

Influence of Ageing of Polluted Soils on Bioavailability of Phenanthrene

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Résumé — Influence du vieillissement des sols pollués sur la biodisponibilité du phénanthrène —

Les hydrocarbures aromatiques polycycliques (HAP) sont des composés aromatiques persistant dans l'environnement de par leur caractère hydrophobe entraînant une faible solubilité en milieu aqueux et une forte adsorption sur les sols et sédiments. Généralement, les bactéries ne sont pas capables de dégrader le phénanthrène adsorbé sur les particules de sol mais seulement le phénanthrène en solution. L'objectif du travail ici présenté est d'évaluer la biodisponibilité du phénanthrène pour les bactéries capables de dégrader le polluant en fonction du vieillissement du sol en présence de phénanthrène.

Les expériences de vieillissement et de biodégradation ont été réalisées en *batch* en présence de sol contaminé stérile H ou d'un solide modèle KF2 (kaolinite recouverte de fioul lourd) auxquels est incorporé du phénanthrène marqué au ¹⁴C. Les échantillons solides sont mis en contact avec le polluant dans l'obscurité pendant 2, 48 h, 1 semaine, 1, 2 et 4 mois pour le sol H et pendant 2, 48 h et 2 mois pour KF2. Après chaque temps de maturation, une solution contenant une suspension bactérienne apte à la dégradation du phénanthrène et du milieu nutritif est ajoutée dans chaque fiole. Le ¹⁴CO₂ dégagé lors de la minéralisation de la molécule et le ¹⁴C résiduel dans la phase liquide sont analysés en scintillation liquide sur une durée totale de 25 jours. Les métabolites formés lors de la dégradation du phénanthrène ont été analysés par HPLC/UV.

Une adsorption du phénanthrène sur le sol H et sur KF2 est mise en évidence par comparaison entre l'essai sans maturation et les essais à différents temps de maturation. Pour le sol H, 8, 40 et 55 % du phénanthrène est absorbé après respectivement 2 et 48 h et 2 mois de maturation. Après ajout des bactéries dégradantes, 25 % du phénanthrène est minéralisé lorsque le sol H a été vieilli pendant 2 h et seulement 8 % du phénanthrène est minéralisé après un temps de maturation de 4 mois. Ceci met en évidence une limitation de la quantité du substrat disponible pour les bactéries. Dans le cas de KF2, l'adsorption du phénanthrène sur le solide est plus rapide que dans le sol H car, après 2 h de mise en contact du solide et du phénanthrène, 55 % du produit est adsorbé. Ceci s'explique par la nature différente de la matière organique entre KF2 (un seul type de matière organique et beaucoup de sites d'adsorption libres) et le sol H (hétérogénéité de la matière organique et de la disponibilité des sites d'adsorption). Comme dans le cas du sol H, la quantité de polluant adsorbé atteint 60 % après 2 mois de maturation de KF2. De plus, le taux de minéralisation du phénanthrène par les bactéries dégradantes est de 20 % après 2 et 48 h de maturation et de 6 % après 2 mois de maturation. Un palier est atteint après 15 jours de suivi de la cinétique de minéralisation. Ces expériences mettent en évidence une diminution de la disponibilité du phénanthrène pour les bactéries lors du vieillissement du sol.

Abstract—Influence of Ageing of Polluted Soils on Bioavailability of Phenanthrene—Polycyclic aromatic hydrocarbons (PAH) are aromatic compounds persistent in the environment because of their hydrophobic nature, low water solubility and high adsorption onto soils and sediments. Bacteria can degrade phenanthrene dissolved in water but not when adsorbed onto soil particles. The bioavailability of phenanthrene for bacteria used for pollutant degradation was evaluated as a function of ageing of a soil polluted by phenanthrene.

Ageing and biodegradation were studied in batches containing a sterile contaminated soil (soil H) and kaolinite coated with heavy fuel oil (KF2), both spiked with ^{14}C -labelled phenanthrene. The solid matrix was kept in contact with the pollutant for 2, 48 h, 1 week, and 1, 2 and 4 months for soil H, and 2, 48 h and 2 months for KF2. After each contact time, a bacterial culture able to degrade phenanthrene and the nutrient medium was added. For 25 days, $^{14}\text{CO}_2$ produced and ^{14}C -residual activity in the liquid phase were monitored using liquid scintillation. The metabolites formed during biodegradation were determined by HPLC/UV.

Adsorption of phenanthrene on soil H or KF2 was shown by comparing assay without ageing and those with different ageing times. For Soil H, 8%, 40% and 55% of the phenanthrene had been adsorbed after 2, 48 h and 4 months of ageing, respectively. After the addition of degrading bacteria, 25% of phenanthrene had been mineralized in non-aged soil H and only 8% after a 4-month ageing time. This indicates that the amount of substrate available is a limiting factor for bacterial activity. For KF2, phenanthrene adsorption onto the solid was more rapid than that observed with the soil H since 55% of the pollutant had been adsorbed after only 2 h of ageing. This is due to the different nature of the organic matter in KF2 (only one type of organic matter and numerous available adsorption sites) and in the soil H (heterogeneous organic matter and adsorption sites). For both soil H and KF2, 60% of phenanthrene had been adsorbed after 2 months of ageing. With KF2, the percentage of phenanthrene mineralized reached 20% after 2 and 48 h of ageing and decreased to 6% after 2 months of ageing. These experiments show that the availability of the pollutant for bacteria decreased with soil ageing.

INTRODUCTION

The fate of polycyclic aromatic hydrocarbons (PAH) in nature is of great environmental concern because of their toxic, mutagenic, and carcinogenic properties. These compounds are anthropogenic, produced mainly by pyrolysis and combustion processes used in industries such as coking-plants, gas works and wood-preserving facilities [1].

Polycyclic aromatic hydrocarbons exhibit low solubility and are resistant to microbial degradation when they contain more than 4 aromatic cycles [2, 3]. In nature, after a hydrocarbon contamination, PAHs are sorbed onto soil particles and become resistant to biodegradation because of a lower availability. This ageing process implies a sequestration of the pollutant molecule by the solid matrix, leading to a decrease in its bioavailability [4]. Soil-compound interactions have been shown to be influenced by a number of factors including soil organic matter, soil inorganic constituents, microorganisms and pollutant concentration [5]. PAH sorption onto soils is highly dependent on the soil organic fraction [6], especially the composition of the organic matter, which, according to Saada and Gaboriau [7], is the primary factor controlling phenanthrene retention by the soil. Pores must also be taken into account in the decreased bioavailability of aged phenanthrene [8] because one of the results of ageing is the movement of dissolved compounds from accessible soil compartments (macropores) into less

accessible or inaccessible compartments (micropores) [5]. A knowledge of the specific interactions of soils with PAHs is crucial if we wish to model their transfer, evaluate their natural attenuation, or develop a cleaning procedure [9]. This understanding is also essential to assess the risks associated with a contaminated soil.

In order to be degraded by microorganisms, PAHs must be dissolved (either added as such, or desorbed due to biological activity-release of a surfactant or other extracellular compounds, physical contact with hydrophobic components on the surface of an organism) [8]. Numerous microorganisms are known to have the enzymatic capacity to oxidize 4 ring-PAHs in the aqueous phase. Some microorganisms, such as *Rhodococcus* sp., are able to degrade PAHs (pyrene, fluoranthene) via an interfacial uptake mechanism [10]. A variety of heterotrophic bacteria isolated from soils are able to utilize a selected 3-ring PAH, phenanthrene, as a carbon and energy source, e.g. *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus* strains [11, 12]. The main factor limiting PAH biodegradation is their bioavailability, and one of the main factors increasing their availability for microorganism attack is their sorption onto soil particles [13, 14]. Various metabolites resulting from bacterial degradation of phenanthrene have been identified. 1-hydroxy-2-naphthoate, *o*-phtalate and protocatechuate were formed using *Nocardioides* sp. strain KP7 [15], and *Pseudomonas* sp. though a meta-cleavage pathway [16].

Phenanthrene *trans*-9,10-dihydrodiol, found in the neutral extract of the growth medium, was identified during degradation by *Mycobacterium* strain S1 [15], but there is little in the literature about the influence of ageing on adsorption and metabolite formation. Our work focused, therefore, on the bioavailability of phenanthrene after various ageing times for a bacterial population able to metabolise this compound. Mineralization and metabolite formation were monitored as a function of ageing time.

1 MATERIALS AND METHODS

1.1 Soil Source

Soil H was a PAH-polluted coking-plant soil from Eastern France. The soil was very heterogeneous and contained 13.7% of total organic carbon (TOC) [5]. It was washed with toluene for 24 h in a Soxhlet apparatus and then sterilised by autoclaving (3 cycles of 1 h at 100°C at 24-h intervals). Sterilisation efficiency was about 99% [17].

The second soil was a model soil expected to have a very high affinity towards PAHs—a fuel oil no. 2-coated kaolinite prepared and washed with toluene in order to prevent any pollutant release [7] and hereafter called KF2. The main interest of this sample was that it contained only one type of organic matter (fuel oil no. 2) and no PAH-degrading bacteria. Its TOC content was 2.0%.

1.2 Chemicals

The selected PAH was phenanthrene, one of the 16 PAHs identified as priority pollutants by the *US Environmental Protection Agency (EPA)* due to the frequency of their detection in water [18]. Stock solutions of phenanthrene were prepared in methanol from cold phenanthrene (98% pure, *Sigma-Aldrich*) and phenanthrene labelled with ^{14}C (>98% pure, *Sigma-Aldrich*). Phenanthrene-9- ^{14}C has a specific activity of $4.59 \cdot 10^{11} \text{ Bq mol}^{-1}$.

1.3 Bacterial Source

A phenanthrene-degrading consortium was isolated from the PAH-contaminated soil H, and maintained by successive subculturing in a nutrient medium with phenanthrene as the sole carbon and energy source ($400 \text{ mg}\cdot\text{l}^{-1}$) under shaking conditions at 25°C. 10 ml of this bacterial solution was transferred to 100 ml of fresh nutrient medium supplemented with $400 \text{ mg}\cdot\text{l}^{-1}$ of phenanthrene.

The nutrient medium consisted of 680 mg KH_2PO_4 , 890 mg Na_2HPO_4 , 350 mg $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 100 mg NO_3NH_4 , 0.005 mg $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 0.01 mg H_3BO_3 , 0.01 mg $\text{MnSO}_4\cdot 7\text{H}_2\text{O}$, 0.01 mg $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 0.01 mg $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$, 0.01 mg

$\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 20 mg CaCl_2 , and 0.4 mg FeSO_4 with a final pH of 6.8 [19].

The phenanthrene-degrading capabilities of the bacterial consortium were evaluated using the technique developed by Kiyohara *et al.* [20]. The bacterial solution was spread onto a plate containing mineral salts, agar, and an acetone solution of phenanthrene. The plate was then incubated at 28°C for 1 week. Colonies showing phenanthrene degradation were surrounded by clear zones on the opaque plate.

1.4 Ageing Experiments

Experiments were carried out in 250-ml glass bottles tightly closed by Teflon stoppers at $20 \pm 2^\circ\text{C}$ and the determinations were made in triplicate. 1 ml of methanolic solution of phenanthrene and 100 ml of CaCl_2 ($10^{-3} \text{ mol}\cdot\text{l}^{-1}$) were placed in the glass bottles. The bottles were shaken for 24 h in the dark on a rotary stirrer. 1 ml of the solution was then sampled and analysed by liquid scintillation counting (LSC) in order to determine the true radioactivity and consequently the true initial concentration applied before contact with solids, keeping in mind that artefacts can be associated with the adsorption of phenanthrene on different components of the reactor [21]. The solid was then added—80 mg of soil H or 20 mg of KF2 to 100 ml of solution. The quantities of solid to be added were determined by means of a preliminary adsorption test, so as to have approximately 50% of phenanthrene sorbed onto the solid and 50% free in solution after 48 h of contact time [7]. The bottles were stirred as previously described for 2, 48 h, 1 week, and 1, 2 and 4 months in the dark at 20°C for soil H and 2 h, 48 h and 2 months for KF2.

1.5 Biodegradation Experiments

The bacterial solution was centrifuged at 10 000 rpm for 15 min. The supernatant was removed and replaced by fresh mineral medium without phenanthrene. This operation was repeated 3 times so that the bacterial solution used in the biodegradation experiments was free of phenanthrene. This washed bacterial solution was prepared just before it was added to the phenanthrene-maturated soil.

The initial bacterial density was $10^6 \text{ cells}\cdot\text{ml}^{-1}$ in each batch sample. The glass bottles were shaken for 2 h in order to homogenize the medium. Degradation experiments were conducted under orbital shaking at 25°C, in the dark. 27 bottles were prepared for each solid studied and each maturation time. Periodically and until the end of the degradation experiment, the air contained in the bottles was flushed for 15 min in order to replace the $^{14}\text{CO}_2$ produced with fresh air. The $^{14}\text{CO}_2$ was trapped in 2 vials connected to the bottle, each containing 8 ml of 1 M sodium hydroxide. 4 ml of the 2 sodium hydroxide traps were analysed by LSC and the cumulative mineralization was calculated.

Periodically, 3 bottles were removed from the shaker and centrifuged 15 min at 2000 rpm. The supernatant was sampled for determination of ^{14}C by LSC and metabolite by HPLC/UV.

1.6 Analytical Techniques

$^{14}\text{CO}_2$ determination: 10 ml of fluoran safe scintillation liquid were added to 4 ml of NaOH trap. The mixture was homogenized for 2-3 min and stored at $+4^\circ\text{C}$ for at least 2 h before being analysed by scintillation counter Tri-Carb 2300 TR (Packard Instruments).

^{14}C determination: 10 ml of fluoran safe scintillation liquid were added to 1 ml of the liquid phase. The mixture was homogenized for 2-3 min and stored at $+4^\circ\text{C}$ for at least 2 h before being analysed by scintillation counter Tri-Carb 2300 TR (Packard Instruments).

Phenanthrene and metabolite determinations: these compounds were recovered by a liquid/liquid extraction with 3×20 ml of CH_2Cl_2 . Two extractions were done—one at a pH of around 1 to remove acid metabolites and phenanthrene and another at neutral pH to remove neutral metabolites and phenanthrene. These organic solutions were concentrated under vacuum using a Rotavapor (Buchi Rotavapor R-205) and stored at -20°C . The extracts were then dissolved in methanol before undergoing another concentration step under a nitrogen flow to obtain a final volume of 200 μl . The extracted metabolites were analysed by HPLC/UV. The HPLC was equipped with a Supelguard LC 18 guard column (Supelco) followed by a Supelcosil LC-PAH column (Supelco, 15 cm \times 4.6 mm) and a UV detector (wavelengths 200 to 400 nm). The mobile phase (1 ml/min) was 100% eluent B and increased linearly to 50% eluent A (7 min) and then 100% eluent A (35 min) and finally to 100% eluent B (40 min). The mobile phase was then maintained at 100% eluent B for 10 min in order to recover the initial conditions for the next analysis. Eluent B was composed of 810/90/100 of mQ water/methanol/acetic acid (v/v) and eluent A was composed of 900/100 of methanol/acetic acid (v/v). The column temperature was 30°C . Helium was used to flush the mobile phase and remove air. Three known phenanthrene metabolites are commercially available: phtalic acid, 2-2 diphenic acid and 1-hydroxy-2 naphthoic acid. Phenanthrene and its metabolites were detected at an excitation wavelength of 254 nm.

2 RESULTS AND DISCUSSION

2.1 Phenanthrene-Degrading Microbial Consortium

The PAH-degrading bacterial consortium, hereafter called B1, was obtained from the contaminated soil H by a standard

culture enrichment technique with phenanthrene as the sole source of carbon and energy. When colonies were grown on phenanthrene-coated agar plates, clear zones appeared, indicating phenanthrene degradation. Selected colonies grown on solid media were isolated and each was found to be gram-negative. Mineralization of phenanthrene was monitored by measuring the conversion of ^{14}C -radiolabelled phenanthrene to $^{14}\text{CO}_2$. The B1 consortium alone (without soil) was able to mineralize 25% of the phenanthrene in 28 days (Fig. 1).

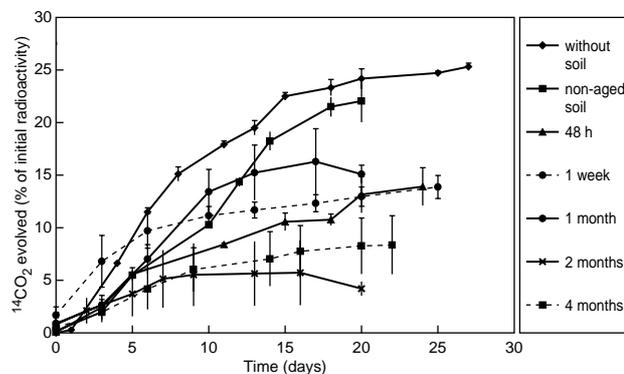


Figure 1

Evolution of phenanthrene mineralization (^{14}C) by pollutant-degrading bacteria. Experiments performed with and without soil H after different ageing times (0, 2 days, 1 week, 1, 2, and 4 months). Mean of 3 replicates with the standard deviation.

2.2 Bioavailability of Phenanthrene as a Function of Ageing

2.2.1 Soil H Experiments

The concentration of dissolved phenanthrene decreased with time (Fig. 2). This loss of bioavailability could not be attributed to biodegradation since this soil had been sterilised. An aliquot sampled in the flask at the end of ageing period was examined under an optical microscope and no microorganisms were found. ^{14}C -activity measured after 2 h of ageing was the same as that measured at time 0 for the non-aged soil experiment. This is probably why only 92% of the ^{14}C -phenanthrene was recovered in the liquid phase. For ageing times between 2 days and 2 months, about 48% of the phenanthrene remained in solution before biodegradation tests. After 4 months of ageing, only 27% of the phenanthrene was detected in the liquid phase before the biodegradation assay. These losses could be attributed to 2 successive phenanthrene adsorption steps onto solid because the soil had been previously sterilised. 2 adsorption steps have been observed by other researchers [21, 22]. The first adsorption step is probably due to rapid

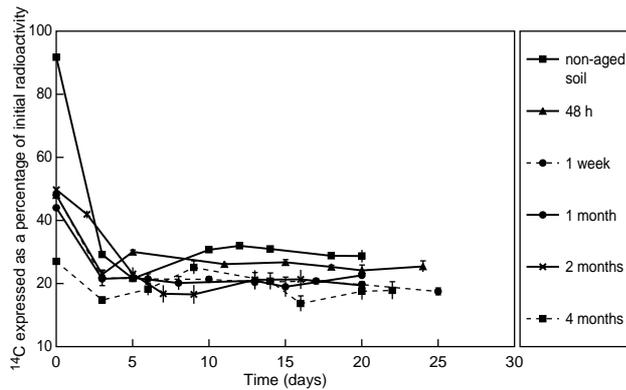


Figure 2

Evolution of ^{14}C activity into the liquid phase. Experiments performed with and without soil H after different ageing times (0, 2 days, 1 week, 1, 2, and 4 months). Mean of 3 replicates with the standard deviation.

adsorption onto easily accessible sites whereas the slower step is due to diffusion of the previously adsorbed PAHs from the soil surface to its pores, thus freeing the easily accessible sites.

For the non-aged soil H, 68% of the phenanthrene had been removed from the liquid phase after 3 days of incubation with the phenanthrene-degrading bacteria (Fig. 1). This loss is due mainly to the fact that the equilibrium of the initial, rapid adsorption step had not been reached at the beginning of the biodegradation experiment. At the end of the biodegradation experiments (3 weeks), only 28.6% of the radiolabelled compounds were recovered in the liquid phase. In this case, the loss was attributed to both microbial degradation and adsorption onto the soil H.

When non-aged soil was added to the bacterial consortium, the level of phenanthrene mineralization remained identical to that obtained without any addition of soil H, and the extent of mineralization declined with ageing (Fig. 1). These results are in agreement with those of Talley *et al.* [23] who found that PAHs associated with the coal-derived fraction appeared to be strongly bound because only 8% of the PAHs in the 63-250 μm residue fraction had been released after 100 days of ageing. This agrees with White *et al.* [24] who found that the percentage of mineralization in a silt loam soil spiked with $[9-^{14}\text{C}]$ phenanthrene decreased from 48% for 3 days of ageing to 28% for a 30-day-aged soil after 21 days of incubation with *Pseudomonas* isolate P5-2. A decrease in the mineralization of phenanthrene has also been observed after an ageing period of 13 days [25]. Various nonpolluted soils spiked with $[9-^{14}\text{C}]$ -phenanthrene aged for 0, 20, 60, 120 and 200 days showed a decrease in mineralization from 44 to 66% for nonaged soils, whereas 28 to 50% of mineralization was recorded for soils aged for 200 days [26]. These variations could not be due entirely to

soil properties such as organic matter or clay content because no correlation was found between the percentage of mineralization and the physicochemical properties of soils. The nature of organic matter is more important than its quantity for a decrease in bioavailability of pollutants in soil [9, 6].

The remaining radioactivity in the liquid phase decreased with increased ageing. Seventeen to 21% of the radiolabelled compounds were still present in the liquid phase at the end of biodegradation for soil H aged 1 week to 4 months (Fig. 2). After 4 months of ageing, 9% of the radioactive loss was found between initial time and final time and it can be attributed to phenanthrene mineralization by bacteria. This is in good agreement with the amount of $^{14}\text{CO}_2$ evolved (around 8%) in the 4-month ageing experiment at the end of the degradation (Fig. 1).

2.2.2 KF2 Experiments

Based on the mineralization curve obtained with soil H, 3 ageing times corresponding to 3 significantly different mineralization maxima were chosen for KF2 experiments. Figure 3 shows the ^{14}C -activity remaining in the liquid phase. At time 0, the ^{14}C -activity in the non-aged KF2 experiment was 54%, which means that 46% of the ^{14}C -phenanthrene to which KF2 had been added had adsorbed onto the organic and mineral particles during the 2 h of mixing before seeding. This difference in phenanthrene adsorption between soil H and KF2 was attributed to the nature of the organic matter (OM) present in these two matrices. Even though there was less OM in KF2 (2.0%) than in the soil H (13.7%), the OM in the KF2 is more highly reactive than the OM in the soil H [7], which is more or less reactive with phenanthrene (plant residues, coal, coal-tar, humic acids, etc.).

The adsorption of phenanthrene onto KF2 after 2 days of ageing was not statistically different from that observed after 2 months of ageing. During the first 3 days of biodegradation, the ^{14}C -activity decreased in the liquid phase due to both mineralization (Fig. 3) and adsorption onto solid particles. Thereafter, the residual ^{14}C -activity tended to stabilise at a level between 18 and 26% (Fig. 3). As for soil H, ageing time has an influence on phenanthrene bioavailability (Fig. 4). The bacterial consortium was able to mineralize around 25% of the phenanthrene in the absence of any solid after 26 days of degradation. When the bacteria were in the presence of non-aged KF2, the percentage of mineralization had decreased to 20% by the end of the 20 days of biodegradation assay. However, since mineralization had not yet ended at this time, the cumulative mineralization percentage might reach that of the control (without soil) after 26 days of incubation. After 2 days and 2 months of ageing, these percentages decreased to 14% and 8%, respectively (Fig. 4).

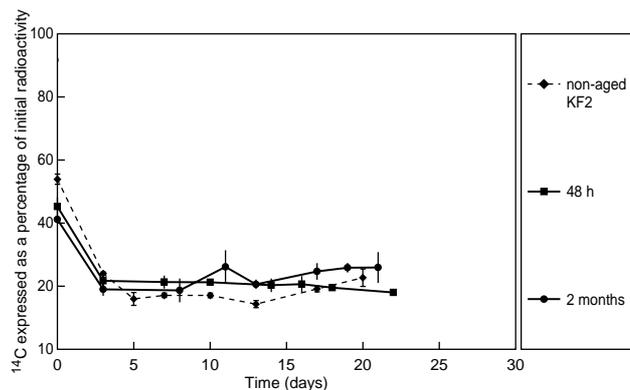


Figure 3

Evolution of ^{14}C activity in the liquid phase. Experiments performed with and without KF2 after different ageing times (0, 2 days, 2 months). Mean of 3 replicates with the standard deviation.

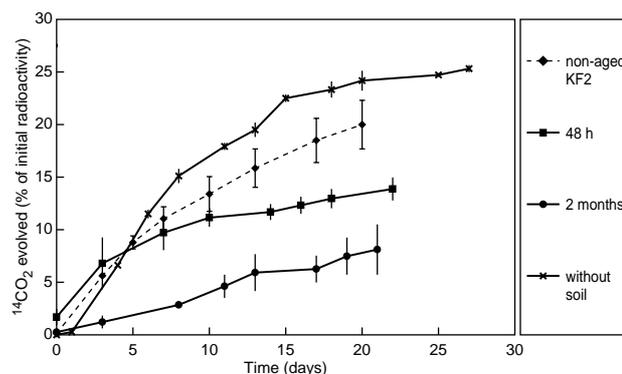


Figure 4

Evolution of phenanthrene mineralization (^{14}C) by pollutant-degrading bacteria. Experiments performed without soil and with KF2 after different ageing times (0, 2 days, 2 months). Mean of 3 replicates with the standard deviation.

2.3 Metabolites Formed

Metabolites were determined in the liquid phase for the experiments “without soil H” and after 2 days of ageing on soil H. Without soil, phenanthrene was the only compound initially detected (Fig. 5) and its concentration decreased rapidly with time. After 3 days of incubation, 69% of the phenanthrene had degraded. After 13 days, 76% of the phenanthrene had been transformed and 2 metabolites, C8 (retention time (RT) = 16.1 min) and C16 (RT = 22.4 min), were detected by HPLC/UV (Fig. 5). The quantity of C8 increased after 2 days and then decreased until the end of the experiments. The same was observed for C16, but to a lesser extent (Fig. 5). Naphtic acid was detected at 15, 20 and 23 days. According to the literature, this compound is a final metabolite of the phenanthrene degradation pathway [27, 28].

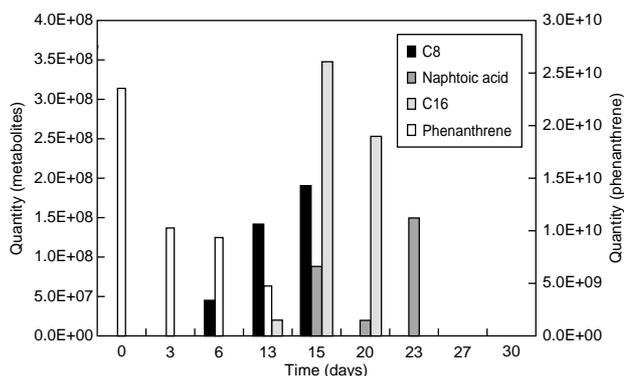


Figure 5

Evolution of phenanthrene and metabolites concentrations, determined by HPLC/UV, in phenanthrene biodegradation experiments without soil H.

These results seem to indicate that the formation rates of these two main metabolites, C8 and C16, are greater than their degradation rates. They seem to be transient compounds coming from phenanthrene metabolism.

After 48 h of ageing and at time 0 of the biodegradation experiment, HPLC analysis showed that only 50% of the phenanthrene remained in solution. This confirms the data obtained from ^{14}C measurements. The decrease in the liquid phase is due to the adsorption of phenanthrene onto the soil matrix. After 7 days of biodegradation, only 41% of the phenanthrene was in solution and 3 days later, no phenanthrene was detected by HPLC/UV (Fig. 6). C8 and C16 were detected as previously but at concentrations 10 times lower than in the experiment “without soil” (Fig. 6). Phenanthrene and 3 other compounds were detected at time 0 of biodegradation. These 3 compounds, C2 (RT = 12.45 min),

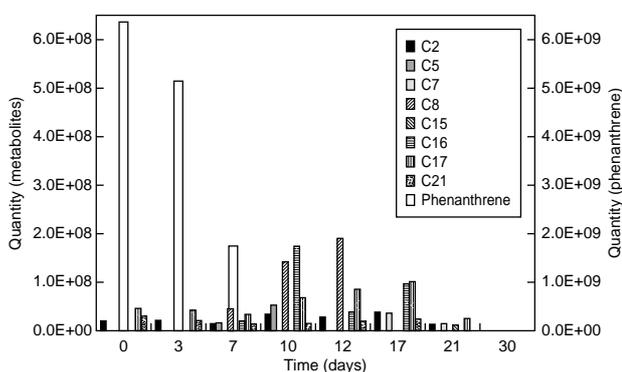


Figure 6

Evolution of phenanthrene and metabolites formed during phenanthrene biodegradation experiments with 2-day-aged soil H.

C17 (RT = 23 min) and C21 (RT = 29.14 min), were found during all the biodegradation experiments (Fig. 6). No trend could be identified in the evolution of C2 and C21. The C17 concentration, however, gradually increased between 7 and 17 days and then decreased. These compounds are currently being identified with HPLC/MS. In the literature, the following phenanthrene ring fission products were found in the acid extracts: phenanthrene *cis*-3,4-dihydrodiol, phenanthrene *cis*-9,10-dihydrodiol, 1-hydroxy-2-naphthoic acid, 2,2'-diphenic acid and phthalic acid [29]. Two possible reasons why we detected no phthalic acid or 2,2' diphenic acid are:

- an alternative phenanthrene degradation pathway after the formation of 1-hydroxy-2-naphthoic acid;
- and an HPLC detection level higher than the trace amounts of these compounds.

CONCLUSION

We observed a decrease in bioavailability to bacteria after only short periods of ageing for both solids studied, and a more rapid phenanthrene adsorption on KF2 than on soil H. For soil H, the phenanthrene detected in the liquid phase in experiments with non-aged soil decreased from 90% to 50% after 48 h of ageing. This led to a decrease in mineralization from 23% to 15% as a result of competition between phenanthrene adsorption and mineralization. For KF2, the quantities of phenanthrene adsorbed were nearly the same in the experiments with non-aged KF2 and those after 48 h of ageing. In addition, the mineralization rate was the same. For both solids, a second adsorption step occurred after 2 months of ageing, probably due to the sequestration of the pollutant in small pores, causing a decrease in phenanthrene availability, as suggested by the lower biodegradation. HPLC/UV supernatant analyses showed that there was an immediate decrease in the phenanthrene concentration and no phenanthrene was detected after 2 weeks of biodegradation for experiments without soil and with 2-day-aged soil H. The metabolites formed are now being identified by mass spectrophotometry.

The decrease in the proportion of soil-associated phenanthrene available for mineralization and the maximum rate of mineralization observed here show that an increase in the ageing time causes the labile compound fraction to decrease while the non-labile fraction increase. This should be taken into account when estimating the bioavailable fraction of a pollutant in a contaminated soil in risk assessment studies.

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