Postprint of: Chemosphere (193): 118-125 (2018)

Combined use of microbial consortia isolated from different agricultural soils and cyclodextrin as a bioremediation technique for herbicide contaminated soils.

Villaverde, J.[a,*], Rubio-Bellido, M.[a], Lara, A.[a], Merchan, F.[b], Morillo, E.[a].

[a] *Instituto de Recursos Naturales y Agrobiología (IRNAS-CSIC), Apartado 1052, 41080, Sevilla, Spain*

[b] *Departamento de Microbiología y Parasitología, Universidad de Sevilla, Facultad de Farmacia, C/ profesor García González, 2, 41012. Sevilla Spain.*

*Corresponding author:

E-mail: j.villaverde@csic.es

ABSTRACT

The phenylurea herbicide diuron is persistent in soil, water and groundwater and is considered to be a highly toxic molecule. The principal product of its biodegradation, 3,4–dichloroaniline, exhibits greater toxicity than diuron and is persistent in the environment. Five diuron degrading microbial consortia (C1–C5), isolated from different agricultural soils, were investigated for diuron mineralization activity. The C2 consortium was able to mineralize 78.6% of the diuron in solution, while consortium C3 was only able to mineralize 22.9%. Isolated consortia were also tested in soil slurries and in all cases, except consortium C4, DT_{50} (the time required for the diuron concentration to decline to half of its initial value) was drastically reduced, from 700 days (non–inoculated control) to 546, 351, and 171 days for the consortia C5, C2, and C1, respectively. In order to test the effectiveness of the isolated consortium C1 in a more realistic scenario, soil diuron mineralization assays were performed under static conditions (40% of the soil water–holding capacity). A significant enhancement of diuron mineralization was observed after C1 inoculation, with 23.2% of the herbicide being mineralized in comparison to 13.1% for the control experiment. Hydroxypropyl–β–cyclodextrin, a biodegradable organic enhancer of pollutant bioavailability, used in combination with C1 bioaugmentation in static conditions, resulted in a significant decrease in the DT_{50} (214 days; 881 days, control experiment). To the best of our knowledge, this is the first report of the use of soil–isolated microbial consortia in combination with cyclodextrins proposed as a bioremediation technique for pesticide contaminated soils.

Keywords: diuron; contaminated soil; microbial degrading consortia; bioremediation; cyclodextrin.

1. Introduction

Agricultural or industrial soils possess large and often highly diverse microbial communities that can potentially exhibit many degradative properties. When these capacities are fully and rapidly deployed, organic chemicals can be readily destroyed. However, many synthetic compounds persist for some time in these environments, even though their molecules are biodegradable, and the question has been asked whether inoculation might appreciably enhance the decomposition of these compounds.

In polluted soils in which the time taken for degradation of the chemical pollutant is not important, it is likely that bioaugmentation is not warranted, because the initial microbial population will multiply to destroy the unwanted chemical (Thompson et al., 2005). However, when rapid destruction is important, it may not be appropriate to rely on the natural response of the indigenous community. It is also clear that microorganisms that act on certain pollutants are not present in some sites. A compound that is metabolized by many species will likely encounter one or several species in any given microbial community that is able to transform it (Mrozik and Piotrowska–Seget, 2010). The use of microbial consortia to enhance the efficiency of biodegradation has increased due to their capacity for synergistic metabolism. The metabolic intermediate of one bacteria can be utilized by another for efficient degradation, thus accelerating biodegradation and avoiding potential toxic effects of the metabolites formed (Li et al., 2017; Villaverde et al., 2017).

The use of pesticides constitutes a critical aspect of modern agriculture, and they are absolutely necessary. However, excessive and continuous use of pesticides results in damage to the environment. About 90 percent of applied agricultural pesticides never reach their target organisms. These compounds are dispersed and are frequently detected in air, soil, and water. Moreover, pesticides can easily pass into the tissues of living organisms and give rise to bioaccumulation. Bioremediation methodologies to treat pesticides in soil have gained

considerable attention (Morillo and Villaverde, 2017), and bioremediation has proved to be an efficient tool to decontaminate the pesticides polluted sites in environment.

The harmful effects of phenylurea herbicides have brought about the need to understand the processes that control their behavior and the fate of such herbicides in the soil–water system within agricultural environments. In the soil compartment, phenylurea herbicides can be transformed into metabolites and even fully mineralized through a range of abiotic and biotic processes, although biodegradation is considered to be the main process responsible for their natural attenuation in the environment (Hussein et al., 2015). Phenylurea bioavailability is not considered to be a major issue affecting biodegradation (Sorensen et al., 2003); however, with aging, herbicide bioavailability may decrease due to diffusion into micro– and macropores or sequestration within soil organic matter or mineral matrices (Johannesen et al., 2003).

On those occasions when bioremediation treatments do not achieve satisfactory results, one of the most important factors is attributable to the high adsorption capacity of many pesticides to soil particles. This is mainly due to their low water solubility that limits their availability to microorganisms, which is a potential problem for the bioremediation of contaminated soils. The ability of cyclodextrins (CDs) to form inclusion complexes with a wide variety of hydrophobic guest molecules has been used in agriculture. The ability of CDs to alter the physical, chemical, and biological properties of guest molecules has been used for the preparation of new formulations of pesticides. CDs form complexes with a wide variety of agricultural chemicals including herbicides, insecticides, fungicides, repellents, pheromones, and growth regulators (Ginés et al., 1996; Villaverde et al., 2005; Yáñez et al., 2012). From an environmental point of view, CDs have been proposed as an alternative agent to enhance the water solubility of hydrophobic compounds (Morillo et al., 2004; Villaverde, 2007; Sánchez–Trujillo et al., 2014, 2013; Trellu et al., 2016) and hence, their bioavailability. The mechanism of action is based on the fact that CDs have a hydrophobic cavity within the molecules, in which organic compounds of appropriate shape and size can form inclusion complexes (Gómez et al., 2010). CDs present

several advantages over organic solvents and non-ionic surfactants, such as improved desorption, non-toxicity to microorganisms, greater biodegradability, and negligible sorption to the solid phase. For these reasons, they have emerged as a useful tool for chemical removal from soils.

The objective of this study was to test an effective bioremediation tool based on the inoculation of microbial degrading consortia isolated from agricultural soils with a record of pesticide application (> 10 years), coupled with the use of HPBCD (hydroxypropyl-β-cyclodextrin), which is capable of increasing the bioavailability of the herbicide diuron, in artificially contaminated soils. To the best of our knowledge, the proposed bioremediation technique for the clean-up of pesticide contaminated soils is reported for the first time.

2. Materials and Methods

2.1. Chemicals, cyclodextrin and soil

Technical grade (98%) diuron [N-(3,4-dichlorophenyl)-N,N-dimethylurea] was provided by Presmar S.L. (Seville, Spain). Analytical grade (99%) 3,4-dichloroaniline (DCA) was purchased from Sigma-Aldrich. Radiolabeled [ring-^{14}C]-diuron was purchased from the Institute of Isotopes, Budapest, Hungary (36 mCi mmol^{-1}, purity = 99.9%, and radiochemical purity 100%). HPBCD was supplied by Cyclolab, Budapest, Hungary. An agricultural soil from south-western Spain with a pH of 7, 2.0% $CaCO_3$, 2.1% organic matter and a particle size distribution of 31.6% sand, 53.6% silt and 14.8% clay (silt loam texture) was selected for this study. The sample was taken from the superficial horizon (0–20 cm) and was air-dried for 24 h and passed through a 2 mm sieve. The particle size distribution was measured with a Bouyoucos densimeter; organic matter was measured by $K_2Cr_2O_7$ oxidation; the pH was determined in a 1:2.5 soil/water extract; and the total carbonate content was measured using the manometric method (Demolon et al., 1952).

2.2. Diuron degrader enrichment

The microbial consortia were isolated from five different agricultural soils (S1, supplementary information) that had been managed with pesticides for more than 10 years. 10 g of each soil were added to sterilized 250 mL Erlenmeyer flasks, (autoclave Auster-G, P-Selecta with one cycle at 120 °C, inlet pressure of 103 kPa, for 20 min) containing 50 mL of a mineral salt medium (MSM) spiked with 40 mg L^{-1} diuron as the only source of C and energy. In all tests, diuron was added to the mineral medium together with a solution of micronutrients (NS) (mg L^{-1}): 75.0 $MnCl_2$ $4H_2O$; 37.5 $FeSO_4$ $7H_2O$; 25.0 $SnCl_2$ $2H_2O$; 12.5 $ZnSO_4$ $7H_2O$; 12.5 $Al_2(SO_4)_3$ $18H_2O$; 12.5 $NiCL_2$ $6H_2O$; 12.5 $CoCl_2$ $2H_2O$; 10.0 $CaSO_4$ $2H_2O$; 3.75 KBr; 3.75 KCl; 2.50 LiCl. The medium was also sterilized by autoclaving at 120 ºC for 20 min. Cultures were incubated with orbital shaking (150 rpm) at 30ºC, and every 15 days 10 mL of the culture was transferred to another flask containing the same sterile mineral medium and incubated again.

After four enrichment transfers (60 days), 100 µL of the culture was plated in R2A-agar medium (0.5 g L^{-1}, $MgSO_4$ 7H2O; peptone; casaminoacids; yeast extract; glucose; starch; K_2HPO_4; sodic pyruvate and 20 g L^{-1} agar) and incubated for 72 h at 30 ºC.

Five different isolated consortia which potentially had diuron degrading activity were sown from R2A-agar petri dishes **and subsequently stored in Microbank™ cryovi**als (2 mL microtubes containing R2A **medium and 20 porous spheres of 3 mm diameter) and kept at −80** °C (Villaverde et al., 2012).

2.3. Microbial consortium identification.

C1 consortium strain identification was accomplished by extracting DNA from the liquid culture and amplifying the 16S rRNA genes by PCR using universal oligonucleotide primers (Lane, 1991): 16F27 (annealing at position 8 – 27, *E. coli* numbering) and 16R1488 (annealing

at the complement of position 1511 – 1488). The PCR products were cloned in a T/A vector (PGEMT easy vector from PROMEGA). After colonies were analyzed by PCR, plasmid DNA from selected colonies was purified and the insert was sequenced with T7 and SP6 universal primers. Finally, the 16S rRNA gene sequences (1450 bp) were compared by BLAST searching with the EzBioCloud database.

2.4. Mineralization and biodegradation experiments

Mineralization of ^{14}C-labeled diuron in (1) solution and (2) soil slurry suspension (under continuous mechanical agitation at 120 rpm) or (3) soil in static media was measured (in triplicate) via the evolution of $^{14}CO_2$. All of the microcosm components were sterilized before the assays except the investigated soil.

The mineralization assays were carried out in respirometers (modified 250 mL Erlenmeyers) into which (1) 50 mL of mineral salts medium (MSM) were placed and ^{14}C-ring-labelled (450 Bq per flask) and unlabeled diuron were added to obtain a final concentration of 10 mg L^{-1}; (2) 50 mL of MSM were placed and ^{14}C-ring-labelled (450 Bq per flask) and unlabeled diuron were added to 10 g of soil to obtain a final concentration of 50 mg kg^{-1}; (3), ^{14}C-ring-labelled (450 Bq per flask) and unlabeled diuron were added to 10 g of soil to obtain a final concentration of 50 mg kg^{-1} and MSM was added until 40% of the soil water holding capacity was reached. The herbicide concentration was selected in order to simulate an accidental spillage of herbicide (Rubio-Bellido et al., 2015).

For the experiments with soil (2 and 3), 0.25 mL of a 2.000 mg L^{-1} diuron stock solution in acetone, which also contained ^{14}C-labelled diuron (450 Bq), was initially added to 2.5 g of soil (25% of the total soil used, 10 g). Thereafter, soil was maintained at room temperature under the fume hood during the time necessary to evaporate any traces of acetone measured by weight loss until constant values (approximately 24 h). The remaining 75 % was then added and mixed,

to avoid damage to the soil indigenous microorganisms. The flasks were inoculated with the different consortia (Bioaugmentation) and micronutrients (NS, Biostimulation) and closed with Teflon-lined stoppers before incubation at 20 ± 1°C.

In experiment (3) a solution of HPBCD, with a concentration corresponding to 10 times that of the diuron previously added in soil mineralization experimental flasks (2.14×10^{-2} mmol), was also employed to enhance the herbicide bioavailability. A preliminary experiment was performed in order to determine the HPBCD concentration needed to obtain the most effective diuron extraction from soil, and for this purpose, a range of the extractant concentrations were employed on the soil contaminated with 50 mg kg^{-1} diuron. From these results it was concluded that the extractant capacity at a concentration equivalent to 10 fold the molar amount of the herbicide initially added to soil was similar to those obtained when higher HPBCD concentrations were tested.

In the different mineralization experiments, 1 mL of each microbial consortium with an initial inoculum density of 10^8 colony-forming units (CFU) mL^{-1} was added. In the experiments in solution, (1) and (2), the final density of the inoculum was 2×10^6 CFU mL^{-1}. Non-inoculated microcosm controls were also prepared. Production of $^{14}CO_2$ was measured as radioactivity appearing in the alkali trap of the biometer flasks, which contained 1mL 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 Perkin Elmer, Inc., USA) and the mixture was kept in darkness for about 24 h for dissipation of chemiluminescence. Radioactivity was then measured in a liquid scintillation counter (Beckman Instruments Inc., Fullerton, California, model LS5000TD).

Biodegradation experiments were performed in the same way as the mineralization assays in solution (1), but in this case, only non-radiolabeled diuron was used, and the main metabolite DCA was analyzed at different time points using HPLC (LC-2010A HT Shimadzu). The chromatographic column was a Kromasil C18 reverse phase column, the mobile phase was

acetonitrile and 0.01% formic acid (60:40, v/v), and detection was performed using a photodiode array detector at 220 nm. The HPLC retention time and photodiode array spectra of DCA standard was used to identify this compound.

Enumeration of viable bacteria in this experiment (potential diuron bacterial strains degraders) for each consortium was determined by counting the CFUs. Bacterial enumeration was carried out using 100 µL of the inoculated solution, which were applied on agar–agar plates prepared from a LB medium. CFUs were counted after 48 h.

2.5. Model of mineralization kinetics

All diuron mineralization curves were fitted to the best kinetic model, employing an excel file provided by the FOCUS (2006) workgroup on degradation kinetics, to facilitate kinetic analysis of the degradation of the parent compound using rate curves and the solver tool (Microsoft statistical package). The mineralization curves were fitted to two kinetic models: a simple first–order model (SFO) and a biphasic first–order sequential model (Hockey–Stick, HS) as described in Rubio–Bellido et al. (2015). These models were selected for consideration based on their relative simplicity and their potential to best fit the measured dissipation kinetic datasets for diuron that appear to be monophasic or biphasic (Beulke et al., 2005).

3. Results and discussion

3.1. Diuron mineralization in solution

The diuron mineralization curves obtained for mineralization in solution using the different isolated consortia are shown in Figure 1. All mineralization curves fit to a biphasic first–order kinetic sequential model (Hockey–Stick, HS). Biphasic behavior consists of two sequential first–order curves (K_1 and K_2) (Rubio–Bellido et al., 2015). K_1 can be explained from the point of view of bioavailability, in a scenario where diuron and/or its metabolite molecules produced

would be completely available to degraders. On the other hand, K_2 represents the difficulty of continued diuron mineralization by the different studied consortia, reaching a plateau although not all of the pesticide is mineralized. The possible explanation could be the different toxicity threshold of the microorganisms present in each inoculated consortium towards the highly toxic aniline principal intermediate, DCA, formed in the degradation of diuron, giving rise to different mineralization profiles with different extents of diuron mineralization for each investigated consortium (Villaverde et al., 2017). The accumulated DCA formed could provoke that the diuron catabolic pathway reactions which lead to its mineralization were inhibited (Chakraborty et al., 2017). Key metabolic enzymes are often inhibited by the end products of the pathway they control.

With the aim of confirming such hypothesis, diuron biodegradation assays in the presence of the different investigated consortia were carried out, where DCA and bacterial enumeration were measured at different times, in order to determine the potential toxic effect of the metabolite on the bacterial strains that compose each consortium studied, (Figure 2 and Table 1). A similar DCA formation profile, reaching a plateau about 4 mg L^{-1} DCA concentration after 20 days of experiment, was observed for all inoculations (Figure 2). Simultaneously, a significant increasing in CFU concentration with respect to the concentration initially added ($2x10^6$ CFU/mL) could be determined in all cases just after diuron mineralization reached a plateau (about 36 days), which led to a further increase after 50 days when no progress on mineralization rate for all the consortia was observed (Table 1). It indicates the lack of a toxic effect on the different microbial consortia by the studied metabolite. Therefore, it can be concluded that if the accumulation of DCA provoked the drastic diuron mineralization rate decrease, new diuron catabolic pathways are still active to degrade the herbicide without achieving its mineralization.

The mineralization parameters determined from the kinetic model are shown in Table 2. As seen in Figure 1 and Table 2, different results were found for each consortium. The consortium C3

showed the lowest diuron mineralization capacity of 22.9%, while C2 showed the best mineralization results, both in the extent of mineralization, 81.6% and with regard to the DT_{50}, 29.7 days. It is also worth noting that significant mineralization was observed for all the isolated consortia, showing the capacity of the investigated consortia for diuron mineralization. Mineralization of pesticides is very difficult for a single isolate and consortia of bacteria are often required for complete degradation (Bhatt et al., 2007). It is likely that 100% herbicide mineralization was not achieved by any of the consortia, which is explained based on the two categories of transformations that may occur. In the first, the biodegradation provides C and energy to support growth, and the process therefore is considered as growth-linked. In the second category, biodegradation is not linked to multiplication as for instance, in scenarios where the main C source is found in a very low concentration, such as in aquifers, groundwater, etc. (Wang et al., 2015; Sorensen et al., 2007), and C is only used to obtain energy in order to maintain biomass activity or when the pollutant is cometabolised. In our case the most probable scenario would be growth-linked transformation, since the concentration employed in the mineralization assays was 10 mg L^{-1} similar to the concentration found in the soil solution in a contaminated soil (50 mg kg^{-1}). This scenario would be in agreement with the total extent of mineralization observed, where part of the ^{14}C-diuron will be incorporated to biomass as intermediates but not mineralized, as previously commented (Alexander, 2000).

3.2. Diuron mineralization in soil suspension

The aim of these experiments was to determine the effectiveness of bioaugmentation with each isolated consortium on diuron mineralization in soil. The soil chosen was that from which consortium C5 was isolated. The endogenous soil flora was evaluated for diuron mineralization (control, Fig. 3), and the global extent of mineralization was only 7.35%. This result indicated the need for bioaugmentation in the case of diuron contamination of this soil.

Diuron mineralization curves obtained in MSM medium and in the presence of micronutrients (NS), after the inoculation of the different microbial consortia are shown in Figure 3. All consortia were tested, except C3, which was not used as it showed the worst percentage of diuron mineralization in solution (Fig. 1). Mineralization kinetic parameters obtained after modelling the data from Figure 3 are shown in Table 3. For three consortia, C5, C2, and C1, the DT_{50} was significantly reduced from 700 days (control) to 546, 351, and 171 days, respectively, but DT_{50} was not reduced on inoculation with consortium C4. When the soil was inoculated with C5, the consortium obtained from the same soil, the best mineralization results were not observed, although a more reduced competition with endogenous flora and acclimation period would be expected. Bioaugmentation is still considered to be a procedure that has unpredictable outcomes. The reason for this is that many abiotic and biotic factors affect its final result. Among the biotic factors, the most important seem to be the interactions between autochthonous and inoculated microorganisms such as predation and the competition for nutrients and niches (Cycon et al., 2017). From the good results observed in bioaugmentation with some of the isolated consortia it can be concluded that the specific diuron degrading strains within each isolated consortium are quite specific, and when this herbicide is the main source of C, no competition with the rest of the soil flora exists (Singer et al., 2005), except in C4, where its flora was not able to acclimatize to the endogenous flora of the soil. Besides, the biodegradation process is considered to be microorganism dependent, which means that the conditions in soil would be probably more unfavorable for the consortium C5 (from the investigated soil) than for consortia C1. Natural conditions (e.g. temperature) are difficult to control to maintain optimal biodegradation, since the enrichment process was quite far from realistic conditions in soil. In summary, from diuron mineralization in the slurry system it can be concluded that the consortium C1 would be the best choice for soil bioaugmentation in diuron contaminated agricultural soils.

The global extent of diuron mineralization in the presence of the studied soil was always lower than that determined in solution. It is widely accepted that sorption of pollutants to soil particles may affect biodegradation/dissipation by modifying chemical bioavailability (Chung and Alexander, 2002; Huesemann et al., 2003; Crampon et al., 2014; Reid et al., 2013; Guo et al., 2016). Protection from microbial attack may arise from the formation of bound residues (covalent interactions between the compound and soil particles), reducing bioaccessibility to the micropores (Kah et al., 1993). It should be noted that during the mineralization assays (100 days) a diuron sorption equilibrium is reached and the aging process will also occur. Semple et al. (2007) stated that bioavailability can be used as a descriptor for the rate and extent of biodegradation in the bioremediation of organic contaminants in soil. Feng and Boyd (2005) emphasized that the contact time between contaminants and soil particles (aging) is a critical factor influencing the bioavailability of organic compounds. Aging increases the sorption of the pollutant in the soil because the chemical has more time to enter organic or mineral matrices and, therefore, to sorb into microporous material (Villaverde et al., 2009; Morillo et al., 2014).

3.3. Diuron mineralization in soil: Static conditions

Most of the diuron degrading consortia isolated from different agricultural soils were effective for diuron bioremediation in solution and in soil suspension, especially, in the case of the C1 consortium with which the best mineralization results were obtained, although significantly reduced effectiveness in the soil slurry system was observed (Table 3). Static mineralization assays were performed in order to examine the effectiveness in a more realistic scenario. The bioavailability of the herbicide should be significantly reduced because contact between the molecules of diuron and the soil particles is increased as a result of an increase in the soil:solution ratio. The extent to which degradation occurs is often used as an indicator of chemical bioavailability in soil. An increase in diuron bioavailability to microorganisms is considered to be one of the main causes of higher mineralization (Giaccomazzi and Cochet, 2004). As fungi and many bacteria have diameters greater than 1000 nm, and no free-living

organism is smaller than 100 nm, a molecule within these smaller nanopores is not available for metabolism, so long as it retained within the soil pores and does not diffuse into larger pores inhabited by microorganisms (Pignatello and Xing, 1996).

Soil diuron mineralization curves were obtained in the non-sterilized soil using biostimulation with nutrients. The non-treated control, bioaugmentation with consortium C1 and/or application of HPBCD as bioavailability enhancer are shown in Figure 4. HPBCD is broadly recognized as an environmentally friendly tool for organic pollutant bioremediation because of its capacity to form inclusion complexes between hydrophobic molecules and its hydrophobic cavity, which increases their hydrosolubility and makes them more bioavailable for biodegradation (Morillo et al., 2012). In the present study, when HPBCD solution was applied a significant improvement in the extent of mineralization could be observed (27.3%) as well as a decrease in the DT_{50} (745 days), in comparison to the soil without treatment (Table 4). HPBCD application made that a higher amount of diuron was bioavailable, thus accelerating its biodegradation (Villaverde et al., 2013).

When bioaugmentation was applied using C1, the most effective diuron degrading microbial consortium tested in the slurry system, a significant enhancement was also observed compared with that mediated by the endogenous flora, reaching 23.2% of global mineralization, with a particularly noteworthy decreasing in the DT_{50} (355 days). Jacques et al. (2007) evaluated the capacity of a defined microbial consortium (formed by five different specific degrader strains) isolated from PAHs contaminated farmland to mineralize four different concentrations of anthracene, phenanthrene, and pyrene in soil. For pesticides, Lopes et al. (2013) assessed the potential of microbial inoculation to reduce soil molinate contamination in paddy field soils, and Lima et al. (2009) evaluated a potential clean-up tool based on bioaugmentation with specific microbial atrazine degraders in a soil contaminated with an atrazine commercial formulation.

With the aim of determining potential pesticide microorganism degraders in C1, the components of the inoculum were identified by 16S rRNA gene analysis. Three different strains were identified belonging to the genera *Pseudoxanthomonas* and *Bacillus*: *Pseudoxanthomonas indica*, *Bacillus anthracis* and *Bacillus cereus*. Ma et al. (2014) isolated a new imidacloprid degrading bacterium, identified as *Pseudoxanthomonas indica* by 16S rRNA gene analysis. This isolate was able to degrade 70.1% of the insecticide in contaminated soil in six days. The Bacillus species *Bacillus cereus and Bacillus spp1* degraded diuron by 21% and 19% of the initially applied concentration, respectively, after 35 days of incubation in liquid culture media (Ngigi et al., 2011).

The effect of HPBCD application in combination with C1 inoculation in the soil is also shown in Figure 4. With these treatments, the extent of mineralization was 42.2%, and a large decrease in DT_{50} was also observed (214 days). HPBCD provoked a substantial improvement in diuron mineralization due to the formation of an inclusion complex with diuron, increasing its solubility, and hence, its bioavailability for the microbial degrader consortium (Villaverde et al., 2013). The combination of bioaugmentation with a bioavailability enhancer, such as cyclodextrin or surfactants, increases the likelihood of success (Odukkathil and Vasudevan, 2013). Garon et al. (2004) used fungal strains and cyclodextrins in order to degrade fluorene and optimize fluorene bioavailability and degradation in soil, and the results of that study indicated that *A. cylindrospora* and maltosyl–cyclodextrin could be used successfully in fluorene bioremediation systems. Simpanen et al. (2016) investigated the bioremediation of 16 PAHs in historically creosote–contaminated soil using both laboratory and field–scale experiments, and they found that nutrient amendments and the circulation of methyl–BCD solution improved soil microbial biodegradation. But, as far as we know, no works have reported the use of cyclodextrins and bioaugmentation for pesticide bioremediation.

4. Conclusions

Five diuron degrading microbial consortia were isolated from five different agricultural soils that had been managed with herbicides for at least ten years. Inoculating specific degraders obtained from soil enrichment back to the contaminated environment still remains problematic because of several factors, being bioavailability one of the main limitations which affect the *in situ* bioremediation process. The capability of such microbial consortia to mineralize the herbicide diuron was explored in solution, in soil slurries and in a more realistic scenario using soil static systems.

In this work, the combination of bioaugmentation using different diuron degrader consortia and biostimulation (essential nutrients and a bioavailability enhancer, cyclodextrin) resulted in a successful strategy to accelerate soil diuron bioremediation. Both indigenous and exogenous microorganisms benefited greatly from biostimulation using a nutrient solution. The bioavailability enhancer, HPBCD, provoked a substantial improvement in diuron mineralization due to the formation of an inclusion complex with diuron, which increased its solubility and hence its bioavailable fraction after application.

In the more effective consortium (C1), three different bacterial species were identified by 16S rRNA gene analysis, which had been previously reported as diuron degraders. Currently, new molecular studies are being performed with the aim of determining the presence of related diuron–genes that encode the main enzymes involved in phenylurea herbicide metabolism in each of the studied consortia to help further elucidate the diuron biodegradation pathway in different environmental scenarios.

Acknowledgments

The authors are indebted to Presmar S.L. for providing the technical grade diuron. This work was supported by the Spanish Ministry of Economy and Competitiveness (co–funded by the Fondo Europeo de Desarrollo Regional, FEDER), under the research project CTM2013–42599–

R and Junta de Andalucía Proyect RNM 894, which made possible the recruitment of Dr. Marina Rubio Bellido.

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Table

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Table 1. Colony-forming units (CFUs) concentration determined for each isolated microbial consortium after 36 and 50 days of diuron biodegradation experiment.

Microbial Consortia	CFU mL^{-1} (36 days)*	CFU mL^{-1} (50 days)*
C1	$5.3x10^7$	$5.7x10^7$
C2	$3.3x10^7$	$1.7x10^8$
C3	$4.3x10^7$	$1.8x10^8$
C4	$4.0x10^6$	$4.3x10^6$
C5	$1.7x10^7$	$1.8x10^7$

** CFU mL^{-1} at the beginning of the experiment: $2.0x10^6$.*

Table

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Table 2. Kinetic parameters (*) obtained from diuron mineralisation in solution (100 days) after inoculation with different microbial consortia.

Microbial consortia Inoculation	Kinetic model	K_1 (day^{-1})	K_2 (day^{-1})	tb (days)	Acclimation period (days)	DT_{50} (days)	Extent of mineralization (%)
Control (non inoculated)	SFO	$0{,}5\ 10^{-7}$	-	-	-	40387	0,34
C1	HS	$2{,}3\ 10^{-2}$	$4{,}0\ 10^{-4}$	24,9	24,9	30,4	65,0
C2	HS	$7{,}1\ 10^{-2}$	$2{,}1\ 10^{-2}$	39,4	25,2	29,7	81,6
C3	HS	$2{,}0\ 10^{-2}$	$5{,}0\ 10^{-4}$	31,1	18,3	955	22,9
C4	HS	$1{,}0\ 10^{-1}$	$9{,}6\ 10^{-3}$	52,1	30,0	36,6	83,2
C5	HS	$2{,}4\ 10^{-2}$	$6{,}1\ 10^{-3}$	50,4	21,2	49,8	65,4

*(*) K : mineralization rate **constants.***

tb : time at which rate constant changes.

***DT_{50}** : time required for the concentration to decline to half of the initial value.*

Table
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Table 3. Kinetic parameters (*) obtained from Diuron mineralization in soil suspension after inoculation with different microbial consortia.

Microbial consortia Inoculation	Kinetic model	K_1 (day^{-1})	K_2 (day^{-1})	tb (days)	Acclimation period (days)	DT_{50} (days)	Extent of mineralization (%)
Non-inoculated (Control)	SFO	$6,5\ 10^{-4}$	-	-	68,72	700	8,35
C1	HS	$7,9\ 10^{-4}$	$5,45\ 10^{-3}$	47,65	47,4	171	37,0
C2	HS	$1,42\ 10^{-3}$	$3,23\ 10^{-3}$	79,9	70,0	351	15,2
C4	SFO	$6,9\ 10^{-4}$	ND	ND	ND	1000	6,07
C5	HS	$9,2\ 10^{-4}$	$1,65\ 10^{-3}$	71,2	53,15	546	17,9

(*) K: mineralization rate constants.
tb : time at which rate constant changes.
***DT_{50}** : ttime required for the concentration to decline to half of the initial value.*

Table
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Table 4. Diuron mineralization kinetic parameters (*) in static soil (40% soil water holding capacity) and after inoculation with the C1 consortium and/or HPBCD application.

Treatment	Kinetic model	K_1 (day^{-1})	K_2 (day^{-1})	tb (days)	Acclimation period (days)	DT_{50} (days)	Extent of mineralization (%)
Soil	HS	$8,0\ 10^{-4}$	ND	ND	8,4	881	13,1
Soil + HPBCD	HS	$5,4\ 10^{-3}$	$4,0\ 10^{-4}$	35,6	10	745	27,3
Soil + C1	HS	$3,4\ 10^{-3}$	$4,6\ 10^{-4}$	60,4	15	355	23,2
Soil+ C1 + HPBCD	HS	$0,8\ 10^{-3}$	$6,2\ 10^{-3}$	63,2	12	214	42,2

*(*) K : mineralization rate **constants**.*
tb : time at which rate constant changes (HS).
***DT_{50}** : time required for the concentration to decline to half of the initial value.*

Figure captions.

Figure 1. Diuron mineralisation curves in solution in the presence of the different investigated microbial degrading consortia: C1 (■), C2 (●), C3 (♦), C4 (▲), C5 (x), and in non-inoculated control (+). Solid lines show model fitting to the experimental results (symbols).

Figure 2. 3,4-dichloroaniline (DCA) formation curves in solution in the presence of the different microbial diuron degrading consortia: C1 (■), C2 (●), C3 (♦), C4 (▲), C5 (x), and in non-inoculated control (+).

Figure 3. Diuron mineralisation curves in non-sterilised soil suspension in the presence of the investigated microbial degrading consortia: C1 (■), C2 (●), C4 (▲), C5 (x), and in non-inoculated control (+). Solid lines show model fitting to the experimental results (symbols).

Figure 4. Diuron mineralisation curves in non-sterilised contaminated soil after application of different treatments: non-treated control (+), bioavailability enhancer, HPBCD (▲), degrading microbial consortium, C1 (■) and combined HPBCD + C1 (■). Solid lines show model fitting to the experimental results (symbols).

Figure

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Figure 1.

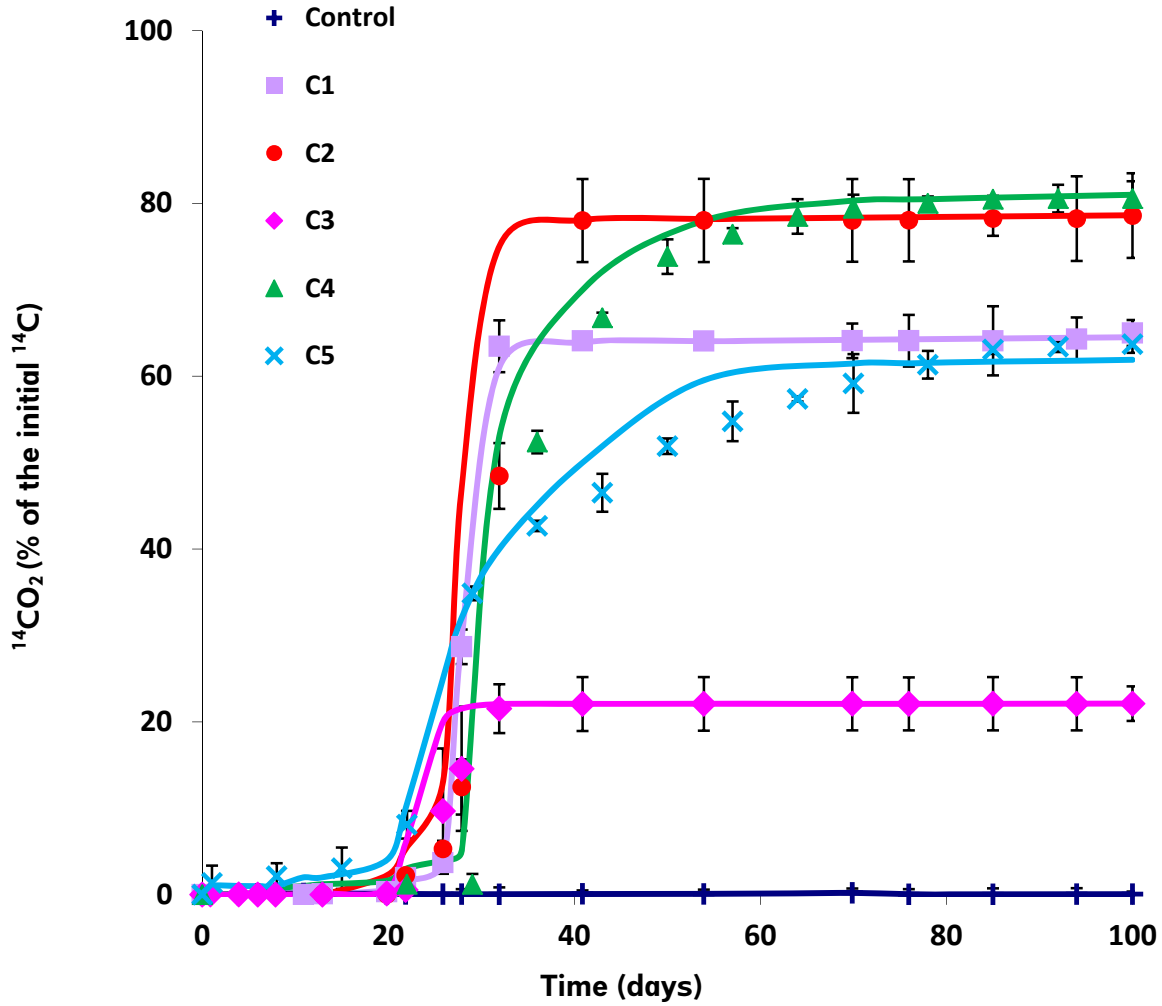

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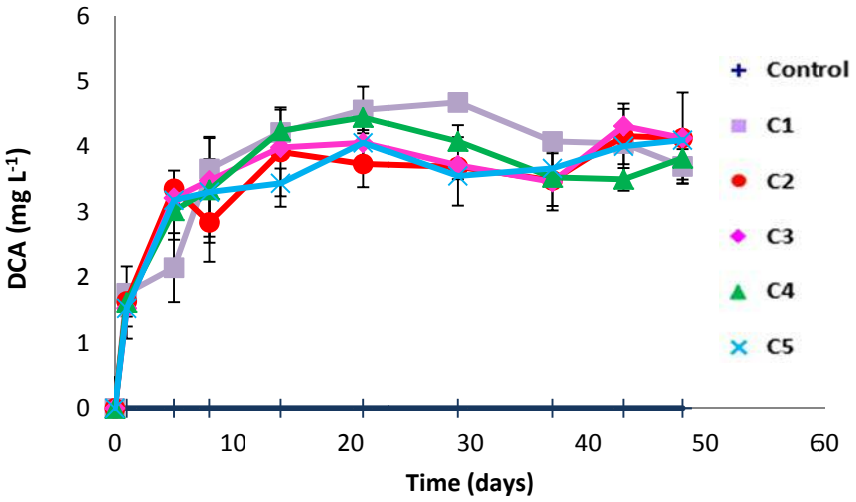
Figure 2.



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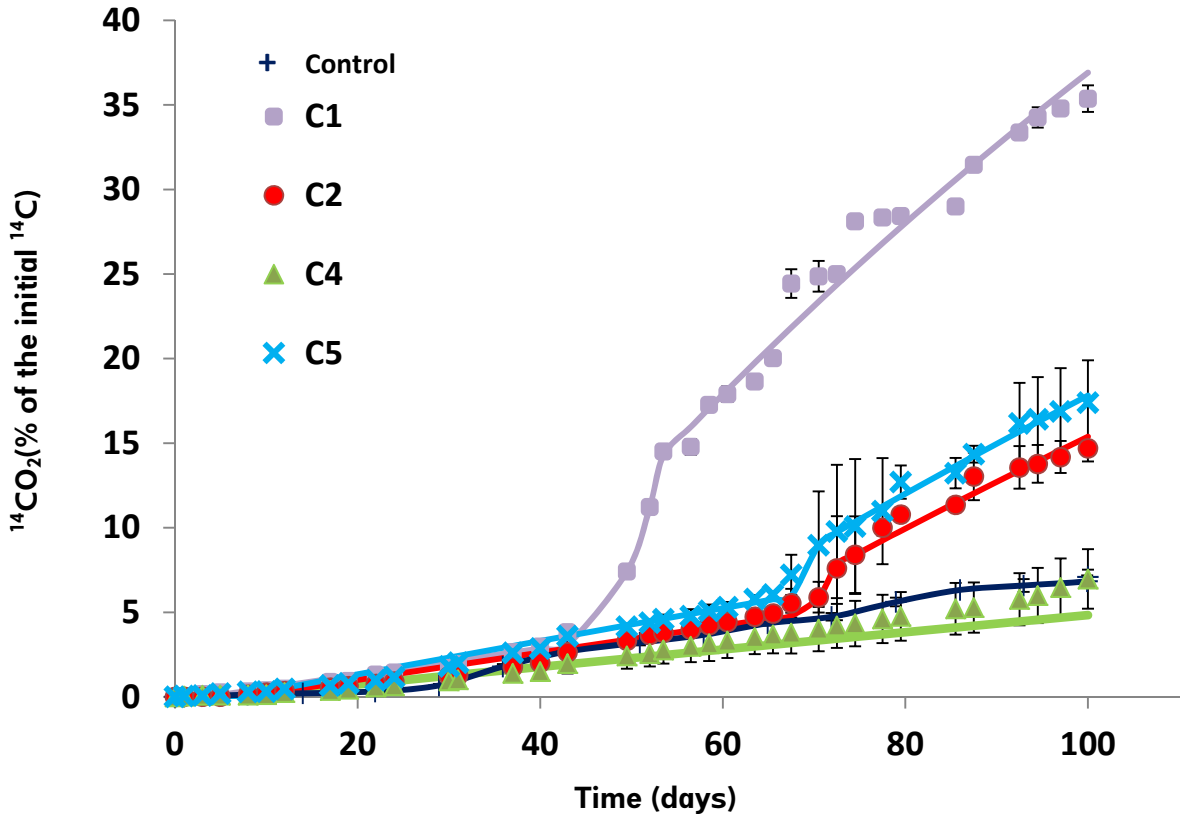

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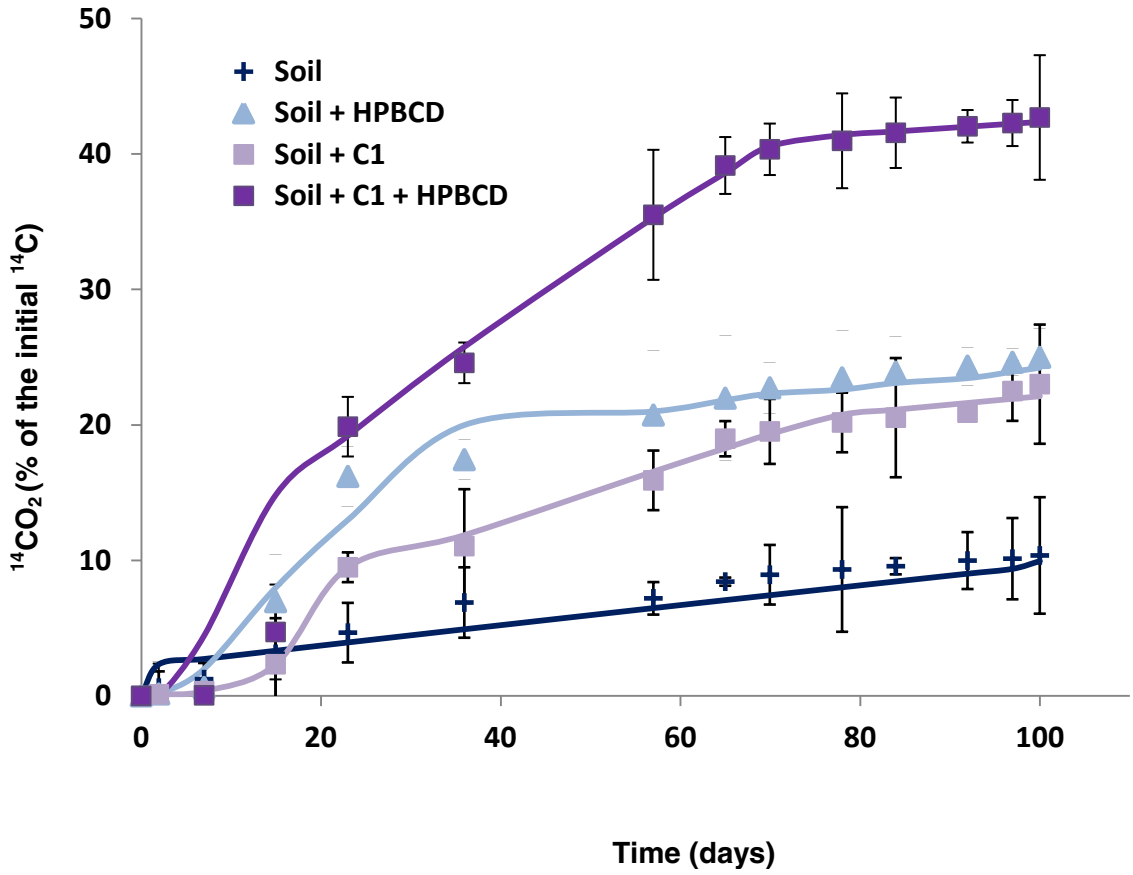

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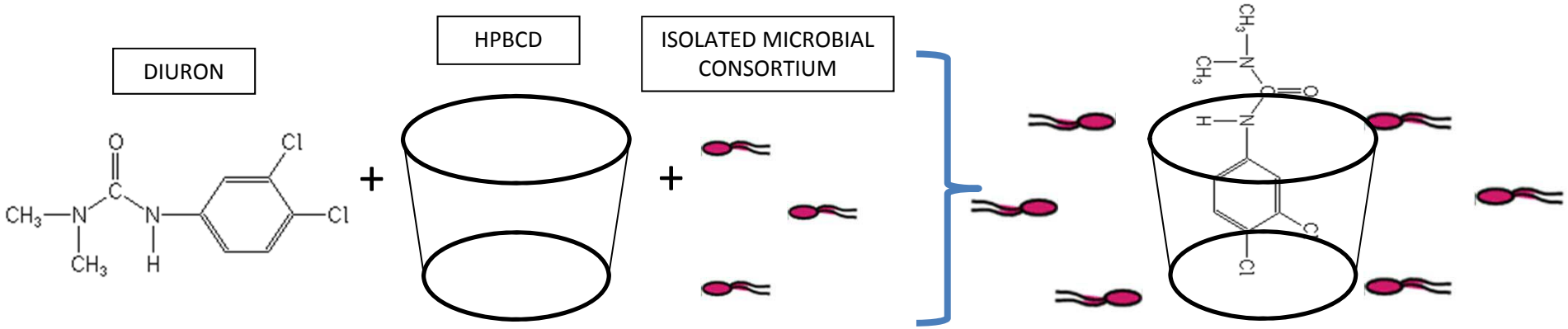
Author(s):	Villaverde, J.,*, Rubio-Bellido, M., Lara, A., Merchan, F., Morillo, E
Format:	American English
Style guide:	Chemosphere at http://www.elsevier.com/journals/chemosphere/0045-6535/guide-for-authors

Graphical Abstract

Primary goal: Accelerated Diuron mineralisation



Solution **Soil suspension** **Soil (40% WHC)**