbicide with DT_{50} in the range of 97–270 days in laboratory studies, and DT_{50} in the range of 39.8–187 days in field studies (PPDB 2023). Preparation 2 promoted the shell pea growth. Gul Jan et al. (2019) and Verma et al. (2019) analyzed the influence of *Y. lipolytica* on the growth of maize under salt stress. They found that inoculation with this yeast promoted the growth of maize plants through controlled metabolism and hormonal secretions (abscisic acid and IAA).

D. hansenii tolerates extreme environmental stress conditions, such as a low pH and a high osmotic pressure. It is able to grow at low temperatures, below 14 °C, and it also exhibits a killer phenotype in the range of 10–20 °C (Czarnecka et al. 2019). *D. hansenii* is commonly used in the food industry, but in recent years, it has also been used in the agriculture. It supports plants in the fight against pathogenic fungi in fruit and vegetable crops by secreting killer toxins (BioPuls Cinderella Label 2023). It can degrade biogenic amines, benzo(a)pyrene, and n-dodecane (Bäumlisberger et al. 2015; Padilla-Garfias et al. 2022; Azimian 2023). In our study, preparation 3, containing *D. hansenii*, was able to degrade fluazifop-P-butyl in the soil (at the maximum level of 33.3% on day 3). For other pesticides, no statistically significant differences were found. The high effect on the shell pea germination was determined in the soil containing preparation 2. This parameter was even higher than for the clean soil. Fernandez-San Millan et al. (2020) found that *D. hansenii* Dh-67 promoted the growth and the chlorophyll content in maize. *D. hansenii* was also reported to mitigate arsenic stress in rice, resulting in the improved growth and the nutrient status of the plant (Kaur et al. 2020).

The content of chlorophyll a and b and carotenoids was lower in the shell pea grown on the soil which contained the pesticide mixture and the yeast preparation, except for chlorophyll b and preparation 1. Lower values of pigments could result from the faster shell pea growth.

The results of our research indicate that yeast preparations have a positive effect on both soil remediation and the plant growth. The use of such preparations complements chemical protection, and should be recommended to enhance the quality and safety of the environment.

5 Conclusions

Biological preparations containing yeasts are recommended in the agriculture to increase the biological soil activity and fertility, support plants in fighting pathogenic fungi, induce resistance to pathogens, and promote plant growth and health. The novelty of this article lies in verification if

such yeast preparations could reduce soil contamination by herbicides, even the persistent ones. In the literature, the information about the effect of yeast preparations on the pesticides degradation is very scarce. The results of our research indicate that yeast preparations have a positive effect on both soil remediation and supporting plant growth. The use of those preparations should be recommended in the agricultural production, to enhance the quality and safety of the environment and, simultaneously, to protect human health against chemical contaminants. The yeast preparations containing *Y. lipolytica* can be most advantageous in the case of the soil contaminated with herbicides.

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Author Contribution E.S. prepared conceptualization; E.S. and P.K.-T. performed the experiments on herbicides dissipation; E.S. performed the analysis of seed germination; P.K.-T. and E.B. performed the analysis of DHA and pigments contents; M.S.-B. performed the analysis of herbicide residues in plants; E.S. and P.K.-T. analyzed the results; E.S. wrote the original draft; E.S. and P.K.-T. performed the review and editing.

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Data Availability The data that support the findings of this study have been presented in the manuscript.

Materials Availability No.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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Supplementary information

The influence of commercial yeast preparations on the degradation of herbicide mixtures in the soil and the effect on the shell pea (*Pisum sativum* L.) cultivation

Ewa Szpyrka, Paulina Książek-Trela, Ewelina Bielak, Magdalena Słowik-Borowiec

Table S1 Retention times and ion transitions for herbicides and TPP.

Active substance	Retention time (min)	Quantifier transition (m/z*)	Qualifier transition (m/z*)	Collision energy (V)
Clomazone	13.9	124.8 → 88.9	204 → 107 124.8 → 98.9	15, 20, 15
Propyzamide	14.5	172.8 → 145	174.9 → 146.7 172.8 → 109	15, 15, 30
Metribuzin	16.8	197.9 → 81.9	197.9 → 55.2 143.8 → 126.9	15, 30, 10
Pendimethalin	21.5	252 → 162	252 → 161.1 162 → 161.1	10, 15, 10
Fluazifop-P-butyl	25.5	282.2 → 91	383.2 → 282.2 282.2 → 238	20, 10, 20
TPP	27.8	326.1 → 228.4	326.1 → 128	15, 15

*m/z – the mass-to-charge ratio

Table S2 Validation parameters for herbicide analyses in soil samples (average values for spiking levels: 0.01 mg/kg and 1 mg/kg)

Active substance	**Average recovery (%)**	**Precision (RSD*) (%)**	**Linearity (R**)**
Clomazone	99	8	0.997
Propyzamide	95	3	0.995
Metribuzin	96	9	0.998
Pendimethalin	94	4	0.995
Fluazifop-P-butyl	85	2	0.998

*RSD - relative standard deviation, **R - correlation coefficient

Załącznik 1 do Uchwały nr 118/07/2022
Rady Naukowej Kolegium Nauk
Przyrodniczych UR z dnia 12 lipca 2022

Rzeszów, dnia 06.03.2024

Imię i nazwisko **mgr inż. Paulina Książek-Trela**

Jednostka **Instytut Biotechnologii**

Promotor **dr hab. inż. Ewa Szpyrka, prof. UR**

Promotor pomocniczy **dr Leszek Potocki**

OŚWIADCZENIE

W związku z przygotowywaniem przeze mnie rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów, oświadczam niniejszym, że wkład mojej pracy naukowej (w ramach dyscypliny Biotechnologia), a tym samym pracy pozostałych współautorów w opublikowaniu poniższych artykułów, które zamierzam przedstawić jako własną dysertację doktorską jest następujący:

I. Szpyrka E., Książek-Trela P., Bielak E., Słowik-Borowiec M. 2024. The influence of commercial yeast preparations on the degradation of herbicide mixtures in the soil and the effect on the shell pea (Pisum sativum L.) cultivation. Journal of Soil Science and Plant Nutrition. DOI: 10.1007/s42729-024-01671-7.

- Przeprowadzenie doświadczenia nad zanikaniem herbicydów w glebie razem z E.S.;
- Oznaczenie aktywności enzymatycznej w glebie razem z E.B.; oznaczanie zawartości barwników w roślinach.
- Analiza wyników razem z E.S.;
- Obowiązki autora korespondencyjnego, w tym wykonanie korekty razem z E.S.

Paulina Książek-Trela

podpis

Oświadczenia współautorów:

1. **Ewelina Bielak**
   Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Oznaczenie aktywności enzymatycznej w glebie razem z P.K.-T., oznaczanie zawartości barwników w roślinach.

Bielak Ewelina
.....
Podpis

2. **Ewa Szpyrka**

Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Opracowanie koncepcji pracy;
- Przeprowadzenie doświadczenia nad zanikaniem herbicydów w glebie razem z P.K.-T;
- Analiza kiełkowania nasion;
- Analiza wyników razem z P.K.-T.;
- Przygotowanie manuskryptu;
- Obowiązki autora korespondencyjnego, w tym wykonanie korekty razem z P.K.-T.

.....

Podpis

3. **Magdalena Słowik-Borowiec**
Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Analiza pozostałości herbicydów w roślinach.

Magdalena Słowik-Borowiec
Podpis

molecules

MDPI

Article

Effect of Three Commercial Formulations Containing Effective Microorganisms (EM) on Diflufenican and Flurochloridone Degradation in Soil

Paulina Książek-Trela *, Ewelina Bielak, Dominika Wężka and Ewa Szpyrka *

Department of Biotechnology, Institute of Biology and Biotechnology, University of Rzeszow, 1 Pigonia St., 35-310 Rzeszow, Poland; eb107477@stud.ur.edu.pl (E.B.); dw107523@stud.ur.edu.pl (D.W.)
* Correspondence: pksiazek@ur.edu.pl (P.K.-T.); eszpyrka@ur.edu.pl (E.S.)

Abstract: The aim of this study was to determine the influence of effective microorganisms (EM) present in biological formulations improving soil quality on degradation of two herbicides, diflufenican and flurochloridone. Three commercially available formulations containing EM were used: a formulation containing *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bacillus*, and *Rhodopseudomonas* bacteria and the yeast *Saccharomyces cerevisiae*; a formulation containing *Streptomyces*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Cellulomonas*, *Arthrobacter*, *Paenibacillusa*, and *Pseudonocardia* bacteria; and a formulation containing eight strains of *Bacillus* bacteria, *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. coagulans*, *B. laterosporus*, *B. mucilaginosus*, and *B. polymyxa*. It was demonstrated that those formulations influenced degradation of herbicides. All studied formulations containing EM reduced the diflufenican degradation level, from 35.5% to 38%, due to an increased acidity of the soil environment and increased durability of that substance at lower pH levels. In the case of flurochloridone, all studied EM formulations increased degradation of that active substance by 19.3% to 31.2% at the most. For control samples, equations describing kinetics of diflufenican and flurochloridone elimination were plotted, and a time of the half-life of these substances in laboratory conditions was calculated, amounting to 25.7 for diflufenican and 22.4 for flurochloridone.

Keywords: pesticides; diflufenican; flurochloridone; effective microorganisms; biodegradation

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1. Introduction

Effective microorganisms (EM) are a composition of interacting microorganisms of natural origin which are safe for the environment. They are used for production of natural and organic fertilizers and agents for revitalizing and nourishing the soil. EM improve the soil structure and fertility [1], as well as biodiversity, by enhancing the activity of soil microorganisms. They facilitate uptake of nutrients, thus influencing plant growth and development. They can increase crop yields and improve crop quality as well as accelerate the breakdown of organic matter from crop residues [2] and accelerate the decomposition of organic waste and pesticide residues and the composting process [3,4].

EM presence in the soil also contributes to a decrease in need for the use of chemical plant protection agents. This can be observed in cases where pesticides are replaced with microorganisms in the process of biological plant protection against pesticides in natural and organic agriculture [5]. Pests and pathogens are eliminated or controlled with natural strategies using beneficial microorganisms, which, e.g., initiate natural mechanisms of plant immunity, act antagonistically, or compete for space and nutrients [6,7].

The main EM are the bacteria *Bacillus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Lactococcus* spp. (*Lactobacteria*), *Rhodopseudomonas* spp., and *Rhodobacter* spp. (photosynthesizing species), yeasts such as *Saccharomyces* spp. and *Candida* spp., Actinobacteria (*Streptomyces* spp.), and molds (*Aspergillus* spp.) [8].

According to many authors, the microorganisms may significantly contribute to the degradation of pesticides' active substances [9–11]. Microorganisms are essential in bioremediation of pesticides, a cost-effective method for removal of pollutants from the environment. In biodegradation processes, pesticides are transformed into non-toxic metabolites used by microorganisms in metabolic processes. Enzymes catalyzing biochemical reactions, such as hydrolases, peroxidases, and oxygenases, play a key role in biotransformation mechanisms [12,13]. The degradation process efficiency depends on weather conditions, including soil temperature, pH, humidity, and composition. Pesticide elimination through biodegradation positively influences the fertility of agricultural soils [14,15]. Recently, many scientists isolated and cultured various microorganism species from the environment to conduct studies on pesticide degradation. The main types of microorganisms include *Bacillus*, *Pseudomonas*, *Flavobacterium*, *Micrococcus*, *Acinetobacter*, *Aerobacter*, *Alcaligenes, Flavobacterium*, *Clostridium*, *Actinomycetes*, *Penicillium*, *Aspergillus*, *Fusarium*, and *Trichoderma* [11,16–18].

The studies regarding persistent contaminants in soil, their fate, and dissipation are very important for environmental and human health. These pollutants could reach surface and ground water and also food products and then constitute a threat for consumers. It is very important to obtain knowledge of new, safe methods for soil remediation and environment protection.

Diflufenican (IUPAC name *N*-(2,4-difluorophenyl)-2-[3-(trifluoromethyl)phenoxy] pyridine-3-carboxamide) is an herbicide widely used to control grasses and broad-leaved weeds in the cultivation of field peas, lentils, lupins, and winter cereals. According to the World Health Organization (WHO), it belongs to class III—slightly hazardous pesticide, but it could be harmful to aquatic life with long lasting effects [19,20].

Flurochloridone (3-chloro-4-(chloromethyl)-1-[3-(trifluoromethyl)phenyl] pyrrolidin-2-one) is a pre-emergence herbicide used to control a range of weeds in umbelliferous crops, cereals, and potatoes. It is classified as a slightly hazardous pesticide (WHO), but it could be toxic to aquatic organisms. This xenobiotic is suspected of damaging fertility or the unborn child [19,20].

The aim of this study was to present the effects of commercially available formulations containing EM on diflufenican and flurochloridone degradation in soil. Dissipation parameters and kinetic equations with correlation coefficients and half-lives ($t_{1/2}$) were also presented. Additionally, changes in pH, oxidoreduction potential, and dehydrogenase activity (DHA) in the soil were analyzed. Soil was also screened for possible metabolites generated by the decomposition of these herbicides.

2. Results

The effects of commercially available formulations containing EM on degradation of active substances of two studied herbicides in the soil were described. Dissipation parameters and kinetic equations with correlation coefficients and half-lives ($t_{1/2}$) were also presented. Additionally, metabolites generated by the decomposition of herbicides and the influence of soil microorganisms on the studied pesticides were also analyzed.

2.1. Diflufenican

Diflufenican is a pyridine carboxamide herbicide belonging to Herbicide Resistance Action Committee (HRAC) Group 12 (carotenoid biosynthesis inhibitors) (Figure 1). It is a moderately persistent herbicide, non-mobile in soil.

Its possible metabolites found in the soil are 2-(3-trifluoromethylphenoxy) nicotinamide and 2-(3-trifluoromethylphenoxy) nicotinic acid. Another known metabolite is 2,4-difluoroaniline (Anaerobic) [20,21]. During a gas chromatography–mass spectrometry (GC-MS) full scan analysis of soil extracts (both non-derivatized and derivatized), none of these substances were found.

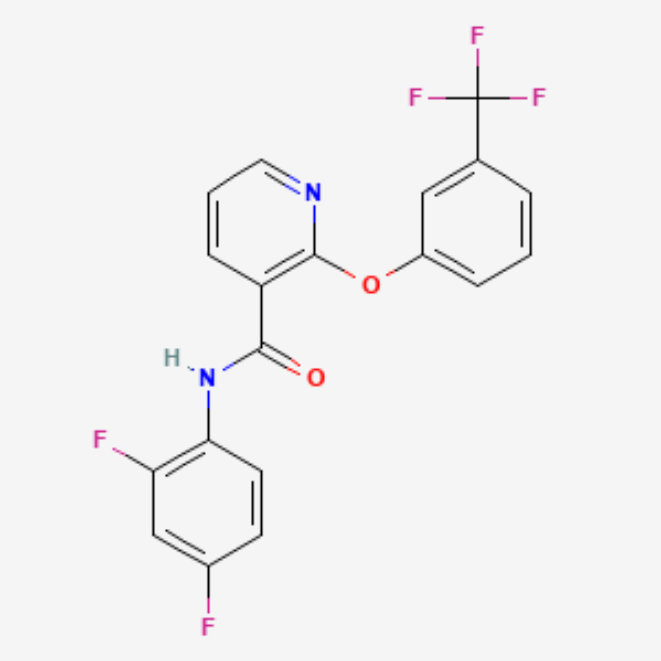

Figure 1. Chemical structure of diflufenican [19].

Figure 2 presents diflufenican's dissipation in time. Diflufenican dissipated according to the equation: $y = 0.4879e^{-0.027x}$ ($R = 0.970$). Its half-life was calculated, amounting to 25.7 days.

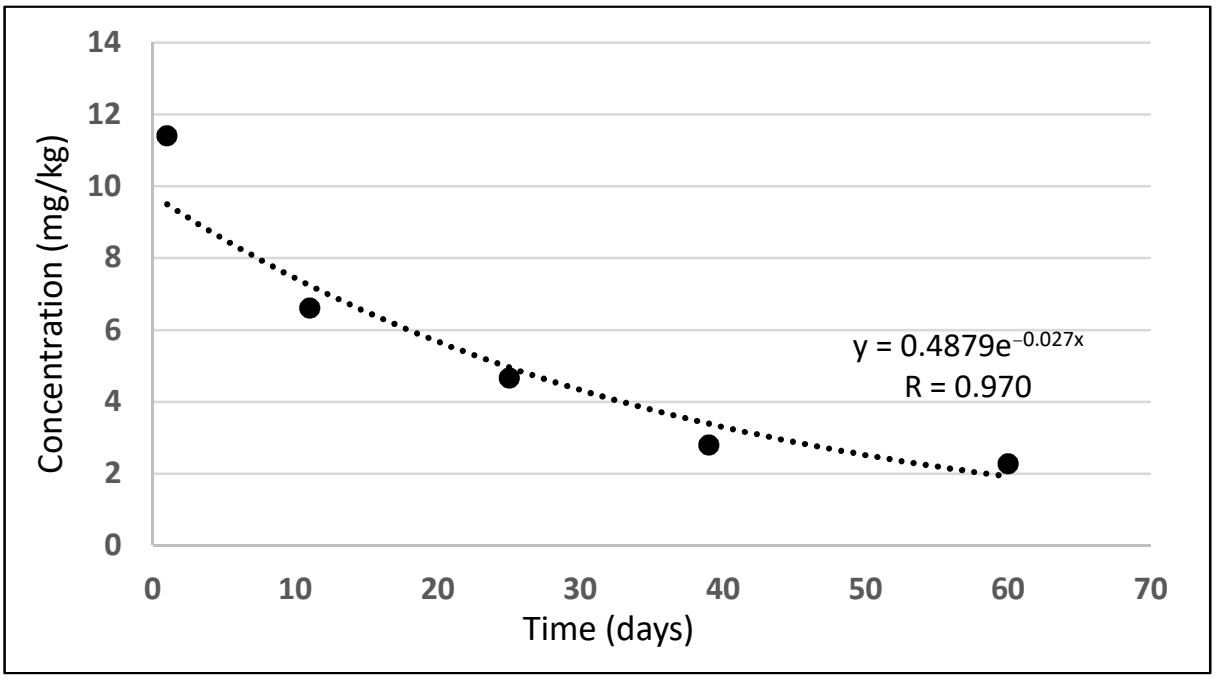

Figure 2. Diflufenican dissipation in the soil.

Table 1 presents percentage degradation of diflufenican after 11, 25, 39, and 60 days from the application of EmFarma Plus™, Rewital PRO+, and BACILLUS VIP Probiotic Microorganisms versus the control.

Table 1. Percentage diflufenican degradation following application of formulations containing EM versus control samples (the content of herbicide in control samples, on each sampling day, was assumed as 100%; '−' means that the degradation rate decreased).

Number of Days after Formulation Application	Degradation (%)		
	EmFarma Plus™	Rewital PRO+	BACILLUS VIP Probiotic Microorganisms
11	−36.5 **	−35.5	−32.6
25	7.2	1.4	7.2
39	3.5	−25.0 *	−38.0
60	2.4	−9.1	−10.0

$p < 0.05$ (*) and $p < 0.01$ (**).

Figures S1–S3 (Supplementary Material) present values of diflufenican levels in individual soil samples and its dissipation following application of formulations containing EM versus control samples. The control samples were soil samples with pesticide but without addition of EM.

2.2. Flurochloridone

Flurochloridone belongs to pyrrolidine 12 group (carotenoid biosynthesis inhibitors) according to the HRAC (Figure 3). It is a moderately persistent herbicide. There is no information about its mobility in soil.

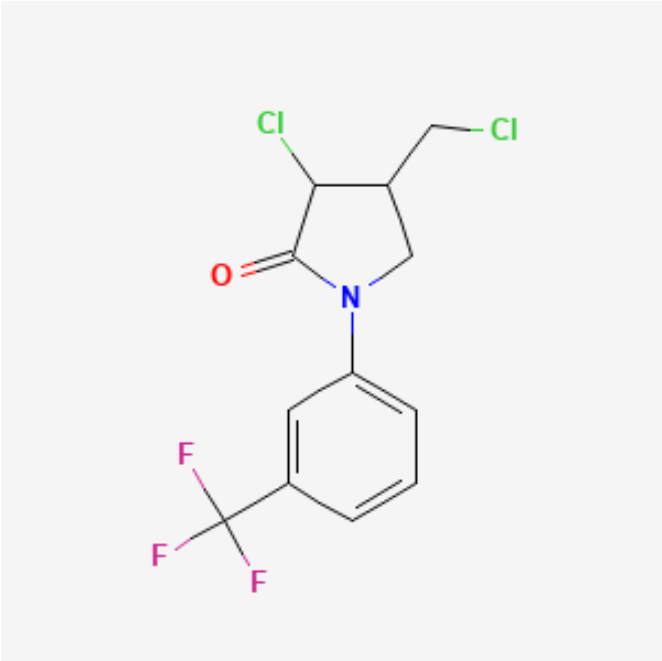

Figure 3. Chemical structure of flurochloridone [19].

Its possible soil metabolites are 4(chloromethyl)-3-hydroxy-1[3(trifluoromethyl)phenyl] pyrrolidin-2-one and 4-(chloromethyl)-1-[3(trifluoromethyl)phenyl] pyrrolidin-2-one. Another known metabolite is (4*R*,*S*)-4(chloromethyl)-1-[3-(trifluoromethyl)phenyl]pyrrolidin-2-one [20,21]. During a GC-MS full scan analysis of soil extracts (both non-derivatized and derivatized), none of these substances were found.

Figure 4 presents flurochloridone dissipation in time on Days 1, 11, 25, 39, and 60 of the study. Flurochloridone dissipated according to the equation: $y = 0.6022e^{-0.031x}$ ($R = 0.925$), achieving its half-life of $t_{1/2} = 22.4$ days.

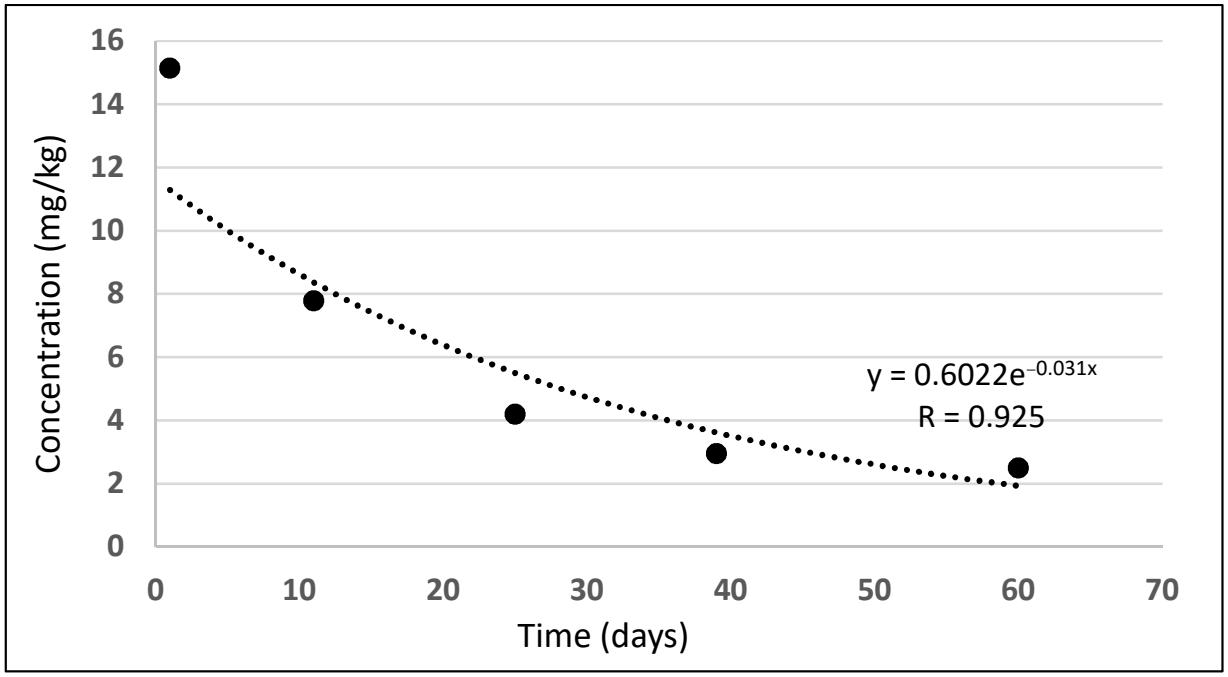

Figure 4. Flurochloridone dissipation in the soil.

Table 2 presents a percentage degradation of flurochloridone after 11, 25, 39, and 60 days from the application of EmFarma Plus™, Rewital PRO+, and BACILLUS VIP Probiotic Microorganisms versus the control.

Table 2. Percentage degradation of flurochloridone following application of formulations containing EM versus control samples (the content of herbicide in control samples, on each sampling day, was assumed as 100%).

Number of Days after Formulation Application	Degradation (%)		
	EmFarma Plus™	Rewital PRO+	BACILLUS VIP Probiotic Microorganisms
11	18.3	7.1	22.0
25	4.9	27.7	29.8
39	4.4	17.9	17.8
60	19.3	30.5 *	31.2 *

$p < 0.05$ (*).

Figures S4–S6 (Supplementary Material) present values of flurochloridone levels in individual soil samples and its dissipation following application of formulations containing EM versus control samples.

2.3. GC-MS Full Scan Analysis

After derivatization of soil extracts, TMS derivatives of palmitic acid were found in analyzed samples and in the blank sample. In all analyzed soil samples, TMS derivatives of palmitoleic acid, oleic acid, glyceryl monopalmitate, and glyceryl monostearate were found and were not detected in the blank soil sample.

2.4. Soil Parameters: pH, Oxidoreduction Potential, and DHA

On the first day, the pH of all soil samples was 7.4 for diflufenican and 7.3 for flurochloridone. When EM formulations were added to samples with diflufenican, the pH of all studied samples decreased significantly, even by 1.2 pH units. In samples with flurochloridone, after EM formulations were added, pH was on the same level as in the control

samples. The last measurement, on Day 60, is an exception, as a significant drop in pH was observed for all samples, caused by the long experiment and closing of samples in containers (3 weeks from the last measurement).

In all samples with diflufenican, the oxidoreduction potential was at a slightly higher level in the study samples versus the control ones, except for Day 60, and ranged from 266 (Day 25, control sample) to 358 (Day 60, sample containing Rewital PRO+). In samples with flurochloridone, the oxidoreduction potential was higher when compared to the control samples on Day 11; on Days 25 and 39, it remained at a similar level as that in the control samples, and ranged from 266 (Day 25, sample with EmFarma Plus™) to 346 (Day 60, sample with Rewital PRO+).

The water content was measured in the samples on Day 1 and amounted to 67%. Then, on successive days, the water content was 70–74% for control and study samples.

DHA activity changes are presented in Figures 5 and 6. Application of diflufenican and flurochloridone resulted in increases in DHA activity in control samples, especially on the 25th and 39th days in the case of diflufenican and on the 11th and 25th days in the case of flurochloridone. On the 25th day, DHA activity of soil samples with herbicides and EM preparations was significantly higher than in control samples. On the 39th day, DHA activity in soil with diflufenican and EM preparations decreased compared to control samples. It confirms our results that diflufenican disappeared more slowly in soil with the addition of EM preparations, probably due to the change in soil pH. On the 39th day, DHA activity in soil with flurochloridone and EM preparations was on the same level as in the control samples. On the 60th day, DHA activity decreased in samples with herbicides. The enzyme activity in samples with herbicides and EmFarma Plus™ and Rewital PRO+ preparations was significantly higher than in the control samples.

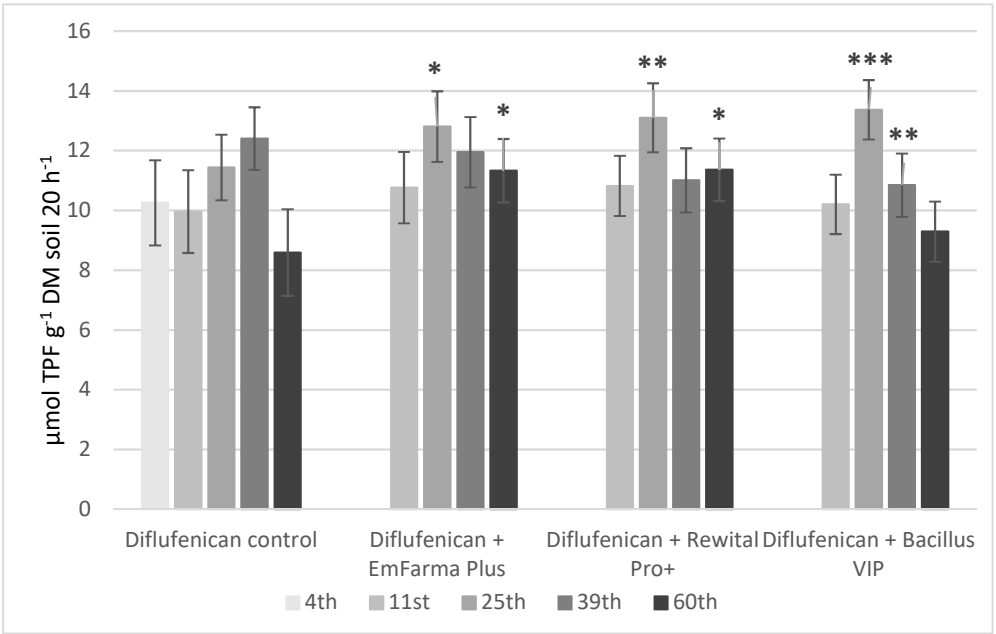

Figure 5. Changes in the DHA of the soil with diflufenican and following application of formulations with EM in time. Statistically significant p values are shown as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Our results confirm that the herbicide or its metabolites may stimulate the activity of microorganisms in the first period, while the activity of DHA decreases with dissipation of the active substance [22].

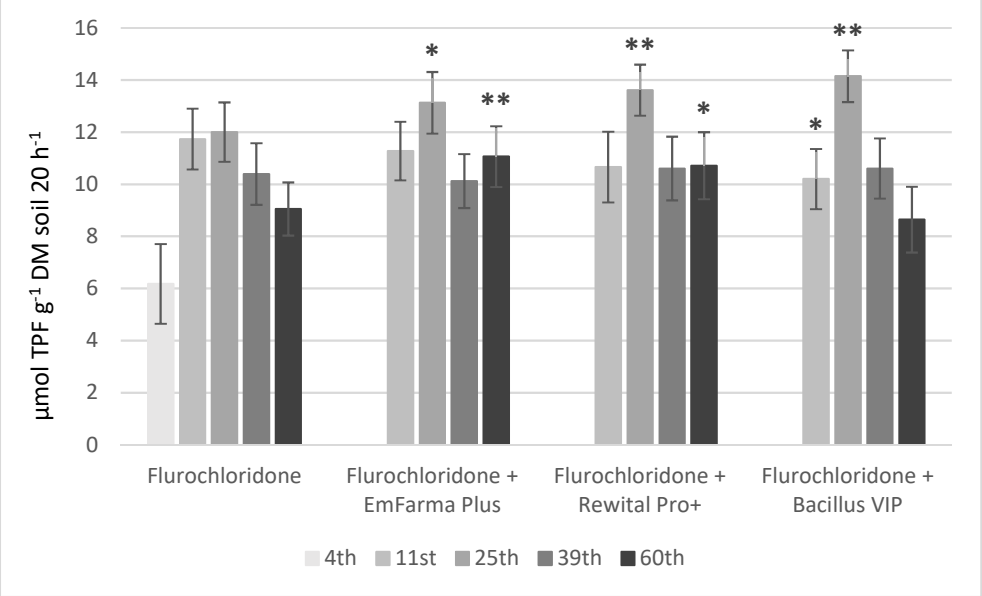

Figure 6. Changes in the DHA of the soil with flurochloridone and following application of formulations with EM in time. Statistically significant p values are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

3. Discussion

3.1. Kinetics of Active Substances Diflufenican and Flurochloridone and Their Disspation and Half-Lives in Soil

3.1.1. Diflufenican

The experiments were conducted using formulation Legato 500 SC, containing diflufenican as an active substance in the quantity of 500 g of active substance/L.

One day after application, diflufenican residues amounted to 11.401 mg/kg and dissipated in accordance with an exponential equation: $y = 0.4879e^{-0.027x}$ ($R = 0.970$), achieving a half-life of $t_{1/2} = 25.7$ days. On the last day of the study, diflufenican residues amounted to 2.267 mg/kg.

The database of pesticide properties specifies that half-lives of diflufenican in the soil are 41.4–318 days in laboratory conditions and 224–621 days in the field [20].

Rouchaud et al. [23] calculated the half-life of diflufenican in the soil in the field, and it amounted to 101 days, while Rouchaud et al. [24] specified the half-life of the herbicide in the soil in a pear orchard, and it amounted to about 65 days for the soil treated with diflufenican for the first time and every year for 3 years.

The diflufenican decomposition rates in the soil differed in pH, coming from Kirton (soil of pH 7.8) and from Wellesbourne (soil of pH 6.8). DT25 of the pesticide was determined, and for two soils it amounted to 29.5 weeks. Diflufenican degraded very slowly, without a change in the degradation rate over time [25].

Conte et al. [26] found no acceleration in diflufenican metabolism with the increase in the pesticide level. The herbicide was used in two fields, before sowing, every year for 4 years. Soil samples were analyzed directly after application every year and at 6 and 12 months after application. Each year, wheat and maize were cultivated in the fields as the main crop and the succeeding crop, respectively. The analysis did not show any residues in any of the wheat or maize plant parts in quantities exceeding the limit of quantification (0.001 mg/kg). No herbicide accumulation year after year was demonstrated. The DT50 value was calculated, amounting to ca. 14 days.

In our experiments, diflufenican dissipated faster (lower $t_{1/2}$ value) than reported by other researchers, and this may have resulted from the experimental conditions differing from the field ones, i.e., the temperature maintained at a level of 22 ± 1 °C, and especially, a high moisture content of 67–74%.

3.1.2. Flurochloridone

The experiments were conducted using formulation Racer 250 EC, containing flurochloridone as an active substance in the quantity of 2500 g of active substance/L.

One day after the formulation application, flurochloridone residues amounted to 15.141 mg/kg and dissipated according to the exponential equation: $y = 0.6022e^{-0.031x}$ ($R = 0.925$), achieving its half-life of $t_{1/2} = 22.4$ days. On the last day, the 60th day of the study, flurochloridone residues amounted to 2.487 mg/kg. The flurochloridone dissipation parameters achieved in our experiments are correlated with data obtained by other researchers.

The database of pesticides properties specifies that half-lives of diflufenican in the soil are 22.8–180 days in laboratory conditions and 11–65 days in the field [20].

Rouchaud et al. [27] calculated its half-life in the soil in the field, and it amounted to 41 days. The next year, they repeated this study in the same field and the $t_{1/2}$ increased to 50 days.

In another experiment, flurochloridone dissipation in potatoes and soil on China's Qinghai plateau (Haixi, Haibei and Haidong) was studied using the modified QuEChERS method combined with liquid chromatography—mass spectrometry (LC-MS/MS). The flurochloridone half-life was calculated, and it amounted to 25.7, 27.7, and 19.3 days in the soil and 8.1, 10.2, and 7.0 in potato leaves in three districts (Haibei, Haixi, and Haidong), respectively. The pesticide degraded at the fastest rate in the Haidong region, in which rainfalls were relatively heavy. Final flurochloridone residues amounted to <0.001, 0.005–0.013, <0.001 and 0.007–0.023 mg/kg in potato leaves, roots, tubers, and soil, respectively, and did not exceed the maximum acceptable level of residues (0.1 mg/kg) [28].

Sharipov et al. [29] assessed the half-life for flurochloridone in three soils from different agricultural regions of the Czech Republic where sunflower are cultivated. The soils differed in pH values, $CaCO_3$ content, organic matter, and contents of clay, silt, and sand. The half-life, $t_{1/2}$, amounted to 38 days for the soil of the Haplic Chernozem type (Suchdol), 46 days for the soil of the Haplic Fluvisol type (Dobromerice), and 51 days for the soil of the Arenic Regozem type (Volarna). The highest degradation of the herbicide occurred in the Arenic Regozem soil, characterized by the highest pH and the highest $CaCO_3$ and sand content when compared to other studied types of soil. That soil was also characterized by the lowest organic matter and silt content.

3.2. Influence of EM in Formulations on Herbicide Degradation in the Soil in Laboratory Conditions

Following a treatment with formulations containing EM, EmFarma Plus™, Rewital PRO+, and BACILLUS VIP Probiotic Microorganisms, the influence of those microorganisms on herbicide degradation in the soil was studied. The literature review concerning decomposition of pesticide residues indicates a great interest in the use of biological methods for degradation of active substances by bacteria, fungi, and yeasts.

3.2.1. Diflufenican

After application of diflufenican and formulation EmFarma Plus™, a degradation at a level of 2.4% to −36.5% was observed compared to the control samples. When Rewital PRO+ was applied, the degradation ranged from 1.4% to −35.5%, while after application of BACILLUS VIP Probiotic Microorganisms, the degradation ranged from 7.2% to −38%. A statistically significant difference was noted for EmFarma Plus™ and Rewital PRO+. No statistically significant differences were observed for BACILLUS VIP Probiotic Microorganisms (Table 1 and Figures S1–S3 Supplementary Material). The results of the conducted experiment indicate that EM had a significant effect on the diflufenican degradation in the soil, but they proved to be opposite to the expected ones.

To this date, there have been several reports on the influence of organic fertilizers applied to soil on diflufenican degradation and one paper on microorganism influence on the diflufenican decomposition.

The degradation was probably inhibited by a significant drop in the soil pH following application of the biological formulations (by 1 pH unit versus the control). According to Houot et al. [30], soil of pH below 6.5 is characterized by a slower metabolic degradation.

Similar results were obtained during a study performed in winter wheat in Belgium. To analyze diflufenican dissipation in field conditions, before sowing, the soil was treated with green fertilizer, cow manure, or pig slurry. The herbicide formulation was used at a dose of 250 g/ha. The diflufenican residues were tested 11 times during 281 days after the treatment. The diflufenican half-life was calculated, and it was 101, 116, 215, and 176 days for the control soil, and the soil treated with green fertilizer, pig slurry, and cow manure, respectively. Organic fertilizers increased durability of the herbicide and its metabolites 2(2-[3-triflurомethyl) phenoxy]-3-pyridinecarboxylic acid, 3(*N*-2,4-difluorophenyl)-2-hydroxy-3-pyridincarboxyamide, and 4,2-hydroxy-3-carboxypyridine. This study confirms the results of our research—increased persistent of diflufenican after application of biological preparations to the soil. After 6 months, the effect of the organic fertilizers was less pronounced and diflufenican and its metabolites in the soil were at similarly low levels [23].

Diflufenican dissipation in field conditions was analyzed in the soil on which winter wheat was sown. Herold® formulation (Spain) was used, containing 20% diflufenican and 40% flufenacet. The field was divided into plots: control soil (S), soil treated with the herbicide (S + H), control soil with spent mushroom substrate treatment (S + SMS), soil treated with the herbicide and SMS (S + H + SMS), soil treated with green compost (S + GC), and soil treated with the herbicide and GC (S + H + GC). Pesticide residues were analyzed 0, 45, 145, 229, and 339 days after the herbicide application. At the beginning of the experiment, diflufenican levels in the control and in the treated soil ranged between 2.24 and 2.81 µg/g of dry matter. The diflufenican levels decreased in S and S + GC 45 days after the treatment; however, no significant reduction was noted in S + SMS, probably due to a different initial diflufenican absorption by the soil. After 339 days, diflufenican residues in the topsoil were >65% [31].

Other studies were conducted in two types of soil, clay loam and sandy loam. The soils were enriched with different types of organic material, using municipal solid waste (MSW) and cow manure (CM). In both cases, the diflufenican content decreased gradually. The study was conducted for 250 days. At the end of the experiment period, the diflufenican content decreased significantly, by 25.5% in clay loam and by 41.2% for the soil treated with CM and MSW, while in the sandy loam, the diflufenican content dropped by 32.5% and 50.2% for CM and MSW, respectively, versus the control group [32]. These results also confirm faster dissipation of diflufenican in soil with lower organic content.

The influence of fungi, *Fusarium oxysporum* PP0030, *Paecilomyces variotii* PP0040, and *Trichoderma viride* PP0050 on dissipation of pesticides, including diflufenican, was also studied. These species proved to be valuable as active microorganisms decomposing pesticides and having a very high capability for biotransformation of target pesticides. The highest level of diflufenican degradation was achieved by *F. oxysporum*, followed by *P. variotii* and *T. viride*. After 120 days, the maximum degradation of diflufenican by *F. oxysporum* amounted to 74.7% [33].

3.2.2. Flurochloridone

After application of flurochloridone and formulation EmFarma Plus™, degradation at a level of 4.4% to 19.3% was observed compared to the control samples. When Rewital PRO+ was applied, the degradation ranged from 7.1% to 30.5%, while after application of BACILLUS VIP Probiotic Microorganisms, the degradation ranged from 17.8% to 31.2%. A statistically significant difference was noted for Rewital VIP+ and BACILLUS VIP Probiotic Microorganisms. No statistically significant differences were observed for EmFarma Plus™ (Table 2 and Figures S4–S6 Supplementary Material).

To this day, only one report has been published on the influence of organic fertilizers on flurochloridone degradation.

Rouchaud et al. [27] studied the flurochloridone dissipation in field conditions in Melle, Belgium. The herbicide (Racer 25CS, Zeneca, Cambridge, UK) at a dose 500 g/ha on the soil was applied 6 months before planting potatoes. Then, the field was divided into plots, and each plot was treated with a different natural fertilizer: cow manure, pig slurry, or green manure (yellow mustard, Emergo type). Control plots were not treated with the organic fertilizers. All treatments (the plots treated with the fertilizers and the control plots) were performed in four fields each (four replicates). The studies were conducted for 2.5 months. The flurochloridone half-life was calculated and it amounted to 48, 67, and 74 for the soil treated with green manure, pig slurry, and cow manure, respectively. In the control plot, $t_{1/2}$ amounted to 41 days. The experiment was repeated in the same field in the subsequent year. Each plot was treated with the same organic fertilizer. The herbicide half-life was verified again. The $t_{1/2}$ increased and amounted to 50 days for the control, and 58, 80, and 70 days for the soil treated with green manure, pig slurry, and cow manure, respectively. In both studies, the highest increase in the herbicide degradation rate was achieved with the green manure. After the potatoes were harvested, the soil in all plots was examined, and flurochloridone was not found again (limit of quantification of 0.01 mg/kg). This results confirm our findings that EM significantly contribute to dissipation of flurochloridone in soil.

4. Materials and Methods

Three commercially available formulations containing EM were used in the study:

EmFarma Plus™ (ProBiotics, Bratuszyn, Poland)—a formulation revitalizing the soil. A natural biostimulator of plant growth and development. It effectively accelerates decomposition of organic matter and increases availability of nutrients, mainly nitrogen, to plants. Microorganisms contained in the formulation, including phototropic bacteria (more resistant to sun rays), interact with each other and replace pathogenic microflora. Its main component is stock cultures of live microorganisms, SCD ProBio Plus®. These are concentrated mixes of live microorganisms produced in a natural fermentation process with participation of beneficial microorganisms commonly found in the environment. The formulation includes bacteria: *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bacillus*, and *Rhodopseudomonas* and yeast *Saccharomyces cerevisiae*, organic sugar cane molasses, revitalized non-chlorinated water, rock salt, and a mineral complex. Molasses is a medium for microorganisms and is rich in numerous components, including micronutrients, that may have an advantageous effect on plant growth [34].

Rewital PRO+ (BIOGEN, Warszawa, Poland)—a formulation revitalizing the soil—contains beneficial bacteria for restoring and maintaining microbiological balance in the soil degraded by, e.g., the use of chemical plant protection agents. The formulation includes bacteria: *Streptomyces*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Cellulomonas*, *Arthrobacter*, *Paenibacillus*, and *Pseudonocardia* and the starter medium. These bacteria facilitate nutrient absorption by plants, improve the soil structure, and support mineralization of residues in the soil, reducing incidence of plant diseases. Microorganisms included in the formulation support and appropriately control processes of the organic matter decomposition in the soil, which significantly influence the nitrogen, phosphorus, and sulfur compound management in the soil [35].

BACILLUS VIP Probiotic Microorganisms (AGROBIOS, Nowy Tomyśl, Poland) is a formulation that improves soil fertility by stimulating plant immunity and growth. The formulation contains eight bacteria strains from the *Bacillus* genus which are highly resistant to the environmental stress caused, e.g., by high temperature, exposure to UV light, lack of water, and high salt content: *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. coagulans*, *B. laterosporus*, *B. mucilaginosus*, and *B. polymyxa*, as well as organic sugar cane molasses and revitalized water. Each of eight bacteria strains in the formulation provide numerous benefits. The most of them include increased bioavailability of nutrients: phosphorus, nitrogen, potassium, and iron, production of growth substances (amino acids, enzymes, phytohormones), stimulation of plant and root growth and plant

germination, and increasing plant resistance to pests and diseases (production of antibiotics or fungicides) [36].

4.1. Reagents

The reagents used for analyses of herbicides and their metabolites in soil included: hexane and acetone of the analytical grade (Witko, Łódź, Poland), petroleum ether for GC (Chempur, Piekary Śląskie, Poland), analytical standards of herbicides (Sigma-Aldrich, St. Louis, MO, USA), sorbents for extraction by the QuEChERS method (magnesium sulfate, $MgSO_4$; sodium chloride, NaCl; sodium citrate, $Na_3C_6H_5O_7$; disodium citrate sesquihydrate, $C_{12}H_{18}Na_4O_{17}$) (Chempur, Poland), and sorbents for clean-up by the dispersive solid phase extraction according to the QuEChERS method (primary and secondary amines PSA (Agilent, Santa Clara, CA, USA) and $MgSO_4$ (Chempur, Poland), Silylating mixture II according to Horning (BSA + TMCS + TMSI 3:2:3) for the GC derivatization (Sigma-Aldrich, USA), and phosphate buffer (60 mM, pH = 7) (Chempur, Poland). The reagents used for DHA analysis included: methanol LC-MS (Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany), 2,3,5-Triphenyltetrazolium chloride (Sigma-Aldrich, USA), 1,3,5-Triphenyl tetrazolium formazan (Tokyo Chemical Industry, Tokyo, Japan).

4.2. Soil Sample Preparation

The soil used in experiment was a universal one, recommended for horticultural crops. It consists of low moor and high moor peat, pine bark, sand, perlite, dolomite, and mineral fertilizers. Soil parameters: pH 6.0–7.3 and a salt level of 0.5–1.0 g KCl/dm^3 (PPUH Zielona Oaza I, Brzozów, Poland).

The experiment was conducted for t2 months, from October to December, at a constant ambient temperature of 22 ± 1 °C and the constant soil humidity of 67–74% (Figures S7–S12, Supplementary Material).

400 g samples of soil were weighed into transparent propylene containers of 2 L each. 80 mL of the formulation Legato 500 SC (ADAMA, Warszawa, Poland) [37], containing diflufenican as an active substance, were added to each of twelve soil samples, and 80 mL of the formulation Racer 250 EC (ADAMA, Warszawa, Poland) [38], containing flurochloridone as an active substance, to each of twelve other soil samples. Legato 500 SC is used to control weeds in cereals before or soon after their emergence. It is absorbed through leaves and partly through plant roots. Racer 250 EC is used against mono- and dicotyledon annual weeds in winter cereals and in vegetables. The plant absorbs it through roots and cotyledons of germinating weeds.

Currently, diflufenican and flurochloridone are registered and approved for use as an active substance of herbicides in the European Union [39].

Legato 500 SC (active substance diflufenican 500 g/L) at a dose 0.1 mL/L and Racer 250 EC (flurochloridone 2500 g/L) at a dose 0.2 mL/L were applied in the form of spraying and mixing with the soil [37,38]. All samples were prepared and analyzed in three replicates (three containers per each sample).

On the next day, 12 h after the treatment, soil samples were collected and analyzed for pesticide residues. Then, on Day 4 of the experiment, formulations containing effective microorganisms, EmFarma Plus™, Rewital PRO+, and BACILLUS VIP Probiotic Microorganisms were added to the samples in accordance with the study plan provided in Table 3. Water was added to control samples, containing only studied pesticides. Samples were mixed thoroughly. All samples were prepared and analyzed in three replicates (three containers per each sample). The blank samples were soil with water.

The samples for pesticide analyses were collected 1, 11, 25, 39, and 60 days after the herbicide application. Before sampling, each soil was mixed thoroughly with a laboratory spoon. In soil samples, water content was measured by a weighing method after drying at 105 °C (S-40, Alpina, Konin, Poland) [40]. Additionally, during sampling, pH, and the oxidoreduction potential were measured with a digital meter ORP/pH AD14 (ADWA,

Szeged, Hungary). The pesticide concentration was calculated for the soil dry mass. All soil samples were stored in stable laboratory conditions.

Table 3. Study plan.

Samples Number	Title 3
1–3	soil + diflufenican
4–6	soil + diflufenican + EmFarma Plus™
7–9	soil + diflufenican + Rewital PRO+
10–12	soil + diflufenican + BACILLUS VIP Probiotic Microorganisms
13–15	soil + flurochloridone
16–18	soil + flurochloridone + EmFarma Plus™
19–21	soil + flurochloridone + Rewital PRO+
22–24	soil + flurochloridone + BACILLUS VIP Probiotic Microorganisms

Figures S7–S12 (Supplementary Material) present changes in pH, the oxidoreduction potential, and the moisture content for the soil samples during successive days of the experiment.

4.3. GC-MS Analysis of Pesticides Residues and Possible Metabolites

Herbicides in the soil were analyzed using the modified standardized method PN-EN 15662:2018-06 entitled "Foods of plant origin. Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE. Modular QuEChERS-method" [41,42]. The modified method was developed and validated in our laboratory for food matrices and soil samples. Validation was carried out with the determination of such parameters as: linearity, recovery, precision, limits of quantification, the working range of the method, and its uncertainty [42].

In brief, 5 g of soil were weighed into a 50-mL polypropylene centrifuge tube, and 10 mL of water and 10 mL of acetone:hexane (1:4 v/v) mixture were added. The tube content was vortexed (BenchMixerTM, Benchmark, Sayreville, NJ, USA) for 1 min. Then, a mixture of QuEChERS extraction salts was added. The tube content was vortexed in a mixer for 1 min and then centrifuged at >4000 rpm (5804R, Eppendorf, Hamburg, Germany) for 5 min. 5 mL of the organic phase were transferred to a 15 mL polypropylene centrifuge tube that contained sorbents for cleanup by the dispersive solid phase extraction method.

A 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a mass detector, model 7000 (GC-MS/MS QqQ), was used to analyze herbicide residues in soil extracts and to determine possible metabolites in Quechers extracts and after its derivatization by the TMS solution.

Before the chromatographic analysis, triphenyl phosphate (TPP) was added to the samples as an internal standard. The samples were monitored in the dynamic multiple reaction monitoring (dMRM) mode. For diflufenican, quantitative determination was performed for fragmentation 266 $\rightarrow$ 238.1 m/z (collision cell energy 15 eV), and qualitative confirmation was performed for fragmentations 266 $\rightarrow$ 246 m/z (15 eV) and 394 $\rightarrow$ 266 m/z (10 eV). The following fragmentation reactions were monitored for flurochloridone: 311 $\rightarrow$ 173.9 m/z (collision cell energy 15 eV), 186.8 $\rightarrow$ 158.9 m/z (10 eV, quantitative ion) and 145.1 $\rightarrow$ 95.1 m/z (15 eV) [42]. The chromatograms of soil samples are shown in Figures S13 and S14 (Supplementary Material).

Additionally, possible metabolites of pesticides were determined in the soil samples. The analysis was performed for Quechers extracts and for extracts subjected to the derivatization process. The sample extract was evaporated under the nitrogen stream, and 100 µL of Silylating mixture II was added according to Horning (BSA + TMCS + TMSI 3:2:3) for GC derivatization (Sigma-Aldrich, USA). The sample was left at the room temperature for 30 min. Then, 500 µL of hexane was added and it was thoroughly mixed for 30 s on the vortex; 1 mL of phosphate buffer (60 mM, pH = 7) was added, and the sample was shaken manually for 1 min. The samples were left for 30 min until separation of the phases.

The top layer (the hexane phase) was transferred into the autosampler vial and analyzed in the full scan mode in the range 40–450 m/z. The following analysis parameters were used: injection of samples in a splitless mode, injected volume—1 µL, carrier gas—helium (flow 1 mL/min), ionization mode—electron (−70 eV), and the temperatures of 250 °C for the injector and transfer line, 300 °C for the ion source, 150 °C for the quadrupoles, and 70–280 °C for the oven.

4.4. Assessment of DHA

The DHA activity was determined in the soil samples according to Casida et al. [43], Tabatabai [44] and Wołejko et al. [22]. Briefly, 6 g of soil was weighed into 50 mL propylene centrifuge tube, 4 mL of distilled water and 1 mL of 3% aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) were added. Samples were incubated in the dark at 37 °C for 20 h. After incubation, 20 mL of methanol was added to the samples, vortexed for 1 min (BenchMixerTM, Benchmark, USA), centrifuged (5804R, Eppendorf, Hamburg, Germany), and filtered. The DHA activity was measured at 485 nm with spectrophotometer Cary 300 Bio (VARIAN, Palo Alto, CA, USA). All results were expressed in micromoles of 1,3,5-triphenyltetrazolium formazan (TPF) per gram of dry soil per 20 h.

4.5. Statistical Analysis of Results

The Student test (Excel Microsoft 365 program) was used to determine statistically significant differences between samples, with and without biological preparation added, for each sampling day. Statistically significant p values are shown in Figure 5, Figure 6 and Figures S1–S6 (Supplementary Material) as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

5. Conclusions

Generally, EM are considered as biodegradation factors for different chemical pollutants. They are used to accelerate dissipation of organic contamination and to remediate and revitalize soil. In our study, we obtained divergent results for two different pesticides. It was demonstrated that commercial EM intended for soil revitalization significantly influenced the degradation of herbicides. For diflufenican, the obtained results were opposite to the expected ones. All studied formulations containing EM reduced the diflufenican degradation level, from 35.5% to 38%, due to an increased acidity of the soil environment and increased durability of that substance at lower pH levels. In the case of flurochloridone, all studied EM formulations increased the degradation of that active substance by 19.3% to 31.2% at the most. The results of our study confirm the need to continue and develop research on the influence of individual EM on the fate of xenobiotics in the environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27144541/s1, Figure S1: Diflufenican levels in individual soil samples and its dissipation following application of EmFarma Plus™ versus the control samples. Statistically significant p value is shown as $p < 0.01$ (**); Figure S2: Diflufenican levels in individual soil samples and its dissipation following application of Rewital PRO+ versus the control samples. Statistically significant p value is shown as $p < 0.05$ (*); Figure S3: Diflufenican levels in individual soil samples and its dissipation following application of BACILLUS VIP Probiotic Microorganisms versus the control samples. No statistically significant differences were observed; Figure S4: Flurochloridone levels in individual soil samples and its dissipation following application of EmFarma Plus™ versus the control samples. No statistically significant differences were observed; Figure S5: Flurochloridone levels in individual soil samples and its dissipation following application of Rewital PRO+ versus the control samples. Statistically significant p value is shown as $p < 0.05$ (*); Figure S6: Flurochloridone levels in individual soil samples and its dissipation following application of BACILLUS VIP Probiotic Microorganisms versus the control samples. Statistically significant p value is shown as $p < 0.05$ (*); Figure S7: Changes in the soil pH levels with diflufenican and following application of formulations containing effective microorganisms during successive days of the experiment; Figure S8: Changes in the soil pH levels with flurochloridone and following application of formulations containing effective microorganisms during successive days of the experiment; Figure S9: Changes in a moisture

content of the soil with diflufenican and following application of formulations with EM in time; Figure S10: Changes in a moisture content of the soil with flurochloridone and following application of formulations with EM in time; Figure S11: Changes in the oxidoreduction potential of the soil with diflufenican and following application of formulations with EM in time; Figure S12: Changes in the oxidoreduction potential of the soil with flurochloridone and following application of formulations with EM in time; Figure S13: Chromatogram of soil sample with diflufenican (retention time for diflufenican—27.854 min, and internal standard—triphenyl phosphate, TPP—27.794 min); Figure S14: Chromatogram of soil sample with flurochloridone (retention time for flurochloridone—20.515 min, and internal standard—triphenyl phosphate, TPP—27.794 min).

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Supplementary material

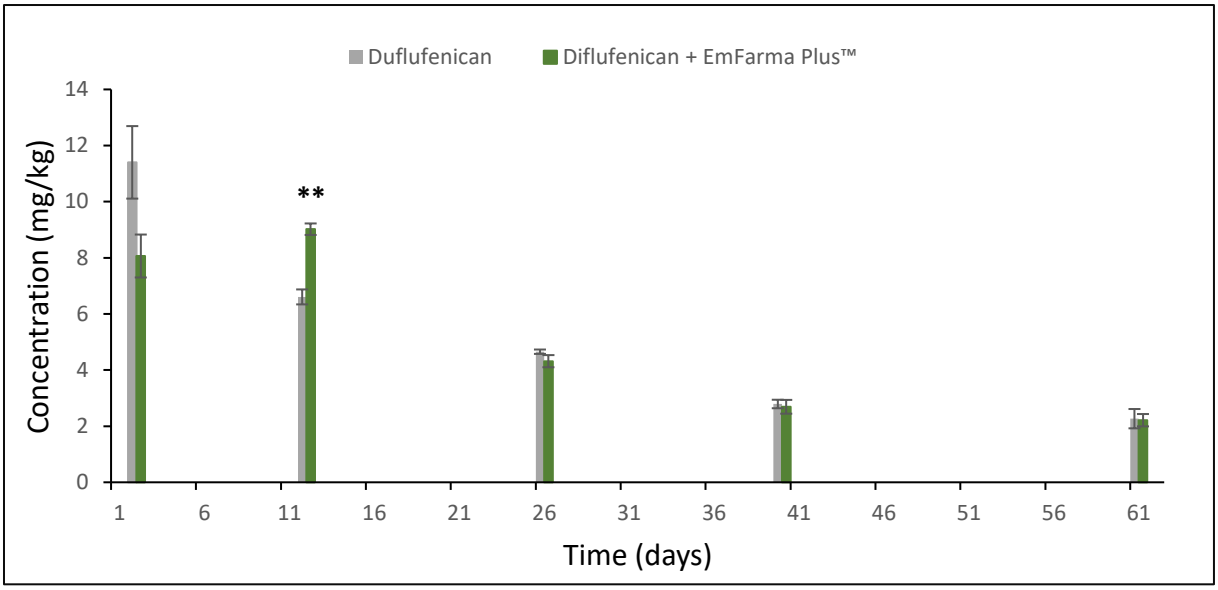


Figure S1: Diflufenican levels in individual soil samples and its dissipation following application of EmFarma Plus™ versus the control samples. Statistically significant p value is shown as $p < 0.01$ (**).

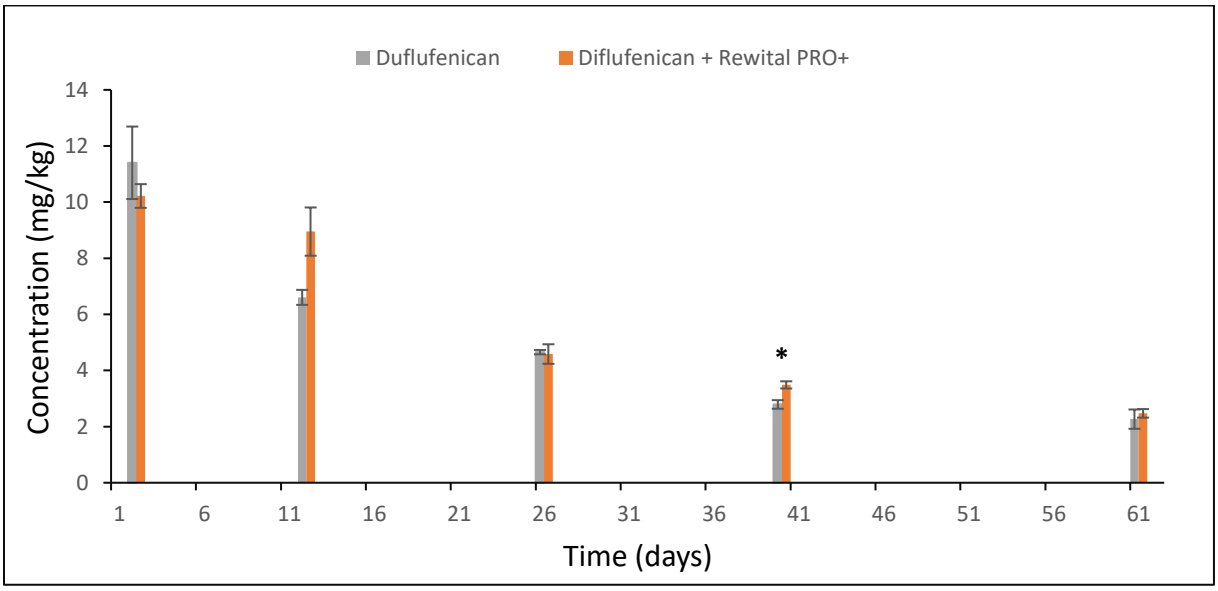


Figure S2: Diflufenican levels in individual soil samples and its dissipation following application of Rewital PRO+ versus the control samples. Statistically significant p value is shown as $p < 0.05$ (*).

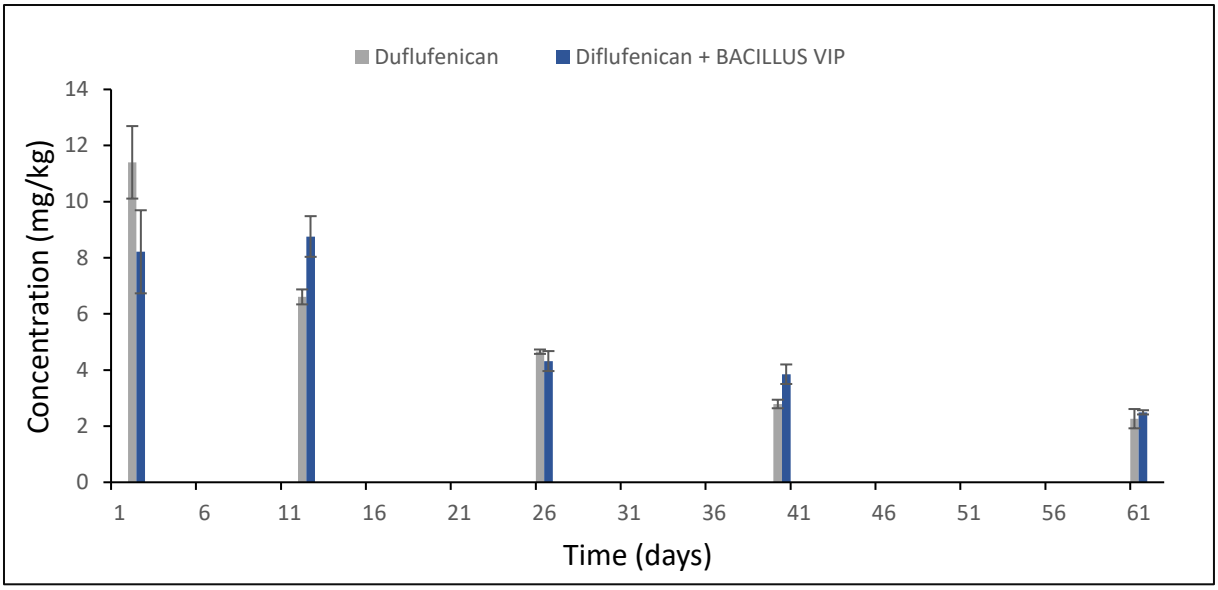


Figure S3: Diflufenican levels in individual soil samples and its dissipation following application of BACILLUS VIP Probiotic Microorganisms versus the control samples. No statistically significant differences were observed.

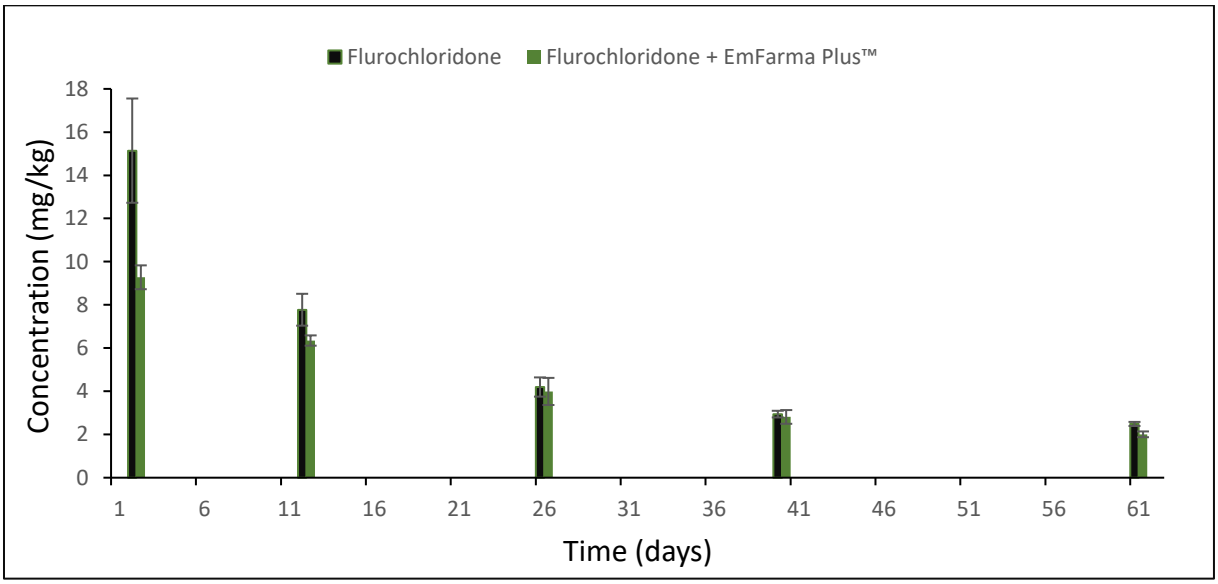


Figure S4: Flurochloridone levels in individual soil samples and its dissipation following application of EmFarma Plus™ versus the control samples. No statistically significant differences were observed.

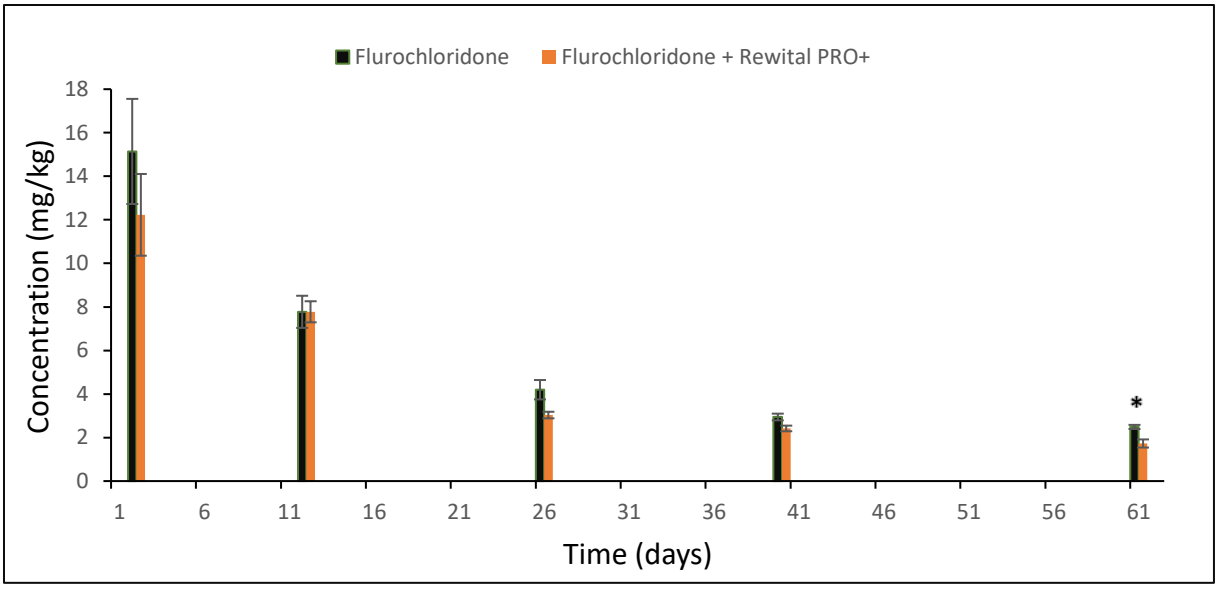


Figure S5: Flurochloridone levels in individual soil samples and its dissipation following application of Rewital PRO+ versus the control samples. Statistically significant p value is shown as $p < 0.05$ (*).

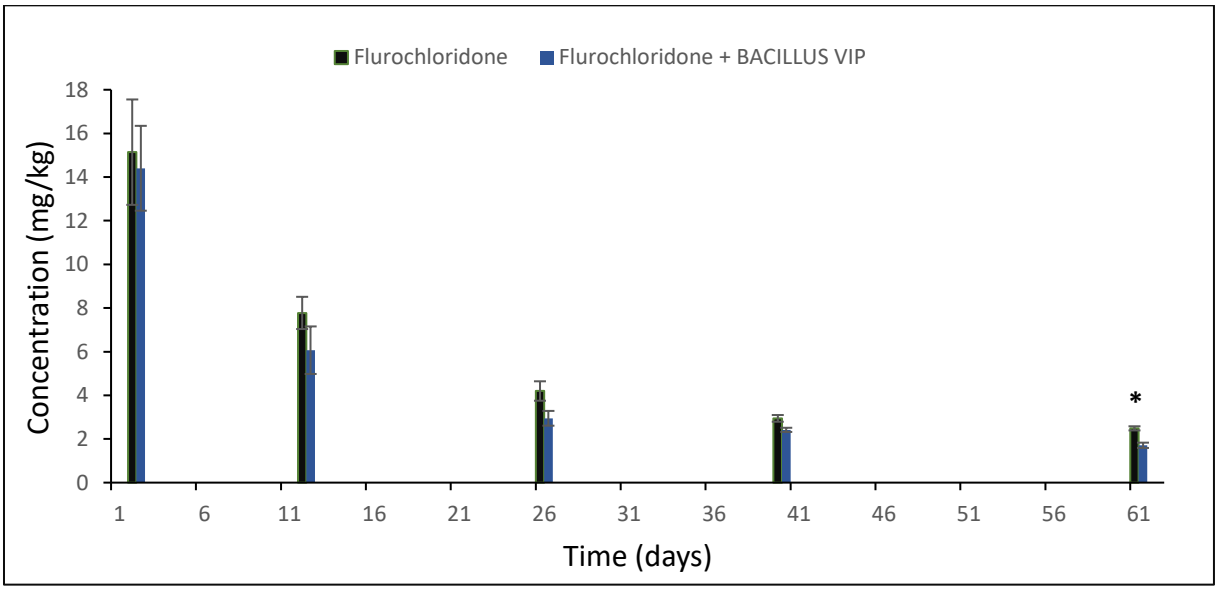


Figure S6: Flurochloridone levels in individual soil samples and its dissipation following application of BACILLUS VIP Probiotic Microorganisms versus the control samples. Statistically significant p value is shown as $p < 0.05$ (*).

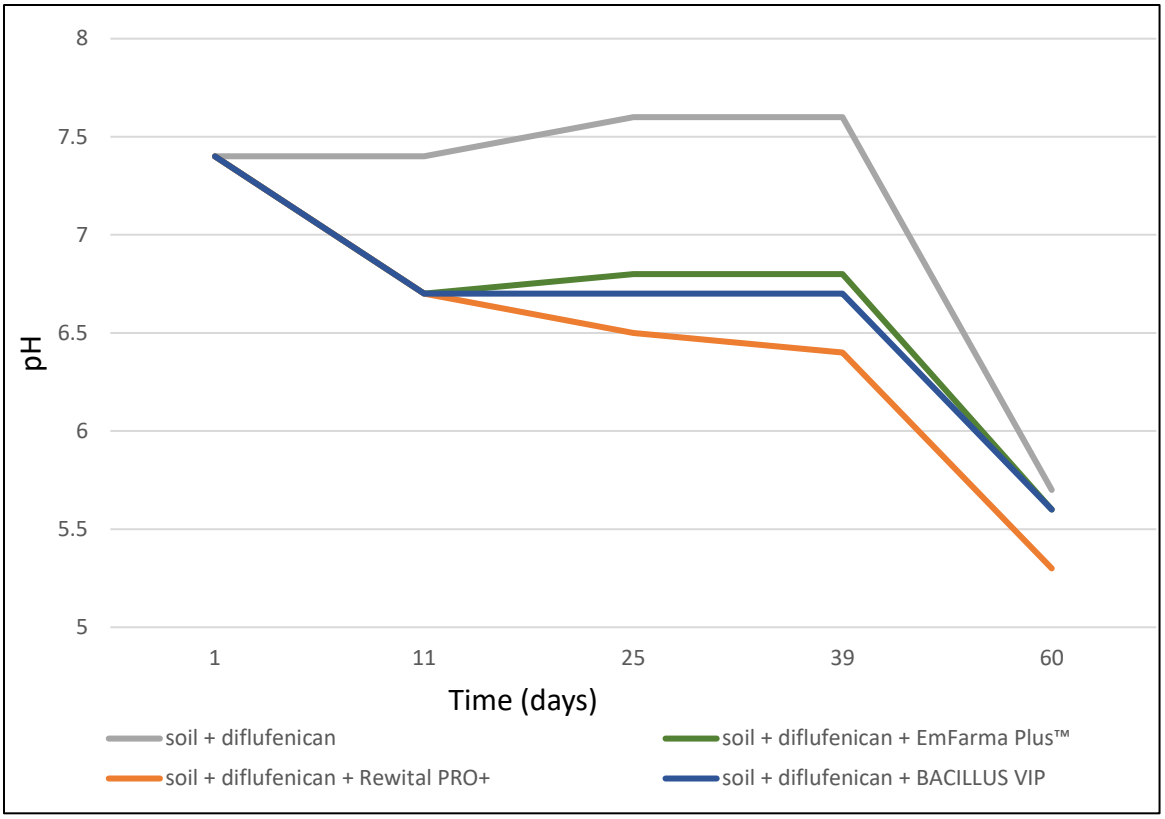


Figure S7: Changes in the soil pH levels with diflufenican and following application of formulations containing effective microorganisms during successive days of the experiment.

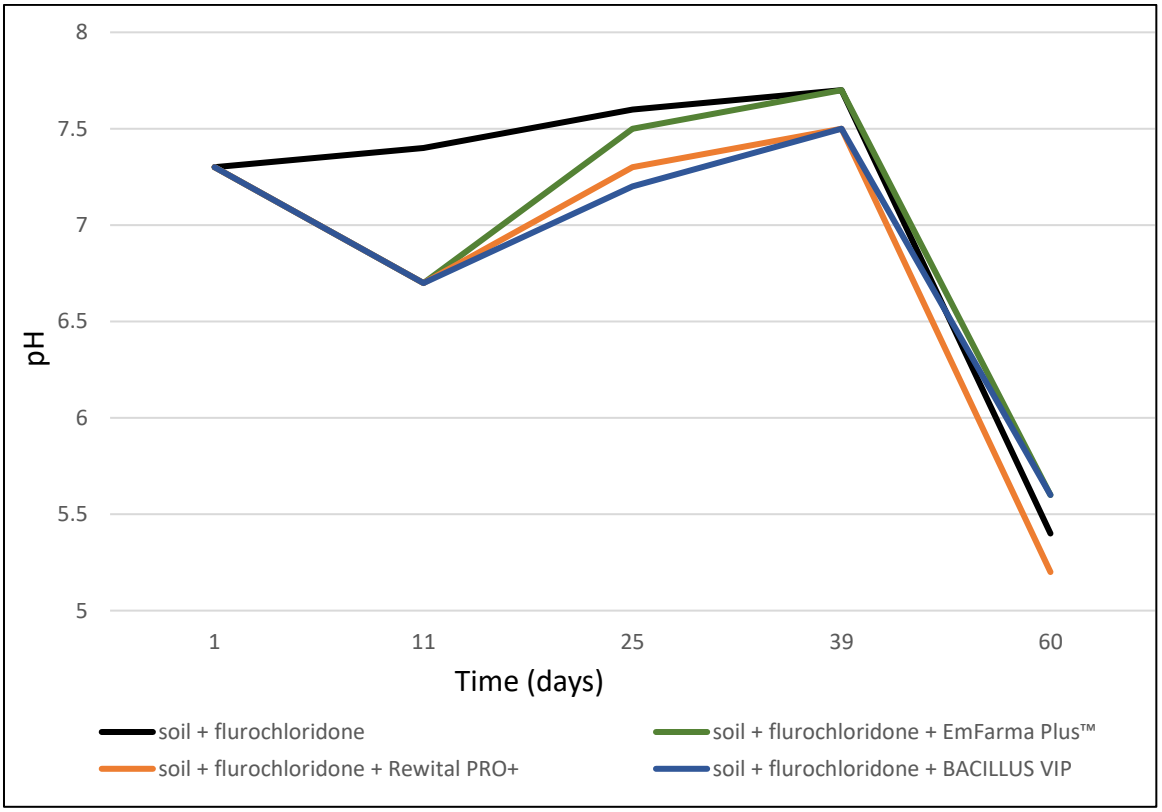


Figure S8: Changes in the soil pH levels with flurochloridone and following application of formulations containing effective microorganisms during successive days of the experiment.

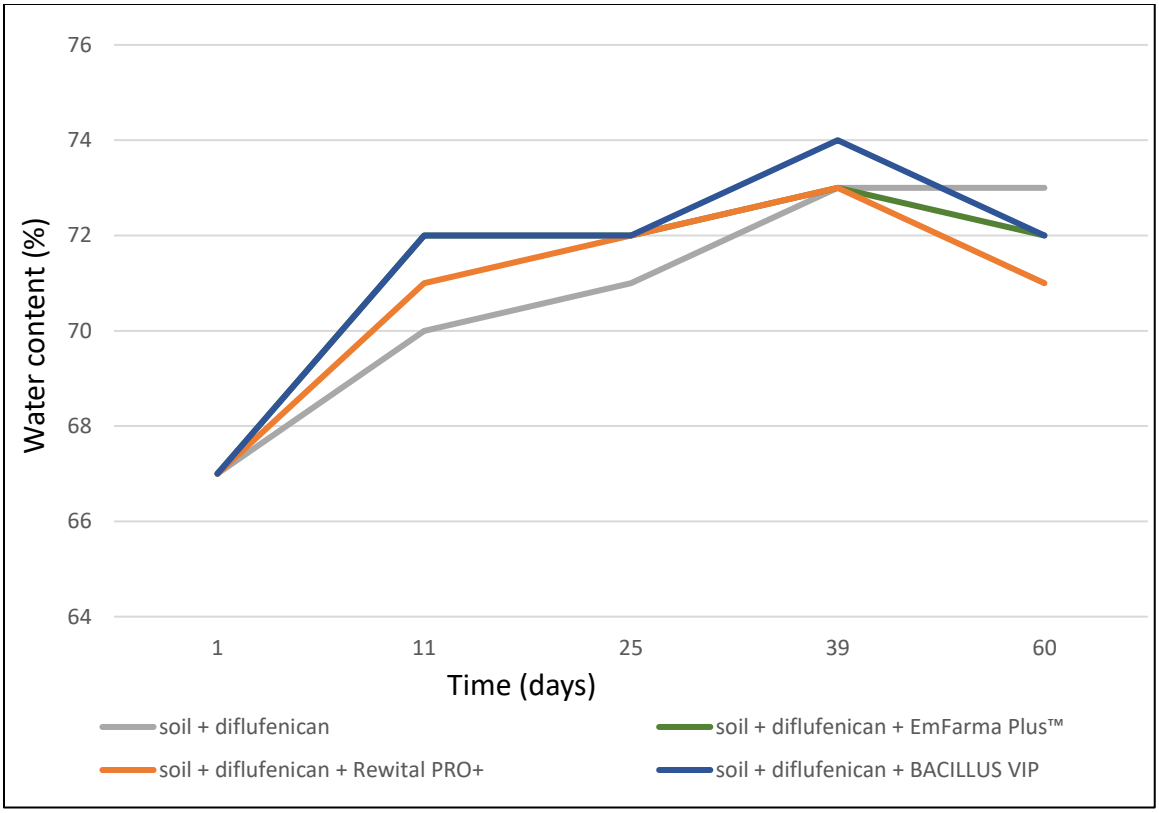


Figure S9: Changes in a moisture content of the soil with diflufenican and following application of formulations with EM in time.

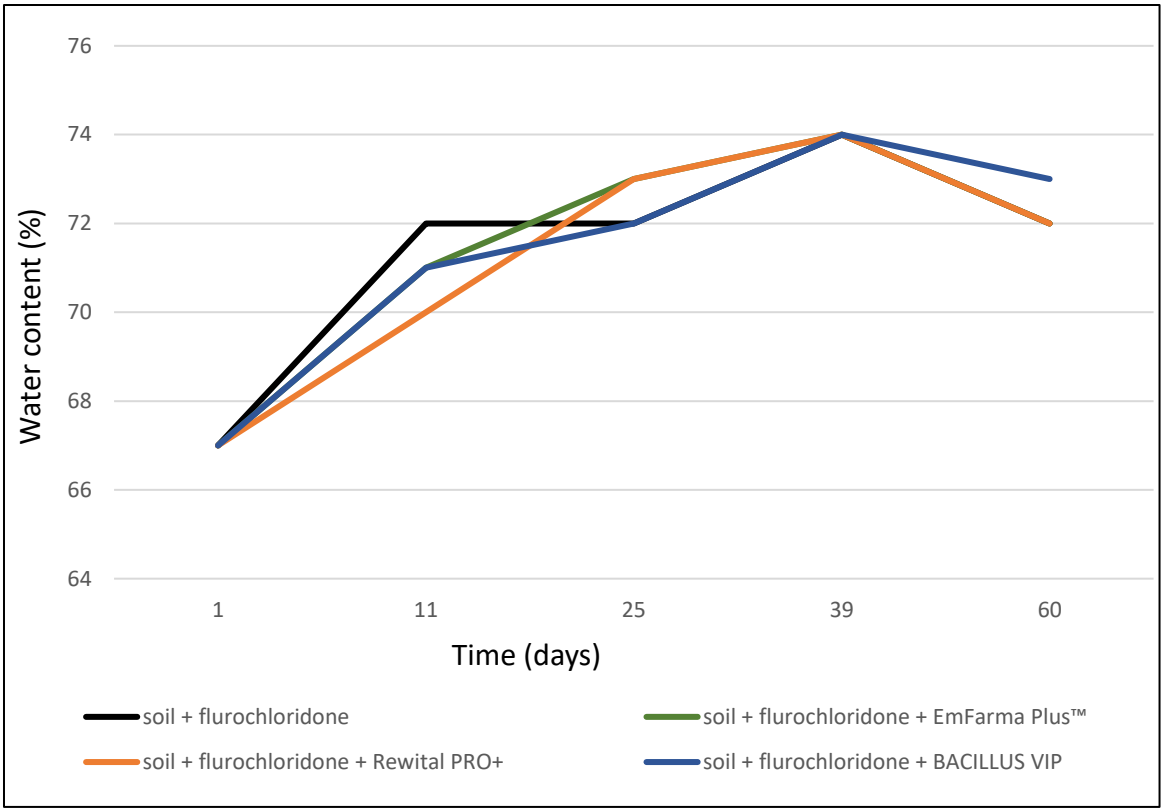
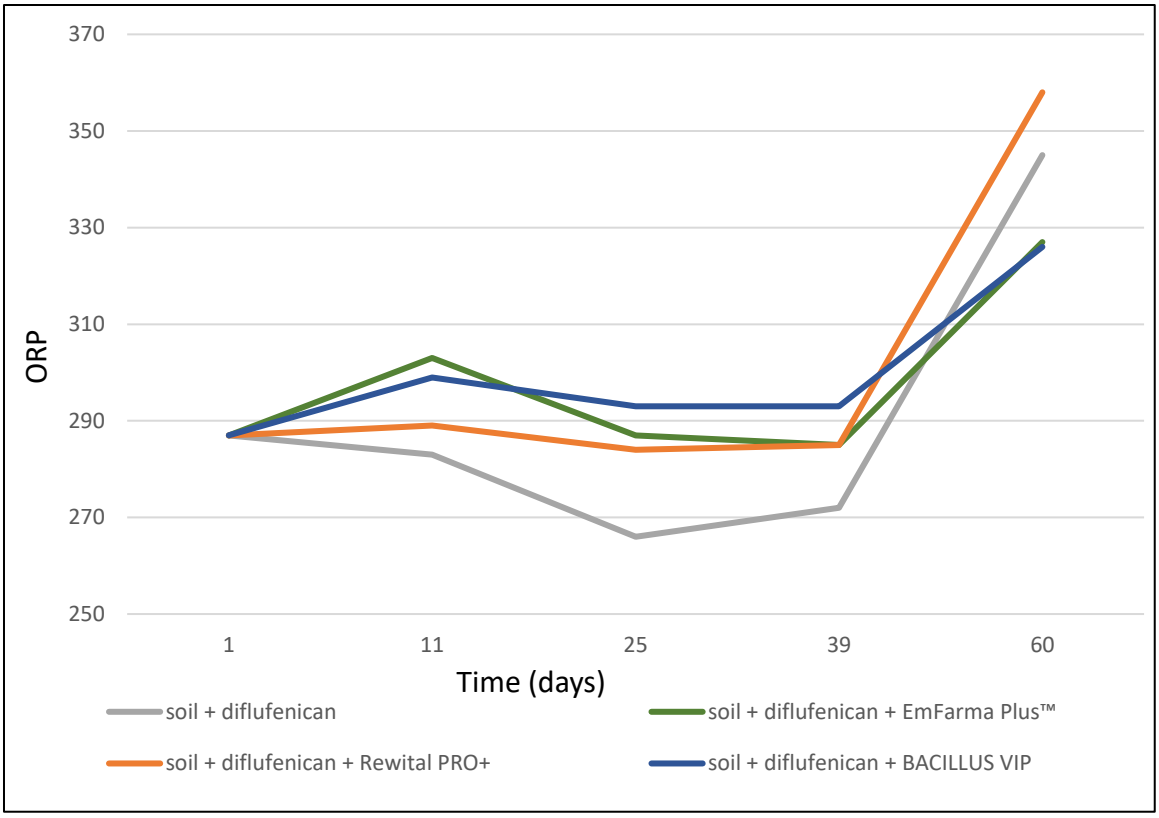
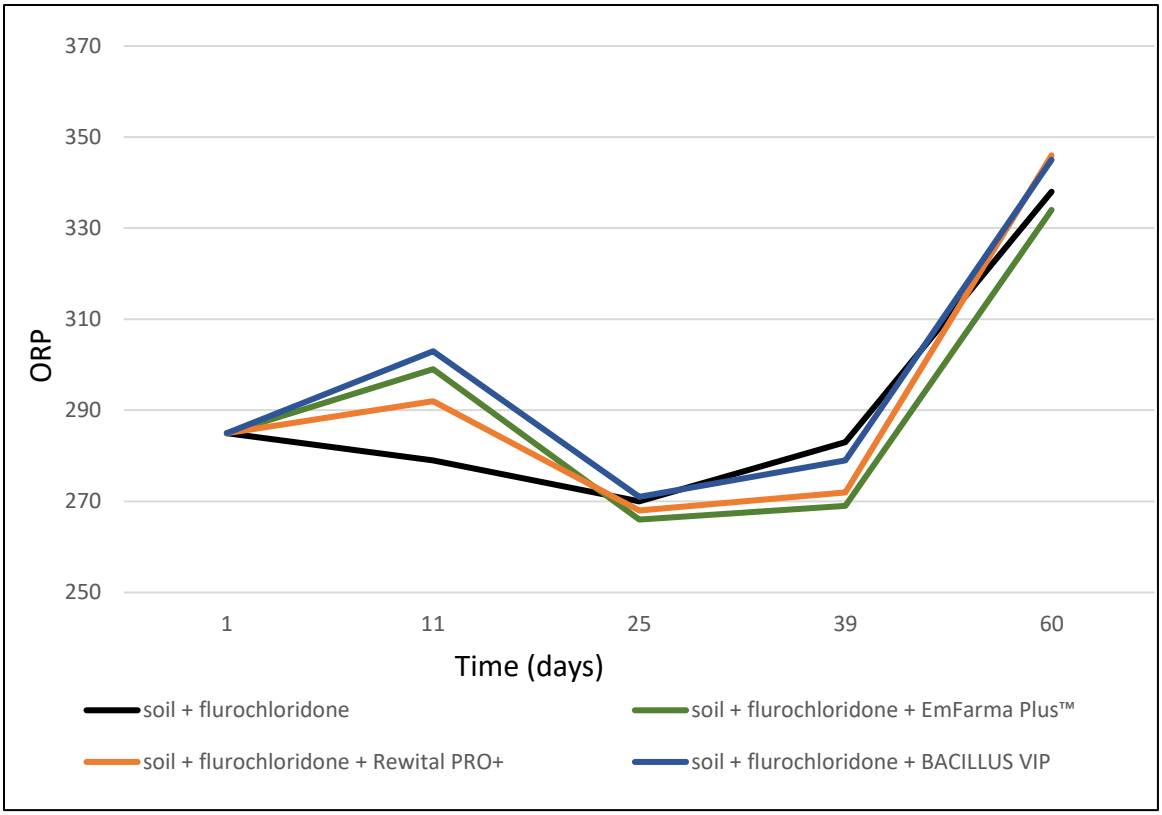


Figure S10: Changes in a moisture content of the soil with flurochloridone and following application of formulations with EM in time.



ORP — oxidation-reduction potential

Figure S11: Changes in the oxidoreduction potential of the soil with diflufenican and following application of formulations with EM in time.



ORP — oxidation-reduction potential

Figure S12: Changes in the oxidoreduction potential of the soil with flurochloridone and following application of formulations with EM in time.

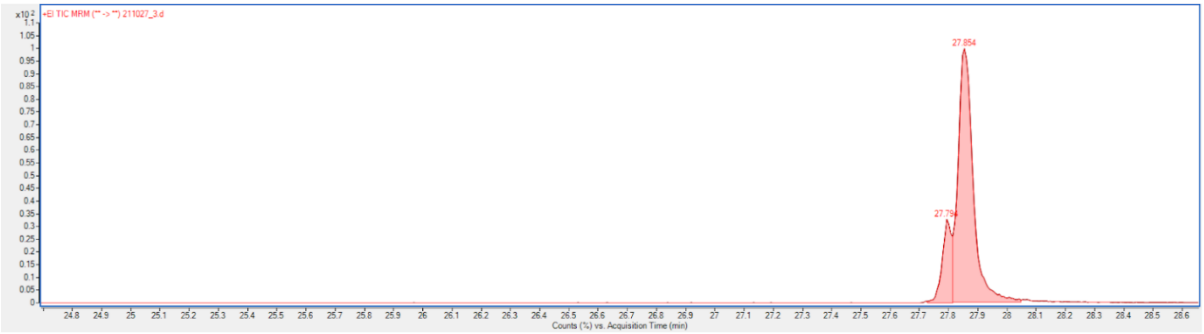


Figure S13: Chromatogram of soil sample with diflufenican (retention time for diflufenican – 27.854 min, and internal standard - triphenyl phosphate, TPP – 27.794 min).

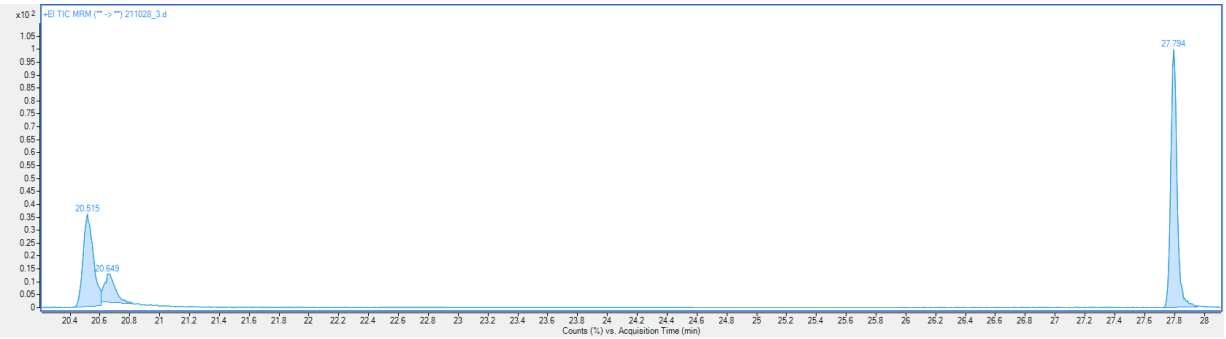


Figure S14: Chromatogram of soil sample with flurochloridone (retention time for flurochloridone – 20.515 min, and internal standard - triphenyl phosphate, TPP – 27.794 min).

Załącznik 1 do Uchwały nr 118/07/2022
Rady Naukowej Kolegium Nauk
Przyrodniczych UR z dnia 12 lipca 2022

Rzeszów, dnia ...06.03.2024.....

Imię i nazwisko **mgr inż. Paulina Książek-Trela**

Jednostka **Instytut Biotechnologii**

Promotor **dr hab. inż. Ewa Szpyrka, prof. UR**

Promotor pomocniczy **dr Leszek Potocki**

OŚWIADCZENIE

W związku z przygotowywaniem przeze mnie rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów, oświadczam niniejszym, że wkład mojej pracy naukowej (w ramach dyscypliny Biotechnologia), a tym samym pracy pozostałych współautorów w opublikowaniu poniższych artykułów, które zamierzam przedstawić jako własną dysertację doktorską jest następujący:

I. Książek-Trela P., Bielak E., Wężka D., Szpyrka E. 2022. Effect of three commercial formulations containing effective microorganisms (EM) on diflufenican and flurochloridone degradation in soil. Molecules 27(14), 4541. DOI: 10.3390/molecules27144541.

- Przeprowadzenie analiz techniką GC-MS/MS razem z E.S.;
- Przygotowanie ekstraktów próbek gleby metodą QuEChERS do oznaczania pozostałości herbicydów techniką GC-MS/MS razem z E.B. i D.W.;
- Analiza, zestawienie i interpretacja wyników;
- Opis i dyskusja wyników;
- Przygotowanie manuskryptu;
- Obowiązki autora korespondencyjnego razem z E.S.

Paulina Książek-Trela
.....
podpis

Oświadczenia współautorów

1. **Ewelina Bielak**
   Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Przygotowanie ekstraktów próbek gleby metodą QuEChERS do oznaczania pozostałości herbicydów techniką GC-MS/MS.

Bielak Ewelina
.....
Podpis

2. **Dominika Wężka**
   Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Przygotowanie ekstraktów próbek gleby metodą QuEChERS do oznaczania pozostałości herbicydów techniką GC-MS/MS.

Dominika Wężka
.....
Podpis

3. **Ewa Szpyrka**

Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Opracowanie koncepcji i metodologii pracy;
- Przeprowadzenie analiz techniką GC-MS/MS razem z P.K.-T;
- Edycja manuskryptu razem z P.K.-T.;
- Obowiązki autora korespondencyjnego razem z P.K.-T.

.....

Podpis

Research Article

Paulina Książek-Trela*, Damian Figura, Dominika Wężka, Ewa Szpyrka*

Degradation of a mixture of 13 polycyclic aromatic hydrocarbons by commercial effective microorganisms

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Abstract: The study focused on the contribution of effective microorganisms (EM) and their consortia, used in commercial biological preparations and formulations for soil revitalization, to the degradation of a mixture of 13 polycyclic aromatic hydrocarbons (PAHs) commonly found in the soil environment. PAHs, diverse forms of which are present in the environment, never occur individually but always as a part of a chemical mixture. Therefore, the research presented in this article, focusing on the EM impact on the mixture of PAHs, reflects the conditions most similar to natural ones. On Day 35 of the experiment, PAH levels decreased by 75.5–95.5%. The highest PAHs degradation efficiency was achieved for fluorene, with a preparation containing eight bacteria strains from the *Bacillus* genus: *B. coagulans, B. amyloliquefaciens, B. laterosporus, B. licheniformis, B. mucilaginosus, B. megaterium, B. polymyxa,* and *B. pumilus*. All tested preparations containing bacterial consortia and a preparation with the yeast *S. cerevisiae* intensified the PAHs degradation more effectively than formulations including only the yeast *Yarrowia lipolytica* or a mixture of *Debaryomyces hansenii* and *Bacillus*. The designed and proposed research will contribute to the development of biotechnological methods – bioremediation by microorganisms that are safe for the human and environment health.

*** Corresponding author: Paulina Książek-Trela,** Department of Biotechnology, Institute of Biotechnology, University of Rzeszow, 1 Pigonia St., 35-310 Rzeszow, Poland, e-mail: pksiazek@ur.edu.pl
*** Corresponding author: Ewa Szpyrka,** Department of Biotechnology, Institute of Biotechnology, University of Rzeszow, 1 Pigonia St., 35-310 Rzeszow, Poland, e-mail: eszpyrka@ur.edu.pl
Damian Figura: Department of Biotechnology, Institute of Biotechnology, University of Rzeszow, 1 Pigonia St., 35-310 Rzeszow, Poland, e-mail: damianfigura100@gmail.com
Dominika Wężka: Department of Biotechnology, Institute of Biotechnology, University of Rzeszow, 1 Pigonia St., 35-310 Rzeszow, Poland, e-mail: doma.wezka@gmail.com

Keywords: polycyclic aromatic hydrocarbons, effective microorganisms, bioremediation, biodegradation, mixed chemicals

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds that contain at least two joined aromatic rings of a flat surface structure (Figure 1) [1]. They form a group consisting of several hundred chemically related compounds, persistent in the environment, of variable toxicity, and having different structures [2]. PAHs are characterized by their carcinogenic, toxic, and mutagenic effects, they are also very strong immunosuppressants. It is thought that their toxicity mechanism is based on the fact that they disturb functions of cell membranes and enzymatic systems [3].

PAH are relatively inert hydrophobic compounds that in mammals can be transformed in metabolic processes into highly reactive dihydrodiol epoxides. These diol epoxides react with single strand and double strand DNA, and they may intercalate between base pairs [5]. PAHs can originate from natural and artificial sources, such as oil and gas. PAHs are produced as a result of biological processes and can be formed as a product of burning organic matter, such as wood and food [6]. PAHs are considered to be omnipresent in our environment and are found in soil, water, atmosphere, and air, as well as during food processing and cooking [1,7]. The sources of PAHs found in the soil include, for example, vehicle exhaust gases. Some PAHs come from more distant sources and were transported in the air to their final destination. PAHs at increased concentrations or at levels exceeding specified limits (amounting to 2.979 mg/kg) are observed in soil samples collected in the vicinity of transportation routes and in locations affected by tourist traffic and transport [8]. The majority of PAHs in the soil are bound with its particles, and this influences their mobility. Compounds of a low molecular mass are characterized by their relative

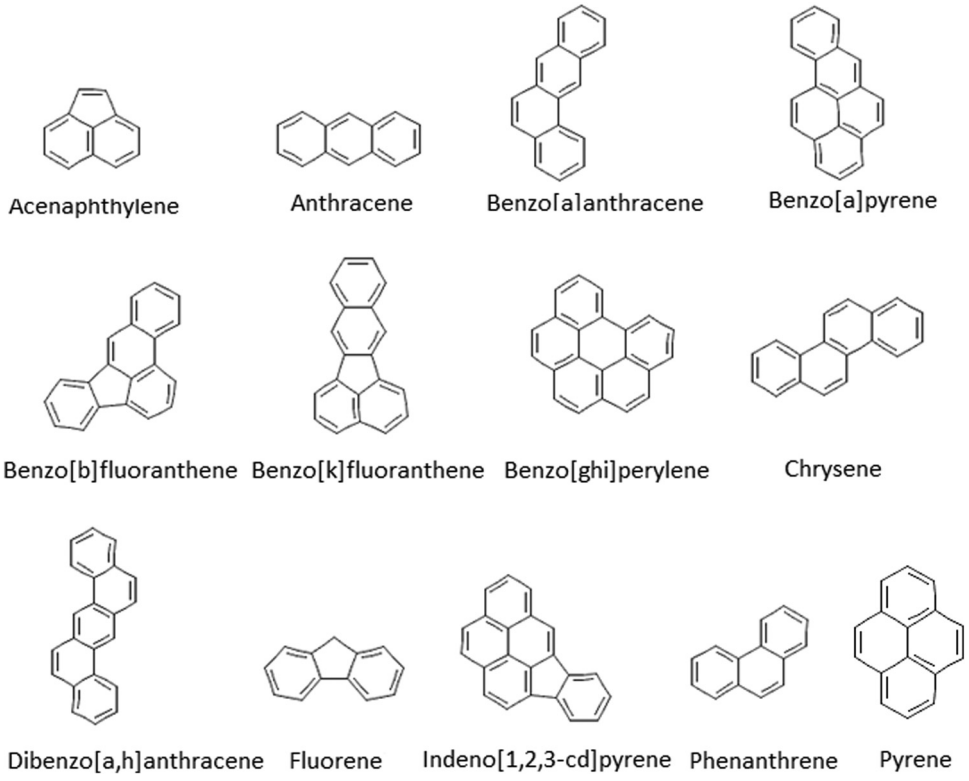

Figure 1: Names and chemical structures of studied PAHs [4].

mobility in the soil and bioavailability to soil microorganisms. However, it is the compounds of a high molecular mass that are more hazardous, because they are stable and hydrophobic, as well as have a low solubility in water [3,9]. Also, the soil properties influence PAHs susceptibility to sorption on the soil particles. Soil conductivity is an important factor influencing PAHs mobility [10]. These compounds are classified as persistent environmental pollutants, and they often occur as a mixture [11].

PAHs are removed from the environment by several methods, including biodegradation, photochemical degradation, photooxidation, washing out, bioaccumulation, or adsorption. Individual processes influence PAHs in different ways, as each compound has a unique structure and different chemical, biological and physical properties [2,12]. Biodegradation of persistent pollutants and soil bioremediation belong to those biotechnological interventions that are most advantageous, in terms of economic and environmental aspects [13–17]. Biodegradation is a preferred and main way for removing PAHs from polluted environments, because it is cost effective and enables complete removal of those compounds [18]. During last decades, a great progress has occurred in studies on PAHs bioremediation [19]. This process involves anaerobic or aerobic biochemical decomposition of organic compounds into simple inorganic compounds by saprobionts, such as bacteria and fungi, but also by yeasts, algae, and protozoa. The bacteria are regarded as key microorganisms for the PAHs degradation [20,21]. Usually, microorganisms are not able to directly decompose PAHs, so the microflora present in a given environment needs to adapt to the PAHs degradation. This is particularly important, taking into account low PAHs solubility in water that results in their low bioavailability to microorganisms [3]. Microorganisms also need time to develop the ability to produce necessary enzymes, depending on a type of microorganisms and properties of PAHs [22]. An individual bacterium or fungus is not able to degrade all contaminations. Thus, biodegradation is a multistage process occurring with a support of a large number of microorganisms that act synergistically [23]. PAHs of a low molecular weight (containing two or three fused benzene rings) can be easily degraded by microorganisms. PAHs of a high molecular weight, containing at least four rings, are resistant to biodegradation; therefore, they accumulate in the ecosystem [19,24,25]. Microorganisms degrading PAHs can metabolize PAHs as a sole source of energy and carbon in the soil [26].

Many studies on PAHs biodegradation focus on bacteria from genera *Nocardia, Pseudomonas, Gordonia, Micrococcus, Rhodococcus, Arthrobacter, Mycobacterium, Flavobacterium,*

Corynebacterium, Klebsiella, Alcaligenes, and *Bacillus*, as well as fungi from genera *Penicillium, Aspergillus, Trichoderma, Candida,* and *Fusarium* [27–31]. While there are many reports on the PAHs degradation by microorganisms, including bacteria and fungi, the publications reporting the successful degradation of PAHs using yeasts are relatively scarce. The effect of yeasts on the degradation of crude oil or PAHs from petroleum-contaminated sites was analyzed in a few studies on *Saccharomyces cerevisiae, Yarrowia lipolytica, Hanseniaspora valbyensis, H. opuntiae,* and *Debaryomyces hansenii* [32–35]. Abioye and Ferreira [32,33] found that yeasts are effective in biodegradation of crude oil, while Mandal [34,35] analyzed decomposition of two PAHs – – benzo[*a*] pyrene and benzo[*ghi*]perylene by a yeast consortium, and its effectiveness was at a level of 76 and 64%, respectively.

PAH bioremediation involves enzymes from bacteria, fungi, yeasts, and other living organisms. Biodegradation using enzymes is efficient and selective, due to higher reaction rates and the capability to catalyze reactions at a wide range of temperatures and pH values [3]. Enzymes responsible for the PAHs degradation include oxygenase, dehydrogenase, lignin peroxidase, manganese peroxidase, laccases, and phenoloxidases [36,37]. Dehydrogenase is an enzyme found in all viable microbial cells. Its activity is a measure of the metabolic state of soil microorganisms [38]. The dehydrogenase activity (DHA) is one of the most adequate, important, and sensitive bioindicators, related to the soil fertility [39]. This activity depends on the same factors that influence the abundance and activity of microorganisms. Besides, it is well known that pesticides, PAHs, and other persistent soil pollutants inhibit DHA [39–41]. Several environmental factors such as the soil moisture content, the oxidation–reduction potential (ORP), the pH, the temperature, the organic matter content, contamination with PAHs or pesticides, and soil fertilization, can significantly affect DHA in the soil [39]. The ORP is an important environmental factor, which reflects the tendency of an environment to receive or supply electrons in a solution [42]. ORP plays a crucial role in regulating the microbial activity, and affects the soil enzymatic activity, especially DHA [43]. Generally, the activity of enzymes tends to increase with the soil pH [44,45]. It was shown that the pH within the acidic range resulted in a strong DHA inhibition, when compared to alkaline soils [39]. The pollution levels are frequently linked to the pH of contaminated sites, as microorganisms may not be able to transform PAHs under acidic or alkaline conditions. The extreme pH values that can be observed in some soils negatively influence the ability of microbial populations to degrade PAHs [46].

The aim of this experiment was to demonstrate the effect of six commercial formulations with effective microorganisms (EM) and one with a mixture of yeasts on the degradation in the soil of 13 substances belonging to PAHs. Furthermore, the analyses focused on shifts in the soil pH, the ORP, and the activity of dehydrogenases (DHA). The novelty of the approach used in this study lies in the analysis of biodegradation for the mixture of PAHs, instead of individual substances, and in the use of commercial preparations. These preparations promote the pro-environmental method of agricultural production – organic farming, and are easily accessible and safe for the environment and humans; therefore, they can be generally used. To this date, the influence of various microorganisms on the degradation of one or several PAHs has been studied. There are many different PAH compounds in the natural environment which never occur individually, but as a chemical mixture. Therefore, the research presented in this article, focusing on the EM influence on the mixture of 13 PAHs, reflects the conditions most similar to natural ones. The presented study also shows differences in the decomposition of individual PAHs by single microorganisms and by consortia of bacteria and yeasts. To our knowledge, no data and publications are available on the influence of tested EM formulations on the degradation of a PAHs mixture in the soil. The proposed research would be advantageous for the development of bioremediation of soils polluted with PAHs. The obtained results can significantly contribute to the development of such disciplines as environmental protection, biotechnology, agronomy, toxicology, and microbiology.

2 Materials and methods

Six commercial formulations containing EM and a mixture of formulations containing yeasts were used in the study. These are biological preparations and formulations for soil revitalization, having a potential to degrade persistent environmental pollutants. They are easily accessible and safe for the environment and humans; therefore, they can be generally used.

Table 1 provides the most important information about the formulations.

2.1 Reagents

Acetone, *n*-hexane of analytical grade, and petroleum ether for GC were obtained from Chempur, Poland. Salts used for extraction using the QuEChERS method included sodium chloride, magnesium sulfate, disodium citrate sesquihydrate,

Table 1: Names and composition of EM formulations [47–52]

Formulation name (name of the manufacturer, country of origin)	Formulation composition
Formulation 1 – EmFarma Plus™ (ProBiotics, Poland)	Bacteria from the genus: *Bifidobacterium*, *Lactococcus*, *Lactobacillus*, *Bacillus*, *Rhodopseudomonas*, and *Streptococcus*, yeasts *S. cerevisiae*, revitalized water, rock salt, organic sugar cane molasses, and a mineral complex
Formulation 2 – Rewital PRO+ (BIOGEN, Poland)	Bacteria from the genus: *Streptomyces*, *Bacillus*, *Pseudomonas*, *Cellulomonas*, *Rhodococcus*, *Pseudonocardia*, *Arthrobacter*, and *Paenibacillus*, starter medium
Formulation 3 – BACILLUS VIP Probiotic Microorganisms (AGROBIOS, Poland)	Eight bacteria strains from the *Bacillus* genus: *B. coagulans*, *B. amyloliquefaciens*, *B. laterosporus*, *B. licheniformis*, *B. mucilaginosus*, *B. megaterium*, *B. polymyxa*, and *B. pumilus*, revitalized water and organic sugar cane molasses
Formulation 4 – Myco Sin® (Biocont, Poland)	Aluminum sulfate tetradecahydrate, inactive, ground, dried yeast – *S. cerevisiae*, and dry horsetail extract (*Equisetum arvense* L.)
Formulation 5 – Biopuls Fusion® (Microlife, Poland)	Yeast strains *Y. lipolytica*
Formulation 6 – Biopuls Cinderella (Microlife, Poland)	Yeast *D. hansenii* and its metabolites, bacteria from *Bacillus* genus
Formulation 7	Mix of yeast Formulations 4, 5, and 6

trisodium citrate (Chempur, Poland), together with sorbents used for clean-up – primary and secondary amines, PSA (Agilent, USA), and magnesium sulfate (Chempur, Poland). A certified mixture of standard solutions, EPA 525 PAHs Mix B, was obtained from Sigma-Aldrich, USA. Furthermore, methanol LC-MS (Honeywell, USA), 2,3,5-triphenyltetrazolium chloride – TTC (Sigma-Aldrich, USA) and 1,3,5-triphenyl tetrazolium formazan – TPF (Tokyo Chemical Industry, Japan) were used for the DHA analysis.

2.2 Soil samples preparation

In the experiment, a commercial universal soil recommended for the horticultural crop was used, containing high and low moor peat, sand, pine bark, dolomite, perlite, and mineral fertilizers. The soil had the salt content at the level of 0.5–1.0 g KCl/dm^3 (the pH of 6.0–7.3) (PPUH Zielona Oaza I, Brzozów, Poland).

The studies were performed in the constant conditions of the ambient temperature of 21 ± 1°C and the soil humidity of about 70–74%.

Soil samples of 200 g were weighted into transparent 2 L polypropylene containers. About 40 mL of PAHs aqueous solution at 0.5 mg/kg was then added to each container. The samples were thoroughly mixed. On Day 4 of the experiment, PAHs content in each container was analyzed. These values were taken as initial and treated as 100% for calculating degradation on Day 35 of the experiment. Then, 40 mL of the EM formulations was added to each sample, as shown in the study plan presented in Table 2. The control samples, which contained only the studied PAHs, were spiked with water. All samples were mixed and analyzed in three replicates.

The samples for analyses of PAHs were taken 4, 14, and 35 days after the PAHs application. The water content was measured using the weighing method, after drying at 105°C (S–40, Alpina, Poland). The pH and the ORP were determined using a digital meter ORP/pH AD14 (ADWA, Poland).

Table 2: Study plan and EM formulation doses

Sample number	Sample content	Formulation dose
1–3	Soil + PAHs	—
4–6	Soil + PAHs + Formulation 1	200 mL/L
7–9	Soil + PAHs + Formulation 2	10 mL/L
10–12	Soil + PAHs + Formulation 3	8 mL/L
13–15	Soil + PAHs + Formulation 4	50 g/L
16–18	Soil + PAHs + Formulation 5	100 mL/L
19–21	Soil + PAHs + Formulation 6	10 g/L
22–24	Soil + PAHs + Formulation 7	50 g/L, 100 mL/L, 10 g/L

Figures S14 and S15 show shifts in pH values and the ORP in the soil samples on Days 4, 14, and 35 of the experiment.

2.3 Chromatographic analysis

Samples for PAHs analyses were prepared using a modification of the method described in PN–EN 15662: 2018-06. Foods of plant origin [53,54]. In brief, 5 g of the soil with 10 mL of water and 10 mL of the acetone: hexane mixture (1:4 v/v) were vortexed for 1 min (BenchMixerTM, Benchmark, USA). Next, extraction salts: 1 g of sodium chloride, 4 g of magnesium sulfate, 0.5 g of disodium citrate sesquihydrate, and 1 g of trisodium citrate were added. Then, samples were vortexed for 1 min and centrifuged at 3,500 rpm (5804R, Eppendorf, Hamburg, Germany) for 5 min. Then, 5 mL aliquots of the sample extract were poured into 15 mL polypropylene centrifuge tubes, containing sorbents for cleanup (150 mg of PSA and 900 mg of magnesium sulfate). The samples were vigorously shaken for 0.5 min and then centrifuged at 3,500 rpm for 5 min. PAHs in the soil extracts were analyzed with a 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with a mass detector, model 7000 (GC-MS/MS QqQ). Software Mass Hunter, B.07.06, was used for data processing and data acquisition [54].

2.4 Assessment of DHA

DHA determination in the soil samples was conducted according to Casida et al., Tabatabai, and Wołejko et al. [55–57]. In brief, 4 mL of water and 1 mL of 3% aqueous solution of TTC were added to 6 g soil samples. Samples were then incubated in the dark at 37°C for 20 h. Next, 20 mL of methanol were added, vortexed for 1 min, centrifuged, and filtered. The DHA measurements were conducted at 485 nm with the spectrophotometer Cary 300 Bio (VARIAN, USA). The results were presented as micromoles of TPF/1 g of dry soil per 20 h.

2.5 Statistical analysis of results

Statistically significant differences between samples with and without biological formulations were established with the Student's test (Excel Microsoft 365 program) for each individual sampling day. p values that are statistically significant, are presented in Figures S1, S2, S4–S8, S10, S12, S13, and S16 as $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

3 Results and discussion

The effect that commercial formulations containing EM have on the degradation of 13 PAHs in the soil is described. Additionally, the analyses covered shifts in the soil pH, the ORP, and the DHA.

Microorganisms that are indigenous to a petroleum contaminated site were reported to be more effective in remediation of the environment after spills of oil and other pollutants, than the nonindigenous ones [58–62]. Many studies concern PAHs biodegradation by microorganisms isolated from different sites contaminated with crude oil. The tested organisms included bacteria belonging to genera *Nocardia, Pseudomonas, Gordonia, Micrococcus, Rhodococcus, Arthrobacter, Mycobacterium, Flavobacterium, Corynebacterium, Klebsiella, Alcaligenes,* and *Bacillus,* and fungi such as *Penicillium, Aspergillus, Trichoderma*, *Candida*, and *Fusarium* [27–31].

3.1 EM formulations influence on PAHs degradation

The study analyzed the effect of EM microorganisms from the following formulations: EmFarma Plus™, Rewital PRO +, BACILLUS VIP, Myco Sin®, Biopuls Fusion®, and Biopuls Cinderella, and a mix of preparations: Myco Sin®, Biopuls Fusion®, and Biopuls Cinderella, on the degradation of 13 PAHs in the soil. Below, we present a review of the literature on the PAHs degradation by microorganisms. Reports concerning the degradation of individual PAHs by microorganisms or by consortia of microorganism in the soil are available. However, to this date, there are no studies concerning the influence of microorganisms found in the studied formulations on the degradation of a mixture of 13 PAHs in the soil.

Table 3 shows a percentage degradation of the studied PAHs after EM formulations were used, on Day 35 of experiment, when compared to the control samples. For some of PAHs, in the case of Formulations 5, 6, and 7, the degradation was slightly lower than for control, but these differences were not statistically important at the end of the experiment on Day 35. This could be caused by a decrease in pH in soil samples after the application of

Table 3: Percentage PAHs degradation (%) after formulations with EM were applied on Day 35 versus the initial concentrations

Analyzed substance	Control	Formulation 1	Formulation 2	Formulation 3	Formulation 4	Formulation 5	Formulation 6	Formulation 7
Acenaphthylene	82.6	91.9	92.7	91.8	91.3	90.7	89.2	87.5
Anthracene	90.3	94.5	93.9	95.1	92.2	92.3	91.4	92.9
Benzo[*a*]anthracene	85.2	90.7	90.4	90.8	88.9	89.6	90.3	88.2
Benzo[*a*]pyrene	77.5	87.3	85.1	83.6	85.2	85.3	82.0	86.2
Benzo[*b*]fluoranthene	79.0	88.4	86.4	84.9	86.6	86.2	78.5	83.8
Benzo[*k*]fluoranthene	75.1	86.8	85.1	83.3	84.9	84.5	75.5	81.5
Benzo[*ghi*]perylene	79.8	86.3	82.7	82.9	81.4	79.3	79.3	76.6
Chrysene	75.9	85.5	80.8	82.0	81.7	80.3	80.8	82.6
Dibenzo[*a,h*]anthracene	80.5	87.9	85.4	82.0	85.6	83.2	77.5	84.0
Fluorene	92.6	94.6	93.5	95.5	93.3	92.0	91.8	91.8
Indeno[1,2,3-*cd*] pyrene	70.0	85.1	83.7	85.6	85.3	87.0	84.4	83.3
Phenanthrene	85.3	90.8	87.8	88.6	87.8	83.6	83.1	83.8
Pyrene	84.2	91.8	90.1	90.7	89.3	88.9	85.9	87.3

preparations 5, 6, and 7, compared to the other formulations. According to Pawar [46], extremes in the pH, which can be observed in some soils, can negatively influence the ability of microbial populations to degrade PAHs.

Figures S1–S13 present PAHs decomposition on Day 4 (before application of EM formulation) and in the samples spiked with EM formulations on Days 14 and 35 of the experiment.

The results and the literature review of the 13 PAHs studied are presented below. No correlation was found between the number of rings and the partition coefficient LogP, and the rate of the tested PAHs degradation. The results were presented according to the molecular mass.

3.1.1 Acenaphthylene

On Day 4 of the study, the acenaphthylene concentration ranged between 0.052 and 0.089 mg/kg. On Day 14 of the study, the acenaphthylene concentration was the highest in the control samples and amounted to 0.02 mg/kg, while it reached the lowest level in the samples with Formulations 4 and 5, amounting to 0.013 mg/kg. On the last, 35th day of the study, the acenaphthylene concentration was also the highest in the control samples (0.014 mg/kg), and it dropped to the minimum level in the samples treated with Formulations 4 and 5 (0.005 mg/kg) (Figure S1).

The use of all EM formulations accelerated the acenaphthylene degradation, when compared to the control. The achieved degradation was the lowest with Formulation 7 (yeast mix – 87.5%) and the highest (92.7%) with Formulation 2, containing bacteria from *Arthrobacter, Bacillus, Cellulomonas, Paenibacillus, Pseudonocardia, Streptomyces, Pseudomonas*, and *Rhodococcus* genera (it increased the rate of the acenaphthylene decomposition by 10.1% versus the control) (Table 3).

Rocha et al. analyzed the influence of *Pleurotus ostreatus* (the oyster fungus, edible mushroom) on the acenaphthylene degradation in the sandy soil. *P. ostreatus* degraded 57.7% of this compound in the soil enriched at the level of 30 mg/kg, and 65.8% of acenaphthylene when the soil was enriched at the level of 60 mg/kg, after the incubation period of 15 days [63].

Barnes et al. investigated the degradation of crude oil and associated PAHs using ten fungal cultures isolated from the aquatic environment: *Penicillium citrinum, Acremonium sclerotigenum, Aspergillus polyporicola, Aspergillus versicolor, Fusarium equiseti, Fusarium* sp., *Aspergillus* sp., *Aspergillus favus*, and *Aspergillus sydowii*. The studies were conducted in 20 mL of the mineral salt medium (MSM) containing 1% (w/v) crude oil as the sole source of carbon for isolates. The experimental

flasks were incubated at 28°C with constant shaking at 80 rpm, for 23 days. Among the ten isolates studied, 100% of acenaphthylene was removed from six isolates [64].

3.1.2 Fluorene

On Day 4, the fluorene concentration ranged from 0.031 to 0.072 mg/kg. On Day 14, the noted substance concentration was the highest in the control samples, amounting to 0.013 mg/kg, and the lowest in the samples treated with Formulations 4 and 5, of 0.008 mg/kg. On Day 35 of the experiment, fluorene was at the highest concentration, of 0.004 mg/kg, in the control samples and after application of Formulations 1 and 2, and at the lowest level, of 0.002 mg/kg, in the samples treated with Formulation 3 (Figure S10).

Fluorene proved to be a persistent substance. After the use of Formulations 5, 6, and 7, its degradation was lower versus the control (91.8–92.0%). The highest degradation was obtained for Formulation 3, at the level of 95.5% (Table 3).

Barnes et al. studied the degradation of the fluorene using ten fungal cultures, isolated from the aquatic environment: *P. citrinum, A. sclerotigenum, A. polyporicola, A. versicolor, F. equiseti, Fusarium* sp., *Aspergillus* sp., *A. favus*, and *A. sydowii.* Among the ten isolates studied, the degradation level ranged from 53.7 to 100% with *Aspergillus* sp., after the incubation period of 23 days [64].

Bankole et al. investigated the fluorene degradation efficiency of the marine derived filamentous fungus, *Mucor irregularis*, bpo1 strain. Optimization of the vital constituents of the MSM used in the study resulted in the fluorene degradation at the rate of 79.8%. The enhanced fluorene degradation efficiency (82.5%) was recorded when the optimized process variables were subjected to growth-linked validation experiments. Furthermore, the activity of enzymes, including laccase, manganese peroxidase, and lignin peroxidase, was demonstrated [65].

Nam et al. researched the influence of *Sphingobacterium* sp. KM-02, isolated from the soil polluted with PAHs near a mine-impacted area, on the fluorene degradation. During culturing of microorganisms with fluorene as the only source of carbon, decomposition of 78.4% of that PAH occurred within 120 h. Additionally, *Sphingobacterium* sp. KM-02 ability to biodegrade fluorene at a level of 100 mg/kg in the soil in the laboratory conditions was verified. During 20 days of the experiment, 65.6% of fluorene was decomposed [66].

Forty-seven fungal strains were isolated from the soil polluted with PAHs and the fluorene degradation rate was verified. The most productive of isolated strains was *Absidia cylindrospora*, and it was found that over 90% of fluorene was degraded within 288 h, while the process required 576 h when no microorganisms were present [67].

3.1.3 Anthracene

Another studied substance was anthracene, and on Day 4, its concentration was in the range from 0.028 to 0.072 mg/kg. On Day 14, the noted substance concentration was the highest in the control samples, amounting to 0.017 mg/kg, while it was the lowest, of 0.008 mg/kg, in the samples treated with Formulation 5. On the last day of the study, anthracene also was at the highest concentration in the control samples, of 0.005 mg/kg, and at the minimum level of 0.002 mg/kg in the samples treated with Formulations 3, 6, and 7 (Figure S2).

Also in the case of anthracene, EM formulations accelerated its degradation, and in their presence, it ranged between 91.4 and 95.1%. The anthracene degradation was the highest following the treatment with Formulation 3, which contained eight bacteria strains from the *Bacillus* genus (Table 3).

The influence of microorganisms isolated from the soil long polluted with creosote oil on the anthracene degradation was studied by Smułek et al. The level of this compound degradation ranged from 30 to 70%. The highest degradation was achieved for *Pseudomonas mosselii* and *Pseudomonas mendocina* [68].

Krivobok et al. isolated from the soil 39 strains of *Micromycetes* fungi, described as effectively degrading PAHs. Nineteen of them degraded at least 50% of anthracene. The highest degradation was obtained for *Rhizopus arrhizus* – 95% and *Cryphonectria parasitica* – 96%. Furthermore, studies were also conducted on other fungal species able to degrade anthracene, including *Rhizoctonia solani* (86%), *Ceriporiopsis subvermispora* (88%), *Oxysporus* sp. (94%), *Cladosporium herbarum* (85%), *Drechslera spicifera* (79%), *Verticillium lecanii* (77%), *Coniothyrium sporulosum* (57%), and *Cunninghamella* spp. (78–87%) [69].

Two strains of bacteria able to degrade anthracene – *Ralstonia pickettii* JANC1A and *Thermomonas haemolytica* JANC2B, were isolated and identified. They were isolated using a method for enrichment of the contaminated soil in the mineral medium. The initial anthracene concentration amounted to 100 mg/kg. It was demonstrated that 50 and 75% of anthracene was degraded after 8 and 20 days, respectively [70].

Wu et al. investigated the anthracene degradation by fungi isolated from the environment of PAH-contaminated mangrove sediments in Ma Wan, Hong Kong. Anthracene at the concentration of 50 mg/L was added to the MSM for initial screening of PAH-degrading fungi, and finally two

fungal species capable of using the studied PAH as the sole source of carbon were isolated and identified as *Fusarium solani* – MAS2 and MBS1 strains. Anthracene removal reached 40% of the added amount after 40 days of incubation in samples with MAS2 strain [71].

3.1.4 Phenanthrene

On Day 4, the phenanthrene concentration ranged from 0.03 to 0.067 mg/kg. On Day 14, the highest substance level was 0.016 mg/kg and it was noted in the control samples, while its concentration was the lowest, of 0.009 mg/kg, in Formulations 4 and 5. On Day 35 of the study, phenanthrene reached the highest concentration of 0.008 mg/kg in the control samples, and the lowest, of 0.004 mg/kg, in the samples treated with Formulation 4 (Figure S12).

The use of Formulations 5, 6, and 7 slowed the phenanthrene degradation process versus the control level; however, these differences were not statistically significant for Day 35. The remaining formulations accelerated the degradation. The highest degradation, of 90.8%, was achieved with Formulation 1 (Table 3).

The phenanthrene biodegradation by *Bacillus. licheniformis* STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03, microorganisms that were isolated from an oil spill site, was studied. The phenanthrene concentration amounted to 50 mg/kg of the soil. After the experiment period of 60 days, 91.43, 84.83, and 83.97% of phenanthrene were decomposed by *B. licheniformis*, *B. subtilis*, and *P. aeruginosa*, respectively. When *B. licheniformis* and *B. subtilis* were used, 90.34% of phenanthrene was decomposed [72].

Nzila et al. isolated and characterized two bacterial strains able to degrade phenanthrene – *Pseudomonas citronellolis* PHC3Z1A and *Stenotrophomonas maltophilia* JPHC3Z2B. The initial phenanthrene concentration (C_0 = 100 ppm) was degraded in 50% after 7 days and in 75% after 15 days [70].

A fungus, *Fusarium* sp., was isolated from soils polluted with crude oil (Liaohe Oil Field, China). The influence of that microorganism on the phenanthrene and pyrene degradation was studied. For both PAHs, four different initial concentrations, 10, 50, 100, and 200 mg/kg, were used. It was demonstrated that when higher initial concentrations were used, of 100 and 200 mg/kg, the phenanthrene degradation was higher, at the level of 83.7 and 70%, respectively (after 350 h), than when its initial levels were lower. For pyrene, biodegradation was the highest, of 74.6%, for the initial pyrene level of 100 mg/kg, and the lowest, of 32.1%, when it was at the level of 200 mg/kg [73].

Barnes et al. investigated the phenanthrene degradation using ten fungal cultures isolated from the aquatic environment: *P. citrinum, A. sclerotigenum, A. polyporicola, A. versicolor, F. equiseti, Fusarium* sp., *Aspergillus* sp., *A. favus,* and *A. sydowii.* Among the ten isolates studied, the degradation ranged from 22.1 to 100% after 23 days of incubation. The highest degradation level was achieved with *F. equiseti* [64].

The microbial degradation of the phenanthrene by screened fungi found in the natural environment was conducted, to select fungi for the phenanthrene bioremediation. The highest degradation level, of 72%, was obtained when *Trichoderma* sp. S019 was incubated for 30 days after 0.1 mM of phenanthrene were added to the liquid medium, while it reached 31% when 1 mM of the studied PAH was added [74].

3.1.5 Pyrene

On Day 4 of the study, the highest pyrene concentration amounted to 0.05 mg/kg, and the lowest amounted to 0.019 mg/kg. On Day 14 of the study, pyrene reached its highest level of 0.011 mg/kg in the control samples, and the lowest level, of 0.006 mg/kg, in the samples treated with Formulation 5. On the last day of the study, pyrene was at the highest level, of 0.006 mg/kg, in the control samples, and at the lowest concentration of 0.003 mg/kg in the samples treated with Formulations 3, 4, 5, and 6 (Figure S13).

It was observed for pyrene that all studied EM formulations accelerated its degradation, which ranged between 85.9 and 91.8%. The degradation was the highest after treatment with Formulation 1, and the lowest for Formulation 6 (Table 3).

The studies on the influence of the immobilized (a hybrid carrier consisting of polyvinyl alcohol with sodium alginate and activated carbon) fungi, *Aspergillus niger*, *Trichoderma* sp., and *Fusarium* sp., on the pyrene degradation were conducted by Wang et al. The initial pyrene concentration was 100 mg/kg. After 240 h of incubation, the pyrene degradation amounted to 63% for *Trichoderma* sp., 49% for *A. niger*, and 69% for *Fusarium* sp. In the case of the synergistic effect of *A. niger* and *Fusarium* sp., the highest pyrene degradation of 81% was recorded [75].

A strain of the bacterium *Achromobacter xylosoxidans* PY4 was isolated from a polluted area (Jubail, Saudi Arabia) and characterized. It was demonstrated that this bacterium is able to metabolize and use pyrene as its only source of carbon. The PY4 strain was capable of decomposing 80% of pyrene at the initial level of 100 mg/L in 25 days [76].

Barnes et al. analyzed the pyrene degradation using ten fungal cultures, isolated from the aquatic environment, of *P. citrinum, A. sclerotigenum, A. polyporicola, A. versicolor,*

F. equiseti, Fusarium sp., *Aspergillus* sp., *A. favus,* and *A. sydowii.* Among the ten isolates studied, the degradation ranged from 45.7 to 100% after 23 days of incubation. The maximum degradation level was observed when *F. equiseti* and *P. citrinum* were used [64].

The APC5 strain of *Coriolopsis byrsina,* white rot fungi, was isolated in the Surguja district of Chhattisgarh, India, and used in the studies on the pyrene biodegradation. The maximum degradation reached the level of 96.1%. *C. byrsina* produced a significant amount of ligninolytic enzyme in the mineral salt broth (MSB) containing pyrene [77].

Hadibarata et al. studied the pyrene degradation by *Candida* sp. S1, the yeast isolated from the tropical rain forest. Biodegradation was at the level of 35% after 15 days, but the percentage of pyrene decomposition increased up to 75% with 24 g/L of sodium chloride added, and decreased as the salinity increased. Under the acidic conditions, biodegradation increased up to 60% at the pH of 5. It was also found that higher glucose concentrations, exceeding 10 g/L, had no significant effect on the pyrene biodegradation, while agitation proved to have greater influence [31].

3.1.6 Chrysene

On Day 4 of the study, the highest chrysene concentration amounted to 0.043 mg/kg, and the lowest amounted to 0.021 mg/kg. On Day 14, the substance was at the maximum concentration of 0.012 mg/kg in the samples treated with Formulation 7, and at the lowest, of 0.004 mg/kg, in the samples containing Formulation 5. On Day 35 of the study, chrysene level was the highest, of 0.008 mg/kg, in the control samples, and the lowest, of 0.004 mg/kg, in the samples treated with Formulations 4, 5, and 6 (Figure S8).

The use of all EM formulations accelerated the chrysene decomposition. The highest degradation, of 85.5%, was obtained for Formulation 1, while it was the lowest, of 80.3%, for Formulation 5 (yeast strains *Y. lipolytica*) (it accelerated the chrysene decomposition by 9.6% versus the control) (Table 3).

A fungus, *Polyporus* sp. S133, isolated from the soil polluted with crude oil, was used in the studies on the chrysene biodegradation in a culture without and with a synthetic surfactant (Tween 80). The maximum degradation intensity, of 86%, was achieved for the fungus incubated with 0.5% Tween 80 solution for 30 days. When the microorganism was incubated without that surfactant, the degradation was only at a level of 30% [78].

Similar studies were conducted in a liquid MSB. The maximum rate of the chrysene degradation, of 65%, was achieved when *Polyporus* sp. S133 was used with an addition of polypeptone as a source of vitamins, carbon, and amino acids for the microorganisms, when compared to the 24% degradation in the pure MSB medium [79].

Other research focused on the influence of a bacterial consortium on the chrysene degradation in the soil polluted with crude oil. The consortium consisting of *Bacillus cereus* and *Pseudomonas putida* 10 and 15% bacteria with ratios – 2:3, 1:1, 3:2 was added into a slurry bioreactor. The initial chrysene concentration amounted to 24.48 ng/μL. After 49 days of the experiment, the chrysene degradation was at the level 67.01, 69.1, and 64.54%, in the case of the 10% bacterial consortium, and of 89.39, 93.58, and 91.73%, for the 15% bacterial consortium [80].

In yet another study, Vaidya et al. developed a bacterial consortium consisting of *Rhodococcus* sp., ASDC1; *Bacillus* sp. ASDC2; and *Burkholderia* sp. to study the chrysene degradation. Chrysene was utilized by the consortium as a sole source of carbon and energy, with the maximum degradation rate of 1.5 mg/L/day and the maximum growth rate of 0.125/h, under optimized conditions of the pH of 7.0, the temperature of 37°C under aeration of 150 rpm, on gyrating shaking. The maximum degradation of 96% was obtained in the polluted, non-sterile soil sediment [81].

3.1.7 Benzo[*a*]anthracene

On Day 4, the benzo[*a*]anthracene level in the soil samples was between 0.016 and 0.041 mg/kg. On Day 14 of the experiment, the maximum concentration was noted in the control samples and following treatment with Formulation 1, amounting to 0.008 mg/kg, while it was the lowest, of 0.004 mg/kg, after treatment with Formulations 3, 4, and 5. On the last day of the study, the benzo[*a*]anthracene level was the highest in the control samples and amounted to 0.005 mg/kg, while it was the lowest after treatment with Formulations 4, 5, and 6, and amounted to 0.002 mg/kg (Figure S3).

The benzo[*a*]anthracene decomposition was accelerated by the use of all formulations. It was the highest, of 90.8%, after treatment with Formulation 3, and the lowest, of 88.2%, with Formulation 7 (Table 3).

The potential of benzo[*a*]anthracene biodegradation by *Panebacillus* sp. strain HD1PAH, a new strain isolated from the soil polluted with crude oil, was studied by Deka and Lahkar. The study was conducted in laboratory conditions for 144 h, and samples were collected seven times. Benzo[*a*]anthracene was the only source of carbon, at the level of 10 mg/L in the MSM. The maximum degradation of that PAH by the *Panebacillus* sp. strain HD1PAH was 82.01% during 144 h of incubation [82].

Rocha et al. also analyzed the *P. ostreatus* influence on the benzo[*a*]anthracene degradation in the soil. *P. ostreatus* degraded about 90% of this compound in the

soil enriched at the level of 30 mg/kg, and 80% of benzo[*a*] anthracene when the soil was enriched at the level of 60 mg/kg after a period of incubation of 15 days [63].

Barnes et al. analyzed the degradation of benzo[*a*] anthracene using ten fungal cultures, isolated from the aquatic environment: *P. citrinum, A. sclerotigenum, A. polyporicola, A.versicolor, F. equiseti, Fusarium* sp., *Aspergillus* sp., *A. favus,* and *A. sydowii.* Among the ten isolates studied, nine isolates achieved a 100% removal of benzo[*a*]anthracene after incubation of 23 days [64].

Wu et al. studied the degradation of benzo[*a*]anthracene by fungi isolated from the environment of PAH-contaminated mangrove sediments in Ma Wan, Hong Kong. Benzo [*a*]anthracene at the concentration of 20 mg/L was added to the MSM for initial screening of PAH-degrading fungi; and finally, two fungal species capable of using the studied PAH as the sole source of carbon were isolated and identified as *F. solani* – MAS2 and MBS1 strains. Benzo[*a*]anthracene removal reached 60% of the added amount after 40 days of incubation in the samples containing the MBS1 strain [71].

3.1.8 Benzo[*a*]pyrene

On Day 4 of the study, the benzo[*a*]pyrene level was the highest of 0.039 mg/kg, and the lowest of 0.015 mg/kg. On Day 14 of the study, the compound concentration was the highest in the control samples and those with Formulation 1 (0.01 mg/kg), and the lowest in the samples spiked with Formulation 5 (0.004 mg/kg). On the last day of the study, the benzo[*a*]pyrene concentration was the highest, of 0.007 mg/kg, in the control samples, while it is at the minimum level of 0.003 mg/kg in the samples treated with Formulations 4, 5, 6, and 7 (Figure S4).

The use of all EM formulations accelerated the degradation of benzo[*a*]pyrene, when compared to the control, and it ranged between 82.0 and 87.3%. Formulation 1, containing bacteria from *Bifidobacterium, Bacillus, Lactococcus, Lactobacillus, Rhodopseudomonas,* and *Streptococcus* genera, and yeasts *S. cerevisiae*, most strongly accelerated the decomposition of this substance (it accelerated the benzo[*a*]pyrene degradation by 9.8% versus the control) (Table 3).

Su et al. studied the influence of two microorganisms, *Bacillus* sp. SB02 and *Mucor* sp. SF06, on the benzo[*a*] pyrene degradation in the soil. Both SF06 and SB02 were isolated from the soil collected from the Shenfu Irrigation Area, China. The researchers studied and compared characteristics of the benzo[*a*]pyrene degradation, by free and by co-immobilized microorganisms. The level of the benzo [*a*]pyrene degradation was verified five times, on Days 7, 14, 21, 28, and 42. A higher degradation of that compound was achieved for microorganisms in the co-immobilized system. The highest degradation level was 79.6% for free microorganisms, and 95.3% for co-immobilized microorganisms [83].

Su et al. conducted similar studies on the benzo[*a*] pyrene degradation in the soil by the fungus, *Mucor* sp., free and immobilized. This microorganism was isolated from the soil contaminated with PAHs from the Shenfu Irrigation Area. The initial concentration of the studied PAH was 50 mg/kg. The degradation rate was higher for the immobilized fungi. The maximum degradation amounted to 68% for the immobilized fungi and 52% for free microorganisms on Day 42 of the experiment [84].

The influence of two bacteria, *Bacillus flexus* S1I26 and *Paenibacillus* sp. S1I8, isolated from the soil polluted with crude oil was studied. It was demonstrated that both isolates were able to secrete biosurfactant that increased benzo[*a*]pyrene solubility. On Day 21 of the experiment, *B. flexus* S1I26 degraded 70.7% of benzo[*a*]pyrene, while *Paenibacillus* sp. S1I8 achieved a higher degradation level, amounting to 76.8%. The results suggest that isolates producing biosurfactants can be a potential tool for PAHs biodegradation in the soil and they can be used to bioremediate sites polluted with those compounds [85].

The studies concerning the influence of fungi, *Trichoderma* sp., *Fusarium* sp., and *A. niger*, on the benzo[*a*]pyrene degradation were conducted. A hybrid carrier consisting of polyvinyl alcohol, sodium alginate, and activated carbon was used to immobilize the microorganisms. The initial concentration of benzo[*a*]pyrene was 100 mg/kg. After 240 h of incubation, the benzo[*a*]pyrene degradation amounted to 34% for *Trichoderma* sp., 29% for *A. niger*, and 37% for *Fusarium* sp. The degradation levels significantly exceeded those obtained for free fungi. Additionally, the synergistic effect of those microorganisms was verified, and for benzo[*a*]pyrene the highest degradation was obtained with *A. niger* and *Fusarium* sp., amounting to 43% [75].

Mandal et al. analyzed the benzo[*a*]pyrene degradation by a yeast consortium including *Rhodotorula* sp. NS01, *H. opuntiae* NS02, *D. hansenii* NS03, and *H. valbyensis* NS04, isolated from the contaminated soil. The maximum degradation amounted to 76% after 6 days in the aqueous medium under optimized conditions of pH 7.0, temperature of 30°C, shaking speed of 130 rpm, an inoculum dose of 3% (w/v), and the initial benzo[*a*]pyrene concentration of 50 mg/L [35].

3.1.9 Benzo[*b*]fluoranthene

On Day 4, the benzo[*b*]fluoranthene concentration was in the range from 0.013 to 0.039 mg/kg. On Day 14 of the study,

the benzo[*b*]fluoranthene concentration was the highest in the control samples and amounted to 0.01 mg/kg, while it was the lowest in those with Formulation 5, amounting to 0.004 mg/kg. On the last day of the study, the studied substance was at the highest level, of 0.006 mg/kg, in the control samples, and at the minimum level of 0.003 mg/kg in the samples treated with Formulations 3, 4, 5, 6, and 7 (Figure S5).

The benzo[*b*]fluoranthene degradation was higher following treatment with EM formulations, excluding Formulation 6 (the yeast *D. hansenii* and its metabolites, bacteria from *Bacillus* genus), for which the degradation was similar to the control, at a level of 78.5%. The highest degradation, of 88.4%, was achieved for Formulation 1 (it accelerated the benzo[*b*]fluoranthene decomposition by 9.4% versus the control) (Table 3).

Kumari et al. demonstrated the ability of *Ochrobactrum anthropi, S. maltophilia, P. mendocina, P. aeruginosa,* and *Microbacterium esteraromaticum* to biodegrade several PAHs, including benzo[*b*]fluoranthene, during the bacteria exposure to crude oil. The benzo[*b*]fluoranthene concentration in the sample of oil collected from the Digboi refinery in India amounted to 6.5 mg/L. The experiments were conducted in laboratory conditions. The samples were tested on Days 15, 30, and 45. The degradation was the lowest, at the level of 34.8%, for *P. mendocina*, and the highest, of 61.2%, for *P. aeruginosa*. However, the consortium of the described bacteria achieved the enhanced benzo(b)fluoranthene degradation of 72.8% [86].

Treviño-Trejo et al. isolated and selected bacteria able to degrade benzo[*b*]fluoranthene. The isolates ability to tolerate concentrations of 50 and 75 mg/L of the studied PAH in the liquid medium was evaluated. The selected isolates were identified as belonging to *Bacillus, Gordonia, Pseudomonas, Rhodococcus, Ochrobactrum,* and *Amycolatopsis* genera. All isolates tolerated and grew at the benzo[*b*]fluoranthene concentrations tested. The most prominent was *Amycolatopsis* sp. Ver12, which removed 47% of benzo[*b*] fluoranthene; furthermore, with the addition of the yeast extract, it removed 59% of the studied compound [87].

3.1.10 Benzo[*k*]fluoranthene

On Day 4 of the study, the highest benzo[*k*]fluoranthene concentration amounted to 0.043 mg/kg, and the lowest amounted to 0.013 mg/kg. On Day 14 of the study, the concentration was the at the highest level, of 0.011 mg/kg, for the control samples and the samples treated with Formulation 1, and the lowest, of 0.004 mg/kg, following treatment with Formulation 5. On the last, 35th day of the study, the studied substance was at the highest level, of 0.008 mg/kg, in the control samples, and at the lowest level, of 0.003 mg/kg, in the samples treated with Formulations 4, 5, 6, and 7 (Figure S6).

The use of all EM formulations accelerated the benzo [*k*]fluoranthene decomposition. The degradation was the lowest for Formulation 6 (75.5%), and the highest for Formulation 1 (86.6%) (it accelerated the benzo[*k*]fluoranthene decomposition by 11.7% versus the control) (Table 3).

The results for benzo[*b*]fluoranthene and benzo[*k*] fluoranthene are very similar, due to the similar structure of these two PAHs.

Arulazhagan et al. studied the benzo(k)fluoranthene degradation by *S. maltophilia* strain AJH1, bacteria isolated from soil samples collected from different areas owned by the Saudi Arabian Mining Company. Strain AJH1 was able to degrade the benzo(k)fluoranthene at the concentration of 10 mg/L in the acidophilic MSM at the pH of 2. The maximum degradation level was 79% after 11 days [88].

Maeda et al. reported the studies on the benzo(k) fluoranthene degradation by the soil bacterial isolates *Sphingobium* sp. strain KK22. Benzo(k)fluoranthene concentration was reduced by 70% in 20 days of the experiment [89].

3.1.11 Dibenzo[*a,h*]anthracene

On Day 4 of the study, the highest dibenzo[*a,h*]anthracene concentration amounted to 0.041 mg/kg, and the lowest amounted to 0.014 mg/kg. On Day 14 of the experiment, the highest dibenzo[*a,h*]anthracene concentration, of 0.013 mg/kg, was found after treatment with Formulation 1. The lowest level, of 0.006 mg/kg, was noted in the samples treated with Formulations 5, 6, and 7. On Day 35 of the study, the substance was at its highest level, of 0.006 mg/kg, in the control samples, while it was at the lowest concentration, of 0.003 mg/kg, in the samples treated with Formulations 4, 5, 6, and 7 (Figure S9).

The use of EM formulations accelerated the dibenzo [*a,h*]anthracene degradation when compared to the control, except for Formulation 6 (77.5%). The highest degradation, of 87.9%, was achieved with Formulation 1 (Table 3).

The influence of eight fungal species *A. praecox, Agrocybe dura, Kuehneromyces mutabilis, Hypholoma capnoides, S. rugosoannulata, Stropharia coronilla, S. cubensis,* and *S. hornemannii,* on the dibenzo[*a,h*]anthracene degradation was studied by Steffen et al. The highest degradation of that compound, of 84%, was obtained for *S. coronilla* [90].

Dibenzo(a,h)anthracene biodegradation by indigenous strains of aerobic heterotrophic bacteria and cyanobacteria isolated from the Bodo creek, a moderately salty aquatic site polluted with crude oil, was monitored.

Dibenzo(*a*,*h*)anthracene levels were reduced to 0 mg/L in all treatment options on Day 56 [91].

3.1.12 Benzo[*ghi*]perylene

Benzo[*ghi*]perylene was yet another studied substance. On Day 4, this substance was at the highest concentration of 0.052 mg/kg, and at the lowest level of 0.020 mg/kg. On Day 14 of the study, the benzo[*ghi*]perylene concentration was the highest in the control samples and amounted to 0.019 mg/kg, while it was the lowest in the samples spiked with Formulations 5 and 7, amounting to 0.011 mg/kg. On Day 35, the concentration was the highest, of 0.008 mg/kg, in the control samples, and the lowest, of 0.004 mg/kg, in the samples treated with Formulation 6 (Figure S7).

After treatment with Formulations 5 and 6, the benzo [*ghi*]perylene degradation was comparable to the control (ca. 79%), and for Formulation 7 the degradation was lower, of 76.6%. The highest degradation was obtained for Formulation 1, of 86.3% (Table 3).

Studies were conducted on the benzo[*ghi*]perylene biodegradation by *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03, which were isolated from wood chips, coal tar, and an oil spill site. In the experiment, mono-septic cultures or co- and augmented cultures were used. The benzo[*ghi*]perylene concentration was 25 mg/kg of the soil. After 60 days of the experiment, 52.73, 40.50 and 58.42% of benzo[*ghi*]perylene were biodegraded by *B. licheniformis*, *B. subtilis*, and *P. aeruginosa*, respectively. The highest rate of the benzo[*ghi*]perylene degradation, of 60.76%, was reached with *B. licheniformis* and *B. subtilis* [72].

Mandal et al. studied the benzo[*ghi*]perylene degradation by the yeast consortium YC04, consisting of three isolates – *Rhodotorula* sp. NS01, *D. hansenii* NS03, and *H. valbyensis* NS04 in the MSM. The YC04 efficiency in benzo[*ghi*]perylene remediation was tested in a presence of ZnO nanoparticles and produced a biosurfactant in the growth medium. The maximum benzo[*ghi*]perylene biodegradation was found to be 63.83% at central values of all factors, a pH of 7.0, at a temperature of 30°C, and at a shaking speed of 130 rpm in the presence of 2 g/L of ZnO nanoparticles, using 3% inoculum doses of the yeast consortium YC04 after 6 days of incubation [34].

3.1.13 Indeno[1,2,3-*cd*] pyrene

On Day 4, the highest determined indeno[1,2,3-*cd*] pyrene level was 0.035 mg/kg, while the lowest concentration amounted to 0.015 mg/kg. On Day 14, the highest level of the studied substance was 0.031 mg/kg in the control samples, while the lowest concentration was found in the samples treated with Formulation 7 (0.014 mg/kg). On the last day of the experiment, the highest indeno[1,2,3-*cd*]pyrene level of 0.008 mg/kg was found in the control samples, while its level was the lowest (0.002 mg/kg) following application of Formulation 6 (Figure S11).

The use of EM formulations significantly accelerated the indeno[1,2,3-*cd*]pyrene degradation versus the control. The highest degradation, of 87%, was obtained for Formulation 5, while it was the lowest, of 83.3%, for Formulation 7 (it accelerated the indeno[1,2,3-*cd*]pyrene decomposition by 17% versus the control) (Table 3).

Two bacterial strains, *Acinetobacter baumannii* INP1 and *Pseudomonas taiwanensis* PYR1, were isolated from Liaohe, China, contaminated with PAHs. These microorganisms were able to degrade 55.3% of indeno[1,2,3-*cd*] pyrene after a period of 30 days. These strains' ability to degrade pyrene was also studied, and it was found that they degraded 58.2% of pyrene [92].

Barnes et al. analyzed the degradation of indeno[1,2,3-*cd*]pyrene using ten fungal cultures, isolated from the aquatic environment – *P. citrinum, A. sclerotigenum, A. polyporicola, A.versicolor, F. equiseti, Fusarium* sp., *Aspergillus* sp., *A. favus,* and *A. sydowii.* All tested isolates degraded almost 100% of indeno[1,2,3-*cd*]pyrene [64].

3.2 Soil parameters: pH, the ORP, and DHA

On Day 1, the soil pH was 5.1, and on Day 4, after PAHs application, the pH of all soil samples was about 5.3. On Day 14, the pH of all studied samples increased, and this increase was the highest for the soil containing bacterial preparations. On Day 35, the pH decreased slightly or remained at the same level. On Days 14 and 35, in the samples with preparation 3 added, the soil pH value was the highest, of 5.9 and 5.6, respectively. The highest PAHs degradation was obtained for these samples. In acidic samples, the tested PAHs were more persistent (Figure S14). According to Pawar [46], the pollutants are frequently linked with the pH of contaminated sites, and microorganisms may not be able to transform PAHs under the acidic or alkaline conditions. Most heterotrophic bacteria and fungi favor a nearly neutral pH, with fungi being more tolerant of acidic conditions. Extremes in the pH, which can be observed in some soils, can negatively influence the ability of microbial populations to degrade PAHs.

On Day 14, following application of EM formulations, the ORP increased in all studied samples, when compared

to the control samples, and was in a range from 295 (the sample containing Formulation 1) to 351 (the sample with Formulation 6). On Day 35 of the study, the ORP increased again and reached the highest value of 379 for the sample containing Formulation 7. On Day 35, ORP increased by almost 100, when compared to Day 1. On each day of testing, ORP for all samples with microorganisms was significantly higher versus the control samples (Figure S15). The ORP plays a crucial role in regulation of the microbial activity, and affects the soil enzymatic activity, which has an impact on the PAHs degradation [43].

The initial DHA value in the control samples was 26.5 µM TPF/g DM soil 20 h. The changes in the DHA are presented in Figure S16. The use of PAHs resulted in the DHA decrease in all samples on Day 14 versus Day 1. According to Karaca et al., Wolińska and Stępniewska, and Järvan et al. [39–42], it is well known that pesticides, PAHs, and other persistent soil pollutants have inhibiting effects on the DHA. On Day 14, DHA in the samples with biological preparations was higher than in controls, except for Formulations 2 and 3. On Day 35, for Formulation 3 (which had the best PAHs bioremediation results), DHA increased from 12.8 µM TPF/g DM soil 20 h to 24.4 µM TPF/g DM soil 20 h after decomposition of these pollutants. The addition of the biological formulations significantly changes the enzymatic activity of the soil, and this was still visible on Day 35 [57].

4 Conclusions

Formulation 1, containing bacteria from *Bacillus*, *Bifidobacterium*, *Lactococcus*, *Lactobacillus*, *Rhodopseudomonas*, and *Streptococcus* genera, and yeasts *S. cerevisiae* was demonstrated to have the highest effectiveness in the biodegradation of the studied PAHs. When this formulation was used, eight PAHs: benzo[*a*]pyrene, benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, benzo[*ghi*]perylene, chrysene, dibenzo[*a,h*]anthracene, phenanthrene, and pyrene were degraded with the highest intensity. The biological formulations containing bacteria proved to be more effective than yeast formulations. The presented studies show differences in the PAHs degradation by commercially available individual microorganisms and consortia of bacteria and yeasts. Commercial preparations are easily accessible and safe for the environment and humans; therefore they can be generally used. The proposed research would be advantageous for the development of bioremediation of soils contaminated with PAHs. The studies on applications of biological preparations should be continued, because other parameters such as the soil type or environmental conditions could also influence the PAHs degradation.

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Supplementary material

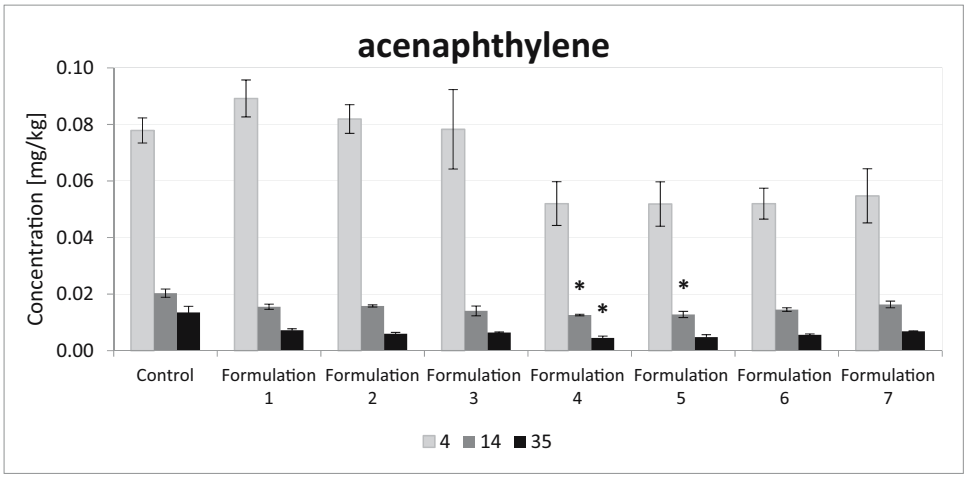

Figure S1: Acenaphthylene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p value is shown as $p < 0.05$ (*).

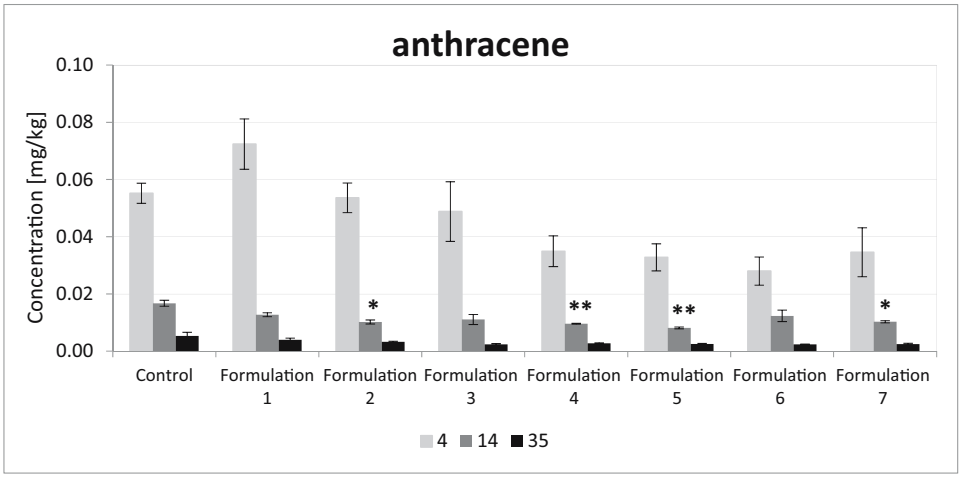

Figure S2: Anthracene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p values are shown as $p < 0.01$ (**) and $p < 0.05$ (*).

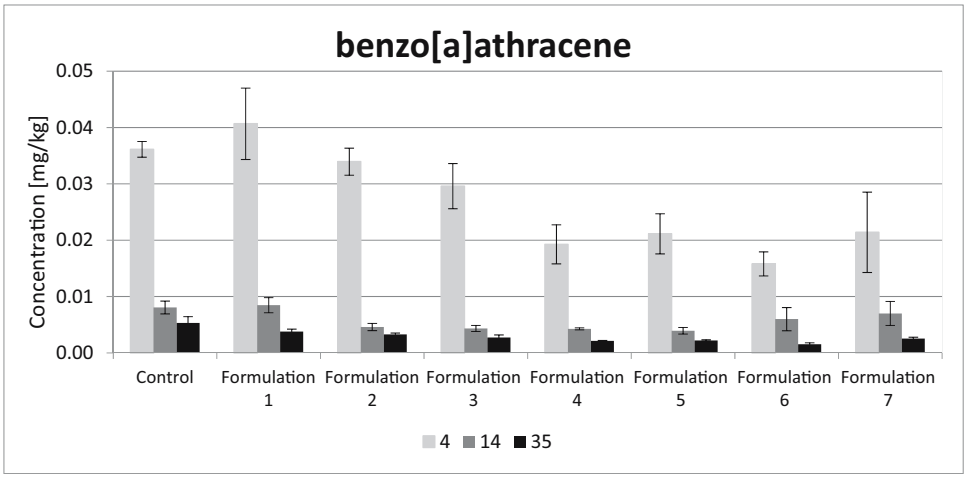

Figure S3: Benzo[a]anthracene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). No statistically significant differences were observed.

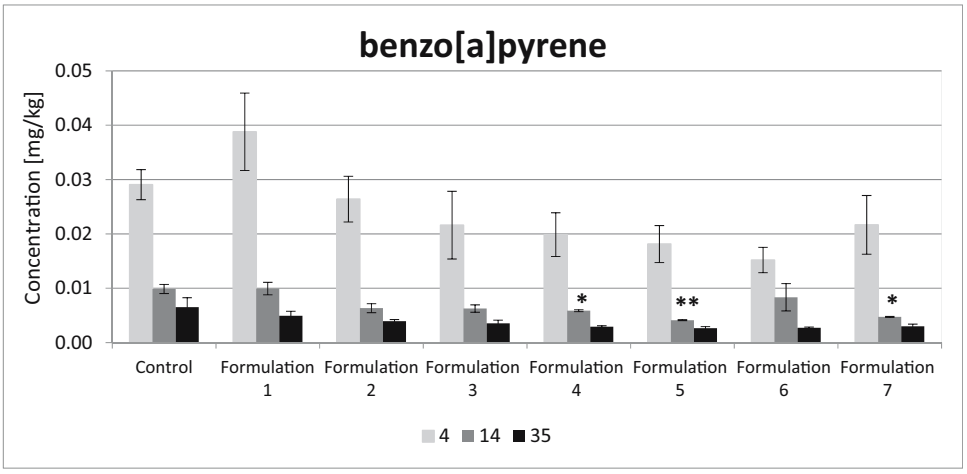

Figure S4: Benzo[a]pyrene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p values are shown as $p < 0.01$ (**) and $p < 0.05$ (*).

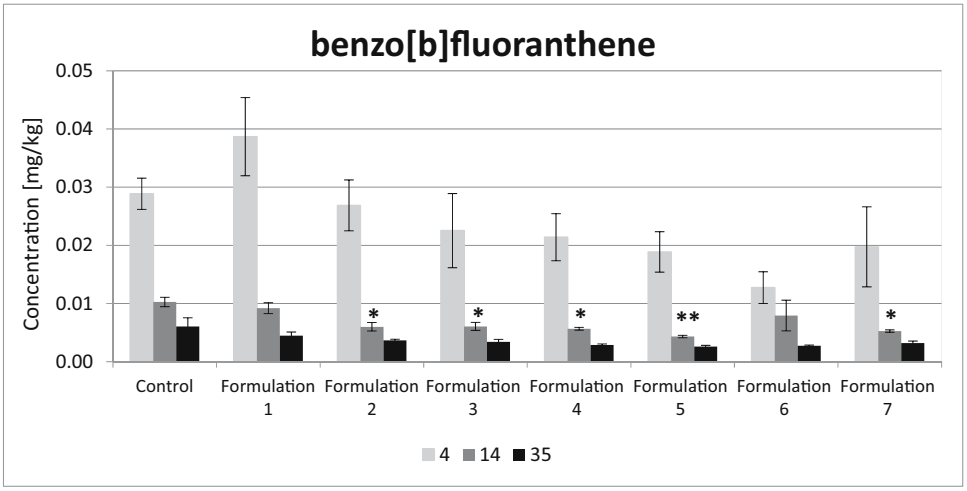

Figure S5: Benzo[b]fluoranthene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p values are shown as $p < 0.01$ (**) and $p < 0.05$ (*).

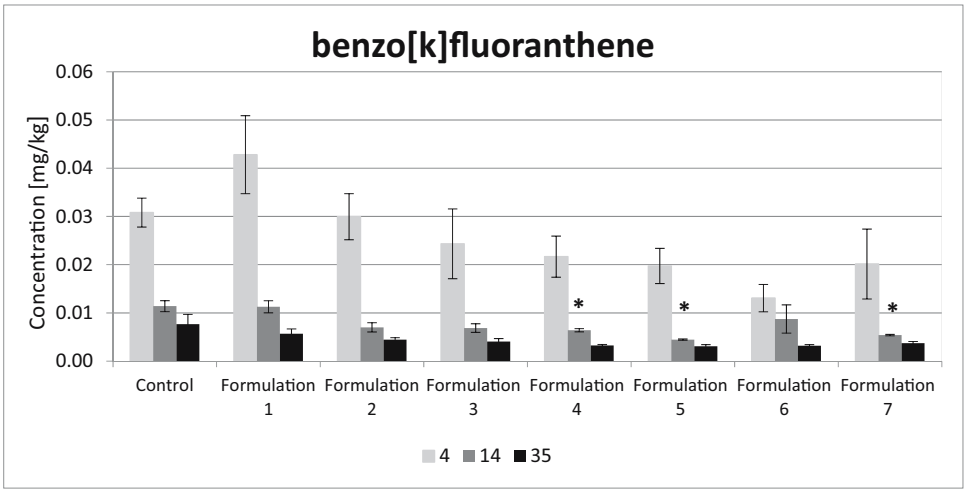

Figure S6: Benzo[k]fluoranthene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p value is shown as $p < 0.05$ (*).

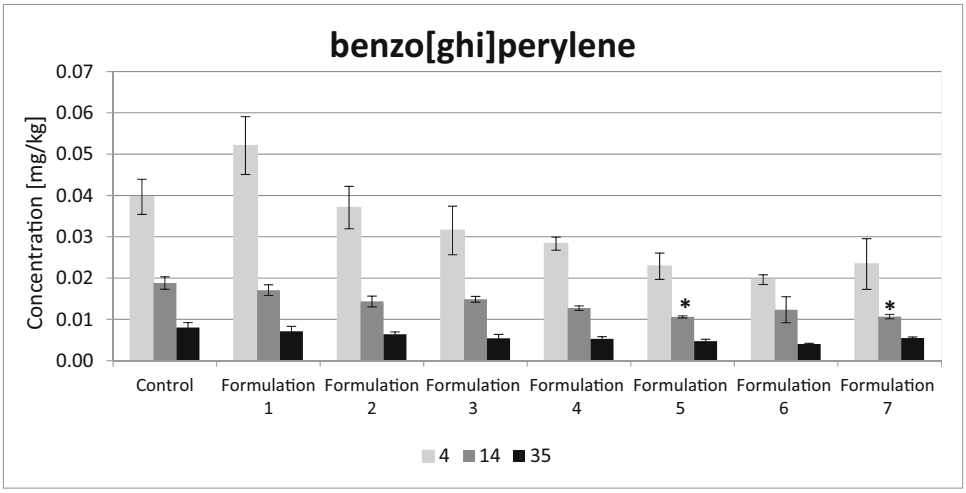

Figure S7: Benzo[ghi]perylene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p value is shown as $p < 0.05$ (*).

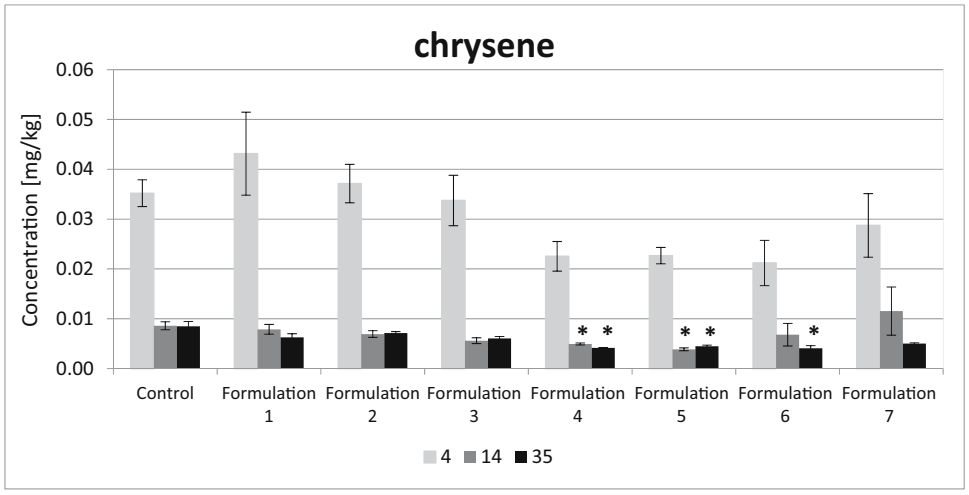

Figure S8: Chrysene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p value is shown as $p < 0.05$ (*).

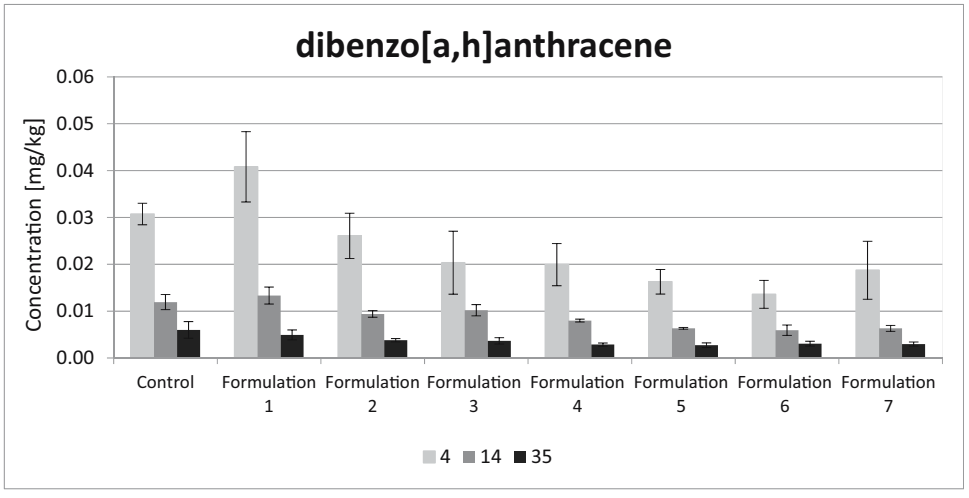

Figure S9: Dibenzo[a,h]anthracene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). No statistically significant differences were observed.

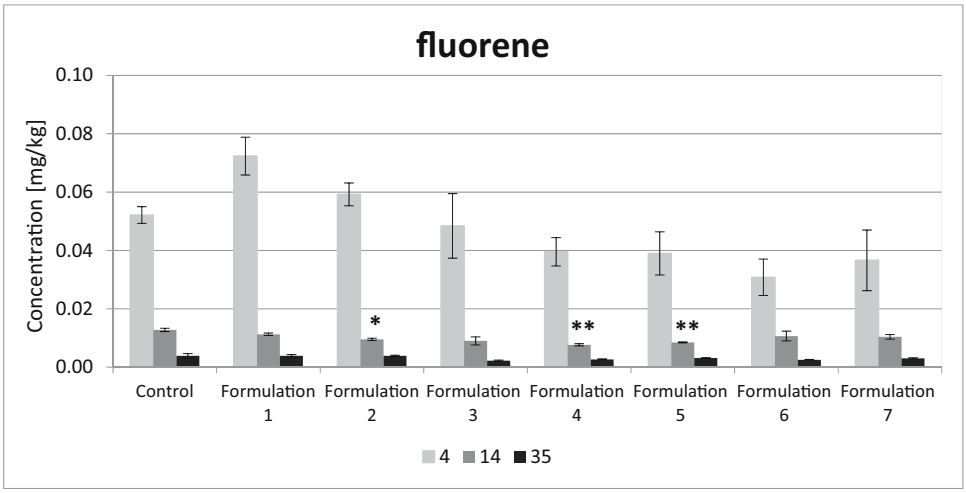

Figure S10: Fluorene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p values are shown as $p < 0.01$ (**) and $p < 0.05$ (*).

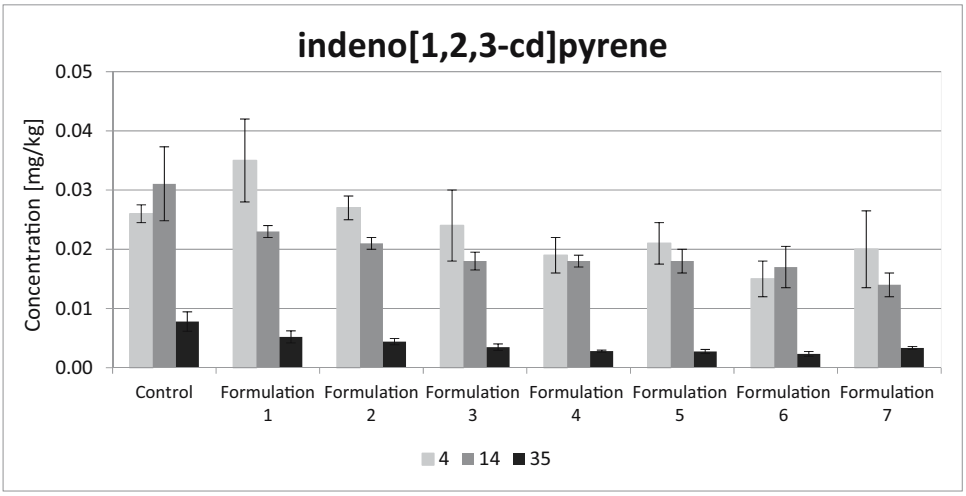

Figure S11: Indeno[1,2,3-cd]pyrene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). No statistically significant differences were observed.

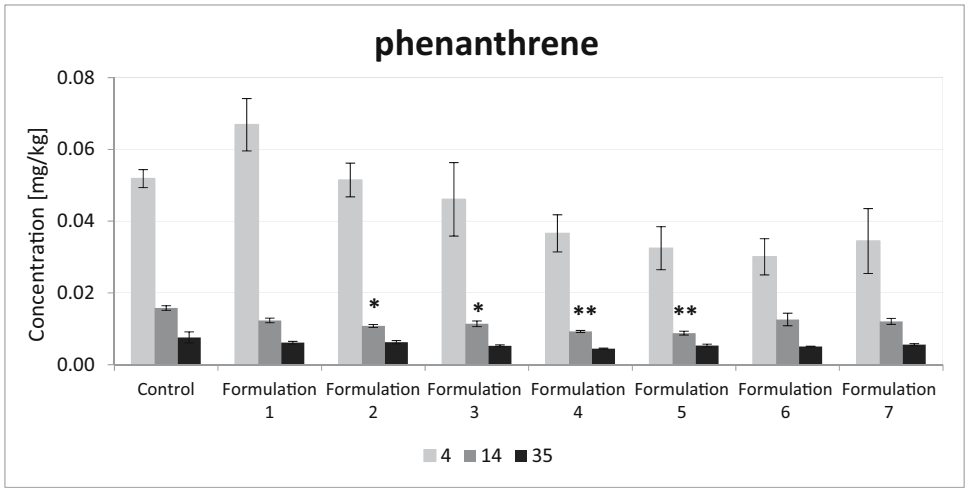

Figure S12: Phenanthrene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p values are shown as $p < 0.01$ (**) and $p < 0.05$ (*).

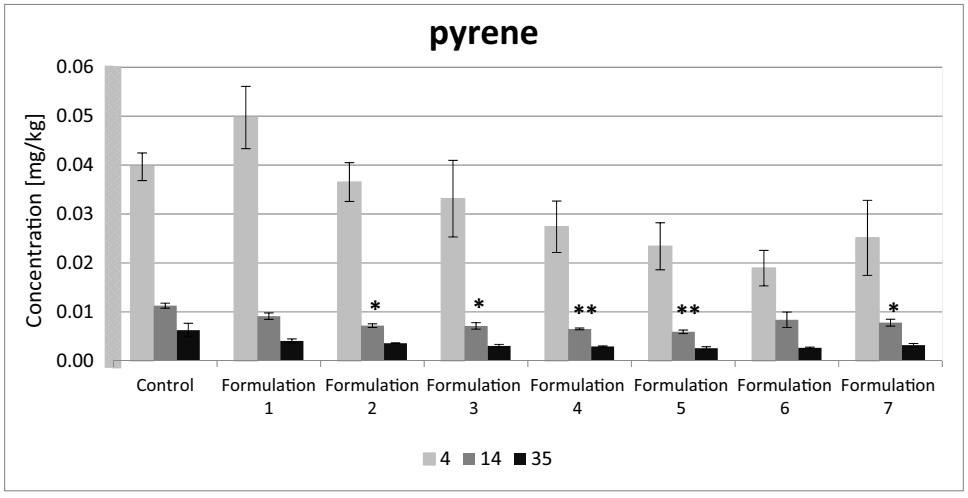

Figure S13: Pyrene concentrations on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). Statistically significant p values are shown as $p < 0.01$ (**) and $p < 0.05$ (*).

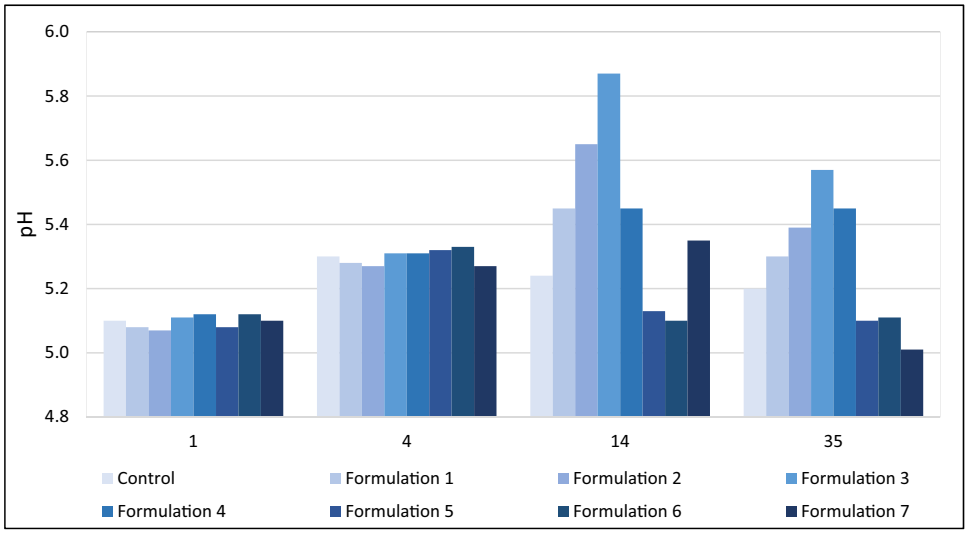

Figure S14: Shifts in the soil pH levels with PAHs and following treatments with EM formulations on successive days of the study, on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM). ORP — oxidation-reduction potential.

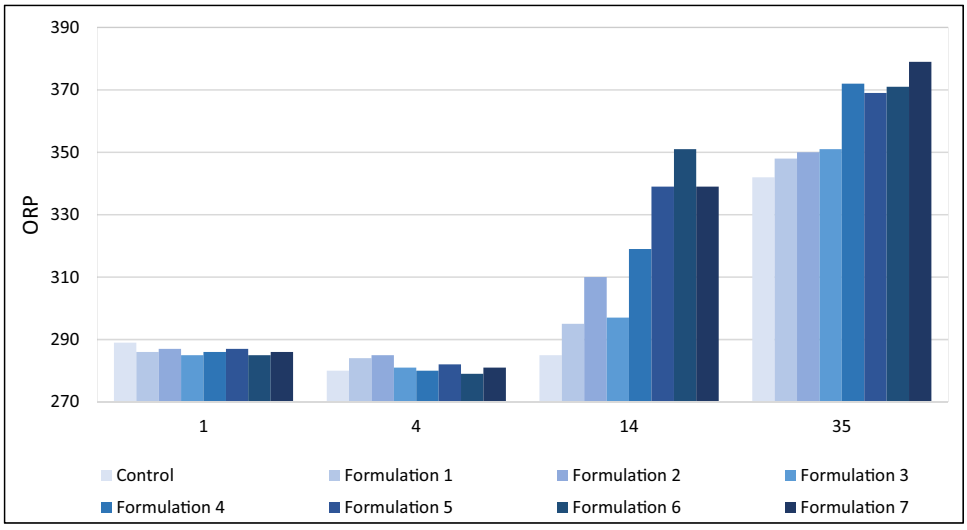

Figure S15: Shifts in the soil oxidation-reduction potential with PAHs and after treatment with EM formulations over time, on Day 4 of experiment (before application of EM), and on Days 14 and 35 of experiment (after application of EM).

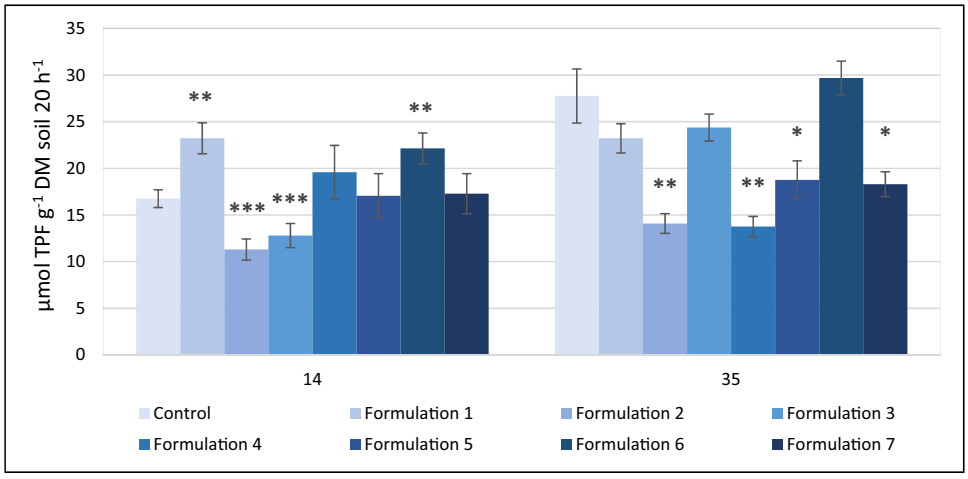

Figure S16: Shifts in the soil DHA with PAHs and after treatment with EM formulations over time. Statistically significant *p* values are shown as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Initial value of DHA was 26.5 µM TPF/g DM soil * 20 h.

Załącznik 1 do Uchwały nr 118/07/2022
Rady Naukowej Kolegium Nauk
Przyrodniczych UR z dnia 12 lipca 2022

Rzeszów, dnia 06.03.2024

Imię i nazwisko **mgr inż. Paulina Książek-Trela**

Jednostka **Instytut Biotechnologii**

Promotor **dr hab. inż. Ewa Szpyrka, prof. UR**

Promotor pomocniczy **dr Leszek Potocki**

OŚWIADCZENIE

W związku z przygotowywaniem przeze mnie rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów, oświadczam niniejszym, że wkład mojej pracy naukowej (w ramach dyscypliny Biotechnologia), a tym samym pracy pozostałych współautorów w opublikowaniu poniższych artykułów, które zamierzam przedstawić jako własną dysertację doktorską jest następujący:

I. Książek-Trela P., Figura D., Węzka D., Szpyrka E. 2024. Degradation of a mixture of 13 polycyclic aromatic hydrocarbons (PAHs) by commercial effective microorganisms. Open Life Sciences 19(1), 20220831. DOI: 10.1515/biol-2022-0831.

- Przygotowanie ekstraktów próbek gleby metodą QuEChERS do oznaczania WWA razem z D.F.,
- Wykonanie analiz aktywności enzymatycznej razem z D.W.;
- Przeprowadzenie analizy wyników razem z E.S;
- Opis i dyskusja wyników;
- Przygotowanie manuskryptu;
- Obowiązki autora korespondencyjnego, w tym wykonanie korekty razem z E.S.

Paulina Książek-Trela

podpis

Oświadczenia współautorów

1. **Damian Figura**
   Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Przygotowanie ekstraktów próbek gleby metodą QuEChERS do oznaczania WWA razem z P.K.-T., integracja wyników GC-MS razem z E.S.

Figura Damian
.....
Podpis

2. **Dominika Wężka**
Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Oznaczenie aktywności enzymatycznej w glebie razem z P.K.-T.

Dominika Wężka
.....
Podpis

3. **Ewa Szpyrka**
Imię i Nazwisko współautora

Jako współautor akceptuję przedstawiony przez Panią **Paulinę Książek-Trela** udział w przygotowaniu powyżej publikacji naukowej. Jednocześnie oświadczam, że wkład mojej pracy naukowej w opublikowaniu powyższego artykułu jest następujący:

- Opracowanie koncepcji i metodologii pracy;
- Przeprowadzenie analizy wyników razem z P.K.-T.;
- Integracja wyników GC-MS razem z D.F.;
- Edycja manuskryptu;
- Obowiązki autora korespondencyjnego, w tym wykonanie korekty razem z P.K.-T.

Ewa Szpyrka
.....
Podpis

9. Życiorys naukowy

Dane osobowe	
Imię i nazwisko	Paulina Książek-Trela
Miejsce zatrudnienia	Uniwersytet Rzeszowski, Kolegium Nauk Przyrodniczych, Instytut Biotechnologii, stanowisko: młodszy specjalista naukowo-techniczny.

Wykształcenie	
02.2019 – 07.2020	Uniwersytet Rzeszowski, Instytut Biologii i Biotechnologii, Kolegium Nauk Przyrodniczych (obecnie Instytut Biotechnologii, Kolegium Nauk Przyrodniczych, Uniwersytetu Rzeszowskiego), studia drugiego stopnia, kierunek Biotechnologia, specjalność Biotechnologia molekularna, magister.
10.2015 – 02.2019	Uniwersytet Rzeszowski, Wydział Biotechnologii (obecnie Instytut Biotechnologii, Kolegium Nauk Przyrodniczych, Uniwersytetu Rzeszowskiego), studia pierwszego stopnia, kierunek Biotechnologia, specjalność Biotechnologia analityczna, inżynier.

Publikacje wchodzące w skład rozprawy doktorskiej

1. **Książek-Trela P.**, Szpyrka E. 2022. The effect of natural and biological pesticides on degradation of synthetic pesticides. Plant Protection Science 58(4), 273–291. DOI: 10.17221/152/2021-PPS.

IF_{2022} – 1,3 $MEiN_{2022}$ – 100 pkt cytowania: 11

2. Szpyrka E., **Książek-Trela P.**, Bielak E., Słowik-Borowiec M. 2024. The influence of commercial yeast preparations on the degradation of herbicide mixtures in the soil and the effect on the shell pea (Pisum sativum L.) cultivation. Journal of Soil Science and Plant Nutrition. DOI:10.1007/s42729-024-01671-7.

IF_{2022} – 3,9 $MEiN_{2024}$ – 100 pkt cytowania: 0

3. **Książek-Trela P**., Bielak E., Wężka D., Szpyrka E. 2022. Effect of three commercial formulations containing effective microorganisms (EM) on diflufenican and flurochloridone degradation in soil. Molecules 27(14), 4541. DOI: 10.3390/molecules27144541.

IF_{2022} – 4,6 $MEiN_{2022}$ – 140 pkt cytowania: 4

4. **Książek-Trela P**., Figura D., Wężka D., Szpyrka E. 2024. Degradation of a mixture of 13 polycyclic aromatic hydrocarbons (PAHs) by commercial effective microorganisms. Open Life Sciences 19(1), 20220831. DOI: 10.1515/biol-2022-0831.

IF_{2022} – 2,2 $MEiN_{2024}$ – 40 pkt cytowania: 0

Pozostałe publikacje

1. Szpyrka E., Słowik-Borowiec M., **Książek P**., Zwolak A., Podbielska M. 2020. The difference in dissipation of clomazone and metazachlor in soil under field and laboratory conditions and their uptake by plants. Scientific Reports 10, 3747. DOI:10.1038/s41598020-60720-0.

IF_{2020} – 4,4 $MEiN_{2020}$ – 140 pkt cytowania: 8

2. Podbielska M., **Książek P**., Szpyrka E. 2020. Dissipation kinetics and biological degradation by yeast and dietary risk assessment of fluxapyroxad in apples. Scientific Reports 10, 21212. DOI: 10.1038/s41598-020-78177-6.

IF_{2020} – 4,4 $MEiN_{2020}$ – 140 pkt cytowania: 7

3. Szpyrka E., Podbielska M., **Książek-Trela P**. 2022. Simple method for fatty acids determination in food, superfood, and spice samples by GC-MS technique. Acta Universitatis Cibiniensis. Series E: Food Technology 26 (2), 171-182. DOI: 10.2478/aucft-2022-0014.

$MEiN_{2022}$ – 140 pkt

4. Słowik-Borowiec M., Szpyrka E., **Książek-Trela P**., Podbielska, M. 2022. Simultaneous determination of multi-class pesticide residues and PAHs in plant material and soil samples using the optimized QuEChERS method and tandem mass spectrometry analysis. Czasopismo: Molecules 27(7), 2140. DOI: 10.3390/molecules27072140.

IF_{2022} – 4,6 $MEiN_{2022}$ – 140 pkt cytowania: 12

5. Słowik-Borowiec M., Zdeb G., Kuras W., **Książek-Trela P**. 2022. Influence of Bacillus Subtilis Fermentation on Content of Selected Macronutrients in Seeds and Beans. Acta Universitatis Cibiniensis. Series E: Food Technology 26(1), pp.123-138. DOI: 10.2478/aucft-2022-0010.

$MEiN_{2022}$ – 140 pkt

Indeks h: **4**

Sumaryczny Impact Factor: **25,358**

Sumaryczna liczba punktów wg MEiN: **1080**

Sumaryczny liczba cytowań: **42** (wg bazy Scopus – 24-03-2024)

Projekty

05.2023 – 01.2024	Grant dla Młodych Naukowców 2023. Wpływ bakterii wyizolowanych z próbek gleby pobranych na terenie Podkarpacia na rozkład trwałych zanieczyszczań środowiska, stanowisko: kierownik.
10.2019 – 02.2020	Podkarpackie Centrum Innowacji 2019. Ekoinnowacyjna metoda przygotowywania próbek do analizy pozostałości pestycydów i WWA – uczestnik.

Zgłoszenie patentowe

Książek-Trela P., Potocki L., Szpyrka E. Nowe izolaty glebowe oraz ich konsorcjum zdolne do degradacji diflufenikanu. Zgłoszenie zostało przyjęte w Urzędzie Patentowym RP w dniu 15.12.2023 i oznaczone numerem: P.447114 [WIPO ST 10/C PL447114].

Staż naukowy

31.07 – 04.08.2023	Państwowy Instytut Weterynaryjny – Państwowy Instytut Badawczy, Zakład Farmakologii i Toksykologii w Puławach.

Praktyki

01.07 – 30.09.2019	Praktyka Absolwencka w Kronospan Mielec Sp. z o. o.
01.09 – 29.09.2017	Praktyka zawodowa w Oczyszczalni Ścieków w Kolbuszowej.

Konferencje międzynarodowe

1. Bielatowicz G., Kaleniuk E., **Książek P**. XXVII Międzynarodowa Naukowa i Praktyczna Konferencja Młodych Naukowców i Studentów. 2019. Eurointegration Aspirations of Ukraine and Problems of Macroeconomics. Uniwersytet im. Alfreda Nobla w Dnipro, Ukraina.

2. Słowik-Borowiec M., Potocki L., Oklejewicz B., Broda D., Podbielska M., **Książek P**., Szpyrka E. Production of vitamin k2 mk-7 in a process of fermentation of seeds/cereals by bacteria Bacillus subtilis. 2021. 8th International Conference "Human – Nutrition – Environment" University of Rzeszow, Rzeszów, Polska – poster.

3. **Książek-Trela P**., Szpyrka E. Fungicides used in organic agriculture. 2021. 8th International Conference "Human – Nutrition – Environment" University of Rzeszow, Rzeszów, Polska – poster.

4. Cichoński J., Szpyrka E., **Książek P**., Masionis S., Chrzanowski G. Antioxidant activity of essential oils from selected Lamiaceae plants. 2021. 8th International Conference "Human – Nutrition – Environment" University of Rzeszow, Rzeszów, Polska – poster.

5. **Książek-Trela P**., Szpyrka E., Bielak E. Effect of three commercial formulations containing effective microorganisms on flurochloridone degradation in soil. 2023. 9th International Conference "Human-Nutrition-Environment University of Rzeszow, Rzeszów, Polska – poster.

Konferencje krajowe

1. Szpyrka E., **Książek P**., Podbielska M., Słowik-Borowiec M. Efekty matrycowe w analizie pozostałości herbicydów w glebie techniką chromatografii gazowej z detekcją wychwytu elektronów. 2019. XIII SEMINARIUM NAUKOWE „Aktualne Problemy Chemii Analitycznej", Katowice – poster.

2. Książek-Trela P., Szpyrka E. Ogólnopolska Konferencja Naukowa. The effect of natural and biological pesticides on degradation of synthetic pesticides. 2023. National Scientific Conference „e-Factory of Science" – IX edition, Łódź – prezentacja.

Szkolenia i kursy

1. Kompleksowy program rozwoju Uniwersytetu Rzeszowskiego. POWR.03.05.00-00-Z072/18. Szkolenie z języka angielskiego wraz z certyfikacją.

2. Jednolity Program Zintegrowany Uniwersytetu Rzeszowskiego – droga do wysokiej jakości kształcenia. POWR.03.05.00-00-Z050/17-00. „Excel do pracy w biurze/uczelni".

3. Przyjazny nURt – rozwój dostępności UR. POWR.03.05.00-00-A007/19. Szkolenie świadomościowe dotyczące problemów osób z niepełnosprawnością dla pracowników Uniwersytetu Rzeszowskiego.

4. „Course of seminar and specialization laboratory" Uniwersytet Rzeszowski.

5. „Kariera i rozwój" Sanofi Rzeszów.

6. „HORYZONT EUROPA - szansa na sfinansowanie badań naukowych" Podkarpackie Centrum Innowacji – PCI.

7. „Nowe podejście do ochrony danych osobowych po wejściu RODO" Uniwersytet Rzeszowski.

8. „Bezpieczeństwo informacji – security awareness" Uniwersytet Rzeszowski.

9. Liczna szkolenia z obsługi nowoczesnego sprzętu laboratoryjnego wykorzystywanego w badaniach naukowych w Instytucie Biotechnologii.

Język obcy

22.06.2023	Kompetencje językowe – język angielski na poziomie B2 według Europejskiego Systemu Opisu Kształcenia Językowego.

Badania komercyjne

1. Górka A., **Książek-Trela P**. „Oznaczanie mykotoksyny DON oraz metali – kadmu i ołowiu w mące żytniej”, na zlecenie Zakładów Zbożowych Ocieka Tuszyma Spółka z o.o., Tuszyma (20.04.2022).

2. Górka A., **Książek-Trela P**. „Analiza zanieczyszczeń kąpieli Zn-Ni (kapiel alkaliczna) – oznaczanie chromu, kadmu, ołowiu, miedzi i żelaza” na zlecenie: Collins Aerospace Poland Sp. z o.o., Krosno (analizy od 06.2022 do 03.2024) – 19 raportów.

Promocja Instytutu Biotechnologii

Od 28.03.2023 – Członek Zespołu do spraw Promocji Instytutu Biotechnologii.

Noc Biologów na Uniwersytecie Rzeszowskim w latach 2020 – 2023.

Zajęcia dla Akademii Tutorial College Sp. z o.o., uczniowie liceów i gimnazjów - uczestnicy warsztatów „Biochemia - Co kryje się w tłuszczach?” (13 – 14.04.2019).

Zajęcia w ramach promocji IBB dla uczniów liceum z Sędziszowa Małopolskiego (26.10.2021) oraz z Jarosławia (08.12.2022).