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Simultaneous Biodegradation and Detoxification of the Herbicides 2,4-Dichlorophenoxyacetic Acid and 4-Chloro-2-Methylphenoxyacetic Acid in a Continuous Biofilm Reactor

Ana Julieta González · María Susana Fortunato · Alfredo Gallego · Sonia Edith Korol

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Abstract The herbicides 2,4-diclorophenoxiacetic and 4-chloro-2-methylphenoxyacetic acids (2,4-D and MCPA) are widely used in agricultural practices worldwide. Not only are these practices responsible of surface waters contamination, but also agrochemical industries through the discharge of their liquid effluents. In this investigation, the ability of a 2,4-D degrading *Delftia* sp. strain to degrade the related compound MCPA and a mixture of both herbicides was assessed in batch reactors. The strain was also employed to remove and detoxify both herbicides from a synthetic effluent in a continuous reactor. Batch experiments were conducted in a 2-L aerobic microfermentor, at 28 °C. Continuous experiments were carried out in an aerobic downflow fixed-bed reactor. Bacterial growth was evaluated by the plate count method. Degradation of the compounds was evaluated by UV spectrophotometry, gas chromatography (GC), and chemical oxygen demand (COD). Toxicity was assessed before and after the continuous process by using *Lactuca sativa* seeds as test organisms. *Delftia* sp. was able to degrade 100 mg L^{-1} of MCPA in 52 h. When the biodegradation assay was carried out with a mixture of 100 mg L^{-1} of each herbicide, the process was accomplished in 56 h. In the continuous reactor, the strain showed high efficiency in the simultaneous removal of 100 mg L^{-1} of each herbicide. Removals of 99.7, 99.5, and 95.0% were achieved for 2,4-D, MCPA, and COD, respectively. Samples from the influent of the continuous reactor showed high toxicity levels for *Lactuca sativa* seeds, while toxicity was not detected after the continuous process.

Keywords Continuous reactors · Detoxification · 2,4-d Herbicide mixtures · MCPA

1 Introduction

2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid(MCPA) are persistent and toxic compoundswith herbicidal properties widely used in agricultural practices for the control of broadleaf weeds in cereal crops worldwide (WHO 2003). In order to improve efficiency in weed control, commercial herbicide formulations usually contain different combinations of these compounds (Smejkal et al. 2001). Therefore, several herbicides such as 2,4-D and MCPA are released together into the environment. Moreover, as

A. J. González
Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Cátedra de Salud Pública e Higiene Ambiental, Junín 956, 1113 Ciudad Autónoma de Buenos Aires, Argentina

A. J. González (✉) · M. S. Fortunato · A. Gallego · S. E. Korol
Univresidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Salud Pública e Higiene Ambiental, Junín 956, Ciudad Autónoma de Buenos Aires 1113, Argentina
e-mail: julietagonzalez@ffyb.uba.ar

phenoxy herbicides have high water solubility and low soil sorption capacity, they can be transported to surface waters from agricultural soils (Önneby et al. 2014). Not only agricultural practices are responsible of surface waters contamination with these toxic compounds, but also agrochemical industries through the discharge of their liquid effluents, as phenoxy herbicides had shown to be refractory to conventional biological treatment processes (González et al. 2006; Marrón-Motiel et al. 2006). Therefore, it is necessary to improve these biological processes in order to prevent contamination of aquatic ecosystems and protect living organisms. A strategy that has generated increasing interest in this field is the use of selected degrading-microorganism in biodegradation processes. Several MCPA-degrading strains have been described in the literature, such as *Alcaligenesdenitrificans*, *Delftiaacidovorans*, and *Sphigomonas* sp. (Tett et al. 1997; Hoffman and Müller 2006; Önneby et al. 2010). Also, microorganisms such as *Ralstoniaeutropha*, *Burkholderia* sp., *Alcaligenesxylosoxidans*, and *Sphingomonas* sp. have shown to be 2,4-D degraders (Don and Pemberton 1981; Tonso et al. 1995; Ka et al. 1994). Particularly the ability of *Delftia* sp. to degrade a broad spectrum of phenoxy herbicides has been described (Müller et al. 1999). This ability could be exploited to the biodegradation of herbicide mixtures. However, few investigations have been conducted to study the biodegradation and detoxification of these mixtures. In the present investigation, the capability of an indigenous 2,4-D-degrading *Delftia* sp. strain previously isolated to degrade MCPA and a mixture of both compounds was studied. The strain was also employed to remove and detoxify both herbicides from a synthetic effluent in a continuous biofilm reactor.

2 Materials and Methods

2.1 Bacterial Strain

The 2,4-D degrading bacterial strain used in biodegradation assays was previously isolated from water samples of a polluted freshwater stream by the enrichment technique in a fed-batch reactor. It was identified as *Delftia* sp. by the 16S rRNA gene sequencing. Selection and identification procedures were described by González et al. (2012).

2.2 Chemicals

The herbicides 2,4-D and MCPA (purity >98%) were purchased from Sigma-Aldrich Co. Herbicide solutions were aseptically prepared by dissolving the necessary amount of each compounds in sterile 0.1 N NaOH.

2.3 Degradation of MCPA and a Mixture of MCPA and 2,4-D by *Delftia* sp.

The ability of the 2,4-D degrading *Delftia* sp. strain to degrade the structurally related compound MCPA and a mixture of both herbicides was assessed in batch reactors.

In order to obtain the inoculum for biodegradation assays, the microorganism was pre-exposed to 50 mg L^{-1} of MCPA as the sole carbon source in Erlenmeyer flasks with 100 mL of minimal medium. The composition of the culture medium was described by Korol et al. (1989). The flasks were incubated in a water bath shaker at 28 °C until the compound disappeared from the culture medium.

Two batch experiments were performed: experiment I, with MCPA as single substrate and experiment II, with a mixture of MCPA and 2,4-D. Both experiments were carried in a 2-L New Brunswick Multigen™aerobic microfermentor (New Brunswick Scientific, N.J., U.S.A.) containing 1.25 L of minimal medium added with 100 mg L^{-1} of MCPA as the sole carbon source, or 100 mg L^{-1} of each herbicide in the case of experiment II. 12.5 mL of the abovementioned culture were used as inoculum to get an initial cell concentration of 10^6 CFU mL^{-1} in biodegradation assays. The incubation temperature was 28 °C. Samples of 10 mL were periodically removed from the reactors to determine the remaining concentration of the herbicides and bacterial growth.

Batch reactors without inoculating were also used as controls in order to assess abiotic losses of the compounds.

2.4 Detoxification of a Synthetic Effluent that Contains a Mixture of MCPA and 2,4-D

The ability of *Delftia* sp. to detoxify a synthetic effluent that contains MCPA and 2,4-D was studied in a downflow fixed-bed biofilm reactor, with polyurethane foam cubes as support material.

Inoculation was carried out with 100 mL of the pre-exposed microorganism in aerobic fed-batch reactors,with effective volume of 1 L, containing the polyurethane foam cubes. The feeding of the reactors

consisted in minimal medium supplemented with 100 mg L^{-1} of each herbicide. These reactors operated during a month in order to promote biofilm formation in the support material.

The design of the continuous reactor was previously described by Gallego et al. (2008). It consisted in a PVC column (100 cm length × 10 cm internal diameter) filled with the polyurethane foam cubes containing the biofilm (Fig. 1). The reactor was continuously fed with a synthetic effluent prepared with free-chlorine tap water supplemented with dipotassium phosphate and ammonium sulfate, as phosphorus and nitrogen sources, respectively, and 100 mg L^{-1} of each herbicide. The synthetic effluent percolates through the support material by gravity. The flow rate was maintained at a constant value of 1 L day^{-1} over the operation period by means of a peristaltic pump, and the hydraulic retention time (HRT) was 5 min. Samples at the inlet and the outlet of the reactor were taken in order to evaluate herbicides, chemical oxygen demand (COD), and toxicity removals.

A second reactor with uninoculated polyurethane foam cubes was used as control reactor. Both reactors operated under environmental conditions.

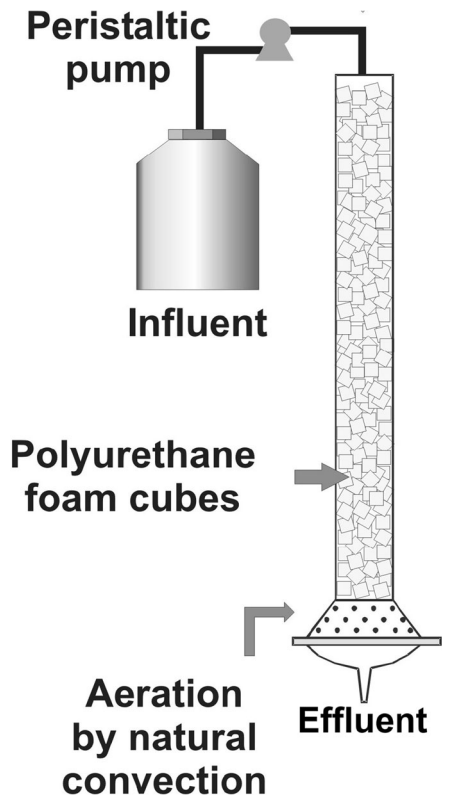

Fig. 1 Scheme of the continuous flow fixed-bed biofilm reactor

2.5 Analytical Methods

Samples from batch and continuous experiments were centrifuged at 4000 rpm and analyzed by UV spectrophotometry (Metrolab UV 1700 Spectrophotometer). The absorbance of the samples was measured at 280 nm. The concentration of MCPA was determined by extrapolating absorbance values in a calibration curve. The biodegradation of the compounds in the mixture was expressed as *percentage of remaining chlorophenoxyacetic acids*, which is the percentage of remaining absorbance with respect to the initial absorbance of the mixture. Bacterial growth in batch experiments was evaluated by the plate count method in triptone soy agar plates, according to APHA (2012).

Batch experiments were carried out in triplicate. For each analytical determination, arithmetic mean and standard deviation were calculated. Percentages of remaining MCPA and remaining chlorophenoxyacetic acids, as well as bacterial counts were plotted in function of time. Bacterial growth curves were analyzed by non-linear regression and fitted to an exponential growth model, using the GraphPad Prism software (version 5.0).

In order to corroborate, the complete degradation of the compounds samples from batch and continuous experiments were also analyzed by gas chromatography-mass spectrometry (GC-MS). The samples were previously filtered through a cellulose acetate membrane with a pore size of 0.45 μm. Filtrates were subjected to a liquid-liquid extraction process with chloroform. Organic extracts were then evaporated to dryness under reduced pressure and redissolved in 1 mL of pyridine. Derivatization was performed with 100 μL of N,O-bis (trimethylsilyl) trifluouoroacetamide (BSTFA). Analysis was carried out on a gas chromatograph Agilent 6890 equipped with a selective mass detector. A HP-5 MS capillary column (30 m length × 0.25 mm internal diameter) was used. Samples of 1 μL were injected in the column at 280 °C and the temperature gradient was the following: from 50 °C to110 °C with an increment of 30 °C min^{-1}, then from 110 °C to 300 °C with an increment of 10 °C min^{-1}, and finally at 300 °C during 6 min. 2,4-D and MCPA standards solutions of 0.5 and 1 mg mL^{-1}, respectively (Accustandard, Connecticut, USA), were used for quantification.

Chemical oxygen demand (COD) was determined before and after biodegradation processes by the colorimetric method of the closed reflux, according to APHA (2012).

Toxicity was evaluated in samples taken at the influent and effluent of the continuous reactor by the standardized bioassay of root elongation of *Lactuca sativa,* according to EPA 600/3–88 (1989). Lettuce seeds were exposed to different sample dilutions on wet filter papers for 120 h at 24 °C ± 2 °C in the dark. Two controls were used: a positive control to evaluate organisms' sensitivity, with zinc sulfate as reference toxic compound, and a negative control with distilled water. Three replicates per dilution and for each control were used. Results were expressed in effective concentration 50 (EC_{50}), concentration which provokes a 50% inhibition of lettuce root elongation compared to the negative control after exposure. EC_{50} values were calculated by plotting the percentage inhibition of root elongation in function of the dilution sample. The dose-response curve was analyzed by linear regression with the GraphPad Prism software (version 5.0).

3 Results

3.1 Degradation of MCPA and a Mixture of MCPA and 2,4-D by *Delftia* sp.

Degradation of MCPA by *Delftia* sp. was studied in batch reactors. Results are shown in Fig. 2. *Delftia* sp. was able to degrade 100 mg L^{-1} of the compound in 52 h, with a lag phase of 8 h. The specific growth rate (μ) was 0.08 h^{-1}. When the biodegradation experiment was conducted with a mixture of 100 mg L^{-1} of MCPA and 100 mg L^{-1} of 2,4-D, the process was accomplished in 56 h and a lag phase of 8 h was observed too (Fig. 3). The specific growth rate (μ) was 0.04 h^{-1}. Chromatography results of samples taken at the end of the biodegradation processes confirm the complete degradation of the compounds (data not shown). The efficiency of the biodegradation process in experiment I was 99.9 and 73.1% expressed as MCPA and COD removal, respectively. In experiment II removals of 99.9 and 99.8% were achieved for 2,4-D and MCPA, respectively, while a reduction of 94.9% of the initial COD was reached after biodegradation process (Table 1).

3.2 Detoxification of a Synthetic Effluent that Contains a Mixture of MCPA and 2,4-D

The use of *Delftia* sp. to remove simultaneously high concentrations of 2,4-D and MCPA (100 mg L^{-1} of each one) from a synthetic effluent was also studied in a continuous biofilm reactor. After 20 days of continuous operation, the reactor reached removal efficiencies of 99.7 and 99.5% for 2,4-D and MCPA, respectively. In addition, a reduction of 95.0% of the initial COD was achieved (Table 2). The maximum COD removal rate was 34.8 g m^{-3} day^{-1}. Chromatography results of samples taken at the outlet of the reactor confirm the complete degradation of the compounds (data not shown). From these results, it can be concluded that *Delftia* sp. was able to degrade the mixture of herbicides in both batch and continuous reactors with similar efficiencies.

In order to evaluate the continuous process, toxicity bioassays were performed. High toxicity levels were observed in the synthetic effluent. EC_{50} in samples taken at the inlet of the reactor was 0.008% *v/v*. Detoxification was successfully achieved after biodegradation process since

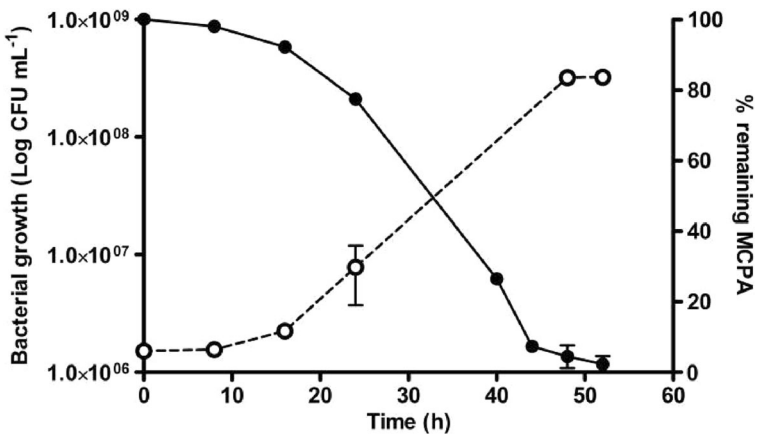

Fig. 2 Degradation of 100 mg L^{-1} of MCPA by *Delftia* sp. in batch reactor. *White circle* denotes bacterial growth; *black circle* symbolizes % remaining MCPA

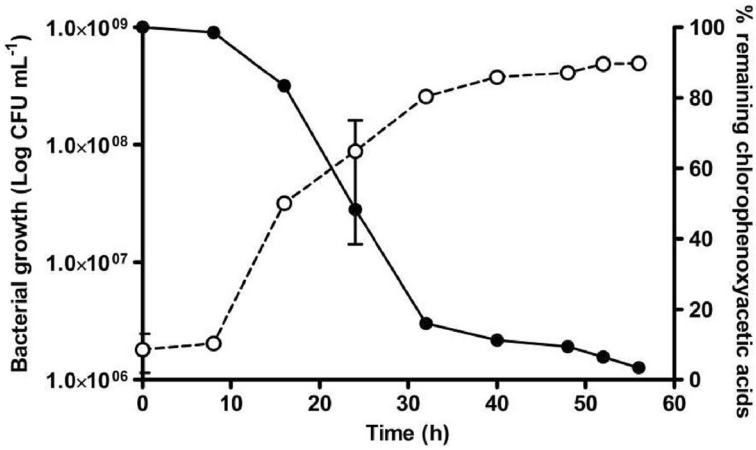

Fig. 3 Simultaneous degradation of 100 of 2,4-D and 100 mg L^{-1} of MCPA by *Delftia* sp. in batch reactor. *White circle* denotes bacterial growth; *black circle* symbolizes % remaining chlorophenoxyacetic acids

toxicity was not detected in samples taken at the outlet of the reactor ($EC_{50} > 90\%$ *v/v*). Results are showed in Fig. 4.

4 Discussion

Studies about the biodegradation of herbicide mixtures are of great environmental importance because commercial formulations employed in agricultural practices usually contain different combinations of these compounds; therefore they are released together into the environment (Smejkal et al. 2001). However, these studies are still scarce. Haugland et al. (1990) studied the degradation of a 2,4-D and 2,4,5-T mixture by a mix culture of a 2,4-D degrading *Alcaligeneseutrophus* strain and a 2,4,5-T degrading *Pseudomonas cepacia* strain. The *A. eutrophus* strain showed little if any 2,4,5-T degradation ability, while the *P. cepacia* strain showed weak activity toward 2,4-D as single substrate. As a result, removals of 2,4-D and 2,4,5-T in the mixture were 45 and 44%, respectively. In addition to the low biodegradation efficiency, the authors observed an accumulation of 2,4-dichlorophenol and 2,4,5-trichlorophenol as degradation products. Conversely, in the present investigation, high degradation efficiency was achieved by using a single *Delftia* sp. strain with the ability to degrade both herbicides in the mixture. Furthermore, no degradation products were detected. Similar results were obtained by Marriott et al. (2000) with a single *Alcaligenesdenitrificans* strain with the ability to degrade the individual compounds 2,4-D and MCPP, and efficiently remove both herbicides in a mixture.

Detoxification of a 2,4-D and MCPA mixture was also studied in a downflow fixed-bed biofilm reactor, with polyurethane foam as support material. This biodegradation experiment was conducted in order to simulate the continuous biological treatment of a synthetic effluent that contains both herbicides. These mixtures could produce serious problems in biological treatment plants (Haugland et al. 1990) due to the persistence and toxicity of the compounds present in the effluent, which inhibit the growth of degrading microorganisms (Wagner and Loy 2002). In this regard, the use of specifically selected microorganisms in biofilm reactors is a strategy that could improve the conventional effluent treatment processes. These reactors have high efficiency in the removal of toxic compounds by virtue of their high biomass concentration and the protective effect of the biofilm (de Mello et al. 2010). However, few studies about the use of biofilm reactors for the biodegradation

Table 1 Biodegradation efficiency in batch reactors

	Experiment I			Experiment II		
Parameter	Initial	Final	% removal	Initial	Final	% removal
MCPA concentration (mg L^{-1})	105.7	0.06	99.9	101.4	0.16	99.8
2,4-D concentration (mg L^{-1})	–	–	–	99.6	0.13	99.9
COD (mg L^{-1})	157.8	42.4	73.1	298	15	94.9

Table 2 Biodegradation efficiency in the continuous biofilm reactor

Parameter	Influent	Effluent	% removal
MCPA concentration (mg L^{-1})	100.8	0.47	99.5
2,4-D concentration (mg L^{-1})	101.2	0.27	99.7
COD (mg L^{-1})	188	9.4	95.0

of chlorophenoxyacetic acid mixtures have been reported in the literature. González et al. (2006) studied the degradation of a mixture of chlorophenoxyacetic and chlorophenoxypropionic acids in a biofilm reactor by microorganisms from a freshwater stream. After 24 days of continuous operation, the reactor reached the complete degradation of 10 μg L^{-1} of MCPA and 10 μg L^{-1} of MCPP. Then, 2,4-D and 2,4-DCP were added to the feeding of the reactor in a concentration of 10 μg L^{-1}.

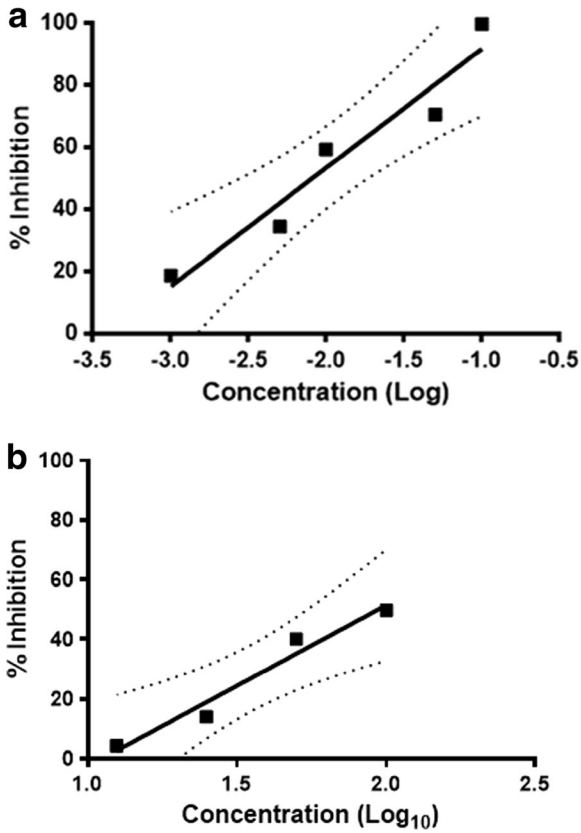

Fig. 4 Root elongation toxicity test with *Lactuca sativa* seeds. Dose-response curves with 95% confidence intervals. **a** Inlet of the continuous reactor, r^2 = 0.9347, and **b** outlet of the reactor, r^2 = 0.9608

Seven days later, the reactor showed a complete degradation of all the compounds. It should be noted that the microorganisms employed by these authors had not been pre-exposed to the compounds. In this investigation, conversely, the use of a specifically selected microorganism previously exposed to the compounds enabled the complete degradation of high concentrations of the compounds in similar periods of time.

It should be noted that degradation of the herbicides does not necessarily ensure the elimination of effluent toxicity. The use of toxicity tests is an important complement to chemical analysis that assesses the presence of toxic metabolites produced during biodegradation (Atlas and Bartha 2002; Arias-Barreiro et al. 2010). For this reason, toxicity bioassays were employed to evaluate the continuous process. The selection of the most appropriate test organism was based on their sensitivity to the compounds under study, in order to ensure the correct evaluation of detoxification. Hence, considering the herbicide properties of 2,4-D and MCPA, *Lactuca sativa* seeds were used as test organisms. Detoxification was successfully achieved after the biodegradation process.

5 Conclusions

A single *Delftia* sp. strain able to degrade the phenoxy herbicides 2,4-D and MCPA as individual compounds could efficiently degrade a mixture of 100 mg L^{-1} of each herbicide in batch reactors. The strain was also employed to detoxify a synthetic effluent that contained the mixture in a continuous biofilm reactor. Detoxification was assessed by the toxicity bioassay of root elongation of *Lactuca sativa*. High efficiencies of 2,4-D, MCPA, and COD removals were achieved in the continuous reactor. The absence of biodegradation products that might confer toxicity to the treated effluent was confirmed not only by chromatographic techniques but also by means of toxicity assays using *Lactuca sativa* seeds as test organisms, considering the herbicidal properties of the compounds under study. The use of herbicide degrading-microorganisms in biofilm reactors is a promising biotechnological tool for the removal of these toxic compounds from liquids effluents.

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