



# Dissipation of a mix of priority PAHs in soils by using availability enhancers. Effect of aging and pollutant interactions



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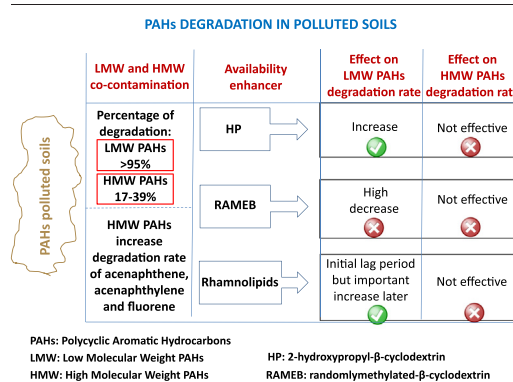
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## HIGHLIGHTS

- More than 95% of 7 PAHs with lower hydrophobicity ( $\log K_{ow} \leq 5.2$ ) degraded after 120d.
- Only 17–39% of 7 PAHs with higher molecular weight degraded after 120d.
- The presence of heavier PAHs increased the degradation rate of only the 3 lightest PAHs.
- Incubation time and soil type are the main factors that influence PAHs dissipation.
- Hydroxypropyl- $\beta$ -CD and rhamnolipid increased degradation rate of only the lightest PAHs.

## GRAPHICAL ABSTRACT



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## ABSTRACT

A remediation strategy using three non-toxic availability enhancers (two cyclodextrins and a rhamnolipid biosurfactant) was applied to various soils artificially contaminated with a mix of Polycyclic Aromatic Hydrocarbons (PAHs) considered priority pollutants at two levels of contamination: only with 7 low molecular weight PAHs (LMW PAHs, 5 with 3-ring and 2 with 4-ring - fluoranthene and pyrene) or with 14 PAHs (from 3 to 6 rings). Natural attenuation of PAHs in all soils showed degradation capacity for the LMW PAHs, with a final content of LMW PAHs <5% of their initial concentration. Conversely, the rest of PAHs (high molecular weight PAHs, HMW) remained in the soils (61% - 83.5%), indicating abiotic dissipation of HMW PAHs due to formation of non-extractable residues in soils. The influence of the presence of HMW PAHs on the degradation of the 7 LMW PAHs was also tested, showing a general decrease in the time to obtain 50% dissipation ( $DT_{50}$ ), statistically significant for acenaphthene, acenaphthylene and fluorene. Availability enhancers showed different effects on PAHs dissipation. 2-hydroxypropyl- $\beta$ -cyclodextrin (HP) decreased  $DT_{50}$  of some of the lighter PAHs, whereas the rhamnolipid (RL) caused a slight  $DT_{50}$  increase due to its initial toxicity on native soil microorganisms, but showing later high degradation rate for LMW PAHs. On the contrary, randomly methylated- $\beta$ -cyclodextrin (RAMEB) slowed down PAHs degradation due to its high adsorption onto soil surface, blocking the desorption of PAHs from the soils. The high number of experimental factors not studied simultaneously before (soil type, co-contamination, availability enhancers and incubation time) allowed to conduct a statistical analysis which supported the conclusions reached. Principal Component Analysis separated the studied PAHs in 3 groups, in relation with their molecular weight and  $K_{ow}$ . The first principal component was related with LMW PAHs, and separate the inefficient RAMEB from the other availability enhancers.

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## 1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a wide number of chemical substances characterized by two or more fused benzene rings. The aromatic structure of these rings causes very stable compounds (Poater et al., 2018), thus, these compounds show a low degradability and high persistence in the environment. They have been classified by the Stockholm Convention as persistent organic pollutants (Stockholm Convention on Persistent Organic Contaminants (POPs), 2011), and were proposed for their prohibition and disposal. PAHs are originated from natural and anthropogenic sources (incomplete wood combustion, petroleum processing industry, volcanic eruptions, etc.). Furthermore, they are easily transported in the atmosphere (Madrid et al., 2016), so they have been detected almost all around the world (Lara et al., 2022). Many PAHs have high capacity to concentrate in living organisms (bioaccumulation) and they are considered mutagenic, carcinogenic, endocrine disruptors and reproductively toxic compounds (Weber et al., 2015) by different international organisms as the European Environment Agency (EEA) and United States Environmental Protection Agency (USEPA), being considered priority pollutants in the different environmental compartments.

According to their molecular weight, PAHs can be grouped in Low Molecular Weight PAHs (LMW, 2–3 rings) and High Molecular Weight PAHs (HMW, 4 or more rings), showing different properties. Overall, PAHs show low polarity and hydrophobic properties, however, LMW PAHs are relatively volatile and easier to be dissipated from soils by volatilization or biodegradation (Madrid et al., 2019a), contrary to HMW PAHs, which tend to get strongly sorbed onto the hydrophobic surface of the soil organic matter (OM) and are more recalcitrant to microbial attack (Leech et al., 2020). This different behavior affects to the capacity to remediate PAHs polluted soils. Many indigenous soil microorganisms have been reported to be capable to degrade LMW PAHs, and hence, bioremediation techniques have been applied to remove these pollutants (Sopeña et al., 2014; Kong et al., 2018; Tian et al., 2019; Villaverde et al., 2019), although most research focuses on the removal of individual compounds. On contrary, HMW PAHs are hardly biodegradable and remediation of soils polluted with these PAHs is usually unsuccessful (Ghosal et al., 2016; Madrid et al., 2019a; Méndez García and García de Llasera, 2021). This difference is mainly attributable to the lower solubility and bioavailability of HMW what provokes that microorganisms cannot access these pollutants to use them as C sources.

The most important process to obtain an increase in organic contaminants biodegradation in soil is their mobilization, in order to increase their bioavailability for soil microorganisms (Semple et al., 2004). For this purpose, extractants and solubilizing agents are used, being the most common synthetic surfactants, organic solvents, emulsifiers, complex-forming agents, micelle-forming agents, dendrimers, etc. (Molnár et al., 2009), but also non-toxic solubility enhancers such as cyclodextrins (CDs) and biosurfactants (BSs) have been increasingly used in the last years (Lau et al., 2014; Molnár et al., 2019). CDs are cyclic oligomers formed by 6 or more glucose units. They show an internal cavity with hydrophobic properties, and a hydrophilic external part (Martín del Valle, 2004; Crini et al., 2018). These characteristics facilitate the formation of inclusion complexes of hydrophobic compounds, what increase their aqueous solubility (Ginés et al., 1996; Pérez-Martínez et al., 2000; Villaverde et al., 2005; Morillo et al., 2014; Sánchez-Trujillo et al., 2014). In this way, CDs have been used frequently to increase bioavailability of hydrophobic pollutants in soil-water systems (Zhang et al., 2022) in order to be applied in bioremediation techniques (Morillo and Villaverde, 2017; Morillo et al., 2020; Lara-Moreno et al., 2021). BSs are biodegradable surface active compounds that have been also used in the last decades to increase solubility and bioavailability of hydrophobic contaminants (Bezza and Chirwa, 2017; Madrid et al., 2019b) by reducing interfacial tension among interfaces of different polarities (Xia et al., 2014). BSs are naturally synthesized by different microorganisms, mainly yeast, fungi and bacteria (Shekhar et al., 2015). They are composed by a wide range of substances, as for example rhamnolipids (RL), sophorolipids, glycolipids,

lipopeptides, phospholipids, etc. They show very low toxicity compared to most of synthetic surfactants, so they are preferable to be used in environmental applications such as soil bioremediation (Liu et al., 2018; Wang et al., 2021).

Other aspect that has to be taken into consideration when studying the biodegradation of a pollutant is the presence of other toxic compounds in the media, what is known as co-contamination (Ye et al., 2017). The simultaneous presence of different pollutants and the combined toxicity that they may cause, may be increased or decreased compared to their effects when they act separately (Ali et al., 2022). This has to be previously known when bioremediation practices are applied to polluted soils. In the case of soils contaminated with PAHs, a significant number of studies have been carried out focused on individual representative PAHs, particularly phenanthrene (PHE) and pyrene (PYR). However, it is often overlooked the influence that the presence of other contaminants, even other PAHs with different physico-chemical nature, may have on their respective dissipation in soils, including the mutual effect on their bioavailability for soil microorganisms. Only a few studies have shown the influences of mixtures of PAHs interactions on their biodegradation in soils, and most of them studied only mixtures composed by just a few PAHs (Couling et al., 2010; Li et al., 2013; Wang et al., 2014; Madrid et al., 2016).

In the present study, microcosms experiments were conducted on three soils with different properties and two types of contamination (soils polluted with 7 PAHs -five with 3 aromatic rings and two with 4 rings-, and soils polluted with 15 PAHs, from 2 to 6 rings) to investigate: i) the effect of the soil properties on the dissipation of 7- versus 15-PAH; ii) the impact that the presence of HMW PAHs can have on the dissipation of the 7 lighter MW PAHs; iii) the effect of three bioavailability enhancers (two cyclodextrins and a rhamnolipid BS) in the dissipation of the 7- versus 15 PAH in the polluted soils. It is important to remark that, as far as we know, this high number of experimental factors (soil type, co-contamination (7 or 15 PAHs), availability enhancer and incubation time) has not been simultaneously studied previously. In this way, a multi-way ANOVA study was carried out to identify the influence of selected factors on PAHs dissipation in soils, whereas Principal Components Analysis (PCA) was used to ascertain the existence of possible associations among variables in order to get a dimensionality reduction.

## 2. Materials and methods

### 2.1. Soils

Three agricultural soils (CR, CN and TM) of different textures and properties were selected. They were sampled from different areas of Southwest Spain (CR: 37°16'59.0" N/ 8°3'58.8" W; CN: 37°51'56.5" N/ 5°36'29.2" W; TM: 37°30'21.1" N/ 5°57'59.8" W) showing a background of application of several pesticides for weed treatment although no source of PAHs was identified. Soils were sampled from the 0–10 cm layer, dried at room temperature, sieved by 2 mm, and stored at 4 °C until analysis. Some of their physicochemical characteristics are shown in Table 1. The three soils were selected because they showed a microbial flora capable of provoking the natural attenuation of PYR when they were artificially contaminated (Fig. 1S). In preliminary tests the concentration of PAHs in these soils was negligible.

### 2.2. Chemicals and reagents

The mix of PAHs was supplied by Supelco (Sigma-Aldrich, CRM47930, naphthalene, acenaphthylene (ACL), acenaphthene (ACE), anthracene (ANT), fluorene (FLU), PHE, fluoranthene (FLT), PYR, benzo[a]anthracene (BANT), chrysene (CHR), benzo[a]pyrene (BPYR), benzo[b]fluoranthene (BFLT), dibenzo[a,h]anthracene (DBANT), benzo[g,h,i]perylene (BPER), and indeno[1,2,3-c,d]pyrene (IPYR)). The mix has a concentration of 2 mg/mL of each component dissolved in CH<sub>2</sub>Cl<sub>2</sub>. Individual PAHs (ACL, ACE, ANT, FLU, PHE, FLT, and PYR) were supplied by Sigma-Aldrich, with purities higher than 98%.

**Table 1**  
Physico-chemical characteristics of the soils used.

Soil	pH	CO <sub>3</sub> <sup>2-</sup> (%)	OM (%)	Water field capacity (%)	Sand (%)	Silt (%)	Clay (%)	Textural class
CR	8.70	17.0	1.04	46.5	59.6	19.4	21.0	Sandy Clay
CN	5.5	1.1	1.91	39.3	50.5	39.3	10.2	Loam
TM	7.84	25.4	2.36	41.4	6.8	33.6	59.6	Clay

Two chemically modified CDs, 2-hydroxypropyl- $\beta$ -cyclodextrin (HP) and randomly methylated- $\beta$ -cyclodextrin (RAMEB) (purity 97%), were supplied by Cyclolab (Budapest, Hungary). The rhamnolipid BS JBR-425 (RL) was supplied by Jeneil Biosurfactants Co. (TX, USA) as an aqueous solution (25% w/w concentration), and with a critical micelle concentration (CMC) value around 30 mg L<sup>-1</sup> (Madrid et al., 2019b). The nutrients solution used contained (mg L<sup>-1</sup>): 12.5 CoCl<sub>2</sub>·2H<sub>2</sub>O, 25.0 SnCl<sub>2</sub>·2H<sub>2</sub>O, 10.0 CaSO<sub>4</sub>·2H<sub>2</sub>O, 12.5 ZnSO<sub>4</sub>·7H<sub>2</sub>O, 12.5 Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·16 H<sub>2</sub>O, 12.5 NiCl<sub>2</sub>·6H<sub>2</sub>O, 3.75 KBr, 3.75 KCl, 75.0 MnCl<sub>2</sub>·4H<sub>2</sub>O, 37.5 FeSO<sub>4</sub>·7H<sub>2</sub>O and 2.5 LiCl (Fenlon et al., 2011). These compounds were purchased from GPR RECTAPUR® (VWR International). Naphthalene-d<sub>8</sub>, acenaphthene-d<sub>10</sub>, phenanthrene-d<sub>10</sub>, chrysene-d<sub>12</sub> and perylene-d<sub>12</sub> (deuterated surrogate PAHs) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Anthracene-d<sub>10</sub> used as internal standard, by Dr. Ehrenstorfer (Augsburg, Germany). Solvents used for extraction and analytical determinations were supplied by Merck (Germany).

### 2.3. Soil contamination and incubation assays

Incubation experiments were carried out in glass containers (300 mL) in triplicate for each treatment. Two different contamination types were used, a mixture of 15 PAHs or a mixture of 7 PAHs, each one prepared as described below. 15 PAHs mix commercial solution was diluted in acetone (6 mL in 250 mL, final concentration 768 mg L<sup>-1</sup>  $\Sigma$ PAHs, 51.2 mg L<sup>-1</sup> of each individual PAH), and 20 mL of this solution was applied over 100 g of the soil. Solvent was evaporated in fume hood for 24 h, and soil was disaggregated with glass rod, and mixed carefully with the other 300 g amount of soil (total 400 g of soil). In this way soil spiking was done preserving original soil natural microorganisms (Madrid et al., 2016). Final concentration of soil pollutants added was 40 mg kg<sup>-1</sup>  $\Sigma$ 15PAHs (2,7 mg kg<sup>-1</sup> each individual PAH).

To observe the influence of HMW PAHs on the dissipation of the LMW ones, the three soils were contaminated only with those 7 PAHs with lower MW (ACL, ACE, ANT, FLU, PHE, FLT, and PYR) using a similar procedure and maintaining the total concentration of contaminants as in the  $\Sigma$ 15 PAHs experiment in order to obtain a similar influence on soil endogenous microorganisms. A unique solution was prepared (concentration 768 mg L<sup>-1</sup>,  $\Sigma$ 7PAHs, 109,7 mg L<sup>-1</sup> of each individual PAH) in acetone, and 20 mL were added to 100 g of soil, solvent was evaporated for 24 h, and mixed carefully with 300 g of soil, to obtain a global concentration of 40 mg kg<sup>-1</sup>  $\Sigma$ 7 PAHs (5,7 mg kg<sup>-1</sup> each individual PAH).

The final moisture was adjusted to 60% of the water field capacity of each soil, and was reached adding 5 mL of the nutrient solution to each bottle plus water in the control treatments, and in the rest of treatments 5 mL of the nutrient solution plus aqueous solutions of HP, RAMEB or RL to obtain a final concentration of 1% on dry soil weight basis. The glass containers were incubated under greenhouse at ambient temperature to simulate natural conditions of real contaminated soils. Moisture was gravimetrically checked twice per week throughout the experiment adding water when needed. Samples were taken at 1, 15, 30, 60 and 120 days after incubation began.

### 2.4. Soil extraction and analysis of PAH

PAHs were extracted from soils and analyzed following the methods proposed by Madrid et al. (2019a). Briefly, PAHs extraction was carried

out with 20 mL hexane/dichloromethane (1:1) after spiking 0.2 g of soil with 1  $\mu$ g of the deuterated surrogate PAHs in Corex glass tubes, which were vortexed and placed for 10 min in an ultrasonic bath. After centrifugation, the supernatant was evaporated to 2 mL at 20 °C under nitrogen stream. After that, the sample was cleaned up using 4 g alumina CHROMABOND® SPE cartridges (pore volume 0.90 ml/g, particle size 60–150  $\mu$ m, specific surface 150 m<sup>2</sup>/g, Sorbtech, USA) previously conditioned with 40 mL hexane:dichloromethane (1:1). Two mL of the extract were added to the column and eluted with 40 mL hexane:dichloromethane (1:1), and the extract evaporated under nitrogen stream to 1.5 mL. 1  $\mu$ g of anthracene-d<sub>10</sub> was added as internal standard and the analysis was carried out by gas chromatography coupled to mass spectrometry (GC–MS, Agilent GC 6890 coupled with a quadrupole mass spectrometer Agilent 5973 N, CA, USA). Electron ionization mode was used at 70 eV. The column was a HP-5 MS (30 m  $\times$  0.25 mm I-D and 0.25  $\mu$ m film thickness) coated with 5%-phenyl-methylpolysiloxane (J&W Scientific, CA, USA). The temperature programme was: 60 °C (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 8 °C/min. The sample was injected in splitless mode (2  $\mu$ L) and the valve was closed for 1.5 min. The carrier gas was helium at 1.9 mL/min. Temperatures for the injector, ion source and transfer line, respectively, were 280, 230 and 270 °C. Time scheduled selected ion monitoring (SIM) was used with four retention time windows. For each compound three ions were monitored. The software used, MSD ChemStation 5.0.0.0 (Agilent, CA, USA).

### 2.5. Kinetic dissipation model

PAHs dissipation curves were adjusted to the kinetic model that best described them using the FOCUS work group guidance recommendations (FOCUS, 2006). Dissipation kinetics were fitted to a simple first-order (SFO) kinetic model ( $C = C_0 e^{-kt}$ ), being C the PAH concentration remaining in soil at time t, C<sub>0</sub> the initial PAH concentration, and k (day<sup>-1</sup>) the dissipation rate. As indicators of the goodness of fit, the chi-square ( $\chi^2$ ) test was calculated. Taking into account the number of observations and degrees of freedom, values below 15.507 indicated that  $\chi^2$  test is fulfilled. The time to obtain 50% dissipation (DT<sub>50</sub>) was also calculated in order to compare the different dissipation curves.

### 2.6. Dehydrogenase activity

It was determined according to the method of Trevors (1984) modified by García et al. (1993). Summarizing, 1 g of soil was mixed with 0.2 mL of iodinitrotetrazolium chloride (INT) solution (0.4%) and 0.4 mL of distilled water. As control, 1 g of soil was incubated with 0.6 mL of water in absence of INT. After 20 h of incubation in darkness at room temperature, methanol was added (10 mL), and the solution agitated in vortex 30 s and filtered. The iodinitrotetrazoliumformazan formed was quantified spectrophotometrically at 490 nm (Madrid et al., 2016).

### 2.7. Cyclodextrin adsorption-desorption onto soils

20 mL of 1 mM solution of each CD (HP and RAMEB), was agitated with 10 g of each soil (CR, TM and CN) in centrifuge tubes. Similar solutions without CDs were prepared as control. All the adsorption experiments were carried out in triplicate. The tubes were shaken on a horizontal plate shaker at 200 rpm at a constant temperature of 20 °C. After 24 h, the suspension was centrifuged and supernatant was separated. Residual CDs in solutions were quantified measuring dissolved organic carbon (DOC) in a Total Organic Carbon analyzer Shimadzu TOC-V SCH. Adsorbed CDs was then calculated by subtracting DOC in the solutions with CDs minus solutions prepared as control for each soil. Desorption experiments were performed after finishing adsorption process by removing half of the supernatant after centrifugation, replacing it by 10 mL of 0.01 M Ca(NO<sub>3</sub>)<sub>2</sub> solution, allowing equilibration for an additional 24 h period, and after that operating as in the adsorption experiment. Desorption process was repeated twice more.

### 2.8. PAHs soil bioavailability

PAH bioavailability was determined one day after adding the treatments according to the method proposed by Reid et al. (2000). In Corex-glass centrifuge tubes, 1 g of soil was extracted with 20 mL of a 50 mM HP solution at room temperature in an orbital shaker at 150 rpm for 20 h. Afterwards, the supernatant was separated by centrifugation and PAHs analyzed as described above. Bioavailability experiments were carried out in triplicate for each treatment.

### 2.9. Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics v.25. Data were analyzed using General Linear Model (GLM) for analyzing Principal Effects and the interactions among the experimental factors: type of contamination (7 or 15 PAHs), type of soil (CR, CN, TM), availability enhancer (control, HP, RAMEB, RL) and incubation time (1, 15, 30, 60, 120 days). Four-way ANOVA, F-test and Bonferroni test for average comparison were employed. Data were tested for normality with the Kolmogorov–Smirnov test and for homogeneity of variance with the Levene test. The Tukey test was applied to significant test results for identification of important contrasts. Varimax-rotated principal components analysis (PCA) was used to ascertain the existence of possible associations among variables in order to get a dimensionality reduction.

## 3. Results and discussion

### 3.1. Natural attenuation of mixed PAHs in three agricultural soils

The percentage of PAHs remaining in the selected soils after 120 days of incubation (control) related to their content just at the beginning of the experiments is shown in Table 2. Although naphthalene was also included in the mix of 15 PAHs added to the soils, it was not studied due to its high volatility (11 Pa at 25° C), which implied that only between 10% and 30% of

NAP was present in the soils (and not homogeneously for each soil) just after their contamination and evaporation of the solvent in fume hood (24 h) before starting the incubation process. Therefore, we have considered that at the beginning of the incubation process the soils were contaminated with a mix of 14 PAHs. The three soils present natural attenuation capability of some PAHs after 120 days of incubation when they were artificially contaminated with this mix. The natural attenuation occurs mainly as a result of biotic degradation, considered as the principal process for PAHs dissipation in the soil environment (Kuppusamy et al., 2017), indicating that the selected soils already had degrader microorganism populations preadapted to the presence of compounds with structures similar to those of PAHs. It could be related with their agricultural origin and the agronomic practices to which they had been previously subjected, such as the use of pesticides received during years, or antique burning of crop residues, an agricultural practice widely used in Southwestern Spain. Furthermore, the natural attenuation can also partially occur, although to a much lesser extent, through abiotic dissipation, which includes volatilization, chemical transformation, or formation of non-extractable residue in soils (Liu et al., 2021).

It is clearly observed in Table 2 the great difference in the control treatment between the residual content of the 7 PAHs with the lowest molecular weights (ACL, ACE, FLU, PHE, ANT, FLT, PYR), which were highly degraded, and the rest of PAHs in the mix. Among the 7 PAHs highly degraded, ACL and ACE were completely dissipated in all soils, and the remaining percentages of the rest was lower than 5.3%, and very close to 0% for some of them. In contrast, the extracted concentrations of the majority of the heavier PAHs were very close to their initial content. This LMW dissipation clearly affected the percentages of Σ14 PAHs remaining in soils (% Σ14 PAHs: 37.5% in TM, 42.2% in CN, 49.7% in CR), which were significantly much higher than the remaining PAHs when only those 7 lighter were considered in the mix of 14 PAHs (% Σ7 PAHs: 3.6% in TM, 3.18% in CN, 1.4% in CR). These last values were very similar to the remaining percentages when a mix of only the 7 lighter PAHs was incubated (Table 2). Among the 7 PAHs highly degraded, 5 of them contain 3

**Table 2**

Residual content (%) of individual PAHs and Σ PAHs in soils contaminated with the mix of 14 PAHs (Σ 14 PAHs, Σ 7 lighter (LMW) PAHs and Σ 7 heavier (HMW) PAHs) and with the mix of 7 lighter PAHs (Σ7 LMW PAHs) after 120 days of incubation in the control experiment and after addition of different availability enhancers (RL, HP and RAMEB). Values in parenthesis are standard deviation. For each PAH and each soil, data followed by a different letter are significantly different ( $p < 0.05$ ).

SOIL	CR				CN				TM			
	CONTROL	RL	HP	RAMEB	CONTROL	RL	HP	RAMEB	CONTROL	RL	HP	RAMEB
<b>14 PAHs</b>												
ACL	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a
ACE	0.2 (0.3)a	0.0 (0.0)a	0.0 (0.0)a	0.4 (0.5)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	3.0 (0.2)b
FLU	5.3 (1.1)a	5.3 (1.0)a	5.6 (0.3)a	90.0 (1.4)b	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	76.6 (1.5)b	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	92.0 (1.2)b
PHE	5.1 (0.0)b	13.3 (1.0)c	0.5 (0.1)a	39.1 (0.1)d	1.4 (1.1)a	11.5 (0.2)b	2.2 (1.1)a	48.4 (1.4)c	5.0 (0.2)a	5.2 (0.0)a	4.3 (0.1)a	67.7 (1.7)b
ANT	4.6 (0.0)a	3.0 (0.7)a	18.2 (3.0)b	83.2 (2.0)c	2.7 (0.2)a	4.7 (0.6)a	6.2 (0.0)a	47.8 (3.4)b	2.2 (0.2)a	2.3 (0.2)a	3.8 (0.4)a	78.0 (1.2)b
FLT	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	86.3 (3.7)a	0.7 (0.2)a	6.2 (0.1)b	5.7 (0.2)b	56.7 (8.2)c	5.2 (0.4)a	3.1 (0.1)a	4.2 (0.1)a	85.8 (0.8)b
PYR	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.0 (0.0)a	0.7 (0.0)a	5.9 (0.1)b	5.3 (0.3)b	57.3 (8.4)c	4.9 (0.3)a	3.2 (0.1)a	3.4 (1.3)a	67.6 (1.4)b
BANT	75.7 (1.2)a	92.3 (2.7)b	71.6 (3.7)a	91.4 (1.4)b	66.3 (2.9)a	90.8 (0.8)b	92.5 (5.3)b	94.6 (4.6)b	47.9 (1.3)a	54.1 (3.9)a	46.7 (0.8)a	60.6 (0.4)b
CHR	91.7 (3.5)a	99.5 (2.6)a	97.8 (6.2)a	98.1 (3.8)a	91.5 (8.1)a	92.6 (3.2)a	90.5 (7.5)a	91.3 (8.3)a	65.1 (1.9)a	73.4 (9.1)a	86.8 (3.4)b	93.6 (0.8)b
BFLT	91.7 (3.0)a	92.8 (3.7)a	45.3 (0.5)b	16.6 (0.6)c	89.1 (4.1)b	91.4 (1.9)b	94.1 (5.0)b	79.5 (3.2)a	49.3 (0.5)b	48.6 (4.4)b	53.7 (0.3)b	29.2 (2.2)a
BPYR	52.8 (0.3)c	78.2 (4.9)d	21.0 (1.1)a	36.9 (2.4)b	30.7 (0.6)a	45.7 (0.2)b	35.5 (2.0)a	72.7 (2.4)c	41.1 (1.6)b	51.6 (3.2)c	19.5 (0.2)a	43.5 (1.0)b
DBANT	89.1 (1.5)a	90.6 (1.1)a	94.9 (3.0)a	93.4 (0.4)a	91.5 (5.8)a	93.1 (5.6)a	98.0 (6.1)a	95.5 (0.7)a	78.1 (3.2)b	88.4 (0.8)c	69.8 (1.5)a	75.3 (1.0)b
IPYR	86.5 (4.0)a	88.4 (1.4)a	83.3 (2.2)a	89.2 (2.6)a	84.5 (4.4)a	91.0 (2.4)a	88.7 (4.9)a	95.4 (2.3)a	73.3 (5.1)a	85.1 (1.1)b	71.2 (0.4)a	71.3 (0.5)a
BPER	89.8 (1.3)a	83.1 (1.5)a	89.9 (1.9)a	101 (2.9)b	83.9 (4.4)a	91.5 (2.3)a	87.4 (4.8)a	90.9 (2.2)a	73.0 (5.1)a	81.1 (0.8)a	71.7 (1.5)a	70.6 (1.0)a
<b>% Σ14 PAHs</b>	49.7 (6.6)	49.0 (7.7)	47.3 (9.0)	63.2 (7.6)	42.2 (13.0)	51.7 (7.5)	53.4 (14.4)	72.9 (15.8)	37.5 (8.5)	38.1 (11.4)	33.2 (4.3)	60.5 (4.2)
<b>% Σ7 LMW (mix 14 PAHs)</b>	1.4 (1.19)	1.4 (1.2)	3.5 (3.0)	51.6 (4.4)	3.18 (2.0)	4.74 (0.60)	6.28 (3.11)	45.8 (12.3)	3.6 (0.7)	2.3 (0.2)	2.6 (1.4)	62.7 (3.0)
<b>% Σ7 HMW (mix 14 PAHs)</b>	83.5 (6.5)	89.3 (7.6)	72.0 (8.4)	75.2 (6.2)	76.8 (12.8)	85.2 (7.5)	83.8 (14.1)	88.6 (10.8)	61.1 (8.4)	68.9 (11.4)	59.9 (4.1)	63.4 (3.0)
<b>% Σ7 LMW (mix 7 PAHs)</b>	1.1 (1.3)	1.2 (2.7)	1.6 (1.1)	65.6 (10.8)	10.1 (3.4)	7.0 (8.0)	3.0 (0.1)	48.4 (4.7)	2.8 (2.2)	2.4 (0.9)	3.6 (2.2)	70.4 (5.9)

aromatic rings, and are considered to belong to the group of LMW PAHs, which have been demonstrated to be easily biodegraded in contaminated soils by other researchers (Johnsen et al., 2005; Liu et al., 2021) due to their higher bioavailability. However, the other two, FLT and PYR, are 4-ring PAHs, and are considered as HMW PAHs, but actually their behavior was more similar to 3-ring PAHs, and quite different from the other two 4-ring PAHs, BANT and CHR (Table 2). In fact, FLT and PYR disappeared completely from CR soil, almost completely from CN soil, and only remained about 5% in TM soil. On the contrary, for BANT and CHR more than 76% was extracted from CR, more than 66% from CN, and more than 48% from TM. This could be related with the lower molecular weight and  $K_{ow}$  (octanol-water partition coefficient) of PYR and FLT. This behavior has been observed also in other laboratory studies in which soils were contaminated with just a few PAHs. In soils contaminated with PHE, FLU, ANT, FLT and PYR, Bouchez et al. (1999) observed that FLT and PYR were mineralized by a consortium from a PAH-contaminated soil at the same level than the 3-ring PAHs. Also Colombo et al. (2011) observed an extent of degradation for FLT and PYR similar to that of PHE in a soil contaminated with PHE, ANT, PYR and FLT, and even their degradation was faster than that of ANT. They also observed an inhibitory effect of FLT and PYR on PHE degradation in inoculated slurries when they were present in dual mixture with PHE. A very interesting study was that of Banach-Szott et al. (2015) who found that, in 5 soils contaminated with FLU, ANT, PYR and CHR, 3-ring PAHs decomposed faster than 4-ring ones. However, the extent of degradation of PYR after 360 days of incubation was quite similar to that of FLU in all the studied soils, but CHR content was much higher than that of PYR in 3 of the 5 soils, concluding that the soil quality parameters were essential for the degradation process.

In contrast, when only those 7 PAHs of HMW are taken into account, an average up to 83.5% was extracted from CR soil after 120 days, 76.8% from CN soil and 61.1% from TM soil. These results indicate also that the type of soil plays an important role in the dissipation of PAHs. This influence of the type of soil in PAHs dissipation is observed precisely in those with higher molecular weight, since, in general, they are dissipated in higher percentages in soil TM. It can be due to a microbial community in this soil with capacity to degrade such heavier molecules, since the effect is observed for all of them, but particularly for BANT, CHR, BFLT and BPYR. But abiotic dissipation has to be also taken into account in these soils, and even could be the main cause in the case of the heaviest PAHs, because their extractable concentration is inversely related to the OM content of each soil, probably due to the formation of non-extractable residues as higher is the OM content of the soils (Wang et al., 2015).

It should be noted that the extractable percentages of BANT and especially BPYR were lower for the three soils than other HMW PAHs, indicating that they can be also partially degraded by the soil endogenous microorganisms. In the case of soil CR, the extractable content of the HMW PAHs was in the range between 89.1% and 98.7%, but those of BANT and BPYR were 75.7% and 52.8%, respectively. For the soil CN, most of the HMW PAHs were in the range 83.9%–91.5%, but for BANT and BPYR their extractable content was only 66.3% and 30.7%, respectively, and in the case of soil TM it was 47.9% for BANT and 41.1% for BPYR.

BPYR is considered one of the PAHs with a high stability against biodegradation. Due to its carcinogen character, it is classified by USEPA among the top ten priority pollutants of great concern (ATSDR's Substance Priority List), being of fundamental concern its removal from the environment. However, few studies have focused on BPYR biodegradation, and most of them have been carried out using bacterial consortia or co-metabolism. Several studies have revealed that biodegradation of BPYR is promoted by using LMW PAHs (usually PHE, but also PYR and FLT) as growth substrate for co-metabolism (Zhao and Wong, 2010; Nzila and Musa, 2021). Juhasz and Naidu (2000) observed an enhanced degradation of BPYR in the presence of LMW PAHs in comparison with in their absence, and proposed that LMW PAHs were acting as co-substrates, increasing metabolic activity through induction of enzymes for BPYR degradation. Also Wang et al. (2014) studied the dissipation of BPYR and observed that in

soils co-contaminated with PYR the dissipation of BPYR was promoted remarkably. In the present study, the presence of LMW PAHs could have facilitated the degradation of BPYR.

### 3.2. Influence of HMW PAHs on the dissipation of LMW PAHs

In order to observe if the presence of the HMW PAHs could influence the dissipation of the LMW ones, the three soils under study were contaminated only with the 7 PAHs with lower MW. The remaining percentage of  $\Sigma$  of these 7 PAHs at the end of the incubation time in all soils is shown in Table 2 (individual percentages for each PAH not shown). These percentages were quite similar to those obtained when LMW PAHs were applied together with the rest of HMW PAHs in the control experiments. However, differences were observed when the remaining concentrations of PAHs in the three soils as a function of time were fitted to SFO model and kinetic parameters calculated. It was observed that  $DT_{50}$  data were higher if only 7 PAHs were applied to the soils in comparison to  $DT_{50}$  data for the same 7 PAHs but added in a mix of 14 PAHs (Table 3, the complete kinetic data are given in Table 1S, including the confidence interval for  $DT_{50}$  values). It indicated that there is an enhanced dissipation rate of the lighter PAHs in the three soils studied in the presence of the heavier PAHs. This trend is more or less general for the lighter PAHs and for the three soils, and seems to indicate that the presence of the HMW PAHs increased their kinetic of dissipation, especially in the cases of ACL, ACE and FLU. There is an exception in PHE in the control and RL samples, whose dissipation seems to be favored in absence of HMW PAHs.

These results are contrary to the conclusions reached by Couling et al. (2010) who observed that, in PAH mixtures in soil, microbes mineralized faster structurally larger molecules than when they were added as individual substrate, but the biodegradation of the lighter ones could be reduced. However, there are other studies that support our results. White et al. (1999) and White and Pignatello (1999) observed an increased availability of aged PHE in soils by competitive displacement with PYR for sorption sites. They suggested that PYR excluded PHE from less mobile sorption domains in the soil OM. Humel et al. (2017) also observed an increase in accessibility and mobility of PAHs in an industrial contaminated soil due to the presence of toluene as sorbate, which competed with them for high-affinity sorption sites in the soil. In our study, the competitive effect may occur just after the mix of 14 PAHs entered into contact with the soils. The high-affinity adsorption sites were first occupied by the HMW PAHs, leaving only the less affinity sites free to be occupied by the LMW PAHs. From these sites, the bioavailability of the lighter PAHs was higher than when only 7 PAHs were used to contaminate the soils.

### 3.3. Effect of availability enhancers addition on the dissipation of mixed PAHs in soils

PAHs bioavailability has significant implications for the remediation of contaminated soils and for the risk assessment. Increasing PAHs bioavailability leads to a higher concentration of the contaminant which can be taken up and degraded by soil microbiota. The three availability enhancers selected (HP, RAMEB and RL) show results quite different, as reflected in the residual content of each PAH (Table 2) and by the data of  $DT_{50}$  reported in Table 3 for the 7 lighter PAHs. The ratio between  $DT_{50}$  values of RL, HP and RAMEB experiments and their respective control experiments (expressed as  $\Delta$ ) has been also included in Table 3 in order to observe more easily the changes due to the addition of the availability enhancers.

The results of PAHs dissipation in the three soils using HP showed similar values to those of the control experiment in the residual content of the different PAHs after 120 days of incubation (Table 2), but a large difference can be observed in the dissipation rate, since  $DT_{50}$  values (Table 3) obtained for some of the 7 LMW PAHs were much lower than the corresponding ones in control experiments ( $\Delta$  values  $<1$ ), especially in the cases of PHE, FLT and PYR. For the rest of PAHs,  $DT_{50}$  values obtained were similar to those of the control or only slightly above them. It implies that the use of HP as availability enhancer could have advantages for the remediation of soils

**Table 3**

DT<sub>50</sub> values (50% dissipation) calculated from the first order degradation kinetics of PAHs in contaminated soil in mixtures with 7 or 14 PAHs in original soils (control) or soils treated with biosurfactant (RL) or cyclodextrins (HP and RAMEB), and the ratio between DT<sub>50</sub> values of the treated soils with respect to the DT<sub>50</sub> in the control experiments (Δ).

PAH	SOIL	Σ PAHs	CONTROL	RL	Δ RL	HP	Δ HP	RAMEB	Δ RAMEB
ACL	CR	7	11.3	17.4	1.54	9.1	0.80	31.5	2.79
		14	3.2	10	3.13	4.8	1.50	23.3	7.28
	CN	7	8.8	10.9	1.24	10.6	1.20	20.2	2.30
		14	4.7	11.8	2.51	8.5	1.80	12.4	2.64
	TM	7	8.8	17.0	1.93	11.5	1.31	32.0	3.64
		14	4.0	13.6	3.40	8.4	2.10	28.2	7.05
ACE	CR	7	17.2	21.0	1.22	12.1	0.70	30.1	1.75
		14	8.3	12.3	1.48	10.2	1.23	23.9	2.88
	CN	7	9.9	15.6	1.58	17.0	1.72	22.3	2.25
		14	6.3	15.4	2.44	12.1	1.92	18.9	3.00
	TM	7	13.9	15.9	1.14	12.1	0.87	36.2	2.60
		14	8.6	20.2	2.35	9.3	1.08	31.1	3.62
FLU	CR	7	19.4	35.5	1.83	20.2	1.04	310	16.0
		14	17.4	24.7	1.42	24.9	1.43	1011	58.1
	CN	7	15.8	18.5	1.17	18.2	1.15	188	11.9
		14	10.4	15.8	1.52	19.4	1.86	173	16.6
	TM	7	15.2	21.4	1.41	18.3	1.20	1843	121
		14	11.9	15.7	1.32	17.3	1.45	1775	149
PHE	CR	7	21.6	34.0	1.57	15.1	0.70	103	4.77
		14	27.3	37.3	1.37	15.6	0.57	74.9	2.74
	CN	7	16.8	30.2	1.80	12.8	0.76	57.8	3.44
		14	19.3	36.5	1.89	13.3	0.69	102	5.28
	TM	7	17.0	22.6	1.33	16.0	0.94	745	43.8
		14	19.4	26.3	1.35	17.1	0.88	192	12.5
ANT	CR	7	58.1	34.2	0.59	36.2	0.62	958	16.5
		14	28.7	22.2	0.77	63.6	2.21	2693	93.8
	CN	7	21.1	9.9	0.47	27.7	1.31	960	45.5
		14	13.4	11.5	0.86	27.3	2.04	138	10.3
	TM	7	19.0	29.5	1.55	42.9	2.26	500	26.3
		14	18.6	22.9	1.23	36.2	1.95	166	8.92
FLT	CR	7	54.1	39.9	0.74	19.2	0.35	147	2.72
		14	49.1	35.9	0.73	21.3	0.43	160	3.26
	CN	7	105	49.5	0.47	33.4	0.32	305	2.90
		14	59.8	48.8	0.81	40.3	0.67	402	6.72
	TM	7	30.7	48.4	1.57	22.8	0.74	1063	34.6
		14	27.7	29.4	1.06	25.4	0.92	1038	37.5
PYR	CR	7	50.0	39.3	0.79	17.6	0.35	28.9	0.58
		14	49.4	35.6	0.72	17.0	0.34	15.1	0.31
	CN	7	109	47.8	0.44	32.9	0.30	506	4.64
		14	58.9	47.8	0.81	38.1	0.65	309	5.24
	TM	7	32.4	48.8	1.51	24.1	0.74	1153	35.6
		14	29.6	29.6	1.00	24.0	0.81	679	22.9

contaminated by a mix of PAHs if those of LMW are present in high proportion, such in the case of diesel contaminated soils. In those soils with endogenous microorganisms capable of degrade PAHs, the use of HP can shorten the time of the bioremediation, resulting a less time consuming process.

In the case the other CD added, RAMEB, the results obtained were quite different. The residual content after 120 days of incubation of most of the 14 PAHs increased greatly (Table 2), with remaining percentages higher than 60%, even for the lighter PAHs, whose dissipation was rather slower for all soils studied, increasing their DT<sub>50</sub> values and giving as result Δ values compared to control > 1 (Table 3). This increasing trend is less pronounced for ACL and ACE, those less voluminous PAHs, than for the rest (Table 3), and at the end of the incubation process practically 100% of both PAHs were degraded (Table 2). The other PAHs of this group of 7 PAHs, with higher MW, present extremely high DT<sub>50</sub> values (Table 3) and the use of RAMEB was not favored in comparison to the control experiments (Table 2). Although the formation of complexes of RAMEB with some PAHs gives as result a great increase in their solubility (up to 535, 477 or 390-fold higher for ANT, PHE and FLU, respectively, Morillo et al., 2012), it is clear that the use of RAMEB as availability enhancer is not adequate in soils contaminated by PAHs. These results contrast with previous studies carried out using RAMEB as solubilizing agents to increase the biodegradation in soil of transformer oil (Molnár et al., 2005, 2009) and diesel

oil (Leitgeb et al., 2008; Fenyvesi et al., 2009). However, our results agree with those of Leonardi et al. (2008) who studied the degradation of seven PAHs (PHE, ANT, FLT, PYR, CHR, BKFLT, BPYR) in a spiked soil after an incubation of 6 weeks. A higher residual concentration of most of them remained in the presence of RAMEB than in the control experiment.

The reason for this behavior was not related to a possible toxicity after the addition of RAMEB to the soils, because the dehydrogenase activity measured at different incubations periods (Fig. 1) showed that it was very similar to that corresponding to HP amendment, increasing both of them at the beginning of the incubation period and decreasing with the time. Another possibility could be that the complexes formed between RAMEB and the different PAHs were so strong that it was very difficult to destroy such complexes to liberate the PAHs to the soil solution. Nevertheless, although RAMEB complexes with certain PAHs presented apparent stability constants higher than those complexes with HP, the differences between both CDs were not very high (Morillo et al., 2012). A third possibility is related to the interaction of RAMEB with the soils under study. Jozefaciuk et al. (2003) observed that RAMEB addition to soils strongly modified their surface, pore size and their aggregation properties, increasing the amount of coarser soil particles. They also observed stronger interactions of RAMEB with the soil solid phase, the higher was the clay content of the soils. Fenyvesi et al. (2002) also observed a significant retention of RAMEB in the upper layers of soil columns when RAMEB solutions were applied. In order to check this possibility in our soils, an adsorption experiment of RAMEB and HP on the three soils was carried out and the result was that HP was not adsorbed at all, but, on the contrary, the adsorption of RAMEB was very high (Table 2S, Supporting Information). The adsorption followed the sequence TM (40.1%) > CN (22.4%) ≈ CR (21.8%). After three consecutive desorption cycles, only 15%, 24.1% and 36.3% of RAMEB previously adsorbed could be desorbed from soils TM, CR and CN, respectively. This sequence agrees with the clay content of these soils (Table 1), being more difficult its desorption as higher is the soil clay content. Such strong adsorption to soils and difficult desorption could probably block the surface of the soils preventing the release of the PAHs to the soil solution, and therefore their biodegradation.

The use of the third availability enhancer, RL, did not provoke a decrease in PAHs content in soils after 120 days of incubation in comparison with the control experiments (Table 2), and even in some cases the residual content increased, especially in the case of HMW PAHs. With respect to DT<sub>50</sub> values for the 7 lighter PAHs (Table 3), they were in general only slightly above those of the control, with the exception of the three of higher MW (ANT, FLU, PYR) in soils CR and CN. In principle, it seems to indicate that the use of RL as availability enhancer did not make any substantial improvement in the dissipation of PAHs in these soils where a degrader microbial community already exists. But the behavior observed when dehydrogenase activity was measured in the different points of these experiments over time is quite interesting and can explain that treatments with RL have delayed slightly the degradation of PAHs. As observed in Fig. 1 for RL treatment, the dehydrogenase activity corresponding to the soil TM was quite low at the beginning of the incubation process, but increased from the day 15 and continued active from that day until the end of the process. But the cases of soils CR and CN are especially remarkable, since the dehydrogenase activity was practically negligible until day 30, where it was drastically activated and maintained with decreasing steps until the end of the incubation process. This is the reason why the profiles of the dissipation curves corresponding to RL treatments are quite different from those of control or HP curves, as it can be observed as an example for the case of PHE in the three soils in Fig. 2. In RL treatments, PAHs dissipation is slowed down during the first days of incubation due to certain toxicity provoked by the addition of this biosurfactant (demonstrated by the suppressed dehydrogenase activity), but after this period of time the dissipation of the PAHs is carried out with a much higher rate than in the control.

One reason for the toxicity observed when using RL could be a higher extraction and bioavailability of PAHs in the system as a consequence of the addition of RL, so their concentration in the soil solution could be toxic for the microbial flora. Madrid et al. (2019a) observed a slight

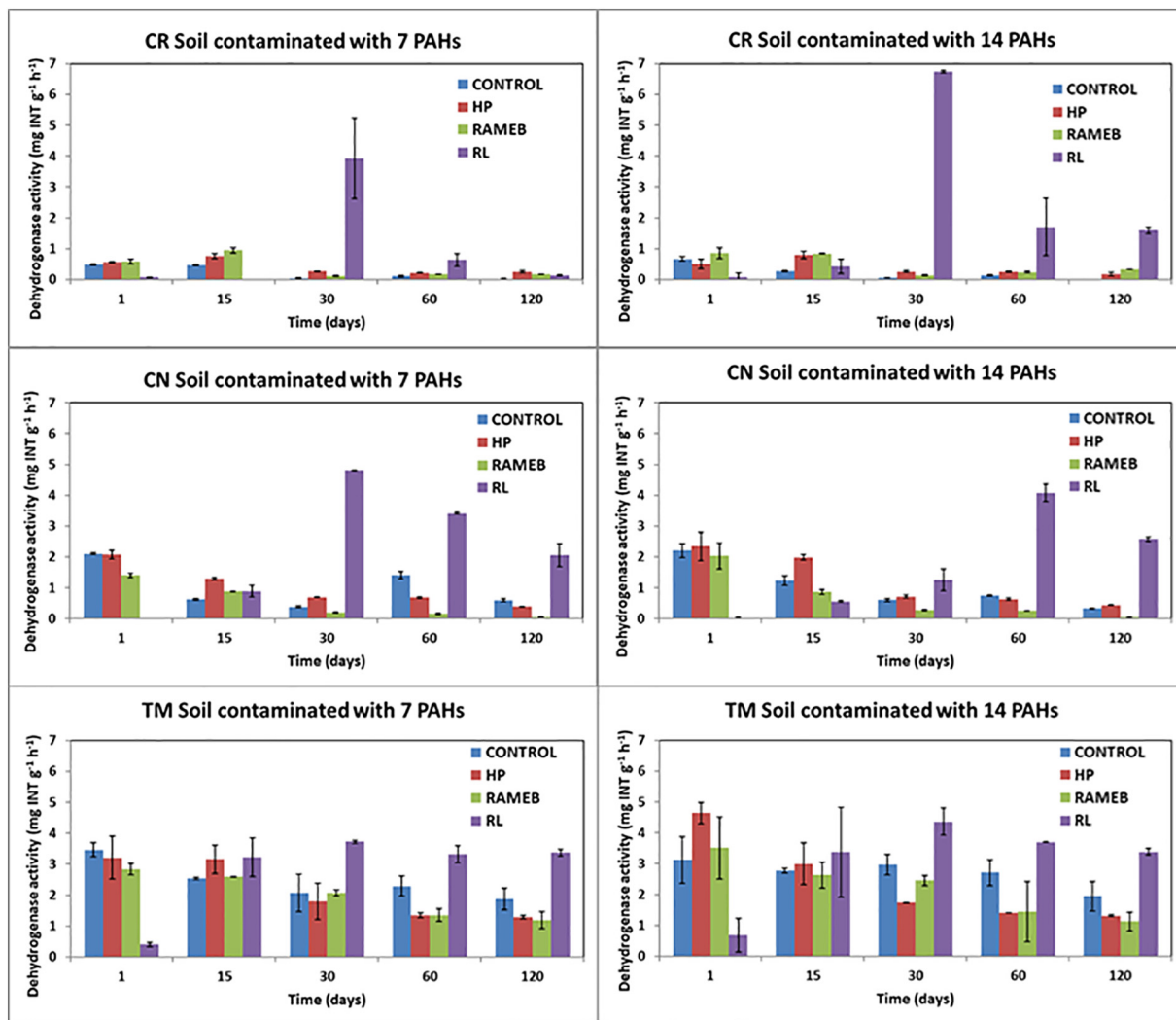


Fig. 1. Dehydrogenase evolution in the 3 soils during the incubation time.

increment in PAHs bioavailability in an industrial soil when RL 1% (on dry soil weight basis) was added, a concentration similar to that added in the present study. For this reason, the bioavailable content of PAHs in soils CR and TM one day after RL addition was measured (Table 3S, Supporting Information). The bioavailable contents of each individual PAH were very low for the soils studied before and after addition of RL, concluding that this is not the reason for the observed toxicity at the beginning of the incubation.

However, the toxicity of RL itself has to be also taken into account. Vatsa et al. (2010) observed antimicrobial effects of a rhamnolipid biosurfactant against specific fungi, bacteria, algae and viruses. Sydow et al. (2018) observed an increased toxicity in extracts from a creosote contaminated soil in presence of RLs, which was associated to both an increased bioavailability of toxic contaminants as well as the toxicity of RLs. Below CMC ( $30 \text{ mg L}^{-1}$ ), RL did not exhibit significant toxicity, but it increased with concentrations beyond CMC. In the present study, the concentration of RL added (1% on dry soil weight basis at a moisture of 60% of the water field capacity) was always well above CMC.

After the period of time where toxicity due to RL addition could be observed, the dissipation of PAHs was carried out even with a much higher rate than in the control (Fig. 2). It could be due to the rapid biodegradation that RL can suffer under aerobic conditions since they serve also as carbon source (Zeng et al., 2018). As it can be observed in Fig. 1, the dehydrogenase activity was quickly increased in soil TM and later in soils CR and CN, indicating that the soil microbial community was activated, and

probably it preferred the use of RL as carbon source. Ghosh and Mukherji (2016) found that RL was degraded preferentially in comparison to PYR, and Zeng et al. (2011) observed that the previous biodegradation of RL further enhanced the degradation of hexadecane. It could be the reason why the dehydrogenase activity was increased (Fig. 1) and delayed the PAHs dissipation with a slower rate than in the control (Fig. 2). As a conclusion, it is necessary to find a balance between the concentration of RLs to use, their biodegradability, and their toxic effects on PAHs dissipation.

#### 3.4. Significant parameters and factors affecting the dissipation of the PAHs

PCA is useful for treating a large amount of data as in this assay. For this analysis, individual PAH contents at every incubation time only for the treatments with soils contaminated with 14 PAHs have been included (a similar analysis for the assay with 7 LMW PAHs, did not success separating principal components). PCA grouped the PAHs into three principal components (PCs) that explain 82.0% of the total variance observed (Table 4). The first component PC1, explained 39.8% of the variance and was associated with 3-rings PAHs (FLU, ACE, ANT, ACL and PHE) and two 4-rings PAHs (FLT, PYR). In this way, among the PAHs studied, PC1 has grouped those 7 with  $\log K_{ow} \leq 5.2$  (Sverdrup et al., 2002) which have shown higher dissipation rates. It means that the behaviors of these 7 compounds were related and probably their dissipation processes were affected by similar factors. It is interesting to note that they are the same PAHs that were initially selected for the 7-PAHs contamination assay. All other PAHs included

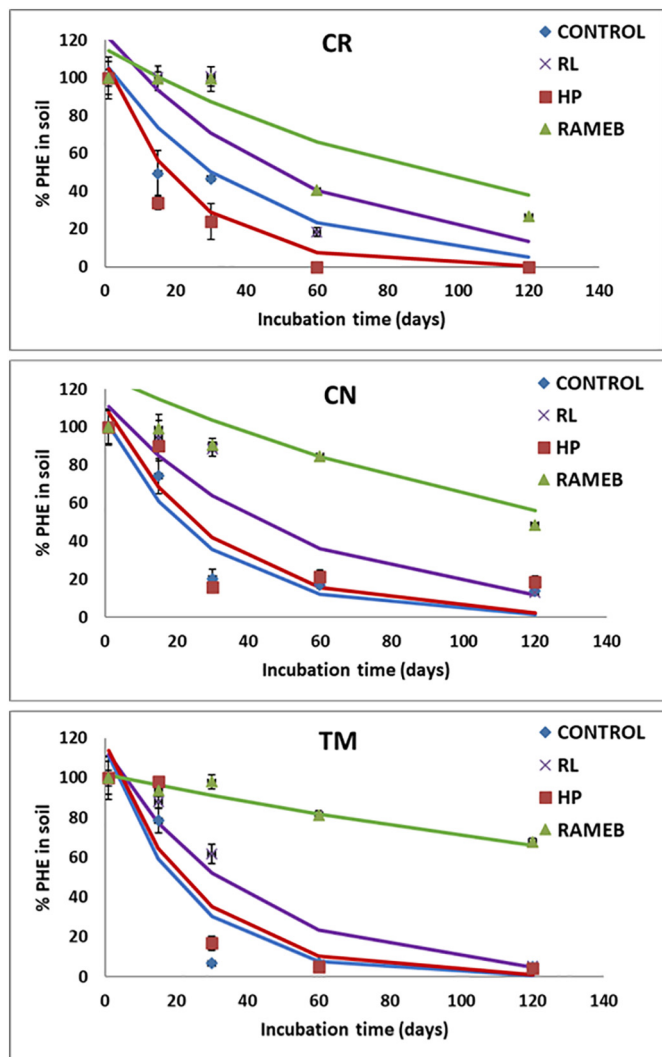


Fig. 2. Phenanthrene evolution content (dots) and SFO kinetic model fitting (lines) in the 3 soils contaminated with PAHs.

in the analysis have  $\log K_{ow} \geq 5.6$  (Sverdrup et al., 2002), and were mainly associated to the other PCs. PC2 explained 27.8% of the variance and grouped the heavier PAHs, those that have 5- (BFLT, BPYR, DBANT) or 6-rings (IPYR, BPER) and  $\log K_{ow} \geq 6.2$ . This could be related with the high adsorption capacity of these highly hydrophobic PAHs onto soils. In this group of PAHs, BPYR showed a lower correlation value with PC2 and a higher value with PC1 than the others, probably because of the moderate dissipation that has been shown in the 3 soils, as has been discussed previously. On the contrary, the other PAHs grouped by PC2 have shown very low dissipation rate after 120 days. Finally, the third Principal Component extracted (PC3) explains 14.4% of the variance and directly groups the other 4-rings PAHs not included in PC1, CHR and BANT. This two 4-rings PAHs, although have shown only some degree of dissipation (mainly in TM soil), in contrast to PYR and FLT (the other 4-rings PAHs studied which were almost completely dissipated), their behaviors have been more erratic and the kinetic models used could not be fitted to their dissipation curves (data not shown). This different dissipation rate among 4-rings PAHs could be related with their much lower water solubility and higher  $\log K_{ow}$  of CHR and BANT in comparison to PYR and FLT (Sverdrup et al., 2002). Golobocanin et al. (2004) applied PCA analysis to a wide set of PAHs contaminated soils. These authors also found an influence of the number of rings in the distribution of the different PAHs among the extracted PC, although they were not so clearly separated as in our PCA, probably

Table 4

Correlation coefficients between the content of PAHs (in three soils studied contaminated with 14 PAHs and treated with different bioavailability enhancers) and the first three principal components resulting from PCA, eigenvalues and percentage of variance accounted for.

PAH	Component			Communalities
	PC1	PC2	PC3	
Fluorene	<b>0.914</b>	0.044	0.097	0.846
Acenaphthene	<b>0.889</b>	0.087	-0.133	0.815
Fluoranthene	<b>0.872</b>	0.205	0.173	0.833
Anthracene	<b>0.858</b>	-0.020	0.250	0.798
Acenaphthalene	<b>0.842</b>	0.057	-0.104	0.722
Pyrene	<b>0.818</b>	0.168	0.125	0.713
Phenanthrene	<b>0.803</b>	0.374	-0.212	0.830
Indene[123-cd]pyrene	0.065	<b>0.958</b>	0.033	0.922
Dibenzo[ah]anthracene	0.005	<b>0.890</b>	0.238	0.848
Benzo[ghi]perylene	0.060	<b>0.877</b>	0.049	0.776
Benzo[b]fluoranthene	0.211	<b>0.775</b>	0.138	0.664
Benzo[a]pyrene	0.556	<b>0.719</b>	-0.067	0.831
Chrysene	-0.100	0.070	<b>0.959</b>	0.934
Benzo[a]anthracene	0.229	0.255	<b>0.909</b>	0.944
Eigenvalue	5.567	3.890	2.020	
Variance (%)	39.764	27.784	14.427	
Cumulative variance (%)	39.764	67.548	81.975	

Values in bold indicate the variables with the highest loadings for each component.

due to a very wide range PAHs concentration in their samples since they were collected from real contaminated sites affected by industrial activities.

In order to describe the global effect of the availability enhancers used for every soil, Fig. 3 shows the mean scores of each treatment plotted on the plane PC1 vs. PC2. It is observed that RAMEB treatments (the amendment that usually retarded the dissipation of PAHs) show the highest values in PC1 for the three soils. On contrary, other treatments (HP and RL and control), with higher dissipation rates of the lighter 7-PAHs, show negative mean scores in this component for the 3 soils. In summary, lower values in PC1 seem to be related with decreasing PAH content along the incubation period. It is corroborated in Fig. 2S representing the evolution of the centroid values at every incubation time of the scores obtained for the three soils and for all treatments simultaneously plotted in the plane PC1 versus PC2. Decreasing values can be observed in PC1 of centroids as incubation time increases, from the highest value at time 1 to negative values in this component at 30, 60 and 120 days.

Otherwise, the effect of soil characteristics is mainly shown in PC2 (Fig. 4, mean scores of each treatment plotted on the plane PC2 vs. PC3), where all treatments with soil CR (the soil with the lowest OM) showed positive data in this PC2 and are clearly separated from treatments with soil TM (the soil with the highest OM), that show negative values in all cases. Although OM is the main soil component responsible of increasing adsorption capacity of hydrophobic pollutants (Huang et al., 2003; Morillo et al., 2014) diminishing their bioavailability and reducing their dissipation

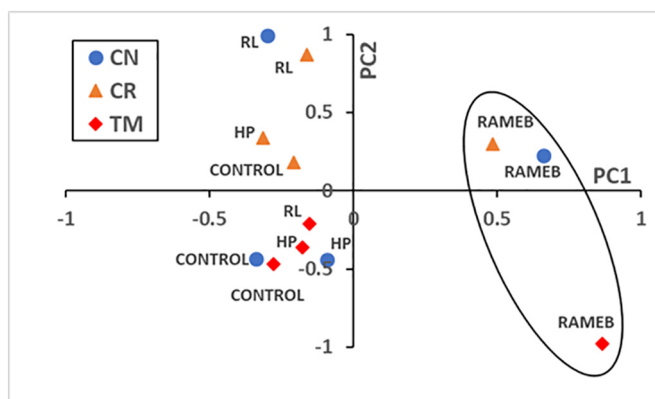


Fig. 3. Scatter of the data obtained in the Varimax rotated PCA for each soil and treatment on the plane of the first two principal components (PC1 vs PC2).



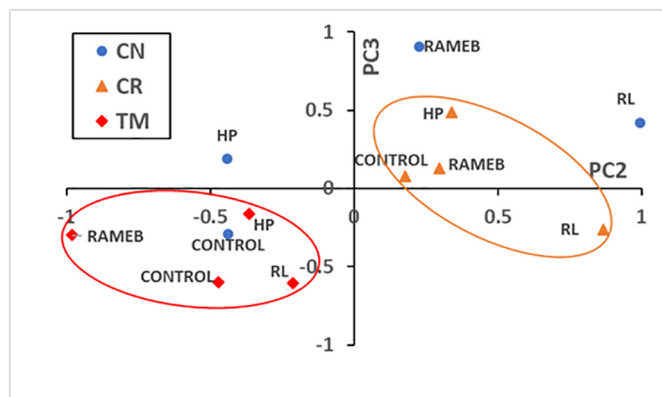


Fig. 4. Scatter of the data obtained in the Varimax rotated PCA for each soil and treatment on the plane of principal components 2 and 3 (PC2 vs PC3).

rates, our results (higher dissipation rates in TM) indicate that other soil properties have to be taken into account, such as probably those related to native degrader microorganisms. The points corresponding to treatments with soil CN (acidic pH, low clay and medium OM content), have more disperse values and the different treatments are not separated in PC2 from the other two soils. The importance of these two experimental factors (incubation time described above by PC1, Fig. 2S, and soil type by PC2, Fig. 4) in PAH dissipation agrees with results of Wu et al. (2014) that applied conjoint statistical analysis to PAHs amended contaminated soils. They also found these characteristics as the most significant factors in PAHs bioavailability.

The second statistical analysis carried out was an analysis of variance (ANOVA) according to the GLM. In this case, the assay with soils contaminated only with 7-PAHs was also included to study the effect of the presence and absence of HMW PAHs on the dissipation of the LMW 7-PAHs that showed the higher dissipation rate. So, not only the effects of the experimental factors soil, availability enhancer and incubation time were studied (the 3 factors already included in PCA), but also co-contamination effect is described. Thus, a 4-way ANOVA was applied.

GLM confirms that the presence of HMW PAHs significantly affects the contents of only some of the 7 PAHs studied, namely ACL, ACE and FLU ( $p$  value  $\leq 0.05$ ) (Table 5). These are the 3 PAHs with the lowest molecular weight (3 rings, 12–13C atoms). This result confirms the results previously observed in  $DT_{50}$  values for these 3 PAHs (Table 3), with faster dissipation when HMW PAHs are present in the system. On the contrary, PHE and ANT (3 rings, 14C atoms), FLT and PYR (4 rings, 16C atoms) were not

statistically affected by the presence of HMW PAHs ( $p$  value  $>0.05$ ). In this way, although PCA has grouped the 7 PAHs with lower MW, GLM separates them in two subgroups, according to the effect of HMW PAHs. Nevertheless, the other 3 factors studied (incubation time, soil and availability enhancer) do statistically affect the 7 PAHs ( $p$ -value  $<0.05$ ).  $Eta_p^2$  value indicates the proportion of variability in the observed data that is accounted for by each factor. Values close to 1 indicate a high relation between the variable and the factor studied (Table 5). It is observed that for the 7 PAHs the most influencing factor in their dissipation in soils was the incubation time, with  $Eta_p^2$  values ranging from 0.961 to 0.773. The second most important factor was the presence of an availability enhancer, with  $Eta_p^2$  values ranging from 0.893 to 0.363. The decreasing  $Eta_p^2$  value when HMW PAHs factor is taking into account from ACL to PYR confirms that the influence of co-contamination decreases as the molecular weight of the 7 lighter PAHs increases. The four-way ANOVA study shows significant interactions among certain experimental factors ( $p$ -value  $<0.05$ , Table 5). 2-factor interactions that were significant for the dissipation of 7 LMW PAHs are SOIL x TIME and AE x TIME, what means that the effect of incubation time depends on soil characteristics and availability enhancer used for these 7 PAHs.

#### 4. Conclusions

The results obtained in this study of soils contaminated with a mix of PAHs indicated that 3-ring PAHs (ACL, ACE, FLU, PHE, ANT) and FLT and PYR (4-ring PAHs), those with  $Kow \leq 5.2$ , could be highly degraded in soils with microbial flora preadapted to the presence of compounds with similar structures. For the rest of PAHs with 4, 5 or 6 rings (BANT, CHR, BPYR, BFLT, DBANT, BPER, IPYR), biodegradation is considered almost negligible, except for BPYR and BANT in the three soils, and the effect of type of soil plays an important role for these high molecular weight PAHs, since their dissipation increased as the organic matter of the soils increased, indicating that abiotic dissipation due to formation of non-extractable residues has to be also taken into account. Moreover, the presence of HMW PAHs increased degradation rate of the most lighter PAHs ACE, ACL and FLU, showing statistically significant decreases in  $DT_{50}$ , confirmed by 4-way ANOVA.

The use of availability enhancers on PAHs degradation rate in the soils studied showed quite different effects. The cyclodextrin HP decreased  $DT_{50}$  of PHE, FLT and PYR due to a positive effect as availability enhancer, but the presence of HP was not necessary for the degradation of the lighter PAHs (ACL, ACE, FLU, ANT), nor has it increased the dissipation of the HMW PAHs. Therefore, HP could have advantages to shorten the time in the remediation of those soils contaminated principally by LMW PAHs.

Table 5

Estimated main effects and interactions of experimental factors (presence of HMW PAHs, type of soil, availability enhancer and incubation time) on the dissipation of the 7 lighter PAHs according to four-way ANOVA.

Factor	ACL		ACE		FLU		PHE		ANT		FLT		PYR	
	p-value	$Eta_p^2$	p-value	$Eta_p^2$	p-value	$Eta_p^2$	p-value	$Eta_p^2$	p-value	$Eta_p^2$	p-value	$Eta_p^2$	p-value	$Eta_p^2$
HMW PAHs (HMW)	<b>0.000</b>	0.239	<b>0.000</b>	0.225	<b>0.002</b>	0.081	0.987	0.000	0.172	0.015	0.354	0.007	0.267	0.010
Soil (SOIL)	<b>0.002</b>	0.096	<b>0.028</b>	0.058	<b>0.000</b>	0.152	<b>0.000</b>	0.165	<b>0.000</b>	0.146	<b>0.007</b>	0.08	<b>0.000</b>	0.177
Availability enhancer (AE)	<b>0.000</b>	0.516	<b>0.000</b>	0.534	<b>0.000</b>	0.893	<b>0.000</b>	0.748	<b>0.000</b>	0.722	<b>0.000</b>	0.644	<b>0.000</b>	0.363
Incubation time (TIME)	<b>0.000</b>	0.953	<b>0.000</b>	0.961	<b>0.000</b>	0.926	<b>0.000</b>	0.897	<b>0.000</b>	0.773	<b>0.000</b>	0.817	<b>0.000</b>	0.822
HMW x SOIL	<b>0.008</b>	0.077	<b>0.000</b>	0.132	0.894	0.002	0.843	0.003	0.359	0.017	0.269	0.022	0.993	0.000
HMW x AE	0.081	0.054	<b>0.015</b>	0.083	0.179	0.040	0.227	0.035	<b>0.012</b>	0.087	0.115	0.048	0.134	0.045
HMW x TIME	<b>0.000</b>	0.372	<b>0.000</b>	0.261	0.829	0.012	0.403	0.033	<b>0.002</b>	0.131	0.836	0.012	0.235	0.045
SOIL x AE	<b>0.020</b>	0.116	<b>0.002</b>	0.161	0.128	0.078	0.053	0.097	<b>0.000</b>	0.289	<b>0.000</b>	0.191	<b>0.000</b>	0.436
SOIL x TIME	<b>0.003</b>	0.173	<b>0.036</b>	0.125	<b>0.012</b>	0.147	<b>0.000</b>	0.225	<b>0.000</b>	0.265	<b>0.000</b>	0.434	<b>0.000</b>	0.282
AE x TIME	<b>0.000</b>	0.552	<b>0.000</b>	0.543	<b>0.000</b>	0.561	<b>0.000</b>	0.636	<b>0.000</b>	0.79	<b>0.000</b>	0.623	<b>0.000</b>	0.526
HMW x SOIL x AE	0.244	0.063	0.200	0.068	0.538	0.041	0.077	0.089	<b>0.039</b>	0.103	0.268	0.06	0.390	0.050
HMW x SOIL x TIME	<b>0.006</b>	0.159	<b>0.006</b>	0.16	0.724	0.042	0.157	0.092	0.194	0.087	0.081	0.108	0.239	0.081
HMW x AE x TIME	<b>0.004</b>	0.205	<b>0.003</b>	0.211	0.903	0.049	0.905	0.049	<b>0.045</b>	0.158	0.762	0.064	0.670	0.072
SOIL x AE x TIME	<b>0.010</b>	0.281	<b>0.004</b>	0.299	<b>0.000</b>	0.408	0.226	0.198	<b>0.000</b>	0.468	<b>0.000</b>	0.368	<b>0.000</b>	0.399
HMW x SOIL x AE x TIME	0.096	0.226	<b>0.010</b>	0.281	0.975	0.090	0.730	0.138	0.307	0.186	0.630	0.149	0.484	0.165

Values in bold means that the experimental factor (or factor interaction) is significant for the corresponding PAHs ( $p < 0.05$ ).  $Eta_p^2$  value indicates the proportion of variability in the observed data that is accounted for by each factor. Values close to 1 indicate a high relation between the variable and the factor studied.

On the contrary, the cyclodextrin RAMEB showed higher PAHs contents in all soils in comparison to their natural attenuation, due to the very high adsorption of RAMEB in the three soils, blocking their surfaces and preventing the release of PAHs to the soil solution. Therefore, RAMEB is not appropriate as availability enhancer in PAHs contaminated soils.

In the case of the rhamnolipid biosurfactant (RL), the content of PAHs at the end of the incubation process (120 days) was not so different to the natural attenuation treatment, but the DT<sub>50</sub> values obtained were higher, due to an initial RL toxicity, what cause that the native microorganisms need an acclimation period before degrading the PAHs. After this period, a higher degradation rate than in the control treatment was achieved. The use of RL needs further research to select adequate concentrations to minimize its toxicity and the acclimation period of soil degrader microorganisms, but seems to be a good choice as availability enhancer for LMW contaminated soils.

These conclusions are supported by Principal Components Analysis, which divided PAHs in three components. The first one (PC1) included the 7 PAHs with the lowest MW and lower Kow, those with the higher degradation rate, what suggest similarities in their dissipation processes. PC1 separated RAMEB treatments from the other availability enhancers, due to their negative effect on the degradation rate of the PAHs. Heavier PAHs are included in the other components, 5- and 6-rings PAHs are grouped in PC2, whereas CHR and BANT (4 rings) are in PC3.

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## Abbreviations

ACE	Acenaphthene
ACL	Acenaphthylene
ANOVA	Analysis of variance
ANT	Anthracene
BANT	Benzo[a]anthracene
BFLT	Benzo[b]fluoranthene
BPER	Benzo[g,h,i]perylene
BPYR	Benzo[a]pyrene
BSs	Biosurfactants
CDs	Cyclodextrins
CHR	Chrysene
CMC	Critical micelle concentration
DBANT	Dibenzo[a,h]anthracene
DOC	Disolved organic carbon
EEA	European Environment Agency
FLT	Fluoranthene
FLU	Fluorene
HMW	High molecular weight
HP	2-hydroxypropyl-β-cyclodextrin
INT	Iodonitrotetrazolium chloride
IPYR	Indeno[1,2,3-c,d]pyrene
LMW	Low molecular weight
PHE	Phenanthrene
OM	Organic matter
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
POPs	Persistent organic pollutants
PYR	Pyrene
RAMEB	Randomlymethylated-β-cyclodextrin
RL	Rhamnolipid
SFO	Simple first order
USEPA	United State Environmental Protection Agency

## Declaration of competing interest

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.

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**Florido M.C.:** Investigation, statistical analysis, review.

**Rubio-Bellido M.:** Investigation, methodology.

**Villaverde J.:** Data curation and kinetic modelling, funding acquisition, review.

**Morillo E.:** Conceptualization, design of experiment, writing, review & editing, funding acquisition.

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