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

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Abstract:	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.
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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*.

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3. The "**Revised manuscript**"

Yours sincerely

Dr. J. Villaseñor

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

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Abstract

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.

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. ~~Thus, crop productivity increased and, consequently, the World population. Despite the necessity of using pesticides to solve this kind of issues, t~~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

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(ecosystems, or human health). In the case of organochlorinated 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. ~~Therefore, e~~Electrokinetic phenomena (such as electrophoresis, electromigration and electroosmosis) mobilize and allow contact between microorganisms, nutrients and pollutants towards the soil ~~improving contact between them~~ (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.

Because of the above-mentioned advantages and limitations of bioremediation and EK, a recent combination of both technologies, known as electro-bioremediation, is becoming more attractive in matter of *in-situ* decontamination of low permeable polluted soils. Electro-bioremediation (EBR) couples the most interesting advantages of both technologies (Yeung and Gu, 2011). EBR improves the contact between pollutants and microorganisms, achieving the biodegradation of the pollutants *in situ* by the action of microorganisms present in soil (Semple et al., 2007; Wick et al., 2007).

There are different options of combining bioremediation and electroremediation to remove organic pollutants from soils (Gill et al., 2014). Previously, the authors of the present work studied several alternatives of such combination, and they optimized different parameters in the process in the case of soils polluted with oxyfluorfen (Barba et al., 2017; Barba et al., 2019a). The present work is focused on the optimization of the electro-bioremediation process of a 2,4-D polluted clay soil. The influences of two different variables have been evaluated: (i) the frequency in the electrode polarity reversal (PR) and (ii) the operation time of the process. Batch experiments were conducted at bench-scale using an EK experimental cell at room temperature under 1 V cm^{-1}. An EK-biostimulation strategy was used, that is the polluted soil already contains an acclimated microbial culture adapted to 2,4-D biodegradation. To the author's knowledge, electro-bioremediation studies for *in-situ* biodegradation of hazardous pesticides in polluted soils are still scarce.

2. Materials and methods

2.1. Materials

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

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2017). Soil was artificially polluted with the pesticide (20 mg of 2,4-D per kg of wet soil) following the procedure explained in section 2.3.

The chemical product 2,4-dichlorophenoxyacetic acid (2,4-D) was selected as model of polar pesticide, supplied by *Alfa Aesar* (98% assay). 2,4-D possible loss by volatilization was checked 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.

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

To start the acclimation process it was selected an inoculum from a biological reactor of an oil-refinery wastewater treatment plant (Puertollano, Spain). Bushnell-Hash Broth (BHB) was used as inorganic nutrients source for microorganisms. This culture media contains 0.20 g L^{-1} Mg SO_4, 0.02 g L^{-1} $CaCl_2$, 1.00 g L^{-1} KH_2PO_4, 1.00 g L^{-1} $(NH_4)_2HPO_4$, 0.05 g L^{-1} $FeCl_3$ and 1.00 g L^{-1} KNO_3. 200 mg L^{-1} of 2,4-D was employed as sole carbon source. Once the acclimation process was achieved, the species of microorganisms contained in the microbial culture were identify by means of MALDI TOF Mass Spectrometry (AXIMA-Assurance Biotech technology, SHIMADZU, Germany). The species identified in the 2,4-D-degrading microbial culture were *Rhodococcus ruber* and *Ochrobactrum anthropic*.

2.2 Experimental set-up

Figure 1 shows the experimental set-up scheme employed in EBR experiments. The installation consists of a bench scale EK cell made of transparent methacrylate, which is divided into five compartments. In the central one is placed the 2,4-D polluted soil. The electrodic compartments are located at both sides separated from soil by a nylon mesh

(0.5 mm mesh size), and they contained the electrodes. The electrodes used are made of graphite with dimensions of 10x10x1 cm, supplied by Carbosystem (Madrid, Spain), which are connected to a power supply (HQ Power, Gavere, Belgium). The electrolyte used is a synthetic inorganic medium with the following composition: 80.75 mg L^{-1} of Na_2SO_4, 70.00 mg L^{-1} of $NaHCO_3$, 30.36 mg L^{-1} of $NaNO_3$, which tries to simulate groundwater. Because of microorganisms can consume the nutrients or due to their removal by electromigration and electroosmosis, excess inorganic nutrients (nitrate, ammonium and phosphate) were provided to avoid nutrient limitations that can occur during the EBR process. To collect the electroosmotic flow (EOF) moved during the process, there are two collector compartments contiguous to electrode ones. It is important to remark that the EOF will be collected at both sides because of the polarity reversal applied in all EBR experiments of this work (Mena et al., 2016).

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2.3. Experimental procedure

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

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

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

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

2. Effect of operation time: Based on the previous results (i), 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). Simultaneously to each EBR experiment using different operation times, a reference test ~~Experiments~~ without electric current (only biological contribution) ~~for each operation time were simultaneously~~was always performed. Reference tests were done under ~~the same~~identical experimental conditions than EBR experiments (that is, the same installation, electrolyte addition, microbial inoculation), but without using electric current, and they would help to ~~as reference tests to~~ check the influence of electrokinetics.

2.4. Sampling and analyses

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

Vis photometer (Gallery, Thermo Scientific). The concentration of 2,4-D was measured by HPLC (Jasco, Japan), using a column model Kinetex 5 µm Biphenyl 100 Å, 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 220 nm. The injection volume was 20 µL.

Soil samples were taken only at the beginning before placing the soil on the set-up, and at the end of the experiments (*post-mortem* analysis, once the experiment was finished). This procedure was followed not to modify the compaction of soil avoiding preferential paths for EOF (Ruiz et al., 2014). For carrying out the *post-mortem* analysis, it is necessary to divide the soil properly after the experiment. Then, the soil was divided into sections as follows: four longitudinal positions (positions 1 to 4), where position 1 corresponds to nearest zone to anodic well, and position 4 to nearest to cathodic well (at t = 0). Each longitudinal section was in turn divided into four parts: two upper parts (left and right) and two bottom parts (left and right), according to previous works (Ramírez et al., 2015).

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

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

3. Results and discussion

3.1. Selection of polarity reversal frequency

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

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previous works when using non-polar pollutants (Barba et al., 2017). However, it was not observed a clear influence of the *f* value on the studied range (between 1 and 6 d^{-1}).

Figure 2b shows the profiles of electrical conductivity towards the soil at the end of experiments. It can be observed that after the three experiments, carried out at different *f* values, electrical conductivity profiles are similar, and again a significant influence of *f* value was not observed. On the contrary, in the case of the reference test (*f* =0) a decrease in the soil conductivity can be observed. This result can be related to the faster removal of ions from the system when no PR is applied. Moreover, it has been previously reported that EOF decreases and relatively high current density value remains when PR is used (Mena et al., 2016; Barba et al., 2017). The application of PR implies that the ions retained in the soil can remain longer because both electromigration and EOF move them alternatively in both directions. Consequently, it allows not only pH control but also maintaining adequate values of inorganic nutrients concentrations, current density and electrical conductivity in soil during the remediation process. Soil temperature (results not shown) kept practically constant during all the experiments carried out (both EBR experiments and reference tests without electricity), around 30 ºC, that is adequate temperature for microbial activity. It was noted again that ohmic heating was negligible at such low voltages at bench scale (Barba et al., 2019a). Again, changes in *f* values in the range between 1 and 6 d^{-1} did not affect soil temperature. Figure 3a shows the concentration of microorganisms in soil at the beginning (discontinuous line) and at the end (continuous line) of experiments under different *f* values. As it can be observed, the microorganisms' concentration kept practically constant in all experiments, that is, a homogeneous profile was observed towards the soil and no microbial decay was observed. pH control is critical for such result. Mena et al. (2015), reported that in the case of not applying periodic polarity reversal, the concentration of active microorganisms in soil at the end of the process is null caused by the negative effect of extreme pH in soil. As it occurs with the other parameters above described, it was not observed difference in microorganisms' concentration depending on the different frequencies studied. Figure 3b shows the results of 2,4-D removal from soil for each experiment conducted. As it can be observed, in only two weeks of treatment it was achieved the completely removal of the pollutant in soil in the three experiments at different *f* values. This fact shows that the removal of 2,4-D is easier than the removal of non-polar compounds, probably because of its polar nature and low sorption in soil, which implies higher mobility through all over the soil and better contact with microorganisms (Barba et al., 2019a). Nevertheless, results shown in Figures 2 and 3 do not allow us to select an optimum value of polarity reversal frequency. For this reason, and because of the frequency value has no economical cost implications in the electro-bioremediation process, it was selected a frequency of 2 d^{-1} as in the previous works carried out by the same authors when using non-polar pollutants (Barba et al., 2017).

3.2. Effect of the operation time

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

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

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

Figure 6 shows the 2,4-D removal efficiencies under the different operation times tested in the present work. Additionally, each 2,4-D percentage removal value is compared with the value obtained in the corresponding reference test, in which no electric field was applied to the soil to be treated, and only biodegradation without the contribution of EK phenomena was the responsible of pollutant removal. It is important to note that 2,4-D was not detected in electrode wells, and no volatilization occurred, thus only biodegradation (with or without the help of EK) is the responsible of pollutant removal efficiencies in Fig.6. Moreover, 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). As it can be observed, almost 50% of 2,4-D was removed in only 3 days in electro-bioremediation experiments, and nearly 100% was removed in 10 days. These removal results are very efficient in comparison with the results obtained in bioremediation reference tests, in which only about 20% have been removed from soil in 10 days. It proves that EK enhances mobility and contact between the species involved in the biological mechanisms. The electro-bioremediation results obtained in the present work are very promising compared with previous studies when using diesel hydrocarbons as model pollutant, where up to 30% removal was obtained after two weeks (Mena et al. 2016) or compared with the results by Barba et al. (2018) where approximately 40% removal of oxyfluorfen was obtained after 11 weeks. Both previous studies were focused on the removal of non-polar pollutants from clay soil by EBR, using acclimated cultures to avoid limitations because of low biodegradability. Solubility, and thus mobility of pollutants, is critical to the success of EBR. Additionally, adequate experimental conditions for microbial activity (such as pH, temperature and nutrients availability) are always necessary.

Conclusions

Removal of 2,4-D from polluted clay soil was successfully reached in 10 days. It is assumed that solubility of the pollutant is a critical factor to ensure high removal efficiencies. 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.

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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 Å, 150 x 4.5 mm (Phenomenex, USA), with a mobile phase of H3PO4 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 30ºC? 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:

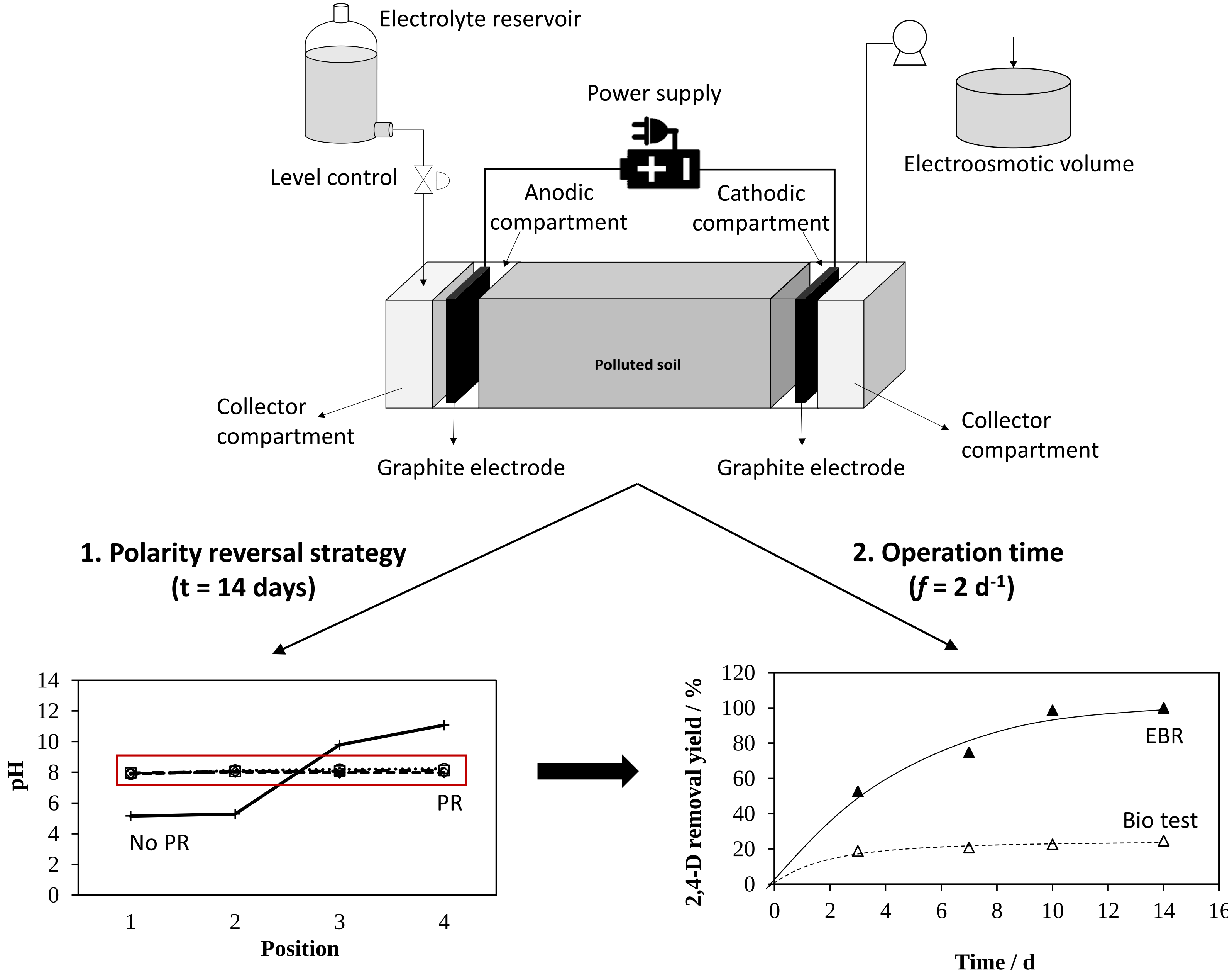
The manuscript by Silvia Barba et. al., describes a research on electro-bioremediation of soil polluted with 2,4-dichlorophenoxyacetic acid, it was studied the influence of the frequency applied in polarity and the time of treatment. Experimental results indicate that combining electro-bioremediation, 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
- Electrobioremediaton improved efficiency compared to conventional bioremediation

Graphical Abstract



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

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Abstract

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.

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 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.

Because of the above-mentioned advantages and limitations of bioremediation and EK, a recent combination of both technologies, known as electro-bioremediation, is becoming more attractive in matter of *in-situ* decontamination of low permeable polluted soils. Electro-bioremediation (EBR) couples the most interesting advantages of both technologies (Yeung and Gu, 2011). EBR improves the contact between pollutants and microorganisms, achieving the biodegradation of the pollutants *in situ* by the action of microorganisms present in soil (Semple et al., 2007; Wick et al., 2007).

There are different options of combining bioremediation and electroremediation to remove organic pollutants from soils (Gill et al., 2014). Previously, the authors of the present work studied several alternatives of such combination, and they optimized different parameters in the process in the case of soils polluted with oxyfluorfen (Barba et al., 2017; Barba et al., 2019a). The present work is focused on the optimization of the electro-bioremediation process of a 2,4-D polluted clay soil. The influences of two different variables have been evaluated: (i) the frequency in the electrode polarity reversal (PR) and (ii) the operation time of the process. Batch experiments were conducted at bench-scale using an EK experimental cell at room temperature under 1 V cm^{-1}. An EK-biostimulation strategy was used, that is the polluted soil already contains an acclimated microbial culture adapted to 2,4-D biodegradation. To the author's knowledge, electro-bioremediation studies for *in-situ* biodegradation of hazardous pesticides in polluted soils are still scarce.

2. Materials and methods

2.1. Materials

The soil employed at this work is a clean clayey soil supported by Millas Hijos Ceramics (Toledo, Spain). Its characteristics has been described in previous works (Barba et al., 2017). Soil was artificially polluted with the pesticide (20 mg of 2,4-D per kg of wet soil) following the procedure explained in section 2.3. The chemical product 2,4-dichlorophenoxyacetic acid (2,4-D) was selected as model of polar pesticide, supplied by *Alfa Aesar* (98% assay). 2,4-D possible loss by volatilization was checked 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.

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

To start the acclimation process it was selected an inoculum from a biological reactor of an oil-refinery wastewater treatment plant (Puertollano, Spain). Bushnell-Hash Broth (BHB) was used as inorganic nutrients source for microorganisms. This culture media contains 0.20 g L^{-1} Mg SO_4, 0.02 g L^{-1} $CaCl_2$, 1.00 g L^{-1} KH_2PO_4, 1.00 g L^{-1} $(NH_4)_2HPO_4$, 0.05 g L^{-1} $FeCl_3$ and 1.00 g L^{-1} KNO_3. 200 mg L^{-1} of 2,4-D was employed as sole carbon source. Once the acclimation process was achieved, the species of microorganisms contained in the microbial culture were identify by means of MALDI TOF Mass Spectrometry (AXIMA-Assurance Biotech technology, SHIMADZU, Germany). The species identified in the 2,4-D-degrading microbial culture were *Rhodococcus ruber* and *Ochrobactrum anthropic*.

2.2 Experimental set-up

Figure 1 shows the experimental set-up scheme employed in EBR experiments. The installation consists of a bench scale EK cell made of transparent methacrylate, which is divided into five compartments. In the central one is placed the 2,4-D polluted soil. The electrodic compartments are located at both sides separated from soil by a nylon mesh (0.5 mm mesh size), and they contained the electrodes. The electrodes used are made of graphite with dimensions of 10x10x1 cm, supplied by Carbosystem (Madrid, Spain), which are connected to a power supply (HQ Power, Gavere, Belgium). The electrolyte used is a synthetic inorganic medium with the following composition: 80.75 mg L^{-1} of Na_2SO_4, 70.00 mg L^{-1} of $NaHCO_3$, 30.36 mg L^{-1} of $NaNO_3$, which tries to simulate groundwater. Because of microorganisms can consume the nutrients or due to their removal by electromigration and electroosmosis, excess inorganic nutrients (nitrate, ammonium and phosphate) were provided to avoid nutrient limitations that can occur during the EBR process. To collect the electroosmotic flow (EOF) moved during the process, there are two collector compartments contiguous to electrode ones. It is important to remark that the EOF will be collected at both sides because of the polarity reversal applied in all EBR experiments of this work (Mena et al., 2016).

2.3. Experimental procedure

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

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

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

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

2. Effect of operation time: Based on the previous results (i), 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). Simultaneously to each EBR experiment using different operation times, a reference test without electric current (only biological contribution) was always performed. Reference tests were done under identical experimental conditions than EBR experiments (that is, the same installation, electrolyte addition, microbial inoculation), but without using electric current, and they would help to check the influence of electrokinetics.

2.4. Sampling and analyses

During the experiments, liquid samples were taken from electrodic wells, from the EOF collector compartment and from the liquid medium in soil. Due to the electrode polarity changes during the process, it is important to remark that EOF is collected alternatively in both collector compartments depending on the cathode position. Moreover, the temperature of soil and current electricity were monitored throughout all the duration of the experiments. Conductivity and pH were measured by using a multiparameter probe (SENSLON, HACH). Inorganic nutrient concentrations were analysed by means of UV-Vis photometer (Gallery, Thermo Scientific). The concentration of 2,4-D was measured by HPLC (Jasco, Japan), using a column model Kinetex 5 µm Biphenyl 100 Å, 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 220 nm. The injection volume was 20 µL.

Soil samples were taken only at the beginning before placing the soil on the set-up, and at the end of the experiments (*post-mortem* analysis, once the experiment was finished). This procedure was followed not to modify the compaction of soil avoiding preferential paths for EOF (Ruiz et al., 2014). For carrying out the *post-mortem* analysis, it is necessary to divide the soil properly after the experiment. Then, the soil was divided into sections as follows: four longitudinal positions (positions 1 to 4), where position 1 corresponds to nearest zone to anodic well, and position 4 to nearest to cathodic well (at t = 0). Each longitudinal section was in turn divided into four parts: two upper parts (left and right) and two bottom parts (left and right), according to previous works (Ramírez et al., 2015).

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

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

3. Results and discussion

3.1. Selection of polarity reversal frequency

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

Figure 2b shows the profiles of electrical conductivity towards the soil at the end of experiments. It can be observed that after the three experiments, carried out at different *f* values, electrical conductivity profiles are similar, and again a significant influence of *f* value was not observed. On the contrary, in the case of the reference test ($f=0$) a decrease in the soil conductivity can be observed. This result can be related to the faster removal of ions from the system when no PR is applied. Moreover, it has been previously reported that EOF decreases and relatively high current density value remains when PR is used (Mena et al., 2016; Barba et al., 2017). The application of PR implies that the ions retained in the soil can remain longer because both electromigration and EOF move them alternatively in both directions. Consequently, it allows not only pH control but also maintaining adequate values of inorganic nutrients concentrations, current density and electrical conductivity in soil during the remediation process. Soil temperature (results not shown) kept practically constant during all the experiments carried out (both EBR experiments and reference tests without electricity), around 30 ºC, that is adequate temperature for microbial activity. It was noted again that ohmic heating was negligible at such low voltages at bench scale (Barba et al., 2019a). Again, changes in *f* values in the range between 1 and 6 d^{-1} did not affect soil temperature. Figure 3a shows the concentration of microorganisms in soil at the beginning (discontinuous line) and at the end (continuous line) of experiments under different *f* values. As it can be observed, the microorganisms' concentration kept practically constant in all experiments, that is, a homogeneous profile was observed towards the soil and no microbial decay was observed. pH control is critical for such result. Mena et al. (2015), reported that in the case of not applying periodic polarity reversal, the concentration of active microorganisms in soil at the end of the process is null caused by the negative effect of extreme pH in soil. As it occurs with the other parameters above described, it was not observed difference in microorganisms' concentration depending on the different frequencies studied. Figure 3b shows the results of 2,4-D removal from soil for each experiment conducted. As it can be observed, in only two weeks of treatment it was achieved the completely removal of the pollutant in soil in the three experiments at different *f* values. This fact shows that the removal of 2,4-D is easier than the removal of non-polar compounds, probably because of its polar nature and low sorption in soil, which implies higher mobility through all over the soil and better contact with microorganisms (Barba et al., 2019a). Nevertheless, results shown in Figures 2 and 3 do not allow us to select an optimum value of polarity reversal frequency. For this reason, and because of the frequency value has no economical cost implications in the electro-bioremediation process, it was selected a frequency of 2 d^{-1} as in the previous works carried out by the same authors when using non-polar pollutants (Barba et al., 2017).

3.2. Effect of the operation time

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

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

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

Figure 6 shows the 2,4-D removal efficiencies under the different operation times tested in the present work. Additionally, each 2,4-D percentage removal value is compared with the value obtained in the corresponding reference test, in which no electric field was applied to the soil to be treated, and only biodegradation without the contribution of EK phenomena was the responsible of pollutant removal. It is important to note that 2,4-D was not detected in electrode wells, and no volatilization occurred, thus only biodegradation (with or without the help of EK) is the responsible of pollutant removal efficiencies in Fig.6. Moreover, 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). As it can be observed, almost 50% of 2,4-D was removed in only 3 days in electro-bioremediation experiments, and nearly 100% was removed in 10 days.

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

Conclusions

Removal of 2,4-D from polluted clay soil was successfully reached in 10 days. It is assumed that solubility of the pollutant is a critical factor to ensure high removal efficiencies. 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.

Acknowledgements

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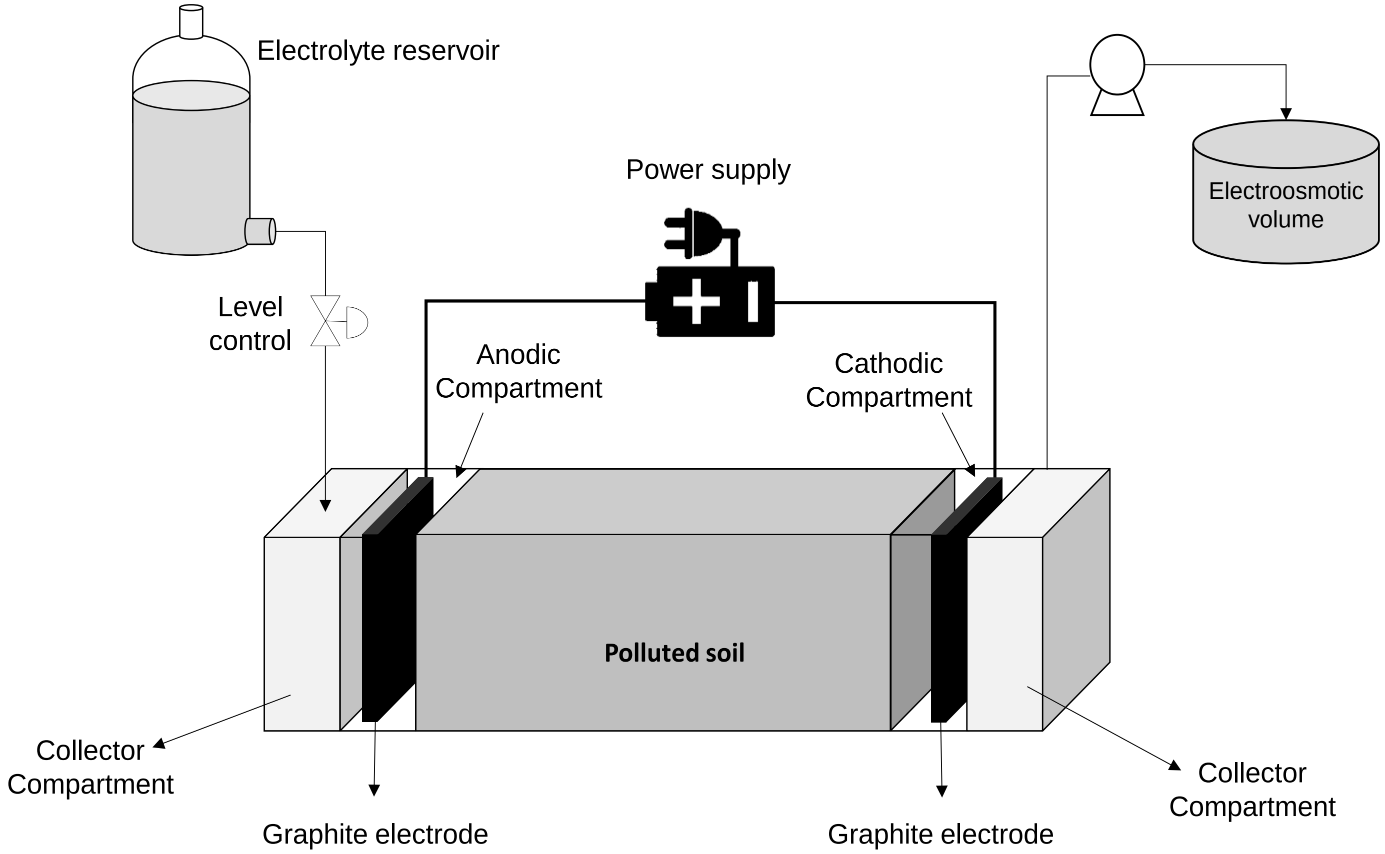
Figure 1



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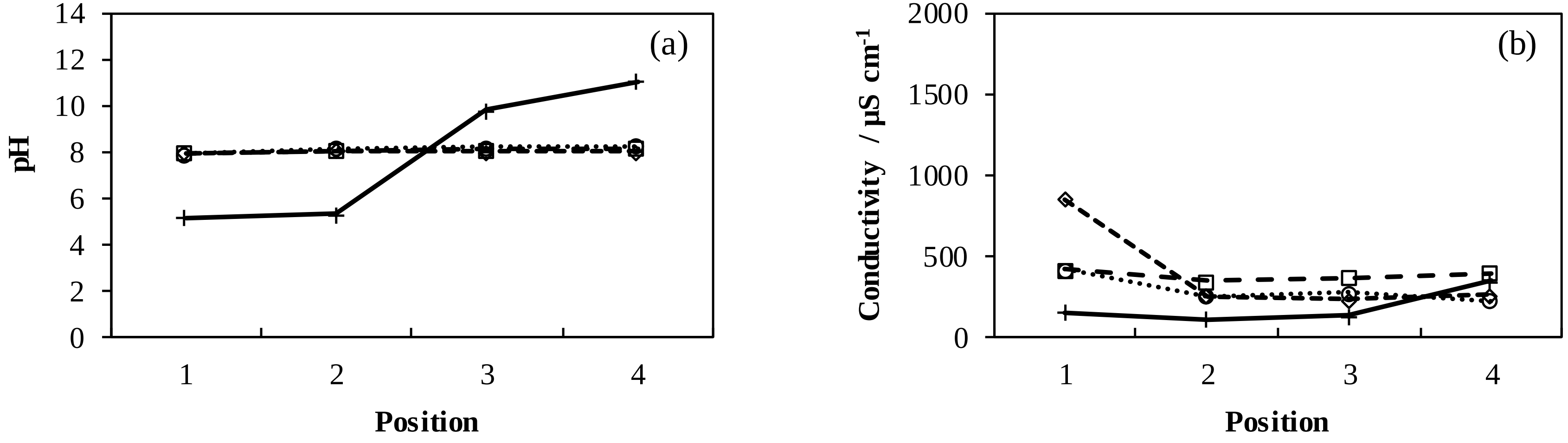

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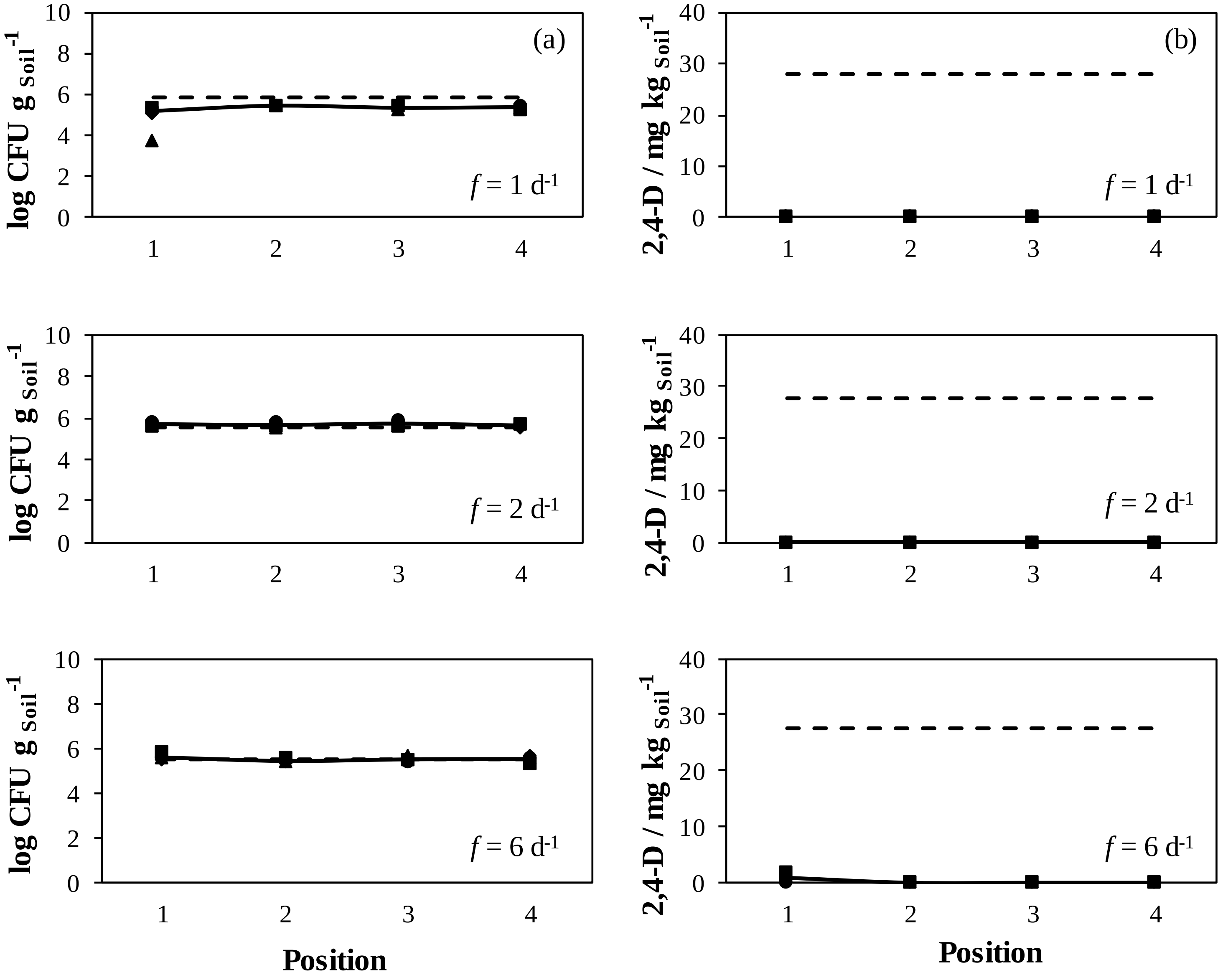

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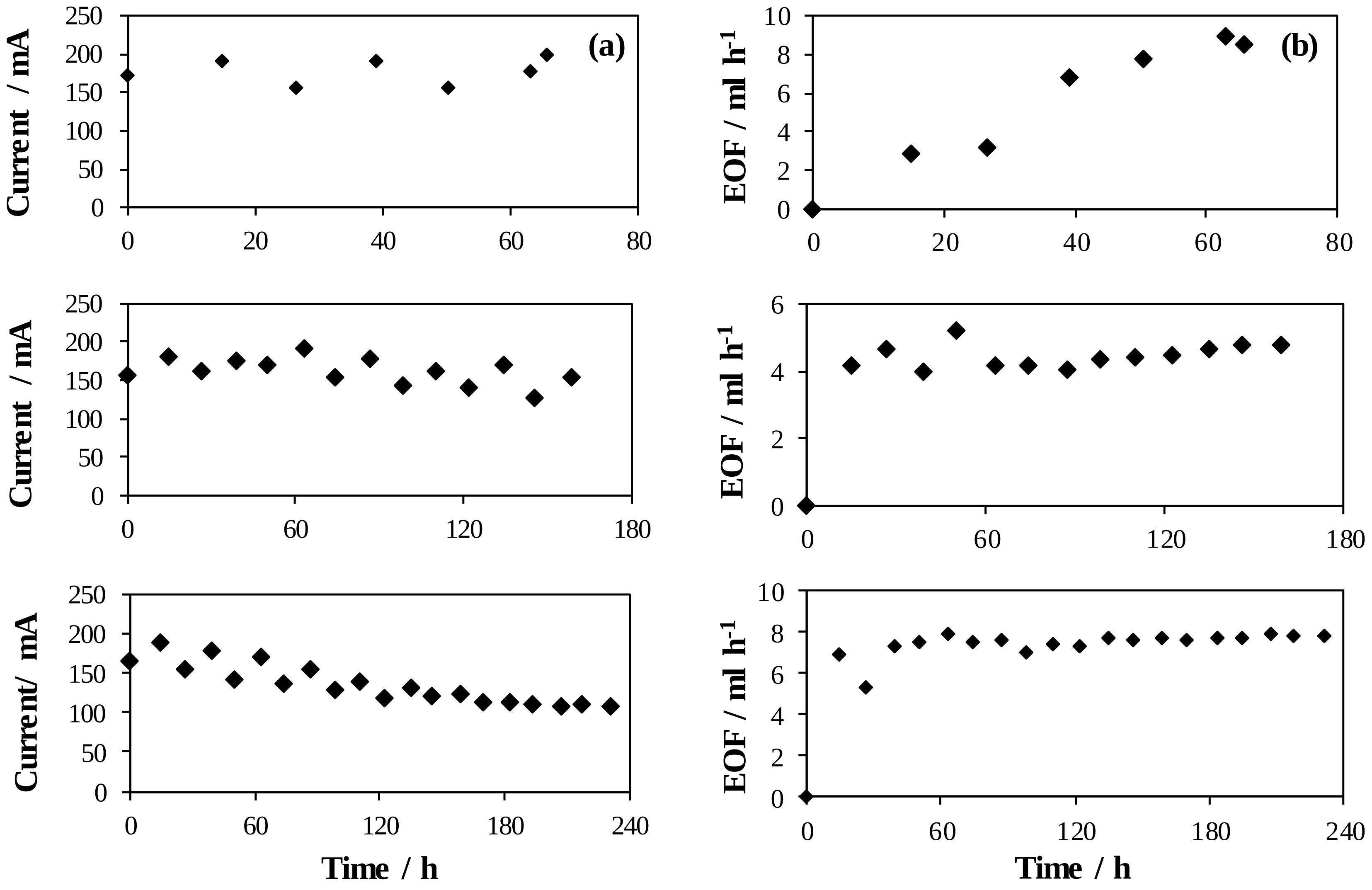

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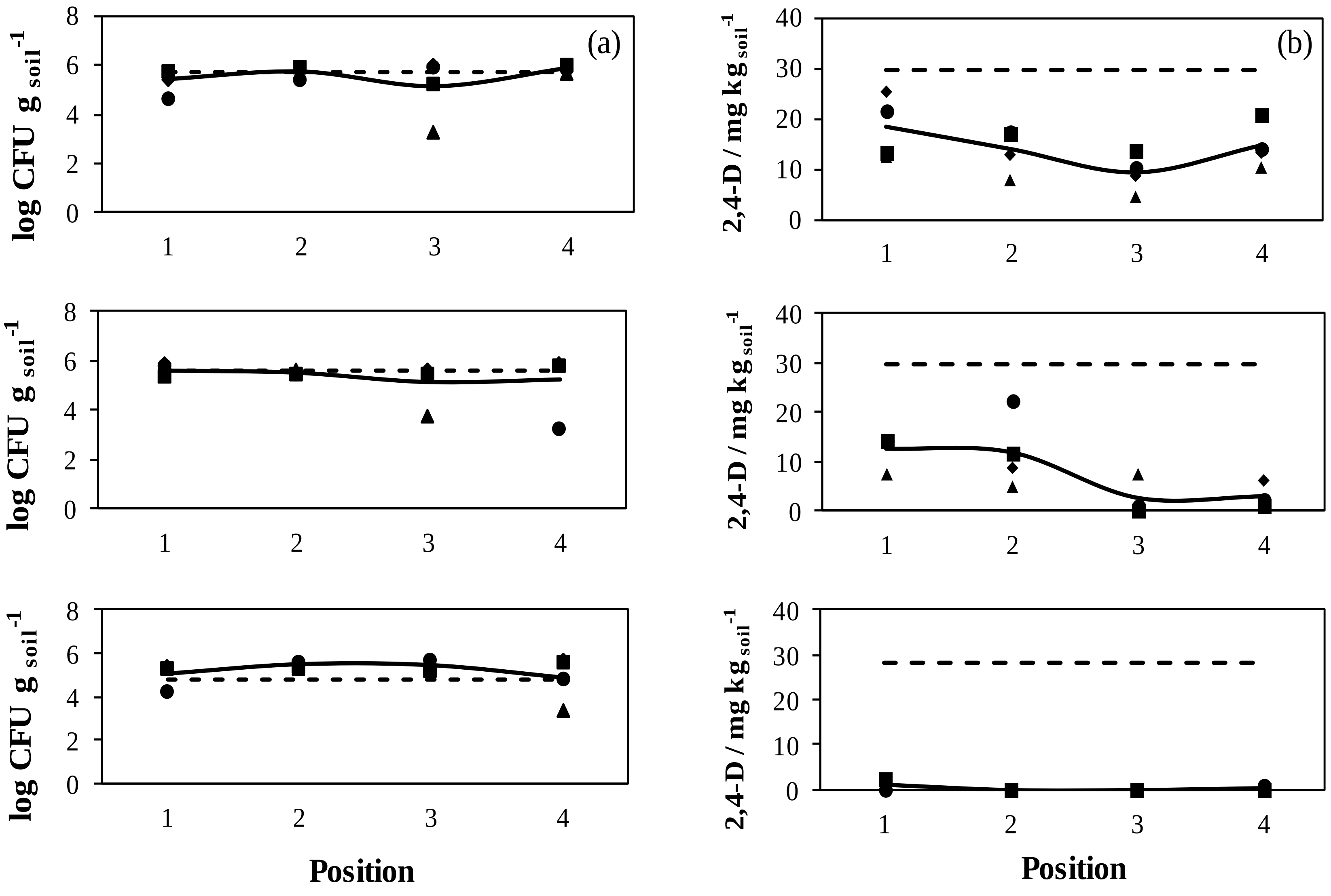

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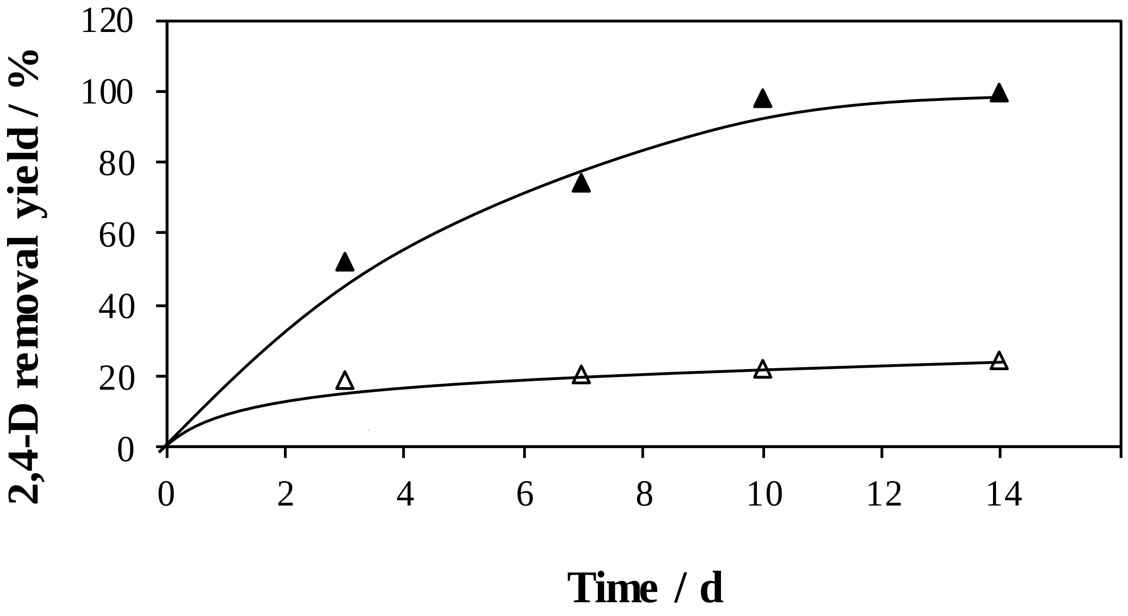

Figure 6

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

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:

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.