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Research article

Bioremediation of diuron contaminated soils by a novel degrading microbial consortium

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A B S T R A C T

Diuron is a biologically active pollutant present in soil, water and sediments. It is persistent in soil, water and groundwater and slightly toxic to mammals and birds as well as moderately toxic to aquatic invertebrates. Its principal product of biodegradation, 3,4-dichloroaniline, exhibits a higher toxicity than diuron and is also persistent in the environment. On this basis, the objective of the study was to determine the potential capacity of a proposed novel diuron-degrading microbial consortium (DMC) for achieving not only diuron degradation, but its mineralisation both in solution as well as in soils with different properties. The consortium was tested in a soil solution where diuron was the only carbon source, and more than 98.8% of the diuron initially added was mineralised after only a few days. The consortium was composed of three diuron-degrading strains, *Arthrobacter sulfonivorans*, *Variovorax soli* and *Advenella* sp. JRO, the latter had been isolated in our laboratory from a highly contaminated industrial site. This work shows for the first time the potential capacity of a member of the genus *Advenella* to remediate pesticide-contaminated soils. However, neither of the three strains separately achieved mineralisation (ring-^{14}C) of diuron in a mineral medium (MSM) with a trace nutrient solution (NS); combined in pairs, they mineralised 40% of diuron in solution, but the most relevant result was obtained in the presence of the three-member consortium, where complete diuron mineralisation was achieved after only a few days. In the presence of the investigated soils in suspension, the capacity of the consortium to mineralise diuron was evaluated, achieving mineralisation of a wide range of herbicides from 22.9 to 69.0%.

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1. Introduction

Microbial degradation is the main process affecting the environmental persistence of pesticides (Buchanan et al., 2012). Dissipation of unwanted residues depends on the presence, number and enzymatic capability of soil microorganisms. The phenylurea herbicide diuron is widely used and, consequently, frequently detected as a major soil and water contaminant in areas where there is extensive use. Biodegradation appeared to be the major cause of diuron dissipation (Hussain et al., 2015). Due to its high persistence, aged residues are found in many environments such as soil, sediments and/or water (Giaccomazzi and Cochet, 2004). Diuron is generally found at trace concentrations (from ng L^{-1} to μg L^{-1}) (Sorensen et al., 2013), but higher concentrations (mg L^{-1}) have been found in, drainage water from agricultural soils and in rainfall runoff from fruit orchards. Besides this latter case is associated to a point contamination where repeated pesticide application, accidental pollution, farmyard activities, direct contamination, and overspray, among others (Pose-Juan et al., 2015) could involve to reach a higher pesticide contamination than that due to diffuse sources of soil pollution include spray drift, run-off, leaching, etc., (Álvarez-Martín et al., 2016). In the research on remediation of diuron contaminated soil, the concentrations found are in the order of mg kg^{-1} (Rosas et al., 2014; Vicente et al., 2012). In solution, diuron water remediation, the diuron concentrations investigated are in the order of mg L^{-1} (Badawi et al., 2009; Rodríguez et al., 2012).

Bioremediation is the intentional use of biodegradation to eliminate environmental pollutants from sites where they have been released (Chishti et al., 2013). Bioremediation technology uses the physiological potential of microorganisms and plants for the degradation of pollutants. Biodegradation involves the breakdown of pesticides or any other organic compounds, usually by

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microorganisms, to less complex compounds and ultimately to water and CO_2 and oxides or mineral salts of other elements present. Clearly, many toxic metabolites can be microbiologically produced, which may represent also a significant threat to the health of humans and a variety of animals and plants. Therefore, it is not sufficient to merely measure the persistence and disappearance of the parent substance. Especially in the case of diuron, where its principal metabolite, 3,4-dichloroaniline (3,4-DCA), is considered as highly toxic to different micro- and macroorganisms (Giaccomazzi and Cochet, 2004). In fact, diuron is considered a "Priority Hazardous Substance" and has been included in the European Commission's list of priority substances for European freshwater resources (Directive 2000/60/EC) and in the U.S. Contaminant candidate List 4 (Environmental Protection Agency, 2015).

The complete breakdown of pesticides into inorganic components is termed mineralisation. In some cases, degradation leads to formation of less complex and less toxic organic compounds, which is referred to as partial biodegradation. The pesticide thus transformed or degraded by microorganisms is used as a carbon source, nitrogen source, any other mineral source or a final electron acceptor in the respiratory chain. Understanding pesticide metabolism in microorganisms is necessary for developing bioremediation strategies for contaminated soils.

Microorganisms with a broad array of catabolic activities are widespread. Soils, sediments and water bodies possess large and highly diverse microbial communities that potentially exhibit many degradative capacities; when these capacities are expressed, organic chemicals are readily degraded. Nevertheless, many synthetic compounds persist for some time in these same environments, even though these molecules are biodegradable, hence the question has been whether inoculation might significantly enhance the dissipation of these compounds. Such inoculation is termed bioaugmentation (Alexander, 1999).

In previous studies (Sorensen et al., 2008; Villaverde et al., 2012), a two-member bacterial consortium formed by the diuron-degrader *Arthrobacter sulfonivorans*, which has the ability to metabolise diuron to its main transformation product DCA in stoichiometric amounts, and the linuron-degrading *Variovorax soli*, with the ability to mineralise DCA, was able to mineralise 50% of phenyl-U-^{14}C diuron to $^{14}CO_2$ in solution.

In this work, a novel diuron-degrading bacterial consortium composed of three diuron-degrading strains including *Arthrobacter sulfonivorans*, *Variovorax soli* and *Advenella* sp. JRO isolated in our laboratory from a highly contaminated industrial site (Sopeña et al., 2014) was tested for diuron mineralisation in solution and in the presence of soils with different properties.

2. Materials and methods

2.1. Materials

Technical grade (98%) diuron [*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea] was provided by Presmar S.L. (Seville, Spain). Radiolabeled [ring-^{14}C]-diuron was purchased from the Institute of Isotopes, Budapest, Hungary (specific activity = 36 mCi $mmol^{-1}$, chemical purity = 99.9%, and radiochemical purity = 100%). Six different soils from southwestern Spain were employed in this study. They were taken from the superficial horizon (0–20 cm). Soil pH values were measured in saturated paste. Total carbonate content was determined by the manometric method, particle size distribution by the hydrometer method, and organic matter (OM) by dichromate oxidation (Madrid et al., 2004). Soil analyses were performed on air-dry soil sieved at 2 mm. These properties are shown in Table 1.

The diuron-degrading organism *Arthrobacter* sp. *N2* was purchased from the Institut Pasteur Collection; *Variovorax* sp. *SRS16* was kindly provided by the Dr. Sebastian R. Sorensen from Geological Survey of Denmark and Greenland (GEUS) (Sorensen et al., 2008) and *Advenella* sp. *JRO* was isolated in our laboratory from a highly contaminated industrial site (Sopeña et al., 2014).

2.2. Inoculum preparation

Upon receipt/isolation of the three bacteria, *A. sulfonivorans, V. soli and Advenella* sp. *JRO*, they were cultivated and subsequently stored in cryovials (Microbank), which are 2 mL microtubes containing a specific culture medium and 20 porous spheres of 3 mm diameter, and kept at −80 °C. Before each experiment, the cryovials were thawed and *A. sulfonivorans* and *Advenella* sp. *JRO* were grown in Luria–Bertani (LB) medium and *V. soli* in an R2A medium (Sorensen et al., 2005, 2013). The bacteria were harvested at the beginning of the stationary phase and subsequently washed twice in a sterile mineral salt (MSM) solution before initiation of the experiments. The final densities of each strain added were approximately 10^7 cells g $soil^{-1}$.

2.3. Bacterial enumeration

Enumeration of viable bacteria (potential diuron-degraders) was performed by determining the colony-forming unit counts g^{-1} of each investigated soil (CFUs g^{-1}). Bacterial enumeration was carried out using 1 g of soil. The soils were whirl-mixed for 30 s with 10 mL of Ringer's solution, sonicated for 1 min and allowed to stand for 2 min. Aliquots (100 μL) of these dilutions were applied on agar plates prepared from an R2a diluted medium (1:40) and the herbicide at a concentration of 40 mg L^{-1} in order to limit the carbon source for the soil endogenous flora and allow the selection of diuron-degraders. After 48 h CFUs were determined.

2.4. Mineralisation and biodegradation experiments

Mineralisation of ^{14}C-labeled diuron in the soil suspension or solution media was measured (in triplicate) through the evolution of the $^{14}CO_2$ produced (Villaverde et al., 2013). All microcosm components were sterilised before the assay, using an autoclave Auster-G, P-Selecta with three cycles at 121 °C with an inlet pressure of 103 kPa for 20 min. Mineralisation assays were carried out in respirometers (modified 250 mL Erlenmeyer flasks) with 50 mL of mineral salt medium (MSM) ^{14}C-ring-labeled and unlabelled diuron to obtain a final concentration of 10 mg L^{-1} in solution, 50 mg kg^{-1} of sterilised or non-sterilised soil in suspensions assays and a radioactivity of approximately 450 Bq per flask. This concentration was selected with the aim of simulating pollution events, such as the disposal or accidental release of high pesticide concentrations, or the accumulation of pesticides through their repeated application as previously commented. The flasks were inoculated, when required, with the specific bacterium or the consortium and a trace of nutrient solution (NS) (Villaverde et al., 2013), closed with Teflon-lined stoppers and incubated at 20 ± 1 °C. Non-inoculated sterile controls were also prepared and no mineralisation was detected. Production of $^{14}CO_2$ was measured as radioactivity appearing in the alkali trap of the biometer flasks which contained 1 mL of 0.5 M NaOH. Periodically, the solution was removed from the trap and replaced with fresh alkali. The NaOH solution was mixed with 5 mL of a liquid scintillation cocktail (Ready safe from PerkinElmer, Inc., USA) and the mixture was kept in darkness for about 24 h for dissipation of chemiluminescence. Radioactivity was measured with a liquid scintillation counter (Beckman Instruments Inc., Fullerton, California, model LS5000TD).

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