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BENEFITS OF ECOTOXICOLOGICAL BIOASSAYS IN THE EVALUATION OF A FIELD BIOTREATMENT OF PAHs POLLUTED SOIL

C. LORS[1,2,*]
F. PERIE[3]
C. GRAND[4]
D. DAMIDOT[1]

[1]*Civil and Environmental Department, Ecole des Mines de Douai*
941 rue Charles Bourseul B.P. 10838, 59508 Douai Cedex, France
[2]*National Center on Polluted Sites and Soils*
930 boulevard Lahure BP 537, 59505 Douai, France
[3]*TOTAL Petrochemicals, R. N. 117 B.P. 34, 64170 Lacq, France*
[4]*ADEME, 2, square la Fayette, 49004 Angers, France*

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*to whom all correspondence should be addressed:
e-mail: lors@ensm-douai.fr

ABSTRACT
The performance of a biological treatment of a PAH-contaminated soil was evaluated with respect to its physicochemical and ecotoxicological properties. After six months, the biological treatment led to an significant reduction of 2- and 3-ring PAHs and to a lesser extent to 4-ring PAHs. As a consequence a significant decrease of the acute ecotoxicity was observed passing from highly ecotoxic before treatment to non-ecotoxic according to *Lactuca sativa* seedling and growth inhibition test and *Eisenia fetida* mortality test. This could be related to the bioavailability of PAHs. Indeed, tests performed on aqueous leachates of the soil showed a strong decrease of 2- and 3-ring PAHs correlated with a significant reduction of acute and chronic ecotoxicity responses. The biological treatment led to the mutagenicity reduction and the genotoxicity disappearance in the leachate. Thus, bioassays are complementary to chemical analyses to evaluate the efficiency of a bioremediation process and to evaluate the bioavailability of the organic pollutants as the total concentration of a contaminant is not the only criterion to consider. The comparison of the ecotoxic responses allowed us to underline the best sensitivity of the earthworm, Microtox, Alga and Ames bioassays among the tested set. These bioassays could thus be good candidates to build a toxicity evaluation procedure for PAHs contaminated/ remediated soils.

KEYWORDS: PAHs, biotreatment, polluted soil, aqueous leachates, bioassays, ecotoxicity, genotoxicity chemical analyses.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic aromatic compounds containing two or more fused phenyl and/or pentacyclic rings in linear, angular or cluster arrangements (Cerniglia, 1992). These compounds are frequently encountered in contaminated industrial soils as a result of incomplete combustion of fossil fuels, coal liquefaction and gasification, creosote production and petroleum refining (Cerniglia, 1992). Because some of them are toxic, mutagenic and carcinogenic, they represent an important environmental concern (White and Claxton, 2004).

Microbial degradation of PAHs has been widely reported and offers an attractive approach to the removal of these compounds from contaminated sites (Bosser and Compeau, 1995; Wilson and Jones, 1993). These bioremediation processes require microorganisms able to degrade PAHs and soil conditions favourable to biodegradation of these contaminants. PAHs degradation in soil is affected by environmental factors (moisture, pH, temperature, O_2 level and PAHs bioavailability) and metabolic constraints of microorganisms such as microbial nutrient requirements and acclimation of the microbial population (Providenti *et al.*, 1993). Some of these factors can be modified to enhance PAHs biodegradation rate.

Bioremediation process is often monitored by following the reduction of contaminant concentrations. Generally, this chemical approach does not allow to identify all the compounds, but only to quantify those which are analysed. The latter are most often related to the initial contaminant and in the case of PAHs to the recommended list of 16 PAHs (US-EPA) (Keith and Telliard, 1979). Moreover, in the case of PAHs, an incomplete biodegradation during bioremediation can lead to the formation of toxic intermediary metabolites, which could increase the soil toxicity (Haeseler *et al.*, 2001). Thus, chemical analysis does not provide information on the bioavailability of the pollutants, neither on synergic or antagonistic phenomena between pollutants, nor on their effect on living organisms. So, chemical analyses alone are not sufficient for biological assessment (Bispo *et al.*, 1999). Alternative methods, which consist in exposing organisms to contaminated samples to measure acute and/or chronic toxicity, can give a better picture of contaminant bioavailability and uptake by test organisms (Bispo *et al*., 1999; Harley and Young, 2000). The ecotoxicological approach, which includes the impact of all the pollutants present in the contaminated soil, can be considered as a necessary complement to chemical analysis in the evaluation of the danger associated with polluted soils. In practice, the comparison of separate sets of chemical and ecotoxicological data may lead to confusing and even contradictory interpretations. This demonstrates the interest to couple (eco)toxicity bioassays with chemical analyses to appreciate the effectiveness of a biological treatment and to evaluate the bioavailability of the organic pollutants, such as the PAHs (Phillips *et al.,* 2000).

This study aimed at further demonstrating the benefits of ecotoxicological bioassays coupled to chemical analysis in the evaluation of the effectiveness of a field biotreatment of PAHs polluted soi**l,** in the particular case of windrow treatment. An additional objective of the study was to compare the sensitivity of bioassays representing different trophic and toxicity levels carried out directly on the soil or on their aqueous leachates, in order to define an optimized procedure.

2. MATERIAL AND METHODS

A windrow treatment was carried out on an industrial site located in the north of France, whose main activity was the distilling of coal tar.

The contaminated soil was sieved and the fraction below 6 mm was mixed with wood shavings in a volumetric ratio of 0.7:0.3. Wood shavings, which had a size about 5-6 mm, enabled to give a specific microstructure for the soil inducing a good venting of the medium. This material was put in place as a windrow of $5x10^3$ tons (Length = 90 m, Width = 5 m, Height = 2.2 m). The solid matrix was turned periodically (once a week during the third months and two times per month during the next months) by a machine. Nitrogenous (agricultural urea) and phosphoric (agricultural super phosphate) nutrients were added at the beginning of the treatment, in order to bring nutrients to allow the growth of the microbiota. Moisture, which was about 17%, was maintained constant by periodic water sprinklings all over of the windrow.

The solid matrix was collected immediately after amending the soil with nutrients at the beginning of biotreatment. Another solid matrix was collected after six months to monitor the biotreatment through physico-chemical and microbiological analyses. All solid matrixes were made by homogenizing five samples corresponding to five locations randomly chosen on the windrow. The solid matrix was initially sieved and the fraction below 4 mm was recovered. This allowed us to remove the wood shafts, in order to only analyze the soil without modifying the results. Indeed, the chemical analyses of the total solid matrix and its fraction below 4 mm gave similar PAHs concentration.

The method used for soil leaching was carried out in accordance with the ISO 21268-2 (2006) reference protocol, without any preliminary filtration. Soil leaching experiments were carried out with a liquid:solid ratio of 10:1 (170 g dry of soil in 1.7 L of distilled water) at 20°C in 2-liter glass flasks for 24 hours on a stirring rate of 60 rpm. After decantation of 15 min, the aqueous phase was centrifuged at 2000 g during 30 min.

The chemical and microbiological analyses were carried out on the recovered leachates.

Soils physico-chemical parameters were measured: pH, moisture content, 16 PAHs (US-EPA), total organic carbon, total organic nitrogen, heavy metals and total cyanides. Turbidity,

pH, dissolved organic carbon, cyanides and 16 PAHs were measured on the leachates obtained after aqueous leaching of the same soils. The pH of soil or leachate samples was measured using a pH-meter (Consort C891, Consort, Turnhout, Belgium) fitted with combined electrode with temperature correction. Soil pH was determined according to the NF ISO 10390 (1994) protocol. Turbidity of leachates was carried out in accordance with the NF EN ISO 7027 method (2000). Total organic carbon of soil and leachate samples was deduced from total carbon and inorganic carbon concentrations, which were determined by a TOC-5000A TOC meter (Shimadzu Corporation, Kyoto, Japan), in accordance with the NF ISO 10694 (1995) protocol for the soil samples. Total organic nitrogen of soils was determined by Kjeldahl method (NF ISO 11261, 1995). Heavy metals (As, Cd, Cr, Cu, Pb, Zn) of soil and leachate samples were analysed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (JY 138 Ultrace, Jobin Yvon, France). For the soil samples, a hot digestion of the solid phase was carried out with hydrofluoric (HF) and perchloric ($HClO_4$) acids (ISO 14869-1, 2001). Total cyanides of soils and leachates were determined according to the ISO 17380 (2004) and ISO 14403 (2002) respectively. After extraction of soil samples by dichloromethane/acetone (50/50, v/v) using an accelerated extractor system (ASE 200, DIONEX Corporation, Sunnyvale, USA), PAH analysis was carried out by High Performance Liquid Chromatography (HPLC Waters 2690, Waters, Milford, USA) fitted with a column (Supelco, C 18 reverse phase, length 250 mm, internal diameter 2.1 mm), coupled to a UV photodiode array detector (Water 996) for soils and to fluorescence detection for leachates (NF ISO 13877, 1999).

The ecotoxicity of soils was assessed by direct contact of the selected organisms to soils in solid phase bioassays: tests of inhibition of the seedling and the growth of lettuce (*Lactuca sativa*) and test of earthworms (*Eisenia fetida*) survival. These bioassays were carried out according to ISO 11269-2 (1995) and ISO 11268-1 (1994) respectively.

Ecotoxity was also evaluated on leachates using liquid phase bioassays. The set of bioassays included tests of acute ecotoxicity (Microtox (ISO 11348-3, 1998) and *Daphnia magna* tests (NF T90-301, 1996)), tests of chronic ecotoxicity (inhibition of the growth of *Pseudokirchneriella subcapitata* (NF T90-375, 1998) and *Brachionus calyciflorus* (NF T90-377, 2000)) and tests of genotoxicity (Fluctuation Ames' test (NF T03-351, 1982) and test of the micronucleus applied to mouse lymphoma cells).

3. RESULTS AND DISCUSSION

3.1 Evolution of soil characteristics during the biotreatment

Untreated soil and soil after six months of biotreatment (called Ti and Tf soils respectively) had a comparable moisture content, about 17% (Table 1). This value indicated the maintenance of moisture at a constant rate during biotreatment.

The pH was around 8 for both Ti and Tf soils. The C:N ratio, which expresses nutritional potentialities of the soil for an optimal biodegradation of PAHs, was determined from contents in total organic nutrient (T.O.N.) and in total organic carbon (T.O.C.). According to Blaine Metting (1992), soils presenting a C:N ratio in the order of 60 offer satisfactory nutritional qualities to ensure optimal PAHs' biodegradation. On this basis, Ti soil, which had a C:N of 56, showed favourable nutritional conditions for optimal biodegradation. It seemed that nutritive conditions of soil at the beginning of biotreatment were favourable to an optimal biodegradation. On the contrary, after six months of biotreatment, the decrease of C:N ratio showed that the conditions were less favourable to the PAHs-biodegradation than at the beginning of biotreatment.

Heavy metals were present in very low contents in the Ti soil, with concentrations similar to the local geochemical background. Ti soil contained a total cyanide concentration always below 1 mg kg^{-1} dry soil (Table 1). Thus, Ti soil was mainly contaminated by organic compounds with 16 PAHs concentration close to 3000 mg kg^{-1} of dry soil (Table 1). It contained mainly 2-, 3- and 4-ring PAHs (Figure 1). 3-ring PAHs were the most concentrated: 44% of the 16 PAHs concentration. 4-ring PAHs represented a smaller proportion (28%). Concentration of 2-ring PAH (naphthalene) was of 20% of the 16 PAHs concentration. 5- and 6-ring PAHs were found at very low contents, 5 and 2% of the 16 PAHs concentration respectively.

Table 1. Physicochemical characteristics of Ti and Tf soils

Soil	pH	C:N	moisture	T.C.	I.C.	T.O.C.	T.O.N.	16 PAHs	CN^-
			% of initial mass				mg kg^{-1} of dry soil		
Ti soil	7.9	56	17.4	11.4	2.2	9.0	1611	2894 ± 54	0.8
	± 0.02		± 0.1	± 0.6	± 0.1				
Tf soil	8.3	26	16.3	8.6	2.9	5.6	2088	801 ± 2	0.5
	± 0.01		± 0.3	± 0.2	± 0.1				

Total carbon (T.C), total organic carbon (T.O.C.), inorganic carbon (I.C.), total organic nitrogen (T.O.N.)

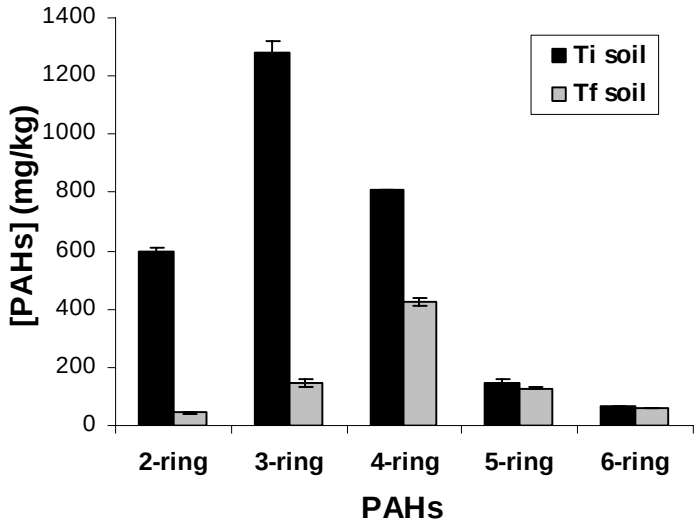

Figure 1. 2-, 3-, 4-, 5-, 6-ring PAHs concentrations (mg kg^{-1} of dry soil) in soil before (Ti) and after (Tf) biotreatment

Six months of biotreatment led to a reduction of 72% of the content of 16 PAHs: [16 PAHs] = 2894 and 801 mg kg^{-1} of dry soil respectively for Ti and Tf soils.

This was mainly related to the decrease of 2-, 3- and 4-ring PAHs concentrations (92%, 89% and 48% respectively) (Figure 1). 5-ring PAHs showed a slight decrease in their content (14%), whereas the content of 6-ring PAHs remained almost constant. Sayles *et al.* (1999) and Mendonça and Picado (2002), who followed a land treatment of PAH-contaminated soil, obtained similar results. This is in agreement with the results of different studies pointing out that biological processes only reduce low molecular weight PAHs (Bossert *et al.*, 1984; Cerniglia, 1992). Neverthelss, as the weak degradation of 5- and 6-ring PAHs, represented an important environmental problem, their removal must be achieved by combined strategies. In our case, a phytotreatment was carried out subsequently to the biotreatment on Tf soil, in order to eliminate the high molecule weight PAHs.

Ecotoxicological results showed that the untreated soil (Ti soil) caused a high level of inhibition on the plant germination and growth (respectively EC_{50} = 29.2 and 16.7 %, LOEC = 5%), whereas the treated soil (Tf soil) displayed no phytotoxicity on *Lactuca sativa* (Table 2). Moreover, Ti soil also exhibited a strong ecotoxicity on the population of earthworms, and caused lethal effects on all the population at the dose of 1%. On the contrary, Tf soil showed no adverse effects and no mortality underlined for the range of tested dose (0.35 to 100%).

The earthworm bioassay was more sensitive than lettuce seedling and growth bioassays. The same response was found by Dorn and Salanitro (2000), which showed that *Eisenia* was the most sensitive of the three utilized methods (earthworm, plant seed germination and growth and modified Microbics Microtox Solid-Phase assays) for a soil contaminated by BTEX.

Table 2. Ecotoxicity of soil before (Ti) and after (Tf) biotreatment

Bioassays	Toxicity measure				Relative response [b]	
	Ti soil		Tf soil		Ti soil	Tf soil
	$E(L)C_{50}$	LOEC	$E(L)C_{50}$	LOEC	inhibition	
	g / 100 g tested soil (%)				%	
Lettuce seedling	29.2 (8.5-88.2)[a]	5	NT	NT	71	0
Lettuce growth	16.7	5	NT	NT	79	0
Earthworm survival	0.6 (0.5-0.6)[a]	0.6	NT	NT	100	0

EC_{50} or EL_{50}: concentration causing 50% inhibition of measured response, LOEC: lowest observed adverse effect concentration, NT (non toxic): observed response statistically indistinguishable from unpolluted soil sampled on the studied site, [a]95% confidence limits, [b]Relative response: inhibition (%) of response at 80 and 100% studied soil respectively for lettuce and earthworm bioassays

3.2. Evolution of leachate characteristics during biotreatment

Results of physico-chemical analyses of leachates issued from Ti and Tf soils (named respectively Ti and Tf leachates) are given in Table 3.

Table 3. Physicochemical characteristics of Ti and Tf leachates

Leachate	pH	turbidity	D.O.C.	CN^-	16 PAHs
			mg L^{-1}		µg L^{-1}
Ti leachate	8.0	28	113.0 ± 7.5	< 0.01	644.7 ± 20.6
Tf leachate	7.9	37	27.4 ± 8.3	< 0.01	99.7 ± 6.2

Ti and Tf leachates had pH values around to 8 that are compatible with the domain of validity of ecotoxicity bioassays. Thus, bioassays were carried out directly without any pH correction. They had a similar turbidity: 28 and 38 respectively for Ti and Tf leachates. Organic carbon content of Tf leachate was lower than that of Ti leachate (27.4 and 113 mg L^{-1} respectively). The organic carbon content of Tf leachate was linked to the weak content of total organic content of Tf soil (c = 5.6 mg kg^{-1} dry soil). Heavy metals and cyanides were not detected in the two leachates.

Ti leachate mainly contained 3-ring PAHs, which represented 82% of the 16 PAHs concentration (Figure 2). Their high quantity in solution was linked to their high concentration in Ti soil (44% of the 16 PAHs content). 4-ring PAHs were also present but in smaller proportion (14%), whereas 5- and 6-ring PAHs were less concentrated (3 and 0.8% respectively).

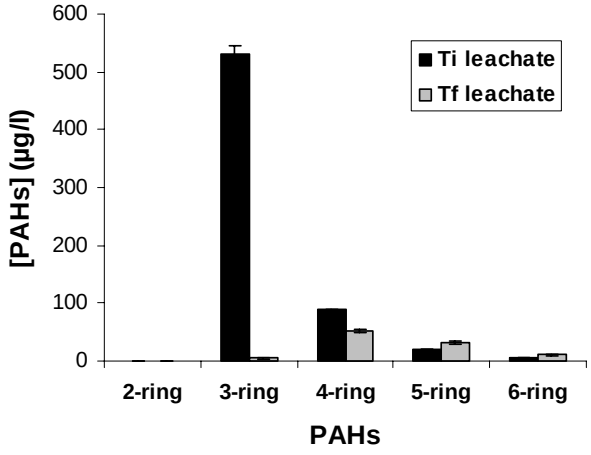

Figure 2. 2-, 3-, 4-, 5-, 6-ring PAHs concentrations in leachates of soil samples before (Ti) and after (Tf) biotreatment

The 16 PAHs concentration of Tf leachate was reduced after six months of biotreatment: 99.7 mg l^{-1} for Tf leachate against 644.7 mg l^{-1} for Ti leachate (Table 3). These results showed that the biotreatment allowed an considerable reduction (85%) of the 16 PAHs concentration leached by water. This reduction mainly concerned 3-ring PAHs, with a very strong reduction in their content in the leachate (99%). 4-ring PAHs also took part in the reduction observed, but in a more moderately (41%). On the other hand, a light increase of the 5-ring and 6-ring PAHs contents was observed, probably due the maturation of the soil and to co-solvatation phenomenon. Surprisingly, naphthalene, the only 2-ring PAH, was detected in very small proportion (c = 0,2 µg l^{-1}) especially in the leachate of Ti soil despite high amounts in this soil. This fact may be due to volatilization of naphthalene during the recovering of the leachate that consisted in several steps as explained previously. The decrease of PAHs bioavailable is higher than the total PAHs concentration decrease. This is a consequence of the higher solubility of lower molecular weight PAHs that are more degraded than the other PAHs.

Ti leachate exhibited a strong acute ecotoxicity response with respect to *V. fischeri* (EC_{50} = 8.1%) (Table 4). A significant effect on the *D. magna* mobility was observed, but it was more moderate than the effect observed on *V. fischeri* (CE_{50} = 62%). On the contrary, Tf leachate presented weak or no ecotoxicity on *V. fisheri* and *D. magna*. The fact that acute ecotoxicity decreased in the same proportion as the 3-ring PAHs content tends to indicate that acute ecotoxicity could be associated to the lightest molecular weight PAHs that have a higher solubility and thus a greater bioavaibility.

Ti leachate also showed a significant inhibition of the growth of *P. subcapitata* and *B. calyciflorus*. However, *P. subcapitata* was more sensitive than *B. calyciflorus* (CE_{50} = 43.8 and 78% respectively) (Table 4). After six months of biotreatment, the chronical ecotoxicity exhibited a significant reduction (CE_{50} = 43.8% against 100% (Alga test); CE_{50} = 78% against non toxic (NT) (*Brachionus* test) between the beginning and the end of biotreatment), but to a lesser extent than acute ecotoxicity.

So, *V. fischeri* and *P. subcapitata* were the most sensitive species to this type of contamination. These results are corroborated by those obtained by Mendonça and Picado (2002), who had ecotoxicologically monitored a landfarming process of a coke oven soil.

Table 4. Ecotoxicity of the leachates of soil samples before (Ti) and after (Tf) biotreatment

Tests	Ti leachate		Tf leachate	
	EC_{50}	Inh.[b]	EC_{50}	Inh.[b]
	%			
Microtox (*V. fischeri*)	8.1 (7.2-9.3)[a]	89.1	> 100	26.7
Daphnia (*D. magna*)	62	100	NT	0
Alga (*P.subcapitata*)	43.8 (32.8-57.3)[a]	93.4	>100 (72.6->100)[a]	43.2
Brachionus (*B. calyciflorus*)	78 (49->100)[a]	57.7	NT	0

EC_{50}: concentration causing 50% inhibition of measured response, LOEC: lowest observed adverse effect concentration, NT: non toxic, [a]95% confidence limits, [b]inhibition (%) of response at the tested highest dose

Ti leachate also induced a significant increase of the number of micronucleus on the mouse lymphoma cells without metabolic activator (LOEC = 25%). However, after six months of biotreatment, the leachate became non-genotoxic (Table 5). The observed genotoxicity of Ti leachate is thus not due to PAHs but certainly to other compounds that are degraded during the biotreatment.

Ti leachate presented a high mutagenic activity with and without metabolic activator for the two tested strains (TA100 and TA98). So, this leachate presented direct and indirect mutagenicity on *S. enterica.* This suggests the occurrence of pollutants with an indirect

mutagenicity, like specific PAHs and pollutants with a direct mutagenicity. Indeed, PAHs are known to be activated into mutagenic metabolites by the S9 rat liver enzymes.

After six months of biotreatment, a significant reduction of direct and indirect mutagenicity on *S. enterica* was also observed on the leachate. However, mutagenicity remained after biotreatment although more moderated. This could be attributed to the remaining PAHs but also to other compounds, such as metabolites formed during the biotreatment. Similar results were obtained with a land treated soil which displayed residual genotoxicity and mutagenicity in the extract assays (Sayles *et al.*, 1999). This observation suggests that these biotreatments alone are not sufficient to eliminate all sources of toxicity and that complementary treatments are necessary to totally reduce the environmental risk.

Despite the increase of the leached concentrations of 5- and 6-ring PAHs after the biotreatment (Figure 2), the leachate presented any genotoxicity with respect to the tested organisms. Moreover, it had a lower indirect mutagenicity. This fact indicated that these heavy PAHs were not very bioavailable for the tested organisms. They were probably fixed on the suspended matter or/and colloids certainly due to their low solubility.

Results obtained with these two bioassays showed that the fluctuation Ames test was more sensitive than micronucleus test. Moreover, the fluctuation method of the Ames test showed a better sensitivity than Ames test using agar solid plates because bacterial exposure was carried out in a hydric medium instead of agar in which bioavailability of pollutants is reduced (Bekaert *et al.*, 1999 ; Eom *et al.*, 2007).

Table 5. Genotoxicity of the leachates of soil samples before (Ti) and after (Tf) biotreatment

	Ti leachate		**Tf leachate**	
Tests	LOEC (%)			
Ames (*S. enterica*)	$S9^-$	$S9^+$	$S9^-$	$S9^+$
TA 98	0.014	0.014	0.10	0.038
TA 100	0.038	0.014	0.10	0.038
Micronucleus (*m.l.* cells)	25	NT	NT	NT

LOEC: lowest observed adverse effect concentration, NT: non toxic, m.l. cells: mouse lymphom cells, $S9^-$ / $S9^+$: absence / presence of liver activation fraction

4. CONCLUSION

The windrow treatment of soils strongly contaminated by PAHs led to a significant reduction in the content of 2-, 3- and 4-ring PAHs, as well as ecotoxicity estimated using bioassays in solid phase. The acute ecotoxicity, evaluated with respect to lettuce and earthworm, was significantly reduced, even completely removed after six months of biotreatment.

Biotreatment also allowed a decrease of the leached PAHs and the associated toxicity. A strong reduction in acute ecotoxicity and chronic ecotoxicity, as well as a significant reduction of the genotoxicity of the leachate was indeed observed. This study also highlighted a probable relationship between acute ecotoxicity and low-molecular weight PAHs that are more soluble.

The comparison of the ecotoxic responses allowed us to underline the best sensitivity of the earthworm, Microtox, Alga and Ames bioassays among the tested set. These bioassays could thus be good candidates to build a toxicity evaluation procedure for PAHs contaminated/remediated soils.

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