Impact of bacterial motility on biosorption and cometabolism of pyrene in a porous medium

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GRAPHICAL ABSTRACT:

Cometabolic-active mobile cells

Salicylate-mobilized cells
ABSTRACT:
The risks of pollution by polycyclic aromatic hydrocarbons (PAHs) may increase in bioremediated soils as a result of the formation of toxic byproducts and the mobilization of pollutants associated to suspended colloids. In this study, we used the motile and chemotactic bacterium *Pseudomonas putida* G7 as an experimental model for examining the potential role of bacterial motility in the cometabolism and biosorption of pyrene in a porous medium. For this purpose, we conducted batch and column transport experiments with $^{14}$C-labelled pyrene loaded on silicone O-rings, which acted as a passive dosing system. In the batch experiments, we observed concentrations of the $^{14}$C-pyrene equivalents well above the equilibrium concentration observed in abiotic controls. This mobilization was attributed to biosorption and cometabolism processes occurring in parallel. HPLC quantification revealed pyrene concentrations well below the $^{14}$C-based quantifications by liquid scintillation, indicating pyrene transformation into water-soluble polar metabolites. The results from transport experiments in sand columns revealed that cometabolic-active, motile cells were capable of accessing a distant source of sorbed pyrene. Using the same experimental system, we also determined that salicylate-mobilized cells, inhibited for pyrene cometabolism, but mobilized due to their tactic behavior, were able to sorb the compound and mobilize it by biosorption. Our results indicate that motile bacteria active in bioremediation may contribute, through cometabolism and biosorption, to the risk associated to pollutant mobilization in soils. This research could be the starting point for the development of more efficient, low-risk bioremediation strategies of poorly bioavailable contaminants in soils.
HIGHLIGHTS:

- Bacterial mobilization of $^{14}$C pyrene was evaluated by column transport experiments
- *Pseudomonas putida* G7 reached the distant pyrene source and cometabolized it
- Salicylate inhibited pyrene cometabolism by *Pseudomonas putida* G7
- Salicylate promoted pyrene mobilization by biosorption
1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic contaminants with two or more aromatic rings bonded in various arrangements (Rocha and Palma, 2019). They originate from unburned products or they are formed during incomplete combustion processes of complex organic materials such as coal, petroleum, gas or waste (Abdel-Shafy and Mansour, 2016). They are considered worldwide as priority pollutants for soils, sediments and waters owing to their toxicity.

Pyrene (PYR) is included in the list of 16 PAHs of the USEPA (United States Environmental Protection Agency) contaminant priority list, and it is representative of high molecular weight (HMW) PAHs, with 4 to 6 rings (Kanaly and Harayama, 2000). Pyrene is highly hydrophobic, with a low water solubility (0.135 mg/L) and tends to sorb to soil organic carbon, which causes a limited bioavailability, and consequently makes it recalcitrant to biodegradation (Gupta et al., 2017).

The bacterial degradation of pyrene was first reported by Heitkamp et al. in 1988, who described a Mycobacterium strain capable of mineralizing pyrene as the sole carbon and energy source (Gupta et al., 2018; Heitkamp et al., 1988). Thereafter, numerous pyrene degraders were identified, most of which belonged to genera such as Sphingomonas, Mycobacterium, Rhodococcus, Bacillus, Burkholderia, Cycloclasticus, Pseudomonas and Stenotrophomonas; the bacterial degradation percentages in a soil system ranged between 32 and 96% with incubation times between 4 and 42 days (Gupta et al., 2017; Peng et al., 2008). Several studies have demonstrated that PYR can also be degraded by bacterial consortia (Gallego et al., 2014; Vaidya et al., 2017; Wanapaisan et al., 2018). In recent years, the pyrene metabolism pathways and the catabolic genes have been identified in Mycobacterium vanbaalenii PYR-1 by genomic, proteomic, and metabolomic analyses (Wanapaisan et al., 2018). Pyrene degradation starts with a dioxygenation and its complete catabolism occurs through phthalate, protocatechuate and tricarboxylic acid cycle intermediates (Kim et al., 2007). Key enzymes in pyrene degradation are the multi-component Rieske-type aromatic ring-hydroxylating oxygenases, which catalyze both the initial and several
steps of the degradation (Chakraborty et al., 2012). Some bacteria, however, are able to transform pyrene through cometabolism (Kazunga and Aitken, 2000). It is important to estimate the products of the incomplete PAH metabolism because of their potential effects on PAH-degrading bacteria or on human exposure (USEPA, 1999). The most studied metabolite is 1-hydroxypyrene (1-HP, molecular weight: 218.1 g/mol), derived from the monohydroxylation of pyrene, which can be detected in urine and sediment samples (Hansen et al., 2008; Li et al., 2000). 1-HP is the preferred biomarker for routine exposure assessments of workers and for PAH-exposure in the environmental exposure assessments of healthy human populations (Hansen et al., 2008). A study conducted by Kazunga and Aitken (2000) identified the main products from the incomplete metabolism of pyrene by four bacterial strains (Kazunga and Aitken, 2000). Pseudomonas stutzeri strain P16 and Bacillus cereus strain P21 transformed pyrene primarily to cis-4,5-dihydro-4,5-dihydroxypyrene (PYRdHD), the first intermediate in the known pathway for aerobic bacterial mineralization of pyrene. Sphingomonas yanoikuyae strain R1 transformed pyrene to PYRdHD and pyrene-4,5-dione (PYRQ). Both strain R1 and Pseudomonas saccharophila strain P15 transform PYRdHD to PYRQ nearly stoichiometrically, suggesting that PYRQ is formed by oxidation of PYRdHD to 4,5-dihydroxypyrene and subsequent autoxidation of this metabolite. A pyrene-mineralizing organism, Mycobacterium strain PYR-1, also transforms PYRdHD to PYRQ at high initial concentrations of PYRdHD (Kazunga and Aitken, 2000).

The literature data suggest therefore that, on one hand, PAHs can be biodegraded and, on the other hand, a successful PAHs bioremediation can be limited by their bioavailability (Posada-Baquero et al., 2019). It is therefore important to develop innovative methods for increasing the bioavailability of these chemicals, making it possible more efficient bioremediation strategies. These methods need to increase biodegradation rates, leading to low PAHs residual concentrations and limit as much as possible the associated environmental risks (Ortega-Calvo et al., 2013). Indeed, research over the last decade has shown that risks of PAH pollution often increase initially in bioremediated soils as a result of an unmodulated biological processing, leading to the formation...
of byproducts eventually more toxic than parent PAHs (Hu et al., 2012). Risks may also arise from the mobilization of PAHs associated to suspended colloids, such as humic acid-clay complexes (Garcia-Junco et al., 2003), and bacterial cells (Stringfellow and Alvarez-Cohen, 1999). In this context, the role of bacterial motility has rarely been considered. In fact, motile bacteria often exhibit chemotactic behaviors, which enable motile cells to move along chemical gradients and to swim toward optimal places for biodegradation (Velasco-Casal et al., 2008), increasing bioavailability and therefore PAHs bioremediation (Krell et al., 2013). Chemotaxis may show an important role in bacterial transport at low flow rates through porous materials, such as sand-filled percolated columns (Velasco-Casal et al., 2008). In this regard, the bacterium *Pseudomonas putida* G7 is able to use the two-ring PAH naphthalene as a growth substrate and responds chemotactically to salicylate, a common metabolic intermediate of PAHs biodegradation, showing smooth movement and positive taxis (Jimenez-Sanchez et al., 2012).

In this study, we used the strain G7 as an experimental model in a new bioremediation scenario, evaluating the potential role of motility in the cometabolism and biosorption of pyrene in a porous medium. For this purpose, we employed a progressive approach by studying the removal of pyrene completely dissolved in the aqueous phase, and in batch and column transport experiments with silicone O-rings with $^{14}$C pyrene, which acted as a passive dosing system. Firstly, we evaluated the role of partitioning of pyrene on the mobilization of this compound in a batch system by bacterial cells and its possible transformation products. Then, in the column transport experiment, we specifically tested whether cometabolic-active, motile cells were capable of accessing a distant source of sorbed pyrene, and, using the same experimental system, whether salicylate-mobilized cells, were able to sorb the compound and mobilize it by biosorption.

2. Materials and methods

2.1. Chemicals
Fine-grained sand was purchased from Panreac Quimica SA (Barcelona, Spain) and sieved to obtain a fraction between 0.25 mm and 0.40 mm of diameter. Silicone rings (O-rings) were obtained from Altec Products Ltd. (Cornwall, U.K.) with an internal diameter of 2.57 mm and a cross section of 1.78 mm. Unlabeled pyrene was obtained from Sigma-Aldrich, Madrid, Spain and [4,5,9,10-14C]-pyrene (58.8 mCi/mmol, radiochemical purity >98%) was purchased from Campro Scientific GmbH (Veenendaal, The Netherlands).

2.2. Cultivation of Bacteria

The bacterium *Pseudomonas putida* G7, able to degrade naphthalene (NAH) was maintained at -80°C until use (Jimenez-Sanchez et al., 2012). The strain G7 is able to use naphthalene and salicylate as a growth substrate, and respond chemotactically (showing smooth movement and positive taxis) to both chemicals. For all experiments, the strain G7 was cultivated in 250 mL Erlenmeyer flasks containing 100 mL of an inorganic salt solution (MM) and sodium salicylate (5 mM) as the sole carbon source (Jimenez-Sanchez et al., 2012). The bacterial cultures were maintained under constant agitation (150 rpm) at 30 °C. At the early stationary phase (48 h after inoculation of the culture; OD 600nm of 0.5 or 5 × 10^8 cells/mL), cells were centrifuged for 10 min at 2,570 rpm and resuspended in a mineral medium (MM) supplemented with or without salicylate.

2.3. Removal of aqueous-phase pyrene

The experiment was performed in batch using the strain G7 in MM with dissolved pyrene, which was measured by fluorimetry. Pyrene had earlier been added to the sterile medium at 45 °C in an acetone solution (0.0001 g/mL) to give a final concentration of 50 ng/mL. This temperature facilitated the fast evaporation of the acetone. Three conditions were tested: MM with a cell suspension of the strain (OD 600nm ~ 0.3, ca. 10^8 cells/mL), MM with the strain G7 at the same cell density and salicylate (at final concentration of 0.001 mM, to allow the detection of the pyrene fluorescence signal), and a control consisting of just MM in order to rule out phenomena of PYR volatilization, photodegradation and/or sorption to the flask walls. Each test was performed in duplicate. The flasks were maintained in an orbital shaker at 100 rpm at room temperature (23 ± 2 °C).
°C). At selected time intervals (10 minutes), 2 mL of a homogeneous sample was taken directly from each flask and transferred to a quartz cuvette (1 cm path length). To determine the disappearance of pyrene from the aqueous solution, synchronous fluorescence spectrophotometry was used with an F-2500 fluorescence spectrophotometer (Hitachi) as previously described (Ortega-Calvo and Gschwend, 2010). After the measurements, the samples were returned to their original flasks to avoid changes in volume as a result of sampling (Tejeda-Agredano et al., 2014).

2.4. Dynamic doping of rings with pyrene

The dynamic doping method (Smith et al., 2012) was used to load the O-rings with ¹⁴C pyrene, as described in detail elsewhere (Jimenez-Sanchez et al., 2018). A maximum of 10 rings were placed on the bottom of a 20 mL glass tube and 1 mL of an acetone solution of ¹⁴C pyrene and an unlabeled pyrene to give a final concentration of 500 mg/L was added. The solution contained the required radioactivity to make each ring to be loaded with approximately 200 000 dpm. The total amount of pyrene initially present in each ring was 50 µg. The same method was used to load the O-rings with unlabeled pyrene only.

2.5. Partitioning of pyrene

The experiment was performed in batch using the strain G7 in a mineral medium with ¹⁴C-labelled pyrene loaded on silicone O-rings (Jimenez-Sanchez et al., 2018). One silicone ring (O-ring), containing sorbed ¹⁴C pyrene (described in detail above), was placed in each flask. Three conditions were tested: MM with a cell suspension (OD ₆₀₀ₙₐ₃ ~ 0.3, ca. 10⁸ cells/mL), MM with bacteria and salicylate (at final concentration of 1 mM, to avoid bacterial excessive growth during extended experimentation) and a control consisting of just MM. Each test was performed in duplicate. The flasks were maintained in an orbital shaker at 80 rpm at room temperature (23 ± 2 °C). Then, the concentration of ¹⁴C pyrene was measured as radioactivity appearing in the aqueous suspension. Each sample (1 mL) was collected from the flask and mixed with 5 mL of a liquid scintillation cocktail (Ultima Gold, Perkin Elmer, Nederland). The radioactivity was measured with a liquid scintillation counter model LS6500 Beckman (Beckman Instruments, Fullerton, California, U.S.A.).
Then, for the measurement of $^{14}$C pyrene biosorption and pyrene total concentration, 2 mL of the inoculated cell suspensions were centrifuged in 20 mL glass test tubes at 15 000 rpm for 5 minutes, the supernatants and bacterial cells were analyzed independently with by HPLC and by liquid scintillation. The HPLC analysis were carried out using a Waters HPLC system (Water 2475 Multi, fluorescence detector and Water 996 photodiode array detector, Water PAH column C 18.5 μm particle size and 4.6×250 mm and 1 mL/min of flow). The mobile phase used was an acetonitrile/milli-Q water gradient. The column was installed in a thermostatic oven at 30 °C.

The predicted fraction of the pyrene freely dissolved in the aqueous phase of bacterial suspensions at the equilibrium ($f_w$), was calculated using the following equation (Sungthong et al., 2015):

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

Where [DOC] is the concentration of dissolved organic carbon (DOC) in Kg/L and $K_{OC}$ is the solid-water distribution coefficient in L/Kg. The DOC was calculated from the biomass of the bacteria transported for mL (169.13 µg prot/mL), assuming that cells protein contains 50% of cell C. The log $K_{OC}$ of pyrene used for this equation was 4.7 (Accardi-Dey and Gschwend, 2002). The maximum capacity for pyrene of the bacterial suspensions was calculated by the ratio between the concentration of freely dissolved pyrene at equilibrium and $f_w$ (Sungthong et al., 2015).

### 2.6. Extraction and analysis of pyrene metabolites

A batch experiment was performed using the same three conditions as described above. One silicone ring (O-ring), containing unlabeled pyrene (described in detail above), was placed in each flask. At the final time of the experiment (408 h) the samples (40 mL) from each condition were collected, and the aqueous solution and bacterial cells were separated by centrifugation (12 000 rpm for 10 minutes). The solution was then extracted five times with equal volumes of ethyl acetate (20 mL), concentrated under a nitrogen flow, and resuspended in 100 µL of ethyl acetate. The degradation products of residual pyrene in the solution were detected by GC-MS Full-Scan (Gas...
Chromatography - Mass Spectrometry). The GC-MS Full-Scan analyses were performed on Thermo Scientific TSQ8000 equipped with ZB-5MS capillary column (30 m length, 0.25 mm diameter, and 0.25-µm film thickness). The temperature program for GC was set as follows: Initial temperature: 50.0 °C. Initial hold time: 1.00 min. Number of ramps: 1. Ramp 01 rate: 7.0 °C/min. Ramp 01 final temperature: 310.0 °C. Ramp 01 hold time: 0.00 min. The flow rate of the carrier gas was 1 mL/min. The following conditions were applied for mass analysis: injector and interface temperatures of 280 °C; ionization mode, detector at 0 eV, 0.25-s scan intervals, and mass range m/z of 50–500. The mass spectra of individual total ion peaks were identified by comparison with a mass spectra database (NIST MS Search 2.0).

Derivatization was carried out to increase the volatility and thermal stability of the compounds during GC analysis (Hadibarata and Kristanti, 2013). Silylation is the classical derivatization method that adds a silyl group [–Si(CH3)3–] to the metabolite by substituting the active hydrogen (e.g., –OH, –SH, –NH4+, –COOH) to generate stable, more volatile, and less polar derivatives of the parental metabolites (Zarate et al., 2016). In our research, silylation was achieved by evaporating the samples and then introducing the trimethylsilyl group (TMS) using N,O-bis-trimethylsilyl acetamide, pyridine and trimethylchlorosilane (final volume 150 µL). The reaction was effected by vortexing and leaving in the darkness for 1 h. The GC-MS Full-Scan analyses of the derivatized samples were performed on Thermo Scientific TSQ8000 equipped with ZB-1MS capillary column (30 m length, 0.25 mm diameter, and 0.25-µm film thickness). The temperature program for GC was set as follows: Initial temperature: 80.0 °C, Initial hold time: 2.00 min, Number of ramps: 2, Ramp 01 rate: 15.0 °C/min, Ramp 01 final temperature: 150.0 °C, Ramp 01 hold time: 0.00 min, Ramp 02 rate: 30.0 °C/min, Ramp 02 final temperature: 320.0 °C, Ramp 02 hold time: 5.00 min. Flow rate of the carrier gas was 1 mL/min. The mass spectra of individual total ion peaks were identified by comparison with a mass spectra database (NIST MS Search 2.0).

2.7. Mineralization experiment
A batch mineralization experiment was performed using the same three conditions of the partitioning experiments described above. One silicone ring (O-ring), containing sorbed $^{14}$C pyrene, was placed in each flask. Each batch was closed with a Teflon-lined stopper equipped with a 2 mL suspended vial, which contained 1 mL of 0.5 M NaOH and was maintained at 23 ± 2 ºC on an orbital shaker (150 rpm). The mineralization of $^{14}$C pyrene was measured as radioactivity incorporated in the alkali trap. The radioactivity was measured by liquid scintillation. At selected times, several samplings were performed to complete an experimental period of 432 hours.

2.8. Column transport experiment

A bacterial transport experiment in percolated sand columns (10 cm length and 0.9 cm inner diameter) with a porosity (estimated gravimetrically) of 0.43, corresponding to a pore volume (PV) of 2.7 mL (Jimenez-Sanchez et al., 2015, 2012) was performed using the same three conditions as described above. In this case, the salicylate concentration was 2.5 mM to optimize the bacterial mobilization through tactic behavior (Jimenez-Sanchez et al., 2015, 2012). The experiment was performed at room temperature (approximately 25 ºC). Each silicone ring, containing $^{14}$C pyrene, was placed two centimeters from the exit of each column in order to recreate a distant pyrene source. Each ring was collected with tweezers and washed separately in milli-Q water before being introduced (once dried with blotting paper) into the column. Mineral medium (control) or a cell suspension (OD$_{600nm}$ ~ 0.3, ca. $10^8$ cells/mL) alone or with salicylate (2.5 mM) was pumped through each column at a constant flow rate of 0.135 ± 0.02 mL/min (hydraulic flow rate 0.50 cm/min). In accordance to this flow rate, the bacteria were exposed to desorbed pyrene during 4 min after passing through the ring and before leaving the column. The column effluent was collected into 20 mL glass test tubes in order to measure cell concentrations with a spectrophotometer (Genesys 10, Spectronic Unicam). The experiment lasted 4 hours with 8 sampling times every 30 minutes. The column transport of the bacteria was followed by comparing the OD$_{600nm}$ of the influent ($C_0$) and effluent ($C$) at fixed time intervals. Total radioactivity was measured by liquid scintillation. For the measurement of total pyrene biosorbed, 1 mL of the control
condition was directly analyzed with a HPLC, 2 mL of the cell suspension of the other two conditions were centrifuged into 20 mL glass test tubes at 15 000 rpm for 5 minutes, the supernatants and bacterial cells (suspended in methanol) were analyzed independently with a HPLC. The results of the control without bacteria are not reported because it was set up in order to check if the column system had worked correctly. The predicted fraction of pyrene dissolved freely in the aqueous phase at the equilibrium ($f_w$), was calculated from the $K_{OC}$ of pyrene as explained above (equation 1), and compared with the experimental $f_w$ obtained by dividing the concentration of pyrene in the effluent of the column detected in the supernatant and the one detected in the supernatant and in the pellet.

3. Results and discussion

3.1. Removal of pyrene from aqueous solution

To test the effect of *P. putida* cells on the removal of aqueous-dissolved pyrene, we examined a simplified system where pyrene losses were followed by synchronous fluorescence in bacterial suspensions with only the dissolved chemical (Figure 1). The data showed rapid and complete loss of dissolved pyrene after 19 h, what indicated that the bacterium was able to degrade the chemical. PYR concentration in the control flasks without bacteria was constant during the experiment, confirming that PYR disappearance was due to bacterial degradation. The losses of pyrene were significantly slower in the presence of salicylate, as evidenced by the statistically higher concentration still remaining after 3.5 h (t-student, $p < 0.05$). At the end of the experimental period, the concentration of dissolved pyrene was in these salicylate-containing suspensions compatible with sorption to the bacterial cells. Indeed, the predicted aqueous phase pyrene concentration at equilibrium resulting from sorption to bacterial cells, based on the $K_{OC}$ value of pyrene and the biomass present in the system, was 2.9 ng/mL, a value which was very close to the final pyrene
concentration reached after 27 h with salicylate, 3.2 ng/mL. These results suggest that the transformation was inhibited in the presence of salicylate.

3.2. Partitioning of pyrene

Batch experiments made it possible to evaluate the influence of bacterial cells on partitioning of pyrene and its possible transformation products, what later would be tested in a porous system. The batch incubations of strain G7 were performed in mineral medium with $^{14}$C-labelled pyrene loaded on silicone rings to control the mass transfer of pyrene to the aqueous phase. The evolution over time of the equivalents of pyrene labeled with $^{14}$C in the liquid cultures revealed an increase in the presence of the G7 strain, reaching at later stages concentrations well above the equilibrium concentration observed in abiotic control (Figure 2A). This phenomenon, which was observed both in the presence and in the absence of salicylate, could only be explained either by sorption to bacterial cells or cometabolic degradation. Both with and without salicylate, the concentration of pyrene equivalents in the bacterial suspension increased during the first 1.5 h at the same rate as abiotic partitioning (Figure 2A). This indicates that the subsequent increase of $^{14}$C in the bacterial suspension, observed until the end of the experiment, was limited by the mass transfer from the silicone ring.

If the phenomenon responsible for the increased mobilization was the sorption to the cells an asymptotic phase would also have been reached, although the asymptote would have occurred at a higher concentration than in the abiotic controls. This removal of pyrene from the aqueous phase, thus caused more pyrene to be released constantly, without the constrain imposed by the theoretical equilibrium concentration, until a new equilibrium was reached. In accordance to our calculations based on the $K_{oc}$ of pyrene, and the amount of bacterial biomass present in the system (Sungthong et al., 2015), the maximum capacity for pyrene of the bacterial suspension in this system at equilibrium was 1,795 ng/mL, a concentration which was very close to the last time point measured at the end of the experimental period, approximately 1,600 ng/mL (Figure 2A). This indicates that
biosorption explained well the observed overall mobilization. However, the fluorimetry results (shown in Figure 1) indicated that the strain G7 was able to remove completely the chemical from the aqueous phase, possibly by cometabolism, occurring in parallel to biosorption, and that the complete removal was prevented, at least for a few hours, by salicylate. The high concentration of pyrene equivalents measured in these batch partitioning experiments as $^{14}\text{C}$ in the aqueous phase, as compared with pyrene concentrations determined by HPLC (Figure 2B), much lower than that observed in abiotic control (Figure 2A), indicate that water soluble metabolites were produced, likely by cometabolism in this strain (as later confirmed by radiorespirometry measurements—see below). The lack of difference of partitioning with and without salicylate can be explained, on the one hand, by the initial constrains imposed by the partitioning rate of pyrene from the silicone rings, and on the other hand, by the possible consumption of salicylate at later stages, thus allowing cometabolism of pyrene to occur. In fact, it was observed that, at the end of the experiments, the cultures acquired a yellow coloration, which indicates the formation of metabolites (Pagnout et al., 2006; Vila et al., 2001).

### 3.3. Identification of 1-hydroxypyrene

The GC-MS analyses showed the presence of a main metabolite, 1-hydroxypyrene (1-HP), only in the extracts of bacterial suspensions. These extracts were studied with and without derivatization. In the extracts without derivatization, the spectrum of this metabolite (molecular mass 218), which had a retention time (tR) of 31.20 min, had significant fragment ions at m/z 189, 109 and 95. This fragmentation pattern, as well as a search of the mass spectral library, indicated that this metabolite was 1-HP (Hadibarata and Kristanti, 2013). A main peak corresponding to a retention time (11.93 min) was observed in the GC chromatogram of the derivatized sample (Figure 3A), when the molecular mass of the silylation derivative of 1-HP was selected (m/z 290). Significant fragment ions at m/z 275, 259, 189 and 73 were observed in the mass spectra of the derivatized sample (Figure 3B), which are characteristic for the silylation of 1-HP (Cochran et al., 2012; Zhong et al.,
2011). Unfortunately, it was not possible to detect other pyrene metabolites formed, probably because they were not at sufficient concentrations for detection.

### 3.4. Mineralization experiment

We tested the capacity of strain G7 to mineralize pyrene loaded on $^{14}$C silicone rings. It was observed that strain G7 could not mineralize pyrene (<0.1%), which indicates that the phenomenon detected in the batch experiment was a cometabolic reaction, which did not give rise to intermediates of the central metabolism (Nzila, 2013). If there was no mineralization, this means that pyrene was not transformed into products that entered into the central metabolism, where the cells obtain energy and precursors of cellular components.

### 3.5. Column transport experiment

The column transport experiments were carried out in order to understand the potential role of bacterial motility in the cometabolism and biosorption of pyrene, processes already examined in batch systems. The results showed that the presence of salicylate promoted an increase in bacterial transport relative to control cells (Figure 4 A). In the absence of salicylate (Figure 4B), the still occurring, but limited transport of bacteria can be attributed to the spontaneous motility exhibited by this bacterium, what increases the probability of interception of cells by the collector surfaces (Jimenez-Sanchez et al., 2012). Without salicylate, we detected a higher concentration of $^{14}$C pyrene, as determined by liquid scintillation, than that analyzed by HPLC (Figure 4 B). This result indicates that the higher value of $^{14}$C pyrene equivalents no longer corresponded to pyrene, but to some product resulting from its transformation into water soluble polar metabolites. Accordingly, the percentage of pyrene biodegraded was calculated by the difference between the pyrene radioactivity measured by liquid scintillation and the pyrene concentration measured by HPLC (Figure 5B), thus evidencing the transformation carried out by the bacteria transported through the column.

As compared to the test without the chemo-effector, the bacterial transport was influenced by salicylate through a change in the motile behavior (Jimenez-Sanchez et al., 2012), resulting in an
enhanced bacterial mobilization (Figure 4 A). Considering that salicylate inhibited the cometabolism of pyrene, these results can be explained by considering biosorption as the dominating process, being in line with those obtained by fluorimetry with batch suspensions, evidencing the role of sorption of pyrene to bacterial cells exposed to salicylate (Figure 1). Therefore, when salicylate was present in the columns, pyrene was absorbed by the cells. This biosorption phenomenon was observed previously, but mainly occurred at the early stages of pyrene biological removal (Liao et al., 2015; Xu et al., 2016). The predicted and measured $f_w$ values of pyrene resulting from biosorption are shown in Figure 5A. We observed that at the start of the arrival of the bacterial front (i.e. at 1 and 2 PV), only few cells had been transported, and this allowed the system to reach the equilibrium faster due to the smaller presence of the adsorbent (constituted by bacterial cells). Then, more bacteria started to adsorb the chemical, but without a sufficient time to equilibrate (as compared with the results shown in Figure 1, evidencing an equilibrium time in the order of hours), what explains the deviation with the $K_{oc}$-predicted values.

4. Conclusions

Our results indicate that the mobilization by the motile bacterial strain G7 of HMW PAHs, such as pyrene, can occur in a porous system through biosorption and cometabolism. We observed that the strain G7 is capable to cometabolize, but not mineralize, pyrene in the absence of salicylate. Moreover, the strain G7 exhibits positive taxis toward salicylate, showing smooth motility without changes in the direction, but this chemical effector causes an inhibition of pyrene cometabolism, leading to sorption of the chemical by the cells. In our study, these two conditions provided completely different scenarios to examine how motile bacteria can contribute to pollutant mobilization through sand columns. The present laboratory experiments differ from field conditions in contaminated soils, where other materials, different to sand, additional sources of dissolved organic carbon, and co-occurring contaminant gradients may exist, leading to additional interactions.
which affect the dispersal of pollutants and bacterial cells. However, our results do provide new insights on how the bioremediation of contaminated areas with the pollutants present at a certain depth can take advantage of motile bacterial strains that can be actively mobilized towards the contamination source, therefore enhancing pollutant transformation. Our results also indicate that bacteria exhibiting flagellar motility can also contribute, through cometabolism and biosorption, to the risk associated to pollutant mobilization in soils during bioremediation. This research could be the starting point for the development of more efficient, low-risk bioremediation strategies with poorly bioavailable contaminants in soils.

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Figure legends

Fig. 1. Effect of salicylate on pyrene biodegradation of aqueous-phase pyrene. The concentration of dissolved pyrene was measured with synchronous fluorescence spectrophotometry. The symbols represent two experimental conditions: mineral medium with a cell suspension of *Pseudomonas putida* G7 (■), and mineral medium with the strain G7 and salicylate (▲). The error bars represent the standard deviation. The asterisk indicates significant difference in concentrations between the two experimental conditions after 3.5 h (p < 0.05).

Fig. 2. Pyrene partitioning experiment by *Pseudomonas putida* G7 measured by liquid scintillation to determine the total concentration of 14C-pyrene equivalents in the suspension (A) and pyrene concentration in the aqueous phase, measured after centrifugation to determine the dissolved chemical only (B). In Figure A, the symbols represent: mineral medium (MM) with a bacterial cell suspension (■), MM with cells and salicylate (▲), and control in MM without bacteria (●). In Figure B, the discontinuous lines represent the concentration of dissolved 14C pyrene equivalents (as measured by liquid scintillation) in inoculated MM with (− −) and without (••) salicylate; the continuous lines represent pyrene concentration in the aqueous phase (as measured by HPLC) in inoculated MM with (▲) and without (■) salicylate. The error bars represent the standard deviation.

Fig. 3. GC chromatogram of the derivatized sample obtained from *Pseudomonas putida* G7 cultures after a period of incubation of 408 hours in presence of pyrene (A) and mass spectrum of the silylation derivative of the metabolite identified as 1-hydroxypyrene (B).

Fig. 4. Experimental results on pyrene transport and adsorption of *Pseudomonas putida* G7 cells in sand-filled percolated columns. A, Bacterial breakthrough in the presence of salicylate and concentration of pyrene and of 14C pyrene equivalents in column effluents measured, respectively, by HPLC and liquid scintillation. B, the same as A, but in the absence of salicylate. The lines
represent the bacterial transport (→), the pyrene concentration measured by HPLC in the supernatant (←) and pellet (··) and the 14C pyrene equivalents total concentration (—··—). The error bars represent the standard deviation.

Fig. 5. A, Fraction of pyrene freely dissolved in the aqueous phase (fw) during the column transport experiment shown in Figure 4A (with salicylate). The symbols represent the Koc-predicted (■) and measured (●) fw values. B, Concentration of pyrene cometabolized in the column effluents with (▲) and without (■) salicylate, calculated from the results shown, respectively, in Figures 4A and 4B.
Fig. 1

![Graph showing pyrene concentration over time](image-url)