INTERFERENCE OF SOIL CONTAMINANTS WITH LACCASE ACTIVITY DURING THE TRANSFORMATION OF COMPLEX MIXTURES OF POLYCYCLIC AROMATIC HYDROCARBONS IN LIQUID MEDIA

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The biotransformation of 16 polycyclic aromatic hydrocarbons (PAH) in mixture was investigated in reactors in the presence of purified laccases of the fungus *Pycnoporus cinnabarinus*, ABTS as a redox mediator, 25% acetonitrile and Tween 20. Several hydrocarbons from a synthetic mixture, such as anthracene and benzo[a]pyrene, were converted up to 80% into quinones, whereas others also belonging to 3- and 5-ring chemicals were less transformed. Chrysene and benzo[k]fluoranthene were not oxidized by the laccase mediator system. Moreover, hydrocarbons extracted from an industrial soil were all recalcitrant to enzymatic attack. This lack of reactivity of the laccases towards the hydrocarbons could be due to the presence of interfering compounds co-extracted from the soil, such as metals.

Keywords polycyclic aromatic hydrocarbons; mixtures; biotransformation; filamentous fungi; laccases; bioavailability

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are hazardous pollutants that are found worldwide in aquatic and terrestrial environments. They are very abundant in soils collected from industrial sites including manufactured gas plants (MGPs) or wood preservative plants, and cokeries. For that reason, remediation of PAH-contaminated soil has been extensively studied for many years. In contrast to some remediation methods such as containment, or thermal, physical and chemical techniques, soil biological treatment is a cheap remediation process, which provides a definitive degradation of the contaminants.[1] Soil fungal treatment appears as a promising recent technology, that offers many advantages over other conventional treatments.[2] Some strains of filamentous fungi, as well as their enzymatic systems, have been studied extensively to investigate their possible use for the transformation of environmental pollutants.[3]

Studies on the enzymatic metabolism of PAHs have been carried out to identify the transformation products formed from PAHs, to understand the mechanisms involved in the reaction, then to evidence the efficiency of the biotreatment. During in *vitro* bench scale experiments, filamentous fungi metabolize PAHs by using two main classes of enzymatic systems.[4] On one hand, PAHs are metabolized by intracellular P450 monooxygenase systems to *trans*-dihydrodiols products that are not mineralized, or to phenols that are further conjugated with sugar or sulfates.[3] On the other hand, a second main system involved in PAH transformation is the lignin degrading system produced by white rot basidiomycetes. It includes lignin peroxidases (LiPs, EC 1.11.1.14),[5] manganese-dependent peroxidases (MnPs, EC 1.11.1.13)[6] and laccases (Lacs, EC 1.10.3.2).[7] These enzymes convert PAHs to quinones. Then, white rot strains partly mineralize these chemicals, suggesting their complete breakdown.[4]

Laccases have been often used for pollutant breakdown.[8] *In vitro* studies intended to evaluate their potential for PAH degradation have generally focused on individual compounds.[7] The laccase degrading capacity was rarely tested mixtures of PAHs,[9] either

synthetic or extracted from polluted media. Because PAHs usually occur as mixtures of compounds at contaminated sites, the later kind of approach remains essential to take into account interactions between substrates themselves, or between pollutants and matrix constituents, all governing the fate of parent compounds.[10] Moreover, it was found that PAHs are generally recalcitrant to biotransformation in manufactured gas plant site soils.[11,12,13] Such a low biotransformation rate may be due to changes in the physico-chemical state of the pollutants because of their aging in the soil, or to an inhibition of microbial growth or activity.

Fungal laccase were rarely found to catalyze the direct oxidation of PAHs *in vitro*.[7] In most cases, the reactions are efficiently enhanced by oxidizable low-molecular-weight compounds, so-called mediators. They are proposed to act as an electron shuttle between the enzyme and the pollutant. 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), or natural compounds, namely cysteine and methionine are known as mediators.[14] The active forms of these compounds are radicals or cation radicals.[15]

Our objectives in the present study were: (i) to determine the efficiency of fungal laccases to transform synthetic mixtures of PAHs, (ii) to assay their use for the transformation of PAHs extracted from an industrial polluted soil and (iii) to evidence the interference of contaminants co-extracted from the polluted soils with PAH transformation.

MATERIAL AND METHODS

Chemicals

All the chemicals, including individual PAHs, [7-^{14}C]benzo[a]pyrene (980 MBq mmol^{-1}, radiochemical purity > 99.5%), 9,10-phenanthrene dione and laccase mediators [2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid): ABTS; 1-hydroxybenzotriazole: HBT] were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). 1,8-naphthalic acid anhydride, 1,2-acenaphthene dione, 9-fluorenone and 9,10-anthracene dione were obtained from Lancaster (Strasbourg, France). Benzo[a]pyrene was enzymatically transformed to diones as published.[16] Solvents of analytical grade were obtained from Carlo Erba (Val de Reuil, France).

Laccase production and purification

The organism used for laccase production was *Pycnoporus cinnabarinus* ss3, a monokaryotic strain originating from the parental dikaryotic strain I-937.[17] The strain was grown in a synthetic medium containing 35 g Glucidex 47 (dextros), 3 g yeast extract, 0.01 mM disodium tartrate, 20 mM diammonium tartrate, 9.8 mM KH_2PO_4, 0.68 mM $CaCl_2$ $2H_2O$, 2 mM $MgSO_4$ $7H_2O$, 0.46 mM $FeSO_4$ $7H_2O$, 0.28 mM $ZnSO_4$ $7H_2O$, 0.23 mM $MnSO_4$ H_2O, 0.45 mM $CuSO_4$ $5H_20$, 1 mL mixed vitamin [18] and 0.05% Tween 80, per L. Culture was carried out at 30°C and stirred at 300 rpm. After 3 days, the culture was induced for laccase expression by addition of 0.5 mM ferulic acid. Culture medium (11.8 L) was collected after 11 days and contained a laccase activity of 41.4 U/mL (determined by the oxidation of ABTS).

Laccases of *P. cinnabarinus* ss3 were purified as published[19]. Briefly, the culture medium was first concentrated by ultrafiltration through a cellulose PLGC membrane (molecular mass cut-off of 10000 Da). Laccases were further concentrated by a two step ammonium sulfate precipitation. In the first step, ammonium sulfate was added with stirring to a 40% (w/v) final concentration, and incubated for 1 h at 4°C. The precipitate was eliminated by a centrifugation at 17000 g for 30 min. The supernatant fluid was then increased to 80% (w/v) saturation with ammonium sulfate, and stored overnight at 4°C. The precipitate was collected by centrifugation (17000 g for 30 min) and dissolved in 100 mL of sodium acetate buffer (0.025 M, pH 5.7). The residual ammonium sulfate was discarded by dialysis. Specific laccase activity was 146 U/mg. At this step, lactose was added to a final concentration of 3 % (w/v), and the laccase extract was freeze-dried over a 24-h period. The resulting powder can be easily manipulated and stored for long periods without any loss of activity.

Laccase activity was determined by monitoring the slope of the increase in A_{420} due to the oxidation of 2 mM ABTS (ε_{420} 36 000 M^{-1} cm^{-1}) in citrate-phosphate buffer (0.1M, pH 3) at 30°C in the dark.[20] Solutions of enzyme were added in a final volume of 1 mL assay media. To assess enzyme inhibition, 250 µL acetonitrile or 150 µL acetonitrile and 100 µL soil extract were added to the assay media containing 2 U laccase. The reaction was started by adding ABTS immediately, or after 6, 24, and 48 h of contact of the organic compounds with the enzyme. The enzyme activity was expressed in international units (U). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS in 1 min.

Preparation of the mixtures of PAHs

Extraction from soil. PAH-contaminated industrial soil was from a pile in a MGP site at Rouen (France). PAHs were Soxhlet-extracted from soil samples (30 g dry weight) in the presence of acetonitrile (100 mL) for 8 hours. After cooling, extracts obtained from about 30 soil samples were pooled and concentrated under vacuum. Final composition of PAH mixture, measured by HPLC (see the analytical procedures below) is shown Table 1.

Synthetic mixture. A synthetic mixture of PAHs was prepared in acetonitrile. Among the PAHs detected in the industrial soil extracts, 16 compounds were selected on the basis of their toxicological relevance according to the Environmental Protection Agency (USA).

Laccase treatment of mixtures of PAHs

Both soil-extracted and synthetic mixtures of PAHs were tested under the same conditions. The reactor used for PAH biodegradation contained 1.5 L 0.1 M citrate-phosphate buffer pH 3 supplemented with 1% (v/v) Tween 20. The mixture of PAHs dissolved in 500 mL acetonitrile was then added. The reaction was started by adding 2 mM mediator (ABTS or HBT) and 4000 U laccase. The reactor was incubated for 48 hours at 30°C in the dark under gentle stirring with a continuous bubbling of air.

The extent of PAH transformation was determined by extracting 20-mL aliquots of the medium four times with 10 mL dichloromethane at the beginning and after 1, 2, 3, 4, 5, 6, 24, and 48 hours of incubation. In these conditions, the mediators remained in the aqueous phase. The organic fractions were pooled, dried over anhydrous Na_2SO_4, concentrated to about 100-µL under slightly reduced pressure, and then diluted with acetonitrile to 8-mL (final volume). After filtration on Millex-GV filter units (0.22 µm), 20-µL aliquots were injected for HPLC analysis.

Preparation of $ABTS^{\bullet+}$ and $ABTS^{++}$

$ABTS^{\bullet+}$ was prepared by incubating 2 mM ABTS with laccase (2 U mL^{-1}) for one hour at 30°C in 0.1 M citrate- phosphate buffer pH 3. $ABTS^{\bullet+}$ was then separated from the enzyme by ultrafiltration through an Amicon YM10 membrane followed by boiling to inactivate any trace of remaining laccase.[21]

$ABTS^{++}$ was prepared from ABTS by oxidation with potassium persulfate as reported.[22] After successive washing in acetonitrile/water, $ABTS^{++}$ precipitate was recovered and resuspended in 10 mM citrate/phosphate solution pH 3 containing 15 % acetonitrile for BaP transformation experiments.

Assays of benzo[a]pyrene transformation

The assays used to study PAH biotransformation were carried out at 30°C under gentle shaking in 0.1 M citrate-phosphate buffer pH 3.

Incubation medium (1 mL final volume) in the presence of the laccase mediator system (LMS) contained 1% (v/v) Tween 20, 2 mM ABTS, 0.1 mM benzo[a]pyrene (dissolved in 50 µL acetonitrile) and 50 µL soil extract when necessary. All assays comprised 4 kBq labeled BaP. The reaction was started for 24 hours by adding 2 U laccase.

The assays (2 mL final volume) used to study the effects of contaminants on PAH oxidation by $ABTS^{++}$ contained 5 mM potassium persulfate ($K_2S_2O_8$), 0.1 mM ABTS, 0.1 mM benzo[a]pyrene (dissolved in 50 µL acetonitrile), 50 µL soil extract when necessary, and 0.075 to 0.375 mM $K_4Fe(CN)_6$.

The extent of benzo[a]pyrene transformation was determined by analyzing 100-µL aliquots of the medium by HPLC using the procedure described hereafter. ABTS concentration was determined by measuring the $ABTS^{\bullet+}$ absorption at 420 nm. When necessary, the medium was diluted before spectrophotometric measurements.

Analytical procedure

Each sample was analyzed twice. HPLC analysis was performed by using an analytical column Supelcosil LC-PAH (15 cm x 4.6 mm, 5 µm, Supelco), maintained at 30°C. The solvent system (flow rate 1.5 mL min^{-1}) was water/acetonitrile 55/45 for 5 min, it was modified to 0/100 during the following 25 min. The last solvent conditions were maintained during 10 min before a return to the initial solvent composition. A_{254} and radioactivity of the column eluate were monitored.

Metabolite identification

The acetonitrile solutions containing the compounds extracted from 48 hours of incubation were submitted to GC-MS analysis. The molecular weight and fragmentation informations of the products were obtained by chemical ionisation (CI-MS) using NH_3 as a reactant gas, and

electron impact (EI-MS, 70 eV) analysis, respectively, using a Nermag R10-10C quadrupole spectrometer with sample introduction by gas chromatography (Varian 3300).

Cyclic voltammetry

Electroanalytical experiments were carried out in 0.1 M KNO_3 pH 5, using a Versastat Voltammetric Analyzer (Princeton Applied Research). An one-compartment cell of 20 mL was used in the voltammetric studies. The electrochemical cell consisted of a saturated calomel reference electrode, a platinum wire counter electrode and a 3 mm diameter glassy carbon working electrode, purchased from Radiometer Tacussel.

RESULTS

Transformation of the synthetic mixture of PAHs

The transformation of a synthetic mixture of PAHs was assayed in the presence of purified laccase of *P. cinnabarinus* and ABTS as a redox mediator, during 48-hour experiments (Figure 1). No significant chemical transformation occurred in the control reactor in the absence of the laccase/ABTS couple. The initial overall concentration was 178 mg L^{-1}, whereas total concentrations of the 16 PAHs remained comprised between 173.2 and 182.3 mg L^{-1} during the experiment. By contrast, the presence of the LMS resulted in a marked decrease from 183.2 to 93.9 mg L^{-1} of the PAHs overall concentration in the mixture. The effect of the LMS was the most important during the first 6-hours of the incubation, before reaching a plateau. Concomitantly, laccase activity decreased rapidly to 36.4% of its initial activity after 6 hours, and was less than 10% at the end of the experiment.

A brown precipitate of insoluble compounds was observed in the reactor at the end of the incubations, which probably resulted from coupling reactions.[7]

The behavior of anthracene (ANT, see Table 1 for abbreviations) and benzo[a]pyrene (BaP), one 3-ring and one 5-ring PAH often used as markers for PAH biotransformation studies, was highlighted. Figure 1 shows that the fate of these two compounds was quite representative of that of the mixture: a rapid degradation during the first 6-hour period is followed by a low degradation rate phase.

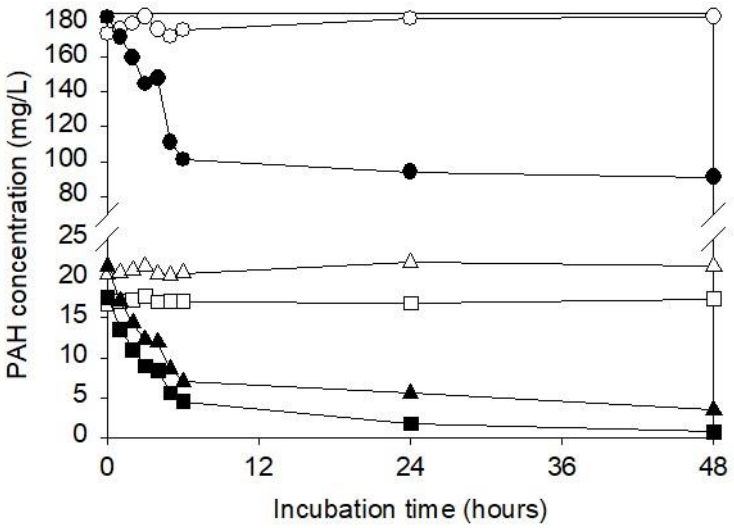

FIGURE 1 Transformation of a mixture of 16 commercial PAHs (circles), ANT (squares) and BaP (triangles) during 48-hour incubations. White symbols refer to controls without the laccase/mediator system, whereas black symbols correspond to the enzymatic incubation.

However, it can be noticed that after 6-hour incubations in the presence of LMS, about 75% of both ANT and BaP were transformed, whereas the overall concentration decreased only to half of its initial value in the PAH mixture.

Extensive HPLC analysis of the extracts from 48-hour aliquots showed that 4 compounds were the most degraded (82.6 to 98.8% of transformation), namely NAP, ACE, ANT and BaP (Table 1). Conversely, 10 compounds (ACY, FLU, PHE, FLA, PYR, BaA, BbF, DaA, BgP and I1P) were less transformed (20.0 to 68.7 %), whereas BkF and CHR, were not oxidized. Parent PAHs were converted to oxygenated compounds, mainly diones, as corroborated by the GC-MS analysis of the orange-colored extracts (strong absorbency at 430-450 nm). Diones were detected by the presence of major ions corresponding

TABLE 1 Common names, abbreviations, initial concentration (mg L^{-1}) and degradation (%, after 48 h) of PAH compounds, and transformation products identified.

Common name	Abbre-viation	PAH		Transformation products identified by GC-MS	Abbre-viation
		Initial conc.	Degradation		
Naphtalene	NAP	10.0	1.2		
Acenaphthylene	ACY	4.0	31.3	1,8-naphthalic acid anhydride	NAA
				1,2-acenaphthene dione	ACQ
Acenaphtene	ACE	3.0	17.4	1,8-naphthalic acid anhydride	NAA
				1,2-acenaphthene dione	ACQ
Fluorene	FLU	5.0	53.7	9-fluorenone	FLQ
Phenanthrene	PHE	25.0	57.2	9,10-phenanthrene dione	PHQ
Anthracene	ANT	17.0	4.3	9,10-anthracene dione	ANQ
Fluoranthene	FLA	22.0	59.3		
Pyrene	PYR	20.0	57.3	1,6- 1,8-pyrene diones	PYQ
Benz[a]anthracene	BaA	9.0	68.6	7,12-benz[a]anthracene-dione	BTQ
Chrysene	CHR	5.0	95.6		
Benzo[b]fluoranthene	BbF	17.0	63.5		
Benzo[k]fluoranthene	BkF	15.0	99.3		
Benzo[a]pyrene	BaP	21.0	16.5	1,6- 3,6- 6,12-benzo[a]pyrene diones	BAQ
Dibenz[a,h]anthracene	DaA	1.0	73.7		
Benzo[ghi]perylene	BgP	1.0	80.0		
Indeno[1,2,3-cd]pyrene	I1P	3.0	71.8		
Σ 16 PAHs	MIX	178.0	49.7		

to *m/z* of [M^+-28] and [M^+-56], resulting from the ejection of two molecules of carbon monoxide.[23] Moreover, spectra were compared with mass data obtained with authentic standards.

No PAH transformation was obtained in similar experiments conducted in the presence of HBT instead of ABTS (data not shown).

Transformation of PAHs extracted from industrial soil

Enzymatic transformation of soil-extracted PAHs was assayed in the presence of the LMS. Overall profiles of the curves showed that total PAH concentrations without or with LMS were quite similar (Figure 2). During the five first hours, PAHs concentration rapidly decreased prior to an abrupt rising to recover their initial concentration after six hours of incubation. Between 6 and 48 hours of incubation, a significant and regular decrease was then observed, with comparable slopes in systems containing or not the LMS. The behavior of two individual PAHs, ANT and BaP, was similar to that of the overall PAH pool, both in the presence and in the absence of the LMS in the same experimental conditions. No PAH metabolites could be detected by HPLC and GC-MS analysis of the extracts.

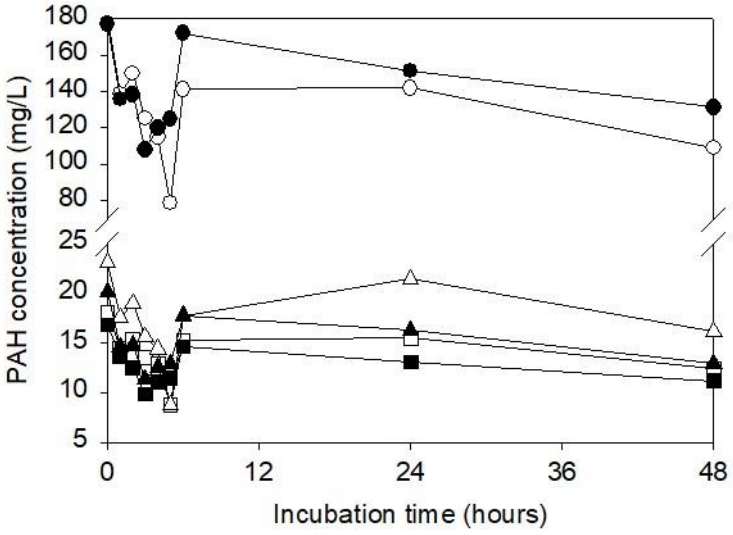

FIGURE 2 Transformation of a mixture of 16 PAHs (circles), ANT (squares) and BaP (triangles) extracted from the MGP site soil and incubated during 48 hours. White symbols refer

to controls without the laccase/ABTS couple, whereas black symbols correspond to the enzymatic incubation.

Concomitant measurements showed that laccase was totally inactivated in the presence of the organic soil extract within 24 hours of incubation. These results suggest that PAH concentration profiles in the reactor do not depend on the presence of the LMS, and that the hydrocarbons are not submitted to any enzymatic transformation. A hypothetic explanation could be related to physico-chemical phenomena. They may govern both the partitioning of PAHs between the liquid phase and the solid or colloidal particles co-extracted from the soil, and their solubilization by shaking in the presence of acetonitrile and surfactant.

Interference of soil contaminants with laccases

Several experiments were performed as an attempt to explain the apparent lack of activity of LMS against PAHs extracted from industrial soil.

First, the different oxidized forms of ABTS were assessed to identify the active species involved in the oxidation of BaP (Table 2). The present results confirm that the 2-step enzymatic oxidation of ABTS to $ABTS^{\bullet+}$, then to $ABTS^{++}$ (the most oxidized form of the mediator) is necessary to transform the synthetic PAHs.[15] Indeed, BaP is not oxidized by ABTS alone whilst a low transformation is observed in the presence of $ABTS^{\bullet+}$. With $ABTS^{++}$, the BaP transformation rate is comparable to that measured in the presence of ABTS and laccase. From the BaP transformation experiments performed in the presence of both laccase and ABTS, it can be noticed that the formation of the stable species $ABTS^{\bullet+}$ proceeded much more quickly than oxidation of BaP, demonstrating the poor reactivity of the hydrocarbon.

In a second set of experiments, BaP and the LMS were incubated in the presence of soil extract (Table 2). Hydrocarbon oxidation was drastically inhibited, in spite of a high $ABTS^{\bullet+}$ formation rate. $ABTS^{\bullet+}$, but above all $ABTS^{++}$, are reduced during incubations in the presence of soil extracts. Some contaminants, such as cyanides and metals,[24] are present in industrial soils and are co-extracted with PAHs and organo-mineral particles during the Soxhlet extraction procedure. They could interfere in the oxido-reduction processes responsible for PAH degradation. The concentrations of six metal species (Fe, Al, Zn, Pb, Cu, Mg) present in the soil extract were measured in the incubation

media. Overall, they amounted to 1.0 mg L^{-1}, with 0.6 mg L^{-1} Fe and 0.24 mg L^{-1} Cu.

TABLE 2 Effects of incubation conditions on benzo[a]pyrene (BaP) oxidation and on ABTS fate.

Incubation conditions	Final metal amount	BaP oxidized	$ABTS^{\bullet+}$ formed	$ABTS^{\bullet+}$ reduced
	mg L^{-1}	nmol h^{-1} mL^{-1}		
ABTS + BaP + 1U laccase	0	9.2	>20.0 10^3	/
ABTS + BaP	0	<0.1	0.2	/
$ABTS^{\bullet+}$ + BaP	0	0.2	1.1*	n.d.
$ABTS^{2+}$ + BaP	0	2.7	n.d.	/
ABTS + BaP + soil extract + 1U laccase	1	<0.1	>20.0 10^3	/
$ABTS^{\bullet+}$ +soil extract	1	/	0	7.8
$ABTS^{2+}$ + soil extract	1	/	13.0	/
$ABTS^{\bullet+}$ +Fe^{++}	1	/	/	6.5 10^3
$ABTS^{\bullet+}$ +Cu^{++}	1	/	/	23

*$ABTS^{\bullet+}$ formed through residual activity of the laccases used for $ABTS^{\bullet+}$ preparation
n.d.: not detected

An efficient reduction of $ABTS^{\bullet+}$ to ABTS was obtained in the presence of both Fe^{++} and Cu^{++} sulfate solutions. As the potential of $ABTS^{++}$ is higher than the potential of $ABTS^{\bullet+}$ (respectively 1.1 V and 0.69 V versus NHE),[25] $ABTS^{++}$ is thus likely to be reduced in the same experimental conditions. The metallic salts concentrations used in this experiment, about 1 mg mL^{-1}, were higher than these measured in soil solutions, but representative of the concentrations found in our polluted soil (more than 1g kg^{-1}).

Another series of experiments was conducted to check the effect of ferrous salts on the oxidation of BaP by $ABTS^{++}$. As cyanide has been found in the contaminated soil used here and is known to form very stable complexes with ferrous ions, we chose to test BaP oxidation in the presence of $K_4Fe(CN)_6$ solutions. $ABTS^{++}$ (0.1 mM) was formed from ABTS oxidation with a 5 mM potassium persulfate solution before the addition of the BaP stock solution. A control experiment

showed that a slight direct oxidation of BaP took place in the presence of the same concentration of $K_2S_2O_8$ alone, after 4 hours of incubation. The cyanide concentration range (less than 0.4 mM) used in these experiments remained lower than this required to induce any laccase inhibitory effect. Indeed, we checked that laccase inhibition was evidenced for cyanide concentrations above 1 mM.

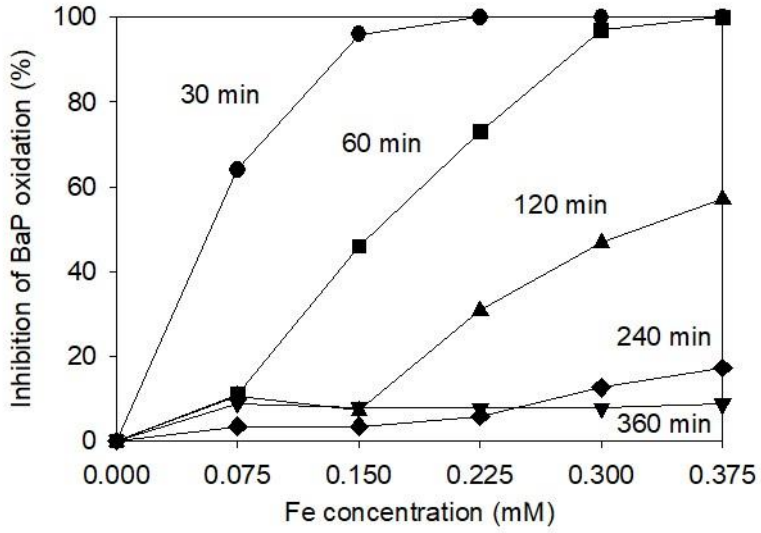

FIGURE 3 Effect of $K_4Fe(CN)_6$ on benzo[a]pyrene (BaP) oxidation by $ABTS^{++}$ after different periods of incubations.

Figure 3 shows the effect of $K_4Fe(CN)_6$ concentration for different incubation times: after 30 minutes, a straight inhibition of BaP oxidation was observed in presence of the inorganic complex, which increased with its concentration. When the complex was present in excess, compared to the ABTS concentration (0.1 mM), allowing a complete reduction of $ABTS^{++}$, BaP oxidation was completely inhibited. However, it seemed that this phenomenon was kinetically controlled since BaP oxidation was found to proceed even at high $K_4Fe(CN)_6$ concentrations, providing the incubation time was long enough. The lag phase before the beginning of BaP oxidation was observed only for the highest ferrocyanide concentrations, showing again that the inhibition is most efficient at high $K_4Fe(CN)_6$ concentrations.

DISCUSSION

For many years, fungal laccases have been studied for their ability to degrade individual PAHs *in vitro*.[6,7,9,14,16,26,27,28] Nevertheless, enzymatic transformation of mixtures of PAHs has been rarely reported (see for example [29]).

Our paper clearly shows that the laccase/ABTS couple oxidizes most of the hydrocarbons present in a mixture of 16 commercial compounds in a water/acetonitrile incubation system, without any correlation between molecular size and degradation extent. The main metabolites formed are typically quinones. PAH losses may also occur, due to a slight evaporation of 2- and 3-ring chemicals, or because of some polymerization reactions, as described.[28] The authors reported the formation of weight-average molecular weight compounds of approximately 1500 Da. In our experiments, we observed some brown compounds, not soluble in water or organic solvents, which are retained by filtration of aliquots of the incubation medium on glass-fiber filters.

The extent of the transformation of the PAHs by fungal oxidases, *i.e.* lignin peroxidases and laccases, has been sometimes expressed as a function of ionization potentials (IP) values of the hydrocarbons.[30] In the case of mixtures of PAHs with a wide range of concentrations, it was no possible establish such a relationship.

Even if direct oxidation of ANT and BaP by laccase alone has been reported, [7,29] the transformation of PAHs remains enhanced by the presence of redox mediators. Two synthetic redox mediators, ABTS and HBT, are commonly used for laccase-mediated transformation of PAHs. ABTS is successively oxidized to the cation radical ($ABTS^{\bullet+}$) and then to the dication ($ABTS^{++}$). The active form of HBT is the radical $HBT^{\bullet}$. The oxidized forms of ABTS are relatively stable and electrochemically reversible, whereas $HBT^{\bullet}$ is not stable and decays to a non-reducible compound.[25] In the case of ABTS, the dication was shown to be the intermediate responsible for the oxidation of non-phenolic compounds, whereas the cation radical reacts only with phenolic structures.[25] Our results confirm that $ABTS^{\bullet+}$ itself cannot oxidize BaP. Others groups [7,31] obtained similar results in the presence of ABTS with anthracene and veratryl alcohol as substrates. The active species towards PAHs is likely $ABTS^{++}$, which is the most oxidized form of ABTS.

In most *in vitro* studies, the concentrations of mediator required for an efficient laccase-mediated oxidation of PAHs, which are non

phenolic compounds, are higher than substrate concentrations. Such a result can be explained on the basis of some thermodynamical data. The oxidation of PAHs, whose normal potentials range between 1.3 V up to 1.8 V versus NHE [28] takes place only in presence of the most oxidized form of ABTS ($ABTS^{++}$), oxidized from ABTS in the presence of laccase. However, oxido-reduction titration of laccase from *Trametes versicolor* have shown that the redox potential of the type-1 Cu(II) site of the enzyme is around 790 mV versus NHE,[32] whereas the redox potential of the couple $ABTS^{\bullet+}/ABTS^{++}$ is E_0=1.1 V. In theory, the redox potential of laccase is therefore not high enough to allow the oxidation of ABTS to $ABTS^{++}$, unless the oxidation should be driven by a high concentration of ABTS. Indeed, according to the Nernst's equation, which accounts for the experimental concentrations, it can be shown that the equilibrium potential in solution is lowered in the presence of a large excess of the reducer of a redox couple, allowing the slow oxidation of ABTS to $ABTS^{++}$. $ABTS^{++}$ further reacts to oxidize PAHs, the reaction being driven by the irreversible formation of the corresponding diones. However, biotransformation of a synthetic mixture of PAHs in the presence of the LMS remains slow and moderate.

When extracted from a MGP site soil, the mixture of the same PAHs remains recalcitrant to the LMS in the same experimental conditions. Several hypotheses can be proposed to explain such a deceiving result. First, biotransformation can be restricted by pollutant bioavailability, as reported in soils.[11,12,13] A similar mechanism can be involved in liquid media, because PAHs adsorb on organo-mineral particles co-extracted from the soil. Previous studies with laccase have shown that the addition of commercial humic acid inhibits BaP oxidation in liquid media, as a function of acid concentration (data not shown). In an attempt to overcome this limitation and to enhance PAH bioavailability in the aqueous media, we supplemented the incubation media with Tween 20 and acetonitrile. However, the presence and the nature of any surfactant may influence PAH transformation. It has been reported that polymeric compounds were formed in the presence of Tween 80, whereas diones accumulated in the absence of the surfactant.[28] Others also found that Tween 80 was more efficient than Tween 20 for PAH oxidation because the reactions can be associated with lipid peroxidation and Tween 80 contains essential unsaturated fatty acid esters.[26] By contrast, high amounts of metabolites have been obtained in the presence of Tween 20 in our study. Acetonitrile was

used here to increase PAH solubility. Besides this favorable effect, a significant drawback of adding a solvent to the incubation medium is the decrease of activity of the enzyme. Indeed, laccase was significantly inactivated after 24-hour incubations in synthetic medium, with a more pronounced effect in the presence of the soil extract. An interesting improvement was proposed to preserve enzyme integrity by entrapment of the enzyme in a solvent-resistant hydrophilic matrix.[33] Laccase activity could be maintained several days in organic solvents in such conditions.

The transformation of PAHs by the laccase/mediator system involves redox reactions. Many compounds present in large amounts in the soil from the MGP sites (organic compounds, cyanides, metals,[12]) can interfere with these chemical reactions and so explain the lack of biotransformation observed in the presence of soil extracts. As an example, indirect effect of Mn^{++} in the presence of organic acids or unsaturated lignin subunit analogs such as 4-methyl-O-isoeugenol are reported.[21] We have shown that some inhibitors (*i.e.* metal ions) are Soxhlet-extracted and recovered in the organic extract constituting the incubation medium. We assayed a range of metals for their ability to inhibit PAH transformation. Among the metal species detected in the contaminated soil, divalent iron was found to be very efficient in reducing $ABTS^{++}$ and $ABTS^{\bullet+}$. Because of the favorable redox potential difference (E_0 $K_3Fe(CN)_6/K_4Fe(CN)_6$ = 0.356 V), the reduction of the dication $ABTS^{++}$ proceed more quickly *via* Fe oxidation than PAH oxidation.

The absence of PAH transformation in the presence of soil extract is the result of additive unfavorable effects. First, it is likely that PAHs are adsorbed onto organo-mineral particles. Second, $ABTS^{++}$ formed by laccase catalysis is preferentially reduced by co-extracted metal species. Third, the dication precipitates because of its low solubility, thus being unavailable for later oxido-reduction reactions. When conditions become more favorable (Fe^{++} fully oxidized and PAHs released in solution after several hours of incubation) laccase has been denaturized by acetonitrile and is no more able to form $ABTS^{++}$.

In conclusion, we have shown that laccases are able to transform several PAHs in a synthetic mixture. The reaction requires the presence of a redox mediator. Our results also establish that the oxidizing system is without any effect on PAHs in media more related to environmental complexity. Consequently, the use of laccases to

degrade environmental pollutants needs to discover natural or synthetic mediators much more efficient at low concentrations, or to increase (by genetic engineering) the ionization potential of the enzyme, thus avoiding the use of a mediator.

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