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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, 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.

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*Cover Letter

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Journal of Environmental Management
Editor

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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".

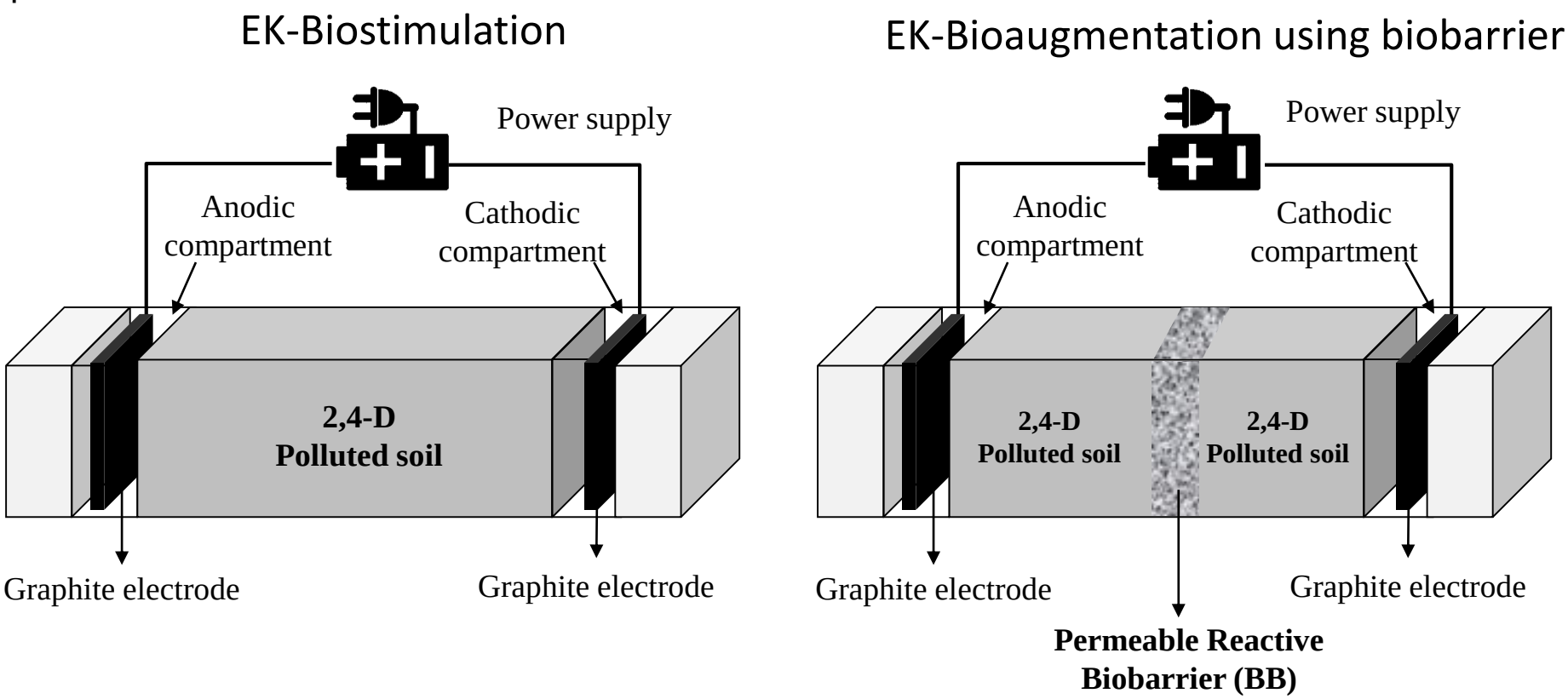
The change has been made (line 378, line 384 and caption of Fig5 in revised manuscript).

Include information about the sterilization of soil by autoclave in M&M section.

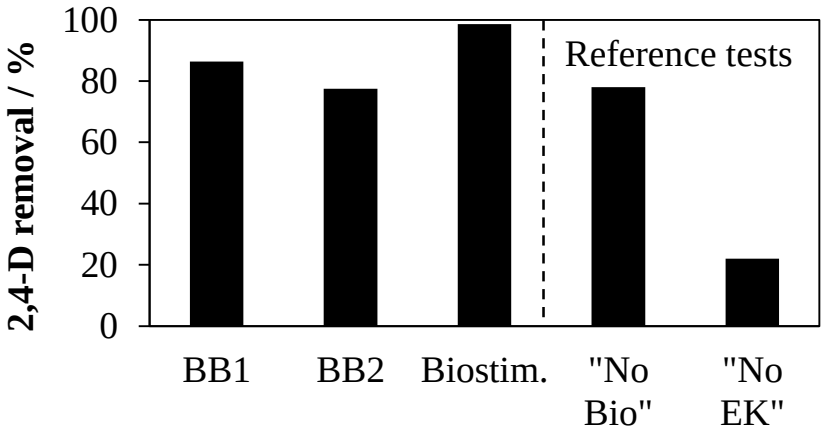
Soil was autoclaved: 121ºC, 15 min.
Information has been included in revised manuscript (line 192)

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.



Three biological strategies for EBR



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

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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, 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

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Keywords

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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).

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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).

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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

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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

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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).

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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).

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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^{-3}	1.65
Electric conductivity/ µS cm^{-1}	1800
pH	7.9
Organic matter	n.d.

Hygroscopic moisture	0.115

n.d.: non detected.

~~Mineralogy~~	
~~Quartz~~	~~12%~~
~~Feldspar~~	~~6%~~
~~Calcite~~	~~1%~~
~~Kaolinite~~	~~23%~~
~~Glauconite~~	~~24%~~
~~Muscovite~~	~~8%~~
~~Montmorillonite~~	~~20%~~
~~Smectite~~	~~-~~
~~Illite~~	~~6~~
~~Other properties~~	
~~Dry density / g cm^{-3}~~	~~1.65~~
~~Hygroscopic moisture~~	~~0.115~~

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

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148 an oil-refinery wastewater treatment plant (Puertollano, Spain), and the culture medium
149 containing inorganic nutrients was Bushnell-Hass Broth (BHB). The composition of
150 BHB per litre of Milli-Q water is 0.20 g Mg SO_4, 0.02 g $CaCl_2$, 1.00 g KH_2PO_4, 1.00 g
151 $(NH_4)_2HPO_4$, 0.05 g $FeCl_3$ and 1.00 g KNO_3. The sole carbon source employed during
152 acclimation was 2,4-D (200 mg L^{-1}). After the acclimation process, microorganisms
153 contained in the microbial culture were identified by using a MALDI TOF Mass
154 Spectrometry AXIMA-Assurance equipment (Biotech technology, SHIMADZU,
155 Germany). The species identified were *Rhodococcus ruber* and *Ochrobactrum*
156 *anthropic*.

157 **2.2 Experimental set-up**

158 The experimental set-up scheme is shown in Figure 1. Fig. 1a corresponds to EK-
159 biostimulation experiment and Fig.1b corresponds to EK-bioaugmentation experiments
160 using biobarriers. The cell is made of transparent methacrylate and divided into five
161 compartments. Soil polluted is placed in the central compartment, while at both sides
162 are located the electrodic wells, which contain the graphite electrodes (10x10x1 cm)
163 supplied by Carbosystem (Madrid, Spain) and connected to the power supply (HQ
164 Power, Gavere, Belgium). Soil is separated from electrodic wells by a nylon mesh (0.5
165 mm mesh size). Contiguous to electrodic wells, there are the collector compartments
166 that collect the electroosmotic flow (EOF) transported during the treatment. As later
167 explained, EOF will be collected at both sides due to the electrode polarity reversal. Fig.
168 1b refers to the EK-bioaugmentation experiments with biobarriers. The experimental
169 set-up is similar to that described above, but the difference in this case is that in the
170 middle of the central compartment is placed the biobarrier separated from soil with a
171 nylon mesh.

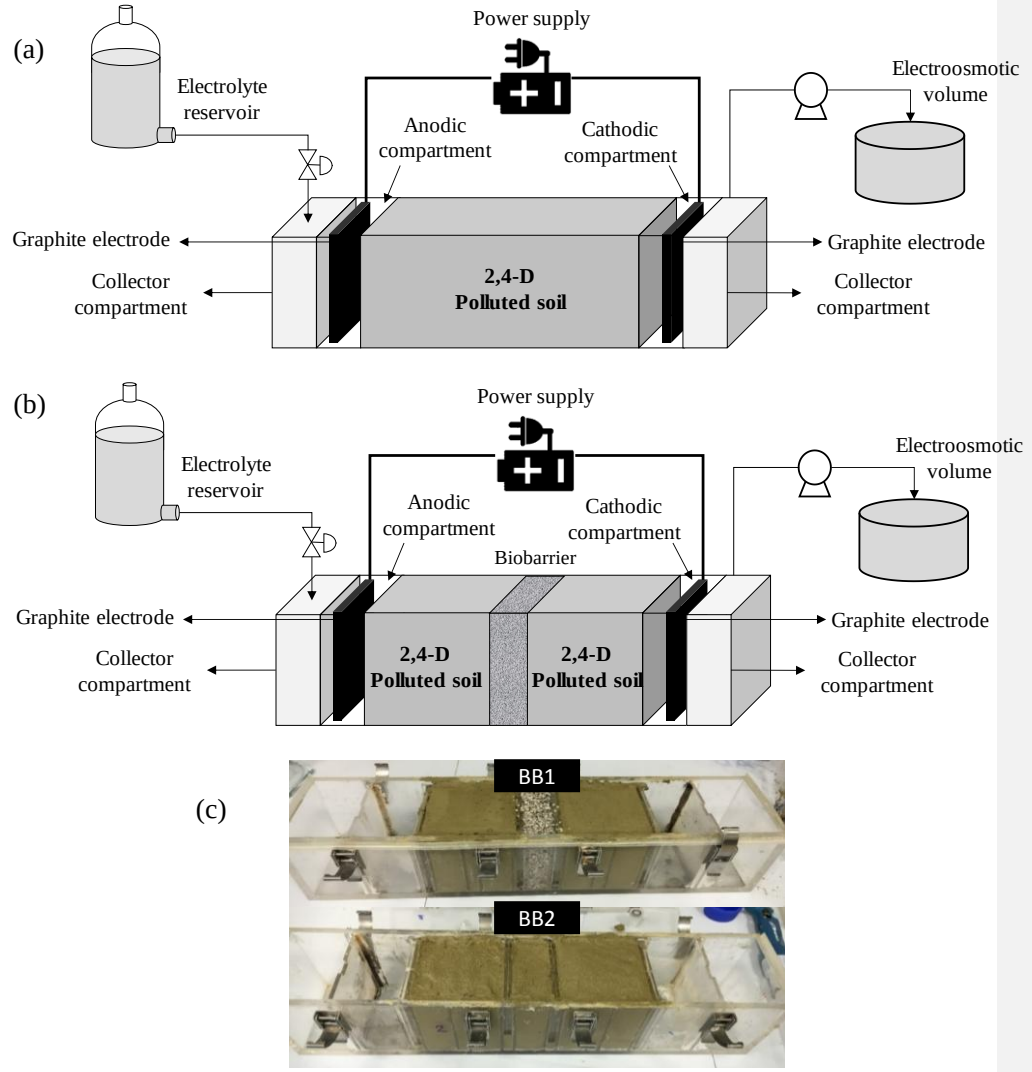

Figure 1. Electro-bioremediation set-up: (a) EK-biostimulation experiment; (b) EK-bioaugmentation experiments using biobarriers; (c) photographs of set-up using biobarriers.

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_2SO_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).

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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

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232 reversal every 12 hours. Temperature of soil and electrical current were monitored
233 during all the treatment.
234 pH and conductivity were measured with multiparameter probe (SENSLON, HACH).
235 To analyse nutrient concentrations, i.e., ammonium, nitrate and phosphate, it was used a
236 photometer Gallery (Thermo Scientific). Soil samples (1 g) were mixed with 2.5 mL of
237 water for the dissolution of 2,4-D. The soil/solvent mixture was agitated vigorously in a
238 vortex agitator for 5 min and centrifuged (15 min, 3800 min^{-1}). Samples were taken
239 from the aqueous supernatant and analysed. 2,4-D was analysed with an HPLC (Jasco,
240 Japan) equipped with a column Kinetex 5 µm Biphenyl 100 Å, 150 x 4.5 mm
241 (Phenomenex, USA). The mobile phase employed was H_3PO_4 0.1%/acetonitrile, 60/40
242 %v/v, with an isocratic flow rate of 0.6 mL min^{-1}. The wavelength of the UV detector
243 was 220 nm and injection volume was 20 µL.
244 Soil samples were taken only at the start of the experiment, before placing it on the
245 installation, and at the end (*post-mortem* analysis) of the treatment, in order to not
246 modify the compaction of soil that could cause preferential ways (Ruiz et al., 2014).
247 The *post-mortem* analysis were conducted in different soil portions as follows: four
248 longitudinal positions were considered (1 to 4, from anode to cathode at time zero) and
249 each one in turn was divided into four sections (two in the upper layer and another two
250 in the bottom layer) according to previous works (Ramírez et al., 2014). Thus,
251 analytical results in each position were the average of 4 measurements. The parameters
252 analysed in soil were moisture, pH, conductivity, microorganisms, nutrients, and 2,4-D
253 concentrations. Moisture was calculated by difference of weights, i.e., an amount of wet
254 soil was dried at 105 ºC for 24h. Weight of evaporated water corresponds to moisture
255 contained in soil. pH and conductivity were measured from dry soil. To do this, it was
256 taken 10 g of dry soil and 25 mL of Milli-Q water was added. Then, it was agitated for

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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.

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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

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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.

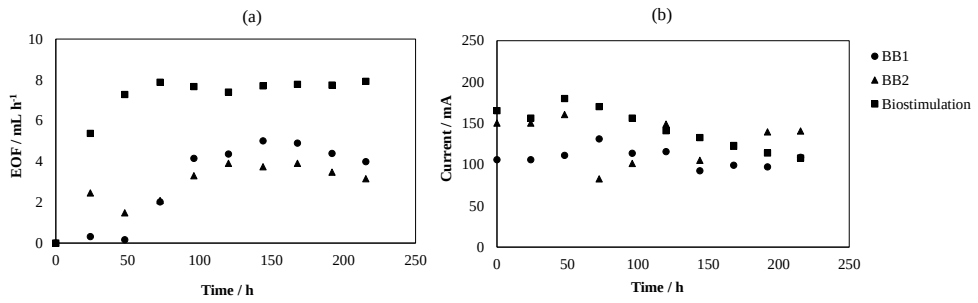
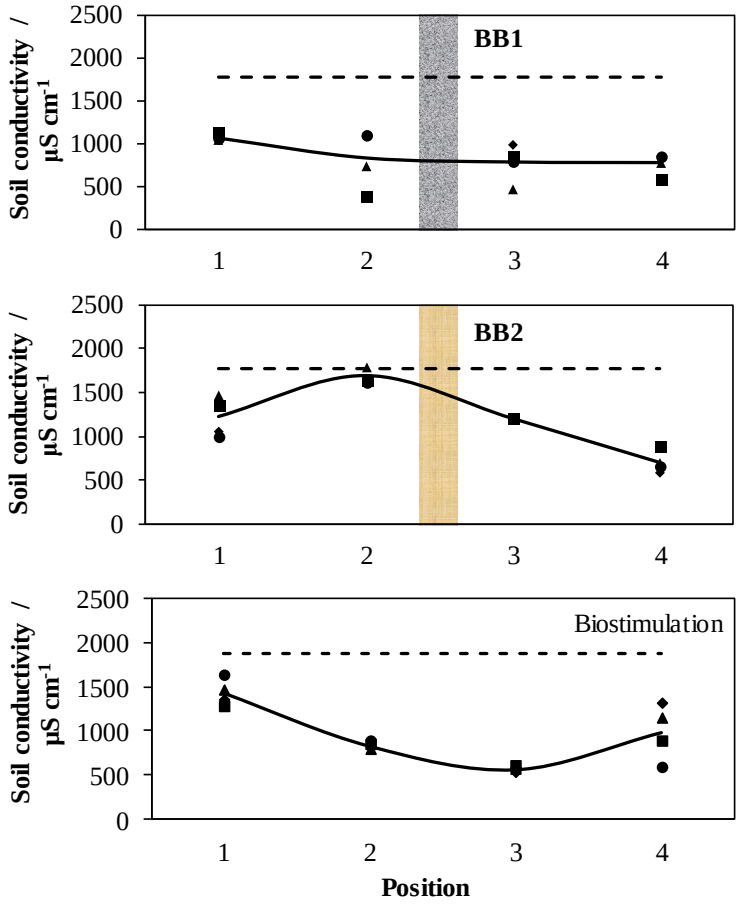


Figure 2. (a) Electroosmotic flow and (b) current intensity through the soil during the EBR experiments.

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.



325

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

330

331 In both figures, 2 and 3, it has been observed that the inclusion of a permeable reactive
332 biobarrier in a polluted soil causes differences in comparison with applying
333 biostimulation strategy. EOF decreases in both bioaugmentation experiments in
334 comparison with biostimulation experiment. It is a fact that the EOF is directly
335 proportional to the voltage applied in system, and thus to the zeta potential which
336 depends on the ionic concentration, and proportional to the dielectric constant of fluid,

337 and inversely proportional to the viscosity of the fluid (Reddy and Cameselle, 2009).
338 Moreover, EOF in low permeability regions is significantly higher than the EOF in
339 regions with upper porosity. As it is explained above soil permeability is higher when
340 biobarriers are applied. Thus, the introduction of biobarriers in the experimental system
341 at the present work generated an important EOF decrease, which could be associated to
342 the decrease in the current density and soil conductivity. Nevertheless, a slight
343 contradiction was observed when comparing BB1 and BB2 results (biobarrier from fix-
344 bed biofilm reactor and clean soil-microorganisms mixture, respectively). EOF is
345 slightly higher using BB1 versus BB2 despite the greater porosity (BB1 is made by
346 gravel particles). It can be considered that variables such as ionic concentration, related
347 to soil conductivity and current density, can influence experiments performance,
348 causing the lower value in BB2 experiment. It is important to remain that variables such
349 as voltage gradient, fluid dielectric constant and viscosity keep constant during all the
350 experiments carried out.
351 Many authors have previously studied the electroremediation process inserting a
352 permeable reactive barrier (PRB). For example, Wan et al. (2010a) reported that the
353 insertion of Pd/Fe PRB caused EOF decrease 1.8 times in an electroremediation process
354 for hexachlorobenzene-polluted soil. Kebria et al. (2016) reported similar results in
355 electroremediation of PCE polluted soil by using Fe^0 particles as PRB. On the other
356 hand, same authors (Wan et al., 2010b) reported that coupling a Cu/Fe PRB in
357 electroremediation of hexachlorobenzene-polluted soil caused that EOF increased. The
358 authors of the present work also reported results related to the application of biobarriers,
359 comparing the performance of BB1 and BB2 in electro-bioremediation of diesel
360 polluted soil, and they found that the application of biobarrier type BB1 causes a higher
361 EOF (Mena et al., 2016) and the EOF was higher when no biobarrier was inserted in

362 soil (biostimulation) (Ramírez et al., 2015). Additionally, similar behaviour to that
363 observed in the present work was reported in EBR of oxyfluorfen polluted clay soil
364 (Barba et al., 2019a). There are some variables which could simultaneously influence
365 the performance of BioPRBs and additional research efforts still need to be made.
366 Figure 4 shows the initial and final average values of soil temperature and pH. In Fig.
367 4a it can be observed that the temperature of soil during the three experiments keeps
368 practically constant around 25-28°C, which is an optimal value for the activity of the
369 microbial culture employed in this work. Related to pH in soil (Fig. 4b), it can be
370 observed that in all the cases the pH has been controlled correctly, i.e., it has been
371 cushioned the extreme pH fluctuations due to the electrolysis of water thanks to polarity
372 reversal strategy (Barba et al., 2017). Yeung and Gu (2011), reported different strategies
373 to control pH in electroremediation processes. One of the most used in recent years is
374 so-called periodic polarity reversal strategy, employed in the present work. Several
375 authors reported the effect of using periodical changes in the polarity of the system and
376 showed beneficial effects in pH, temperature and moisture of soil at the end of treatment
377 for the proper activity microbial culture in electro-bioremediation process (Li et al.,
378 2015; Li et al., 2016). Both temperature and pH show a homogeneous distribution
379 profile throughout the treated soil in all the cases studied. Thus, these conditions are
380 considered to be adequate for microbial activity in soil during the three EBR processes
381 conducted at this work.
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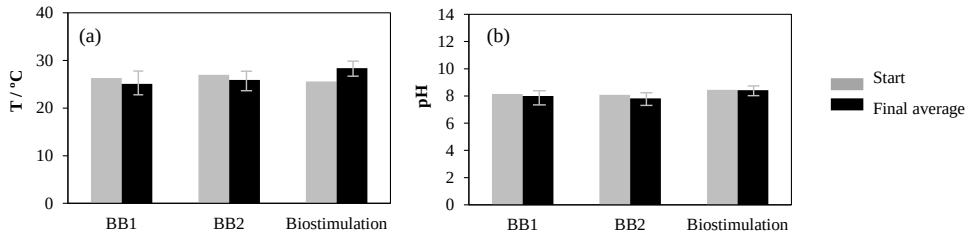

Figure 4.

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~~ 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 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~~ 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

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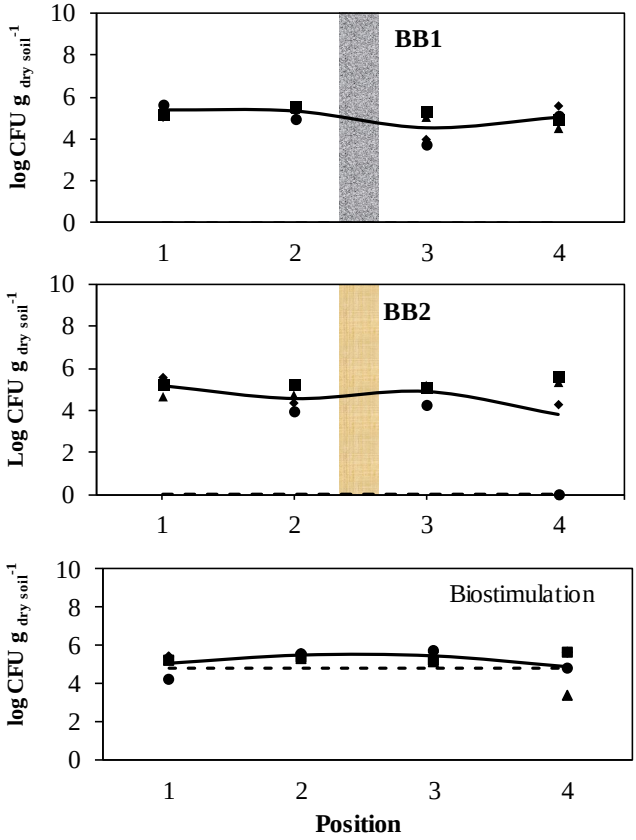
404 microorganisms in the zones near to electrodes, and it indicates adequate conditions in
405 soil for microbial activity.



406

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

411 Figure 6 (a-c) shows the average values of 2,4-D concentrations in soil at the start and at
412 the end of the treatment. Figure 6d shows a comparative about the 2,4-D percentage
413 removal efficiencies in the three experiments carried out, including also the removal
414 efficiencies of the two reference tests. As it can be observed, removal of 2,4-D by using
415 biobarriers is quite effective, so in both cases (BB1 and BB2) it was achieved between
416 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 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.

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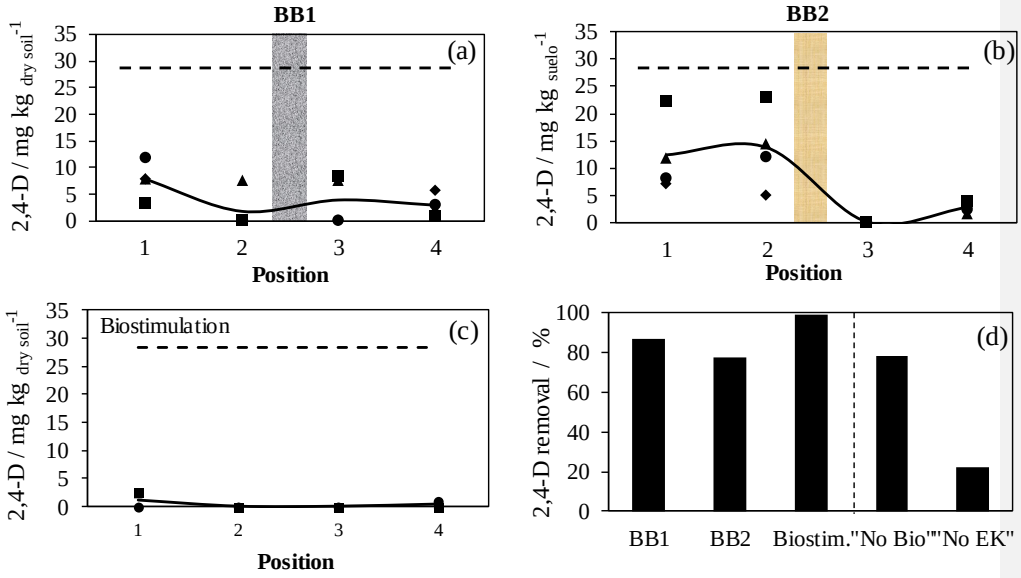

Figure 6. (a-c) 2,4-D concentration profiles in soil at the start (- - -) and at the end (¯¯¯) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (◆), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only. (d) 2,4-D removal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

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References

Barba, S., Villaseñor, J., Cañizares, P., Rodrigo, M.A., 2019a. Strategies for the

485 electrobioremediation of oxyfluorfen polluted soils. Electrochimica Acta 297, 137-144.
486 Barba, S., Carvela, M., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2019b. Fixed-bed
487 biological barrier coupled with electrokinetics for the *in situ* electrobioremediation of
488 2,4-dichlorophenoxyacetic acid polluted soil. Journal of Chemical Technology and
489 Biotechnology 94, 2684–2692.Barba, S., Villaseñor, J., Rodrigo, M.A., Cañizares, P.,
490 2018a. Can electro-bioremediation of polluted soils perform as a self-sustainable
491 process? Journal of Applied Electrochemistry 48, 579-588.
492 Barba, S., López-Vizcaíno, R., Saez, C., Villaseñor, J., Cañizares, P., Navarro, V.,
493 Rodrigo, M., 2018b. Electro-bioremediation at the prototype scale: What it should be
494 learned for the scale-up. Chemical Engineering Journal 334, 2030-2038.
495 Barba, S., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2017. Effect of the polarity
496 reversal frequency in the electrokinetic-biological remediation of oxyfluorfen polluted
497 soil. Chemosphere 177, 120-127.
498 Cameselle, C., 2014. Electrokinetic Remediation, Cost Estimation. in: Kreysa, G., Ota,
499 K.-i., Savinell, R.F. (Eds.). Encyclopedia of Applied Electrochemistry. Springer New
500 York, New York, NY, pp. 723-725.
501 Chowdhury, A., Pradhan, S., Saha, M., Sanyal, N., 2008. Impact of pesticides on soil
502 microbiological parameters and possible bioremediation strategies. Indian Journal of
503 Microbiology 48, 114-127.
504 de Vidales, M.J.M., Castro, M.P., Sáez, C., Cañizares, P., Rodrigo, M.A., 2018.
505 Radiation-assisted electrochemical processes in semi-pilot scale for the removal of
506 clopyralid from soil washing wastes. Separation and Purification Technology.
507 DeFlaun, M.F., Condee, C.W., 1997. Electrokinetic transport of bacteria. Journal of
508 hazardous materials 55, 263-277.
509 Gill, R., Harbottle, M.J., Smith, J., Thornton, S., 2014. Electrokinetic-enhanced

510 bioremediation of organic contaminants: A review of processes and environmental
511 applications. Chemosphere 107, 31-42.
512 Kebria, D.Y., Taghizadeh, M., Camacho, J.V., Latifi, N., 2016. Remediation of PCE
513 contaminated clay soil by coupling electrokinetics with zero-valent iron permeable
514 reacive barrier, Environ. Earth Sci. 75 699-709.
515 Li, T., Guo, S., Wu, B., Zhang, L., Gao, Y., 2015. Effect of polarity-reversal and
516 electrical intensity on the oil removal from soil, J. Chem. Technol. Biotechnol. 90, 441-
517 448.
518 Li, T., Wang, Y., Guo, S., Li, X., Xu, Y., Wang, Y., Li, X., 2016. Effect of polarity-
519 reversal on electrokinetic enhanced bioremediation of Pyrene contaminated soil,
520 Electrochimica Acta, 187, 567-575.Mao, X., Wang, J., Ciblak, A., Cox, E.E., Riis, C.,
521 Terkelsen, M., Gent, D.B., Alshawabkeh, A.N., 2012. Electrokinetic-enhanced
522 bioaugmentation for remediation of chlorinated solvents contaminated clay. Journal of
523 Hazardous Materials 213– 214, 311– 317.
524 Mena, E., Villaseñor, J., Cañizares, P., Rodrigo, M.A., 2014. Effect of a direct electric
525 current on the activity of a hydrocarbon-degrading microorganism culture used as the
526 flushing liquid in soil remediation processes. Separation and Purification Technology
527 124, 217-223.
528 Mena, E., Ruiz, C., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2015. Biological
529 permeable reactive barriers coupled with electrokinetic soil flushing for the treatment of
530 diesel-polluted clay soil. Journal of hazardous materials 283, 131-139.
531 Mena, E., Villasenor, J., Rodrigo, M.A., Canizares, P., 2016a. Electrokinetic
532 remediation of soil polluted with insoluble organics using biological permeable reactive
533 barriers: Effect of periodic polarity reversal and voltage gradient. Chemical Engineering
534 Journal 299, 30-36.

535 Mena, E., Villaseñor, J., Cañizares, P., Rodrigo, M., 2016b. Influence of electric field
536 on the remediation of polluted soil using a biobarrier assisted electro-bioremediation
537 process. Electrochimica Acta 190, 294-304.
538 Mena, E., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2016c. Electrokinetic
539 remediation of soil polluted with insoluble organics using biological permeable reactive
540 barriers: Effect of periodic polarity reversal and voltage gradient. Chemical Engineering
541 Journal 299, 30-36.
542 Moliterni, E., Jiménez-Tusset, R., Rayo, M.V., Rodriguez, L., Fernández, F., Villasenor,
543 J., 2012. Kinetics of biodegradation of diesel fuel by enriched microbial consortia from
544 polluted soils. International Journal of Environmental Science and Technology 9, 749-
545 758.
546 Morillo, E., Villaverde, J., 2017. Advanced technologies for the remediation of
547 pesticide-contaminated soils. Science of The Total Environment 586, 576-597.
548 Paillat, T., Moreau, E., Grimaud, P., Touchard, G., 2000. Electrokinetic phenomena in
549 porous media applied to soil decontamination. IEEE Transactions on Dielectrics and
550 Electrical Insulation 7, 693-704.
551 Ramírez, E.M., Camacho, J.V., Rodrigo, M.R., Cañizares, P.C., 2014. Feasibility of
552 electrokinetic oxygen supply for soil bioremediation purposes. Chemosphere 117, 382-
553 387.
554 Ramírez, E.M., Jiménez, C.S., Camacho, J.V., Rodrigo, M.A.R., Cañizares, P., 2015.
555 Feasibility of coupling permeable bio-barriers and electrokinetics for the treatment of
556 diesel hydrocarbons polluted soils. Electrochimica Acta 181, 192-199.
557 Reddy, K.R., Cameselle, C., 2009. Overview of Electrochemical Remediation
558 Technologies, in: K. R. Reddy, C. Cameselle, (Eds.), Electrochemical Remediation
559 Technologies for Polluted Soils, Sediments and Groundwater. Eds. John Wiley & Sons,

560 Inc., Hoboken, New Jersey, 2009, pp. 3-29.
561 Rodrigo, M., Oturan, N., Oturan, M., 2014. Electrochemically assisted remediation of
562 pesticides in soils and water: a review. Chemical reviews 114, 8720-8745.
563 Ruiz, C., Mena, E., Canizares, P., Villasenor, J., Rodrigo, M.A., 2014. Removal of
564 2,4,6-Trichlorophenol from Spiked Clay Soils by Electrokinetic Soil Flushing Assisted
565 with Granular Activated Carbon Permeable Reactive Barrier. Industrial & Engineering
566 Chemistry Research 53, 840-846.
567 Semple, K.T., Doick, K.J., Wick, L.Y., Harms, H., 2007. Microbial interactions with
568 organic contaminants in soil: Definitions, processes and measurement. Environmental
569 Pollution 150, 166-176.
570 Spanish Presidential Ministry (2005) Royal Decree 9/2005, of 14 January, which
571 establishes the list of activities that potentially contaminate the soil and criteria and
572 standards for the declaration of polluted soils. Span Off Bull 15:1833–1843 (in spanish).
573 Wan, J., Li, Z., Lu, X., Yuan, S., 2010a. Remediation of a hexaclorobenzene
574 contaminated soil by surfactant-enhanced electrokinetics coupled with microscale Pd/Fe
575 PRB, J. Hazard. Mater. 184, 184-190.
576 Wan, J., Li, Z., Yuan, S., Lu, X., 2010b. Removal of hexaclorobenzene from kaolin by
577 electrokinetics coupled with Cu/Fe PRB, J. Hazard. Toxic Radioact. Waste, 15, 175-
578 179.
579 Wick, L.Y., Shi, L., Harms, H., 2007. Electro-bioremediation of hydrophobic organic
580 soil-contaminants: A review of fundamental interactions. Electrochimica Acta 52, 3441-
581 3448.
582 Yeung, A.T., Gu, Y.-Y., 2011. A review on techniques to enhance electrochemical
583 remediation of contaminated soils. Journal of hazardous materials 195, 11-29.
584

BIOSTIMULATION VERSUS BIOAUGMENTATION FOR THE ELECTRO-BIOREMEDIATION 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, 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.

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

101 located into the soil and it acts as a fixed bed biofilm reactor for pollutant
102 biodegradation when groundwater moves across it (Gill et al., 2014). When using EK, it
103 is recommended to place the biobarrier in the centre of the soil portion in order to avoid
104 the extreme pH values near electrodes. This configuration helps to keep the
105 microorganisms inserted alive, and the pollution plume passes through it by
106 electrokinetic transport phenomena allowing the pollutant biodegradation (Mena et al.,
107 2016a).
108 The authors of the present work have previously studied different alternatives of
109 combining conventional bioremediation with electrokinetic remediation in the case of
110 hydrocarbon-polluted soils (Ramírez et al., 2015) or pesticide-polluted soils (Barba et
111 al., 2019a). The present work is focused on the study of different biological strategies in
112 an electro-bioremediation process of a 2,4-D clayey polluted soil. Three different
113 situations were evaluated: (i) EK-biostimulation: EK is applied to the polluted soil that
114 already contains a 2,4-D degrading microbial culture, (ii) EK-bioaugmentation (using
115 biobarrier named as BB1): consists in applying electrokinetics in the polluted soil which
116 contains a fix-bed biofilm bioreactor as biobarrier, which was previously and externally
117 developed to the biodegradation of 2,4-D, and (iii) EK-bioaugmentation (using
118 biobarrier named as BB2): similar situation as (ii), but in this case the biobarrier
119 consists of a mixture of a clean soil portion with the microbial suspension. Thus, the
120 present work it is a proposal for the improvement of *in situ* techniques for
121 organochlorines polluted soils remediation. It is expected that results would contribute
122 to know the feasibility of the in situ EK-enhanced bioremediation technology for the
123 treatment of polluted soil. Under the author's knowledge, 2,4-D is a hazardous pollutant
124 and no previous research (exception of previous works in our research group) has been
125 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.

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^{-3}	1.65
Electric conductivity/ μS cm^{-1}	1800
pH	7.9
Organic matter	n.d.

Hygroscopic moisture	0.115

n.d.: non detected.

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*.

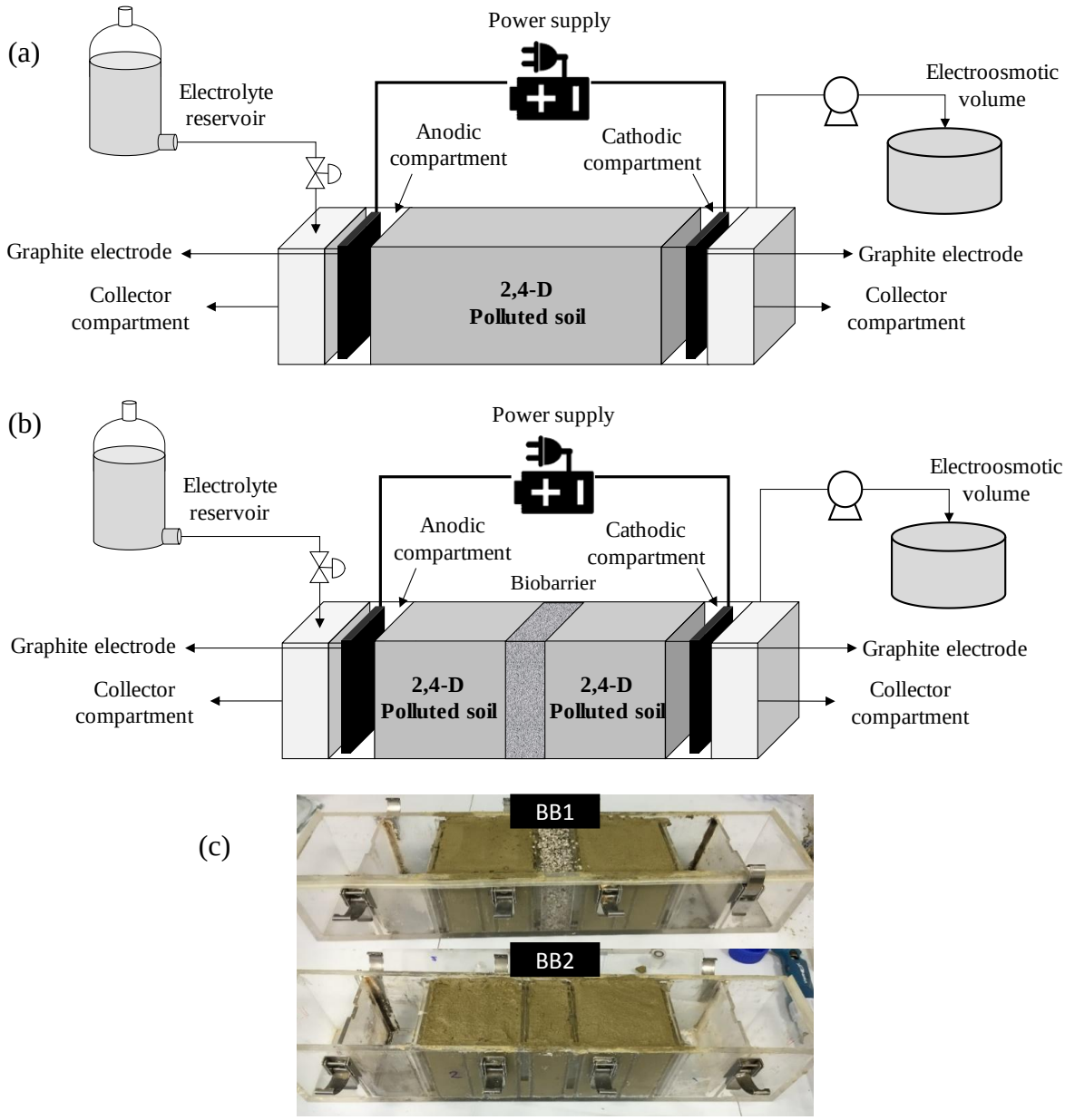
Microbial culture

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_4, 0.02 g $CaCl_2$, 1.00 g KH_2PO_4, 1.00 g $(NH_4)_2HPO_4$, 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-

158 biostimulation experiment and Fig.1b corresponds to EK-bioaugmentation experiments
159 using biobarriers. The cell is made of transparent methacrylate and divided into five
160 compartments. Soil polluted is placed in the central compartment, while at both sides
161 are located the electrodic wells, which contain the graphite electrodes (10x10x1 cm)
162 supplied by Carbosystem (Madrid, Spain) and connected to the power supply (HQ
163 Power, Gavere, Belgium). Soil is separated from electrodic wells by a nylon mesh (0.5
164 mm mesh size). Contiguous to electrodic wells, there are the collector compartments
165 that collect the electroosmotic flow (EOF) transported during the treatment. As later
166 explained, EOF will be collected at both sides due to the electrode polarity reversal. Fig.
167 1b refers to the EK-bioaugmentation experiments with biobarriers. The experimental
168 set-up is similar to that described above, but the difference in this case is that in the
169 middle of the central compartment is placed the biobarrier separated from soil with a
170 nylon mesh.



171

172 **Figure 1.** Electro-bioremediation set-up: (a) EK-biostimulation experiment; (b) EK-
173 bioaugmentation experiments using biobarriers; (c) photographs of set-up using
174 biobarriers.

175

176 The electrolyte employed in electrode wells and in soil to provide a proper electrical
177 conductivity is a simulated groundwater, whose composition per litre of Milli-Q water
178 is 80.75 mg of Na_2SO_4, 70.00 mg of $NaHCO_3$, 30.36 mg of $NaNO_3$. Additionally,
179 inorganic nutrients (ammonium, phosphate and nitrate) were supplied in excess to the
180 soil by using BHB media, in order to avoid nutrient limitations that could happen during

181 the treatment because of biological consumption or because of EK transport to the
182 external compartments (Mena et al., 2016b).

183 **2.3. Electro-bioremediation experimental procedure**

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

188 Experiment 1 (EK-biostimulation, Fig. 1a): an inoculum from the acclimated microbial
189 culture was grown in a batch reactor using BHB as culture media supplemented with
190 2,4-D. After 4 days, the obtained culture was centrifuged and suspended again in BHB.
191 Then, it was added to the 2,4-D polluted soil and mixed homogeneously obtaining a
192 final moisture of 25%. The mixture of polluted soil and microorganisms was manually
193 compacted into the central compartment of the installation simulating an autochthonous
194 microbial culture in soil for 2,4-D degradation. Both electrodic wells were filled with
195 electrolyte solution and the direct current was connected.

196 Experiment 2 (EK-bioaugmentation, Fig 1b, by using a portion of a fixed-bed biofilm
197 reactor as permeable biological barrier or "BB1"): The polluted soil was moistened with
198 the electrolyte solution and compacted into the central compartment as in experiment 1.
199 In this case, microorganisms were not inoculated through all over the soil (soil was
200 previously autoclaved at 121ºC and 15 min) but were added to soil by means of the
201 biobarrier (BB1, which is a portion of a fix-bed bioreactor previously developed as
202 reported by Barba et al., 2019b) in the central position of the soil to be remediated. A
203 central portion of soil was removed and replaced by the biobarrier (5 cm length) and
204 separated from the soil by a nylon mesh. Moreover, in order to ensure the properly
205 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

231 reversal every 12 hours. Temperature of soil and electrical current were monitored
232 during all the treatment.
233 pH and conductivity were measured with multiparameter probe (SENSLON, HACH).
234 To analyse nutrient concentrations, i.e., ammonium, nitrate and phosphate, it was used a
235 photometer Gallery (Thermo Scientific). Soil samples (1 g) were mixed with 2.5 mL of
236 water for the dissolution of 2,4-D. The soil/solvent mixture was agitated vigorously in a
237 vortex agitator for 5 min and centrifuged (15 min, 3800 min^{-1}). Samples were taken
238 from the aqueous supernatant and analysed. 2,4-D was analysed with an HPLC (Jasco,
239 Japan) equipped with a column Kinetex 5 µm Biphenyl 100 Å, 150 x 4.5 mm
240 (Phenomenex, USA). The mobile phase employed was H_3PO_4 0.1%/acetonitrile, 60/40
241 %v/v, with an isocratic flow rate of 0.6 mL min^{-1}. The wavelength of the UV detector
242 was 220 nm and injection volume was 20 µL.
243 Soil samples were taken only at the start of the experiment, before placing it on the
244 installation, and at the end (*post-mortem* analysis) of the treatment, in order to not
245 modify the compaction of soil that could cause preferential ways (Ruiz et al., 2014).
246 The *post-mortem* analysis were conducted in different soil portions as follows: four
247 longitudinal positions were considered (1 to 4, from anode to cathode at time zero) and
248 each one in turn was divided into four sections (two in the upper layer and another two
249 in the bottom layer) according to previous works (Ramírez et al., 2014). Thus,
250 analytical results in each position were the average of 4 measurements. The parameters
251 analysed in soil were moisture, pH, conductivity, microorganisms, nutrients, and 2,4-D
252 concentrations. Moisture was calculated by difference of weights, i.e., an amount of wet
253 soil was dried at 105 ºC for 24h. Weight of evaporated water corresponds to moisture
254 contained in soil. pH and conductivity were measured from dry soil. To do this, it was
255 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.

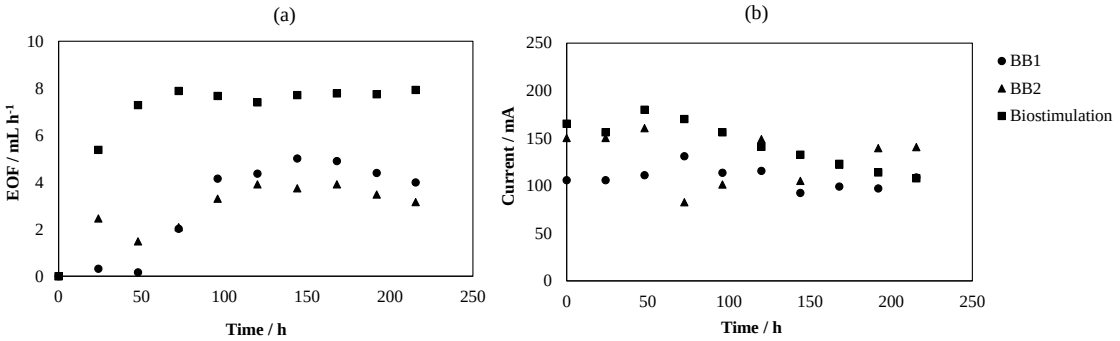


Figure 2. (a) Electroosmotic flow and (b) current intensity through the soil during the EBR experiments.

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.

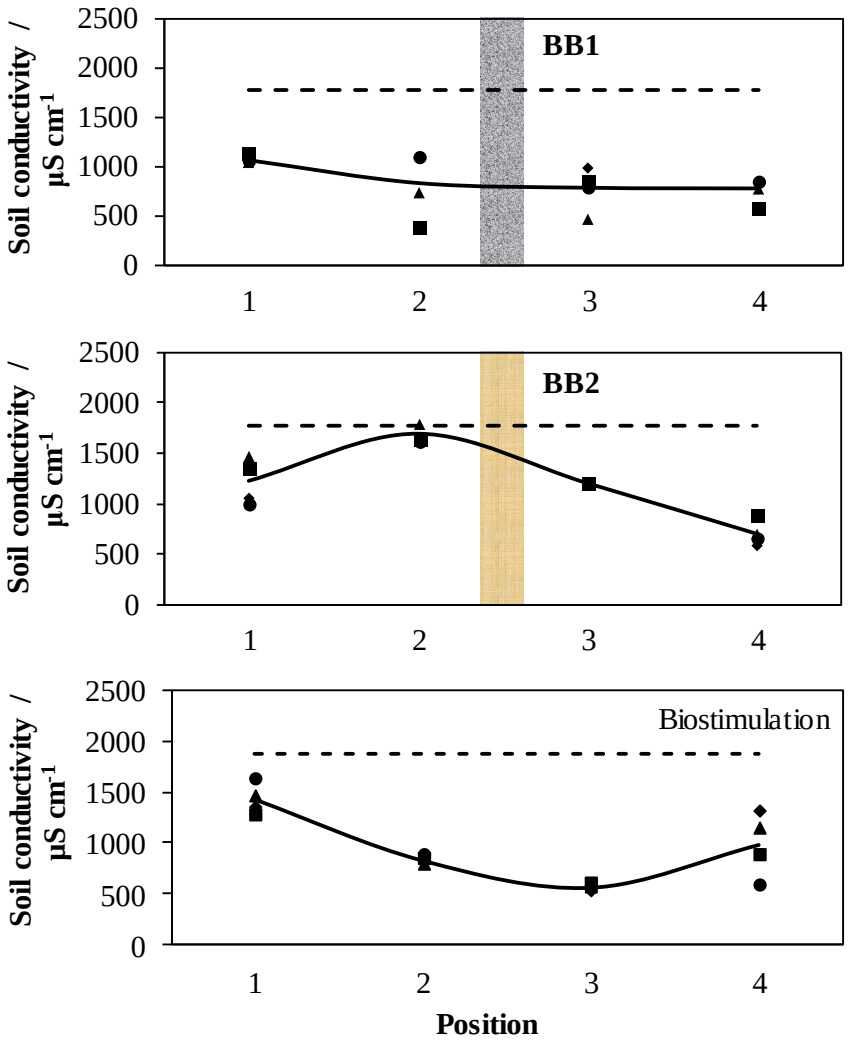


Figure 3. Soil conductivity profile in soil at the start (- - -) and at the end (‾‾) of the EBR experiments. Lines are the average of the four values in the different axial positions (top right (◆), top left (●), bottom right (■) and bottom left (▲)) and they mean trends only.

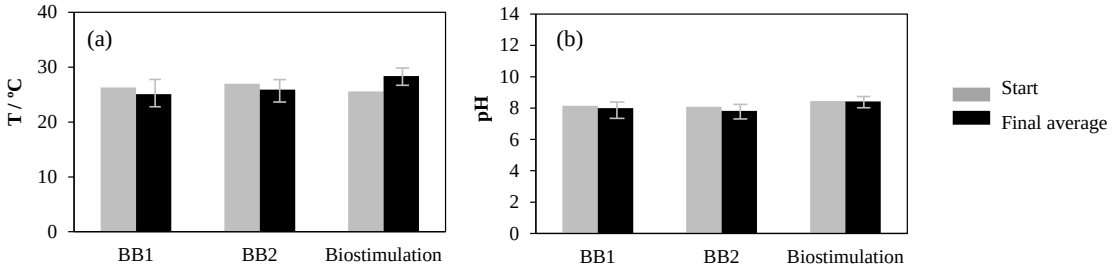
In both figures, 2 and 3, it has been observed that the inclusion of a permeable reactive biobarrier in a polluted soil causes differences in comparison with applying biostimulation strategy. EOF decreases in both bioaugmentation experiments in comparison with biostimulation experiment. It is a fact 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 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 Fe^0 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.

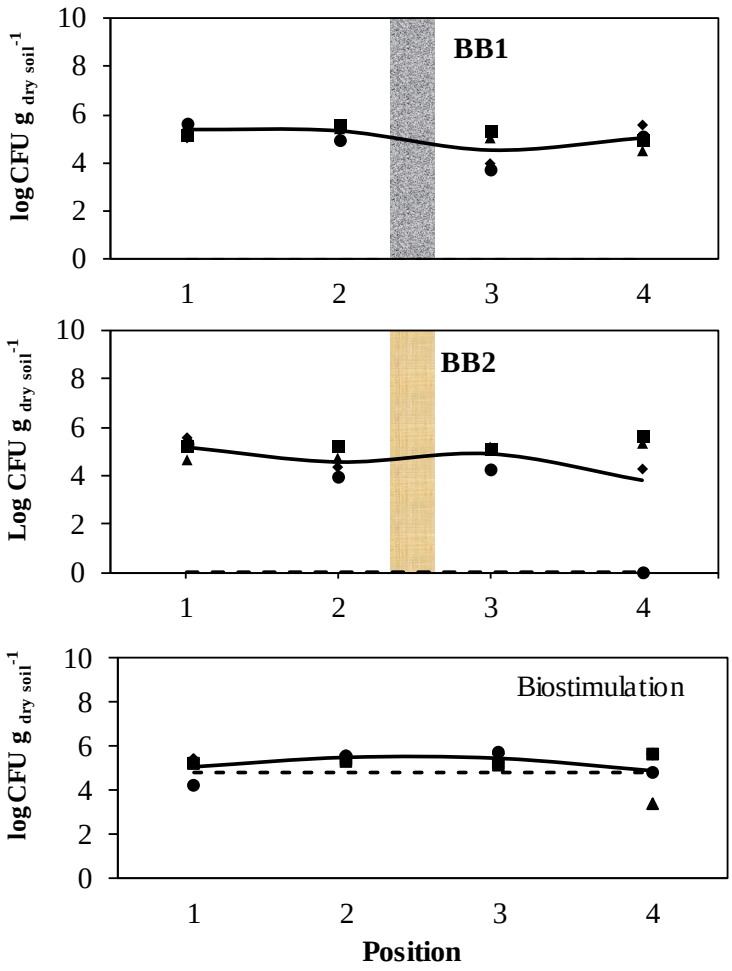


382 **Figure 4.**

383 **Figure 4.** Average values of soil (a) temperature and (b) pH at the start and at the end of
384 EBR experiments. Grey bars represent initial conditions while black bars represent final
385 average value.

386 Figure 5 shows the microorganisms' population profile in soil before and after the EBR
387 treatment. As it can be observed, only in the experiment when using biostimulation as
388 biological strategy the initial concentration (dashed line) is presented in the figure
389 because in the two experiments with biobarriers, the soil at the start was autoclaved and
390 the microorganisms were only inoculated through the biobarrier. From this figure, it can
391 be extracted two main conclusions: the first one is that in all the experiments carried
392 out, the microorganisms' population has similar concentration values at the end of EBR
393 treatment in the three cases. The second one is that there exists homogeneous
394 distribution of microorganisms in all the soil at the end of the treatment. This behaviour
395 can be explained because of the biofilm detachment from biobarrier and movement of
396 microorganisms from the central location to the rest of soil positions thanks to the
397 electrophoresis and electroosmotic flow passing thought it (DeFlaun and Condee,
398 1997). A similar result was also observed by the same authors when non polar pesticide
399 was used as model pollutant (Barba et al., 2019a). Due to the application of polarity
400 reversal strategy in EBR experiments, a correct control of pH has been achieved as it
401 was explained above. Thus, it was not observed harmful effects because of extreme pH
402 which would cause a decrease in the concentration of microorganisms in the zones near

403 to electrodes, and it indicates adequate conditions in soil for microbial activity.



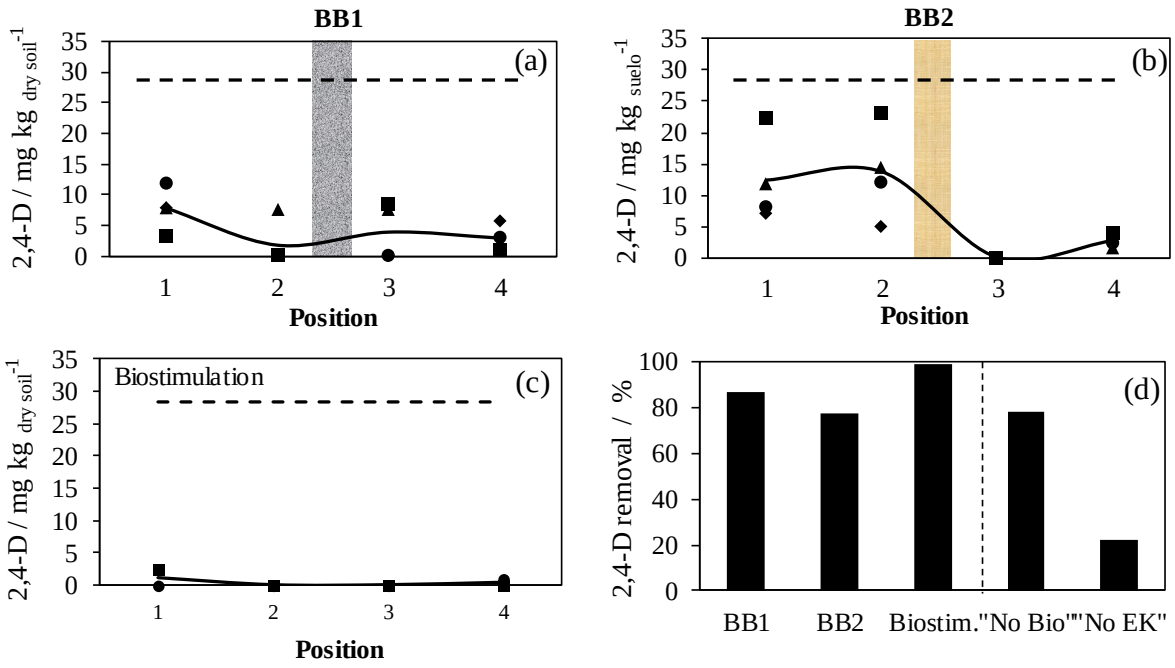
404

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

409 Figure 6 (a-c) shows the average values of 2,4-D concentrations in soil at the start and at
410 the end of the treatment. Figure 6d shows a comparative about the 2,4-D percentage
411 removal efficiencies in the three experiments carried out, including also the removal
412 efficiencies of the two reference tests. As it can be observed, removal of 2,4-D by using
413 biobarriers is quite effective, so in both cases (BB1 and BB2) it was achieved between
414 75-85% of 2,4-D removal in only 10 days of treatment. Nevertheless, EBR with
415 biostimulation strategy offers a complete 2,4-D elimination in soil and a homogeneous

416 removal profile. The lower 2,4-D removal rate when using biobarriers could be directly
417 related to EOF decrease due to the higher porosity in the biobarriers zones, and
418 consequently the current intensity and soil conductivity also decrease, as it was above
419 explained. The slightly differences of 2,4-D removal rates between two biobarriers
420 evaluated, and taking into account that experimental conditions for microbial activity
421 are practically identical in all the experiments (i.e., pH, temperature, nutrients and
422 microorganisms' concentrations) could be explained again because of the lower EOF of
423 EBR by using BB2 in comparison with BB1 related to the lower mixture effect between
424 nutrients, pollutant and microorganisms in the process of remediation.
425 Despite offering biostimulation option better results of pollutant elimination, the use of
426 biobarriers in EBR process is a great advantage because, in the case that the soil does
427 not contain microorganisms adapted to the degradation of such pollutant, this would be
428 the most optimal way to introduce the microbial culture into the soil. Studies about EK-
429 bioaugmentation are scarce. Mao et al. [49] studied EK-enhanced bioaugmentation for
430 remediation of clays contaminated with chlorinated solvents but they did not use
431 BioPRB: the microbial culture solution was added to the electrode compartments and to
432 a central injection well. They found that the microbial distribution within the clay
433 suggested that electrokinetic microbial transport was primarily driven by
434 electroosmosis, the injected bacteria were able to survive and grow, and complete
435 effective dechlorination of chlorinated ethene was observed after 94d.
436 Additionally, in Fig. 6d the results obtained in the three EBR experiments were
437 compared with two reference tests: test "No EK" and test "No Bio". Test "No Bio"
438 means only EK treatment without microbial activity, and the 2,4-D removal result from
439 this reference test is quite similar to ones obtained using biobarriers. However it is
440 important to note that, when using only EK, the pollutant is moved to the electrodic

441 wells, and then it is necessary to treat the contaminated water by external techniques,
442 e.g., electro-oxidation (de Vidales et al., 2018). Comparing the *in situ* removal EBR
443 treatments and the reference test "No EK" (that is, only *in situ* bioremediation without
444 EK) it can be observed that the 2,4-D removal percentages reached up in three
445 experiments of EBR are much higher than in the test "No EK". This behaviour prove
446 that electrokinetics acts as a mixer improving the contact and transfer matter between
447 pollutant, microorganisms and nutrients contained in soil (Mena et al., 2016c; Barba et
448 al., 2017). It is important to remark that the microbial culture is able to successfully
449 degrade high pesticide concentrations in relatively short retention times (as previously
450 reported by the same authors, Barba et al., 2019b) and thus the success, or not, of the
451 subsequent EBR technology would not be limited by the biological response, that is, the
452 biodegradation mechanism will not be considered as the limiting step in the possible
453 removal of 2,4-D in soil when this culture was used. Thus, the main conclusion that can
454 be extracted from figure 6d is that coupling electrokinetic processes with biological
455 treatment improve the *in situ* removal of 2,4-D from soil.



456

457 **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

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References

Barba, S., Villaseñor, J., Cañizares, P., Rodrigo, M.A., 2019a. Strategies for the electrobioremediation of oxyfluorfen polluted soils. Electrochimica Acta 297, 137-144.

Barba, S., Carvela, M., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2019b. Fixed-bed biological barrier coupled with electrokinetics for the *in situ* electrobioremediation of 2,4-dichlorophenoxyacetic acid polluted soil. Journal of Chemical Technology and Biotechnology 94, 2684–2692.Barba, S., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2018a. Can electro-bioremediation of polluted soils perform as a self-sustainable process? Journal of Applied Electrochemistry 48, 579-588.

Barba, S., López-Vizcaíno, R., Saez, C., Villaseñor, J., Cañizares, P., Navarro, V., Rodrigo, M., 2018b. Electro-bioremediation at the prototype scale: What it should be learned for the scale-up. Chemical Engineering Journal 334, 2030-2038.

Barba, S., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2017. Effect of the polarity reversal frequency in the electrokinetic-biological remediation of oxyfluorfen polluted soil. Chemosphere 177, 120-127.

Cameselle, C., 2014. Electrokinetic Remediation, Cost Estimation. in: Kreysa, G., Ota, K.-i., Savinell, R.F. (Eds.). Encyclopedia of Applied Electrochemistry. Springer New York, New York, NY, pp. 723-725.

Chowdhury, A., Pradhan, S., Saha, M., Sanyal, N., 2008. Impact of pesticides on soil microbiological parameters and possible bioremediation strategies. Indian Journal of Microbiology 48, 114-127.

de Vidales, M.J.M., Castro, M.P., Sáez, C., Cañizares, P., Rodrigo, M.A., 2018. Radiation-assisted electrochemical processes in semi-pilot scale for the removal of clopyralid from soil washing wastes. Separation and Purification Technology.

DeFlaun, M.F., Condee, C.W., 1997. Electrokinetic transport of bacteria. Journal of hazardous materials 55, 263-277.

Gill, R., Harbottle, M.J., Smith, J., Thornton, S., 2014. Electrokinetic-enhanced bioremediation of organic contaminants: A review of processes and environmental

509 applications. Chemosphere 107, 31-42.
510 Kebria, D.Y., Taghizadeh, M., Camacho, J.V., Latifi, N., 2016. Remediation of PCE
511 contaminated clay soil by coupling electrokinetics with zero-valent iron permeable
512 reacive barrier, Environ. Earth Sci. 75 699-709.
513 Li, T., Guo, S., Wu, B., Zhang, L., Gao, Y., 2015. Effect of polarity-reversal and
514 electrical intensity on the oil removal from soil, J. Chem. Technol. Biotechnol. 90, 441-
515 448.
516 Li, T., Wang, Y., Guo, S., Li, X., Xu, Y., Wang, Y., Li, X., 2016. Effect of polarity-
517 reversal on electrokinetic enhanced bioremediation of Pyrene contaminated soil,
518 Electrochimica Acta, 187, 567-575.Mao, X., Wang, J., Ciblak, A., Cox, E.E., Riis, C.,
519 Terkelsen, M., Gent, D.B., Alshawabkeh, A.N., 2012. Electrokinetic-enhanced
520 bioaugmentation for remediation of chlorinated solvents contaminated clay. Journal of
521 Hazardous Materials 213– 214, 311– 317.
522 Mena, E., Villaseñor, J., Cañizares, P., Rodrigo, M.A., 2014. Effect of a direct electric
523 current on the activity of a hydrocarbon-degrading microorganism culture used as the
524 flushing liquid in soil remediation processes. Separation and Purification Technology
525 124, 217-223.
526 Mena, E., Ruiz, C., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2015. Biological
527 permeable reactive barriers coupled with electrokinetic soil flushing for the treatment of
528 diesel-polluted clay soil. Journal of hazardous materials 283, 131-139.
529 Mena, E., Villasenor, J., Rodrigo, M.A., Canizares, P., 2016a. Electrokinetic
530 remediation of soil polluted with insoluble organics using biological permeable reactive
531 barriers: Effect of periodic polarity reversal and voltage gradient. Chemical Engineering
532 Journal 299, 30-36.
533 Mena, E., Villaseñor, J., Cañizares, P., Rodrigo, M., 2016b. Influence of electric field

534 on the remediation of polluted soil using a biobarrier assisted electro-bioremediation
535 process. Electrochimica Acta 190, 294-304.
536 Mena, E., Villaseñor, J., Rodrigo, M.A., Cañizares, P., 2016c. Electrokinetic
537 remediation of soil polluted with insoluble organics using biological permeable reactive
538 barriers: Effect of periodic polarity reversal and voltage gradient. Chemical Engineering
539 Journal 299, 30-36.
540 Moliterni, E., Jiménez-Tusset, R., Rayo, M.V., Rodriguez, L., Fernández, F., Villasenor,
541 J., 2012. Kinetics of biodegradation of diesel fuel by enriched microbial consortia from
542 polluted soils. International Journal of Environmental Science and Technology 9, 749-
543 758.
544 Morillo, E., Villaverde, J., 2017. Advanced technologies for the remediation of
545 pesticide-contaminated soils. Science of The Total Environment 586, 576-597.
546 Paillat, T., Moreau, E., Grimaud, P., Touchard, G., 2000. Electrokinetic phenomena in
547 porous media applied to soil decontamination. IEEE Transactions on Dielectrics and
548 Electrical Insulation 7, 693-704.
549 Ramírez, E.M., Camacho, J.V., Rodrigo, M.R., Cañizares, P.C., 2014. Feasibility of
550 electrokinetic oxygen supply for soil bioremediation purposes. Chemosphere 117, 382-
551 387.
552 Ramírez, E.M., Jiménez, C.S., Camacho, J.V., Rodrigo, M.A.R., Cañizares, P., 2015.
553 Feasibility of coupling permeable bio-barriers and electrokinetics for the treatment of
554 diesel hydrocarbons polluted soils. Electrochimica Acta 181, 192-199.
555 Reddy, K.R., Cameselle, C., 2009. Overview of Electrochemical Remediation
556 Technologies, in: K. R. Reddy, C. Cameselle, (Eds.), Electrochemical Remediation
557 Technologies for Polluted Soils, Sediments and Groundwater. Eds. John Wiley & Sons,
558 Inc., Hoboken, New Jersey, 2009, pp. 3-29.

559 Rodrigo, M., Oturan, N., Oturan, M., 2014. Electrochemically assisted remediation of
560 pesticides in soils and water: a review. Chemical reviews 114, 8720-8745.
561 Ruiz, C., Mena, E., Canizares, P., Villasenor, J., Rodrigo, M.A., 2014. Removal of
562 2,4,6-Trichlorophenol from Spiked Clay Soils by Electrokinetic Soil Flushing Assisted
563 with Granular Activated Carbon Permeable Reactive Barrier. Industrial & Engineering
564 Chemistry Research 53, 840-846.
565 Semple, K.T., Doick, K.J., Wick, L.Y., Harms, H., 2007. Microbial interactions with
566 organic contaminants in soil: Definitions, processes and measurement. Environmental
567 Pollution 150, 166-176.
568 Spanish Presidential Ministry (2005) Royal Decree 9/2005, of 14 January, which
569 establishes the list of activities that potentially contaminate the soil and criteria and
570 standards for the declaration of polluted soils. Span Off Bull 15:1833–1843 (in spanish).
571 Wan, J., Li, Z., Lu, X., Yuan, S., 2010a. Remediation of a hexaclorobenzene
572 contaminated soil by surfactant-enhanced electrokinetics coupled with microscale Pd/Fe
573 PRB, J. Hazard. Mater. 184, 184-190.
574 Wan, J., Li, Z., Yuan, S., Lu, X., 2010b. Removal of hexaclorobenzene from kaolin by
575 electrokinetics coupled with Cu/Fe PRB, J. Hazard. Toxic Radioact. Waste, 15, 175-
576 179.
577 Wick, L.Y., Shi, L., Harms, H., 2007. Electro-bioremediation of hydrophobic organic
578 soil-contaminants: A review of fundamental interactions. Electrochimica Acta 52, 3441-
579 3448.
580 Yeung, A.T., Gu, Y.-Y., 2011. A review on techniques to enhance electrochemical
581 remediation of contaminated soils. Journal of hazardous materials 195, 11-29.
582

Figure 1
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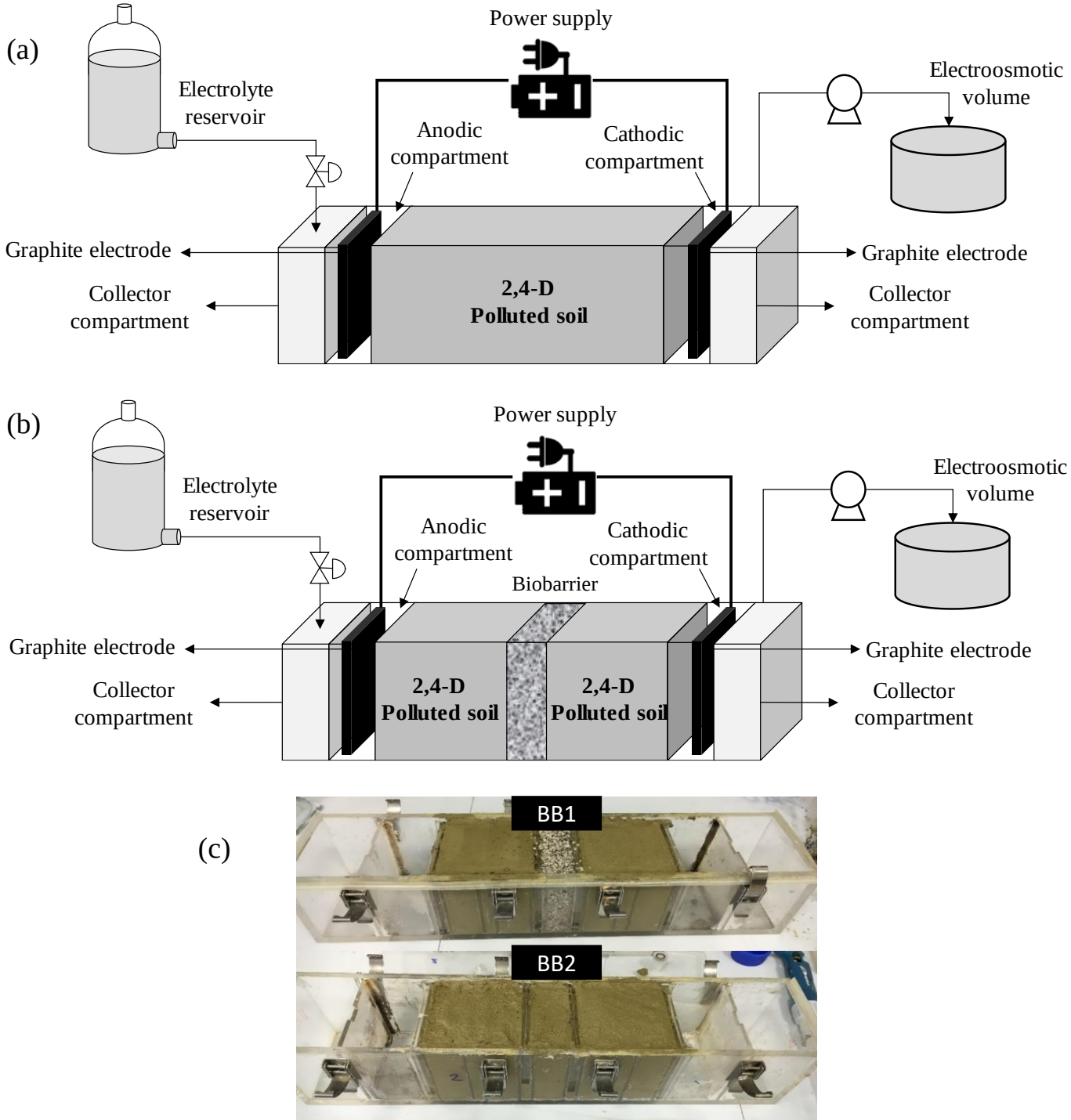

Figure 1.

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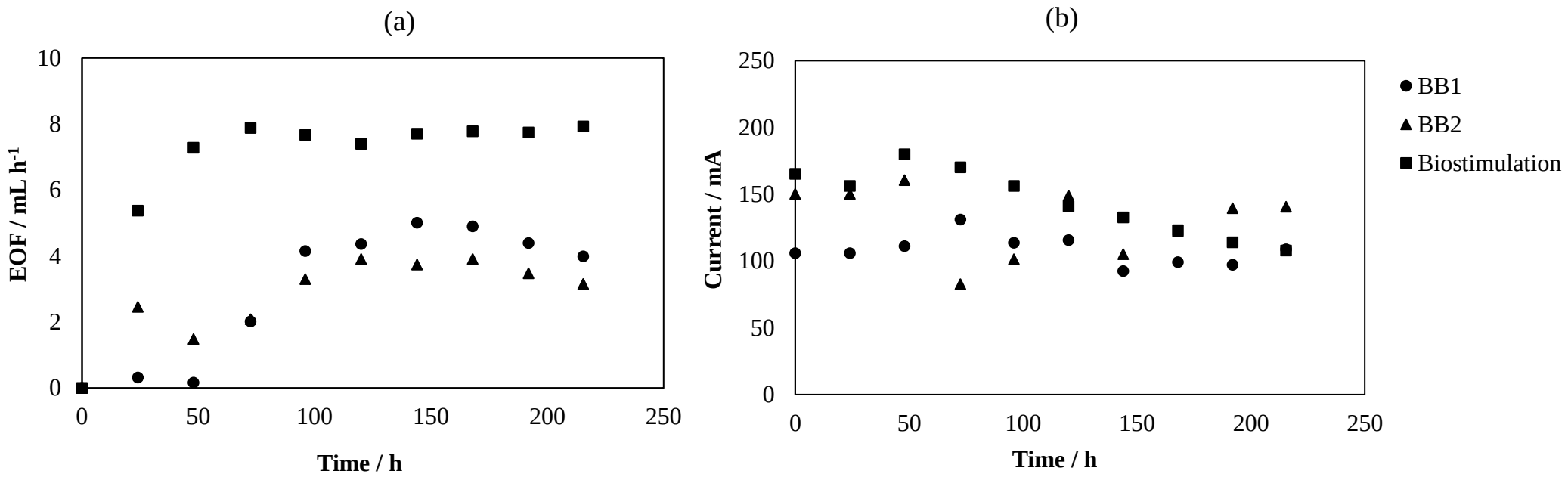

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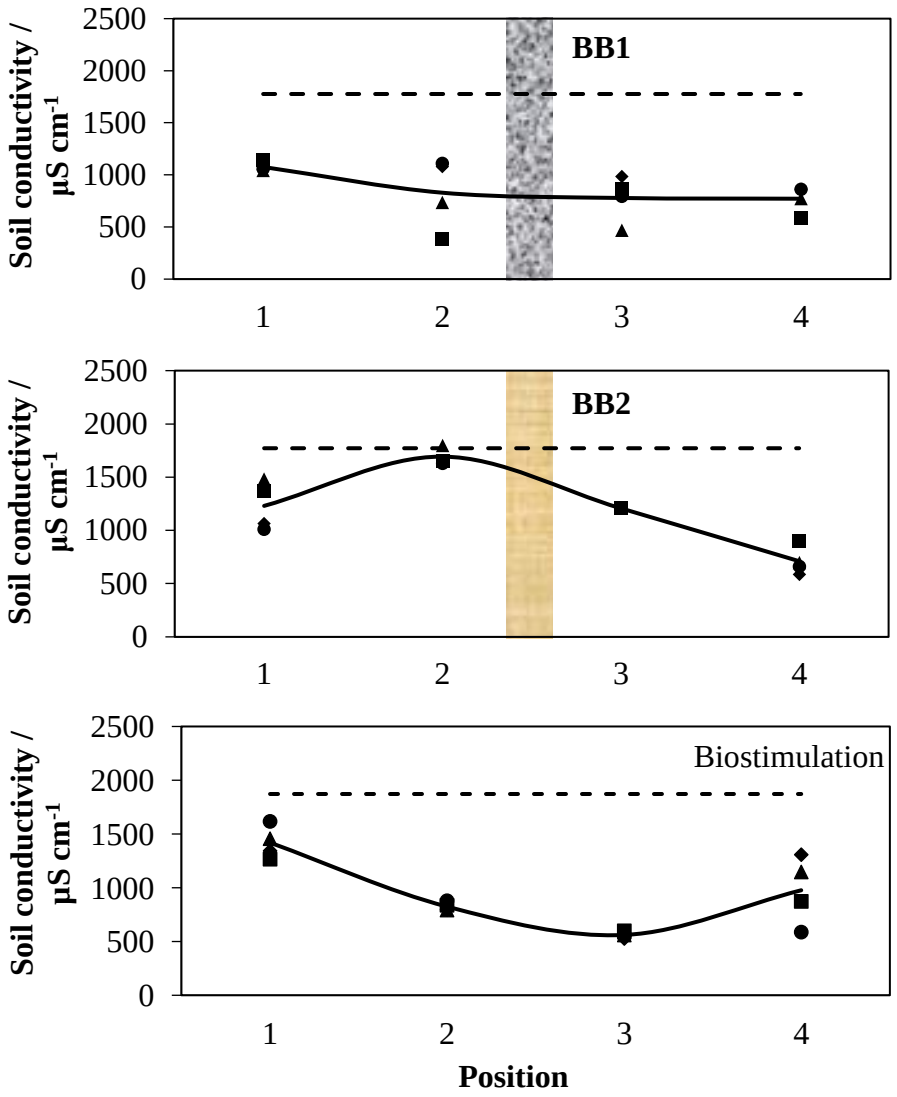

Figure 3.

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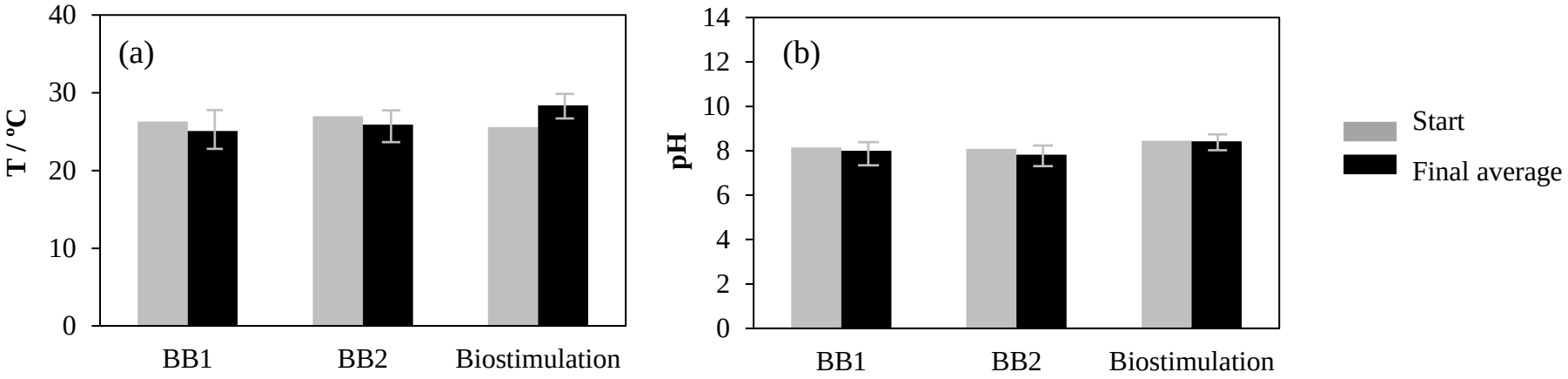

Figure 4.

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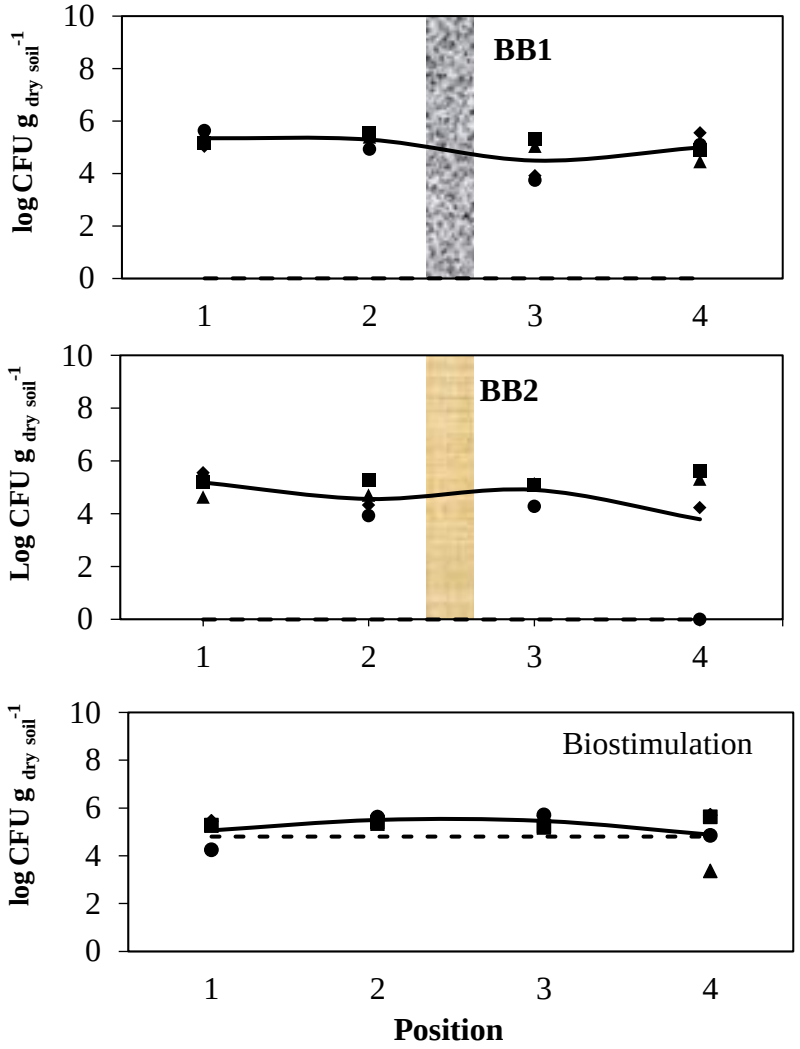

Figure 5.

Figure 6
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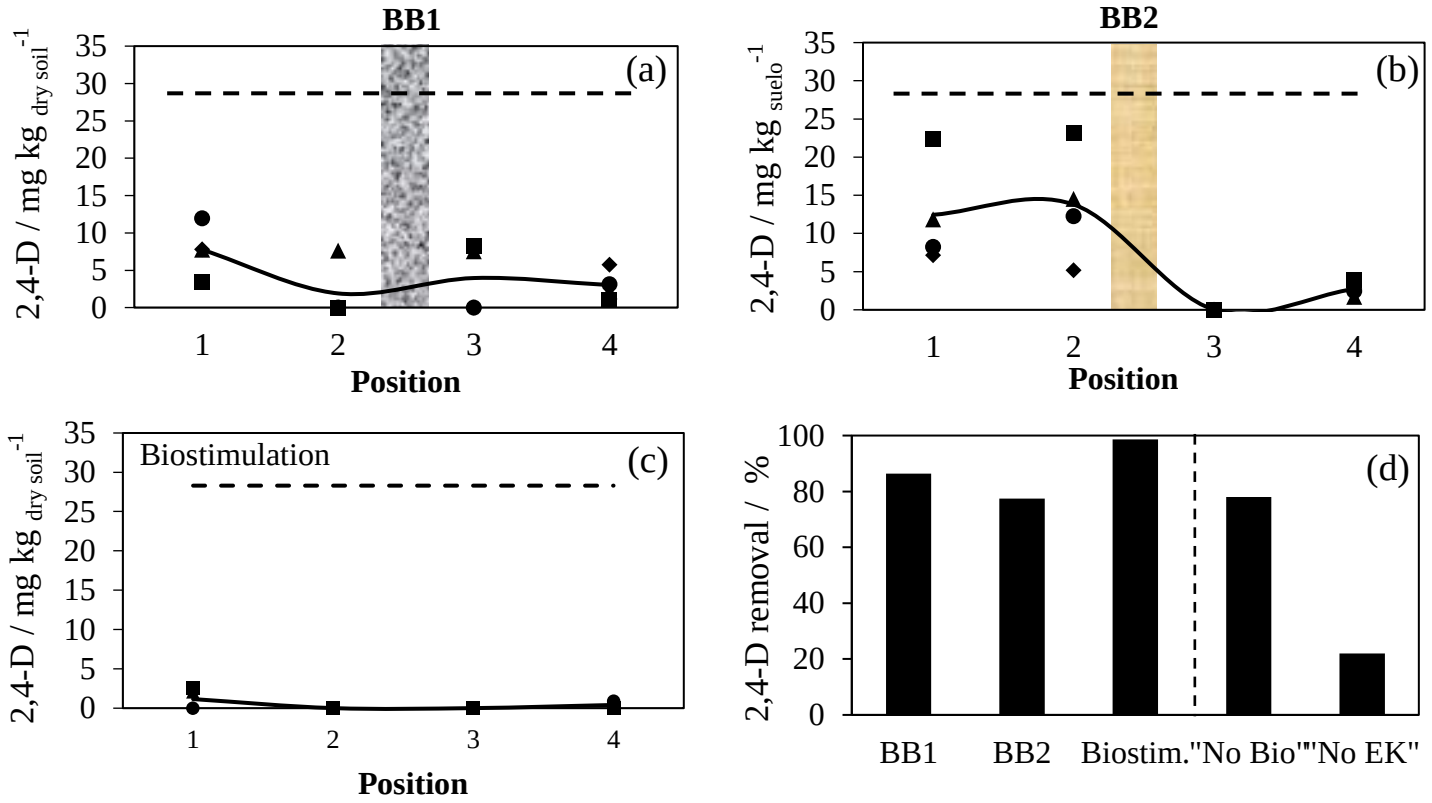

Figure 6.

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.

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^{-3}	1.65
Electric conductivity/ µS cm^{-1}	1800
pH	7.9
Organic matter	n.d.
Hygroscopic moisture	0.115

n.d.: non detected.

***Declaration of Interest Statement**

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:

Silvia Barba: Experimental work in laboratory. Calculations. Figures preparation.

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.