Biodegradation of Phenanthrene by *Pseudomonas putida* and a Bacterial Consortium in the Presence and in the Absence of a Surfactant

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Abstract This study describes the biodegradation of phenanthrene in aqueous media in the presence and in the absence of a surfactant, Brij 30. Biodegradations were performed using either *Pseudomonas putida* DSMZ 8368 or a bacterial consortium Pyr01 isolated from one PAHs-polluted site. *P. putida* degraded phenanthrene to form 1-hydroxy-2-naphthoic acid (1H2Na) as the major metabolite. LC–MS analysis revealed the production of complementary intermediates in the presence of Brij 30, showing intense ions at mass-to-charge ratios (m/z) 97 and 195. Higher phenanthrene biodegradation rate was obtained in the presence of Brij 30. Conversely, in the case of Pyr01consortium, the addition of Brij 30 (0.5 g L^{-1}) had a negative effect on biodegradation: no phenanthrene biodegradation products were detected in the medium, whereas a production of several intermediates (m/z 97, 195 and 293) was obtained without surfactant. New results on phenanthrene metabolism by *P. putida* DSMZ 8368 and Pyr01 consortium in the presence and in the absence of Brij 30 we obtained. They confirm that the knowledge of the effect of a surfactant on bacterial cultures is crucial for the optimization of surfactant-enhanced PAHs biodegradation.

Keywords Phenanthrene · Surfactant · Biodegradation · *P. putida* · Consortium

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Introduction

PAHs are among the most common organic pollutants. As invasive clean-up techniques are often expensive and damaging to the environment, bioremediation, which exploits the ability of microorganisms to degrade recalcitrant xeno-biotic compounds, has attracted particular attention as safe and cost-effective [1]. The biodegradation pathway of low molecular weight PAHs (having two or three rings) has been demonstrated by a number of researchers [2, 3]. Phenanthrene, a three-ring PAH, taken as a model in the present study, is generally degraded by bacteria through one of two different pathways. The majority of initial steps of phenanthrene biodegradation lead to the formation of 1-hydroxy-2-naphthoic acid (1H2Na), which is further metabolized either via the naphthalene pathway to salicylic acid, which can be later transformed to catechol, or via the phthalate pathway [4]. However, in most of studies the accumulation of only or mainly 1H2Na in culture liquids was

observed during phenanthrene biodegradation by pure or mixed bacterial cultures [4–8]. Indeed, the rapid transformation of PAHs to polar intermediates may have adaptive significance. In an uncontrolled open system this kind of metabolism is advantageous because the formed hydrophobic aromatic compounds which could potentially disrupt membrane functions [9] are converted to water-soluble products which can be easily evacuated from bacterial cells. Thus, PAHs may be rendered innocuous without being completely degraded [10], and their incomplete mineralization may therefore represent a detoxification strategy.

Low water solubility of PAHs is one of the major problems affecting their biodegradation efficiency. That is why intensive studies of the application of surfactants in PAHs degradation processes have been carried out. Nevertheless, adverse effects of surfactants addition on biodegradation have been reported [11–18]. On the one hand, surfactants may increase the solubility and the bioavailability of PAHs. On the other hand, they may be consumed preferentially to PAHs or form a layer preventing the direct contact between microorganisms and PAHs. Thus it is important to have a better understanding of the interaction of surfactants with microorganisms during degradation processes. Zhao et al. [19] showed that the use of mixed anionic-nonionic surfactants resulted in a synergetic solubility enhancement for phenanthrene. The mixed surfactants exhibited no inhibitory effect on biodegradation of phenanthrene. Moreover, in soil–water systems the use of mixed surfactants may be advantageous over the use of individual surfactants because they could be employed over a wider range of temperature, salinity and hardness conditions [20]. Nevertheless, in the present work we decided to use a single surfactant because a mixture of surfactants could have a more significant effect on the composition and the equilibrium of a Pyr01 bacterial consortium then the use of a single one. The present study extends earlier studies on bacterial metabolism of PAHs and proposes a closer insight on the effect of a surfactant on pure and mixed bacterial cultures during phenanthrene biodegradation.

Experimental

Strains

In the present study *P. putida* DSMZ 8368 pure strain known to degrade PAHs or a consortium of microorganisms Pyr01 isolated from a natural site polluted with PAHs (Fensch river sediment, Moselle, France) were used. The consortium Pyr01 contained mostly *Gammaproteobacteria*, *Actinobacteria* and *Alphaproteobacteria*.

Surfactant

The surfactant retained for this study was Brij 30 (Sigma, France). The particular efficiency of Brij 30 in increasing of the solubility of phenanthrene was reported by Aitken et al. [21]. In the case of PAHs, their bioavailability increases with the enhancement of their solubility [22]. So, in order to obtain the highest solubility of phenanthrene, the effectiveness of three surfactants (Tween 80, Brij 30 and rhamnolipids JBR 204) was compared and Brij 30 was chosen as the most effective (see Supplementary Material, Fig. SM1). A concentration of 0.5 g L^{-1} was chosen since at higher Brij 30 concentrations a slight negative effect on the growth of *P. putida* DSMZ 8368 is noted [14].

Strain's Cultures

The culture of *P. putida* DSMZ 8368 was performed in Erlenmeyer flasks of 250 mL containing 60 mL of mineral medium (Brunner) MMB No. 457 [23] supplemented with 5 g L^{-1} of ammonium sulfate to promote the growth of biomass and autoclaved during 20 min at 121 °C. All chemicals were purchased from Sigma (France). Glucose (50 g L^{-1}) added after autoclaving was used as carbon source in pre-culture flasks (see Supplementary material, Fig. SM2) and phenanthrene (50 mg L^{-1}) was used as carbon source in culture flasks. The culture of *P. putida* DSMZ 8368 was carried out on an agitation table at 28 °C, at 200 rpm. The cell growth was determined by OD_{600} measurements with the help of a Hitachi U-2000 spectrophotometer (Science Tec).

The culture of Pyr01 consortium was carried out in the same conditions, but without pre-culture on glucose.

Identification of Phenanthrene and of its Catabolites

The identification of phenanthrene and of its catabolites was done by means of liquid chromatography–mass spectrometry (LC–MS). LC–MS (Thermo scientific LTQ Orbitrap XL, San Jose, CA, USA) was performed in electrospray ionization (negative) mode. Data were processed using Xcalibur 2.1 software. The operational parameters of the mass spectrometer were as shown below. The spray voltage was—5 kV and the temperature of the heated capillary was set at 300 °C. The flow rates of sheath gas, auxiliary gas and sweep gas were set (in arbitrary units min^{-1}) to 40, 10 and 10, respectively. Capillary voltage was—22 V, tube lens was −52 V. All parameters were optimized using 1H2Na in mobile phase MilliQ water with HCOOH (0.1 %)/pure acetonitrile (50/50) at flow rate of 0.2 mL min^{-1}. The optimization was done using 1H2Na because other studied PAHs responded in a similar way. Standard solutions of phenanthrene and of its catabolites

Table 1 Values of principal parameters of phenanthrene biodegradation by *P. putida* DSMZ 8368 in the presence and in the absence of Brij 30

	$OD_{600\ max}$ (17 h)	$OD_{600\ final}$ (60 h)	μ_{max} (h^{-1})	q_{phe} $(\mu M\ h^{-1})$
With Brij 30 (0.5 g L^{-1})	1.67	0.31	0.35	−5.7
Without Brij 30	1.59	1.14	0.35	−0.7

were prepared in pure acetonitrile (HPLC quality, Carlo Erba, France). The column used was Vydac 201TP5215, 0.21 × 15 cm. The injection volume was 1–10 μL. These mobile phases were chosen in order to have a good response of the analyzed molecules in ESI negative mode. Generally, ions are seen in $[M-H]^-$ i.e. deprotoned form. UV detector was used. Phenanthrene and its catabolites were extracted from the medium with acetonitrile. The unknown molecules were determined using full scan analysis (50→500 *m/z*). The single-stage MS scan (MS1) is a full scan (50→500 *m/z*) that permits to observe parent ions. MSn (in the present study MS2 and MS3) is the fragmentation of ions that permits to carry out a filiation study: MS2 results in derivative ions of the first generation and MS3 gives derivative ions of the second generation. Standard molecules of PAHs were analyzed via SIM (Single Ion Monitoring) for catechol and 2-naphtol and SRM (Single Reaction monitoring) for phthalic acid, protocatechol, salicylic acid and 1H2Na. Phenanthrene did not give a response in mass analysis and was analyzed using the UV detector.

Quantitative Analysis of Phenanthrene and of its Catabolites

Quantitative determination of phenanthrene and of its catabolites was performed by high-pressure liquid chromatography (HPLC) (Shimadzu, Japan), using the same column and conditions as for their identification by LC–MS described above. Phase A was MilliQ water acidified to pH = 2 with H_3PO_4; phase B was pure acetonitrile. The injection volume was 1 μL. Two detectors (fluorescence and UV) were used.

Results and Discussion

Biodegradation of Phenanthrene by *P. putida* DSMZ 8368

In order to study the effect of Brij 30 on biodegradation of phenanthrene by *P. putida* DSMZ 8368, cultures were carried out at 28 °C, 200 rpm with and without Brij 30. When Brij 30 (0.5 g L^{-1}) was used, a preliminary solubilization of phenanthrene at 28 °C during 24 h was carried out before inoculation. Kinetics of biomass growth,

phenanthrene degradation and catabolites production were compared. The obtained values for the main parameters of these cultures concerning *P. putida* DSMZ 8368 biomass growth and phenanthrene degradation are shown in Table 1. These results show that the growth of *P. putida* DSMZ 8368 ($OD_{600\ max}$ and μ_{max}) was similar with or without Brij 30, although a toxic effect was observed $OD_{600\ max}$ being attained. At this moment all soluble phenanthrene was degraded in both cases. The phenanthrene degradation rate was much higher when Brij 30 (0.5 g L^{-1}) was used, proving the efficiency of this surfactant in phenanthrene biodegradation by *P. putida* DSMZ 8368. This may be explained by the fact that phenanthrene partitioned into micelles of Brij 30 is directly bioavailable [24]. Similar results were reported by Liu et al. [25] for the biodegradation of naphthalene in the presence of Brij 30.

The study of the production of phenanthrene catabolites was deepened using LC–MS and HPLC. The LC–MS screening of 7 standards of PAHs, able to be either the substrate or its classical catabolites (phenanthrene, 1H2Na, salicylic acid, catechol, protocatechol, phthalic acid, 2-naphtol) showed the presence of an important quantity of 1H2Na and a trace quantity of residual phenanthrene in samples taken at 24 h of culture performed with *P. putida* DSMZ 8368 in the presence of Brij 30. HPLC measurements allowed to determine that their concentrations were about 90 μM and less than 6 μM, respectively. Other standard compounds were not detected in the medium. Note however that LC–MS analysis revealed the presence of some unidentified ions, most intense of them at *m/z* 97 and 195. In order to elucidate the structure of the two ions mentioned above, the MS1 scan was followed by MS2 and MS3 scans. It should be noted that the ions at *m/z* 97 and 195 came out just after the column dead time, that is to say, they were almost not retained. Therefore, their separation on the basis of retention time was not possible. So it was decided to establish the correlation between these ions referring to the results on full scan MS and fragmentations MSn. An example of the obtained UV and MS spectra is shown on Fig. 1.

Therefore, it was suggested that the ion at *m/z* 97 was most probably a cyclic compound having one –OH group in its structure. The ion with *m/z* 195 is supposed to have two C6 cycles in its structure, one of which being mono-unsaturated, and two –OH groups. The proposed hypothetic structures of the corresponding ions and their fragmentation pathway are presented on Fig. 2. Note however that the proposed structure
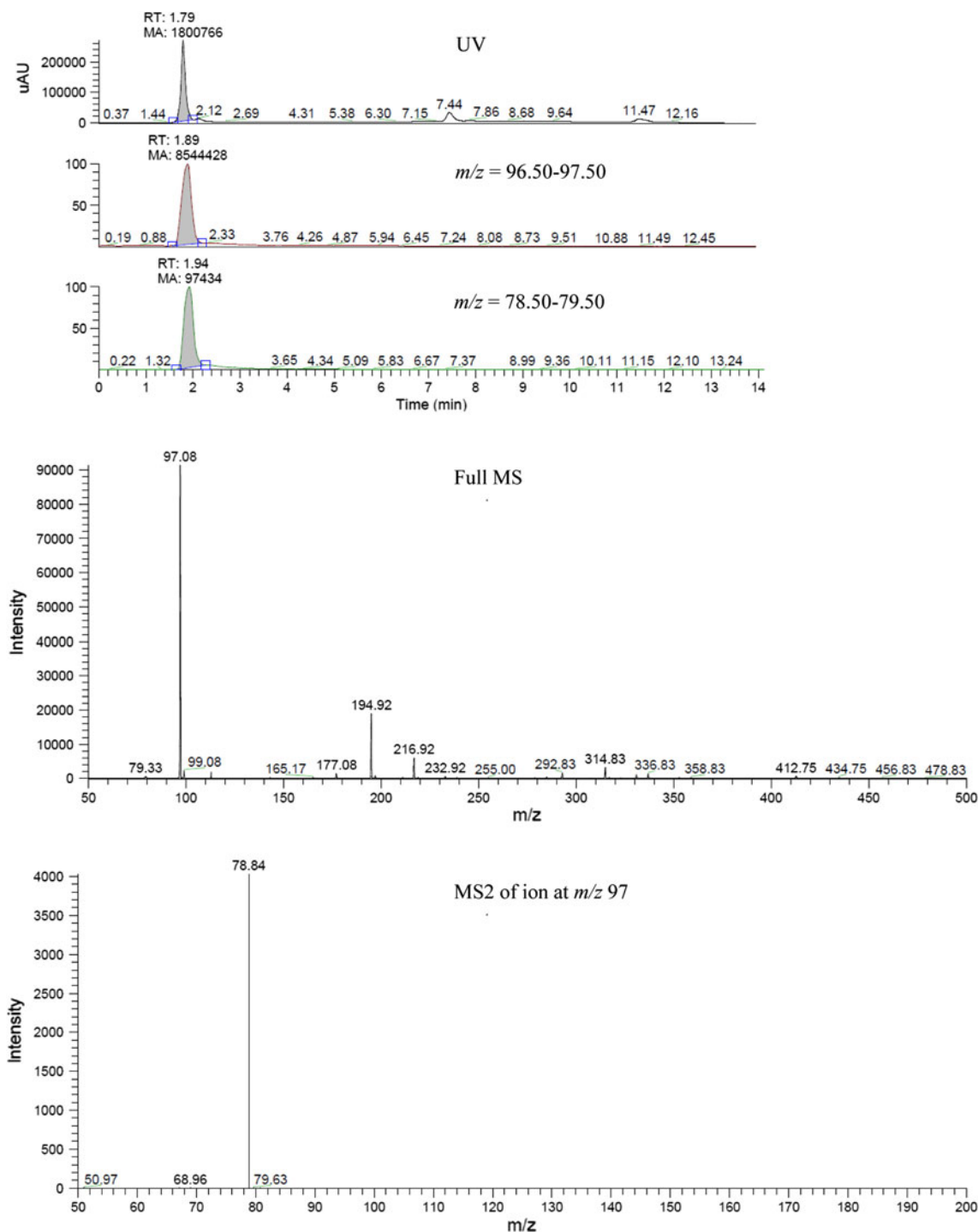
Fig. 1 UV and MS spectra of samples of culture medium taken at 24 h of phenanthrene biodegradation by *P. putida* DSMZ 8368 in the presence of Brij 30 (0.5 g L^{-1}). Focus on the ion detected at the retention time $RT = 1.9$ min (ion at m/z 97)

of the core of the ions is hypothetic, but the fragmentation pathway of the ions (for example, dehydration of parent ions) is most probably as it is shown on Fig. 2. It should be also noted that though some of the proposed structures are similar to the products observed during photodegradation of PAHs, abiotic controls set up for all degradation experiments showed that abiotic loss of phenanthrene was negligible (<2 %) and no new molecules appeared in the medium during such controls. This suggests that the abovementioned ions giving intense response in LC–MS were not the products of photodegradation, but metabolites produced by *P. putida* DSMZ 8368.
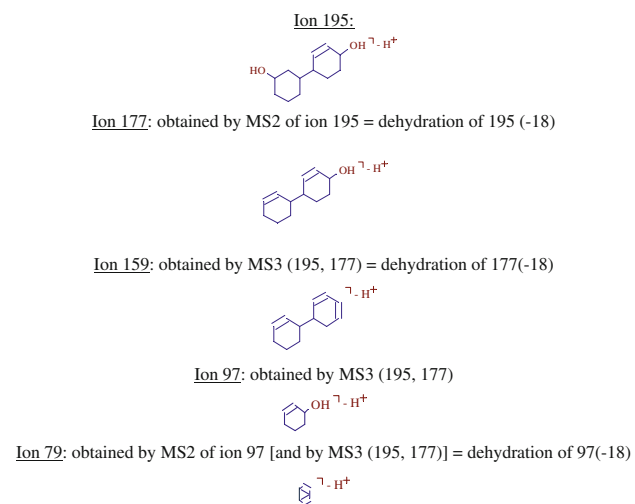
Ion 195:

Ion 177: obtained by MS2 of ion 195 = dehydration of 195 (-18)

Ion 159: obtained by MS3 (195, 177) = dehydration of 177(-18)

Ion 97: obtained by MS3 (195, 177)

Ion 79: obtained by MS2 of ion 97 [and by MS3 (195, 177)] = dehydration of 97(-18)

Fig. 2 Proposed structures and fragmentation pathway of ions detected in samples taken at 24 h of phenanthrene biodegradation by *P. putida* DSMZ 8368 in the presence of Brij 30 (0.5 g L^{-1})

Fig. 3 OD$_{600}$ and phenanthrene concentration curves during culture of Pyr01 consortium in the presence of Brij 30 (0.5 g L^{-1})

It should be underlined that though the MS3 fragmentation of the parent ion at *m/z* 195 gives the ion at *m/z* 97, it was still supposed that the ion at *m/z* 97 seen on the full scan was really present in the medium as a product and not as a derivative of fragmentation in the apparatus. Indeed, this ion is largely the most intense ion on the full scan. Besides, it is not the major derivative ion obtained after MS2 of the ion at *m/z* 195. So, the fragmentation of the ion at *m/z* 195 in the apparatus is not likely to be the only reason of such an important quantity of the ion at *m/z* 97. These results extend earlier studies on *P. putida* DSMZ 8368 metabolism of PAHs.

In the absence of Brij 30, only 1H2Na, present in much smaller concentration than that obtained in the presence of Brij 30 (about 30 against 90 μM), and residual phenanthrene were detected in the medium at the end of culture. It should be noted that the amount of intermediates released into liquid media depends on their diffusivity, on the concentration gradient between cell membranes and on the thickness of cell membranes [26]. In fact, it was shown by Ahn et al. [26] that, comparing to other phenanthrene catabolites, 1H2Na has a higher diffusivity and is therefore more easily released from bacterial cells into the culture medium. This probably explains that with or without Brij 30, 1H2Na is the major phenanthrene catabolite detected in the medium. Besides, a rapid transformation of phenanthrene to 1H2Na has an adaptive significance to bacteria [10].

Biodegradation of Phenanthrene by Pyr01 Consortium

The biodegradation of phenanthrene by Pyr01 consortium in the presence of Brij 30 was then studied. HPLC and
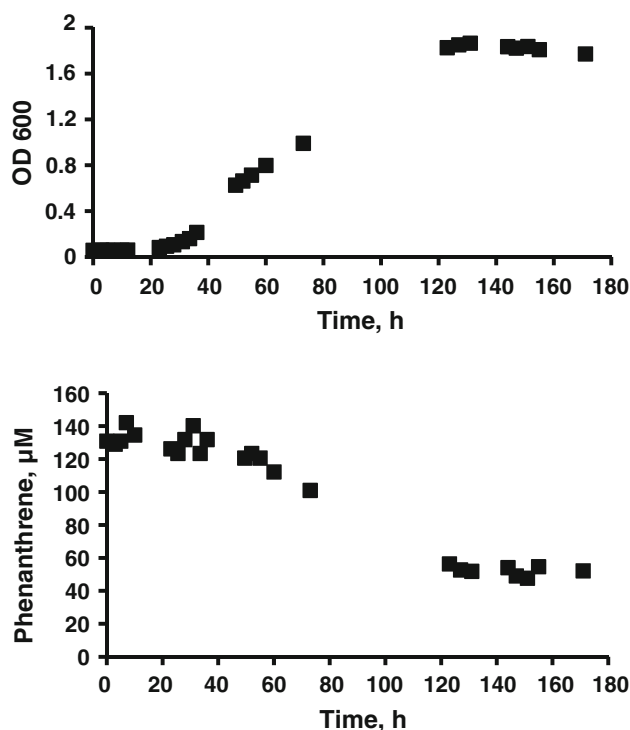
LC–MS analysis performed on samples of the culture medium showed that in the presence of Brij 30 no phenanthrene catabolites, including 1H2Na, were detected in the medium during and at the end of culture (180 h). It was therefore supposed that Brij 30 was consumed preferentially to phenanthrene by Pyr01 consortium. Indeed, preferential consumption of surfactants by certain bacteria has already been reported [27]. This suggestion was also supported by the fact that though no phenanthrene catabolites were produced, soluble phenanthrene concentration reduced in time (Fig. 3), that can be due to the decreasing surfactant concentration in the medium. A complementary experiment demonstrated that in the presence of Brij 30 as a sole carbon source a growth of Pyr01 consortium was observed, thus showing that Brij 30 can be utilized by bacteria as a carbon source. Therefore, as Brij 30 (0.5 g L^{-1}) and phenanthrene were the only carbon sources of the medium and the latter was not biodegraded, the growth of Pyr01 consortium was most probably the result of the consumption of Brij 30 that confirms the suggestion its preferential utilization.

In the absence of Brij 30 the screening of standards of 7 PAHs mentioned above revealed the presence of small quantities of phenanthrene in samples taken at the end of culture (180 h) and the absence of other standard PAHs molecules. The presence of intense ions at *m/z* 97, 195 and 293 was detected in the medium. Therefore, phenanthrene

degradation pathway of Pyr01 consortium has several common catabolites with that of *P. putida* DSMZ 8368. It should be, however, noted that, contrary to *P. putida* DSMZ 8368 biodegradation of phenanthrene, the above-mentioned common ions were already detectable in the culture medium of the Pyr01 consortium in the absence of Brij 30. This may be due to the fact that the consortium produces its proper surface-active molecules that render phenanthrene catabolites more soluble. Indeed, the release of biosurfactants and extracellular polymeric substances by microbial consortia during biodegradation of PAHs has already been mentioned [28, 29]. Nevertheless, concentrations of phenanthrene intermediates, estimated by the area of the corresponding peaks, produced by the Pyr01 consortium and common with those of *P. putida* DSMZ 8368 were much lower than in the case of phenanthrene biodegradation by *P. putida* DSMZ 8368 in the presence of Brij 30 (0.5 g L^{-1}). It can be, therefore, concluded that even if there are some similarities in phenanthrene biodegradation of *P. putida* DSMZ 8368 and the Pyr01 consortium, the effect of Brij 30 on them is quite different.

Conclusion

It was demonstrated that the interaction of Brij 30 surfactant with *P. putida* DSMZ 8368 and mixed consortium of microorganisms is not the same and may either enhance or retard phenanthrene biodegradation. In the case of *P. putida* DSMZ 8368, the biodegradation of phenanthrene was enhanced, though a toxic effect on biomass occurred. As for the mixed Pyr01 bacterial consortium, Brij 30 was most probably preferentially consumed rather than phenanthrene. These results extend earlier studies on bacterial metabolism of PAHs in the presence and in the absence of a surfactant. They suggest that a close study of the interaction of surfactants with pure or mixed bacterial cultures is crucial for the optimization of PAHs biodegradation processes.

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