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# Efficiency of defined strains and of soil consortia in the biodegradation of polycyclic aromatic hydrocarbon (PAH) mixtures

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**Abstract**

The microbiological characteristics of the bacterial degradation of mixtures of five polycyclic aromatic hydrocarbons (PAH), phenanthrene, fluorene, anthracene, fluoranthene and pyrene, were investigated. Three pure bacterial strains using one or several of these PAH as carbon sources were selected. The interactions between PAH during the degradation of PAH pairs by each of these strains were studied and their effects on the kinetics and the balance of degradation were characterised. Competition between PAH and degradation by cometabolism were frequently observed. Mixed cultures of two or three strains, although possessing the global capacity to mineralise the set of five PAH, achieved limited degradation of the mixture. In contrast, a consortium from a PAH-contaminated soil readily mineralised the five-PAH mixture. The results suggested that soil consortia possessed a wider variety of strains capable to compensate for the competitive inhibition between PAH as well as specialised strains that mineralised potentially inhibitory PAH metabolites produced by cometabolism.

## Introduction

Detailed knowledge of the degradation of pollutants in the environment is critical, in particular, to assess the persistence of these chemicals in the environment (Logan & Rittmann 1998). One of the main characteristics of pollutant hydrocarbons is that they are most often constituted of mixtures of numerous homologous compounds. It is known that the biodegradation of these complex mixtures in the environment involves various interactions between the components of the mixtures and the diverse strains constituting the degradative microfloræ (Alexander 1965; Perry 1979). However, our understanding of the modalities and of the importance of these interactions is still quite imprecise. Detailed information on these aspects is necessary to characterise the degradative microfloræ and evaluate the fate of these hydrocarbon mixtures.

Polycyclic aromatic hydrocarbons (PAH) constitute an important class of such compounds. Because of their genotoxicity and of their ubiquitous distribution, their biodegradation is an actively investigated field (Cerniglia 1993; Bouchez et al. 1996b). Bacterial strains, using as sole carbon and energy sources, PAH possessing up to 4 cycles, have been isolated, and it has been shown that each of these strains was capable of using a limited, although variable, range of PAH as carbon sources (Boldrin et al. 1993; Bouchez et al. 1995; Kästner et al. 1994; Mueller et al. 1990). Considering a given strain, PAH not used as substrates were rather frequently found to interact with the degradation of the PAH used as carbon source, in particular, through competitive inhibition and cometabolism (Bouchez et al. 1995; Mueller et al. 1990; Stringfellow & Aitken 1995). The present work is aimed at a better understanding of the degradation

of PAH mixtures, by comparing the performance of defined mixed cultures with that of natural consortia. We studied the effects of PAH interactions in the degradation of a mixture of five PAH. Three bacterial strains with different ranges of PAH substrates covering together the whole set of PAH studied were selected. For all strains, the overall performance and the kinetics of degradation of their PAH substrates, as well as the effects of adding another PAH of the set, were systematically characterised. The degradation of a five-PAH mixture by defined mixed cultures was then studied and compared to the degradation of the same mixture by natural soil consortia.

## Materials and methods

### *Microorganisms*

The strains used were isolated from former manufactured gas plant sites as described by Bouchez et al. (1995) for their capacities to use a defined PAH as sole carbon and energy source: *Pseudomonas* sp. *S Flu Au1* on fluorene, *Pseudomonas* sp. *S Phe Na1* on phenanthrene, strain *S Ant Mu5* (a new isolate), on anthracene. They were maintained on a vitamin-supplemented salt medium (MSM) with the PAH used for isolation as carbon source, as described by Bouchez et al. (1995).

Consortium *C Ge* was a microflora from a former manufactured gas plant site. The soil contaminated with coal tar was stored at room temperature. The capacity of the soil consortium to degrade all individual PAH used in the study, was checked by the microplate method of Stieber et al. (1994).

### *Cultures on PAH in respirometric flasks*

Studies of PAH biodegradation were performed in stirred-flask reactors monitored under constant oxygen pressure by electrolytic respirometry and giving continuous time-course recordings of oxygen consumed during growth on PAH. The apparatus was a D-12S Sapromat (Voith, Heidenheim, Germany) equipped with twelve 500-ml flasks placed in a thermostatic water bath at 30 °C (Bouchez et al. 1997).

The cultures were performed at pH 7.1 in 240 ml of MSM medium. Unless stated otherwise, the flasks were inoculated with 5% (v/v) of a preculture or a mixture of equal volumes of precultures of each tested strain, grown on the PAH used for its isolation. For experiments with soil consortium *C Ge*, a soil sample was humidified and layered in a tube under air for one week at room temperature before being used as inoculum. The flasks were directly inoculated with 1 g of this soil sample. PAH were used as sole carbon and energy sources. They were supplied as crystals obtained by evaporation in the empty flasks of a defined amount of a diethyl ether solution of the PAH used. The amounts of PAH added (between 280 and 340 mg $L^{-1}$ for each individual PAH) were precisely determined by analysis in control flasks (Bouchez et al. 1995). The concentrations of accumulated metabolites and of residual PAH in respirometric flasks were determined at the end of the incubation time indicated by the end of oxygen consumption.

### *Analytical procedures*

Residual PAH in flasks were quantified after extraction with cyclohexane, using a gas chromatograph with flame ionisation detection equipped with a DB5 30 m $\times$ 0.32 mm (internal diameter) column from J&W Scientific, in the conditions described by Bouchez et al. (1995). Overall accumulation of metabolites in the culture media was evaluated on the acidified supernatants of the centrifuged cultures after elimination of dissolved $CO_2$, by determination of the total soluble organic carbon with a carbon analyser (DC 80 from Xertex, Dorhman Division) as previously described (Bouchez et al. 1996a).

## Results

### *PAH carbon sources of the selected strains*

The set of PAH studied included phenanthrene, anthracene, fluorene, pyrene and fluoranthene. The capacities of utilising PAH as carbon sources are summarised in Table 1 for each of the three strains used. The selected strains had quite different ranges of PAH substrates, two of them, *Pseudomonas S Phe Na1* and *Pseudomonas S Flu Au1* utilising each only one PAH, phenanthrene and fluorene respectively, as carbon source whereas strain *S Ant Mu5* had a wide spectrum of PAH substrates, only fluorene not being used as a carbon source.

### *Degradation of a mixture of two PAH*

Detailed degradation studies were carried out in respirometric flasks which allowed precise monitoring of metabolic activity and observation of kinetic effects

*Table 1.* Utilisation of individual PAH as sole carbon and energy sources by the strains used

| Strain tested | PAH degraded | | | | |
|---|---|---|---|---|---|
| | Phenanthrene | Fluorene | Anthracene | Pyrene | Fluoranthene |
| *S Phe Na1* | + | – | – | – | – |
| *S Flu Au1* | – | + | – | – | – |
| *S Ant Mu5* | + | – | + | + | + |

Each strain was incubated in 20 mL of MSM medium containing 200 to 300 mg $L^{-1}$ of the tested PAH in closed penicillin flasks under air with shaking at 30 °C using a preculture on solid nutrient medium.Growth responses were scored as positive when turbidity was visible after up to one month of incubation of the second subculture on the tested PAH.

by continuous determination of oxygen consumption (Bouchez et al. 1997). The interactions between PAH during the degradation of PAH binary mixtures by strain *S Ant Mu5* were first analysed. The kinetic effects and the final degradation data are presented in Figure 1 and Table 2 respectively. Dual carbon source utilisation for PAH pairs of anthracene, with pyrene, phenanthrene or fluoranthene as a second PAH, took place, but with clearly different patterns. Extensive degradation was observed for the pair, anthracene plus phenanthrene, in a one-phase time course but the kinetics was clearly faster than that of anthracene alone. Anthracene and pyrene were also well degraded but with a global velocity, comparable to that of anthracene alone. Fluoranthene had a quite different effect: mixtures of anthracene and fluoranthene were degraded with a velocity, somewhat below that of anthracene alone, and the final degradation rates were only around 60% for each PAH. Fluorene decreased the velocity and the final rate (70%) of anthracene degradation and underwent 30% degradation, a process involving cometabolism since fluorene could not be used as a carbon source. Oxygen consumption data have been shown to be well related to PAH mineralisation (Bouchez et al. 1996a). Here, in the case of anthracene, as sole PAH, the extent of mineralisation expressed by the molar ratio of oxygen consumed to the carbon of PAH degraded ($O_2$/C), was found to be good, with an average value of 0.65 mol/mol, the data for a pure strain growing on its PAH carbon source, reported by Bouchez et al. (1996a) usually ranging between 0.77 and 0.85. The addition of a second PAH lowered the overall $O_2$/C ratios, and thus the rate of mineralisation.

A similar experiment was completed for the degradation of PAH pairs by *Pseudomonas S Phe Na1* and by *Pseudomonas S Flu Au1*. The kinetic and final degradation results are presented in Figure 2 and Table 3 respectively. Considering first strain *S Phe Na1*, complete degradation of its substrate, phenanthrene, took place with a high rate of mineralisation ($O_2$/C of 0.73 mol/mol). Degradation was partially inhibited (final rate 65%) by fluorene which underwent limited attack (20%) by cometabolism. Inhibition might be due to products of fluorene cometabolism, since it was not apparent before 6 hours (Figure 2). Other PAH did not modify phenanthrene degradation (kinetic data not shown), whether, as shown in Table 3, they were cometabolised (in the case of anthracene and fluoranthene), or not (in the case of pyrene). Cooxidation of anthracene, however, lowered the overall $O_2$/C ratio, in accordance with the limited mineralisation resulting from degradation by cometabolism.

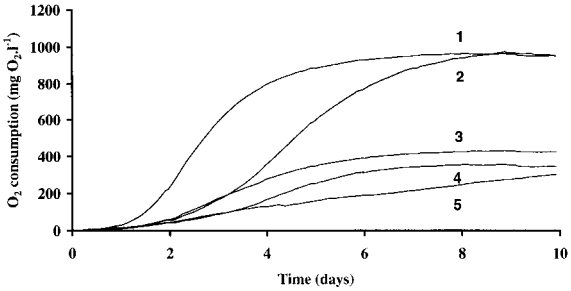


*Figure 1.* Degradation of anthracene with and without a second PAH by strain *S Ant Mu5*: Time courses of oxygen consumption. Experiments were carried out using an electrolytic respirometry apparatus as described in Materials and Methods. pH changes in cultures did not exceed 0.3 during incubation. Oxygen consumption by inoculated controls without PAH was below 20 mg $L^{-1}$. Continuous recordings of $O_2$ consumption for one of the replicates on: anthracene + phenanthrene (1); anthracene + pyrene (2); anthracene (3); anthracene + fluoranthene (4); anthracene + fluorene (5).

Concerning strain *S Flu Au1*, no cooxidation of other PAH took place during degradation of its substrate, fluorene, and the $O_2$/C ratios were fairly high.

*Table 2.* Degradation of binary PAH mixtures during growth of strain *S Ant Mu 5* on anthracene

| PAH mixture | Final degradation (%) for Anthracene | | Second PAH | | $O_2/C$[a] mol/mol | |
|---|---|---|---|---|---|---|
| Anthracene | 100 | (0.4) | | | 0.65 | (0.17) |
| Anthracene + phenanthrene | 80 | (7) | 100 | (1) | 0.49 | (0.02) |
| Anthracene + fluorene | 70 | (6) | 30 | (3) | 0.54 | (0.05) |
| Anthracene + pyrene | 90 | (2) | 96 | (3) | 0.52 | (0.07) |
| Anthracene + fluoranthene | 60 | (22) | 57 | (25) | 0.49 | (0.07) |

The data are given for the experiment of Figure 1 after 15 days of incubation. The cultures were carried out at least in triplicate. The average deviations are indicated in parentheses.
[a] mol of oxygen consumed/mol of carbon of PAH degraded.

*Table 3.* Degradation of binary PAH mixtures by strain *S Phe Na1*, by strain *S Flu Au1* and by a mixed culture of both strains

| Strains tested | Final degradation (%) for First PAH | | | Second PAH | | | $O_2/C$[a] mol/mol | |
|---|---|---|---|---|---|---|---|---|
| *S Phe Na1* | Phenanthrene | 100 | (0) | | | | 0.73 | (0.03) |
| *S Phe Na1* | Phenanthrene | 65 | (6) | Fluorene | 20 | (9) | 0.66 | (0.04) |
| *S Phe Na1* | Phenanthrene | 100 | (0) | Anthracene | 40 | (7) | 0.55 | (0.02) |
| *S Phe Na1* | Phenanthrene | 95 | (4) | Pyrene | 2 | (3) | 0.72 | (0.02) |
| *S Phe Na1* | Phenanthrene | 92 | (1) | Fluoranthene | 31 | (10) | 0.6 | (0.02) |
| *S Flu Au1* | Fluorene | 100 | (0) | | | | 0.7 | (0.01) |
| *S Flu Au1* | Fluorene | 0 | (7) | Phenanthrene | 0 | (8) | n.d.[b] | |
| *S Flu Au1* | Fluorene | 87 | (9) | Anthracene | 4 | (2) | 0.61 | (0.01) |
| *S Flu Au1* | Fluorene | 98 | (0.4) | Pyrene | 4 | (6) | 0.61 | (0.02) |
| *S Flu Au1* | Fluorene | 98 | (0.4) | Fluoranthene | 5 | (1) | 0.66 | (0.02) |
| *S Phe Na1* + *S Flu Au1* | Phenanthrene | 100 | (0) | Fluorene | 100 | (0) | 0.65 | (0.01) |

The data are given for the experiment of Figure 2 after 10 days of incubation. The cultures were carried out in 2, 3 or 4 replicates (average deviations are given in parentheses).
[a] As in Table 2.
[b] n.d.: not determinable.

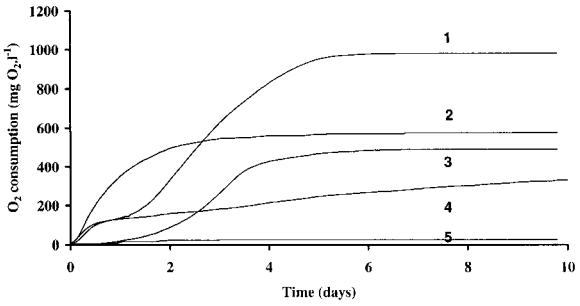


*Figure 2.* Degradation by strains *S Phe Na1* and *S Flu Au1* of their PAH substrate with and without a second PAH: Time courses of oxygen consumption. General conditions as in Figure 1. Continuous recordings of $O_2$ consumption for one of the replicates of: Strain *S Phe Na1* + strain *S Flu Au1* with phenanthrene + fluorene (1); strain *S Phe Na1* with phenanthrene (2); strain *S Flu Au1* with fluorene (3); strain *S Phe Na1* with phenanthrene + fluorene (4); strain *S Flu Au1* with fluorene + phenanthrene (5).

Fluorene degradation however, was completely inhibited by phenanthrene. In this case, the inhibitor was likely to be phenanthrene itself, as inhibition took place immediately. Other PAH, anthracene, pyrene or fluoranthene, did not modify fluorene degradation (kinetic data not shown).

Using a mixed culture of strains, *S Phe Na1* and *S Flu Au1* completely removed the blockage of the activity of strain *S Flu Au1* and both phenanthrene and fluorene were completely degraded with a high overall $O_2/C$ ratio. The kinetics during the first day was identical to that of the degradation of the binary mixture by strain *S Phe Na1* alone. Then, degradation proceeded at a higher rate, with no evidence of sequential utilisation of phenanthrene and fluorene. A possibility is that, because of the limited solubility of

phenanthrene (1 mg $L^{-1}$), its consumption strongly lowered its steady-state concentration in the aqueous phase during degradation, before it became exhausted, thus relieving inhibition of strain *S Flu Au1*.

*Degradation of a mixture of five PAH*

The degradation of a more complex mixture including all five PAH was studied. The degradation performance of mixed cultures of two strains (*S Ant Mu5* and *S Flu Au1*) and of three strains (*S Ant Mu5, S Flu Au1* and *S Phe Na1*) were assessed in comparison with that of a natural soil consortium from a PAH-contaminated site. The kinetic and final degradation results are presented in Figure 3 and Table 4 respectively. Quite limited degradation was observed in the case of the mixed culture of strains *S Ant Mu5* and *S Flu Au1*. The results indicated that the performance of strain *S Ant Mu5* was low, possibly because of joint inhibition by fluoranthene and fluorene, strain *S Flu Au1* being then probably inhibited by phenanthrene as observed above (Table 3). Indeed, when strain *S Phe Na1* was added to the mixed culture, the overall performance was clearly improved, fluorene and phenanthrene being well degraded by the three-strain mixed culture. The situation resembled that observed above for the degradation of these two PAH by the mixed culture of strains *S Flu Au1* and *S Phe Na1*. In the present case however, the efficiency of strain *S Ant Mu5* in degrading anthracene, pyrene and fluoranthene, appeared to be low, possibly because of inhibition by fluoranthene. In contrast, the performance of soil consortium *C Ge* was remarkable, resulting in almost total degradation of all PAH of the mixture. The efficiency of microfloræ of former manufactured gas plants for the degradation of the same and of more complex PAH mixtures was consistently observed (data not shown).

Important differences between mixed cultures and soil consortium *C Ge* were observed concerning PAH mineralisation and production of metabolites. As shown in Table 4, the final concentration of total organic carbon in the aqueous phase was higher in the case of the mixed culture of the three strains than for the soil consortium, suggesting a possible inhibition of the mixed culture by accumulated metabolites of incomplete PAH degradation, originating in particular of cometabolism. Limited degradation (40%) of the same five-PAH mixture with accumulation of metabolites (amounting to 40% of PAH degraded) was also observed with a different mixed culture of five strains (data not shown). The percentages of the carbon of

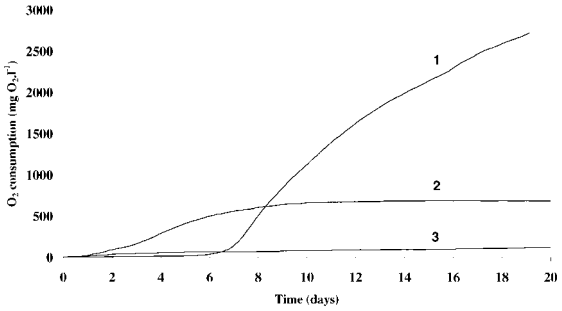


*Figure 3.* Degradation of the mixture of five PAH by defined mixed cultures and by a soil consortium: Time courses of oxygen consumption. General conditions as in Figure 1. Continuous recordings of $O_2$ consumption for one of the replicates of: Soil consortium *C Ge* (1); mixed culture of strains *S Phe Na1 + S Flu Au1 + S Ant Mu5* (2); mixed culture of strains *S Flu Au1 + S Ant Mu5* (3).

degraded PAH in the metabolite fraction were much lower for soil consortia as illustrated in the case of soil consortium *C Ge*, showing its higher efficiency for the mineralisation of PAH metabolites. The $O_2$/C values clearly confirmed the high efficiency of PAH mineralisation by consortium *C Ge*, in contrast to the performance of the mixed cultures. In accordance with these observations, we isolated from a similar soil consortium, a strain of *Pseudomonas aeruginosa* that persisted in the microflora from the soil consortium after repeated subcultures in liquid medium on a mixture of PAH as sole carbon source, although it could not use any of them as a carbon source. However, it was able to grow on such compounds as *o*-phtalic and salicylic acids (Arino et al. 1998), that are known degradation metabolites of phenanthrene and of naphthalene, respectively (*o*-phtalic acid was observed in cultures of strain *S Phe Na1*).

## Discussion

Two characteristic features of the degradation of PAH mixtures by individual strains and defined mixed cultures, were observed in this study: antagonism or competition between PAH, and cometabolism. Both types of interactions, which are strain-specific but quite widespread, contribute in making the degradation of PAH mixtures difficult, even for mixed cultures possessing the capacities to degrade the whole range of individual PAH involved. During the degradation of phenanthrene with a second PAH, Stringfellow & Aitken (1995) observed competitive inhibition in all cases, whether the second PAH was used as a growth

*Table 4.* Degradation of a mixture of five PAH by defined mixed cultures and by a soil consortium

| Inoculum | Final degradation (%) for | | | | | Metabolites accumulated | | $O_2/C$[d] |
|---|---|---|---|---|---|---|---|---|
| | PHE[a] | FLU[a] | ANT[a] | PYR[a] | FLT[a] | mg C $L^{-1}$ [b] | % [c] | mol/mol |
| Defined mixed culture | | | | | | | | |
| *S Ant Mu5 + S Flu Au1* | 16 (5) | 13 (1) | 11 (2) | 8 (0.5) | 9 (1) | 44 (23) | 23 (8) | 0.32 (0.5) |
| Defined mixed culture | | | | | | | | |
| *S Ant Mu5 + S Flu Au1 + S Phe Na1* | 72 (2) | 82 (2) | 13 (2) | 8 (1) | 11 (3) | 136 (16) | 23 (3) | 0.46 (0.05) |
| Soil consortium *C Ge* | 99 (0.5) | 100 (0.3) | 97 (4) | 95 (5) | 97 (3) | 40 (8) | 3 (0.5) | 0.89 (0.02) |

The data are given for the experiment of Figure 3 after 20 days of incubation. Cultures were carried out in 4 or 5 replicates (average deviations in parentheses). [a] PHE: phenanthrene; FLU: fluorene; ANT: anthracene; PYR: pyrene; FLT: fluoranthene.
[b] mg $L^{-1}$ of total organic carbon in aqueous phase.
[c] Total organic carbon in aqueous phase in % of total carbon degraded.
[d] See [a] of Table 2.

substrate or cooxidized, and such competition can be expected when PAH are metabolised by a common enzyme system. In addition, inhibition of the degradation of a PAH by a competing PAH that was not transformed has also been reported (Bouchez et al. 1995). Bacterial strains that degrade four or five individual PAH have been described (Grifoll et al. 1995; Kästner et al. 1994; Mueller et al. 1990; Walter at al. 1991; Weissenfels et al. 1991) and Mueller et al. (1990, 1997) observed quite substantial degradation of creosote PAH by some particular isolates. In the present study, the fact that strain *S Ant Mu5* had a wide spectrum of substrate PAH, did not make its performance really satisfactory for the degradation of their mixtures, as antagonism between anthracene and fluoranthene was observed. In addition, if growth on anthracene of this strain was quite reliable, we found (data not shown) that repeated subculturing on its other PAH substrates led to erratic growth. This behaviour, already observed for a similar strain (Bouchez et al. 1995), might be related to a lower capacity of the PAH concerned, to induce their degradation pathways. As a whole, the results suggested that soil consortia might possess a variety of strains wide enough to compensate for the multiple competitions between PAH, susceptible to occur in individual strains.

Cometabolism was observed in the present study in multiple instances and was possibly involved in particular in the inhibition of strain *S Phe Na1* by fluorene. The high frequency of cometabolism is also apparent in the literature (Keck et al. 1989; Walter et al. 1991; Weissenfels et al. 1991; Bouchez et al. 1995; Ye et al. 1996). The high degradative capacity of soil consortia observed here, is in accordance with the reports of Mueller et al. (1989) and of Wiesel et al. (1993) on the degradation of PAH of creosote or anthracene oil by microfloræ from PAH-contaminated soils. Our results suggest that in soil bacterial consortia, cometabolism promotes rather than inhibits PAH degradation. These consortia can be complex (Wiesel et al. 1993) and, as noted above, they appear to contain strains which are not PAH degraders, but utilise PAH metabolites. Thus, besides avoiding accumulation of metabolites and consequential inhibition of degradation, optimal organisation of cooxidation also provides a plausible mechanism for the degradation of higher PAH such as benzo(*a*)pyrene in soil communities, as observed by various authors (Heitkamp and Cerniglia, 1988; Keck et al. 1989; Ye et al. 1996). Finally, the efficiency of soil bacterial consortia in degrading metabolites is important, in view of the recurrent question of the possible risk resulting from production of potentially toxic metabolites during bioremediation and natural attenuation of PAH-contaminated soils.

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