**FIXED-BED BIOLOGICAL BARRIER COUPLED WITH
ELECTROKINETICS FOR THE IN-SITU
ELECTROBIOREMEDIATION OF 2,4-
DICHLOROPHENOXYACETIC ACID POLLUTED SOIL**

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Revision Notes: Response to Reviewers

This document shows detailed responses to the reviewer`s comments. The responses indicate also the changes made in the revised manuscript. The changes are easily identifiable in the highlighted revised manuscript (revised manuscript changes marked document). The location of changes (page/line details in the responses) always refer to the highlighted revised manuscript **MS Word file**. Please note that, after the changes, it is possible that the PDF generation process through the on-line editorial system could move the lines.

COMMENTS FROM REVIEWERS

Reviewer #1:

COMMENTS FOR AUTHORS

This is an interesting paper, that presents data which should be of wide interest to the journal readership. The authors address an important issue, and make an incremental contribution to knowledge. Methods are well-designed and conclusions supported by the results presented. Overall I recommend this manuscript for publication.

Reviewer #2:

COMMENTS FOR AUTHORS

1-More description need for the process and figures.

RESPONSE:

Additional information has been included in the revised manuscript to deep in the discussion section in three different parts:

A) Discussion, regarding the performance of the biofilm reactor and figure 3, has been improved in section 3.1. See revised manuscript:

Page 12 line 17-20: “The biosorption process has been also reported to occur in the removal of organic and inorganic pollutants from industrial wastewater ^{25,26} and specifically, Benoit et al.²⁷ reported 2,4-D biosorption by microbial biomass.”

Page 13 line 20-25: “Previous research regarding 2,4-D removal from wastewater by using biofilm reactor has been reported. ^{28,29} These works showed that 2,4-D removal rates depended on retention times and biomass concentration attached to the solid carrier particles. High removal efficiencies (between 90 and 100%) were always reported under aerobic conditions and different bacterial strains. The main difference in the present work is that high removal efficiencies have been obtained also under non-oxygenated conditions.”

Additional references supporting the discussion have been included (no self-citations):

25. Bhatti H N, Sadaf S and Aleem A, Treatment of Textile Effluents by Low Cost Agricultural Wastes: Batch Biosorption Study. *J Animal Plant Sci* **25**: 284–289 (2015).
26. Ay C O, Ozcan A S, Erdogan Y and Ozcan A, Characterization of *Punica granatum L.* peels and quantitatively determination of its biosorption behavior towards lead(II) ions and Acid Blue 40. *Colloids Surf B* **100**: 197–204 (2012). <https://doi.org/10.1016/j.colsurfb.2012.05.013>
27. Benoit P, Barriuso E and Calvet R, Biosorption characterization of herbicides, 2,4-D and atrazine, and two chlorophenols on fungal mycelium. *Chemosphere* **37**: 1271-1282 (1998). [https://doi.org/10.1016/S0045-6535\(98\)00125-8](https://doi.org/10.1016/S0045-6535(98)00125-8)
28. González A J, Fortunato M S, Gallego A and Korol S E, Simultaneous Biodegradation and Detoxification of the Herbicides 2,4-Dichlorophenoxyacetic Acid and 4-Chloro-2-Methylphenoxyacetic Acid in a Continuous Biofilm Reactor. *Water Air Soil Pollut* **228**: 300-306 (2017). DOI 10.1007/s11270-017-3468-4
29. González-Cuna S, Galíndez-Mayer J, Ruiz-Ordaz N, Murugesan S, Piña-Escobedo A, García-Mena J, Lima-Martínez E, and Santoyo-Tepole F, Aerobic biofilm reactor for treating a commercial formulation of the herbicides 2,4-D and dicamba: Biodegradation kinetics and biofilm bacterial diversity. *Int Biodet Biodeg* **107**: 123-131 (2016). <http://dx.doi.org/10.1016/j.ibiod.2015.11.014>

B) Discussion, regarding the experimental conditions in the soil (electro osmotic flow EOF) under electrobioremediation treatment (section 3.2), has been improved. See revised manuscript:

Page 15 lines 2-14: “This EOF value is low compared to similar reported experiments without using BioPRB.¹⁶ It is well known that EOF is directly proportional to the applied voltage gradient, to the zeta potential (that, in turn, depends on the ionic concentration) and the dielectric constant of the fluid, and inversely proportional to the fluid viscosity⁴ and that EOF through low permeability regions is significantly greater than the EOF achieved in higher porosity regions. It has been assumed that BioPRB increased porosity in the central position, and porosity can be one of the main factors that influences such low EOF values. Some authors previously studied the influence of the insertion of a permeable reactive barrier (PRB) in electrokinetic remediation of organochlorines polluted soil, and different results were reported. Wang et al.³⁰ reported that a Pd/Fe PRB insertion caused a clear EOF decrease in an electrokinetic remediation process of organochlorines-polluted soil. On the contrary, the same authors³¹ reported that a Cu/Fe PRB insertion caused that EOF increased.”

30. Wang J, Li Z, Lu X and Yuan S, Remediation of a hexachlorobenzene contaminated soil by surfactant-enhanced electrokinetics coupled with microscale Pd/Fe PRB. *J Hazard Mater* **184**: 184-190 (2010). <https://doi.org/10.1016/j.jhazmat.2010.08.022>
31. Wang J, Li Z, Yuan S and Lu X, Removal of hexachlorobenzene from kaolin by electrokinetics coupled with Cu/Fe PRB, *J Hazard Toxic Radioact Waste* **15**: 175-179 (2011). DOI: 10.1061/(ASCE)HZ.1944-8376.0000054

C) Discussion regarding the electrobioremediation efficiency (section 3.2 and figure 7):

Authors additionally discuss that “EBR is a more efficient process because the increase in removal rate (from 25% to 85%) only would need an increase of 15% approximately in operating costs because of electric energy consumption, according to the estimations by

Athmer.³² This statement has been included in the revised manuscript (page 17 line 4) and also the new reference.

Note that the present response is also used to discuss and support the higher efficiency of EBR as requested by reviewer 3

32. Athmer CJ, Cost estimates for electrokinetic remediation, in: Reddy KR, Cameselle C. (Eds.), Electrochemical remediation technologies for polluted soils, sediments and groundwater, John Wiley & Sons, pp. 583-587 (2009).

2- Literature must be completed.

RESPONSE:

According to the response to comment 1, new literature is cited in the revised manuscript. Moreover, there are no self-citations (see last comment of reviewer 3).

Reviewer #3:

COMMENTS FOR AUTHORS

The main conclusion of this work is that EBR treatment of 2,4-D polluted soil reports a higher degradation rate of the pollutant when compared to biological degradation.

This is not surprising at all giving the state of the art of electro-bioremediation. It is hypothesized that EK improves contact between pollutant and microorganisms and this leads to the faster degradation rate.

However, in its present form the manuscript is too descriptive with no advancing quantitative description of the phenomena responsible for the results. **A table with the experimental details of the systems under study should be provided allowing comparison of conditions.**

RESPONSE:

According to this reviewer's comment, authors think that he/she refers to the "comparison of conditions" between the Electrobioremediation experiment (EBR) and the reference test using only biological degradation (Bio test).

Thus, a new table (Table 1) has been included in the revised manuscript. The table compares experimental details and conditions of both batch 10d duration experiments. It has been cited in materials and methods section (page 8 line 12) and also at the end of discussion section (page 16 line 19) in order to better understand the differences observed in figure 7.

Table 1. Experimental details and conditions of both batch 10d soil remediation experiments

Experiment	EBR experiment	Biological reference test
Initial 2,4D concentration in soil (mg kg ⁻¹)	20	20
Average soil temperature (°C)	22 ± 1.5	22 ± 1.5
Average soil pH	7.8	7.6
Inorganic medium in soil	Electrolyte solution and BHB medium into the BioPRB	BHB medium
Microorganisms inoculation and distribution	Microbial culture attached (biofilm) into de BioPRB	Microbial culture distributed all over the soil
Initial microorganisms' concentration	No microorganisms in the soil. 8.7 g _{VS} L ⁻¹ in the BioPRB	10 ⁵ CFU g ⁻¹ in soil
Electrochemical parameters:	Voltage (V cm ⁻¹)	-
	Polarity reversal frequency (d ⁻¹)	-
	Average current density (mA)	-
	Average EOF (ml h ⁻¹)	-

The **mechanism and kinetics of 2,4-D degradation in both approaches** should be analysed and discussed. And the energy intensity of both processes should be calculated and compared to support “the higher efficiency concluded by the authors”.

RESPONSE:

Regarding degradation mechanism, 2,4-D removal was caused only by biological degradation because no volatilization occurred, and no 2,4-D was detected in the electrode compartments. This statement has been completed in revised manuscript (page 17 line 10).

No kinetics were measured during the soil remediation experiments because only post-mortem analyses were done as indicated in section 2.4.

Regarding “the higher efficiency concluded by the authors”, authors just concluded in the original manuscript that “EK transport phenomena improved contact between pollutant and microorganisms and clearly increased the 2,4-D removal rate”. The results clearly show that electrokinetics accelerated the bioremediation process. Moreover, authors also consider that EBR is a more efficient process because the increase in removal rate (from 25% to 85%) only would need an increase of 15% approximately in operating costs because of electric energy consumption, according to the estimations by Athmer.³² This statement has been included in the revised manuscript (page 17 line 4) and also the new reference.

32. Athmer CJ, Cost estimates for electrokinetic remediation, in: Reddy KR, Cameselle C. Electrochemical remediation technologies for polluted soils, sediments and groundwater: John Wiley & Sons, 2009, pp. 583-587

Minor comments:

The small size of the graphs makes difficult differentiation of experimental data; experimental error is not included.

RESPONSE:

All figures have been revised

Why a concentration of 2,4-D as high as 1200 mg.L⁻¹ has been selected in the experiments?

RESPONSE:

The aim of using such a high pesticide concentration was just to prove the robustness of the biological reactor. It was observed that the biofilm reactor was able to successfully degrade high pesticide concentrations in relatively short retention times and the main conclusion derived from this result was that the success, or not, of the electrobioremediation technology would not be limited by the biological response. It was later confirmed that the mass transport mechanisms promoted by electrokinetics played a crucial role in the bioremediation process. Additional explanation has been included in the revised manuscript (page 11 line 23; page 14 line 12).

The literature has a very high number of self-citations; it needs revision.

RESPONSE:

New literature has been included regarding the improvement of discussion (see responses to reviewer 2), but no self-citations.

In summary in its present form it doesn't meet the quality requirements of the JCTB and I recommend rejection.

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FOR THE IN-SITU ELECTROBIOREMEDIATION OF 2,4-
DICHLOROPHENOXYACETIC ACID POLLUTED SOIL**

Short Title:
**ELECTROBIOREMEDIATION OF 2,4-DICHLOROPHENOXYACETIC ACID
POLLUTED SOIL**

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Conflict of Interest: The authors declare that they have no conflict of interest.

Abstract

Background: This work studies the in-situ electrobioremediation (EBR) of clay soil polluted with 2,4-Dichlorophenoxyacetic acid (2,4-D) in a two-stage method. First, a fixed-bed biofilm reactor able to biodegrade 2,4-D in wastewater was developed; Second, a portion of the biofilm-covered bed was included into the polluted soil and used as biological permeable reactive barrier (BioPRB), whereas electrokinetics were used to promote the contact between pollutant and microorganisms into the soil.

Results: The fixed-bed biofilm reactor successfully treated wastewater polluted with 2,4-D concentrations up to 1200 mg L⁻¹ and under non oxygenated conditions because of the culture ability to use nitrate as electron acceptor. A portion of the stationary biofilm was later used as BioPRB into the polluted soil to perform in-situ EBR study. The study was performed in a bench scale setup using potentiostatic conditions (1.0 V cm⁻¹), room temperature and periodic polarity reversal (2 d⁻¹) in a 10 d batch experiment. The feasibility of the proposed technology was checked by the comparison with a conventional in-situ bioremediation test without electrokinetics. The EBR treatment, coupling electrokinetics with the previously developed BioPRB, offered 85% removal efficiency of 2,4-D after 10 d. According to the low removal efficiency observed in the reference test, it was proved that the electrokinetic transport phenomena improved contact between pollutant and microorganisms and clearly increased 2,4-D removal rate.

Conclusions: According to these results it is considered that the combination of electrokinetics and biobarriers could be a promising technology to achieve remediation of low permeable soils polluted with pesticides.

Keywords

2,4-Dichlorophenoxyacetic acid, soil pollution, electrobioremediation, biobarrier, biofilm reactor.

1. Introduction

One of the most extended pesticides nowadays used in agriculture for broadleaf weed control is 2,4-dichlorophenoxyacetic acid (2,4-D), which is a systemic hormonal herbicide because it attacks directly to the hormonal system of plants inhibiting their growth. This compound belongs to organochlorinated hydrocarbons group, which are very persistent pollutants in environment, soil, water or air, because of their chemical structure and composition.¹ Due to the soil is a non-renewable source, and because 2,4-D can produce seriously effects on animals or humans that could have been in contact with this pesticide, it is necessary to remove this pollutant from soils.²

There are currently many different technologies available to remediate polluted soils, based on physical, thermal, biological or electrochemical techniques. Nowadays, the study of electrochemical technologies for remediating polluted soils is increasing because of its greatest advantages versus conventional treatments.³ One of the most important advantages is that electrochemical techniques can be used for *in situ* remediation processes, which means that the soil pollutants can be removed from the site without the need to dig the soil and treat it externally by other techniques. This advantage supposes a clear cost-effective alternative for polluted soils remediation.⁴

Electroremediation or electrokinetic remediation (EK) of soils consists of applying an electric field between a pair of electrodes inserted in the soil producing electrokinetic mass transport phenomena, which mobilize different species retained in the soil such as ions, microorganisms or pollutants, improving the contact between them and removing pollutants from soil. Subsequently, the soil washing wastewater obtained has to be treated by external technologies.^{3,5} In soils with low permeability, where conventional pump methods are not effective to move the pollutant along the soil,⁴ electroremediation has been proved as a cost-effective *in situ* technology achieving successful results in removal

of pollutants.⁶

Nevertheless, EK remediation has limitations such as the low mobility of non-polar contaminants, the heating of soil (due to the Joule effect whereby part of applied energy is transformed in heat because of the ohmic resistance), and one of the most significant limitations is the formation of extreme pH zones in areas near the electrodes, among others. To solve these limitations, EK treatment can be coupled with different complementary technologies.⁷ One of these combinations that is becoming more interesting in recent years is electrobioremediation (EBR), which couples the electrokinetic phenomena with conventional bioremediation mechanisms. The main hypothesis of EBR is that microorganisms in soil are able to biodegrade *in situ* the organic pollutants because the EK mass transport phenomena move water, pollutants, microorganisms and nutrients into the soil keeping all of them in contact.^{8,9}

One option included in the electrobioremediation technology is coupling electrokinetics with a Permeable Reactive biological Barrier (BioPRB) inserted in the soil to be remediated.¹⁰ A biological barrier or “biobarrier” consists of a fixed bed of solid particles that contains pollutant-degrading microorganisms adhered on its surface, forming which is known as biofilm, and thus it acts like a fixed-film bioreactor. The aim of the biological barriers is to eliminate the pollution plume, which is moved through it by electrokinetic mechanisms.¹¹ Applying a biobarrier in EK remediation of polluted soils is a good way to achieve EK-*bioaugmentation*, that is, a biological alternative that allows soil inoculation of external microorganisms adapted to the specific biodegradation of soil pollutant.¹² Because of the extreme pH values that could be produced in zones near the electrodes, the biobarrier should be placed in a central position far from the electrodes in order to avoid such pH values and keep the microorganisms alive for the biodegradation activity.

The external development of a functional BioPRB, before it would be subsequently placed in a polluted soil, includes two stages. The first one is a microbial acclimation stage. It consists of adapting the microbial culture to use the soil pollutant as organic substrate. Once it is achieved, the second stage is the microbial inoculation into the bioreactor and the biofilm development. Both stages can take several weeks. Previous works have reported the treatment of wastewater polluted with different types of pesticides using biofilm reactors or biobarriers,^{13,14} and many of them are related to 2,4-D as a pollutant.¹³⁻¹⁵

The aim of the present work is to study the feasibility of using a biobarrier as biological strategy in electrokinetic-biological remediation of 2,4-D polluted real clayey soil. The proposed technology has two particular scopes. First, the development of an external biofilm reactor for biodegradation of 2,4-D in water, which its stationary performance is monitored. Second, a portion of this biofilm is placed into the polluted soil and used as a biobarrier while electric field is applied through all over the soil to improve the contact between microorganisms, nutrients and 2,4-D. Additionally, a conventional in-situ bioremediation test without electric current (Test "Bio") was used as reference test. As previously stated, despite the serious environmental problem related to this type of herbicides in soil, no previous research about electrobioremediation of 2,4-D polluted soils by using biobarriers have been found.

2. Materials and methods

2.1. Materials

The soil employed in this work is a real clayey soil supplied by Millas e Hijos Ceramics (Toledo, Spain). Its characteristics have been described in previous works by the same authors.¹⁶ As the soil provided was not polluted, it was necessary to pollute it artificially previously to the EBR treatment. To do that, the soil was mixed homogeneously with a

2,4-D pesticide water solution. After this process, the concentration of 2,4-D in wet soil was 20 mg per kg (26.7 mg kg⁻¹ on dry soil) and the moisture of the soil reached approximately 25%, near to saturation conditions.

The pesticide used in this study as model of polar pollutant is 2,4-dichlorophenoxyacetic acid (2,4-D) which was supplied by *Alfa Aesar* with 98% assay. 2,4-D belongs to organochlorinated compounds group, commonly used in agricultural activity for pest control and weed growth.

A microbial consortium was acclimated to the degradation of 2,4-D following the procedure described in previous works.¹⁷ First, activated sludge from an oil-refinery wastewater treatment plant (Puertollano, Spain), was used as inoculum for this process. The culture medium used for providing inorganic nutrients to microorganisms was Bushnell-Hass Broth medium (BHB), which composition per litre of Milli-Q water is 0.20 g Mg SO₄, 0.02 g CaCl₂, 1.00 g KH₂PO₄, 1.00 g (NH₄)₂HPO₄, 0.05 g FeCl₃ and 1.00 g KNO₃, and 2,4-D was employed as sole carbon source (200 mg L⁻¹). Once the microbial culture was acclimated to 2,4-D biodegradation, the microorganisms contained were identified by MALDI TOF Mass Spectrometry AXIMA-Assurance (Biotech technology, SHIMADZU, Germany).

2.2. Biobarrier development and performance

A fix-bed bioreactor for 2,4-D biodegradation in water was developed as follows. A glass 2.4L reactor was filled with porous solid particles of pumice stone (supporting material, average diameter 3 mm), synthetic wastewater containing 2,4-D, and a seed of acclimated microbial culture for the removal of 2,4-D was inoculated. Then, wastewater was circulated through the bed (Figure 1a). Sequencing batch cycles of seven days duration with an initial concentration of 200 mg L⁻¹ of 2,4-D, and using BHB culture medium, were conducted till the steady state was reached up. After that, the reactor performance

was checked under increasing organic loadings. To do this, three batch experiments with different initial concentrations of 2,4-D (200, 800 and 1200 mg L⁻¹) were conducted. Once the bioreactor was developed, a portion of the fix-bed biofilm was later used as Permeable Reactive Biobarrier (BioPRB) in the electro-bioremediation study (section 2.4).

2.3 Electro-bioremediation set-up

Figure 1b shows the bench scale set-up used in the electro-bioremediation study. The installation is made of transparent methacrylate and divided into five compartments. In the central one (25 cm) is placed the polluted soil, and the biobarrier (5 cm wide) is inserted in central position of this compartment and separated by a nylon mesh (0.5 mm mesh size). Anodic and cathodic compartments are located at both sides of the polluted soil, where the graphite electrodes (Carbosystem, Madrid, Spain) are inserted and connected to a power supply (HQ Power, Gavere, Belgium). The water electroosmotic flow (EOF) moved during the process is collected in the external compartments contiguous to both electrode compartments. Note that EOF direction changes (because the electrode polarity will be changed periodically during the treatment) and thus there are two collector compartments at both sides.

The electrolyte used in electrolyte compartments is a synthetic groundwater (80.75 mg L⁻¹ Na₂SO₄, 70.00 mg L⁻¹ NaHCO₃, 30.36 mg L⁻¹ NaNO₃) and the soil moisture at the start was 25%. As reported in previous works,¹⁸ nutrient limitations to the biological process can occur. To avoid nutrient limitations, ammonium, phosphate and nitrate were supplied in excess concentration by adding BHB medium in the biobarrier.

2.4. Electro-bioremediation experimental procedure

As it has been described above, the 2,4-D polluted soil was placed in the central compartment of the set-up. Then, a central soil portion was removed and replaced by the BioPRB. At the start, the electrode compartments were filled with the electrolyte solution,

and it was connected a control level system in the anodic compartment to replace the liquid that was removed out of the set-up by electroosmosis during the process in order to keep the moisture and ensure enough electrical conductivity in soil.

Once the experimental installation was prepared, it was conducted an electro-bioremediation experiment. The study was carried out under potentiostatic conditions at 1.0 V cm⁻¹ and room temperature (22±1.5°C). Periodic electrode polarity reversal was applied in this test with a frequency in the change of 2 d⁻¹, which means change every 12 hours. The duration of the experiment was 10 d.

Moreover, a reference test was carried out in order to evaluate the elimination of 2,4-D only by biological mechanisms without the contribution of electrokinetics (*Test "Bio"*, a conventional in-situ bioremediation test using acclimated microorganisms previously added to the polluted soil). Table 1 shows experimental details and conditions of both remediation experiments (electrobioremediation experiment and the reference "Bio" test).

2.4. Sampling and analyses

Bioreactor development and performance of BioPRB

During the stationary operation of the fixed-bed biofilm reactor and during the batch experiments, 2,4-D concentration, inorganic nutrients concentrations, and microbial biomass concentration were measured. 2,4-D concentration in the liquid medium was analysed by HPLC (Jasco, Japan) using a column Kinetex 5 µm Biphenyl 100 Å, 150 x 4.5 mm (Phenomenex, USA). The mobile phase was H₃PO₄ 0.1%/acetonitrile, 60/40 %v/v, with an isocratic flow rate of 0.6 mL min⁻¹. The wavelength of the UV detector was 220 nm and 20 µl of the injection volume. The concentration of microbial biomass, supported as biofilm on the surface of solid carrier, was measured by Volatile Suspended Solids method.¹⁷ Once the biofilm was developed on the fix-bed bioreactor surface,

several images were taken by FESEM (Field Emission Scanning Electron Microscopy).

Electro-bioremediation study

Electrical conductivity, pH, and nutrient and 2,4-D concentrations in the electrode compartments and electroosmotic flow were measured during the experiment.

Soil samples were taken at the beginning, before placing it in the set-up, to analyse the initial characteristics of the soil to be treated. Once the electrobioremediation experiment was finished, the soil was characterized by *post-mortem* analyses in order to not modify permeability properties of the soil during the process.¹⁹ To do this, soil samples were taken in four longitudinal positions that correspond to intermediate points between anode and cathode compartments, where the position 1 is nearest to anode ($t=0$), and position 4 is nearest to cathode ($t=0$). In addition, each position considers four different sample points divided in different axial positions (Figure 1c). More details regarding sampling procedure have been previously reported.²⁰ The experimental points plotted in Figs. 5 and 6 indicate the concentrations of the four samples at each longitudinal position.

The concentration of 2,4-D in soil was analysed by HPLC as it has been described above.

The moisture was calculated by difference of weight after drying step: it was taken an amount of wet soil and was dried during 24h at 105°C, and the weight loss related to the evaporated water was calculated. pH and conductivity of soil was measured following the next procedure: it was taken 10 g of dry soil and 25 mL of Milli-Q water was added. Then, it was agitated vigorously during 30 min and it was left decant during 2 hours. After that, the supernatant liquid was filtered with 0.2 μm nylon filters and measured by using a multiparameter probe (SENSLON, HACH). To analyse nutrient concentrations, it was used a photometer Gallery (Thermo Scientific). To determine the concentration of microorganisms in soil expressed as Colony Forming Units (CFU) per gram of dry soil,²¹ samples were prepared as follows: it was taken 1 g of wet soil and was added 10 mL of

NaCl solution (0.9%). Then, the mixture was agitated during 3 min using a vortex agitator, and aliquots of supernatant of 100 μL were taken and put on Petri dishes. They contained LB media (10.0 g L^{-1} NaCl, 5.0 g L^{-1} yeast extract and 10.0 g L^{-1} casein peptone, 15 g L^{-1} of European Bacteriological Agar and 10.0 g L^{-1} of glucose as carbon source) as solid culture media for microorganisms. The incubation time for inoculated Petri dishes was 24h at 26.5°C. Colonies were counted after incubation.

3. Results and discussion

3.1 Biofilm reactor performance

Prior to the biobarrier development, the acclimation step enabled to obtain an acclimated mixed microbial culture to degrade 2,4-D. Sequencing batch microbial growth cycles using the initial microbial seed and increasing the initial concentrations of 2,4-D from 100 to 200 mg L^{-1} in the liquid medium were carried out. The acclimation process finished when the biodegradation time required for removal of 200 mg L^{-1} of 2,4-D was constant. After that, microbial species contained in the acclimated culture were identified by using a MALDI TOF equipment (Biotech technology, SHIMADZU, Germany). Two species (*Rhodococcus ruber* and *Ochrobactrum anthropic*) were identified with a minimum of 80% confidence threshold.

Then, the acclimated culture was inoculated into the fixed-bed reactor and 2,4-D polluted wastewater was treated. During the first three sequential batch cycles, an initial concentration of 2,4-D of 200 mg L^{-1} in wastewater was introduced. Since the biodegradation process it was observed to occur very quickly, it was increased the 2,4-D initial concentration of the following cycles up to 400 mg L^{-1} , obtaining also a satisfactory result. The concentration of microbial biomass attached to the bed particles increased slowly, and after two months it reached an approximate value of 11 g of attached volatile solids (VS) per kg of solid carrier, i.e., 8,7 g_{VS} L^{-1} . This stationary biomass concentration

in the biofilm reactor is comparable to that obtained in previously works by the same authors using the herbicide oxyfluorfen as model pollutant.²² Moreover, a low amount biomass in suspension, which corresponded to the excess biomass that was detached from the biofilm, was accumulated in the deeper part (settler) of the reactor and it was removed from the reactor at the end of each batch cycle. The temperature and electrical conductivity remained practically constant (26.5°C and 2500 $\mu\text{S cm}^{-1}$), and the pH was controlled using the buffer capacity of the BHB medium, maintaining a pH of 7.5, ideal for the development and activity of this type of microorganisms.

After a period of approximately two months, because of the sequencing batch cycles offered similar repetitive results, it was considered that a steady state had been reached, and it was observed that 2,4-D was completely eliminated in a residence time of less than 4 days. For this reason, it was considered that this biofilm reactor could be successfully used as permeable biobarrier for the biodegradation of 2,4-D in a subsequent *in situ* process of electro-bioremediation of contaminated soils.

Fig. 2 shows two FESEM images of the stationary biofilm reactor using different zoom levels: 400 μm (left) and 100 μm (right). A homogenous biofilm that completely covered the solid support was not observed because biomass flocs were distributed in different locations on the surface. It can be distinguished the clean surface of the solid carrier in different zones and the microbial biofilm particles (indicated with circles) at different points of the surface.

Once the development of the biofilm was reached up, it was checked the bioreactor performance under different 2,4-D loads. To do this, several batch experiments were conducted using different initial concentrations (200, 800 and 1200 mg L^{-1}) of 2,4-D. The aim of using such a high pesticide concentration was just to prove the robustness of the biological reactor. The results obtained are shown in Figures 3 and 4. Figure 3 shows the

concentration of 2,4-D (left axis) and soluble COD (right axis) in each of the three cases studied. According to the 2,4-D concentration profiles observed in these experiments, two stages could be considered. The first one corresponds to a fast decrease in the pesticide concentration, in which approximately 200 mg L^{-1} are eliminated in an approximate period of about 20 hours, and it was observed to occur in the three cases. At a second stage, it can be observed that the removal of the pesticide is rather slow. In case (a) the 2,4-D concentration that remains after 20 h is very low, but in cases (b) and (c) there is still a high 2,4-D concentration and a linear decrease is observed. According to the results observed in cases (a) and (b) it is estimated that also a complete elimination of 2,4-D would be observed after additional time in case (c). The behaviour observed in the first stage could be related to the bioadsorption of 2,4-D on the biological flocs, which would correspond to the fast elimination of this contaminant in water. Later, once the bioadsorption step finished (according to the biological adsorption capacity of the biomass, approximately 200 mg L^{-1} in all cases), the biodegradation of 2,4-D until its complete removal would be a slower process, which would correspond to the second stage. The rapid bioadsorption of organic compounds, such as pesticides, on a biological sludge has already been observed in previous works within our research group.^{23,24} The biosorption process has been also reported to occur in the removal of organic and inorganic pollutants from industrial wastewater^{25,26} and specifically, Benoit et al.²⁷ reported 2,4-D biosorption by microbial biomass. Regarding the behaviour of the COD concentrations, it was observed in all cases that a residual COD value remained at the end of experiments (around 40 mg L^{-1}). Since the biodegradation of 2,4-D was complete, the residual COD concentration may be mainly related to organic matter produced from endogenous metabolism or cell lysis, or in part to intermediates (undetected) that could be generated due to the biodegradation of 2,4-D.

Figure 4 shows the concentrations of dissolved oxygen (DO) and nitrates during the three experiments performed. In the first case, Figure 4a, a drop in the concentration of dissolved oxygen in water from 2.5 to approximately 0.5 mg L⁻¹ is observed in a time of 20 h, and it increased later to values of approximately 1 mg L⁻¹ for a time of 40 h. Then, the oxygen concentration remained practically constant around this value for times greater than 40 hours. On the other hand, it is observed that the concentration of nitrate did not vary throughout the experiment. The sharp decrease in DO was caused by the rapid consumption of the organic pollutant until it was practically been eliminated (Figure 3a). In the second experiment, Figure 4b, it can be observed again a very rapid decrease in the DO concentration during the first hours, related with the rapid elimination of the organic pollutant. Then, it is observed that the DO continued to be consumed slowly until reaching concentration values around 1 mg L⁻¹. Regarding the concentration of nitrate, it was observed an important consumption of nitrate by microorganisms that started when oxygen was scarce in the medium. Finally, Figure 4c shows a similar behaviour, that is, a sudden initial drop in the DO concentration, with a slight rebound and a slow subsequent fall. A decrease in nitrate concentration was also observed when oxygen began to be scarce in the medium. In general, it is observed that once DO is eliminated, microorganisms can work under anoxic conditions using nitrate as electron acceptor. Nitrate consumption was not observed in case (a) because the organic pollutant concentration was not so high as in cases b and c. Previous research regarding 2,4-D removal from wastewater by using biofilm reactor has been reported.^{28,29} These works showed that 2,4-D removal rates depended on retention times and biomass concentration attached to the solid carrier particles. High removal efficiencies (between 90 and 100%) were always reported under aerobic conditions and different bacterial strains. The main difference in the present work is that high removal efficiencies have been obtained also

under non-oxygenated conditions. The microbial ability to use nitrate instead of oxygen as electron acceptor is very important in the study of biological soil remediation techniques. Note that soil is a non-aerated heterogeneous medium and a portion of this biofilm reactor will be later used as BioPRB in the EBR process, and the oxygen availability is notably low in the soil as observed in a previous work.²⁰ Thus, it is expected that when the concentration of oxygen in soil decreases, microorganisms will be able to use nitrate as an electron acceptor, thus maintaining the active bioremediation process. Regarding the inorganic nutrients concentrations (ammonium and phosphate) during the batch cycles, they were always sufficiently high to ensure that there were no nutrient limitations, and only a slight decrease from their initial values (approximately 130 mgL⁻¹ of ammonium and 550 mgL⁻¹ of phosphate) was observed.

According to these results, it was concluded that the biofilm reactor was able to successfully degrade high pesticide concentrations in relatively short retention times and thus the success, or not, of the subsequent electrobioremediation technology (when a portion was located as BioPRB in the soil EBR experiment) would not be limited by the biological response, that is, t~~According to the successful results of the performance of the biofilm reactor,~~ the biodegradation mechanism ~~was~~will not ~~be~~ considered ~~to be~~as the limiting step in the possible removal of 2,4-D in soil when this culture was used. ~~Then, a portion was located as BioPRB in the soil EBR experiment.~~

3.2 Electro-bioremediation experiment results

Experimental conditions of the electrobioremediation experiment using the BioPRB were daily monitored during the process in electrode wells, and also in the soil at the end of the experiment. Figure 5 (parts a and b) shows the current intensity and the EOF monitored during the experiment. As it can be observed, current intensity kept practically constant although EOF displaced water and ions from soil. This is because the electrolyte level

control system used in anodic compartment replaced the electrolyte removed from the system by EOF, and thus it allowed to maintain the conductivity and intensity properly in soil. The EOF increased during the first 90 hours of treatment and then it was maintained approximately constant at 4 mL h⁻¹. This EOF value is low compared to similar reported experiments without using BioPRB.¹⁶ It is well known that EOF is directly proportional to the applied voltage gradient, to the zeta potential (that, in turn, depends on the ionic concentration) and the dielectric constant of the fluid, and inversely proportional to the fluid viscosity⁴ and that EOF through low permeability regions is significantly greater than the EOF achieved in higher porosity regions. It has been assumed that BioPRB increased porosity in the central position, and porosity can be one of the main factors that influences such low EOF values. Some authors previously studied the influence of the insertion of a permeable reactive barrier (PRB) in electrokinetic remediation of organochlorines polluted soil, and different results were reported. Wang et al. reported that a Pd/Fe PRB insertion caused a clear EOF decrease in an electrokinetic remediation process of organochlorines-polluted soil.³⁰ On the contrary, the same authors reported that a Cu/Fe PRB insertion caused that EOF increased.³¹

Once the EBR experiment was finished (10 d), it was conducted the *post-mortem* analysis (Fig. 5, parts c and d). Figure 5c shows the pH profile in the soil at the start and the end. As it can be observed, the value of pH was constant around 7-8 in all positions, which is a very important condition to keep the microorganisms alive. It was proved the success of the polarity reversal strategy to cushion the pH fluctuations caused by water electrolysis, previously studied by the same authors.¹⁶ Soil electrical conductivity (Figure 5d) decreased at the end in comparison with the start of the treatment despite the electrolyte level control system in the anodic well replaced electrolytes. Even so, the value of electrical conductivity in soil was adequate enough to ensure the effectivity of

electrokinetic phenomena in soil.

Figure 6 shows the concentration of microorganisms and 2,4-D in soil at the start and the end of the EBR treatment. Horizontal dashed line refers only to the initial 2,4-D concentration because there were not microorganisms in the soil at the start of the experiment (the soil to be remediated was autoclaved to ensure that microbial activity was only provided by bioaugmentation because of the inclusion of the central biobarrier). As it can be observed, the solid line related to final microbial concentration shows a homogeneous distribution of microorganisms through all over the soil after 10 days. This important behaviour could be explained because of the microorganisms' movement from the BioPRB caused by the biofilm detachment and the electrokinetic transport phenomena, such as electrophoresis and electroosmosis as it has been reported in previous works.¹⁸ This transport occurs in both directions because of the changes in the electrode polarity.

Regarding 2,4-D soil concentration (Figure 6b), it can be observed that the present strategy based on bioaugmentation by the inclusion of a BioPRB offers interesting results of 2,4-D removal from soil in an electrobioremediation process. 85% removal percentage was achieved in only 10 days of EBR treatment. These results offer, clearly, good advances in the in-situ technology for removal of polar pesticides from soil.

Figure 7 shows the comparison of 2,4-D removal efficiencies obtained in the electro-bioremediation experiment when using biobarriers, and the additional "Bio" reference test whose experimental details are indicated in Table 1. The reference test was done to evaluate the in-situ 2,4-D removal from soil only by microbial activity, and this test indicates that only bioremediation (with no options to improve the mass transport phenomena into the soil) offered low 2,4-D removal efficiencies when compared to the electro-bioremediation experiment that combined electrokinetics and biological

treatment. On the one hand, although the biodegradability of 2,4-D was not the limiting factor as it was shown above (Section 3.1), the removal of 2,4-D in the reference test did not exceed 25% despite microorganisms were distributed homogeneously all over the soil. This behaviour can be explained due to the mass transfer limitation and low contact between the species (pollutants, nutrients and microorganisms) in this kind of low permeability soil if no EK was used. According to these results it could be considered that EBR is a more efficient process because the increase in removal rate (from 25% to 85%) only would need approximately an increase of 15% in operating costs because of electric energy consumption, according to the estimations by Athmer.³² Despite the high mobility of 2,4-D due to its polar nature, it was not observed any pesticide concentration in electrode wells when using electro-bioremediation because of polarity reversal maintained the pollutant in soil during the process. Moreover, no volatilization of 2,4-D occurred, thus biodegradation was considered the only mechanism for 2,4-D removal.

Conclusions

A biofilm reactor treating 2,4-D polluted wastewater was successfully developed. It was able to work under 2,4-D concentrations up to 1200 mg L⁻¹ and also under non oxygenated conditions because of the culture ability to use nitrate as electron acceptor. According to these results it was expected that the biodegradation mechanism would not be the limiting step in the possible removal of 2,4-D in soil.

The EBR treatment, coupling EK and the previously developed BioPRB, offered 85% removal efficiency of 2,4-D after 10 d. According to the results of the reference test, it was proved that the EK transport phenomena improved contact between pollutant and microorganisms and clearly increased the 2,4-D removal rate.

According to these results it is considered that the combination of electrokinetics and biobarriers could be a promising technology to achieve remediation of low permeable

soils polluted with pesticides.

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**FIXED-BED BIOLOGICAL BARRIER COUPLED WITH ELECTROKINETICS
FOR THE IN-SITU ELECTROBIOREMEDIATION OF 2,4-
DICHLOROPHENOXYACETIC ACID POLLUTED SOIL**

Short Title:
**ELECTROBIOREMEDIATION OF 2,4-DICHLOROPHENOXYACETIC ACID
POLLUTED SOIL**

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Abstract

Background: This work studies the in-situ electrobioremediation (EBR) of clay soil polluted with 2,4-Dichlorophenoxyacetic acid (2,4-D) in a two-stage method. First, a fixed-bed biofilm reactor able to biodegrade 2,4-D in wastewater was developed; Second, a portion of the biofilm-covered bed was included into the polluted soil and used as biological permeable reactive barrier (BioPRB), whereas electrokinetics were used to promote the contact between pollutant and microorganisms into the soil.

Results: The fixed-bed biofilm reactor successfully treated wastewater polluted with 2,4-D concentrations up to 1200 mg L⁻¹ and under non oxygenated conditions because of the culture ability to use nitrate as electron acceptor. A portion of the stationary biofilm was later used as BioPRB into the polluted soil to perform in-situ EBR study. The study was performed in a bench scale setup using potentiostatic conditions (1.0 V cm⁻¹), room temperature and periodic polarity reversal (2 d⁻¹) in a 10 d batch experiment. The feasibility of the proposed technology was checked by the comparison with a conventional in-situ bioremediation test without electrokinetics. The EBR treatment, coupling electrokinetics with the previously developed BioPRB, offered 85% removal efficiency of 2,4-D after 10 d. According to the low removal efficiency observed in the reference test, it was proved that the electrokinetic transport phenomena improved contact between pollutant and microorganisms and clearly increased 2,4-D removal rate.

Conclusions: According to these results it is considered that the combination of electrokinetics and biobarriers could be a promising technology to achieve remediation of low permeable soils polluted with pesticides.

Keywords

2,4-Dichlorophenoxyacetic acid, soil pollution, electrobioremediation, biobarrier, biofilm reactor.

1. Introduction

One of the most extended pesticides nowadays used in agriculture for broadleaf weed control is 2,4-dichlorophenoxyacetic acid (2,4-D), which is a systemic hormonal herbicide because it attacks directly to the hormonal system of plants inhibiting their growth. This compound belongs to organochlorinated hydrocarbons group, which are very persistent pollutants in environment, soil, water or air, because of their chemical structure and composition.¹ Due to the soil is a non-renewable source, and because 2,4-D can produce seriously effects on animals or humans that could have been in contact with this pesticide, it is necessary to remove this pollutant from soils.²

There are currently many different technologies available to remediate polluted soils, based on physical, thermal, biological or electrochemical techniques. Nowadays, the study of electrochemical technologies for remediating polluted soils is increasing because of its greatest advantages versus conventional treatments.³ One of the most important advantages is that electrochemical techniques can be used for *in situ* remediation processes, which means that the soil pollutants can be removed from the site without the need to dig the soil and treat it externally by other techniques. This advantage supposes a clear cost-effective alternative for polluted soils remediation.⁴

Electroremediation or electrokinetic remediation (EK) of soils consists of applying an electric field between a pair of electrodes inserted in the soil producing electrokinetic mass transport phenomena, which mobilize different species retained in the soil such as ions, microorganisms or pollutants, improving the contact between them and removing pollutants from soil. Subsequently, the soil washing wastewater obtained has to be treated by external technologies.^{3,5} In soils with low permeability, where conventional pump methods are not effective to move the pollutant along the soil,⁴ electroremediation has been proved as a cost-effective *in situ* technology achieving successful results in removal

of pollutants.⁶

Nevertheless, EK remediation has limitations such as the low mobility of non-polar contaminants, the heating of soil (due to the Joule effect whereby part of applied energy is transformed in heat because of the ohmic resistance), and one of the most significant limitations is the formation of extreme pH zones in areas near the electrodes, among others. To solve these limitations, EK treatment can be coupled with different complementary technologies.⁷ One of these combinations that is becoming more interesting in recent years is electrobioremediation (EBR), which couples the electrokinetic phenomena with conventional bioremediation mechanisms. The main hypothesis of EBR is that microorganisms in soil are able to biodegrade *in situ* the organic pollutants because the EK mass transport phenomena move water, pollutants, microorganisms and nutrients into the soil keeping all of them in contact.^{8,9}

One option included in the electrobioremediation technology is coupling electrokinetics with a Permeable Reactive biological Barrier (BioPRB) inserted in the soil to be remediated.¹⁰ A biological barrier or “biobarrier” consists of a fixed bed of solid particles that contains pollutant-degrading microorganisms adhered on its surface, forming which is known as biofilm, and thus it acts like a fixed-film bioreactor. The aim of the biological barriers is to eliminate the pollution plume, which is moved through it by electrokinetic mechanisms.¹¹ Applying a biobarrier in EK remediation of polluted soils is a good way to achieve EK-*bioaugmentation*, that is, a biological alternative that allows soil inoculation of external microorganisms adapted to the specific biodegradation of soil pollutant.¹² Because of the extreme pH values that could be produced in zones near the electrodes, the biobarrier should be placed in a central position far from the electrodes in order to avoid such pH values and keep the microorganisms alive for the biodegradation activity.

The external development of a functional BioPRB, before it would be subsequently placed in a polluted soil, includes two stages. The first one is a microbial acclimation stage. It consists of adapting the microbial culture to use the soil pollutant as organic substrate. Once it is achieved, the second stage is the microbial inoculation into the bioreactor and the biofilm development. Both stages can take several weeks. Previous works have reported the treatment of wastewater polluted with different types of pesticides using biofilm reactors or biobarriers,^{13,14} and many of them are related to 2,4-D as a pollutant.¹³⁻¹⁵

The aim of the present work is to study the feasibility of using a biobarrier as biological strategy in electrokinetic-biological remediation of 2,4-D polluted real clayey soil. The proposed technology has two particular scopes. First, the development of an external biofilm reactor for biodegradation of 2,4-D in water, which its stationary performance is monitored. Second, a portion of this biofilm is placed into the polluted soil and used as a biobarrier while electric field is applied through all over the soil to improve the contact between microorganisms, nutrients and 2,4-D. Additionally, a conventional in-situ bioremediation test without electric current (Test "Bio") was used as reference test. As previously stated, despite the serious environmental problem related to this type of herbicides in soil, no previous research about electrobioremediation of 2,4-D polluted soils by using biobarriers have been found.

2. Materials and methods

2.1. Materials

The soil employed in this work is a real clayey soil supplied by Millas e Hijos Ceramics (Toledo, Spain). Its characteristics have been described in previous works by the same authors.¹⁶ As the soil provided was not polluted, it was necessary to pollute it artificially previously to the EBR treatment. To do that, the soil was mixed homogeneously with a

2,4-D pesticide water solution. After this process, the concentration of 2,4-D in wet soil was 20 mg per kg (26.7 mg kg⁻¹ on dry soil) and the moisture of the soil reached approximately 25%, near to saturation conditions.

The pesticide used in this study as model of polar pollutant is 2,4-dichlorophenoxyacetic acid (2,4-D) which was supplied by *Alfa Aesar* with 98% assay. 2,4-D belongs to organochlorinated compounds group, commonly used in agricultural activity for pest control and weed growth.

A microbial consortium was acclimated to the degradation of 2,4-D following the procedure described in previous works.¹⁷ First, activated sludge from an oil-refinery wastewater treatment plant (Puertollano, Spain), was used as inoculum for this process. The culture medium used for providing inorganic nutrients to microorganisms was Bushnell-Hass Broth medium (BHB), which composition per litre of Milli-Q water is 0.20 g Mg SO₄, 0.02 g CaCl₂, 1.00 g KH₂PO₄, 1.00 g (NH₄)₂HPO₄, 0.05 g FeCl₃ and 1.00 g KNO₃, and 2,4-D was employed as sole carbon source (200 mg L⁻¹). Once the microbial culture was acclimated to 2,4-D biodegradation, the microorganisms contained were identified by MALDI TOF Mass Spectrometry AXIMA-Assurance (Biotech technology, SHIMADZU, Germany).

2.2. Biobarrier development and performance

A fix-bed bioreactor for 2,4-D biodegradation in water was developed as follows. A glass 2.4L reactor was filled with porous solid particles of pumice stone (supporting material, average diameter 3 mm), synthetic wastewater containing 2,4-D, and a seed of acclimated microbial culture for the removal of 2,4-D was inoculated. Then, wastewater was circulated through the bed (Figure 1a). Sequencing batch cycles of seven days duration with an initial concentration of 200 mg L⁻¹ of 2,4-D, and using BHB culture medium, were conducted till the steady state was reached up. After that, the reactor performance

was checked under increasing organic loadings. To do this, three batch experiments with different initial concentrations of 2,4-D (200, 800 and 1200 mg L⁻¹) were conducted. Once the bioreactor was developed, a portion of the fix-bed biofilm was later used as Permeable Reactive Biobarrier (BioPRB) in the electro-bioremediation study (section 2.4).

2.3 Electro-bioremediation set-up

Figure 1b shows the bench scale set-up used in the electro-bioremediation study. The installation is made of transparent methacrylate and divided into five compartments. In the central one (25 cm) is placed the polluted soil, and the biobarrier (5 cm wide) is inserted in central position of this compartment and separated by a nylon mesh (0.5 mm mesh size). Anodic and cathodic compartments are located at both sides of the polluted soil, where the graphite electrodes (Carbosystem, Madrid, Spain) are inserted and connected to a power supply (HQ Power, Gavere, Belgium). The water electroosmotic flow (EOF) moved during the process is collected in the external compartments contiguous to both electrode compartments. Note that EOF direction changes (because the electrode polarity will be changed periodically during the treatment) and thus there are two collector compartments at both sides.

The electrolyte used in electrolyte compartments is a synthetic groundwater (80.75 mg L⁻¹ Na₂SO₄, 70.00 mg L⁻¹ NaHCO₃, 30.36 mg L⁻¹ NaNO₃) and the soil moisture at the start was 25%. As reported in previous works,¹⁸ nutrient limitations to the biological process can occur. To avoid nutrient limitations, ammonium, phosphate and nitrate were supplied in excess concentration by adding BHB medium in the biobarrier.

2.4. Electro-bioremediation experimental procedure

As it has been described above, the 2,4-D polluted soil was placed in the central compartment of the set-up. Then, a central soil portion was removed and replaced by the BioPRB. At the start, the electrode compartments were filled with the electrolyte solution,

and it was connected a control level system in the anodic compartment to replace the liquid that was removed out of the set-up by electroosmosis during the process in order to keep the moisture and ensure enough electrical conductivity in soil.

Once the experimental installation was prepared, it was conducted an electro-bioremediation experiment. The study was carried out under potentiostatic conditions at 1.0 V cm⁻¹ and room temperature (22±1.5°C). Periodic electrode polarity reversal was applied in this test with a frequency in the change of 2 d⁻¹, which means change every 12 hours. The duration of the experiment was 10 d.

Moreover, a reference test was carried out in order to evaluate the elimination of 2,4-D only by biological mechanisms without the contribution of electrokinetics (*Test "Bio"*, a conventional in-situ bioremediation test using acclimated microorganisms previously added to the polluted soil). Table 1 shows experimental details and conditions of both remediation experiments (electrobioremediation experiment and the reference "Bio" test).

2.4. Sampling and analyses

Bioreactor development and performance of BioPRB

During the stationary operation of the fixed-bed biofilm reactor and during the batch experiments, 2,4-D concentration, inorganic nutrients concentrations, and microbial biomass concentration were measured. 2,4-D concentration in the liquid medium was analysed by HPLC (Jasco, Japan) using a column Kinetex 5 µm Biphenyl 100 Å, 150 x 4.5 mm (Phenomenex, USA). The mobile phase was H₃PO₄ 0.1%/acetonitrile, 60/40 %v/v, with an isocratic flow rate of 0.6 mL min⁻¹. The wavelength of the UV detector was 220 nm and 20 µl of the injection volume. The concentration of microbial biomass, supported as biofilm on the surface of solid carrier, was measured by Volatile Suspended Solids method.¹⁷ Once the biofilm was developed on the fix-bed bioreactor surface,

several images were taken by FESEM (Field Emission Scanning Electron Microscopy).

Electro-bioremediation study

Electrical conductivity, pH, and nutrient and 2,4-D concentrations in the electrode compartments and electroosmotic flow were measured during the experiment.

Soil samples were taken at the beginning, before placing it in the set-up, to analyse the initial characteristics of the soil to be treated. Once the electrobioremediation experiment was finished, the soil was characterized by *post-mortem* analyses in order to not modify permeability properties of the soil during the process.¹⁹ To do this, soil samples were taken in four longitudinal positions that correspond to intermediate points between anode and cathode compartments, where the position 1 is nearest to anode ($t=0$), and position 4 is nearest to cathode ($t=0$). In addition, each position considers four different sample points divided in different axial positions (Figure 1c). More details regarding sampling procedure have been previously reported.²⁰ The experimental points plotted in Figs. 5 and 6 indicate the concentrations of the four samples at each longitudinal position.

The concentration of 2,4-D in soil was analysed by HPLC as it has been described above.

The moisture was calculated by difference of weight after drying step: it was taken an amount of wet soil and was dried during 24h at 105°C, and the weight loss related to the evaporated water was calculated. pH and conductivity of soil was measured following the next procedure: it was taken 10 g of dry soil and 25 mL of Milli-Q water was added. Then, it was agitated vigorously during 30 min and it was left decant during 2 hours. After that, the supernatant liquid was filtered with 0.2 μm nylon filters and measured by using a multiparameter probe (SENSLON, HACH). To analyse nutrient concentrations, it was used a photometer Gallery (Thermo Scientific). To determine the concentration of microorganisms in soil expressed as Colony Forming Units (CFU) per gram of dry soil,²¹ samples were prepared as follows: it was taken 1 g of wet soil and was added 10 mL of

NaCl solution (0.9%). Then, the mixture was agitated during 3 min using a vortex agitator, and aliquots of supernatant of 100 μL were taken and put on Petri dishes. They contained LB media (10.0 g L^{-1} NaCl, 5.0 g L^{-1} yeast extract and 10.0 g L^{-1} casein peptone, 15 g L^{-1} of European Bacteriological Agar and 10.0 g L^{-1} of glucose as carbon source) as solid culture media for microorganisms. The incubation time for inoculated Petri dishes was 24h at 26.5°C. Colonies were counted after incubation.

3. Results and discussion

3.1 Biofilm reactor performance

Prior to the biobarrier development, the acclimation step enabled to obtain an acclimated mixed microbial culture to degrade 2,4-D. Sequencing batch microbial growth cycles using the initial microbial seed and increasing the initial concentrations of 2,4-D from 100 to 200 mg L^{-1} in the liquid medium were carried out. The acclimation process finished when the biodegradation time required for removal of 200 mg L^{-1} of 2,4-D was constant. After that, microbial species contained in the acclimated culture were identified by using a MALDI TOF equipment (Biotech technology, SHIMADZU, Germany). Two species (*Rhodococcus ruber* and *Ochrobactrum anthropic*) were identified with a minimum of 80% confidence threshold.

Then, the acclimated culture was inoculated into the fixed-bed reactor and 2,4-D polluted wastewater was treated. During the first three sequential batch cycles, an initial concentration of 2,4-D of 200 mg L^{-1} in wastewater was introduced. Since the biodegradation process it was observed to occur very quickly, it was increased the 2,4-D initial concentration of the following cycles up to 400 mg L^{-1} , obtaining also a satisfactory result. The concentration of microbial biomass attached to the bed particles increased slowly, and after two months it reached an approximate value of 11 g of attached volatile solids (VS) per kg of solid carrier, i.e., 8,7 g_{VS} L^{-1} . This stationary biomass concentration

in the biofilm reactor is comparable to that obtained in previously works by the same authors using the herbicide oxyfluorfen as model pollutant.²² Moreover, a low amount biomass in suspension, which corresponded to the excess biomass that was detached from the biofilm, was accumulated in the deeper part (settler) of the reactor and it was removed from the reactor at the end of each batch cycle. The temperature and electrical conductivity remained practically constant (26.5°C and 2500 $\mu\text{S cm}^{-1}$), and the pH was controlled using the buffer capacity of the BHB medium, maintaining a pH of 7.5, ideal for the development and activity of this type of microorganisms.

After a period of approximately two months, because of the sequencing batch cycles offered similar repetitive results, it was considered that a steady state had been reached, and it was observed that 2,4-D was completely eliminated in a residence time of less than 4 days. For this reason, it was considered that this biofilm reactor could be successfully used as permeable biobarrier for the biodegradation of 2,4-D in a subsequent *in situ* process of electro-bioremediation of contaminated soils.

Fig. 2 shows two FESEM images of the stationary biofilm reactor using different zoom levels: 400 μm (left) and 100 μm (right). A homogenous biofilm that completely covered the solid support was not observed because biomass flocs were distributed in different locations on the surface. It can be distinguished the clean surface of the solid carrier in different zones and the microbial biofilm particles (indicated with circles) at different points of the surface.

Once the development of the biofilm was reached up, it was checked the bioreactor performance under different 2,4-D loads. To do this, several batch experiments were conducted using different initial concentrations (200, 800 and 1200 mg L^{-1}) of 2,4-D. The aim of using such a high pesticide concentration was just to prove the robustness of the biological reactor. The results obtained are shown in Figures 3 and 4. Figure 3 shows the

1 concentration of 2,4-D (left axis) and soluble COD (right axis) in each of the three cases
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1 concentration of 2,4-D (left axis) and soluble COD (right axis) in each of the three cases
2 studied. According to the 2,4-D concentration profiles observed in these experiments, two
3 stages could be considered. The first one corresponds to a fast decrease in the pesticide
4 concentration, in which approximately 200 mg L^{-1} are eliminated in an approximate
5 period of about 20 hours, and it was observed to occur in the three cases. At a second
6 stage, it can be observed that the removal of the pesticide is rather slow. In case (a) the
7 2,4-D concentration that remains after 20 h is very low, but in cases (b) and (c) there is
8 still a high 2,4-D concentration and a linear decrease is observed. According to the results
9 observed in cases (a) and (b) it is estimated that also a complete elimination of 2,4-D
10 would be observed after additional time in case (c). The behaviour observed in the first
11 stage could be related to the bioadsorption of 2,4-D on the biological flocs, which would
12 correspond to the fast elimination of this contaminant in water. Later, once the
13 bioadsorption step finished (according to the biological adsorption capacity of the
14 biomass, approximately 200 mg L^{-1} in all cases), the biodegradation of 2,4-D until its
15 complete removal would be a slower process, which would correspond to the second
16 stage. The rapid bioadsorption of organic compounds, such as pesticides, on a biological
17 sludge has already been observed in previous works within our research group.^{23,24} The
18 biosorption process has been also reported to occur in the removal of organic and
19 inorganic pollutants from industrial wastewater^{25,26} and specifically, Benoit et al.²⁷
20 reported 2,4-D biosorption by microbial biomass. Regarding the behaviour of the COD
21 concentrations, it was observed in all cases that a residual COD value remained at the end
22 of experiments (around 40 mg L^{-1}). Since the biodegradation of 2,4-D was complete, the
23 residual COD concentration may be mainly related to organic matter produced from
24 endogenous metabolism or cell lysis, or in part to intermediates (undetected) that could
25 be generated due to the biodegradation of 2,4-D.

Figure 4 shows the concentrations of dissolved oxygen (DO) and nitrates during the three experiments performed. In the first case, Figure 4a, a drop in the concentration of dissolved oxygen in water from 2.5 to approximately 0.5 mg L⁻¹ is observed in a time of 20 h, and it increased later to values of approximately 1 mg L⁻¹ for a time of 40 h. Then, the oxygen concentration remained practically constant around this value for times greater than 40 hours. On the other hand, it is observed that the concentration of nitrate did not vary throughout the experiment. The sharp decrease in DO was caused by the rapid consumption of the organic pollutant until it was practically been eliminated (Figure 3a). In the second experiment, Figure 4b, it can be observed again a very rapid decrease in the DO concentration during the first hours, related with the rapid elimination of the organic pollutant. Then, it is observed that the DO continued to be consumed slowly until reaching concentration values around 1 mg L⁻¹. Regarding the concentration of nitrate, it was observed an important consumption of nitrate by microorganisms that started when oxygen was scarce in the medium. Finally, Figure 4c shows a similar behaviour, that is, a sudden initial drop in the DO concentration, with a slight rebound and a slow subsequent fall. A decrease in nitrate concentration was also observed when oxygen began to be scarce in the medium. In general, it is observed that once DO is eliminated, microorganisms can work under anoxic conditions using nitrate as electron acceptor. Nitrate consumption was not observed in case (a) because the organic pollutant concentration was not so high as in cases b and c. Previous research regarding 2,4-D removal from wastewater by using biofilm reactor has been reported.^{28,29} These works showed that 2,4-D removal rates depended on retention times and biomass concentration attached to the solid carrier particles. High removal efficiencies (between 90 and 100%) were always reported under aerobic conditions and different bacterial strains. The main difference in the present work is that high removal efficiencies have been obtained also

under non-oxygenated conditions. The microbial ability to use nitrate instead of oxygen as electron acceptor is very important in the study of biological soil remediation techniques. Note that soil is a non-aerated heterogeneous medium and a portion of this biofilm reactor will be later used as BioPRB in the EBR process, and the oxygen availability is notably low in the soil as observed in a previous work.²⁰ Thus, it is expected that when the concentration of oxygen in soil decreases, microorganisms will be able to use nitrate as an electron acceptor, thus maintaining the active bioremediation process. Regarding the inorganic nutrients concentrations (ammonium and phosphate) during the batch cycles, they were always sufficiently high to ensure that there were no nutrient limitations, and only a slight decrease from their initial values (approximately 130 mgL⁻¹ of ammonium and 550 mgL⁻¹ of phosphate) was observed.

According to these results, it was concluded that the biofilm reactor was able to successfully degrade high pesticide concentrations in relatively short retention times and thus the success, or not, of the subsequent electrobioremediation technology (when a portion was located as BioPRB in the soil EBR experiment) would not be limited by the biological response, that is, the biodegradation mechanism will not be considered as the limiting step in the possible removal of 2,4-D in soil when this culture was used.

3.2 Electro-bioremediation experiment results

Experimental conditions of the electrobioremediation experiment using the BioPRB were daily monitored during the process in electrode wells, and also in the soil at the end of the experiment. Figure 5 (parts a and b) shows the current intensity and the EOF monitored during the experiment. As it can be observed, current intensity kept practically constant although EOF displaced water and ions from soil. This is because the electrolyte level control system used in anodic compartment replaced the electrolyte removed from the system by EOF, and thus it allowed to maintain the conductivity and intensity properly

in soil. The EOF increased during the first 90 hours of treatment and then it was maintained approximately constant at 4 mL h^{-1} . This EOF value is low compared to similar reported experiments without using BioPRB.¹⁶ It is well known that EOF is directly proportional to the applied voltage gradient, to the zeta potential (that, in turn, depends on the ionic concentration) and the dielectric constant of the fluid, and inversely proportional to the fluid viscosity⁴ and that EOF through low permeability regions is significantly greater than the EOF achieved in higher porosity regions. It has been assumed that BioPRB increased porosity in the central position, and porosity can be one of the main factors that influences such low EOF values. Some authors previously studied the influence of the insertion of a permeable reactive barrier (PRB) in electrokinetic remediation of organochlorines polluted soil, and different results were reported. Wang et al. reported that a Pd/Fe PRB insertion caused a clear EOF decrease in an electrokinetic remediation process of organochlorines-polluted soil.³⁰ On the contrary, the same authors reported that a Cu/Fe PRB insertion caused that EOF increased.³¹

Once the EBR experiment was finished (10 d), it was conducted the *post-mortem* analysis (Fig. 5, parts c and d). Figure 5c shows the pH profile in the soil at the start and the end. As it can be observed, the value of pH was constant around 7-8 in all positions, which is a very important condition to keep the microorganisms alive. It was proved the success of the polarity reversal strategy to cushion the pH fluctuations caused by water electrolysis, previously studied by the same authors.¹⁶ Soil electrical conductivity (Figure 5d) decreased at the end in comparison with the start of the treatment despite the electrolyte level control system in the anodic well replaced electrolytes. Even so, the value of electrical conductivity in soil was adequate enough to ensure the effectivity of electrokinetic phenomena in soil.

Figure 6 shows the concentration of microorganisms and 2,4-D in soil at the start and the

1 end of the EBR treatment. Horizontal dashed line refers only to the initial 2,4-D
2 concentration because there were not microorganisms in the soil at the start of the
3 experiment (the soil to be remediated was autoclaved to ensure that microbial activity
4 was only provided by bioaugmentation because of the inclusion of the central biobarrier).
5 As it can be observed, the solid line related to final microbial concentration shows a
6 homogeneous distribution of microorganisms through all over the soil after 10 days. This
7 important behaviour could be explained because of the microorganisms' movement from
8 the BioPRB caused by the biofilm detachment and the electrokinetic transport
9 phenomena, such as electrophoresis and electroosmosis as it has been reported in previous
10 works.¹⁸ This transport occurs in both directions because of the changes in the electrode
11 polarity.

12 Regarding 2,4-D soil concentration (Figure 6b), it can be observed that the present
13 strategy based on bioaugmentation by the inclusion of a BioPRB offers interesting results
14 of 2,4-D removal from soil in an electrobioremediation process. 85% removal percentage
15 was achieved in only 10 days of EBR treatment. These results offer, clearly, good
16 advances in the in-situ technology for removal of polar pesticides from soil.

17 Figure 7 shows the comparison of 2,4-D removal efficiencies obtained in the electro-
18 bioremediation experiment when using biobarriers, and the additional "Bio" reference
19 test whose experimental details are indicated in Table 1. The reference test was done to
20 evaluate the in-situ 2,4-D removal from soil only by microbial activity, and this test
21 indicates that only bioremediation (with no options to improve the mass transport
22 phenomena into the soil) offered low 2,4-D removal efficiencies when compared to the
23 electro-bioremediation experiment that combined electrokinetics and biological
24 treatment. On the one hand, although the biodegradability of 2,4-D was not the limiting
25 factor as it was shown above (Section 3.1), the removal of 2,4-D in the reference test did

not exceed 25% despite microorganisms were distributed homogeneously all over the soil. This behaviour can be explained due to the mass transfer limitation and low contact between the species (pollutants, nutrients and microorganisms) in this kind of low permeability soil if no EK was used. According to these results it could be considered that EBR is a more efficient process because the increase in removal rate (from 25% to 85%) only would need approximately an increase of 15% in operating costs because of electric energy consumption, according to the estimations by Athmer.³² Despite the high mobility of 2,4-D due to its polar nature, it was not observed any pesticide concentration in electrode wells when using electro-bioremediation because of polarity reversal maintained the pollutant in soil during the process. Moreover, no volatilization of 2,4-D occurred, thus biodegradation was considered the only mechanism for 2,4-D removal.

Conclusions

A biofilm reactor treating 2,4-D polluted wastewater was successfully developed. It was able to work under 2,4-D concentrations up to 1200 mg L⁻¹ and also under non oxygenated conditions because of the culture ability to use nitrate as electron acceptor. According to these results it was expected that the biodegradation mechanism would not be the limiting step in the possible removal of 2,4-D in soil.

The EBR treatment, coupling EK and the previously developed BioPRB, offered 85% removal efficiency of 2,4-D after 10 d. According to the results of the reference test, it was proved that the EK transport phenomena improved contact between pollutant and microorganisms and clearly increased the 2,4-D removal rate.

According to these results it is considered that the combination of electrokinetics and biobarriers could be a promising technology to achieve remediation of low permeable soils polluted with pesticides.

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Table 1. Experimental details and conditions of both batch 10d soil remediation experiments

Experiment	EBR experiment	Biological reference test
Initial 2,4D concentration in soil (mg kg ⁻¹)	20	20
Average soil temperature (°C)	22 ± 1.5	22 ± 1.5
Average soil pH	7.8	7.6
Inorganic medium in soil	Electrolyte solution and BHB medium into the BioPRB	BHB medium
Microorganisms inoculation and distribution	Microbial culture attached (biofilm) into de BioPRB	Microbial culture distributed all over the soil
Initial microorganisms concentration	No microorganisms in the soil. 8.7 g _{VS} L ⁻¹ in the BioPRB	10 ⁵ CFU g ⁻¹ in soil
Electrochemical parameters:	Voltage (V cm ⁻¹)	-
	Polarity reversal frequency (d ⁻¹)	-
	Average current density (mA)	-
	Average EOF (ml h ⁻¹)	-

Figure 1. Experimental set-up scheme. a) Fix-bed bioreactor; b) Electrobioremediation cell (b1: scheme and b2: photograph); c) Soil sample points.

Figure 2. Images of the biofilm in the stationary period taken with FESEM.

Figure 3. Concentrations of 2,4-D (●), and COD (○) over time in batch experiments under different 2,4-D initial concentrations: a) 200 mg L⁻¹; b) 800 mg L⁻¹; and c) 1200 mg L⁻¹.

Figure 4. Concentrations of dissolved oxygen (●), and nitrates (○) over time in batch experiments under different 2,4-D initial concentrations: a) 200 mg L⁻¹; b) 800 mg L⁻¹; and c) 1200 mg L⁻¹.

Figure 5. Experimental conditions monitored during the EBR process: (a) electric current through the soil; (b) EOF through the soil. *Post-mortem* analysis: (c) pH and (d) conductivity in soil at the start and the end of EBR process. Horizontal dashed lines: initial value; Solid lines: final values obtained as the average of different measurements at different axial positions in the soil (data points). The grey vertical bar in (c) and (d) indicates the central BioPRB position.

Figure 6. (a) Microorganisms concentration profile in soil; (b) 2,4-D concentration profile in soil. Horizontal dashed lines: initial value; Solid lines: final values obtained as the average of different measurements at different axial positions in the soil (data points). The grey vertical bar indicates the central BioPRB position.

Figure 7. 2,4-D removal efficiency compared to the “Bio” reference test at 10 days of duration treatment. BioPRB: Electro-bioremediation by BioPRB and Test “Bio”: Conventional in situ bioremediation.

Figure 1

Figure 1. Experimental set-up scheme. a) Fix-bed bioreactor; b) Electrobioremediation cell (b1: scheme and b2: photograph); c) Soil sample points.

338x190mm (150 x 150 DPI)

Figure 2

Figure 2. Images of the biofilm in the stationary period taken with FESEM.

338x190mm (150 x 150 DPI)

Figure 3

Figure 3. Concentrations of 2,4-D (●), and COD (○) over time in batch experiments under different 2,4-D initial concentrations: a) 200 mg L⁻¹; b) 800 mg L⁻¹; and c) 1200 mg L⁻¹.

204x279mm (150 x 150 DPI)

Figure 4

Figure 4. Concentrations of dissolved oxygen (●), and nitrates (○) over time in batch experiments under different 2,4-D initial concentrations: a) 200 mg L⁻¹; b) 800 mg L⁻¹; and c) 1200 mg L⁻¹.

196x276mm (150 x 150 DPI)

Figure 5

Figure 5. Experimental conditions monitored during the EBR process: (a) electric current through the soil; (b) EOF through the soil. Post-mortem analysis: (c) pH and (d) conductivity in soil at the start and the end of EBR process. Horizontal dashed lines: initial value; Solid lines: final values obtained as the average of different measurements at different axial positions in the soil (data points). The grey vertical bar in (c) and (d) indicates the central BioPRB position.

338x190mm (150 x 150 DPI)

Figure 6

Figure 6. (a) Microorganisms concentration profile in soil; (b) 2,4-D concentration profile in soil. Horizontal dashed lines: initial value; Solid lines: final values obtained as the average of different measurements at different axial positions in the soil (data points). The grey vertical bar indicates the central BioPRB position.

190x275mm (150 x 150 DPI)

Figure 7

Figure 7. 2,4-D removal efficiency compared to the "Bio" reference test at 10 days of duration treatment.
BioPRB: Electro-bioremediation by BioPRB and Test "Bio": Conventional in situ bioremediation.

338x190mm (150 x 150 DPI)