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Par

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ECOTOXICITE D'UN SOL DE COKERIE CONTAMINE PAR DES HYDROCARBURES AROMATIQUES POLYCYCLIQUES [HAP]

Devant un jury composé de :

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Laboratoire d'Ecotoxicite, Sante Environnementale (ESE)

ECOTOXICITY OF POLYCYCLIC AROMATIC HYDROCARBON (PAH)-CONTAMINATED SOIL FROM AN ANCIENT COKERY

Ecotoxicité d'un Sol de Cokerie Contaminé par Des Hydrocarbures Aromatiques Polycycliques [HAP]

A Dissertation

by

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To my Parents, Wife Yoon-kyung and Daughter So-mok

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LIST OF ABBREVIATIONS

AAS : Atomic Absorption Spectroscopy
ADEME : French Energy and Environment Agency
AFNOR : French Standards Association
ATSDR : Agency for Toxic Substances and Disease Registry
BaP : Benzo[a]pyrene
BAF : Biological Accumulation Factor
BCF :Biological Concentration Factor
BRGM : French Geological Survey
BSAF : Biota-Soil Accumulation Factor
CEC : Cation Exchange Capacity
CI : Confidence Interval
CNRS : French National Center for Scientific Research
DCM : Dichloromethane
ECx : x% Effective Concentration
EC SCF: European Commission, Scientific Committee on Food
EEA : European Environment Agency
EHC : Environmental Health Criteria
U.S. EPA : U. S. Environmental Protection Agency
GC/MS : Gas Chromatography / Mass Spectrometry
GISFI : French Scientific Interest Group-Industrial Wasteland
HPLC : High-Perfomance Liquid Chromatography
IARC : International Agency for Research on Cancer
IF : Induction Factor
INERIS : French Environment and Risk Institute
INRA : French National Institute for Agricultural Research
ISO : International Organization for Standardization
IRIS : Integrated Risk Information System
IPCS : International Programme on Chemical Safety
Koc : Organic Carbon Partition coefficient
Kow : Octanol-water coefficient
LC : Lethal Concentration
LOEC : Lowest Observed Effective Concentration
NOEC : No Observed Effective Concentration
OECD : Organization for Economic Cooperation and Development
PAH : Polycyclic Aromatic Hydrocarbon
QL : Quantification Limit
QSAR : Quantitative Structure-Activity Relationship
SCF : Shoot Concentration Factor
TEF : Toxic Equivalent Factor
TEQ : Toxic Equivalent Quantity
TOC : Total Organic Content
TU : Toxic Unit
WHC : Water Holding Capacity

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RESUME DE LA THESE (In French)

Ecotoxicité d'un Sol de Cokerie Contaminé par les Hydrocarbures Aromatiques Polycycliques [HAP])

(Ecotoxicity of a polycyclic aromatic hydrocarbon [PAH]-contaminated soil from an ancient cokery)

Les tableaux et figures (tirés du document anglais) illustrant ce résumé sont numérotés en chiffres romains ; les intitulés sont en français

1. Introduction

L'ampleur du problème posé par les sols et sites pollués dans les pays industrialisés est à l'origine de la mobilisation des instances gouvernementales et européennes, pour la gestion des sites contaminés et la prévention de nouvelles contaminations. La France a pris la dimension du problème dans les années 75-80 avec le premier inventaire des points noirs au niveau national. Mais c'est surtout à partir de 1992 et à l'initiative de l'ADEME[1] qu'ont été entrepris l'inventaire systématique par région des sites pollués et leur caractérisation. Le BRGM[2] s'est impliqué très vite dans le développement des approches pour l'évaluation des risques. En 1996, le CNRSSP[3] a été créé spécifiquement à l'initiative de l'Ecole des Mines de Douai et avec le soutien du Ministère de l'Environnement pour répondre aux besoins nationaux de recherche exprimés dans le cadre de la politique nationale de gestion, classification et réhabilitation des sites potentiellement pollués. L'INERIS[4], l'IRSN[5] et d'autres instituts et équipes de recherche se sont également investis dans l'étude des problèmes posés par les contaminations, notamment le comportement des polluants dans le sol et les nappes, la modélisation des transferts sol-eau, sol-plantes en relation avec la santé humaine et leur effets sur les écosystèmes.

Au niveau national, la production d'énergie et la sidérurgie viennent en tête des secteurs d'activités responsables de la contamination des sols, suivies de près par la pétrochimie et la chimie fine (EEA, 2005). Le chiffre de 100 000 sites contaminés était avancé en 1998, dont 20 000 à nettoyer (Lecomte, 1998). Le Nord-Pas de Calais, la Lorraine, la Région de Saint-Etienne, de par leur passé minier figurent au premier plan des régions concernées par ces questions. En Lorraine s'est créé un Groupement d'Intérêt Scientifique sur la problématique des friches industrielles (GIS F.I.) au sein duquel s'inscrit cette thèse sur un sol de cokerie (2002).

En 2005, l'Agence Européenne de l'Environnement (EEA) a dressé un bilan de la situation sur (i) les principaux secteurs d'activité contribuant à la pollution des sols dans les Etats Membres Européens, (ii) le coût de la décontamination à la charge des pouvoirs publics, (iii) les progrès réalisés par chaque Etat membre au cours de ces dernières années en terme de contrôle et de rémédiation de la contamination des sols, (iv) et les principales classes de polluants responsables de

[1] Agence de l'Environnement et de la Maîtrise de l'Energie
[2] Bureau de Recherches Géologiques et Minières
[3] Centre National de Recherche sur les Sites et Sols Pollués
[4] Institut National de l'Environnement industriel et des Risques
[5] Institut de Radioprotection et de Sureté Nucléaire

la contamination, et susceptibles de menacer la qualité des eaux souterraines et la santé humaine (www.eea.eu.int)

Les principales familles de contaminants recherchés dans les sols sont les métaux lourds, les hydrocarbures aromatiques, hydrocarbures halogénés, hydrocarbures polycycliques aromatiques (HAP), dioxines et furanes, polychlorobiphényles, phénols, pesticides,...(BRGM, 2001). La pollution par les métaux et métalloïdes est sans doute la plus étudiée, sinon la mieux connue, compte tenu des méthodes d'analyse performantes actuellement disponibles pour l'étude de ces contaminants à un coût raisonnable.
La pollution organique est plus difficile à appréhender, du fait de sa complexité aggravée encore par la présence de produits de (bio)dégradation probables.

Dans cette catégorie, la pollution des sols par les HAP figure parmi les sujets de préoccupation actuelle, parce que ce type de pollution est complexe, mal connu et que les congénères de poids moléculaire élevé sont classés cancérogènes pour l'Homme et les mammifères. Ces préoccupations expliquent les journées d'étude et ateliers organisés par le CNRSSP et l'ADEME en 2005 spécifiquement sur le sujet (www.cnrssp.org). Les questions posées concernent la mobilité des HAPs dans les sols via les eaux d'infiltration, leur biodisponibilité dans des sols anciennement pollués, et leur toxicité sur les organismes aquatiques et terrestres. Les réponses à ces questions sont nécessaires à la gestion des sites et à la prise de décision d'une réhabilitation. Les mêmes questions se posent d'ailleurs à la suite des traitements de rémédiation qui impliquent de vérifier l'efficacité de la dépollution au plan toxicologique et écotoxicologique.

Les HAPs constituent une vaste catégorie d'hydrocarbures à plusieurs cycles aromatiques accolés, générés lors de la combustion incomplète des matières carbonées par les activités anthropiques de manière générale, mais en particulier par les activités industrielles de type cokeries, pétrochimie, sidérurgie. Les premiers termes de la série sont très faiblement hydrosolubles, volatiles et biodégradables ; ces caractères s'estompent avec l'augmentation du poids moléculaire, notamment pour les molécules à plus de 4 noyaux aromatiques. Ce qui explique le caractère ubiquiste de la pollution par les HAP dans les secteurs fortement anthropisés. Leur rémanence dans l'environnement est d'autant plus préoccupante que les congénères à plus de 4 noyaux sont biotransformés chez les vertébrés en métabolites plus réactifs et plus toxiques que la molécule de départ. Les métabolites formés chez les organismes exposés sont responsables de la

mutagénicité et de la cancérogénicité des HAP. Ces effets ont été très étudiés chez les vertébrés, par contre la toxicité des HAP chez les invertébrés est peu connue.

2. Objectif de la thèse

La recherche concerne l'évaluation de l'écotoxicité d'échantillons de sol d'une ancienne cokerie en Lorraine et contaminés par les HAP. Il s'agit donc d'étudier un sol contaminé sur le terrain, et non un sol artificiel ou naturel dopé en laboratoire par les HAP.
Les objectifs sont de :

- **caractériser la nature et le degré de la contamination chimique du sol analysé ;**
- **étudier la mobilité à l'eau des 16 HAP dont le suivi est recommandé par l'US-EPA, la biodisponibilité, la toxicité à court et long terme des polluants vis-à-vis des organismes aquatiques et terrestres, et les relations doses-effets-temps ;**
- **identifier dans la mesure du possible la fraction de la pollution responsable de la toxicité enregistrée ;**
- **modéliser les transferts sol-plantes et sol-invertébrés des 16 HAP lors d'exposition à long terme.**

Les relations entre la contamination des sols, la contamination des lixiviats et leur toxicité ont été étudiées à l'aide de bioessais en laboratoire sur des organismes représentatifs des différents niveaux d'organisation biologique des milieux aquatiques. La fraction de la pollution mobilisable par l'eau susceptible d'affecter la vie aquatique par des effets de toxicité aiguë, chronique ou des effets mutagènes a été évaluée à l'aide des modèles classiques :

- pour la toxicité à court terme : bactéries luminescentes *Vibrio fischeri* (test Microtox), et microcrustacés *Daphnia magna* (inhibition de la mobilité à 24 et 48h),
- pour la toxicité à moyen terme : *Ceriodaphnia dubia* (inhibition de la reproduction sur 7 jours), et microalgues *Pseudokirchneriella subcapitata* (inhibition de la croissance des suspensions algales sur 72h)
- pour la mutagénicité : mutants bactériens *Salmonella typhimurium* (test d'Ames et test Umu) et mutants non luminescents *Vibrio fischeri* (test Mutatox)

La biodisponibilité des polluants du sol sur les espèces terrestres a été évaluée par la mesure de la toxicité à moyen et long terme des échantillons de sol sur :

- les plantes supérieures (germination et croissance) : *Avena sativa* (avoine), *Brassica chinensis* (chou chinois), *Lactucca sativa* (laitue)
- les collemboles *Folsomia candida* (survie et reproduction)
- et les vers de terre *Eisenia fetida* (survie des adultes et des juvéniles, et reproduction).

Le transfert des HAP du sol aux végétaux et aux invertébrés a été quantifié après exposition prolongée de 28 jours des plantes et de 24 semaines des populations de vers de terre générées à la suite des essais de reproduction.

Les relations doses-effets ont été étudiées en testant le sol contaminé à différentes concentrations obtenues par mélange avec un sol artificiel standard. La discussion engagée au niveau international sur la représentativité des sols artificiels et sur l'intérêt qu'il y aurait à utiliser des sols naturels dans les essais, nous a conduits à étudier ce paramètre et déterminer s'il pouvait influencer significativement les résultats d'écotoxicité terrestre. Une comparaison a donc été faite entre des essais utilisant comme contrôle un sol artificiel et une terre végétale provenant d'un sol de prairie vierge de tout traitement chimique, de type argilo-limoneux.

3. Présentation du document

Le mémoire de thèse comporte 5 grandes parties à la suite de l'introduction classique :

- **Etude bibliographique** portant sur les propriétés physicochimiques et (éco)toxicologiques des HAPs dans les sols ; les données écotoxicologiques des HAP lourds (> 4 noyaux) sont assez pauvres, s'agissant en particulier de sols « naturellement » contaminés et non dopés. Les congénères les plus étudiés pour la biodégradation et leur comportement dans l'environnement concernent le naphtalène, le phénanthrène, l'anthracène, dans une moindre mesure le fluorène et le pyrène. Les données bibliographiques sont présentées sous forme de tableaux de synthèse relatifs aux effets sur les organismes aquatiques et terrestres des sols et déchets contaminés par les HAP, et des HAP étudiés séparément dans des matrices dopées.
- **Objectifs de la recherche**
- **Matériels et Méthodes**
- **Résultats structurés en trois articles soumis pour publication**

Table I. Summary of the tests selected to assess the toxicity of soils and soil leachates to soil and aquatic organisms respectively.
Essais mis en œuvre pour l'évaluation de la toxicité des échantillons du sol de cokerie et des lixiviats

Test organisms	Tests & Species	Durations	Measured parameters	Reference
Microoraganisms	Microtox® (*Vibrio fischeri*)	15, 30 min	Luminescence inhibition	ISO 11348-3 (1999)
	Mutatox® (*Vibrio fischeri* M169)	24 hrs	Genotoxicity	Microbics (1993)
	Ames (*Salmonella typhimurium* TA98, TA100)	72 hrs	Genotoxicity	Environment Canada (1993)
	UMU (*Salmonella typhimurium* TA1535/ PSK 1002)	4 hrs	Genotoxicity	ISO 13829 (1999)
Algae	Algae (*P. subcapitata*)	72 hrs	Growth inhibition	ISO 8692 (1989)
Invertebrates	Daphnid (*Daphnia magna*)	24, 48 hrs	Immobilization	ISO 6341 (1989)
	Ceriodaphnid (*Ceriodaphnia magna*)	7 days	Reproduction	AFNOR T90-376 (2000)
	Collembola (*Folsomia candida*)	28 days	Mortality, Reproduction	ISO 11267 (1998)
	Earthworm (*Eisenia fetida*)	14 days	Mortality	ISO 11268-1 (1994)
		56 days	Reproduction	ISO 11268-2 (1998)
Plants	Oat, Lettuce, Chinese cabbage	14 ~ 21 days	Emergence, growth	ISO 14269-1 (1993)

1. Ecotoxicité d'un sol de cokerie contaminé par les HAP sur les organismes aquatiques et terrestres

2. Influence du sol contrôle, sol artificiel ISO ou sol naturel de prairie, sur l'écotoxicité en milieu terrestre du sol contaminé par les HAPs

3. Transfert des HAP du sol aux plantes supérieures et aux invertébrés terrestres

- **Discussion générale** sur les approches utilisées et les résultats obtenus

-Conclusion

-Annexes sur les résultats du transfert dans les plantes des HAP du sol de cokerie mélangé au sol naturel (résultats non inclus dans l'article 3 ; les répétitions sont en cours).

4. Méthodes

Les essais d'écotoxicité ont été réalisés selon les méthodes normalisées au niveau international (ISO) d'une part (tableau I), et d'autre part, selon des protocoles non standardisés mais pouvant être proposés ultérieurement à la normalisation en vue de compléter ou améliorer des normes existantes.

Les invertébrés aquatiques (daphnies et cériodaphnies) et terrestres (collemboles et vers de terre) utilisés dans les essais sont élevés au laboratoire ESE dans des conditions standardisées.

Les élevages et les essais de toxicité sur *Daphnia magna Strauss* sont réalisés selon la norme ISO 6341.

Les cériodaphnies, *Ceriodaphnia dubia*, sont élevées en milieu Evian-Volvic (1/4 v/v) additionné d'un mélange de vitamine (tetramin-HCl, thiamine, B12) à la température de 25°C en pots fermés de 150 mL contenant 125 mL de milieu (pour une trentaine d'adultes). La nourriture est composée d'algues chlorophycées (*P. subcapitata*) à raison de $21x10^7$ cellules par litre apportées trois fois par semaine lors des changements de milieu, en même temps que 2,3 mL/L de cérophylle et 2,3 mL/L de tétramine (solutions à 5g/L bullée, décantées et conservées à -18°C). Les essais sont réalisés avec des petits âgés de 18-24h selon la norme AFNOR NF T90-376, modifiée pour ce qui concerne la composition du milieu qui est identique au milieu d'élevage. Les petits sont dénombrés tous les jours à partir du 3è jour de l'essai en même temps que le renouvellement du milieu.

Les invertébrés terrestres sont cultivés de manière à pouvoir disposer d'animaux synchrones c'est-à-dire aux mêmes stades de leur cycle de vie, œufs, juvéniles et adultes.

Les élevages de collemboles sont cultivés à la température de 20°-22°C selon la norme ISO 11267 dans un milieu charbon actif-plâtre de Paris (120g) à 70-80% d'humidité (15g/120g/130g d'eau) en bac cristal à usage unique de 1 litre, sous une lumière < 800 lux. La nourriture est la levure de boulanger lyophilisée (Briochin) apportée toutes les semaines à raison de 2 fois 1g par semaine, la levure étant renouvelée en même temps que la réhumidification du milieu. L'élevage est renouvelé par transfert sur un milieu neuf tous les mois. Pour la réalisation d'un essai, un transfert est réalisé sur milieu neuf pour favoriser les pontes qui surviennent dans les 72h et les œufs sont récupérés 7 jours plus tard. Après la première éclosion, on compte 48 h avant d'éliminer les œufs non éclos. Les petits éclos pendant ces 48 h seront utilisés 10 jours plus tard dans l'essai sur la viabilité et la reproduction.

Les vers de terre sont élevés dans un substrat composé de tourbe et de crottin de cheval (50/50) d'origine contrôlée et exempt de micropolluants et de pesticides et maintenu humide par aspersion d'eau. Le milieu est enrichi périodiquement de petites quantités de son de blé et de flocons d'avoine d'origine biologique. Des ajouts de $CaCO_3$ sont réalisés pour maintenir le pH autour de 6.0. L'élevage est conduit à une température de 18 à 20°C et en lumière naturelle tamisée, en bacs contenant 5 kg de milieu et des animaux de même âge (à 2 à 3 semaines près), juvéniles ou adultes. La densité maximale est de 300 adultes par bacs. Les cocons sont prélevés toutes les semaines pour éviter la surpopulation et synchroniser les populations.

Le sol étudié provient du site d'Homécourt. Il a été prélevé et fourni par le GIS F.I. aux différentes équipes de recherche travaillant sur la contamination des sols de cokeries. Les prélèvements de sol ont été homogénéisés, tamisés à 4 mm. Plusieurs échantillons du stock ainsi constitué et conservé à l'obscurité en containers fermés et à température de 13 ± 2°C ont été analysés. Ce stock a été utilisé pour les essais de lixiviation et les essais d'écotoxicité aquatique, ainsi que pour les essais d'écotoxicité terrestre. Les essais de lixiviation ont été conduits selon la norme ISO 12457-2 (1999).

les lixiviats, les sols, les plantes et les vers de terre ont été effectuées par les laboratoires agréés COFRAC pour les analyses concernées, laboratoire de

Table II. Physico-chemical characteristics of the PAH-contaminated soil studied and pollutant concentration and leaching capacity of the PAH-contaminated soil water extracts I and II

Caractéristiques physico-chimiques et concentrations en métaux et HAP des échantillons du sol de cokerie et des lixiviats I et II.

	QL[a]	Contaminated soil	QL[b]	Water extracts[c]		*Ratio [water extr]/[soil]*	
				I	II	I	II
Soil texture							
Clay (%)		10.7					
Silt (%)		22.1					
Sand (%)		67.2					
pH (H_20)		9.6					
Water-holding capacity (%)		53					
Organic matter (%)		17.3					
Total organic carbon (%)		10					
Heavy metals (mean ± sd)[e]	(mg/kg)	(mg/kg)	(µg/L)	(µg/L)			
Cd	0.02	6.7 ± 0.6	0.1	0.1	<0.1	1.5×10^{-5}	$<1.5\times10^{-5}$
Cr	0.08	54 ± 0.9	0.4	1.2	1.0	2.2×10^{-5}	1.8×10^{-5}
Cu	0.1	26 ± 0.6	0.5	195	188	7.5×10^{-3}	7.2×10^{-3}
Hg	0.002	12 ± 0.3	0.01	<0.3	<0.3	$<2.5\times10^{-5}$	$<2.5\times10^{-5}$
Pb	0.3	120 ± 4	1.5	<1	<1	$<8.3\times10^{-6}$	$<8.3\times10^{-6}$
Zn	0.02	347 ± 6	0.1	9	5	2.6×10^{-5}	1.4×10^{-5}
Ni	0.24	23 ± 0.3	1.2	32	30	1.4×10^{-3}	1.3×10^{-3}
PAHs (mean ± sd)[e]	(µg/kg)	(mg/kg)	(ng/L)	(µg/L)			
Naphthalene	10	5.9 ± 2	5	2.9	<0.10	4.9×10^{-4}	$<2\times10^{-5}$
Acenaphthylene	1	4.6 ± 0.3	5	1.3	<0.80	2.8×10^{-4}	$<1.7\times10^{-4}$
Acenaphthene	1.7	46 ± 4	5	2.6	0.42	5.7×10^{-5}	0.9×10^{-6}
Fluorene	3.7	103 ± 9	5	2.4	0.97	2.3×10^{-5}	0.9×10^{-6}
Phenanthrene	2.9	380 ± 46	5	2.8	1.9	7.4×10^{-6}	4.9×10^{-6}
Anthracene	0.1	186 ± 39	5	0.59	0.27	3.2×10^{-6}	1.4×10^{-6}
Fluoranthene	1.7	561 ± 49	3	1.6	0.2	2.9×10^{-6}	0.4×10^{-6}
Pyrene	1.2	379 ± 28	5	1.1	0.7	2.9×10^{-6}	1.8×10^{-6}
Benzo[a]anthracene	0.1	230 ± 19	5	0.47	<0.05	2.0×10^{-6}	$<2.1\times10^{-7}$
Chrysene	0.1	196 ± 16	5	0.34	<0.05	1.7×10^{-6}	$<2.5\times10^{-7}$
Benzo[b]fluoranthene	0.1	146 ± 8	2	0.4	<0.05	2.7×10^{-6}	$<3\times10^{-7}$
Benzo[k]fluoranthene	0.1	89 ± 6	0.5	0.23	<0.05	2.6×10^{-6}	$<5.6\times10^{-7}$
Benzo[a]pyrene	1.1	145 ± 8	1	0.43	<0.05	3.0×10^{-6}	$<3.5\times10^{-7}$
Dibenzo[ah]anthracene	0.1	15 ± 0.8	5	0.08	<0.05	5.2×10^{-6}	$<3.2\times10^{-6}$
Benzo[ghi]perylene	0.1	65 ± 3	3	0.23	<0.10	3.5×10^{-6}	$<1.5\times10^{-6}$
Indeno[1,2,3-cd]pyrene	0.1	82 ± 5	0.5	0.28	<0.10	3.4×10^{-6}	$<1.2\times10^{-6}$
∑16 PAHs		**2634 ± 241**		**17.7**	**4.5**		

[a] QL: Quantification limits in µg/kg.d.w, (soil), [b] QL: quantification limits in ng/L (water extract)

[c] PAH concentrations of water extracts were determined in two times independently (I, II).

[d] *R*: Ratio of PAH concentration in water extracts to concentration in soil [(µg/L)/ (µg/kg)].

[e] mean ± standard deviation (n=3).

Rouen et IRH-Environnement de Nancy. Les analyses de métaux ont été réalisées par le laboratoire LBFE de l'Université de Metz.

5. Résultats et discussions

5.1. Ecotoxicité du sol provenant d'une ancienne cokerie contaminé par les HAP

► Plusieurs échantillons du stock de sol de la cokerie d'Homécourt prélevé et préparé par le GIS-F.I. ont été analysés pour leur teneur en micropolluants organiques (HAP) et en métaux.

. La contamination du sol par les 16 HAP de la liste de l'US-EPA s'élevait à 2634 ± 241 mg/kg de sol exprimé en poids sec comprenant 28 % d'HAP légers (2 et 3 cycles), 52 % de congénères à 4 cycles (fluoranthène, pyrène, chrysène, benzo[a]anthracène) et 21% d'HAP à 5 et 6 cycles aromatiques accolés (tableau II).

. Des métaux ont été trouvés également mais à concentration faible (chrome, cuivre, nickel) de l'ordre des niveaux géologiques en Lorraine (Bonnefoy et Bourg, 1985) ou à des niveaux moyens de contamination (cadmium, plomb, zinc) selon les valeurs guides néerlandaises à l'exception du mercure dont la teneur est plus élevée (BRGM, 2000).

La faible variabilité des résultats analytiques enregistrée entre les différents échantillons témoigne d'une homogénéité satisfaisante du stock de sol étudié.

► Plusieurs lixiviats préparés indépendamment ont été analysés pour leur toxicité à court terme sur les bactéries luminescentes (Microtox) et sur les daphnies, et à plus long terme sur les cériodaphnies et les algues.

. Une toxicité élevée sur la reproduction des cériodaphnies et la croissance des suspensions algales a été enregistrée. Par contre, la bioluminescence bactérienne et la survie des daphnies à court terme ne sont affectées que pour des concentrations relativement élevées des lixiviats dans le milieu d'essai. La figure I présente les valeurs de toxicité exprimées en unités de toxicité (TU = 100/CI(E)50) obtenues avec les différents essais et la relative sensibilité des différents modèles biologiques vis à vis des échantillons testés.

. Ces résultats soulignent la sensibilité des deux essais de toxicité chronique, sur la reproduction des cladocères et la division algale, dans le cadre de l'évaluation de l'écotoxicité en milieu aquatique.

. Une excellente homogénéité des résultats d'écotoxicité a été obtenue sur les cinq à six lixiviats analysés.

► Deux lixiviats (« *water extracts* » I et II) ont été testés pour leur caractère mutagène à l'aide des tests de mutagénicité Ames, Mutatox et Umu.

. Un caractère mutagène du lixiviat I (water extract I) a été enregistré avec les deux souches TA98 et TA100 de *S. typhimurium* his- utilisées dans le test d'Ames, ainsi qu'avec le test Mutatox. Les valeurs de LOEC (concentrations de lixiviat la plus faible induisant un effet mutagène) indiquent une sensibilité élevée du test d'Ames, notamment dans les essais effectués avec activation métabolique (+ S9). Un caractère mutagène plus élevé après activation métabolique (+ S9) que sans activation (- S9) est logique sur la base de l'hypothèse que les HAP sont responsables de la mutagénicité. En effet, les HAP deviennent génotoxiques après activation métabolique. Aucune réponse n'a été enregistrée avec le test Umu, ce qui peut s'expliquer par la sensibilité plus faible de cet essai comparé aux deux autres méthodes.

. Le deuxième extrait s'est révélé nettement moins mutagène avec le test d'Ames, et seule la souche TA100 a donné une réponse positive. Par contre, la réponse du test Mutatox est plus élevée qu'avec le lixiviat I.

L'écotoxicité de ces deux lixiviats évaluée avec les tests Microtox, daphnies, cériodaphnies, algues s'inscrivait dans le cadre des valeurs moyennes enregistrées avec les différents échantillons, sans qu'aucune différence ne puisse être enregistrée entre les lixiviats I et II.

► L'analyse des micropolluants des deux lixiviats a révélé des concentrations en métaux similaires, mais des différences importantes au plan des concentrations en HAP : la teneur totale des 16 HAP dans le lixiviat I était de 18 µg/L contre 4.5 µg/L pour le lixiviat II., ce que l'on peut relier avec la réponse plus faible du test d'Ames pour l'échantillon II (tableau II).

. L'examen des teneurs en micropolluants des extraits aqueux montre qu'elles correspondent à des concentrations 10^5 à 10^6 fois plus faibles que celles des sols pour tous les métaux analysés à l'exception du cuivre. Les pourcentages de cuivre extraits par l'eau sont cent fois plus élevés que ceux des autres éléments : avec un ratio des concentrations lixiviat/sol de 7.2×10^{-3} à 7.5×10^{-3} pour les deux lixiviats, contre des ratios de l'ordre de 10^{-5} pour les autres métaux (tableau II).

. Les concentrations de cuivre correspondant aux valeurs de CI50 des bioessais expliquent à elles-seules la toxicité sur la survie des daphnies et

sur la reproduction des Cériodaphnies : la CE50-48h du cuivre sur la survie de *D. magna* se situerait entre 7 et 87 µg/L en fonction des conditions expérimentales (alcalinité, température, pH) (IPCS EHC 200, 1998) et est de 32 ± 3 µg/L dans les conditions du test ISO selon Bossuyt et Janssen (2005) ; la CE50 sur la reproduction des Cériodaphnies est de l'ordre de 5 µg/L à pH 8 et inférieure à 5 µg/L à pH plus bas (Gagneten et Vila, 2001), ce qui correspond à la gamme de pH 7.5-8.0 dans nos essais entre deux changements de milieu.

. Le taux d'extraction par l'eau des HAP se situe entre 10^{-4} et 10^{-7} selon le nombre de cycle des différents congénères, les HAP légers étant plus facilement mobilisés par l'eau. Les profils de répartition des 16 HAP dans le sol et les lixiviats, montre que la distribution des HAP retrouvés dans les extraits coïncide avec leur hydrosolubilité et non avec leur niveau de concentration dans le sol.

► Les résultats d'écotoxicité terrestre, sur plantes, collemboles et vers de terre exprimés en unités de toxicité (TU) sont présentés dans la figure II.

. Ils témoignent d'une toxicité élevée du sol étudié sur la reproduction des collemboles et des vers de terre, ainsi que sur la survie des deux invertébrés, à la condition de considérer les vers de terre **juvéniles**.

. En effet, la survie des vers de terre adultes n'est affectée qu'à des concentrations élevées, supérieures à 40% de sol dans le milieu test. Aucun ver de terre adulte ne survit toutefois dans le sol contaminé sans dilution.
Par contre, la reproduction des vers de terre adultes est sévèrement inhibée, et ce à des dilutions du sol n'affectant pas la viabilité des géniteurs. La production de cocoons mesurée après 28 jours d'exposition est un indicateur sensible de toxicité, presque aussi sensible que le nombre de juvéniles éclos après 56 jours d'essai.

. En ce qui concerne les effets sur les collemboles, l'inhibition de la reproduction apparaît due majoritairement à la diminution de la survie des adultes : les valeurs de CE50 sur la reproduction après 28 jours sont de 5.7 % en considérant l'ensemble des juvéniles produits. La CE50 calculée sur la base du nombre de juvéniles par adulte est de l'ordre de 10 %, soit proche de la LC50 de 11% sur la survie.

. Les deux plantes testées ne sont pas apparues très sensibles aux polluants du sol comparées aux invertébrés. La germination n'est pas affectée et les valeurs de CE50 basées sur la mesure de l'inhibition de croissance par

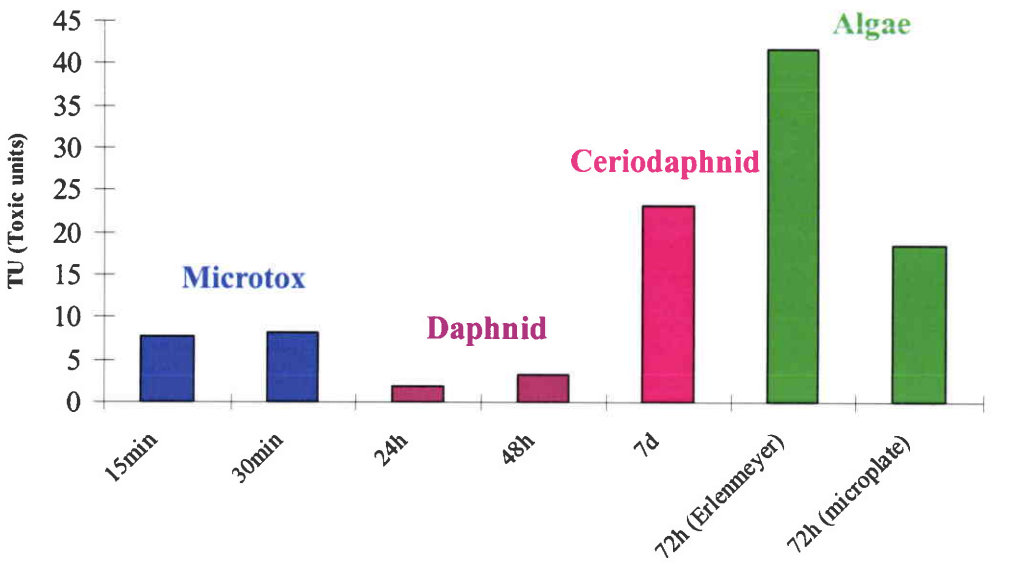

Fig. I. *Sensibilité relative des essais Microtox, daphnies (viabilité), Cériodaphnies (reproduction) et algues (croissance des suspensions en microplaque et en Erlenmeyer) réalisés sur les lixiviats du sol contaminé par les HAP et réponses exprimées en unités toxiques (Toxic Units : TU=100/ EC_{50})*

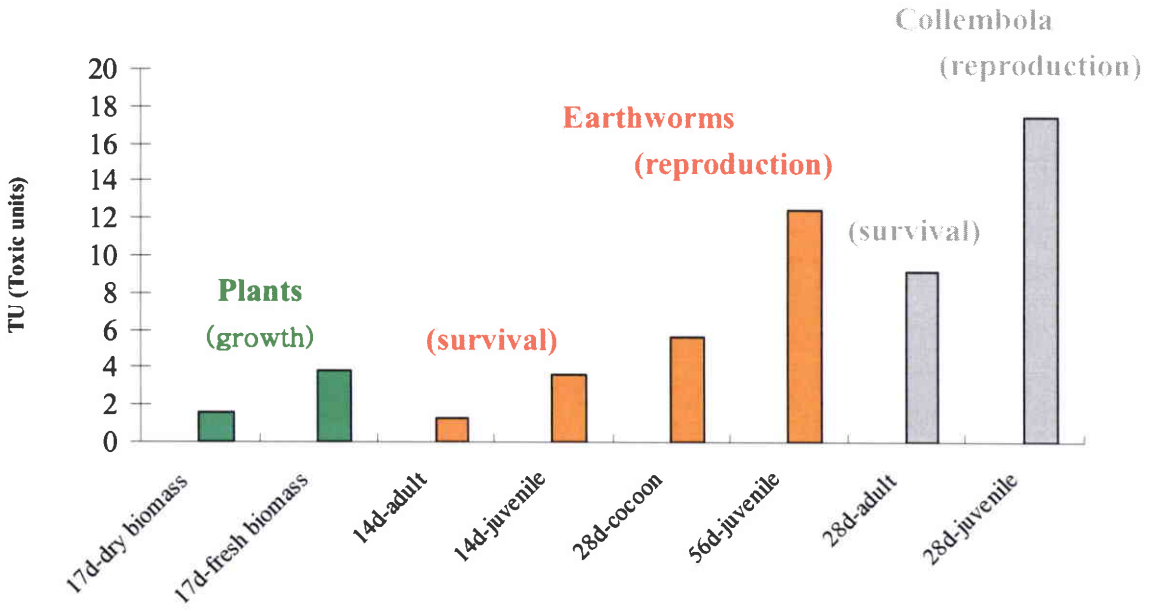

Fig. II. *Sensibilité relative des essais sur les plantes (croissance), les vers de terre (survie des adultes et des juvéniles, et reproduction) et les collemboles (survie et reproduction) exposés au sol contaminé par les HAP et réponses en unité toxiques (Toxic Units : TU=100/ EC_{50})*

rapport aux témoins et sur le poids frais des parties aériennes sont de 26 % pour la laitue et 27 % pour le chou. Les valeurs de CE50 sont de 55 % et 71% respectivement, si l'on considère le poids sec.

► L'examen des concentrations de chacun des micropolluants analysés dans les milieux testés sur les espèces terrestres, et leur comparaison aux concentrations rapportées dans la littérature, montre que la présence des métaux dans les sols n'explique pas la toxicité sur les collemboles et les vers de terre.

. Par contre si l'on considère les concentrations de chacun de HAPs, il apparaît que les concentrations de phénanthène, fluoranthène et pyrène correspondent aux valeurs de LC50 de chacun de ces hydrocarbures testés en sol artificiel par Sverdrup et al. (2001) sur les collemboles *Folsomia fimetaria*, pour la même durée d'exposition et par Sorensen et Holmstrup (2005) pour le pyrène et le fluorène sur *Folsomia candida*.

. Il en est de même de la concentration des trois congénères précédents - phénanthène, fluoranthène, pyrène -, et de celle du fluorène, dans les essais de reproduction des vers de terre : ces concentrations sont du même ordre de grandeur que les valeurs de CE50 de chacun des hydrocarbures testés sur la reproduction d'*Eisenia veneta* par Sverdrup et al. (2002a).

► **En conclusion,**

1. Ces résultats soulignent l'intérêt d'inclure dans une batterie d'essais l'étude de la toxicité chronique - sur la reproduction des cladocères et la division algale -, pour l'évaluation de l'écotoxicité des échantillons hydriques.
L'étude toxicologique couplée à l'analyse physico-chimique des lixiviats a permis d'identifier le cuivre comme responsable de la toxicité sur daphnies, du fait de la mobilité de cet élément.

2. Les résultats obtenus à la suite des essais d'écotoxicité terrestre sont importants car ils mettent l'accent sur 2 points essentiels, quant à la nécessité d'étudier systématiquement :

- la reproduction dans les essais d'écotoxicité terrestre,
- la survie des juvéniles, dans les essais sur vers de terre : la norme ISO qui préconise l'utilisation d'organismes adultes serait à revoir en ce sens.

Notons que les essais de viabilité sont généralement réalisés sur des organismes âgés de moins 24h dans les essais (cério)daphnies, ou des juvéniles pour le test sur collemboles. Le protocole d'étude de l'inhibition de la viabilité des vers de terre adulte selon la norme en cours est un anachronisme qui mériterait d'être corrigé.

Table III. Main physicochemical characteristics of the PAH-contaminated soil and the two control soils (natural soil, ISO) used in this study.

Caractéristiques physico-chimiques et teneurs en métaux et HAP du sol contaminé et des deux sols contrôles utilisés, sol artificiel ISO et sol naturel, pour la préparation des différentes concentrations testées.

	QL[a]	Contaminated soil	Natural soil	Artificial ISO (10% peat)
Texture				
Soil type		Sandy loam	Silty clay loam	Sandy loam
Clay (%)		10.7	19.7	10.7
Silt (%)		22.1	61.4	8.2
Sand (%)		67.2	18.9	81.1
pH (H_20)		9.6	6.5	6.0 ± 0.5
Water-holding capacity (%)		53	58	59
C/N		46.1	9.6	39.9
Organic carbon (%)		10.0	2.3	3.4
Organic matter (%)		17.3	3.9	5.9
Heavy metals (mean±sd) [b]	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Cd	0.02	6.7 ± 0.6	0.2	0.1
Cr	0.08	54 ± 0.9	7.4	1.0
Cu	0.1	26 ± 0.6	16	1.2
Hg	0.002	12 ± 0.3	< 0.1	< 0.05
Pb	0.3	120 ± 4	13	46
Zn	0.02	347 ± 6	61	5.3
Ni	0.24	23 ± 0.3	20	0.3
PAHs (mean±sd) [b]	(µg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Naphthalene	10	5.9 ± 2	< 0.01	< 0.01
Acenaphthylene	1	4.6 ± 0.3	< 0.01	< 0.01
Acenaphthene	1.7	46 ± 4	< 0.01	< 0.01
Fluorene	3.7	103 ± 9	< 0.01	< 0.01
Phenanthrene	2.9	380 ± 46	0.02	< 0.01
Anthracene	0.1	186 ± 39	< 0.01	< 0.01
Fluoranthene	1.7	561 ± 49	0.12	< 0.01
Pyrene	1.2	379 ± 28	0.10	< 0.01
Benzo[a]anthracene	0.1	230 ± 19	0.05	< 0.01
Chrysene	0.1	196 ± 16	0.05	< 0.01
Benzo[b]fluoranthene	0.1	146 ± 8	0.06	< 0.01
Benzo[k]fluoranthene	0.1	89 ± 6	0.03	< 0.01
Benzo[a]pyrene	1.1	145 ± 8	0.06	< 0.01
Dibenzo[ah]anthracene	0.1	15 ± 0.8	< 0.01	< 0.01
Benzo[ghi]perylene	0.1	65 ± 3	0.04	< 0.01
Indeno[1,2,3-cd]pyrene	0.1	82 ± 5	0.05	< 0.01
Σ16 PAHs		**2634 ± 241**	**0.63**	-[c]

[a] QL: Quantification limits in µg/kg d.w.

[b] Mean ± SD: mean ± standard deviation.

[c] - : < Quantification limits.

3. Les HAP légers de type phénanthène, fluoranthène, pyrène et fluorène, pourraient expliquer la toxicité à court et à long terme du sol de cokerie étudié. **Un modèle de toxicité des sols contaminés par les HAP pourrait être proposé en utilisant les concentrations de ces quatre congénères comme indicateurs d'écotoxicité terrestre.**

5.2. Influence du sol de dilution, ISO (artificiel) ou sol de prairie (naturel), sur les résultats des essais d'écotoxicité terrestre du sol contaminé par les HAP.

Plusieurs auteurs ont récemment exprimé des recommandations en matière d'évaluation de l'écotoxicité terrestre, visant à utiliser un sol contrôle naturel plutôt qu'un sol artificiel critiqué souvent pour son manque de représentativité (Spurgeon et al., 2002 ; Van Gestel et Weeks, 2004). Cette recommandation s'ajoutait à celle d'étudier des sols contaminés sur sites plutôt que des sols dopés en laboratoire.

Le sol artificiel ISO préconisé dans la norme est composé de kaolin (20%) de tourbe (10%) et de sable de fontainebleau (70%) (ISO 11268-1, 1994). Nous avons étudié l'influence éventuelle de la nature du sol contrôle mélangé au sol contaminé pour préparer la gamme des concentrations testées. La comparaison des résultats d'essais du sol de cokerie mélangé au sol ISO ou à un sol naturel de prairie (historiquement vierge de tout traitement) a été faite pour les essais sur plantes et invertébrés - collemboles et vers de terre.

Les caractéristiques des deux sols témoins ont été analysées (tableau III)

► Aucune influence significative du sol contrôle n'a été enregistrée lors des essais sur plantes. La croissance des plantes, chou chinois et laitue, est inhibée pour des concentrations du sol de cokerie supérieures à 25 % quel que soit le sol de dilution, milieu artificiel ISO ou sol naturel de prairie. Pour le chou, la croissance est meilleure en présence du sol de prairie, pour la laitue elle est meilleure en présence du sol ISO, mais le pourcentage d'inhibition de la croissance par rapport au témoin ne diffère pas.

► En ce qui concerne les vers de terre, aucun effet du sol témoin de dilution n'est noté sur la survie des vers de terre adultes ou juvéniles après 14 jours d'essais.

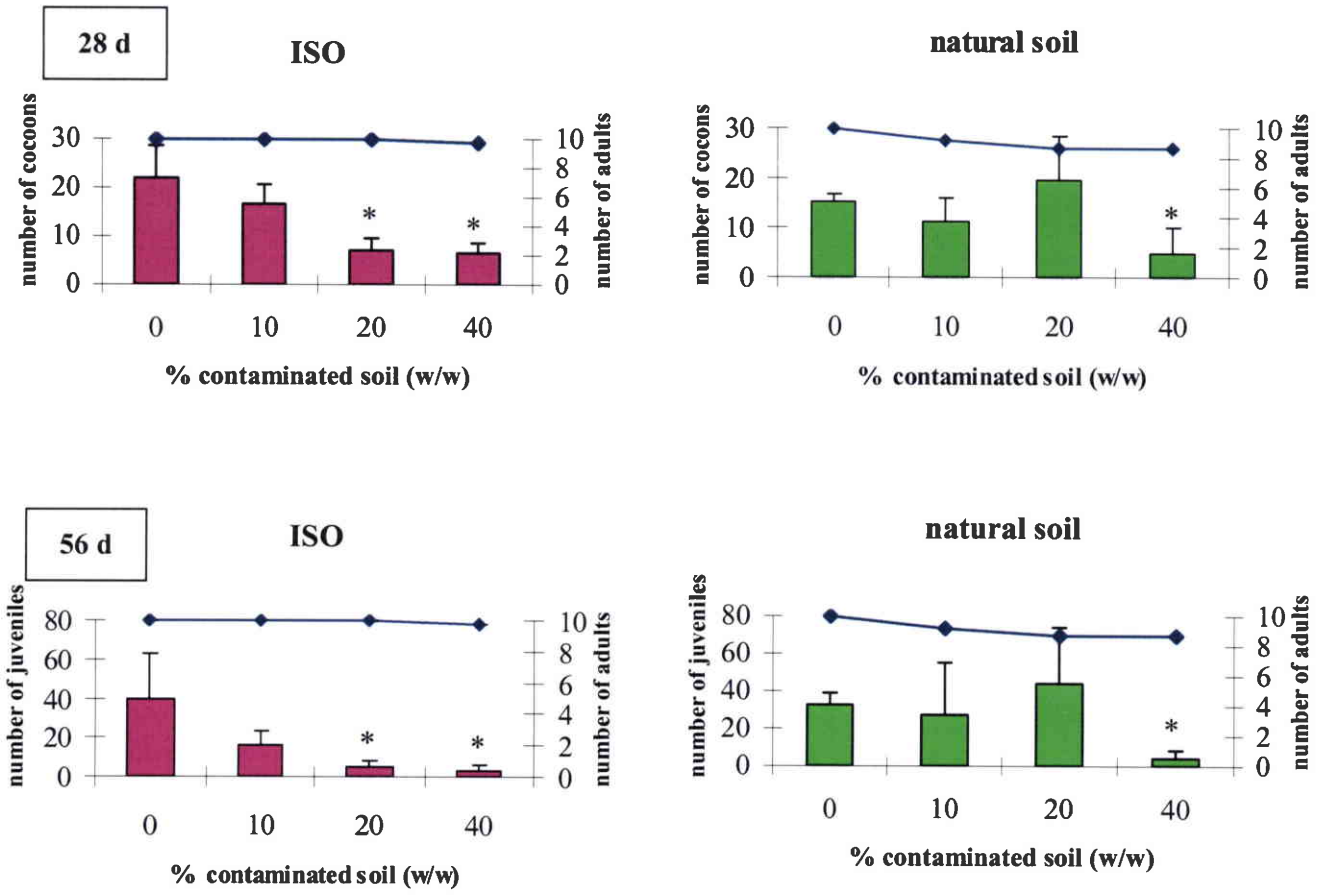

Fig. III. *Nombre de cocons après 28 jours et de juvéniles produits après 56 jours d'exposition des vers de terre E. fetida à différentes concentrations du sol contaminé par les HAP dans le sol contrôle ISO ou le sol naturel. La survie des adultes est indiquée par la courbe (axe de droite). Le nombre de cocons et de juvéniles est la moyenne ± écart-type des 4 réplicats.*
** Différence significative par rapport aux contrôles respectifs (P<0.05).*

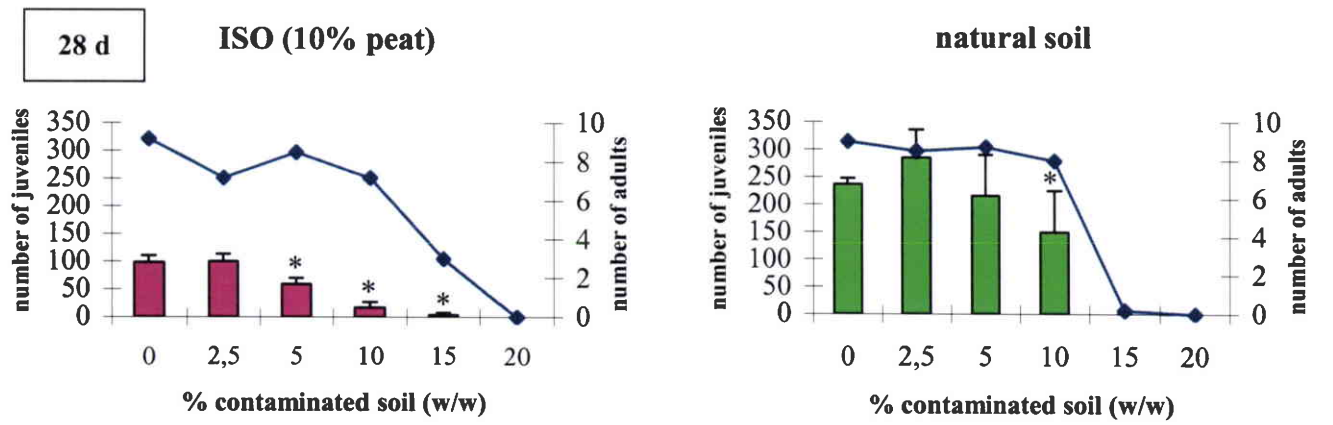

Fig. IV. *Reproduction (barres) et survie (courbe) des collemboles F. candida exposés à différentes concentrations du sol contaminé par les HAP dans le sol contrôle ISO ou le sol naturel (moyenne et écart-type des 4 réplicats).*
** Différence significative par rapport aux contrôles respectifs (P<0.05).*

Par contre, une influence a été notée sur la reproduction. La reproduction évaluée par le nombre de cocons après 28 jours est diminuée significativement par le sol contaminé par les HAP à la concentration de 20% dans le milieu ISO, alors qu'aucune inhibition n'est notée à la même concentration dans le sol de prairie. Dans ce dernier cas, l'inhibition n'est significative qu'à la concentration de 40% du sol de cokerie mélangé au sol naturel.
La même tendance est observée à 56 jours d'essai sur la base du nombre total de juvéniles, avec une inhibition plus marquée avec le sol de dilution ISO qu'avec le sol naturel (figure III)

► Pour les essais sur collemboles, la différence entre les deux sols témoins est nette, avec d'une part un meilleur taux de reproduction en présence du sol naturel, et une inhibition de la reproduction (CE50) pour des concentrations plus faibles du sol contaminé en sol ISO qu'en sol naturel : ce qui témoigne d'une sensibilité plus élevée des collemboles dans le sol artificiel par rapport au sol de prairie utilisé ici (figure IV).

En conclusion,

► Les résultats des essais ont montré des différences, faibles certes mais significatives lors de l'utilisation d'un sol témoin artificiel ou d'un sol naturel.

Ces résultats militeraient en faveur de l'utilisation d'un sol naturel pour l'évaluation de l'écotoxicité des sols contaminés sur invertébrés.

Compte tenu de la variété des sols naturels, il serait utile d'explorer d'autres types de sols que le sol de prairie utilisé ici, de manière à approfondir l'étude de la relation entre l'expression de la toxicité des micropolluants des sols contaminés et la composition du sol de dilution.

Soulignons cependant la difficulté de trouver des sols témoins dont on peut certifier l'absence de traitement antérieur, notamment de traitement par des pesticides, et ce même pour des sols provenant de station expérimentale agronomique.

► L'étude ne permettait pas d'identifier quel(s) facteur(s) étai(en)t à l'origine de la diminution de toxicité en sol naturel. La teneur en carbone organique du sol est donnée comme un paramètre pouvant influencer la biodisponibilité des polluants métalliques et leur toxicité, une faible teneur étant associée à plus forte biodisponibilité. Cependant dans le cas présent, la teneur en carbone organique et

celle en matières organiques se sont révélées à l'analyse, supérieures dans le sol artificiel par rapport au sol de prairie.

Il faut trouver l'explication à la diminution de toxicité en sol de prairie, vraisemblablement dans la nature de la matière organique représentée par les acides humiques et fulviques aux capacités de complexation élevées vis-à-vis des micropolluants. Comme ces paramètres n'ont pas été mesurés, il est difficile d'aller aujourd'hui, au-delà de la formulation d'hypothèses.

5.3. Etude du transfert des HAP du sol de cokerie dans les invertébrés (vers de terre) et les plantes (chou chinois).

Le transfert des HAP du sol aux biocénoses, notamment la biosorption des congénères de poids moléculaire élevé (> 4 cycles aromatiques) est considéré comme très faible. Le transfert éventuel des HAP du sol de cokerie de composition proche de celui utilisé ici avait été étudié en 2003 dans les plantes légumières, salade, carottes et tomates (Charissou et al., 2003) dans le cadre du programme Sarcartom piloté par l'ADEME, pour apprécier le risque posé par la contamination des aliments cultivés dans un sol contaminé. D'autres auteurs se sont intéressés à la fraction des HAP biosorbés sur les racines, ou présents dans la partie comestible des végétaux. Le transfert des HAP sol-végétaux a été notamment étudié dans le cas de plantes mycorrhizées afin d'apprécier l'intérêt de la mycorrhization des racines dans la dégradation des HAPs et les conséquences sur le transfert des hydrocarbures vers les végétaux (Joner et al., 2004). Des programmes de recherche ont été développés aux Etats-Unis afin d'analyser l'efficacité de la phytorémédiation des sols contaminés par les HAP, et mettant en œuvre différentes plantes aux systèmes racinaires très développés et mycorrhizées, herbe des Bermudes (*Cynodon dactylon*), trêfle blanc (*White clover*) et fétuque élevée (*Festuca arundinacea)* (Fiorenza et al., *2000).* Un transfert des HAP dans les plantes a été observé, mais il reste très faible.
Dans la cadre de cette thèse, nous avons étudié le transfert des 16 HAP du sol de cokerie mélangé au sol ISO dans les parties aériennes du chou chinois après 28 jours d'exposition. La partie racinaire n'a pas été étudiée, du fait des biais signalés par plusieurs auteurs liés à la difficulté d'éliminer les petites particules de terre des racines. Les essais ont été effectués en sol naturel également, mais comme les essais de répétitions sont en cours les résultats préliminaires seront présentés uniquement en annexe.

A notre connaissance, le transfert des HAP du sol aux invertébrés a été très peu étudié. Nous avons donc prolongé les essais de reproduction sur vers de terre

Table IV. Bioaccumulation factor (BAFs) and Biota soil accumulation factor (BSAFs) for PAHs in population of juvenile earthworms exposed during 24 weeks to PAH-contaminated soil mixed at different concentrations in either ISO soil or natural soil.
Facteurs de bioaccumulation (BAF) et facteurs de bioaccumulation sol-biota (BSAF, normalisé par rapport au carbone organique du sol et à la teneur en lipide des organismes) des HAP mesurés dans les vers de terre exposés pendant 24 semaines à différentes concentrations du sol contaminé, en sol ISO ou en sol naturel.

	No. of rings	**ISO**		**natural soil**	
PAHs (µg/kg MS)		BAF	BSAF	BAF	BSAF
Naphthalene	2	0.533 ± 0.583	1.264 ± 1.320	0.094 ± 0.088	0.194 ± 0.184
Acenaphthylene	3	0.075 ± 0.017	0.204 ± 0.047	0.086 ± 0.016	0.200 ± 0.087
Acenaphthene	3	0.006 ± 0.005	0.015 ± 0.012	0.007 ± 0.004	0.016 ± 0.009
Fluorene	3	0.017 ± 0.025	0.038 ± 0.056	0.003 ± 0.003	0.008 ± 0.007
Phenanthrene	3	0.001 ± 0.001	0.004 ± 0.004	0.003 ± 0.002	0.007 ± 0.007
Anthracene	4	0.013 ± 0.010	0.032 ± 0.020	0.005 ± 0.004	0.012 ± 0.010
Fluoranthene	4	0.004 ± 0.004	0.012 ± 0.010	0.006 ± 0.005	0.015 ± 0.014
Pyrene	4	0.009 ± 0.002	0.025 ± 0.001	0.008 ± 0.006	0.020 ± 0.018
Benzo[a]anthracene	4	0.027 ± 0.013	0.070 ± 0.022	0.015 ± 0.006	0.036 ± 0.021
Chrysene	4	0.062 ± 0.039	0.160 ± 0.073	0.031 ± 0.012	0.071 ± 0.034
Benzo[b]fluoranthene	5	0.099 ± 0.020	0.275 ± 0.080	0.126 ± 0.083	0.287 ± 0.182
Benzo[k]fluoranthene	5	0.110 ± 0.042	0.290 ± 0.059	0.095 ± 0.062	0.215 ± 0.135
Benzo[a]pyrene	5	0.050 ± 0.032	0.142 ± 0.093	0.088 ± 0.065	0.199 ± 0.140
Dibenzo[ah]anthracene	5	0.129 ± 0.046	0.341 ± 0.059	0.176 ± 0.150	0.404 ± 0.319
Benzo[ghi]perylene	6	0.042 ± 0.018	0.111 ± 0.026	0.049 ± 0.013	0.107 ± 0.031
Indeno[1,2,3-cd]pyrene	6	0.032 ± 0.024	0.093 ± 0.069	0.041 ± 0.030	0.104 ± 0.091
Σ16 PAHs		0.027 ± 0.007	0.058 ± 0.003	0.026 ± 0.015	0.060 ± 0.036
Σ2 rings PAH (n=1)		0.533 ± 0.583	1.264 ± 1.320	0.094 ± 0.088	0.194 ± 0.184
Σ3 rings PAH (n=4)		0.005 ± 0.004	0.012 ± 0.010	0.004 ± 0.003	0.010 ± 0.007
Σ4rings PAH (n=5)		0.017 ± 0.007	0.045 ± 0.011	0.011 ± 0.006	0.026 ± 0.018
Σ5rings PAH (n=4)		0.085 ± 0.013	0.232 ± 0.048	0.107 ± 0.074	0.295 ± 0.194
Σ6rings PAH (n=2)		0.037 ± 0.008	0.092 ± 0.024	0.044 ± 0.013	0.105 ± 0.059

[a] Data given mean ± standard deviation (n=3; 10%, 20%, 40% contaminated soils).

[b] BAFs calculated by the PAH concentration ratio earthworm tissue to the tested medium.

[c] BSAFs expressed as the lipid normalized PAH content to the organic carbon normalized contaminant content in the soil.

Eisenia fetida au-delà des 56 jours d'essai. Les vers de terre adultes avaient été retirés du milieu dès la production des cocons. Nous avons analysé le niveau de contamination des juvéniles éclos dans le sol contaminé après 24 semaines d'exposition. Avant analyse, un massage manuel de chacun des vers est réalisé afin de vider le tube digestif de son contenu. Les animaux sont ensuite laissés 24 h dans un milieu constitué de silice humidifiée, et la vidange manuelle du tube digestif est effectuée à nouveau afin de parfaire l'épuration.
Le transfert des HAP du sol de cokerie a été étudié en présence de sol ISO ou de sol naturel utilisé comme milieu de dilution.

► Les essais d'exposition à très long terme ont confirmé les résultats obtenus lors des essais de reproduction à proprement parlé sur 28 et 56 jours, à savoir :

. L'absence de toxicité du sol contaminé par les HAP aux concentrations inférieures à 40% dans le sol de prairie, sur la croissance des vers de terre juvéniles, et ce même après 24 semaines d'exposition. Par contre, la diminution de croissance est significative pour les concentrations de 10, 20 et 40% du sol mélangé au milieu ISO.

► Pour ce qui concerne le sol de cokerie mélangé au sol naturel, les résultats analytiques mettent en évidence une contamination des vers de terre par les HAP lourds de 5 et 6 cycles aromatiques, nettement supérieure à celle par les HAP à 4 et 3 cycles, ce qui donne par ordre décroissant d'accumulation :

HAP à 5 cycles aromatiques > 2 cycles > 6 cycles > 4 cycles > 3 cycles

. L'accumulation est plus élevée dans les organismes exposés aux concentrations de 10 et 20% du sol contaminé qu'à la concentration de 40%, ce qui s'explique par la toxicité enregistrée à cette concentration.

► La même tendance est observée avec le sol contaminé mélangé au milieu ISO, avec des facteurs d'accumulation toutefois moins élevés que pour le mélange avec le sol naturel. Les facteurs d'accumulation des HAP vont décroissant selon l'ordre suivant :

HAP à 2 cycles aromatiques > 5 cycles > 6 cycles > 4 cycles > 3 cycles

. Les facteurs de bioaccumulation (BAF) sont présentés dans le tableau IV. Une normalisation par rapport au carbone organique et par rapport au taux de lipides d'Eisenia a été effectuée, mais cette correction des BSAF (biota-sol accumulation factor) ne modifie pas les résultats : les valeurs de BSAF sont proches des valeurs brutes de BAF.

Table V. Shoot concentration factor (SCFs) of PAHs, on a dry wet weight basis, for plant (Chinese cabbage) after 28 days exposed to the PAH-contaminated soil mixed with artificial ISO soil.

Facteurs de concentration des HAP dans les pousses (SCF) du chou Chinois après 28 jours d'exposition au sol contaminé testé à différentes concentrations (0, 1, 10, 15%) en sol ISO.

	No. of rings	**PAH concentration in shoots** (mg/kg d.w) % contaminated soil in ISO test medium (w/w)				**SCF[a]**		
PAHs (µg/kg MS)		0 (control)	1	10	15	1	10	15
Naphthalene	2	0.040	<0.001	0.099	0.083	<0.001	0.168	0.094
Acenaphthylene	3	<0.010	<0.010	<0.010	<0.010	<0.001	<0.001	<0.001
Acenaphthene	3	0.001	0.002	0.030	0.017	0.005	0.007	0.002
Fluorene	3	0.002	0.002	0.014	0.008	0.002	0.001	0.001
Phenanthrene	3	0.027	0.004	0.416	0.238	0.012	0.011	0.004
Anthracene	4	0.002	0.007	0.142	0.101	0.004	0.008	0.004
Fluoranthene	4	0.029	0.006	1.280	1.040	0.011	0.023	0.012
Pyrene	4	0.005	0.011	0.230	0.167	0.003	0.006	0.003
Benzo[a]anthracene	4	0.003	0.004	0.273	0.073	0.002	0.012	0.002
Chrysene	4	0.005	0.003	0.210	0.059	0.002	0.011	0.002
Benzo[b]fluoranthene	5	0.002	0.002	0.185	0.029	0.001	0.013	0.001
Benzo[k]fluoranthene	5	0.001	<0.001	0.108	0.016	<0.001	0.012	0.001
Benzo[a]pyrene	5	0.001	0.001	0.201	0.027	0.001	0.014	0.001
Dibenzo[ah]anthracene	5	<0.001	<0.001	0.011	0.002	<0.001	0.007	0.001
Benzo[ghi]perylene	6	0.003	0.014	0.134	0.018	0.020	0.021	0.002
Indeno[1,2,3-cd]pyrene	6	<0.001	<0.001	0.091	0.014	<0.001	0.011	0.001
Σ16 PAHs		**0.123**	**0.153**	**3.424**	**1.891**	**0.006**	**0.013**	**0.005**
Σ2 rings PAH (n[b] =1)		0.040	<0.001	0.099	0.083	<0.001	0.168	0.094
Σ3 rings PAH (n=4)		0.030	0.049	0.460	0.263	0.009	0.009	0.003
Σ4rings PAH (n=5)		0.045	0.087	2.135	1.440	0.005	0.014	0.006
Σ5rings PAH (n=4)		0.004	0.003	0.505	0.074	0.001	0.013	0.001
Σ6ings PAH (n=2)		0.004	0.014	0.225	0.032	0.009	0.015	0.001

[a] SCFs calculated by the PAH concentration ratio plant shoot to the tested medium. [b] n = number of individual PAH.

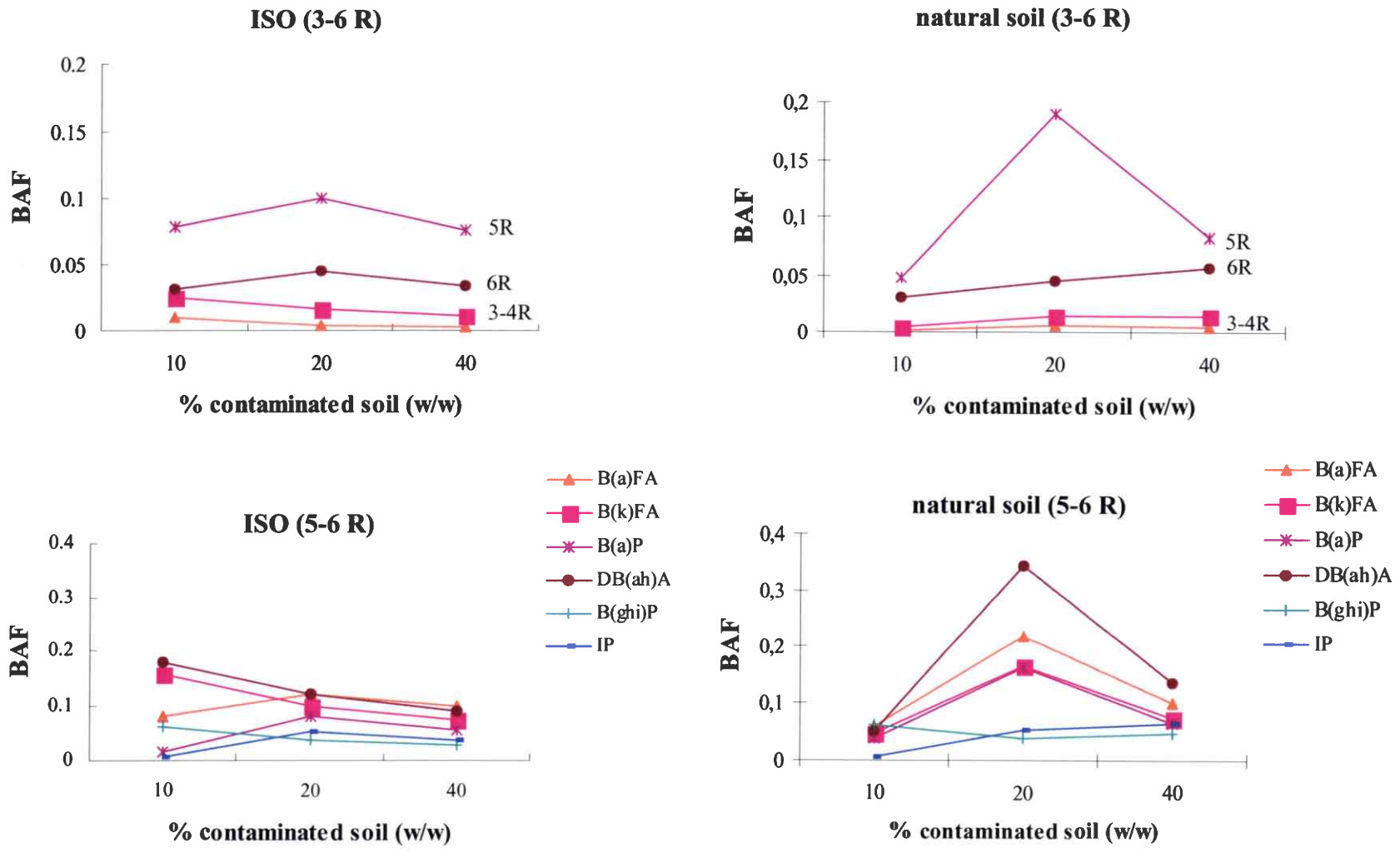

Fig. V. *Comparaison des facteurs de bioaccumulation (BAF) des HAP dans les vers de terre juvéniles exposés 24 semaines à différentes concentrations (10%, 20%, 40%) du sol contaminé mélangé au sol contrôle ISO ou au sol naturel.*

. Pour les HAP à 5 et 6 noyaux aromatiques, les facteur de transfert restent largement inférieur à 1 et dans la gamme de 0.3 à 0.01 pour les HAP de 5 à 3 noyaux aromatiques, respectivement.

Il ne semble pas que la volatilité des HAP légers explique les différences d'accumulation notées entre les molécules, puisque le naphtalène, l'hydrocarbure le plus volatile, est aussi l'hydrocarbure dont les concentrations sont les plus élevées. L'hypothèse qu'une métabolisation des HAP à 3 et 4 cycles a lieu chez les vers de terre peut être avancée pour expliquer les niveaux d'accumulation beaucoup plus faibles de ces dérivés. Les conditions expérimentales, sol contrôle ISO ou sol naturel n'influencent pas les résultats.

► Le facteur de transfert des HAP est très faible dans les parties aériennes du chou.

. Dans ce cas, le transfert est très faible pour les HAP comprenant 3, 4, 5 ou 6 noyaux aromatiques (0.001 à < 0.02). Un facteur de transfert pouvant atteindre 0.17 est noté pour le naphtalène. Une plus forte accumulation des dérivés naphtaléniques dans les plantes a également été observée lors des essais de biorémédiation utilisant la fétuque élevée et l'herbe des Bermudes (Fiorenza et al., 2000).

. Des facteurs d'accumulation plus élevés dans le mélange du sol contaminé avec le sol naturel que dans le mélange avec le sol ISO ont été mesurés dans le chou. Ces résultats préliminaires sont à prendre avec réserve, tant que les résultats des essais de répétition (en cours) ne sont pas connus.

En conclusion

► Il ne semble pas que le transfert des HAP du sol à la biomasse végétale ou à celle des invertébrés pose des problèmes d'accumulation, compte tenu des facteurs enregistrés nettement inférieurs à 1.

►Une accumulation des HAPs lourds chez les vers de terre a cependant été mise en évidence, ce qui n'avait jamais été rapporté antérieurement. En effet la plupart des études sur les HAP ont été réalisées avec les congénères légers.
Les résultats enregistrés mettent en évidence un comportement différent des HAP lourds (5 et 6 cycles) par rapport aux HAP plus légers (3 et 4 cycles) avec une bioaccumulation plus marquée. Cette accumulation peut résulter d'une élimination plus lente que celle des congénères à 3 et 4 noyaux.

Le naphtalène semble se distinguer des 16 autres HAP par l'accumulation la plus élevée, chez les vers de terre.

► Chez les plantes, le facteur de transfert est minime, se situant entre 0.001 et 0.02, si l'on exclut le naphtalène.

6. Conclusion

► La biodisponibilité des polluants dans des sols anciennement contaminés est difficile à prédire par l'analyse des sols. Comme l'extraction par solvants apolaires des polluants organiques permet d'extraire des dérivés fortement liés à la matrice des sols et peu biodisponibles, la prédiction des risques biologiques peut être surévaluée par l'extrapolation des résultats analytiques. Les approches biologiques sont donc nécessaires pour appréhender les effets de la fraction des polluants mobilisables par l'eau pour les espèces aquatiques d'une part, et les effets des polluants du sol biodisponibles pour les espèces terrestres d'autre part.

Les résultats sur les lixiviats ont confirmé le bien-fondé de la batterie d'essais utilisée incluant les bioessais de toxicité chronique sur la reproduction des invertébrés (Cériodaphnies) et la croissance (division cellulaire) des suspensions algales. L'étude de la variabilité des résultats biologiques et analytiques a montré que l'extraction des métaux mobilisables par l'eau était sujette à de très faibles variations comparées à celle des HAP. La fraction hydrosoluble inorganique était responsable majoritairement de la toxicité à court ou plus long terme, tandis que la fraction des HAP extractibles par l'eau participait à la génotoxicité des lixiviats.

► L'originalité du travail portait plus sur les réponses à (très) long terme des organismes terrestres aux échantillons du sol de cokerie. Les études de reproduction sur invertébrés terrestres ont confirmé leur sensibilité et leur intérêt dans la perspective d'une extrapolation des réponses aux populations *in situ*. Les collemboles se sont distingués pour leur sensibilité, quel que soit le critère étudié, survie ou reproduction.

Les résultats obtenus sur vers de terre infirment les déclarations selon lesquels ces invertébrés seraient peu sensibles aux polluants. En effet, si l'on étudie la survie des juvéniles et non des adultes, il s'avère que la survie est affectée à des niveaux de concentrations des polluants faibles, à peine supérieurs à ceux inhibant la reproduction.
La reproduction constitue le critère de choix pour juger des effets à long terme. La numération des cocons après 28 jours d'exposition des adultes apparaît un

indicateur prédictif et très sensible du nombre de juvéniles éclos à 56 jours pour le type de sol étudié.
Il a été possible d'expliquer la toxicité des échantillons de sol contaminés par les HAP sur la viabilité et la reproduction des collemboles, par la présence des congénères de faible poids moléculaire, de type phénanthrène, fluorène, pyrène, fluoranthène. Il serait utile de vérifier sur d'autres types d'échantillons contaminés par les HAP que la toxicité sur les invertébrés peut être prédite par ces seuls hydrocarbures.

Les essais de bioaccumulation sur vers de terre ont montré une biosorption des HAP de cinq cycles et plus. Les résultats ont montré que les HAP lourds (5 cycles et plus) étaient plus fortement accumulés que les légers (3 et 4 cycles), naphtalène exclu. Comme les HAP lourds sont hydrophobes, ces résultats démontrent que ce n'est pas uniquement la fraction hydrosoluble des hydrocarbures qui est biodisponible, comme l'ont conclu plusieurs auteurs. L'absorption des xénobiotiques par la voie digestive suite à l'ingestion de sol est à prendre en considération, en particulier pour les vers de terre. La lipophilie des molécules peut expliquer la biosorption, mais l'intervention d'autres mécanismes de biosorption est possible. Il reste aussi à étudier si les HAP lourds sont génotoxiques chez ces invertébrés et dans quelle mesure ils participent à la toxicité à long terme.

► Les essais sur plantes confirment que la germination est peu, sinon pas, affectée par la toxicité des polluants et qu'il faut impérativement étudier la croissance pour identifier des effets de phytotoxicité potentiels.

► La comparaison des réponses des invertébrés aux polluants du sol de cokerie étudié fait apparaître une sensibilité plus élevée en sol artificiel ISO qu'en sol naturel. En d'autres termes, les conditions standardisées ISO pourraient constituer un pire cas dans l'évaluation des dangers et des risques posés par les sols contaminés. L'utilisation d'un sol contrôle plus représentatif des conditions environnementales serait tout à fait appropriée à une évaluation des risques reflétant une situation locale. La tendance mise en évidence avec les invertébrés est celle d'une sensibilité moindre aux polluants toxiques en présence d'un sol favorisant la croissance des populations.
Il serait très utile d'analyser plus avant cette tendance, voire la confirmer, en testant une variété de sols contrôles naturels de composition différente.

► En ce qui concerne le transfert des HAP dans la chaîne alimentaire, il apparaît qu'une absorption par les végétaux et les vers de terre a bien lieu à partir d'un sol

contaminé, mais cette absorption est très faible. Chez les végétaux, le phénomène de translocation à partir des racines aboutit à des concentrations infimes dans les pousses, et ne semble pas mettre en danger la santé des consommateurs dans le cas de sols anciennement contaminés par les HAP. La voie de transfert des HAP sur les parties aériennes des plantes par dépôt des poussières atmosphériques n'a pas été étudiée dans cette étude. Or, c'est une voie majeure de contamination des végétaux qui ne peut être occultée et qui est à prendre en compte in situ pour l'évaluation des expositions.

CHAPTER I. INTRODUCTION

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Abandoned waste sites present a risk to human health. The Agency for Toxic Substances and Disease Registry (ATSDR) found that some heavy metals, volatile organic compounds, and other specific substances occur at levels of health concern in the bodies of exposed people. ATSDR concluded that "uncontrolled hazardous waste sites and unplanned releases of hazardous substances that constitute emergency events are a major environmental threat to human health" (www.atsdr.cdc.gov).

According to the latest estimates there might be more than two million sites across Europe potentially contaminated from localized pollution sources, with an estimated 100 000 considered as needing remediation. The largest concentrations of sites are estimated to be around the old industrialized heartlands (European Environment Agency, www.eea.eu.int).

In the 1970s, France was probably one of the first European countries to carry out inventories of polluted sites, but limited attention was paid to the problems of land pollution until the beginning of the 1990s (www.sites-pollues.ecologie.gouv.fr). Since 1993 the French Ministry for Ecology and Sustainable Development has run a National Register, referring to sites that have been reported by the local authorities as being in fact or potentially polluted and needing specific administrative action. By December 1997 it referred to 896 contaminated sites and 125 sites that had already been restored with or without any limitation in land use. (www.ecologie.gouv.fr). Apart from the investigation of operating sites, the ministry conducts a systematic national survey of old and abandoned industrial sites. These inventories are managed on a regional scale. As an indication, in the Lorraine region for this study, north-east France, some 20 000 old sites were identified through research into public and private archives (French Geological Survey, www.brgm.fr).

The US EPA (ORD, Office of Research and Development) identified contaminated soils as one of four broad research topic areas related to environmental problems to cover the full range of waste-related research. The US EPA concluded the focus of research activities of contaminated soil is on the issues of improved exposure and risk assessment of soils, the application and

management of natural and accelerated process for remediation, and the demonstration and verification of innovative characterization and remediation technologies in soils.

In France, the various public institutions (CNRS: French National Center for Scientific Research, www.cnrs.fr; INERIS: French Environment and Risk Institute, www.ineris.fr, etc.) have involved a number of international and european coordinating programmes for research and technological development in contaminated-land management. The French Energy and Environment Agency has developed inventories of rehabilitation methods for contaminated sites and established a national research project on the methods most commonly applied to the main types of polluted sites encountered (ADEME, www.ademe.fr).

Polycyclic aromatic hydrocarbons (PAHs) occur naturally, notably in oil and coal deposits. PAHs are also products of incomplete combustion of organic compounds, and they are released into the environment both by the human activities and by natural events, e.g. forest fires and volcanic activity. PAHs are of particular concern because of the carcinogenic and/or mutagenic properties of some congeners of high molecular weight (>4 rings). Inevitably, there are questions about possible harmful effects on ecosystems that are exposed to high levels of them (Walker, 2001).

The effect and fate of PAHs in nature are of great environmental and human health concerns due to their widespread occurrence, persistence in terrestrial ecosystems, which have resulted in numerous studies on contaminated soils. Nevertherless as to PAHs, there still exists a lack of information on ecotoxicological data in soil. Indeed, actual environmental hazard assessment of soils has been usually performed by chemical analysis.

Our study is based on these research needs. The major focus of this study was to integrate chemical analysis with a battery of bioassays and to assess the bioavailability, toxicity, and genotoxicity of complex PAH mixtures in a contaminated soil from a former cokery site.

CHAPTER II. LITERATURE REVIEW

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II.1. Polycyclic Aromatic Hydrocarbons (PAHs): Sources and Exposures

Polycyclic aromatic hydrocarbons (PAHs) are produced by natural sources, such as volcanic eruptions and grass and forest fires, and by a number of man-made sources including: motor vehicles, coal combustion, steel, iron, and aluminum production, residential heating and cooking using wood and coal-based products, and incineration of refuse. PAHs are commonly found at sites from formerly manufactured gas plants, wood-treating facilities, municipal and industrial landfills, coal, petroleum, and petrochemical processing and manufacturing plants, and `military facilities. Numerous PAHs are ubiquitous in the environment and biota due to discharges to water, soil, and sediments from industrial and domestic waste and wastewater effluents, urban runoff, and discharges to atmosphere from incomplete combustion of fossil fuels (coal, fuel oil, gasoline) and subsequent wet and dry atmospheric deposition (IPCS EHC 202, 1998).

The US EPA has identified sixteen PAHs as priority pollutants due to their particular toxicity to mammals and aquatic organisms. These priority pollutant PAHs include: *naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-c,d]pyrene.*

PAHs commonly encountered as environmental pollutants have two to six fused aromatic rings, and molecular weights (Mol Wt) ranging from 128-278 g/mol. Their physicochemical properties, represented by parameters such as water solubility, octanol-water (K_{ow}) and organic carbon partition coefficient, regulate their distribution between the atmosphere, hydrosphere, and biosphere (Fig. 1, Table 1).

As PAH are hydrophobic with low solubility in water, their affinity for the aquatic phase is very low; however, in spite of the fact most PAHs are released into the environment via the atmosphere, considerable concentrations are also found in the hydrosphere because of their low Henry's law constants. As the affinity of PAH for organic phases is greater than that for water, their partition

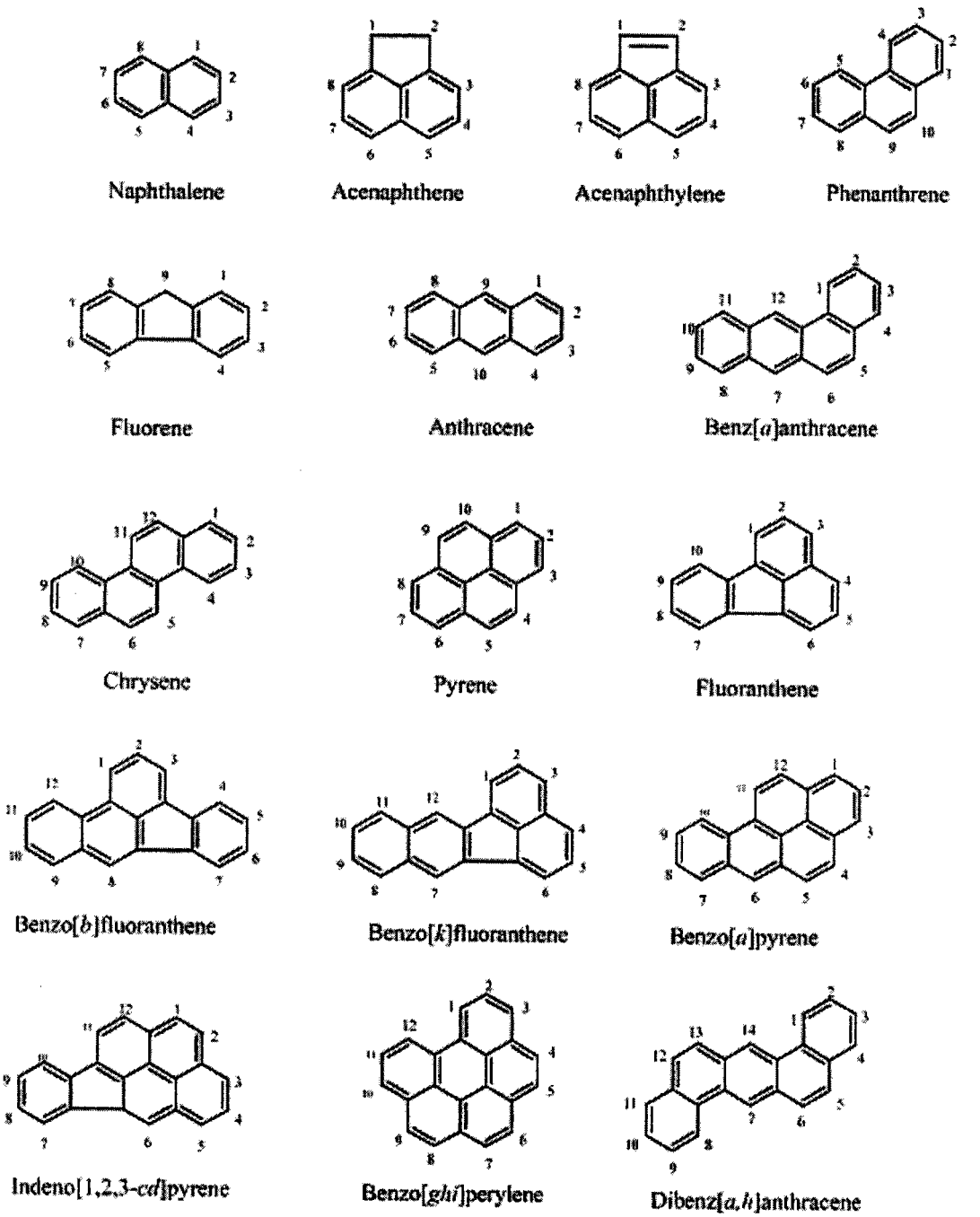

Fig. 1. Chemical structures of the 16 PAHs on the EPA priority pollutant list.

Table 1. Physicochemical properties of 16 PAHs on the EPA priority pollutant list (US EPA, 1984).

PAH compound	No. of Rings	Abbreviation	CAS No.	Mol Wt [a] (g/mol)	Log K_{oc} [b] (unitless)	Solubility [a] (mg/L)	Log K_{ow} [a] (unitless)	Vapour pressure [a] (Pa at 25°C)
Naphthalene	2	NA	91-20-3	128	3.11	31.7	3.4	10.4
Acenaphthylene	3	ACL	208-96-8	152	3.83	-	4.07	8.9×10^{-1}
Acenaphthene	3	AC	83-32-9	154	3.79	3.93	3.92	2.9×10^{-1}
Fluorene	3	FL	86-73-7	166	4.15	1.98	4.18	8.0×10^{-2}
Phenanthrene	3	PHE	85-01-8	178	4.22	1.29	4.6	1.6×10^{-2}
Anthracene	3	ATR	120-12-7	178	4.41	0.073	4.5	8.0×10^{-4}
Fluoranthene	4	FA	206-44-0	202	4.74	0.260	5.22	1.2×10^{-3}
Pyrene	4	PY	129-00-0	202	4.82	0.135	5.18	6.0×10^{-4}
Benzo[a]anthracene	4	BaA	56-55-3	228	5.25	0.014	5.61	2.8×10^{-5}
Chrysene	4	CHR	218-01-9	228	5.37	0.002	5.91	8.4×10^{-5}
Benzo[b]fluoranthene	5	BbFA	205-99-2	252	5.89	0.001	6.12	6.7×10^{-5}
Benzo[k]fluoranthene	5	BkFA	207-08-9	252	5.89	0.0008	6.84	1.3×10^{-8}
Benzo[a]pyrene	5	BaP	50-32-8	252	5.71	0.0038	6.50	7.3×10^{-7}
Dibenzo[a,h]anthracene	5	DBahA	53-70-3	278	5.97	0.0005	6.50	1.3×10^{-8}(20°C)
Benzo[g,h,i]perylene	6	BghiP	191-24-2	276	-	0.00026	7.10	1.4×10^{-8}
Indeno[1,2,3-c,d]pyrene	6	IP	193-39-5	276	6.14	0.0062	6.58	1.3×10^{-8}(20°C)

[a] Data from Environmental Health Criteria 202 (IPCS, 1998).

[b] Data from Sverdrup et al., (2002b).

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Benzo[b]fluoranthene	5	BbFA	205-99-2	252	5.89	0.001	6.12	6.7×10^{-5}
Benzo[k]fluoranthene	5	BkFA	207-08-9	252	5.89	0.0008	6.84	1.3×10^{-8}
Benzo[a]pyrene	5	BaP	50-32-8	252	5.71	0.0038	6.50	7.3×10^{-7}
Dibenzo[a,h]anthracene	5	DBahA	53-70-3	278	5.97	0.0005	6.50	1.3×10^{-8}(20°C)
Benzo[g,h,i]perylene	6	BghiP	191-24-2	276	-	0.00026	7.10	1.4×10^{-8}
Indeno[1,2,3-c,d]pyrene	6	IP	193-39-5	276	6.14	0.0062	6.58	1.3×10^{-8}(20°C)

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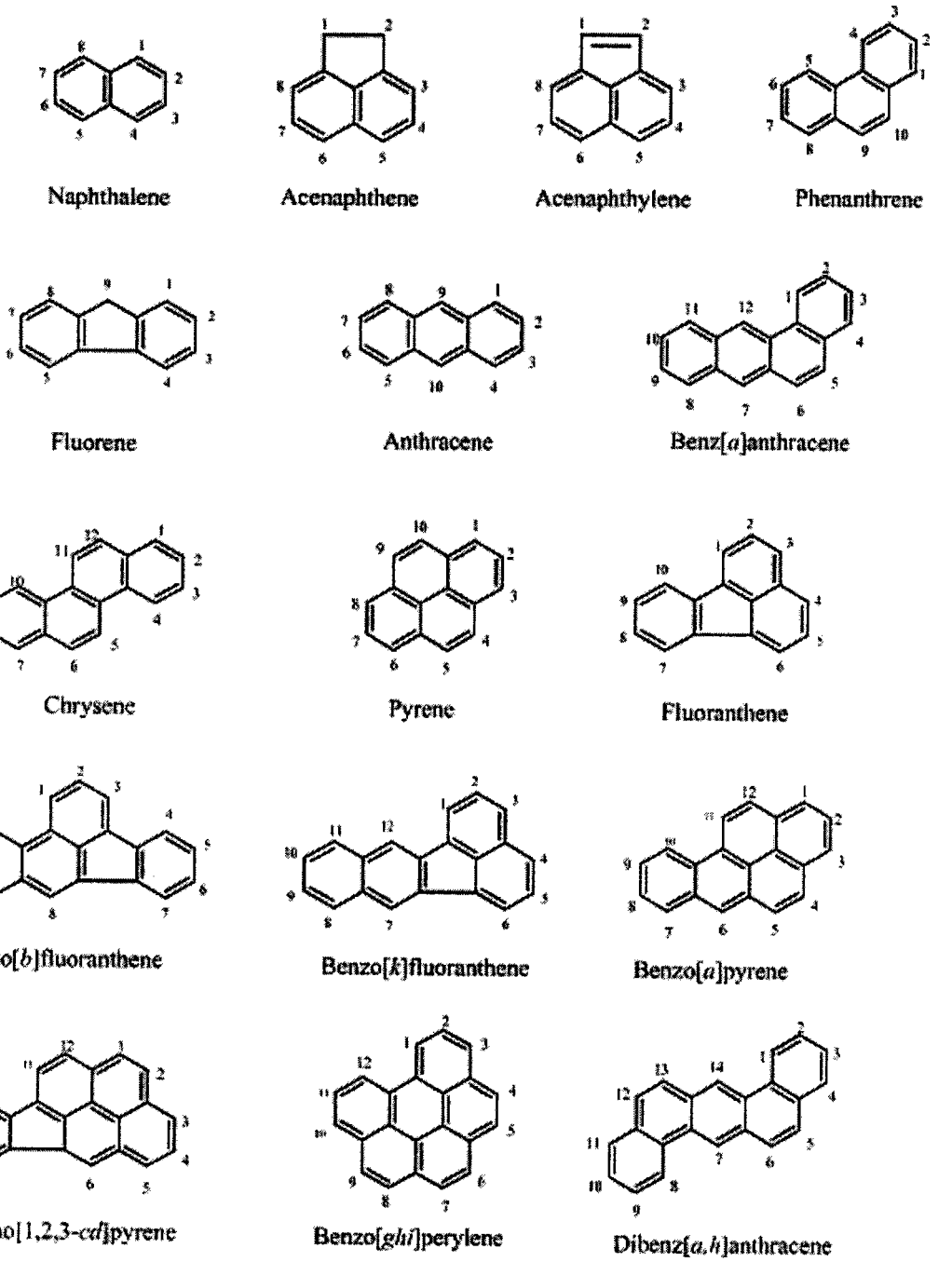

Fig. 1. Chemical structures of the 16 PAHs on the EPA priority pollutant list.

Table 2. Genotoxicity and carcinogenicity characteristics of 16 PAHs on the EPA priority pollutant list.

PAH compound	No. of Rings	TEFs [a]	Carcinogenicity Classification [b]		Genotoxicity Classification [d]
			(IARC) [b]	(US EPA) [c]	(SCF)
Naphthalene	2	0.001			PNG
Acenaphthylene	3	0.001		D	DI
Acenaphthene	3	0.001			DI
Fluorene	3	0.001	3	D	DI
Phenanthrene	3	0.001	3	D	E
Anthracene	3	0.01	3	D	L
Fluoranthene	4	0.001	3	D	E
Pyrene	4	0.001	3	D	NG
Benzo[a]anthracene	4	0.1	2A	B2	G
Chrysene	4	0.01	3	B2	G
Benzo[b]fluoranthene	5	0.1	2B	B2	G
Benzo[k]fluoranthene	5	0.1	2B	B2	G
Benzo[a]pyrene	5	1	2A	B2	G
Dibenzo[a,h]anthracene	5	5	2B	B2	G
Benzo[g,h,i]perylene	6	0.01	3	D	G
Indeno[1,2,3-c,d]pyrene	6	0.1	2B	B2	G

[a] Toxic equivalent factor in B(a)P according to Nisbet and LaGoy (1992).

[b] IARC (International Agency for Research on Cancer):

- 2A: probably carcinogenic to human
- 2B: possibly carcinogenic to human
- 3: not classifiable.

[c] US-EPA, IRIS (Intergrated Risk Information System):

- B2: probably carcinogenic to human
- D: not classifiable.

[d] EC, SCF (Scientific Committee on Food) (2002):

- PNG: probably not genotoxic
- DI: database inadequate for evaluation
- L: limited evidence
- E: equivocal
- NG: not genotoxic.
- G: genotoxic.

coefficients between organic solvents, such as octanol, and water are high. Their affinity for organic fractions in sediment, soil, and biota is also high, and PAH thus accumulate in organisms in water and sediments (IPCS EHC 202, 1998). The toxicity of individual PAHs to soil organisms can be described by their lipophilicity (log K_{ow}) and soil sorption properties (i.e., log K_{oc} combined with information on the organic carbon content of the soil used (Sverdrup et al., 2002b).

The main sources of PAH in soil are atmospheric deposition, carbonization of plant material, and deposition from sewage and particulate waste. The extent of pollution of soil depends on factors such as its cultivation, porosity, and content of humic substances. Near industrial sources, individual PAH levels of up to 1 g/kg soil have been found. The concentrations in soil from other sources such as automobile exhaust, are in the range 2 - 5 mg/kg. In polluted areas, the PAH levels were 5 - 100 μg /kg soil dw (IPCS EHC 202, 1998).

Food can be contaminated by environmental PAH that are present in air (by deposition), soil (by transfer) or water (by deposition and transfer), or during processing and cooking (EC SCF, 2002). The levels of individual PAHs in meat, fish, dairy products, vegetables and fruits, cereals and their products, sweets, beverages, and animal and vegetable fats and oils were within the range 0.01 - 10 μg/kg dw (IPCS EHC 202, 1998).

Occupational exposure to PAHs also could be significant since many products, such as asphalt, soot, coal tar, petroleum, and cutting oils, contain and release PAHs (EC SCF, 2002). Of the sixteen priority PAHs pollutants, the US EPA has characterized seven as probable human carcinogens (Group B2). The seven PAHs that are likely to be carcinogenic are identified in Table 2. These compounds pose a significant potential health threat to human and ecological populations due to their toxicity and widespread occurrence. The remaining PAHs in Table 2 are not classifiable as to human carcinogenicity (Group D) based on inadequate evidence for carcinogenicity in animals, and mammals especially. Details regarding the noncarcinogenic and carcinogenic hazard potential of PAHs can be found on-line at the US EPA Integrated Risk Information System (IRIS) database (www.epa.gov/iris).

II.2. PAH Bioactivation and Genotoxicity

PAHs constitute a major class of chemical carcinogens present in the environment. These compounds require activation to electrophilic metabolites to exert

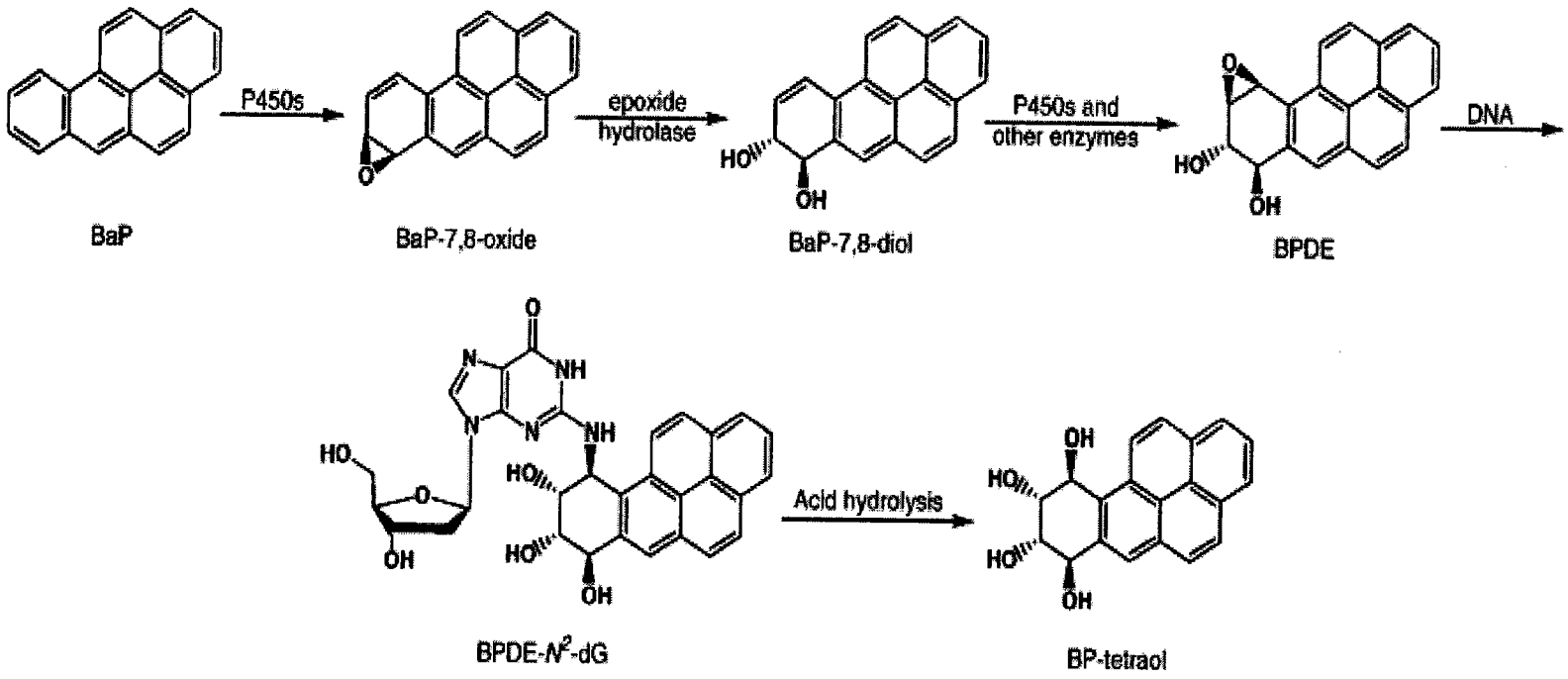

Fig. 2. The major established pathway of metabolic activation of BaP (Benzo[a]pyrene), leading to the formation of BPDE-N^2-dG, shown as the nucleoside obtained by enzymatic hydrolysis of DNA. There is convincing evidence for the presence of this DNA adduct in tissues of laboratory animals and humans exposed to BaP. The adduct results from the reaction with DNA of BPDE. BPDE is one of four possible stereoisomers of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. BPDE is the most carcinogenic of these isomers and the most reactive with DNA. BPDE also reacts with hemoglobin and serum albumin to form protein adducts. Serum albumin adducts of the opposite enantiomer to BPDE have also been observed. Hydrolysis of BPDE-N^2-dG or BPDE-protein adducts leads to BP-tetraols. The BP-tetraol isomer shown is the major one that has been observed in most studies. P450s: cytochrome P450s. (from Boysen & Hecht, 2003)

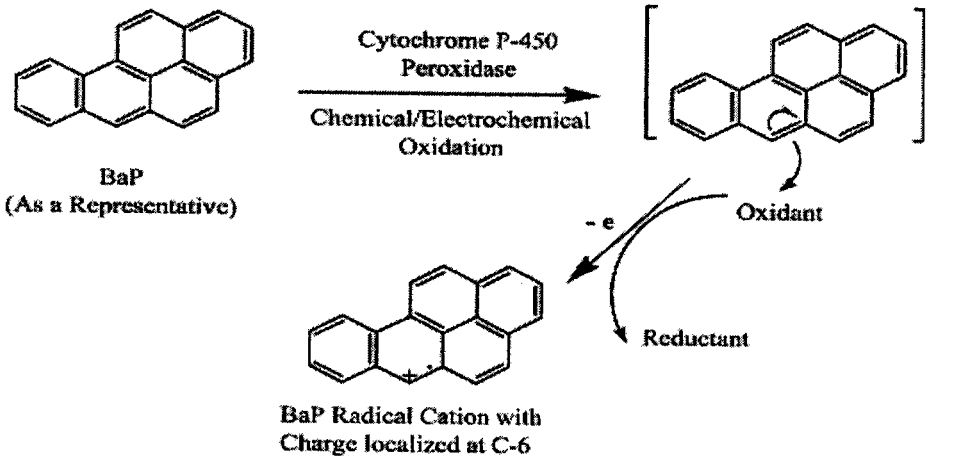

Fig. 3. Formation of PAH radical cation (from Xue & Warshwsky, 2005).

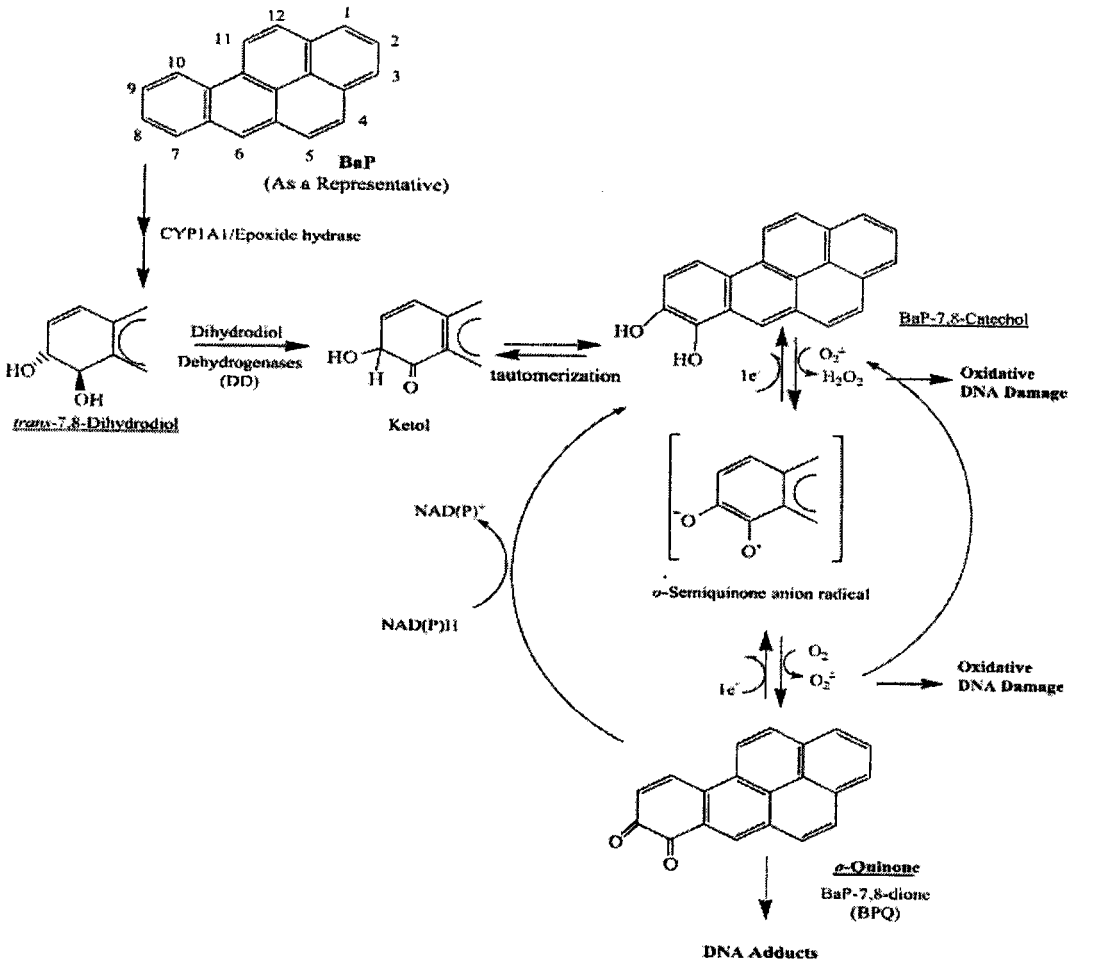

Fig. 4. Metabolic activation pathway of PAH via *o* -quinone (from Xue & Warshwsky, 2005).

their mutagenic or carcinogenic effects. There are three principal pathways currently proposed for metabolic activation of PAH: the pathway via bay region dihydrodiol epoxide by cytochrome P450 enzymes (CYPs), the pathway via radical cation by one-electron oxidation, and the ortho-quinone pathway by dihydrodiol dehydrogenase (DD). In addition to these major pathways, a brief description of a minor metabolic activation pathway, sulfonation, for PAHs that contain a primary benzylic alcoholic group of secondary hydroxyl group(s) comprehensively reviewed (Xue & Warshwsky, 2005).

A widely accepted pathway of PAH activation involves formation of electrophilic diol epoxides. Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation as the first step. The resultant epoxides or phenols might get detoxified in a reaction to produce glucoronides, sulfates or glutathione conjugates. Some of the epoxides might metabolize into dihydrodiols, which in turn, could undergo conjugation to form soluble detoxification products or be oxidized to diol-epoxides. Many PAHs contain a bay-region as well as a K-region, both of which allow metabolic formation of bay-and K-region epoxides, which are highly reactive (Samanta et al., 2002). The bioactivation via the diol-epoxide pathway of benzo[a]pyrene (BaP), a representative PAH, is presented in Fig. 2.

It is known that the radical cation of PAH is formed by removal of one electron from the π electron system of the molecule through one electron oxidation. Radical cations are electrophilic in nature, capable of interacting with nucleophilic centers in cellular macromolecules. Therefore, the formation of radical cation in metabolic oxidation process catalyzed by P450 peroxidase was proposed to be one of the pathways of PAH activation shown in Fig. 3 (Cavalieri & Rogan, 1995).

The formation of *o*-quinones by dihydrodiol dehydrogenases (DD)-catalyzed oxidation was proposed in the late 1980s of the last century as the 3rd major metabolic activation pathway of PAH (Penning et al., 1999). The redox active PAH-*o*-quinone can be reduced to reform catechol by a nonenzymatic two-electron reduction or the semiquinone anion radical (SQ) via a one-electron enzymatic reduction. In the metabolic process, reactive oxygen species (ROS) are generated. Therefore, *o*-quinone formation is now considered to be a metabolic activation pathway rather than detoxification (Penning et al., 1999) (Fig. 4).

For anthracene, not classifiable as carcinogenic, the evidence of genotoxicity is limited and mainly based on results obtained *in vitro*. Further studies, especially

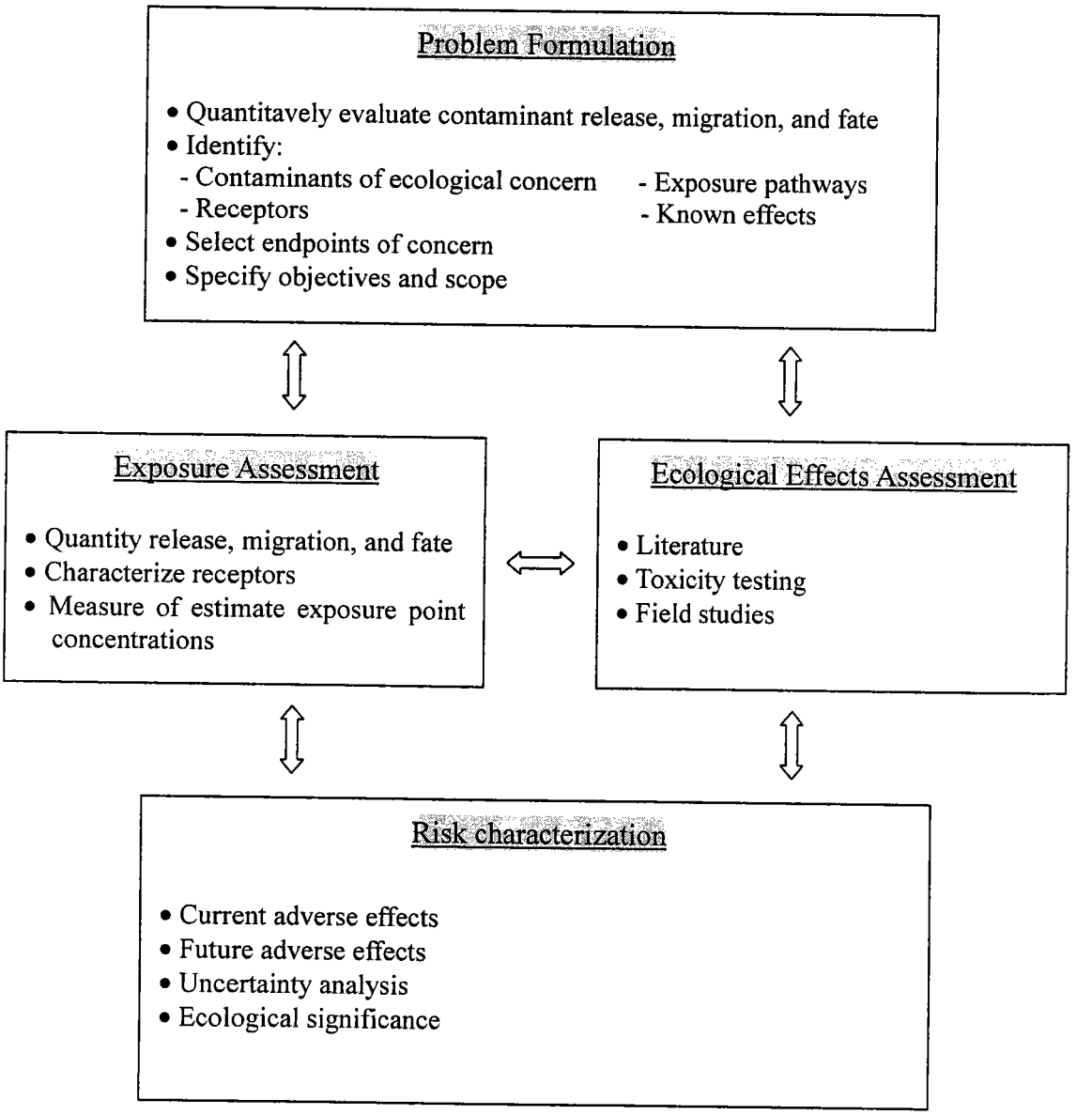

Fig. 5. Components of environmental risk assessment paradigm (from US EPA, 1996).

in vivo, are warranted to clarify the genotoxic potential of these PAH. Equivocal or contradictory data are available for five other compounds (acenaphthene, acenaphthylene, fluorene, phenanthrene, fluoranthene), which cannot be properly evaluated for genotoxicity. Finally, three compounds (naphthalene, anthracene, pyrene) gave totally or mainly negative results in a variety of short-term tests (EC SCF, 2002) (Table 2)

II.3. Risk Assessment of PAH-Contaminated soils

Risk assessment is the process whereby biological, dose-response, and exposure data are combined to produce a qualitative evaluation and quantitative estimate of an adverse outcome from a defined activity and chemical agent. The risk assessment paradigm, first presented by the US National Research Council (NRC, 1983), can be viewed as a four-step process with the concepts or components of problem formulation (hazard identification), exposure assessment, ecological effects assessment, and risk characterization (Fig. 5). Problem formulation (hazard identification) is the most critical stage in ecological risk assessment.This stage involves identifying the actual environmental values to be protected (assessment endpoints) and selecting ways in which these can be measured and evaluated (measurement endpoints) (US EPA, 1996).

The key information required during this phase of the risk assessment process are as follows:

(a) potential/actual contaminant of concern,

(b) source of contaminant ; current and historic use,

(c) mode of action of the contaminant,

(d) contaminant characteristics (e.g. physical/chemical properties and environmental environmental behaviour, persistence in the ecosystem, transformation products and bioaccumulation),

(e) ecosystem potentially at risk and

(f) areas of uncertainty (Iscan, 2004).

Further stages of risk assessment – *exposure and effects assessment, then risk Characterization*–require additional information and can include other tools such as measures of exposed populations of animals and plants, long-term laboratory or field bioassays, toxicity identification evaluations, etc. Finally, risk characterization builds upon the results of the analysis phase to develop an estimate of risk (Fent, 2003).

Toxicological risk assessment of PAHs usually focuses on their genetic or genotoxic health effects. This is because their carcinogenic effects in human and ecological receptors are assumed to result from long-term, low-dose exposures through diet, occupation, and environment that tend to over shadow noncarcinogenic toxic effects that occur at much higher doses and are not typically observed with environmental exposures. In addition, noncarcinogenic toxic endpoints tend to be transitory and dependent on exceeding dose thresholds. Genotoxicity, and particularly DNA damage to gametes, potentially have a greater negative impact on populations since such effects could be transmitted between generations (IPCS EHC 202, 1998).

PAH contaminated soils are typically characterized by very complex mixtures of chemicals (Sverdrup et al., 2002b). Lack of information about the behavior of toxic substances in complex mixtures is often avoided by assuming that the toxicity of a mixture is simply the sum of the expected effects from each mixture component, i.e. no synergistic or antagonistic interactions (US EPA, 1989).
Although this assumption is supported by research investigating non-genotoxic endpoints, the literature describing the behavior of genotoxic substances in complex mixtures is sparse and, occasionally, contradictory (White, 2002). White (2002) found that the actual mutagenic hazard posed by an unsubstituted and homocyclic PAH mixture is likely to be equal to, or lower than, the sum of the hazards posed by each mixture component. This could be due to chemical interactions or due to the presence of unidentified chemicals, such as alkyl PAHs or high molecular weight PAHs that are not included in the standard risk assessment procedure (Donnelly et al., 2004).

Toxicity of some major groups of structurally-related compounds (e.g., PAHs) may be assessed using a toxicity equivalent factor (TEF) approach. The TEF approach is used in several european countries (CARACAS, 1998). The relative potency was expressed as toxic equivalency factors (TEF) for individual PAH (relative to benzo[a]pyrene) with the purpose of summarizing the contributions from individual PAH in a mixture into a total benzo[a]pyrene equivalent dose, assuming additivity in their carcinogenic effects (Nisbet & Lagoy, 1992). The TEF approach is used to normalize exposures to chemicals with the same mechanism of action (common mechanism chemicals) with different potencies to yield a total equivalent exposure (TEQ) to one of the chemicals, the "index compound" (EC SCF, 2002).

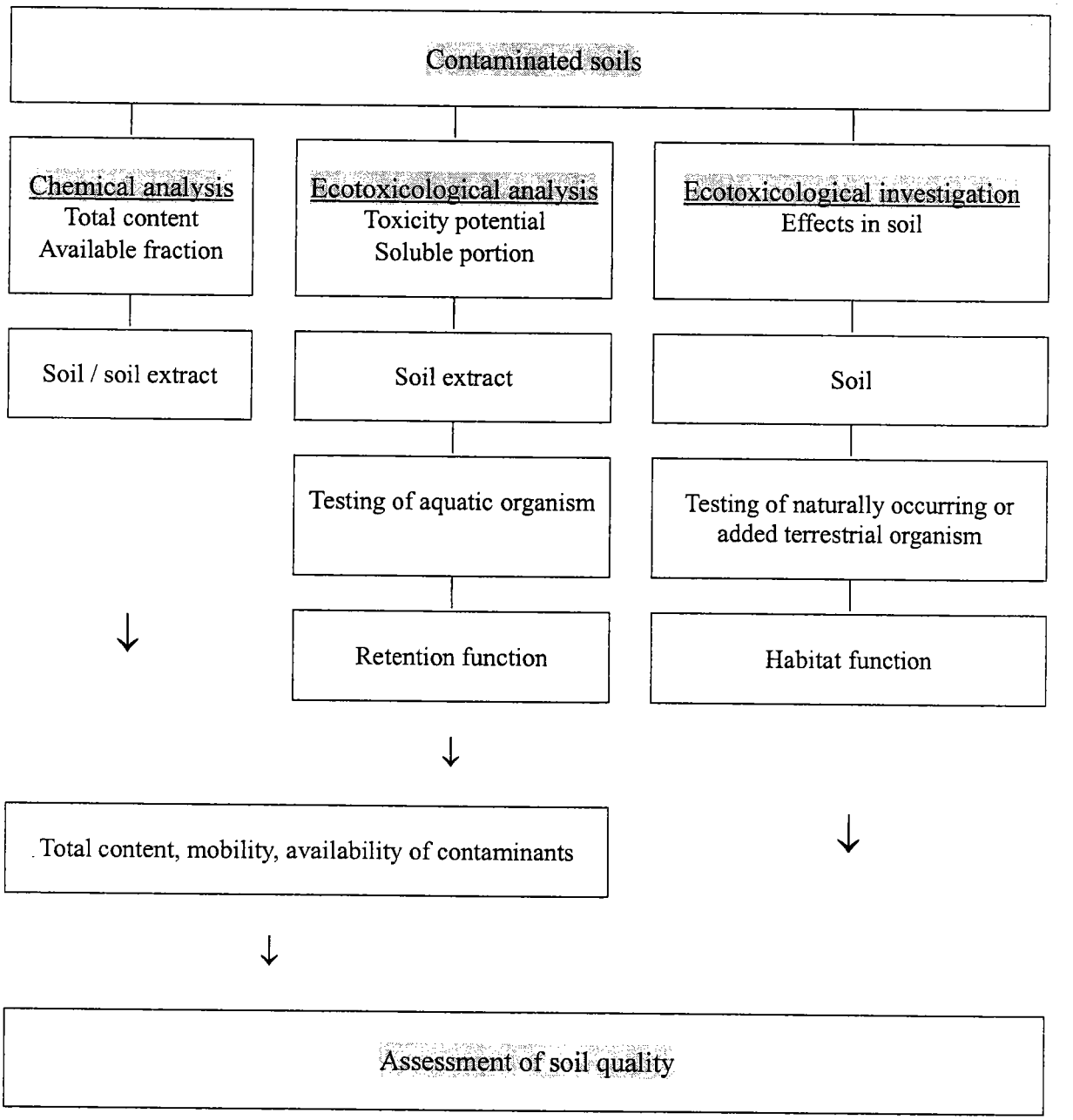

Fig. 6. Test strategy for the assessment of contaminated soils (from Debus & Hund, 1997).

II.4. Ecotoxicological Assessment of Contaminated soils

Actual environmental hazard assessment of soils is usually performed exclusively by chemical analysis. However, not all the present contaminants in the soil, including metabolites or transformation by-products, may be considered during the analysis, resulting in an underestimation of the real environmental risk. Furthermore, the chemical analysis does not allow an integration of the combined effects produced by the chemical mixture present at a polluted site. In addition, total concentrations can overestimate the real risk, as aging processes can strongly reduce the bioavailability and, subsequently, the toxicity of pollutants. Bioassays try to integrate these effects and help in evaluating the risk associated with exposure to bioavailable substances present in a soil (Fernandez et al., 2005).

Soil quality tests using bacteria, plants and on invertebrates are promising tools for risk-based correction action. The effects-based assessment provides more realistic targets for cleanup criteria than non-specific measurements such as total petroleum hydrocarbons (TPH) (Plaza et al., 2005b).

Debus & Hund (1997) proposed the strategy for assessing contaminated soils (Fig. 6). Tests with aquatic organisms in soil extracts (generally aqueous) are suitable for determining the potential toxicity in soils. To evaluate the habitat function, tests are performed on organisms occurring directly in the soil. In case where this is not feasible, terrestrial organisms may be added to the test soils. With this kind of procedure, the assessment of the soil quality is based on the results regarding mobility and availability of the contaminants, the toxicity potential in soil extracts and the harmful effects on soil organisms.

The intentional and accidental discharges of toxic pollutants into the lithosphere results in soil contamination. In some cases (e.g. wood preserving wastes, coal-tar, air-born combustion by-products), the contaminated soil constitutes a genotoxic hazard. There is a paucity of information about the sources and potential hazards of mutagens in soil. Thus, there is a need to investigate the mutagenic hazards of contaminated soils and incorporate the use of well-established assays in assessments of potential hazard (White & Claxton, 2004).

II.5. Bioavailability and Bioaccumulation of Contaminants in Soils

Biological availability is a key word that is often encountered in current

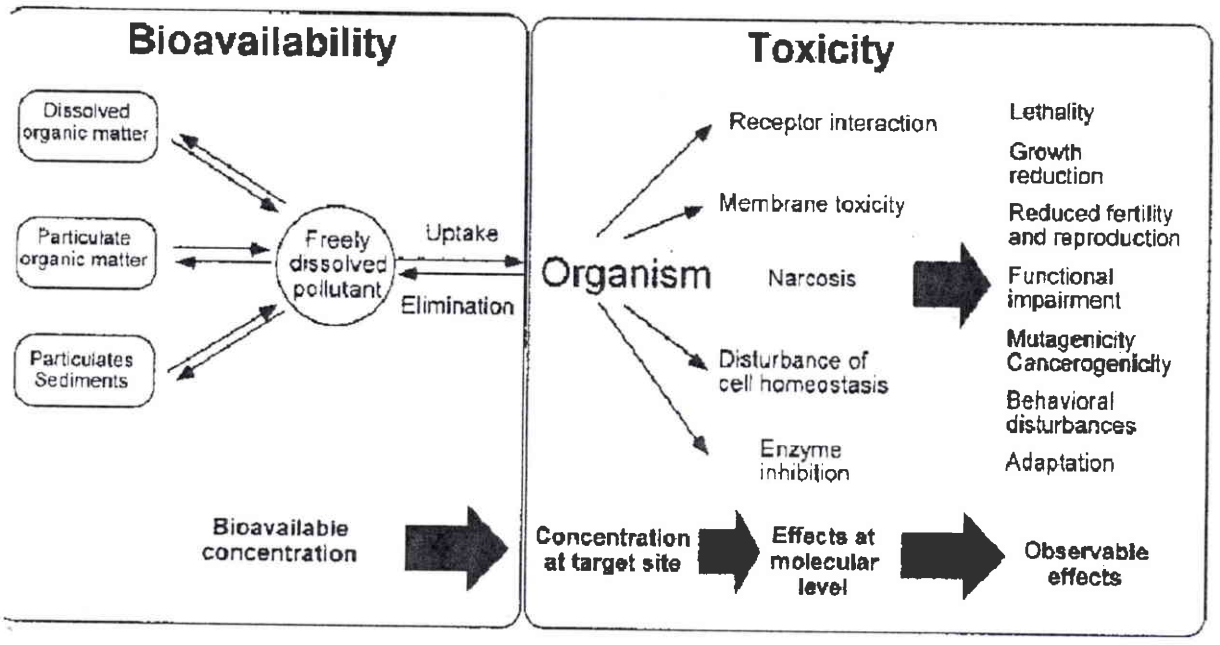

Fig. 7. Ecotoxicological effects are dependent on the bioavailable fraction of pollutants, and concentrations at the target sites induce molecular effects that propagate to a variety of toxic manifestations in organisms (from Fent, 2003).

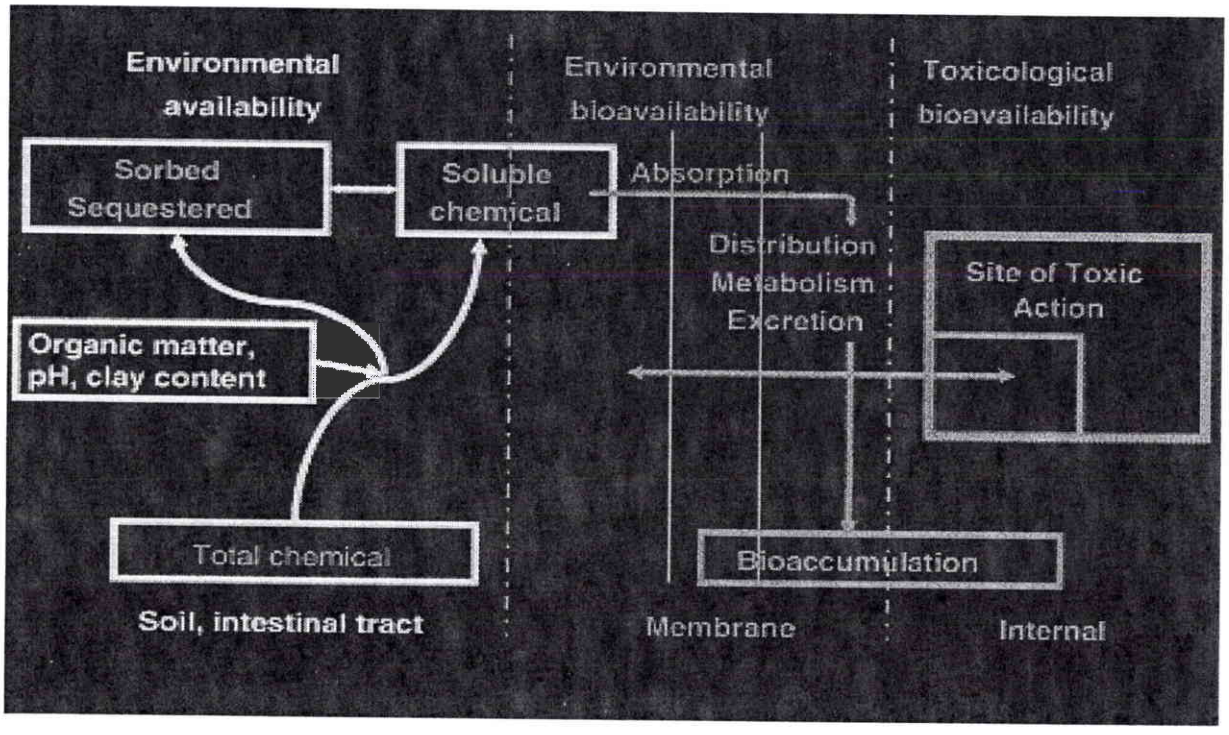

Fig. 8. Schematic model of bioavailability (from Lanno et al., 2004).

publications on the fate of pollutants in ecosystems and their efforts on individual species, populations, and ecosystem levels (Peijnenburg et al., 2002). Beck et al., (1996), defined bioavailability as 'the fraction of a chemical that is absorbed and available for metabolism, storage or excretion by the animal'. The bioavailability of chemicals, which is dependent on biogeochemical and physiological process, is an important factor, often neglected in ecotoxicological evaluation and hazard assessment. The bioavailable fraction is critical for uptake, and ultimately, for the concentration at the target site in organism (Fig. 7) (Fent, 2003). To environmental scientists, bioavailability represents the accessibility of a chemical for assimilation and possible toxicity (Alexander, 2000).

Alexander (2000) reviewed informations on differences of bioavailability among organism species, environments (soil types), and compounds in soils. Two possible mechanisms of the sequestration (often designated "aging") and resultant decline in bioavailability of organic chemicals in soil were also discussed: partitioning of compounds into soil organic matter and entrapment of molecules in micropores. According to the former, chemicals sorb rapidly to external surfaces of soil and then slowly partition into interior regions of the solid organic material. The author concluded that the bioavailability of organic pollutants in soil decline with time (aging or weathering) and that current analytical methods (a vigorous extraction of soils with organic solvents), because they measure total and not bioavailable concentrations, may overestimate the magnitude of the environmental and societal problem from these pollutants.

A role for organic matter content of soil in reducing the bioavailability of aged phenanthrene for microbial degradation has been proposed, although organic matter may not be the only soil property that is important (White et al., 1997; Nam et al., 1998; Chung & Alexander, 1998; 2002). Sequestration of compounds in soils could be considered as the decrease in availability of the compound for uptake by a living organism and for extraction (Nam et al., 1998). Aging of phenanthrene in sterile samples of dissimilar soils showed that this compound became less available with time to bacteria, earthworms, or both. Aging could be markedly affected by soil properties such as aggregate size, organic matter content, clay content and environmental factors (White et al., 1997). Soil texture and structure appeared to play a major role in PAH availability and biodegradation (Amellal et al., 2001). The organic matter content of soil is a major determinant of soil aging (sequestration) (White et al., 1997; Nam et al., 1998). Chung & Alexander, (2002),

analyzed sixteen soils with markedly different properties to determine their porosity in the range of 7 nm-10μm, cation-exchange capacity (CEC), surface area and clay mineralogy. Correlations were sought between soil characteristics and four methods of measuring sequestration of phenanthrene. They found that some but not all measures of phenanthrene sequestration was highly correlated with organic carbon content, nanoporosity or CEC but not other properties of the soils.

The soil pore-water fraction is a major exposure route for chemicals for several soil-inhabiting animals, which implies that the bioavailability of contaminants will depend mainly on the sorption equilibrium between soil and pore water. For earthworms, sorptions data can be used to estimate bioavailability and toxicity of organic contaminants (Smit et al., 1997). The sublethal bioassays using snail, a terrestrial invertebrate, have been proposed to evaluate the potential impact of pollutant present in the soil via oral and dermal exposure. Under these conditions, the intoxication of the snail can result from soil ingestion but also from epithelial absorption of the contaminants (Gomot-de Vaufleury & Bispo, 2000).

In a review of crop plant uptake and microbial biodegradation of organic compounds from sludge-amended soils it was concluded that the vast majority of organic compounds in sludge are so strongly sorbed by the soil-sludge matrix that they exhibit low bioavailability to crop plants and so they accumulate at very low concentrations in the edible portion of food crops (O'Connor, 1996) and there may be a reduction in the bioavailability due to increased sorption potential associated with greater organic matter contents (Beck et al., 1996).

A study was conducted to determine the extent of reduction in availability for genotoxicity of mutagens, such as PAHs, sorbed for short periods by soils differing appreciably in all range of properties. If soils with <0.7% organic matter were not considered, correlations were evident between organic carbon content and the bioavailability of the three PAHs (r values of 0.90 or higher). The results also showed that a mild solvent extraction (n-butanol) might provide the basis for a chemical assay to determine the quantity of sorbed compound that is available to exert a genotoxic effect. However, vigorous solvent extraction, as carried out by Soxhlet extraction (hexanes), is not correlated with availability (Alexander & Alexander, 2000)

The potential of a substance to bioaccumulate in the aquatic environment into an organism depends particularly on its lipophilicity and its metabolism within the organism. Bioaccumulation is an exposure-related parameter and will determine

the body burden, but it is not an 'effect' or hazard in itself. Therefore, when appropriate, bioaccumulation should be included as an exposure related parameter in the environmental risk assessment of substances (Feijtel et al., 1997; Amorim et al., 2002). Bioaccumulation tests can become important in order to estimate the accumulation of contaminants in the food web as may eventually prove to be dangerous to predatory animals (mammals, birds) who prey on organisms living in the soil (Kordel & Rombke, 2001). The bioavailability of chemicals to earthworms can be modified dramatically by soil physical/chemical characteristics, yet expressing exposure as total chemical concentrations does not address this problem. In order to understand the effects of modifying factors on bioavailability, one must measure and express chemical bioavailability to earthworms in a consistent, logical manner. This can be accomplished by direct biological measures of bioavailability (e.g., bioaccumulation, critical body residues), indirect biological measures of bioavailability (e.g. biomarkers, reproduction, toxicity, physiological effects), or indirect chemical measures of bioavailability (e.g., chemical or solid-phase extracts of soil). Direct biological measures of bioavailability, such as bioaccumulation, are determinations of the actual amount of chemical taken up by the earthworm and may provide the most accurate measure of bioavailability since they integrate all of the biotic (e.g., metabolism) and abiotic (e.g., pH, organic matter content of soil) modifying factors of chemical bioavailability (Lanno et al., 2004).

The conceptual model of bioavailability for earthworm is presented in Fig. 8. Bioavailability should be handled as a dynamic process that comprises two distinct phases: a physicochemically driven desorption process (also referred to as "environmental availability") and a physiologically driven uptake process (also referred to as "environmental bioavailability"). Since the internal concentration of the organism is related with organ-effect levels (also referred to as "toxicological bioavailability"), it is the determinant for the actual bioavailability (Peijnenburg et al., 1997). Some portion of the environmentally bioavailable fraction of the chemical may be absorbed across the external membranes of the earthworm (e.g., epidermis, gastrointestinal tract). Once absorbed, the chemical may be metabolized and excreted, accumulated in other tissues, sequestered internally, or transported in the organism to the sites of toxic action (STA). Toxicological bioavailability refers to that portion of absorbed chemical that reaches and interacts with the STA. In this model, bioaccumulation is associated with chemical residues accumulated

at the site of toxic action but below a toxic threshold, as well as residues in other tissues containing no site of toxic action (e.g., storage lipid) and represents an intermediate between environmetal bioavailability and toxicological bioavailability (Lanno et al., 2004). The ultimate way of assessing the bioavailability of chemical substances, is by actually measuring the amount of chemicals accumulated within organisms, preferably at the site of toxic action (Peijnenburg et al., 2002).

II.6. Bioassay For Quality Assessment of a Contaminated Soil Quality

An integrated approach considering environmental, chemical, toxicological, and ecological concepts is needed for the understanding of ecotoxicological effects in contaminated ecosystems. A more prospective approach is based on investigations of potential toxicological effects in laboratory assays that may be used for extrapolation to the field. Bioassays play an important role in this process. They are valuable tools in the characterization of the toxic action of chemicals and in the understanding of associated toxicity (Fent, 2003). The chemical analysis does not allow an integration of the combined effects produced by the chemical mixture present at a polluted site. In addition, total concentrations can overestimate the real risk, as aging processes can strongly reduce the bioavailability and, subsequently, the toxicity of pollutants. Bioassays try to integrate these effects and help in evaluating the risk associated with exposure to bioavailable substances present in a soil (Fernandez et al., 2005).

Actually, the biological approach enables to measure the consequence of several factors:

(1) different chemical forms present in the medium,

(2) phenomenon of synergy and of antagonism between compounds,

(3) bioavailability of compounds of the medium, that-is-to-say, their ability to penetrate into the body by digestive, branchial and dermal absorption, this biodisponibility is dependent on solubility of pollutants in the medium but also in the physiological liquids,

(4) ability of contaminants to be metabolized by the concerned organism or to be physico-chemically damaged (Bekaert et al., 2002).

Not only can pollutants pass from contaminated soils to plants (through root absorption and contamination with soil particles) and to the water-bearing stratum but this transfer may also be observed into the ambient air and to plants

(via degassing), as well as into surface water (by way of drainage discharge and erosion). It must be established that biological testing methods are available for the investigation of surface waters (e.g. with bacteria, algae or water fleas) for estimating the quality of soils (Kordel & Rombke, 2001).

Development of screening-level benchmarks for ecological risk assessment (ERA) of contaminated soils has become a critical need in recent years, primarily due to the large number of polluted sites undergoing environmental assessments in the USA (Kuperman et al., 2004). The US EPA is developing Ecological Soil Screening Level (Eco-SSL) benchmarks for the contaminants, such as PAHs and metals, most frequently found at Superfund sites. Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will theoretically be protective of terrestrial ecosystems from unacceptable harmful effects. The US EPA recommended soil toxicity tests such as plants or soil invertebrates (earthworms, Collembola etc.) to generate the data for deriving Eco-SSLs (US EPA, 2003). The toxicity tests available for assessing polluted sites were first described by the US EPA (1992). Several authors reviewed toxicity tests available for ecotoxicity assessment of contaminated soils (Keddy et al., 1995; Wang & Freemark, 1995; Cortet et al., 1999).

Hund-Rinke et al., (2002), performed a round-robin test to monitor the comparability of the results from ecotoxicological test methods on soils and to facilitate the standardization of corresponding test method. Contaminated soils were tested with the standardized test procedure for collembolae (*F. candida*), enchytraeids (*E. crypticus*) and lumbricids (*E. fetida*). They found that the different test methods investigated in the soil fauna round-robin test differed from each other in terms of effort, feasibility and sensitivity. By considering these properties, contaminated soils should possibly always be tested using a combination of different test methods ('test set'), whereby reproduction tests should be applied due to the sensitivity and validity necessary for this soil assessment.

The intentional and accidental discharges of toxic pollutants into lithosphere result in soil contamination. In some cases (e.g., wood preserving wastes, coal-tar, airborne combustion by-products), the contaminated soil constitutes a genotoxic hazard. The published information on soil mutagenicity were comprehensively reviewed. The majority of the assessments (38%) employed the Salmonella mutagenicity test with strains TA98 and/or TA100. Additional analyses showed significant empirical relationships between S9-activated TA98 mutagenicity and

Table 3. Aquatic and terrestrial bioassays used for evaluating contaminated soils and solid wastes from the literature.

Contaminants	Sample	Bioassays	Species	Measured endpoints	Criteria	Results obtained (relative sensitivities of species)	Reference
Petroleums (hydrocarbons)	Soil	Microtox® (solid phase) Earthworm Plant	*Vibrio fisheri* *Eisenia fetida* Corn, oat	Bioluminescence Mortality Germination, growth	EC_{50}, LC_{50}	- Earthworm 14d: most sensitive	Dorn et al., 1998 ; Dorn & Salanitro, 2000
	Leachate	Microtox® Spirotox Umu-test Plant	*Vibrio fisheri* *S. ambiguum* *S. typhimurium* Lettuce, rye etc.	Bioluminescence Mortality Genotoxicity Germination, root elongation	EC_{50}, LC_{50}	- Spirotox: most sensitive - Organic extracts more toxic - Lettuce most sensitive plant species	Plaza et al., 2005
	Leachate Soil	Microorganism RTG-2 fish cell Daphnid Algae Plant Earthworm	 *Daphnia magna* *C. vulgaris* Wheat, clover etc. *E. fetida*	Carbon transformation Cytotoxicity Immobilization Growth inhibition Germination, growth Survival	EC_{20}	- Microorganisms and plants most sensitive - Daphnids least sensitive - Plant growth more sensitive than germination	Fernandez et al., 2005
	Soil	Earthworm Plant	*E. fetida* Lettuce, corn etc.	Mortality, reproduction Germination, growth, root length	NOEC	- Reproduction and shoot growth more sensitive - mustard and lettuec more sensitive species	Saterbak et al., 2004
Explosives (2,4,6-TNT)	Leachate Soil	Microtox® Mutatox® Plant Collembola	*E. coli* *Vibrio fisheri* Cress *Folsomia candida*	Bioluminescence Mutagenicity Early seedling growth Reproduction	EC_{20}	- Sensitivity order : microtox > mutatox, cress growth > collembola reproduction	Frische, 2003
	Leachate Soil	Microtox® Algae SOS chromotest Plant Earthworm	*Vibrio fisheri* *S. capricornutum* *E. coli* Lettuce, barle *E. andrei*	Bioluminescense Growth (microplate) Genotoxicity Germination, growth Mortality, reproduction	EC_{50}, EC_{25}, LOEC, NOEC	- SOS chromotest more sensitive than toxicity (algae. Microtox) - No effect on plants - Lethal and sublethal effect on earthworm	Robidoux et al., 2004

Table 3 (Continued).

Contaminants	Sample	Bioassays	Species	Measured endpoints	Criteria	Results obtained (sensitivity of assays)	Reference
Heavy metals	Soil	Collembola Earthworm Enchytraeid	*F. candida* *E. fetida* *Enchytraeus. crypticus*	Survival, reproduction Survival, reproduction Survival, reproduction	EC_{50}, EC_{20}, LOEC	Different sensitivy depending on metal type	Lock & Janssen, 2001, 2002 Kuperman et al., 2004
Solid waste (landfill)	Leachate	Microtox Daphnid Ceriodaphnid Algae	*Vibrio fisheri* *D. magna* *C. dubia* *S. capricornutum*	Bioluminescence Immobilization Immobilization Growth inhibition	EC_{50}, LC_{50}	Algae and *C. Dubia* more sensitive than bacteria, daphnids	Lambolez et al., 1994 Bernard et al., 1996 Ward et al., 2002 Isidori et al., 2002
Solid waste (incineration ash)	Leachate Soil	Microtox® Daphnid Ceriodaphnid Algae Plant	*Vibrio fisheri* *D. magna* *C. dubia* *P. subcapitata* Lettuce, oat etc.	Bioluminescence Immobilization Reproduction Growth inhibition Germination, growth	EC_{50}, EC_{20} LOEC	- Algae: most sensitive - *C. dubia* more sensitive than *D. magna* - plant germination: least sensitive	Ferrari et al., 1999 Lapa et al., 2002
Waste (oil)	Leachate Soil	MetPLATE Microtox Mutatox® Plant Collembola Enchytraeid	*E. coli* *Vibrio fisheri* *Vibrio fisheri* Red clover etc. *F. candida* *E. crypticus*	Enzyme inhibition Bioluminescence Mutagenicity Germination, growth Survival, reproduction Survival, reproduction	EC_{50}	- Metplate no response - Microtox solid phase more sensitive than water extract method - Collembola more sensitive than enchytraeid	Juvonen et al., 2000

soil PAH concentration, suggesting that much of the mutagenic activity in contaminated soils is S9-activated frameshift activity (White & Claxton, 2004).

II.7. Use of a Battery of Bioassays for Soil and Solid Waste Quality Assessments

Risk assessment of contaminated soils is mainly based on chemical analyses of a priority list of toxic substances, corrected for the total amount of organic and inorganic matter in the soil. This analytical approach does not allow for mixture toxicity, nor does it take into account the bioavailability of the pollutants present. In this respect bioassays provide an alternative because they constitute a measure for environmentally relevant toxicity, i.e. the effects of a bioavailable fraction of interacting pollutants set in a complex environmental matrix (Bierkens et al., 1998).

Increasing evidence suggests that the use of a single bioassay will never provide a full picture of the quality of the environment. Only a test battery, composed of bioassays of different animal and plant species from different trophic levels will reduce uncertainty, allowing an accurate assessment of the quality of the environment (Table 3).

Several authors compared different bioassays of varying biological endpoints and proposed a battery of biotests (Keddy et al., 1995; Rojickova-Padrtova et al., 1998; Bierkens et al., 1998).

Keddy et al., (1995) evaluated whole organism bioassays for their application in the assessment and remediation of contaminated sites in Canada. They recommended a battery of tests for soil quality assessment of soil using soil leachates or elutriates and soil samples. Plant seedling emergence, earthworm and anthropod (*Folsomia candida etc.*), bacterial test and algal growth inhibition tests were considered to use and eligible for the test battery. The earthworm and springtails reproduction tests might be appropriate to replace survival test.

Rojickova-Padrtova et al., (1998) selected a battery of three to four alternative assay on the basis of the statistical analyses, sensitivity comparisons and general conditions for the selection of a test battery member like incorporation of different trophic levels or complementation of assays in a battery. They found that miniaturized algal assay (called microbiotest), rotifer or crustacean microbiotest, bacterial test and possibly protozoan microbiotest could represent an optimal battery of alternative assays for the toxicity evaluation of fifty environmental samples studied.

The ultimate goal of composing a test battery for soil quality is to create a quantitative tool to assess the quality of soil samples. In this respect, the relative sensitivity of the bioassays is but one criterion that can be looked at. Cost-effectiveness, accuracy, economy of the test system are as many others. Still, understanding the relative sensitivity of the different bioassays is a prerequisite to arrive at a coherent, normalized test battery (Bierkens et al., 1998).

Until recently the assessment of environmental hazards of remediated sites was mostly based on chemical analyses. However, not all contaminants of concern may be known, and undetected metabolites or compounds may be formed during biogeochemical processes. There is increasing interest for incorporation of toxicity tests (with a battery of different assays) for ecological assessment at hazardous waste sites and for supporting management decisions for remediation. (Dorn et al., 1998; Dorn & Salanitro, 2000; Juvonen et al., 2000; Frische, 2003b; Plaza et al., 2005).

Many studies have also supported the utilization of such a battery with algal, crustacean, and bacterial assays on leachates and terrestrial species in contact tests to assess the hazard to the environment when testing solid wastes in landfills (Lambolez et al., 1994 ; Bernard et al., 1996 ; Ferrari et al., 1999 ; Ward et al., 2002; Isidori et al., 2002 ; Lapa et al., 2002). Férard & Ferrari, (2005), reviewed papers describing the battery of bioassays used for evaluation ecotoxicity of solid waste. Authors reported that during 20 years (1983 - 2003), 37 test batteries were applied to solid wastes, comprising 2 to 9 bioassays. Test batteries with four bioassays were most frequently used. The three most frequently used bioassays were acute *Vibrio fischeri* test (26 times), the acute *Daphnia magna* test (22 times) and the chronic *Pseudokirchneriella subcapitata* test (16 times), confirming that acute tests are used more frequently than chronic ones. Plant as well as genotoxic (including mutagenic) tests are characterized by a large variety of species and methods.

II.8. Aquatic Toxicity of Soil Contaminated with Individual PAH and Complex PAH Mixture

II.8.1. Toxicity of individual PAHs on aquatic organisms

Toxic effects of individual PAHs were evaluated on *Vibrio fisheri* - bioluminescence inhibition (El-Alwai et al., 2002; Loibner et al., 2004) and the

Table 4. Summary of individual PAHs EC (effective concentrations) values for microtox, algae and crustacean tests.

		Test species				
	Water solubility [a]	Daphnid [b]	Microtox [c]		Algae [d]	
		D. magna etc.	*V. fisheri*		*S. subspicatus*	
		LC_{50}	EC_{50}	EC_{10}	EC_{50}	EC_{10}
Endpoints		Immobilization, mortality	Bioluminesence inhibition		Growth inhibition	
Test durations		3 ~ 96 h	30 min		7 days	
Unit			µg/l			
PAHs						
Naphthalene	31700	4777	1890	390	6821	727
Acenaphthylene			860	180		
Acenaphthene	3930		1060	310		
Fluorene	1980		990	330		
Phenanthrene	1290	409	480	130	5024	491
Anthracene	73	145			1004	10
Fluoranthene	260	206				
Pyrene	135	620			19	2.4
Benzo[a]anthracene	14	10				
Benzo[a]pyrene	3.8	98			1.5	0.1
Dibenzo[ah]anthracene	0.5	496				

[a]Data from Environmental Health Criteria 202 (IPCS, 1998).

[b]Data from Sanchez-Bayo, 2005.

[c]Data from Loibner et al., 2004.

[d]Data from Djomo et al., 2004.

green alga, *Scenedesmus subspicatus* -growth inhibition tests (Djomo et al., 2004) (Table 4). Sanchez-Bayo (2005) compared all the available information on acute toxicity of 486 organic pollutants including PAHs to 89 species of planktonic crustaceans to get a better knowledge of the toxicity of these chemicals. The bulk of data in his study was from the AQUIRE database, available online to anyone under the broader ECOTOX database (US EPA, 2003).

The toxicity to the waterflea *D. magna* has been tested on 92% of all organic pollutants in the database. The fact of having been chosen as the preferred species in standard toxicity protocols for registration of agrochemicals in the OECD, US and other countries explains the overwhelming information available on this species alone. The acute toxicity of PAHs to waterfleas ranges from 10 μg/l for benz[a]anthracene to 4.8 mg/l for naphthalene (Sanchez-Bayo, 2005).

El-Alawi et al., (2002) used the luminescent bacterium *V. fisheri* for measuring photoinduced short (15min)- and long (18h)-term toxicity of 12 different PAH congeners and their oxy & nitro derivatives. The experiment was performed in darkness or under simulated solar radiation (SSR) to test phototoxicity. The results of short-term toxicity showed that acenaphthene and acenaphthylene caused increasing inhibition of luminescence with increasing chemical concentration. Acenaphtylene was the most toxic to *V. fisheri* but pyrene and anthracene showed no toxicity at the highest concentration tested. There was no statistical difference of short-term toxicity in darkness or SSR. Comparing the short-term and long-term toxicity test, acenaphtene and acenaphtylene were less toxic in long-term assays. Toxicity was enhanced by light with the long-term assay. Loibner et al., (2004) investigated to which extent the 16 U.S. EPA priority PAHs contribute to the *V. fisheri* bioluminescence inhibition measured by the acute Lumistox® luminescent bacteria test. Five of the 16 PAHs-naphtalene, acenaphtylene, acenaphthene, fluorene, and phenanthrene-revealed inhibiting effects when measuring saturated aqueous solutions of these compounds. Saturated aqueous solutions of these two- and three-ring PAHs with the exception of anthracene exhibited considerable bioluminescence inhibition up to 95%, whereas solutions of PAHs with more than three rings did not show toxicity to *V. fisheri*. The EC_{50} values were estimated in a range (0.48 - 1.89 mg/L) for these low-molecular-weight PAHs. Phenanthrene was the most toxic PAH for *V. fisheri* as expressed by the corresponding EC_{50} value.

Table 5. Aquatic toxicity studies of PAH-contaminated soils from the literature.

Extraction methods	Contaminants (mg/kg dry weight)	Bioassays	Test duration	Results obtained[b]	Reference
Soil/water (1/10)	Creosote (14 PAHs : 23600)	Microtox	30 min	TU: 500	Ahtiainen et al., 2002
Soil/water (1/4)	Gas plant (12 PAHs : 610)	Microtox	30 min	4 < TU < 2	Sasek et al., 2003
Soil/water (1/10)	Gas plant (16 PAHs : 900, 4700)	Microtox	30 min	Max inhibition (%): <20, 50	Haeseler et al., 1999
Soil/water (1/2.5)	Gas plant (16 PAHs : 101 ~ 4202)	Microtox	30 min	Max inhibition (%): 14 - >90 at 50% leachate in test solution	Loibner et al., 2004
Soil/water (1/10), Methanol	Coke oven (16 PAHs : 1251 ~ 2897)	Microtox® *D. magna* Algae (*P. subcapitata*)	30 min 24h 72h	• TU (methanol extract): Microtox®: 169 - 2630, *D. magna*: 184, Algae: 15000 • TU (leachate): Microtox®: 0 - 26, *D. magna*: 0 - 1.2, Algae: 0 - 13	Bispo et al., 1999
W (1/4)	Gas work sites (7 PAHs : 2960)	Algae (*P. subcapitata*)	48h	EC_{10}: 2.8 g soil/l	Baun et al., 2002
Soil/water (1/2), DMSO	Tar (16 PAHs : 108~ 129 µg/l in leachate)	Microtox®	30 min	EC_{50}: 0.9 % extract	Hauser et al., 1997
Soil/water (1/2)	16 PAHs : 816~960	Microtox Algae (*S. subspicatus*) *D. magna*	30 min 72h 48h	Sensitivity of bioassays on water leachate: Microtox > algae > daphnid survival test	Maxam et al., 2000, Rila & Eisentraeger, 2003
Soil/water (1/2.5)	Creosote (13 PAHs : 519)	Lumistox Algae (*P. subcapitata*)	30 min 72h	Toxicity (% inhibition compared to control): Lumistox: 54 Algae (*P. subcapitata*): 91	Joner et al., 2004
Soil/water (1/10)	Coke oven (16 PAHs : 1141)	Microtox® Algae (*P. subcapitata*) *D. magna*	5 min 72h 48h	• EC_{50} (% extract): Microtox: 8.7 , Algae: 3.2, *D. magna*: 83.5	Mendonca & Picado, 2002

[a]TU (Toxic units) ; $1/EC_{50}$.

[b]Results were expressed as $E(L)C_x$ in units % (v/v) extracts in tested medium.

II.8.2. Toxicity of complex PAH mixtures on aquatic organisms

Pollution of historically PAH-contaminated sites always comprises mixtures of numerous PAH congeners. In order to address biological effects to concentrations of compounds in pollutant mixtures, it is necessary to clarify whether interactions of chemical pollutants influence the resulting toxicity. Combined toxic effects depend not only on the pollutants present but also on the organisms affected. Anthracene oil was used to study toxic effects of substances co-occurring with priority PAHs. This oil is a high-boiling fraction of the coal tar distillation process, and besides the 16 U.S. EPA PAHs, it contains a variety of potential toxicants with many of them not yet identified. The anthracene oil extract exhibited higher toxicity than the solution containing only the priority PAHs at maximum dissolvable concentration (Loibner et al., 2004).

Testing of the ecotoxicological potential of water extracts with aquatic test systems like luminescence inhibition assays, crustacean assay, and algal growth inhibition assays has been applied successfully for assessing PAH-contaminated soil quality (Table 5).

The ecotoxicity tests on bioluminescent bacteria (*V. fischeri*) were performed to evaluate efficiency of PAH-contaminated soil remediation process (Haeseler et al., 1999; Ahtianen et al., 2002; Sasek et al., 2003).

The analytically well-characterized soils from four different former manufacturing gas plant sites contaminated by coal tars were used in extensive biodegradation tests of PAHs by bioremediation. The acute *V. fischeri* toxicity test was performed on the soil leachates. The contaminated soils, which had an important leaching capacity, had higher toxicity. Leachate analysis involved determination of PAHs and microtox test. They found that PAH leachability and microtox toxicity test were well correlated to the concentrations of naphtalene and phenanthrene (Haeseler et al., 1999).

Ahtiainen et al., (2002) investigated creosote-contaminated soils using solid-phase modification and water extracts luminescent bacterial test to know that chemical analysis could be supported and even partly replaced by biological toxicity test. Composting was applied to remediate creosote-contaminated soils. The results showed that toxicity tests on water eluates and soils seemed to reflect a reduction in PAHs during the 5-month composting period. However, the elutriate test was less toxic at the end of composting. They explained this by

Table 6. Genotoxicity studies of PAH-contaminated soils from the literature. LOECs (lowest observed effective concentrations) are expressed as % (v/v) of extract in the test medium.

Extraction methods	Contaminants in soil (mg/kg dry weight)	Bioassays	Results obtained (LOEC %)	Reference
DCM (CH_2Cl_2)	Hydrocarbons (PAHs : <5.2)	UMU test SOS chromotest	Negative (with & without S9)	Plaza et al., 2005
DCM (CH_2Cl_2)	Gas plant (12 PAHs : 610)	SOS chromotest	Negative (without S9), positive (with S9)	Sasek et al., 2003
DCM (CH_2Cl_2)	Tar (19 PAHs : 2800)	Ames (agar plate)	Positive (without S9)	Sayles et al., 1999
Soil/water (1/10) Methanol	Coke oven (16 PAHs : 1251~2897)	Mutatox®	Positive (with & without S9), LOEC: 1.5 ~ 22.5 (without S9), 0.2 ~ 12.5 (with S9)	Bispo et al., 1999
Soil/water (1/10)	Gas plant (16 PAHs : 900, 4700)	SOS chromotest	Negative (with & without S9)	Haeseler et al., 1999
Soil/water (1/10)	Cokery (6 PAHs : 238)	Mutatox® Ames (fluctuation, plate)	Positive (with & without S9 in Mutatox, with S9 in only Ames fluctuation) LOEC : 20 (Ames), 37 (Mutatox)	Bekaert et al., 1999
Soil/water (1/2)	Tar (leachate 16 PAHs : 108~129 µg/l in leachate)	Mutatox®	LOEC :1.6 (without S9)	Hauser et al., 1997
Soil/water (1/2)	Coal and Coking (16PAHs: 960)	UMU test SOS chromostest	Positive (with S9 activation) in only UMU	Ehrlichmann et al., 2000
Soil/water (1/10)	Cokery (16 PAHs : <70)	Mutatox® Ames (agar)	Positive (without S9) in only Mutatox	Mouchet et al., 2005
Soil/water (1/10)	Coke oven (16 PAHs: 1141)	Mutatox®	Positive (with & without S9)	Mendonca & Picado, 2002

Table 7. The comparison of different ecotoxicity test results of PAH-contaminated soils collected from the literature.

Reference	PAH conc.	Test species									
		UMU (*Salmonella typhimurium TA1535/PSK 1002*)		SOS Chromotest (*Escherichia coli*)		Mutatox (*Vibrio fisheri*)		Ames (*Salmonella typhimurium*)			
								Plate		Fluctuation	
		No S9	With S9	No S9	With S9	No S9	With S9	No S9	With S9	No S9	With S9
	soils (mg/kg dry weight)										
Bekaert et al., 1999	238					+ (37)	+ (37)	-	-	-	+ (20)
Sasek et al., 2003	610			-	+						
Haeseler., 1999	900			-	-						
Ehrlichmann et al., 2000	960	-	+	-	-						
Bispo et al., 1999	1251							+ (22)	+ (0.2)		
Mendonca & Picado, 2002	1411					+	+				
Bispo et al., 1999	1836							+ (2)	+ (12)		
Sayles et al., 1999	2800							+	-		
Bispo et al., 1999	2897							+ (22)	-		
Haeseler., 1999	4700			-	-						
	extracts (µg/l)										
Mouchet et al., 2005	8.5					+ (3)	-	-	-		
Hauser et al., 1997	108					+ (2)					
Hauser et al., 1997	129					+ (2)					
Mouchet et al., 2005	1655					-	-	-	-		

[a]– indicates that is non-mutagenic, + indicates mutagenicity.
[b]In parenthesis : LOEC (%, v/v) value is the lowest concentration of water extracts in the test medium providing a mutagenic effect.
[c]Ames test was carried out on agar plates (classical method) and in liquid medium (fluctuation test).

water-limited extractability of certain compounds.

Hauser et al., (1997) examined the possibility of using the EC_{50} measured by means of the Microtox test for range-finding of genotoxic concentrations for the Mutatox test, because the Microtox test is less time-consuming and more cost effective than the Mutatox test. The samples tested were from different origin and included samples from a heavily PAH-contaminated tar site. Distilled water and distilled water containing 2% DMSO were used for extraction of soil pollutants. LOEC that give a positive genotoxic response in the mutatox test without S9 were 1.6% for two different leachates. They concluded that EC_{50} deduced from Microtox could not be used for exact prediction of the concentration that would have a genotoxic effect in the Mutatox, but the EC_{50} determination would be useful for a rough estimation of the range of concentrations that should be tested with Mutatox (Hauser et al., 1997).

II.9. Genotoxicity of Complex PAH Mixtures Contaminated Soils

A number of rapid test systems to detect environmental carcinogens have been devised for soil and water analyses. The *umu*, SOS chromotest, mutatox, and Ames tests have been widely employed for PAH-contaminated soil testing (Tables 6 & 7).

Two bacterial rapid bioassays, SOS chromotest and the *umu* test, were used to evaluate the genotoxicity of petroleum hydrocarbon-contaminated soil following bioremediation treatment (Plaza et al., 2005).

Many researchers employ organic solvents to extract and concentrate organic constituents prior to mutagenicity testing. Dichloromethane (DCM) and methanol (MetOH) are the most popular extraction solvents (White & Claxton, 2004).

The mutagenicity of DCM, MetOH extracts of PAH-contaminated soils were studied by Sayles et al., (1999), Sasek et al., (2003) and Plaza et al., (2005a).

The ecotoxicity tests on SOS chromotest were performed to evaluate effects of compost-assisted remediation of a manufactured gas plant soil contaminated with PAHs. All DCM extracts of soil before composting as well as after composting showed indirect (positive with S9 activation) mutagenicity, indicating possibility of insufficient degradation of PAHs or their metabolites (Sasek et al., 2003).

These organic solvent extraction procedures give no information about the water-extractable mutagenic soil-bound compounds, and there is no relation between the extraction conditions and the naturally occurring transport conditions in soil

(Ehrlichmann et al., 1997).

Depending on the kind of mutagenic substances, soil type, and geological conditions mutagenic substances may spread from contaminated sites to the groundwater, which may be source of drinking water. For this reason it is accepted that the water-extractable toxicological potential should be assessed by extracting soil samples with water (Donnelly et al., 1991).

The water-extractable genotoxic potential of PAH-contaminated soils was evaluated (Hauser et al., 1997; Bispo et al., 1999; Bekaert et al., 1999; Haeseler et al., 1999; Ehrlichmann et al., 2000; Mendonca & Picado, 2002; Mouchet et al., 2005).

Three soil samples polluted with PAHs were assessed for genotoxicity (*Vibrio Fischeri* M169, [Mutatox® test]). Bioassays were performed on soil water leachates. The genotoxic responses were recorded with and without S9 metabolic activation on the leachates. They observed that the water leachates were genotoxic, although not toxic with a test battery such as Microtox®, algae etc. The genotoxicity with non activated (without S9) samples could be explained by the molecular forms such as quinone or diol epoxide PAHs responsible for mutagenenesis.
These compounds might be induced during biodegradation processes in soils and linked to heavier molecular PAHs such as benzo[a]pyrene or to PAH by-products generated by degradation processes which have not been analyzed (Bispo et al., 1999).

Two in vitro bacterial assays (Ames test and Mutatox® test) were used to assess the genotoxicity of soils contaminated with PAHs and heavy metals. The concentrations of PAHs in the 0.45μm filtered and non-filtered leachate were in the range 0.6 - 1.6 and 500 - 2900 μg/l, respectively. They found that the bioassay sensitivity was influenced by the experimental conditions such as 0.45μm filtration of leachates according to standard protocol. LOEC (% leachates v/v) values for filtered and non-filtered leachates were 50, 20 and 75, 37 in Ames and Mutatox® tests, respectively. The Ames (*Salmonella typhimurium* his$^-$ reverse mutation) test was carried out on agar plates (classical method) and in liquid medium (fluctuation test). The mutagenic responses of Ames test in agar medium were negative with and without metabolic activation (S9) on all leachates tested. However the Ames test in liquid medium (fluctuation test) gave a positive

response in the presence of S9. They explained the higher sensitivity of the fluctuation test as a result of an increased bioavailability of pollutants in liquid medium (Bekaert et al., 1999).

The land treatment of PAH-contaminated soils from a former wood-treatment site was simulated at a pilot scale. Nineteen two- through six-ring PAHs were monitored (initial total PAHs = 2800 mg/kg). The Ames mutagenicity assay was assessed using organic (dichloromethane) extract with and without the addition of S9. Strains TA97 and TA98 displayed no mutagenicity in soil extracts both with and without S9. Strains TA100 showed weak mutagenic response without S9. For TA102, the mutagenicity was positive both with and without S9. Based on these results, Ames assays using strains TA 100 and TA 102 might refine the understanding of mutagenicity of these samples (Sayles et al., 1999).

The published information on soil mutagenicity was comprehensively reviewed. In total, 1313 assessments of genotoxic activity from 118 works were examined. The majority of the assessments (38%) employed the Ames Salmonella mutagenicity test with strains TA98 and/or TA100 (White & Claxton, 2004).

The aqueous extracts from 6 PAH-contaminated soils in different concentrations were evaluated using three genotoxicity assays: the *umu* using two different strains and SOS Chromotests. They observed genotoxic response only in aqueous extracts of the highest contaminated soil with PAHs at a concentration of 960 PAHs mg/kg. However, after the aqueous extracts were concentrated with a factor of 30, four of six soils were classified as genotoxic. They found also significant differences between three assays. It was recommended that non-concentrated water extract be tested first and afterwards the concentrated water extract, if results of the non-concentrated water extract were negative (Ehrlichmann et al., 2000).

Plant assays for the detection of mutagenic and clastogenic effects have been in existence for many years. White & Claxton, (2004) reviewed plant assays used for soil genotoxicity. Cotelle et al., (1999) evaluated the genotoxicity of contaminated soil using three plant bioassays, the *Vicia faba* (broad bean), the *Allium cepa* (white onion) and the *Tradescantia* (spiderwort) micronuclei tests according to method established by the IPCS and WHO. Micronucleus test on *Vicia faba* has been recently standardized by the French standard association (AFNOR NF T 90-327, 2004).

Table 8. Terrestrial toxicity studies of PAH-contaminated soils from the literature.

Soil Texture	TOC [a] (g/100g)	Soil contaminants (mg/kg dry weight)	Test species	Measured endpoints	Results obtained [c]	Reference
Natural Soil						
Clay, Sandy	1 ~ 2.8	Creosote (16 PAHs: 1300~1500)	*E. fetida*	14-d survival	No survival at 3.1%	Charrois et al., 2001
Sandy	5.1	Gaswork (16 PAHs: 1584~3251)	Higher plants (7 species)	Germination, 60-d growth	Colza, alfalfa, white clover more sensitive species	Henner et al., 1999
Sandy loam	ND	Spiked 5 PAHs: ~1000	Watercress (*L. sativum*)	3-d germination 3-d seeding emergence	• EC_{50}: 150 ~ 300 mg/kg • EC_{50}: 50 ~ 150 mg/kg	Maila & Cloete, 2002
Sandy	3.3	Spiked 7 PAHs: 1000, Tar (16 PAHs : 1600)	Higher plants (7 species)	Germination Growth (12 weeks)	Vegetables species more sensitive than grass species as for toxicity	Smith et al., 2005
Sandy	ND	Gas plant (12 PAHs: 610)	Mustard *E. fetida*	5-d germination 14-d survival	• 5-d seed germination: 76 % • 14-d survival: 73 %	Sasek et al., 2003
Loamy sand	1~3.2	Spiked 4 PAHs: <100	Higher plants (6 species)	15-d root length	• EC_{20}: 130 mg/kg (tomato) • EC_{20}: 116 mg/kg (bean)	Maliszewska-kordybach & Smreczak, 2003
Sandy loam	1.6	Spiked 4 PAHs: <1000	*F. fimentaria*	21-d reproduction	• LC_{50} (mortality) in mg/kg Fluorene: 39, Phenanthrene: 41 Fluoranthene: 81, Pyrene: 53	Sverdrup et al., 2001
Sandy loam	ND	Spiked 2 PAHs (pyrene, fluorene): <250	*F. candida*	21-d reproduction	• EC_{50} in mg/kg Pyrene: 51, Fluorene: 71	Sorensen & Holmstrup, 2005
Sandy loam	1.6	Spiked 16 PAHs: <1000	*F. fimentaria*	21-d reproduction	• 8 PAHs with 2 ~3 rings (Naphthalene, pyrene etc.) EC_{10} (reprodcution): 5~37 mg/kg LC_{50} (mortality): 39~167 mg/kg	Sverdrup et al., 2002a

Table 8 (Continued).

Soil Texture	TOC [a] (g/100g)	Soil contaminants (mg/kg dry weight)	Test species	Measured endpoints	Results obtained	Reference
Natural Soil						
Sandy loam	1.6	Spiked 4 PAHs: ~1000	*E. veneta*	21-d survival	• LC_{50} (mortality) in mg/kg Fluorene: 69, Phenanthrene: 134 Fluoranthene: 416, Pyrene: 155	Sverdrup et al., 2002b
Sandy loam	1.6	Spiked 4 PAHs: ~1000	Higher plants (3 species)	21-d growth	• EC_{20} (fresh weight basis) in mg/kg Fluorene: 55, Phenanthrene: 37 Fluoranthene: 140, Pyrene: 49	Sverdrup et al., 2003
Artificial Soil						
OECD		Spiked Phenanthrene: 200	*E. fetida*	14-d survival Reproduction (10 weeks)	• No mortality • No. of juvenile: 47 % reduction	Shin & Kim, 2001
OECD		Coke oven (16 PAHs: 1141)	Higher plants (lettuce)	5-d germiantion	• EC_{50} (*Lactuca sativa*): 53 %	Mendona & Picado, 2002
OECD		Tar (19 PAHs: 2800)	Higher plants (lettuce, oat) *E. fetida*	5-d germiantion 5-d root elongation 14-d survival	• EC_{50} (lettuce, root elongation): 97 % • EC_{50} (oat, root elongation): 84% • LC_{50} (*E. fetida*): 33%	Sayles et al., 1999
ISO, Loamy	1.6	Spiked PAH (phenanthrene): <800	*F. candida*	33-d reproduction	• EC_{50} (chemical in ISO): 175 mg/kg • EC_{50} (artificially polluted soil) : 69 %	Crouau et al., 1999

[a]TOC ; Total Organic Carbon, [b]ND ; Not documented. [c]Results were expressed as $E(L)C_x$ in units % contaminated soil or mg/kg PAH in tested medium.

Table 9. Summary of results of the toxicity tests on collembolan (*Folsomia fimentaria*), earthworm (*Eisenia veneta)* and higher plants: 50 % effect on mortality (LC_{50}), 10 % effect on reproduction for collembola and on growth for earthworm (EC_{10}) and 20% reduction in fresh weight of seedling for plants (EC_{20}).

		Collembola		**Earthworm**		**Higher plants**		
	Log K_{ow}	*F. fimentaria*		*E. veneta*		(Red clover) *T. pratense*	(Ryegrass) *L. perenne*	(Mustard) *S. alba*
		LC_{50}	EC_{10}	LC_{50}	EC_{10}	EC_{20}		
Endpoints		survival	reproduction	survival	growth	growth (fresh weight basis)		
Test durations		21-d		28-d		21-d		
Unit (mg/kg)								
PAHs								
Naphthalene	3.3	167	20					
Acenaphthylene	4.1	145	23					
Acenaphthene	3.9	107	31					
Fluorene	4.2	39	7.7	69	31	55	380	120
Phenanthrene	4.5	67	23	134	25	37	300	77
Anthracene	4.6	41	5					
Fluoranthene	5.2	53	37	416	113	140	490	650
Pyrene	5.2	81	10	155	38	49	>1000	120
Benzo[a]anthracene	5.7	>980	>980					
Chrysene	5.8	>1030	>1030					
Benzo[b]fluoranthene	6.4	>360	>360					
Benzo[k]fluoranthene	6.4	>560	>560					
Benzo[a]pyrene	6.4	>560	>560					
Dibenzo[ah]anthracene	6.2	>840	>840					
Benzo[ghi]perylene	6.7	>910	>910					
Indeno[1,2,3-cd]pyrene	6.5	>780	>780					

[a]Data from Sverdrup et al., 2002a; 2002b; 2003.

II.10. Terrestrial Toxicity of Individual and Complex PAH Mixtures Contaminated Soils

II.10.1. Earthworms and springtail collembolae

Comparison of aquatic species sensitivity has been successfully performed by several authors (e.g., Sanchez-Bayo, 2005), using the abundant literature data available. In contrast, relatively few ecotoxity studies have been conducted on soil organisms, and most studies have used only one or a few substances and species. Contamination by PAHs is one of the major problems related to industrial soils (Sverdrup et al., 2002b).

It is sometimes stated that toxicity testing with terrestrial animals is simpler than with aquatic animals as only one route of exposure via the gut has to be considered. Although this is the case with widely used tests with rats and mice, this is certainly not the case with more recently developed tests with invertebrates where exposure via the external medium (air, soil) is important also (Walker et al., 1996). Indicator organisms such as springtail collembolae and earthworms have been used to assess the toxicity of various PAHs in soil (Table 8). Earthworms are common test organisms in terrestrial ecotoxicology. Both an acute earthworm toxicity test and a reproduction test with the endpoints mortality, reproduction (cocoon production and juvenile hatching) and adult body weight change are standardized and well described in guidelines (ISO 11268-1/2) (Schaefer, 2003). An internationally standardized collembola reproduction bioassay developed in Europe (ISO 1999) using *F. candida* is sensitive and extremely useful for ecotoxicological risk assessments of contaminated soils (Greenslade et al., 2003).

Works on individual PAHs and terrestrial organisms include toxicity studies carried out with earthworms (Shin & Kim, 2001; Sverdrup et al., 2002a), and springtails (Crouau et al., 1999; Sverdrup et al., 2001, 2002b; Sorensen & Holmstrup, 2005).

Sverdrup et al., (2001, 2002a) evaluated the effects of four PAHs: fluorene, phenanthrene, fluoranthene and pyrene, on the survival and reproduction of the 21-d collembolan *Folsomia fimentaria* and 28-d earthworm *Eisenia veneta* in a sandy loam agricultural soil (Table 9). The estimated concentrations resulting in a 50% reduction of reproductive output (EC_{50}) of *F. fimentaria* were for fluorene 14 mg/kg, phenanthrene 30 mg/kg, fluoranthene 51 mg/kg and pyrene 16 mg/kg. The

E. veneta EC_{50} values of sublethal effects on growth were based on measured initial concentrations and were 50 mg/kg for fluorene, 94 mg/kg for phenanthrene, 166 mg/kg for fluoranthene and 71 mg/kg for pyrene.

The estimated 50 % lethality (LC_{50}, mg/kg) were 39, 41, 81, 53 (*F. fimentaria*) and 69, 134, 416, 155 (*E. veneta*) for fluorene, phenanthrene, fluoranthene and pyrene, respectively. Species sensitivity was examined using LC_{50} and NOEC values for growth or reproduction as endpoints. The LC_{50} values for *E. veneta* were higher than for *F. fimentaria* by a factor of 1.8 - 5.7. *E. veneta* was slightly less sensitive than *F. fimentaria*. For the sublethal toxicity data (growth for *E. veneta* and reproduction for *F. fimentaria*), the range in difference of EC_{50} values was by a factor of 1.4 - 4.4. The collembolan *F. fimentaria* was more sensitive than earthworm *E. veneta* to the toxicity of the four PAHs.

Remediation of soils contaminated with PAHs has been studied using a 14-d earthworm survival bioassay (Sayles et al., 1999; Charrois et al., 2001; Sasek et al., 2003).

The land treatment of PAH-contaminated soil from a former wood-treating site was simulated at a pilot scale. Nineteen two- through six-ring PAHs were monitored (initial total PAHs = 2800 mg/kg). Toxicity was assessed using root elongation, earthworm survival assays. The earthworm survival assay was more sensitive than two plant assays (Sayles et al., 1999).

Total 16 PAHs decreased significantly in the clay natural soil after 52 days of slurry phase biotreatment but the sandy natural soil had not the same reduction of creosote constituents. Unfavorable soil habitat for microorganisms as well as surface area for sorption of contaminants may be influencing degradation and availability of contaminants to microorganism. The dichloromethane extractable organics (DEO) seems to have an essential role in toxicity of PAHs, as expressed in the PAH: DEO ratio. Soils with low PAHs: DEO were less toxic, as measured by earthworm survival days, compared to soils with higher ratios. Potential exists for using the PAHs:DEO ratio for *E. fetida* as a better predictor of toxicity compared to total PAH or DEO concentration (Charrois et al., 2001).

Sverdrup et al., (2002b) measured the effects of individual 16 PAHs on survival and reproduction of the soil dwelling springtail *Folsomia fimetaria* to predict toxicity of PAHs. The results showed that PAHs with log K_{ow} values = 3.3 - 5.2 (i.e., naphthalene, acenaphthene, acenaphthylene, anthracene, phenanthrene, fluorene, pyrene and fluoranthene) significantly affected the survival or reproduction of springtail, whereas eight highly lipophilic PAHs (log K_{ow} values ≥

5.6) were not toxic. Threshold values for the toxicity of the individual PAHs could be estimated as pore-water concentrations by the use of organic carbon-normalized soil-pore-water partitioning coefficients (*Koc* values) using quantitative structure–activity relationship (QSAR). For the PAHs with a 5.6 ≥ log K_{ow} values, a significant relationship was obtained between the log K_{ow} and pore-water toxicity of the eight PAHs, indicating a narcotic mode of toxicity. However, the PAHs with a log K_{ow} values ≥ 5.2 such as chrysene, perylene etc., were not expected to be toxic (i.e., expected EC_{10} < solubility). The absence of toxicity with a higher log K_{ow} could be explained by the limited water solubility. These results indicated that these PAHs do act by narcosis as the mode of toxicity and that their toxicity is governed by concentrations in the soil pore-water.

For PAH chemicals, reproduction was a considerably more sensitive parameter than adult survival (Sverdrup et al., 2001, 2002b ; Sorensen & Holmstrup, 2005).

II.10.2. Higher plants

In recent years, phytotoxicity tests with terrestrial higher plants have been frequently used as a component of ecological risk assessment for the characterization, evaluation, and remediation process monitoring of contaminated soils (Gong et al., 1999).

As part of a battery of bioassays, seed germination and seedling growth tests have been used to monitor the ecotoxicity of soils contaminated with organic chemicals such as PAHs (Table 8).

The effects of individuals or complex PAH mixture on seed emergence, root elongation and early life stage growth of terrestrial plants were studied using natural soils (Henner et al., 1999; Maila & Cloete, 2002; Sverdrup et al., 2003; Maliszewska-kordybach & Smreczak, 2003; Smith et al., 2005).

Maila & Cloete, (2002) investigated the sensitivity of germination of the watercress *Lepidium sativum* to PAHs in soils artificially and historically contaminated with mixtures of PAH. The germination level and fresh weight of *L. sativum* decreased with an increase in the concentration of PAH in the soil. The effective concentration value (EC_{50}) for 3day fresh wet weight seedling was, then, in the range of 50 - 150 ∑5 PAHs mg/kg artificially spiked concentration in sandy loam field soil spiked with naphthalene, pyrene, fluorene, phenanthrene, anthracene.

Sverdrup et al., (2003) tried to determine the relative toxicity of PAHs by testing in the same study (e.g., using the same soil type). The effects of four

polycyclic aromatic hydrocarbon (PAH); fluorene, phenanthrene, fluoranthene and pyrene, on seed emergence and early life-stage growth of three terrestrial plants (two dicotyledon plant species; red clover and mustard, one monocotyledon plant species; rye grass) were studied using a agricultural soil with an organic carbon content of 1.6%. After three weeks of exposure, seed emergence and seedling weight (fresh and wet) were determined. Seedling growth was a more sensitive endpoint than seed emergence for the four PAHs. The concentrations producing a 20% reduction of seedling fresh weight (fresh weight EC_{20} values) of the four PAHs ranged from 37 to 140 mg/kg for red clover (*Trifolium pratense*), from 77 to 650 mg/kg for mustard (*Sinapsis alba*) and from 300 to > 1000 mg/kg for rye grass (*Lolium perenne*). Fluoranthene was slightly less toxic than other PAHs. As compared to the EC_{20} values, there was a rather large difference in sensitivity between the species, and dicotyledonous red clover (*T. pratense*) was the most sensitive of the species tested for the four PAHs. The general rank in sensitivity is red clover (*T. pratense*) > mustard (*S. alba*) > rye grass (*L. perenne*) (Table 9).

Ecotoxicity of soils polluted with polycyclic aromatic hydrocarbons (PAHs) and heavy metals was evaluated in laboratory experiments. Mixture of four PAH compounds (fluorene, anthracene, pyrene and chrysene) was applied at levels of 10 - 100 $\sum$4 PAHs mg/kg. Effect on plants was assessed on the basis of growth at early stage of development. Six different plant species (wheat, oat, maize in the monocotyledonous category and tomato, bean, sunflower in the dicotyledonous category) were used. Contamination of soils with PAH in all monocotyledonous plants rather stimulated than inhibited the growth of the plants in loamy soil (3.2% organic carbon) at 100 $\sum$4 PAHs mg/kg. Dose/effect regression was statistically significant in two cases only; for bean in loamy sand soil (1 % organic carbon) and for tomato in loamy soil (3.2% organic carbon). The sum of 4 PAHs-EC_{20} values were calculated: 116, 130 mg/kg for bean & tomato, respectively. The combined inhibitory effects of PAH and heavy metals on some plants (bean and sunflower) was stronger than in soils amended with heavy metals or PAHs separately (Maliszewska-kordybach & Smreczak, 2003).

Smith et al., (2005) evaluated the germination and subsequent growth of 7 species (4 grass and 3 legume) in soils spiked with a mixture of 7 PAHs (2 - 4 rings: naphthalene, fluorine, acenaphthene, phenanthrene, anthracene, fluoranthene and pyrene). Despite variability in the rate of germination between species, after

10 days, soils with artificially spiked PAHs (Σ7 PAHs: 1000 mg/kg) caused no significant reduction in % germination of any of the plant species. After 12 weeks growth, the soil spiked with the coal tar yielded significantly less dry foliage weight for 4 of the 7 species tested; one grass species and all the three vegetable species. In general, the vegetable species (clover etc.) were affected more severely than the grass species (ryegrass, fescue etc.) in an artificial PAH mixture.

Phytotoxicity was tested with pure PAHs. Under the conditions studied, high molecular weight PAHs such as benzo[a]pyrene and benzo[a]anthracene did not inhibit plant germination and growth. However, plant germination and growth were strongly inhibited by the presence of volatile, water-soluble low molecular-weight hydrocarbons (< 3 rings) such as benzene, toluene, xylene, styrene, indonaphtene, naphthalene. This finding suggested that once low molecular weight aromatic hydrocarbons are removed, e.g., by volatilization, biodegradation, weathering, tillage and fertilizing, plants should be able to grow (Henner et al., 1999).

The PAH contaminated sites were assessed to evaluate the efficiency of remediation techniques or toxicity of ancient industrial fields by phytotoxicity tests (Sayles et al., 1999; Henner et al., 1999; Mendona & Picado, 2002).

Henner et al., (1999) tested 2 moths plant growth with a medium (average Σ16 PAHs: 1584 mg/kg) and a highly (average Σ16 PAHs: 3251 mg/kg) PAH contaminated gaswork soil. Species were colza, alfalfa, white clover, barley, fescue, ryegrass, maize. Two months growth tests showed a strong inhibition of plant height. The most resistant species appeared to be maize and rye grass. The dicotyledonous plant such as Colza, alfalfa and white clover, were more sensitive species to toxicity of contaminated soils.

The vegetable species (white & and red clover, birdsfoot-trefoil) were also affected more severely than the grass species (ryegrass, fescue, cocks-foot) in a PAH-contaminated soil (Smith et al., 2005).

The land treatment of PAH-contaminated soil from a former wood-treatment site was simulated at pilot scale. Nineteen two- through six-ring PAHs were monitored (initial total PAHs = 2800 mg/kg). Toxicity was assessed using two plant assays: germination and root elongation. Comparing the two plant assays, the root elongation assay was more sensitive to the tested soils than the seed germination assay. Root length reduction corresponded to an EC50 of 97% and 84 % soil (w/w) for lettuce and oats, respectively (Sayles et al., 1999).

Table 10. Bioaccumulation factors (BAFs) of Individual PAHs in plants and earthworms collected from the literature.

	TOC [a] (g/100g)	Soil contaminants (mg/kg dry weight)	Test species	Exposure Durations	Results obtained [c] (BCFs for plant, BSAFs for earthworm)	Reference
Plant	1.5	Phenanthrene: 1 ~ 50	Chinese cabbage Ryegrass, Amaranth	45-d	Phe (35~45ppm): 0.016~0.094 (S), 0.053~0.138 (R)	Zhu & Gao, 2004
	ND	Phenanthrene: 5, 10 Anthracene: 5, 10	Tomato	28-d	Phe (10ppm) 0.008 (S), 0.006 (R) Ant (10ppm) 0.017 (S), 0.006 (R)	Harms, 1996
	3.5	12 PAHs: 36.3	Zucchini Cucumber Squash	28-d	Zucchini 0.14 (S), 0.09 (R) Cucumber 0.053 (S), 0.004 (R) Squash 0.011 (S), 0.031 (R)	Parrish et al., 2005
	ND	16 PAHs: 590 ~ 2301	Potato, Carrot	Field harvest	Potato: 0.015 ~ 0.036, Carrot : 0.01 ~ 0.032	Zohair et al., 2005
	2	16 PAHs: 1 ~ 6	Chinese cabbage, Turnip etc. (8 species)	Field harvest	• 8 species mean value: Site A (1ppm) 0.44 (S), 0.07 (R) Site B (6ppm) 0.16 (S), 0.034 (R)	Tao et al., 2004
	1.5	Phenanthrene, Pyrene: 7 ~ 490	Chinese cabbage etc, (12 species)	45-d	• 12 species mean value range (Phe =133, +Pyr = 172ppm): 0.006~1.2 (Phe-S), 0.05~0.67 (Phe-R) 0.006~1.2 (Pye-S), 0.23~4.4 (Pyr-R)	Gao & Zhu, 2004
	11	Anthracene: 100	Wheat, barley	14-d	Wheat: 0.071(S), barley: 0.052 (S)	Tang et al., 1998
Earthworm	0.6 ~ 11	20 PAHs: 1 ~ 186	*Lumbricus terrestris*	15-d	• Lipid and organic matter-normalized mean value: 17 PAHs: 0.13 ~ 0.2 (except for Flu: 0.41, Phe: 0.33, Ant: 0.26)	Krauss et al., 2000
	ND	PAHs	*Lumbricus rebellus*	Field study	• Lipid and organic matter-normalized values (mean, range): 0.1, 0.03 ~ 0.26	Ma et al., 1998
	5.7	Phenanthrene, Fluor-Anthene: 10,100	*Lumbricus rebellus*	14-d, 56-d	Phe (100ppm) 0.970 (14d), 0.027 (56d) Flu (100ppm) 0.183 (14d), 0.092 (56d)	Ma et al., 1995
	11	Anthracene, Pyrene, Flouranthene: 100	*Eisenia fetida*	10-d	Ant Flu Pyr 0d aging 1.84 2.34 2.17 140d aging 1.19 1.50 1.10	Tang et al., 1998
	3.5	12 PAHs: 36.3	*Eisenia fetida* *Lumbricus terrestris*	14-d	• Lipid and organic matter-normalized values : *E. fetida* (0.011), *L. terrestris* (0.007)	Parrish et al., 2005

[a]TOC: Total Organic Carbon, [b]ND: Not documented, [c]S: Shoot, R: Root, [d]Results expressed as BCF (Bioconcentration factor) for plant and BSAF (Biota-soil accumulation factor) for earthworm; the concentration ratio biomass to soil on a fresh or dry weight basis

II.11. Bioaccumulation of Individual and Complex PAH mixtures in Terrestrial organisms

II.11.1. Higher plants

Several studies have been conducted on bioaccumulation of PAHs by plants both from field surveys and laboratory experiments in order to get a better insight into the processes of PAHs plant uptake (Harms et al., 1996; Tang et al., 1998; Tao et al., 2004; Gao & Zhu, 2004; Zhu & Gao, 2004; Parrish et al., 2005; Zohair et al., 2005) (Table 10).

Various tissues of rice plants from a PAH-contaminated site were analyzed at different growth stages of ripening period. No specific gradient trends along roots, stem, ear axes, and grains were observed, suggesting that systematic translocation among them is unlikely. Over the ripening period, PAH concentrations were increased in rice roots and decreased in most of aerial tissues of rice. Significant correlations between PAH and lipid contents can only be observed during full mature stage. There was also a significantly positive correlation between bioconcentration factor (plant over air) and octanol/air partitioning coefficient (K_{oa}) (Tao et al., 2005). Aging of soil also reduced the availability of anthracene to the two plant species (wheat and barley). This was evident in tissue concentrations, percentages taken up, and values for BCF (Tang et al., 1998).

Uptake of non-polar organic compounds by plants depended on the species and physico-chemical properties of the chemicals. The main metabolites being formed are polar conjugates with carbohydrates and amino acids. Cell suspension cultures of monocotyledonous and dicotyledonous plants with anthracene were compared. Different amounts of anthracene and its metabolites were found in all cell wall fractions such as pectine, lignin, hemicellulose, cellulose. Depending on the plant species and especially in monocotyledonous plants, large amounts of the chemicals and/or their metabolites, are frequently incorporated into non-extractable residues. The knowledge about the association of a chemical to individual cell wall components permits conclusions about possible ecotoxicological risks (Harms et al., 1996).

Chiou et al., (2001) proposed a simple partition-limited model to describe the plant uptake of organic contaminants from soil and water. The model enables one to estimate the levels of contaminants in a plant or different plants based on

the contaminant levels in soils. The model is expressed as

$$C_{pt} = \alpha_{pt}[Cs/(f_{som}K_{som})][\ f_{pw}+f_{ch}K_{ch}+f_{lip}K_{ow}]$$

or

$$\alpha_{pt} = K_{som}f_{som}\,(C_{pt}/C_s)/[\ f_{pw}+f_{ch}K_{ch}+f_{lip}K_{ow}]$$

where

C_{pt} is the concentration of a contaminant in the whole plant or in a specific part of the plant;
C_s is the soil contaminant concentration on a dry weight basis;
K_{som} is the contaminant partition coefficient between soil organic matter (SOM) and water;
f_{som} is the SOM content of the soil;
K_{ow} is the octanol-water partition coefficient;
f_{pw}, f_{lip} and f_{ch} are the total weight fractions of the water, the lipid, and the sum of carbohydrates, cellulose, and proteins in plants that are assumed to have approximately the same partition coefficient (K_{ch}).
α_{pt} is a quasi-equilibrium factor.

The performance of the model in predicting the levels of plant contamination by organic pollutants such as PAHs was evaluated. Modeled root concentrations appeared to be more accurate than modeled shoot concentrations. The differences of simulated and experimented concentrations of phenanthrene in roots and shoots of three representative plant species, including ryegrass, flowering Chinese cabbage, and three-colored amaranth, were less than 81% for roots and 103% for shoot (Zhu & Gao, 2004).

A significantly positive correlation was observed between root BCFs of phenanthrene and root lipid contents (Gao & Zhu, 2004; Zhu & Gao, 2004).
Plant uptake and accumulation of PAHs by 12 plant species grown in various treated soils were comparatively investigated. Soils were spiked with a mixture of phenanthrene and pyrene. The BCFs for root of phenanthrene and pyrene mixture for plants grown in contaminated soils with initial phenanthrene concentration of 133 mg/kg and pyrene of 172 mg/kg were 0.05 - 0.67 and 0.23 - 4.44, whereas the BCFs for shoot were 0.006 - 0.12 and 0.004 - 0.12, respectively. For the same

soil-plant treatment, shoot concentrations and BCFs values were generally much lower than root. They found no significant correlations of shoot lipid contents and shoot PAH concentration (Gao & Zhu, 2004).

Parrish et al., (2005) also found that not only is the accumulation of weathered PAHs species-specific but also that the bioavailability of individual PAH constituent is highly variable. However, Tao, et al (2004) reported that the concentration of the 16 PAHs in the aerial part of 8 species vegetables collected from two slightly-contaminated sites (1 - 6 mg/kg) was higher than that in root. The 16PAHs BCFs were 0.16 - 0.44 for roots and 0.034 - 0.07 for aerial parts.

II.11.2. Earthworms

Oligochaete earthworms are functionally important in terrestrial ecosystems as food organisms for avian and mammalian wildlife and as intermediates in nutrient cycling processes. The bioaccumulation potential of toxic chemicals in earthworms provides a useful parameter when attempting to derive adequate soil quality criteria for ecological risk assessment of contaminated areas (Ma et al., 1998).

Several investigators have conducted bioaccumulation studies with earthworms using either field-collected or laboratory-spiked soils. (Ma et al., 1995; 1998; Tang et al., 1998; Krauss et al., 2000) (Table 10).

However, methodology differences (worm species and age, incubation time, worm-to-soil ratio, contaminant concentration and age) and variable data expression (concentrations that may or may not be normalized to lipid content, ratios of lipid normalized to organic carbon normalized content) make direct comparisons problematic.

In calculating the uptake of contaminants by organisms present in soil or sediment, the equilibrium partitioning (EqP) theory has been developed. The accumulation of hydrophobic organic contaminants does not closely correlate with the octanol-water partition coefficient (K_{ow}) because the pronounced bioaccumulation potential of hydrophobic compounds is compensated by their strong sorption to soil organic matter (Connell & Markwell, 1990).

Assuming steady-state conditions, the bioaccumulation of PAHs in earthworm can be described by the BSAF (biota-soil accumulation factor):

$$BSAF = (C_w \times f_{om}) / (C_s \times f_{lip})$$

Where

C_w = concentration in worm (mg/kg fresh weight)
C_s = concentration in soil solid phase (mg/kg)
f_{om} = weight fraction of organic matter (kg/kg)
f_{lip} = weight fraction of lipid (kg/kg) (Ma et al., 1998).

The suitability of applying equilibrium partitioning (EqP) theory to predict the bioaccumulation of PAHs by earthworms was assessed when these are exposed to contaminated soils in the field or laboratory. Results of studies were in good agreement with model prediction that BSAF were independent of the K_{ow} (Ma et al., 1998; Krauss et al., 2000). According to equilibrium partitioning theory, the correction of BSAF with lipid content of the animals and organic matter content of the exposure medium, should eliminate the differences in PAH concentration between different species. The expression of PAH concentrations on a lipid basis and soil concentrations on the basis of soil organic matter is not only useful for comparing species but within a species it gives also an advantage as it may reduce variation caused by differences in lipid content among individuals from the same site (Van Brummelen et al., 1996).

Ma et al., (1998) carried out studies *in situ* in various contaminated floodplain sites. They observed the presence of linear relationships with intercept zero between the lipid-normalized concentration of different PAHs in the earthworm *Lumbricus rubellus* and the organic-matter normalized concentration of the compounds in soil. The BSAF could be considered a constant for compounds with varying log K_{ow} values. The BSAF values of PAHs were determined at the different sites and ranged from 0.03 to 0.26. The average BSAF value was 0.10 (±0.06 SD, n=12).

Krauss et al., (2000) also studied the uptake of 20 PAHs from 25 field-contaminated soils (max. 186 Σ20 PAHs mg/kg) by *Lumbricus terrestris* during 15 days. The average BSAFs of most individual ranged between 0.13 and 0.20, except for fluoranthene (0.41), phenanthrene (0.33), and anthracene (0.26). They observed close and significant relationships between total concentration of PAHs in soils and earthworms with r = 0.77 - 0.94.

The bioconcentration factor (BCF) of low-molecular–weight PAHs, such as phenanthrene, fluoranthene, and pyrene, was derived from kinetic modeling of

water-only experiments and found to be of the same order of magnitude as the bioaccumulation factor in the field when the latter was normalized to calculated concentrations in soil pore water. The authors concluded that the exposure of earthworms to PAHs in soil was mediated through direct contact of the worms with the dissolved interstitial soil-water phase (Ma et al., 1998).

Bioaccumulation was found to be reduced by changes in bioavailability associated with increasing residence time ("aging") of the PAH-amended soil. Aging could limit bioavailability for uptake, and decrease the amount of PAHs uptake by earthworms as shown by lower tissue concentrations, percentages assimilated, and bioconcentration factors (Ma et al., 1995; Tang et al., 1998; Johnson et al., 2002).

Earthworms showed a several times greater bioaccumulation of PAHs under conditions of food limitation. There was no relation with body lipid contents. It was possible that the effect of this food is related to some shift in exposure route. The earthworm uptake of bioavailable organic compounds occured at the interphase of interstitial soil water and earthworm body surface. However, it was possible that earthworms increased their oral intake of soil particles when driven by hunger stress and consequently take up more PAHs via the gastrointestinal tract (Ma et al., 1995).

CHAPTER III. RESEARCH GOAL AND OBJECTIVE

III. RESEARCH GOAL AND OBJECTIVES

Although the toxicity of many individual PAHs is relatively well characterized, information that defines the bioavailability, bioaccumulation, and toxicity potential of complex PAH mixtures is much less available for naturally contaminated soils containing mixtures of PAH congeners, their derivatives, and metabolites. Contaminants present at polluted soils occur as mixtures: therefore, interactions between individual compounds may be of importance.

In field-contaminated soils, bioavailability of pollutants may be reduced with aging. Therefore, environmental risk assessment from chemical analyses of individual 16 PAH is critical. Moreover, a number of PAH derivatives and degradation products are present in mixture and their effects are unknown. These considerations argue for a biological evaluation of toxic effects of the whole soil matrix rather than analysis.

The risk associated with industrially contaminated soils should be evaluated to protect human health and the environment. Toxic or genotoxic compounds may endanger either soil or aquatic ecosystems by directly affecting soil biota or indirectly, after runoff and percolation, affecting groundwater biota.

This study focused on a soil from an ancient cokery on the wasteland of Homécourt in Lorraine region (north-east France). The issue of requalification of degraded industrial sites is related to major industrial changes in recent years in Lorraine Region that especially concern steel and coal activities, textiles, small industries etc. In addition to their economic consequences, these changes have resulted in the creation of thousands of hectares of industrial wasteland, the renovation of which almost always involves highly complex pollution problems. The degraded zones, which include mining, industrial, railway, military and urban, represent considerable areas in Europe (for example, over 6000 ha in Lorraine Region). The GISFI (French Scientific Interest Group-Industrial Wasteland) is now developing synergy between different skills, based around a scientific and technological project dedicated to acquisition of knowledge for sustainable requalification of degraded

sites polluted by past industrial activities (www.gisfi.prd.fr).

The goal of this research was to study the utility of an integrated approach using chemical analyses and a battery of bioassays to investigate and assess the bioavailability, potential aquatic toxicity & genotoxicity, terrestrial toxicity of complex PAH mixtures in a soil contaminated on the field.

The specific objectives of this study were to:

1. Characterize the nature and extent of organic and inorganic chemical contaminants in polluted soils from a former coke plant site,

2.
   - Assess the aquatic toxicity of the water extractable fraction of the PAH-contaminated soils using a battery of bioassays, including microtox®, (cerio)daphnids, and algae tests, allowing us to investigate acute, chronic toxicity to bacteria, invertebrates, and photosynthetic species.

   - Evaluate the genotoxicity of water extractable fraction of the PAH-contaminated soils using the *in vitro* bacterial assays (*umu*, Mutatox®, and Ames tests)

   - Assess the PAH-contaminated soil by the solid-phase direct effects on plants (Oat, Lettuce, Chinese cabbage), earthworms (*Eisenia fetida*) and collembola (*Folsomia candida*) (**ref. Results V.1**).

3. Compare the effect of the PAH-contaminated soil on germination, growth of higher plant, and survival, reproduction of earthworm and springtail collembola with two control soil types: the artificial ISO or the loamy natural soil (**ref. Results V.2**).

4. Determine the transfer and uptake of the PAHs from contaminated soil to biota (earthworm and higher plant) (**ref. Results V.3**).

CHAPTER IV. MATERIALS AND METHODS

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IV.1. Study Field Site Description

Our test soils studied originated from an experimental station of the GISFI at Homécourt located at a about 30km from the northeast city of METZ in France (Fig. 9).

IV.2. Soil Samples

The contaminated soils were collected from the site of Homécourt, a former industrial site that has stopped its activity more than thirty years ago and was polluted by cokery residues. The site was mainly contaminated by PAHs and slightly by heavy metals. The soil samples were sieved at 4 mm and homogenized, then stored at 13 °C until use (Fig. 10 & 11).

Two different soils were used for dilution with contaminated soils as test substrate (control soils) in this study: the artificial ISO or the natural soil.

Artificial ISO soil was prepared according to the ISO guideline and consists of 70% quartz sand and 20% kaolin clay and 10% finely ground 1-mm sieved sphagnum peat (% based on dry weight). Calcium carbonate ($CaCO_3$) was used to adjust pH to 6.0 ± 0.5. The soil pH and water-holding capacities (WHC) were determined according to ISO methods 10390 (1994) and 11267 (1998), respectively.

The natural soil, an agricultural loamy soil, from grassland at Hommarting situated to the northeast in France, were taken from the 0 - 25 cm surface layer, air dried and sieved (4 mm).

IV.3. Soil Water Extraction (leaching) Methods

The leaching procedure for soil extracts (leachates) was carried out according to the ISO standard (12457-2, 1999), comprising a 24 ± 0.5 h mixing with deionized water (soil: water ratio of 1 kg: 10 l) in two-liter polyethylene bottles at a temperature of 20 ± 5°C. The bottles were placed in a rotating apparatus working at 10 rpm. Then a settling period was maintained for 12 ± 0.5 h at 4 ± 1°C. After

Table 11. Institutions involved in physicochemical analyses.

Sample type		Components	Institution	Methods
Leachates		Heavy metals	Laboratoire BFE, Univ. of Paul Verlaine METZ	AAS, ICP
		PAHs	Laboratoire de Rouen/ETSA	NF T 90-115 (HPLC/F)
Soils	Homécourt	Heavy metals	IRH (Institute of hydrological research, Nancy)	AAS, ICP
		PAHs		NF ISO 13877 (X 31-417, HPLC/F)
		Soil physico-chemical Characterization (TOC, CEC, Texture etc.)	INRA (Arras)	French, ISO standards
	ISO, Natural	Heavy metals	Laboratoire de Rouen/ETSA	AAS, ICP
		PAHs		XP X 33-012 (GC/MS)
		Soil physico-chemical Characterization (TOC, CEC, Texture etc.)		French, ISO standards
Earthworms		PAHs	Laboratoire de Rouen/ETSA	GC/MS
Plants		PAHs	Laboratoire de Rouen/ETSA	XP X 33-012 (GC/MS)

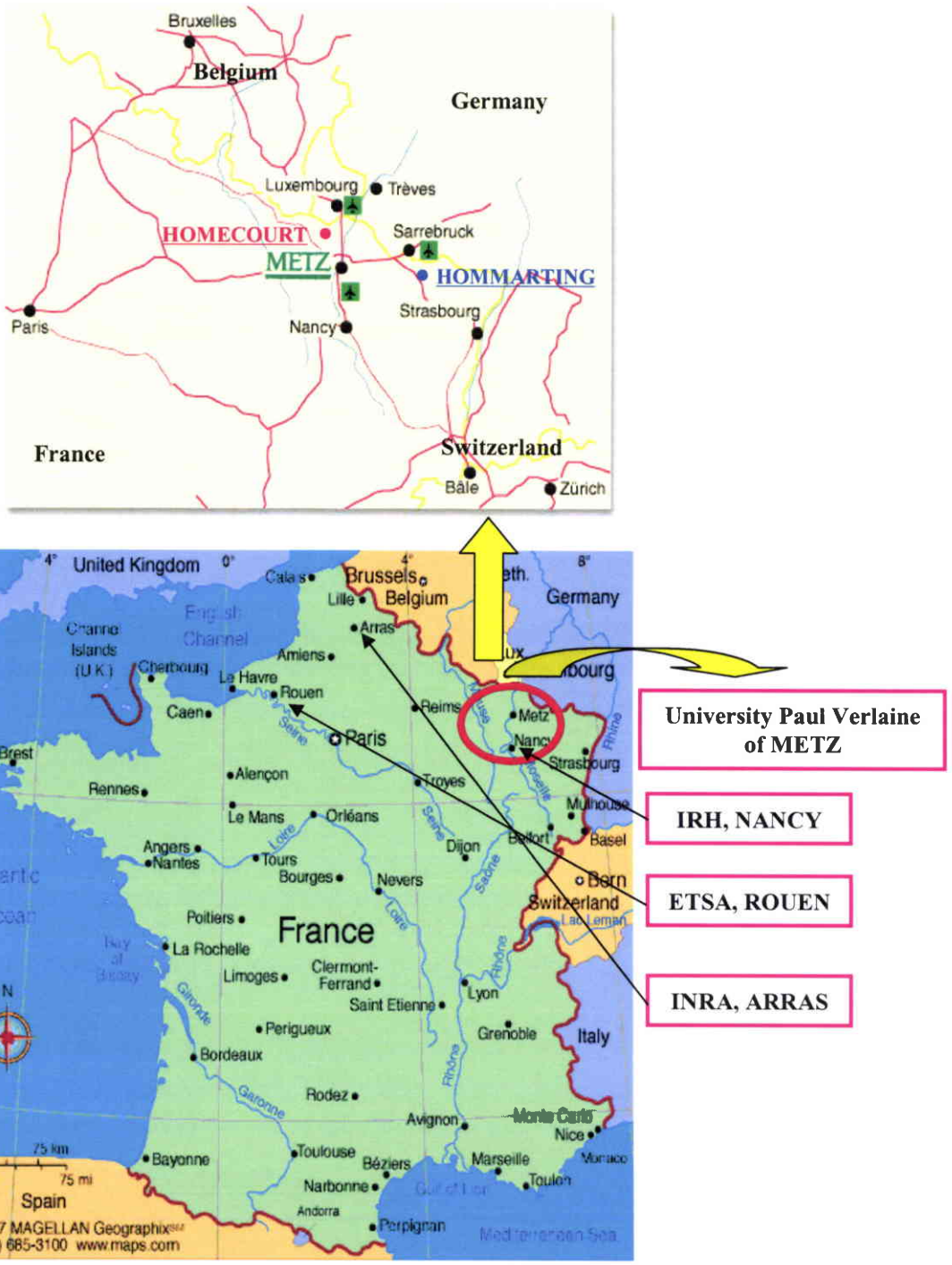


Fig. 9. Geographic location of our study site. The site of Homécourt located at a about 30km in the northwest of the city of METZ in Lorraine (France).

Fig. 10. The study area on the industrial wasteland of the *Homécourt* (North-East France) originally occupied by coking plant.

Fig. 11. Soils sampling and sieving.

centrifugation (1500g, 15min) and paper filtration, the supernatant was stored at 4°C without filtration at 0.45 μm and used for bioassay for 72h. A membrane filtration step (porosity 0.45μm) and pH correction was not carried out in our study.

It is extremely important by which methods the clean up of the soil extracts is performed prior to chemical analysis and ecotoxicological testing. The performance of the elution with a soil-water ratio of 1:2 and subsequent centrifugation and filtration is a compromise between the demands of the test systems and the search for realistic path-specific extraction conditions (Wahle & Kordel, 1997). The chemical and biological results demonstrated that the 0.45 μm filtration in the preparation of leachates from soils and solid wastes was not relevant for ecotoxicological studies. Indeed, this filtration eliminates many potentially genotoxic micropollutants adsorbed on suspended solids (Békaert et al., 1999).

IV.4. Physico-chemical Analyses

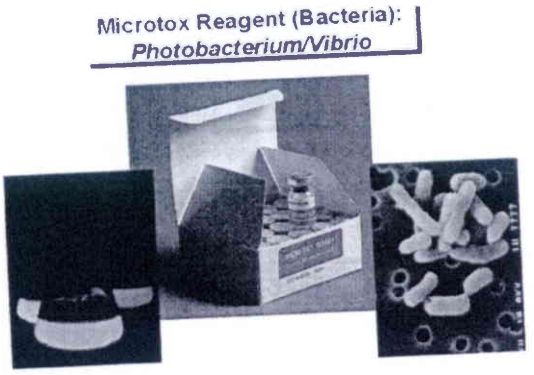
The institutions involved in analyses of organic and inorganic components of soils, soil extracts and earthworm are indicated in Table 11. The contents of 16 PAHs congeners listed as priority pollutants by the US- EPA in soils were determined by HPLC/fluorescence method in the IRH Environment (Institute of Hydrological Research, Nancy, France). The quantification of PAHs from soils was performed as follows. Soil samples were dried at 40 °C until constant mass. Extraction of PAHs was conducted by ASE extractor (Accelerated Solvent Extractor 200, from DIONEX, Sunnyvale, USA), allowing a solid/liquid extraction at 100 °C and under a pressure of 136 bars, with dichloromethane and hexane as extraction solvents (ratio 50:50; v:v). After solvent evaporation in a nitrogen atmosphere, extracts were dissolved in acetonitrile. PAHs were separated and detected by reversed high performance liquid chromatography (HPLC), coupled with a spectrofluorimeter and a UV diode array detector.

Total PAH concentrations corresponded to the sum of the concentrations of the 16 analyzed PAHs. TEQs (Toxic Equivalent Factors) were expressed as defined by Nisbet and Lagoy (1992) for genotoxicity studies.

PAH contents in soil water extracts were analysed by GC/MS (capillary gas chromatography/mass spectrometry) according to the French standard (NF T 90-115) method in the ETSA (Expertises Technologies & Services-Analyses) laboratory (www.laborouen.com, Rouen, France).

Table 12. Summary of the test selected to assess the toxicity of soils and soil leachates to soil and aquatic organisms.

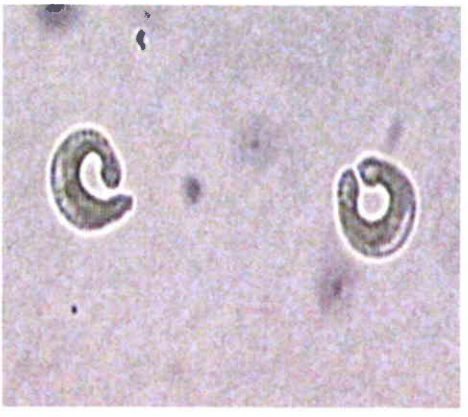
Test organisms	Tests & Species	Durations	Measured parameters	Reference
Microorganisms	Microtox® (*Vibrio fischeri*)	15, 30 min	Luminescence inhibition	ISO 11348-3 (1999)
	Mutatox® (*Vibrio fischeri* M169)	24 hrs	Genotoxicity	Microbics (1993)
	Ames (*Salmonella typhimurium* TA98, TA100)	72 hrs	Genotoxicity	Environment Canada (1993)
	UMU (*Salmonella typhimurium* TA1535/ PSK 1002)	4 hrs	Genotoxicity	ISO 13829 (1999)
Algae	Algae (*P. subcapitata*)	72 hrs	Growth inhibition	ISO 8692 (1989)
Invertebrates	Daphnid (*Daphnia magna*)	24, 48 hrs	Immobilization	ISO 6341 (1989)
	Ceriodaphnid (*Ceriodaphnia dubia*)	7 days	Reproduction	AFNOR T90-376 (2000)
	Collembola (*Folsomia cadida*)	28 days	Mortality, Reproduction	ISO 11267 (1998)
	Earthworm (*Eisenia fetida*)	14 days 56 days	Mortality Reproduction	ISO 11268-1 (1994) ISO 11268-2 (1998)
Plants	Oat, Lettuce, Chinese cabbage	14 ~ 21 days	Emergence, growth	ISO 14269-1 (1993)



Vibrio fischeri

Daphnia magna

Ceriodaphnia magna



Algae (*Pseudokirchneriella subcapitata*)

Collembola (*F. candida*)

Higher plant (oat)

Earthworm (*E. fetida*)

Fig. 12. Several test species used for the battery of ecotoxicity bioassays used in the present research.

PAHs contents in earthworm tissues and plants were also analysed by GC/MS and French standard (XP X 33-012) in the ETSA laboratory (Rouen, France).

Metals in soils and soil extracts were analysed by AAS (atomic absorption spectroscopy) or in BFE. laboratory (www.univ-metz.fr, University of P. V. METZ, France) and IRH Environment (www.irh.fr).

Total organic carbon, pH in H_2O, CEC, soil texture characterization, and moisture content were also measured according to French and ISO standards by INRA (www.inra.fr: National Institute for Agricultural Research, Arras, France).

IV.5. Ecotoxicological Bioassays

A set of different bioassays was used to assess the toxicity of soil and soil water extracts to soil and aquatic organisms, respectively (Fig. 12). The tests were carried out in accordance with the French (AFNOR) and ISO (International Standardization Organization) standard, when available (Table 12).

IV.5.1. Aquatic Toxicity Tests Protocols

Toxicity of soil extracts to aquatic organisms was determined on the water extract samples to assess both acute and chronic effects.

The acute toxicity tests were performed by measuring mobility inhibition of the crustacean *Daphnia magna* (ISO 6341, 1989) and luminescence inhibition of the bacterium *Vibrio fischeri* using Microtox® 500 system (Microbics company) according to ISO 11348-3 (1999).

Chronic toxicity tests were also performed to assess the following parameters:

- Population growth inhibition of *Ceriodaphnia magna* in 7 days (AFNOR T90-376, 2000),
- Growth inhibition of the freshwater alga *Pseudokirchneriella subcapitata* in a 3 days batch culture (ISO 8692, 1989).

Algae toxicity test were performed with the classical procedure (Erlenmeyer flask) and in parallel with the 96-well microplate.

IV.5.1.1. Microtox® Test Protocol

The bioluminescence inhibition assay is based on a marine gram negative

bacterium, *Vibrio fischeri* (earlier referred as *Photobacterium phosphoreum*). The specific strain, NRRL B-11177, has been widely used for acute toxicity estimation and several commercial test kits, i.e., Microtox, LUMIStox and ToxAlert are based on this strain (Farré & Barcelo, 2003). Light production is directly proportional to the metabolic activity of the bacterial cell and inhibition of enzymatic activity, especially the ones involved in energy production, causes a rapid decrease in bioluminescence. The assay provides a measure of sub-lethal response (Parvez et al., 2005).

The method is based on reduction of bioluminescence of the bacterium, *V. fischeri,* following direct exposure of the sample to the bacterial suspension. The test reagent was purchased as a freeze-dried bacterial form, stored at -20°C and reconstituted with ultrapure water at the time of the assay according to the procedure described by Microbics company (now Azur Environmental). 2% sodium chloride solution was used for the sample dilution. The vial containing the fresh bacteria culture and sample was maintained at 15°C and measured using a sensitive Microtox 500 photometer. The luminescence inhibition after 15 and 30min exposure was taken as endpoint.

IV.5.1.2. *Daphnia magna* Test Protocol

This bioassay was carried out with the cladoceran *D. magna*: immobilization test according to ISO standard method (ISO 6341, 1989). The experiment was initiated with neonates (< 24-h-old), born between the 3rd and 5th broods, which were obtained from bulk group cultures. Ten neonates were placed in individual glass tube with 10ml of the diluted sample solution without light. Assay was done in four replicates for each concentration. All experimental replicates were checked for immobilized individuals at 24 and 48h, which were counted for posterior determination of ECx values.

IV.5.1.3. *Ceriodaphnia dubia* Test Protocol

C. dubia was cultivated and maintained at 25 ± 1°C under a 16h light/ 8h dark photoperiod. The culture water was 20% *Evian* spring water and 80% *Volvic* spring water (v/v) (Ferrari & Ferard, 1995; Tatarazako et al., 2002). In each test, green algae (*P. subcapitata*), cerophyll and TetraMin© were used as food for *C. dubia.*

C. dubia survival and reproduction tests were run for seven days by French standard methods (AFNOR T90-376, 2000). Percent survival and the mean number of young produced per female were calculated, deleting the ones of dead adults. A minimum five concentrations were prepared with diluents. Sample dilutions were made with fresh culture solution. Ten replicate polyethylene vessels (25ml), each containing one organism, were used for each test concentration. These vessels were closed tightly using caps to minimize volatilisation of pollutants.
Culture solutions for test were renewed daily. For a test to be valid, one required a minimum 80% survival of control animals and ≥ 15 young per female over the seven-day test period.

IV.5.1.4. Algae Test Protocol

Algae toxicity tests were performed in *Erlenmeyer flasks* (125ml) according to ISO 8692 (1989). The algal strain used is unicellular chlorophyea *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*). Algae were maintained routinely and precultured according to standard method. Algal growth medium was the ISO medium, containing various nutrients and microelements. Algae were cultured in axenic conditions and only cultures in logarithmic phases were used for inoculations. Each flask contained 45ml of the tested sample and 5ml of inoculum to ensure a final algal concentration of 10^4 cells/ml. The flasks were illuminated with a constant light (4000 lux) and maintained at 23 °C with stirring (100 rpm/min). The assay was performed in triplicates. After 72h of incubation, cell counts were performed with a Coulter counter (Beckman Z1).

The 96-well *microplate* assays were performed in parallel using identical liquid media, samples, algal-precultures and incubation conditions with the following modifications: 96-well transparent polystyrene microplate with 2 ml liquid in each well used for fluorometric measurements, inoculum to ensure a final algal concentration of 2×10^4 cells/ml and incubation without agitation. These plates were sealed with their covers with a polyethylene sheet. Fluorescence measurement of microplate was performed using instrument (Fluostar, BMG LabTechnologies, Germany) with a 485-nm excitation filter and 640-nm emission filter.

The applications with microplate-based toxicity test using algae have been reviewed by Blaise and Vasseur (2005a).

IV.5.2. Genotoxicity Tests Protocols

All genotoxicity tests were applied with and without metabolic activation (S9). Microsomal enzymes were obtained from rats induced with Aroclor 1254. The supernatant fraction of the liver was homogenized and centrifuged at 9000g for 20min. The supernatant of hepatic homogenate (S9) containing metabolizing enzymes was used in genotoxicity bioassays for the detection of pro-mutagens.

IV.5.2.1. Mutatox® Test Protocol

The Mutatox test, which was developed by the Microbics company (now Azur Environmental), uses dark mutants of the luminescent bacteria *Vibrio fisheri* (strain M169). The assay was carried out with lyophilized reagents kept at -20°C and reconstituted at the time of the assay, according to the procedure described by Microbics. In the presence of mutagenic compounds mutants can reverse and recover their luminescence. Luminescence was measured with a bioluminometer after different times of exposure (16, 20 and 24 h) of the bacteria to the test medium at 27°C. A concentration which induced the luminescence intensity at least twice that of the negative control was declared positive. The sample was considered mutagenic when at least two successive concentrations were positive.

IV.5.2.2. Ames (Fluctuation) Test Protocol

The *Salmonella typhimurium his⁻ reverse mutation* test consists in measuring the growth of bacteria in a histidine-deficient medium. The bacterial mutants are initially unable to synthesize histidine and to grow in a medium devoid of this aminoacid. However, they recover this property after exposure to mutagens. The classical procedure was applied in *agar* medium. The *fluctuation* method carried out in liquid medium was applied for this study.

To a volume of 2.5 ml of medium consisting of Davis-Mingioli salts, D-glucose, D-biotine, L-histidine and bromocresol purple, was added with sterile distilled water containing the sample and 5 µl of an overnight culture grown in Oxoid broth. A 200 µl volume of the mixture was dispensed into 96-well microplates. The plates were sealed with their covers with a polyethylene sheet and incubated for 5 days at 37°C. Two microplates were used for each concentration of

the tested sample. The reversion of mutants was confirmed by a yellow color of the wells. The pH indicator, bromocresol purple, turned yellow due to the acidification of the test medium resulting from the growth of reverse mutants. For each concentration, the number of yellow wells in each microplate was counted and compared to negative controls. Chi-square analysis (Gilbert, 1980) was used for statistical evaluation of the treated plates versus the control plates. A sample is considered mutagenic at a tested concentration when there is a significant increase of the number of positive wells in treated plates over the negative control plates. A dose-effect relationship is useful to attest positivity of a sample. In order to eliminate bacterial contaminations the aqueous samples must be sterilized by membrane filtration (0.45 μm).

Genotoxicity was expressed by means of the LOEC which was the lowest dilution (or concentration) tested which induced a positive response.

Incubation of bacteria in a liquid medium (*fluctuation* method) enhanced the sensitivity of the Ames test, by increasing the bioavailability of pollutants in comparison with the agar plate method (Békaert et al., 1999).

IV.5.2.3. *umu* Test Protocol

Salmonella typhimurium TA1535 carrying a multicopy plasmid pSK1002 with fused *umu*C-lacZ genes and the gene for resistance to ampiciline was used as the tester strain. Monitoring of *umu*C expression was performed by measuring the cellular β-galactosidase activity, which reflects *umu*C induction.

All concentrations were tested in triplicate, with each set of experiments usually repeated two or more times. After 2h of exposure, the bacterial suspension was diluted 5-fold and 10-fold with warm TCA medium, followed by a subsequent additional incubation period of 2h. At the end of treatment and post-treatment incubation, the bacterial growth was measured as turbidity (600 nm) and the level of β-galactosidase activity (420 nm) was assayed by the colorimetric method using ONPG as a substrate. The genotoxic activities were expressed in β-galactosidase units and in enzyme induction ratios (IR) related to control samples. Induction rations above 1.5-fold are scored as sufficient positive results, estimated as minimal concentrations of genotoxins required to produce statistically significant increases from background controls.

IV.5.3. Terrestrial Organism Toxicity Tests

A set of different bioassays was used to assess the toxicity of contaminated soils to terrestrial organisms, respectively (Table 12). Three soil toxicity tests were carried out for this study according to the ISO standardized methods:

1) 17-day germination and early seedling growth higher plant test,
2) 14-day acute lethality and 56-day chronic reproduction (cocoon & juvenile production) earthworm test, and
3) 28-day collembola reproduction test.

IV.5.3.1. Earthworm Test Protocol

Acute and chronic toxicity tests with *Eisenia fetida* were carried out according to the ISO standardized methods (11268-1 & 2, 1994 & 1998). Survival and growth in biomass of adult earthworms were determined after 14days of exposure, whereas reproduction parameters, including cocoon production, hatching, juvenile survival, and growth were measured at 28 days and 56 days.

The test organism was *E. fetida* originating from our synchronized laboratory culture according to the ISO 11268-1 (1994) (Annex A: Example of breeding technique for *E. fetida*). To ensure comparable conditions in all tests, calcium carbonate ($CaCO_3$) was added to adjust pH (H_2O) to 6.0 ± 0.5 and distilled water was given to reach 60% of maximum water-holding capacity. Water content was readjusted weekly and test containers were incubated in climate chamber at 20 ± 2°C in a light: dark cycle of 16: 8 h at 400-800 lux. Four replicates were applied for both the acute and reproduction tests.

In the *acute* toxicity test (ISO 11268-1, 1994), ten adult worms with a fully developed clitellum were placed in 1 L glass vessels filled with 500g wet weight of test substrate. To prevent the worms from escaping, test containers were covered with a polyethylene sheet with 10 holes (Ø 1 mm) to ensure optimal ventilation. After 14 days of incubation, surviving worms were extracted by hand-sorting. The test endpoint was mortality.

In the *chronic* toxicity tests (ISO 11268-2, 1998), ten adult earthworms with a fully developed clitellum per vessel were also exposed. Finely ground cattle manure supplied weekly 5g per vessel for 4 weeks. Surviving worms were hand-sorted after 28 days, and the number of cocoons produced counted and further

incubated for another 28 days (total incubation time 56 days). The number of cocoons and juveniles were recorded for each of the four replicates at 28 days and 56 days, respectively. The test endpoints were 28-d cocoon production and 56-d numbers of hatched juveniles. The biomass of each individual was determined at the beginning and end of each test.

To obtain juvenile *E. fetida* for 14 day acute toxicity, cocoons from laboratory culture were reared in containers with the same substrate as culture for 5 weeks. Newly emerged earthworms were sieved from soil, weighed and used.

IV.5.3.2. Earthworm PAH Uptake Test

To determine PAHs bioaccumulation in earthworm tissues, *Eisenia fetida* juveniles after 56-d reproduction toxicity test were used and cultured for an additional period of 24 weeks. To ensure growth during the experiment, worms were supplied with cattle manure as a source of food. Manure was supplied per container at a ratio of 1g (dry weight) per 1g biomass (wet weight) each week. Cluzeau et al., (1989), concluded that 350 mg/g worm per week of suitable food would be sufficient to allow unrestricted growth, maturation in *E. andrei*.

After 24 weeks culture, the worms were removed from the soil by digging and hand sorting and washed with tap water. One-fourth of the posterior part was massaged to expel the content of the lower gut. The guts of the earthworms were then evacuated by incubation in pre-extracted silica (silicon dioxide) for 24h.
Earthworms were once more massaged, rinsed with distilled water and stored at -20°, and freeze-dried. And earthworms were then sent to the analytical lab for PAHs Soxhlet extraction of earthworm tissues and chemical analyses.

IV.5.3.3. Collembola Test Protocol

The springtail *Folsomia candida* test was carried out according to the ISO standardized methods (11267, 1998).

In the chronic toxicity tests, ten adults synchronized collembola 10 ~ 12 days old were exposed per 0.1l glass vessel containing 30 g wet weight of soil. The reproduction assay with *F. candida* lasted for 4 weeks. Granulated dry yeast was added weekly to the soil surface as food. At the end of test, the number of adults, and juveniles were counted after extraction by floatation

The springtail *F. candida* (Collembola; Isotomidae) was cultured in the laboratory, in plastic containers with substrates of plaster of Paris mixed with powdered activated charcoal, at 20 ± 2°C and at 400-800 lux. The breeding containers were tightly closed and high moisture content of the substrate was maintained by periodically adding a few drops of water. Cultures were fed with granulated dry yeast (Baker' Yeast) twice a week in small amounts to avoid spoilage by fungi. Synchronized cultures of juvenile collembolae (10 - 12 day old) were obtained by transferring egg clusters from breeding containers to a freshly prepared breeding substrate using a fine hair-brush. After 48 h of starting to hatch, the non-hatched eggs were removed. Juvenile collembola were collected after a further 10 days of incubation.

The collembola reproduction test determines the effects of different concentrations of test substances on the reproduction of 10 to 12-day old collembola in artificial substrate or test soils (ISO 11267, 1998). The numbers of offspring produced by the collembola were determined after 28 days. Milli-Q water was added to achieve 50% of the water holding capacity. Each test container, which was 0.1L glass jars (55mm diameter×60 mm high), was filled with 30g wet mass of the test substrate. For each dilution series and control, four replicates were prepared. Ten juvenile collembola were placed in each test container and 2 mg of granulated dry yeast were added. Test containers were covered with a polyethylene sheet and placed in an environmental cabinet at 20 ± 2°C with lighting at 400-800 lux and a 16:8 h light-dark photoperiod twice a week for aeration. At the end of test, the number of adults and juveniles were counted from a photograph of the surface after extraction them by floatation. Indigo blue dye was added to the water to improve contrast between the collembola and the water surface and floating debris.

IV. 5.3.4. Plant Toxicity Test Protocol

In a higher plant toxicity tests, three plant species were used: one monocotyledon (*Avena sativa* L., common name: oat) and two dicotyledons (*Lactuca sativa* L., common name: lettuce, *Brassica chinensis* J., common name: Chinese cabbage). The seeds were obtained from a commercial supplier. Before being used for bioassays, their germination potential was examined and germination over 90% guaranteed the viability of the seeds.

The continuous seed germination and seedling growth tests were performed according to the ISO 11269-2 (1994). Assays were conducted in four replicate

pots (disposable plastic, diameter 9 cm, height 6.5 with some drain holes on the bottom). A petridish (9 cm diameters) was placed underneath each pot. Fifteen seeds for oat and thirty seeds for Chinese cabbage and lettuce were sown uniformly in each pot containing 160 g of dry weight soil. At the beginning of each experiment, the soil was rehydrated to 70 % for oats and 80 % for lettuce12 and Chinese cabbage. Water evaporation was determined by daily weighing of pots and compensated by addition of distilled water. Plants were grown in a chamber under controlled conditions at a temperature of 20 ± 2°C and with irradiation of 2000 lux under a 16/8 h (light/dark) photoperiod. Seed germination was determined by visual seedling emergence and was recorded daily until the termination of the test. After 50 % of the seeds had germinated in the control soils, the test was terminated 14 - 21 days later. The seedling shoots were then cut above the soil surface and the total fresh biomass of each pot (five shoots or more which were uniform) was immediately weighed. The dry biomass was also measured after oven drying at 70 - 80°C for 16 h.

IV. 5.3.5. Plant PAH Uptake Test

To determine PAHs bioaccumulation in plants, tests was performed under the same conditions as procedures described in the ISO standard method. After 50% of the seeds had germinated in the control soils, the seedlings harvest was conducted 28 days later. Afterwards, the plant samples were freeze-dried, weighed, and stored at - 20°C and sent to the analytical lab for PAH analyses.

IV.6. Quality Control

The toxicity tests included negative controls (no sample or toxic substances added). Positive controls (reference toxicants according to protocols) were performed concurrently with the sample as a quality control in each series of assays. The validity of ecotoxicological analyses was also insured according to the established criteria (e.g., ISO). The sensitivity of the test organisms was proven using reference toxicants according to standard methods. Adult mortality, growth, and reproduction endpoints were verified against laboratory in-house control data.

IV.7. Statistical Analysis and Result Expressions

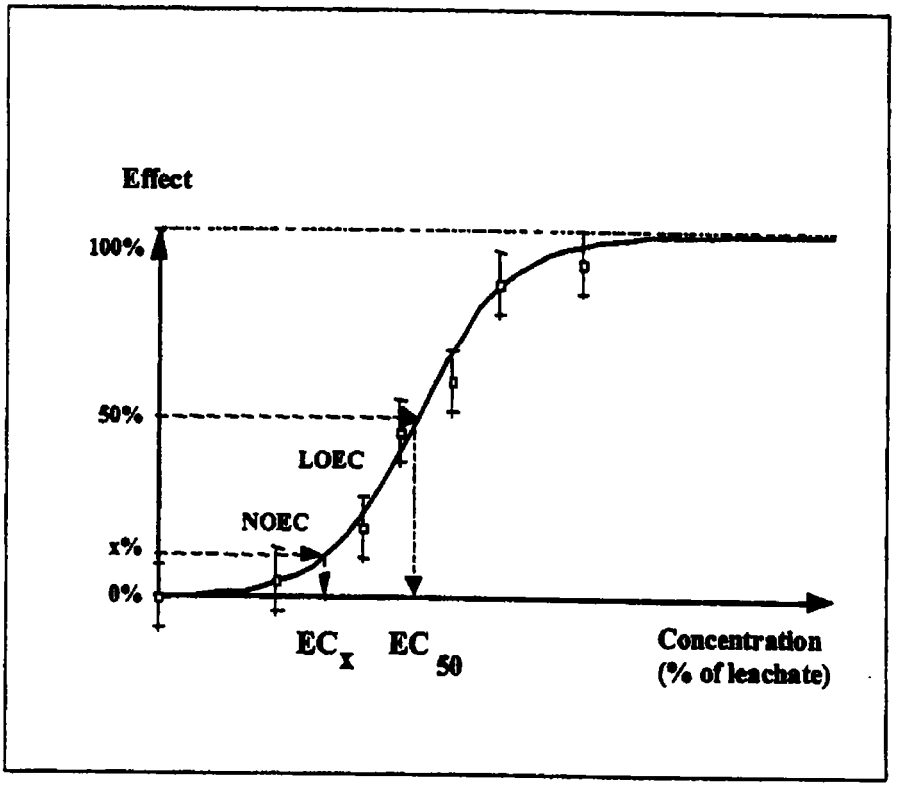


Figure 13. Example of a typical concentration-response curve obtained from a bioassay and the associated measurement endpoints (NOEC, LOEC and EC*x*) calculated by a statistical methods (from Férard & Ferrari, 2005).

Ecotoxicity testing of water extract (or tested soil) samples obtained at different liquid-to-solid (or solid-to-solid) aims at measuring effects on species representing various levels of biological organization as a function of dilution rate while controls without water extract (or tested soil) were used as reference. In order to express results in a synthetic form, raw data obtained from concentration-response curves are transformed into a summary criterion corresponding to a specific measurement endpoint (e.g., EC_{50}, EC_x, NOEC, LOEC, etc) for each test (Fig. 13) (Férard & Ferrari, 2005).

Isnard et al., (2001), collected twenty-seven sets of chronic data (algae, daphnia, fish) and analyzed by ANOVA and regression models. They reported the EC_x depend on the regression models and that their accuracy decreases in the low effect zone and EC_{50} are the most precise values to estimate on a concentration-response curve, but they are clearly different from the NOEC and their use would require a modification of existing assessment factors. The log-logistic model provided the best fits among the restricted set of models considered, as well as the most precise determinations of EC_x (confidence intervals from a bootstrap procedure).

The EC_x that produces a specific percent reduction (e.g., x = 10, 20 or 50%) in ecotoxicity testing can be estimated by an equation directly derived from the logistic model, as follows:

$$EC_x = EC50 \times [\, x / (100 - x)]^{1/Hn}$$

Where

EC50 is the estimated concentration which causes 50% of reproduction inhibition and Hn is the estimated Hill number corresponding to the slope of the sigmoid curve; x corresponds to the x% level of effect compared to the control.

Estimation of each parameter of such a logistic model and their confidence intervals associated can be performed with any statistical analysis software. Calculations can be programmed into Microsoft Excel® solver. An Excel® macro called REGTOX has been elaborated and can be downloaded form the Internet (http://eric.vindimian.9online.fr/en_index.html). REGTOX is freely distributed with the moral obligation to cite its source whenever its use results in publications. The advantage of REGTOX is that other regression models, such as Weibull and Log-

normal models can also be used (Férard & Ferrari, 2005).

Data were also analysed with a nonparametric Kruskall-wallis ANOVA to identify difference among samples. When differences among the means were found, a Mann-Whitney U-test for independent samples was used to compare the results ($P \leq 0.05$; STATISTICA 5.1 for windows, Statsoft, Tulsa, OK, USA).

For a more convenient graphical expression and interpretation of the toxicity data, all median toxicity values were converted into Toxic Units (TU), i.e. the inverse of the EC_{50} expressed in %, using the formula: $TU = (100 / EC_{50})$. This expression is the dilution factor which must be applied to the water extract or soil samples so as to obtain a 50% effect, and is directly proportional to toxicity.

CHAPTER V. RESULTS

CHAPTER V. RESULTS

V.1. Ecotoxicity of a Polycyclic Aromatic Hydrocarbon (PAH)-Contaminated Soil

Samples of soil polluted by PAHs were assessed for their toxicity to terrestrial and aquatic organisms and their mutagenicity.

- Physico-chemical analyses of soils and water extracts were conducted in order to quantify (in)organic pollutants including PAHs (polycyclic aromatic hydrocarbons), heavy metals and to determine the degree of PAHs water-extractability from soils. A standard leaching protocol was used for water extraction.
- Toxicity of water-extractable pollutants from contaminated soil samples was evaluated using acute (*Vibrio fischeri*; Microtox® test, *Daphnia magna*) and chronic (*Pseudokirchneriella subcapitata*, *Ceriodaphnia dubia*) bioassays.
- Genotoxicity of water extracts was also assessed by means of the Ames (fluctuation), *umu*, and Mutatox® tests.
- Direct effects of contaminated soils on terrestrial organisms such as earthworms (*Eisenia fetida*), collembolae (*Folsomia candida*) and higher plants (lettuce: *Lactuca sativa* & chinese cabbage: *Brassica chinensis*), were tested using endpoint parameters such as survival, reproduction and growth. The juvenile earthworm 14-d survival test was also argued for its use as ecotoxicological endpoint.
- Bioassays were performed under standard (ISO) conditions. Effective concentration (EC_x) values based on concentration-response relationships were estimated and compared.
- We integrated the results of chemical analyses and biological assays in an attempt to identify pollutants responsible for toxicity of the PAH-contaminated soils and water extracts.

Ecotoxicity of a polycyclic aromatic hydrocarbon (PAH)-contaminated soil

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Abstract

Soil samples from an ancient cokery site polluted by PAHs (polycyclic aromatic hydrocarbons) were assessed for their toxicity to terrestrial and aquatic organisms and their mutagenicity. Total concentration of the 16 PAHs listed as priority pollutants by the US Environmental Protection Agency (EPA) was 2634 ± 241 mg/kg d.w in soil samples. Toxicity of water-extractable pollutants from contaminated soils was evaluated using acute (*Vibrio fischeri*; Microtox® test, *Daphnia magna*) and chronic (*Pseudokirchneriella subcapitata*, *Ceriodaphnia dubia*) bioassays and the EC values were expressed as % water extract in the test media (v/v). Algal growth (EC50-3d = 2.4±0.2 % of the water extracts) and reproduction of *C dubia* (EC50-7d = 4.3±0.6 %) were the most severely affected, compared to bacterial luminescence (EC50-30 min = 12±3%) and daphnid viability (EC50-48h = 30±3%). The Ames and Mutatox□ tests indicated mutagenicity of water extracts, while no response was found with the *umu test.*

Toxicity of the soil samples was assessed on survival and reproduction of earthworms (*Eisenia fetida) and* collembolae (*Folsomia candida*), and on germination and growth of higher plants (*Lactuca sativa* L.: lettuce & *Brassica chinensis* J.: chinese cabbage). The EC50 values expressed as % contaminated soil in ISO soil test medium (w/w) indicated severe effects on reproduction of the collembola *F. candida* (EC50-28d = 5.7% soil) and *E. fetida* (EC50-28d = 18% soil and EC50-56d = 8% soil based on cocoon and juvenile production respectively). Survival of collembolae was already affected at low concentration of the contaminated soil (EC50-28d = 11%); Viability of juvenile earthworms was inhibited at much lower concentrations of the cokery soil (EC50-14d = 28%) than viability of adults (EC50-14d = 74%). Only plant growth was inhibited (EC50-17d = 26% soil) while germination was not.

Chemical analyses of water extracts allowed us to identify inorganic water extractible pollutants as responsible for toxicity on aquatic species, and especially copper for effects on *D magna* and *C dubia.* Toxicity of soils on collembolae and earthworms could be explained by the 4 PAH congeners, fluorene, phenanthrene, pyrene and fluoranthene.

Keywords: Water extract, PAHs, Mutagenicity, Bioassay, Contaminated soil, Earthworm, Collembola, Plant

1. Introduction

Soils are increasingly becoming sinks for a wide range of hazardous chemicals generated by human activities. These include polycyclic aromatic hydrocarbons (PAH) residues which result from coke production, petroleum refining, combustion and other high-temperature industrial processes (Bispo et al., 1999). Hazard and risk assessment of polluted soil samples are usually performed by means of physical and chemical measurements, but chemical analysis alone may not be sufficient for biological assessment (Fernandez et al., 2005). Analysis of all the chemical pollutants contributing to toxicity and of their synergistic and antagonistic effects using chemical data is not possible (Juvonen et al., 2000). Therefore, a biological approach is required.

Bioassays can provide a measure of the whole toxic impact, due to complex mixture of chemicals integrating different factors, such as pH, solubility, antagonism, synergism, bioavailibility, etc. The use of a battery of bioassays involving different bioindicator species at different trophic levels is an efficient and essential tool for predicting environmental hazards to the aquatic ecosystem (Farré and Barcelo, 2003).

The use of a set of tests on species at different levels of biological organization and biological approaches in complement to physico-chemical analyses are needed and recommended in ecotoxicological studies for a refined evaluation of environmental risk (Debus and Hund, 1997; Bierkens et al., 1998; Bispo et al., 1999; Békaert et al., 1999; Farre and Barcelo, 2003; Rila and Eisentraeger, 2003; Fernandez et al., 2005). The three most frequently used bioassays in ecotoxicity testing are the acute *Vibrio fischeri* test, the acute *Daphnia magna* test and the chronic *Pseudokirchneriella* subcapitata test, yet chronic toxicity tests are by far less frequently performed (Férard and Ferrari, 2005). While soil leachates have often been used to assess soil ecotoxicity with aquatic organisms, the main focus of soil quality testing should concern soil-dependent organisms (Keddy et al., 1995). Relatively few ecotoxicity studies have been conducted on soil organisms, and such studies have used only one or a few substances and species (Sverdrup et al., 2002).

Terrestrial plant and invertebrate (earthworm, collembola) tests should be selected on the basis of their ability to measure chemical toxicity to ecologically relevant test species during chronic assays and include at least one reproductive

Table 1. Summary of the bioassays selected to assess the toxicity of a PAH-contaminated soil and their water extracts to aquatic and terrestrial organisms.

	Tests (species)	Durations	Measured parameters	Test procedure
Microorganisms	Microtox® (*Vibrio fischeri*)	15, 30 min	Luminescence inhibition	ISO 11348-3 (1999)
	Mutatox® (*Vibrio fischeri* M169)	24 hrs	Genotoxicity	Microbics (1993)
	Ames (*Salmonella typhimurium* TA98, TA100)	72 hrs	Genotoxicity	Environment Canada (1993)
	UMU (*Salmonella typhimurium* TA1535/PSK1002)	4 hrs	Genotoxicity	ISO 13829 (1999)
Algae	Algae (*P. subcapitata*)	72 hrs	Growth inhibition	ISO 8692 (1989)
Aquatic invertebrates	Daphnid (*Daphnia magna*)	24, 48 hrs	Immobilization	ISO 6341 (1989)
	Ceriodaphnid (*Ceriodaphnia dubia*)	7 days	Reproduction	AFNOR T90-376 (2000)
Terrestrial invertebrates	Collembola (*Folsomia candida*)	28 days	Survival, Reproduction	ISO 11267 (1998)
	Earthworm (*Eisenia fetida*)	14 days	Survival	ISO 11268-1 (1994)
		28, 56 days	Reproduction	ISO 11268-2 (1998)
Terrestrial plants	Lettuce (*Lactuca sativa*) Chinese cabbage (*Brassica chinensis*)	14 - 21 days	Germination, growth	ISO 14269-1 (1993)

component among the measured endpoints (Gong et al., 1999; Kuperman et al., 2004). Springtails (Collembola) are among the most abundant of soil arthropods (Hopkin, 1997) and collembolae were found to be more sensitive species than other terrestrial invertebrates, such as earthworms, enchytraeids, to PAH compounds (Sverdrup et al., 2002). *Folsomia candida* is a species commonly used to assess the effects of chemicals on nontarget soil arthropods (Walker et al., 2006). The *F. candida* reproduction test appeared to be a useful assay that should be included for soil ecotoxicological testing of xenobiotic compounds (Crouau et al., 1999).

Bioavailability is a key word that is often encountered in current publications on the fate of pollutants in ecosystems and their effects on individual species, populations and ecosystem levels (Peijnenburg et al., 2002). Several authors reported that bioavailability of PAH compound such as phenanthrene was markedly affected by soil properties and environmental factors such as, organic matter, clay content, aging (or weathering), wetting and drying of soils, and other properties of soil (White et al., 1997; Nam et al., 1998; Chung & Alexander, 2002).

Although PAHs are responsible for the contamination of a wide range of industrial soils, ecotoxicological data on PAH-contaminated soils are sparse. The few ecotoxicity studies conducted so far concerned soils contaminated with creosote (Charrois et al., 2001), tar (Sayles et al., 1999), residues of coke oven activity (Mendona & Picado, 2002) and gas plant (Sasek et al., 2003). Effects on higher plants, earthworms and collembolae were assessed..Other studies have been conducted using individual PAH-spiked soils in which bioavailability of PAHs may be different from field contaminated soils. Debate on bioavailability of PAHs in ancient polluted sites requires that biological investigations are conducted using field contaminated soil samples. This study was based on these research needs.

The aims of this study were: (i) to evaluate PAH-contaminated soil from an ancient cokery which stopped its activities more than thirty-years-ago, using ecotoxicological and chemical analyses, and (ii) to compare and assess the sensitivity of acute, chronic, and genotoxic bioassays using biological models representing different trophic levels, - microorganisms, algae, aquatic and terrestrial invertebrates and plants - to soil water extracts or contaminated soil samples (Table I).

2. Experimental

2.1. Soil samples and water extraction procedure

The soil studied was sampled from a site polluted by cokery residues and contaminated by polycyclic aromatic hydrocarbons (PAHs). The soil sample was sieved at 4 mm and homogenized. Fresh sieved soil was stored at 13°C. Three independent samples of the sieved soil were analysed. The soil water extraction procedure was carried out according to the ISO standard (12457-2, 1999), comprising a 24 ± 0.5 h mixing with ultrapure water (soil: water ratio of 100g/1 L) in two-liter polyethylene bottles at a temperature of 20 ± 2°C. The bottles were placed in a rotating apparatus working at 10 rpm and a settling period was maintained for 12 ± 0.5 h at 4 ± 1 °C. After centrifugation of the liquid phase (1500g, 15min) and paper filtration, the supernatant was tested for ecotoxicity and stored at 4 °C. All the bioassays were undertaken within 72h after preparation. Several water extractions of different samples of the soil were carried out independently. Water extracts I and II were analyzed in parallel for chemical pollutants and for their mutagenicity and their ecotoxicity. A 0.45 μm filtration was carried out for mutagenicity testing with the Ames and umu tests.

2.2. Physico-chemical analyses

PAH concentrations in soil extracts were analysed by HPLC/fluorescence method according to French standard (NF T 90-115) in the Laboratoire de Rouen/ETSA (www.laborouen.com, France) and PAHs in soils were determined by french standard XP X 33-012 (HPLC/UV) method after ASE extraction in the IRH Environment laboratory (Nancy, France). Metals in water extracts and soils were analysed by AAS (atomic absorption spectroscopy). Total organic carbon, pH, soil texture, and moisture content were also measured according to the ISO standards by INRA (www.inra.fr, Arras, France).

Concentrations of the sum of the 16 PAHs were integrated in a TEQ index (toxic equivalent quantities to Benzo(a)pyrene) for genotoxicity purposes. TEQ was calculated using the TEF (toxic equivalent factor) values of each PAH proposed by Nisbet and LaGoy (1992). For each PAH, concentration was ponderated with genotoxic potential and multiplied by the corresponding TEF in order to obtain TEQ. Then, the sum of the 16 TEQ values was calculated as an indicator of genotoxicity potential of the whole mixture.

2.3. Ecotoxicological bioassays

2.3.1. Aquatic toxicity tests

Toxicity of water extractable pollutants to aquatic organisms was determined to assess both acute and chronic effects. The acute toxicity tests were performed with mobility inhibition of the crustacean *Daphnia magna* (ISO 6341, 1989) and luminescence inhibition of the bacterium *Vibrio fischeri* (Microtox® 500 system, Microbics©) according to the ISO 11348-3 (1999). Chronic toxicity tests were carried out measuring parameters indicators of population development : reproduction inhibition of *Ceriodaphnia magna* in 7 days (AFNOR T90-376, 2000) and growth inhibition of the freshwater alga *Pseudokirchneriella subcapitata* in a 3 days batch culture (ISO 8692, 1989).

Algal toxicity tests were performed in Erlenmeyer flask according to the classical procedure and in 96-well microplate ("microbiotest") in parallel. Fluorescence measurement in microplates was performed using Fluostar (BMG LabTechnologies, Germany) with a 485-nm excitation filter and 640-nm emission filter.

In *C. dubia* reproduction test, the water used for culture and testing was a mineral water, a mixture of *Evian-Volvic* mineral drinking waters (1/4 vol/vol) (Férrari and Ferard, 1995; Tatarazako et al., 2002).

2.3.2. Genotoxicity tests

Mutatox test which was developed by the Microbics Company, uses dark mutants of the luminescent bacteria *Vibrio fischeri* M169. The assay was carried out according to the procedure described by Microbics© (1993).

The Ames *Salmonella typhimurium his*$^-$ reverse mutation test was conducted with the bacterial strains TA98 and TA100 in liquid medium in 96-well microplates according to fluctuation method (Environment Canada, 1993).

The *umu* test with the bacterium *Salmonella typhimurium* TA1535/pSK1002 was carried out according to ISO (13829, 1999). All genotoxicity tests were performed with and without S9 metabolic activation from livers of Aroclor 1254-treated rats. In order to eliminate bacterial contaminations, the aqueous samples for the Ames and *umu* tests were sterilized by membrane filtration (0.45 μm).

2.3.3. Terrestrial organism toxicity tests

The artificial soil used as test substrate was composed as prescribed by the respective ISO protocols (ISO 11268-1, 2, 1994; 1998), of 70% sand and 20% kaolinite clay and 10% 1-mm sieved finely ground sphagnum peat, and adjusted to pH 6.0 ± 0.5 with $CaCO_3$. Earthworm and collembola tests were carried out at a soil moisture content of 40-60 % of water holding capacity and during exposure, all test vessels were kept at 20 ± 2°C in a light (400-500 lux): dark cycle of 16:8 h.

2.3.3.1. Plant toxicity test

In a higher plant toxicity tests, two species were used: *Lactuca sativa L.*, common name: lettuce, *Brassica chinensis J.*, common name: Chinese cabbage. Several concentrations of the cokery soil in ISO artificial soil were tested : 100, 50, 25, 10, 1% weight per weight (w/w) with 100% ISO soil as control.

The early seedling growth tests were carried out in a chamber at 20 ± 2°C, which was maintained at the following conditions: lighting 16h; light intensity 2000 lux. Soils (with moisture content of 70-80 % of water holding capacity) of 160g were placed in a disposable plastic pot (diameter: 9 cm [top]) and distilled water was refilled to continuously maintain available water as needed. Fresh and dry shoot biomass of plants were weighed at 17 days exposure after 50% of the seeds had germinated in the control soils.

2.3.3.2. Earthworm test

Acute and chronic toxicity tests with *Eisenia fetida* were carried out according to the standardized methods (ISO 11268-1, 2, 1994; 1998). Survival and growth in biomass of adult and juvenile earthworms were determined after 14 days of exposure, whereas reproduction parameters, including cocoon production at 28 days, juvenile production were measured at 56 days.

Several concentrations of the cokery soil mixed with the ISO artificial soil in the range 100%-10% were tested. Ten adult worms with a fully developed clitellum were exposed per 1 L glass vessel containing 500 g wet weight of soil for 4 weeks. Finely ground cow dung was supplied weekly as food (5 g per vessel) and then surviving earthworm were hand sorted out from soils. The number of cocoons and

juveniles were recorded for each of the four replicates at 28 days and 56 days, respectively.

To obtain juvenile *E. fetida* for survival test, cocoons from laboratory culture were reared in containers with the same substrate as culture for 5 weeks. Newly emerged worms were sieved from soil, weighed and used for the acute 14-day toxicity test. Ten individuals were exposed in 1 L glass vessel containing 200 g wet weight of soils for 14 days. Other experimental conditions were the same as described in the standard method ISO (11268-1, 1994) for adult survival.

2.3.3.3. Collembola test

The springtails *Folsomia candida* test was carried out according to the standardized method (ISO 11267, 1998). Ten adults with synchronized collembola 10 - 12 days old were exposed per 0.1 L glass vessel containing 30 g wet weight of soil. The range of the tested concentrations of the cokery soil were 20, 15, 10, 5, 2.5% (w/w) in the ISO soil, with 100% ISO as control. The reproduction assay with *F. candida* lasted 4 weeks. Granulated dry yeast was added two times, at the start of the test and two weeks later, to the soil surface as food. At the end of the test after 28 days, the numbers of adults and juveniles were counted from a photograph of the surface after extraction by floatation.

2.4. Quality control

The toxicity test included negative (no sample or toxic substances added) and positive (reference toxicants according to protocols) controls concurrently with the sample as a quality control in each series of assays. The validity of bioassays was ensured according to the established criteria (e.g., ISO standards) and test results were complied with the validity criteria for negative controls defined in the respective ISO test protocols.

2.5. Statistical analysis and data expression

Toxicity endpoints such as effective concentrations values (EC_x) that were used for the earthworm, plant, Microtox and algal tests etc, were estimated using the bootstrap method in the REGTOX Excel® macro (E.Vindimian, INERIS, France),

Table 2. Main physico-chemical characteristics of the PAH-contaminated soil studied.

	$LogK_{ow}$ [a]	QL [b]	Contaminated soil
Soil texture			
Clay (%)			10.7
Silt (%)			22.1
Sand (%)			67.2
pH (H_20)			9.6
Water-holding capacity (%)			53
Organic matter (%)			17.3
Total organic carbon (%)			10
Heavy metals (mean ± sd)[c]		(mg/kg)	(mg/kg)
Cd		0.02	6.7 ± 0.6
Cr		0.08	54 ± 0.9
Cu		0.1	26 ± 0.6
Hg		0.002	12 ± 0.3
Pb		0.3	120 ± 4
Zn		0.02	347 ± 6
Ni		0.24	23 ± 0.3
PAHs (mean ± sd)[c]		(µg/kg)	(mg/kg)
Naphthalene	3.4	10	5.9 ± 2
Acenaphthylene	4.07	1	4.6 ± 0.3
Acenaphthene	3.92	1.7	46 ± 4
Fluorene	4.18	3.7	103 ± 9
Phenanthrene	4.6	2.9	380 ± 46
Anthracene	4.5	0.1	186 ± 39
Fluoranthene	5.22	1.7	561 ± 49
Pyrene	5.18	1.2	379 ± 28
Benzo[a]anthracene	5.61	0.1	230 ± 19
Chrysene	5.91	0.1	196 ± 16
Benzo[b]fluoranthene	6.12	0.1	146 ± 8
Benzo[k]fluoranthene	6.84	0.1	89 ± 6
Benzo[a]pyrene	6.50	1.1	145 ± 8
Dibenzo[ah]anthracene	6.50	0.1	15 ± 0.8
Benzo[ghi]perylene	7.10	0.1	65 ± 3
Indeno[1,2,3-cd]pyrene	6.58	0.1	82 ± 5
Σ16 PAHs			**2634 ± 241**

[a] Octanol-water partition coefficients: data from EHC 202 (IPCS, 1998).

[b] QL: Quantification limits in µg/kg dw.

[c] mean ± sd: mean ± standard deviation (n=3).

Table 3. Contaminant characterization and leaching capacity of the PAHs-contaminated soil water extracts I and II .

	QL[a]	Water extracts[b]		*R*[c]	
		I	II	I	II
Heavy metals	(μg/L)	(μg/L)			
Cd	0.1	0.1	<0.1	1.5×10^{-5}	$<1.5\times10^{-5}$
Cr	0.4	1.2	1.0	2.2×10^{-5}	1.8×10^{-5}
Cu	0.5	195	188	7.5×10^{-3}	7.2×10^{-3}
Hg	0.01	<0.3	<0.3	$<2.5\times10^{-5}$	$<2.5\times10^{-5}$
Pb	1.5	<1	<1	$<8.3\times10^{-6}$	$<8.3\times10^{-6}$
Zn	0.1	9	5	2.6×10^{-5}	1.4×10^{-5}
Ni	1.2	32	30	1.4×10^{-3}	1.3×10^{-3}
PAHs	(ng/L)	(μg/L)			
Naphthalene	5	2.9	<0.10	4.9×10^{-4}	$<2\times10^{-5}$
Acenaphthylene	5	1.3	<0.80	2.8×10^{-4}	$<1.7\times10^{-4}$
Acenaphthene	5	2.6	0.42	5.7×10^{-5}	0.9×10^{-6}
Fluorene	5	2.4	0.97	2.3×10^{-5}	0.9×10^{-6}
Phenanthrene	5	2.8	1.9	7.4×10^{-6}	4.9×10^{-6}
Anthracene	5	0.59	0.27	3.2×10^{-6}	1.4×10^{-6}
Fluoranthene	3	1.6	0.2	2.9×10^{-6}	0.4×10^{-6}
Pyrene	5	1.1	0.7	2.9×10^{-6}	1.8×10^{-6}
Benzo[a]anthracene	5	0.47	<0.05	2.0×10^{-6}	$<2.1\times10^{-7}$
Chrysene	5	0.34	<0.05	1.7×10^{-6}	$<2.5\times10^{-7}$
Benzo[b]fluoranthene	2	0.4	<0.05	2.7×10^{-6}	$<3\times10^{-7}$
Benzo[k]fluoranthene	0.5	0.23	<0.05	2.6×10^{-6}	$<5.6\times10^{-7}$
Benzo[a]pyrene	1	0.43	<0.05	3.0×10^{-6}	$<3.5\times10^{-7}$
Dibenzo[ah]anthracene	5	0.08	<0.05	5.2×10^{-6}	$<3.2\times10^{-6}$
Benzo[ghi]perylene	3	0.23	<0.10	3.5×10^{-6}	$<1.5\times10^{-6}$
Indeno[1,2,3-cd]pyrene	0.5	0.28	<0.10	3.4×10^{-6}	$<1.2\times10^{-6}$
Σ16 PAHs		**17.7**	**4.5**		

[a] Given as μg/L to metals & ng/L to PAHs.

[b] PAHs concentrations of water extracts were determined in two times independently (I, II).

[c] *R*: Ratio of PAH concentration in water extracts to concentration in soil [(μg/L)/ (μg/kg)].

which is available from http://eric.vindimial.9online.fr/en_index.html). Data were analysed with a nonparametric Kruskall-Wallis ANOVA to identify difference among samples and exposed organisms. When differences among the means were found, a Mann-Whitney U-test was used to determine the differences between independent samples ($P<0.05$; STATISTICA 5.1 for windows, Statsoft, Tulsa, OK, USA). The NOEC is the highest concentration tested that did not significantly (risk $\alpha = 5\%$) differ from the control. For a more convenient graphical expression and interpretation of the toxicity data, all median toxicity values were converted in Toxic Units (TU), i.e. the inverse of the EC_{50} expressed in %, using the formula: $TU = (100 / EC_{50})$. This expression is the dilution factor which must be applied to the water extract or soil samples so as to obtain a 50% effect, and is directly proportional to toxicity. Isnard et al., (2001), reported the EC_x depends on the regression models and that their accuracy decreases in the low effect zone and EC_{50} are the most precise effect values.

3. Results

3.1. Chemical analyses of the cokery soil and water extracts I and II.

The physical and chemical characteristics of soil are presented in Table 2. The soil studied appeared principally contaminated with PAHs and slightly with heavy metals. The sum of the concentrations of the 16 PAHs listed by US-EPA for environmental survey was determined to 2634 ± 241 mg/kg d.w. from three independent soil samplings composed of 28% of 2 & 3 rings PAH, 52% of 4 rings and 21% of 5 & 6 rings congeners. The heavy metal concentrations in soil were at low (Cr, Cu, Ni) or medium (Cd, Pb, Zn) levels in the range of respective regional reference values in the Lorraine country (France) where the cokery was located. Mercury levels were higher than reference values.

The total concentrations of the16 PAHs in the two water extracts, prepared independently, were 17.7 µg/l for the water extract I and 4.5 µg/l for the water extract II (Table 3). No major difference in metal concentrations was noted between the two extracts. Copper and nickel were removed at higher concentrations than chromium, zinc or cadmium, the latter element was only found in extract I. No mercury and lead were present in both water extracts I and II (< quantification limits).

Table 4. Toxicity expressed as NOEC, $E(L)C_{10}$, $E(L)C_{20}$, $E(L)C_{50}$ of different water extracts independently (n>5) prepared from the PAH-contaminated soil to aquatic species.

	Microtox®		***D. magna***		***C. dubia***	**Algae**	
Test duration	15 min	30 min	24 h	48 h	7 d	3 d (*Erlenmeyer flask*)	3 d (*Microplate*)
Endpoints	Luminescence inhibition		Immobilization		reproduction	Population growth	
NOEC		< 5		22 ± 5	2.6 ± 0.9	0.6 ± 0.5	3.1 ± 1
$E(L)C_{10}$ (% dilution)	3.9 ± 2	3.4 ± 2	39 ± 15	20 ± 2	3.0 ± 0.7	1.0 ± 0.2	2.7 ± 1
$E(L)C_{20}$ (% dilution)	5.6 ± 0.5	5.1 ± 1	46 ± 10	23 ± 1	3.4 ± 0.7	1.5 ± 0.1	3.6 ± 1
$E(L)C_{50}$ (% dilution)	13 ± 3	12 ± 3	55 ± 9	30 ± 3	4.3 ± 0.6	2.4 ± 0.2	6.0 ± 2
N	6	6	5	5	5	5	5
Toxicity (TU)	7.7	8.3	1.8	3.3	23	42	17

[a] Data given as average ± standard deviation.
[b] EC_x: effective concentration in % water extracts (v/v) in the test medium causing x % inhibition of measured endpoints.
[c] Toxicity expressed in toxic units(TU) according to the following formula; $TU = 100/ EC_{50}$.
[d] N: number of soil water extracts prepared independently for bioassays.
[e] In *Ceriodaphnid* test, the water mediums used for cultures and tests were an *Evian-Volvic* medium.

Table 5. LOECs of the genotoxicity tests carried out on the PAH-contaminated soil water extracts I & II.

	Σ16 PAHs (ΣTEQs)	LOEC (%, v/v)							
		Ames test				Mutatox® test		*umu* test	
	µg / L (µg B(a)P/ L)	TA 98 -S9	TA 98 +S9	TA 100 -S9	TA 100 +S9	-S9	+S9	-S9	+S9
Water extract I	17.7 (1.091)	negative	25	50	6	25	negative	negative	negative
Water extract II	4.5 (0.007)	negative	negative	75	75	6	50	negative	negative

[a] LOEC: Lowest observed effect concentration in % water extracts in the test medium.
[b] TEQ : Toxic equivalent quantities in µg of benzo(a)pyrene per L of extract according to Nisbet and LaGoy (1992).
[c] The Ames test was carried out in liquid medium (fluctuation test) and water extracts were filtered on 0.45 µm membrane filters.

Table 6. EC_{50} and EC_{20}values of aquatic organisms for the PAH-contaminated soil water extracts I & II.

$EC_{50, 20}$ (%, v/v)	**Microtox** (30 min)	***Daphnia magna*** (24 h)	***Daphnia magna*** (48 h)	***Ceriodaphnia dubia*** (7 days)		**Algae** (3 days) (Erlenmeyer flask)		(microplate)	
	EC_{50}	EC_{50}	EC_{50}	EC_{50}	EC_{20}	EC_{50}	EC_{20}	EC_{50}	EC_{20}
Water extract I	12 (11-13)	58 (53-63)	32 (26-38)	4.7 (4-5)	3.9 (3-5)	2.2 (2-2.5)	1.2 (1-1.4)	7.3 (6-9)	3.6 (2-5)
Water extract II	15 (13-17)	60 (57-63)	29 (27-31)	3.6 (3-4)	2.6 (2-3)	2.4 (2-2.6)	1.2 (1-1.2)	3.4 (3-4)	2.1 (2-3)

[a] EC_x: effective concentration in % water extracts (v/v) in the test medium causing x % inhibition of measured endpoints.
[b] 95% confidence interval in parenthesis.

3.2. Relative sensitivity of acute and chronic bioassays for water extracts of the PAH-contaminated soil

The results of bioassays on six water extracts prepared independently from the soil are presented in Table 4. The concentrations of water extracts, expressed as % volume (v/v) in the tested medium reducing by 50% bacterial luminescence (EC50-30 min) was 12±3% and viability of *D. magna* was 30±3% (EC50-48h) as compared to controls. Algal growth (EC50-3d = 2.4±0.2 %) and reproduction of *C dubia* (EC50-7d = 4.3±0.6 %) were more severely affected by the water-extracted pollutants. The NOEC value (mean ± standard deviation) for *D. magna* after 48h was 22 ± 5 % for all the water extracts, while NOEC-7d for *C. dubia* was 2.6 ± 0.9 % and NOEC-3d for *P. subcapitata* in Erlenmeyer flask was 0.6 ± 5 %.

The acute and chronic toxicity values expressed in toxic units (TU = 100/CE50) indicated higher sensitivity of the algal (*P. subcapitata*) and ceriodaphnid (*C. dubia*) chronic tests applied to soil water extracts, compared to the Microtox and *D. magna* acute tests (Figure 4).

3.3. Genotoxicity and ecotoxicity of the water extracts I and II of the PAH-contaminated soil

The genotoxicity results of water extracts performed on three different tests, with and without S9 metabolic activation, are presented in Table 5. A genotoxic response of the two extracts was recorded with the Ames and Mutatox® tests. In contrast, the umu test was negative with the two extracts tested with or without metabolic activation. The Ames test in liquid medium using the strain TA 100 indicated a higher mutagenic potential of water extract I compared to extract II, on the basis of the lowest concentration of the water extract inducing a mutagenic response (LOEC). Mutagenicity was higher after metabolic activation, suggesting implication of PAH metabolites. The Mutatox test gave also a positive response with extracts I and II without metabolic activation, extract II being more mutagenic than extract I.

The response of the Ames test was in line with chemical analyses which indicated higher PAHs concentrations in water extract I (Σ 16 PAH = 17.7 µg/L) than in water extract II (Σ 16 PAH = 4.5 µg/L). The TEQs values were also two orders of magnitude higher in extract I than extract II.

Table 7. Toxicity data summary (NOEC, $E(L)C_{10}$, $E(L)C_{20}$, $E(L)C_{50}$) of terrestrial organisms (plant, earthworm and collembola) exposed to PAH-contaminated soil mixed with artificial ISO soil.

	Plants						**Earthworm (*E. fetida*)**				**Collembola (*F. candida*)**		
Endpoints	germination		growth (dry biomass)		growth (fresh biomass)		survival		reproduction		survival	reproduction	
Exposure duration (days)	(17 d)		(17 d)		(17 d)		(14 d)		cocoon (28 d)	Juvenile (56 d)	(28 d)	juvenile/replicate (28 d)	juvenile/adult (28 d)
	lettuce	chinese cabbage	lettuce	chinese cabbage	lettuce	chinese cabbage	juvenile	adult					
NOEC	> 100	50	10	10	10	10	20	60	10		5		
$E(L)C_{10}$ (%, w/w)			6	4	6	7	23	66	4.5	3.5	4.3	3.0	6.0
CI			(2–13)	(1–10)	(3–10)	(4–10)	(21–30)	(54–76)	(1–11)	(6–9)	(2–7)	(2–4)	(4–9)
$E(L)C_{20}$ (%, w/w)	> 100	> 100	13	11	11	11	25	69	7.3	4.6	6.1	3.8	7.2
CI			(6–23)	(4–22)	(7–15)	(8–15)	(22–31)	(60–77)	(2–14)	(5–9)	(4–9)	(3–5)	(5–10)
$E(L)C_{50}$ (%, w/w)	> 100	> 100	55	71	27	26	28	74	18	8	11	5.7	9.9
CI			(40–74)	(46–100)	(22–33)	(22–30)	(24–33)	(68–79)	(10–28)	(0-14)	(8–14)	(5–6)	(8–12)

[a] EC_x: effective concentration in % contaminated soils (w/w) in the test medium causing x % inhibition of measured endpoints.

[b] CI: 95 % confidence interval in parenthesis.

Ecotoxicity responses obtained with water extracts I and II are presented in Table 6. The toxicity values for microtox, *D. magna*, *C. dubia*, and algae indicated no statistically significant difference between the two water extracts. The EC_{50} of 7-d *C. dubia* were 4.7% for water extract I and 3.6% for water extract II. Algae (*P. subcapitata*) showed the same EC_{50} value (1.2%) for two water extracts. The results were also within the confidence intervals of the EC_{50} or EC_{20} values obtained on the whole water samples (Table 4).

3.4. Toxicity of PAH-contaminated soil on plants and terrestrial invertebrates (earthworm and collembola)

The toxicity results of the PAH-contaminated soil on plants (chinese cabbage, lettuce), earthworm *E. fetida* , and collembola *F. candida*, are summarized in Table 7.

In the plant bioassay, three endpoints namely seed germination, fresh and dry shoot biomass were measured on Chinese cabbage and lettuce after 17 days of exposure. No inhibition of lettuce germination was observed in the cokery soil tested without any dilution, while a low inhibition (< 20%) was noted with the chinese cabbage. Yet, chinese cabbage will show higher seedling development ability than lettuce. Plant growth was a more sensitive endpoint than germination. A statistically significant inhibition of fresh seedling development was observed at the concentration of 25 % of the contaminated soil in test medium with both species (Figure 1). We observed no significant difference between the two plant species, with EC_{50}-17d values of 27% and 26% for lettuce and Chinese cabbage, respectively. The NOECs values for growth based on fresh and dry biomass was 10 % in both species (Table 7). Compared to dry shoot biomass, the fresh shoot biomass was a more sensitive endpoint.

Earthworm survival of *E. fetida* after 14 days was tested using adults and juveniles. The concentrations of the cokery soil in the test substrate reducing viability by 50% (EC_{50}-14d) was 74 % for adults and 28 % for juveniles, showing a much higher sensitivity of juveniles to the PAH-contaminated soil. In the reproduction test, the mean number of cocoons produced after 28 days and the mean number of juveniles alive after 56 days in the control soil were 22 and 40 per replicate (containing 10 individuals each) respectively, which gave a mean of 2.2±0.7 cocoons

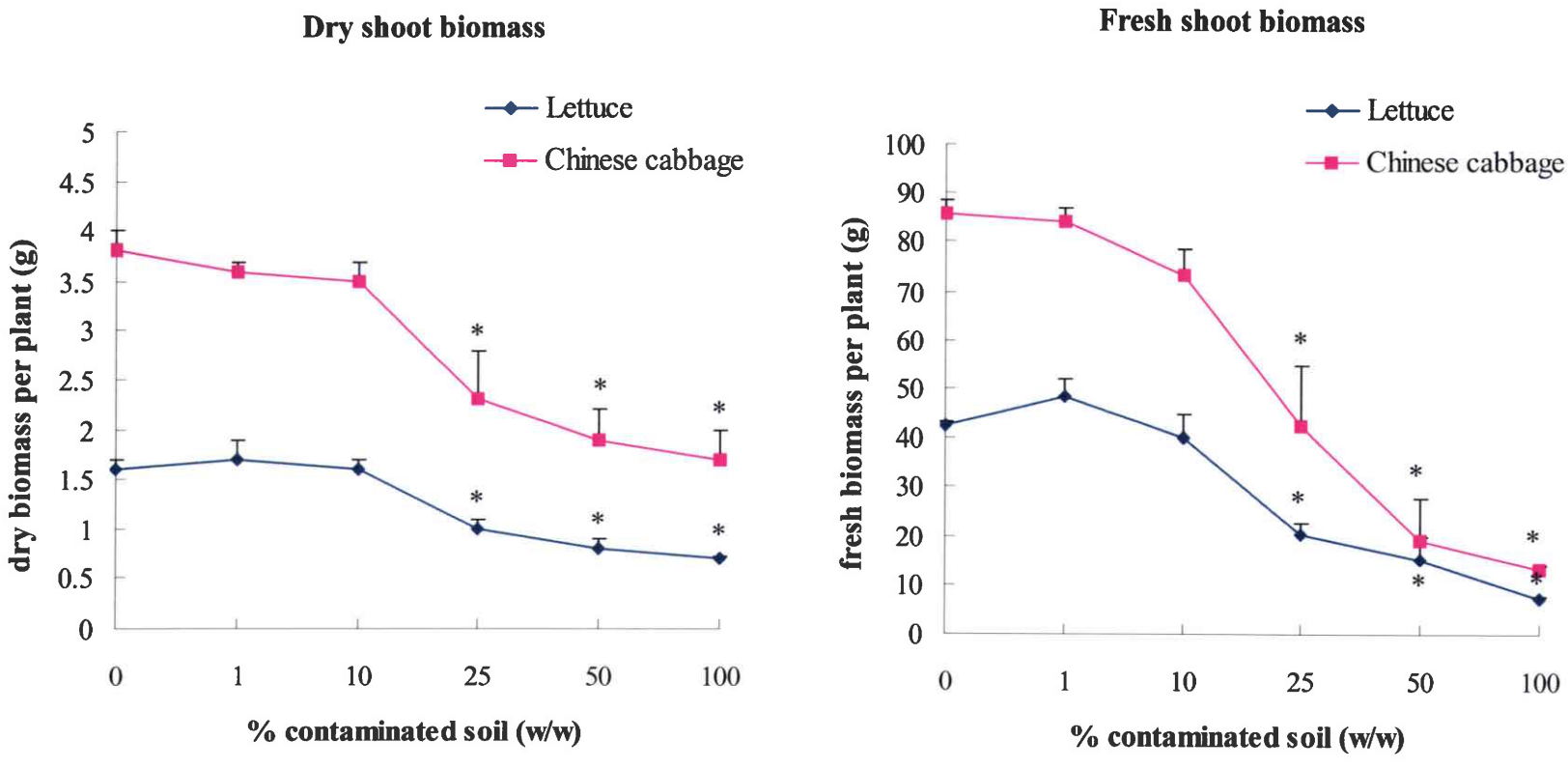
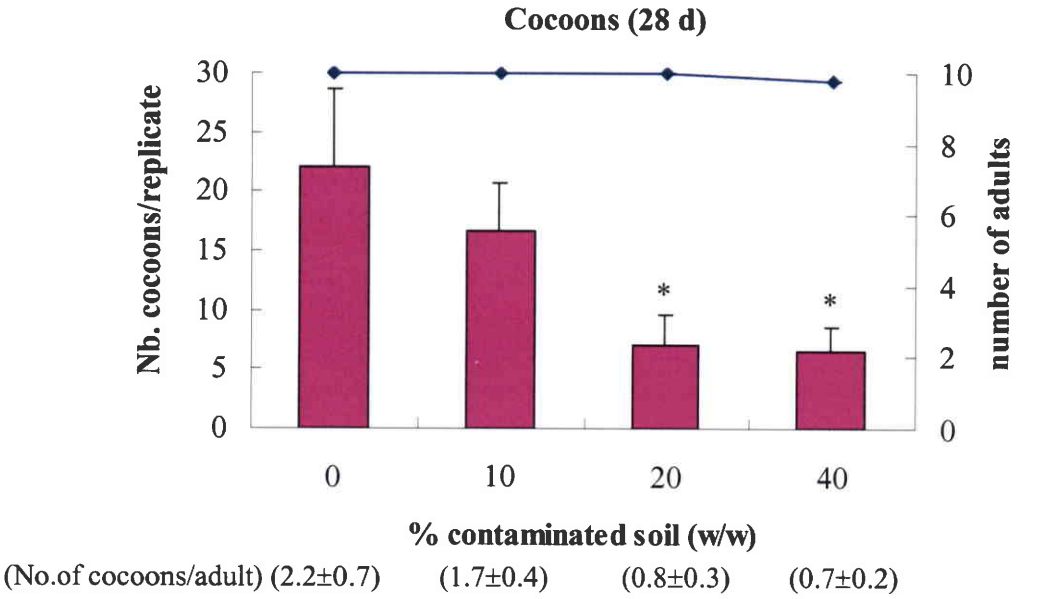


Fig. 1. Dry and fresh biomass of early seedling shoots of two plant species grown in artificial ISO soil for 17 days. Means and standard deviations (error bars) were calculated from four replicates. * Significantly different from control with <0.05



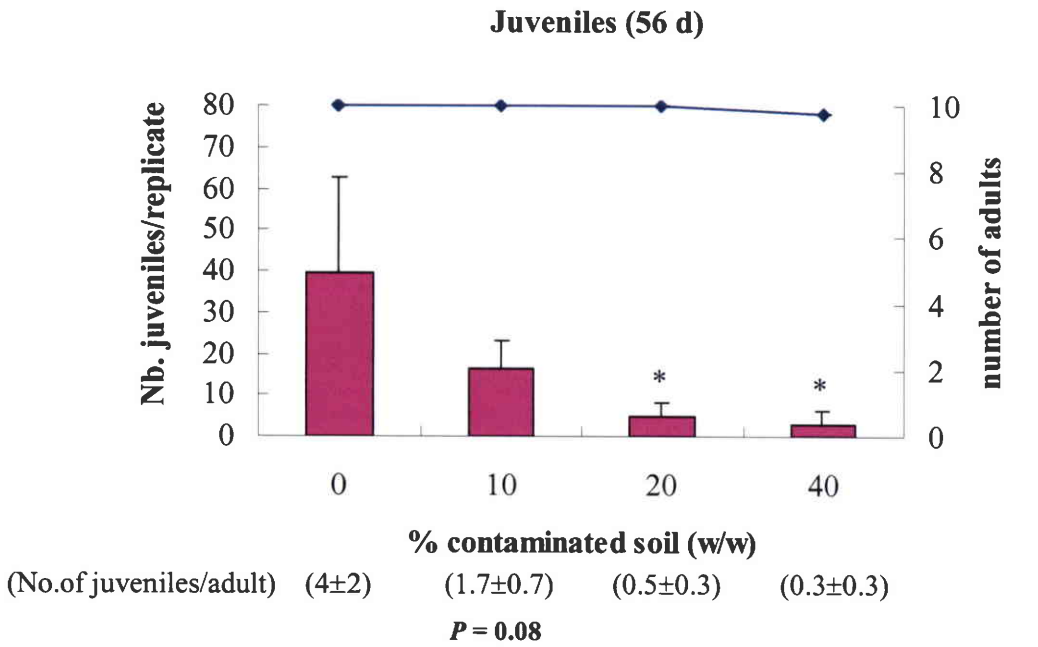


Fig. 2. Population development (bar; left *Y*-axis) and adult survival (curve; right *Y*-axis) of the earthworm *E. fetida* exposed for 28 days (cocoons) and 56 days (juveniles) to the PAH-contaminated soil mixed with artificial ISO soil at the different concentrations. Values are means per replicate (10 adults/replicate) ± standard deviation on four replicates. * : significantly different from control with $P<0.05$.
control with $P<0.05$.

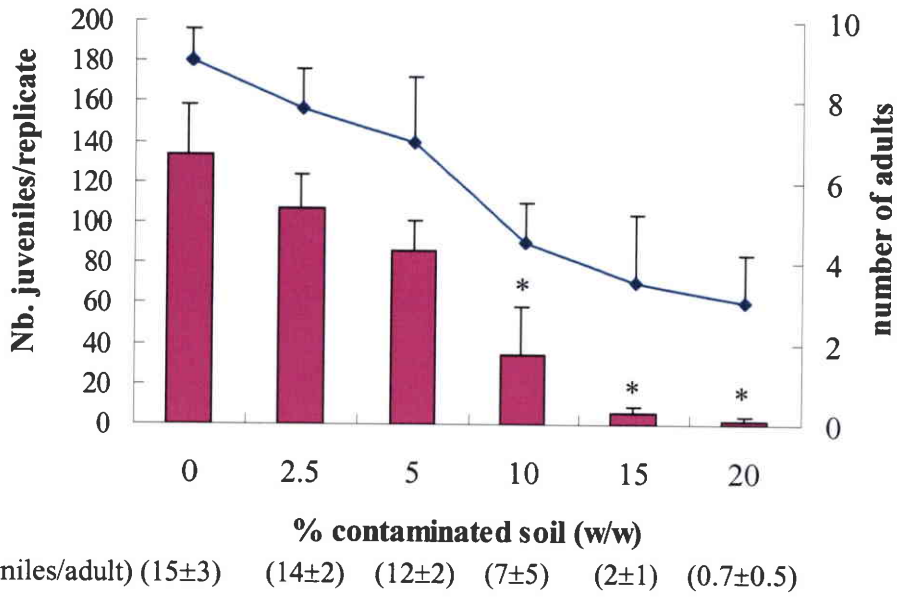


Fig. 3. Population development (bar; left *Y*-axis) and adult survival (curve; right *Y*-axis) of the collembola *F. candida* exposed for 28 days to the PAH-contaminated soil mixed with artificial ISO soil at the different concentrations. Values are means per replicate (10 adults/replicate) ± standard deviation for four replicates. * Significantly different from control with $P<0.05$.

and 4.0±2.0 juveniles per adult. At the highest concentration tested of 40% of the cokery soil in the test medium, cocoon production was reduced by 70% as compared to controls.

The concentrations of the PAH-contaminated soil in the test medium reducing cocoon production by 50% after 28 days was 18 % (EC_{50}-28d) and the EC_{50}-56d value for juvenile production was 8 % (Table 7). The lowest concentration of the cokery soil reducing significantly cocoon and juvenile production was 20 % (Figure 2). The juvenile production appeared already affected at a concentration of 10 % but the difference with controls was not statistically significant due to large variations between replicates ($p > 0.05$).

In the collembola test *using F. candida*, the mean juvenile production number of controls was 134 per replicate. The *F. candida* EC_{50}-28d values were 11 % for adult survival and 5.7 % for juvenile production (Table 7). The *F. candida* survival was already significantly affected at the concentration of 10 % of the contaminated soil in the test medium. At the highest concentration of 20%, *F. candida* survival and juvenile number were significantly reduced by 68% and 98%, respectively, as compared to controls (Figure 3).

4. Discussion

In this study, we evaluated acute and chronic bioassays for aquatic and terrestrial organisms of a soil from a cokery site and water extracts. We compared the sensitivity of the different ecotoxicity tests. We also attempted to identify pollutants responsible for the toxicity of PAH-contaminated soils by interpreting bioassay results together with pollutant concentrations of the soil and water samples, despite the fact that relationships between contaminants and toxicity results are difficult to establish due to interaction between contaminants, by-products of degradation processes, and pollutants not identified (Bispo et al., 1999; Békaert et al., 1999; Donnelly et al., 2004).

The results of bioassays demonstrated the different sensitivities of test organisms to water-extracted contaminants. Although the various endpoints clearly have not the same ecotoxicological significance (e.g., acute vs chronic), we can compare responses of the different species used in testing. The sensitivity

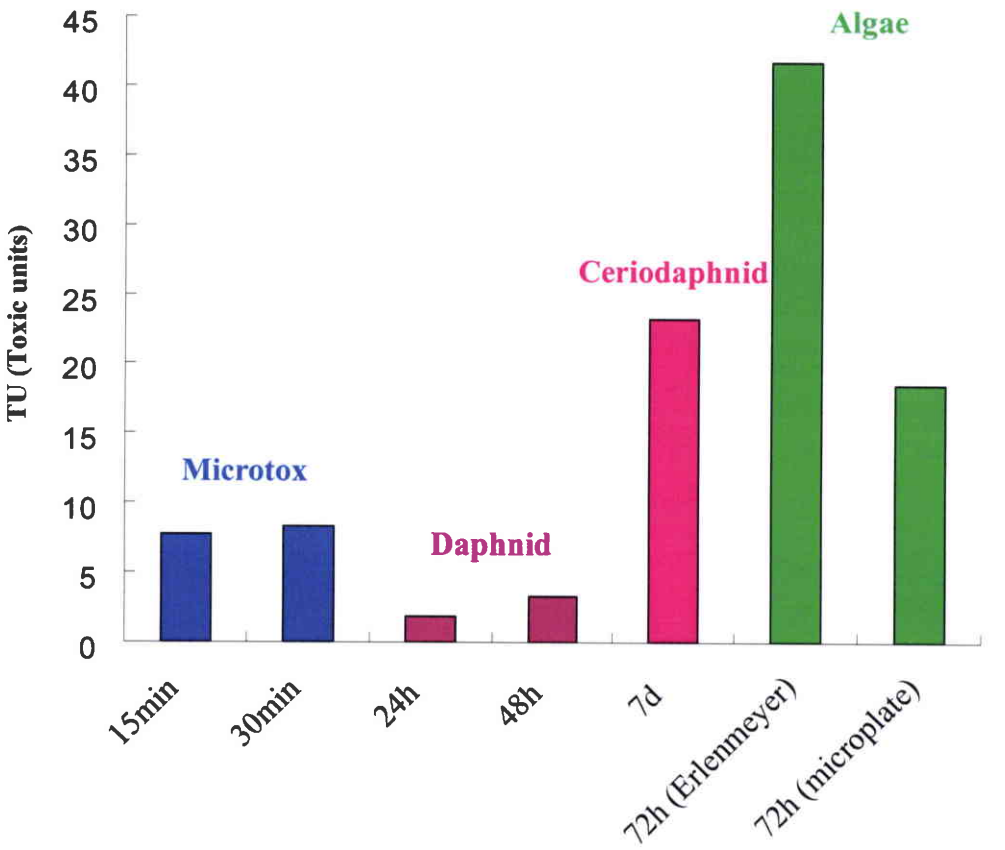


Fig. 4. Relative sensitivity of bioassays performed on water extracts of the PAH-contaminated soil.

[a]TU (Toxic Units): 100/ EC_{50}.

[b]Algal tests performed in classical Erlenmeyer flask and microplate procedures in parallel.

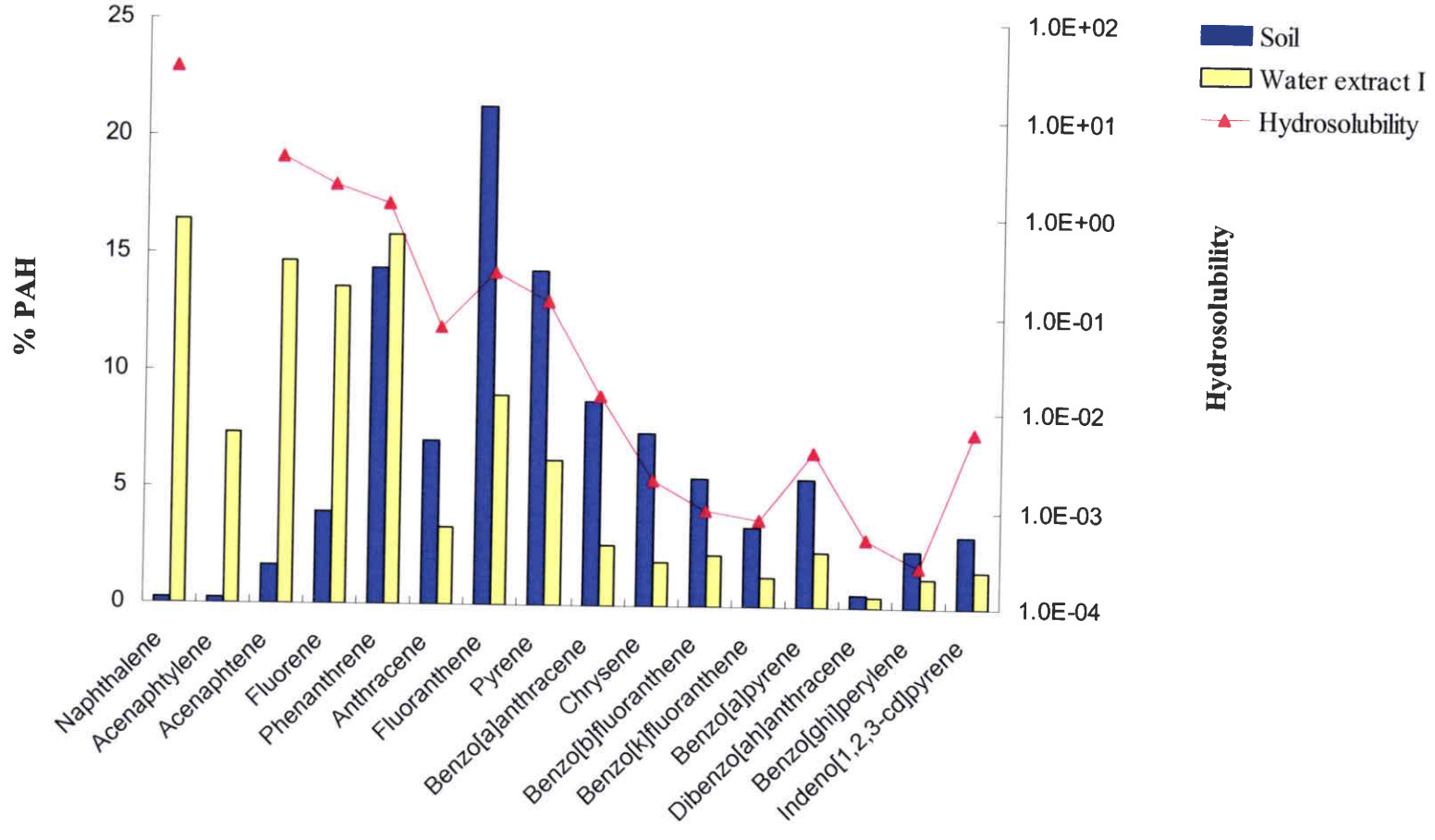


Fig. 5. PAH water solubility (▲, right *Y*-axis), Relative percentage of the individual 16 PAH in the soil (bar in blue, left *Y*-axis, w/w) and the water extract I (bar in yellow, left *Y*-axis, v/v).

ranking for non direct toxicity of the water extracts in the present study followed this decreasing order : algae $_{\text{Erlenmeyer flask method}}$ > *C. dubia* > Microtox > *D. magna* as shown in Figure 4. These results are consistent with previously published literature (Rojickoa-Padrtova et al., 1998; Bispo et al., 1999; Mendonça and Picado, 2002).

Because microplate-based phytotoxicity tests could be alternatives to classical flask-based tests, several studies were conducted to demonstrate the concordance of toxicity responses between the two procedures. Some authors reported a good agreement (Rojickova et al., 1998; Rojickoa-Padrtova et al., 1998; Eisentraeger et al., 2003). In our study, the classical Erlenmeyer flask test showed higher sensitivity than the microplate procedure. Although results do not strictly coincide with results in Erlenmeyers, use of microplates can be advised as they offer several advantages in ecotoxicity screening due to small volume requirement, incubator space economy, disposable microplates and increased bio-analytical output (Blaise and Vasseur, 2005b).

In this study, standard water extraction protocol gave very low (in)organic contaminants extraction yields (10^{-4} - 10^{-6} for PAHs and 10^{-3} - 10^{-5} for metals). Despite the low concentrations removed in water, substantial toxicity to reproduction of the cladoceran *Ceriodaphnia*, cell division of photosynthetic species and genotoxicity were recorded (Table 3). The more soluble PAHs such as naphtalene, acenaphtylene, fluorene with 2-3 rings were found relatively at the highest concentrations in the water extracts (Fig. 5). PAHs concentrations in water extracts paralleled hydrosolubility, as expected, but were not correlated with soil concentrations, as illustrated with naphthalene found at high concentrations in water while poorly present in the soil.

Differences in PAHs concentrations were found between water extracts I and II, with lower levels of PAHs in the latter extract. Despite the low pollutant concentrations, mutagenicity was recorded with Ames and Mutatox tests, with a profile of response of the two tests to the water extracts slightly different. The sensitivity of the mutants *Salmonella typhimurium* his- to PAHs metabolites is well established. The response obtained with TA100 after metabolic activation suggested that PAHs were responsible for mutagenicity of water extracts. The Mutatox test has been applied to environmental mutagenicity testing more recently than the Ames test and pollutants inducing mutant reversion are not yet clearly identified. PAHs have been found to induce positivity without metabolic activation in our lab. This may explain why positive responses were obtained in extract I without S9 in the present study. It should also be noted that the Mutatox test was carried out on extracts which had not been

sterilized on a 0.45 µm membrane. Therefore, mutagenicity may also result from the pollutant fraction adsorbed on particles and eliminated by membrane filtration, which may explain differences with the Ames test response. Indeed, the Ames and Umu tests, whose responses are based on measurements of bacterial growth, require necessarily elimination of bacteria before mutagenicity testing, while Mutatox, based on a luminescence recovery by dark mutants, do not. Mutatox is a useful mutagenicity test for this reason and appears complementary to the Ames test for environmental mutagenicity assessment.

Negative results of the umu test are in line with the fact that this test is less sensitive to mutagens than the Ames test (fluctuation test), due to nutrient richness of the bacterial medium used in the umu standard procedure.

White et al. (2002) reviewed the published information on soil mutagenicity. The authors reported that the majority of the assessments employed the *Salmonella* mutagenicity test using agar solid plates with strains TA98 and/or TA100. The fluctuation method in Ames test showed a better sensitivity, since bioavailability of pollutants is increased in liquid medium (Békaert et al., 1999). We also confirmed their results. Based on our results, the assays for genotoxicity were ranked for their sensitivities to the PAHs contaminated soils: Ames (Fluctuation) with TA 100 ≥ mutatox® > Ames (Fluctuation) with TA 98 > *umu* test. Thus, the Ames test (fluctuation method) has a better sensitivity than two other test in this study.

Phytotoxicity tests, such as early seedling growth tests, were recommended as part of a test battery in order to develop a comprehensive toxicity profile for hazardous sites (Wang and Freemark, 1995). Sensitivity among endpoints in plant tests were said to vary across species (Salanitro et al., 1997; Gong et al., 1999; Saterbak et al., 1999; Maliszewska-Kordybach an Smreczak, 2003; Sverdrup et al., 2003). Yet, germination was often reported as poorly affected by chemical pollution and germination studies alone would not predict the effects on subsequent growth of the species tested (Gong et al., 1999; Henner et al., 1999). Plant seedling growth was considered to be a more sensitive endpoint than seed emergence (Keddy et al., 1995; Sverdrup et al., 2003).

Several terrestrial invertebrate toxicity tests, for which standardized protocols have been developed, were also recommended to assess contaminated soil (Cortet et al., 1999). In the present study we demonstrated interest of juvenile survival in testing

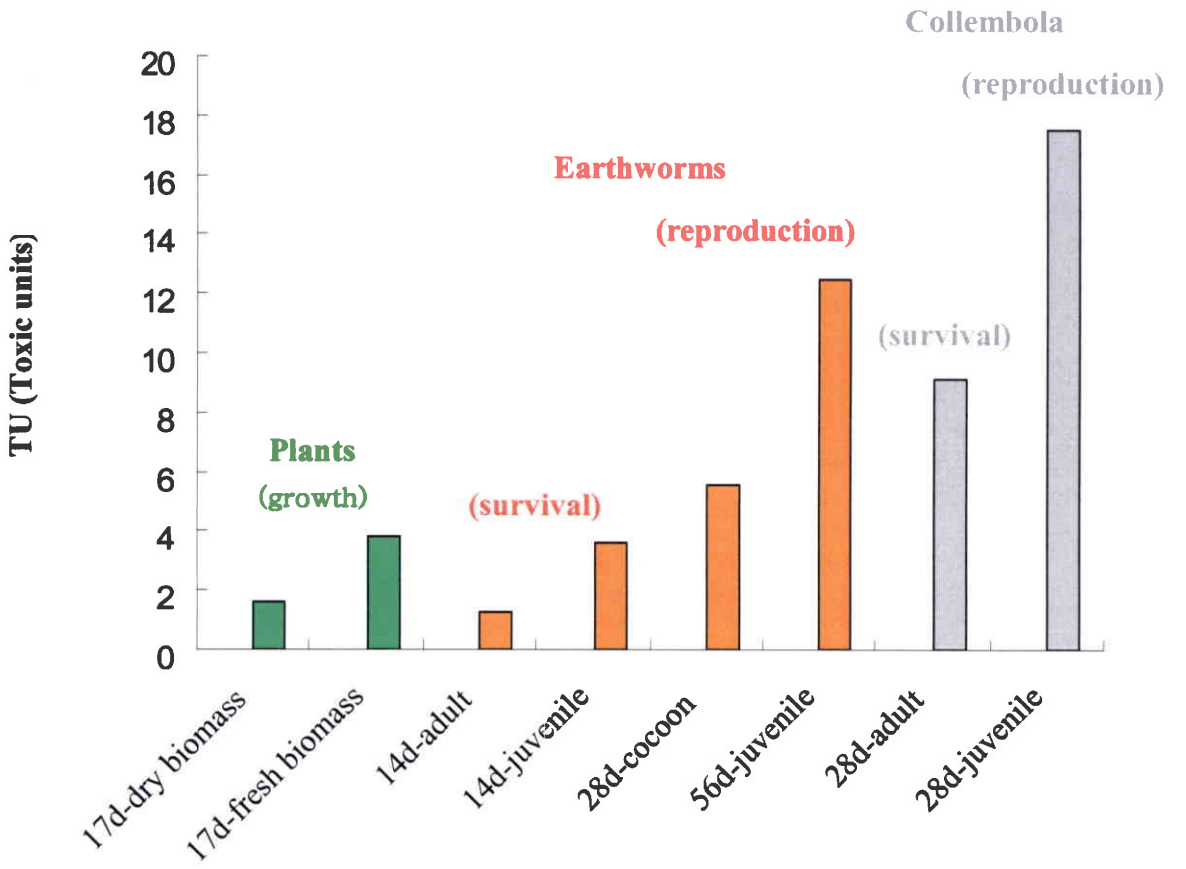


Fig. 6. Relative sensitivity of terrestrial organisms (plants, invertebrates; earthworms and collembole) exposed to the PAH-contaminated soil.

[a] TU (Toxic Units): 100/ EC_{50}.

Table 8. Concentration of metals or PAH congeners in µg/l in the water extracts I & II corresponding to the E(L)C 50 values measured with aquatic bioassays.

	Microtox (EC_{50}-30min)		**Daphnia** (EC_{50}-24 h)		**Ceriodaphnia** (EC_{50}-7 d)		**Algae** (EC_{50}-7 d)	
Water extract No.	I	II	I	II	I	II	I	II
Metals (µg/l)								
Cd	0.01	0.02	0.06	0.06	0.00	0.00	0.00	0.00
Cr	0.14	0.15	0.70	0.60	0.06	0.04	0.03	0.02
Cu	23	28	**113***	**113***	**9.2***	**6.8***	4.3	4.5
Hg	0.04	0.05	0.17	0.18	0.01	0.01	0.01	0.01
Pb	0.12	0.15	0.58	0.60	0.05	0.04	0.02	0.02
Zn	1.1	0.75	5.2	3.0	0.42	0.18	0.20	0.12
Ni	3.8	4.5	19	18	1.50	1.08	0.70	0.72
PAHs (µg/l)								
Naphthalene	0.348	<0.015	1.682	<0.060	0.136	<0.004	0.064	<0.002
Acenaphthylene	0.156	<0.120	0.754	<0.480	0.061	<0.029	0.029	<0.019
Acenaphthene	0.312	0.063	1.508	0.252	0.122	0.015	0.057	0.010
Fluorene	0.288	0.146	1.392	0.582	0.113	0.035	0.053	0.023
Phenanthrene	0.336	0.285	1.624	1.140	0.132	0.068	0.062	0.046
Anthracene	0.071	0.041	0.342	0.162	0.028	0.010	0.013	0.006
Fluoranthene	0.192	0.030	0.928	0.120	0.075	0.007	0.035	0.005
Pyrene	0.132	0.105	0.638	0.420	0.052	0.025	0.024	0.017
Benzo[a]anthracene	0.056	<0.008	0.273	<0.03	0.022	<0.002	0.010	<0.001
Chrysene	0.041	<0.008	0.197	<0.03	0.016	<0.002	0.007	<0.001
Benzo[b]fluoranthene	0.048	<0.008	0.232	<0.03	0.019	<0.002	0.009	<0.001
Benzo[k]fluoranthene	0.028	<0.008	0.133	<0.03	0.011	<0.002	0.005	<0.001
Benzo[a]pyrene	0.052	<0.008	0.249	<0.03	0.020	<0.002	0.009	<0.001
Dibenzo[ah]anthracene	0.010	<0.008	0.046	<0.03	0.004	<0.002	0.002	<0.001
Benzo[ghi]perylene	0.028	<0.015	0.133	<0.06	0.011	<0.004	0.005	<0.002
Indeno[1,2,3-cd]pyrene	0.034	<0.015	0.162	<0.06	0.013	<0.004	0.006	<0.002
Σ16 PAHs	**2.13**	**0.669**	**10.3**	**2.68**	**0.834**	**0.161**	**0.391**	**0.107**

*Concentration of contaminants in water extracts (in bold letters) when corresponding to toxic values to the species according to literature data.

Table 9. Concentration of metals or PAHs congener in mg/kg in contaminated soil corresponding to the E(L)C values measured with the terrestrial bioassays.

	Collembola (28 d)		**Earthworm**		**Plant** (17 d)
	LC_{50} (survival)	NOEC (reproduction)	LC_{50} (survival-14 d)	NOEC (cocoons-28 d)	NOEC (growth)
Metals (mg/kg)					
Cd	0.7	0.3	5.0	0.7	0.7
Cr	6	2.7	40	5	5
Cu	2.9	1.3	19	2.6	2.6
Hg	1.3	0.6	9	1.2	1.2
Pb	13	6	89	12	12
Zn	38	17	257	35	35
Ni	2.5	1.2	17	2.3	2.3
PAHs (mg/kg)					
Naphthalene	0.6	0.3	4.4	0.6	0.6
Acenaphthylene	0.5	0.2	3.4	0.5	0.5
Acenaphthene	5	2.3	34	4.6	4.6
Fluorene	11	5	**76***	10	10
Phenanthrene	**42***	**19***	**281***	38	**38***
Anthracene	20	9	138	17	19
Fluoranthene	**62***	**28***	**415***	56	**56***
Pyrene	**42***	**19***	**280***	38	**38***
Benzo[a]anthracene	25	11	170	23	23
Chrysene	22	10	145	20	20
Benzo[b]fluoranthene	16	7	108	15	15
Benzo[k]fluoranthene	10	4.5	66	9	9
Benzo[a]pyrene	16	7	107	15	15
Dibenzo[ah]anthracene	1.7	0.8	11	1.5	1.5
Benzo[ghi]perylene	7	3.3	48	6	6
Indeno[1,2,3-cd]pyrene	9	4.1	61	8	8
Σ16 PAHs	**290**	**132**	**1949**	**263**	**263**

*Concentration of contaminants in water extracts (in bold letters) when corresponding to toxic values to the species according to literature data.

Table 10. Literature review of toxicity studied on aquatic and terrestrial organisms to exposed to individual PAH.

	Microtox (30min)	**Daphnids** (24h-48h)	**Ceriodaphnids** (7d)	**Algae** (3d-7d)	**Collembola** (reproduction)	**Earthworm** (survival)	**Earthworm** (reproduction-cocoon)	**Plants** (growth)
	EC_{50} (µg/l)				$E(L)C_{50}$ (mg/kg soil)			
Metals								
Cadmium (Cd)	2200±600[1]	30-80[2]		40[3]	180-800 (LC_{50}) 5 (NOEC)[14]			140-214[2]
Chromium (Cr)	10400[4]	200-6400[9]	66[9]					
Copper (Cu)	200±30[5]	10-60[8] 32[15]	5[25]	47[6]		683[8]	191 (3 weeks)[8]	950 (LOEC)[8]
Mercury (Hg)					3.2 (EC_{50}), 1.8 (NOEC)[24]	194 (NOEC)[23]	9.2 (EC_{50}), 10 (NOEC)[24]	30 12 (NOEC)[22]
Lead (Pb)		300-450[10]						
Nickel (Ni)		320 (64h)[11]		900[11]				≥ 50[11]
Zinc (Zn)	400±50[1]	0.07-0.25[12]	22 (NOEC)[12]	50[7]	727 (LC_{50}), 266 (EC_{50})[26]	662-1010[12]	276 (EC_{50})[13] 199 (NOEC)[13]	500- >1000[12] 165 (NOEC)[12]
PAHs								
Naphthalene	1890[17]	4777[16]		6821[18]	20 (EC_{10})[19]			
Acenaphthylene	860[17]				23 (EC_{10})[19]			
Acenaphthene	1060[17]				31 (EC_{10})[19]			
Fluorene	990[17]				7.7 (EC_{10})[19]	69[20]	31 (EC_{10}-growth)[20]	55-380[21]
Phenanthrene	480[17]	409[16]		5024[18]	23 (EC_{10})[19]	134[20]	25 (EC_{10}-growth)[20]	37-300[21]
Anthracene	>10000[17]	145[16]		1004[18]	5 (EC_{10})[19]			
Fluoranthene	>10000[17]	206[16]			37 (EC_{10})[19]	416[20]	113 (EC_{10}-growth)[20]	140-650[21]
Pyrene	>10000[17]	620[16]		19[18]	10 (EC_{10})[19]	155[20]	38 (EC_{10}-growth)[20]	49->1000[21]
Benzo[a]anthracene	>10000[17]	10[16]			>980 (EC_{10})[19]			
Benzo[a]pyrene	>10000[17]	98[16]		1.5[18]	>560 (EC_{10})[19]			
Dibenzo[ah]anthracene	>10000[17]	496[16]			>840 (EC_{10})[19]			
Dibenzo[1,2,3,-cd]pyrene	>10000[17]							

and confirmed sensitivity of cocoon and juvenile production in reproduction tests. Spurgeon and Hopkin, (1996) had underlined that survival of juveniles *E.fetida* was more sensitive to metal-contaminated soils than mature earthworms. The earthworm reproduction endpoints were already reported to be more sensitive than survival and were recommended for soil assessment (Saterbak et al., 1999; Hund–Rinke et al., 2002; Davies et al., 2003; Schaefer, 2003; Van Gestel and Weeks, 2004).

The toxic effects varied substantially depending on terrestrial test species and endpoint parameters. In our study, the sensitivity ranking for direct toxicity based on EC_{50} values for endpoints (established in this study for PAH-contaminated soils), in decreasing order, was collembola (reproduction) > collembola (survival) ≥ earthworm (reproduction) ≥ plant growth (fresh shoot biomass) ≅ earthworm (juvenile survival) >> plant growth (dry shoot biomass) ≅ earthworm (survival) > plant germination (Figure 6). *F. Candida* was the most sensitive species.

Finally, we integrated the results of chemical and biological assays in an attempt to identify pollutants responsible for toxicity. We estimated concentrations of each metal or PAH congener corresponding to the $E(L)C_{50}$ values in the water extracts and soil samples used in this study (Table 8 & 9) and compared to literature studies of individual metal or PAH toxicity (Table 10). When comparing concentrations to the corresponding toxic values to the test species, we found that copper concentrations could explain toxicity to cladoceran. Indeed, the 48-h EC_{50} of copper to *D. magna* survival was 32 ± 3 µg/L under ISO protocol test according to Bossuyt & Jansen (2005). Also, the EC_{50} values on reproduction of *C. dubia was* 5 µg Cu/L at pH 8.0, and below at acidic pH, which corresponded to the same conditions as our pH range (7.5-8.0) (Gagneten and Vila, 2001).

As for invertebrate responses to the contaminated soils, results of Sverdrup et al., (2002a; 2002b) obtained on soils spiked with individual PAHs contaminants and tested on the springtail *Folsomia fimetaria* and the earthworm *Eisenia veneta* allowed us to hypothetize that PAHs with 3 and 4 rings explain toxicity registered with the cokery soil studied. On the hypothesis that bioavailability was the same in spiked soils and in the cokery soil and that sensitivity between *F. fimetaria* and *F. candida* on one part, and *E. veneta* and *E. fetida* on the other part, fluorene, fluoranthene, phenanthrene and pyrene might explain toxicity on terrestrial organisms observed in our study. Indeed, concentrations of these congeners at the CE_{50} of the cokery soil registered on survival of *E fetida* coincided with the EC_{50}

values registered by these authors on *E veneta*. Likewise, phenanthrene, fluoranthene and pyrene concentrations explained toxicity registered on survival and reproduction of *F candida*. It would be necessary to confirm this hypothesis with other soil studies, before proposing a model based on these four PAH indicators (i.e. fluorene, phenanthrene, fluoranthene, pyrene) for predicting terrestrial toxicity of PAH-contaminated soil samples.

5. Conclusion

In the present study, we evaluated acute and chronic toxicity of water extracts of the PAH-contaminated coke-soil samples using a test battery including Microtox®, (cerio)daphnids, algae. As compared to direct soil assessment bioassays, this procedure provides a quick and inexpensive characterization of soil hazard. Although PAHs contents and TEQs expressed in B(a)P equivalent in soil water extracts were negligible, our results showed genotoxic responses. So, a bioassay for genotoxicity should be considered as one of the battery tests. The use of a set of tests on species from different trophic levels of organisms is recommended in ecotoxicological studies for more refined evaluation of environmental risk.

This study confirmed sensitivity to PAH-contaminated soils of the springtail *F candida*, and. the earthworm *E. fetida* if reproduction and survival of juveniles are considered. Reproduction endpoints in terrestrial ecotoxicity testing are of ecological relevance.

The coupling of the two approaches, chemical and biological, to characterize the effects of contaminated soils is recommended as the biological assays provide invaluable informations that cannot be obtained from chemical analyses. Chemical analyses may also shed light on the types of the bioavailable pollutants responsible for toxicity, the ones which should be worth to include in models for predicting toxicity.

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V.2. Influence of Control Soil, ISO Artificial Soil or Natural Soil, on Terrestrial Ecotoxicity of a Polycyclic Aromatic Hydrocarbon (PAH)-Contaminated Soil

A PAHs-contaminated soil was assessed for its toxicity to terrestrial organisms using two control soils, either ISO artificial soil or a natural clay loamy soil from a pristine grassland.

- Direct effects of contaminated soils on terrestrial organisms, such as earthworm (*Eisenia fetida*), collembola (*Folsomia candida*), higher plants (lettuce: *Lactuca sativa*, chinese cabbage: *Brassica chinensis,* & oat: *Avena sativa*), were tested using endpoints such as survival, reproduction and growth.
- Juvenile earthworm 14-d survival test was also included.
- Toxicity tests were conducted in standard ISO conditions, the control soil substrate excepted.
- Effective concentration (EC_x) values based on concentration-response relationships were estimated and compared between the two different control soils.
- Integration of chemical and biological results to explain toxicity of the PAH-contaminated soil on terrestrial organism was conducted.

Influence of Control Soil, ISO Artificial Soil or Natural Soil, on Terrestrial Ecotoxicity of a Polycyclic Aromatic Hydrocarbon (PAH)-Contaminated Soil

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Abstract

The ecotoxicity of a PAH-contaminated soil, sampled from an ancient cokery site, was assessed using terrestrial organisms - earthworms (*Eisenia fetida)*, collembolae (*Folsomia candida)* and higher plants (*Avena sativa, Lactuca sativa, Brassica chinensis*). Different concentrations of the contaminated soil mixed with ISO artificial soil or a natural clay loamy soil from a pristine grassland, were tested to determine the influence of the control soil on toxicity results. Germination of seeds in the contaminated soil was little affected (inhibition <10% for oat and lettuce and 43 % for Chinese cabbage) while growth was more severely inhibited. The sensitivity of the three species was similar. Soil toxicity on plant growth did not significantly differ depending on the control soils with EC50-17d of 27% and 24% for lettuce, and of 26% and 30% for Chinese cabbage, in ISO and natural soil, respectively.

The nature of the control soil significantly influenced toxicity results on reproduction of earthworms and collembolae exposed to the contaminated soil, but not survival results. The toxicity of the cokery soil was lower using the natural soil than using ISO soil as a control. At a concentration of 20% of the cokery soil in the natural loamy soil, no toxicity was registered on cocoon production of *E. fetida* after 28 days ot on juvenile production after 56 days, while 93% and 96% inhibition of cocoon and juvenile numbers, respectively, were registered in ISO control soil at the same concentration. Toxicity of the PAH-contaminated soil on reproduction of *F. candida* also significantly increased using ISO soil as a control (EC50-28d = 5.7% in ISO, versus 10% in natural soil).

While the percentage of organic matter (% OM) or organic carbon (% OC) was inversely correlated with toxicity in artificial soil, the measurement of these parameters alone did not explain reduced toxicity in the natural soil

Keywords : Earthworm, Collembola, Plant, Bioavailability, PAHs. Contaminated soil.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are compounds with two or more fused benzene rings produced by the incomplete combustion of organic substances involved in natural and anthropogenic processes. The main anthropogenic source of PAHs is the combustion of coal, oil, gas and wood [1]. Contamination by polycyclic aromatic hydrocarbons (PAHs) is one of the main problems affecting industrial soils [2]. There is increasing interest in the incorporation of toxicity tests in a battery of different bioassays for ecological assessment at hazardous waste sites. Chemical analysis does not permit an integration of the combined effects produced by the mixture of chemicals present at a polluted site. Bioassays integrate these effects and help to evaluate the risk associated with exposure to bioavailable pollutants present in a soil [3]. Biological assays have been developed by the Organization for Economic Cooperation and Development (OECD) and the U.S. Environmental Protection Agency (U.S. EPA) [4]. Acute and chronic toxicity of contaminated soils are assessed using different soil organisms including earthworms (*Eisenia sp.*) [5], collembolae (*Folsomia sp.*) [6-7], and higher plants [8-9].

Soil properties have a major influence on chemical bioavailability, making it difficult to predict toxicity associated with soil properties. Often, well-defined test soils are used in bioassays for standardization between laboratories and a better comparison of test results obtained with different species [10]. For instance, an artificial soil substrate made of sand, peat, and kaolin clay is used for earthworm and collembola toxicity tests standardized by OECD and ISO. From the comparison of test results in artificial soils and in natural soils, it has appeared that chemicals were invariably much more bioavailable to worms in the artificial medium [11]. Understanding how soil characteristics interact with chemicals and influence toxicity to animals becomes important for the extrapolation of data to real environments [12]. The 3rd International Workshop on Earthworms (IWE) recommended that natural soils be selected for ecotoxicity testing. As a result of soil properties on the (bio)availability of chemical compounds, results obtained in artificial test substrates, such as OECD artificial soil, cannot be transferred directly to field soils [13]. Several authors have recommended that natural soil be selected for ecotoxicity testing [13, 14]. However, few studies have been published regarding the ecotoxicity of PAH in field-contaminated soils and effects depending

on soil type.

In this study, we tested the toxicity of PAH-contaminated soil samples, originating from a former coking plant site on terrestrial species. Toxicity was assessed by measuring the direct effects of PAH-contaminated soil on plants (oat, lettuce, Chinese cabbage), earthworms (*Eisenia fetida*), and springtail collembolae (*Folsomia candida*). Three soil toxicity tests were carried out following ISO standardized methods: 1) the 17-day germination and early seedling plant growth test, 2) the 14-day acute survival and 56-day chronic reproduction (cocoon and juvenile production) earthworm test, and 3) the 28-day collembolan reproduction test. Survival of juvenile earthworms, not standardized yet, was also assessed using a method derived from the ISO 11268-1 method [15] used for acute toxicity on adult earthworm.

As the bioavailability of pollutants in soil depends on soil properties [10, 13], we examined the influence of the control soil used in toxicity testing. Toxicity of PAH-contaminated soil on higher plants and invertebrates was assessed using two different control soils, either an artificial ISO or a natural clay loamy soil from a pristine grassland. The question of the influence of the percentage of peat in the ISO soil was also addressed on collembolae.

MATERIALS AND METHODS

Test soils

The contaminated soil was collected from a site polluted by a former cokery and mainly contaminated with polycyclic aromatic hydrocarbons (PAHs), slightly contaminated with heavy metals. The soil samples were sifted at 4 mm, homogenized, and stored at 13 °C until used for toxicity testing and physico-chemical analyses. Two different control soils for the dilution of the contaminated soil and the preparation of the test substrate were used in this study: the artificial ISO soil and a natural soil. The artificial ISO soil was prepared according to ISO guideline and consisted of 70% quartz sand and 20% kaolin clay and 10% finely ground 1-mm sieved sphagnum peat (% based on dry weight). Calcium carbonate (CaCO3) was used to adjust pH to 6.0 ± 0.5. The soil pH and water-holding capacities (WHC) were determined according to ISO methods 10390 [16] and 11267 [17], respectively. The natural soil, a loamy clay soil from a pristine grassland at Hommarting (situated in the northeast of France) was sampled from

the 0 - 25 cm surface layer, air-dried and sieved (4 mm). We also checked for the absence of contaminants in both ISO and natural soil.

Physicochemical analyses

16 PAH congeners listed as priority pollutants by the US- EPA were analysed according to French standard methods XP X 33-012 [18] . The quantification of PAHs was conducted as follows. The extraction of PAHs was using ASE extractor (Accelerated Solvent Extractor 200, DIONEX, Sunnyvale, USA), allowing a solid/liquid extraction with hexane and acetone as extraction solvents (ratio 50/50; volume/volume). After silica purification, extracts were analysed by means of reversed high performance liquid chromatography (HPLC), coupled with a spectrofluorimeter and a UV diode array detector. PAH were separated on a C18 column (4.6 mm x 250 mm x 5 µm) using a gradient of acetonitrile/water solvent. Concentrations were expressed using mean values and standard deviation of independent measurements carried out on several samples of the contaminated or control soils. Total PAH concentrations corresponded to the sum of the concentrations of the 16 PAHs analyzed.

Metals in soils were analysed by AAS (atomic absorption spectroscopy). Total organic carbon, pH, grain size, soil texture and moisture content were also measured according to the ISO standards.

Plant toxicity tests

Three plant species were used: one monocotyledon (*Avena sativa* L., common name: oat) and two dicotyledons (*Lactuca sativa* L., common name: lettuce, *Brassica chinensis* J., common name: Chinese cabbage). The seeds were obtained from a commercial supplier (oat: Dalem, France; lettuce: Apex, Magasin Vert, Lachapelle sur Erdre Cedex, France; Chinese cabbage: SARL Ferme de Sainte Marthe, Cormeray, France). The test was performed according to the ISO 11269-2 [19]. Assays were performed in pots (disposable plastic, diameter 9 cm, height 6.5 cm) and four replicates were used. Sixteen seeds for oat and thirty seeds for lettuce or Chinese cabbage were sown in each pot containing 160 g of dry weight soil. Several concentrations of the cokery soil in the control soils were tested : 100, 50, 25, 10, 1% weight per weight (w/w) with ISO or natural soil, and 100% ISO or

natural soil as a control. At the beginning of each experiment, the soil was rehydrated to 70 % for oat and 80 % for lettuce and chinese cabbage. Water evaporation was determined by weighing pots daily and compensated by the addition of distilled water. Plants were grown in a chamber under controlled conditions at a temperature of 20 ± 2°C and with irradiation of 2000 lux under a 16/8 h (light/dark) photoperiod. The test lasted 17 days after 50 % of the seeds had germinated in the control soils. At the end of the test period, the seedling shoots were cut above the soil surface and the total fresh biomass of each pot was immediately weighed. The dry biomass was also measured after oven drying at 70 °C for 16h. Cumulative germination was recorded daily throughout the test.

Earthworm tests

The test organism was *Eisenia fetida,* originating from our synchronized laboratory culture according to the ISO 11268-1 [15]. To ensure comparable conditions in all tests, calcium carbonate ($CaCO_3$) was added to adjust pH (H_2O) to 6.0 ± 0.5, and distilled water to reach 60% of maximum water-holding capacity. Water content was readjusted weekly and test containers were incubated in the climate chamber at 20 ± 2°C in a light/dark cycle of 16/8 h at 400 - 500 lux. Four replicates were used for both the acute and reproduction tests. Acute and chronic toxicity tests with *E. fetida* were carried out according to the ISO standard methods11268-1 [15] and 11268-2 [20]. In the acute toxicity test [15], ten adult earthworms with a fully developed clitellum were placed in 1 l glass containers filled with test substrate. To prevent the worms from escaping, test containers were covered with a polyethylene sheet with holes ensuring good aeration. After 14 days of incubation, surviving worms were sorted by hand. The test endpoint was survival. In the acute toxicity tests, concentrations of the cokery soil tested in ISO or natural soil were in the range of 40-100%.

In the chronic toxicity test [20], the concentrations of contaminated soil tested were 40, 20, 10% (w/w) in either ISO or natural soils, with 100% ISO or natural soils as a control. As incubation time was longer than for the acute toxicity test, additional food (5 g ground cattle manure/test container) was given to the earthworms weekly. Surviving worms were hand-sorted after 28 days, and cocoons produced were returned and further incubated for another 28 days (total incubation time 56 days). The test endpoints were cocoon production after 28 days

and the number of hatched juveniles after 56 days. The biomass of each individual was determined at the beginning and end of each test.

To obtain juvenile *E. fetida* for the survival test, cocoons from laboratory cultures were reared in containers filled with the same substrate as culture, for 5 weeks. Ten juvenile earthworms with an individual wet weight of 13 ± 0.5 mg were used for the 14-day acute toxicity test and placed in 1 L glass containers filled with a total 200 g of test substrate. The concentrations of cokery soil tested were 60, 40, 20, 10, 5% (w/w) in ISO or natural soil, with 100% ISO or natural soil as a control.

Collembola tests

The springtail *Folsomia candida* (Collembola; Isotomidae) was grown in the laboratory, in plastic containers with substrates of plaster of Paris mixed with powdered activated charcoal, at 20 ± 2°C and 500 lux. The breeding containers were tightly closed and the high moisture content of the substrate was maintained by periodically adding a few drops of water. Cultures were fed with granulated dry yeast (Baker's yeast) twice a week in small amounts to avoid spoilage by fungi. Synchronized cultures of juvenile collembola (10 - 12 days old) were obtained by transferring egg clusters from breeding containers to a freshly-prepared breeding substrate using a fine hair brush. Forty eight hours after hatching had started, the non-hatched eggs were removed. Juvenile collembolae were collected after a further 10 days of incubation.

The collembola reproduction test determines the effects of different concentrations of test substances on the reproduction of 10 to 12-day old collembolae in artificial substrate or test soils according to ISO 11267 [17]. The number of offspring produced by collembolae was determined after 28 days. Ultrapure water was added to reach 50% of the water holding capacity. Each test container, which was a 0.1 L glass jar (55mm diameter × 60 mm high), was filled with 30g wet mass of the test substrate. For each dilution series and control, 4 replicates were prepared. Ten juvenile collembolae were placed in each test container. Two mg of granulated dry yeast were added as food at the start of the test and two weeks later. The containers were placed in an environmental cabinet at 20 ± 2°C with lighting at 400 - 500 lux and a 16:8 h light-dark photoperiod, and aeration twice a week. At the end of the test, the number of adults and juveniles was counted from a

photograph of the surface after their extraction by flotation. Indigo blue dye was added to the water to improve contrast between the collembola and the water surface and floating debris.

The contaminated soil was tested at the following concentrations: 20, 15, 10, 5, 2.5% (w/w) in a control soil. Toxicity results were compared when the cokery soil was mixed with the ISO soil containing 5% or 10% peat, and the natural loamy soil.

Quality control

In our study, the validity of bioassays was ensured in accordance with established criteria defined by ISO 11268-1 [15], 11268-2 [20], and ISO 11267 [17].

Statistical analysis and data expression

Toxicity endpoints such as effective concentration values (EC50 and EC20) on plants, earthworms, and collembola, were estimated using the bootstrap method in the REGTOX Excel® macro developed by Vindimian (INERIS, France). This programme can be downloaded from the web site (http://eric.vindimian.9online.fr/en_index.html). Data was analysed with a non-parametric Kruskall-Wallis ANOVA to identify differences between control soils. When differences between means were found, a Mann-Whitney U-test was used to determine the differences between independent samples ($P \leq 0.05$; STATISTICA 5.1 for windows, Statsoft, Tulsa, OK, USA). The NOEC is the highest concentration tested that did not significantly (risk $\alpha = 5\%$) differ from the control.

RESULTS

Chemical analyses

The physical and chemical characteristics of different soil samples are presented in Table 1. The cokery soils studied were highly contaminated with PAHs ($\sum$16 US-EPA PAHs: 2634 ± 241 mg/kg dry weight). The heavy metal concentrations in soil fell within the range of corresponding regional reference values, with the exception of mercury whose concentration was higher. The artificial ISO and the loamy natural soils, those were used as controls for the dilution of the cokery soil,

Table 2. Germination & growth of plants in the PAH-contaminated soil mixed for testing with two different control soils : ISO or natural soil. % inhibition of germination and effective concentration reducing growth by 20% (EC_{20}) and 50% (EC_{50}) after 17 days of exposure are expressed.

Species	Control soil	Inhibition % of seed germination[a]	Effects on plant growth			
Endpoints			Fresh biomass		Dry biomass	
			EC_{50}	EC_{20}	EC_{50}	EC_{20}
Avena sativa	ISO	NT[c]	NT[c]		NT[c]	
(oat)	natural	15	30	13	51	14
			(24–38)	(24–38)	(41–62)	(8–21)
Lactuca sativa	ISO	8	27	11	55	13
(lettuce)			(22–33)	(7–15)	(40–74)	(6–24)
	natural	0	24	6	68	12
			(19 – 30)	(4–9)	(54–86)	(6–19)
Brassica chinensis	ISO	43	26	11	71	11
(chinese cabbage)			(22 – 30)	(8–15)	(46–100)	(4–22)
	natural	40	30	13	67	11
			(26–35)	(10–17)	(48–91)	(4–19)

[a] % inhibition of seed germination in the tested soil (100% of contaminated soils) compared to control.

[b] 95% confidence interval in parenthesis.

[c] NT : not tested.

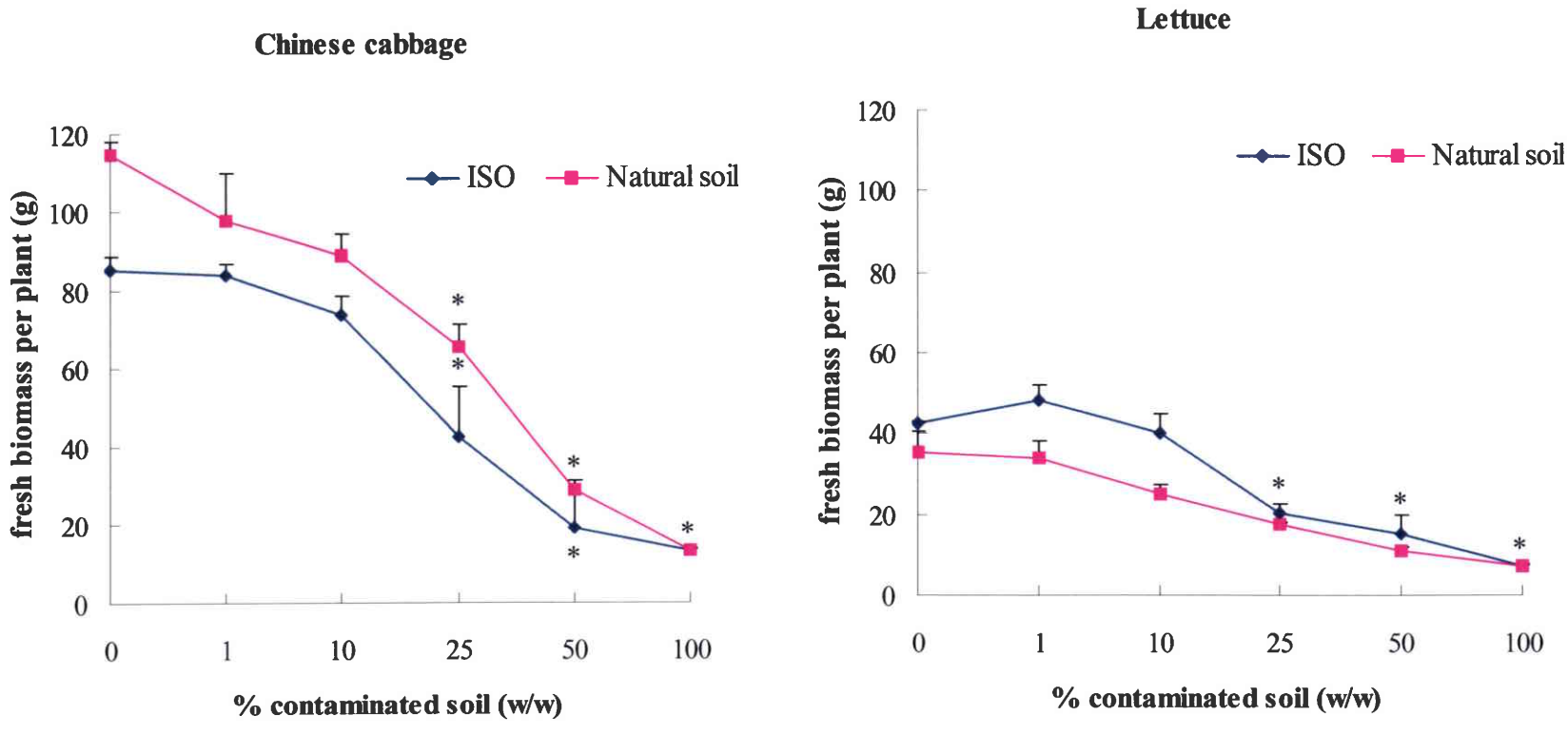

Fig. 1. Fresh biomass of early seedling shoots of two plant species exposed during 17 days to different concentrations (in %, w/w) of the PAH-contaminated soil in two different control soils: ISO or loamy natural soil. Means and standard deviations (error bars) were calculated from four replicates. Asterisks indicate significant difference from the respective controls (0%) at 95% (*) confidence.

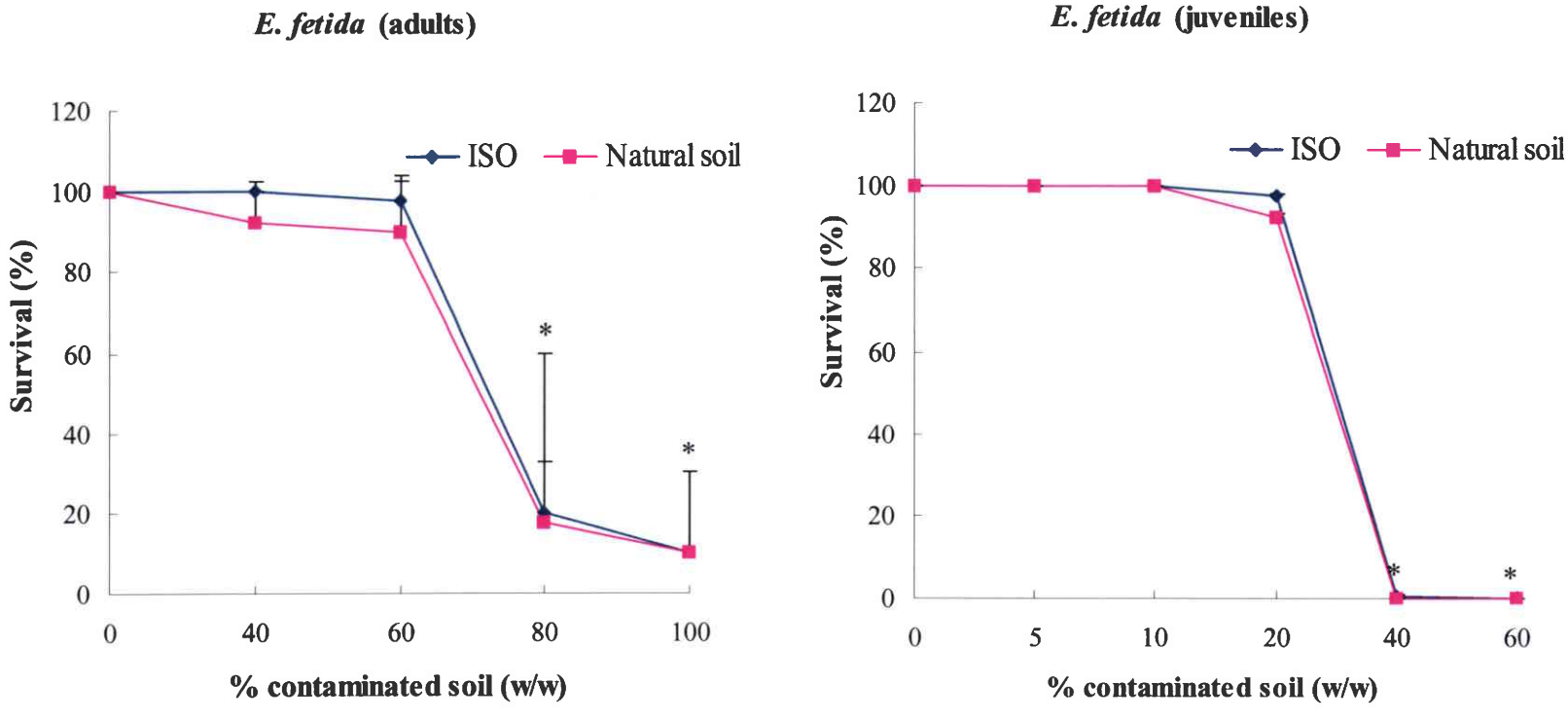

Fig. 2. Survival of adults and juveniles *E. fetida* after 14 days of exposure to different concentrations (in %, w/w) of the PAH-contaminated soil mixed with different control soils: ISO or loamy natural soil. Means and standard deviations (error bars) were calculated from four replicates. Asterisks indicate significant difference from the respective controls (0%) at 95% (*) confidence.

showed no PAH contamination and contained low concentrations of metals, falling within the range typical for an "uncontaminated" soil

Plant test

Germination of seeds in the cokery soil (100% concentration) was little affected. Inhibition was inferior to 10% for oat and lettuce, and was 43 % for Chinese cabbage. Growth was more severely inhibited. When EC50 values were compared, the fresh shoot biomass of 17-day early seedlings showed more sensitivity than dry shoot biomass and seed germination. Sensitivity of the three species was similar. Soil toxicity did not significantly differ from the control soils with EC50-17d on growth of 27% and 24% for lettuce, and of 26% and 30% for Chinese cabbage, in ISO and natural soil, respectively (Table 2). Although Chinese cabbage showed a higher biomass development in the natural soil, and lettuce in the artificial ISO soil, the differences in plant growth between control soils were not significant (Fig. 1).

Earthworm test

The adult *E. fetida* showed 18 % survival at the concentration of 80 % cokery soil after a 14-day exposure, but survived (> 90%) at the concentration of 60%, whatever the nature of the control soil (Fig. 2). On the other hand, the juvenile *E. fetida* showed no survival at 40 % contaminated soil in ISO or natural soil, which underlined the fact that its sensitivity to soil pollutants was much higher than adult. Toxicity of the PAH-contaminated soil to earthworm survival after 14 days was not significantly modified by the nature of the control soil, - ISO or natural soil.

However, reproduction appeared to be influenced by the control soil. Toxicity of the cokery soil was lower using the natural soil than using ISO soil as a control. At a concentration of 20% cokery soil in the natural loamy soil, no toxicity was registered on cocoon production of *E. fetida* after 28 days, or on juvenile production after 56 days, while 93% and 96% inhibition of cocoon and juvenile numbers respectively, were registered in ISO control soil at the same concentration of cokery soil (Fig 3). An increase in production of cocoons and juveniles of around 20% was even registered at this concentration of 20 %

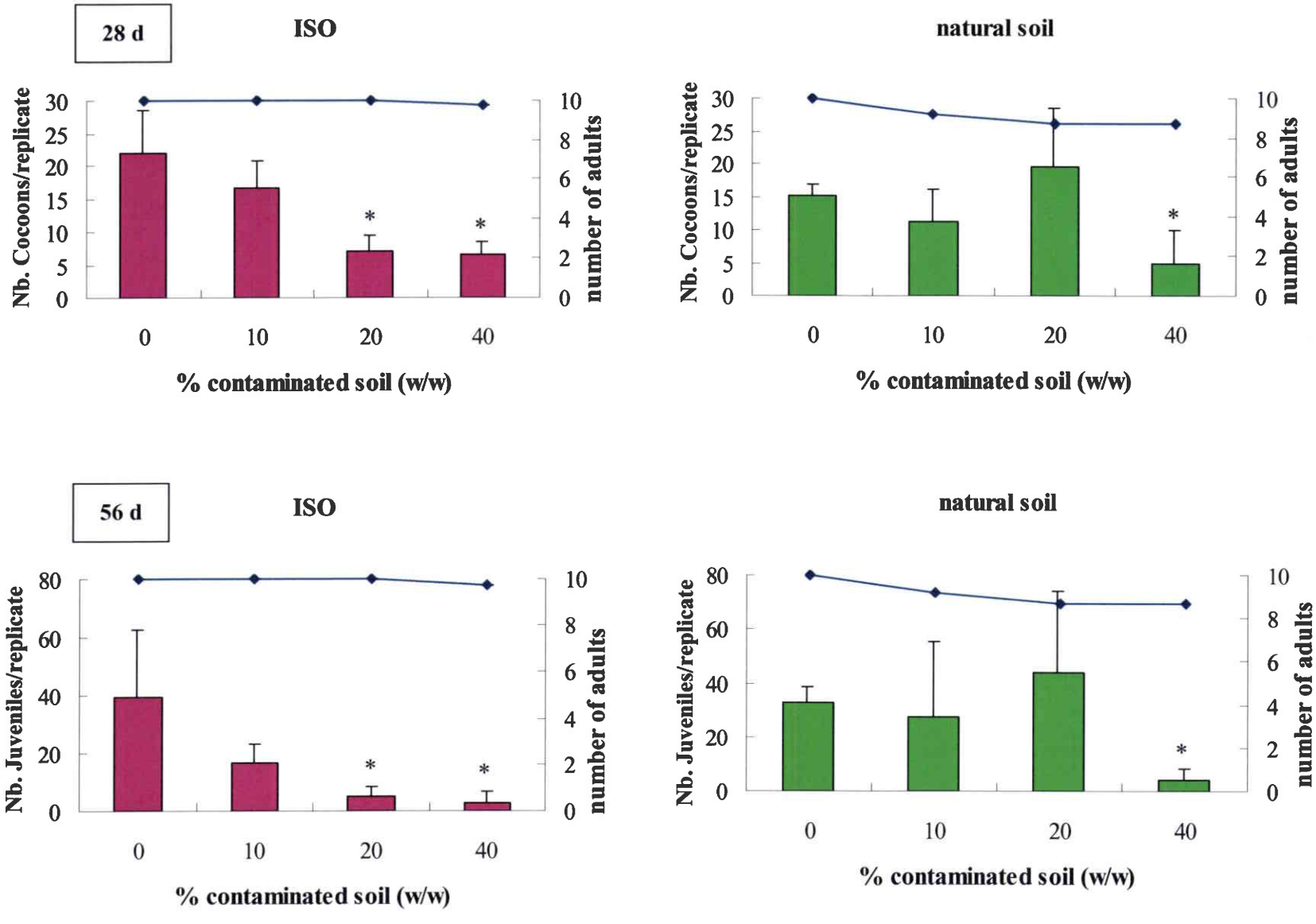

Fig. 3. Number of cocoons (bars; left *Y*-axis) after 28 days and juveniles (bars; left *Y*-axis) produced after 56 days of exposure to different concentrations of the PAH-contaminated soil in two different control soils: ISO or loamy natural soil. Survival of adults *E. fetida* was indicated as curve; right *Y*-axis. Number of cocoons and juveniles are means ± standard deviations from four replicates. Asterisks indicate significant difference from the respective controls (0%) at 95% (*) confidence.

Table 3. Summary of toxicity results on *E. fetida* survival and reproduction parameters. % inhibition, EC_{50}, and EC_{20} Values for measured parameters are reported after 28 or 56 days of exposure to the PAH-contaminated soil mixed with two different control soils: ISO or natural soil

Measured parameters	exposure duration (days)	ISO contaminated soil (w/w) 0%	ISO contaminated soil (w/w) 40%	ISO I%[b]	ISO EC_{50}	ISO EC_{20}	Natural soil contaminated soil (w/w) 0%	Natural soil contaminated soil (w/w) 40%	Natural soil I%[b]	Natural soil EC_{50}	Natural soil EC_{20}
% Adult survival/replicate	28	100	97 ± 5	-			100	87 ± 10	-		
% increase in adult growth (biomass)	28	30 ± 6	23 ± 5	23			28 ± 6	27 ± 9	-		
No. of cocoons/replicate	28	22 ± 7	6.5 ± 2*	70	18 (10-28)	7.3 (2-14)	15 ± 2	5 ± 5*	67	38 (22-48)	33 (10-39)
% cocoon hatchability	56	74 ± 8	41 ± 16*	44			64 ± 7	37 ± 36	42		
mg biomass/juveniles	56	7.6 ± 2	3.6 ± 2*	53			11 ± 5	9.8 ± 5	-		
No. of juveniles/replicate	56	40 ± 23	3 ± 3*	92	8 (0-14)	4.6 (5-9)	33 ± 6	4.2 ± 4*	87	20-40	20-40
No. of juveniles/hatched cocoon	56	2.4 ± 1	1.1 ± 1	54			3.4 ± 1	2.3 ± 0.3	32		

[a] Data expressed as average ± standard deviation in the control or the highest concentration tested (40% w/w) of contaminated soil in the test medium depending parameters measured.

[b] Mean % inhibition of the concentration of 40% (w/w) in the test medium compared to the control.

[c] EC_x and 95% confidence interval in parenthesis.

[c] *: significant different from controls at 95% confidence.

Table 4. Concentrations (in %, w/w) of the PAH-contaminated soil in the ISO (with 5% or 10% peat) or loamy natural control soil reducing survival of the collembola *F. candida* by 50% (LC_{50}) and reproduction by 50% (EC_{50}) and 20% (EC_{20}) after 28 days of exposure.

Control soil	Total organic carbon contents (%)	Peat contents in ISO (%)	Survival (adults)	Reproduction (juveniles)	
			LC_{50}	EC_{50}	EC_{20}
ISO	1.7	5	8.9*	3.7	2.0
			(7–10)	(2–6)	(1–5)
	3.4	10	14	5.7	3.8
			(11–17)	(5–6)	(3–5)
natural	2.3		12$^{\bullet}$	10$^{\nabla}$	9.4$^{\nabla}$
			(11–14)	(9–11)	(7–10)

[a] 95% confidence interval in parenthesis.

* Significantly different from LC_{50} 10% peat ISO soil.

$^{\bullet}$ Significantly different from LC_{50} in 5% peat ISO soil.

$^{\nabla}$ Significantly different from ISO values (5% and 10% peat).

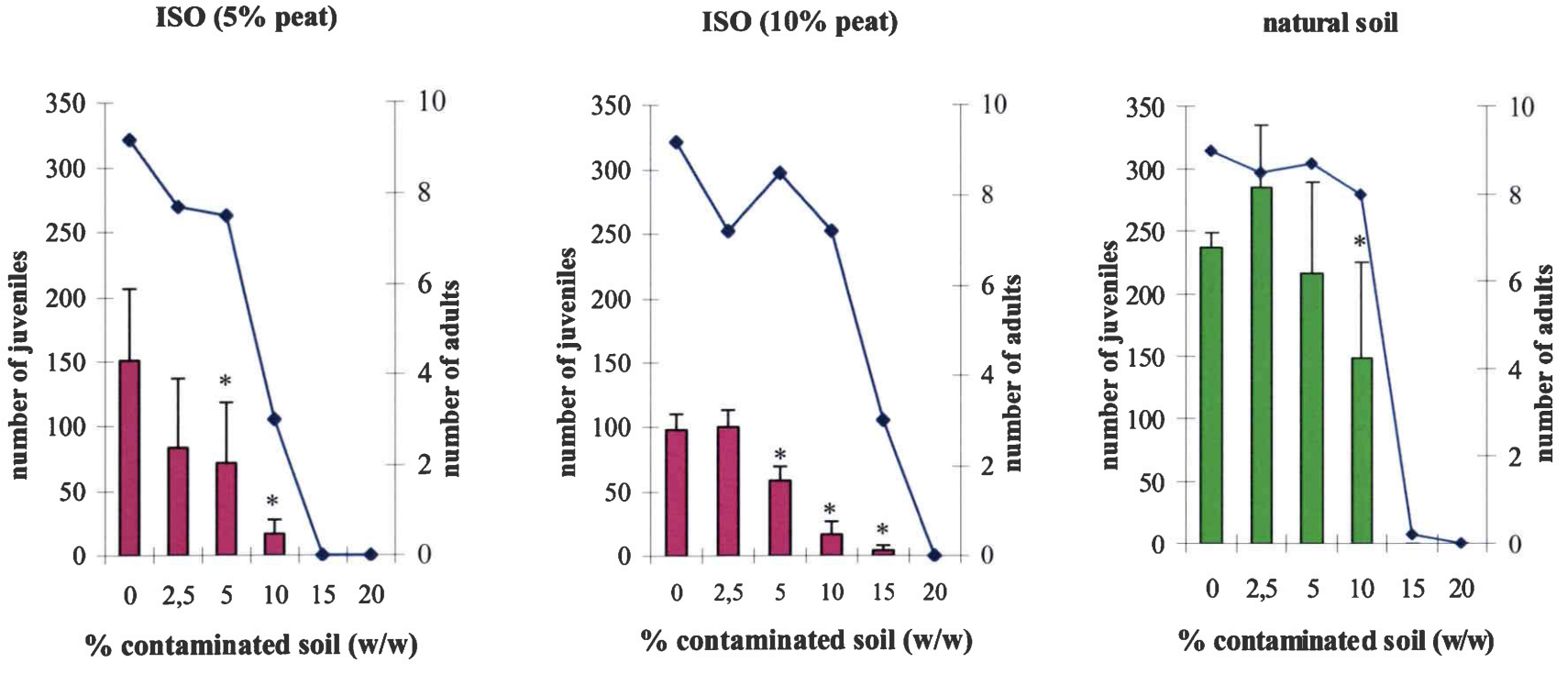

Fig. 4. Reproduction (bars; left *Y*-axis) and survival of adults *F. candida* (curve; right *Y*-axis) exposed to different concentrations of the PAH-contaminated soil in two different control soils: ISO containing 5% & 10% peat or loamy natural soil (means and standard deviations from four replicates). Asterisks indicate significant difference from the respective controls (0%) at 95% (*) confidence.

contaminated soil in natural soil compared to the control (Fig. 3): the mean number of cocoons in each replicate of 10 individuals was 15 ± 2 in controls and 19 ± 9 in the medium containing 20% cokery soil in the natural soil.

The results of the earthworm *E. fetida* reproduction test (28-d and 56-d) are summarized in Table 3. Cocoon production and juvenile production were more sensitive endpoints than the other chronic parameters, - cocoon hatchability or biomass -. The concentrations of cokery soil inhibiting cocoon production of *E. fetida* (EC50-28d) by 50% were 18 % in ISO and 38 % in the loamy natural soil. The EC50-56d value for juvenile production was 8 % in ISO soil, and within the range of concentrations 20 - 40 % contaminated soil in loamy soil (with corresponding % inhibition of juvenile production of 0% - 98%). No inhibition of juvenile production was registered at the 20% cokery soil concentration in the natural soil, as noted above.

Collembola test

Toxicity results of the PAH-contaminated soil on the collembola *F. candida* using different peat contents of the ISO control soil (10% in accordance with ISO protocol and 5% tested here) showed that survival was significantly reduced at 5% peat compared to 10% peat (Table 4). A tendency towards higher toxicity on reproduction with the 5% peat ISO soil also appeared from the comparison of EC50-28d values for juvenile production, but the difference was not significant (Table 4).

The comparison of toxicity of the contaminated soil mixed with the ISO soil (10% peat, 5.9 % organic matter) or the loamy natural soil (3.8% organic matter) showed that toxicity was significantly lower in the loamy natural soil that in ISO. Reproduction was influenced by the control soils, but survival of adults was not. The EC20 or EC50 values based on juvenile production were significantly higher when the test medium incorporated natural soil. Reproduction performances were also significantly higher in the natural soil. This was indicated by the mean juvenile numbers produced in controls per replicate of 10 individuals: 98 ± 12 in ISO (10% peat), 150 ± 56 in ISO (5% peat) and 230 ±11 in the natural soil (Figure 4). At the 10 % contaminated soil concentration, juvenile production of *F. candida* was reduced by 83% in the 10% peat ISO medium and by 89% in the 5% peat ISO, versus 37% in the natural soil compared to corresponding

Table 5. Summary of toxicity data (NOEC, $E(L)C_{20}$, $E(L)C_{50}$) to plants (oat, lettuce, Chinese cabbage), terrestrial invertebrates (earthworms, collembola) exposed to the PAH-contaminated soil mixed with two different control soils: ISO or natural soil.

	Control soil	**Plants**			**Earthworm** (*Eisenia fetida*)				**Collembola** (*Folsomia candida*)	
		Fresh shoot biomass			Survival		Reproduction		Survival	Reproduction
		oat	lettuce	chinese cabbage	juveniles	adults	Cocoons	juveniles	adults	juveniles
Exposure duration (days)		17 d			14 d		28 d	56 d	28 d	
NOEC	ISO						10	10		2.5
	natural						20	20		5
$E(L)C_{20}$	ISO	NT[c]	11 (7–15)	11 (8–15)	25 (22–31)	69 (60–77)	7.3 (2–14)	4.6 (5–9)	5.4 (3–8)	3.8 (3–5)
	natural	13 (10–18)	6 (4–9)	13 (10–17)	21 (21–22)	66 (60–75)	33 (10–39)	20-40	11 (10–13)	9.4 (7–10)
$E(L)C_{50}$	ISO	NT[c]	27 (22–33)	26 (22–30)	28 (24–33)	74 (68–79)	18 (10–28)	8 (0–14)	14 (11–17)	5.7[d] (5–6)
	natural	30 (24–38)	24 (19–30)	30 (26–35)	23 (22–25)	73 (68–78)	38 (22–48)	20-40	12 (11–14)	10[d] (9–11)
										p = 0.004

[a] $E(L)C_x$: effective concentration in % contaminated soil (w/w) in the tested medium causing x % inhibition of measured endpoints.

[b] 95 % confidence interval in parenthesis, [c] NT: not tested.

[d] Values significantly different between ISO & natural soils ($< p = 0.05$).

controls. The NOEC values of the cokery soil on reproduction of *F. candida* were determined as 2.5% in ISO and 5% in the natural soil.

DISCUSSION

The toxicity data of the cokery soil on three plant species (oat, lettuce, Chinese cabbage), earthworm (*E. fetida*) and collembola (*F. candida*), expressed as EC20 and EC50 values is summarized in Table 5. Comparison of sensitivity on the basis of sublethal toxicity data (fresh shoot biomass for plants and reproduction for *F. candida* and *E. fetida*), indicates fairly small differences between arthropods, oligochaetes, and plants. The collembola *F. candida* appeared to be more sensitive to cokery soil pollutants than the plant species or the earthworm *E. fetida.* The survival of adult earthworms and plant germination were the least sensitive endpoints in this study. It is worth noting that the earthworm 14-d survival test using juveniles showed much more sensitivity than one using adults. It would be necessary to review the ISO standard method on earthworms so as to integrate this ecologically-relevant point.

Sensitivity among endpoints in plant tests have been said to vary across species [4, 9, 21-23]. Generally, plant seedling growth is considered to be a more sensitive endpoint than seed emergence [23]. This was confirmed by the present study, in which no relationship was noted between germination success and plant growth inhibition in oat, lettuce and Chinese cabbage seeded in the cokery soil. According to some authors, the more water soluble, and low-molecular weight PAHs are sources of phytotoxicity [8, 23]. Sverdrup et al. [23] recommended that phytotoxicity studies focused on seedling fresh weight rather than dry weight as endpoints, as EC values were in many cases lower when calculated on the basis of fresh weight compared to dry weight. We also found that growth inhibition based on fresh weight was higher than that based on dry weight. We have no valid explanation for this observation, other than the hypothesis that water transfer and the metabolism of organic carbon in plant cells are disturbed to a greater extent than cation and mineral metabolisms in response to the soil pollutants. In the present study we focused on food plants, but other species could be studied depending on the remediation of the polluted sites envisaged. Saterbak et al. [4] emphasized that the selection of plant test species should be based on documented exposure pathways and plant receptors appropriate for the present and future use of the site

rather than on the most sensitive species, by default.

It has already been mentioned that earthworm reproduction endpoints were more sensitive than survival endpoint and should be systematically studied for soil quality assessment [4, 13]. The decrease in earthworm reproduction performances found in the present study did not result from a decrease in survival. Indeed, no mortality occurred in adult earthworms during the 28 days of experiments at the highest concentration tested (40% of the contaminated soil). In collembolae, this was not observed and the decrease in juvenile production paralleled decrease in survival, indicating that mortality of adults explained in part inhibition of reproduction.

Sensitivity of collembola reproduction to PAHs has already been mentioned by Sverdrup et al. [24], who showed that collembola was the most sensitive species to PAH compounds of terrestrial invertebrates tested, including earthworms and enchytraeids. The influence of the physico-chemical characteristic of the samples studied, such as organic matter content on toxic effects to the *F. candida,* was reported [25, 26]. In our study, we also observed with the ISO artificial soil (5% and 10% peat) a decrease in toxicity of the cokery soil to collembolae when the organic matter content, i.e. peat, of the soil matrix was higher. Yet, this relationship failed to explain why long term toxicity of the cokery soil in the natural loamy soil to *F. candida* and *E. fetida* was reduced, compared to the artificial ISO soil. Indeed, the content in organic carbon and organic matter was higher in the ISO soil (10% peat), than in the loamy soil. The organic composition of natural soils obviously plays an essential role in pollutant adsorption capacities, but qualitative parameters do not seem to be reflected very well by traditional measurements of organic carbon and organic matters. It is questionable whether the rules established in artificial soils are also valid in natural soils, because the bioavailability of (in)organic pollutants may differ.

Indeed, the results of laboratory spiking experiments showed large differences in the toxicity of oil contamination depending on type of soil [27]. The fractions of the PAHs that were leachable were higher in the spiked soil than in field-contaminated soil. The soil pore water fraction has been said to be a major exposure route of chemicals for several soil-inhabiting animals, which implies that the bioavailability of contaminants will depend mainly on the sorption equilibrium between soil and pore water [7, 10]. In their studies with invertebrates and plants, Sverdrup et al. [23, 24] found a significant relationship between pore-water toxicity and lipophilicity (log K_{ow}) of the lower molecular-weight PAHs. Yet, the

Table 6. Literature review of toxicity studies on terrestrial organisms (plants, earthworm, and collembola) exposed to the PAH-contaminated soil from field or spiked in the laboratory.

Soil substrate (TOC %)	**PAH contaminants** (mg/kg dry weight)	**Test species**	**Endpoints**	**Results obtained** (PAH mg/kg dry weight)	**Reference**
Field contaminated soil					
Clay, Sandy (1-2.8)	Creosote (∑16 PAHs : 1300-1500)	*E. fetida*	14-d survival (adults)	• no survival : 40-45	Charrois et al., 2001
Sandy (ND)[b]	Gas plant (12 PAHs : 610)	Mustard (*B.alba*)	5-d germination	• EC_{50} : >610	Sasek et al., 2003
		E. fetida	14-d survival (adults)	• LC_{50}: > 610	
Clay loam/ISO (2.3/3.4)	Coking Plant (∑16 PAHs : 2634)	Plants (lettuce, oat chinese cabbage)	17-d germination	• EC_{50} : >2630	This study
			17-d fresh seedling growth	• EC_{50} : 630-790, EC_{20} : 160-340	
		E. fetida	14-d survival (adults)	• LC_{50} : 1920-1950, LC_{20} : 1740-1820	
			14-d survival (juveniles)	• LC_{50} : 605-740, LC_{20} : 553-658	
			28-d reproduction (cocoons)	• EC_{50} : 474-1027, EC_{20} : 192-869	
			56-d reproduction (juveniles)	• EC_{50} : 210, EC_{20} : 121	
		F. candida			
			28-d survival (adults)	• LC_{50} : 320-370 LC_{20} : 290-140	
			28-d reproduction (juveniles)	• EC_{50} : 150-263, EC_{20} : 100-248	
OECD	Coke oven (∑16 PAHs :1141)	Higher plants (lettuce)	5-d germination	• EC_{50} : 690	Mendona & Picado, 2002
OECD	Tar (∑19 PAHs :2800)	Plants (lettuce, oat)	5-d germination	• EC_{50} : >2800	Sayles et al., 1999
			5-d root elongation	• EC_{50} : 2352-2716	
		E. fetida	14-d survival	• LC_{50} : 924	

Table 6 (Continued).

Spiked natural and artificial soils					
Clay loam (ND)[b]	∑5 PAHs (naphthalene, pyrene, phenanthrene, fluorene, anthracene) <1000	Watercress (*L. sativum*)	3-d germination 3-d fresh seedling growth	• EC_{50} : 150-300 • EC_{50} : 50-150	Maila & Cloete, 2002
Loamy sand (1-3.2)	∑4 PAHs (pyrene, fluorene, anthracene, chrysene): <100	Higher plants (bean, tomato)	15-d root length	• EC_{20} : 116-130	Maliszewska-kordybach & Smreczak, 2003
Sandy loam (1.6)	Individual 4 PAHs : <250	*F. fimentaria*	21-d reproduction	• EC_{50} : Fluorene : 14, Phenanthrene : 30 Fluoranthene : 51, Pyrene : 16	Sverdrup et al., 2001
Sandy loam (ND) [b]	Individual 2 PAHs : <250	*F. candida*	21-d reproduction	• EC_{50} : Pyrene : 51, Fluorene : 71	Sorensen & Holmstrup, 2005
Sandy loam (1.6)	Individual 16 PAHs: <1000	*F. fimentaria*	21-d survival (adults) 21-d reproduction (juveniles)	• 2-4 rings low molecular 8 PAHs (naphthalene, pyrene etc.); LC_{50}: 39-167 • 4-6 rings high molecular 8 PAHs (chrysene etc.); LC_{50}: >360 • 2-4 rings low molecular 8 PAHs (naphthalene, pyrene etc.); EC_{10}: 5-37 • 4-6 rings high molecular 8 PAHs (chrysene etc.); EC_{10}: >560	Sverdrup et al., 2002a
Sandy loam (1.6)	Individual 4 PAHs: <1000	*E. veneta*	21-d survival (adults) 21-d growth (adults)	• LC_{50} : Fluorene : 70, Phenanthrene : 130 Fluoranthene : 420, Pyrene : 150 • EC_{50} : Fluorene : 50, Phenanthrene : 90 Fluoranthene : 170, Pyrene : 70	Sverdrup et al., 2002b
Sandy loam (1.6)	Individual 4 PAHs: <1000	Plants (red clover, ryegrass, mustard)	21-d fresh seedling growth	• EC_{20} : Fluorene : 55-380, Phenanthrene : 37-300 Fluoranthene : 140-650, Pyrene : 49->1000	Sverdrup et al., 2003
Loam (1.6)	Phenanthrene: 800	*F. candida*	33-d reproduction	• EC_{50} : 70	Crouau et al., 1999
ISO	Phenanthrene: <380	*F. candida*	33-d reproduction	• EC_{50} : 170	Crouau et al., 1999

relationship failed when the authors considered PAHs of high molecular weight (5 and 6 rings), the latter congeners presenting high lipophilicity, but no toxicity to collembola [28]. In earthworms, routes of chemical uptake are not fully elucidated, although pore-water-mediated uptake from the gut appears to dominate dermal uptake [29]. Feeding behaviour should be considered, especially for earthworms which ingest soil, in the assessment of toxicity from chemical exposure.

Haeseler et al. [30] found no correlation between leachable and total PAHs in several industrially-contaminated soils. This may be due to the different organic matter contents of the soils as the former acts as a strong sorbent for hydrophobic substances [31]. For most non-ionic organic chemicals the content of organic carbon is considered to be the determining factor governing soil adsorption [32]. In spiked soils, the bioavailability of PAH was shown to decrease with increasing organic carbon content in soil [33]. In an aged phenanthrene-spiked soil, the binding to the soil organic matter has been suggested to reduce bioavailability of the hydrocarbon for microbial degradation [34]. The sequestration and changes in bioavailability of phenanthrene was markedly affected by organic matter, clay content, aging (or weathering), wetting and drying of soils, nanoporosity, and other properties of soil [34-36]. A number of studies on PAH contamination in soils have been conducted using spiked natural or artificial soils, but only a few studies have been carried out in field-contaminated soils (Table 6). Although total organic carbon or organic matter contents are generally good indicators to quantify soil adsorption properties of spiked soils, other characteristics of the soil organic fraction should be taken into consideration for contaminant bioavailability in natural soils.

We tried to explain the toxicity of the PAH-contaminated soil by comparing the results with chemical analyses. We estimated the concentration of each metal and PAH congener corresponding to the E(L)C50 and NOEC values in the soil samples tested, then compared them with literature data on individual metal and PAH toxicity. Metal concentrations in the test medium corresponding to effective toxicity values were far lower than the concentrations reported to induce toxicity in collembola and earthworm, when survival and reproduction were considered. Although zinc was the metal with the highest concentration in the cokery soil, its concentrations at the EC50s and NOECs were far below the toxic concentrations in *F candida* [44-45].

According to Sverdrup et al. [7] the 21-d EC50 value of PAHs to *F.*

fimetaria reproduction were 14 mg/kg for fluorene, 30 mg/kg for phenanthrene, 51 mg/kg for fluoranthene, and 16 mg/kg for pyrene in an agricultural soil test medium. The 21-d LC50 values on survival of *E. veneta* were reported to be 70 mg/kg for fluorene, 130 mg/kg for phenanthrene, 420 mg/kg for fluoranthene, and 150 mg/kg for pyrene in the same test medium [24]. In the present study, the concentrations of individual PAHs in the cokery soil reducing reproduction of *F. candida* by 50% after 28 days deduced from the EC50-28d were estimated to be 10 mg/kg for fluorene, 38 mg/kg for phenanthrene, 56 mg/kg for fluoranthene, and 38 mg/kg for pyrene. The concentrations of individual PAHs reducing survival of *E. fetida* by 50% after 14 days (LC50-14d) were calculated to be 76 mg/kg for fluorene, 281 mg/kg for phenanthrene, 415 mg/kg for fluoranthene, and 280 mg/kg for pyrene. The concentrations of fluorene, phenanthrene, fluoranthene, and pyrene calculated from the measured E(L)C50 of the cokery soil, were close to the concentration of each of the four PAH congeners inducing alone 50% toxicity as reported by these authors. This indicated that the sum of the 16 PAHs in the contaminated soil induced toxicity (50% effects) equivalent to the ones produced by each of the four PAHs - fluorene, phenanthrene, fluoranthene, and pyrene- tested alone in spiked soils. This demonstrated that toxicity of the field-contaminated soil was much lower than the toxicity deduced from experiments with spiked soils. This may be due to a reduced bioavailability of PAHs in the soil of the ancient cokery.

CONCLUSION

1. Toxic effects of PAH-contaminated soils from cokery sites to terrestrial invertebrates and higher plants varied between the species, endpoints measured, and to some extent, depending on the soil matrix tested (ISO and loamy natural soils).
2. Reproduction endpoints (especially, cocoon and juvenile production of earthworms, juvenile population development of collembola) were suitable and sensitive parameters to assess the sublethal effects of PAH-contaminated soils on terrestrial invertebrates.
3. PAH-contaminated soils were more toxic to the collembola *F. candida* than to the earthworm *E. fetida* or higher plant species used in this study.
4. Plant seedling growth based on fresh shoot biomass was a much more sensitive endpoint than plant seedling growth based on dry shoot biomass

and seed germination.

5. Artificial ISO soil differs from a loamy natural soil in several ways (soil texture, clay content, organic matter content, etc.). In our study, toxicity of PAH-contaminated soil tended to be lower in the natural loamy soil tested than in ISO. The difference was observed when reproduction was studied in collembola and earthworm. Therefore, it can be expected that "a worst case" scenario, i.e. an over-estimated environmental risk assessment of contaminated sites, results from testing using an artificial soil. Hence, it is necessary to consider conducting studies with natural soils for an environmentally–relevant ecotoxicity testing.

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V.3. Transfer of Polycyclic Aromatic Hydrocarbons (PAHs) from Contaminated Soil to Biota (Earthworm and Plant)

Polycyclic aromatic hydrocarbons (PAHs) are pollutants frequently found in soils, particularly in industrial areas, which can be taken up by biota.

- In this part, we studied transfer of PAHs from the cokery soil to terrestrial organism in the long-term experiments.
- Two terrestrial organisms, earthworm (*Eisenia fetida*) and a higher plant (*Chinese cabbage*), were used for studing PAH transfer to biota.
- Chinese cabbage was cultivated for 28 days.
- Juvenile earthworm produced after 56 days of chronic toxicity test were grown for an additional 24 week period.
- Uptake of PAH of the cokery soil was studied at different concentrations using two different control soils: the artificial ISO or the loamy natural soil from a pristine grassland.
- BAF (bioaccumulation factor), ratio of the concentration in earthworm to concentration in soil & BSAF (biota-soil accumulation factor), defined as the ratio of lipid normalized PAH concentration in earthworm and organic carbon normalized soil, were calculated to assess the transfer ability for PAHs to earthworm.
- SCF (shoot concentration factor), ratio of the concentration in plant shoot to concentration in soil, were also calculated and compared.

Transfer of polycyclic aromatic hydrocarbons (PAHs) from contaminated soil to biota (earthworm and plant)

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are pollutants frequently found in soils, particularly in industrial areas. The PAHs can be taken up into biota. Experiments were conducted to assess the transfer (i.e. bioavailability) of PAHs in contaminated soil from an old cokery site to terrestrial organisms: earthworm (*Eisenia fetida*) and plant (chinese cabbage). Juvenile earthworms were cultivated for the long-term of exposure (24 weeks) to the PAH-contaminated soil samples. Chinese cabbage was grown for 28 days. PAH uptake results by biota were also compared to the ones obtained using two different control soils: the artificial ISO or the loamy natural soil from rural grassland. The mean biota accumulation factors (BAFs) of individual 16 PAHs for earthworm cultivated in contaminated soil were 0.001-0.533 and 0.003-0.176, when mixed with ISO or natural soil, respectively. The mean biota-soil accumulation factors (BSAFs) were in the range of 0.012-1.264 for ISO and 0.007-0.404 for natural soil test medium, respectively. The BAFs for naphthalene with 2 aromatic rings and higher-molecular-weight PAHs with 5–6 aromatic rings were higher than for PAHs with 3-4 aromatic rings. The shoot concentration factors (BCFs) of the individual 16 PAHs for chinese cabbage were 0.001-0.023 (when excluding naphthalene: 0.094-0.168) under the concentration of 15% contaminated soil in ISO test medium, showing relatively low transfer capacity for PAHs in contaminated soil.

Keywords : Contaminated soil, Earthworm, Plant, PAHs, Uptake, BAF, BSAF, SCF

Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of hundreds of congeners that may be released during incomplete combustion or pyrolysis of organic matter. PAHs are lipophilic compounds having poor water solubility. Water solubility generally decreases with increasing molecular mass and octanol-water partition coefficient, K_{ow}. The main sources of PAH in soil are atmospheric deposition, carbonization of plant material, deposition from sewage and particulate waste and industrial activities (*1*, *2*). Contaminants at large contaminated sites often share critical properties such as toxicity, high environmental persistence and high lipophilicity increasing probability of bioaccumulation in food webs. PAHs do not bioaccumulate in vertebrates due to biotransformation into polar metabolites which are rapidly eliminated regarding congeners of high molecular weight; some intermediates are more toxic than the parent compound. In invertebrates, elimination is not so fast as in vertebrates, and bioaccumulation has been described in aquatic species (*3*). The bioaccumulation process in earthworm has been mostly studied in case of metal exposure (*4*), but fate of PAHs is poorly known. In plants, bioavailability of PAHs has been questioned (*5*, *6*).

Bioaccumulation is an exposure-related parameter determining the body burden, but not an 'effect' or hazard in itself. Therefore, when appropriate, bioaccumulation should be included as an exposure related parameter in the environmental risk assessment of substances (*7*). Soil properties have a major influence on chemical bioavailability making it difficult to predict the toxicity associated with any particular type of soil (*8*). In particular, factors such as the percent organic matter, humidity of the soil, soil structure, aging of contaminated soil might be important. Often, well defined test soils are used in bioassays to allow for a standardization between laboratories or a better comparison of test results obtained with different species (ISO, OECD). Accumulation of hydrophobic compounds in earthworms from field-contaminated soil is less efficient than in lab-contaminated OECD artificial soil (*9*). Moreover, mixture of pollutants in environmental samples is the rule and interactions between individual compounds may be of importance (*10*).

Bioavailability is a complicated phenomenon that is difficult to predict. The fraction of a compound that is available for uptake may differ between organisms, and between exposure routes such as dermal contact or oral ingestion (*11*).

Although the degradation of low molecular weight PAHs (2-4 rings) has been shown to be effective, the degradation of 5-6 rings PAHs is limited by low aqueous solubility and stronger adsorption to soil components (*12*). An increase in toxicity with increasing log K_{ow} is expected for chemicals acting by narcosis because lipophilicity can be causatively linked to the bioaccumulation process in many organisms (*13*). Bioaccumulation is a process whereby chemical residues are accumulated in tissues, especially in the lipid fraction in the case of lipophilic compounds (*14*). Several studies have investigated uptake of PAHs from (spiked)-contaminated soils by plants (*15*, *16*) and by earthworms (*11*, *18*). However, as far as PAHs are concerned, studies are still scarce which evaluate terrestrial organism uptake, accumulation and bioavailability.

In the present work, we studied the transfer of PAHs from a contaminated soil originating from an ancient coking site to earthworms (*Eisenia fetida*) and plants (Chinese cabbage). Transfer was studied over one generation of earthworms and extended to 24 weeks in juvenile produced by adults that had been exposed to the PAH-contaminated soil. Transfer of PAHs to earthworm was studied at concentrations not affecting survival and growth of populations in the long term or inducing low toxicity (< 20% mortality in adults). Concentrations were obtained by mixing the soil sample with a control soil, either the ISO artificial soil, or a loamy natural soil, in order to determine if the soil type could influence the process. The shoot transfer of PAHs in Chinese cabbage seeded in the ISO soil mixed with the cokery soil was measured after 28 days following germination. The concentrations tested ranged between non-toxic concentrations to concentrations inducing 15% decrease in plant growth.

Methods

Soils

The contaminated soil for this study was collected from a site polluted by cokery residues and was contaminated by polycyclic aromatic hydrocarbons (PAHs). The soil samples were sifted at 4 mm, homogenized, and analysed for physicochemical characterization and pollutants, then stored at 13 °C until use for uptake testing. Two different soils for dilution with contaminated soils as test substrate were used in this study: an artificial ISO soil and a natural soil. Artificial

ISO soil was prepared according to the ISO protocol and consisted of 70% quartz sand and 20% kaolin clay and 10% finely ground 1-mm sieved sphagnum peat (% based on dry weight). Calcium carbonate ($CaCO_3$) was used to adjust pH to 6.0 ± 0.5. The soil pH and water-holding capacities (WHC) were determined according to ISO methods 10390 (1994) and 11267 (1998), respectively. The natural soil, a grassland loamy soil from a Hommarting situated to the northeast in France, were taken from the 0 – 25 cm surface layer, air dried and sieved (4 mm).

Physico-chemical Analyses

The PAH contents of soils were determined by IRH Environment (www.irh.fr, Nancy, France). The 16 PAHs congeners listed as priority pollutants by the US-EPA were analyzed. The quantification of PAHs was performed according to standard method (NF ISO 13887, 1999). Extraction of PAHs was conducted by ASE extractor (Accelerated Solvent Extractor 200, Dionex, Sunnyvale, USA). PAHs were separated and detected by reversed high performance liquid chromatography (HPLC), coupled with a spectrofluorimeter and a UV diode array detector. Concentrations were expressed by the mean values and standard deviation of independent measurements. Total PAH concentrations corresponded to the sum of the concentrations of the 16 analysed PAHs. The PAHs contents in earthworms and plants were analysed by capillary gas chromatography/mass spectrometry (XP X 33-012, 2000) in the laboratoire de Rouen/ETSA (www.laborouen.com, Rouen, France). Total organic carbon, pH in H_2O, grain size and moisture content were also measured according to ISO standards by INRA (www.inra.fr, Arras, France).

PAHs Uptake by Earthworms

The test organism was *Eisenia fetida* originating from our synchronized laboratory culture according to the ISO 11268-1 (1994) (Annex A) (*19*). To ensure comparable conditions in all tests, calcium carbonate ($CaCO_3$) was adjusted pH to 6.0 ± 0.5 and distilled water was added to reach 60% of maximum water-holding capacity. Water content was readjusted weekly and test containers were incubated in a climate chamber at 20 ± 2°C in a light: dark cycle of 16:8 h at 400-500 lux. Four replicates were applied in the reproduction test. The 56-d chronic toxicity tests with *E. fetida* were carried out according to the ISO standardized methods

(ISO 11268-2, 1998) (*20*). The mixing treatments were dilutions of the 100% contaminated soils: 40, 20, 10% (w/w) with ISO or natural soils, 100% ISO or natural soils as a control. The test endpoints were cocoon production after 28 days and the numbers of hatched juveniles after 56 days. Surviving worms were hand-sorted after 28 days, and the number of cocoons produced returned and further incubated for another 28 days (total incubation time 56 days).

To determine PAH uptake by earthworm, juveniles *E. fetida* produced after 56 days of chronic toxicity test were used and cultured an additional 24 weeks. During the additional 24 weeks, earthworms were supplied with cattle manure as a source of food to ensure growth during the experiment. Cattle manure was supplied per container at a ratio of 1 g (dry weight) per 1 g biomass (wet weight) each week. Cluzeau and Fayolle (1989) reported that 50 mg/g worm per day of suitable food would be sufficient to allow unrestricted growth, maturation in *Eisenia andrei* (*21*). After 24 weeks culture, the worms were removed from the soil by digging and hand sorting and washed with tap water. One-fourth of the posterior part was massaged to expel the content of the lower gut. The guts of the earthworms were evacuated by incubation in pre-extracted silica (silicon dioxide) for 24h and Earthworms were again submitted to massage to remove silica substrate from the gut, rinsed with distilled water, stored at -20°, and freeze-dried. Earthworms were then sent to the analytical lab for PAH solvent extraction of earthworm and chemical analysis

PAHs Uptake by Plant (Chinese cabbage)

The plant species, a dicotyledon (*Brassica chinensis* J., common name: Chinese cabbage), was used. The seeds were obtained from a commercial supplier (SARL, Cormeray, France). The test was performed according to the ISO 11269-2 (1994) (*22*). Assays were conducted in four replicate pots (disposable plastic, diameter 9 cm, height 6.5 cm). The mixing treatments were dilutions of the 100% contaminated soils: 15, 10, 1% (w/w) with ISO or natural soils, 100% ISO or natural soils as a control. Thirty seeds were sown in each pot containing 160 g of dry weight soil. At the beginning of each experiment, the soil was rehydrated to 80 % of the water holding capacity.

Water evaporation was determined by daily weighing of pots and compensated by addition of distilled water. Plants were grown in a chamber under controlled conditions

at a temperature of 20 ± 2°C and with irradiation of 2000 lux under a 16/8 h (light/dark) photoperiod. The seedlings harvest was conducted 28 days later After 50% of the seeds had germinated in the control soils. The seedling shoots were then cut above the soil surface. Afterwards, the plant shoot samples were weighed, freeze-dried, stored at - 20°C, weighed again, and analyzed for PAH contents.

Data Expressions (BSAF, BAF, SCF)

The uptake results for earthworm were expressed as a biological accumulation factor (BAF). The BAF could be expressed in the ratio of concentration of the PAH in earthworms to the concentration of PAH in soil at any given soil concentration. The results were also expressed as biota-soil accumulation factors (BSAF) values. BSAF were calculated as the lipid normalized PAH content to the organic carbon normalized contaminant content in the soil: (concentration in earthworms/lipid content) ÷ (concentration in soil/TOC). TOC was calculated by considering the proportions of the contaminated & control soils and their respective organic contents. The presence of such an isometric relationship indicates that the BSAF, as represented by the regression coefficient for the slope, can be considered a constant for compounds with different log *K*ow values (*17*, *18*). We used a lipid content 1.8% for *E. fetida* from Wagman et al., (*23*).

Shoot concentration factors (SCFs) for plant (Chinese cabbage) were described as ratio of the concentration in plant to soil / concentration in soil (*16*, *24*).

Concentration of PAHs in plant and earthworm biomass and soil are reported on a dry weight basis for each concentration tested of the cokery soil samples.

Statistical Analysis

Data were analysed with a nonparametric Kruskall-Wallis ANOVA to identify differences among soil samples. When differences among the means were found, a Mann-Whitney U-test was used to test for significant difference between samples ($P \leq 0.05$; STATISTICA 5.1 for windows, Statsoft, Tulsa, OK, USA).

Table 1. Main physicochemical characteristics of the PAH-contaminated soil and two control soils (natural soil, ISO) used in this study.

	LogK_{ow} [a]	QL [b]	Contaminated soil	natural soil	Artificial ISO
Texture					
Soil type			Sandy loam	Silty clay loam	Sandy loam
Clay (%)			10.7	19.7	10.7
Silt (%)			22.1	61.4	8.2
Sand (%)			67.2	18.9	81.1
pH (H_20)			9.6	6.5	6.0 ± 0.5
Water-holding capacity (%)			53	58	59
C/N			46.1	9.6	39.9
Organic carbon (%)			10.0	2.3	3.4
Organic matter (%)			17.3	3.9	5.9
Heavy metals (AVG ± SD) [c]		(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Cd		0.02	6.7 ± 0.6	0.2	0.1
Cr		0.08	54 ± 0.9	7.4	1.0
Cu		0.1	26 ± 0.6	16	1.2
Hg		0.002	12 ± 0.3	< 0.1	< 0.05
Pb		0.3	120 ± 4	13	46
Zn		0.02	347 ± 6	61	5.3
Ni		0.24	23 ± 0.3	20	0.3
PAHs (AVG ± SD) [c]		(µg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Naphthalene	3.4	10	5.9 ± 2	< 0.01	< 0.01
Acenaphthylene	4.1	1	4.6 ± 0.3	< 0.01	< 0.01
Acenaphthene	3.9	1.7	46 ± 4	< 0.01	< 0.01
Fluorene	4.2	3.7	103 ± 9	< 0.01	< 0.01
Phenanthrene	4.6	2.9	380 ± 46	0.02	< 0.01
Anthracene	4.5	0.1	186 ± 39	< 0.01	< 0.01
Fluoranthene	5.2	1.7	561 ± 49	0.12	< 0.01
Pyrene	5.2	1.2	379 ± 28	0.10	< 0.01
Benzo[a]anthracene	5.6	0.1	230 ± 19	0.05	< 0.01
Chrysene	5.9	0.1	196 ± 16	0.05	< 0.01
Benzo[b]fluoranthene	6.1	0.1	146 ± 8	0.06	< 0.01
Benzo[k]fluoranthene	6.8	0.1	89 ± 6	0.03	< 0.01
Benzo[a]pyrene	6.5	1.1	145 ± 8	0.06	< 0.01
Dibenzo[ah]anthracene	6.5	0.1	15 ± 0.8	< 0.01	< 0.01
Benzo[ghi]perylene	7.1	0.1	65 ± 3	0.04	< 0.01
Indeno[1,2,3-cd]pyrene	6.6	0.1	82 ± 5	0.05	< 0.01
∑16 PAHs			**2634 ± 241**	**0.63**	- [d]

[a]Octanol-water partition coeffiecients: data from EHC 202 (IPCS, 1998).

[b]QL: Quantification limits in µg/kg d.w.

[c]AVG ± SD: Average ± standard deviation.

[d]- : < Quantification limits.

Table 2. Growth parameters of juvenile earthworm population generated by adults after a 56 day-reproduction test using the PAH-contaminated soil. Juveniles were exposed for an additional 24 week period to the PAH-contaminated soil mixed with ISO or natural soil at different concentrations.

	Exposure duration (weeks)	ISO % contaminated soil in test medium (w/w)				natural soil			
		0 (control)	10	20	40	0 (control)	10	20	40
Biomass/worm (mean ± SD, mg)	0	7.6 ± 2	6.6 ± 2	4.6 ± 2	3.6 ± 2	11 ± 5	10 ± 2	7.9 ± 3	9.8± 5
Total number of juveniles		160	68	20	12	132	108	176	16
Total biomass (g)		1.2	0.45	0.092	0.044	1.4	1.1	1.4	0.16
Biomass/worm (mean ± SD, mg)	4	39 ± 11	36 ± 11	40 ± 10	29 ± 4	38 ± 15	51 ± 19	29 ± 5	36 ± 19
Total number of juveniles		261	172	68	57	168	124	212	14
Total biomass (g)		10	6.2	2.7	1.6	6.4	6.4	6	0.5
Biomass/worm (mean ± SD, mg)	8	59 ± 18	48 ± 12	77 ± 11	56 ± 5	84 ± 35	111 ± 49	67 ± 24	81 ± 41
Total number of juveniles		263	176	68	60	168	124	212	14
Total biomass (g)		16	8.4	5.2	3.2	14	14	14	1.1
Biomass/worm (mean ± SD, mg)	12	96 ± 30	84 ± 21	127 ± 19	91 ± 9	178 ± 66	244 ± 110	148 ± 54	164 ± 61
Total number of juveniles		268	176	72	64	168	124	208	14
Total biomass (g)		26	15	9.2	6	30	30	31	2.3
Biomass/worm (mean ± SD, mg)	16	196 ± 53	185 ± 44	241 ± 55	163 ± 24	267 ± 99	359 ± 135	236 ± 67	246 ± 90
Total number of juveniles		264	136	72	64	160	120	200	14
Total biomass (g)		52	25	17	9.2	43	43	47	3.4
Mean growth rate (mg day^{-1})	16	1.6	1.4	2.0	1.4	2.5	3.4	2.1	2.3
Final biomass/worm (mean, mg)	24	220	286	323	222	270	292	183	294
Total number of juveniles		216	112	64	52	152	104	184	18
Final total biomass (g)		47	32	21	12	41	31	33	5.3

[a] Mean ± SD: mean fresh weight (mg) ± standard deviation (n = 4)

[b] Fresh weights are given from the mean weight of surviving earthworms at the each time (weeks) measured. Mean growth rate is determined as the slope of the regression for fresh weight over the early linear stage (16 weeks) of fresh weight increase.

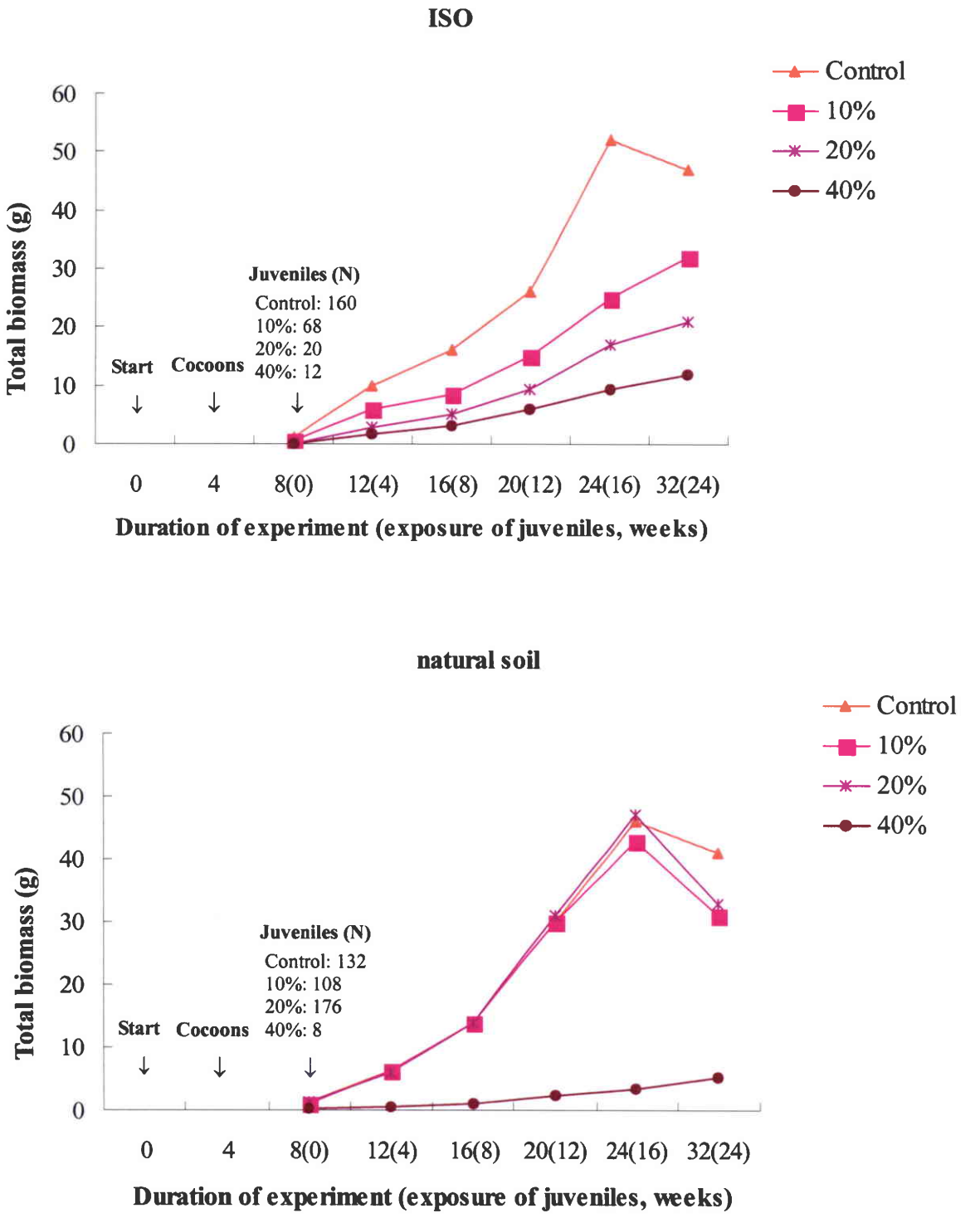

Fig. 1. Total biomass growth patterns of juveniles *E. fetida* exposed to PAH-contaminated soil for 24 weeks to the different concentrations (10%, 20%, 40%) of the PAH-contaminated soil mixed with a control soil either ISO or natural soil.

[a] N: total number of juveniles at the start of the exposure of juveniles at o week in different % contaminated soil.

Table 3. Bioaccumulation factor (BAFs) and Biota soil accumulation factor (BSAFs) for PAHs in population of juvenile earthworms exposed to the PAH-contaminated soil during 24 weeks in two different control soil types: ISO or natural soil.

PAHs (µg/kg MS)	No. of rings	**ISO**		**natural soil**	
		BAF	BSAF	BAF	BSAF
Naphthalene	2	0.533 ± 0.583	1.264 ± 1.320	0.094 ± 0.088	0.194 ± 0.184
Acenaphthylene	3	0.075 ± 0.017	0.204 ± 0.047	0.086 ± 0.016	0.200 ± 0.087
Acenaphthene	3	0.006 ± 0.005	0.015 ± 0.012	0.007 ± 0.004	0.016 ± 0.009
Fluorene	3	0.017 ± 0.025	0.038 ± 0.056	0.003 ± 0.003	0.008 ± 0.007
Phenanthrene	3	0.001 ± 0.001	0.004 ± 0.004	0.003 ± 0.002	0.007 ± 0.007
Anthracene	4	0.013 ± 0.010	0.032 ± 0.020	0.005 ± 0.004	0.012 ± 0.010
Fluoranthene	4	0.004 ± 0.004	0.012 ± 0.010	0.006 ± 0.005	0.015 ± 0.014
Pyrene	4	0.009 ± 0.002	0.025 ± 0.001	0.008 ± 0.006	0.020 ± 0.018
Benzo[a]anthracene	4	0.027 ± 0.013	0.070 ± 0.022	0.015 ± 0.006	0.036 ± 0.021
Chrysene	4	0.062 ± 0.039	0.160 ± 0.073	0.031 ± 0.012	0.071 ± 0.034
Benzo[b]fluoranthene	5	0.099 ± 0.020	0.275 ± 0.080	0.126 ± 0.083	0.287 ± 0.182
Benzo[k]fluoranthene	5	0.110 ± 0.042	0.290 ± 0.059	0.095 ± 0.062	0.215 ± 0.135
Benzo[a]pyrene	5	0.050 ± 0.032	0.142 ± 0.093	0.088 ± 0.065	0.199 ± 0.140
Dibenzo[ah]anthracene	5	0.129 ± 0.046	0.341 ± 0.059	0.176 ± 0.150	0.404 ± 0.319
Benzo[ghi]perylene	6	0.042 ± 0.018	0.111 ± 0.026	0.049 ± 0.013	0.107 ± 0.031
Indeno[1,2,3-cd]pyrene	6	0.032 ± 0.024	0.093 ± 0.069	0.041 ± 0.030	0.104 ± 0.091
Σ16 PAHs		0.027 ± 0.007	0.058 ± 0.003	0.026 ± 0.015	0.060 ± 0.036
Σ2 rings PAH (n=1)		0.533 ± 0.583	1.264 ± 1.320	0.094 ± 0.088	0.194 ± 0.184
Σ3 rings PAH (n=4)		0.005 ± 0.004	0.012 ± 0.010	0.004 ± 0.003	0.010 ± 0.007
Σ4rings PAH (n=5)		0.017 ± 0.007	0.045 ± 0.011	0.011 ± 0.006	0.026 ± 0.018
Σ5rings PAH (n=4)		0.085 ± 0.013	0.232 ± 0.048	0.107 ± 0.074	0.295 ± 0.194
Σ6rings PAH (n=2)		0.037 ± 0.008	0.092 ± 0.024	0.044 ± 0.013	0.105 ± 0.059

[a] Data given mean ± standard deviation (n=3; 10%, 20%, 40% contaminated soils).

[b] BAFs calculated by the PAH concentration ratio earthworm tissue to the tested medium.

[c] BSAFs expressed as the lipid normalized PAH content to the organic carbon normalized contaminant content in the soil.

Results

Physico-chemical analysis

The three replicate soil analyses confirmed the presence of PAHs at a total concentration of 2634 ± 241 mg/kg 16 U.S. EPA PAHs as major contaminants in soil from an old coking plant site (Table 1). The results of chemical analyses on two "control" soils used for dilution with contaminated soil: an ISO and a loamy natural soils, showed nondetectable contaminant concentrations. The total organic carbon (TOC) content ranged from 2.3 to 10% (w/w) for all of the soil samples used this study.

PAH Uptake by Earthworm

The juvenile earthworms produced after 56-d reproduction test were studied for rate of growth and uptake of PAHs from contaminated soils for an additional 24 weeks. We observed no significant change in mortality of juveniles at the tested concentrations (max. 40% contaminated soils; w/w) during the course of the uptake test (Table 2). We found in an earlier toxicity test that juveniles were sensitive during the 14-day acute and 56-day chronic tests, indicating significant reduction of juvenile number and afterwards total biomass (Fig. 1). We determined growth rate of juveniles using a linear regression with no intercept constant for data over the period when fresh weight was increasing (Table 2). Results of calculated growth rate (g day^{-1}) after 16-week exposure indicate significant difference ($P < 0.05$) between control soil types tested. Juveniles showed higher growth rate in loamy natural soils.

The mean total 16 PAH content and standard deviation of *E. fetida* in ISO and natural soil after 24 weeks of exposure at the 10% to 40% concentration of the PAH-contaminated soil were 16 ± 6 μg/g for ISO and 19 ± 14 μg/g for natural soil, respectively. The mean BAF and BSAF values for the 16 PAHs in earthworms after 24 weeks of exposure to PAH-contaminated soils were calculated (Table 3). As to earthworms grown in ISO test medium, BAFs for individual 16 PAHs were in the range of 0.001-0.533. The BAFs for individual 16 PAHs were 0.006–0.176 in natural soil test medium.

BSAFs, defined as the ratio of lipid normalized PAH concentration in earthworm

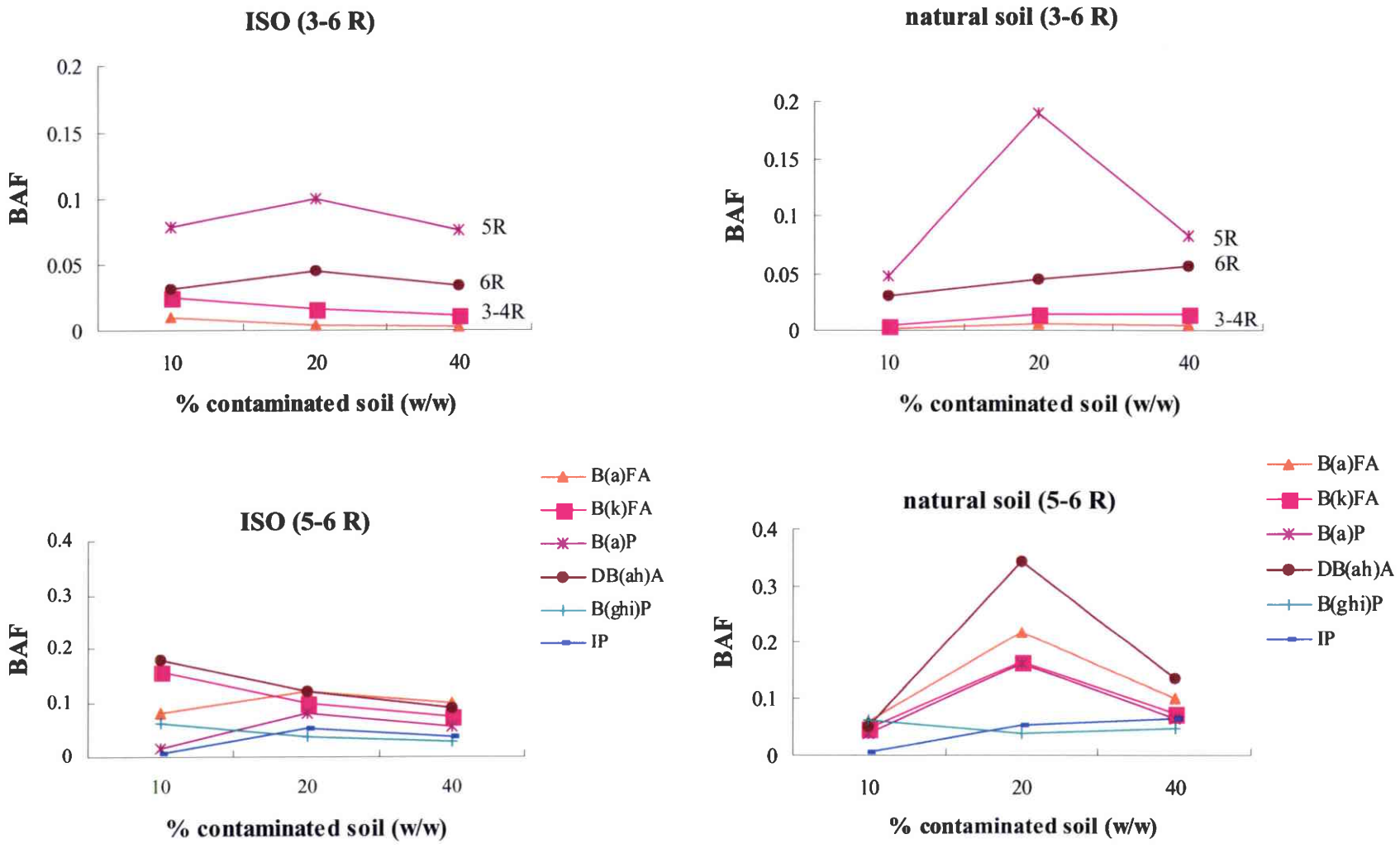

Fig. 2. Comparison of BAFs of juvenile earthworms exposed for 24 weeks to the different concentrations(10%, 20%, 40%) of the PAHs-contaminated soil mixed with a control soil either ISO or natural soil.

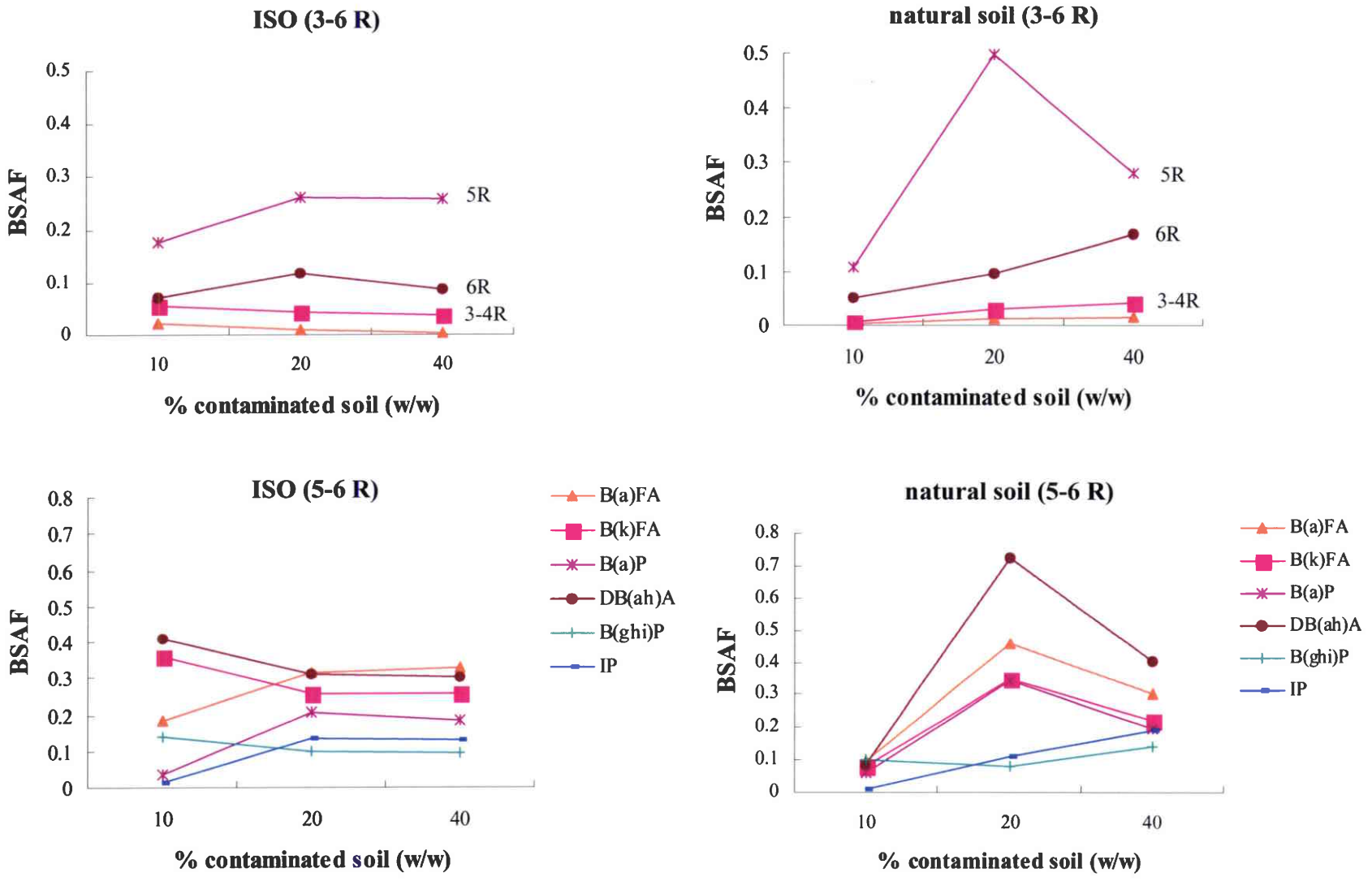

Fig. 3. Comparison of BSAFs for juvenile earthworms exposed for 24 weeks to the different concentrations (10%, 20%, 40%) of the PAHs-contaminated soil mixed with a control soil either ISO or natural soil.

Table 4. Shoot concentration factor (SCFs) of PAHs, on a dry wet weight basis, for plant (Chinese cabbage) after 28 days exposed to the PAH-contaminated soil mixed with artificial ISO soil.

	No. of rings	**PAH concentration in Shoot** (mg/kg d.w) % contaminated soil in ISO test medium (w/w)				**SCF**[a]		
PAHs (µg/kg MS)		0 (control)	1	10	15	1	10	15
Naphthalene	2	0.040	<0.001	0.099	0.083	<0.001	0.168	0.094
Acenaphthylene	3	<0.010	<0.010	<0.010	<0.010	<0.001	<0.001	<0.001
Acenaphthene	3	0.001	0.002	0.030	0.017	0.005	0.007	0.002
Fluorene	3	0.002	0.002	0.014	0.008	0.002	0.001	0.001
Phenanthrene	3	0.027	0.004	0.416	0.238	0.012	0.011	0.004
Anthracene	4	0.002	0.007	0.142	0.101	0.004	0.008	0.004
Fluoranthene	4	0.029	0.006	1.280	1.040	0.011	0.023	0.012
Pyrene	4	0.005	0.011	0.230	0.167	0.003	0.006	0.003
Benzo[a]anthracene	4	0.003	0.004	0.273	0.073	0.002	0.012	0.002
Chrysene	4	0.005	0.003	0.210	0.059	0.002	0.011	0.002
Benzo[b]fluoranthene	5	0.002	0.002	0.185	0.029	0.001	0.013	0.001
Benzo[k]fluoranthene	5	0.001	<0.001	0.108	0.016	<0.001	0.012	0.001
Benzo[a]pyrene	5	0.001	0.001	0.201	0.027	0.001	0.014	0.001
Dibenzo[ah]anthracene	5	<0.001	<0.001	0.011	0.002	<0.001	0.007	0.001
Benzo[ghi]perylene	6	0.003	0.014	0.134	0.018	0.020	0.021	0.002
Indeno[1,2,3-cd]pyrene	6	<0.001	<0.001	0.091	0.014	<0.001	0.011	0.001
Σ16 PAHs		**0.123**	**0.153**	**3.424**	**1.891**	**0.006**	**0.013**	**0.005**
Σ2 rings PAH (n[b] =1)		0.040	<0.001	0.099	0.083	<0.001	0.168	0.094
Σ3 rings PAH (n=4)		0.030	0.049	0.460	0.263	0.009	0.009	0.003
Σ4rings PAH (n=5)		0.045	0.087	2.135	1.440	0.005	0.014	0.006
Σ5rings PAH (n=4)		0.004	0.003	0.505	0.074	0.001	0.013	0.001
Σ6ings PAH (n=2)		0.004	0.014	0.225	0.032	0.009	0.015	0.001

[a] SCFs calculated by the PAH concentration ratio plant shoot to the tested medium.

[b] n = number of individual PAH.

and organic carbon normalized soil, were 0.012-1.264 and 0.007-0.404 for ISO and natural soil test medium, respectively. We observed no statistical difference between control soil types tested: ISO or loamy natural soil. Generally, the BAFs and BSAFs for naphthalene with 2 aromatic rings and higher-molecular-weight PAHs with 5-6 aromatic rings were relatively higher than for the 3-4 ring PAHs (Fig. 2 & 3).

Plant PAH Uptake

The 16 PAH contents in Chinese cabbage after 28 days of exposure to the PAH-contaminated soil and shoot concentration factors (SCFs) were shown in Table 4. Content of total 16 PAH in plant shoot was in the range of 0.1-3.4 μg/g at the tested 1-15% concentrations of the PAH-contaminated soil mixed with ISO. As to the chinese cabbage grown in ISO test medium, SCFs for total 16 PAHs were 0.005-0.013 in the contaminated soil concentration tested (1-15%). Naphthalene with higher hydrosolubility showed the highest SCF value. The SCFs for Chinese cabbage at 10% concentration of the PAH-contaminated soil was higher than at the other tested concentration.

We observed higher PAH transfer ability from contaminated soil to Chinese cabbage in the loamy natural soil (results not shown). We are performing additional testing to confirm these results. The results for PAH uptake testings in natural soil test medium will be presented in the next paper.

Discussion

In the testing of earthworm population development, we found that most of juvenile earthworms died during the first 4 weeks of the experiment when exposed for 24 weeks to the PAH-contaminated soil. Spurgeon & Hopkin (1996), also reported this death pattern of juvenile exposed to metal-contaminated soils and explained as the particular sensitivity of juveniles (*25*).

The literature studies on the uptake of PAHs by earthworm species show that methodology differences (worm species and age, incubation time) and variable data expression (concentrations that may or may not be normalized to lipid content, ratios of lipid normalized to organic carbon normalized content) make direct comparisons between studies difficult (*6*, *27*).

Krauss et al., (2000), reported biota-to-soil accumulation factors (BSAFs for *L. terrestris*) of individual PAHs, expressed as the lipid normalized PAH content to the organic carbon normalized contaminant content in the soil, of 0.13-0.2 except for Flu (0.41), Phe (0.33), and Ant (0.26) after short term exposure (*18*). BSAFs of the total PAHs were observed for *L. rubellus* (average 0.1, range: 0.03-0.26) in different field sites (Ma et al., 1998) (*17*). Parrish et al., (2006) calculated the BSAFs calculated of total PAHs for *E. foetida* was 0.011 in the short-term test but authors found no accumulation of 3, 4, or 6 ring PAH (*6*).

In our study, the BSAFs values of the 16 PAHs for *E. fetida* were in the range of 0.007-0.404 under the experimental condition of the long-term exposure to the PAH-contaminated soil mixed with natural soil. Alternatively, Matschko et al., (2002) observed relatively low BSAFs of 0.02 on average for polycyclic aromatic compounds including 12 PAHs from an aged contaminated soils after 19-day exposure but this value is based on soil organic matter content, instead of carbon (*27*).

The average BSAFs of the PAHs for earthworm are almost independent of the K_{ow} (*18*). There are two major uptake routes by which PAHs can be accumulated by earthworms. The first is simple diffusion across the earthworm' dermis. This passive dermal uptake is facilitated by the hydrophobic nature of the PAHs, resulting in a partitioning of soil-associated PAHs in the lipid-rich earthworm tissues. Secondly, when PAHs are ingested together with soil, this leads to the diffusion of the contaminants across the gastrointestinal tract and accumulation in the earthworm tissue (*11*, *18*, *26*). It is demonstrated in the scientific literature that uptake of contaminants by earthworms may be correlated with the total content in soil, but in most cases only part of the this total content is available for uptake (*28*). The earthworm uptake assay is probably the most useful measure of the relative bioavailability of PAHs in soil because it is reliable, inexpensive, and sensitive at meaningful soil concentrations (*11*).

The exposure of earthworms to low-molecular-weight PAHs in soil is mediated through direct contact of the earthworms with the dissolved interstitial soil-water phase (*17*). Indeed, It was of interest to note that the higher-molecular weight PAHs with 5 and 6 aromatic rings and naphthalene showed higher BASFs values in this long-term study. The BSAFs ranking depending on classification in the number of aromatic rings, in decreasing order, was 5 rings ≥ 2 rings > 6 rings >> 3 & 4 rings. The previous studies demonstrated that factors reducing the bioaccumulation of PAH

by earthworms might consist of sorption to soil, matrix effects, aging and food limitation etc., which could significantly reduce bioavailability for uptake (*11*, *26*, *29*, *30*). The BSAFs of the PAHs from field-contaminated soils to earthworm is independent of the octanol-water partitioning coefficient (K_{ow}) (*17*, *18*). Because the lipid contents were similar between species, the major factor affecting the BSAFs values was the soil total organic carbon. The PAH-contaminated soil had higher BSAFs than the laboratory-spiked soil (*11*).

We observed no significant difference in PAH uptake capacity for earthworm between control soil types tested: ISO or loamy natural soil. We could explain our results as same earthworm species and close TOC contents between control soil types (3.4% for ISO, 2.3% for natural soil).

Studies attempting to quantify the soil to plant transfer from PAHs-contaminated soils are uncommon (16). Gao & Zhu, (2004), reported shoot bioconcentration factors (BCFs) of spiked-phenanthrene and pyrene for Chinese cabbage ranged from 0.02 to 0.03 and 0.01 to 0.02, respectively after 45-day (*16*). The BCFs by tomato plants after 4 weeks were 0.01 for anthracene and 0.02 for phenanthrene by laboratory spiking (*31*). In the current study, SCFs for Chinese cabbage were calculated in the range of 0.001-0.023 for most PAHs, excluding naphthalene. Although there were differences of experimental conditions, our results were also in the previous finding ranges, showing lower transfer ability of PAH from contaminated soil. Jourdain et al., (2003), also reported shoot bioconcentration factors (SCFs) of PAHs for plants in contaminated soil from a coking site were very small, showing in the range of 10^{-3}-10^{-5} (*32*).

Uptake depends on the plant species and on the physico-chemical properties of the chemicals (*31*). The vast majority of organic compounds in sludge are so strongly sorbed by the soil-sludge matrix that they exhibit low bioavailability to crop plants and so they accumulate at very low concentrations in the edible portion of food crops (*33*). The main metabolites being formed are polar conjugates with carbohydrates and amino acids. PAHs are partly converted to oxygenated derivatives some of which are known to be even more toxic (*31*). Transport of compounds from roots to shoots usually was the major pathway of shoot accumulation (*16*). For plants with high water contents the plant-water phase acts as the major reservoir for highly water-soluble contaminants. By contrast, lipid in a plant, even at small amounts, is usually the major reservoir for highly water-insoluble contaminants (*34*). The most water-soluble naphthalene

showed the most content in chinese cabbage in this study. Wild et al., (1992) reported that substituted naphthalenes were present in the highest concentrations in the shoot samples of fescue but the concentrations of all PAHs with more than two rings were much higher in the roots than the shoots under field studies (35). The very low concentrations of PAHs in the fescue shoots, despite the high concentrations in the roots, suggested that only the low-molecular-weight polarity compounds show any propensity for translocation from root to shoot (36). Some authors found that the shoot uptake of PAHs from the ambient air, possibly originally volatized from the soils, was an important pathway for these PAH intake by vegetable aerial part (*15*, *16*, *37*). Because the various treated pots were randomized in the small glass greenhouse side by side and exchanged places frequently, the PAH concentrations in the air would quickly become homogenuous after volatilizing from soils (*16*). Zhu et al., (2004), attributed the phenanthrene concentration in shoot of plants in the control groups as direct absorption of PAH from contaminated air in the greenhouse (*38*). We also observed negligible PAH accumulation (total 16 PAH: 0.12 μg/g d.w) in plant shoot in the controls.

Conclusion

The results in this study confirmed the limited transfer capacity of PAHs from contaminated soils to terrestrial organisms such as earthworm and plant. The earthworm BSAFs of PAHs from contaminated soil were 0.012 - 1.264 and 0.007 - 0.404 for ISO and natural soil test medium, respectively. The high-molecular-weight PAHs with 5 & 6 aromatic rings showed higher contents in earthworm after 24 weeks exposure than PAHs with 3 & 4 rings. No significant difference in BASFs for earthworm was found between tested control soil types: the ISO or loamy natural soil. Plant Chinese cabbage showed very lower shoot concentration factors (10^{-2} - 10^{-3} for SCF). Generally, uptake by biota is known to dependent on several factors such as soil type, chemicals tested, test organism species etc. The previous studies were conducted under different experimental conditions. All of these factors make it very difficult to compare the relatively few published studies. It should be stressed that more data and research are needed to establish and develop a better insight into the process of uptake of PAHs by biota and to provide quantitative description of different pathways of transfer, bioavailability and accumulation.

bioavailability and accumulation.

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CHAPTER VI. DISCUSSION

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In this study, ecotoxicity of the water extracts of the PAH-contaminated soil from a cookery site was evaluated by using a battery of bioassay, involving aquatic and terrestrial species.

Various single-species screening test are often employed to detect possible harmful effects of chemicals on biotic systems. In the environment, however, different species and complex food webs are exposed (*Juvonen et al., 2000*).

The use of a set of tests on species at different levels of organization and integrating physico-chemical and biological approaches are needed and recommended in ecotoxicological studies for a refined evaluation of environmental risk (*Debus et al., 1997; Bispo et al., 1999; Békaert et al., 1999; Farre & Barcelo, 2003; Rila & Eisentraeger, 2003; Fernandez et al., 2005*).

Soil quality assessment with a battery of bioassays using plant, invertebrates (earthworm, anthropod reproduction), bacteria, algae, other soil organisms (e.g. nematodes) was recommended (*Keddy et al., 1995*; *Plaza et al., 2005*).

The preference for a test battery approach could be explained by the need to assess hazard at different levels of biological organization so as not to underestimate toxicity. Contaminants could demonstrate "trophic-level specificity" or adverse effects at multiple levels. When test battery approach was used, bioassays were mostly conducted with two, three or four trophic levels (*Blaise & Férard, 2005b*). The three mostly used bioassays were the acute *Vibrio fischeri* test, the acute *Daphnia magna* test and the chronic *Pseudokirchneriella subcapitata* test (*Férard & Ferrari, 2005*).

Water extractability of pollutants may affect aquatic species. Bioassays carried out at different trophic level of aquatic chains are useful.

Soil is composed of particles, pore air, and pore water, and soil organisms may be exposed to substances through ingestion of soil particles or absorption from pore air or pore water (*Sverdrup et al., 2001*). For aquatic species, the large quantity of literature data to various contaminants are available. In contrast, relatively few ecotoxicity studies have been conducted on soil organism (*Sverdrup et al., 2002a*) The reason of this could be explained that the sufficient number of validated test

methods have only become available recently (*Kordel & Rombke, 2001*). While soil leachates could always be made and have often been used to assess soil toxicity with aquatic organisms, the main focus of soil quality testing should concern soil-dependent organisms (*Keddy et al., 1995*). For the assessment of contaminated soils, ecotoxicological tests are not only performed on the retention function of soils (determined by tests with aqueous soil extracts) but the habitat function (determined by tests with soil) for soil organisms also must fundamentally be evaluated (*Kordel & Rombke, 2001; Hund-Rinke et al., 2002*).

The integration of two approaches, chemical and biological assays, could be recommended to evaluate the toxicity of contaminated soil. Assessment of soil quality based exclusively on chemical analyses would be not sufficient.

Chemical analysis does not allow an integration of the combined effects produced by the chemical mixture present at a polluted site. In addition, total concentrations can overestimate the real risk, as aging processes can strongly reduce the bioavailability and subsequently, the toxicity of pollutants. Bioassays try to integrate these effects and help in evaluating the risk associated with exposure to bioavailable substances present in a soil. Bioassays may also be used as screening tools in order to identify polluted soil or water samples and to reduce the number of samples that require full chemical and toxicological analysis (*Fernandez et al 2005*). Toxicity tests have also good potential for applications as environmental monitoring tools to assess the efficiency of remediation technologies for site cleanup (*Plaza et al., 2005*).

As waste elutriates often represent complex mixtures of organic and inorganic contaminants, correlations of ecotoxicological effects with single compounds are often impossible (*Hauser et al., 1997*). Because of interaction phenomena between the contaminants such as synergistic and antagonistic effects, the lack of data in metal speciation, possible presence of natural substances extracted, other pollutants not measured, and by-products by degradation process, the relationships between the contaminants and the genotoxicity results is difficults to establish (*Bispo et al., 1999; Békaert et al., 1999; Donnelly et al., 2004*).

At historically PAH-contaminated sites, pollution always comprises numerous individual PAHs (i.e. pollutant mixture) (*Haeseler et al., 1999; Loibner et al., 2004*). Unidentified components or mixture interactions may contribute significantly

to the carcinogenic potency of a mixture. The toxicity of complex mixtures could not be accurately predicted based on chemical analysis alone. This could be due to chemical interactions or due to the presence of unidentified chemicals, such as alkyl PAHs or high molecular weight PAHs that are not included in the standard risk assessment procedure (*Donnelly et al., 2004*).

As organic compounds persist with aging, they become progressively less available for uptake by organisms, for exerting toxic effects, and for biodegradation and bioremediation by microorganisms. This declining bioavailability is not reflected by currently used methods for chemical analysis of soils for determining concentrations of organic pollutants. As a result, such method overestimate exposure, and thus risk, from toxic chemicals in contaminated sites (*Alexander, 2000*). Sorption and aging of soils spiked with phenanthrene showed less toxicity to algal growth over time (*Baun et al., 2002*). Aged coking soils did not show any significant inhibition on plant growth (*Henner et al., 1999*).

We observed that the chronic algal (*P. subcapitata*) and microcrustacean *C. dubia* tests were more sensitive than microtox and *D. magna* tests for acute effect to water extract of the PAH-contaminated soil.

Results of bioassays demonstrated different sensitivities of the test organisms to the various contaminants (*Bierkens et al., 1998; Rojickova-Padrtova et al., 1998; Maxam et al., 2000*).

It seems to us that the chronic algal (*P. subcapitata*) and microcrustacean *C. dubia* tests may be appropriate for evaluating PAH-contaminated soils. *D. magna test* was the least sensitive test. This is consistent with results of published literature (*Rojickoa-Padrtova et al., 1998; Bispo et al., 1999; Mendonca & Picado, 2002*). It is of interest to compare our results to data obtained on industrial and municipal solid waste incineration bottom ash and landfill leachates. The ecotoxicity sensitivity profile follows, in the all cases, the order algal test > microtox > *D. magna* test. (*Lambolez et al., 1994; Ferrari et al., 1999; Lapa et al., 2002; Ward et al., 2002*). Daphnids were also the least sensitive tested organisms for leachates of soils containing metals and petroleum hydrocarbons (*Fernandez et a.,l 2005*). As the endpoints are cell division and reproduction, these bioassays are ecologically relevant. Results also indicate that toxicity of sample is detected at lower level of contamination with these two tests than with the short term test

(Microtox & Daphnia), which can be expected from exposure to pollutants with high lipophilicity or long term effects.

Some authors confirmed that microtox test with *Vibrio fischeri* can be used for screening tests. However, this bioassay has certain limitations due to the high influence of physico-chemical parameters on the test responses (*Isidori et al., 2003*). Among bacterial bioassays, *V. fischeri* luminescence inhibition assay is often chosen as the first test in a test battery based on speed and cost consideration for acute toxicity screening and assessment. Based on the literature review, *V. fischeri* test was sensitive test, cost effective, rapid, reproducible, easy to operate for acute toxicity prediction (*Parvez et al., 2006*). One biotest is adequate, if only process monitoring is concerned. This screening test should be fast, user-friendly, and cost-effective to test a large quantity of samples (*Juvonen et al., 2000*).

Small-scale toxicity test (also called "micro-scale toxicity tests" or "micro-biotests") with 96-well microplate using algae were reviewed. Several advantages such as small volume requirement, incubator space economy, disposable microplates and increased bio-analytical output, were outlined. Some of these advantages will also hold true for microplate-based toxicity tests conducted with different cells, microorganisms or small organisms (*Blaise & Vasseur, 2005a*). Because microplate-based phytotoxicity tests could be alternatives to classical flask-based tests, several studies were conducted to demonstrate the concordance of toxicity responses between the two procedures. Some authors reported a good interprocedural agreement (*Rojickova et al., 1998; Rojickoa-Padrtova et al., 1998; Eisentraeger et al., 2003*).

The earthworm reproduction tests were more sensitive than toxicity tests for survival (*Saterbak et al., 1999; Hund–Rinke et al., 2002*). Acute earthworm test does not seem appropriate to evaluate environmentally relevant toxic levels of contaminants (*Davies et al., 2003*). Reproduction tests should be applied for this purpose due to the sensitivity and validity necessary for soil assessment (*Saterbak et al., 1999; Hund–Rinke et al., 2002; Van Gestel & Weeks, 2004*). The cocoon production is the more sensitive parameter (*Davies et al., 2003; Schaefer*, 2003). *Saterbak et al., (1999)* found a considerably less variability in the cocoon endpoint than mortality.

The collembola reproduction test was considerably more sensitive and more reliable parameter for PAH contamination than adult survival (*Sverdrup et al., 2002a; Sorensen & Holmstrup, 2005*). The collembola was the most sensitive

species of three terrestrial invertebrate test (earthworms, enchytraeids) to PAH compounds (*Sverdrup et al., 2002a*). The *F. candida* reproduction test appears to be a very useful bioassay and should be included for soil ecotoxicological testing of xenobiotic compounds (*Crouau et al., 1999*). Impact on population development of *F. candida* is the result of effects on reproduction and on mortality of adults and juveniles (*Crouau et al., 2002*). It was observed that the physico-chemical characteristics of the wastes such as pH, humidity and organic matter content played a role on toxic effects of *F. candida* (*Crommentuijn et al., 1997; Crouau et al., 2002*).

Sensitivity among endpoints in the plant tests varied across species (*Salanitro et al., 1997; Gong et al., 1999; Saterbak et al., 1999; Maliszewska-Kordybach & Smreczak, 2003; Sverdrup et al., 2003*). Plant seedling growth was a far more sensitive endpoint than seed emergence and root elongation test if non or low-water-soluble toxic constituents are present in soil (*Keddy et al., 1995; Sverdrup et al., 2003*).

Some authors confirmed the hypothesis that water soluble, volatile compounds such as low-molecular weight PAHs are a source of phytotoxicity (*Henner et al., 1999; Sverdrup et al., 2003*). Germination studies alone would not predict the effect of subsequent growth of the species tested (*Gong et al., 1999; Henner et al., 1999; Smith et al., 2005*). Seed germination that depends on the energy reserves in cotyledons is a less sensitive endpoint than the early seedling growth (*Gong et al., 2001*). *Sverdrup et al., (2003)*, recommended that phytotoxicity studies focus on seedling fresh weight rather than dry weight as endpoints, as EC values were in many cases lower when calculated on the basis of fresh weight as compared to dry weight. *Saterbak et al., (1999)*, emphasized that plant test species should be selected based upon the documented exposure pathways and plant receptors appropriate for the present and future use of the site rather than based on the most sensitive species, by default.

When incorporating ecotoxicity test methods into site assessments, differences between endpoints within a species, among species in a taxa, and among taxa should be expected. These results support the hypothesis that different taxa respond differently to contaminants and that a "universal" contaminant parameter that can be used to predict toxic effects on soil communities has not yet been identified (*Saterbak et al., 1999*). These differences in species sensitivity stress that a valid assessment of the environmental hazard of contaminated soils can only be obtained on the

basis of test systems covering different key organisms (*Fernandez et al., 2005*). However, the final choice of bioassays for a certain polluted site must also depend on other factors such as applicability in relation to soil characteristics (e.g. pH) and ecological relevance (*Sverdrup et al., 2003*).

We confirmed the usefulness of juveniles in terrestrial ecotoxicity. It is interesting to note that acute survival tests using juvenile earthworms proved to be highly sensitive which argue for using juvenile survival as ecotoxicological endpoints.

The survival test using juveniles *Eisenia fetida* was more sensitive to metal-contaminated soils than was that using mature earthworms. Juvenile growth rate and sexual development are important life history parameters for earthworms, since they may modify the dynamics of earthworm populations in the field. Results of juvenile development experiments should therefore be considered when proposing a safe environmental concentration for pollutants (*Spurgeon & Hopkin, 1996*).

In general, water extracts of soil have very low leaching capacity compared to solvent extracts but are more representative of leaching process in the environment.

Most genotoxic substances are lipopilic and it is obvious that they are underrepresented in the leachates considering the design of the leaching procedure. Some authors did not observe mutagenic activity on leachates, but tested organic or lyophilized concentrated fractions to improve assay sensitivity (*Blaise & Ferard, 2005b*). The standard test systems using water extraction are not sensitive enough to detect to most of the mutagenic substances (*Ehrlichmann et al., 2000*).

Some authors used the organic solvents for soil extractions in order to identify pollutants present in a complex matrix (*Bispo et al., 1999; Bekaert et al., 1999*).

The widely used protocols of federal and state regulatory agencies (USA) rely on analytical methods that entail vigorous extraction of soils and sediments with organic solvents. The aim is to remove all, or as much as possible, of the pollutant form the environmental sample. However, bioavailability may decline with little or no reduction in the concentration as determined by procedures that

rely on initial vigorous extractions. Hence, such methods are often not relevant for prediction of potential exposures to and thus risk from contaminated soils or sediment (*Alexander, 2000*).

The standardized leachate procedure with 0.45 μm membrane filtration underestimates the potential hazard of contaminated soil to water as, in the environment, solid particles can be carried along either by run-off to surface waters or by infiltration into ground-water (*Bekaert et al., 2002*). Soil extraction with waters seems to be the appropriate approach to assess the degree of mutagenicity with respect to the path of soil to groundwater (*Wahle et al & Kordel, 1997*).

In our study, we had relatively good sensitivity in Ames (Fluctuation method) test for mutagenicity.

The water extracts of the PAH-contaminated soil were found not to present acute and chronic toxicity on several aquatic organisms but were genotoxic. So, a genotoxicity test should also be included into a test battery to take DNA damage in account (*Bispo et al., 1999*). *White & Claxton* (*2004*) reviewed the soil mutagenicity literatures using classical Ames agar plate method. He reported that much of the mutagenic potency on soil extracts was obtained using TA98 with S9 (58%) and without S9 (30%) metabolic activation mixture. This suggests that much of the mutagenicity in industrial contaminated soils was S9-activated. The fluctuation method in Ames test showed a better sensitivity than the classical procedure on agar plate, since bioavailability of pollutants is increased in liquid medium (*Békaert et al., 1999*).

There have been few studies investigating the ecotoxicity of the PAH-contaminated soil. Most of them were conducted with laboratory-spiking method in artificial substrate (e.g., OECD soil).

In ecotoxicological testing of chemicals for soil organisms, well-defined test soils are recommended to allow for the standardization between laboratories or to compare test results obtained with different species. For instance, an artificial soil substrate made of sand, peat, and kaolin clay is used for earthworm and collembola toxicity tests (*OECD, ISO*). The higher the peat proportion of ISO soil in the mixture the higher the organic matter content and the lower the

bioavailability of toxic compounds with a high affinity for organic matter. So, for testing polluted soil it would be better, if possible, to use as a blank, and for dilution of the polluted soil, the same soil but without contamination (*Crouau et al., 2002*). The 3rd International workshop on earthworm (IWE) recommended that natural soils might be selected for ecotoxicity testing. As a result of soil properties on the (bio)availability of chemical compounds, results obtained in artificial test substrates, such as OECD artificial soil, cannot be translated directly to field soils (*Van Gestel & Weeks, 2004*).

The result of laboratory spiking experiments showed large differences in toxicity of oil contamination depending on soil types (*Dorn et al., 1998*). The fractions of the PAHs that were leachable were higher in the spiked soil than in the contaminated soil. This result is clear evidence of soil sorption and matrix effects on the mobility on PAHs (*Stroo et al., 2000*).

As a matter of course, the ultimate way for assessing the bioavailability of contaminants (or chemicals) is by actually measuring biological response or the accumulated amount within organisms.

Bioaccumulation is associated with chemical residues accumulated at the site of toxic action as well as residues in other tissues containing no site of toxic action (e.g., storage lipid) (*Lanno et al., 2004*). The potential of a compound to bioconcentrate and bioaccumulate should be assessed and accounted for in a risk assessment, when appropriate (*Feijtel et al., 1997*). An increase in toxicity with increasing log K_{ow} is expected for chemicals acting by narcosis as the mode of toxic action because this substance parameter can be causatively linked to the process of bioaccumulation in many organisms (*Sverdrup et al., 2003*). Bioconcentration factors are important in aquatic ecotoxicology where the ambient medium is a major source of organic pollutants. Bioaccumulation factors are critical in terrestrial ecotoxicology, where food (or, in some cases, ingested water) represents a major source of organic pollutants (*Walker et al., 2006*)

Uptake depended on the plant species and on the physico-chemical properties of the chemicals (*Harms, 1996; Parrish et al., 2005*). The vast majority of organic compounds in sludge are so strongly sorbed by the soil-sludge matrix that they exhibit low bioavailability to crop plants and so they accumulate at very low

concentrations in the edible portion of food crops (*O'Connor, 1996*).

The main metabolites being formed are polar conjugates with carbohydrates and amino acids. PAHs are partly converted to oxygenated derivatives some of which are known to be even more toxic (*Harms, 1996*). Transport of compounds from roots to shoots usually was the major pathway of shoot accumulation (*Gao & Zhu, 2004*). Soil-crop bioconcentration factors (BCFs) decreased with increasing log *K*ow for PAHs up to about 4.5, above which no change were discernible for contaminants (*Zohair et al., 2005*). For plants with high water contents the plant-water phase acts as the major reservoir for highly water-soluble contaminants. By contrast, lipid in plant, even at small amounts, is usually the major reservoir for highly water-insoluble contaminants (*Chiou et al., 2001*).

Some authors found that the shoot uptake of PAHs from the ambient air, possibly originally volatized from the soils, was an important pathway for these PAH intake by vegetable aerial part (*Beck et al., 1996; Gao & Zhu, 2004; Tao et al., 2004*). *Tao et al., (2005)*, observed a significant correlation between bioconcentration factor (BCF for plant over air) and octanol/air partitioning coefficient (K_{oa}). Because the various treated pots were randomized in the small glass greenhouse side by side and exchanged places frequently, the PAH concentrations in the air would quickly become homogenuous after volatilizing from soils (*Gao & Zhu, 2004*). *Zhu & Gao, (2004)*, explained the phenanthrene concentration in shoot of plants in the control groups as direct absorption of PAH from contaminated air in the greenhouse.

O'Connor (1996) reviewed the literature dealing with plant uptake of sludge-borne toxic organic chemicals (TOCs). He reported plant bioconcentration factors for most TOCs are small, often < 0.01 dry weight.

There are two major uptake routes by which PAHs can be accumulated by earthworms. The first is simple diffusion across the earthworm'dermis. This passive dermal uptake is facilitated by the hydrophobic nature of the PAHs, resulting in a partitioning of soil-associated PAHs in the lipid-rich earthworm tissues. Secondly, when PAHs are ingested together with soil, this leads to the diffusion of the contaminants across the gastrointestinal tract and accumulation in the earthworm tissue (*Krauss et al., 2000; Stroo et al., 2000; Johnson et al., 2002*)

It is demonstrated in the scientific literature that uptake of contaminants by earthworms may be correlated with the total content in soil, but in most cases only part of this total content is available for uptake (*Van Gestel & Weeks, 2004*). The

earthworm uptake assay is probably useful measure of the relative bioavailability of PAHs in soil because it is reliable, relatively inexpensive, and sensitive at meaningful soil concentrations (*Stroo et al., 2000*).

The chemical availability and bioaccumulation in terrestrial organisms (earthworms) is inversely correlated with the amount of organic carbons in a soil or sediment (*Belfroid & Sijm, 1998; Amorim et al., 2002*).

The previous studies demonstrated that factors reducing the bioaccumulation of PAH by earthworms might consist of sorption to soil, matrix effects, aging and food limitation etc., which could significantly reduce bioavailability for uptake (*Ma et al., 1995; Tang et al., 1998; Stroo et al., 2000; Johnson et al., 2002*). The BSAFs of PAHs from field-contaminated soils to earthworm is independent of octanol-water partitioning coefficient (K_{ow}) according to *Ma et al.*, (1998) & *Krauss et al.*, (2000). Because the lipid contents were similar between earthworm species, the major factor affecting the BSAFs values was the soil total organic carbon. The PAHs-contaminated soil had higher BSAFs than the laboratory-spiked soil (*Stroo et al., 2000*).

Krauss et al., (2000), reported biota-to-soil accumulation factors (BSAFs for *L. terrestris*) of 0.13~0.2 except for Fluorene (0.41), Phenanthrene (0.33), and Anthracene (0.26) in soils containing weathered PAHs. He expressed BSAFs as the lipid normalized PAH content to the organic carbon normalized contaminant content in the soil. The BSAFs of PAHs were comparable to those reported for *L. rubellus* (average 0.1, range: 0.03-0.26) by *Ma et al., (1998)*.

The bioavailability needs to be considered for understanding exposure in soil systems and that biological and chemical measures of bioavailability must be correlated and not developed independently. Estimates of the environmentally available fraction of chemicals should be included in standard laboratory toxicity tests with earthworms or when determining chemical levels in field soils (*Lanno et al., 2004*).

In summary, bioassays can provide a measure of the whole toxic effects in soils contaminated by a complex mixture of chemicals integrating different factors, such as solubility, antagonism or synergism, bioavailability, etc.

The difference observed in the toxicity could be due to the intrinsic physicochemical properties of each compound, such as the octanol-water partitioning

coefficient (K_{ow}), volatility, aqueous solubility, reactivity potential of hydroxylated radicals with PAHs and Home-Lumo (highest occupied molecular orbital-lowest unoccupied molecular orbital) gap explaining the persistence, light absorption and photo-induced toxicity of PAHs (*Djomo et al., 2004*).

Five low molecular weight PAHs with 2-3 rings of the 16 US EPA PAHs revealed inhibiting effects on *Vibrio fischeri* bioluminescence. These results indicated that PAH solubility was an important parameter in determining the extent of bioluminescence inhibition (*Haeseler et al., 1999; Loibner et al., 2004*).

The toxic effect was stronger in the case of soils contaminated with both groups of these pollutants than in soils amended with heavy metals or PAHs separately (*Maliszewska-Kordybach & Smreczak, 2003*).

The soil pore water fraction is a major exposure route for chemicals for several soil-inhabiting animals, which implies that the bioavailability of contaminants will depend mainly on the sorption equilibrium between soil and pore water (*Smit & Van Gestel, 1998; Sverdrup et al., 2001*). In the studies with invertebrate and plants, *Sverdrup* et al., (2002; 2003), found a significant relationship between the soil pore-water toxicity and lipophilicity (log K_{ow}) of the low molecular PAHs as well as N-, S-, O- Polyaromatic compounds, suggesting the same mode of toxic action (narcosis) was generally expected.

The determination of the soil adsorption behaviour of an environmental chemical is very important to the evaluation of potential dangers for man and nature. The most frequently used parameter to indicate the soil mobility of a given species is the soil organic carbon partition coefficient, K_{oc}, (*Gawlik et al., 1997*).
A significant influence of dissolved organic matter in soils and waters was shown on fate and effects on chemical (*Kordel, 1997*). It is well-known that for most of the non-ionic organic chemicals the content and nature of organic carbon is the determining factor of soil adsorption (*Gawlik et al., 1997*). If soil organisms are mainly exposed through pore-water and all PAHs have a narcotic mode of toxic action, the toxicity of individual PAHs to soil organisms can be described by their lipophilicity (log K_{ow}) and soil sorption properties (i.e., log K_{oc} combined with information on the organic carbon content of the soil used) (*Sverdrup et al., 2002b*).

Haeseler et al., (1999), did find not any good correlation between leachable and total PAHs in several industrially contaminated soils. This reason may be found in the varing organic matter content of the soil as it acts as strong sorbent for

hydrophobic substances (*Alexander, 2000*). The reduced bioavailability varied markedly with the soil and the compound. The bioavailability of the three PAHs was correlated with organic carbon content (*Alexander & Alexander, 2000*).

A role for organic matter content of soil in reducing the bioavailability of aged phenanthrene for microbial degradation has been proposed (*Nam et al., 1998*). The sequestration and changes in bioavailability of phenanthrene is markedly affected by soil properties and environmental factors such as, organic matter, clay content, aging (or weathering), wetting and drying of soils, nanoporosity, cation exchage capacity, and other properties of soil (*White et al., 1997; Nam et al., 1998; Chung & Alexander, 2002*). The biodegradation of a mixture of PAHs in soil is also controlled by soil parameters such as the nature of the soil particle fractions and aggregates in which the PAHs were associated (*Amellal et al., 2001*).

CHAPTER VII. CONCLUSION AND PERSPECTIVES

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The goal of this research was to use an integrated approach using chemical analyses and a battery of bioassays to investigate and assess the potential aquatic toxicity, genotoxicity, terrestrial toxicity of PAH-contaminated soil. *This research demonstrated that an integrated ecotoxicological evaluation approach using a battery of bioassays, with the simultaneous characterization of soil physicochemical properties, is more effective than that using only conventional chemical analysis in assessing the toxicity, and bioavailability of PAH-contaminanted soil.* The conclusions derived from this research included:

Objective : Characterize the nature and extent of organic and inorganic chemical contaminants in polluted soils from a former coke plant site:

The nature and extent of organic and inorganic chemical contaminants in soil samples from former coking sites were characterized. A complex mixture of US EPA priority pollutants PAHs and heavy metals were analyzed in the soil samples. Total mean 16 PAHs concentration was determined to **2634** mg/kg d.w. from three independent samplings PAHs as a major contaminants in soils. The heavy metals concentration in soil were in the range of respective regional reference values excluding mercury (**ref. Appendix B**)

Objective : Assess the aquatic toxicity of the water extractable fraction of PAHs-contaminated soils using a battery of *in vitro* bioassays, including microtox®, daphnids, and algae tests, allowing us to investigate acute, chronic toxicity to bacteria, invertebrates, and photosynthetic species:

The standard water extraction protocol showed low (in)organic contaminants transfer capacity to water (10^{-4} - 10^{-6} when expressed as ratio of PAH concentration in water extracts to concentration in soil [µg/L]/[µg/kg]). The acute and chronic toxicity of PAHs-contaminated soils was evaluated using a battery of bioassays including *Vibrio fischeri* (Microtox®), *Daphnia magna*, algae (*Pseudokirchneriella subcapitata*) and *Ceriodaphnia dubia*. The algae, *P. subcapitata*,

and microcrustacean *C. dubia* for chronic effects appeared to be sensitive and appropriate bioassays for measuring toxicity in PAHs-contaminated soils. The *D. magna* bioassay was less sensitive than the other bioassays. Analytical results of water extracts allowed us to explain toxicity registered with the aquatic species. It appeared that aquatic toxicity was related with water extractable metals, especially copper. In assessing soil aquatic toxicity, the coupling of two approach, physicochemical analyses and a battery of bioassays, to characterize the effects of contaminated soil is recommended for complementarity of toxicity and physico-chemical approach- PAHs were released in water extracts in line with the hydrosolubility.

Objective : Evaluate the genotoxicity of water extractable fraction of PAHs-contaminated soils using the *in vitro* bacterial assays (*umu*, Mutatox®, and Ames tests).

Genotoxicity of soil water extracts were evaluated by means of the Ames, *umu*, and Mutatox® tests using soil water extracts. Toxicity of PAHs in water extract was expressed with TEQ (toxic equivalent quantities in Benzo[a]pyrene) which corresponded to **7 & 1091 ng TEQ/L** in the two water extracts analyzed, showing significant differences of two orders of magnitude. The difference of genotoxic responses between these extracts was significant. The soil water extract (No. I) induced a positive genotoxic response both with and without metabolic activation, when tested with Ames (fluctuation method) with TA 100 strain and Mutatox® tests. Yet, the *umu* test showed the least sensitivity to water extracts from PAHs-contaminated soils, indicating no genotoxic responses in all cases. In assessing soil aquatic toxicity, the microbial genotoxicity bioassays, coupled with chemical analysis, should be performed to assess contaminated soils effects on human and ecological health. Genotoxicity responses were related with the level of TEQs of the water extracts

Objective : Assess the PAHs-contaminated soils by comparing the Direct effects on plants (Oat, Lettuce, Chinese cabbage), earthworms (*Eisenia fetida*) and collembola (*Folsomia candida*).

Direct effects of PAH-contaminated soils on terrestrial organisms were tested using endpoints such as survival, reproduction and growth. Collembolan *F.*

candida was the most sensitive organisms. Bioassays on earthworms showed that it would be worth studying survival of juveniles instead of adults in a 14 days test. Reproduction of earthworm cocoon produced with 28 days & juvenile hatched within 56 days and juvenile population development of collembola are sensitive and suitable endpoints, when assessing sublethal effects of PAHs-contaminated soils on terrestrial invertebrates. Plant growth on a basis of fresh biomass was also more sensitive endpoint than dry biomass growth and germination for PAHs-contaminated soils. Results obtained in this study indicated that the soil toxicity testing should include reproductive endpoints as well as acute endpoints.

Objective : Compare the effect of PAH-contaminated soil sample on growth of plants and survival, the reproduction of earthworm and springtail collembola when mixed with the artificial ISO or a loamy natural soil.

Toxicity of PAH-contaminated soils on the collembolan *F. candida* was significantly higher when mixed with an artificial ISO soil compared than when mixed with a loamy natural soil. Yet no statistically significant differences observed between control soil types in earthworm and higher plant tests. In this study, although the differences in toxicity of PAHs-contaminated soils on terrestrial organisms between the control soil types were not significant, A trend was noted in earthworm testing of the PAH-contaminated soil when mixed with the ISO soil compared to the control loamy soil, indicating also a slightly higher toxicity could be observed on the whole data sets. So, it would be useful to perform studies with natural soils in terrestrial ecotoxicity testing. To more sophisticated conclusions, use of natural soils with varing physical and chemical parameters as control soils also needs to be taken into account in ecological risk assessment.

Objective : Determine the transfer of PAHs from contaminated soil sample to earthworm and higher plant.

Two terrestrial organisms, earthworm (*Eisenia fetida*) and plant (chinese cabbage), were used to assess the uptake from PAH-contaminated soil to biota in the long-term. Juvenile earthworms and Chinese cabbage were exposed to PAHs-contaminated soils for 24 weeks and 28 days. Uptake results were compared to the

ones obtained using two different control soils: an artificial ISO soil or a loamy natural soil from agricultural field. The PAH contents in earthworm and plant shoot in contaminated soil sample were elevated with the increase of their soil concentration. Earthworm (*E. fetida*) uptaked total 16 PAH 9-22 μg/g d.w and 3-28 μg/g d.w, when exposed to contaminated soil mixed with ISO and natural soil in tested dilution series (w/w), respectively. Chinese cabbage shoot took up total 16 PAHs to yield residues of 0.2-3.4 μg/g d.w at the tested concentrations (1-15% contaminated soil, w/w). The biota-soil accumulation factors (BSAFs), normalized with lipid in earthworm and organic carbon in soil, of PAHs from contaminated soils to earthworm were 0.012-1.264 and 0.007-0.404 using two different control soils; ISO and natural soils, respectively, but without statistically significant difference between control soil types. The plant shoot concentration factors (SCFs) of PAHs from contaminated soils to plant were 0.005-0.013, when mixed with ISO.

The current study suggests an integrated ecotoxicological evaluation approach, chemical analyses coupled with biological assays, is useful. However, additional future research is needed.

- Chinese cabbage showed higher uptake ability for PAHs in natural soil than in ISO (**ref. Appendix A**). Yet, this difference between ISO & natural soil need to be confirmed with additional experiments (work in progress).

- The 1st generation (juveniles) produced by adults earthworm & collembola were exposed to the contaminated soil samples in this study. It was interesting to note that pollutant effects on earthworms were similar after 56 days of exposure than after 16 and 24 weeks later. Yet it would be worth studying effects on the next generations to check that population dynamics were not affected more sevely over time.

- In the present study, a test for evaluating genotoxicity of solid-phase contaminated soil samples was not included. However, several studies reported that these biological endpoints (or biomarker such as DNA damage) were more sensitive than the conventional environmental effect assessment technique in direct ecotoxicity measurements. It is noteworthy that we observed the higher transfer ability of higher-molecular-weight PAHs with 5-6 aromatic rings than lower-

molecular-weight PAHs with 3-4 aromatic rings in our earthworm PAHs uptake testing. Indeed, sublethal effects are believed to occur early in the cascade of toxic events and at relatively low levels of genotoxicants. Now, future research of our laboratory would focus on the more subtle and specific endpoint using the comet assay (single cell gel electrophoresis, SCGE) for genotoxicity to the earthworm (*Eisenia fetida*).

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APPENDICES

Appendix A.

Shoot concentration factor (SCFs) of PAHs, on a dry wet weight basis, for plant (Chinese cabbage) after 28 days exposed to the PAH-contaminated soil mixed with loamy natural soil.

	No. of rings	PAH concentration in Shoot (mg/kg d.w) % contaminated soil in natural soil test medium (w/w)				SCF[a]		
PAHs (μg/kg MS)		0 (control)	1	10	15	1	10	15
Naphthalene	2	0.052	0.091	0.110	0.198	1.542	0.186	0.224
Acenaphthylene	3	<0.010	<0.010	<0.010	<0.010	<0.001	<0.001	<0.001
Acenaphthene	3	0.011	0.016	0.029	0.055	0.035	0.006	0.008
Fluorene	3	0.014	0.029	0.040	0.062	0.028	0.004	0.004
Phenanthrene	3	0.134	0.206	0.314	0.455	0.054	0.008	0.008
Anthracene	4	0.049	0.074	0.150	0.220	0.040	0.008	0.008
Fluoranthene	4	0.246	0.450	0.845	1.260	0.079	0.015	0.015
Pyrene	4	0.165	0.300	0.553	0.860	0.077	0.015	0.015
Benzo[a]anthracene	4	0.050	0.107	0.286	0.453	0.045	0.012	0.013
Chrysene	4	0.062	0.130	0.290	0.460	0.065	0.015	0.016
Benzo[b]fluoranthene	5	0.019	0.050	0.200	0.318	0.033	0.014	0.014
Benzo[k]fluoranthene	5	0.012	0.032	0.126	0.210	0.035	0.014	0.016
Benzo[a]pyrene	5	0.015	0.043	0.195	0.312	0.028	0.013	0.014
Dibenzo[ah]anthracene	5	0.002	0.005	0.030	0.044	0.034	0.020	0.020
Benzo[ghi]perylene	6	0.005	0.033	0.114	0.188	0.048	0.017	0.019
Indeno[1,2,3-cd]pyrene	6	0.005	0.028	0.104	0.178	0.032	0.013	0.014
Σ16 PAHs		**0.842**	**1.594**	**3.386**	**5.273**	**0.006**	**0.013**	**0.013**
Σ2 rings PAH (n[b] =1)		0.052	0.091	0.110	0.198	1.542	0.186	0.224
Σ3 rings PAH (n=4)		0.159	0.092	0.383	0.572	0.047	0.007	0.007
Σ4rings PAH (n=5)		0.572	0.489	2.124	3.253	0.067	0.014	0.014
Σ5rings PAH (n=4)		0.048	0.082	0.551	0.884	0.032	0.014	0.015
Σ6ings PAH (n=2)		0.011	0.061	0.218	0.366	0.039	0.014	0.017

[a] SCFs calculated by the PAH concentration ratio plant shoot to the tested medium, [b] n = number of individual PAH.

Appendix B.

Comparison of heavy metal contaminant levels in the PAH-contaminated soil with reference values from the Netherlands and regional values in Lorraine, France.

	Soil samples used for this study	Reference values in the Netherlands [a]			Regional contamination levels in soil [a]	
Heavy metals : mg/kg		A [b]	B [b]	C [b]	mean	maximum
Cd	6.7	1	5	20	2	5
Cr	54	100	250	800	80	500
Cu	26	50	100	500	15	50
Hg	**12** [c]	0.5	2	10	0.5	2
Pb	120	50	150	600	25	100
Zn	347	200	500	3000	120	500
Ni	23	50	100	500	30	100

[a] Data from BRGM (2000).

[b] Contamination level index : A- low, B- medium, C- high.

[c] Contamination level above maximum reference values in bold letter.

Résumé

L'écotoxicité d'un sol contaminé par les HAP et provenant d'une ancienne cokerie a été étudiée. La contamination du sol par les 16 HAP s'élevait à 2634 ± 241 mg/kg de sol exprimé en poids sec. Des métaux ont été trouvés également mais à concentration faible, de l'ordre des niveaux géologiques en Lorraine, à l'exception du mercure. Les résultats de toxicité des lixiviats soulignent la sensibilité des deux essais de toxicité chronique - sur la reproduction des cladocères et la division algale. Un caractère mutagène du lixiviat a été enregistré avec le test d'Ames et le test Mutatox ; mais aucune réponse n'a été notée avec le test Umu moins sensible. La germination des plantes testées n'est pas affectée par le sol de cokerie, contrairement à la croissance. Les résultats des essais d'écotoxicité terrestre témoignent d'une toxicité élevée du sol étudié sur la survie et la reproduction des collemboles, sur la reproduction des vers de terre et la survie des juvéniles. L'influence du sol de dilution utilisé pour tester différentes concentrations du sol contaminé a été étudiée en comparant un sol contrôle artificiel (ISO) et un sol naturel de prairie. La comparaison des réponses des invertébrés au sol de cokerie fait apparaître une sensibilité plus élevée en sol artificiel ISO qu'en sol naturel. Il ne semble pas que le transfert des HAP du sol à la biomasse végétale ou à celle des invertébrés pose des problèmes d'accumulation, compte tenu des facteurs enregistrés nettement inférieurs à 1. Les essais de bioaccumulation sur vers de terre ont montré que les HAP lourds (5 cycles et plus) étaient plus fortement accumulés que les légers (3 et 4 cycles).

Abstract

A cokery soil polluted by PAHs was assessed for its toxicity and genotoxicity to soil and aquatic organisms. Total mean 16 PAH concentration was determined to 2634 ± 241 mg/kg d.w. The heavy metals were in the range of the respective regional reference values. The effective concentration (EC) values based on concentration-response relationships were calculated. Toxicity of water extracts in chronic bioassays (algal growth and reproduction of *C. dubia*) was high compared to acute toxicity (bacterial luminescence and daphnid viability). The Ames and Mutatox tests indicated mutagenicity of water extracts, while no response was found with the Umu test showing lower sensitivity. Germination of the tested plant species was not affected, contrary to plant growth. Fresh biomass was a more sensitive parameter than dry biomass. The results of terrestrial toxicity showed that toxic effect of contaminated soil was high, when survival and reproduction of collembola, reproduction of earthworm and survival of juvenile earthworm were measured. Different concentrations of the contaminated soil mixed with the artificial ISO or loamy natural soil were tested to determine influence of the control soil on toxicity. There was a significant trend that toxicity of contaminated soil was lower in the natural soil tested than in ISO, when reproduction of collembola and earthworm was studied. The transfer of PAHs from contaminated soil to biota (earthworm and plant) was inferior to 1, indicating no major problem in trophic chains. The earthworm bioaccumulation of high-molecular-weight PAHs with 5-6 aromatic rings was higher than the one of low-molecular-weight PAHs with 3-4 aromatic rings.