1 Natural and Assisted dissipation of Polycyclic Aromatic Hydrocarbons in a long-
2 term co-contaminated soil with Creosote and Potentially Toxic Elements

3 Madrid F.^a, Rubio-Bellido M.^a, Villaverde J.^a, Peña A.^b, Morillo E.^{a*}

4 ^a Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Apdo.
5 1052, 41080 Sevilla, Spain.

6 ^b Instituto Andaluz de Ciencias de la Tierra (CSIC-UGR). Avda. de las Palmeras 4.
7 18100-Armilla, Granada, Spain.

8 * Corresponding author: morillo@irnase.csic.es

9 ABSTRACT

10 An enhanced bioremediation strategy was applied to an industrial soil co-contaminated
11 with Polycyclic Aromatic Hydrocarbons (PAHs) and Potentially Toxic Elements
12 (PTEs). Hydroxypropyl- β -cyclodextrin (HPBCD) and a natural mixture of two
13 rhamnolipids (RL) were added to increase PAHs bioavailability, and combined with a
14 microbial consortium (MC) to biodegrade soil PAHs. Bioavailability of only six PAHs
15 (3-, 4-ring PAHs) increased when using HPBCD, with a maximum increase about 2.8-
16 fold higher. The highest dose of HPBCD (5%) enhanced PAH degradation, with the
17 best results for 4-ring PAHs with treatments of HPBCD + MC (up to 48% degradation
18 for pyrene and 43% for fluoranthene), whereas dissipation for 5-ring PAHs was very
19 low and for 6-ring was negligible. The use of RL increased the bioavailability of 13 of
20 the 16 PAHs studied, reaching up to 60-fold higher values for phenanthrene or 18-fold
21 higher for acenaphthene. RL addition did not show degradation improvement in any
22 situation, and even inhibited the scarce degradation observed in the control treatment.
23 The high increase in availability of both PAHs and mainly PTEs when using RL as

amendment could make them toxic for microorganisms. In fact, Microtox Acute Toxicity test using *Allivibrio fischeri* and the absence of colony forming units (CFUs) of indigenous bacteria demonstrated the extremely high levels of toxicity in RL treated soil.

Keywords: Cyclodextrin, rhamnolipid, soil remediation, bioavailability enhancer agents.

1. INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are a group of products extensively distributed in the environment. They are highly persistent and many of them known as carcinogenic and/or mutagenic agents. The main source of soil PAHs is considered the incomplete combustion of wood or fossil fuels, but other sources are also possible, such as volcanic ashes, open fires, crude oil extraction and refining, engine exhaust emissions, manufactured gas plants by-products and domestic heating systems (Von Lau et al., 2014). Creosote is a carbonaceous product used as preservative of outdoor wood structures, and has been widely used for decades for railroad ties, power line poles and ship's hull to prevent rot. The main components of creosote oil are aromatic hydrocarbons, including PAHs, which can make up to 85% of creosote composition (Mueller et al., 1989). In this way, many ancient facilities of wood treatment with creosote are nowadays brownfield of contaminated soil by PAHs. These sites are considered as hotspots of PAHs endangering nearby groundwater quality.

PAHs can be divided into two groups with different properties and behaviour in soils: low molecular weight (LMW) and high molecular weight (HMW) PAHs. The first one is formed by compounds with 2 or 3 benzene condensed rings; they are relatively volatile, mobile and bioavailable in soils, and can be usually degraded by soil microorganisms. On the contrary, HMW PAHs (4 or more rings) show very low volatility, mobility and bioavailability, and are highly persistent in soils due to their hydrophobicity and stability.

Different methods have been proposed to remediate PAHs contaminated soils: biological, thermal, physical, chemical and solidification (Kalantary et al., 2014). Among these techniques, biological ones, based on PAH degradation by plants and/or microorganisms are considered cost effective and environmentally friendly (Haritash and Kaushik, 2009; Sopena et al., 2014; Kuppusamy et al., 2017). However, remediation technologies have to be optimized for each case, depending on soil type, contaminant, origin, etc. Not only physical, chemical and biological characteristics of the contaminated soil, but also the 'ageing' of the contaminant have a definitive influence on the progress of the remediation process. The result of this process is the sequestration of compounds in the soil matrix by different mechanisms that remain not completely understood (Semple et al., 2003; Morillo et al., 2014), declining their bioavailability and reducing the biodegradation of the contaminants. Ageing is especially important in hydrophobic compounds as PAHs. Although many studies have been published about PAHs dissipation in soils artificially contaminated in laboratory conditions (Madrid et al., 2016; Morillo et al., 2014), very few investigations have been conducted on aged contaminated soils with PAHs. Wei et al. (2017) compared bioremediation of freshly contaminated soils with long term aged contaminated soils,

obtaining reductions in PAHs dissipation from 95.0 and 84.5 % (fresh contamination) to 10.2 and 0 % (aged contamination) for the same soils.

In order to avoid this sharp decrease of PAHs degradation rates with ageing, Shi et al. (2017) described an assisted bioremediation strategy that consisted in adding *Fusarium* sp. ZH-H2 (a PAHs potentially degrading fungus strain) that manages to increase the removal rate of aged PAHs. This strategy, named bioaugmentation, involves inoculating microorganisms capable of degrading the target contaminants, and has been frequently used for increasing dissipation rates of Persistent Organic Pollutants (POPs) in soils (Villaverde et al., 2017). Another strategy, named biostimulation, refers to the improvement of soil conditions (moisture, aeration, pH, ...) and/or the application of nutrients and electron acceptors to enhance the growth of degrading microbial populations (Garbisu et al., 2017).

The reduction of bioavailability due to the ageing effect can be also avoided with the use of amendments that increase the solubility of the contaminants. Increasing solubility is a key step to increase bioavailable fractions of soil contaminants (Stroud et al., 2009). Recently, biogenic compounds as cyclodextrins (CDs) or biosurfactants (BSs) have been used in environmental applications to improve remediation of polluted soils, because these compounds increase the solubility of hydrophobic organic contaminants (Villaverde et al., 2006; Morillo et al., 2012; Flaherty et al., 2013; Trellu et al., 2016; Morillo and Villaverde, 2017).

CDs form inclusion complexes with a wide range of guest hydrophobic molecules, which are located in the interior cavity of the CDs. The hydrophilic properties of the outer zone of the CDs solubilize the resulting complex, increasing its bioavailability. It has been previously demonstrated that hydroxypropyl- β -cyclodextrin (HPBCD) is a

good extractant for PAHs in soils (Sánchez-Trujillo et al., 2013; 2014) increasing their dissipation (Madrid et al., 2016). CDs are formed by glucose monomers, so microorganisms could also use CDs as a biostimulant.

Surface active compounds, such as BSs, can also be used to increase hydrophobic compounds bioavailability (Bezza and Chirwa, 2016, 2017; Szulc et al., 2014). BSs addition is an alternative strategy to increase bioavailability by dissolving and emulsifying contaminants, as well as releasing the trapped hydrocarbons in pores of the soil (Xia et al., 2014). The amphiphilic properties of biosurfactants favour the interaction with interfaces of various polarities reducing interfacial tension and increasing solubility and bioavailability of hydrophobic compounds (Hazra et al., 2012; Xia et al., 2014). These properties, combined with their biodegradability have increased the interest on BSs as agents for assisted bioremediation of hydrophobic contaminants (Shavandi et al., 2011). Biosurfactants are mainly naturally produced by bacteria or yeast, and also are available from plants and animals (Paria, 2008). Rhamnolipids (RLs) are a type of BSs synthesized by *Pseudomonas aeruginosa*, a microorganism that has been found in consortia of cultures grown in PAHs enriched media (Bezza and Chirwa, 2016) and petroleum contaminated soils (Dong et al., 2017). Due to their special hydrophilic and lipophilic moieties, RLs have enhanced PAH bioaccessibility through micellar solubilization (Mohanty and Mukherji, 2013).

In a previous study (Madrid et al., 2019) several CDs and the rhamnolipid JBR 425 were used to extract the 16 priority PAHs defined by the US-EPA from a soil highly contaminated with creosote for almost 100 years. Up to 15.4% of the $\Sigma 16$ PAHs were extracted using RLs, while with CDs the maximum extraction reached was only 2% in the case of HPBCD.

The aim of this paper is to use HPBCD and the rhamnolipid JBR 425 to study their effect not only as extractants to increase the bioavailability of PAHs in the same creosote contaminated soil, but also as stimulant agents. Fenyvesi et al. (2005) observed the complete biodegradation of HPBCD in soils of different characteristics, likely because several plant- and fungi-associated bacteria metabolised HPBCD as a carbon source. Biosurfactants also stimulate microbial biomass growth to decompose the contaminants (Bezza and Chirwa, 2017). The addition of HPBCD or the rhamnolipid (extraction and biostimulation) combined with a potentially pyrene degrader consortium (bioaugmentation) has been selected as the strategy for an enhanced bioremediation that could increase the chance for a successful decontamination of this long-term polluted soil.

The selected soil is co-contaminated with Potentially Toxic Elements (PTEs), due to the industrial activities related with railway maintenance tasks. Unlike PAHs, PTE cannot be degraded, but differences in solubility and availability were observed especially when rhamnolipids were used for PAHs extraction (Madrid et al., 2019). During bioremediation processes, increases in PTE availability could increase their mobility through the soil profile endangering groundwater. For this reason, monitoring the effect of the amendments used for the bioremediation of co-contaminated soils on PTEs availability is a key step to avoid the risk of dispersion of such contaminants in the environment.

2. MATERIALS AND METHODS

2.1. Materials

Soil was collected from an antique facility located in Andújar (South-eastern Spain: 38°01'39''N, 4°03'58''W), where railway crossbar were treated with creosote and inorganic wood preservatives for almost 100 years. The soil was sampled from the top 0–20 cm layer, air-dried and crushed to pass through a 2-mm mesh sieve before analysis. The soil has not received any remediation treatment since this activity was abandoned. The soil was analysed for pH in a water:soil extract at the ratio 2.5:1, total carbonate content, particle size distribution, organic matter (OM) content and PAHs and PTEs contents. Detailed soil description is reported elsewhere (Madrid et al., 2019). General properties are: pH 8.14, 8.11 % OM, 23.2 % CaCO₃; 74.8 % sand, 14.8 % silt and 10.4 % clay (sandy loam texture).

Hydroxypropyl-β-cyclodextrin (HPBCD) was supplied by Sigma-Aldrich (Germany). The rhamnolipid JBR-425 is a commercial mixture of mono- and di-rhamnolipids supplied by Jeneil Biosurfactant (USA). JBR-425 has been already described in detail elsewhere (Ozdemir and Malayoglu, 2004).

PAHs and PTEs analytical standards were supplied by Sigma Aldrich and VWR International (Spain) respectively. Other chemical reagents used were always analytical grade quality.

2.2. Methods

2.2.1. Pyrene microbial consortium (MC)

Assays of ¹⁴C-labeled pyrene (PYR) and phenanthrene (PHE) in this soil were conducted to check the potential of the soil for their mineralization due to the presence of a population of degrading microorganisms preadapted to compounds with structures similar to those of PYR and PHE. Mineralization was measured (in triplicate) by the production of ¹⁴CO₂ according to Madrid et al. (2016). Figure 1S shows the kinetics of

PYR and PHE mineralization by the endogenous soil microflora. Therefore, a degrading MC was isolated to be used as bioaugmentation (inoculation with specific microbial PAH degraders) in some of the incubation experiments. The MC was isolated from native soil as described in Villaverde et al. (2018). Briefly, 10 g of soil were added to sterilised 250 mL Erlenmeyer flasks, (autoclave Auster-G, P-Selecta with one cycle at 121 °C, inlet pressure of 103 kPa, during 20 min) containing 50 mL of a mineral salt medium (MSM) and a solution of micronutrients (mg L^{-1} , 75.0 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 37.5 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 25.0 $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$; 12.5 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 12.5 $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$; 12.5 $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$; 12.5 $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$; 10.0 $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$; 3.75 KBr; 3.75 KCl; 2.50 LiCl). Both MSM and solution of micronutrients had been also previously sterilized. Afterwards, the suspension was spiked with 40 mg L^{-1} PYR as the only source of C and energy. PYR, but not PHE, was selected as the only source of C due to its higher content in the selected soil and its higher molecular weight (4-rings). Microbial consortia able to degrade 4-ring PAHs should be also able to degrade 3-ring PAHs. The culture was incubated with orbital shaking (150 rpm) at 30°C, and each 15 days, 10 mL of the culture were transferred to another flask containing the same sterile mineral medium and incubated again. After four enrichment transfers (60 days), 100 μL of the culture was plated in R2A-agar medium (0.5 g L^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; peptone; casaminoacids; yeast extract; glucose; starch; K_2HPO_4 ; sodic pyruvate and 20 g L^{-1} agar) and incubated for 72 h at 30 °C. The isolated consortium, with potential PYR degrading activity, was sown and subsequently stored in Microbank criovials (2 mL microtubes containing specific culture medium and 20 porous spheres of 3 mm diameter) and kept at -80°C . To prepare the solution to spike the soil, this criovial was grown in R2A medium (Villaverde et al., 2012). The initial cell inoculum density of the MC added to soil was 10^9 CFU g^{-1} .

2.2.2. Remediation microcosms

Ten different treatments were prepared for incubation as shown in Table 1. 500 g of dry soil were weighed in 500 mL glass bottles, and each bottle of fresh soil received one or more of the following solutions according to its corresponding treatment. A Nutrient Solution (NS) was added to all treatments: 21.2 g of NH_4NO_3 and 7.19 g of KH_2PO_4 were dissolved in 100 mL of deionized water, and 6.2 mL of this solution was added to each bottle of treatments. HP treatments: 50% w/v solution of HPBCD was prepared. A volume of 50 mL of this solution was added to each bottle of HP 5% treatments and 10 mL were added to bottles of HP 1% treatments. RL treatments: The commercial solution of JBR-425 was directly used to obtain RL 1% and 5% treatments. Microbial consortium (MC) treatments: 5 mL of the 10^9 CFU g^{-1} initial inoculum solution freshly prepared as described above were added to each bottle of MC treatments. Finally, deionized water was added to each bottle to reach 60% of soil water holding capacity. After the addition of the different solutions, bottles were closed and homogenized by end-over-end agitation during 3 days. Then the soil content of each bottle was divided into two replicates (250 g of soil), and incubation was started (day 0). Treatments were incubated in semi-closed glass containers placed in a greenhouse at room temperature for 120 days of ageing (from June to October, 2017) to simulate as close as possible a contamination scenario. The climate of the area is Mediterranean, with an average annual temperature of approximately 18 °C and sunshine for 2800 h year^{-1} . Moisture was controlled gravimetrically and water was added when necessary. Immediately after the beginning of the experiment and at periodic intervals (1, 30, 60, 120 days) the soil from each container was mixed and soil samples were taken for their analysis.

214

215 2.2.3. PAH soil content

216 PAHs soil content has been analysed according to QuEChERS methodology with minor
217 modifications to get a quantitative extraction of analytes (Cvetkovic et al., 2016). Five
218 grams of soil were spiked with Internal Standards (NAP-d8, ACE-d10, PHE-d10, PYR-
219 d10, CHR-d12, BaPYR-d12 and PERY-d12) and 5 mL of water were added. The
220 mixture was homogenized by Vortex agitation, and 10 mL of acetonitrile were added
221 and homogenized again. Three cycles of agitation (5 min)-sonication (15 min) were
222 carried out, and a salt mixture kit was added (MgSO₄, NaCl, sodium citrate). After
223 agitation and centrifugation, 3 mL of the organic phase were cleaned up by dispersive
224 solid phase extraction (dSPE) and PAHs content was determined by Gas
225 Chromatography (Agilent 7890B) coupled with mass spectrometer detection (Agilent
226 7000C). Electron ionization mode was used at 70 eV. A HP-5 MS column coated with
227 5%-phenyl-methylpolysiloxane of 30 m x 0.25 mm I.D and 0.25 µm film thickness
228 (J&W Scientific, CA, USA) was used with the following temperature programme: 60°C
229 (1 min) to 175°C at 6 °C/min (4 min) to 235°C at 3°C/min (5 min) and raised to 315°C at
230 8°C/min. 2 µl of sample was injected in splitless mode and the valve was closed for 1.5
231 min. Helium was used as a carrier gas at 1.9 mL/min. Temperatures for the injector,
232 transfer line and ion source were 280, 270 and 230°C, respectively. Time scheduled
233 selected ion monitoring (SIM) was used with four retention time windows. Three ions
234 were monitored for each compound to improve the sensitivity and selectivity. The
235 software used was MSD ChemStation 5.0.0.0 (Agilent, CA, USA). The quantification
236 procedure with deuterated surrogate standard is explained in a previous study (Sánchez-
237 Trujillo et al., 2013). The extraction was effective with recovery rates from 88±6 to
238 115±8% and relative standard deviation (RSD) lower than 15%.

239

240 2.2.4. PAH soil bioavailability

241 PAH bioavailability in soil just after adding the treatments was determined according to
242 the method proposed by Reid et al. (2000). In Corex-glass centrifuge tubes, 1 g of soil
243 was extracted with 20 mL of a 50 mM HPBCD solution at room temperature in an
244 orbital shaker (New Brunswick Scientific model G25) at 150 rpm for 20 h. Afterwards,
245 the supernatant was separated by centrifugation and PAHs analysed as described below.

246

247 2.2.5. PTE soil availability

248 The availability of PTEs in the soil at the end of the incubation period was studied and
249 compared with that of the original soil (control treatment). Soil samples were extracted
250 with 0.05 M ethylenediaminetetraacetic acid (EDTA) at pH= 7.00 according to Ure et
251 al. (1993). Briefly, 25 mL of EDTA 0.05 M were added to 2.5 g of soil, and agitated for
252 1 h. The soil suspension was centrifuged for 10 min at 5000 rpm and filtered through
253 Whatman paper number 2. The PTE content in the soil extract was determined by ICP-
254 OES (Varian 720-ES).

255

256 2.2.6. Bacterial enumeration

257 Enumeration of viable bacteria at different incubation periods for each treatment was
258 achieved by counting the colony forming units (CFUs) per gram of soil. Bacterial
259 enumeration was carried out using 1 g of soil which was whirl-mixed for 30 s with 10
260 mL of Ringer's solution, and then sonicated for 1 min and allowed to stand for 2 min.

Aliquots (100 µL) of these dilutions were applied on agar–agar plates prepared from a LB medium. CFUs were counted after 48 h.

2.2.7. Ecotoxicity bioassays

The inhibitory effects of soil extracts on the light emission of freeze-dried luminescent bacteria *Aliivibrio fischeri* were determined with the Microtox Acute toxicity test, according to ISO 11348-3 (2007). Soils were mixed with deionized water at a 1:10 ratio (w/v) and shaken during 24 h, at room temperature. The supernatants were separated by centrifugation and then filtered through 0.45 µm PVDF filters (Scharlau, Spain) to remove remaining particulate matter (Alvarenga et al., 2008; Manzano et al., 2014). Soil extracts and their dilutions with NaCl at 2%, 6.25%, 12.5%, 25% and 50% (v/v), were tested and compared with the control. The decrease of luminescence was measured in duplicate after 15 min contact using an Optocomp I luminometer (MGM Instruments, Gomensoro, Spain). For each soil sample the EC₅₀ value, which corresponds to the soil extract concentration (% v/v) having a toxic effect on 50% of the bacterial population, was calculated. Toxicity values were also expressed in toxic units, using the formula $TU = 100/EC_{50}$.

2.2.8. Statistical analysis

The one-way ANOVA with three replicates was performed to study the significant differences among treatments. Statistical analysis was carried out by means of the SPSS (v. 21) statistical package.

3. RESULTS AND DISCUSSION

Initial analysis of soil PAH contents (Table 2) showed a relative low amount of 2 and 3-ring PAHs, compared to 4, 5 and 6-ring PAHs. This profile of PAHs in creosote contaminated soil is typical of aged contamination, because LMW PAHs are easily degradable, and they are dissipated in a short-medium period of time. Otherwise, HMW PAHs are more hydrophobic and recalcitrant in soil and show less bioavailability. This is the reason why detected contents of HMW PAH in aged contaminated soils are usually similar to those presented here when soil was originally contaminated (Realp et al., 2008), whereas LMW PAHs show lower contents. According with this, bioremediation of this aged contaminated soil would need to focus on HMW PAHs (Lladó et al., 2015). The consortium isolated to inoculate the contaminated soil (bioaugmentation strategy) was grown with a 4 ring-PAH (PYR) as the only source of carbon, so that the consortium could be at least a potential degrader for 4-ring PAH, the most abundant in this contaminated soil (40.9% related to $\Sigma 16$ PAHs).

Furthermore, increase PAH bioavailability is a key step to improve the access of microorganism to PAHs, improving the chances of success to get dissipation of these PAHs. HPBCD and RL form stable complexes with different PAHs (Sánchez-Trujillo et al., 2013; Madrid et al., 2016; Mohanty and Mukherji, 2013), so their addition to soil may be a good alternative to increase bioavailability of these contaminants during a decontamination assay.

3.1. PAH bioavailability

The effect of the different amendments used on the bioavailable content of PAHs in soil at the beginning of the incubation is shown in Table 3. The selected method by Reid et

al. (2000) based on the extraction using HPBCD has been proposed to more closely mimic the mass transfer mechanisms that govern the bioavailability of non-polar organic contaminants, in particular of PAHs. A wide range of studies that have compared HPBCD extraction to the bioavailability of PAHs in soil biota have reported strong correlations (Khan et al., 2011; Rubio-Bellido et al., 2018). However, the use of HPBCD has limitations because its cavity maybe suitable for LMW PAHs, but is not large enough for the HMW PAHs, resulting in their lower extraction efficiency (Lal et al., 2015).

Control treatment showed in general very low content of bioavailable PAHs, with percentages lower than 2% for each individual PAH content except NAP and ANT with values 3.08 and 2.22%, respectively. DANT, IND and BPER were not bioavailable according to the method employed (Reid et al., 2000). In relation to Control treatment, bioavailability increases are statistically significant ($p \leq 0.05$) for six PAHs (ACE, FLU, PHE, FLT, BaANT, CHR) when using HPBCD as amendment, but only in case of the high rate (HP5%), with a maximum increase about 2.8-fold higher with HP5% treatment for ACE. But the great bioavailability increment was obtained with RL, especially at the high rate (RL5%). 13 of the 16 PAHs studied increased their bioavailability, both for RL5% and for RL1%, reaching up to 60-fold higher values for PHE or about 18-fold higher for ACE with RL5% in relation to control treatment. That is fully in accordance with the results obtained in the previous paper of Madrid et al. (2019), which revealed a much higher $\Sigma 16$ PAHs extraction using RLs (15.4% of the total content in the soil), while with CDs the maximum extraction reached was only 2% with HPBCD. In principle, with these results it seemed reasonable to assume that both amendments, but especially RL, could be appropriate for a bioremediation enhancement of the creosote-polluted soil.

333

334 **3.2. PAHs evolution in soil microcosms**

335 Dissipation data obtained for 16 PAHs included by USEPA and EU in their priority lists
336 of contaminants are shown in Table 4 for the Control and HPBCD treatments.
337 Dissipation rate (%) is calculated measuring PAH concentration at the end of the
338 incubation, in relation with the initial one. It can be observed that 2- and 3-ring PAHs,
339 PAHs with the lowest molecular weight, show very low dissipation rate for most of the
340 treatments or even none at all, with similar contents at the beginning and at the end of
341 the incubation. This could be related with the ageing process that has suffered this soil
342 for many years in the presence of the contaminants. In consequence, the bioavailable
343 fraction of these LMW PAHs would have been greatly reduced before remediation
344 treatments were applied. Certain LMW PAHs, such as PHE or NAP, are compounds
345 that originate in the metabolic pathways of degradation of HMW PAHs (Hussein,
346 2008), and it is possible that their content at certain incubation times does not only
347 come from their initial content. In spite of this, their contents were very low even at the
348 end of the incubation period (data not shown). The low degradation rate recorded also
349 agrees with the low PAHs bioavailability data (mg kg^{-1}) showed in Table 3.

350 The highest dissipation percentages are observed for 4-ring PAHs, but dissipation
351 profiles among them and depending on the treatment are quite different. The evolution
352 of these PAHs is also shown in Figure 1. No dissipation of FLT was observed in the
353 Control treatment, with contents at the end of the incubation very similar to the initial
354 one (Figure 1), but the addition of the MC was successful to dissipate FLT (75.7%
355 remaining after 120 days). The addition of HPBCD (1% or 5%) decreased the
356 remaining FLT till 65-70%. The combination of microorganism and HPBCD at higher

rate (HP5%) led to the highest FLT decay at the end of the incubation with a relative final content of 56.8%. On the contrary, the lower rate of HPBCD (HP 1%) showed similar dissipation with and without addition of MC.

PYR dissipation profile shows some differences with FLT. In this case, the Control treatment reached a dissipation extent around 30% of the initial content. Addition of the degrader consortium or HPBCD separately did not improve PYR dissipation, but the simultaneous addition of both agents at the higher rate of the cyclodextrin (5%) succeeded in obtaining a final PYR relative content of 51.8%. Hence, it can be concluded that both, a high HPBCD rate (5%), which seems to have mobilized a fraction of PYR that initially was not bioavailable, and bioaugmentation with the PYR microbial degrader consortium used, are necessary to get PYR dissipation in this creosote polluted soil.

BaANT Control treatment showed low amount dissipated (11%), and the degrader consortium addition did not improve dissipation. In contrast, HPBCD addition was successful, decreasing BaANT content at about 70%. The combined addition of the degrader consortium and HPBCD did not improve BaANT dissipation compared with HPBCD alone.

In the case of CHR, there is no significant difference among the dissipation extents observed for the different treatments and the Control, with no influence of HPBCD or the degrader consortium. At the end of the treatments about 30% of CHR is dissipated in all cases.

As described above, 4-ring PAHs showed, in general, higher degradation percentages than their corresponding estimated bioavailable fractions (Table 3). It agrees with Doick et al. (2006), who also observed that HPBCD extractions slightly under-estimated

pyrene mineralization. It can be related to the fact that the hydrophobic cavity of HPBCD is too small for a good complexation of larger molecules such as 4-ring PAHs, and of course 5- and 6-ring PAHs.

Dissipation results for 5-ring PAHs are in general very low, in agreement with the very low bioavailable content (Table 3). Control treatment did not show any degradation, whereas HP 1% showed the best results that ranged from 8-21%. A higher rate of HPBCD or degrader consortium addition did not improve PAHs degradation, and these treatments showed dissipation rates even lower than HP 1%. Dissipation results for BPER and IND (6-ring PAH) were negligible for all treatments, what agrees with their bioavailable content, that was below the detection limit of the method used (Table 3).

In general, the best results of MC addition have been observed for the high rate of HPBCD. In case of no HPBCD addition or low rate the consortium did not enhance PAHs degradation observed in the Control treatment. This could indicate that a high rate of HPBD is necessary to increase the original PAHs bioavailable fraction, allowing the consortium to access a larger quantity of pollutant to be degraded. This is in agreement with the results in Table 3, which indicate that only with HP5% a bioavailability enhancement could be observed for 3- and 4-ring PAHs, but not for 5- and 6-ring. 4-ring PAHs showed the best degradation rates, probably because the consortium was obtained with PYR (4 rings) as the only source of carbon, and, according to Wu et al. (2010), it is also due to their higher contents in relation with the other PAHs in this soil.

Data for treatments with rhamnolipid addition are shown as supporting information in Table S1. Although RL amended soil enhanced significantly PAH bioavailability (Table 3), these treatments not only did not enhance PAHs dissipation, but even inhibited the

scarce degradation observed in the control treatment. RLs generally increase desorption and bioavailability of PAHs and hydrophobic compounds, but their effect on PAH degradability is contradictory. Some authors have described increases in PAHs dissipation (Gotfried et al., 2010; Wang et al., 2016), while others have reported no effect or even inhibition of PAH degradation (Szczepaniak et al., 2016; Adrion et al., 2016). Degradation is frequently related with solubilisation, desorption and/or increase in enzymatic activities, whereas inhibition is associated with toxicity, disruption of bacterial membrane or changes of cell surface hydrophobicity (Liang et al., 2017). RL antibiotic activity against some bacteria and fungus has also been recently described (Patoway et al., 2017). Shin et al. (2005) described different toxic mechanisms of the same RL used in the present study, JBR-425, to different phenanthrene degrading strains in liquid culture media. At the conditions used in this assay, with a higher RL JBR-425 rate added than that used by Shin et al. (2005), negative effects on microbiota would have been the likely cause of the negligible effect of RL on PAHs biodegradation, although this RL had been reported to increase PAH extraction in this soil (Madrid et al., 2019). Two possibilities could be responsible of these results: 1) JBR-425 is toxic as it has been discussed above, or 2) increases in PAHs/PTEs availability in this polluted soil after RL treatment are so important that these pollutants are toxic for the microorganisms responsible of PAHs degradation. High PAHs bioavailability due to RL treatments has been previously demonstrated as shown in Table 3, and the other possibilities are discussed below.

3.3. Potentially Toxic Elements availability

Increases in PTE availability could increase their mobility through the soil profile endangering groundwater or having a toxic effect on soil microorganisms. Unlike PAHs, PTE cannot be degraded during incubation, so differences in their contents are not expected, but differences in availability could be observed due to the different treatments applied (HPBCD or RL).

Available (EDTA extractable) contents of PTE in the contaminated soil under study before HPBCD or RL treatments (Control) at the beginning of the incubation experiment are shown in Table 5. These data are typical of a highly contaminated soil with medium-high metal availability. PTEs availability one day after application of HPBCD treatments is slightly higher than that of the Control, but is the same for both HPBCD concentrations used (1 and 5%). Interaction between PTE and HPBCD in soil systems is not clear. Some authors have reported no effect on PTE extractability (Khodadoust et al., 2005), whereas other ones have found modest increments of Pb and Cu recoveries (Ehsan et al., 2007). At the end of the incubation with HPBCD, PTE availability decreased, with values more similar to those of the unamended soil. It seems that the PTE-HPBCD interaction is not a lasting effect, maybe due to the biodegradable character of this amendment. In this way, it is not expected that HPBCD may mobilize PTE during PAHs bioremediation, what would endanger groundwater quality.

Unlike HPBCD, interactions among the rhamnolipid and many PTEs have been described, and conditional stability constants for these complexes have been reported (Tan et al., 1994; Ochoa-Loza et al., 2001; Hogan et al., 2017). Even more, RL has been used as mobilizing agent for metal removal in soils and sediments in washing and electrokinetic treatments (Herman et al., 1995; Lu et al., 2017; Tian et al., 2017). RL mainly mobilize metals in exchangeable, carbonate-bound or Fe-Al-Mn oxide-bound fractions (Chen et al., 2017).

In the case of RL, one day after treatments metal availability was higher than in the control, and the increment was much higher when the concentration of RL increased to 5%, especially for Cr and Ni, with increments in availability of 468% and 191%, but also for Cu and As (67% and 75% increased availability).

After 120 days of incubation, the observed PTE availability depended on each element. For Pb, Zn and Cd availability decreased, reaching values close to those in the control. On the contrary, for Cr, Ni, As and Cu availability remained higher than in the Control treatment after 120 days, with higher increases found for RL5% treatment (213%, 109%, 56% and 29%, respectively). The results obtained are in agreement with those previously found in Madrid et al. (2019). Although there are differences because in that study the availability was measured after the extraction of part of PTEs from the soil, the remaining PTEs also presented high increases in the availability of Cr, Ni and As. The increases in availability in this soil may imply a mobilizing effect of the PTEs, and probably it causes in part the toxicity that prevents the biodegradation of PAHs that should be carried out by the microbial population. Moreover, it would be a limiting aspect which has to be controlled in order not to endanger the quality of groundwater.

3.4. Toxicity bioassays

Toxicity analysis with *A. fischeri* applies to any kind of liquid waste discharged in natural waters and, by extension, also to leachates/percolates from waste dumps and from contaminated soils. Toxicity assessment of the leachates (Table 6) from this contaminated soil after the application of different amendments can provide a direct assessment of the hazard to the microbial flora. Concerning the hazard Toxic Units (TU) developed by Persoone et al. (2003), leachates from this creosote-polluted soil before

amendment treatments (Control, day 1) could be classified as belonging to Class III, presenting Acute toxicity ($1 < TU < 10$), which does not change after HPBCD (1% and 5%) treatment nor after 120 days of incubation. The leachates coming from soils treated with RL1% were Class IV, showing high acute toxicity ($10 < TU < 100$), which decreased to Acute toxicity after 120 days of incubation. The toxicity changed to Class V ($TU > 100$) when using RL5% (very high acute toxicity), and decayed to High acute toxicity after incubation. The higher toxicity in RL treated soil could be related to an increased PAHs bioavailability and also PTEs available content, because both contaminants are more available after RL amendment (Tables 3 and 5). Toxicity of rhamnolipid extracts obtained from creosote-contaminated soil have been also observed by Sydow et al. (2018). In general, it can be concluded that the treated soil after 120 days exhibited less toxicity towards *A. fischeri* (higher EC_{50} values) in all the cases. Moreover, the toxicity of the RLs itself should also be taken into consideration, as previously mentioned (Shin et al., 2005; Rodrigues et al., 2017). An experiment was carried out to check this possibility using natural soils with different characteristics not contaminated by organic contaminants or PTEs. Results obtained are shown in Table S2. Only two of the four soils studied increased their toxicity when using RL 1%, but the four soils presented ‘very high acute toxicity’ (Persoone et al., 2003) when the concentration used was 5%, indicating that the toxicity of the biosurfactant should be also considered as an important factor.

In parallel with *A. fischeri* toxicity assays, cell numbers were enumerated (colony forming units, CFUs) after different incubations periods for the HP and RL treatments (Figure 2).

The number of CFUs in the contaminated soil (Control, Figure 2a) decayed completely after RL5% application (Figure 2b), eliminating any microbial activity in the soil. On

the contrary, the CFU after HP5% treatment (Figure 2c) is quite similar to the Control, indicating little toxic effects towards microbial population. These results are in good agreement with the previous ecotoxicity studies using *A. fischeri*. However, when RL was applied in conjunction with the MC, the microbial activity was not completely eliminated (Figure 2d), but PAH biodegradation did not occur either. Although the toxicity was enhanced as RL concentration increased, it was not able to inhibit the MC growth, probably due to its inoculation at very high rates (Cycon et al., 2017). Despite the microbial activity of MC was not completely eliminated due to the presence of RL, PAHs were not degraded at all. One possible explanation involves stimulation of the bacterial growth in MC that degrade preferably RL which serve as a primary carbon source instead of PAHs, as it has been previously described by Chrzanowski et al. (2012) and Lawniczak et al. (2013), because RL can be rapidly biodegraded under aerobic conditions.

4. CONCLUSIONS

The majority of remediation studies on contaminated soils are carried out to remove only organic or inorganic contaminants, but the effect of the selected treatment on the rest of other contaminants present in the soil is frequently overlooked. In the present study the effect of two biodegradation treatments, using a cyclodextrin (HPBCD) or rhamnolipids (RL) combined with a microbial consortium, were applied to degrade PAHs in an industrial soil co-contaminated with PTEs. HPBCD addition was effective for the degradation of 3- and 4-ring PAHs, but the availability of PTEs was not affected, demonstrating its feasibility to accelerate their biodegradation in PAHs + PTE co-contaminated soils. On contrary, RL addition to the same soil prevented PAH

degradation and increased PTE metal availability. RL produced a high extraction not only of PAHs but also of PTEs, giving as a result an increased toxicity in the treated soil which inhibited the PAH bioremediation process. Results obtained from this investigation indicated that RLs are not a good choice as extractant in such co-contaminated soils. Research to elucidate if the toxic effects derived from RL application are related to the extremely high availability of only PAHs or PTEs, or PAHs + PTEs is currently being performed, as well as the specific role of RL as a toxic agent.

5. ACKNOWLEDGEMENTS

The authors are indebted to ADIF (Ministerio de Fomento) for facilitating the sample collection of creosote-polluted soil and for the information supplied. This work was supported by the Spanish Ministry of Economy and Competitiveness (co-funded by the Fondo Europeo de Desarrollo Regional, FEDER), under the research project CTM2013-42599-R.

References

- Adrion, A.C., Nakamura, J., Shea, D., Aitken, M.D., 2016. Screening nonionic surfactants for enhanced biodegradation of polycyclic aromatic hydrocarbons remaining in soil after conventional biological treatment. *Environ. Sci. Technol.* 50, 3838-3845. DOI: 10.1021/acs.est.5b05243.
- Alvarenga, P., Palma, P., Gonçalves, A.P., Baiao, N., Fernandes, R.M., de Varennes, A., Vallini, G., Duarte, E., Cunha-Queda, A.C., 2008. Assessment of chemical,

549 biochemical and ecotoxicological aspects in a mine soil amended with sludge of either
 550 urban or industrial origin. *Chemosphere* 72, 1774-1781. DOI:
 551 10.1016/j.chemosphere.2008.04.042.

552 Bezza, F.A., Chirwa, E.M.N., 2016. Biosurfactant-enhanced bioremediation of aged
 553 polycyclic aromatic hydrocarbons (PAHs) in creosote contaminated soil. *Chemosphere*
 554 144, 635-644. DOI: 10.1016/j.chemosphere.2015.08.027.

555 Bezza, F.A., Chirwa, E.M.N., 2017. The role of lipopeptide biosurfactant on microbial
 556 remediation of aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil.
 557 *Chem. Eng. J.* 309, 563-576. DOI: 10.1016/j.cej.2016.10.055.

558 Chen, W., Qu Y., Xu Z., He F., Chen Z., Huang S., Li Y., 2017. Heavy metal (Cu, Cd,
 559 Pb, Cr) washing from river sediment using biosurfactant rhamnolipid. *Environ Sci.*
 560 *Pollut. Res.* 24, 16344–16350. DOI: 10.1007/s11356-017-9272-2.

561 Chrzanowski, L., Dziadas, M., Ławniczak, L., Cyplik, P., Białas, W., Szulc, A.,
 562 Lisiecki, P., Jelen, H. 2012. Biodegradation of rhamnolipids in liquid cultures: Effect of
 563 biosurfactant dissipation on diesel fuel/B20 blend biodegradation efficiency and
 564 bacterial community composition. *Biores. Technol.* 111, 328-335. DOI:
 565 10.1016/j.biortech.2012.01.181.

566 Cvetkovic, J.S., Mitic, V.D., Stankov Jovanovic, V. P., Dimitrijevic, M. V., Petrovic, G.
 567 M., Nikolic-Mandic S.D., Stojanovic G.S. 2016. Optimization of the QuEChERS
 568 extraction procedure for the determination of polycyclic aromatic hydrocarbons in soil
 569 by gas chromatography-mass spectrometry. *Anal. Methods* 8, 1711-1720. DOI:
 570 10.1039/c5ay03248b.

571 Doick K.J., Clasper P.J., Urmann K., Semple K.T., 2006. Further validation of the
 572 HPCD-technique for the evaluation of PAH microbial availability in soil. Environ.
 573 Pollut. 144, 345–354. DOI: 10.1016/j.envpol.2005.10.054.

574 Dong W., He C., Li Y., Huang C., Chen F., Ma Y., 2017. Complete genome sequence
 575 of a versatile hydrocarbon degrader, *Pseudomonas Aeruginosa* DN1, isolated from
 576 petroleum-contaminated soil. Gene Reports 7, 123-126. DOI:
 577 10.1016/j.genrep.2017.04.001.

578 Ehsan S., Prasher S.O., Marshall W.D., 2007. Simultaneous mobilization of heavy
 579 metals and polychlorinated biphenyl (PCB) compounds from soil with cyclodextrin and
 580 EDTA in admixture. Chemosphere 68, 150-158. DOI:
 581 10.1016/j.chemosphere.2006.12.018.

582 Fenyvesi E., Gruiz K., Verstichel S., De Wilde B., Leitgib L., Csabai K., Szaniszlo N.,
 583 2005. Biodegradation of cyclodextrins in soil. Chemosphere 60, 1001–1008. DOI:
 584 10.1016/j.chemosphere.2005.01.026.

585 Flaherty, R.J., Nshime, B., Delamarre, M., Dejong, S., Scott, P., Lantz, A.W., 2013.
 586 Cyclodextrins as complexation and extraction agents for pesticides from contaminated
 587 soils. Chemosphere 91, 912–920. DOI: 10.1016/j.chemosphere.2013.02.005.

588 Garbisu C., Garaiyurrebaso O., Epelde L., Grohmann E., Alkorta I., 2017. Plasmid-
 589 mediated bioaugmentation for the bioremediation of contaminated soils. Front.
 590 Microbiol. 8, 1966. DOI: 10.3389/fmicb.2017.01966.

591 Gottfried, A., Singhal, N., Elliot, R., Swift, S., 2010. The role of salicylate and
 592 biosurfactant in inducing phenanthrene degradation in batch soil slurries. Appl.
 593 Microbiol. Biotechnol. 86, 1563-1571. DOI: 10.1007/s00253-010-2453-2.

594 Haritash A.K., Kaushik C.P., 2009. Biodegradation aspects of Polycyclic Aromatic
 595 Hydrocarbons (PAHs): A review. J. Hazard. Mater. 169, 1-15. DOI:
 596 10.1016/j.jhazmat.2009.03.137.

597 Hazra, C., Kundu, D., Chaudhari, A., 2012. Biosurfactant-assisted bioaugmentation in
 598 bioremediation. In Microorganisms in Environmental Management: Microbes and
 599 Environment. Eds: Satyanarayana, T., Johri, B.N., Anil P., Springer, Netherlands, pp.
 600 631–664.

601 Herman D.C., Artiola J.F., Miller R.M., 1995. Removal of cadmium lead, and zinc from
 602 soil by a rhamnolipid biosurfactant. Environ. Sci. Technol. 29, 2280–2285. DOI:
 603 10.1021/es00009a019.

604 Hogan D.E., Curry J.E., Pemberton J.E., Maier R.M., 2017. Rhamnolipid biosurfactant
 605 complexation of rare earth elements. J. Hazard. Mater. 340, 171-178. DOI:
 606 10.1016/j.jhazmat.2017.06.056.

607 Husein S., 2008. Identification of pyrene-degradation pathways: Bench-scale studies
 608 using *Pseudomonas fluorescens* 29L. Remediation 18, 119-142. DOI:
 609 10.1002/rem.20176.

610 Kalantary RR., Mohseni-Bandpi A., Esrafil A., Nasser S., Ashmagh F.R., Jorfi S.,
 611 Jafari M., 2014. Effectiveness of biostimulation through nutrient content on the
 612 bioremediation of phenanthrene contaminated soil. J. Environ. Health Sci. Eng. 12(1),
 613 1. DOI: 10.1186/s40201-014-0143-1.

614 Khan, M., Cheema, S., Shen, C., Zhang, C., Tang, X., Malik, Z., 2011. Assessment of
 615 pyrene bioavailability in soil by mild hydroxypropyl- β -cyclodextrin extraction. Arch.
 616 Environ. Contam. Toxicol. 60, 107–115. DOI: 10.1007/s00244-010-9517-2.

617 Khodadoust A.P., Reddy K.R., Maturi K., 2005. Effect of different extraction agents on
 618 metal and organic contaminant removal from a field soil. *J. Hazard. Mat.* 117, 15-24.
 619 DOI: 10.1016/j.jhazmat.2004.05.021.

620 Kuppusamy S., Thavamani P., Venkateswarlu K., Lee Y.B., Naidu R., Megharaj M.,
 621 2017. Remediation approaches for polycyclic aromatic hydrocarbons (PAHs)
 622 contaminated soils: Technological constraints, emerging trends and future directions.
 623 *Chemosphere* 168, 944-968. DOI: 10.1016/j.chemosphere.2016.10.115.

624 Lal, V., Peng, C., Ng, J., 2015. A review of non-exhaustive chemical and bioavailability
 625 methods for the assessment of polycyclic aromatic hydrocarbons in soil. *Environmental*
 626 *Technology and Innovation* 4, 159-167. DOI: 10.1016/j.eti.2015.07.001.

627 Ławniczak, L., Marecik, R., Chrzanowski, L. 2013. Contributions of biosurfactants to
 628 natural or induced bioremediation. *Appl. Microbiol. Biotechnol.* 97, 2327-2339. DOI
 629 10.1007/s00253-013-4740-1.

630 Liang X., Guo C., Liao C., Liu S., Wick L.Y., Peng D., Yi X., Lu G., Yin H., Lin Z.,
 631 Dang Z., 2017. Drivers and applications of integrated clean-up technologies for
 632 surfactant-enhanced remediation of environments contaminated with polycyclic
 633 aromatic hydrocarbons (PAHs). *Environ. Pollut.* 225, 129-140. DOI:
 634 10.1016/j.envpol.2017.03.045.

635 Lladó, S., Covino, S., Solanas, A.M., Petruccioli, M., D'annibale, A., Viñas, M., 2015.
 636 Pyrosequencing reveals the effect of mobilizing agents and lignocellulosic substrate
 637 amendment on microbial community composition in a real industrial PAH-polluted soil.
 638 *J. Hazard. Mater.* 283, 35-43. DOI: 10.1016/j.jhazmat.2014.08.065.

Lu Y., Luo D., Liu L., Tan Z., Lai A., Liu G., Li J., Long J., Huang X., Chen Y., 2017. Leaching variations of heavy metals in chelator-assisted phytoextraction by *Zea mays* L. exposed to acid rainfall. *Environ. Sci. Pollut. Res.* 24, 24409–24418. DOI: 10.1007/s11356-017-0065-4.

Madrid F., Rubio-Bellido M., Villaverde J., Tejada M., Morillo E., 2016. Natural attenuation of fluorene and pyrene in contaminated soils and assisted with hydroxypropyl- β -cyclodextrin. Effect of co-contamination. *Sci. Total Environ.* 571, 42-49. DOI: 10.1016/j.scitotenv.2016.07.110.

Madrid, F., Ballesteros, R., Lacorte, S., Villaverde, J., Morillo, E. 2019. Effect of cyclodextrins and rhamnolipids on the extraction of PAHs and potentially toxic elements from an aged creosote-polluted soil. *Sci. Total Environ.* 653, 384-392. DOI: 10.1016/j.scitotenv.2018.10.316.

Manzano, R., Esteban, E., Peñalosa, J.M., Alvarenga, P., 2014. Amendment application in a multi-contaminated mine soil: effects of soil enzymatic activities and ecotoxicological characteristics. *Environ. Sci. Pollut. Res.* 21, 4539-4550. DOI: 10.1007/s11356-013-2412-4.

Mohanty, S., Mukherji, S., 2013. Surfactant aided biodegradation of NAPLs by *Burkholderia multivorans*: comparison between Triton X-100 and rhamnolipid JBR-515. *Colloids Surf. B: Biointerfaces.* 102, 644–652. DOI: 10.1016/j.colsurfb.2012.08.064.

Morillo, E., Sánchez-Trujillo, M.A., Moyano, J.R., Villaverde J., Gómez-Pantoja, M.E., Pérez-Martínez, J.I., 2012. Enhanced solubilisation of six PAHs by three synthetic cyclodextrins for remediation applications: Molecular modelling of the inclusion complexes. *Plos One* 7 (9) e44137. DOI: 10.1371/journal.pone.0044137.

663 Morillo, E., Sánchez-Trujillo, M.A., Villaverde, J., Madrid, F., Undabeytia, T., 2014.
 664 Effect of contact time and the use of hydroxypropyl- β -cyclodextrin in the removal of
 665 fluorene and fluoranthene from contaminated soils. *Sci. Total Environ.* 496, 144–154.
 666 DOI: 10.1016/j.scitotenv.2014.07.027.

667 Morillo, E., Villaverde, J., 2017. Advanced technologies for the remediation of
 668 pesticide-contaminated soils. *Sci. Total Environ.* 586, 576-597. DOI:
 669 10.1016/j.scitotenv.2017.02.020.

670 Mueller J.G., Chapman P.J., Pritchard P.H., 1989. Creosote-contaminated sites. Their
 671 potential for bioremediation. *Environ. Sci. Technol.* 23, 1197–1201. DOI:
 672 10.1021/es00068a003.

673 Ochoa-Loza F.J., Artiola J.F., Maier R.M., 2001. Stability constants for the
 674 complexation of various metals with a rhamnolipid biosurfactant. *J. Environ. Qual.* 30,
 675 479–485. DOI:10.2134/jeq2001.302479x.

676 Ozdemir, G., Malayoglu, U. 2004. Wetting characteristics of aqueous rhamnolipids
 677 solutions. *Colloids Surf. B* 39, 1-7. DOI: 10.1016/j.colsurfb.2004.08.006.

678 Paria, S. 2008. Surfactant-enhanced remediation of organic contaminated soil and water.
 679 *Adv. Colloid Interf.* 138, 24–58. DOI: 10.1016/j.cis.2007.11.001.

680 Patowary, K., Patowary R., Kalita M.C., Deka S., 2017. Characterization of
 681 biosurfactant produced during degradation of hydrocarbons using crude oil as sole
 682 source of carbon. *Frontiers in Microbiol.* 8, 279. DOI: 10.3389/fmicb.2017.00279.

683 Persoone, G., Marsalek, B., Blinova, I., Törökne, A., Zarina, D., Manusadzianas, L.,
 684 Nalecz-Jawecki, G., Tofan, L., Stepanova, N., Tothova, L., Kolar, B., 2003. A practical

685 and user-friendly toxicity classification system with microbiotests for natural waters and
 686 wastewaters. *Environ. Toxicol.* 18, 395-402. DOI: 10.1002/tox.10141.

687 Realp E., Doménech J.A., Martínez-García R., Restrepo C., LLadó S., Viñas M.,
 688 Solanas A.M. 2008. Ensayo piloto de biorremediación por la tecnología de la biopila
 689 dinámica para la descontaminación de suelos contaminados por creosotas provenientes
 690 de las actividades dedicadas a la preparación de la madera. *Residuos.* 103. 38-49 (In
 691 Spanish).

692 Rodrigues A.I., Gudiña E.J., Teixeira, J.A., Rodrigues, L.R. 2017. Sodium chloride
 693 effect on the aggregation behaviour of rhamnolipids and their antifungal activity.
 694 *Scientific Reports* 7, Article number 12907 (Open Access). DOI: 10.1038/s41598-017-
 695 13424-x.

696 Rubio-Bellido M., Morillo E., Villaverde J., 2018. Assessment of soil diuron
 697 bioavailability to plants and microorganisms through non-exhaustive chemical
 698 extractions of the herbicide. *Geoderma* 312, 130–138. DOI:
 699 10.1016/j.geoderma.2017.09.031.

700 Sánchez-Trujillo, M.A., Lacorte, S., Villaverde, J., Barata, C., Morillo, E., 2014.
 701 Decontamination of polycyclic aromatic hydrocarbons and nonylphenol from sewage
 702 sludge using hydroxypropyl- β -cyclodextrin and evaluation of the toxicity of lixiviates.
 703 *Environ. Sci. Pollut. Res.* 21, 507–517. DOI: 10.1007/s11356-013-1930-4.

704 Sánchez-Trujillo, M.A., Morillo, E., Villaverde, J., Lacorte, S. 2013. Comparative
 705 effects of several cyclodextrins on the extraction of PAHs from an aged contaminated
 706 soil. *Environ. Pollut.* 178, 52–58. DOI: 10.1016/j.envpol.2013.02.029.

707 Semple K.T., Morriss A.W.J., Paton G.I., 2003. Bioavailability of hydrophobic organic
 708 contaminants in soils: fundamental concepts and techniques for analysis. Eur. J. Soil
 709 Sci. 54, 809-818. DOI: 10.1046/j.1351-0754.2003.0564.x.

710 Shavandi, M., Mohebbi, G., Haddadi, A., Shakarami, H., Nuhi, A., 2011.
 711 Emulsification potential of a newly isolated biosurfactant-producing bacterium,
 712 *Rhodococcus* sp. Strain TA6. Colloids Surf. B: Biointerfaces 82, 477-482. DOI:
 713 10.1016/j.colsurfb.2010.10.005.

714 Shi, W., Zhang, X.N., Jia, H.B., Feng S.D., Yang Z.X., Zhao, O.Y., Li, Y.L., 2017.
 715 Effective remediation of aged HMW-PAHs polluted agricultural soil by the
 716 combination of *Fusarium* sp. and smooth brome grass (*Bromus inermis* Leyss.). J.
 717 Integrative Agr. 16(1), 199-209. DOI: 10.1016/S2095-3119(16)61373-4.

718 Shin, K.H., Ahn, Y., Kim, K.W., 2005. Toxic effect of biosurfactant addition on the
 719 biodegradation of phenanthrene. Environ. Toxicol. Chem. 24: 2768-2774. DOI:
 720 10.1897/05-071R1.1

721 Sopena, F., Laiz, L., Morillo, E., Sanchez-Trujillo, M.A., Villaverde, J., Jurado, V.,
 722 Saiz-Jimenez, C. 2014. Phenanthrene biodegradation by *Pseudomonas xanthomarina*
 723 isolated from an aged contaminated soil. Clean - Soil, Air, Water 42, 785-790. DOI:
 724 10.1002/clen.201300247.

725 Stroud, J.L., Tzima, M., Paton, G.I., Semple, K.T., 2009. Influence of hydroxypropyl- β -
 726 cyclodextrin on the biodegradation of ¹⁴C-phenanthrene and ¹⁴C-hexadecane in soil.
 727 Environ. Pollut. 157, 2678–2683. DOI: 10.1016/j.envpol.2009.05.009.

728 Sydow, Z., Lisiecki, P., Staninska-Pięta, J., Olejnik, A., Cyplik, P., Zgoła-Grześkowiak,
 729 A., Marecik, R., Nowak, J., Glazar, K., Piotrowska-Cyplik, A. Multidimensional

730 toxicity of rhamnolipid extracts obtained from creosote-contaminated soil. *Clean - Soil,*
731 *Air, Water* 46, Article number 1800053. DOI: 10.1002/clen.201800053.

732 Szczepaniak, Z., Czarny, J., Staninska-Pięta, J., Lisiecki, P., Zgoła-Grzeskowiak, A.,
733 Cyplik, P., Chrzanowski, Ł., Wolko, Ł., Marecik, R., Juzwa, W., Glazar, K.,
734 Piotrowska-Cyplik, A., 2016. Influence of soil contamination with PAH on microbial
735 community dynamics and expression level of genes responsible for biodegradation of
736 PAH and production of rhamnolipids. *Environ. Sci. Pollut. Res.*, 23, 23043-23056. DOI:
737 10.1007/s11356-016-7500-9.

738 Szulc, A., Ambrozewicz, D., Sydow, M., Lawniczak, L., Piotrowska-Cyplik, A.,
739 Marecik, R., Chrzanowski, L., 2014. The influence of bioaugmentation and bio-
740 surfactant addition on bioremediation efficiency of diesel-oil contaminated soil:
741 feasibility during field studies. *J. Environ. Manag.*, 132: 121–128. DOI:
742 10.1016/j.jenvman.2013.11.006.

743 Tan H., Champion J.T., Artiola J.F., Brusseau M.L., Miller R.M., 1994. Complexation
744 of cadmium by a rhamnolipid biosurfactant. *Environ. Sci. Technol.*, 28, 2402–2406.
745 DOI: 10.1021/es00062a027.

746 Tian Y., Boulangé-Lecomte C., Benamar A., Giusti-Petrucciani N., Duflot A., Olivier
747 S., Frederick C., Forget-Leray J., Portet-Koltalo F., 2017. Application of a crustacean
748 bioassay to evaluate a multi-contaminated (metal, PAH, PCB) harbour sediment before
749 and after electrokinetic remediation using eco-friendly enhancing agents. *Sci. Tot.*
750 *Environ.* 607-608, 944-953. DOI: 10.1016/j.scitotenv.2017.07.094.

751 Trellu, C., Mousset, E., Pechaud, Y., Huguenot, D., Van Hullebusch, E.D., Esposito, G.,
752 Oturan, M.A., 2016. Removal of hydrophobic organic pollutants from soil

753 washing/flushing solutions: a critical review. *J. Hazard. Mater.*, 306, 149-174. DOI:
 754 10.1016/j.jhazmat.2015.12.008.

755 Villaverde, J., Maqueda, C., Morillo, E., 2006. Effect of the simultaneous addition of β -
 756 cyclodextrin and the herbicide norflurazon on its adsorption and movement in soils. *J.*
 757 *Agric. Food Chem.* 54: 4766–4772. DOI: 10.1021/jf060548a

758 Villaverde, J. Posada-Baquero, R., Rubio-Bellido, M., Laiz, L., Saiz-Jimenez, C.,
 759 Sanchez-Trujillo, M.A., Morillo, E., 2012., Enhanced mineralization of diuron using a
 760 cyclodextrin-based bioremediation technology. *J. Agric. Food Chem.* 60, 9941-9947.
 761 DOI: 10.1021/jf3021909.

762 Villaverde, J., Rubio-Bellido, M., Merchán, F., Morillo, E., 2017. Bioremediation of
 763 diuron contaminated soils by a novel degrading microbial consortium. *J. Environ.*
 764 *Manag.* 188, 379-386. DOI: 10.1016/j.jenvman.2016.12.020.

765 Villaverde, J., Rubio-Bellido, M., Lara-Moreno, A., Merchán, F., Morillo, E., 2018.
 766 Combined use of microbial consortia isolated from different agricultural soils and
 767 cyclodextrin as a bioremediation technique for herbicide contaminated soils.
 768 *Chemosphere* 193, 118-125. DOI: 10.1016/j.chemosphere.2017.10.172.

769 Von Lau, E., Gan, S., Kiat Ng, H., Eong Poh, P. 2014. Extraction agents for the removal
 770 of polycyclic aromatic hydrocarbons (PAHs) from soil in soil washing technologies.
 771 *Environ. Pollut.* 184, 640-649. DOI: 10.1016/j.envpol.2013.09.010.

772 Wang, L., Li, F., Zhan, Y., Zhu, L., 2016. Shifts in microbial community structure
 773 during in situ surfactant-enhanced bioremediation of polycyclic aromatic hydrocarbon-
 774 contaminated soil. *Environ. Sci. Pollut. Res.*, 23, 14451-14461. DOI: 10.1007/s11356-
 775 016-6630-4.

776 Wei R., Ni J., Li X., Chen W., Yang Y., 2017. Dissipation and phytoremediation of
777 polycyclic aromatic hydrocarbons in freshly spiked and long-term field-contaminated
778 soils. *Environ. Sci. Pollut. Res.* 24, 7994-8003. DOI: 10.1007/s11356-017-8459-x.

779 Wu J., Gong Z., Zheng L., Yi Y., Jin J., Li X., Li P., 2010. Removal of high
780 concentrations of polycyclic aromatic hydrocarbons from contaminated soil by
781 biodiesel. *Frontiers of Environ. Sci. Engin. China*, 4, 387-394. DOI: 10.1007/s11783-
782 010-0269-z.

783 Xia, W., Du, Z., Cui, Q., Dong, H., Wang, F., He, P., Tang, Y., 2014. Biosurfactant
784 produced by novel *Pseudomonas* sp. WJ6 with biodegradation of n-alkanes and
785 polycyclic aromatic hydrocarbons. *J. Hazard. Mater.* 276, 489–498. DOI:
786 10.1016/j.jhazmat.2014.05.062.

787

788 **Figure legends**

789 Figure 1. Evolution of 4-ring PAHs (Pyrene, Fluoranthene, Benzo(a)anthracene,
790 Chrysene) during the incubation of the creosote-contaminated soil under different
791 remediation treatments using HPBCD.

792 Figure 2. Evolution of the colony forming units (CFUs) through the incubation period
793 under different treatments.

Graphical abstract

Fig. 1. Evolution of 4-ring PAHs (Pyrene, Fluoranthene, Benzo(a)anthracene, Chrysene) during the incubation of the creosote-contaminated soil under different remediation treatments using HPBCD.

Fig. 2. Evolution of the colony forming units (CFUs) through the incubation period under different treatments.

Table 1. Treatments applied in [soil remediation](#).

Treatment	Description
Control (C)	Soil amended with nutrient solution (NS)
Microbial Consortium (MC)	Soil amended with MC and NS
HP 5%	Soil amended with HPBCD at 5% and NS
HP 1%	Soil amended with HPBCD at 1% and NS
HP 5% + MC	Soil amended with HPBCD at 5%, NS and MC
HP 1% + MC	Soil amended with HPBCD at 1%, NS and MC
RL 5%	Soil amended with JBR-425 at 5% and NS
RL 1%	Soil amended with JBR-425 at 1% and NS
RL 5% + MC	Soil amended with JBR-425 at 5%, NS and MC
RL 1% + MC	Soil amended with JBR-425 at 1%, NS and MC

Table 2. Content of PAHs in the contaminated soil (mg kg⁻¹) and percentage of individual PAHs related to Σ 16 PAHs (determined after exhaustive extraction). Standard deviation in parenthesis.

PAHs	mg kg ⁻¹	%
Naphtalene (NAP)	1.72 (0.15)	0.41 (0.04)
Acenaphthylene (ACL)	5.20 (0.70)	1.26 (0.17)
Acenaphthene (ACE)	0.78 (0.22)	0.19 (0.05)
Fluorene (FLU)	0.57 (0.12)	0.14 (0.03)
Phenanthrene (PHE)	7.04 (2.04)	1.70 (0.50)
Anthracene (ANT)	10.1 (0.36)	2.44 (0.09)
Fluoranthene (FLT)	53.9 (4.70)	13.0 (1.13)
Pyrene (PYR)	46.1 (2.50)	11.1 (0.60)
Benzo(a)anthracene (BaANT)	24.5 (1.71)	5.92 (0.41)
Chrysene (CHR)	45.1 (5.78)	10.9 (1.40)
Benzo(b)fluoranthene (BbFLT)	77.0 (15.0)	18.6 (3.67)
Benzo(k)fluoranthene (BkFLT)	18.9 (5.07)	4.56 (1.23)
Benzo(a)pyrene (BaPYR)	32.3 (7.35)	7.80 (1.78)
Dibenzo(a,h)anthracene (DANT)	21.2 (2.66)	5.12 (0.64)
Indeno(1,2,3-cd)pyrene (IND)	36.7 (7.19)	8.86 (1.74)
Benzo(g,h,i)perylene (BPER)	33.0 (5.42)	7.97 (1.31)
Σ 16 PAHs	414.1	100

Values taken from Madrid et al. (2019).

Table 3. Effect of the different amendments used on PAHs bioavailability enhancement compared to the bioavailable PAHs in the Control treatment (all measured one day after amendments treatment). Standard deviation in parenthesis.

PAHs	Control		Bioavailability enhancement			
	mg kg ⁻¹	% [□]	HP1%	HP5%	RL1%	RL5%
NAP	0.053 (0.003)	3.08 ab	0.82 a	0.86 a	1.36 b	5.42 c
ACL	0.015 (0.001)	0.29 a	0.86 a	1.03 a	2.08 b	8.43 c
ACE	0.007 (0.001)	0.90 a	1.07 a	2.76 b	6.21 c	18.4 d
FLU	0.007 (0.001)	1.23 a	0.62 a	2.83 b	3.91 b	13.7 c
PHE	0.009 (0.001)	0.13 a	0.41 a	2.08 b	9.44 c	60.2 d
ANT	0.224 (0.047)	2.22 ab	0.86 a	1.65 ab	1.91 b	4.96 c
FLT	0.386 (0.086)	0.72 a	0.58 a	2.02 b	4.91 c	11.6 d
PYR	0.342 (0.035)	0.74 a	0.71 a	1.58 a	4.93 b	11.1 c
BaANT	0.244 (0.028)	1.00 a	0.82 a	1.69 b	3.10 c	3.11 c
CHR	0.865 (0.046)	1.92 a	0.62 a	1.59 bc	1.91 c	6.40 d
BbFLT	0.414 (0.023)	0.54 a	0.87 a	1.12 a	1.85 b	7.34 c

PAHs	Control		Bioavailability enhancement			
	mg kg ⁻¹	% [□]	HP1%	HP5%	RL1%	RL5%
BkFLT	0.110 (0.011)	0.58 a	0.82 a	1.04 a	1.69 b	6.72 c
BaPYR	0.444 (0.017)	1.37 a	0.89 a	1.00 a	1.64 b	5.17 c
DANT	n.d.	—	n.d.	n.d.	n.d.	n.d.
IND	n.d.	—	n.d.	n.d.	n.d.	n.d.
BPER	n.d.	—	n.d.	n.d.	n.d.	n.d.

n.d. not detected.

The same letter in the same row means no significant differences ($p \leq 0.05$) among treatments.

□

Percentage of bioavailable PAH in relation to their total content in the soil.

Table 4. Percentage of individual PAHs remaining in the contaminated soil after an incubation period of 120 days (relative to their total content determined by exhaustive extraction) using HPBCD (1 and 5%) and/or a microbial consortium (MC) extracted from this contaminated soil. Standard deviation in parenthesis.

PAHs	Control	Control + MC	HP1%	HP1% + MC	HP5%	HP5% + MC
NAP	90.0 (12.4)	97.7 (5.2)	104 (4.1)	102 (6.9)	95.9 (6.2)	98.0 (5.3)
ACL	105 (6.9)	96.8 (6.3)	103 (3.2)	93.2 (7.2)	104 (0.2)	91.4 (1.8)
ACE	101 (0.4)	93.0 (5.3)	98.3 (7.0)	92.6 (5.8)	87.4 (3.5)	96.5 (7.0)
FLU	101 (2.7)	108 (0.2)	98.3 (3.4)	107 (2.5)	70.4 (1.5)	110 (1.6)
PHE	97.2 (4.7)	103 (4.1)	96.2 (8.8)	98.0 (6.0)	96.1 (3.3)	91.4 (0.2)
ANT	99.7 (1.0)	108 (1.5)	89.4 (0.1)	105 (5.1)	105 (4.3)	98.1 (0.9)
FLT	99.0 (5.6)	75.7 (1.6)	64.5 (6.0)	72.3 (5.9)	70.0 (4.7)	56.8 (0.2)
PYR	70.71 (8.0)	75.2 (1.8)	64.2 (9.6)	70.3 (5.2)	70.1 (2.6)	51.8 (1.7)
BaANT	88.7 (6.1)	84.2 (0.7)	68.3 (0.4)	70.8 (6.2)	71.3 (0.6)	65.0 (1.6)
CHR	76.7 (9.4)	69.5 (6.8)	68.9 (2.3)	69.8 (7.3)	63.8 (4.4)	67.9 (5.0)
BbFLT	100 (5.8)	84.4 (6.2)	88.0 (0.5)	95.8 (5.2)	92.7 (4.4)	89.2 (4.0)
BkFLT	100 (3.4)	99.8 (4.7)	78.7 (2.5)	97.4 (7.1)	92.8 (3.0)	97.8 (2.4)
BaPYR	105 (3.1)	96.6 (9.7)	81.5 (7.0)	98.5 (8.8)	101 (3.2)	98.8 (2.7)
DANT	103 (7.3)	99.2 (8.9)	91.7 (1.6)	98.9 (6.3)	94.2 (4.9)	100 (6.1)
IND	102 (9.5)	94.8 (10.2)	93.4 (1.3)	101 (0.6)	96.3 (4.8)	101 (4.0)
BPER	99.2 (6.1)	94.6 (9.2)	96.1 (3.0)	96.1 (6.6)	93.6 (6.5)	98.9 (3.9)

Table 5. Available (EDTA) PTEs (mg kg⁻¹) in the soil after 1 and 120 days of incubation using HPBCD or RL as amendments. Standard deviation in parenthesis.

	Control	HP1%	HP5%	RL1%	RL5%
Day 1					
Cd	0.270 (0.059)	0.327 (0.018)	0.343 (0.065)	0.353 (0.044)	0.388 (0.046)
Cu	35.3 (3.7)	46.7 (3.5)	40.7 (0.4)	46.8 (0.7)	59.0 (1.8)
Pb	266 (17)	303 (16)	299 (2)	343 (6)	302 (24)
Zn	42.3 (6.4)	51.9 (1.0)	51.5 (2.9)	56.3 (0.7)	53.0 (5.4)
As	1.61 (0.37)	1.86 (0.03)	1.56 (0.04)	2.14 (0.19)	2.81 (0.83)
Cr	0.206 (0.029)	0.307 (0.032)	0.267 (0.015)	0.332 (0.025)	1.171 (0.248)
Ni	0.327 (0.074)	0.435 (0.023)	0.438 (0.015)	0.465 (0.026)	0.953 (0.233)
Day 120					
Cd	0.312 (0.002)	0.313 (0.023)	0.312 (0.034)	0.360 (0.052)	0.320 (0.012)
Cu	38.9 (0.2)	39.6 (1.1)	42.2 (2.7)	52.6 (4.9)	50.0 (4.3)
Pb	277 (12)	283 (15)	247 (35)	301 (28)	281 (14)
Zn	36.1 (1.7)	35.8 (2.4)	33.5 (4.1)	40.4 (3.23)	41.0 (2.1)
As	1.29 (0.02)	1.11 (0.28)	1.25 (0.15)	1.64 (0.13)	2.01 (0.21)
Cr	0.277 (0.026)	0.261 (0.024)	0.266 (0.007)	0.467 (0.085)	0.866 (0.025)
Ni	0.301 (0.024)	0.331 (0.065)	0.352 (0.055)	0.479 (0.051)	0.628 (0.009)

Table 6. Acute toxicity test towards *A. fischeri* after 1 and 120 days of incubation of the soil with HPBCD (HP, 1 and 5%) and rhamnolipids (RL, 1 and 5%).

	Day 1			Day 120		
	EC ₅₀	TU	Toxicity ^a	EC ₅₀	TU	Toxicity ^a
Control	12.7	7.9	Acute toxicity	30.7	3.3	Acute toxicity
HP1%	23.1	4.3	Acute toxicity	35.1	2.8	Acute toxicity
HP5%	23.6	4.2	Acute toxicity	25.8	3.9	Acute toxicity
RL1%	6.7	14.8	High acute toxicity	17.1	5.9	Acute toxicity
RL5%	0.17	592	Very high acute toxicity	5.4	18.5	High acute toxicity

TU: Toxic units; EC₅₀: concentration having a toxic effect on 50% of the bacterial population.

^a

According to Persoone et al. (2003).

Supplementary data to this article can be found online at
<https://doi.org/10.1016/j.scitotenv.2018.12.376>