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Enhanced Mineralization of Diuron Using a Cyclodextrin-Based Bioremediation Technology

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ABSTRACT: The phenylurea herbicide diuron [*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea] is widely used in a broad range of herbicide formulations and, consequently, it is frequently detected as a major soil and water contaminant in areas where there is extensive use. Diuron has the unfortunate combination of being strongly adsorbed by soil organic matter particles and, hence, slowly degraded in the environment due to its reduced bioavailability. *N*-Phenylurea herbicides seem to be biodegraded in soil, but it must be kept in mind that this biotic or abiotic degradation could lead to accumulation of very toxic derived compounds, such as 3,4-dichloroaniline. Research was conducted to find procedures that might result in an increase in the bioavailability of diuron in contaminated soils, through solubility enhancement. For this purpose a double system composed of hydroxypropyl-β-cyclodextrin (HPBCD), which is capable of forming inclusion complexes in solution, and a two-member bacterial consortium formed by the diuron-degrading *Arthrobacter sulfonivorans* (*Arthrobacter* sp. N2) and the linuron-degrading *Variovorax soli* (*Variovorax* sp. SRS16) was used. This consortium can achieve a complete biodegradation of diuron to CO_2 with regard to that observed in the absence of the CD solution, where only a 45% biodegradation was observed. The cyclodextrin-based bioremediation technology here described shows for the first time an almost complete mineralization of diuron in a soil system, in contrast to previous incomplete mineralization based on single or consortium bacterial degradation.

KEYWORDS: *cyclodextrin, diuron, 3,4-dichloroaniline, soil contamination, biodegradation, bacterial consortium*

INTRODUCTION

Diuron is a biologically active pollutant present in soil, water, and sediments. This substituted urea herbicide inhibits photosynthesis by preventing oxygen production[1] and blocks the electron transfer at the level of photosystem II of photosynthetic microorganisms and plants. Diuron has the unfortunate combination of being strongly adsorbed on soil organic matter particles and, hence, slowly degraded in the environment due to its reduced bioavailability.

Diuron is considered a Priority Hazardous Substance by the European Commission[2] because its degradation in soil leads to 3,4-dichloroaniline (3,4-DCA), a very toxic compound, which has a high tendency to accumulate in the environment with carcinogenic and mutagenic properties.[3−6] Consequently, diuron 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 3.[7]

Biodegradation has been described as the primary mechanism for diuron dissipation in soils and waters,[8] although dispersion of this compound in agriculture leads to pollution of the aquatic environment by soil leaching[9−11] and runoff.[12]

In this work, some studies were conducted to find procedures that might result in an increase in the bioavailability of diuron in a contaminated soil, through solubility enhancement using biodegradable molecules. These molecules are cyclodextrins (CDs), which are cyclic oligosaccharides, containing 6 (α-CD), 7 (β-CD), or 8 (γ-CD) *R*-(1,4)-linked glucose units, formed from the enzymatic degradation of starch by bacteria. It is well-known that they are capable of forming inclusion complexes both in solution and in solid state with a variety of guest molecules, which are placed in their hydrophobic interior cavity.[13] A large number of papers describing the complexation of CDs with pesticides can be found in the literature. Most pesticide−CD complexes were aimed to improve their solubility in water.[14−18] However, no research has been reported with the aim of finding correlations between this increase in solubility, desorption percentage from soil, and bioavailability by means of mineralizing assays, confirming the complete dissipation of the pesticides. However, an increase of bioavailability only will not be enough to reach a significant soil diuron dissipation, because, although several diuron-degrading bacteria have been isolated from different agricultural soils[3,8,19,20] and river waters,[20] none of them has been identified as capable of reaching a complete diuron mineralization in the presence of soil. Sorensen et al.[5] used a two-member diuron-mineralizing consortium, which gave better results by combining the cooperative degradation capacities of two bacteria. In this work, we attempt to enhance mineralization of diuron using a cyclodextrin-based bioremediation technology involving a bacterial consortium, which resulted in an effective diuron mineralization system thanks to the increased bioavailability.

The development of an in situ and environmentally friendly soil decontamination technique, which could give rise to a complete diuron mineralization by means of increasing the bioavailability of the pollutant and employing specific chemical

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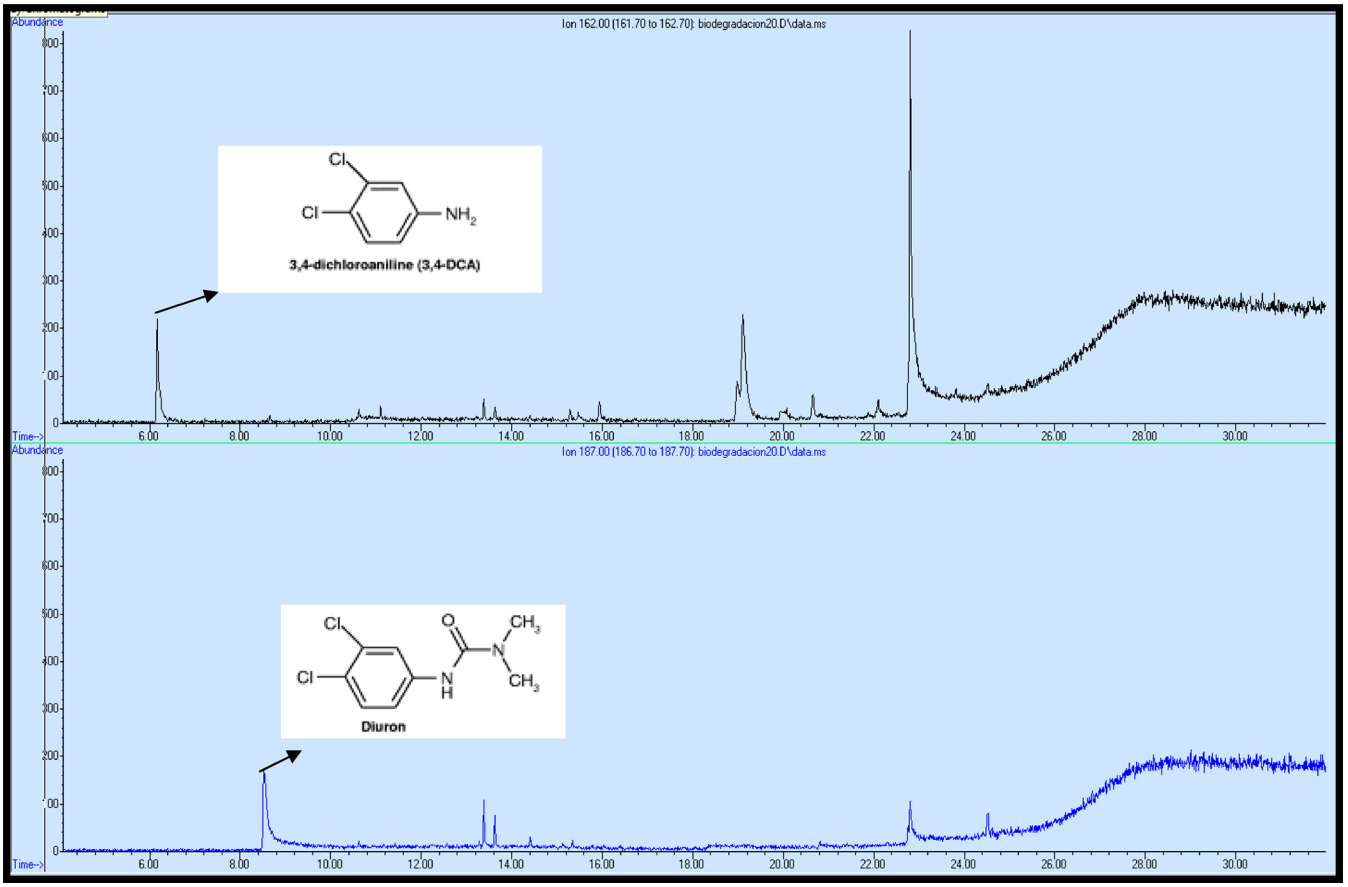

Figure 1. GC-MS ion chromatograms of diuron and 3,4-dichloroaniline.

bacterium degraders, would involve an improvement from both economical and environmental points of view.

MATERIALS AND METHODS

Materials. Technical grade (98%) diuron [*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea] was provided by Presmar S.L. (Seville, Spain). Radiolabeled [*ring*-U-^{14}C]-diuron was purchased from Institute of Isotopes, Budapest, Hungary (specific activity = 36 mCi mmol^{-1}, chemical purity = 99.9%, and radiochemical purity = 100%). The cyclodextrins employed were *β*-CD (BCD), hydroxypropyl-*β*-CD (HPBCD), *γ*-CD (GCD), and hydroxypropyl-*γ*-CD (HPGCD) (all from Cyclolab, Budapest, Hungary, and with a chemical purity of 97%).

The CD hydroxypropyl-*β*-CD (HPBCD) from Cyclolab was selected because in previous tests (data not shown) it was demonstrated that this CD is not used as a carbon source by the bacteria tested, eliminating its use as a growth substrate by the strains studied. Likely, this CD showed the best complexation parameters obtained from the solubility studies.

A southwestern Spain loamy sandy soil with a pH of 8.7, 6.9% of $CaCO_3$, 1% of organic matter, and a particle size distribution of 82.3% sand, 4.1% silt, and 13.5% clay was selected for this study. The sample was taken from the superficial horizon (0−20 cm), air-dried for 24 h, and sieved through 2 mm, to remove stones, plant materials, etc. The soil was analyzed for particle size distribution, measured by a Bouyoucos densimeter, organic matter, measured by $K_2Cr_2O_7$ oxidation, pH, determined in the 1:2.5 soil/water extract, and total carbonate content, measured by the manometric method.[21]

The diuron-degrading organism *Arthrobacter* sp. N2[3] was purchased from the Institut Pasteur Collection. *Variovorax* sp. SRS16 was kindly provided by S. R. Sorensen.[5] The identification of phylogenetic neighbors was carried out by applying BLAST[22] to the GenBank sequence database and the EzTaxon database.[23] *Arthrobacter* sp. N2 showed a 99.567 pairwise similarity with *Arthrobacter sulfonivorans* (AF235091), whereas *Variovorax* sp. SRS16 yielded a 98.780 pairwise similarity with *Variovorax soli* (DQ432053). In this study we will refer to these strains as *A. sulfonivorans* and *V. soli*.

Methods. *Diuron Desorption Studies from Soil.* Prior to desorption studies, triplicate batch adsorption experiments were performed by mixing 5 g of the soil with 10 mL of 0.01 M $Ca(NO_3)_2$ solution, containing various concentrations (5, 10, and 15 mg L^{-1}) of diuron, in 50 mL polypropylene centrifuge tubes. The samples were shaken for 24 h at 20 ± 1 °C. This time of reaction was chosen from preliminary kinetic studies (not shown), which showed that adsorption had reached pseudoequilibrium. After shaking (on an orbital shaker), the suspensions were centrifuged, and the concentration of diuron in the supernatant was determined by using a Shimadzu HPLC equipped with a UV detector. The difference in herbicide concentration between the initial and final equilibrium solutions was assumed to be due to sorption, and the amount of diuron retained by the adsorbent was calculated.

Desorption experiments were performed after adsorption equilibrium had been reached by removing half of the supernatant after centrifugation, replacing it by 5 mL of the extractant solution, allowing equilibration for an additional 24 h period, and, after that, operating as in the adsorption experiment. This process was repeated twice. Desorption experiments were carried out using 0.01 M $Ca(NO_3)_2$ solution (named $Ca(NO_3)_2$ solution) and the same solution plus HPBCD with a final concentration of 50 mM (named HPBCD solution). The percentage of diuron desorbed with respect to that previously adsorbed during the adsorption process (%D) was calculated for all of the desorption experiments.

Inoculum Preparation. After both bacteria had been received, *A. sulfonivorans* and *V. soli* were stored in criovials 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

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experiment, the criovials were thawed, and then *A. sulfonivorans* was grown in Luria–Bertani (LB) medium and *V. soli* was grown in an R2A medium.[24] The bacteria were harvested at the beginning of the stationary phase and, afterward, washed twice in a sterile mineral salts (MS) solution[24] before initiation of the experiments. The initial cell densities of the bacteria in the degradation experiments were 10^7 CFU mL^{-1}. For diuron degradation, the bacteria were grown in a MS medium supplemented with 40 mg L^{-1} diuron, as described by Sorensen et al.[24]

Solubility in the Aqueous Phase in the Presence of Different CDs. Phase solubility studies were performed according to the method reported by Villaverde et al.[16] An excess of diuron (5 mg) was added to aqueous solutions (20 mL) that contained various concentrations of CDs (0–0.012 M for BCD; 0–0.05 M for GCD; and 0–0.1 M for HPBCD and HPGCD). The flasks were shaken at 25 °C for 1 week. The suspensions were subsequently filtered through a 0.22 μm Millipore glass-fiber membrane, and the concentration of diuron was determined using HPLC. The apparent stability constants of the different diuron–CD complexes (K_c) were determined from the straight line obtained in the phase solubility diagram according to the equation proposed in Villaverde et al.[16]

$$K_c = \text{slope}/S_0(1 - \text{slope}) \quad (1)$$

where S_0 is the diuron equilibrium concentration in aqueous solution in the absence of the CD and slope refers to the slope of the phase solubility diagram. Another parameter that can be obtained from the data of the solubility diagram is the solubilization efficiency (S_e), which is defined as the increment of diuron apparent solubility at the highest CD concentration with respect to its solubility.

Mineralization and Biodegradation Experiments. Mineralization of ^{14}C-labeled diuron in the soil was measured (in triplicate) through the evolution of $^{14}CO_2$ produced.[25] Soil was sterilized using an autoclave Auster-G, P-Selecta with three cycles at 121 °C, inlet pressure of 103 kPa, during 20 min. The mineralization assays were carried out in respirometers: modified 250 mL Erlenmeyers into which 10 g of soil together with 50 mL of mineral salts medium (MMK) was placed. Acetone stock solution containing ^{14}C-labeled and unlabeled diuron was added to the soil to obtain a final concentration of 50 mg kg^{-1} and a radioactivity of approximately 900 Bq per flask. The flasks were inoculated with the specific bacterium or the consortium, prepared as described above, and were closed with Teflon-lined stoppers and incubated at 20 ± 1 °C. Noninoculated soil sterile controls and noninoculated soil sterile with HPBCD addition controls were also prepared, and no mineralization 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 liquid scintillation cocktail (Ready safe from PerkinElmer, Inc., USA) and the mixture kept in darkness for about 24 h for dissipation of chemiluminescence. Radioactivity was measured as described by Posada-Baquero et al.[26]

Biodegradation experiments were performed in parallel in the same way as mineralization ones, but in this case, only nonradiolabeled diuron was used, and the main metabolite 3,4-dichloroaniline and the parent compound were analyzed at different time points by CG-MS. A gas chromatograph 6890N from Agilent Technologies coupled to an Agilent Automass quadrupole mass spectrometer 5975A was used. Injection was in splitless mode with the split valve closed for 48 s. Helium was employed as gas carrier. A Hewlett-Packard DB 17 ms (30 cm × 0.25 i.d. × 0.17 μm film thickness) capillary column was used. The temperature program for the chromatographic run was as follows: injector temperature, 70 °C (hold for 0.5 min), followed by a 100 °C min^{-1} ramp to 300 °C (hold for 15 min). For mass spectrometric detection, a potential of 70 eV was initially imposed in total ion scan or full scan (m/z 45–300). Then, acquisition was performed under time-scheduled selected ion monitoring (SIM) using the following product ion qualifers: 74, 88, 97, 124, 159, and 187, the precursor ion being m/z 187 for diuron quantification detected as intermediate 1,4-dichloro-2-isocyanatobenzene.[27] In the case of the main metabolite (3,4-DCA) the precursor ion was m/z 162 (Figure 1). The MS detector was kept at 200 °C. To avoid saturation and to preserve it, the analyzer was switched off for 4 min during solvent elution and after the last eluting analyte determination. The limit of quantification for the metabolite was 1 μg/g of soil. The organic solvent selected to carry out the extractions from aqueous supernatant was hexane (1:1 ratio MMK medium/organic solvent, mL) and for extractions from soil pellet dichloromethane was used (1:1 ratio soil/organic solvent). Recoveries were 91–102%.

Both mineralization and biodegradation experiments were incubated for 120 days.

Effect of Cyclodextrin Application on Soil Mineralization and Biodegradation. A HPBCD solution, with a concentration corresponding to 10 times the millimoles of diuron previously added in soil degradation experiments flasks (50 mg kg^{-1}), was employed to enhance the herbicide bioavailability and increase its biodegradation rate. This solution was incorporated into either biodegradation and mineralization experiments to determine the increase in bioavailability of the herbicide.

Model of Mineralization Kinetics. Mineralization data [expressed as the percentage (P) of the initial activity converted to $^{14}CO_2$ as a function of time (t)] were fitted to a first-order equation of the following form:[28]

$$P = P_{max}(1 - e^{-kt}) \quad (2)$$

Nonlinear regression analysis (Sigmaplot v. 8.0) was used to estimate the parameters P_{max} (overall extent of ^{14}C mineralization) and k (first-order mineralization rate).

The parameters derived from this model accurately describe the mineralization kinetics of nonsorbed diuron in systems under equilibrium (instantaneous sorption and/or desorption) and pseudoequilibrium (desorption rates much slower than degradation rates) conditions.

RESULTS AND DISCUSSION

Diuron Solubility in the Aqueous Phase in the Presence of Different Cyclodextrins. The phase solubility diagrams of diuron in the presence of the different CDs used in this work are shown in Figure 2. The initial purpose of this experiment was to test whether the interaction of diuron with the different CDs produced the formation of inclusion complexes in solution. The increase in diuron hydrosolubility in the presence of CDs indicates that the herbicide forms inclusion complexes with them. A solubility limit could not be obtained in the range of CD concentrations used in any of the

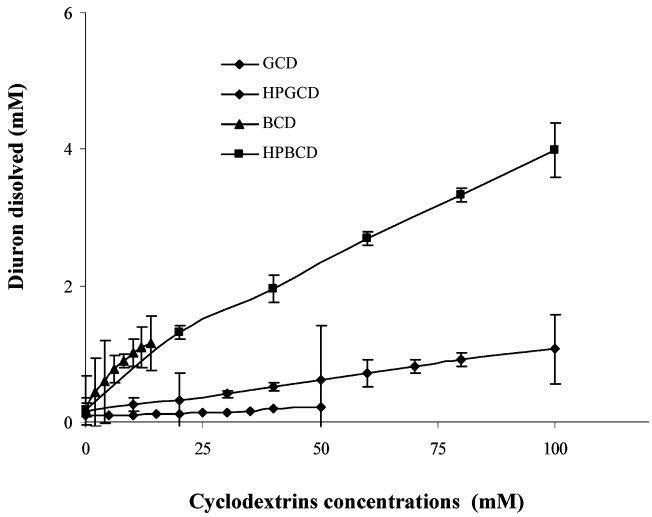

Figure 2. Phase solubility diagrams of diuron in the presence of the cyclodextrins studied.

cases, which is in agreement with an A_L classification.[16] The inclusion complexation parameters for all CDs tested are shown in Table 1. The straight lines of the phase solubility diagrams

Table 1. Diuron Apparent Stability Constants (K_c)[a] and Solubilization Efficiency (S_e)[b] Obtained from the Phase Solubility Diagrams

CD	S_e	K_c (M^{-1})	R^2
HPBCD	23.27 ± 1.33	207.70 ± 3.55	0.9886
BCD	6.76 ± 1.02	175.86 ± 4.21	0.9577
HPGCD	6.79 ± 0.88	58.68 ± 1.11	0.9336
GCD	2.27 ± 0.36	25.74 ± 2.85	0.9985

[a]Calculated according to eq 1. [b]Diuron solubility calculated for the highest CD concentration studied.

exhibited a slope of <1, which is ascribed to the formation of a 1:1 complex stoichiometry in solution. The apparent formation constants of the inclusion complexes formed (K_c) were calculated according to eq 1. A comparative study of the K_c values shows that the highest values were obtained when HPBCD and BCD were used. The lowest solubilization efficiency (S_e) and lowest K_c values corresponded to GCD and its derivative, HPGCD. This result reflects the effect of the size of the CD cavity (BCD and GCD contain 7 and 8 α-(1,4)-linked glucose units, respectively, which form the toroidal ring) on the formation of the different inclusion complexes because the larger size of the internal cavity diameter of GCD would result in an easy path for the diuron molecule to escape the GCD cavity. Although the K_c value for BCD was high, which indicates a strong tendency to form a complex with diuron, the S_e value is low because of the low solubility of this CD (16 mM).

Diuron Desorption Experiments Using 0.01 M HPBCD as Extractant Solution. The desorption percentages (%D) values obtained for the soil under study when the HPBCD solution was employed as extractant in comparison to $Ca(NO_3)_2$ solution are shown in Table 2. In this soil for all

Table 2. Percentage of Diuron Desorbed from the Soil

	extractant solutions	
diuron initial concn (mg L^{-1})	0.01 M Ca $(NO_3)_2$	0.01 M HPBCD
5	51.58 ± 0.66	94.64 ± 2.01
10	84.01 ± 1.02	89.79 ± 3.33
15	87.20 ± 2.55	100 ± 1.09

diuron initial concentrations about 90% could be desorbed with HPBCD solution. The results obtained indicate the high extracting power of HPBCD toward the herbicide previously adsorbed on the soils in comparison to the percentages extracted with $Ca(NO_3)_2$ solution, due to the formation of water-soluble inclusion complexes between diuron and HPBCD. Similar results have been obtained in previous papers using HPBCD and CDs as extractant solutions for the herbicide 2,4-D and norflurazon from soil.[14,15,29−31] In general, low-polarity pesticides have a high tendency to be adsorbed on soil surfaces, leading to their inactivation and low bioavailability and, sometimes, to soil contamination. If these pesticides are able to form inclusion complexes with CDs and, as a consequence, to increase their solubility, the application of CD solutions to soils containing a high concentration of pesticide residues adsorbed can increase their removal and pass to the soil solution, where they become bioavailable.

Diuron Mineralization and Biodegradation Experiments in Soil Inoculated with *A. sulfonivorans*, *V. soli*, and Their Bacterial Consortium. The principal product of diuron biodegradation, 3,4-DCA, exhibits a high toxicity and is also persistent in soil, water, and groundwater. Diuron indirectly possesses a significant amount of toxicity and could be a potential poisoning herbicide contaminant of groundwater. Therefore, the ultimate objective of this work was to obtain a complete diuron mineralization in a soil−water system. The chosen scenario was a loamy sandy soil from an agricultural site.

A two-member diuron-mineralizing consortium, combining the cooperative degradation capacities of the diuron-degrading bacteria *A. sulfonivorans* and the linuron-mineralizing bacteria *V. soli*, was used, in comparison to the individual bacteria.

The effects on the mineralization of diuron in soil (50 mg kg^{-1}) after inoculation of *A. sulfonivorans* and *V. soli*, individually or in a coculture, were determined (Figure 3). The overall extent of ^{14}C mineralization was estimated using the first-order production eq 1,[28] (Table 3). Inoculation with *A. sulfonivorans* resulted in a mineralization of 6.86%. The metabolite 3,4-DCA was detected in the parallel biodegradation experiments only in the samples inoculated with this bacterium, and the amount was equivalent to 8.51% of the initially added diuron. Inoculation with *V. soli* alone resulted in a diuron mineralization in the soil of only 5.22%. Inoculation with the coculture resulted in rapid diuron mineralization, and an important mineralization was observed (45.25% of the added ^{14}C diuron was metabolized to $^{14}CO_2$ during the experiment, after 120 days) (Figure 3), and no metabolite was determined after the experiment (Table 3). The highest mineralization rate values corresponded to the soil slurries inoculated with the bacterial consortium, k values of 5 and 6 times higher than those of *A. sulfonivorans* and *V. soli*, used separately (Table 3). The time necessary to reach mineralization values >5% (lag phase) was only 12.53 days for systems inoculated with both selected bacteria, in comparison to 120 and 97 days for *A. sulfonivorans* and *V. soli*, respectively, when grown alone.

In Table 3, the residual diuron measured at the end of the parallel biodegradation experiments is also shown, being remarkable that in the *A. sulfonivorans* inoculated system a 60.52% of the diuron initially added was still present, confirming that about 40% of diuron is biodegraded, but not mineralized, as can be observed in the mineralization experiments results (6.86%), remaining in the form of the toxic metabolite 3,4-DCA (8.51%) or other intermediate species. A similar result could be observed for the system inoculated with *V. soli*, but in this case, the percentage of diuron remaining at the end of the biodegradation experiment was lower (38.78%), from which only 5.22% was mineralized. However, the presence of the toxic metabolite was much lower (0.61%). Finally, the percentage of diuron measured after soil biodegradation experiment in the presence of the two-member bacterial consortium was the lowest, only 21.73%, confirming again the need to use the two bacteria to reduce drastically the real risk in a diuron-contaminated soil. Moreover, there is an extremely high increase in the percentage of diuron mineralization, from about 5−6% for the individual bacteria to 45% when the consortium was used. In addition, the toxic metabolite was not detected. It is also important to highlight that the lag phase is reduced by 120 or 97 days (only one degrader) to 12 days in the presence of the consortium, which

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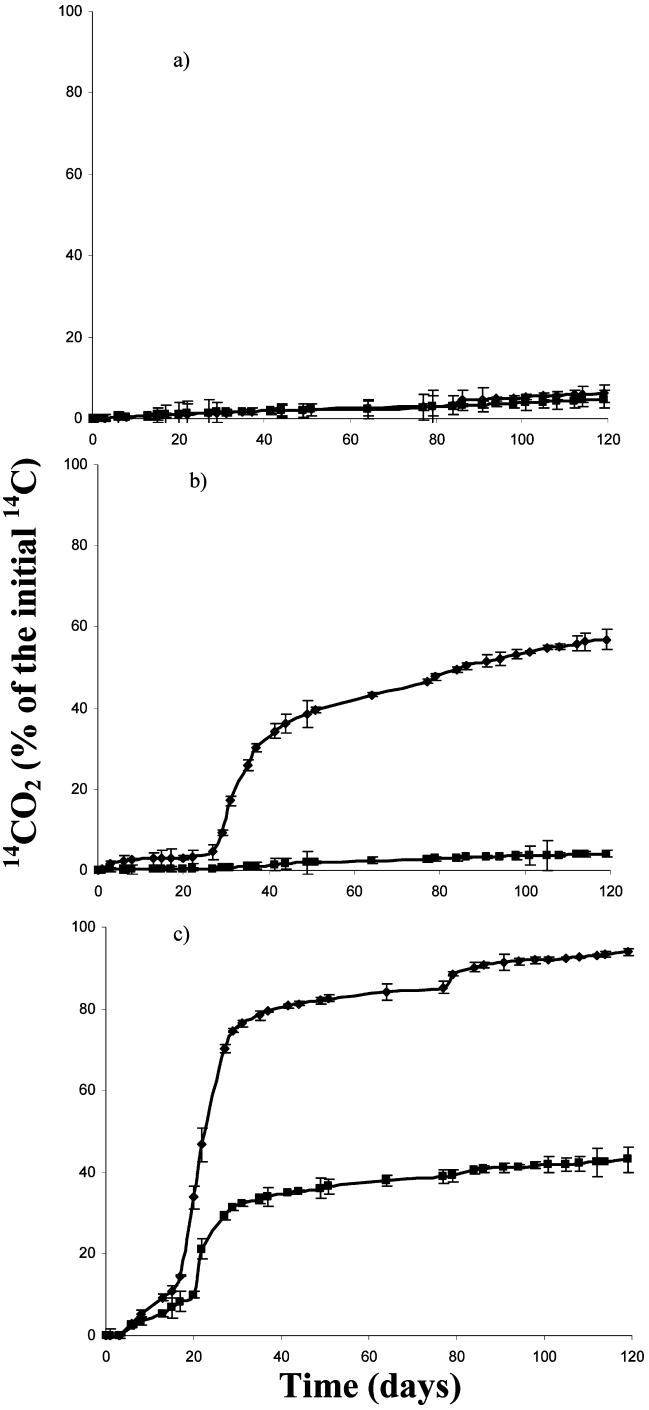

Figure 3. Mineralization of ^{14}C-labeled diuron in soil (50 mg kg^{-1}) and inoculated with (a) *Arthrobacter sulfonivorans*, (b) *Variovorax soli*, and (c) a bacterial consortium, in the presence (◆) and absence (■) of the HPBCD solution.

denotes that the cooperative diuron degradation of the consortium increases the mineralization rate (0.28 days^{-1}), clearly turning out to be an effective in situ bioremediation tool.

From these results it can be concluded that this type of chemical (organochloride persistent compounds) will require a more complex and conscientious analysis about the best strategy to reach an optimal bioremediation of a contaminated soil, especially for those chemical having main metabolites that are suspected of having unwanted effects on nontarget microorganism, such as the scenario investigated in this work.

Diuron Mineralization and Biodegradation Experiments in the Presence of a HPBCD Solution in Soil Inoculated with *A. sulfonivorans* and *V. soli* and Their Bacterial Consortium. HPBCD seems to be a good choice for being applied as decontamination technique in the case of the herbicide diuron. Similar results were previously reported for naphthalene and phenanthrene by Badr et al.[32] For this reason diuron mineralization experiments employing soil slurries in the presence of HPBCD solution were performed with the aim of enhancing diuron bioavailability and its subsequent dissipation, ensuring that the potentially bioavailable herbicide fraction passes to the soil solution in a faster way, because, as demonstrated above, higher desorption percentages of diuron from the soil studied were obtained with the HPBCD solution employed as diuron extractant. The ability of soils to release (desorb) pollutants determines their susceptibility to suffer microbial degradation, thereby influencing the effectiveness of the bioremediation process. The degradation of adsorbed contaminants can presumably occur via microbially mediated desorption of contaminants and the development of a steep gradient between the solid phase and interfacial contaminant.[33]

The use of CD solutions as enhancers in pollutant dissipation in soil has been postulated as a promising in situ decontamination tool due to its capacity for pesticide soil desorption. Previously, our group has reported numerous results using different types of CD solutions for enhancing soil desorption of the different herbicides with CDs naturally originated and their derivates and different aging periods,[14,17,31] but no works correlating the CD solution desorption effect with microbial biodegradation enhancing have been carried out yet.

In Figure 3, diuron mineralization curves obtained from inoculated soil slurries in the presence of HPBCD in solution at a concentration equivalent to 10 times the amount of diuron initially spiked are shown. In the case of inoculation with *A. sulfonivorans* (Figure 3a) mineralization was very low (8.55%, Table 3) despite the fact that this bacterium is a diuron degrader.[3] Then, the addition of the HPBCD solution, which provokes a slight increase in herbicide bioavailability, provokes also a slight increase of the main metabolite after degradation, as confirmed with the data obtained for the 3,4-DCA analysis, 10.26% (Table 3). When the HPBCD solution was applied on the soil slurries inoculated with *V. soli* (Figure 3b), a clear increase in herbicide bioavailability was translated into a higher percentage of diuron mineralized, reaching an overall extent of ^{14}C mineralization of 57.87%. Finally, in Figure 3c the diuron mineralization curve, when the HPBCD solution was added, shows a new significant increase in herbicide mineralization regarding that observed in the absence of the CD solution, reaching a maximum percentage of mineralization of 98.67% and the highest value determined for mineralization rate of 3.08 days^{-1} (Table 3), demonstrating once again that the increase in the amount of herbicide that passes to the soil solution is unequivocally connected with the mineralization rate, especially when diuron mineralization is managed by an effective bacterial consortium where cometabolism is necessary. Likewise, in the presence of the HPBCD solution, diuron biodegradation assays showed the almost complete disappearance of diuron at the end of the experiment when the bacterial consortium was applied, only 6.85% of the initial amount remaining.

Comparison of the Cyclodextrin-Based Bioremediation with Other Diuron-Degrading Technologies. *A. sulfonivorans* (=*Arthrobacter* sp. N2) was isolated and

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Table 3. First-Order Diuron Mineralization Kinetic Parameters for *Arthrobacter sulfonivorans*, *Variovorax soli*, and the Two-Strain Consortium, in the Absence or Presence of Cyclodextrin[a]

diuron degrader	lag phase (days)	mineralization rate ($days^{-1}$), $K \times 10^2$	overall extent of ^{14}C mineralization (%)	diuron (% at the end of expt)	3,4-DCA (% at the end of expt)
A. sulfonivorans	120.22 ± 0.99	5.32 ± 0.21	6.86 ± 0.84	60.52 ± 1.15	8.51 ± 0.55
V. soli	97.41 ± 1.01	4.81 ± 0.52	5.22 ± 0.88	38.78 ± 0.99	0.61 ± 0.05
bacterial consortium	12.53 ± 0.88	28.1 ± 1.1	45.25 ± 2.27	21.73 ± 0.91	0.00 ± 0.03
A. sulfonivorans + HPBCD solution	28.33 ± 1.03	6.42 ± 2.52	8.55 ± 0.56	22.81 ± 0.02	10.26 ± 0.02
V. soli + HPBCD solution	194.13 ± 2.25	45.6 ± 6.1	57.87 ± 1.21	24.34 ± 1.84	0.00 ± 0.01
bacterial consortium + HPBCD solution	7.62 ± 0.55	308 ± 9	98.67 ± 1.02	6.85 ± 0.44	0.00 ± 0.01

[a]Percentages of diuron and 3,4-DCA at the end of the parallel biodegradation experiments.

characterized by Widehem et al.[3] from a soil by diuron enrichment procedures. These authors observed that this bacterium was capable of metabolizing diuron to the degradation product 3,4-DCA, and this metabolite was produced in stoichiometric amounts; however, no mineralization was observed. On the other hand, Sorensen et al.[24] isolated from a Danish agricultural soil enriched with linuron a *Variovorax* sp. strain SRS16 (=*V. soli*). This was a linuron-mineralizing bacterium able to use the herbicide as a carbon, nitrogen, and energy source. Approximately 60−70% of ^{14}C-linuron was metabolized to $^{14}CO_2$ within 10 days with only low concentrations of 3,4-DCA detected. Satsuma[34] isolated *Variovorax* sp. strain RA8 from Japanese river sediments able to mineralize 70% of linuron and detected a trace amount of 3,4-DCA.

Furthermore, Sorensen et al.[5] constructed a linuron- and diuron-mineralizing two-member consortium by combining the cooperative degradation capacities of the diuron-degrading organism *Arthrobacter globiformis* and the linuron-mineralizing organism *V. soli*. Neither of the strains mineralized diuron alone in a mineral medium, but combined, the two strains mineralized 31−62% of the added [*ring*-U-^{14}C]-diuron to $^{14}CO_2$. These results are similar to those obtained in the present paper when using our two-member consortium, but this two-member consortium reached an almost complete mineralization of [*ring*-U-^{14}C]-diuron (98.67%) without traces of 3,4-DCA when HPBCD was used to enhance diuron bioavailability. In conclusion and on the basis of these results, the use of a HPBCD solution at a very low concentration of only 10 times the diuron equimolar concentration in soil will act as a bioavailability enhancer, accelerating the pass of the diuron desorbing fraction from the soil particle surface to the soil solution and improving microorganism accessibility to the herbicide, being necessary the use of a co-metabolism to reach a complete mineralization of the desorbing fraction, avoiding the presence in the soil solution of its main toxic and also persistent metabolite 3,4-DCA.

The cyclodextrin-based bioremediation technology here described shows for the first time an almost complete mineralization of diuron in a soil system, in contrast to previous incomplete mineralization (>60%) based on single or consortium bacterial degradation. The results obtained indicate the potential use of HPBCD for in situ remediation of pesticide-contaminated soils.

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