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TOWARDS THE OPTIMIZATION OF ELECTRO-BIOREMEDIATION OF SOIL POLLUTED WITH 2,4-DICHLOROPHENOXYACETIC ACID

--Manuscript Draft--

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Abstract:	<p>The aim of this work is to study the optimization of electro-bioremediation (EBR) treatment of a 2,4-dichlorophenoxyacetic acid (2,4-D) polluted clay soil. The influence of two different variables were evaluated trough batch experiments in a bench-scale electrokinetic setup using previously acclimated microbial cultures for 2,4-D biodegradation. First, it was studied the influence of the frequency applied in polarity reversal (PR): frequencies under study were 1, 2 and 6 d-1, i.e., polarity changed every 24, 12 and 4 hours respectively. The duration of experiments were 14 days and the electric field applied was 1.0 V cm⁻¹ (20 V) at room temperature. The second variable under study was the operation time, and based on the previous results, the selected frequency of PR was 2 d-1 and three additional EBR experiments were conducted using different operation times (3, 7 and 10 days). Experiments without electric current (only biological contribution) for each operation time were simultaneously performed under the same experimental conditions as reference tests to check the influence of electrokinetics. Removal of 2,4-D from polluted clay soil was completed in 10 days. It was observed that solubility of the pollutant is a critical factor to ensure high removal efficiencies. Moreover, polarity reversal contributed to the successful results by maintaining correct pH values and reducing the removal of electrolytes from soil. By comparing the EBR results with the reference tests (without the contribution of EK phenomena), it was proved that the combination of bioremediation and electrokinetics has positive effects in the remediation of low permeable polluted soil.</p>
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Dear Editor:

Attached you will find the REVISED form of the manuscript ETI_2020_1011 “*Towards the optimization of electro-bioremediation of soil polluted with 2,4-dichlorophenoxyacetic acid*”, by Silvia Barba, José Villaseñor, Manuel Andrés Rodrigo and Pablo Cañizares (corresponding author: jose.villasenor@uclm.es), in order to be reviewed for a possible publication as original research paper in *ET&I*.

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**”

Yours sincerely

Dr. J. Villaseñor

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1 **TOWARDS THE OPTIMIZATION OF ELECTRO-BIOREMEDIATION OF**
2 **SOIL POLLUTED WITH 2,4-DICHLOROPHENOXYACETIC ACID.**

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11 **Abstract**

12 The aim of this work is to study the optimization of electro-bioremediation (EBR)
13 treatment of a 2,4-dichlorophenoxyacetic acid (2,4-D) polluted clay soil. The influence
14 of two different variables were evaluated trough batch experiments in a bench-scale
15 electrokinetic setup using previously acclimated microbial cultures for 2,4-D
16 biodegradation. First, it was studied the influence of the frequency applied in polarity
17 reversal (PR): frequencies under study were 1, 2 and 6 d⁻¹, i.e., polarity changed every
18 24, 12 and 4 hours respectively. The duration of experiments were 14 days and the electric
19 field applied was 1.0 V cm⁻¹ (20 V) at room temperature. The second variable under study
20 was the operation time, and based on the previous results, the selected frequency of PR
21 was 2 d⁻¹ and three additional EBR experiments were conducted using different operation
22 times (3, 7 and 10 days). Experiments without electric current (only biological
23 contribution) for each operation time were simultaneously performed under the same
24 experimental conditions as reference tests to check the influence of electrokinetics.
25 Removal of 2,4-D from polluted clay soil was completed in 10 days. It was observed that

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26 solubility of the pollutant is a critical factor to ensure high removal efficiencies.
27 Moreover, polarity reversal contributed to the successful results by maintaining correct
28 pH values and reducing the removal of electrolytes from soil. By comparing the EBR
29 results with the reference tests (without the contribution of EK phenomena), it was proved
30 that the combination of bioremediation and electrokinetics has positive effects in the
31 remediation of low permeable polluted soil.

32 **Keywords**

33 2,4-dichlorophenoxyacetic acid, electro-bioremediation, polluted soil, herbicide, periodic
34 polarity reversal.

35 **1. Introduction**

36 Since the last century, the use of pesticides has become more extensive, mostly in
37 agricultural industry. ~~Thus, crop productivity increased and, consequently, the World~~
38 ~~population. Despite the necessity of using pesticides to solve this kind of issues, these~~
39 compounds have several disadvantages such as the high persistence in environment and
40 thus the health problems that can cause in animals or humans being in contact with these
41 substances (Rodrigo et al., 2014; Verma et al., 2014; Geed et al., 2017). 2,4-
42 dichlorophenoxyacetic acid or commonly known as 2,4-D, is a systemic hormonal
43 herbicide, it means that can affect to hormonal system in plants to avoid its growth. 2,4-
44 D is framed within the group of organochlorinated pesticides, which are known for being
45 very persistent in water, air and soils (Chowdhury et al., 2008). Due to the environmental
46 problems associated to the use of pesticides, and because the soil is a non-renewable
47 resource, it is necessary to remediate pesticide-polluted soils. For these reasons, national
48 regulation in Spain is becoming harder regarding soil contamination, stablishing low
49 pollutant limit concentrations in soil (Spanish Presidential Ministry, 2005). These limits,
50 known as reference pollution levels, are different depending on the impact receptors

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51 (ecosystems, or human health). In the case of organochlorinated pollutants such as 2,4-
52 D, the maximum allowed concentration in soil is 1.0 mg per kg of soil.

53 To remediate polluted soils, there are several strategies based on biological, physical,
54 thermal or electrochemical technologies. One of the most applied techniques is
55 conventional bioremediation because of its low cost (Juwarkar et al., 2010). However, in
56 case of *in-situ* treatments (that is, soil is treated on its original location without the need
57 for excavation and transport to external treatment facilities) bioremediation requires high
58 operation times as the mass transfer phenomena necessary to contact pollutant,
59 microorganisms and nutrients are very slow, especially in low permeable soils (Barba et
60 al., 2018).

61 Regarding this limitation, in recent years, electrokinetic remediation (EK) has been
62 increasing as a clear cost-effective alternative for *in-situ* soil remediation (Reddy and
63 Cameselle, 2009). EK remediation consists of the application of a direct electric current

64 across electrodes placed in the polluted soil. ~~Therefore, electrokinetic phenomena (such~~
65 ~~as electrophoresis, electromigration and electroosmosis) mobilize and allow contact~~
66 ~~between~~ microorganisms, nutrients and pollutants towards the soil ~~improving contact~~
67 ~~between them~~ (Paillat et al., 2000; Rodrigo et al., 2014). EK treatment is mainly

68 recommended for low permeability polluted soils where conventional pump and treat
69 methods do not allow moving groundwater neither the transport of contaminants along
70 the soil (Reddy and Cameselle, 2009). Previous research (Yeung and Gu, 2011;
71 Cameselle, 2014) achieved great removal results demonstrating that EK is a successful
72 technology for remediating low permeable polluted soils. However, EK technology also
73 presents some limitations during the operation time such as the soil heating by Joule
74 effect, extreme pH zones near electrodes or the low mobility of non-polar pollutants
75 through the soil.

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76 Because of the above-mentioned advantages and limitations of bioremediation and EK, a
77 recent combination of both technologies, known as electro-bioremediation, is becoming
78 more attractive in matter of *in-situ* decontamination of low permeable polluted soils.
79 Electro-bioremediation (EBR) couples the most interesting advantages of both
80 technologies (Yeung and Gu, 2011). EBR improves the contact between pollutants and
81 microorganisms, achieving the biodegradation of the pollutants *in situ* by the action of
82 microorganisms present in soil (Semple et al., 2007; Wick et al., 2007).
83 There are different options of combining bioremediation and electroremediation to
84 remove organic pollutants from soils (Gill et al., 2014). Previously, the authors of the
85 present work studied several alternatives of such combination, and they optimized
86 different parameters in the process in the case of soils polluted with oxyfluorfen (Barba
87 et al., 2017; Barba et al., 2019a). The present work is focused on the optimization of the
88 electro-bioremediation process of a 2,4-D polluted clay soil. The influences of two
89 different variables have been evaluated: (i) the frequency in the electrode polarity reversal
90 (PR) and (ii) the operation time of the process. Batch experiments were conducted at
91 bench-scale using an EK experimental cell at room temperature under 1 V cm^{-1} . An EK-
92 biostimulation strategy was used, that is the polluted soil already contains an acclimated
93 microbial culture adapted to 2,4-D biodegradation. To the author's knowledge, electro-
94 bioremediation studies for *in-situ* biodegradation of hazardous pesticides in polluted soils
95 are still scarce.

96 2. Materials and methods

97 2.1. Materials

98 The soil employed at this work is a clean clayey soil supported by Millas Hijos Ceramics
99 (Toledo, Spain). Its characteristics has been described in previous works (Barba et al.,

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7 100 2017). Soil was artificially polluted with the pesticide (20 mg of 2,4-D per kg of wet soil)
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9 101 following the procedure explained in section 2.3.
10
11 102 The chemical product 2,4-dichlorophenoxyacetic acid (2,4-D) was selected as model of
12
13 103 polar pesticide, supplied by *Alfa Aesar* (98% assay). 2,4-D possible loss by volatilization
14
15 104 was checked during the preparation of polluted soil. It was proved that 2,4-D
16
17 105 volatilization in sterilized polluted soil after 1 week at the same temperature than EBR
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19 106 experiments was negligible.
20 107 Previously to the EBR experiments, it was carried out the acclimation process of the
21
22 108 microorganisms for the biodegradation of 2,4-D in order to get a strong microbial culture
23
24 109 following the procedure describe in previous works by Moliterni et al. (2012).
25
26 110 To start the acclimation process it was selected an inoculum from a biological reactor of
27
28 111 an oil-refinery wastewater treatment plant (Puertollano, Spain). Bushnell-Hash Broth
29
30 112 (BHB) was used as inorganic nutrients source for microorganisms. This culture media
31
32 113 contains 0.20 g L⁻¹ Mg SO₄, 0.02 g L⁻¹ CaCl₂, 1.00 g L⁻¹ KH₂PO₄, 1.00 g L⁻¹ (NH₄)₂HPO₄,
33
34 114 0.05 g L⁻¹ FeCl₃ and 1.00 g L⁻¹ KNO₃. 200 mg L⁻¹ of 2,4-D was employed as sole carbon
35
36 115 source. Once the acclimation process was achieved, the species of microorganisms
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38 116 contained in the microbial culture were identify by means of MALDI TOF Mass
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40 117 Spectrometry (AXIMA-Assurance Biotech technology, SHIMADZU, Germany). The
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42 118 species identified in the 2,4-D-degrading microbial culture were *Rhodococcus ruber* and
43
44 119 *Ochrobactrum anthropic*.

44 120 **2.2 Experimental set-up**

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46 121 Figure 1 shows the experimental set-up scheme employed in EBR experiments. The
47
48 122 installation consists of a bench scale EK cell made of transparent methacrylate, which is
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50 123 divided into five compartments. In the central one is placed the 2,4-D polluted soil. The
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52 124 electrodic compartments are located at both sides separated from soil by a nylon mesh
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125 (0.5 mm mesh size), and they contained the electrodes. The electrodes used are made of
126 graphite with dimensions of 10x10x1 cm, supplied by Carbosystem (Madrid, Spain),
127 which are connected to a power supply (HQ Power, Gavere, Belgium). The electrolyte
128 used is a synthetic inorganic medium with the following composition: 80.75 mg L⁻¹ of
129 Na₂SO₄, 70.00 mg L⁻¹ of NaHCO₃, 30.36 mg L⁻¹ of NaNO₃, which tries to simulate
130 groundwater. Because of microorganisms can consume the nutrients or due to their
131 removal by electromigration and electroosmosis, excess inorganic nutrients (nitrate,
132 ammonium and phosphate) were provided to avoid nutrient limitations that can occur
133 during the EBR process. To collect the electroosmotic flow (EOF) moved during the
134 process, there are two collector compartments contiguous to electrode ones. It is
135 important to remark that the EOF will be collected at both sides because of the polarity
136 reversal applied in all EBR experiments of this work (Mena et al., 2016).

137 **2.3. Experimental procedure**

138 The biological strategy coupled to EK employed in this work was biostimulation, which
139 consists of adding nutrients to a polluted soil already containing acclimated
140 microorganisms for pollutant biodegradation. Nutrients addition and EK application are
141 expected to stimulate biological metabolism and, thus, improve the biodegradation of
142 pollutants. Thus, the previous procedure to the electro-bioremediation experiments was
143 as follows: an inoculum of 2,4-D-degrading microbial culture was grown in a batch
144 reactor using BHB culture media as inorganic nutrients source during 4 days; the culture
145 was subsequently centrifuged and suspended in new BHB media, and added to a 2,4-D
146 solution.; then, the suspension of microorganisms and 2,4-D solution was homogeneously
147 distributed into the clayey soil, obtaining a final moisture of soil around 25%, and a
148 concentration of 2,4-D of 20 mg per kg of wet soil (26.7 mg per kg of dry soil).

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149 The inoculated and polluted soil was placed and compacted manually in the central
150 compartment of the experimental set-up, and the electrodic compartments were filled with
151 electrolyte solution. Direct current was connected, and electro-bioremediation batch
152 experiments were conducted under 1.0 V cm^{-1} (20 V) at room temperature.

153 The experimental planning to study the influence of variables was as follows:

154 1. Effect of electrode polarity reversal frequency: In this case, three batch electro-
155 bioremediation experiments (14 d duration each) were carried out. The frequencies under
156 study were 1, 2 and 6 d^{-1} , i.e., polarity changed every 24, 12 and 4 hours respectively.

157 2. Effect of operation time: Based on the previous results (i), the selected frequency of
158 PR was 2 d^{-1} and three additional EBR experiments were conducted using different
159 operation times (3, 7 and 10 days). Simultaneously to each EBR experiment using
160 different operation times, a reference test Experiments without electric current (only
161 biological contribution) for each operation time were simultaneously was always
162 performed. Reference tests were done under the same identical experimental conditions
163 than EBR experiments (that is, the same installation, electrolyte addition, microbial
164 inoculation), but without using electric current, and they would help to as reference tests
165 ~~to~~ check the influence of electrokinetics.

166 2.4. Sampling and analyses

167 During the experiments, liquid samples were taken from electrodic wells, from the EOF
168 collector compartment and from the liquid medium in soil. Due to the electrode polarity
169 changes during the process, it is important to remark that EOF is collected alternatively
170 in both collector compartments depending on the cathode position. Moreover, the
171 temperature of soil and current electricity were monitored throughout all the duration of
172 the experiments. Conductivity and pH were measured by using a multiparameter probe
173 (SENSLON, HACH). Inorganic nutrient concentrations were analysed by means of UV-

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174 Vis photometer (Gallery, Thermo Scientific). The concentration of 2,4-D was measured
175 by HPLC (Jasco, Japan), using a column model Kinetex 5 μm Biphenyl 100 \AA , 150 x 4.5
176 mm (Phenomenex, USA), with a mobile phase of H_3PO_4 0.1%/acetonitrile, 60/40 %v/v,
177 and an isocratic flow rate of 0.6 mL min^{-1} , and the wavelength of UV detector was 220
178 nm. The injection volume was 20 μL .

179 Soil samples were taken only at the beginning before placing the soil on the set-up, and
180 at the end of the experiments (*post-mortem* analysis, once the experiment was finished).

181 This procedure was followed not to modify the compaction of soil avoiding preferential
182 paths for EOF (Ruiz et al., 2014). For carrying out the *post-mortem* analysis, it is
183 necessary to divide the soil properly after the experiment. Then, the soil was divided into
184 sections as follows: four longitudinal positions (positions 1 to 4), where position 1
185 corresponds to nearest zone to anodic well, and position 4 to nearest to cathodic well (at
186 $t = 0$). Each longitudinal section was in turn divided into four parts: two upper parts (left
187 and right) and two bottom parts (left and right), according to previous works (Ramírez et
188 al., 2015).

189 The following parameters were measured in soil: moisture, pH, conductivity,
190 microorganisms, inorganic nutrients and 2,4-D concentrations. Moisture was calculated
191 by weights difference from wet soil sample and dry one: an amount of wet soil was dried
192 at 105°C for 24 hours and the weight of evaporated water was related to the moisture of
193 the soil. Soil pH and conductivity were measured from the soil dried. Thus, once it was
194 dried as it has been described above, it was taken 10 g of dry soil, and 25 mL of Milli-Q
195 water were added. The mixture soil-water was agitated during 30 min by magnetic stirring
196 and then, it was left decant a couple of hours at least. The liquid supernatant was filtered
197 with nylon filters of $0.2 \mu\text{m}$ and measured by using the multiparameter probe. Inorganic
198 nutrients concentrations were measured as well from dry soil with a photometer. On the

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199 other hand, 2,4-D concentration was measured from wet soil employing the same HPLC
200 method above described.

201 The concentration of microorganisms expressed as Colony Forming Units (CFU) per
202 gram of dry soil was done following the procedure described in previous works (Ramírez
203 et al., 2015), i.e., it was taken 1 g of wet soil and it was added 10 mL of saline solution
204 (0.9% NaCl). Then, it was agitated during 3 min by using a vortex agitator. Once it was
205 vigorous mixed, an aliquot of 100 µL of supernatant was taken and placed on Petri dishes,
206 which contained Luria Bertani (LB) solid culture medium for microbial growth. The
207 composition of LB medium for 1 L of Milli-Q water is 10.0 g of NaCl, 5.0 g of yeast
208 extract and 10.0 g of casein peptone, 15 g of European Bacteriological Agar and 10.0 g
209 of glucose acting as carbon source. Finally, Petri dishes were incubated for 24h at 26.5
210 °C and colonies grown were counted.

211 **3. Results and discussion**

212 **3.1. Selection of polarity reversal frequency**

213 Figure 2a shows the pH ~~profile-values at different soil positions towards the soil~~ after
214 electro-bioremediation experiments ~~for every PR frequency studied using different PR~~
215 ~~frequencies~~. Left part of the Figure 2a corresponds to the anode position and right part
216 corresponds to cathode position (at t=0). Additionally, as reference test using no PR ($f=$
217 0), the figure also includes the results previously reported by our research group (Vieira
218 dos Santos et al., 2016) who studied 2,4-D behaviour under abiotic electrokinetics using
219 exactly the same soil and experimental conditions. As it can be observed in Figure 2a, the
220 pH next to anodic and cathodic wells in the case of not applying polarity reversal ($f=0$)
221 is acid and basic, respectively, while pH was maintained neutral in soil when EBR
222 experiments were finished. Similar behaviour was also reported by the same authors in

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7 223 previous works when using non-polar pollutants (Barba et al., 2017). However, it was not
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9 224 observed a clear influence of the f value on the studied range (between 1 and 6 d⁻¹).
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11 225 Figure 2b shows the profiles of electrical conductivity towards the soil at the end of
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13 226 experiments. It can be observed that after the three experiments, carried out at different
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15 227 f values, electrical conductivity profiles are similar, and again a significant influence of f
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17 228 value was not observed. On the contrary, in the case of the reference test ($f=0$) a decrease
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19 229 in the soil conductivity can be observed. This result can be related to the faster removal
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21 230 of ions from the system when no PR is applied. Moreover, it has been previously reported
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23 231 that EOF decreases and relatively high current density value remains when PR is used
24
25 232 (Mena et al., 2016; Barba et al., 2017). The application of PR implies that the ions retained
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27 233 in the soil can remain longer because both electromigration and EOF move them
28
29 234 alternatively in both directions. Consequently, it allows not only pH control but also
30
31 235 maintaining adequate values of inorganic nutrients concentrations, current density and
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33 236 electrical conductivity in soil during the remediation process. Soil temperature (results
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35 237 not shown) kept practically constant during all the experiments carried out (both EBR
36
37 238 experiments and reference tests without electricity), around 30 °C, that is adequate
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39 239 temperature for microbial activity. It was noted again that ohmic heating was negligible
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41 240 at such low voltages at bench scale (Barba et al., 2019a). Again, changes in f values in
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43 241 the range between 1 and 6 d⁻¹ did not affect soil temperature. Figure 3a shows the
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45 242 concentration of microorganisms in soil at the beginning (discontinuous line) and at the
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47 243 end (continuous line) of experiments under different f values. As it can be observed, the
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49 244 microorganisms' concentration kept practically constant in all experiments, that is, a
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51 245 homogeneous profile was observed towards the soil and no microbial decay was
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53 246 observed. pH control is critical for such result. Mena et al. (2015), reported that in the
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55 247 case of not applying periodic polarity reversal, the concentration of active

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248 microorganisms in soil at the end of the process is null caused by the negative effect of
249 extreme pH in soil. As it occurs with the other parameters above described, it was not
250 observed difference in microorganisms' concentration depending on the different
251 frequencies studied. Figure 3b shows the results of 2,4-D removal from soil for each
252 experiment conducted. As it can be observed, in only two weeks of treatment it was
253 achieved the completely removal of the pollutant in soil in the three experiments at
254 different f values. This fact shows that the removal of 2,4-D is easier than the removal of
255 non-polar compounds, probably because of its polar nature and low sorption in soil, which
256 implies higher mobility through all over the soil and better contact with microorganisms
257 (Barba et al., 2019a). Nevertheless, results shown in Figures 2 and 3 do not allow us to
258 select an optimum value of polarity reversal frequency. For this reason, and because of
259 the frequency value has no economical cost implications in the electro-bioremediation
260 process, it was selected a frequency of 2 d^{-1} as in the previous works carried out by the
261 same authors when using non-polar pollutants (Barba et al., 2017).

262 3.2. Effect of the operation time

263 Results in section 3.1 indicate that 14 d duration treatment is not necessary and operation
264 time can be reduced in order to optimize the process. Thus, lower operation times (3, 7
265 and 10 d) were tested. Figure 4 shows the current intensity (Fig. 4a) and EOF (Fig. 4b)
266 through the soil during the experiments using lower operation times. As it can be observed
267 in Fig. 4a, for operation times lower than 7 days current intensity keeps practically
268 constant during the experiment (around 150 mA), while higher operation times cause a
269 decrease of intensity from 150 to 100 mA. This behaviour can be associated to the
270 removal of ions from soil by electromigration and electroosmosis, or because of the
271 electrodes wear down (Reddy and Cameselle, 2012).

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272 On the other hand, in Fig. 4b it can be observed that EOF shows similar trend in the three
273 electro-bioremediation experiments. It was observed that the EOF increases during the
274 first hours of treatment, and then, it stabilizes around a constant value for the rest of the
275 experiment. In three cases, the stationary EOF is around 5-8 mL h⁻¹, and the low
276 differences between EOF in the experiments may be due to differences in manual soil
277 compaction in each one. Note that changes in soil particle size or porosity implies changes
278 in EOF (Reddy and Cameselle, 2009).

279 Fig. 5 shows the microorganisms' concentration 2,4-D concentration (Fig. 5a) and 2,4-D
280 concentration microorganisms' concentration (Fig. 5b) profiles in soil at the start
281 (discontinuous line) and at the end (continuous line) of the EBR experiments at different
282 operation times evaluated of the treatment. As it was observed in section 3.1, complete
283 removal of 2,4-D from soil was achieved after 14d. Figure 5a shows that only 10 days is
284 time enough to remove almost completely the initial amount of 2,4-D in soil. Regarding
285 microorganisms' concentration, it can be observed that microorganisms kept alive during
286 all the process, and the concentration at the start of the treatment is similar to the final
287 one, which confirms that pH, moisture, conductivity and nutrients availability in soil have
288 been suitable for the microbial activity.

289 Figure 6 shows the 2,4-D removal efficiencies under the different operation times tested
290 in the present work. Additionally, each 2,4-D percentage removal value is compared with
291 the value obtained in the corresponding reference test, in which no electric field was
292 applied to the soil to be treated, and only biodegradation without the contribution of EK
293 phenomena was the responsible of pollutant removal. It is important to note that 2,4-D
294 was not detected in electrode wells, and no volatilization occurred, thus only
295 biodegradation (with or without the help of EK) is the responsible of pollutant removal
296 efficiencies in Fig.6. Moreover, metabolites were not detected by HPLC. A previous

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297 [research by the same authors showed that 2,4-D is readily biodegradable and oxidized as](#)
298 [the organic matter concentration \(measured as COD\) was nearly completely removed](#)
299 [\(Barba et al., 2019b\)](#). As it can be observed, almost 50% of 2,4-D was removed in only 3
300 days in electro-bioremediation experiments, and nearly 100% was removed in 10 days.
301 These removal results are very efficient in comparison with the results obtained in
302 bioremediation reference tests, in which only about 20% have been removed from soil in
303 10 days. It proves that EK enhances mobility and contact between the species involved
304 in the biological mechanisms. The electro-bioremediation results obtained in the present
305 work are very promising compared with previous studies when using diesel hydrocarbons
306 as model pollutant, where up to 30% removal was obtained after two weeks (Mena et al.
307 2016) or compared with the results by Barba et al. (2018) where approximately 40%
308 removal of oxyfluorfen was obtained after 11 weeks. Both previous studies were focused
309 on the removal of non-polar pollutants from clay soil by EBR, using acclimated cultures
310 to avoid limitations because of low biodegradability. Solubility, and thus mobility of
311 pollutants, is critical to the success of EBR. Additionally, adequate experimental
312 conditions for microbial activity (such as pH, temperature and nutrients availability) are
313 always necessary.

314 **Conclusions**

315 Removal of 2,4-D from polluted clay soil was successfully reached in 10 days. It is
316 assumed that solubility of the pollutant is a critical factor to ensure high removal
317 efficiencies. Polarity reversal contributed to the successful results by maintaining correct
318 pH values and reducing the removal of electrolytes from soil. By comparing the EBR
319 results with the reference tests (without the contribution of EK phenomena), it was proved
320 that the combination of bioremediation and electrokinetics has positive effects in the
321 remediation of low permeable polluted soil.

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322 **Acknowledgements**

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325 Competitiveness, and EQC2018-004240-P from Ministry of Science, Innovation and
326 Universities is gratefully acknowledged. The FPI grant BES-2014-069662 is also
327 acknowledged.

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Revision Notes: Response to Reviewers

This document shows detailed responses to the reviewer's comments. The responses indicate also the changes made in the revised manuscript. The changes are easily identifiable in the highlighted revised manuscript (revised manuscript changes marked document). The location of changes (page/line details in the responses) always refer to the highlighted revised manuscript MS Word file. Note that it is possible that the PDF generated by EES move lines.

Reviewers' comments:

Reviewer #1:

The aim of the manuscript is to present a study about the optimization of electro-bioremediation (EBR) treatment of a 2,4-dichlorophenoxyacetic acid (2,4-D) polluted clay soil. The paper is interesting, presenting promising results that should be further studied. It has clear objectives and the methods used, as well as the data obtained, seems relevant. The assumptions and analyses presented seem as a whole valid, as well as the extent to which the interpretations are supported by the data. The paper is well organized, concise, clearly written, using correct grammar and syntax. The title, graphical abstract and highlights are informative and a reflection of the content. Keywords are provided and appropriate. It can be considered for publication after minor revision.

In the following I present some questions and suggestions:

Lines 34-37: "Thus, crop productivity increased and, consequently, the World population. Despite the necessity of using pesticides to solve this kind of issues, these." This sentence is not clear for me. Please revise.

Authors consider that this sentence in introduction section is not relevant. It has been removed.

Lines 64-67: "Therefore, electrokinetic phenomena such as electrophoresis, electromigration and electroosmosis mobilize microorganisms, nutrients and pollutants towards the soil improving contact between them (Paillat et al., 2000; Rodrigo et al., 2014)." This sentence is not clear for me. Please revise.

The sentence has been revised (lines 64-67 revised manuscript).

Lines 155-157: “Experiments without electric current (only biological contribution) for each operation time were simultaneously performed under the same experimental conditions as reference tests to check the influence of electrokinetics.” Please explain the chemical medium added in this experiment. Have been also added the electrolytes? This is not completely clear.

The sentence has been revised. The conditions of reference tests were exactly the same than EBR experiments but without using electricity (lines 159 – 165 revised manuscript).

Lines 166-170: “The concentration of 2,4-D was measured 167 by HPLC (Jasco, Japan), using a column model Kinetex 5 μm Biphenyl 100 \AA , 150 x 4.5 mm (Phenomenex, USA), with a mobile phase of H_3PO_4 0.1%/acetonitrile, 60/40 %v/v, and an isocratic flow rate of 0.6 mL min^{-1} , and the wavelength of UV detector was 170 nm.” Please explain if any metabolites have detected and/or been analysed after treatment?

Metabolites were not detected by HPLC. A previous research by the same authors showed that 2,4-D is readily biodegradable and oxidized as the organic matter concentration (measured as COD) was nearly completely removed (Barba et al., 2019b).

This information has been included in revised manuscript (lines 296-299) and a new reference:

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. *J. Chem. Technol. Biotechnol.* 94, 2684–2692.

Lines 205-206 “Figure 2a shows the pH profile towards the soil after electro-bioremediation experiments for every PR frequency studied.” This sentence is not clear for me. Please revise.

The sentence has been revised (lines 213-215).

Line 283. "No volatilization occurred". Please include some additional explanation and some data to support this affirmation.

2,4-D volatilization was already known not to occur because of auxiliary measurements during the preparation of polluted soil. It was proved that 2,4-D volatilization in sterilized polluted soil after 1 week at the same temperature than EBR experiments was negligible. This information has been included in revised manuscript (lines 103-106).

Finally, I think it is necessary explain specifically in more detail the experimental conditions of the biodegradation without EK (reference test). The geometry of the system, is it exactly the same arrangement? Were added the same electrolyte compounds? Was heated the experimental system at 30°C? How was the heat transferred to the soil? In my opinion this is a part really essential to understand the conclusions obtained in this paper.

As previously indicated, the conditions of reference tests were exactly the same than EBR experiments but without using electricity, thus the geometry of the system, electrolyte addition, microbial inoculation, etc, was the same (This information has been included in revised manuscript, lines 159-165). Regarding temperature, no differences were observed between EBR experiments and reference tests because ohmic heating was negligible at bench scale (this information has been included in revised manuscript, lines 237-240).

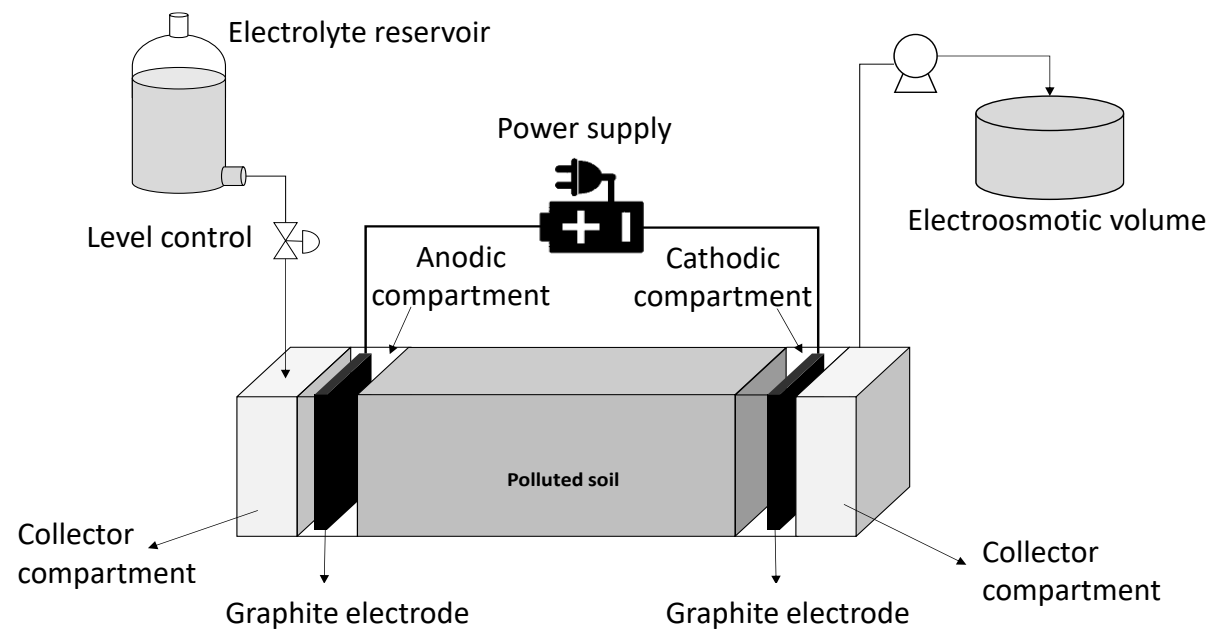
Reviewer #2:

The manuscript by Silvia Barba et. al., describes a research on electro-bioremediation of soil polluted with 2,4-dichlorophenoxyacetic acid, it was studied the influence of the frequency applied in polarity and the time of treatment. Experimental results indicate that combining electro-bioremediation, a significant abatement of 2,4-D contamination is achieved in short times. It may be interesting to underline that, an 2,4-D contamination of such low hydraulic permeability matrix could not be remediated by other methods. Soil removal could be the only practical solution of this environmental problem. From this point of view, the manuscript is certainly interesting. The manuscript is well organized, and the experiments have been well conceived and described. I think that it could be published with minor revisions, consisting of a better connection with text and figures, and of some language revision.

Once the manuscript has been revised according to reviewer 1 suggestions, the whole text has been checked for grammar and also for the correct connections between text, figures and figure captions. Note that a mistake regarding Figure 5 description has been corrected (revised manuscript, lines 279-282).

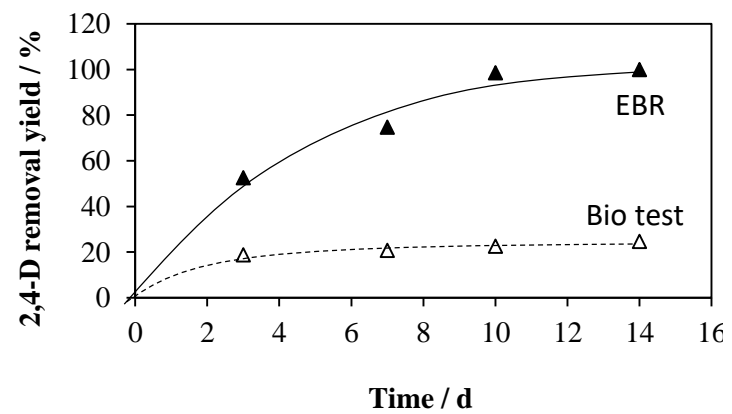
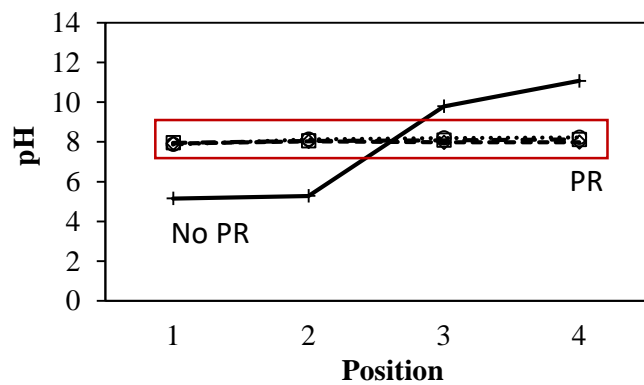
HIGHLIGHTS

- 2,4-D polluted clay soil was treated by electro-bioremediation technology
- Removal of 2,4-D from polluted clay soil was successfully reached in 10 days
- Polarity reversal maintained properly pH and reduced electrolytes removal from soil
- Electrobioremediation improved efficiency compared to conventional bioremediation



1. Polarity reversal strategy
($t = 14$ days)

2. Operation time
($f = 2 \text{ d}^{-1}$)



1 **TOWARDS THE OPTIMIZATION OF ELECTRO-BIOREMEDIATION OF**
2 **SOIL POLLUTED WITH 2,4-DICHLOROPHENOXYACETIC ACID.**

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10

11 **Abstract**

12 The aim of this work is to study the optimization of electro-bioremediation (EBR)
13 treatment of a 2,4-dichlorophenoxyacetic acid (2,4-D) polluted clay soil. The influence
14 of two different variables were evaluated trough batch experiments in a bench-scale
15 electrokinetic setup using previously acclimated microbial cultures for 2,4-D
16 biodegradation. First, it was studied the influence of the frequency applied in polarity
17 reversal (PR): frequencies under study were 1, 2 and 6 d⁻¹, i.e., polarity changed every
18 24, 12 and 4 hours respectively. The duration of experiments were 14 days and the electric
19 field applied was 1.0 V cm⁻¹ (20 V) at room temperature. The second variable under study
20 was the operation time, and based on the previous results, the selected frequency of PR
21 was 2 d⁻¹ and three additional EBR experiments were conducted using different operation
22 times (3, 7 and 10 days). Experiments without electric current (only biological
23 contribution) for each operation time were simultaneously performed under the same
24 experimental conditions as reference tests to check the influence of electrokinetics.
25 Removal of 2,4-D from polluted clay soil was completed in 10 days. It was observed that

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solubility of the pollutant is a critical factor to ensure high removal efficiencies. Moreover, polarity reversal contributed to the successful results by maintaining correct pH values and reducing the removal of electrolytes from soil. By comparing the EBR results with the reference tests (without the contribution of EK phenomena), it was proved that the combination of bioremediation and electrokinetics has positive effects in the remediation of low permeable polluted soil.

Keywords

2,4-dichlorophenoxyacetic acid, electro-bioremediation, polluted soil, herbicide, periodic polarity reversal.

1. Introduction

Since the last century, the use of pesticides has become more extensive, mostly in agricultural industry. These compounds have several disadvantages such as the high persistence in environment and thus the health problems that can cause in animals or humans being in contact with these substances (Rodrigo et al., 2014; Verma et al., 2014; Geed et al., 2017). 2,4-dichlorophenoxyacetic acid or commonly known as 2,4-D, is a systemic hormonal herbicide, it means that can affect to hormonal system in plants to avoid its growth. 2,4-D is framed within the group of organochlorinated pesticides, which are known for being very persistent in water, air and soils (Chowdhury et al., 2008). Due to the environmental problems associated to the use of pesticides, and because the soil is a non-renewable resource, it is necessary to remediate pesticide-polluted soils. For these reasons, national regulation in Spain is becoming harder regarding soil contamination, stablishing low pollutant limit concentrations in soil (Spanish Presidential Ministry, 2005). These limits, known as reference pollution levels, are different depending on the impact receptors (ecosystems, or human health). In the case of organochlorinated

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pollutants such as 2,4-D, the maximum allowed concentration in soil is 1.0 mg per kg of soil.

To remediate polluted soils, there are several strategies based on biological, physical, thermal or electrochemical technologies. One of the most applied techniques is conventional bioremediation because of its low cost (Juwarkar et al., 2010). However, in case of *in-situ* treatments (that is, soil is treated on its original location without the need for excavation and transport to external treatment facilities) bioremediation requires high operation times as the mass transfer phenomena necessary to contact pollutant, microorganisms and nutrients are very slow, especially in low permeable soils (Barba et al., 2018).

Regarding this limitation, in recent years, electrokinetic remediation (EK) has been increasing as a clear cost-effective alternative for *in-situ* soil remediation (Reddy and Cameselle, 2009). EK remediation consists of the application of a direct electric current across electrodes placed in the polluted soil. Electrokinetic phenomena (such as electrophoresis, electromigration and electroosmosis) mobilize and allow contact between microorganisms, nutrients and pollutants towards the soil (Paillat et al., 2000; Rodrigo et al., 2014). EK treatment is mainly recommended for low permeability polluted soils where conventional pump and treat methods do not allow moving groundwater neither the transport of contaminants along the soil (Reddy and Cameselle, 2009). Previous research (Yeung and Gu, 2011; Cameselle, 2014) achieved great removal results demonstrating that EK is a successful technology for remediating low permeable polluted soils. However, EK technology also presents some limitations during the operation time such as the soil heating by Joule effect, extreme pH zones near electrodes or the low mobility of non-polar pollutants through the soil.

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74 Because of the above-mentioned advantages and limitations of bioremediation and EK, a
75 recent combination of both technologies, known as electro-bioremediation, is becoming
76 more attractive in matter of *in-situ* decontamination of low permeable polluted soils.
77 Electro-bioremediation (EBR) couples the most interesting advantages of both
78 technologies (Yeung and Gu, 2011). EBR improves the contact between pollutants and
79 microorganisms, achieving the biodegradation of the pollutants *in situ* by the action of
80 microorganisms present in soil (Semple et al., 2007; Wick et al., 2007).
81 There are different options of combining bioremediation and electroremediation to
82 remove organic pollutants from soils (Gill et al., 2014). Previously, the authors of the
83 present work studied several alternatives of such combination, and they optimized
84 different parameters in the process in the case of soils polluted with oxyfluorfen (Barba
85 et al., 2017; Barba et al., 2019a). The present work is focused on the optimization of the
86 electro-bioremediation process of a 2,4-D polluted clay soil. The influences of two
87 different variables have been evaluated: (i) the frequency in the electrode polarity reversal
88 (PR) and (ii) the operation time of the process. Batch experiments were conducted at
89 bench-scale using an EK experimental cell at room temperature under 1 V cm^{-1} . An EK-
90 biostimulation strategy was used, that is the polluted soil already contains an acclimated
91 microbial culture adapted to 2,4-D biodegradation. To the author's knowledge, electro-
92 bioremediation studies for *in-situ* biodegradation of hazardous pesticides in polluted soils
93 are still scarce.

94 **2. Materials and methods**

95 **2.1. Materials**

96 The soil employed at this work is a clean clayey soil supported by Millas Hijos Ceramics
97 (Toledo, Spain). Its characteristics has been described in previous works (Barba et al.,
98 2017). Soil was artificially polluted with the pesticide (20 mg of 2,4-D per kg of wet soil)

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99 following the procedure explained in section 2.3. The chemical product 2,4-
100 dichlorophenoxyacetic acid (2,4-D) was selected as model of polar pesticide, supplied by
101 *Alfa Aesar* (98% assay). 2,4-D possible loss by volatilization was checked during the
102 preparation of polluted soil. It was proved that 2,4-D volatilization in sterilized polluted
103 soil after 1 week at the same temperature than EBR experiments was negligible.

104 Previously to the EBR experiments, it was carried out the acclimation process of the
105 microorganisms for the biodegradation of 2,4-D in order to get a strong microbial culture
106 following the procedure describe in previous works by Moliterni et al. (2012).

107 To start the acclimation process it was selected an inoculum from a biological reactor of
108 an oil-refinery wastewater treatment plant (Puertollano, Spain). Bushnell-Hash Broth
109 (BHB) was used as inorganic nutrients source for microorganisms. This culture media
110 contains 0.20 g L⁻¹ Mg SO₄, 0.02 g L⁻¹ CaCl₂, 1.00 g L⁻¹ KH₂PO₄, 1.00 g L⁻¹ (NH₄)₂HPO₄,
111 0.05 g L⁻¹ FeCl₃ and 1.00 g L⁻¹ KNO₃. 200 mg L⁻¹ of 2,4-D was employed as sole carbon
112 source. Once the acclimation process was achieved, the species of microorganisms
113 contained in the microbial culture were identify by means of MALDI TOF Mass
114 Spectrometry (AXIMA-Assurance Biotech technology, SHIMADZU, Germany). The
115 species identified in the 2,4-D-degrading microbial culture were *Rhodococcus ruber* and
116 *Ochrobactrum anthropic*.

117 **2.2 Experimental set-up**

118 Figure 1 shows the experimental set-up scheme employed in EBR experiments. The
119 installation consists of a bench scale EK cell made of transparent methacrylate, which is
120 divided into five compartments. In the central one is placed the 2,4-D polluted soil. The
121 electrodic compartments are located at both sides separated from soil by a nylon mesh
122 (0.5 mm mesh size), and they contained the electrodes. The electrodes used are made of
123 graphite with dimensions of 10x10x1 cm, supplied by Carbosystem (Madrid, Spain),

124 which are connected to a power supply (HQ Power, Gavere, Belgium). The electrolyte
125 used is a synthetic inorganic medium with the following composition: 80.75 mg L⁻¹ of
126 Na₂SO₄, 70.00 mg L⁻¹ of NaHCO₃, 30.36 mg L⁻¹ of NaNO₃, which tries to simulate
127 groundwater. Because of microorganisms can consume the nutrients or due to their
128 removal by electromigration and electroosmosis, excess inorganic nutrients (nitrate,
129 ammonium and phosphate) were provided to avoid nutrient limitations that can occur
130 during the EBR process. To collect the electroosmotic flow (EOF) moved during the
131 process, there are two collector compartments contiguous to electrode ones. It is
132 important to remark that the EOF will be collected at both sides because of the polarity
133 reversal applied in all EBR experiments of this work (Mena et al., 2016).

134 **2.3. Experimental procedure**

135 The biological strategy coupled to EK employed in this work was biostimulation, which
136 consists of adding nutrients to a polluted soil already containing acclimated
137 microorganisms for pollutant biodegradation. Nutrients addition and EK application are
138 expected to stimulate biological metabolism and, thus, improve the biodegradation of
139 pollutants. Thus, the previous procedure to the electro-bioremediation experiments was
140 as follows: an inoculum of 2,4-D-degrading microbial culture was grown in a batch
141 reactor using BHB culture media as inorganic nutrients source during 4 days; the culture
142 was subsequently centrifuged and suspended in new BHB media, and added to a 2,4-D
143 solution.; then, the suspension of microorganisms and 2,4-D solution was homogeneously
144 distributed into the clayey soil, obtaining a final moisture of soil around 25%, and a
145 concentration of 2,4-D of 20 mg per kg of wet soil (26.7 mg per kg of dry soil).

146 The inoculated and polluted soil was placed and compacted manually in the central
147 compartment of the experimental set-up, and the electrodic compartments were filled with

148 electrolyte solution. Direct current was connected, and electro-bioremediation batch
149 experiments were conducted under 1.0 V cm^{-1} (20 V) at room temperature.

150 The experimental planning to study the influence of variables was as follows:

151 1. Effect of electrode polarity reversal frequency: In this case, three batch electro-
152 bioremediation experiments (14 d duration each) were carried out. The frequencies under
153 study were 1, 2 and 6 d^{-1} , i.e., polarity changed every 24, 12 and 4 hours respectively.

154 2. Effect of operation time: Based on the previous results (i), the selected frequency of
155 PR was 2 d^{-1} and three additional EBR experiments were conducted using different
156 operation times (3, 7 and 10 days). Simultaneously to each EBR experiment using
157 different operation times, a reference test without electric current (only biological
158 contribution) was always performed. Reference tests were done under identical
159 experimental conditions than EBR experiments (that is, the same installation, electrolyte
160 addition, microbial inoculation), but without using electric current, and they would help
161 to check the influence of electrokinetics.

162 **2.4. Sampling and analyses**

163 During the experiments, liquid samples were taken from electrodic wells, from the EOF
164 collector compartment and from the liquid medium in soil. Due to the electrode polarity
165 changes during the process, it is important to remark that EOF is collected alternatively
166 in both collector compartments depending on the cathode position. Moreover, the
167 temperature of soil and current electricity were monitored throughout all the duration of
168 the experiments. Conductivity and pH were measured by using a multiparameter probe
169 (SENSLON, HACH). Inorganic nutrient concentrations were analysed by means of UV-
170 Vis photometer (Gallery, Thermo Scientific). The concentration of 2,4-D was measured
171 by HPLC (Jasco, Japan), using a column model Kinetex $5 \mu\text{m}$ Biphenyl 100 Å, 150×4.5
172 mm (Phenomenex, USA), with a mobile phase of H_3PO_4 0.1%/acetonitrile, 60/40 % v/v,

173 and an isocratic flow rate of 0.6 mL min^{-1} , and the wavelength of UV detector was 220
174 nm. The injection volume was $20 \text{ }\mu\text{L}$.

175 Soil samples were taken only at the beginning before placing the soil on the set-up, and
176 at the end of the experiments (*post-mortem* analysis, once the experiment was finished).

177 This procedure was followed not to modify the compaction of soil avoiding preferential
178 paths for EOF (Ruiz et al., 2014). For carrying out the *post-mortem* analysis, it is
179 necessary to divide the soil properly after the experiment. Then, the soil was divided into
180 sections as follows: four longitudinal positions (positions 1 to 4), where position 1
181 corresponds to nearest zone to anodic well, and position 4 to nearest to cathodic well (at
182 $t = 0$). Each longitudinal section was in turn divided into four parts: two upper parts (left
183 and right) and two bottom parts (left and right), according to previous works (Ramírez et
184 al., 2015).

185 The following parameters were measured in soil: moisture, pH, conductivity,
186 microorganisms, inorganic nutrients and 2,4-D concentrations. Moisture was calculated
187 by weights difference from wet soil sample and dry one: an amount of wet soil was dried
188 at 105°C for 24 hours and the weight of evaporated water was related to the moisture of
189 the soil. Soil pH and conductivity were measured from the soil dried. Thus, once it was
190 dried as it has been described above, it was taken 10 g of dry soil, and 25 mL of Milli-Q
191 water were added. The mixture soil-water was agitated during 30 min by magnetic stirring
192 and then, it was left decant a couple of hours at least. The liquid supernatant was filtered
193 with nylon filters of $0.2 \text{ }\mu\text{m}$ and measured by using the multiparameter probe. Inorganic
194 nutrients concentrations were measured as well from dry soil with a photometer. On the
195 other hand, 2,4-D concentration was measured from wet soil employing the same HPLC
196 method above described.

197 The concentration of microorganisms expressed as Colony Forming Units (CFU) per
198 gram of dry soil was done following the procedure described in previous works (Ramírez
199 et al., 2015), i.e., it was taken 1 g of wet soil and it was added 10 mL of saline solution
200 (0.9% NaCl). Then, it was agitated during 3 min by using a vortex agitator. Once it was
201 vigorous mixed, an aliquot of 100 μ L of supernatant was taken and placed on Petri dishes,
202 which contained Luria Bertani (LB) solid culture medium for microbial growth. The
203 composition of LB medium for 1 L of Milli-Q water is 10.0 g of NaCl, 5.0 g of yeast
204 extract and 10.0 g of casein peptone, 15 g of European Bacteriological Agar and 10.0 g
205 of glucose acting as carbon source. Finally, Petri dishes were incubated for 24h at 26.5
206 $^{\circ}$ C and colonies grown were counted.

207 **3. Results and discussion**

208 **3.1. Selection of polarity reversal frequency**

209 Figure 2a shows the pH values at different soil positions after electro-bioremediation
210 experiments using different PR frequencies. Left part of the Figure 2a corresponds to the
211 anode position and right part corresponds to cathode position (at $t=0$). Additionally, as
212 reference test using no PR ($f=0$), the figure also includes the results previously reported
213 by our research group (Vieira dos Santos et al., 2016) who studied 2,4-D behaviour under
214 abiotic electrokinetics using exactly the same soil and experimental conditions. As it can
215 be observed in Figure 2a, the pH next to anodic and cathodic wells in the case of not
216 applying polarity reversal ($f=0$) is acid and basic, respectively, while pH was maintained
217 neutral in soil when EBR experiments were finished. Similar behaviour was also reported
218 by the same authors in previous works when using non-polar pollutants (Barba et al.,
219 2017). However, it was not observed a clear influence of the f value on the studied range
220 (between 1 and 6 d^{-1}).

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221 Figure 2b shows the profiles of electrical conductivity towards the soil at the end of
222 experiments. It can be observed that after the three experiments, carried out at different
223 f values, electrical conductivity profiles are similar, and again a significant influence of f
224 value was not observed. On the contrary, in the case of the reference test ($f=0$) a decrease
225 in the soil conductivity can be observed. This result can be related to the faster removal
226 of ions from the system when no PR is applied. Moreover, it has been previously reported
227 that EOF decreases and relatively high current density value remains when PR is used
228 (Mena et al., 2016; Barba et al., 2017). The application of PR implies that the ions retained
229 in the soil can remain longer because both electromigration and EOF move them
230 alternatively in both directions. Consequently, it allows not only pH control but also
231 maintaining adequate values of inorganic nutrients concentrations, current density and
232 electrical conductivity in soil during the remediation process. Soil temperature (results
233 not shown) kept practically constant during all the experiments carried out (both EBR
234 experiments and reference tests without electricity), around 30 °C, that is adequate
235 temperature for microbial activity. It was noted again that ohmic heating was negligible
236 at such low voltages at bench scale (Barba et al., 2019a). Again, changes in f values in
237 the range between 1 and 6 d⁻¹ did not affect soil temperature. Figure 3a shows the
238 concentration of microorganisms in soil at the beginning (discontinuous line) and at the
239 end (continuous line) of experiments under different f values. As it can be observed, the
240 microorganisms' concentration kept practically constant in all experiments, that is, a
241 homogeneous profile was observed towards the soil and no microbial decay was
242 observed. pH control is critical for such result. Mena et al. (2015), reported that in the
243 case of not applying periodic polarity reversal, the concentration of active
244 microorganisms in soil at the end of the process is null caused by the negative effect of
245 extreme pH in soil. As it occurs with the other parameters above described, it was not

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246 observed difference in microorganisms' concentration depending on the different
247 frequencies studied. Figure 3b shows the results of 2,4-D removal from soil for each
248 experiment conducted. As it can be observed, in only two weeks of treatment it was
249 achieved the completely removal of the pollutant in soil in the three experiments at
250 different f values. This fact shows that the removal of 2,4-D is easier than the removal of
251 non-polar compounds, probably because of its polar nature and low sorption in soil, which
252 implies higher mobility through all over the soil and better contact with microorganisms
253 (Barba et al., 2019a). Nevertheless, results shown in Figures 2 and 3 do not allow us to
254 select an optimum value of polarity reversal frequency. For this reason, and because of
255 the frequency value has no economical cost implications in the electro-bioremediation
256 process, it was selected a frequency of 2 d^{-1} as in the previous works carried out by the
257 same authors when using non-polar pollutants (Barba et al., 2017).

258 **3.2. Effect of the operation time**

259 Results in section 3.1 indicate that 14 d duration treatment is not necessary and operation
260 time can be reduced in order to optimize the process. Thus, lower operation times (3, 7
261 and 10 d) were tested. Figure 4 shows the current intensity (Fig. 4a) and EOF (Fig. 4b)
262 through the soil during the experiments using lower operation times. As it can be observed
263 in Fig. 4a, for operation times lower than 7 days current intensity keeps practically
264 constant during the experiment (around 150 mA), while higher operation times cause a
265 decrease of intensity from 150 to 100 mA. This behaviour can be associated to the
266 removal of ions from soil by electromigration and electroosmosis, or because of the
267 electrodes wear down (Reddy and Cameselle, 2012).

268 On the other hand, in Fig. 4b it can be observed that EOF shows similar trend in the three
269 electro-bioremediation experiments. It was observed that the EOF increases during the
270 first hours of treatment, and then, it stabilizes around a constant value for the rest of the

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271 experiment. In three cases, the stationary EOF is around 5-8 mL h⁻¹, and the low
272 differences between EOF in the experiments may be due to differences in manual soil
273 compaction in each one. Note that changes in soil particle size or porosity implies changes
274 in EOF (Reddy and Cameselle, 2009).

275 Fig. 5 shows the microorganisms' concentration (Fig. 5a) and 2,4-D concentration (Fig.
276 5b) profiles in soil at the start (discontinuous line) and at the end (continuous line) of the
277 EBR experiments at different operation times evaluated. As it was observed in section
278 3.1, complete removal of 2,4-D from soil was achieved after 14d. Figure 5a shows that
279 only 10 days is time enough to remove almost completely the initial amount of 2,4-D in
280 soil. Regarding microorganisms' concentration, it can be observed that microorganisms
281 kept alive during all the process, and the concentration at the start of the treatment is
282 similar to the final one, which confirms that pH, moisture, conductivity and nutrients
283 availability in soil have been suitable for the microbial activity.

284 Figure 6 shows the 2,4-D removal efficiencies under the different operation times tested
285 in the present work. Additionally, each 2,4-D percentage removal value is compared with
286 the value obtained in the corresponding reference test, in which no electric field was
287 applied to the soil to be treated, and only biodegradation without the contribution of EK
288 phenomena was the responsible of pollutant removal. It is important to note that 2,4-D
289 was not detected in electrode wells, and no volatilization occurred, thus only
290 biodegradation (with or without the help of EK) is the responsible of pollutant removal
291 efficiencies in Fig.6. Moreover, metabolites were not detected by HPLC. A previous
292 research by the same authors showed that 2,4-D is readily biodegradable and oxidized as
293 the organic matter concentration (measured as COD) was nearly completely removed
294 (Barba et al., 2019b). As it can be observed, almost 50% of 2,4-D was removed in only 3
295 days in electro-bioremediation experiments, and nearly 100% was removed in 10 days.

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296 These removal results are very efficient in comparison with the results obtained in
297 bioremediation reference tests, in which only about 20% have been removed from soil in
298 10 days. It proves that EK enhances mobility and contact between the species involved
299 in the biological mechanisms. The electro-bioremediation results obtained in the present
300 work are very promising compared with previous studies when using diesel hydrocarbons
301 as model pollutant, where up to 30% removal was obtained after two weeks (Mena et al.
302 2016) or compared with the results by Barba et al. (2018) where approximately 40%
303 removal of oxyfluorfen was obtained after 11 weeks. Both previous studies were focused
304 on the removal of non-polar pollutants from clay soil by EBR, using acclimated cultures
305 to avoid limitations because of low biodegradability. Solubility, and thus mobility of
306 pollutants, is critical to the success of EBR. Additionally, adequate experimental
307 conditions for microbial activity (such as pH, temperature and nutrients availability) are
308 always necessary.

31 32 **Conclusions**

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310 Removal of 2,4-D from polluted clay soil was successfully reached in 10 days. It is
311 assumed that solubility of the pollutant is a critical factor to ensure high removal
312 efficiencies. Polarity reversal contributed to the successful results by maintaining correct
313 pH values and reducing the removal of electrolytes from soil. By comparing the EBR
314 results with the reference tests (without the contribution of EK phenomena), it was proved
315 that the combination of bioremediation and electrokinetics has positive effects in the
316 remediation of low permeable polluted soil.

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Figure 1

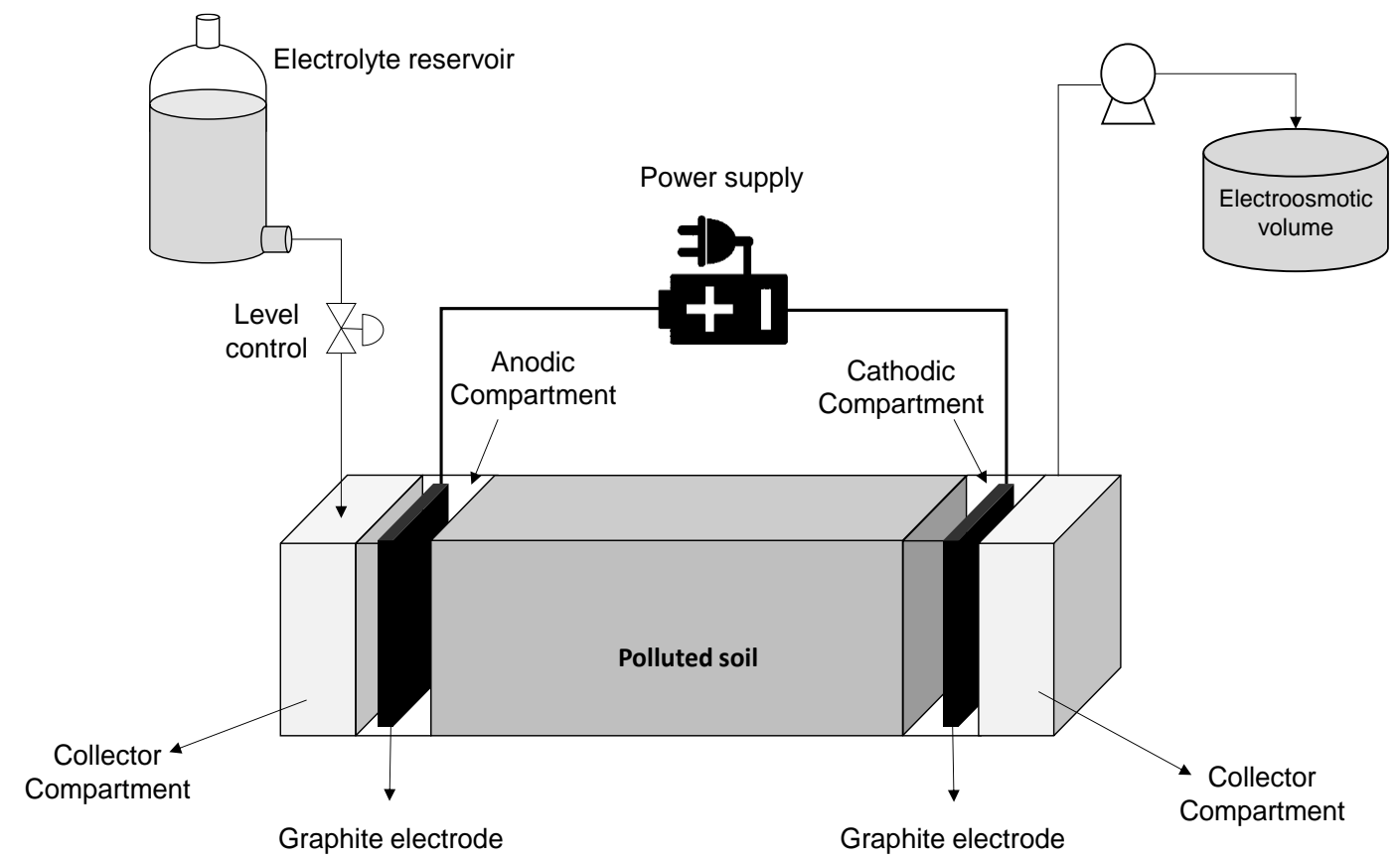


Figure 1.

Figure 2

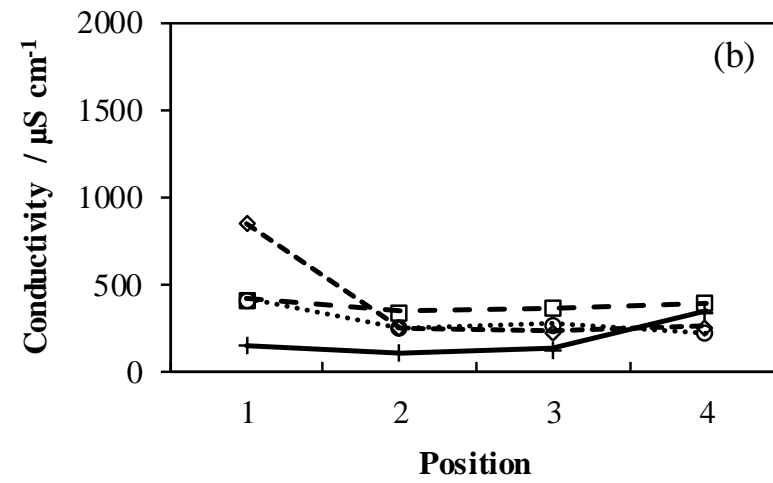
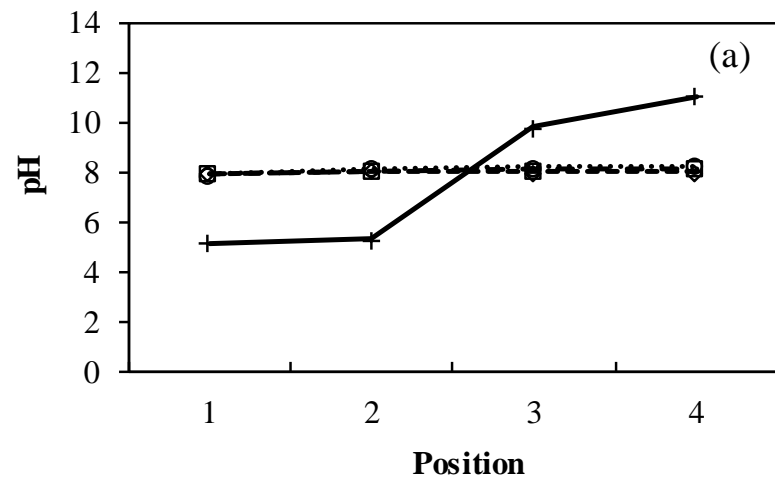


Figure 2.

Figure 3

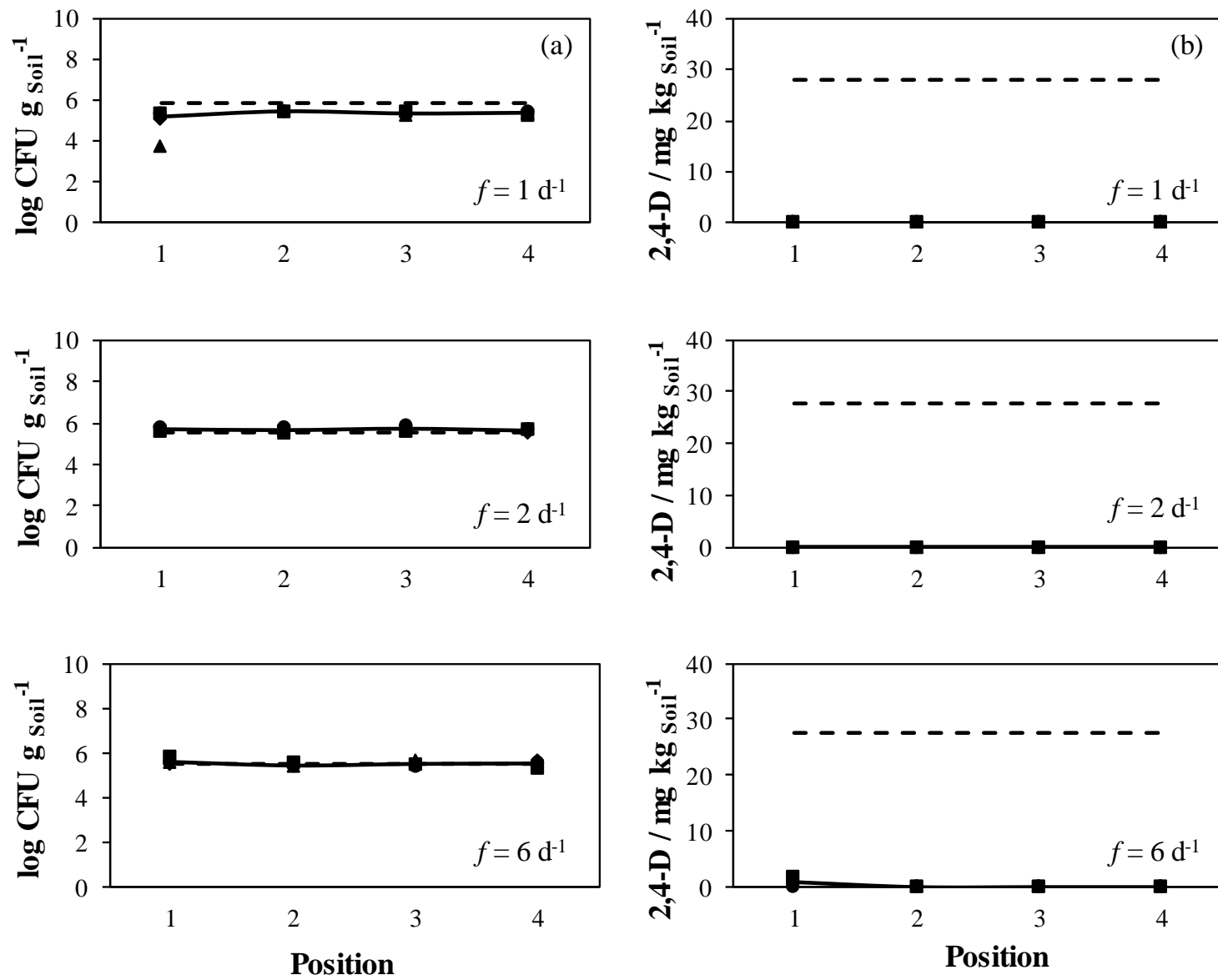


Figure 3.

Figure 4

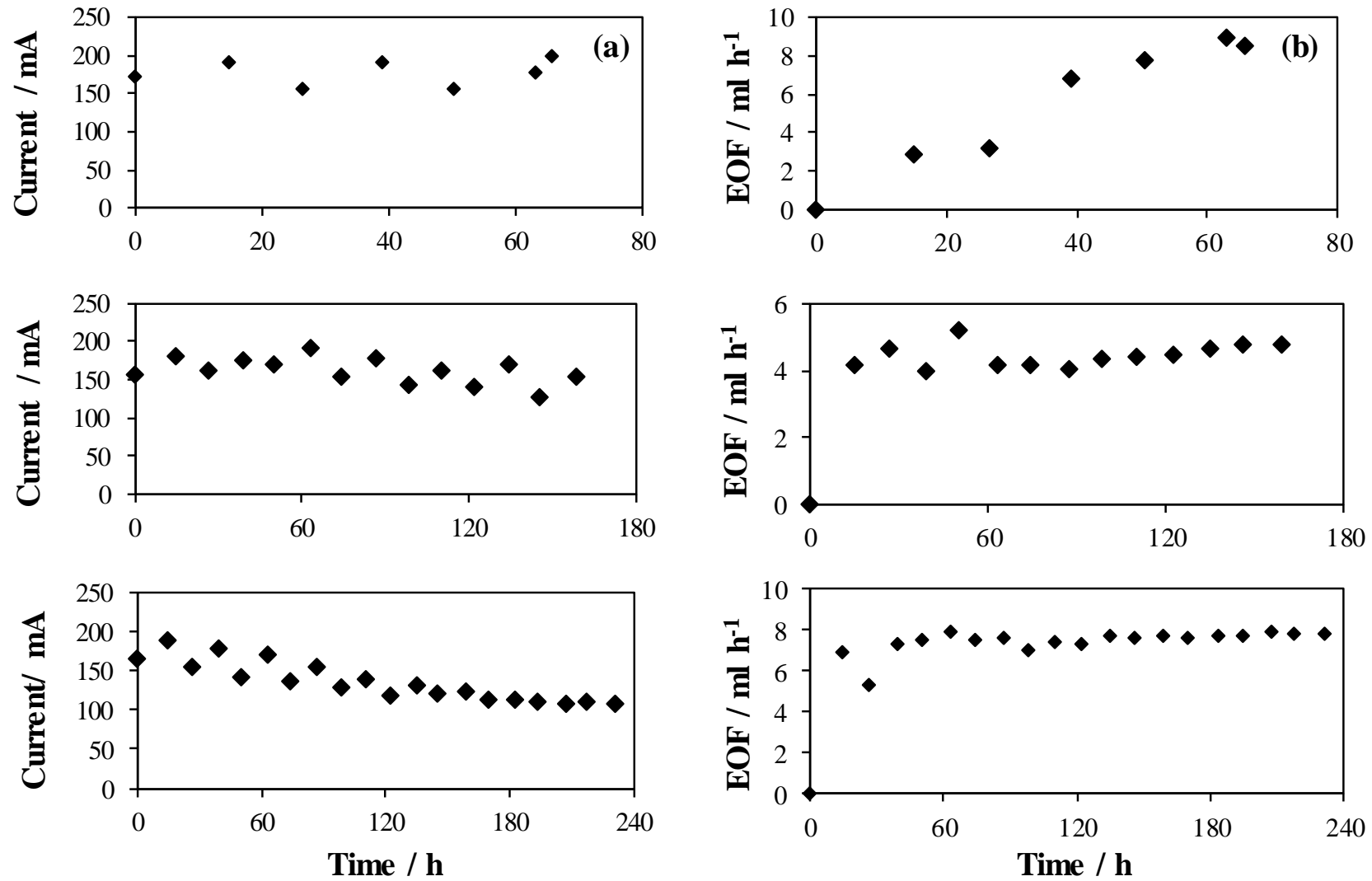


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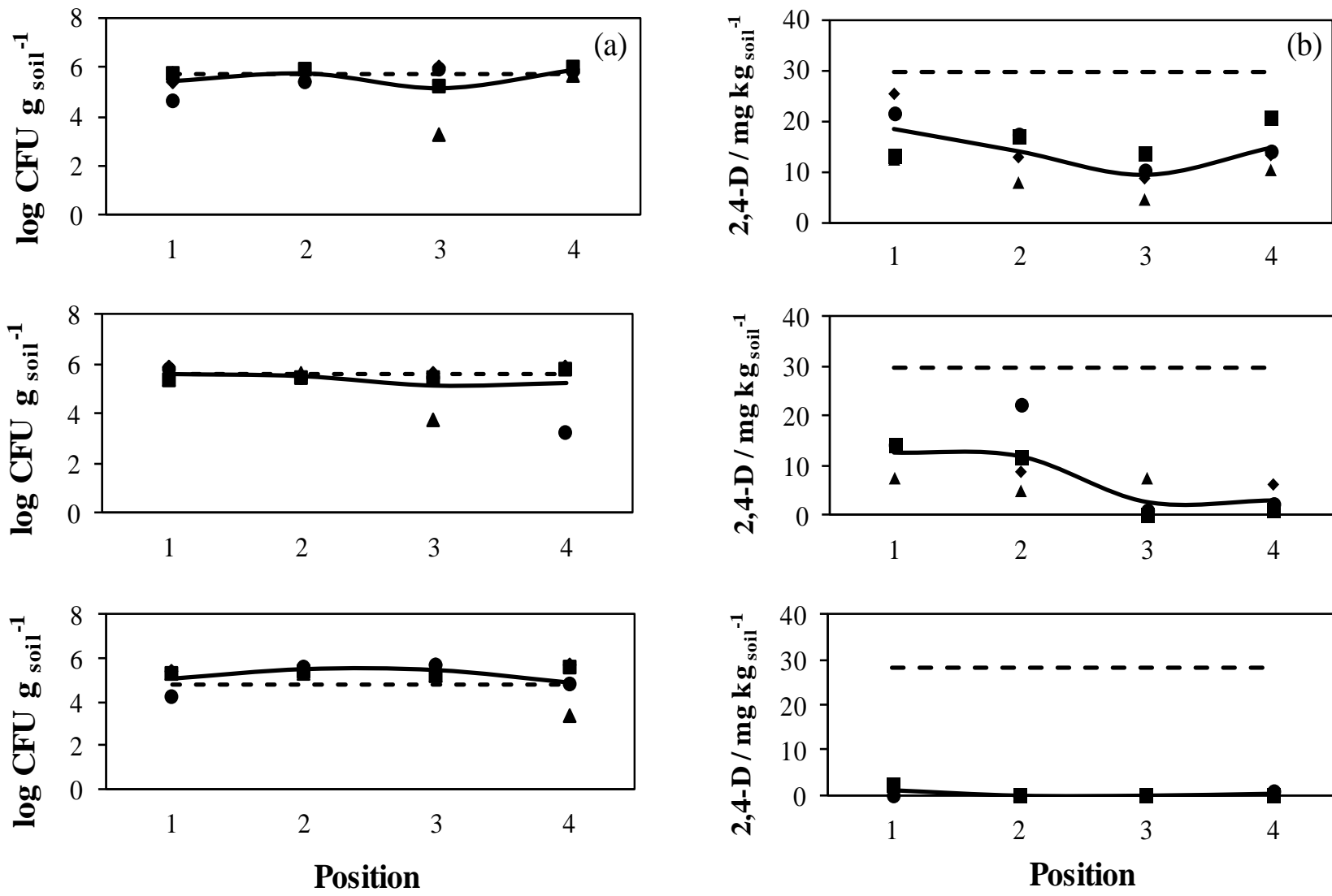


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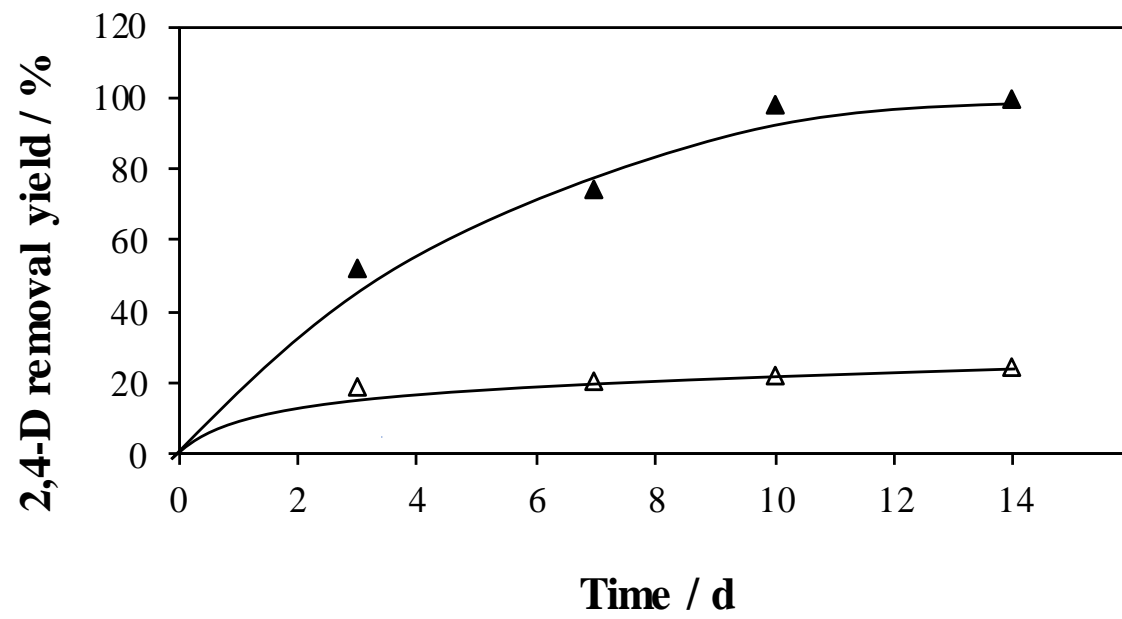


Figure 6

Figure 1. Electro-bioremediation bench scale set-up.

Figure 2. Influence of polarity reversal in (a) soil pH and (b) soil conductivity after EBR experiments. Polarity reversal frequency values: (○) 1 d⁻¹, (◇) 2 d⁻¹, (□) 6 d⁻¹, (+) no PR.

Figure 3. (a) Microorganisms concentration and (b) 2,4-D concentration profiles in soil at the start (- - -) and at the end (—) of the EBR experiments at different f evaluated. Lines are the average of the four values in the different axial positions (top right (◇), top left (●), bottom right (■) and bottom left (▲)).

Figure 4. (a) Current intensity and (b) EOF profiles in EBR experiments at different operation times evaluated.

Figure 5. (a) Microorganisms concentration and (b) 2,4-D concentration profiles in soil at the start (- - -) and at the end (—) of the EBR experiments at different operation times evaluated. Lines are the average of the four values in the different axial positions (top right (◇), top left (●), bottom right (■) and bottom left (▲)).

Figure 6. 2,4-D removal yield at different operation times. (▲) Electro-bioremediation tests and (Δ) conventional bioremediation tests (reference tests).

Declaration of interests

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:

TOWARDS THE OPTIMIZATION OF ELECTRO-BIOREMEDIATION OF SOIL POLLUTED WITH 2,4-DICHLOROPHENOXYACETIC ACID.

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.