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Biodegradation kinetics of 2,4-D by bacterial strains isolated from soil

Research Article

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Abstract: The aim of the study was to characterize the 2,4-dichlorophenoxyacetic acid (2,4-D) degradative potential of three bacterial strains identified by MIDI-FAME profiling as *Burkholderia cepacia* (DS-1), *Pseudomonas* sp. (DS-2) and *Sphingomonas paucimobilis* (DS-3) isolated from soil with herbicide treatment history. All strains were capable of using herbicide as the only source of carbon and energy when grown in mineral salt medium (MSM) containing 2,4-D (50 mg/l). Over a 10 day incubation period, 69%, 73% and 54% of the initial dose of 2,4-D were degraded by strains DS-1, DS-2 and DS-3, respectively. Analysis of 2,4-dichlorophenol (2,4-DCP) concentration, the main metabolite of 2,4-D degradation, revealed that strains DS-1 and DS-2 may also have the potential to metabolize this compound. The percentage of 2,4-DCP removal was 67% and 77% in relation to maximum values of 9.5 and 9.2 mg/l determined after 4 and 2 days for MSM+DS-1 and MSM+DS-2, respectively. The degradation kinetics of 2,4-D (50 mg/kg) in sterile soil (SS) showed different potential of tested strains to degrade 2,4-D. The times within which the initial 2,4-D concentration was reduced by 50% (DT_{50}) were 6.3, 5.0 and 9.4 days for SS+DS-1, SS+DS-2 and SS+DS-3, respectively.

Keywords: *2,4-D • 2,4-DCP • Biodegradation kinetics • Burkholderia cepacia • Pseudomonas sp. • Sphingomonas paucimobilis • Soil*

1. Introduction

2,4-dichlorophenoxyacetic acid (2,4-D) is the active ingredient of herbicides applied widely since the 1940s. It is recommended for the control of broadleaf weeds in cereal cultures, lawns, rangeland, and pastures. As a synthetic auxinic pesticide, 2,4-D causes an uncontrolled auxin response in sensitive plants leading to the disturbance of plant regulatory mechanisms. The toxic effects of 2,4-D involve various growth abnormalities such as leaf epinasty, stem twisting, stem and root thickening, and ultimately chlorosis and disruption of cellular ultrastructure [1,2].

In soil, 2,4-D has a moderate persistence with a relatively short half-life. Dissipation studies conducted in various soils showed that 2,4-D had an apparent soil half-life of 5 days with a range of 1.7 to 13.1 days [3-5]. Phenoxy herbicides, such as 2,4-D, are potentially mobile and can be spread by leaching [6]. In soil, the fate and persistence of 2,4-D is dependent upon many environmental factors including the content of organic matter, clay, pH, water potential, temperature, oxygen saturation and it undergoes many processes such as runoff, adsorption-desorption, abiotic transformations and photodecomposition [7,8]. However, a key factor controlling its movement and dissipation is microbial degradation [6,9,10]. Many bacterial strains isolated from both soils with a history of herbicide treatment and pristine soils are known to be 2,4-D degraders. On the basis of their phylogeny and the sequences

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of genes encoding the 2,4-D-degrading enzymes these bacteria have been divided into three groups [11]. Bacteria of I class belonging to the subdivision of *β*- and *γ*-Proteobacteria have been isolated mainly from herbicide treated soils [12,13], whereas in pristine environmental conditions, strains belonging to III class have been found [14,15]. Class III degraders include oligotrophic, slow-growing strains belonging to the *Bradyrhizobium–Agromyces–Nitrobacter–Afipia* (BANA) cluster of α-Proteobacteria. Class II includes bacteria from α-Proteobacteria belonging to the genus *Sphingomonas* [16]. The biodegradative capacity of some isolates degrading 2,4-D has been extensively studied both in liquid media and soils [7,17,18]. The aim of this study was to characterize the 2,4-D degradative potential of bacterial strains isolated from soil with pesticide treatment history. For this purpose, the kinetics of 2,4-D degradation in both liquid media and sterile soil inoculated with a single bacterial strain were studied.

2. Experimental Procedures

2.1 Soils

Composite soil samples collected from the top layer (0–20 cm) of commercial fields located in Upper Silesia, southern Poland were used in this experiment. A sandy loam soil (SL) treated with 2,4-D annually for the last six years was used for the isolation of 2,4-D-degrading bacteria, while a loamy sand soil (LS) with no known history of previous 2,4-D applications was used for the inoculation studies with the isolated bacteria. Detailed physico-chemical properties of the soils and methods of their determination are presented in Table 1. In the laboratory, the soils were gently air-dried to the point of soil moisture suitable for sieving. After sieving to a maximum particle size of <2 mm, the soils were immediately used for the experiment.

2.2 Chemicals and media

Certified standards of 2,4-D (99.6% chemical purity) and 2,4-DCP (99.7% chemical purity) were purchased from Institute of Industrial Organic Chemistry (Warsaw, Poland). Chemical structures of both compounds are presented in Figure 1. All other chemicals were of analytical grade and purchased from Merck, Germany. A stock solution of 2,4-D, dissolved at 5 g/l in deionised water, was sterilized by filtration through 0.22 µm-pore size Millipore membranes and used for the preparation of 2,4-D containing media. For the bacteria isolation procedures and biodegradation studies, mineral salt medium (MSM) was used. The medium contained 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.001 g of $FeSO_4 \cdot 7H_2O$, 1.5 g of $Na_2HPO_4 \cdot 12H_2O$, and 1.5 g of KH_2PO_4 per litre of deionised water. The final pH value was adjusted to 7.2. After autoclaving (121°C, 20 min) and cooling, the medium was supplemented with 2,4-D.

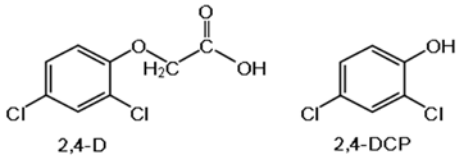

Figure 1. Chemical structures of 2,4-D and 2,4-DCP.

Parameter	Soil classification*		Method of determination	Reference
	Sandy loam (SL)	Loamy sand (LS)		
Sand (2000-50 µm) (%)	69.0 ± 3.2	85.0 ± 2.9	Sedimentation and sieving method	ISO 11277:2009
Silt (<50-2 µm) (%)	21.0 ± 1.3	12.0 ± 2.2	Sedimentation and sieving method	ISO 11277:2009
Clay (<2 µm) (%)	10.0 ± 1.0	3.0 ± 0.3	Sedimentation and sieving method	ISO 11277:2009
Density g cm^{-3}	1.4 ± 0.2	1.1 ± 0.1	Core method	ISO 11272:1998
$pH_{(in\ water)}$ (1:5)	7.1 ± 0.2	6.8 ± 0.2	Measurement with glass electrode	ISO 10390:2005
CEC (cmol/+kg)[a]	13.5 ± 2.3	8.0 ± 0.4	Modified Gillman method	ISO 11260:1994
WHC (%)[b]	36.0 ± 1.9	33.6 ± 2.1	Gravimetric method	ISO 14239:1997
C_{org} (%)	1.4 ± 0.2	1.0 ± 0.1	Oxidation in the presence of H_2SO_4	ISO 14235:1998
N_{tot} (%)	0.11 ± 0.02	0.08 ± 0.02	Modified Kjeldahl method	ISO 11261:1995
MB (mg/kg d.w.)[c]	786 ± 41	627.0 ± 28	Substrate-induced respiration (SIR)	ISO 14240-1:1997

Table 1. Properties of the soils used in the experiment. The values are the means of three replicates with the standard deviation which was within 5% of the mean.

**According to US/FAO System, [a] CEC: cation exchange capacity, [b] WHC: water holding capacity, [c] MB: microbial biomass*

2.3 Isolation and identification of bacterial strains

The enrichment culture technique was used for the isolation of 2,4-D-degrading bacteria. For this purpose, 10 g of soil previously treated with 2,4-D (sandy loam soil, SL) was added to 300 ml flasks with 100 ml of MSM supplemented with 2,4-D (50 mg/l). Samples were incubated for 72 h on a rotary shaker (120 rpm) in a darkened thermostatic chamber maintained at 30±1°C. After this time, 1 ml of soil suspension was transferred into flasks containing the fresh medium supplemented with the same concentration of 2,4-D and incubated for an additional 72 h under the same conditions. After five subsequent transfers into the same medium, serial dilution of the flask samples were plated onto MSM agar plates supplemented with 2,4-D (50 mg/l) for isolation of individual colonies. Isolates exhibiting distinct colony morphologies were purified by repeated streaking on the same agar medium. For further analyses, three 2,4-D-degrading strains designated as DS-1, DS-2 and DS-3 were used.

Identification of bacterial isolates was based on the profile of fatty acid methyl esters (FAMEs) analysed by gas chromatography (GC) using the Sherlock Microbial Identification System (MIDI Inc., Newark, USA). FAMEs were extracted from each isolate using the standard and recommended procedure, consisting of saponification, derivatization, extraction, and final base washing. Cellular FAME was separated by Hewlett Packard 6890 GC equipped with a capillary column Ultra 2-HP and flame ionization detector (FID) using hydrogen as a carrier gas. In addition, the biochemical properties of the isolates and the substrate utilization patterns using the API 20 NE Systems (bioMérieux Inc., France) were determined. Biochemical identification was performed according to the manufacturer's recommendation.

2.4 Inoculum preparation

Bacteria were cultured in 100 ml Erlenmeyer flasks containing 20 ml MSM medium supplemented with 50 mg/l 2,4-D. At the exponential phase, the bacteria were pelleted by centrifugation (5 min, 10,000 *g*). The pellet was washed twice with 0.85% sterile NaCl medium, resuspended in the same medium to obtain the bacterial suspension at a concentration of approximately 6×10^8 cells/ml. The cell density (OD 550 nm) was measured using a Densimat® (bioMérieux, France) photometer. One ml of bacterial suspension was transferred into 200 ml of MSM or 200 g of soil to give a final concentration of approximately 3×10^6 cells/ml or g, respectively.

2.5 Growth of bacterial isolates and 2,4-D degradation in MSM

Degradation studies were performed in 500 ml Erlenmeyer flasks containing 200 ml of sterile MSM supplemented with 2,4-D (50 mg/l) as the sole carbon source and inoculated with respective bacterial strain. Triplicate samples of MSM+2,4-D, MSM+DS-1, MSM+DS-2 or MSM+DS-3 were used as controls. Flasks were incubated on a rotary shaker (120 rpm) in a darkened thermostatic chamber maintained at 30±1°C. Samples of MSM were periodically removed aseptically for bacterial growth rate and pH determinations, as well as for chemical analyses to determine 2,4-D and 2,4-DCP concentrations. The growth of isolates was recorded spectrophotometrically by measuring the OD at 660 nm using a UV-VIS spectrophotometer (Varian, USA), while the pH values of MSM were measured with a glass electrode using a Jenway pH-meter.

2.6 Studies on 2,4-D dissipation in soil

Prior to use, soil was sterilized by autoclaving three times for 1 h at 121°C. To study the disappearance dynamics, the water solution of 2,4-D was sprayed on the surface of 200 g of soil by means of a micro-syringe that dispensed very small droplets and ensured thorough mixing. The applied amount of 2,4-D corresponded to a soil concentration of 50 mg/kg. Next, the bacterial suspension of each 2,4-D-degrading isolate was introduced into the soil (in triplicate) to give a final bacterial count of approximately 3×10^6 cells/g soil. In order to study the 2,4-D dissipation rate under abiotic condition, the triplicate samples of sterile soil (SS), without bacteria were kept as controls. Additionally, the same 2,4-D dose was applied to the non-sterile soil samples (nSS), non-contaminated earlier with pesticide to study the degradative potential of the autochthonous microorganisms. The water content of the soil samples was adjusted to 50% of the maximum water holding capacity by the addition of sterile deionised water. All soil samples were incubated in a darkened thermostatic chamber maintained at 30±1°C. Throughout the incubation period, the sterile deionised water was added to the soil treatments to compensate for any water losses exceeding 5% of the initial amount added. Samples of soil treatments (10 g) were periodically removed aseptically for determination of 2,4-D concentrations.

2.7 Chemical analyses

Concentrations of chemicals were measured by high performance liquid chromatography (HPLC). For 2,4-D and 2,4-DCP concentration determination in MSM, 10 ml samples were acidified with HCl and filtered using solid phase extraction (SPS) column (ENVI-18), previously conditioned with 2 × 5 ml of acetone, 2 × 5 ml of methanol and 2 × 5 ml distilled water (pH 2). After the sample was dried, determined chemicals were eluted from the column with 2 × 5 ml of methanol, and reserved

for chromatographic analysis. For 2,4-D concentration determination in soil, 10 g samples were extracted with 5 ml of acetone-water mixture (20:50 *v*/*v*, pH 2) and 30 ml of diethyl ether on a rotary shaker for 10 min and 1 h, respectively. Next, the organic phase was dehydrated with anhydrous Na_2SO_4, evaporated to dryness under a stream of N_2 at 40°C using a rotary evaporator (IKA, RV05 Basic, Janke & Kunkel-Ika Labortechnik, Germany), and finally reconstituted in 10 ml of the mobile phase, and reserved for chromatographic analysis.

HPLC analyses were performed using a Varian ProStar System (Varian, Inc., USA) equipped with a UV-VIS detector (ProStar 325), solvent delivery module (ProStar 210), and reverse-phase column (Microsorb-MV 100-5 C18 (250 mm × 4.6 mm × 5 µm)). For chemical detection, the mobile phase was acetonitrile-universal buffer pH 2.36 (60:40, *v*/*v*) injected at a flow rate of 1.0 ml/min and detected at 285 nm. The obtained data were analysed with Chromatography Workstation Software (Star, LC WS, Ver. 6.2). Retention times under these chromatographic conditions were 4.7 and 5.6 min for 2,4-D and 2,4-DCP, respectively. To evaluate the usefulness of this chemical determination method, validation studies to determine the linear range of the calibration curves, recoveries, and limits of detection (LOD) and quantification (LOQ) were performed. The calibration curve proved to be linear within the range of 0.1–20 mg/l with R^2=0.9998 and 0.9999 for 2,4-D and 2,4-DCP, respectively. LOD, LOQ, and recovery of 2,4-D were 0.01 mg/l, 0.05 mg/l, and 96.4±1.8%; and 0.05 mg/kg, 0.09 mg/kg, and 92.3±1.1% for MSM and soil, respectively. LOD, LOQ, and recovery for the concentration determination of 2,4-DCP in MSM were 0.005 mg/l, 0.01 mg/l, and 98.2±1.2%, respectively.

2.8 Kinetics and statistics analyses

Disappearance of 2,4-D from liquid medium or soil was fitted to a first-order kinetic model. The rate constant *k* (day) was determined using the algorithm $C_t/C_0 = e^{-kt}$, where C_0 is the amount of pesticide in MSM or soil at time zero, C_t is the amount of pesticide in MSM or soil at time *t* (day). Time in which the pesticide concentration in MSM or soil was reduced by 50% (DT_{50}) was calculated from the linear equation obtained from the regression between $\ln(C_t/C_0)$ of the chemical data and time (*t*). The results from three replicates of each treatment were also evaluated using analysis of variance and statistical analysis. The significance ($P<0.05$) of differences were assessed by *post hoc* comparison of means using the least significant differences (LSD) test using the Statistica 6.0 PL software package. The data obtained for 2,4-D degradation kinetics were analysed statistically by one-way ANOVA, considering the effect of treatment, while the results concerning the pH values were analysed by two-way ANOVA, considering the effects of inoculum type and time.

3. Results and Discussion

3.1 Isolation and characterization of 2,4-D-degrading strains

Using a soil enrichment procedure, three bacterial strains identified by MIDI-FAME profiling as *Burkholderia cepacia* (DS-1), *Pseudomonas* sp. (DS-2) and *Sphingomonas paucimobilis* (DS-3) were screened and found to be capable of degrading 2,4-D. The similarity index (SIM) calculated by the MIDI system was determined to be 0.859, 0.899 and 0.876 for strains DS-1, DS-2 and DS-3, respectively, which indicate good matches (Table 2). Analysis of biochemical patterns by the API 20 NE test system also supported reliable identification of the isolates with high identity (Table 2).

Selection pressure is a common approach to isolate toxicant-degrading organisms and has previously been

Strain	FAME		Biochemical pattern (API 20 NE)[a]																				% ID[d]	Identification
	%ID[b]	SIM[c]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
DS-1	99.6	0.859	+	–	+	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	99.9	*Burkholderia cepacia*
DS-2	99.3	0.899	+	–	–	+	–	–	+	–	+	+	+	+	+	–	+	+	–	+	+	–	+	99.9	*Pseudomonas* sp.
DS-3	99.9	0.876	+	–	–	–	–	+	–	+	+	+	+	–	+	+	+	–	–	+	+	–	+	99.2	*Sphingomonas paucimobilis*

Table 2. Bacterial strains isolated from 2,4-D contaminated sandy loam (SL) soil.

[a] Active ingredients: 1 – potassium nitrate (NO3), 2 – L-tryptophane (TRP), 3 – D-glucose fermentation (GLU), 4 – L-arginine (ADH), 5 – urea (URE), 6 – esculin (ESC), 7 – gelatin (GEL), 8 – 4-nitrophenyl-β-D-galactopyranoside (PNPG), 9 – D-glucose assimilation (GLU), 10 – L-arabinose (ARA), 11 – D-mannose (MNE), 12 – D-mannitol (MAN), 13 – N-acetyl-glucosamine (NAG), 14 – D-maltose (MAL), 15 – potassium gluconate (GNT), 16 – capric acid (CAP), 17 – adipic acid (ADI), 18 – malic acid (MLT), 19 – trisodium citrate (CIT), 20 – phenylacetic acid (PAC), 21 – oxidase (OX). [b] Percent of identified fatty acids, [c] Similarity index based on the Microbial Identification System (MIS) Sherlock 6.1 (MIDI Inc., Newark, USA), [d] Percent of identity based on the apiwebTM system.

applied to the screening of soil bacteria and fungi capable of degrading various types of pesticides [19-21]. The number and diversity of reported bacterial isolates that are capable of 2,4-D degradation is high. The ability to metabolize of 2,4-D both in liquid media and soil have been identified in several bacteria from various genera including *Arthrobacter* [6], *Sphingomonas* [12], *Alcaligenes* [12,13], *Pseudomonas* [12,18], *Acinetobacter* [18], *Ralstonia* [22] and *Comamonas* [18,23]. Much is also known about the genes and enzymes involved in the degradation of 2,4-D. Genes responsible for 2,4-D degradation are predominantly located on plasmids with the most studied gene, *tfd*A, encoding an *α*-ketoglutarate-dependent 2,4-dioxygenase that is responsible for the cleavage of the acetate side chain and the conversion of 2,4-D to 2,4-DCP [15,24-26]. Considering evidences showing the catabolic plasmid transfer between different species the ability for phenoxyacetic herbicides degradation within microbial population may be very high [12,27]. Chong and Chang [28] suggested that even the measurement of plasmid quantity was a sufficient method for the estimation of potential 2,4-D degradation by soil microorganisms.

3.2 Degradation of 2,4-D and 2,4-DCP in mineral salt medium

Culturing bacteria in medium containing 2,4-D as the sole organic compound revealed that all strains were capable of using applied pesticide as a source of carbon and energy to grow, and confirmed the degradation of 2,4-D and subsequent production of the main metabolite 2,4-DCP (Figure 2). The results showed differences in growth kinetics of individual bacterial isolates in MSM+2,4-D. Bacterial growth was the most rapid during the first four days of incubation. The growth curve for isolates attained a maximum OD 660 nm after 8 days, while respective controls (MSM+2,4-D, MSM+DS-1, MSM+DS-2 and MSM+DS-3) showed no change in OD 660 nm for 10 days of incubation (Figure 2A). During the 10 days of the experiment, 69%, 73% and 54% of the initial dose of 2,4-D was degraded in MSM inoculated with strains DS-1, DS-2 and DS-3, respectively. The 2,4-D degradation pattern showed that the most efficient degradation occurred within the first incubation period (0–4 days) (Figure 2B). Kinetic data showed that the degradation process observed for strains DS-1 and DS-2 was characterized by the similar rate constants (*k*) of 0.117 and 0.132/day, and disappearance rates (*V*) of 3.29 and 3.55 mg/kg/day, respectively (Table 3). Strain *Sphingomonas paucimobilis* (DS-3) was characterized by a lower degradative potential (Table 3) resulting in the least amount of 2,4-D catabolism in the same incubation period (Figure 2B). The times within which the initial

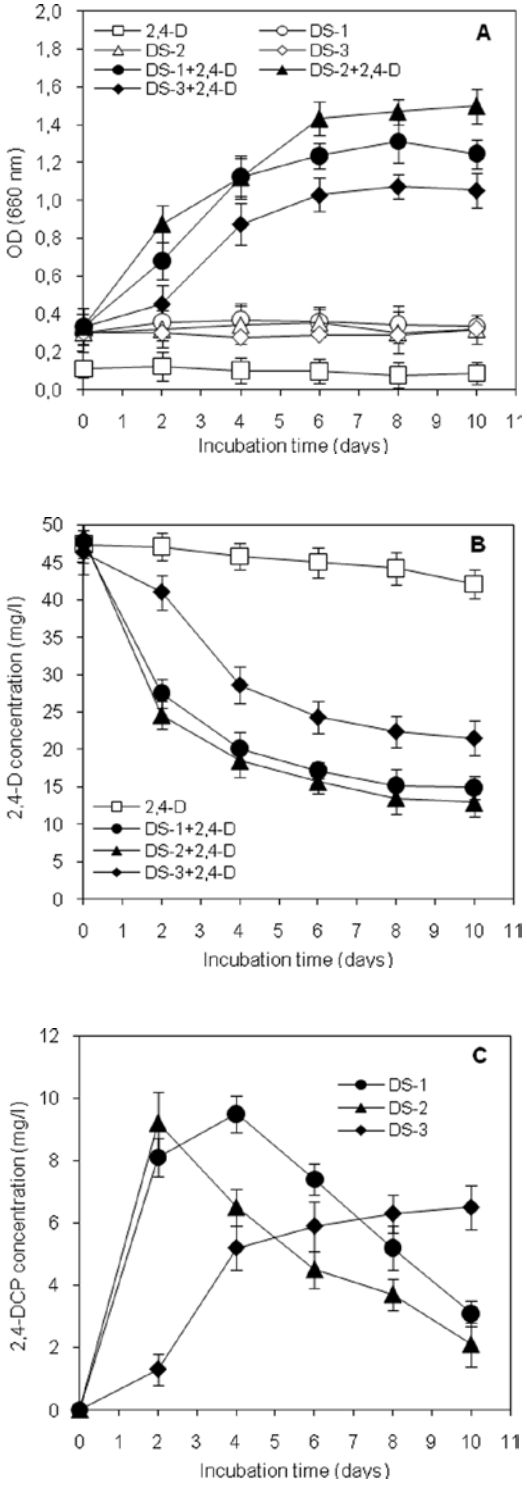

Figure 2. Bacterial growth (A), degradation of 2,4-D (B) and subsequent production of 2,4-DCP (C) in mineral salt medium inoculated with *Burkholderia cepacia* (DS-1), *Pseudomonas* sp. (DS-2) or *Sphingomonas paucimobilis* (DS-3). Symbols are the means of three replicates. Error bars represent the standard deviation which was within 5% of the mean.

2,4-D concentration was reduced by 50% (DT_{50}) were 4.1, 3.4 and 7.8 days for strains DS-1, DS-2 and DS-3, respectively (Table 4). In the growth curve no lag phase was observed, demonstrating the ability of strains to rapidly adapt to the culture medium. Similar phenomena were observed by Silva *et al*. [29], who studied the potential to degrade 2,4-D among 25 strains isolated from soil regularly treated with herbicide for three years.

As a consequence of microbial growth and 2,4-D metabolism, a decrease of medium pH was measured. For example, in MSM+2,4-D inoculated with strain DS-2, the pH of medium dropped from 7.20 to 5.00 during 10 days of incubation (Table 5). The chemical properties of 2,4-D indicate that high pH increases the rate of its transformation, whereas the acidic conditions increase the herbicide stability and its resistance to chemical and microbial degradation [30]. Moreover, low pH may decrease the activity of bacteria and/or enzymes involved in the pesticide transformation resulted in the inhibition of 2,4-D degradation [21,31]. These effects may be reflected by the curves obtained for bacterial growth (Figure 2A) and 2,4-D disappearance (Figure 2B) that reached plateau phase after 8 days of incubation. It has been reported that pH has a marked influence on pesticide degradation by the bacterial isolates, and their increased stability under acidic conditions was observed for pesticides from various chemical groups [32,33]. The pH measurements in MSM during the experimental period showed that this parameter was dependent on the bacterial inoculum ($P<0.0001$), time ($P<0.0001$), and interaction between these factors ($P<0.0001$) (Table 5).

Chemical analysis of the 2,4-D degradation product revealed significant differences ($P<0.05$) in the concentration of 2,4-DCP between the respective

Parameter	Treatments	Time intervals (days)					Average (0 – 10 days)
		0 – 2	2 – 4	4 – 6	6 – 8	8 – 10	
k (day)	MSM + DS-1	0.276	0.157	0.078	0.062	0.010	0.117 ± 0.019[a]
	MSM + DS-2	0.338	0.143	0.082	0.075	0.023	0.132 ± 0.022[a]
	MSM + DS-3	0.061	0.180	0.081	0.041	0.021	0.077 ± 0.012[b]
V (mg/kg/day)	MSM + DS-1	10.15	3.70	1.45	1.00	0.15	3.29 ± 0.15[a]
	MSM + DS-2	11.90	3.05	1.40	1.10	0.30	3.55 ± 0.18[a]
	MSM + DS-3	2.65	6.20	2.15	0.95	0.45	2.48 ± 0.13[b]

Table 3. Values of degradation rate constant (*k*) and rate of disappearance (*V*) for 2,4-D in mineral salt medium. The values are the means of three replicates with the standard deviation which was within 5% of the mean. Different letters for a given parameter indicate significant differences between treatments ($P<0.05$, LSD test).

MSM: mineral salt medium; DS-1: Burkholderia cepacia; DS-2: Pseudomonas sp.; DS-3: Sphingomonas paucimobilis.

Treatments	Regression equation (1st-order)	R^2	DT_{50} (days)
MSM + DS-1	$\ln(C_t/C_0) = -0.1109t - 0.2376$	0.8543	4.1 ± 0.8[a]
MSM + DS-2	$\ln(C_t/C_0) = -0.1225t - 0.2814$	0.8509	3.4 ± 0.9[a]
MSM + DS-3	$\ln(C_t/C_0) = -0.0830t - 0.0417$	0.9178	7.8 ± 0.7[b]
SS	$\ln(C_t/C_0) = -0.0102t + 0.0297$	0.9750	70.9 ± 2.9[a]
SS + DS-1	$\ln(C_t/C_0) = -0.1692t + 0.3724$	0.8941	6.3 ± 0.9[b]
SS + DS-2	$\ln(C_t/C_0) = -0.2022t + 0.3190$	0.9587	5.0 ± 0.8[b]
SS + DS-3	$\ln(C_t/C_0) = -0.1212t + 0.4419$	0.9469	9.4 ± 1.2[c]
nSS	$\ln(C_t/C_0) = -0.0844t + 0.4252$	0.9546	13.3 ± 1.5[d]

Table 4. Disappearance time (DT) for 2,4-D in liquid medium and loamy sand (LS) soil calculated from the linear equation obtained from the regression between $\ln(C_t/C_0)$ of the chemical data and time (*t*). The values are the means of three replicates with the standard deviation which was within 5% of the mean. Different letters indicate significant differences between treatments ($P<0.05$, LSD test).

MSM: mineral salt medium; DS-1: Burkholderia cepacia; DS-2: Pseudomonas sp.; DS-3: Sphingomonas paucimobilis; SS: sterile soil; nSS: non-sterile soil without bacteria inoculum.

bacterial cultures and provided evidence for different degradative capacities of the bacterial strains (Figure 2). The highest concentrations of 2,4-DCP, were measured at 9.5 and 9.2 mg/l in MSM inoculated with strains DS-1 and DS-2 after 4 and 2 days of the incubation, respectively. After this time, the concentration of 2,4-DCP gradually declined, and after 10 days of incubation the percentage of its removal was 67% and 77% for MSM+DS-1 and MSM+DS-2 in relation to maximum values determined after 4 and 2 days, respectively (Figure 2C). The initial increase of the 2,4-DCP concentration as a result of 2,4-D degradation and its depletion during the experimental period might suggests the ability of strains DS-1 and DS-2 to utilize 2,4-DCP. This effect was not observed for culture of strain DS-3, in which the concentration of 2,4-DCP increased gradually reaching the maximum value of 6.5 mg/l after 10 days of incubation.

Revealed by several studies, 2,4-DCP has been found to be a main metabolite of 2,4-D degradation as mediated by microorganisms [6,10,22]. Moreover, numerous bacterial and fungal strains have also been reported to utilize 2,4-DCP as the sole carbon and energy source, such as *Achromobacter* sp., *Aeromonas* sp., *Bacillus insolitus*, *Pseudomonas* sp., *Streptomyces viridosporus*, *Flavimonas oryzihabitans*, *Chryseomonas luteola*, *Aspergillus penicilloides*, *Mortierella isabelina*, *Chrysosporium pannorum* and *Mucor genevensis* [34-39]. Some of these studies showed that high concentrations of 2,4-DCP were found to increase the duration of the lag phase and decrease the rate of biodegradation. However, no sound kinetic models were suggested for the inhibitory effects of 2,4-DCP on the rate and extent of biodegradation for a wide range of 2,4-DCP concentrations [40]. In our studies, we observed incomplete degradation of 2,4-D in MSM inoculated with individual bacterial strains. One possible explanation for this is the toxicity of 2,4-DCP, as this chemical has been reported to be more toxic than 2,4-D and its accumulation to be inhibitory to cell growth and to 2,4-D degradation [41,42]. For example, Baarschers *et al*. [43] found that the fungi *Trichoderma viride*, *Mortierella isabellina* and *Saprolegnia parasitica* were 8–35 times more sensitive to 2,4-DCP than to 2,4-D. However, in our studies the incomplete degradation of 2,4-D was linked with an increased herbicide resistance and decreased bacterial activity under acidic conditions, rather than toxicity of 2,4-DCP, as indicated by data obtained from the 2,4-DCP disappearance curves (Figure 2C).

3.3 Degradation dynamics of 2,4-D in soil

The degradation kinetics of 2,4-D in soil revealed that there were differences in the 2,4-D degradation potential between bacterial isolates (Figure 3). In sterile soil (SS) inoculated with *Pseudomonas* sp. (SS+DS-2) the complete degradation of 2,4-D occurred within 24

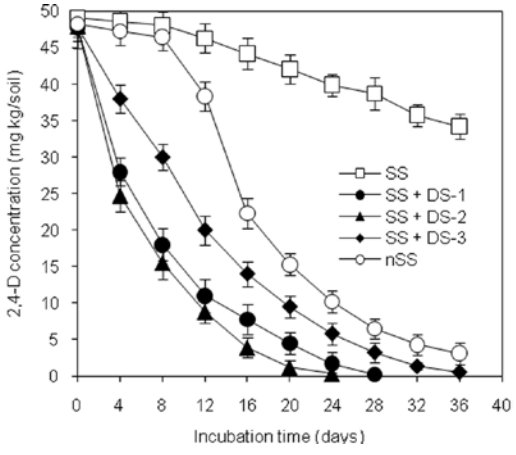

Figure 3. The disappearance dynamics of 2,4-D in loamy sand (LS) soil inoculated with *Burkholderia cepacia* (SS + DS-1), *Pseudomonas* sp. (SS + DS-2) or *Sphingomonas paucimobilis* (SS + DS-3). SS: non-inoculated sterile soil; nSS: non-sterile soil without bacteria inoculum. Symbols are the means of three replicates. Error bars represent the standard deviation which was within 5% of the mean.

Days	Treatments						
	MSM+2,4-D	MSM+DS-1	MSM+DS-2	MSM+DS-3	MSM+2,4-D+DS-1	MSM+2,4-D+DS-2	MSM+2,4-D+DS-3
0	7.21 ± 0.03^{a}	7.22 ± 0.02^{a}	7.20 ± 0.03^{a}	7.21 ± 0.03^{a}	7.20 ± 0.02^{a}	7.20 ± 0.02^{a}	7.22 ± 0.02^{a}
2	7.20 ± 0.02^{a}	7.20 ± 0.02^{a}	7.18 ± 0.02^{ai}	7.20 ± 0.02^{a}	6.92 ± 0.02^{b}	6.87 ± 0.03^{c}	6.96 ± 0.03^{b}
4	7.19 ± 0.03^{a}	7.18 ± 0.03^{ai}	7.17 ± 0.02^{ai}	7.18 ± 0.03^{a}	6.83 ± 0.02^{d}	6.75 ± 0.02^{e}	6.85 ± 0.02^{d}
6	7.17 ± 0.03^{a}	7.16 ± 0.04^{ai}	7.15 ± 0.03^{ai}	7.17 ± 0.03^{a}	6.64 ± 0.03^{f}	5.54 ± 0.02^{g}	6.75 ± 0.04^{h}
8	7.17 ± 0.02^{ai}	7.15 ± 0.02^{i}	7.14 ± 0.04^{i}	7.17 ± 0.02^{ai}	6.56 ± 0.03^{j}	5.50 ± 0.03^{gj}	6.64 ± 0.03^{k}
10	7.16 ± 0.03^{ai}	7.14 ± 0.03^{i}	7.13 ± 0.03^{i}	7.15 ± 0.04^{ai}	6.12 ± 0.04^{l}	5.00 ± 0.03^{m}	6.24 ± 0.04^{n}

Table 5. Changes in pH values of MSM during degradation of 2,4-D. The values are the means of three replicates with the standard deviation which was within 5% of the mean. Different letters indicate significant differences, considering effects of inoculum type and time (two-way ANOVA, $P<0.05$, LSD test).

DS-1: Burkholderia cepacia; DS-2: Pseudomonas sp.; DS-3: Sphingomonas paucimobilis.

days, while in soil inoculated with *Burkholderia cepacia* (SS+DS-1), 28 days. Kinetic data showed that the degradation by strains DS-1 and DS-2 was characterized by the similar rate constants (*k*) of 0.196 and 0.211/day, and disappearance rates (*V*) of 1.70 and 1.98 mg/kg/day, respectively (Table 6). In turn, *Sphingomonas paucimobilis* (DS-3) exhibited the lowest degradative potential (Table 6) and resulted in the highest time persistency (36 days) of 2,4-D in soil (Figure 3). The times within which the initial 2,4-D concentration was reduced by 50% (DT_{50}) were 6.3, 5.0 and 9.4 days for SS+DS-1, SS+DS-2 and SS+DS-3, respectively (Table 4).

In these studies, soils were inoculated with 3×10^6 bacterial cells/g soil and this inoculum density appeared to be able to degrade 2,4-D. As indicated by other experiments, inoculum size is an important factor that determines whether or not target pollutants are efficiently degraded. It has been observed that when lower inoculum densities ($<10^6$ cells/g soil) are used, lower numbers of bacteria are able to survive the initial competition, and population decline usually occurs following inoculation [31,44,45]. This effect may be compensated for by using an inoculum with a higher bacterial density [46,47].

Our results on the disappearance kinetics of 2,4-D in SS and nSS confirm the data that microbial degradation is the main mechanism of 2,4-D dissipation in soil [28,48,49]. The chemical data showed that in non-inoculated sterile soil (SS) a significant amount of 2,4-D (70% of the initial dose) still persisted after 36 days (Figure 3). The 2,4-D disappearance process in SS was characterized by a rate constant of 0.010/day, following first-order kinetics, and the DT_{50} calculated from the linear equation was 70.9 days (Table 4). Obtained results revealed that disappearance of 2,4-D was more effective in sterile soil (SS) inoculated with individual isolates than that in non-sterile soil (nSS) with mixed microbial population. Moreover, this process in nSS was characterized by the 8-day lag phase during which only 4% of the applied dose was degraded (Figure 3). After this time disappearance of herbicide increased substantially with a rate constant of 0.076/day (Table 6), giving the final rate of degradation on the level of 93% of the 2,4-D initial dose after 36 days of incubation (Figure 3). This lag phase observed in our studies, might involve the time needed for proliferation of a small, population of pesticide-degrading microorganisms to reach an optimal level for effective degradation of the pesticide [31,33]. This effect may be also linked to a relatively high concentration of the herbicide (50 mg/kg soil), possibly resulting in the initial inhibition of indigenous microflora activity and/or its degradative potential. Moreover, we applied 2,4-D to soil which had not been previously treated with this herbicide. Generally, 2,4-D applied at recommended field rate is non-toxic to bacteria, as they do not have sensitive targets [49]. However, some studies have shown the adverse effects of higher 2,4-D concentrations on soil microorganism activity. For example, Macur *et al.* [50] studying the impacts of 2,4-D application on soil microbial community structure showed that the high 2,4-D treatments (100 and 500 mg/kg soil) significantly reduced the diversity of 2,4-D degrading bacterial strains. By contrast, in our studies the lag phase was not observed in sterilized soil samples inoculated with individual bacterial strains, which were isolated from 2,4-D contaminated soil.

Parameter	Treatments	Time intervals (days)									Average (0 – 32 days)
		0 – 4	4 – 8	8 – 12	12 – 16	16 – 20	20 – 24	24 – 28	28 – 32	32 – 36	
k (day)	SS	0.004	0.008	0.010	0.004	0.012	0.013	0.008	0.019	0.011	0.010 ± 0.003[a]
	SS + DS-1	0.134	0.110	0.123	0.086	0.148	0.243	0.535	-	-	0.196 ± 0.024[b]
	SS + DS-2	0.166	0.117	0.142	0.203	0.295	0.347	-	-	-	0.211 ± 0.027[b]
	SS + DS-3	0.057	0.059	0.101	0.089	0.097	0.123	0.149	0.225	0.239	0.127 ± 0.015[c]
	nSS	0.005	0.004	0.048	0.136	0.094	0.101	0.113	0.103	0.082	0.076 ± 0.010[d]
V (mg/kg/day)	SS	0.20	0.38	0.45	0.20	0.53	0.55	0.30	0.73	0.40	0.41 ± 0.07[a]
	SS + DS-1	4.95	2.50	1.75	0.80	0.83	0.70	0.38	-	-	1.70 ± 0.21[b]
	SS + DS-2	5.80	2.30	1.68	1.23	0.68	0.23	-	-	-	1.98 ± 0.32[b]
	SS + DS-3	2.45	2.00	2.50	1.50	1.13	0.93	0.65	0.48	0.20	1.31 ± 0.17[c]
	nSS	0.25	0.20	2.03	4.03	1.75	1.28	0.93	0.55	0.30	1.26 ± 0.12[c]

Table 6. Values of degradation rate constant (*k*) and rate of disappearance (*V*) for 2,4-D in loamy sand (SL) soil. The values are the means of three replicates with the standard deviation which was within 5% of the mean. Different letters for a given parameter indicate significant differences between treatments ($P<0.05$, LSD test).

SS: non-inoculated sterile soil; DS-1: Burkholderia cepacia; DS-2: Pseudomonas sp.; DS-3: Sphingomonas paucimobilis ; nSS: non-sterile soil without bacteria inoculum

4. Conclusions

Strains of *Burkholderia cepacia* (DS-1), *Pseudomonas* sp. (DS-2) and *Sphingomonas paucimobilis* (DS-3) isolated from soil previously treated with 2,4-D were capable of degrading this herbicide in MSM and use it as the only carbon and energy source. As indicated by the data obtained from chemical analyses, strains DS-1 and DS-2 may additionally possess the potential to metabolize 2,4-DCP. Inoculation studies show that these strains also efficiently degrade 2,4-D in soil. Kinetic data support the view that respective species of soil microorganisms differ in their degradative activity towards pesticides. However, the strains isolated from soils regularly treated with 2,4-D have a considerable potential for bioremediation, since the organisms have already adapted to the presence of pesticide. There is still a need for further research on the biochemical and genetic aspects of 2,4-D degradation by the isolated bacterial strains as well as the various environmental factors affecting their survival and degradative potential.

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