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Risk reductions during pyrene biotransformation and mobilization in a model plant-bacteria-biochar system

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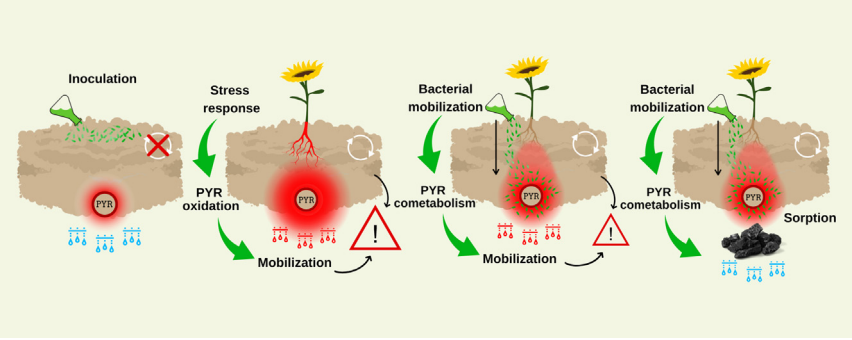
HIGHLIGHTS

- Sunflowers transform distantly located ^{14}C-pyrene in soil.
- Root-mediated bacterial mobilization facilitates cometabolism of this PAH.
- Pyrene metabolites appeared in leachates and in plant tissues.
- Biochar retained metabolites and reduced biotransformation risks.
- This new scenario has implications for risk reduction during bioremediation.

GRAPHICAL ABSTRACT



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ABSTRACT

The productive application of motile microorganisms for degrading hydrophobic contaminants in soil is one of the most promising processes in modern remediation due to its sustainability and low cost. However, the incomplete biodegradation of the contaminants and the formation of the intermediary metabolites in the process may increase the toxicity in soil during bioremediation, and motile inoculants may mobilize the pollutants through biosorption. Therefore, controlling these factors should be a fundamental part of soil remediation approaches. The aim of this study was to evaluate the sources of risk associated with the cometabolism-based transformation of ^{14}C-labeled pyrene by inoculated *Pseudomonas putida* G7 and identify ways to minimize risk. Our model scenario examined the increase in bioaccessibility to a distant source of contamination facilitated by sunflower (*Helianthus annuus* L.) roots. A biochar trap for mobilized pollutant metabolites and bacteria has also been employed. The experimental design consisted of pots filled with a layer of sand with ^{14}C-labeled pyrene (88 mg kg^{-1}) as a contamination focus located several centimeters from the inoculation point. Half of the pots included a biochar layer at the bottom. The pots were incubated in a greenhouse with sunflower plants and *P. putida* G7 bacteria. Pots with sunflower plants showed a higher biodegradation of pyrene, its mobilization as metabolites through the percolate and the roots, and bacterial mobilization toward the source of contamination, also resulting in increased pyrene transformation. In addition, the biochar layer efficiently reduced the concentrations of pyrene metabolites collected in the leachates. Therefore, the combination of plants, motile bacteria and biochar safely reduced the risk caused by the biological transformation of pyrene.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one of the most common groups of contaminants found in the environment. These chemical compounds are very persistent in soils due to their high hydrophobicity and high structural stability (Lawal, 2017; Ma et al., 2022). In this context, the bioavailability of these pollutants is an important subject in soil remediation studies (Ortega-Calvo et al., 2013; Ortega-Calvo et al., 2015). Although the concept of bioavailability is often associated with techniques to increase the solubilization of contaminants, it can also be increased by promoting the dispersal of microorganisms through contaminated soil. In this process, bacterial chemotaxis plays an important role in the mobilization of PAH-degrading bacteria and the biodegradation of these contaminants (Ahmad et al., 2020; Ortega-Calvo et al., 2003).

Research has shown that many *Pseudomonas* species (Harwood et al., 1990; Sampedro et al., 2015), among others, exhibit chemotactic responses to PAHs such as pyrene, anthracene or phenanthrene (Ortega-Calvo et al., 2003). Chemotaxis enhances the accessibility of the low-permeability regions of contaminated soils to bacteria, which may not be accessible by dispersion alone and may increase the residence times of the bacteria in the contamination sites and therefore, biodegradation (Lacal, 2017; Wang et al., 2016). However, the process of dispersing microorganisms in soil may be quite complicated (Harms and Wick, 2006). Chemotaxis is essential in bacterial transport in roots and is established as one of the major vectors to promote bacterial dispersal (Dupuy and Silk, 2016). Kamilova et al. (Kamilova et al., 2005) showed that with the motile bacteria *Pseudomonas fluorescens*, *Pseudomonas putida* and *Aeromonas hydrophila*, tomato root colonization was higher than that of the control with a nonmotile strain (Kamilova et al., 2005). A number of investigations have revealed the importance of bacterial taxis and their interactions with plant root exudates and how they may improve the degradation efficiency (Neal et al., 2012; Sun et al., 2012; Zhang et al., 2013). Exudates include amino acids, organic acids and sugars, and some of them, such as organic acids, may modify the physicochemical properties of the soil and enhance the attraction to the chemotactic bacteria to the pollutant focus (Hartmann et al., 2009). Root exudates can also increase the degradation of PAHs in different ways: desorption of contaminants in soils, increasing bioavailability, can be enhanced by the production of carboxylates; transformation of pollutants by enzymes secreted by plants and stimulation of the activity of microbial communities via C and N secreted by plants (Gkorezis et al., 2016).

In this study, the cometabolic transformation of pyrene carried out by a motile microbial inoculant was taken as an example to assess risk that may arise from the bioremediation of PAH-polluted soils. The application of a cometabolic-competent inoculum based on motile bacteria may constitute an advantage in bioremediation, especially for high-molecular-weight (HMW) PAHs that are only degraded through cometabolism (Fernández-López et al., 2021). However, such biological processing may cause problems if toxic and mobile metabolites are formed, thus constituting an additional source of risk, different from the parent pollutant. For example, Tian et al. (2017) identified different pyrene products derived from the bacterial metabolic activity of the pollutant as toxic to humans. Many of these metabolites produced during the bioremediation process can even lead to changes in soil ecosystems, reducing the diversity of microbial organisms or decreasing seed production and root length in plants (Wang et al., 2021). Motile bacteria can also mobilize pollutants through biosorption (Rolando et al., 2020), increasing toxicity and the risk of contamination in less accessible areas such as groundwater (Kumar et al., 2022). We examined, in a model scenario, how this risk could be reduced through an appropriate arrangement of plants and sorbent materials. In this paper, sunflower (*Helianthus annuus* L.) was used due to its strong tolerance to PAHs (Fernández-López et al., 2021; Ortega-Calvo et al., 2013; Ortega-Calvo et al., 2017). Some studies have reported significant phytoremediation efficiencies of sunflower species in petroleum-contaminated soil (Diab, 2008; Dickson et al., 2020; Sivaram et al., 2018). In addition to its rapid germination and long root system that increases the surface area for bacterial colonization (Maliszewska-Kordybach and Smreczak, 2000), the strong chemotactic response of *P. putida* G7 to sunflower root exudates has been reported (Castilla-Alcantara et al., 2022). Therefore, sunflower and its higher leaf evaporation level have been demonstrated to be adequate for the experimental model system (Fernández-López et al., 2021; Tejeda-Agredano et al., 2013). In addition to the combined use of plants and bacteria, biochar amendment has been established as another reference strategy for the remediation of PAH-contaminated soils and sediments (Bianco et al., 2021; Kong et al., 2018). Due to its eco-friendly use and low cost compared to physical and chemical techniques (Liu et al., 2011; Wu et al., 2017), biochar can be established as an added value to the bioremediation process. When adding biochar, an amount of metabolites could be absorbed onto the biochar, leading to a significant reduction in soil toxicity and biodegradation enhancement (Kumar et al., 2022).

We employed sunflower and the chemotactic bacterium *P. putida* G7 in a new model plant-bacteria-biochar system to understand the potential sources of risk caused by motile inoculants and identify possible methods for risk minimization. This bacterial strain has been well studied in our laboratory under this context for chemotaxis to different substances, such as sunflower plant exudates (Castilla-Alcantara et al., 2022; Jimenez-Sanchez et al., 2018), and its ability to degrade naphthalene and to cometabolize pyrene (Rolando et al., 2020). These characteristics make this bacterium suitable for the purpose of our study. Therefore, we specifically tested whether pyrene polar metabolites were produced in this system under greenhouse conditions and to what extent the potential risk from pyrene biodegradation could be reduced by incorporating biochar as a sequestering agent. Our experimental approach included, on the one hand, sand as a model substrate for plant growth and bacterial inoculation to minimize the eventual interactions of the pollutant, its metabolites and the inoculated bacteria with the substrate. On the other hand, ^{14}C-tracer techniques were employed to facilitate the study of the transfer of pollutant carbon to the substrate, leachates and plant tissues.

2. Materials and methods

2.1. Chemicals

[4,5,9,10-^{14}C]-pyrene (58.8 mCi mmol^{-1}, radiochemical purity >98 %) was purchased from Campro Scientific GmbH (Veenendaal, The Netherlands), and ^{12}C-pyrene (98 % purity) was purchased from Sigma Aldrich (Madrid, Spain). Analytical-grade dichloromethane, acetonitrile, methanol and acetone were supplied by Fischer Chemical (Madrid, Spain). Washed sand was obtained from Scharlab, S.A. (Barcelona, Spain) and had the following characteristics: pH, 5.8; grain size, 300–350 μm; and specific density, 2.66 g cm^{-3}. Commercial biochar produced through pyrolysis of birch, beech or oak wood was obtained from EGoS GmbH (Bottrop, Germany). The material had a carbon content higher than 90 % and an inner surface area of 500 m^2 g^{-1}. The total PAH content was 0.2 mg kg^{-1}, the humidity was 48.3 %, and the pH was 10.

2.2. Cultivation of bacteria

The flagellated bacterium *P. putida G7*, which is able to use naphthalene as the sole carbon and energy source, and degrade pyrene by cometabolism, was cultivated in MSB medium supplemented with salicylate and prepared for greenhouse experiments as described previously (Fernández-López et al., 2021), with some modifications. Briefly, cultures were grown in three stages to obtain a final volume of 7 L. Three bacterial cultures of 100 mL were initially produced and transferred to three Erlenmeyer flasks (1 L) with 400 mL of MSB medium and then incubated for 24 h. The cultures were divided into seven 200-mL portions that were then transferred into individual 2 L flasks, diluted with 800 mL of medium, and incubated for another 24 h to reach the early stationary phase (optical density at 600 nm or OD_{600} of 0.5 or 5 × 10^8 cell mL^{-1}). Cells were then harvested by centrifugation at 5000 rpm for 10 min. The pellet was resuspended in 100 mL of modified MSB (per liter: 670 mg of $Na_2HPO_4{\cdot}12H_2O$, 340 mg of KH_2PO_4, 80 mg of $CaCl_2$, 33 mg of NH_4NO_3, 112 mg of $MgSO_4{\cdot}7H_2O$,

5 mg of $ZnSO_4{\cdot}7H_2O$, 2.5 mg of $Na_2MoO_4{\cdot}2H_2O$, 0.22 mg of $FeCl_3$) without salicylate to give a cell density of 5.58×10^7 cell mL^{-1}. This culture medium was modified to provide the necessary N requirement for plant growth and development, and still allowed a stable cell motility in *P. putida*, which was confirmed by light microscopy. Inoculation of 18 of the 26 total pots of the experiment with these suspensions (see Section 2.3, greenhouse experiment) resulted in a cell concentration of 1.43×10^9 cell kg^{-1} soil.

2.3. Greenhouse experiment

2.3.1. Experimental design

The experiment was carried out in a greenhouse at 23 ± 1 °C using a total of 26 pots. The pots were 18.5 cm high, and the maximum and minimum diameters were 21.5 and 15.0 cm, respectively. A hole at the bottom of each pot allowed for the collection of leachates during the experiment. Each pot received 4.8 kg of sand, which had a height of 11 cm upon packing. The pots were classified into two different groups: those that received biochar as a layer at the bottom and those without biochar. A diagram describing the experiment is shown in SI (Fig. S1). In each group, a subgroup was prepared with pots of sand loaded with ^{12}C- and ^{14}C-pyrene (14 labeled pots, numbers 1–8 to 15–20) and another with only ^{12}C-pyrene (12 unlabeled pots, numbers 9–14 to 21–26). The latter subgroup was used for estimations not requiring ^{14}C-labeling (i.e., soil dehydrogenase (DH) activity, total organic carbon (TOC) measurements, metabolite analysis, and fresh weight of plants). In each subgroup, there were three different treatments, each one performed in duplicate: planted inoculated pots (pots 1–4; 9–10; 15–16; 21–22), planted uninoculated pots (5–6; 11–12; 17–18; 23–24) and unplanted inoculated pots (7–8; 13–14; 19–20; 25–26). Neither the sand, the biochar, the percolation medium or water were sterilised as the pots were maintained in the greenhouse under non-sterile conditions. Because the focus of this study was to monitor the transformation of ^{14}C-pyrene, no control pots were run without the contaminant. Nevertheless, preliminary germination and growth tests on sand with and without pyrene showed no apparent effects of the chemical on the plants at the initial concentration that was used in the greenhouse experiment, either as seed germination, plant development or flowering.

Before pot packing, pyrene was added to 500 g portions of sand that would later be introduced in each individual pot as a contaminated sand layer. For this preparation, 1.5 mL of an acetone solution containing 360,000 dpm of ^{14}C-pyrene and sufficient unlabeled pyrene to give a concentration 88 mg kg^{-1} in the contaminated layer of every pot, was dispensed onto a 10 g sand subsample. When the solvent was completely evaporated, this subsample was mixed thoroughly with 40 g of sand in a glass jar. Then, this portion was homogenized with the rest of the sand portion (450 g) and shaken manually for 10 min to ensure homogenization. In the case of unlabeled pots, the spiking solution had only ^{12}C-pyrene. The contaminated sand was introduced into the pots as a layer at 7 cm from the upper sand surface during packing.

At the beginning of the experiment, the water content in each pot was adjusted to 100 % water holding capacity with 1.2 L of Milli-Q water, due to the rapid percolation capacity of the pure sand. The inoculation of *P. putida* G7 was performed 14 days after of the introduction of the sunflower seeds into the pots, once there was important root development. Then, 100 mL of a modified MSB suspension (described in Section 2.2) of *P. putida* G7 cells was added to each pot. To maintain the moisture, 3 irrigations per week were necessary: 2 times per week with modified MSB medium for plant nutrition and bacterial maintenance (to coincide with the collection of leachate) and once per week Milli-Q water was added to maintain the humidity in the pots. A second inoculation with *P. putida G7* was performed 28 days after seed introduction.

The sunflower seeds were immersed in tap water and stirred for 30 min to eliminate anti- microorganism protection, and immediately, the coats were manually removed. Fifteen of these naked seeds were used per planted pot. Once the germination period was over, seven to thirteen plants grew per pot. Throughout the sunflower development cycle, the percentage of seed germination and the blooming evolution and stem length of plants were separately evaluated for each treatment.

2.3.2. Sample collection

Leachate sampling was performed to determine the ^{14}C- activity at the beginning, after 2 days, and then 2 days per week until the end of the experimental period. Leachates were collected by gravity immediately after each irrigation event. Samples of sand and, if applicable, plants were collected at the end of the experimental period (81 days for both planted and unplanted pots and 133 days for unplanted pots). Five sand samples were extracted with a hand auger from each pot. These samples were then divided into three subsamples and homogenized: top, corresponding to the first 2.5 cm of sand, middle, representing the next 1.5 cm; and bottom, representing remaining sand to 11 cm. In the case of the pots with biochar, the third sample was composed of a mixture of sand and biochar at the bottom of the pots. In unplanted pots, for which the experiment continued after the first sampling, the holes in the pots were refilled with clean sand and marked to avoid additional sampling at those spots. Both the sand and leachate samples were stored at −20 °C until analysis to prevent microbial activity. From each treatment, two complete plants per pot in the case of labeled pots were harvested at the end of the experiment; roots, stems, leaves, flowers and fruits were carefully separated for the oxidizer analysis; and fresh weight of each organ was obtained. All the plants that grew in the unlabeled treatment pots were also harvested at the end of the experiment, and their leaves, stems, roots and fruits were washed with distilled water to calculate the fresh weight of each organ.

An experimental control was run after the second inoculation of the pots (28 days) to establish possible bacterial percolation through irrigation. Determination of the number of colony-forming units (CFUs) of *P. putida* G7 in leachate samples was carried out for planted and unplanted pots with or without biochar. A fraction of the leached liquid of the following two sample collections after inoculation (4 and 7 days) from pots without ^{14}C was collected, inoculated on TSA agar plates, and incubated for 48 h at 30 °C. The results showed that the maximum bacterial recovery in the leachates was at day 4 in planted pots without biochar ($2.81 \pm 0.66 \times 10^5$ CFU mL^{-1}) in comparison with unplanted pots without biochar ($9.45 \pm 4.66 \times 10^4$ CFU mL^{-1}). The presence of the biochar resulted in higher bacterial retention in both cases ($1.37 \pm 0.27 \times 10^5$ CFU mL^{-1} and $1.92 \pm 0.88 \times 10^4$ CFU mL^{-1} for planted and unplanted pots, respectively). However, many of the bacterial cells were retained in the pots because of the high cell density of the inoculum used.

2.3.3. Extraction and analysis of pyrene

To measure the concentration of ^{14}C-pyrene equivalents (PYR_{eq}) in the leachates, an aliquot (10 mL) was mixed with 10 mL of liquid scintillation cocktail (Ultima Gold XR, PerkinElmer). Radioactivity was measured by liquid scintillation (Beckman Instruments, Inc., Fullerton, Calif.; model LS 6500 TD). In accordance to the specific activity of ^{14}C-pyrene in the pots, 1 dpm corresponded to 1×10^{-4} mg PYR_{eq}. To determine ^{14}C in sand and sunflower plant samples, a subsample weighing 1 g in the case of sand samples or 0.2 g in the case of plant samples was combusted in an oxidizer (307 Sample Oxidizer, Perkin Elmer, combustion for 5 min with O_2). The samples obtained after combustion were measured by liquid scintillation in a QUANTULUS 1220 (PerkinElmer) and for these samples the liquid scintillation cocktail used was Permafluor E+ from PerkinElmer. Preliminary determinations with ^{14}C-spiked sunflower plant samples resulted in a recovery of 100 % of the added radioactivity.

The residual concentration of ^{12}C-pyrene was also measured in selected leachate samples by exhaustive solid-liquid extraction using a subsample of 5 g and a mixture of 100 mL of acetone/dichloromethane (1:1 *v*/v) with a Soxtherm extractor (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). Once the extract was obtained, the organic solvent was evaporated with N_2 and redissolved in acetonitrile. Then, before analysis by HPLC, the samples were filtered through 0.45 μm nylon filters. The analytical procedure for HPLC analysis has been described previously (Posada-Baquero et al., 2022).

The maximum capacity for pyrene of the leachates, assuming instantaneous equilibration to reach the solubility of pyrene in water and to sorb to the DOC components, was calculated as explained previously (Rolando et al., 2020). Briefly, the predicted fraction of pyrene freely dissolved in the aqueous phase of bacterial suspensions at equilibrium (f_w) was calculated as

$$f_w = \frac{1}{1 + [DOC] \cdot K_{OC}} \quad (1)$$

where [DOC] is the concentration (in kg L^{-1}) of dissolved organic carbon (DOC) determined experimentally in the leachates with a TOC-VC SH with an ASI-V auto sampler by Shimadzu Corporation (Tokyo, Japan) after filtration through Whatman filter with 0.45 μm pore diameter purchased from Sigma Aldrich (Madrid, Spain). The detection limit of this method was 0.05 mg L^{-1} TOC. K_{OC} is the solid-water distribution coefficient in L kg^{-1}. The log K_{OC} of pyrene used for this equation was 4.7. The capacity for pyrene was calculated as the ratio between the solubility in water of pyrene and f_w.

2.3.4. Analysis of pyrene metabolites

The analysis of leachates was performed with ultrahigh-performance liquid chromatography coupled to mass spectrometry (UHPLC–MS). The equipment setup included a Dionex Ultimate RS binary pump coupled to a quadrupole-Orbitrap QExactive hybrid mass spectrometer (Thermo Fisher Scientific, USA) equipped with an electrospray source (HESI-II) operating in positive mode at 3.5 KV and in negative mode at −3.2 KV. UHPLC analysis was carried out with a reversed-phase analytical column (Acquity UPLC BEH C18 (2.1 × 100 mm) with a 1.7 μm particle size. The flow rate used was 0.4 mL min^{-1}. Chromatographic separation was performed using a binary gradient of water (A) and methanol (B), both containing 0.15 % formic acid (v/v) to promote ionization. The elution profile consisted of 5 % B 1 min, then a linear gradient for 7.5 min until 100 % B was reached. The gradient was maintained for 2 min, and finally, it was returned to the initial conditions and maintained for 0.5 min. The chromatographic column was maintained at 40 °C throughout the analysis. The injection volume was 5 μL. The samples were previously microfiltered with 0.2 μm nylon filters. The acquisition method in the QExactive mass spectrometer was performed using a data-dependent acquisition method (TOP5). Data files acquired by UHPLC were processed by Compound Discoverer 3.2 software to identify unknown metabolites and TraceFinder 5.1 software (Thermo Scientific) to determine compounds using molecular formulas and exact masses.

2.3.5. Calculation of the bioconcentration and translocation factors

The transfer and distribution of organic chemicals in soil-plant systems have been adequately explained by using bioconcentration factors (BCFs) and translocation factors (TFs). These factors are defined as the ratio of the chemical concentrations between plant biomass and soil solution and between leaves, stems and fruit biomass and root biomass, respectively (Doucette et al., 2018; Fernández-López et al., 2021; González García et al., 2018; Lin et al., 2006; Tao et al., 2008; Yang and Zhu, 2007). We hypothesized that any significant differences between treatments in the plant uptake or transfer rates of pyrene were indications of plant uptake of polar metabolites generated by the modified bacterial accessibility and cometabolism of this PAH in soil. Therefore, the BCF for pyrene was calculated based on ^{14}C-pyrene concentrations (see Section 2.3.3) and using the following previously reported equation (Fernández-López et al., 2021; González García et al., 2019) with some modifications:

$$BCF\ (L\ kg^{-1}) = \frac{C_p\ (\text{mg kg}^{-1}\ \text{dry weight})}{C_s\ (mg\ L^{-1})} \quad (2)$$

where C_p is the concentration of pyrene equivalents (PYR_{eq}) in dry (roots stems, leaves and fruits) plant biomass and C_s is the concentration of PYR_{eq} (g L^{-1} soil solution) calculated using the following equation:

$$Cs = \frac{\Sigma(A_f - A_o - A_l)}{\Sigma(V_i - V_l)} \quad (3)$$

where A_o is the amount of PYR_{eq} (mg) in the pots measured at the start of the experiment, A_l is the amount of PYR_{eq} (mg) in the leachate, V_i is the volume of irrigation water (mL), V_l is the volume of leachate (mL), and A_f is the average amount of PYR_{eq} (mg) at the end of the experiment and was calculated taking into account the specific weights in each part of the pots using the following equation:

$$A_f = \Sigma(A_t + A_c + A_b)x\ W \quad (4)$$

where A_f is the concentration of PYR_{eq} (mg kg^{-1}) in the top of the pot multiplied by the layer thickness in the top, A_c is the concentration of PYR_{eq} (mg kg^{-1}) in the center of the pot multiplied by the layer thickness in the center, A_b is the concentration of PYR_{eq} (mg kg^{-1}) in the bottom of the pot multiplied by the layer thickness in the bottom, and W is the total weight of sand in each pot (kg).

The translocation factor (TF) was calculated to evaluate the transfer of the mass of pyrene from roots to leaves, stems and fruits, where a value equal to or lower than 1 is optimal for phytostabilization, which involves the reduction in the mobility of PYR_{eq} in soil. The TF was calculated as the ratio between the mass of pyrene in stems, leaves and fruits and that in roots:

$$TF = \frac{C_{plant}}{C_r} \quad (5)$$

where C_{plant} is the mass of PYR_{eq} (mg) per kg of dry stem, leaf and fruit biomass and C_r is the mass of PYR_{eq} (mg) per kg of dry root biomass.

2.3.6. Microbial and biochemical determinations

The number of *P. putida* cells was determined in sand samples from unlabeled pots at the end of the greenhouse experiment. Subsamples of sand cores at different heights (1 g) were vortexed mixed with 3 mL of MSB medium, and agitated for 30 min at 150 rpm. After a proper time for sand decantation, serial dilutions of the supernatant were plated in MSB agar plates with sterile naphthalene crystals on the lid. Bacterial motility was confirmed using an AxioVert A1 phase contrast inverted microscope (Zeiss, Germany), and several videos were recorded using an Axiocam 305 color (Zeiss, Germany) (interface, Zen software blue edition). To exclude any possible interference in the transformation of pyrene by cross-contamination, samples of non-inoculated pots (pots 11 and 12, Fig. S1) were plated on naphthalene-containing solid medium with substrate samples obtained at the end of the greenhouse experiment. Two main types of colonies different to *P. putida* G7 were identified. This was expected because of the lack of sterility under greenhouse conditions. Nevertheless, the two main types of colonies observed in the plates were isolated and their capacity to transform pyrene was tested by radiorespirometry and partitioning in a passive dosing system (Rolando et al., 2020) in 100 mL flask assays containing 25 mL of MSB and passively dosed ^{14}C-pyrene (100,000 dpm) with a silicone O-ring. One milliliter samples were taken at different time periods from the soda trap and the suspension, and mixed with 5 mL of scintillation liquid to determine radioactivity as described above (see Section 2.3.3) The DH activity of the sand in the pots was determined by the reduction of the tetrazolium salt iodonitrotetrazolium chloride (INT) to yield iodonitrotetrazolium formazan (INTF) as described elsewhere (García et al., 1994; Trevors, 1984)

3. Results

3.1. Plant response

The seed germination percentage, plant development and flowering indicated that sunflower plants tolerated growth conditions well. The seed germination rate was 78.12 % at 6 days from the start of the greenhouse experiment and 100 % at 24 days. As the experiment progressed, the plant length reached an average of 68.83 cm at 44 days and 96.94 cm at day 63. The proportion of flowering plants reached 92.5 % at day 28, and on day 49, all the plants had flowered. No significant differences (Tukey test,

$P \leq 0.05$) among treatments were found regarding fresh weight with or without biochar and inoculation at the end of the assay (Table S1). In accordance with the ontogenetic cycle of sunflower in the assayed greenhouse conditions, the plants started to decline at day 60.

3.2. Mobilization of ^{14}C-pyrene into leachates

The time evolution of the concentration of pyrene equivalents (PYR_{eq}) in the leachates is presented in Fig. 1. Plant development caused a significant increase in the concentration of PYR_{eq} in the leachates of uninoculated pots at day 20 (Fig. 1A), reaching its maximum concentration on day 39. With inoculation (performed at one or two stages, indicated by the arrows in Fig. 1B), the concentration of PYR_{eq} also increased at day 20 in the leachates from planted pots but not to the extent (approximately at 1 mg L^{-1} with two inoculations, Fig. 1B) as with plants only. These increases were not observed in planted pots with a biochar layer at the bottom, with and without inoculation. During many parts of the experimental period, the PYR_{eq} concentrations in the leachates from those pots were statistically lower (Student's *t*-test, $P \leq 0.05$) than those in the corresponding pots without biochar (as indicated by the asterisks in Fig. 1A and B). Two replicates per treatment were used for statistical comparisons. PYR_{eq} mobilization also occurred in the leachates from unplanted inoculated pots (Fig. 1C), but the mobilization was delayed to 40 days and occurred at lower PYR_{eq} maximum concentrations than in planted pots that received no bacterial inoculum. This increase also occurred in unplanted pots with a biochar layer. The sampling of leachates in these two treatments was maintained longer (compared to planted pots, where sunflower plant decay forced the end of the experimental period to secure plant biomass sampling) to follow the mobilization of PYR_{eq} at these later stages.

The maximum PYR_{eq} concentrations detected in the inoculated treatments (Fig. 1 B and C) far exceeded the aqueous solubility of pyrene (0.135 mg L^{-1}), which indicates the presence of hydrophilic metabolites. This was expected, given the capacity of the *P. putida* G7 strain to cometabolize the compound (Fernández-López et al., 2021; Rolando et al., 2020). However, pyrene was also mobilized to a greater extent in planted pots that received no inoculum (Fig. 1A). Sorption to dissolved organic matter (DOM) can also cause pollutant mobilization and an apparent increase in the aqueous concentration (Rolando et al., 2020; Yang and Zhu, 2007; Zhu et al., 2009). Therefore, several sampling times were selected for TOC determinations (employed to estimate DOM levels) to cover the periods of maximum PYR_{eq} mobilization in the different treatments (Fig. 1). The results are shown in Table 1. The TOC concentrations were lower in most of the leachates from biochar pots, confirming the adsorptive capacity of this material. The range of TOC concentrations observed in planted pots without biochar, both with and without inoculation (13 mg L^{-1}–32 mg L^{-1}), was not sufficient to explain the observed mobilization of PYR_{eq} through sorption to DOM. In accordance with our calculations based on the K_{oc} of pyrene and the TOC concentration in the leachates, the enhanced capacity for pyrene eventually caused by sorption to DOM would have only doubled its aqueous solubility, i.e., up to 0.25 mg L^{-1} (Table 2). To confirm experimentally that the mobilized ^{14}C did not fully correspond to the parent compound, these ^{14}C-containing leachate samples were extracted with organic solvents and analyzed by HPLC. This analysis gave a very low concentration of pyrene (Table 2). These results indicate that water-soluble metabolites were produced by the direct action of the plants, thus explaining the high PYR_{eq} concentrations in these leachates.

Table 1
Concentration of total organic carbon (TOC) in selected leachate samples (mg L^{-1}) from pots during the greenhouse experiment.

Treatment	Time (d)	TOC
Planted noninoculated	35	25.37 ± 2.84
	39	17.28 ± 5.58
	49	15.65 ± 7.13
Planted noninoculated + biochar	35	21.26 ± 4.11
	39	7.35 ± 1.39
	49	3.38 ± 2.23
Planted inoculated 2×	25	15.31 ± 1.70
	28	12.71 ± 1.34
	32	14.61 ± 1.97
	35	31.96 ± 6.80
	39	16.97 ± 0.65
	43	15.26 ± 0.97
Planted inoculated 2× + biochar	25	12.99 ± 18.36
	28	3.20 ± 4.52
	32	6.70 ± 2.22
	35	24.63 ± 8.60
	39	7.16 ± 3.04
	43	4.07 ± 3.21
Inoculated 2x	39	8.62 ± 1.05
	70	8.90 ± 0.97
	74	9.40 ± 1.58
	77	8.71 ± 1.27
Inoculated 2x + biochar	39	ND[a]
	70	ND[a]
	74	ND[a]
	77	ND[a]

[a] ND, not detected.

Leachates from the same sampling days in parallel planted treatments that contained non labeled pyrene only were selected in accordance with these results and analyzed by UHPLC-MS. Positive and negative ionization modes were used for the analysis (Fig. 2), obtaining the best responses in negative ion mode. A major metabolite with the molecular formula

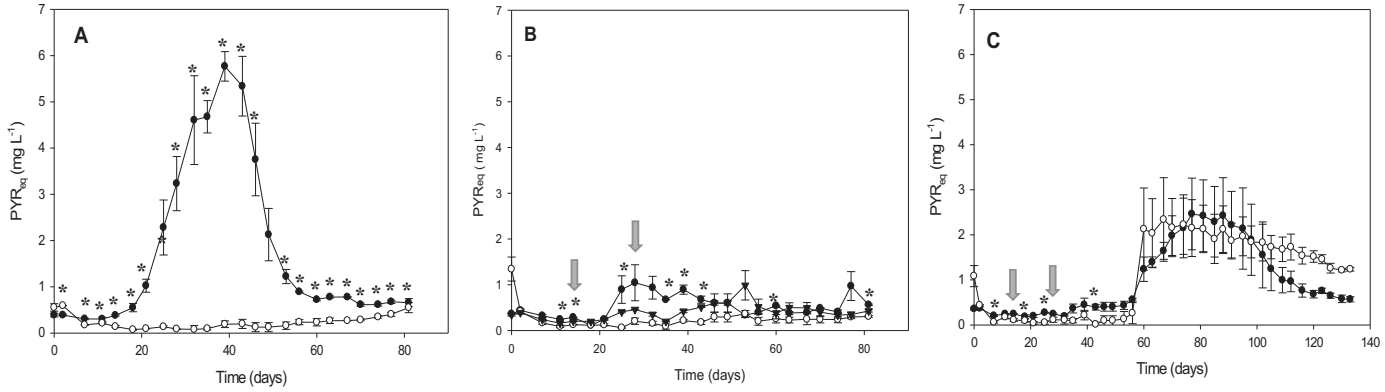

Fig. 1. Evolution of the concentration of equivalents (PYR_{eq}) in leachate samples in planted uninoculated pots (A), planted inoculated pots (B) and unplanted inoculated pots (C). The filled symbols represent the treatments without biochar, and the empty symbols represent the treatments where a biochar layer was also present at the bottom of the pots. All pots in B and C received two inoculations (indicated by the arrows), with the exception of the pots corresponding to the filled triangles in panel B, which received only the first inoculation at 14 days. The asterisks indicate significant differences (Student's *t*-test, $P \leq 0.05$) for each treatment (i.e., planted uninoculated, planted and inoculated 2×, unplanted and inoculated 2×) with and without biochar.

Table 2
Chemical analysis of selected leachate samples from pots during the greenhouse experiment.

Treatment	Sampling day	PYR_{eq} (mg L^{-1})	Pyrene (μg L^{-1})	Maximum capacity pyr (mg L^{-1})[a]	P-4-C peak area[b]
Planted uninoculated	39	5.76 ± 0.64	0.9 ± 0.1	0.25	116,558,521
Planted inoculated 2x	28	1.04 ± 0.79	1.5 ± 0.70	0.22	949,009

[a] Calculated from the total organic carbon concentrations in Table 1.
[b] P-4-C, phenanthrene-4-carboxylic acid.

$C_{15}H_{10}O_2$ was found with a retention time of 6.1 min and molecular mass of 222.06082 (Fig. 2B). To ensure the assignment, a second data processing software was used (TraceFinder), which, using the exact molecular mass, showed the corresponding fragmentation of this metabolite, and the mass spectral library attributed to phenanthrene-4-carboxylic acid (P-4-C, also named 4-phenanthroic acid). This compound gave rise to the adduct $[M-H]^-$ in negative ion mode, corresponding to the loss of the acid group and the appearance of the fragmentation ion at *m/z* 177.07097 (Fig. 2C). Although a detailed quantitative analysis was not possible due to the lack of an appropriate standard, the relative differences in the P-4-C peak areas in these two samples (Table 2) suggest a 100-fold higher concentration of this metabolite in the planted uninoculated treatment than in the planted inoculated treatment. Unfortunately, it was not possible to identify other pyrene metabolites formed in the planted inoculated pots, probably because they were not at sufficient concentrations for detection.

3.3. Mobilization of ^{14}C-pyrene into plants

The distribution of PYR_{eq} in the different plant samples is shown in Table 3. The results have a general concordance with the mobilization of PYR_{eq} observed in the leachates. With the exception of roots, significantly higher PYR_{eq} concentrations were measured in the tissues from plants that grew in pots without inoculum and biochar (uninoculated, see Table 3) than in those from the other treatments. This treatment, as well as the planted pots with two inoculations, was characterized by the absence of significant differences among different tissues, i.e., PYR_{eq} distributed

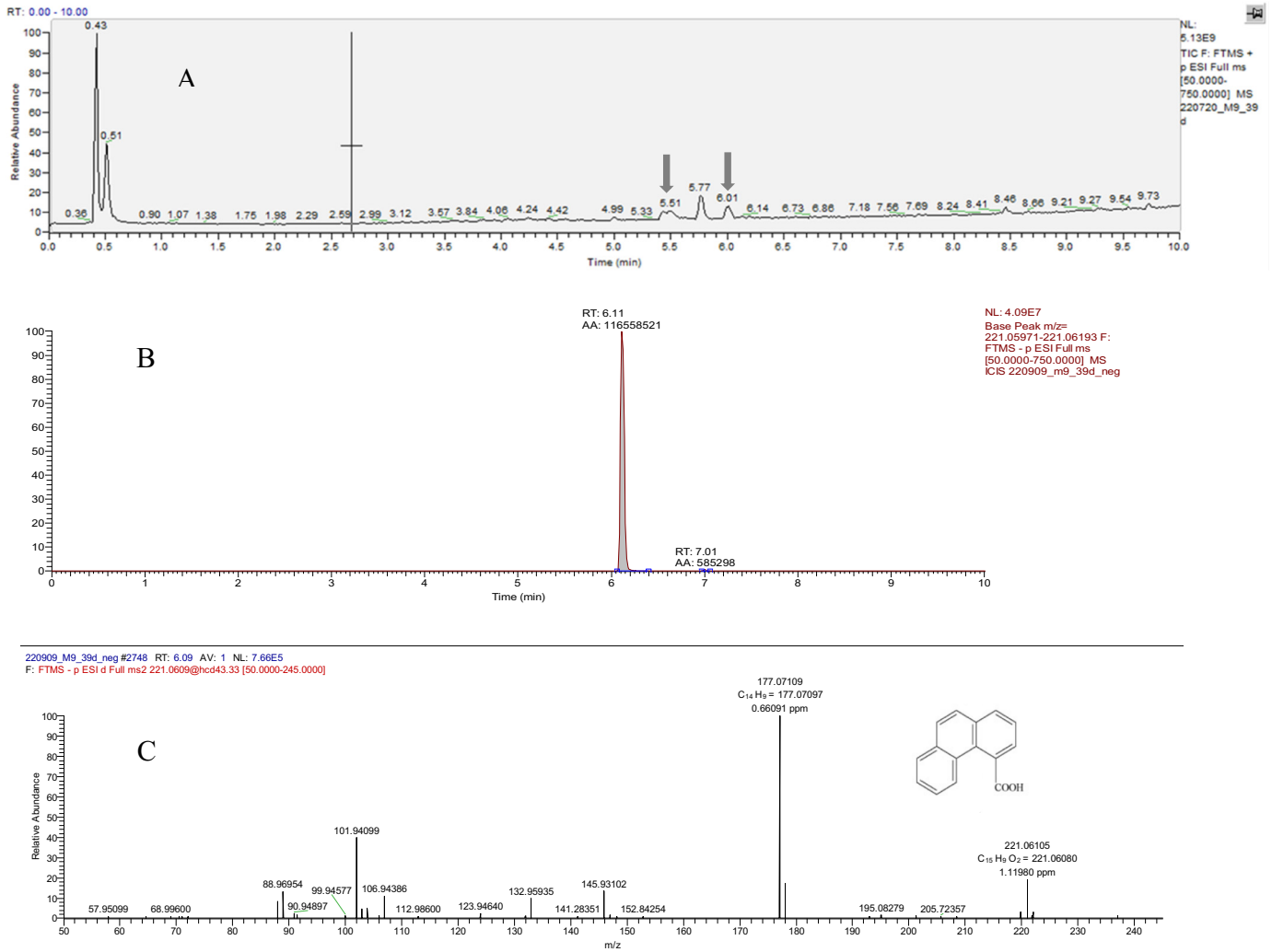

Fig. 2. Analysis of a leachate sample (planted uninoculated pot, 39 d) through UHPLC-MS. A, fragment of the complete chromatogram analized in the positive ionization mode, where the first arrow indicates the peak of pyrene and the second arrow indicates the peak of the identified metabolite. B, *m/z* 221.06 ion chromatogram showing the area and the retention time for the metabolite. C, mass spectrum of the $[M-H]^-$ adduct of the metabolite, identified as phenanthrene-4-carboxylic acid.

Table 3
Concentration of pyrene equivalents in the plant samples (mg kg^{-1} dry weight) under greenhouse conditions.[a,b]

	Uninoculated	Uninoculated + biochar	Inoculated 1×	Inoculated 2×	Inoculated 2× + biochar
Root	88.37 ± 35.65 Aa	159.12 ± 83.67 Aa	256.29 ± 86.68 Aa	91.90 ± 104.28 Aa	166.72 ± 73.37 Aa
Stem	130.21 ± 47.10 Aa	15.04 ± 4.69 Bb	19.70 ± 2.62 Bb	29.26 ± 7.83 Ba	22.97 ± 7.98 Bb
Leaf	92.06 ± 42.92 Aa	17.63 ± 2.59 Bb	17.33 ± 5.65 Bb	25.92 ± 3.71 Ba	27.17 ± 13.76 Bb
Flower/Fruit	64.77 ± 6.90 Aa	21.31 ± 4.01 Bb	26.47 ± 10.14 Bb	19.80 ± 7.57 Ba	31.42 ± 6.77 Bb

[a] Values in a row followed by the same capital letter are not significantly different (ANOVA, $P \leq 0.05$).
[b] Values in a column followed by the same lowercase letter are not significantly different (ANOVA, $P \leq 0.05$).

homogenously through the plants. With the rest of the treatments, significantly higher PYR_{eq} concentrations were observed in the roots than in the stems, leaves and flowers. The BCF and TF values are shown in Table 4. The maximum values of stem and leaf BCF values and all three TFs (C_{stem}/C_{root}, C_{leaf}/C_{root}, and C_{fruit}/C_{root}) were observed in uninoculated treatments, and in most cases, inoculation and biochar (by separate or in combination) significantly reduced ($P \leq 0.05$) these values. These results indicate that the treatments significantly reduced the mobilization of PYR_{eq} into the plants.

3.4. Microbial and biochemical determinations

In the pots without plants, most of the DH enzymatic activity (approximately 90 %) was found in the top sand layer (Table 5). When plants were present, and in the absence of biochar, the DH activity was distributed between the top (approximately 60 %) and middle (approximately 20 %) layers, showing a low activity at the bottom. No DH activity was detected in the bottom layers of the pots containing biochar. Apparently, there was no microbial activity in these samples, although the possibility of biochar interfering with the analytical procedure cannot be discarded. From these results it seems clear that plants helped the introduced bacteria to mobilize and distribute through the substrate. This result is in agreement with previous results on the enhancement of the transport of this strain mediated by taxis to sunflower DOM components at a TOC concentration (16 mg L^{-1}) (Jimenez-Sanchez et al., 2015) comparable to those detected in the leachates from planted pots (Table 1). The direct determination of viable cells in substrate samples showed that *P. putida* G7 cells were active at the end of the experimental period, as they were able to grow on selective agar media (MSB with naphthalene crystals as the sole carbon source) (ca. 10^6 CFU g^{-1}), and even cell swimming motility was maintained (see video S1 in Supporting Information). However, due to the high variability in the CFU counts, no clear trend could be determined in relation to treatment and height in the sand profile.

Because planted pots that received no inoculum developed a significant mobilization of PYR_{eq} in the leachates, it is plausible that this mobilization was caused by the direct transformation of pyrene by the plants. The microbiological control carried out with non-inoculated pots revealed the presence of very low numbers ($<10^3$ CFU g^{-1}) of colonies with morphology different from *P. putida* G7 colonies, in comparison with the high number of colonies observed in the inoculated pots (see above). This very low concentration of other culturable microorganisms suggests that significant pyrene microbial transformation in non-inoculated pots was unlikely, although it does not completely exclude the activity of eventually developed microorganisms that were unculturable. Nevertheless, the two main types of colonies observed in the plates were isolated by plating in TSA medium, and their capacity to transform pyrene was tested in flask assays containing passively dosed ^{14}C-pyrene. Mineralization and partitioning of pyrene in the aqueous phase was measured for both isolates. In the mineralization test (see Fig. S2 for methods and results), $^{14}CO_2$ was not detected in the soda trap for any of the isolates, and pyrene in the aqueous phase showed very similar results to those of the control experiment (abiotic conditions). Therefore, transformation by the plants seems to be the most likely explanation.

4. Discussion

The high capacity of sunflower plants to resist PAH pollution, their possible pollutant transformation ability, and the motility and pyrene cometabolizing capacity of *P. putida* G7 made our system perfect for assessing and controlling the risk from the biodegradation process. Our results indicate that the mechanisms for risk reduction associated with the

Table 4
Bioconcentration factors (BCFs, L kg^{-1}) and translocation factors (TFs) in plant samples under greenhouse conditions.[a]

	Uninoculated	Uninoculated + biochar	Inoculated 1×	Inoculated 2×	Inoculated 2× + biochar
BCF					
Root	1.95 ± 0.77A	8.40 ± 5.11AB	12.37 ± 4.56B	3.38 ± 3.96A	7.65 ± 4.16AB
Stem	2.88 ± 1.00A	0.76 ± 0.18B	0.95 ± 0.15B	1.06 ± 0.31B	1.02 ± 0.31B
Leaf	2.02 ± 0.93A	0.90 ± 0.06B	0.83 ± 0.28B	0.93 ± 0.13B	1.14 ± 0.39AB
Flower/Fruit	1.43 ± 0.25A	1.09 ± 0.12A	1.31 ± 0.62A	0.71 ± 0.25A	1.40 ± 0.34A
TF					
C_{stem}/C_{root}	1.85 ± 1.27A	0.12 ± 0.08B	0.09 ± 0.04B	0.65 ± 0.50AB	0.12 ± 0.08B
C_{leaf}/C_{root}	1.28 ± 0.87A	0.14 ± 0.06B	0.08 ± 0.05B	0.57 ± 0.38AB	0.22 ± 0.21B
C_{fruit}/C_{root}	0.82 ± 0.30A	0.17 ± 0.10B	0.11 ± 0.04B	0.41 ± 0.34AB	0.24 ± 0.16B

[a] Values in a row followed by the same capital letter are not significantly different (ANOVA, $P \leq 0.05$).

Table 5
Dehydrogenase activity (in μg INTF g^{-1} h^{-1}, mean ± standard deviation) in substrate samples from the experimental pots at three different depths (81 d).

	Planted uninoculated[b]	Planted uninoculated + biochar[c]	Planted inoculated 2x[b]	Planted inoculated 2x + biochar[c]	Inoculated 2x[b]	Inoculated 2x + biochar[c]
Top[a]	1.11 ± 0.38 ABa	0.96 ± 0.14 ABa[c]	1.49 ± 0.11 Ba	0.55 ± 0.31 Aa[c]	2.42 ± 0.57 Ca	3.67 ± 0.18 Da[c]
Middle[a]	0.39 ± 0.07 Ab	0.76 ± 0.07 Bb[c]	1.06 ± 0.18 Bb	0.80 ± 0.24 Ba[c]	0.29 ± 0.20 Ab	0.35 ± 0.01 Ab[c]
Bottom[a]	0.23 ± 0.08ABb	ND[d]	0.33 ± 0.04Bc	ND[d]	0.10 ± 0.00 Ab	ND[d]

[a] Values in the row followed by the same capital letter are not significantly different (ANOVA, $P \leq 0.05$).
[b] Values in the column followed by the same lowercase letter are not significantly different (ANOVA, $P \leq 0.05$).
[c] Values in the column followed by the same lowercase letter are not significantly different (Student's *t*-test, $P \leq 0.05$).
[d] ND, not detected.

leaching of pyrene metabolites resided, on the one hand, on plant-microorganism interactions and, on the other hand, on the trapping capacity of the biochar. The presence of sunflower roots also facilitated microbial dispersal to the source of contamination, probably due to the chemotactic ability of the *P. putida* G7 strain to many of the components of the exudates produced by the plant (Fernández-López et al., 2021; Jimenez-Sanchez et al., 2015). Also, irrigation may have physically affected the dispersal in planted pots, particularly as the plant roots grew downward toward the pyrene-sand layer. Taking into account the hazardous nature of many of the substances produced during the pollutant degradation process, the addition of biochar as a sequestering agent reduces the risk associated with the decontamination process. In this way, the novel system proposed in this study may open up new possibilities for the future control of risk associated with bioremediation in contaminated soils. The enhanced concentration of PYR_{eq} observed in the leachates from planted pots after inoculation (Fig. 1B) may reflect the cometabolic activity of *P. putida* G7 cells once they reached the distant pyrene source, which was facilitated by the plants. This promoting effect of sunflower plants on the transport and pyrene-cometabolic activity of *P. putida* G7 cells in soil has already been observed (Fernández-López et al., 2021). Our results extend those findings by showing a significant reduction in PYR_{eq} concentrations in the leachates from planted, inoculated pots equipped with a biochar layer, therefore indicating that the mobilized metabolites were efficiently retained in the pots.

The possible capability of sunflower plants observed in our study to directly transform pyrene is not surprising, given the capacity of higher plants to oxidize PAHs through cytochrome P-450 enzymes (Alagić et al., 2015). Indeed, water-soluble pyrene metabolites were produced by cell cultures of purple foxglove and wheat exposed to pyrene (Hückelhoven et al., 1997) and by poplar cuttings cultivated in sand (Kuhn et al., 2004) and were also detected in leaves from several woody plants exposed to urban atmospheres (Nakajima et al., 1996) . To our knowledge, this is the first report on the direct transformation of pyrene by sunflower plants (Kathi & Khan, 2011). The metabolite identified, P-4-C, is a metabolite described in microbial degradation pathways for pyrene (Al-Shaikh and Jamal, 2020; Hadibarata and Kristanti, 2013; Haritash and Kaushik, 2009), and phenanthrene polar derivatives (different from P-4-C) have been found in a study on pyrene plant metabolism (Kuhn et al., 2004) . These results support that pyrene degradation has taken place in these planted treatments. Our previous studies on the biodegradation of PAHs (including pyrene) in soil with sunflower plants targeted different aspects of their interactions with PAH-degrading bacteria in soil, such as root exudation (Tejeda-Agredano et al., 2013), biosurfactant action (Posada-Baquero et al., 2020) and bacterial transport and cometabolism (Fernández-López et al., 2021). Possibly because of the transparency of the model system employed in this study, involving the cultivation of this plant in sand and inorganic nutrient solution, such direct transformation became evident, unlike in previous studies. Furthermore, the inhibition of transformation by inoculation with *P. putida* G7 (Fig. 1A and B) suggests that the plant was highly sensitive to the presence of this microorganism in the rhizosphere. Such a reaction can be explained by postulating that the direct transformation of pyrene by the plant was the result of a stress response to the culture conditions (including the exposure to pyrene). These unfavorable conditions activated the production of enzymes related to detoxification and antioxidative systems and therefore the storage and transformation of pyrene and its metabolites in root and shoot plant tissues and their translocation. This response was not observed in the presence of the microbial inoculant, reflecting a positive plant-microbe interaction and a reduction in the stress induced by the toxic pollutant. Some microbes possess plant growth-promoting capacities that may, among other positive effects, reduce the production of antioxidative-related enzymes as a consequence of a reduction in toxic adverse effects (e.g., by degrading and/or cometabolizing the pollutants) and/or the improvement of plant fitness (e.g., the release of phytohormones, the production of biosurfactants, or the enhancement of nutrient availability, among others) (Balseiro-Romero et al., 2017; Glick, 2012).

The enhancement observed under greenhouse conditions at later stages in inoculated pots that had no plants can be explained by the activity of the bacteria that were also transported, still to a limited extent, to the contaminated zone. Such an increase was also observed in inoculated pots equipped with the biochar layer, which can be explained by the fact that this material was only stable and therefore retained its sorption capacity during the first two months of experimentation. This may not only be an environmentally sustainable option due to the biodegradability of the product, avoiding the persistence of exogenous components in the soil for long times, it could also provide an ecological niche that allows for controlled bacterial deposition at the source of contamination. In this way, the high availability of the nutrients provided by the biochar can promote the microbial activity of the bacterial cells retained in the product, increasing the degrading capacity of the pollutants present in the material. However, the microbial and biochemical determinations performed at the end of the experimental period indicate that, without plants, most of the bacterial inoculant was retained in the upper layers of the pots. The limited transport of this strain through porous materials and the enhanced mobilization and activity as a result of tactic reactions have been examined in a variety of scenarios, including sand columns (Jimenez-Sanchez et al., 2015; Rolando et al., 2020) and sunflower-planted soil (Fernández-López et al., 2021). Our results extend those findings by showing that mobilized bacteria and metabolites can efficiently be controlled through a combination of plants and adsorbent phases.

5. Conclusions

The present proof-of-concept study shows a new integrated model for bioremediation where the role of bacterial motility combined with sunflower activity generates a synergic response for pyrene degradation. We demonstrated that the action of the plant and the bacteria was effective in the degradation of pyrene as well as that the plant facilitated the dispersal of the motile bacteria. In addition, the use of a biochar-based sequestering trap for the metabolites produced during biodegradation made it possible to reduce the leaching risk associated with the decontamination process. These results provide new insights into the bioremediation of PAH-polluted matrices, where biologically caused environmental risk is considered fundamental in the process. The feasibility of this greenhouse experiment under real field conditions is made effective due to the low cost of the materials used in the process, providing new tools for future application in the remediation sector. Our results suggest improved nature-based solutions for the treatment of soils polluted by PAHs and other hydrophobic pollutants and show that the use of plant-bacteria-sorbent arrangements can constitute a valid alternative to control the risk of such contamination. The results could also be applied in wastewater reuse for soil irrigation where the biological transformation of organic contaminants introduced in soil may cause further environmental and human risk.

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CRediT authorship contribution statement

José Carlos Castilla-Alcantara: Conceptualization, Methodology, Data curation, Writing – original draft. **Rosa Posada-Baquero:** Conceptualization, Methodology, Data curation, Writing – original draft. **Maria Balseiro-Romero:** Conceptualization, Methodology, Data curation. **Carmen Fernández-López:** Conceptualization, Writing – original draft. **José Luis García:** Methodology, Data curation, Writing – original draft. **Alicia Fernandez-Vazquez:** Methodology, Data curation. **John R. Parsons:** Conceptualization, Writing – original draft. **Manuel Cantos:** Conceptualization, Methodology, Data curation, Writing – original draft. **Jose Julio Ortega-Calvo:** Writing – review & editing, Funding acquisition.

Data availability

No data was used for the research described in the article.

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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