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Chemosphere 68 (2007) 259–265
CHEMOSPHERE
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Dissipation of 2,4-D in soils of the Humid Pampa region, Argentina: A microcosm study

Luciano J. Merini, Virginia Cuadrado, Cecilia G. Flocco, Ana M. Giulietti *

Microbiología Industrial y Biotecnología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires Junín 956 (1113), Ciudad Autónoma de Buenos Aires, Argentina

Received 11 August 2006; received in revised form 8 January 2007; accepted 8 January 2007
Available online 21 February 2007

Abstract

Phenoxy herbicides like 2,4-dichlorophenoxyacetic acid (2,4-D) are widely used in agricultural practices. Although its half life in soil is 7–14 d, the herbicide itself and its first metabolite 2,4-dichlorophenol (2,4-DCP) could remain in the soil for longer periods, as a consequence of its intensive use. Microcosms assays were conducted to study the influence of indigenous microflora and plants (alfalfa) on the dissipation of 2,4-D from soils of the Humid Pampa region, Argentina, with previous history of phenoxy herbicides application. Results showed that 2,4-D was rapidly degraded, and the permanence of 2,4-DCP in soil depended on the presence of plants and soil microorganisms. Regarding soil microbial community, the presence of 2,4-D degrading bacteria was detected even in basal conditions in this soil, possibly due to the adaptation of the microflora to the herbicide. There was an increment of two orders of magnitude in herbicide degraders after 15 d from 2,4-D addition, both in planted and unplanted microcosms. Total heterotrophic bacteria numbers were about 1×10^8 CFU g^{-1} dry soil and no significant differences were found between different treatments. Overall, the information provided by this work indicates that the soil under study has an important intrinsic degradation capacity, given by a microbial community adapted to the presence of phenoxy herbicides.

Keywords: 2,4-D; Argentinean Humid Pampa; Dissipation; Humic acids; Microcosms; Rhizosphere

1. Introduction

The so-called Humid Pampa region (Pampa Húmeda) is prime agricultural land in Argentina, characterized by its well drained soils, with adequate pH and inorganic nutrient contents and also rich in organic mater. Intensive agricultural practices are carried out in this region. Plots are chiefly used with an annual rotation scheme of crops such as corn, soybean, and wheat. Pastures for feeding cattle are cultivated as well. In the last two decades, the implementation of the ''no-till system'' (seeding directly on the previous crop residue, without ploughing or disking) became a widespread agricultural practice in this region. Some of the advantages of this practice include seeding on a firm seedbed and reduced labor requirements. However, this practice implies the use of agrochemicals, like herbicides, which became an irreplaceable tool for pre- and post-emergent weed control (Bollich et al., 1988; Street and Kurtz, 2002).

Glyphosate (*N*-phosphonomethylglycine), atrazine and 2,4-D (2,4-dichlorophenoxyacetic acid) are among the most used herbicides in cultivable Argentinean lands. The phenoxy herbicide 2,4-D and its derivatives (i.e. esters, amine salts) are intensively used to control broad leaf weeds in pastures, cotton, tobacco, corn, sugar cane and rice among other cultures (UNESP – Brasil, 1997; Crespín et al., 2001) and also to treat non-agricultural fields that will be used thereafter for planting crops. Particularly in the Pampa Húmeda region, 2,4-D is applied mainly in pasture fields (total area 3.4×10^6 ha; 0.4 l ha^{-1} of 2,4-D a.i. 100%)

* Corresponding author. Tel.: +54 11 964 8269/70; fax: +54 11 4964 8200x8377.
E-mail address: agiule@ffyb.uba.ar (A.M. Giulietti).

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doi:10.1016/j.chemosphere.2007.01.012

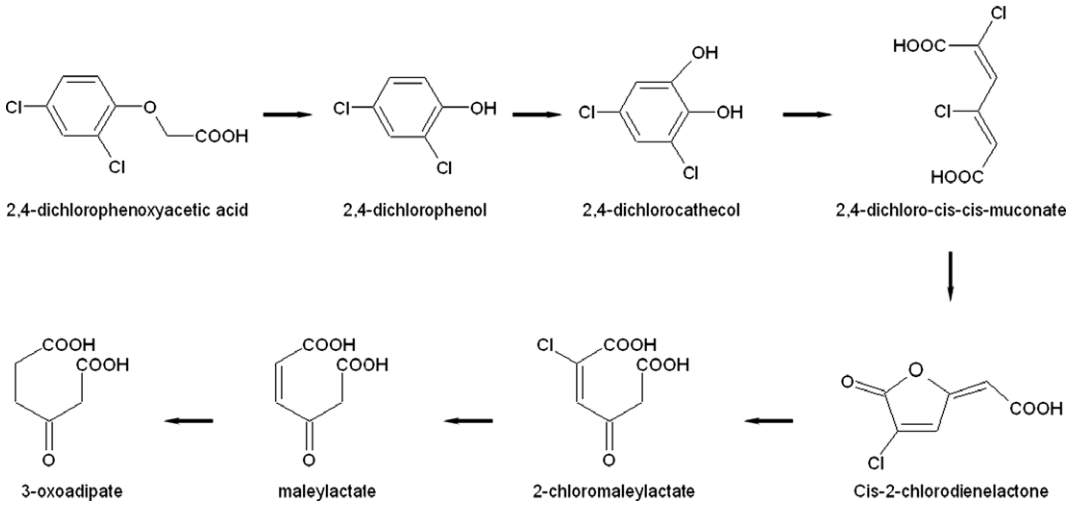

Fig. 1. Bacterial biodegradation pathway of 2,4-D (adapted from Young and Oh, 2006).

(CREA, 2005). In order to extend their half-life and improve their performance, these phenoxy herbicides are usually formulated as inorganic or amine salts, or as esters, and delivered in the form of emulsionable or soluble concentrates. Once in the soil, the derivatives are hydrolyzed to the free acid form, which exerts the herbicide action. Afterwards, 2,4-D is converted into 2,4-dichlorophenol (2,4-DCP), which is its first degradation product (Fig. 1).

The degradation of herbicides in soil is often the result of soil microbial activity and usually the agrochemicals do not persist in the environment (Golovleva et al., 1990). There is evidence that after years of agricultural practice the microbial community is enriched in microorganisms capable to degrade the herbicides. As the catabolic genes for degradation of many herbicides, including 2,4-D, are mainly plasmid-encoded, the possibility of horizontal transference can extend the degradation capability to a high proportion of the soil microflora (Top et al., 1998; Dejonghe et al., 2000). In addition, the plants and their root system can enhance the degradation of the herbicides, either by taking an active role in this process or by improving the conditions of the rhizospheric degradation processes (Anderson et al., 1994; Burken and Schnoor, 1996; Feng and Kennedy, 1997; Poner and Leyval, 2003).

Nevertheless, the intrinsic degradation capacity of a defined soil can be surpassed as result of the excessive or repeated use of herbicides (Colleman et al., 2002). For example, the half life of the formulated 2,4-D in soil is 7–14 d (Starrett et al., 2000). However, 2,4-D and its first metabolite 2,4-DCP can remain in agricultural soils for longer periods, due to its intensive use. After this, an extended soil/herbicide contact is produced, as well as an increased risk of run-off (Felding et al., 1995; Starrett et al., 2000) and/or of leaching to groundwater sources (Tindall and Vencill, 1995; Cox et al., 2000, 2001). Due to the deleterious effects of these chemicals on human health and the serious risk that they represent for the wildlife (HSDB, 2005), it is necessary to reduce their input in the environment. For this reason it becomes necessary to use simple, accurate and reasonable inexpensive methods to assess the levels of these compounds in environmental samples, particularly in developing countries like Argentina.

The aim of this work was to study the dissipation of 2,4-D in soils of the Humid Pampa region, Argentina. In order to simulate the field conditions in the lab, this study was performed in microcosms. These assays were conducted in soil samples with and without 2,4-D, with and without plants (alfalfa), and under sterile and non-sterile conditions, which permitted to dissect the influence of abiotic and biotic factors on herbicide dissipation. Total heterotrophic and 2,4-D degrading bacteria populations were assessed in parallel to the analyses of the remaining 2,4-D and 2,4-DCP.

2. Materials and methods

2.1. Chemicals

The herbicide 2,4-D ($\geqslant$98% purity) used for spiking the soils was supplied by Sigma, the analytical standards 2,4-D (99.9% purity) and 2,4-DCP (99.9% purity) by Chem Service. Methanol and ketone (HPLC grade) were obtained from Sintorgan Argentina, acetic acid from Anedra and phosphoric acid from Merck. Nutritive agar and R2A agar medium (Reasoner and Geldreich, 1985) for bacterial cultures were supplied by Oxoid.

2.2. Agricultural data and soil sampling

The agricultural fields selected for this study are located in the proximities of Colón city, in the Humid Pampa region, in the north of Buenos Aires province, Argentine (33°52′23.33″ S 61°08′10.61″ W). The average temperatures in the region are 8–10 °C in winter and 22–23 °C in summer and the rainfall about 1024 mm yr^{-1}. The fields of the area

under study are classified as a Sandy Loam Argiudoll, not salty and well drained. The pH (water) of this soil is 6.24 and the carbon content is 2.1%. The water content at the moment of sampling averaged $10.50 \pm 0.21\%$. These fields have been mainly used as pasture plots for 20 years, with the application of phenoxy herbicides such as 2,4-D and 2,4-dichlorophenoxybutyric acid to control weeds.

For obtaining representative samples, the agricultural plot was divided in a grid and randomly sampled. Samples were collected from the top 20–25 cm of the soil using a stainless steel tube drill. Duplicate composite samples of the soil were taken (with 10 sub samples each, thoroughly mixed), appropriately packed, labeled and transported to the laboratory. Then they were air-dried, sieved with a 5.6 mm mesh and preserved at 4 °C for further analysis. These soil samples were used for the herbicide recovery studies and microcosms experiments.

2.3. Herbicide tolerance assays

Prior to the microcosms experiment, the tolerance of alfalfa plants to the herbicide 2,4-D was assessed in vitro. Alfalfa (*Medicago sativa, L.*) was selected since it is a common pasture planted in the fields under study. Glass flasks (360 ml) were filled with 100 g of soil and four seeds were planted in each microcosm. They were placed in a growing chamber at 25 °C, 50% humidity under 16 h photoperiod (light intensity 500 μM photons $seg^{-1}\,m^{-2}$). After 14 d, plants were watered with 10 ml of different aqueous solutions of 2,4-D, in order to reach the desired concentrations up to 5 mg kg^{-1} (this concentration range comprises the level of 2,4-D used in farming practices which is approximately 1.5–2.0 mg kg^{-1}). Triplicates were set for each treatment. Tolerance parameters (shoot length, biomass and also visual inspection), were evaluated after 10 d from herbicide addition.

2.4. Microcosms assay

Each experimental unit used for this assay consisted of a 360 ml glass flask with screw fitted transparent plastic cap, containing 100 g of soil. In order to separately assess the influence of microorganisms, plants and abiotic factors on the herbicide dissipation, different sets of microcosms were established according to the scheme described in Fig. 2. For microcosms with plants, alfalfa seeds were surface sterilized using bleach/ethanol and soaked overnight in sterile water at 4 °C to synchronize germination. Next day, four seeds were planted in each microcosm and placed in a growing chamber at 25 °C, 50% humidity under 16 h photoperiod (light intensity 500 μM photons $seg^{-1}\,m^{-2}$). Seedlings emerged 2 d after seeding and were grown in the chamber for eight d. At this time, the experiment was started with the addition of 10 ml of a 10 mg l^{-1} aqueous solution of herbicide, in order to reach a final concentration of 1 mg kg^{-1} of soil. Microcosms without herbicide

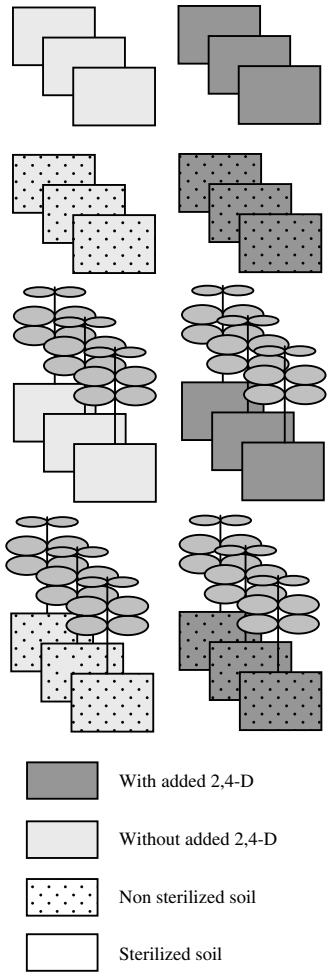

Fig. 2. Scheme of the experimental design of microcosms assay.

were watered with 10 ml of sterile distilled water to keep the moisture in the same range. Herbicide addition as well as watering was carried out under laminar flow, in order to preserve the initial conditions given by the experimental design.

To evaluate the abiotic dissipation, soil was sterilized by autoclaving for 1 h at 121 °C and 101.325 kPa, three times. Between each autoclaving cycle, the flasks remained at 25 °C for 24 h. Triplicate experimental units were set for each sampling time and treatment; sampling was done in a destructive fashion.

Samples were collected at time 0 (T_0) and after 8 h, 1, 5, 15 and 30 d from 2,4-D addition. At every sampling time, triplicate sets of microcosms were separated and samples of soil were collected under laminar flow, for microbiological and chemical analysis (2,4-D and 2,4-DCP content). As all the soil contained in each microcosm with alfalfa plants was in deep contact with its abundant root system, every soil sample coming from planted microcosms was considered as rhizospheric. The non-rhizospheric soil samples were those originated from non-planted microcosms. Soil samples from sterile microcosms were taken and plated into R2A and nutritive agar plates to control their sterility.

2.4.1. Microbiological analysis

Soil samples for analyses of total heterotrophic and 2,4-D degrading bacteria were collected and processed immediately. Total heterotrophic bacteria were plate cultured into R2A agar medium with cycloheximide (50 mg l^{-1}) as fungus inhibitor at 28 °C for 5 d and the number of Colony Forming Units (CFU) per g of dry soil was estimated. Most Probably Number (MPN) technique relying on herbicide degradation was used for 2,4-D degrading bacteria counts (Ka et al., 1994; Ostrofsky et al., 2002). In this way, 7 10-fold serial dilutions of soil samples were prepared and 1 ml of each dilution was inoculated in 3 ml of minimal saline medium with 500 mg l^{-1} of 2,4-D as sole carbon source. The MPN reaction tubes were set by triplicate and cultured on a shaker, at 24 °C for 21 d. After the incubation time, culture media extracts were analyzed by HPLC as described below. The criterion considered for the estimation of 2,4-D degraders number was more than 75% of degradation for positive cultures. Negative dilutions showed no degradation at all. MPN program with 95% confidence intervals was used to analyze the data.

2.4.2. Chemical analysis: herbicide recovery and analysis

The method for herbicide extraction and analysis from soil was adapted from the protocol described by Sutherland et al. (2003). In this way, 15.0 ± 0.1 g of soil were weighted into 250 ml flasks fitted with a Teflon screw cap, to which 30 ml of extraction solvent (ketone/water/acetic acid 80:19:1) were added afterwards. The flasks were tightly capped and shaked overnight at 100 rpm at 25 °C. Next day, they were left to decant for 1 h at 4 °C and then supernatant was transferred to glass vials and centrifuged for 10 min at 2000 rpm. Fifteen milliliters of supernatant were transferred to 25 ml glass vials and evaporated to dryness at 25 °C, under gentle nitrogen stream. The residue was re-suspended in 1 ml of methanol, vortexed for 60 s, filtered through a 0.45 μm Nylon membrane and analyzed by HPLC, as described below.

To analyze the herbicide from the culture medium for MPN assay, 500 μl of it were transferred to an Eppendorf tube and 1 ml of HPLC grade methanol was added. Then, solutions were vortexed and centrifuged at 13 000 rpm for 15 min. The resulting supernatant was filtered through a 0.45 μm Nylon membrane and analyzed by HPLC.

The herbicide and its first metabolite contents in soil extracts and liquid culture media were analyzed by HPLC, using a Jasco HPLC modular device with a LG-980-02 ternary gradient unit, PU-980 intelligent pump equipped with an AS-950 autosampler and UV-975 UV detector, and suited with a 250×4.6 mm RP-18 Xterra column, purchased from Waters. Compounds were analyzed using a methanol/phosphoric acid aqueous solution (pH 2.5) 65:35 mobile phase at a flow rate of 1 ml min^{-1} and detected at 230 nm.

Stock standard solutions of 1 mg ml^{-1} of 2,4-D and 2,4-DCP were prepared in HPLC grade methanol and stored at 4 °C in screw capped amber bottles. These solutions were further diluted with methanol to obtain working solutions at the μg ml^{-1} level.

2.5. Statistical analysis

Homogeneity of variances was tested and one way ANOVA analyses were conducted to find statistical significant differences. Post hoc comparisons were done with Tukey's Honestly Significantly Different (HSD) tests. The software used for the statistical analysis was Statistical Package for Social Sciences (SPSS) for Windows, standard version (release 10.0.1).

3. Results and discussion

The tolerance of alfalfa plants to the herbicide 2,4-D was assessed prior to microcosms experiment. Simple plant tolerance parameters (shoot length, total biomass, and general state of leaves) were evaluated after 10 d from the addition of different concentrations of the herbicide (1–5 mg kg^{-1}). Plants exposed to 1 mg $kg^{-1}$of 2,4-D had the same characteristics than control ones. The only affected parameter was shoot length, with a reduction of about 25% in comparison with control. Concentrations of 2.5 mg kg^{-1} and specially 5 mg kg^{-1} of 2,4-D had deleterious effects on plant growth and physiology: chlorosis, yellow leaves, dry leaves, reduced biomass and shoot length (data not shown). Hence, the chosen 2,4-D concentration for the microcosms assay was 1 mg kg^{-1}, as it was tolerated by alfalfa plants and also it represented concentrations commonly employed in farming practices.

The microcosms experimental design permitted to dissect the influence of abiotic and biotic factors on the herbicide dissipation. Alfalfa plants can improve soil properties, since they promote the symbiotic nitrogen fixation (Aranjuel et al., 2006) and their abundant root system in contact with the soil provides a good niche for the establishment of microorganisms (Walker et al., 2003). Also, as it was indicated before, this plant specie has proved to have bioremediation capability for aromatic compounds, including some herbicides (Wiltse et al., 1998; Liste and Alexander, 2000a,b; Flocco et al., 2002a,b, 2004).

The microcosms were sampled and chemical and microbiological analyses were done in a synchronised way, in order to relate the data obtained. For chemical analysis, modifications applied on the protocol of Sutherland et al. (2003) as was described in Materials and Methods, permitted to improve the recovery of 2,4-D and 2,4-DCP from soils with high humic acids content as well as to reduce the time and costs of the herbicide recovery and analysis.

Using this modified method, it was observed that after 8 h from herbicide application, 100% of the 2,4-D was degraded in the "non-sterile with plants" microcosms. Also, about 80% of the herbicide was degraded in "non-sterile, without plants" and "sterile with plants" treatments (Fig. 3a). This showed that both alfalfa plants and soil microorganisms independently were able to dissipate most

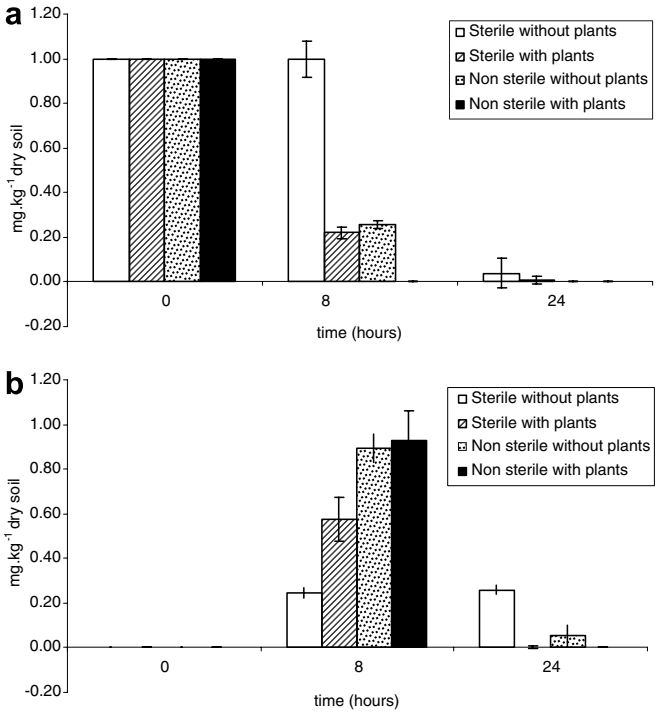

Fig. 3. Contents of 2,4-D (a) and 2,4-DCP (b) of soils corresponding to the different treatments of the microcosms assay. Mean ± SD; $n = 3$. Differences between treatment means were analyzed with ANOVA and Tukey's HSD tests (confidence level 95%).

of the 2,4-D after 8 h. Although the fast dissipation of the substrate (2,4-D) prevented a neat visualization of the events, it is likely that a synergism between plants and microorganisms occurred, since it was observed an enhanced dissipation of the herbicide in the "non-sterile with plants" microcosms (Fig. 3a). When the 2,4-DCP soil content was analyzed at this time, amounts of 0.93 mg kg^{-1} were found in the "non-sterile with plants" and 0.90 mg kg^{-1} in the "non-sterile, without plants" microcosms (93% and 90%, respectively) suggesting a quantitative transformation of the originally added 2,4-D into its first metabolite. About 0.20 mg kg^{-1} of 2,4-DCP were also detected in the control microcosms ("sterile without plants"), which evidenced that abiotic dissipation of the herbicide occurred (Fig. 3b). Indeed, in absence of biotic processes, which are faster and more efficient, the removal of the 2,4-D was almost complete in 24 h. This is in accordance with the results obtained by Cheney et al. (1996), who showed that more than 1000 mg kg^{-1} of 2,4-D can be dissipated in contact with birnessite (δ-MnO_2 used to mimic soil in standard abiotic dissipation tests) in 24 h. In our experiment, the highest metabolite concentration was found in the non-sterile soils, without statistically significant difference between planted and unplanted microcosms ($p > 0.05$). After 1 d from herbicide addition, 2,4-D was completely degraded and for the 7th d, there were no traces of 2,4-D or 2,4-DCP in any treatment, suggesting its degradation to simplest metabolites (Fig. 1).

The faster dissipation of 2,4-D in non-sterile treatments could be attributed to the high population of degrading microflora present in soils with history of use of phenoxy herbicides. This could be also due to the use of the pure free acid form of the herbicide, which makes it more easily accessible for microbial degradation, avoiding the interference of coadjutants present in commercial formulations.

In order to evaluate the microbial response to the herbicide addition and the effect of plants on this process, enumerations of total heterotrophic and 2,4-D degrading bacteria populations were carried out for every treatment and for each sampling time. Total heterotrophic bacteria numbers ranged between 2×10^7 to 5×10^8 CFU g^{-1} dry soil, but not statistically significant differences were found between treatments means and along the time of the experiment (Fig. 4). Nevertheless, the total heterotrophic bacterial counts showed a notorious initial decrease after 1 d from herbicide addition, only in planted microcosms. This can be explained as an acute toxic effect of 2,4-D on the microflora associated with plant roots.

Herbicide degrading bacteria MPN showed an important and significant increase of two orders of magnitude after 15 d from 2,4-D addition, both in planted and unplanted microcosms. It can be seen in Fig. 5 the behavior of 2,4-D degrading population when the herbicide is added to the system, in comparison with the respective controls. The observed increment of the degrading population after 5 d was similar in the presence and absence of plants, with maximum numbers at d 15. The data did show, however, that this population size was significantly higher in planted microcosms as compared to the unplanted soil at the beginning (T_0) and at the end of the study (T_{30}), with numbers 12-fold and 7-fold greater, respectively.

The presence of 2,4-D degrading bacteria was detected even in basal conditions in this soil (from 7×10^1 to 6×10^2), possibly due to the adaptation of the microflora to the contaminant after several years of intensive use of these herbicides in farming practices. The observation of a higher degrading population after 2,4-D addition,

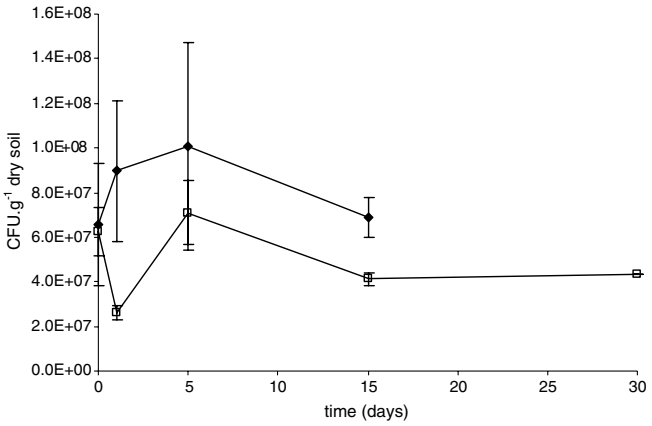

Fig. 4. Total heterotrophic bacteria measured during the microcosm assay. Treatments: (◆) without plants, with herbicide; (□) with plants, with herbicide. Mean ± SD.

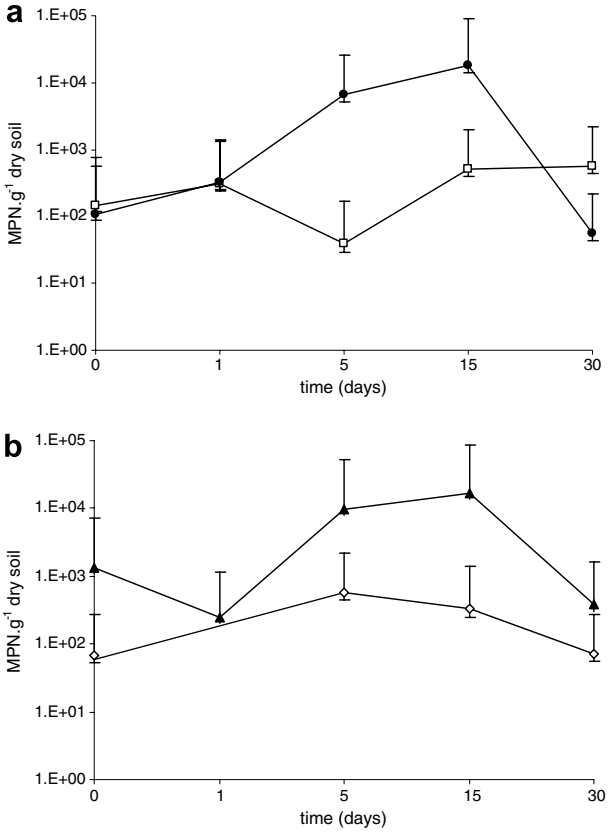

Fig. 5. 2,4-D degrading bacteria MPN measured during the microcosms assay. (a) unplanted microcosms; (b) planted microcosms. Treatments: (□) without plants, without herbicide; (●) without plants, with herbicide; (◇) with plants, without herbicide; (▲) with plants, with herbicide. Bars represent 95% confidence intervals.

without significant changes in the total heterotrophic numbers of bacteria, suggests that a qualitative change occurred in the soil microflora as a consequence of the selective pressure exerted by the herbicide. These results are in accordance with studies carried out with other organic toxic compounds like PAHs (Ringelberg et al., 2001; Flocco et al., 2002b). It is important to point out that the detection of 2,4-D degraders enrichment at d 5 and 15 did not correlate temporally with the herbicide biodegradation. In fact, 100% of the herbicide was already dissipated from the soil before the important increment in the degrader population was detected. This phenomenon was also described by other authors, who reported an enrichment in phenanthrene degraders after most of the compound had already been degraded, both in natural and artificially created rhizospheres (Miya and Firestone, 2000, 2001). The increase of herbicide degrading bacteria numbers in soil after the 2,4-D had been completely depleted, could be ascribed to a better adaptation of the degrading species to soil conditions as a consequence of the herbicide application, which would produce an stimulatory effect either by itself or by its degradation products. Since further metabolites than 2,4-DCP were not assessed, it is possible that other intermediates of the 2,4-D degradation pathway (Fig. 1) still remained in soil after 15 d so that they would provide sources of nutrients or serve as cometabolites for some degrading bacteria species.

As the data did show, the soil with history of phenoxy herbicide application had a proportion of 2,4-D degrading microflora under basal conditions, that was able to degrade rapidly all the herbicide upon its addition. The application of a higher concentration of herbicide, which could possibly not be dissipated so fast, would evidence the biotic factors contribution on herbicide degradation. However, the concentration of herbicide used in this work was based on the aim to reproduce field condition in microcosm systems.

We could analyze the behavior of the indigenous microflora of a soil with history of use of herbicides, and 2,4-D biodegradation was studied in a naturally occurred rhizosphere. Plants, soil microorganisms and abiotic factors could influence the biodegradation process and we could detect their contribution and their interactions. It is important to note that previous reports (Feng and Kennedy, 1997) described also the biodegradation of 2,4-D in the rhizosphere. However, these experiments were conducted with hydroponic cultures of plants and pure cultures of microorganisms, and this does not represent field conditions. Our study was performed with a more realistic model that could predict what would happen in a field bioremediation trial with indigenous microbes and without any genetically modified microorganisms (Top et al., 1998; Dejonghe et al., 2000).

4. Conclusions

Under the experimental conditions assessed in the microcosms assay, the herbicide (2,4-D) was rapidly degraded and the permanence of its first metabolite (2,4-DCP) in soil depended on the presence of plants and soil microorganisms. Biological degradation would be the main component in the dissipation of the herbicide and specific herbicide degrading bacteria population was selected after herbicide addition. Alfalfa plants did show to enhance the 2,4-D degradation under the experimental conditions assessed.

The soils of Humid Pampa Region, under intensive agricultural practices and with history of use of phenoxy herbicides, have an important intrinsic herbicide degradation capacity. This capacity would reduce the harmful effects of 2,4-D over the environment and the human health, as it would prevent the accumulation of the herbicide itself or its degradation products. The high numbers of herbicide degrading bacteria detected by MPN method in these soils reveal the presence of cultivable microorganisms that are potential candidates to be employed in future bioremediation trials, including bioaugmentation strategies.

Acknowledgements

This work was supported by Grant ACCESS (ICA4-CT-2002-10011, European Community) and University of Buenos Aires. Cecilia Flocco was postdoctoral researcher under ACCESS Grant and subsequently researcher from

Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). Virginia Cuadrado and Luciano Merini are fellows from CONICET. The authors are also thankful to F. Flocco, who kindly provided access to soil samples and logistical support for sampling.

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