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The impact of novel bacterial strains and their consortium on diflufenican degradation in the mineral medium and soil

Paulina Książek-Trela✉, Leszek Potocki & Ewa Szpyrka

Four novel bacterial strains isolated from agricultural soil were tested for their ability to degrade the persistent herbicide - diflufenican, in a mineral medium and soil. The presence of three potential diflufenican metabolites, 2,4-difluoroaniline, 2-(3-trifluoromethylphenoxy)nicotinamide, and 2-(3-trifluoromethylphenoxy)nicotinic acid, was analysed. The isolated bacterial species were identified by 16S rRNA gene sequencing as *Pseudomonas* sp. 10Kp8 - A1, *Pseudomonas chlororaphis* subsp. *aureofaciens* strain B19 - A2, *Pseudomonas baetica* strain JZY4-9 - C1, and *Streptomyces atratus* strain ROA017 - D1. The effect of each isolate and its consortia on diflufenican degradation was compared to control samples without microorganisms. In the mineral salt medium (MSM) and soil, strain D1 showed the highest degradation, reaching 70.1% and 79%, respectively. However, the application of a synthetic consortium of four microorganisms resulted in even higher degradation in both environments, achieving 74.4% and 82.2% in the liquid medium and the soil, respectively. Notably, the microorganisms were able to grow in MSM containing diflufenican at concentrations of 60–220 mg/kg, utilizing it as a source of carbon and energy. The quadruple synthetic consortium holds promise as a bioremediation technique for reducing the contamination of ecosystems by this persistent herbicide. These findings demonstrate the diversity of diflufenican-degrading bacteria in agricultural soil ecosystems and provide a promising new approach for bioremediation of diflufenican and other persistent herbicides in the agricultural soil.

Keywords Herbicides, Diflufenican, *Pseudomonas*, *Streptomyces*, Biodegradation, Metabolites

Herbicides, also known as pesticides, are used for weed control in agricultural and horticultural crops. Some of them are persistent organic pollutants which are characterized by their long–term degradation, and include, e.g.,: diflufenican (the disappearance time of 90% of the active substance (DT90) of up to 1900 days), flurochloridone (DT90 of up to 545 days), aclonifen (DT90 of up to 649 days), chlortoluron (DT90 of up to 796 days), clomazone (DT90 of up to 645 days), napropamide (DT90 of up to 1000 days), and glyphosate (DT90 of up to 1600 days)[1].

Diflufenican ($C_{19}H_{11}F_5N_2O_2$) is a globally used pesticide that acts as an herbicide against grasses and broad-leaved weeds. It exhibits low volatility and low solubility in water. Diflufenican is applied to protect crops such as clover-based pastures, field peas, lupins, lentils, and winter cereals. It is very persistent in aquatic systems and moderately persistent in the soil. It shows high toxicity to algae, and moderate toxicity to other aquatic organisms, birds and eathworms[2–4]. Possible metabolites of diflufenican in the soil and ground waters include 2,4-difluoroaniline, 2-(3-trifluoromethylphenoxy)nicotinamide, and 2-(3-trifluoromethylphenoxy)nicotinic acid[1]. Diflufenican is recognized for its slow degradation[1,5,6] and tendency to accumulate[5], and adsorb[7] in soil. Despite this, the European Food Safety Authority (EFSA) continues to approve its use due to its strong sorption to soil, which significantly limits its leaching from agricultural lands[5].Numerous studies have demonstrated that diflufenican sorption is positively correlated with the soil organic matter content[8,9]. Tejada et al. (2011) confirm faster dissipation of diflufenican in the soil with the lower organic mattercontent[8]. According to Svendsen et al. (2020) diflufenican degrades in both agricultural soil and urban gravel. However, its metabolites are significantly more persistent in urban gravel, where sorption is also lower when compared to agricultural soils[7].

Książek-Trela et al. (2022) investigated the effect of three commercial preparations containing different species of microorganisms on diflufenican degradation in soil. The degradation inhibition was observed for all preparations. The degradation was probably inhibited by a significant decrease in the soil pH after the application

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of biological preparations (by 1 pH unit versus the control)[2]. According to Houot et al. (2000), soil with pH below 6.5 is characterized by the slower metabolic degradation[10]. Similar results were obtained during a study conducted on the soil before sowing of winter wheat. The soil was treated with green fertilizer, cow manure, or pig slurry. The diflufenican half-life was calculated, and it was 116, 215 and 176 days, respectively, for soils treated with organic fertilizers. This study confirms the increased persistence of diflufenican after application of microorganisms to the soil. After 6 months, the effect of organic fertilizers was less pronounced, and diflufenican and its metabolites in the soil were at similarly low levels[11].

The widespread use of chemical plant protection products poses a threat to the safety and health of humans, animals, and the natural environment. The biodegradation of persistent pollutants and soil bioremediation are among the most economical and environmentally beneficial biotechnological innovations[12,13]. Therefore, it is crucial to identify new microorganisms capable of degrading persistent pollutants.

The microbial degradation of pesticides is mediated through diverse biochemical mechanisms that collectively contribute to detoxification and environmental remediation. Enzymatic degradation pathways dominate, wherein microorganisms exploit pesticides as sources of carbon, nitrogen, and energy, catalyzing oxidation, reduction, and hydrolysis reactions that mineralize complex organic compounds into innocuous end products such as carbon dioxide, nitrates, phosphates, ammonia, and water[14,15]. Non-enzymatic mechanisms further facilitate degradation through microbially induced environmental modifications, including pH shifts and the production of reactive metabolites that chemically alter pesticide structures[16]. In addition, cometabolic processes—whereby microorganisms transform pesticides via incidental enzymatic activity while metabolizing other primary substrates—play a critical role in the breakdown of recalcitrant compounds. These reactions often generate intermediates that serve as substrates for subsequent microbial taxa, thereby reducing environmental persistence and toxicity; for example, dichlorodiphenyltrichloroethane (DDT) is effectively cometabolized through such pathways[17]. Microbial degradation offers a sustainable approach for pesticide remediation. Traditional studies focused on single-strain degraders; however, recent insights reveal that natural biodegradation is typically a community-driven process, shaped by diverse interspecific interactions[18]. Certain pesticides require multiple enzymatic steps for complete degradation, often exceeding the metabolic capabilities of a single organism. Cooperative degradation allows microbial consortia to partition the degradation pathway among community members[19]. For example, one species may transform a pesticide into an intermediate metabolite, which is further degraded by another. *Pseudomonas* species initiate the hydrolysis of chlorpyrifos, producing 3,5,6-trichloro-2-pyridinol (TCP), which is subsequently degraded by *Cupriavidus* sp. DT-1[20,21]. Competition for pesticide-derived carbon sources can shape community composition. Fast-growing, pesticide-adapted strains often dominate, outcompeting native microbes. This phenomenon can reduce microbial diversity but enhances degradation rates. In atrazine-contaminated soils, the dominance of *Arthrobacter* spp. is associated with higher atrazine degradation rates[22,23]. Pesticide degradation genes are often located on plasmids or transposons, facilitating their rapid dissemination among community members. Horizontal Gene Transfer (HGT) enhances community adaptability to xenobiotics, promoting the emergence of new degradative phenotypes[24]. The Mpd gene encoding methyl parathion hydrolase has been observed transferring among *Burkholderia* and *Pseudomonas* strains in pesticide-contaminated fields[25]. Environmental parameters such as pH, temperature, soil structure, nutrient availability, and pesticide concentration modulate microbial interactions[26]. Biofilm formation is particularly important, as it fosters close microbial contact, enhancing both cooperation and HGT[27]. Synthetic microbial consortia are emerging as promising tools to enhance bioremediation efficiency[28,29].

The literature review reveals that bacteria of the genera *Pseudomonas* spp. and *Streptomyces* spp. have been extensively studied for their potential in biological control of plant diseases. Some *Pseudomonas* spp. strains have demonstrated the ability to protect plants against leaf pathogens through various mechanisms, including systemic resistance, competing for nutrients, or a living space, and producing antimicrobial compounds. *Pseudomonas* spp. strains have also been shown to promote plant growth by enhancing the availability and uptake of essential mineral nutrients through phosphate solubilization or stimulating root development[30]. *Pseudomonas chlororaphis* is a ubiquitous bacterium found in various environmental niches. It is considered safe for use in agricultural practices, particularly in the cultivation of food and feed crops. The United States Environmental Protection Agency has classified *P. chlororaphis* as non-pathogenic to humans, wildlife, and the environment. Commercial products based on *P. chlororaphis* strains are already available and widely used. *P. chlororaphis* produces a variety of bioactive metabolites, i.e., phenazine and lipopeptide compounds, which exhibit valuable antibiotic properties in agriculture. These metabolites contribute to the broad antagonistic activity of *P. chlororaphis* against many plant pathogens[31]. *P. chlororaphis* was investigated for its effectiveness against *Seiridium cardinale*, the fungus responsible for cypress cancer (*Cupressus sempervirens*)[32,33]. In addition, *P. chlororaphis* demonstrated efficacy against *Ceratocystis fimbriata*, the causal agent of black rot disease in sweet potato tuber roots (TRs)[34]. *Streptomyces* spp. represents the most extensively studied bacterial genus among *Actinomycetes* for its the potential application in biological control of plant diseases[35–38]. This genus is widely used in biocontrol programs due to its ability to induce systemic resistance in plants or produce substances that inhibit pathogens growth, such as antibiotics (atramycin, neomycin, streptomycin, cypemycin, grisemycin, bottromycins, and chloramphenicol), and enzymes that degrade the fungal cell wall (cellulases, hemicellulases, chitinases, and glucanases)[39]. Notably, certain commercial biological formulations contain *Streptomyces* spp., have been approved for use in organic agriculture. *Streptomyces atratus*, a bacterium isolated from the soil in Shimoneda, Japan, demonstrated antagonistic activity against various plant pathogens, including *Botrytis cinerea*, *Colletotrichum orbiculare*, *Fusarium oxysporum*, *Phytophthora capsica*, and *Phytophthora infestans*[40]. To this date, no research was conducted on the potential of *Pseudomonas baetica* in biocontrol and plant protection programs, as well as on the effect of the studied bacteria on degradation of pesticides or other persistent compounds.

The aim of this study was to isolate and identify new bacterial strains with a potential to degrade diflufenican. The biodegradation tests were performed both in the MSM and in the soil, using single bacterial isolates and their

synthetic consortium. Additionally, the presence of three potential diflufenican metabolites: 2,4-difluoroaniline, 2-(3trifluoromethylphenoxy)nicotinamide, and 2-(3-trifluoromethylphenoxy)nicotinic acid, was investigated in the samples. The innovative aspect of our research is centered on investigating the diflufenican degradation. To the best of our knowledge, currently there is no information available on bacterial degradation of this herbicide. All available literature data refer to the inhibition of diflufenican degradation, hence the need to search for other microorganisms capable of degrading the studied herbicide. In our study, we successfully isolated four novel bacterial strains capable of degrading diflufenican. The genome sequences have been deposited in GenBank, submitted to the Patent Office of the Republic of Poland on 15/12/2023, and assigned the number: P.447,114 [WIPO ST 10/C PL447114], highlighting their potential biotechnological significance. Another pioneering aspect of our research is the detailed examination of the degradation pathway of diflufenican in both liquid medium and soil environments. We analyzed the formation of diflufenican metabolites. This comprehensive approach provides new insights into the microbial metabolism of diflufenican and contributes to the broader understanding of its environmental fate. There is an urgent need to search for new bacterial environmental isolates capable of degrading pesticides used in agriculture and of other organic pollutant, which can support the improvement of soil "health", as well as the conservation of the natural biodiversity of agricultural ecosystems, and mitigation of environmental impacts. The results of our study will contribute to the effective sustainable management of environmental systems, i.e. bioremediation and the enhancement of quality of contaminated sites.

Materials and methods

Chemicals and reagents

The analytical standard of diflufenican was purchased from Dr. Ehrenstorfer GmbH, Germany; the commercial formulation, Legato 500 SC was bought from ADAMA, Poland. Other chemicals and reagents used included the internal standard Triphenyl Phosphate (TPP), Supelco Inc. from Bellefonte, PA., USA; the analytical standard of diflufenican metabolites: 2,4-difluoroaniline and 2-(3-trifluoromethylphenoxy)nicotinic acid from Sigma-Aldrich, USA; 2-(3-trifluoromethylphenoxy)nicotinamide from HPC Standards GmbH, Germany; the silylating mixture II according to Horning (BSA + TMCS + TMSI 3:2:3) for the GC derivatization from Sigma-Aldrich, USA; the phosphate buffer (60 mM, pH = 7) from Chempur, Poland; chromatographic grade acetone, hexane, and petroleum ether for GC from Chempur, Poland; kits of salts and sorbents for extraction and purification by QuEChERS method from Agilent Technologies (Santa Clara, USA); salts for preparation of MSM, Ringer's solution and Tween 80 from Chempur, Poland; agar and nutrient broth from BTL Sp. z o. o., Poland; and the commercial kit for genomic DNA isolation from EURX Sp. z o. o., Poland.

Soil sample collection

A soil sample used for isolation of diflufenican–degrading bacteria was collected from the arable layer of crop fields (Subcarpathia, Poland), where various herbicide treatments, including diflufenican, are frequently applied. The sample was collected using a sterile laboratory spoon and placed in sterile polypropylene tubes. It was then transported to the laboratory for further analysis.

Isolation of diflufenican degrading bacteria

Enriched culture techniques were used to isolate diflufenican–degrading bacterial strains.

The mineral salts medium (MSM) containing 4.8 g/L KH_2PO_4; 1.2 g/L K_2HPO_4; 1 g/L NH_4NO_3; 0.2 g/L $MgSO_4 \times 7H_2O$; 0.04 g/L $Ca(NO_3)_2 \times 4H_2O$; and 0.001 g/L $Fe(SO_4)_3$ was used for bacteria isolation. The pH of the medium was adjusted to 7.0 to facilitate assessment of biodegradation.

One gram of the soil sample was transferred to a sterile 250 mL Erlenmeyer flask containing 30 mL of enriched MSM supplemented with 160 mg/L of diflufenican. The commercial formulation, Legato 500 SC, contains 500 g/L (42.02%) of diflufenican as the active substance was used in all studies. MSM without the herbicide was used as a control sample. The samples were incubated at 22 °C on a rotary shaker (100 rpm) (GFL 3006, Poland) for 7 days. After 7 days, 5 mL of the soil suspension were transferred into fresh enrichment medium flasks and incubated under the same conditions for a further 7 days. Serial dilutions of the Erlenmeyer flask samples were plated onto MSM agar plates (BTL sp. z o.o., Poland) containing 100 mg/L diflufenican to isolate single colonies. Serial dilutions were prepared using Ringer's solution, containing 8.6 g/L NaCl, 0.3 g/L KCl, and 0.333 g/L $CaCl_2 \times 2H_2O$. All media were sterilized by autoclaving at 121 °C for 20 min.

Bacteria were selected on the basic of phenotypic differences when compared to control bacteria.

DNA isolation and identification of bacterial species

Each bacterial strain was cultured for 24 h in the nutrient broth containing meat extract (2 g/L), yeast extract (2 g/L), peptone (5 g/L), NaCl (4 g/L), and glucose (10 g/L), pH 7.5 (BTL Sp. z o. o., Poland). The bacterial genomic DNA was isolated using a commercial kit (EURX Sp. z o. o., Poland) according to the manufacturer's protocol.

The isolated DNA was sequenced using the Sanger sequencing method (an outsourced service), and the species identified in the isolates were determined by comparing the sequences with those deposited in the National Center of Biotechnology Information Database (NCBI) using BLAST software.

Preparing bacteria and bacterial synthetic consortium

Four bacteria strains were inoculated into the nutrient broth (BTL Sp. z o. o., Poland) and incubated for 24 h in a laboratory shaker at 30 °C.

Before adding bacteria to the experiment samples, the bacteria concentration was determined using a TECAN device (Infinite®, model M200, USA) with i-Control software, at a wavelength of λ=600 nm. The concentration of each bacterial strain was approximately 0.1 (OD 600 nm).

To prepare the bacterial synthetic consortium, equal volumes of the four bacterial suspensions were mixed in a sterile polypropylene tube. The concentration of the synthetic consortium was approximately 0.1 (OD 600 nm).

Determination of bacterial titer

For each sample collected for the herbicide residue analysis, a colony forming unit (CFU) was measured to determine the number of viable and active microorganisms in 1 mL of medium or 1 g of soil. CFU was measured at the beginning of the experiment and at subsequent time points, as shown in Figs. 2C, 3C and 4B.

To determine the bacterial titer, a series of 10-fold dilutions were prepared from the MSM samples and the soil samples containing bacteria. For the soil samples, 1 g of the soil was weighed into sterile 100 mL Erlenmeyer flasks. 9 mL of Ringer's solution with 3% Tween 80 was added, and the flasks were shaken on a rotary shaker (100 rpm) (GFL 3006, Poland) at 22 °C for 3 h. Serial dilutions were made in Ringer's solution. Then, 20 µL samples of each dilution were spotted onto nutrient agar plates in triplicate. The plates were incubated at 30 °C for 24 h. After incubation, the bacterial titer was calculated and expressed as $\log_{10}$/CFU per 1 mL of medium or 1 g of soil.

Biodegradation studies in MSM in long-term culture

The experiment was conducted in sterile 50 mL Erlenmeyer flasks containing MSM supplemented with diflufenican at a concentration of 220 mg/kg. The experiment was performed in triplicate. At the beginning of the experiment, four bacterial strains were inoculated separately and as a synthetic consortium into the medium containing the herbicide. The control was consisted of the mineral medium containing the herbicide. The flasks were shaken on a rotary shaker, at 100 rpm and 22 °C (GFL 3006, Poland) for three weeks. Samples for herbicide residue analysis were collected on the first day of the experiment and then subsequently on days 7, 14, and 21 of the study. Before collecting samples for the diflufenican residue analysis, the bacterial density, expressed as $\log_{10}$/CFU, was determined to assess the number of viable and active microorganisms per 1 mL of medium.

The herbicide residues were determined in MSM following the method described by Podbielska et al. (2020)[41]. Briefly, 100 µL of the samples were transferred into polypropylene tubes, followed by the addition of 5 mL of acetone (Honeywell, Charlotte, NC, USA) and 0.5 g of anhydrous sodium sulfate (Chempur, Piekary Śląskie, Poland). The tubes were vortexed for 1 min (BenchMixerTM, Benchmark Scientific, Inc., Edison, NJ, USA) and then centrifuged at 3500 rpm for 5 min (5804R, Eppendorf, Hamburg, Germany). Next, 200 µL of the acetone extract were transferred to a chromatographic vial, and 800 µL of petroleum ether (Chempur, Piekary Śląskie, Poland) were added. Finally, 50 µL of an internal standard solution containing 1000 µg/mL of TPP (Triphenyl Phosphate, Supelco Inc., Bellefonte, PA, USA) were added. The extracts were analyzed by gas chromatography with mass detection (GC-MS/MS). Herbicide residues in the samples were compared to controls. In addition, the percentage of herbicide degradation was reported.

Biodegradation studies in MSM in short term culture

The short–term culture experiment was conducted using the *Streptomyces atratus* strain ROA017 - D1 and the bacterial synthetic consortium exhibiting the highest degradation rate in the long-term liquid culture experiment.

The experiment was performed in sterile 50 mL Erlenmeyer flasks containing MSM supplemented with diflufenican at a concentration of 60 mg/kg. The experiment was performed in triplicate. Initially, the D1 strain and the synthetic consortium were inoculated into the herbicide containing medium. The control consisted of the mineral medium with the herbicide. The flasks were shaken on a rotary shaker at 100 rpm and 22 °C (GFL 3006, Poland) for 336 h. Samples were collected at the start of the experiment and after 12, 24, 72, 168 and 336 h.

The herbicide residues were analyzed according to the methodology described by Podbielska et al. (2020)[41] and described in Sect. "Biodegradation studies in MSM in long-term culture". The herbicide residues in the samples were compared with those in controls. Additionally, the percentage of herbicide degradation was reported.

Soil Preparation

The experiment was conducted with the universal soil, recommended for the horticultural crop. The soil consisted of high and low moor peat, pine bark, dolomite, sand, perlite, and mineral fertilizers. The soil had the following parameters: pH 6.0–7.3, and salinity 0.5–1.0 g KCl/L (PPUH Zielona Oaza I, Brzozów, Poland). Soil was sterilized by autoclaving at 121 °C for 20 min. The soil water content was in the range of 70–72%.

Biodegradation studies in soil

The experiment was conducted in triplicate using 2 L glass containers, each containing 200 g of the sterilized soil. At the beginning of the experiment, 4 bacterial strains were inoculated separately and as a synthetic consortium into the soil. After 24 h, an aqueous herbicide solution at a concentration of 70 mg/kg was added. The soil with the herbicide was used as a control. The experiment was conducted for 4 weeks, and the glass containers were incubated at 22 °C. Samples for the herbicide residues analysis were collected on 1 and 28 days of the experiment. Before taking samples for the herbicide residue analysis, a measurement of the bacterial density, expressed as $\log_{10}$/CFU per 1 mL, was performed. This measurement indicated the number of viable and active microorganisms in 1 g of soil. Diflufenican in the soil was determined using a modified QuEChERS method, which involve extraction of the analytes with a mixture of acetone and hexane and purification of extracts by the dispersive solid phase extraction (dSPE), as described by Słowik–Borowiec et al. (2022)[42].

Briefly, 5 g of soil samples were weighed into polypropylene tubes to which 10 mL of water and 10 mL of an acetone: hexane mixture (1:4 v/v) were added. The contents of the tubes were vortexed for 1 min (BenchMixerTM, Benchmark Scientific, Inc., Edison, NJ, USA). Next, buffer salts containing 4 g of anhydrous magnesium sulfate ($MgSO_4$), 1 g of sodium chloride (NaCl), 1 g of anhydrous trisodium citrate, and 0.5 g of disodium sesquihydrate citrate were added. The samples were then vortexed using a vortex (BenchMixerTM, Benchmark Scientific, Inc.,

Edison, NJ, USA) for 1 min and centrifuged for 5 min at 4000 rpm in a centrifuge (5804R, Eppendorf, Hamburg, Germany). For sample cleanup 5 mL of the upper organic layer was transferred to a new tube and mixed with a mixture of salts (150 mg of PSA and 900 mg of $MgSO_4$). This mixture was vortexed for 1 min, and centrifuged at 4000 rpm for 5 min. Finally, 1 mL of the purified extract was transferred to a chromatographic vial, and 50 µL of an internal standard solution containing 1000 µg/mL of TPP (Triphenyl Phosphate, Supelco Inc., Bellefonte, PA, USA) were added. The extracts were analyzed by gas chromatography with mass detection (GC-MS/MS).

Diflufenican metabolites

In addition to diflufenican, its metabolites, 2,4-difluoroaniline, 2-(3-trifluoromethylphenoxy)nicotinamide, and 2-(3-trifluoromethylphenoxy)nicotinic acid, were also determined in the MSM and the soil samples. The preparation of the MSM and soil samples followed the methods described by Podbielska et al. (2020)[41] and Słowik–Borowiec et al. (2022)[42], respectively, and outlined in Sects. "Biodegradation studies in MSM in long-term culture" and "Biodegradation studies in soil". For the determination of 2-(3-trifluoromethylphenoxy) nicotinic acid, the extracts underwent a derivatization process according to Książek-Trela et al. (2022)[2]. Briefly, the extracts were evaporated under a stream of nitrogen. Then, 100 µl of a silylating mixture were added, and the sample was left at the room temperature for 30 min. Subsequently, 500 µl of hexane and 1 mL of phosphate buffer (60 mM, pH 7) were added, and the samples were shaken for 1 min. The samples were then analyzed by GC–MS/MS.

Chromatographic analysis

Sample extracts were analyzed using an Agilent Technologies 7890 A gas chromatograph (USA) coupled with a model 7000 triple quadrupole tandem mass spectrometer (GC-MS/MS). The dynamic multiple reaction monitoring (dMRM) mode was employed for the analyses, with three mass transitions for diflufenican and its metabolites. The analyzed compounds were identified based on their retention times and qualitative ions (Table S1, Supplementary Material). The GC–MS/MS analysis conditions are presented in Table S2, Supplementary Material. Chromatograms and spectra for the tested diflufenican metabolites are provided in the Supplementary Material, Figures S1–S6. The limit of quantification for diflufenican was 0.01 mg/kg in soil and in the MSM. The average recoveries were 101.8% for soil and 102.1% for MSM. The method was validated according to the SANTE 11312/2021[43] recommendation.

Statistical analysis of results

All analyzes were performed in triplicate and are presented as mean ± SD. Differences between control and treated samples were analyzed using one-way ANOVA, Dunnett's multiple comparison test and Student's t-test. Statistical significance was evaluated using GraphPad Prism 10. Statistically significant differences are presented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results

Isolation and identification of diflufenican-degrading soil bacteria

Seven bacterial strains were isolated from the crop field soil sample with the enrichment technique. Subsequently, four strains exhibiting the highest diflufenican degradation potential were selected for further analysis. These strains showed a different colony morphology compared to those isolated from the control soil sample (Fig. 1).

Identification of selected bacteria through 16S rRNA sequence analysis showed that the bacterial strains were classified withinthe genus *Pseudomonas* sp. 10 Kp8 - A1, *Pseudomonas chlororaphis* subsp. *aureofaciens* strain B19 - A2, *Pseudomonas baetica* strain JZY4-9 - C1, and *Streptomyces atratus* strain ROA017 - D1 (Table 1).

Bacteria effectively degrade diflufenican in the MSM within 21 days

The ability of four bacterial isolates and synthetic consortium to degrade diflufenican was investigated in the MSM containing the herbicide at a concentration of 220 mg/L.

After 21 days, the highest decrease in the initial concentration of diflufenican, of 220 mg/kg, occurred after the application of the D1 strain alone, of 65.6 mg/kg, and after application of the bacterial synthetic consortium, of 56.2 mg/kg (Fig. 2A).

The D1 bacterial strain exhibited the highest diflufenican degradation efficiency in the MSM, eliminating 70.1% of the herbicide after 21 days of incubation. The bacterial synthetic consortium achieved a slightly higher degradation rate of 74.4% during the same period. These results suggest that the D1 strain is a promising candidate for the bioremediation of diflufenican–contaminated environments (Fig. 2B).

The bacterial density (CFU) was measured at the beginning of the experiment and on days 7, 14, and 21 (Fig. 2C). All MSM samples containing bacterial strains and the synthetic consortium showed a decrease in the CFU/mL density on day 21, therefore this was the last day of sampling.

Diflufenican degrades in the MSM – a short term culture

A controlled experiment was conducted with the D1 strain, which had the best potential to remove diflufenican in the long-term experiment, and the bacterial synthetic consortium, using MSM supplemented with diflufenican at a concentration of 60 mg/L.

After 336 h (14 days), the highest decrease in the initial concentration of diflufenican, of 60 mg/L, occurred after application of the bacterial synthetic consortium, of 22.4 mg/L (Fig. 3A).

The bacterial synthetic consortium demonstrated superior diflufenican degradation capabilities, eliminating 63.1% of the herbicide after 336 h, compared to 51.8% degradation achieved by the D1 strain (Fig. 3B).

The bacterial density measurements were conducted at the beginning of the experiment and at subsequent time points of 12, 24, 72, 168, and 336 h (Fig. 3C).

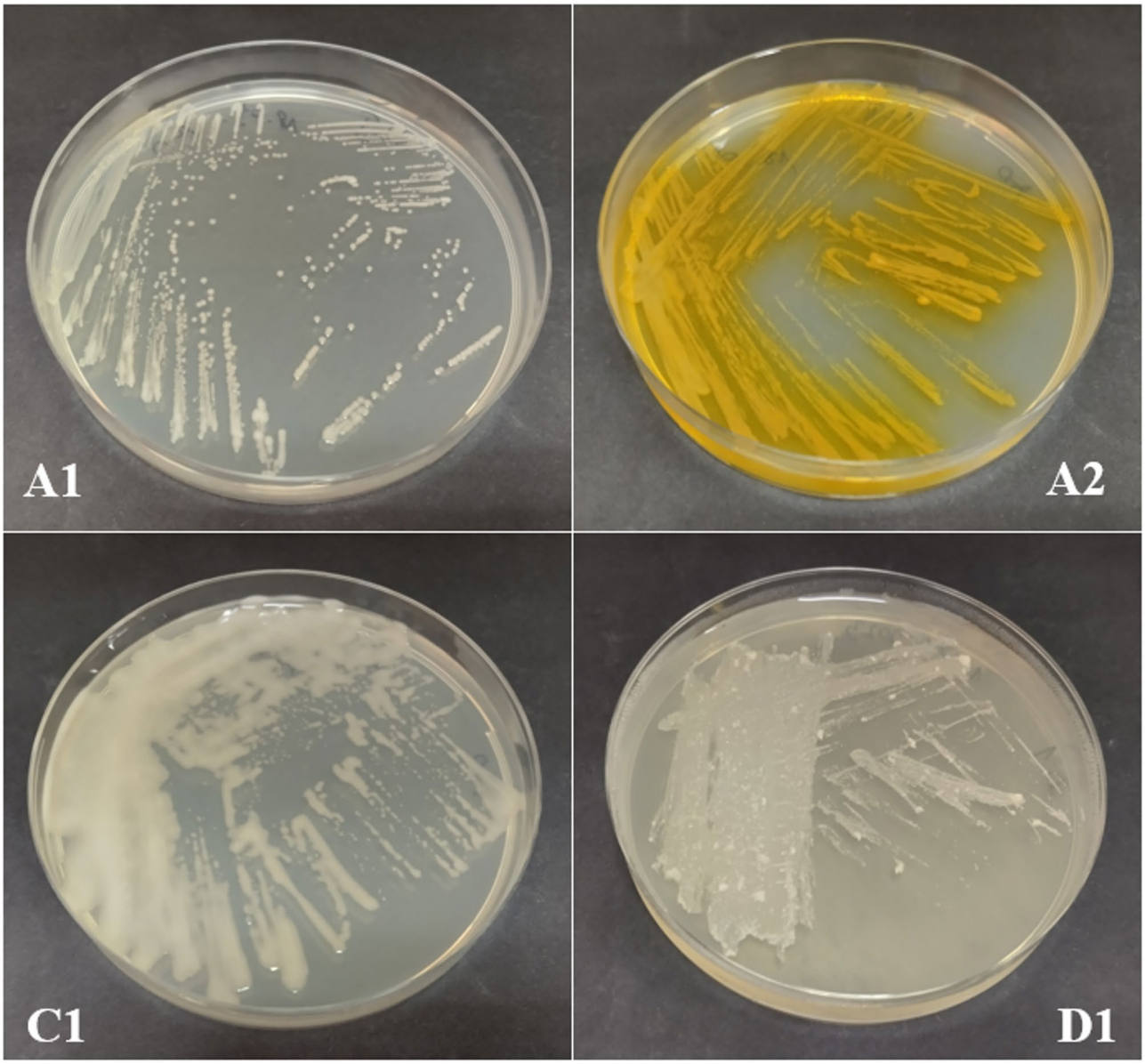

Fig. 1. Selected soil bacterial isolates grown on nutrient agar medium: *Pseudomonas* sp. 10 Kp8 - A1, *Pseudomonas chlororaphis* subsp. *aureofaciens* strain B19 - A2, *Pseudomonas baetica* strain JZY4-9 - C1, and *Streptomyces atratus* strain ROA017 - D1.

No.	Isolates	Identified bacteria	Gene Bank Accession Number	Query cover [%]	Sequence identity [%]
1	A1	*Pseudomonas* sp. 10 Kp8	KT825698.1	100%	99.93%
2	A2	*Pseudomonas chlororaphis* subsp. *aureofaciens* strain B19	KJ145861.1	100%	99.85%
3	C1	*Pseudomonas baetica* strain JZY4-9	MT071381.1	100%	99.55%
4	D1	*Streptomyces atratus* strain ROA017	MT490873.1	100%	99.85%

Table 1. Isolates used in the present study.

These results suggest that the bacterial synthetic consortium may be more promising for bioremediation applications than, the D1 strain alone. Further studies are warranted to investigate the stability and efficacy of the synthetic consortium in diflufenican-contaminated environments.

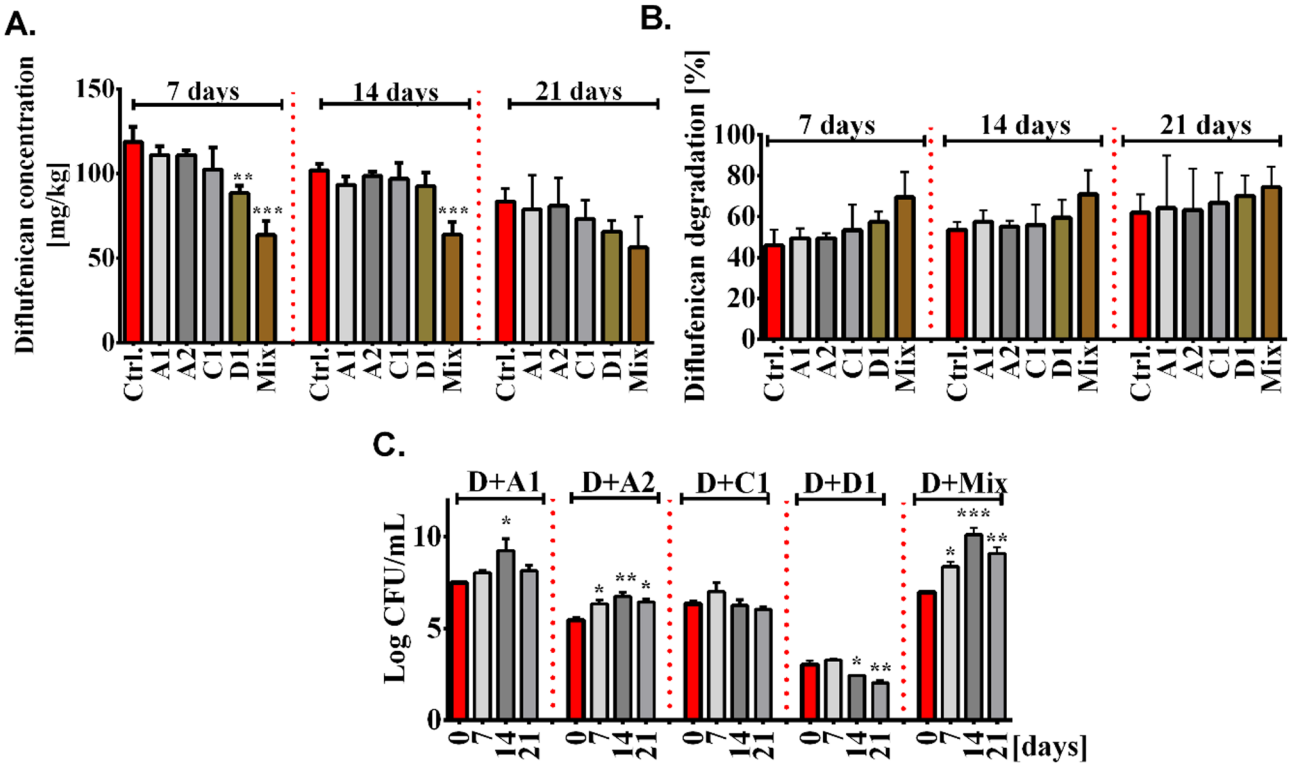

Fig. 2. Bacterial density expressed as log CFU per 1 ml of medium (**C**), diflufenican concentration (**A**) and its degradation (**B**) in MSM in control samples and with A1, A2, C1 and D1 strains separately and in a mixture. Statistically significant differences are presented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

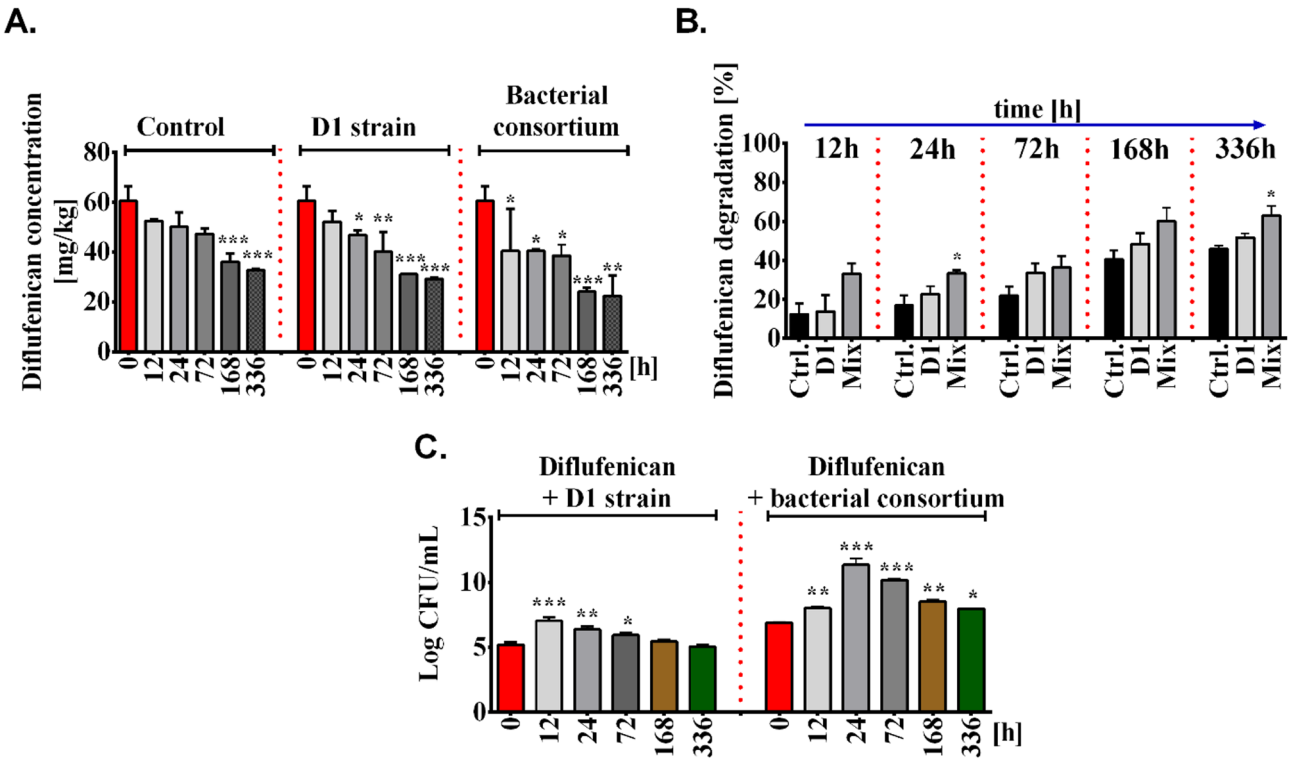

Fig. 3. Bacterial density expressed as log CFU per 1 ml of medium (**C**), diflufenican concentration (**A**) and its degradation (**B**) in MSM medium in control samples, with D1 strain separately and with bacterial synthetic consortium. Statistically significant differences are presented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

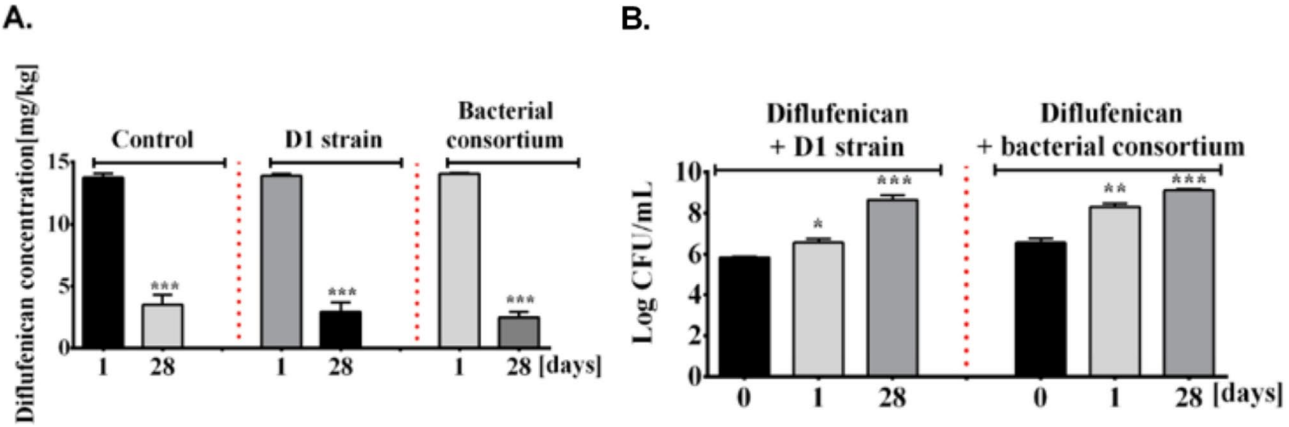

Fig. 4. Bacterial density (**B**), concentration (**A**) of diflufenican in the soil in control samples, after application of the D1 strain and bacterial synthetic consortium. Statistically significant differences are presented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Samples composition	Degradation [%]
Diflufenican - control	75 ± 2.5
Diflufenican + D1 strain	79 ± 4
Diflufenican + bacterial synthetic consortium	82.2 ± 3.7

Table 2. Diflufenican degradation in soil.

Bacteria effectively degrade diflufenican in the soil

Previous studies on diflufenican degradation in the MSM (Sect. "Bacteria effectively degrade diflufenican in the MSM within 21 days") have identified the D1 bacterial strain as the most efficient isolate. Based on these findings, the present study investigated the diflufenican degradation potential in the soil of the D1 strain and the bacterial synthetic consortium consisting of A1, A2, C1, and D1 strains.

To assess diflufenican degradation, the aqueous herbicide solution was added to the soil samples after 24 h, and diflufenican concentrations were measured at regular intervals. The results indicated that both the D1 strain and the bacterial synthetic consortium demonstrated diflufenican degradation capabilities in soil. The synthetic consortium achieved a slightly higher degradation rate, when compared to the D1 strain, suggesting that the synergistic interactions between the synthetic consortium members may enhance diflufenican removal.

After 28 days the concentration of diflufenican in control soil samples was 3.5 mg/kg, compared to 2.9 mg/kg in samples incubated with the D1 strain. Samples with the bacterial synthetic consortium achieved a lower diflufenican concentration of 2.5 mg/kg (Fig. 4A).

The bacterial density was assessed on the initial day of the experiment and subsequently on days 1 and 28 (Fig. 4B).

Table 2 presents the diflufenican degradation after 28 days of experiment in soil samples, compared to the control samples. The bacterial synthetic consortium was the most effective in diflufenican degradation.

Diflufenican metabolites

The metabolite, 2-(3-trifluoromethylphenoxy) nicotinic acid derivative, was detected in all MSM and soil samples analyzed. According to the literature data, in anaerobic environment, mainly this metabolite is produced[44]. Based on these results, it can be concluded that two main enzymes, hydrogenase and hydroxylase, are involved in the diflufenican metabolic pathway[45]. No traces of two other analyzed metabolites, 2,4-difluoroaniline and 2-(3-trifluoromethylphenoxy)nicotinamide, were found in any of the samples.

Discussion

In recent years, there has been a surge in publications on pesticide biodegradation, reflecting growing interest in this field.

Several soil bacterial genera are involved in degradation of hazardous chemicals. *Bacillus* species have the greatest ability among all bacterial genera to efficiently degrade persistent pesticides with the highest efficiency[46]. Zhu et al. (2021) isolated *Bacillus atrophaeus* YQJ-6 from the soil in which atrazine had been used for over 10 years. The rate of atrazine (50 mg/L) degradation by YQJ-6 strain reached approximately 99.2% in 7 days. This strain could tolerate atrazine at the level of at least 1000 mg/L[47]. Zhu et al. (2022) reported that *Bacillus pumilus* TDJ-7 and *Bacillus subtilis* TDJ-9, two isolates soil strains, were capable of degrading terbutylazine (10 mg/L) within 6 days. The maximum degradation rate was 95% and 98%, respectively[48]. Acharya et al. (2014) conducted studies on monocrotophos degradation in MSM medium by the *Bacillus subtilis* KPA-1 strain. The

highest degradation rate was 94.2% under aerobic conditions[49]. Other pesticide-degrading species from *Bacillus* genus include: *B. amyloliquefaciens*[50–52], *B. firmus*[53], *B. pumilus*[52,54], *B. subtilis*[52,55–59], and *B. thuringiensis*[60–62].

While literature reports specifically focusing on the impact of the tested bacteria on pesticide degradation or other persistent compounds are currently lacking, studies demonstrated the potential of different bacteria, including *Pseudomonas* and *Streptomyces* in pesticide degradation[63].

To our knowledge, there is no information of biodegradation of diflufenican by bacteria. The results of our study indicate that bacteria from the genus *Pseudomonas* may be able to degrade diflufenican with a yield of 64.1% for *Pseudomonas* sp. 10 Kp8 - A1, 63.2% for *Pseudomonas chlororaphis* subsp. *aureofaciens* strain B19 - A2, and 66.7% for *Pseudomonas baetica* strain JZY4-9 - C1 in 21 days, in liquid media. The best potential for diflufenican degradation was observed for *Streptomyces atratus* strain ROA017 - D1. With this strain, the degradation of the studied herbicide reached 70.1% and 79% in the liquid medium and the soil, respectively. However, the application of a synthetic consortium of all four microorganisms resulted in even higher degradation in both environments, amounting to 74.4% and 82.2% in the liquid medium and the soil, respectively.

The results of our study are consistent with previous research that has shown the effectiveness of bacteria from the genera *Pseudomonas* and *Streptomyces* in bioremediation applications. The ability of these bacteria to degrade diflufenican in both liquid and soil environments is particularly promising for bioremediation applications and may offer a potential solution to mitigate the environmental impact of diflufenican contamination.

Diflufenican is a persistent organic pollutant with a DT90 up to 1900 days, making it difficult to remove from contaminated sites[1].

The metabolite, 2-(3-trifluoromethylphenoxy)nicotinic acid derivative, was detected in all liquid medium and soil samples analyzed. In aerobic soil lead to the formation of two major metabolites 2-(3-trifluoromethylphenoxy) nicotinic acid and 2-(3-trifluoromethylphenoxy)nicotinamide. Anaerobic diflufenican degradation led to the formation of metabolites 2-(3-trifluoromethylphenoxy) nicotinic acid as a major metabolite[44]. Based on these reports, it can be concluded that two main enzymes, hydrogenase and hydroxylase, are involved in the diflufenican metabolic pathway[45].

The degradation rates observed are comparable to those reported for other persistent herbicides such as chlorpyrifos[64], imidacloprid[65] or quinalphos and carbendazim[66,67].

Previous research also confirms that consortia degrade pollutants faster. Negi et al. (2014) conducted studies on imidacloprid degradation in MSM, soil and soil slurry by *Achromobacter* sp. R-46,660, *Pseudomonas* sp. HY8 N, and *Microbacterium* sp. B-2013, separately and as a consortium. The highest degradation was obtained using the consortium of bacteria – 82% in MSM, 69% in the soil and 75.7% in the soil slurry. When *Pseudomonas* sp. was used separately, degradation was at the level of 64%, 62% and 68%, respectively[68]. Mu et al. (2023) isolated seven bacteria strains from mandipropamide–contaminated soil and identified three isolates with the highest efficiency in pesticide degradation were identified – *Pseudomonas* sp. (M01), *Mycolicibacterium parafortuitum* (MW05), and *Stenotrophomonas maltophilia* (MW09). These strains achieved pesticides degradation ranged from 63.3–73.4%[68].

The literature data indicate that *Pseudomonas* sp. has the ability to degrade pollutants, including pesticides, which means that they may be potential candidates in the bioremediation process[69]. *P. aeruginosa* PAO1 displayed remarkable ability to degrade several pyrethroid insecticides, including etofenprox, bifenthrin, tetramethrin, D-cypermethrin, allethrin, and permethrin with a degradation efficiency reaching over 84% within 36 h at the concentration of 50 mg/L. Additionally, strain PAO1 demonstrated significant potential for soil bioremediation, effectively removing etofenprox across varying concentrations (25–100 mg/kg), with a degradation efficiency over 77% within 15 days[70]. In addition, this strain was capable of efficiently degrading beta-cypermethrin and its major metabolite 3-phenoxybenzaldehyde in water/soil environments. Strain PAO1 could remove 91.4% of beta-cypermethrin (50 mg/L) in mineral salt medium within 120 h. At the same time, it also possesses a significant ability to metabolize its major metabolite[69]. *P. plecoglossicida* MBSB-12, isolated from pesticide contaminated tea garden soil of Assam, Indie reduced 87% of imidacloprid from the treated soil in 90 days compared to the control soil[71]. Jesitha et al. (2015) showed that *P. fluorescens* isolated from soil has the ability to degrade endosulfan at an initial concentration of 350.24 µg/L within 12 days and, after 18 days, complete degradation of the pesticide was achieved[72]. Duhan et al. (2023) reported that *P. fluorescens* strain c50 effectively removed atrazine within 30 days[73]. Similarly, Al-Janabi and Hashim (2021) isolated three chlorpyrifos–degrading strains of *P. putida* – PB1, PB2 and PB3. PB1 strain removed 98% of CP within 6 days. The percentage degradation was higher in first 6 days and rapidly increased thereafter, reaching 100% within 12 days, compared to the control[64]. In another experiment, the effect of *P. fluorescences* on trifluralin degradation in four media types, including media with carbon, nitrogen, carbon plus nitrogen, and carbon-free plus nitrogen, was studied. Results indicated that 72 h after inoculation, the highest biodegradation rate was obtained in carbon plus nitrogen (63.97%), and carbon (45.05%) medium[74]. Essa et al. (2016) isolated *P. aeruginosa* from agricultural drainage ditches. The isolate was grown in a minimal medium with diazinon as a sole source of carbon. The maximum insecticide degradation was 83.6% within 14 day[75]. Studies by Pawar and Mali (2014) and Fang et al. (2010) showed the potential of *Pseudomonas* sp. strains to degrade two pesticide – quinalphos and carbendazim. The first ones isolated fourteen strains from this genus. Only one 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[67]. Whereas Fang et al. (2010) proved the ability of *Pseudomonas* sp. CBW to use carbendazim as the sole source of carbon and energy. The maximum degradation was 99.1% after 3 days of the experiment[66].

Other reports demonstrated the use of *Streptomyces* isolates to degrade pesticides. Khajezadeh et al. (2020) isolated *Streptomyces rimosus* strain NBRC 12,907 capable of degrading deltamethrin. NBRC 12,907 strain significantly reduced the studied pesticide concentration, from 80 mg/L to 55, 27, and 12 mg/L after 24, 48, and 72 h, respectively[76]. Another study concerned the influence of bacteria – *Streptomyces* sp. M7, MC1, A5 and *Amycolatopsis tucumanensis* DSM 45,259 to lindane degradation. In the liquid medium, MC1 strain and the

quadruple consortium of studied bacteria, reached the highest insecticide removal, of 52% and 50%, respectively. In the soil samples, M7, MC1 and DSM 45,259 strains were able to individually remove more than 60% of lindane, while A5 strain showed the ability to remove around 50%[77]. Briceno, Fuentes et al. (2018) reported that *S. albidoflavus* A7-9 was able to diuron degradation, achieving the level of 95% degradation after 5 days[78]. However, Briceno, Vergara et al. (2018) evaluated the removal of mixture of two insecticides – diazinon and chlorpyrifos. After 24 h, degradation of studied pesticides was 45% and 90%, respectively[79]. Bourguignon et al. (2014) isolated four *Streptomyces* strains: A3, A6, A12 and A14, which were able to methoxychlor degradation. The maximum value – 100% was achieved by A14 strain after 7 days of the experiment[80]. Briceno et al. (2012) demonstrated the potential of four *Streptomyces* isolates to degrade the chlorpyrifos. Two of them reached the maximum degradation amounting to 90% after 24 hours[81].

Conclusions

The studied microorganisms demonstrated the ability to accelerate diflufenican degradation in both contaminated environments, the liquid medium and the soil. In the liquid medium, the D1 strain exhibited the highest degradation rate, achieving 70.1% diflufenican removal. However, the application of a bacterial synthetic consortium composed of four microorganisms resulted in an even higher degradation rate of 74.4%.

Similarly, in the soil the D1 strain demonstrated the highest degradation rate among the analyzed individual strains, achieving 79% diflufenican removal. Interestingly, the bacterial synthetic consortium again outperformed the D1 strain, achieving even higher degradation rate of 82.2%. These findings suggest that the synergistic interactions between the four microorganisms in the bacterial synthetic consortium may enhance the efficiency in diflufenican degradation, when compared to individual strains acting alone. Furthermore, the studied microorganisms exhibited the ability to grow and utilize diflufenican as a source of carbon and energy, even at concentrations as high as 220 mg/kg in MSM. Collectively, these findings demonstrate the remarkable potential of the bacterial consortium as a bioremediation tool for effectively decontaminating soils polluted with diflufenican and other organic compounds.

The studied bacteria strains are considered safe for humans and the environment. Notably, certain commercial biological formulations containing *Pseudomonas* spp. and *Streptomyces* spp. have been approved for use in organic agriculture, so isolates can potentially have commercial application in sustainable agriculture.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. The genome sequences have been deposited in the GenBank repository, under accession numbers PV483858 for Pseudomonas sp. 10 Kp8 - A1 (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB15250933), PV483887 for P. chlororaphis subsp. aureofaciens strain B19 - A2 (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB15251214), PV484507 for P. baetica strain JZY4-9 - C1 (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB15251276) and PV484548 for S. atratus strain ROA017 - D1 (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB15251293).

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Conceptualization, E.S., L.P.; methodology, E.S., L.P., P.K.-T.; writing—original draft preparation, P.K.-T.; review and editing, E.S., L.P, P.K.-T.; GC-MS/MS analyses, E.S., P.K.-T.; microbiological analyses, L.P., P.K.-T., formal analyses, P.K.-T.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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