The aim of this work is to compare three biological strategies for the in situ remediation of a 2,4-dichlorophenoxyacetic acid (2,4-D) polluted clayey soil by coupling electrokinetics (EK) and bioremediation (technology named as electrobioremediation, EBR). The first option (i) is EK-biostimulation, in which the activity of microorganisms already present in soil is enhanced by EK phenomena. The second and third options are EK-bioaugmentation, which consist of addition of microorganisms to soil through the inclusion of permeable biological barriers: (ii) using a microbial fixed biofilm reactor as biobarrier (BB1), and (iii) using a mixture of clean soil and a microbial suspension as biobarrier (BB2). Thus, three batch experiments at bench scale were conducted under a constant electric field of 1 V cm⁻¹, and electrode polarity was periodically reversed every 12 h (2 d⁻¹). The duration of each test was 10 days. Two additional tests using only biodegradation or only EK were performed as auxiliary reference tests. A microbial consortium acclimated to 2,4-D biodegradation was employed. Results showed that EK-biostimulation strategy offered the best pollutant removal efficiency (reaching up almost 100%) while biobarriers offered pollutant removal rates between 75-85%. Permeable biobarriers allowed the introduction of microorganism but caused a decrease in the electro-osmotic flow which, in turn, reduced the mobilization and contact between microorganisms and pollutants. These results can contribute to the knowledge and understanding of electrobioremediation of polluted soil and to the feasibility of delivering microorganism to the soil by using biobarriers. Despite biostimulation was found to be the best option, results show that permeable reactive biobarriers may result in a successful alternative for in-situ EK-bioaugmentation when acclimated microbial population is not already present in soil.
BIOSTIMULATION VERSUS BIOAUGMENTATION FOR THE ELECTRO-
BIOREMEDIATION OF 2,4-DICHLOROPHENOXYACETIC ACID
POLLUTED SOILS.

Silvia Barba¹, José Villaseñor¹,*, Manuel A. Rodrigo² and Pablo Cañizares²

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Dear Editor:

Attached you will find the REVISED form of the manuscript JEMA-D-20-03905R1 “Biostimulation versus bioaugmentation for the electro-bioremediation of 2,4-dichlorophenoxyacetic acid polluted soils”, by Silvia Barba, José Villaseñor, Manuel A. Rodrigo and Pablo Cañizares (corresponding author: jose.villasenor@uclm.es), in order to be reviewed for a possible publication as research paper in Journal of Environmental Management.

The following items are included in the new submission:

1. The “Responses to reviewers”: One MS Word document containing the detailed answers to each concrete reviewer’s comments. Each answer indicates the position of the modifications in the highlighted revised manuscript.
2. The “Highlighted revised manuscript”, that is the revised manuscript MS Word file, using the track changes mode, where you can easily find the modifications made to the text.
3. The “Revised manuscript”
4. Table 1 has been modified

Yours sincerely

Dr. J. Villaseñor
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. Note that it is possible that the PDF generated by EES move lines.

Reviewers' comments:

Reviewer #4: The manuscript JEMA-D-20-03905R1 provides an interesting work about the combination of bioremediation a electro kinetic remediation. The first revision improve the manuscript and in my opinion the answer to reviewers were adequate. However, some minor point have to be clarified. The manuscript can be published in JEMA previous revision.

Table 1: Soil characterization is incomplete. Include important (and typical) soil characteristics such as organic matter, pH, electrical conductivity, cation exchange capacity, clay, silt and sand content, ... Some of these parameters were monitored during the assay.

Table 1 has been modified

Section 2.4: 2,4-D analysis is reported but how did you extract the herbicide from soil? Include in this section the extraction procedure of 2,4-D.

Soil samples (1 g) were mixed with 2.5 mL of water for the dissolution of 2,4-D. The soil/solvent mixture was agitated vigorously in a vortex agitator for 5 min and centrifuged (15 min, 3800 min-1). Samples were taken from the aqueous supernatant and analysed. This information has been included in revised manuscript in section 2.4 before 2,4-D analysis description.

The term "concentration" isn’t adequate to denote abundance of microbiota. Use the term "microbial population" instead "microbial concentration".

The change has been made (line 378, line 384 and caption of Fig5 in revised manuscript).
Include information about the sterilization of soil by autoclave in M&M section.

**Soil was autoclaved: 121ºC, 15 min.**
Information has been included in revised manuscript (line 192)

Include description of the two controls used "no Bio" and "no EK" in M&M section

Description has been included at the end of section 2.3 in revised manuscript.
Graphical Abstracts

**EK-Biostimulation**

- Anodic compartment
- Cathodic compartment
- Graphite electrode
- Power supply
- 2,4-D Polluted soil
- Permeable Reactive Biobarrier (BB)

**EK-Bioaugmentation using biobarrier**

- Anodic compartment
- Cathodic compartment
- Graphite electrode
- Power supply
- 2,4-D Polluted soil
- Permeable Reactive Biobarrier (BB)

Three biological strategies for EBR:

- BB1
- BB2
- Biostim.
- "No Bio"
- "No EK"

**2,4-D removal / %**

- 100
- 80
- 60
- 40
- 20
- 0

Reference tests

<table>
<thead>
<tr>
<th>BB1</th>
<th>BB2</th>
<th>Biostim.</th>
<th>&quot;No Bio&quot;</th>
<th>&quot;No EK&quot;</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>
Highlights:

Electrobioremediation of 2,4-D polluted soil was studied at bench scale set-up
The influence of using or not biological barriers was evaluated
EK-biostimulation allowed 100% pollutant removal in 10 d
EK-bioaugmentation by bio-barriers allowed 75-85% pollutant removal in 10 d
Biobarriers successfully included microorganisms but decreased electroosmotic flow
BIOSTIMULATION VERSUS BIOAUGMENTATION FOR THE ELECTRO-
BIOREMEDICATION OF 2,4-DICHLOROPHENOXYACETIC ACID
POLLUTED SOILS.

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Abstract

The aim of this work is to compare three biological strategies for the in situ remediation
of a 2,4-dichlorophenoxyacetic acid (2,4-D) polluted clayey soil by coupling
electrokinetics (EK) and bioremediation (technology named as electrobioremediation,
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constant electric field of 1 V cm⁻¹, and electrode polarity was periodically reversed
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only biodegradation or only EK were performed as auxiliary reference tests. A
microbial consortium acclimated to 2,4-D biodegradation was employed. Results
showed that EK-biostimulation strategy offered the best pollutant removal efficiency (reaching up almost 100%) while biobarrers offered pollutant removal rates between 75-85%. Permeable biobarrers allowed the introduction of microorganism but caused a decrease in the electro-osmotic flow which, in turn, reduced the mobilization and contact between microorganisms and pollutants. These results can contribute to the knowledge and understanding of electrobioremediation of polluted soil and to the feasibility of delivering microorganism to the soil by using biobarrers. Despite biostimulation was found to be the best option, results show that permeable reactive biobarrers may result in a successful alternative for in-situ EK-bioaugmentation when acclimated microbial population is not already present in soil.

Keywords
2,4-dichlorophenoxyacetic acid, permeable reactive biobARRIER, electrobioremediation, polluted soil, pesticide pollution.

1. Introduction

Pesticide pollution is a serious environmental problem in our days due to the mostly use of these compounds in agricultural activities for pest control and weed growth. Within the group of pesticides, 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most commonly used. 2,4-D is a systemic hormonal herbicide, which can affect directly to hormonal system in plants avoiding their growth. Moreover, 2,4-D belongs to the organochlorinated pollutants group, which means to be very persistent compounds in soil, water and air (Chowdhury et al., 2008). Additionally, it can produce a dangerous impact in humans or animals in contact with this pollutant, causing genomic mutations or in the worst of cases, even the death (Morillo and Villaverde, 2017).
Because of it, regulation referring to soil pollution in Spain and E.U. is currently becoming harder in order to control the pollution levels in soil and forcing its remediation if contaminant levels exceed those allowed. Depending on the impact in natural ecosystems or whether it affects to human health, the maximum pollution levels are different, e.g., for organochlorinated pollutants the maximum allowed level in soil is 1.0 mg per kg of soil (Spanish Presidential Ministry, 2005).

According to above mentioned environmental and health cited risks, and because of the soil is a non-renewable natural resource, it is necessary to remediate it. There are several remediation technologies for polluted soils based on biological, chemical, physical, or thermal fundamentals. The in situ remediation treatments are focused on the removal of the pollutant in the polluted site, regardless of the biological, physical or chemical method used, and thus external treatments are not required, which supposes a clear cost-effective alternative for remediating polluted soils (Reddy and Cameselle, 2009).

Bioresidemiation is one of the treatment methods most applied under the in situ option due to the low cost associated, but the main limitation is the high operation times required because of the slow mass transfer phenomena to contact microorganisms, nutrients and pollutants, especially in soils contaminated with non-polar compounds (Barba et al., 2018a).

Alternatively, electrokinetic remediation or electroremediation (EK) is an in situ technique, which consists in applying an electric field through the soil between a couples of electrodes inserted on it. Consequently, electrokinetic transport phenomena appear, mobilizing different species contained in the soil such as microorganisms, pollutants and nutrients, encouraging the contact between them (Paillat et al., 2000; Rodrigo et al., 2014). Electoremediation has been proved as a cost-effective and successful in situ treatment, mainly in low permeability soils, where conventional pump
and treat methods are not indicated to transport the contaminant through all over the soil
(Reddy and Cameselle, 2009; Cameselle, 2014). However, this technology also presents
some limitations during operation time, e.g., soil heating due to the Joule effect, low
mobility of non-polar pollutants in soil, or extreme pH values near the electrode’s
zones.
In recent years it is becoming more attractive the idea of combining biological with
electrochemical technologies. Electrokinetic bioremediation or also called as electro-
bioresmediation (EBR) mixes the conventional in situ bioremediation with EK (Gill et al,
2014). This technology tries to join the most interesting advantages of both techniques
(that is, low-cost biological elimination without excavation and transport to external
treatment systems) and avoiding the limitations that can appear during the in situ
process (Yeung and Gu, 2011). In this manner, the microbial culture contained in soil is
capable of biodegrading the organic contaminant in situ (Semple et al., 2007; Wick et
al., 2007).
The present work is focused on the study of two EBR options: (1) EK-biostimulation
and (2) EK-bioaugmentation. In the first option, electrokinetic phenomena tries to
accelerate the slow biodegradation of pollutants thanks to the mixing between
autochthonous microorganisms and pollutants by adding nutrients which encourage the
microbial activity in soil. In the second case, microorganisms and nutrients are added
into the soil, and one alternative to deliver the microorganisms can be by the inclusion
of a biological permeable reactive barrier (BioPRB) or biobarrier in the soil (Mena et
al., 2015).
A biobarrier consists of a portion of a porous solid bed which acts as a support of
microorganisms acclimated to the biodegradation of the specific pollutant.
Microorganisms attached to the solid particles form a so-called biofilm. The barrier is
located into the soil and it acts as a fixed bed biofilm reactor for pollutant biodegradation when groundwater moves across it (Gill et al., 2014). When using EK, it is recommended to place the biobarrier in the centre of the soil portion in order to avoid the extreme pH values near electrodes. This configuration helps to keep the microorganisms inserted alive, and the pollution plume passes through it by electrokinetic transport phenomena allowing the pollutant biodegradation (Mena et al., 2016a).

The authors of the present work have previously studied different alternatives of combining conventional bioremediation with electrokinetic remediation in the case of hydrocarbon-polluted soils (Ramírez et al., 2015) or pesticide-polluted soils (Barba et al., 2019a). The present work is focused on the study of different biological strategies in an electro-bioremediation process of a 2,4-D clayey polluted soil. Three different situations were evaluated: (i) EK-biostimulation: EK is applied to the polluted soil that already contains a 2,4-D degrading microbial culture, (ii) EK-bioaugmentation (using biobarrier named as BB1): consists in applying electrokinetics in the polluted soil which contains a fixed-bed biofilm bioreactor as biobarrier, which was previously and externally developed to the biodegradation of 2,4-D, and (iii) EK-bioaugmentation (using biobarrier named as BB2): similar situation as (ii), but in this case the biobarrier consists of a mixture of a clean soil portion with the microbial suspension. Thus, the present work it is a proposal for the improvement of in situ techniques for organochlorines polluted soils remediation. It is expected that results would contribute to know the feasibility of the in situ EK-enhanced bioremediation technology for the treatment of polluted soil. Under the author’s knowledge, 2,4-D is a hazardous pollutant and no previous research (exception of previous works in our research group) has been found about electro-bioremediation of 2,4-D-polluted soil.
2. Materials and methods

2.1. Materials

Soil

Millas Hijos Ceramics (Toledo, Spain) supplied the clean clayey soil employed in this work. Table 1 shows soil characteristics (Barba et al., 2017).

Table 1. Properties of the soil used in the experiments.

<table>
<thead>
<tr>
<th>Mineralogy:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz</td>
</tr>
<tr>
<td>Feldspar</td>
</tr>
<tr>
<td>Calcite</td>
</tr>
<tr>
<td>Kaolinite</td>
</tr>
<tr>
<td>Glauconite</td>
</tr>
<tr>
<td>Muscovite</td>
</tr>
<tr>
<td>Montmorillonite</td>
</tr>
<tr>
<td>Smectite</td>
</tr>
<tr>
<td>Illite</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters USCS (Unified Soil Classification System):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasticity index</td>
</tr>
<tr>
<td>USCS Code</td>
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<tr>
<td>Low plasticity clay (CL)</td>
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<table>
<thead>
<tr>
<th>Granulometry</th>
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<tbody>
<tr>
<td>&lt; 4 µm</td>
</tr>
<tr>
<td>4 µm – 200 µm</td>
</tr>
<tr>
<td>&gt; 4 µm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry density / g cm$^{-1}$</td>
</tr>
<tr>
<td>Electric conductivity/ µS cm$^{-1}$</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Organic matter</td>
</tr>
<tr>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 1: Properties of the soil used in the experiments.

- **Mineralogy:**
  - Quartz: 12%
  - Feldspar: 6%
  - Calcite: 1%
  - Kaolinite: 23%
  - Glauconite: 24%
  - Muscovite: 8%
  - Montmorillonite: 20%
  - Smectite: -
  - Illite: 6%

- **Parameters USCS (Unified Soil Classification System):**
  - Plasticity index: 22
  - USCS Code: Low plasticity clay (CL)

- **Granulometry:**
  - < 4 µm: 10%
  - 4 µm – 200 µm: 78%
  - > 4 µm: 12%

- **Other properties:**
  - Dry density / g cm$^{-1}$: 1.65
  - Electric conductivity/ µS cm$^{-1}$: 1800
  - pH: 7.9
  - Organic matter: n.d.
<table>
<thead>
<tr>
<th><strong>Hygroscopic moisture</strong></th>
<th>0.115</th>
</tr>
</thead>
</table>

n.d.: non detected.

### Mineralogy

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz</td>
<td>12%</td>
</tr>
<tr>
<td>Feldspar</td>
<td>6%</td>
</tr>
<tr>
<td>Calcite</td>
<td>4%</td>
</tr>
<tr>
<td>Kaolinite</td>
<td>23%</td>
</tr>
<tr>
<td>Glaucnite</td>
<td>24%</td>
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<td>Muscovite</td>
<td>8%</td>
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<td>20%</td>
</tr>
<tr>
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<td>-</td>
</tr>
<tr>
<td>Illite</td>
<td>6%</td>
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</tbody>
</table>

### Other properties

<table>
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<td>1.65</td>
</tr>
<tr>
<td>Hygroscopic moisture</td>
<td>0.115</td>
</tr>
</tbody>
</table>

### Soil preparation

Soil provided was previously artificially polluted for EBR experiments. The procedure followed was to mix homogeneously clean soil with 2,4-D solution. The 2,4-D soil concentration after this preparation is 20 mg per kg of wet soil (26.7 mg kg$^{-1}$ on dry soil).

### Pesticide

The pesticide selected in this work was 2,4-dichlorophenoxyacetic acid (2,4-D) as polar pesticide model. 2,4-D, 98% assay, was supported by Alfa Aesar.

### Microbial culture

Microorganisms acclimation to the biodegradation of 2,4-D followed the procedure described in previous studies (Moliterni et al., 2012). The inoculum was obtained from
an oil-refinery wastewater treatment plant (Puertollano, Spain), and the culture medium containing inorganic nutrients was Bushnell-Hass Broth (BHB). The composition of BHB per litre of Milli-Q water is 0.20 g Mg SO\textsubscript{4}, 0.02 g CaCl\textsubscript{2}, 1.00 g KH\textsubscript{2}PO\textsubscript{4}, 1.00 g \((NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4}, 0.05 g FeCl\textsubscript{3} and 1.00 g KNO\textsubscript{3}. The sole carbon source employed during acclimation was 2,4-D (200 mg L\textsuperscript{-1}). After the acclimation process, microorganisms contained in the microbial culture were identified by using a MALDI TOF Mass Spectrometry AXIMA-Assurance equipment (Biotech technology, SHIMADZU, Germany). The species identified were *Rhodococcus ruber* and *Ochrobactrum anthropic*.

### 2.2 Experimental set-up

The experimental set-up scheme is shown in Figure 1. Fig. 1a corresponds to EK-biostimulation experiment and Fig.1b corresponds to EK-bioaugmentation experiments using biobarriers. The cell is made of transparent methacrylate and divided into five compartments. Soil polluted is placed in the central compartment, while at both sides are located the electrodic wells, which contain the graphite electrodes (10x10x1 cm) supplied by Carbosystem (Madrid, Spain) and connected to the power supply (HQ Power, Gavere, Belgium). Soil is separated from electrodic wells by a nylon mesh (0.5 mm mesh size). Contiguous to electrodic wells, there are the collector compartments that collect the electroosmotic flow (EOF) transported during the treatment. As later explained, EOF will be collected at both sides due to the electrode polarity reversal. Fig. 1b refers to the EK-bioaugmentation experiments with biobarriers. The experimental set-up is similar to that described above, but the difference in this case is that in the middle of the central compartment is placed the biobarrier separated from soil with a nylon mesh.
Figure 1. Electro-bioremediation set-up: (a) EK-biostimulation experiment; (b) EK-bioaugmentation experiments using biobarrers; (c) photographs of set-up using biobarrers.

The electrolyte employed in electrode wells and in soil to provide a proper electrical conductivity is a simulated groundwater, whose composition per litre of Milli-Q water is 80.75 mg of Na$_2$SO$_4$, 70.00 mg of NaHCO$_3$, 30.36 mg of NaNO$_3$. Additionally, inorganic nutrients (ammonium, phosphate and nitrate) were supplied in excess to the soil by using BHB media, in order to avoid nutrient limitations that could happen during
the treatment because of biological consumption or because of EK transport to the external compartments (Mena et al., 2016b).

2.3. Electro-bioremediation experimental procedure

Once the experimental set-up was ready, the electro-bioremediation experiments were carried out. The three-batch experiments of 10 days-duration were conducted under an electric field of 1.0 V cm\(^{-1}\) (20 V) at room temperature and using 2 d\(^{-1}\) of polarity reversal frequency.

Experiment 1 (EK-biostimulation, Fig. 1a): an inoculum from the acclimated microbial culture was grown in a batch reactor using BHB as culture media supplemented with 2,4-D. After 4 days, the obtained culture was centrifuged and suspended again in BHB. Then, it was added to the 2,4-D polluted soil and mixed homogeneously obtaining a final moisture of 25%. The mixture of polluted soil and microorganisms was manually compacted into the central compartment of the installation simulating an autochthonous microbial culture in soil for 2,4-D degradation. Both electrodic wells were filled with electrolyte solution and the direct current was connected.

Experiment 2 (EK-bioaugmentation, Fig 1b, by using a portion of a fixed-bed biofilm reactor as permeable biological barrier or “BB1”): The polluted soil was moistened with the electrolyte solution and compacted into the central compartment as in experiment 1. In this case, microorganisms were not inoculated through all over the soil (soil was previously autoclaved at 121°C and 15 min) but were added to soil by means of the biobarrier (BB1, which is a portion of a fix-bed bioreactor previously developed as reported by Barba et al., 2019b) in the central position of the soil to be remediated. A central portion of soil was removed and replaced by the biobarrier (5 cm length) and separated from the soil by a nylon mesh. Moreover, in order to ensure the properly concentration of nutrients for the microbial culture, it was filled the biobarrier.
compartment with BHB culture medium solution. Both electrodic wells were filled with
electrolyte solution and the direct current was connected. This configuration were
studied by the authors in previous works (Barba et al., 2019a; 2019b), and details about
the procedure for biobarrier development has been reported there.

Experiment 3 (EK-bioaugmentation, Fig 1b, through the inclusion of a mixture of soil
with microorganisms’ suspension as permeable biological barrier or “BB2”). This
option is similar to the last one, and the only difference is the type of biobarrier used. In
this case, the biological barrier consists of a mixture of clean clayey soil and
microorganisms suspended in BHB culture medium. The mixture soil/microorganisms
was placed in the central position of polluted soil. This option (BB2) is quite easy and
quick to prepare. Both electrodic wells were filled with electrolyte solution and the
direct current was connected.

Additionally, two complementary reference experiments were carried out. The first
reference test was identical to the Experiment 1, but no electric current was applied to
the soil (named as “No EK”). This test would inform about the possible evolution of
pollutant biodegradation without the contribution of electrokinetic phenomena. The
second reference test was an abiotic EK reference test (named as “No Bio”) and it was
carried out by using the same electrokinetic conditions of all experiments (1.0 V cm\(^{-1}\)
and 2.0 d\(^{-1}\) polarity reversal frequency) but using no inoculated soil (no addition of
acclimated 2,4-D removal microorganisms). This test would inform about the possible
removal of pollutant by non-biologically assisted mechanisms.

2.4. Sampling and analyses

Samples were taken and analysed during the operation time in both electrodic
compartments, and in the electroosmotic flow. It is important to remark that EOF was
alternatively collected in both collector compartments due to the electrode polarity
reversal every 12 hours. Temperature of soil and electrical current were monitored
during all the treatment.
	pH and conductivity were measured with multiparameter probe (SENSLON, HACH).

To analyse nutrient concentrations, i.e., ammonium, nitrate and phosphate, it was used a
photometer Gallery (Thermo Scientific). Soil samples (1 g) were mixed with 2.5 mL of
water for the dissolution of 2,4-D. The soil/solvent mixture was agitated vigorously in a
vortex agitator for 5 min and centrifuged (15 min, 3800 min⁻¹). Samples were taken
from the aqueous supernatant and analysed. 2,4-D was analysed with an HPLC (Jasco,
Japan) equipped with a column Kinetex 5 µm Biphenyl 100 Å, 150 x 4.5 mm
(Phenomenex, USA). The mobile phase employed 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 injection volume was 20 µL.

Soil samples were taken only at the start of the experiment, before placing it on the
installation, and at the end (post-mortem analysis) of the treatment, in order to not
modify the compaction of soil that could cause preferential ways (Ruiz et al., 2014).

The post-mortem analysis were conducted in different soil portions as follows: four
longitudinal positions were considered (1 to 4, from anode to cathode at time zero) and
each one in turn was divided into four sections (two in the upper layer and another two
in the bottom layer) according to previous works (Ramírez et al., 2014). Thus,
analytical results in each position were the average of 4 measurements. The parameters
analysed in soil were moisture, pH, conductivity, microorganisms, nutrients, and 2,4-D
concentrations. Moisture was calculated by difference of weights, i.e., an amount of wet
soil was dried at 105 °C for 24h. Weight of evaporated water corresponds to moisture
contained in soil. pH and conductivity were measured from dry soil. To do this, it was
taken 10 g of dry soil and 25 mL of Milli-Q water was added. Then, it was agitated for
30 min and it was left decant around 2 hours. Liquid supernatant was filtered by using nylon filters of 0.2 μm and measured with a multiparameter probe. Nutrient concentrations were also measured from dry soil with the same method. 2,4-D concentration was determined from wet soil by HPLC as described above. Microorganisms concentration is expressed as Colony Forming Units (CFU) per gram of dry soil (Ramírez et al., 2015). To do this, it was taken 1 g of wet soil and then, it was added 10 mL of a solution of 0.9% NaCl. After that, it was mixed and agitated for 3 min with a vortex agitator. An aliquot of 100 μL of supernatant liquid was taken and put on Petri dishes containing LB media as solid culture media for the microbial growth with the following composition: 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. Then, the dishes were incubated for 24h at 26.5 °C.

### 3. Results and discussion

The present work considers two possible real situations in the case of a soil contaminated with 2,4-D. One possible situation considers a recent pesticide spill in a soil which does not contain an adapted microbial population capable of biodegrading 2,4-D and thus bioaugmentation is needed. The authors consider that a good option to include the acclimated microorganisms in such polluted soil is through inserting a biobarrier on it with them (EK-bioaugmentation). To do this, two different types of biobarriers have been proposed: BB1 consists of a portion of fixed-bed biofilm reactor for 2,4-D biodegradation, previously developed in a laboratory, while BB2 is just a mixture of clean clayey soil and a 2,4-D acclimated microorganisms suspension. According to recent works, the extreme pH in electrodi zones can avoid the microbial activity (Mena et al., 2014). Thus, the authors consider that the optimal way to insert the...
biobarrier in soil is in the central position, and this disposition implies to mobilize the pollutant to pass through the barrier by EK.

The other possible situation considers that the polluted soil already contains an autochthonous microbial population adapted to use the organic pollutant (2,4-D) as the carbon source (a possible situation in historically polluted sites) and inorganic nutrients are available. In this case the proposed treatment consists of using electrokinetics for the mobilization of pollutants, nutrients, and microorganisms, in order to improve the contact between them (EK-biostimulation) but trying to keep experimental conditions in suitable values for microbial life.

Figure 2. (a) Electroosmotic flow and (b) current intensity through the soil during the EBR experiments.

Figure 2 shows EOF values and current intensity throughout the duration of the EBR experiments. The EOF profile (Fig. 2a) indicates the movement of system water out of the set-up and, consequently, it is necessary to replace it with an electrolyte solution, which guarantee the correct conductivity in soil for electro-bioremediation process. As it can be observed in Fig. 2a, in all the cases, the EOF increases till a maximum level to keep constant along the treatment. In the experiments of EBR by biobarriers (BB1 and BB2), similar values of EOF, around 4-5 mL h\(^{-1}\) and approximately constant during the
process, are observed. On the other hand, in the case of biostimulation, EOF is higher than in EK-bioaugmentation cases, around 7-8 mL h\(^{-1}\), which is supposed to be caused by a lower soil permeability than in the bioaugmentation experiments because of no central biobarrier is needed in this case. This behaviour is similar to previous works using non-polar pesticides (oxyfluorfen) reported by the same authors (Barba et al., 2019a). Related to current intensity (Fig. 2b), it can be observed that in the case of using biobarrers the value is approximately constant and slightly lower than in the case of biostimulation. This behaviour can be explained because of the higher ohmic resistance due to the inclusion of a biobarrier into the soil. Moreover, current intensity values when using two biobarrers are similar and slight differences can be explained by soil permeability changes due to the introduction of the biobarrers or also because of the manual compaction of soil at the start of the experiments (Mena et al., 2015; Mena et al., 2016b).

Figure 3 shows the soil conductivity profiles obtained in the *post-mortem* analysis and compared to initial values for each experiment carried out. Solid lines show the average values for each longitudinal sample point or soil position (position 1 corresponds to the nearest to anode and position 4 to cathode at t=0). As it can be observed, the electrical conductivity in three cases at the start is high, around 1500-1600 \(\mu S \, cm^{-1}\), and decreases until 700-1000 \(\mu S \, cm^{-1}\) at the end of the treatment. Despite this drop of conductivity, the final average value is also high to secure the proper conductivity in soil for electrokinetics, and simultaneously it is not excessive for biological phenomena.
Figure 3. Soil conductivity profile in soil at the start (---) and at the end (--.) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only.

In both figures, 2 and 3, it has been observed that the inclusion of a permeable reactive biobarrier in a polluted soil causes differences in comparison with applying biostimulation strategy. EOF decreases in both bioaugmentation experiments in comparison with biostimulation experiment. It is a fact that the EOF is directly proportional to the voltage applied in system, and thus to the zeta potential which depends on the ionic concentration, and proportional to the dielectric constant of fluid,
and inversely proportional to the viscosity of the fluid (Reddy and Cameselle, 2009).
Moreover, EOF in low permeability regions is significantly higher than the EOF in regions with upper porosity. As it is explained above soil permeability is higher when biobarriers are applied. Thus, the introduction of biobarriers in the experimental system at the present work generated an important EOF decrease, which could be associated to the decrease in the current density and soil conductivity. Nevertheless, a slight contradiction was observed when comparing BB1 and BB2 results (biobarrier from fix-bed biofilm reactor and clean soil-microorganisms mixture, respectively). EOF is slightly higher using BB1 versus BB2 despite the greater porosity (BB1 is made by gravel particles). It can be considered that variables such as ionic concentration, related to soil conductivity and current density, can influence experiments performance, causing the lower value in BB2 experiment. It is important to remain that variables such as voltage gradient, fluid dielectric constant and viscosity keep constant during all the experiments carried out.

Many authors have previously studied the electroremediation process inserting a permeable reactive barrier (PRB). For example, Wan et al. (2010a) reported that the insertion of Pd/Fe PRB caused EOF decrease 1.8 times in an electroremediation process for hexachlorobenzene-polluted soil. Kebria et al. (2016) reported similar results in electroremediation of PCE polluted soil by using Fe⁰ particles as PRB. On the other hand, same authors (Wan et al., 2010b) reported that coupling a Cu/Fe PRB in electroremediation of hexachlorobenzene-polluted soil caused that EOF increased. The authors of the present work also reported results related to the application of biobarriers, comparing the performance of BB1 and BB2 in electro-bioremediation of diesel polluted soil, and they found that the application of biobarrier type BB1 causes a higher EOF (Mena et al., 2016) and the EOF was higher when no biobarrier was inserted in
soil (biostimulation) (Ramírez et al., 2015). Additionally, similar behaviour to that observed in the present work was reported in EBR of oxyfluorfen polluted clay soil (Barba et al., 2019a). There are some variables which could simultaneously influence the performance of BioPRBs and additional research efforts still need to be made.

Figure 4 shows the initial and final average values of soil temperature and pH. In Fig. 4a it can be observed that the temperature of soil during the three experiments keeps practically constant around 25-28°C, which is an optimal value for the activity of the microbial culture employed in this work. Related to pH in soil (Fig. 4b), it can be observed that in all the cases the pH has been controlled correctly, i.e., it has been cushioned the extreme pH fluctuations due to the electrolysis of water thanks to polarity reversal strategy (Barba et al., 2017). Yeung and Gu (2011), reported different strategies to control pH in electroremediation processes. One of the most used in recent years is so-called periodic polarity reversal strategy, employed in the present work. Several authors reported the effect of using periodical changes in the polarity of the system and showed beneficial effects in pH, temperature and moisture of soil at the end of treatment for the proper activity microbial culture in electro-bioremediation process (Li et al., 2015; Li et al., 2016). Both temperature and pH show a homogeneous distribution profile throughout the treated soil in all the cases studied. Thus, these conditions are considered to be adequate for microbial activity in soil during the three EBR processes conducted at this work.
Figure 4. Average values of soil (a) temperature and (b) pH at the start and at the end of EBR experiments. Grey bars represent initial conditions while black bars represent final average value.

Figure 5 shows the microorganisms’ concentration profile in soil before and after the EBR treatment. As it can be observed, only in the experiment when using biostimulation as biological strategy the initial concentration (dashed line) is presented in the figure because in the two experiments with biobarriers, the soil at the start was autoclaved and the microorganisms were only inoculated through the biobarrier. From this figure, it can be extracted two main conclusions: the first one is that in all the experiments carried out, the microorganisms’ concentration has similar concentration values at the end of EBR treatment in the three cases. The second one is that there exists homogeneous distribution of microorganisms in all the soil at the end of the treatment. This behaviour can be explained because of the biofilm detachment from biobarrier and movement of microorganisms from the central location to the rest of soil positions thanks to the electrophoresis and electroosmotic flow passing thought it (DeFlaun and Condee, 1997). A similar result was also observed by the same authors when non polar pesticide was used as model pollutant (Barba et al., 2019a). Due to the application of polarity reversal strategy in EBR experiments, a correct control of pH has been achieved as it was explained above. Thus, it was not observed harmful effects because of extreme pH which would cause a decrease in the concentration of...
microorganisms in the zones near to electrodes, and it indicates adequate conditions in soil for microbial activity.

![Figure 5](image)

**Figure 5.** Soil microorganisms’ concentration profile in soil at the start (---) and at the end (—) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only.

Figure 6 (a-c) shows the average values of 2,4-D concentrations in soil at the start and at the end of the treatment. Figure 6d shows a comparative about the 2,4-D percentage removal efficiencies in the three experiments carried out, including also the removal efficiencies of the two reference tests. As it can be observed, removal of 2,4-D by using biobarriers is quite effective, so in both cases (BB1 and BB2) it was achieved between 75-85% of 2,4-D removal in only 10 days of treatment. Nevertheless, EBR with
biostimulation strategy offers a complete 2,4-D elimination in soil and a homogeneous removal profile. The lower 2,4-D removal rate when using biobarriers could be directly related to EOF decrease due to the higher porosity in the biobarriers zones, and consequently the current intensity and soil conductivity also decrease, as it was above explained. The slightly differences of 2,4-D removal rates between two biobarriers evaluated, and taking into account that experimental conditions for microbial activity are practically identical in all the experiments (i.e., pH, temperature, nutrients and microorganisms’ concentrations) could be explained again because of the lower EOF of EBR by using BB2 in comparison with BB1 related to the lower mixture effect between nutrients, pollutant and microorganisms in the process of remediation. Despite offering biostimulation option better results of pollutant elimination, the use of biobarriers in EBR process is a great advantage because, in the case that the soil does not contain microorganisms adapted to the degradation of such pollutant, this would be the most optimal way to introduce the microbial culture into the soil. Studies about EK-bioaugmentation are scarce. Mao et al. [49] studied EK-enhanced bioaugmentation for remediation of clays contaminated with chlorinated solvents but they did not use BioPRB: the microbial culture solution was added to the electrode compartments and to a central injection well. They found that the microbial distribution within the clay suggested that electrokinetic microbial transport was primarily driven by electroosmosis, the injected bacteria were able to survive and grow, and complete effective dechlorination of chlorinated ethene was observed after 94d. Additionally, in Fig. 6d the results obtained in the three EBR experiments were compared with two reference tests: test “No EK” and test “No Bio”. Test “No Bio” means only EK treatment without microbial activity, and the 2,4-D removal result from this reference test is quite similar to ones obtained using biobarriers. However it is
important to note that, when using only EK, the pollutant is moved to the electrodic wells, and then it is necessary to treat the contaminated water by external techniques, e.g., electro-oxidation (de Viales et al., 2018). Comparing the in situ removal EBR treatments and the reference test “No EK” (that is, only in situ bioremediation without EK) it can be observed that the 2,4-D removal percentages reached up in three experiments of EBR are much higher than in the test “No EK”. This behaviour prove that electrokinetics acts as a mixer improving the contact and transfer matter between pollutant, microorganisms and nutrients contained in soil (Mena et al., 2016c; Barba et al., 2017). It is important to remark that the microbial culture is able to successfully degrade high pesticide concentrations in relatively short retention times (as previously reported by the same authors, Barba et al., 2019b) and thus the success, or not, of the subsequent EBR technology 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. Thus, the main conclusion that can be extracted from figure 6d is that coupling electrokinetic processes with biological treatment improve the in situ removal of 2,4-D from soil.
Figure 6. (a-c) 2,4-D concentration profiles in soil at the start (---) and at the end (-----) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only. (d) 2,4-D removal percentages after 10 days of treatment.

Conclusions

Different alternatives based on EK-biostimulation and EK-bioaugmentation were tested in electro-bioremediation of 2,4-D polluted soils. Supposing there exists already an autochthonous culture in the soil capable of degrading 2,4-D, the biostimulation strategy practically achieved the complete elimination of the herbicide after 10d. Pollutant removal efficiencies when using biobarriers (bioaugmentation) were successful (75-85%) but lower than efficiency obtained when using biostimulation. The use of biobarriers was found to be a viable strategy to deliver microorganisms if soil does not contain an adapted microbial population. Temperature and pH were correctly controlled in all cases, but the inclusion of biobarriers caused EOF to decrease due to the higher porosity in the biobarriers zones, and consequently the current intensity and soil conductivity also decreased. As a result, the mixture and transport contribution of EK phenomena were lower when using bioaugmentation. Reference tests proved the positive effect of coupling both biological and electrokinetic mechanisms.

Acknowledgements

Financial support from the Spanish Government and European Union through projects CTM2016-76197-R (AEI/FEDER, UE) from Ministry of Economy, Industry and Competitiveness, and EQC2018-004240-P from Ministry of Science, Innovation and Universities is gratefully acknowledged. The FPI grant BES-2014-069662 is also acknowledged.

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BIOSTIMULATION VERSUS BIOAUGMENTATION FOR THE ELECTRO-BIOREMEDIATION OF 2,4-DICHLOROPHENOXYACETIC ACID POLLED SOILS.

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Abstract

The aim of this work is to compare three biological strategies for the \textit{in situ} remediation of a 2,4-dichlorophenoxyacetic acid (2,4-D) polluted clayey soil by coupling electrokinetics (EK) and bioremediation (technology named as electrobioremediation, EBR). The first option (i) is EK-biostimulation, in which the activity of microorganisms already present in soil is enhanced by EK phenomena. The second and third options are EK-bioaugmentation, which consist of addition of microorganisms to soil through the inclusion of permeable biological barriers: (ii) using a microbial fixed biofilm reactor as biobarrier (BB1), and (iii) using a mixture of clean soil and a microbial suspension as biobarrier (BB2). Thus, three batch experiments at bench scale were conducted under a constant electric field of 1 V cm\textsuperscript{-1}, and electrode polarity was periodically reversed every 12 h (2 d\textsuperscript{-1}). The duration of each test was 10 days. Two additional tests using only biodegradation or only EK were performed as auxiliary reference tests. A microbial consortium acclimated to 2,4-D biodegradation was employed. Results
showed that EK-biostimulation strategy offered the best pollutant removal efficiency (reaching up almost 100%) while biobarriers offered pollutant removal rates between 75-85%. Permeable biobars allowed the introduction of microorganism but caused a decrease in the electro-osmotic flow which, in turn, reduced the mobilization and contact between microorganisms and pollutants. These results can contribute to the knowledge and understanding of electrobioremediation of polluted soil and to the feasibility of delivering microorganism to the soil by using biobarriers. Despite biostimulation was found to be the best option, results show that permeable reactive biobars may result in a successful alternative for in-situ EK-bioaugmentation when acclimated microbial population is not already present in soil.

Keywords
2,4-dichlorophenoxyacetic acid, permeable reactive biobARRIER, electrobioremediation, polluted soil, pesticide pollution.

1. Introduction
Pesticide pollution is a serious environmental problem in our days due to the mostly use of these compounds in agricultural activities for pest control and weed growth. Within the group of pesticides, 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most commonly used. 2,4-D is a systemic hormonal herbicide, which can affect directly to hormonal system in plants avoiding their growth. Moreover, 2,4-D belongs to the organochlorinated pollutants group, which means to be very persistent compounds in soil, water and air (Chowdhury et al., 2008). Additionally, it can produce a dangerous impact in humans or animals in contact with this pollutant, causing genomic mutations or in the worst of cases, even the death (Morillo and Villaverde, 2017).
Because of it, regulation referring to soil pollution in Spain and E.U. is currently becoming harder in order to control the pollution levels in soil and forcing its remediation if contaminant levels exceed those allowed. Depending on the impact in natural ecosystems or whether it affects to human health, the maximum pollution levels are different, e.g., for organochlorinated pollutants the maximum allowed level in soil is 1.0 mg per kg of soil (Spanish Presidential Ministry, 2005).

According to above mentioned environmental and health cited risks, and because of the soil is a non-renewable natural resource, it is necessary to remediate it. There are several remediation technologies for polluted soils based on biological, chemical, physical, or thermal fundamentals. The in situ remediation treatments are focused on the removal of the pollutant in the polluted site, regardless of the biological, physical or chemical method used, and thus external treatments are not required, which supposes a clear cost-effective alternative for remediating polluted soils (Reddy and Cameselle, 2009). Bioremediation is one of the treatment methods most applied under the in situ option due to the low cost associated, but the main limitation is the high operation times required because of the slow mass transfer phenomena to contact microorganisms, nutrients and pollutants, especially in soils contaminated with non-polar compounds (Barba et al., 2018a).

Alternatively, electrokinetic remediation or electroremediation (EK) is an in situ technique, which consists in applying an electric field through the soil between a couples of electrodes inserted on it. Consequently, electrokinetic transport phenomena appear, mobilizing different species contained in the soil such as microorganisms, pollutants and nutrients, encouraging the contact between them (Paillat et al., 2000; Rodrigo et al., 2014). Electroremediation has been proved as a cost-effective and successful in situ treatment, mainly in low permeability soils, where conventional pump
and treat methods are not indicated to transport the contaminant through all over the soil (Reddy and Cameselle, 2009; Cameselle, 2014). However, this technology also presents some limitations during operation time, e.g., soil heating due to the Joule effect, low mobility of non-polar pollutants in soil, or extreme pH values near the electrode’s zones.

In recent years it is becoming more attractive the idea of combining biological with electrochemical technologies. Electrokinetic bioremediation or also called as electro-bioremediation (EBR) mixes the conventional in situ bioremediation with EK (Gill et al., 2014). This technology tries to join the most interesting advantages of both techniques (that is, low-cost biological elimination without excavation and transport to external treatment systems) and avoiding the limitations that can appear during the in situ process (Yeung and Gu, 2011). In this manner, the microbial culture contained in soil is capable of biodegrading the organic contaminant in situ (Semple et al., 2007; Wick et al., 2007).

The present work is focused on the study of two EBR options: (1) EK-biostimulation and (2) EK-bioaugmentation. In the first option, electrokinetic phenomena tries to accelerate the slow biodegradation of pollutants thanks to the mixing between autochthonous microorganisms and pollutants by adding nutrients which encourage the microbial activity in soil. In the second case, microorganisms and nutrients are added into the soil, and one alternative to deliver the microorganisms can be by the inclusion of a biological permeable reactive barrier (BioPRB) or biobarrier in the soil (Mena et al., 2015).

A biobarrier consists of a portion of a porous solid bed which acts as a support of microorganisms acclimated to the biodegradation of the specific pollutant. Microorganisms attached to the solid particles form a so-called biofilm. The barrier is
located into the soil and it acts as a fixed bed biofilm reactor for pollutant biodegradation when groundwater moves across it (Gill et al., 2014). When using EK, it is recommended to place the biobarrier in the centre of the soil portion in order to avoid the extreme pH values near electrodes. This configuration helps to keep the microorganisms inserted alive, and the pollution plume passes through it by electrokinetic transport phenomena allowing the pollutant biodegradation (Mena et al., 2016a).

The authors of the present work have previously studied different alternatives of combining conventional bioremediation with electrokinetic remediation in the case of hydrocarbon-polluted soils (Ramírez et al., 2015) or pesticide-polluted soils (Barba et al., 2019a). The present work is focused on the study of different biological strategies in an electro-bioremediation process of a 2,4-D clayey polluted soil. Three different situations were evaluated: (i) EK-biostimulation: EK is applied to the polluted soil that already contains a 2,4-D degrading microbial culture, (ii) EK-bioaugmentation (using biobarrier named as BB1): consists in applying electrokinetics in the polluted soil which contains a fix-bed biofilm bioreactor as biobarrier, which was previously and externally developed to the biodegradation of 2,4-D, and (iii) EK-bioaugmentation (using biobarrier named as BB2): similar situation as (ii), but in this case the biobarrier consists of a mixture of a clean soil portion with the microbial suspension. Thus, the present work it is a proposal for the improvement of in situ techniques for organochlorines polluted soils remediation. It is expected that results would contribute to know the feasibility of the in situ EK-enhanced bioremediation technology for the treatment of polluted soil. Under the author’s knowledge, 2,4-D is a hazardous pollutant and no previous research (exception of previous works in our research group) has been found about electro-bioremediation of 2,4-D-polluted soil.
2. Materials and methods

2.1. Materials

Soil

Millas Hijos Ceramics (Toledo, Spain) supplied the clean clayey soil employed in this work. Table 1 shows soil characteristics (Barba et al., 2017).

Table 1. Properties of the soil used in the experiments.

<table>
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<td>Feldspar</td>
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</tr>
<tr>
<td>Calcite</td>
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<tr>
<td>Kaolinite</td>
<td>23%</td>
</tr>
<tr>
<td>Glauconite</td>
<td>24%</td>
</tr>
<tr>
<td>Muscovite</td>
<td>8%</td>
</tr>
<tr>
<td>Montmorillonite</td>
<td>20%</td>
</tr>
<tr>
<td>Smectite</td>
<td>-</td>
</tr>
<tr>
<td>Illite</td>
<td>6%</td>
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<table>
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<tr>
<td>USCS Code</td>
<td>Low plasticity clay (CL)</td>
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<table>
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<tr>
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<tr>
<td>&lt; 4 µm</td>
<td>10%</td>
</tr>
<tr>
<td>4 µm – 200 µm</td>
<td>78%</td>
</tr>
<tr>
<td>&gt; 4 µm</td>
<td>12%</td>
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<table>
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<td>Dry density / g cm⁻³</td>
<td>1.65</td>
</tr>
<tr>
<td>Electric conductivity/ µS cm⁻¹</td>
<td>1800</td>
</tr>
<tr>
<td>pH</td>
<td>7.9</td>
</tr>
<tr>
<td>Organic matter</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Hygroscopic moisture 0.115

n.d.: non detected.

136 **Soil preparation**
Soil provided was previously artificially polluted for EBR experiments. The procedure followed was to mix homogeneously clean soil with 2,4-D solution. The 2,4-D soil concentration after this preparation is 20 mg per kg of wet soil (26.7 mg kg$^{-1}$ on dry soil).

138 **Pesticide**
The pesticide selected in this work was 2,4-dichlorophenoxyacetic acid (2,4-D) as polar pesticide model. 2,4-D, 98% assay, was supported by Alfa Aesar.

144 **Microbial culture**
Microorganisms acclimation to the biodegradation of 2,4-D followed the procedure described in previous studies (Moliterni et al., 2012). The inoculum was obtained from an oil-refinery wastewater treatment plant (Puertollano, Spain), and the culture medium containing inorganic nutrients was Bushnell-Hass Broth (BHB). The composition of BHB per litre of Milli-Q water is 0.20 g Mg SO$_4$, 0.02 g CaCl$_2$, 1.00 g KH$_2$PO$_4$, 1.00 g (NH$_4$)$_2$HPO$_4$, 0.05 g FeCl$_3$ and 1.00 g KNO$_3$. The sole carbon source employed during acclimation was 2,4-D (200 mg L$^{-1}$). After the acclimation process, microorganisms contained in the microbial culture were identified by using a MALDI TOF Mass Spectrometry AXIMA-Assurance equipment (Biotech technology, SHIMADZU, Germany). The species identified were *Rhodococcus ruber* and *Ochrobactrum anthropic*.

2.2 **Experimental set-up**
The experimental set-up scheme is shown in Figure 1. Fig. 1a corresponds to EK-
biostimulation experiment and Fig. 1b corresponds to EK-bioaugmentation experiments using biobarriers. The cell is made of transparent methacrylate and divided into five compartments. Soil polluted is placed in the central compartment, while at both sides are located the electroductive wells, which contain the graphite electrodes (10x10x1 cm) supplied by Carbosystem (Madrid, Spain) and connected to the power supply (HQ Power, Gavere, Belgium). Soil is separated from electroductive wells by a nylon mesh (0.5 mm mesh size). Contiguous to electroductive wells, there are the collector compartments that collect the electroosmotic flow (EOF) transported during the treatment. As later explained, EOF will be collected at both sides due to the electrode polarity reversal. Fig. 1b refers to the EK-bioaugmentation experiments with biobarriers. The experimental set-up is similar to that described above, but the difference in this case is that in the middle of the central compartment is placed the biobarrier separated from soil with a nylon mesh.
Figure 1. Electro-bioremediation set-up: (a) EK-biostimulation experiment; (b) EK-bioaugmentation experiments using biobarrers; (c) photographs of set-up using biobarrers.

The electrolyte employed in electrode wells and in soil to provide a proper electrical conductivity is a simulated groundwater, whose composition per litre of Milli-Q water is 80.75 mg of Na$_2$SO$_4$, 70.00 mg of NaHCO$_3$, 30.36 mg of NaNO$_3$. Additionally, inorganic nutrients (ammonium, phosphate and nitrate) were supplied in excess to the soil by using BHB media, in order to avoid nutrient limitations that could happen during
the treatment because of biological consumption or because of EK transport to the external compartments (Mena et al., 2016b).

2.3. Electro-bioremediation experimental procedure

Once the experimental set-up was ready, the electro-bioremediation experiments were carried out. The three-batch experiments of 10 days-duration were conducted under an electric field of 1.0 V cm\(^{-1}\) (20 V) at room temperature and using 2 d\(^{-1}\) of polarity reversal frequency.

Experiment 1 (EK-biostimulation, Fig. 1a): an inoculum from the acclimated microbial culture was grown in a batch reactor using BHB as culture media supplemented with 2,4-D. After 4 days, the obtained culture was centrifuged and suspended again in BHB. Then, it was added to the 2,4-D polluted soil and mixed homogeneously obtaining a final moisture of 25%. The mixture of polluted soil and microorganisms was manually compacted into the central compartment of the installation simulating an autochthonous microbial culture in soil for 2,4-D degradation. Both electrodic wells were filled with electrolyte solution and the direct current was connected.

Experiment 2 (EK-bioaugmentation, Fig 1b, by using a portion of a fixed-bed biofilm reactor as permeable biological barrier or “BB1”): The polluted soil was moistened with the electrolyte solution and compacted into the central compartment as in experiment 1. In this case, microorganisms were not inoculated through all over the soil (soil was previously autoclaved at 121ºC and 15 min) but were added to soil by means of the biobarrier (BB1, which is a portion of a fix-bed bioreactor previously developed as reported by Barba et al., 2019b) in the central position of the soil to be remediated. A central portion of soil was removed and replaced by the biobarrier (5 cm length) and separated from the soil by a nylon mesh. Moreover, in order to ensure the properly concentration of nutrients for the microbial culture, it was filled the biobarrier
compartment with BHB culture medium solution. Both electrodic wells were filled with electrolyte solution and the direct current was connected. This configuration were studied by the authors in previous works (Barba et al., 2019a; 2019b), and details about the procedure for biobarrier development has been reported there.

Experiment 3 (EK-bioaugmentation, Fig 1b, through the inclusion of a mixture of soil with microorganisms’ suspension as permeable biological barrier or “BB2”). This option is similar to the last one, and the only difference is the type of biobarrier used. In this case, the biological barrier consists of a mixture of clean clayey soil and microorganisms suspended in BHB culture medium. The mixture soil/microorganisms was placed in the central position of polluted soil. This option (BB2) is quite easy and quick to prepare. Both electrodic wells were filled with electrolyte solution and the direct current was connected.

Additionally, two complementary reference experiments were carried out. The first reference test was identical to the Experiment 1, but no electric current was applied to the soil (named as “No EK”). This test would inform about the possible evolution of pollutant biodegradation without the contribution of electrokinetic phenomena. The second reference test was an abiotic EK reference test (named as “No Bio”) and it was carried out by using the same electrokinetic conditions of all experiments (1.0 V cm⁻¹ and 2.0 d⁻¹ polarity reversal frequency) but using no inoculated soil (no addition of acclimated 2,4-D removal microorganisms). This test would inform about the possible removal of pollutant by non-biologically assisted mechanisms. 2.4. Sampling and analyses

Samples were taken and analysed during the operation time in both electrodic compartments, and in the electroosmotic flow. It is important to remark that EOF was alternatively collected in both collector compartments due to the electrode polarity
reversal every 12 hours. Temperature of soil and electrical current were monitored
during all the treatment.

pH and conductivity were measured with multiparameter probe (SENSLON, HACH).
To analyse nutrient concentrations, i.e., ammonium, nitrate and phosphate, it was used a
photometer Gallery (Thermo Scientific). Soil samples (1 g) were mixed with 2.5 mL of
water for the dissolution of 2,4-D. The soil/solvent mixture was agitated vigorously in a
vortex agitator for 5 min and centrifuged (15 min, 3800 min⁻¹). Samples were taken
from the aqueous supernatant and analysed. 2,4-D was analysed with an HPLC (Jasco,
Japan) equipped with a column Kinetex 5 μm Biphenyl 100 Å, 150 x 4.5 mm
(Phenomenex, USA). The mobile phase employed 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 injection volume was 20 μL.

Soil samples were taken only at the start of the experiment, before placing it on the
installation, and at the end (post-mortem analysis) of the treatment, in order to not
modify the compaction of soil that could cause preferential ways (Ruiz et al., 2014).
The post-mortem analysis were conducted in different soil portions as follows: four
longitudinal positions were considered (1 to 4, from anode to cathode at time zero) and
each one in turn was divided into four sections (two in the upper layer and another two
in the bottom layer) according to previous works (Ramírez et al., 2014). Thus,
analytical results in each position were the average of 4 measurements. The parameters
analysed in soil were moisture, pH, conductivity, microorganisms, nutrients, and 2,4-D
concentrations. Moisture was calculated by difference of weights, i.e., an amount of wet
soil was dried at 105 °C for 24h. Weight of evaporated water corresponds to moisture
contained in soil. pH and conductivity were measured from dry soil. To do this, it was
taken 10 g of dry soil and 25 mL of Milli-Q water was added. Then, it was agitated for
30 min and it was left decant around 2 hours. Liquid supernatant was filtered by using nylon filters of 0.2 µm and measured with a multiparameter probe. Nutrient concentrations were also measured from dry soil with the same method. 2,4-D concentration was determined from wet soil by HPLC as described above. Microorganisms concentration is expressed as Colony Forming Units (CFU) per gram of dry soil (Ramírez et al., 2015). To do this, it was taken 1 g of wet soil and then, it was added 10 mL of a solution of 0.9% NaCl. After that, it was mixed and agitated for 3 min with a vortex agitator. An aliquot of 100 µL of supernatant liquid was taken and put on Petri dishes containing LB media as solid culture media for the microbial growth with the following composition: 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. Then, the dishes were incubated for 24h at 26.5 ºC.

3. Results and discussion

The present work considers two possible real situations in the case of a soil contaminated with 2,4-D. One possible situation considers a recent pesticide spill in a soil which does not contain an adapted microbial population capable of biodegrading 2,4-D and thus bioaugmentation is needed. The authors consider that a good option to include the acclimated microorganisms in such polluted soil is through inserting a biobarrier on it with them (EK-bioaugmentation). To do this, two different types of biobarriers have been proposed: BB1 consists of a portion of fixed-bed biofilm reactor for 2,4-D biodegradation, previously developed in a laboratory, while BB2 is just a mixture of clean clayey soil and a 2,4-D acclimated microorganisms suspension. According to recent works, the extreme pH in electrodic zones can avoid the microbial activity (Mena et al., 2014). Thus, the authors consider that the optimal way to insert the
biobarrier in soil is in the central position, and this disposition implies to mobilize the pollutant to pass through the barrier by EK.

The other possible situation considers that the polluted soil already contains an autochthonous microbial population adapted to use the organic pollutant (2,4-D) as the carbon source (a possible situation in historically polluted sites) and inorganic nutrients are available. In this case the proposed treatment consists of using electrokinetics for the mobilization of pollutants, nutrients, and microorganisms, in order to improve the contact between them (EK-biostimulation) but trying to keep experimental conditions in suitable values for microbial life.

**Figure 2.** (a) Electroosmotic flow and (b) current intensity through the soil during the EBR experiments.

Figure 2 shows EOF values and current intensity throughout the duration of the EBR experiments. The EOF profile (Fig. 2a) indicates the movement of system water out of the set-up and, consequently, it is necessary to replace it with an electrolyte solution, which guarantee the correct conductivity in soil for electro-bioremediation process. As it can be observed in Fig. 2a, in all the cases, the EOF increases till a maximum level to keep constant along the treatment. In the experiments of EBR by biobARRiers (BB1 and BB2), similar values of EOF, around 4-5 mL h\(^{-1}\) and approximately constant during the
process, are observed. On the other hand, in the case of biostimulation, EOF is higher than in EK-bioaugmentation cases, around 7-8 mL h\(^{-1}\), which is supposed to be caused by a lower soil permeability than in the bioaugmentation experiments because of no central biobarrier is needed in this case. This behaviour is similar to previous works using non-polar pesticides (oxyfluorfen) reported by the same authors (Barba et al., 2019a). Related to current intensity (Fig. 2b), it can be observed that in the case of using biobarriers the value is approximately constant and slightly lower than in the case of biostimulation. This behaviour can be explained because of the higher ohmic resistance due to the inclusion of a biobarrier into the soil. Moreover, current intensity values when using two biobarriers are similar and slight differences can be explained by soil permeability changes due to the introduction of the biobarriers or also because of the manual compaction of soil at the start of the experiments (Mena et al., 2015; Mena et al., 2016b).

Figure 3 shows the soil conductivity profiles obtained in the post-mortem analysis and compared to initial values for each experiment carried out. Solid lines show the average values for each longitudinal sample point or soil position (position 1 corresponds to the nearest to anode and position 4 to cathode at t=0). As it can be observed, the electrical conductivity in three cases at the start is high, around 1500-1600 µS cm\(^{-1}\), and decreases until 700-1000 µS cm\(^{-1}\) at the end of the treatment. Despite this drop of conductivity, the final average value is also high to secure the proper conductivity in soil for electrokinetics, and simultaneously it is not excessive for biological phenomena.
Figure 3. Soil conductivity profile in soil at the start (- - -) and at the end (-----) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only.

In both figures, 2 and 3, it has been observed that the inclusion of a permeable reactive biobarrier in a polluted soil causes differences in comparison with applying biostimulation strategy. EOF decreases in both bioaugmentation experiments in comparison with biostimulation experiment. It is a fact that the EOF is directly proportional to the voltage applied in system, and thus to the zeta potential which depends on the ionic concentration, and proportional to the dielectric constant of fluid,
and inversely proportional to the viscosity of the fluid (Reddy and Cameselle, 2009). Moreover, EOF in low permeability regions is significantly higher than the EOF in regions with upper porosity. As it is explained above soil permeability is higher when biobarrriers are applied. Thus, the introduction of biobarrriers in the experimental system at the present work generated an important EOF decrease, which could be associated to the decrease in the current density and soil conductivity. Nevertheless, a slight contradiction was observed when comparing BB1 and BB2 results (biobarrier from fix-bed biofilm reactor and clean soil-microorganisms mixture, respectively). EOF is slightly higher using BB1 versus BB2 despite the greater porosity (BB1 is made by gravel particles). It can be considered that variables such as ionic concentration, related to soil conductivity and current density, can influence experiments performance, causing the lower value in BB2 experiment. It is important to remain that variables such as voltage gradient, fluid dielectric constant and viscosity keep constant during all the experiments carried out.

Many authors have previously studied the electroremediation process inserting a permeable reactive barrier (PRB). For example, Wan et al. (2010a) reported that the insertion of Pd/Fe PRB caused EOF decrease 1.8 times in an electroremediation process for hexachlorobenzene-polluted soil. Kebria et al. (2016) reported similar results in electroremediation of PCE polluted soil by using Fe⁰ particles as PRB. On the other hand, same authors (Wan et al., 2010b) reported that coupling a Cu/Fe PRB in electroremediation of hexachlorobenzene-polluted soil caused that EOF increased. The authors of the present work also reported results related to the application of biobarrriers, comparing the performance of BB1 and BB2 in electro-bioremediation of diesel polluted soil, and they found that the application of biobarrier type BB1 causes a higher EOF (Mena et al., 2016) and the EOF was higher when no biobarrier was inserted in
soil (biostimulation) (Ramírez et al., 2015). Additionally, similar behaviour to that observed in the present work was reported in EBR of oxyfluorfen polluted clay soil (Barba et al., 2019a). There are some variables which could simultaneously influence the performance of BioPRBs and additional research efforts still need to be made.

Figure 4 shows the initial and final average values of soil temperature and pH. In Fig. 4a it can be observed that the temperature of soil during the three experiments keeps practically constant around 25-28°C, which is an optimal value for the activity of the microbial culture employed in this work. Related to pH in soil (Fig. 4b), it can be observed that in all the cases the pH has been controlled correctly, i.e., it has been cushioned the extreme pH fluctuations due to the electrolysis of water thanks to polarity reversal strategy (Barba et al., 2017). Yeung and Gu (2011), reported different strategies to control pH in electroremediation processes. One of the most used in recent years is so-called periodic polarity reversal strategy, employed in the present work. Several authors reported the effect of using periodical changes in the polarity of the system and showed beneficial effects in pH, temperature and moisture of soil at the end of treatment for the proper activity microbial culture in electro-bioremediation process (Li et al., 2015; Li et al., 2016). Both temperature and pH show a homogeneous distribution profile throughout the treated soil in all the cases studied. Thus, these conditions are considered to be adequate for microbial activity in soil during the three EBR processes conducted at this work.
Figure 4. Average values of soil (a) temperature and (b) pH at the start and at the end of EBR experiments. Grey bars represent initial conditions while black bars represent final average value.

Figure 5 shows the microorganisms’ population profile in soil before and after the EBR treatment. As it can be observed, only in the experiment when using biostimulation as biological strategy the initial concentration (dashed line) is presented in the figure because in the two experiments with biobarrriers, the soil at the start was autoclaved and the microorganisms were only inoculated through the biobarrier. From this figure, it can be extracted two main conclusions: the first one is that in all the experiments carried out, the microorganisms’ population has similar concentration values at the end of EBR treatment in the three cases. The second one is that there exists homogeneous distribution of microorganisms in all the soil at the end of the treatment. This behaviour can be explained because of the biofilm detachment from biobarrier and movement of microorganisms from the central location to the rest of soil positions thanks to the electrophoresis and electroosmotic flow passing thought it (DeFlaun and Condee, 1997). A similar result was also observed by the same authors when non polar pesticide was used as model pollutant (Barba et al., 2019a). Due to the application of polarity reversal strategy in EBR experiments, a correct control of pH has been achieved as it was explained above. Thus, it was not observed harmful effects because of extreme pH which would cause a decrease in the concentration of microorganisms in the zones near
to electrodes, and it indicates adequate conditions in soil for microbial activity.

**Figure 5.** Soil microorganisms’ population profile in soil at the start (- - -) and at the end (---) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (◆), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only.

Figure 6 (a-c) shows the average values of 2,4-D concentrations in soil at the start and at the end of the treatment. Figure 6d shows a comparative about the 2,4-D percentage removal efficiencies in the three experiments carried out, including also the removal efficiencies of the two reference tests. As it can be observed, removal of 2,4-D by using biobaroiers is quite effective, so in both cases (BB1 and BB2) it was achieved between 75-85% of 2,4-D removal in only 10 days of treatment. Nevertheless, EBR with biostimulation strategy offers a complete 2,4-D elimination in soil and a homogeneous
removal profile. The lower 2,4-D removal rate when using biobarriers could be directly related to EOF decrease due to the higher porosity in the biobarriers zones, and consequently the current intensity and soil conductivity also decrease, as it was above explained. The slightly differences of 2,4-D removal rates between two biobarriers evaluated, and taking into account that experimental conditions for microbial activity are practically identical in all the experiments (i.e., pH, temperature, nutrients and microorganisms’ concentrations) could be explained again because of the lower EOF of EBR by using BB2 in comparison with BB1 related to the lower mixture effect between nutrients, pollutant and microorganisms in the process of remediation.

Despite offering biostimulation option better results of pollutant elimination, the use of biobarriers in EBR process is a great advantage because, in the case that the soil does not contain microorganisms adapted to the degradation of such pollutant, this would be the most optimal way to introduce the microbial culture into the soil. Studies about EK-bioaugmentation are scarce. Mao et al. [49] studied EK-enhanced bioaugmentation for remediation of clays contaminated with chlorinated solvents but they did not use BioPRB: the microbial culture solution was added to the electrode compartments and to a central injection well. They found that the microbial distribution within the clay suggested that electrokinetic microbial transport was primarily driven by electroosmosis, the injected bacteria were able to survive and grow, and complete effective dechlorination of chlorinated ethene was observed after 94d.

Additionally, in Fig. 6d the results obtained in the three EBR experiments were compared with two reference tests: test “No EK” and test “No Bio”. Test “No Bio” means only EK treatment without microbial activity, and the 2,4-D removal result from this reference test is quite similar to ones obtained using biobarriers. However it is important to note that, when using only EK, the pollutant is moved to the electrode
wells, and then it is necessary to treat the contaminated water by external techniques, e.g., electro-oxidation (de Vidales et al., 2018). Comparing the in situ removal EBR treatments and the reference test “No EK” (that is, only in situ bioremediation without EK) it can be observed that the 2,4-D removal percentages reached up in three experiments of EBR are much higher than in the test “No EK”. This behaviour prove that electrokinetics acts as a mixer improving the contact and transfer matter between pollutant, microorganisms and nutrients contained in soil (Mena et al., 2016c; Barba et al., 2017). It is important to remark that the microbial culture is able to successfully degrade high pesticide concentrations in relatively short retention times (as previously reported by the same authors, Barba et al., 2019b) and thus the success, or not, of the subsequent EBR technology 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. Thus, the main conclusion that can be extracted from figure 6d is that coupling electrokinetic processes with biological treatment improve the in situ removal of 2,4-D from soil.

**Figure 6.** (a-c) 2,4-D concentration profiles in soil at the start (---) and at the end (——)
of the EBR experiments. Lines are the average of the four values in the different axial
positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they
mean trends only. (d) 2,4-D removal percentages after 10 days of treatment.

Conclusions

Different alternatives based on EK-biostimulation and EK-bioaugmentation were tested
in electro-bioremediation of 2,4-D polluted soils. Supposing there exists already an
autochthonous culture in the soil capable of degrading 2,4-D, the biostimulation strategy
practically achieved the complete elimination of the herbicide after 10d. Pollutant
removal efficiencies when using biobarriers (bioaugmentation) were successful (75-
85%) but lower than efficiency obtained when using biostimulation. The use of
biobarriers was found to be a viable strategy to deliver microorganisms if soil does not
contain an adapted microbial population. Temperature and pH were correctly controlled
in all cases, but the inclusion of biobarriers caused EOF to decrease due to the higher
porosity in the biobarriers zones, and consequently the current intensity and soil
conductivity also decreased. As a result, the mixture and transport contribution of EK
phenomena were lower when using bioaugmentation. Reference tests proved the
positive effect of coupling both biological and electrokinetic mechanisms.

Acknowledgements

Financial support from the Spanish Government and European Union through projects
CTM2016-76197-R (AEI/FEDER, UE) from Ministry of Economy, Industry and
Competitiveness, and EQC2018-004240-P from Ministry of Science, Innovation and
Universities is gratefully acknowledged. The FPI grant BES-2014-069662 is also
acknowledged.

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Figure 1

Click here to download Figure: Figure1.pdf
Figure 3

Click here to download Figure: Figure3.pdf

Figure 3.
Figure 4

(a) and (b) illustrate the temperature (T) and pH changes for BB1, BB2, and Biostimulation treatments. The bars represent the start and final averages, with error bars indicating variability.

Figure 4.
Figure 5

Click here to download Figure: Figure5.pdf
Figure 6. 

Graphs showing the distribution of 2,4-D (mg kg dry soil$^{-1}$) across different positions for BB1 and BB2 treatments. The graphs illustrate the impact of biostimulation on 2,4-D removal (in %) for the 'No Bio', 'No EK', and 'Biostim."No Bio""No EK' conditions.
**Figure 1.** Electro-bioremediation set-up: (a) EK-biostimulation experiment; (b) EK-bioaugmentation experiments using biobarriers; (c) photographs of set-up using biobarriers.

**Figure 2.** (a) Electroosmotic flow and (b) current intensity through the soil during the EBR experiments. Grey bars represent initial conditions while black bars represent final average value.

**Figure 3.** Soil conductivity profile in soil at the start (- - -) and at the end (—) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only.

**Figure 4.** Average values of soil (a) temperature and (b) pH at the start and at the end of EBR experiments.

**Figure 5.** Soil microorganisms’ population profile in soil at the start (- - -) and at the end (—) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only.

**Figure 6.** (a-c) 2,4-D concentration profiles in soil at the start (- - -) and at the end (—) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (♦), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only. (d) 2,4-D removal percentages after 10 days of treatment.
Table 1. Properties of the soil used in the experiments.

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n.d.: non detected.
Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
BIOSTIMULATION VERSUS BIOAUGMENTATION FOR THE ELECTRO-BIOREMEDIATION OF 2,4-DICHLOROPHENOXYACETIC ACID POLLUTED SOILS.

Credit Author Statement:


José Villaseñor: Data discussion and interpretation. Writing- Original draft preparation. Submission.

Manuel A. Rodrigo: Discussion of electrochemical aspects. Manuscript revision.

Pablo Cañizares: Discussion of biological aspects. Manuscript revision.