Nancy-Université
Université Henri Poincaré
Faculté des Sciences et Technologies
Ecole Doctorale RP2E

Thèse
présentée pour l'obtention du titre de
Docteur de l'Université Henri Poincaré, Nancy I
en Géosciences
Par ZHOU Xiaobai

Etude expérimentale de quelques paramètres affectant la dissipation des hydrocarbures aromatiques polycycliques (HAP) dans la rhizosphère de plantes mycorhizées

Experimental study of some parameters affecting polycyclic aromatic hydrocarbons (PAHs) dissipation in the rhizosphere of mycorrhizal plants

Soutenance publique le 22 Novembre 2010 devant le jury:

Rapporteurs : Mr Erik JONER, Chercheur, Bioforsk Soil and Environment, Oslo
Mr Thierry LEBEAU, Professeur, UHA Colmar

Examinateurs: Mme Anissa LOUNES HADJ SAHRAOUI, Maître de Conférence, Université du Littoral côte d'Opale
Mme Sylvie Dousset, Professeur, UHP Nancy

Directrice de thèse: Mme Corinne LEYVAL, Directrice de Recherche, CNRS, Nancy

Laboratoire des Interactions Microorganismes- Minéraux- Matière Organique dans les Sols
U M R 7137 Nancy Université-CNRS, Faculté des Sciences
BP 70239, 54506 Vandoeuvre - les - Nancy, Cedex, France

LIMOS

Remerciements

Ce travail de thèse a été réalisé au Laboratoire des Interactions Micro-organismes - Minéraux - Matière organique dans les sols (LIMOS). Ce manuscrit est ainsi le fruit du travail réalisé lors de mon doctorat mais il est également issu de la participation de l'ensemble des personnels du LIMOS, qui chacun à leur manière, ont rendu cette thèse possible.

Je tiens tout d' abord à remercier Corinne Leyval, ma directrice de thèse, directrice du LIMOS, qui m'a donné l'opportunité de venir travailler à Nancy au sein de son laboratoire et d'apprendre toutes les ficelles du métier de chercheur. Elle a su me conseiller et m'orienter pour que je mène à bien mes recherches en me laissant une grande part de liberté et de responsabilités.

Ensuite j'adresse un grand merci aux chercheurs et enseignants chercheurs du LIMOS avec qui j'ai eu des conversations et des conseils de qualité, qui m'ont permis de mieux cerner mon sujet et d'ouvrir des horizons dans d'autres domaines de la science du sol et de la microbiologie. Tout d'abord, je tiens à remercier Thierry Beguiristain et Aurélie Cébron pour leur aide dans la biologie moléculaire qui m'a permis d'avancer dans la compréhension des communautés bactériennes. Je veux aussi remercier Thierry pour son aide dans ma vie pratique. Quand j'ai besoin d'aide, il est toujours là. Ensuite, je veux remercier David Billet et Hervé Marmier, qui m'ont aidée pour l'analyse des échantillons. Je veux remercier aussi Geneviève et Christine, qui m'aident avec mes expériences sur les mycorhizes. Pour continuer, je voudrais remercier tous les autres doctorants, post-docs: Marie-Paule Norini, Paul-Olivier Redon, Emile Boulou Bi, Clarisse Balland, Arsène Sanon, Brice Louvel, Norbert Ondo, Malgorzata Grybos, Antoine Joubert, Isabelle Paskiewicz, Isabelle Auvray, Remy Albrecht, Veronica Landeros, Fabien Laurent, Cecile Caupert, Djihad Ouldkadi etc.. Ils ont contribué au bon déroulement de ce travail. Je tiens à remercier les uns et les autres pour leur aide et leur bonne humeur quotidienne. Je tiens à remercier Christian Mustin, Dominique Goepfer, Chantal Ginsburger, Laetitia Despouy etc. pour leur aide dans mon travail et ma vie à Nancy. De bonnes relations et une ambiance chaleureuse dans le bureau, c'est aussi ce qui m'a permis d'effectuer ma thèse dans les meilleures conditions.

Enfin, je ne peux terminer sans remercier de tout coeur mes amis et ma famille, en particulier mes parents, pour leur soutien affectif et économique, dans les bons comme les mauvais moments, qui m'a permis de mener ce travail à son terme. Merci pour tout votre amour!

SOMMAIRE

ABBREVIATIONS

Abbreviation	Name
AM fungi	arbuscular mycorrhizal fungi
APS	amnonium persulfate
ASE	accelerated solvent extraction
BET	ethidium bromide
BH	Bushnel Haas
Bp	base pair
BSA	bovine serum albumin
BuOH	1-butanol
CTAB	cetyltrimethylammonium bromide
DBA	dibenzo(a,h)anthracene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EPA	environmental protection agency
HMW	high molecular weight
LMW	Low molecular weight
MPN	most probable number
PAH	Polycyclic aromatic hydrocarbon
PCA	principal component analysis
PCR	polymerase chain reaction

PHE	Phenanthrene
PLFA	phospholipid fatty acids
PVPP	Polyvinylpolypyrrolidone
PYR	Pyrene
RHDα gene	PAH-ring hydroxylating dioxygenase genes
RNA	ribonucleic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
TEMED	N, N, N', N' - tetramethyl ethylenediamine
TTGE	temporal temperature gradient electrophoresis
WHC	water holding capacity

LISTE DES TABLEAUX

Synthèse bibiographique

Matériels et méthodes

Résultats et discussion

Table 13. AM fungi colonization, shoot phosphorus concentration in planted treatments. Mycorrizal colonization is expressed as frequency of mycorrhizal roots (F%) and arbuscular abundance in the root systems (A%).

Table 14. PHE, PYE and DBA dissipation per g of root and sorption to roots with the different plant species. PAH dissipation (mg/g root) = PAH content (mg) in unplanted pots-PAH content (mg) in planted pots) / g of roots per pot.

Table 15. Number of 16S, 18S rDNA and PAH- RHDα bacterial gene copies in AM inoculated, non inoculated planted and unplanted (Control) pots.

Chapitre 3:

Table 16. Numbers of cultivable PAH degraders (cfu.g^{-1} soil) in soil samples in PHH (mycorrhizal planted, high water, high phosphorus) treatment and PAH concentration in microplates after two weeks.

Table 17. Numbers of cultivable PAH degraders (cfu g^{-1} soil) in the soil samples of unplanted and mycorrhizal planted pots (Mean ± S.D., n=3) estimated in microplates with PHE, DBA and PHE+DBA as carbon sources.

Table 18. Phenanthrene (PHE) and dibenzo-a,h-anthracene (DBA) concentration (mg kg^{-1}) after two and four weeks of incubation in microplates with soil inoculum from PHH, PHL and CHL treatments

Chapitre 4-1:

Table 19. Plant biomass and AM fungi colonization in different treatment. Mycorrizal colonization expressed as frequency of mycorrhizal roots (F%) and arbuscular abundance in the root systems (A%).

Table 20. Microorganism numbers in rhizosphere soil after 6 weeks.

Chapitre 4-2:

Table 21. Plant biomass and AM fungi colonization of each pot. Mycorrizal colonization expressed as frequency of mycorrhizal roots (F%) and arbuscular abundance in the root systems (A%).

Table 22. Remaining PAH concentration and PAH-RHDα gene copies in the soil of different treatments.

LISTE DES FIGURES

Synthèse bibiographique

Matériels et méthodes

Résultats et discussion

Chapitre 1:

Figure 14. PAH degrading gram positive and gram negative PAH-RHDα gene copy number g^{-1} and percentage relative to the total bacteria (16S rDNA gene copy number g^{-1}) in planted (P) and unplanted (C) pots.

Chapitre 2:

Figure 15. Shoot and root biomass in AM inoculated (+M) and uninoculated (-M) planted pots.

Figure 16. Remaining concentration of PHE, PYR and DBA in spiked soil after 6 weeks' treatment. +AM: mycorrhizal; -AM: non-mycorrhizal.

Chapitre 3:

Figure 17. TTGE gels of DNA from samples incubated for four weeks in microplates with a) DBA and PHE, b) DBA and PHE+DBA as carbons substrates.

Figure 18. Principal component analysis (PCA) of bacterial community structures based on TTGE data from samples incubated for four weeks in microplates with different PAHs . ①PHH soil, PHE substrate; ② PHL soil, PHE substrate; ③CHL soil, PHE substrate; ④PHH soil, DBA substrate ⑤ PHL soil, DBA substrate;⑥ PHH soil, DBA substrate; ⑦PHH soil, PHE+DBA substrate; ⑧PHL soil, PHE + DBA substrate; ⑨ CHL soil, PHE+DBA substrate.

Chapitre 4-1:

Figure 19. Phenanthrene (PHE) and dibenzo[a,h]anthracene (DBA) concentration after 6 weeks in each treatment.

Figure 20. Dissipation of dibenzo[a,h]anthracene (DBA) per gram of root in DBA spiked soil.

Introduction générale

Introduction générale

Les hydrocarbures aromatiques polycycliques (HAP) sont une famille de composés chimiques constitués d'atomes de carbone et d'hydrogène dont la structure des molécules comprend au moins deux cycles aromatiques condensés. La Communauté Européenne et les Etats-Unis (via l'Environmental Protection Agency, EPA) ont classé comme polluants prioritaires 16 HAP formés de 2 à 6 cycles (Keith et Teilliard, 1979), même si plus de cent HAP différents sont aujourd'hui répertoriés. Les HAP sont des composés hydrophobes dont le coefficient de partage octanol/eau, logKow, est relativement élevé, variant de 3.37 à 6.5 (Mackay et al., 1992), et leur solubilité en phase aqueuse est modérée à très faible et diminue lorsque le poids moléculaire des molécules augmente : de 30 mg L^{-1} pour les HAP légers à 2, 6×10^{-4} mg L^{-1} pour les lourds.

Les HAP ont pour origine la combustion incomplète de la matière organique, la diagenèse et la biogenèse. Les activités anthropiques, par exemple la combustion de combustibles fossiles (Wild et Jones, 1995), sont la principale source de HAP dans l'environnement. En raison de leur persistance et du transport à longue distance, les sols et les sédiments sont des sites d'accumulation des HAP. Dans les sols, les HAP d'origine anthropique proviennent des dépôts atmosphériques, du ruissellement, des émissions domestiques et industrielles et du déversement direct de pétrole ou de produits pétroliers. Les HAP ont été détectés dans le monde entier dans les sols et les sédiments depuis les années 1850), avec un pic dans les années 1950/1960s (Juhasz et al., 2000).

Les HAP ont un fort pouvoir cancérigène, mutagène et ont, par conséquent, des effets nocifs pour la santé humaine. La toxicité des HAP est liée à leur masse molaire donc au nombre de cycles du composé considéré : la plupart des HAP de haut poids moléculaire sont connus pour

avoir des effets mutagènes voire cancérigènes, et les HAP de faible poids moléculaire présentent un risque toxique aigu (Luch, 2005). L'exposition humaine aux HAP peut se faire via l'alimentation, par voie pulmonaire ou par voie cutanée (Nielsen et al., 1996).

Les techniques de restauration des sols sont nombreuses et reposent sur des processus physico-chimiques ou biologiques. Les techniques physico-chimiques, par exemple la désorption thermique, sont plus rapides, cependant les techniques biologiques présentent des avantages en termes d'écologie et d'économie. La bioremédiation, qui est liée à la biodégradation microbienne, est largement dépendante des conditions affectant l'activité bactérienne, à savoir la toxicité des polluants, les conditions environnementales, comme la structure du sol (Erickson et al., 2000) ou la biodisponibilité des éléments nutritifs et des polluants. De nombreuses méthodes ont été utilisées pour améliorer l'efficacité de biodégradation.

La présence de plantes dans le sol peut améliorer l'efficacité de la biodégradation des HAP (Reilley et al. 1996). Des effets positifs de la phytoremédiation ont été obtenus dans des expériences en pots et sur le terrain (Liste et Alexander, 2000). Corgié et al (2004) ont montré que la dégradation du phénanthrène augmentait lorsque la distance aux racines diminuait. Cet effet positif des plantes sur la dissipation (diminution des concentrations extractibles) des HAP n'est pas lié à une absorption par la racine (Binet et al., 2000). La première étape de dégradation bactérienne des HAP est une dioxygénation de la molécule, i.e. l'incorporation de deux atomes d'oxygène sur l'un des cycles, ainsi, l'augmentation du transfert d'oxygène par les plantes augmenterait la dégradation des HAP (Juhasz et al., 1997). La présence et l'activité des racines provoquent des changements qualitatifs et quantitatifs du sol qui entoure les racines et de la communauté de micro-organismes, notamment par les exsudats racinaires. Les racines modifient les propriétés physico-chimiques du sol tant au niveau de sa microporosité

que de sa macroporosité (modification de pH, de potentiel redox, de température, d'aération, d'humidité, de salinité) qui conduisent à des modifications des propriétés biologiques et microbiologiques.

La fertilisation est un paramètre important pour augmenter la densité et l'activité de micro-organismes, la croissance des plantes, et donc la dissipation des HAP dans les sols (Li et al., 1997). Joner et al. (2002) ont montré que la fertilisation en phosphore et en azote pouvait augmenter la dégradation des HAP à trois et quatre cycles. Liebig et Cutright (1999) ont également étudié l'effet de macro-et micronutriments sur la dégradation du pyrène et ont montré que l'apport de nutriments améliorait la dégradation des HAP.

La colonisation des racines des plantes par des champignons mycorhiziens à arbuscules (MA) peut aussi augmenter l'accès des plantes aux éléments nutritifs dans la rhizosphère, et augmenter la dissipation des HAP dans la rhizosphère (Joner et Leyval, 2001, 2003a). Les symbioses plante-champignons MA sont très répandues puisque l'on estime que plus de 95% des espèces végétales peuvent former des mycorhizes. Les champignons MA améliorent la croissance des plantes ce qui se traduit d'une manière générale par une augmentation de la biomasse végétale produite. Chang et Corapcioglu (1998) ont montré que le rayon et la longueur de la racine influencent la dégradation du pétrole dans un sol contaminé. Les plantes mycorhizées absorbent plus de phosphore que les plantes non mycorhizées (Sieverding, 1991) avec un taux d'absorption du phosphore par unité de longueur de racine colonisée 2 à 3 fois plus élevé que dans les racines non colonisées et absorbent aussi plus d'azote (Aziza-Chulan et Martin, 1992). De plus, les études de Joner et al. (2001) et Wu et al. (2008a) ont montré que la structure des communautés microbiennes était modifiée en présence de mycorhizes, et que cette microflore associée aux mycorhizes pouvait contribuer à la réduction des concentrations de HAP. Bien qu'aucune preuve n'ait démontré que les champignons MA pouvaient dégrader

les HAP, l'expérience réalisée avec des cultures monoxéniques de Verdin et al. (2006) suggère une contribution positive de l'association symbiotique sur la dissipation de l'anthracène en l'absence d'autres micro-organismes. Corgié et al. (2006c) ont montré que l'expression de l'activité dioxygénase était plus grande dans la mycorhizosphère en présence de *G. mosseae*. Ainsi l'utilisation des plantes en symbiose avec un champignon mycorhizien dans des sols contaminés peut contribuer à réduire la concentration de HAP dans les sols contaminés.

Malgré les nombreuses études sur le sujet, plusieurs questions se posent encore sur le comportement des plantes et des champignons MA dans les sols pollués par les HAP. L' influence des plantes et des champignons MA sur les HAP est complexe, et est-ce que les différentes espèces végétales et de champignons MA ont le même effet vis à vis de la dissipation des HAP dans les sols pollués? L'effet positif du champignon est-il dépendant de l'augmentation de la biomasse végétale dans les sols contaminés par les HAP? Les sites pollués contiennent le plus souvent un mélange de différents types de HAP. Ces effets plante-champignons MA sont-ils dépendants du type de HAP considéré et de l'interaction entre les HAP de différentes tailles?

Pour tenter de répondre à ces différentes questions, le présent travail se subdivise en quatre parties :

(i) La première partie est une synthèse des différents travaux de la littérature scientifique où sont exposées les données relatives aux HAP, à leur devenir dans l'environnement et dans la rhizosphère.

(ii) La deuxième partie décrit le matériel et les méthodes utilisés au cours de ces travaux pour répondre aux questions posées.

(iii) La troisième partie expose les résultats obtenus. Elle comporte quatre chapitres, et est présentée sous forme d'articles en anglais : (a) La première expérience avait pour objectif de tester le facteur fertilisation et en particulier l'effet de la concentration en phosphore et de la teneur en eau sur la dissipation de 3 HAP dans la rhizosphère. Une culture en pot d'un mélange de luzerne (*Medicago sativa cv. Europe*) et de fétuque (*Festuca arundinacea cv. Bariane*) mycorhizées a été réalisée avec un témoin non planté sur un sol artificiellement contaminé avec trois HAP (le phénanthrène, le pyrène et le dibenzo(a,h)anthracène) avec deux niveaux de phosphore et de régime d'arrosage différents. (b) La deuxième expérience est une culture en pots de quatre plantes différentes (luzerne (*Medicago sativa cv. Europe*), fétuque (*Festuca arundinacea cv. Bariane*), ray-grass (*Lolium multiflorum cv. Barclay*) et céleri (*Apium graveolen*)) mycorhizées (*Glomus intraradices*) ou non sur le même sol contaminé en HAP pour comparer l'effet de ces plantes et évaluer l'effet de ce champignon MA sur la dissipation des HAP. (c) Des expériences en micro-plaques de culture ont été menées pour évaluer la capacité des microorganismes cultivables de la rhizosphère des plantes de la première expérience à dégrader des HAP de faible poids et de haut poids moléculaire seuls ou en interaction. (d) La quatrième expérience comprend deux sous-expériences : avec les mêmes plantes - hôtes que dans la première expérience, la première sous-expérience a comparé les effets de deux isolats de champignons MA (*Glomus intraradices* et *Glomus mosseae*) sur la dissipation du phénanthrène et du dibenzo-a,h-anthracène et les communautés microbiennes dans la rhizosphère. La deuxième sous-expérience a comparé les effets des deux plantes cultivées séparément ou en co-culture (luzerne (*Medicago sativa cv. Europe*) et fétuque (*Festuca arundinacea cv. Bariane*)) mycorhizées par *G.intraradices* sur la

dissipation du phénanthrène et du dibenzo-a,h-anthracène.

(iv) Enfin, la quatrième partie constitue une discussion générale synthétique qui met en relation les principaux résultats obtenus dans les différentes expériences et débouche sur une conclusion générale et les perspectives de cette étude. Le document se termine sur les références bibliographiques et quelques annexes.

Partie 1

Synthèse bibliographique

Bibliographic review

Partie 1: Synthèse bibliographique - Bibliographic review

Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant organic pollutants consisting of two or more fused benzene rings in linear, angular, or cluster arrangements (Kanaly and Harayama, 2000). PAHs are among the most problematic substances because they are widely distributed, and have accumulated in the environment, causing adverse effects to human health and the environment by their acute toxicity, mutagenicity or carcinogenity (Rezek et al., 2008).

1.1. Distribution and transportation of PAHs on earth

Both nature and human activity contribute to the generation of PAHs in the environment. PAHs may be indigenous as a result of forest fires, or rapid aromatization of biological compounds during humification processes. They can also reflect contributions from ancient hydrocarbon sources, such as coal residues, provided that the soil has been initially formed by weathering of a petroleum source-rock or coal deposite. Anthropogenic PAHs mainly generate from the inadvertently incomplete combustion of organic matter, for instance home heating, traffic, and waste incineration (Finlayson-Pitts and Pitts, 1997; Fiala et al., 1999).

Owing to the persistence and long-range transport properties of PAHs, these substances are detected worldwide in soils and sediments, even in the most pristine areas such as the Poles, and consequently threaten to the environmental security of the whole globe.

1.1.1 Atmosphere

PAHs are one of the major groups of organic atmospheric constituents. They are semi-volatile substances at atmospheric conditions and frequently occur both in the vapor phase and as attached to particles depending on the vapor pressure of each PAH (Wingfors et al. 2001).

PAHs could transport in the air, and deposit onto soil, water and vegetation. The atmospheric residence time and transport distance depend on the size of the particles to which the PAHs are absorbed and the meteorological conditions, such as temperature, wind speed and humidity (Chetwittayachan et al., 2002).

1.1.2 Water

Low molecular weight (LMW) PAHs, containing two or three fused rings are more water soluble and volatile, and are found predominantly in vapor phase. High molecular weight (HMW) PAHs containing more than four fused rings, which are relativity more hydrophobic, are found mainly adsorbed on particulate material. PAHs have been detected in most lakes and marine in the world, where concentration peaks were observed during the 1960-1980s (Basheer, et al., 2003; Fernández, et al., 2000; Law, et al., 1997).

The transport of PAHs from water to the atmosphere by volatilization depends on Henry's law constants of these compounds (Mackay and Bentzen, 1997). The volatilization of LMW PAHs is much higher than HMW PAHs. Because of their low aqueous solubility and high affinity for organic carbon, PAHs in aquatic systems tend to adsorb to the sediments and surface soils.

1.1.3 Soil

Anthropogenic PAHs enter the soil environment through a variety of routes including atmospheric deposition, river runoff, domestic and industrial outfalls and the direct spillage of petroleum or petroleum products. PAHs have been detected in the soil of both industrial or non industrial countries and regions, and reported background contents of PAHs in soils of North America and Europe ranged from 50 to 500 μg kg^{-1} (Maxin and Kögelknabner, 1995). PAH concentrations in soils from industrial countries have revealed an increasing PAH

burden since the mid 1800 (Juhasz et al., 2000). For example, a study by Jones et al. (1989) on PAH concentrations in soil collected from Rothamsted, UK, reported an increase in BaP concentration of over 20-fold from 1893 to 1987. PAH concentration in soil depends on the distance of the site to the polluting industry, it also relates to the soil properties, climatic conditions, and frequently, to the other pollutants in soil, e.g. the concentration of PAHs in New Orleans displayed linear relationship with the total metal concentration (Mielke. et al., 2001).

1.2 Structure and property of PAHs

More than 100 different PAHs are ubiquitously distributed in the environment, among which 16 PAHs have been identified as "priority pollutants" by the US Environmental Protection Agency (US EPA 2002). The stability and aromaticity of PAHs are structural dependent. Their hydrophobicity generally increases with the number of rings and the degree of ring condensation. The hydrophobicity could promote their accumulation in the solid phases of the terrestrial environment (Johnsena et al., 2005). Besides, ring linkage patterns in PAHs may occur such that the tertiary carbon atoms are centers of two or three interlinked rings, as in the linear kata-annelated PAH anthracene or the pericondensed PAH pyrene (Kanaly and Harayma, 2000). The different linkage patterns between PAHs confer different electron density, symmetry and the active region, and finally result in different biological stabilities: the biochemical persistence increases with cloud density of p-electrons on both sides of the ring structures, making them resistant to nucleophilic attack (Chang et al., 2007) (Table 1).

Table 1. Structures and physicochemical properties of 16 priority PAHs

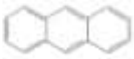
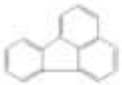
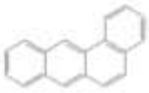
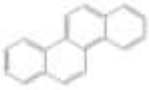
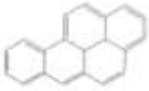
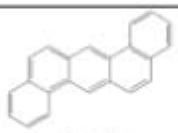
Name	Structure	Number of rings	Molecular weight (g/mol)	Water solubility (g/m^3)	Log Kow
Naphthalene		2	128.2	31	3.37
Acenaphthylene		3	125.2	16.1	4
Acenaphthlene		3	154.2	3.8	3.92
Fluorene		3	166.2	1.9	4.18
Phenanthrene		3	178.2	1.6	4.46
Anthracene		3	178.2	0.045	4.54
Fluoranthene		4	202.3	0.26	5.22
Pyrene		4	202.3	0.132	5.18
Benzo[a]anthracene		4	228.3	0.011	5.91
Chrysene		4	228.3	0.006	5.61
Benzo[b]fluoranthene		5	252.3	0.0015	5.8
Benzo[k]fluoranthene		5	252.3	0.0008	6
Benzo[a]pyrene		5	252.3	0.0038	6.04

Table 1. Structures and physicochemical properties of 16 priority PAHs

Name	Structure	Number of rings	Molecular weight (g/mol)	Water solubility (g/m^3)	Log Kow
Dibenz[a,h]anthracene		5	278.4	0.012	5.97
Benzo[g,h,i]perylene		6	268.4	0.00026	6.5
Indeno[1, 2, 3-c,d]pyrene		6	276.3		

1.3 Toxicity of PAH

PAHs have detrimental effects on the flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains (bioaccumulation and biomagnification) and in some instances, serious health problems of humans (Luch, 2005). The LMW PAHs have a significant acute toxicity (Sims and Overcash, 1983), whereas some of the higher molecular weight PAHs are carcinogenic (Luch, 2005). Isomers (PAHs with the same formula and number of rings) with different structures could vary from being nontoxic to being extremely toxic. Seven PAH compounds have been classified by EPA as probable human carcinogens: benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-c,d]pyrene (US EPA 2002).

PAHs seldom exist singly in the terrestrial ecosystem, and the mixture of PAHs could result in enhanced genotoxicity and carcinogenicity. For example, 1-nitropyrene, a nitrated PAH, is produced during reactions between ketones in products of burning automobile fuel and airborne nitrogen oxides that take place on the surface of hydrocarbon particles in diesel exhaust. In the Ames assay (Salmonella typhimurium), 1-nitropyrene was found to be highly

mutagenic and carcinogenic in presence of pyrene, while this compound is noncarcinogenic and only weakly mutagenic (Pothuluri and Cerniglia, 1994).

Fortunately, the toxicity of PAHs in contaminated sites can be mitigated naturally with time going by bioremediation and also by sequestration in soil organic matter. Sequestration involves slow partitioning of hydrophobic compounds into organic matter or slow diffusion into micropores, and finally decreases PAHs bioavailability (Kelsey et al., 1997).

1.4 PAH extraction and assessment

Classical methods for the determination of PAHs in soil samples are usually based on exhaustive extraction from the matrix (Ramos et al., 2002). However, these exhaustive extraction methods are supposed to overestimate the PAH toxicity in soil (Alexander, 2000; Liste and Alexander, 2002), since toxicity and microbial mineralization of PAHs are shown to decrease as PAH residence time increases owing to the interaction, e.g. partition, adsorption, covalent binding between the PAHs and soil organic matter (Pignatello and Xing, 1996; Weber and Huang, 1996). Thus, methods extracting the bioavailable PAHs rather than the total concentration are required to assess the actual risk of mutagenic and carcinogenic effects.

Traditional methods to evaluate the bioavailability of organic compounds with sensitive organisms such as earthworms and microorganisms are time consuming (Kelsey et al., 1997). Thus a number of newly designed chemical, physical methods, including solid phase extraction (Cuypers et al., 2002), supercritical fluid extraction (Hawthorne and Grabanski, 2000), surfactant extraction (Volkering et al., 1998), persulfate oxidation (Cuypers et al., 2000), solvent extraction (Kelsey et al., 1997; Liste and Alexander, 2002) and cyclodextrin extraction (Reid et al., 2000; Cuypers et al., 2002) were developed and proved efficient for

assessing the bioavailability of organic compounds in soils. One of the potential mild extraction methods for bioavailable PAH extraction was butanol extraction. This chemical substrate was firstly proposed by Kelsey et al. (1997) who found a good correlation between bioavailability and butanol extractability of the PAHs in planted and unplanted soil. Liste and Alexander (2002) confirmed that the amount of chrysene extracted by n-butanol from an aged contaminated soil was not statistically different from the quantities assimilated by earthworms (*Eisenia fetida*) introduced into the soil.

Most of the methods used to quantify PAHs in soil analyse the disappearance of total or bioavailable extractable PAHs. However, the decrease of the extractable PAHs does not mean that they are completely degraded, since they could be only transformed or made unavailable (volatilized, sorbed…). That is why the term dissipation is often used (Reilley et al., 1996; Binet et al., 2000) to express the decrease of the extractable PAH concentration.

1.5 PAH polluted soil remediation methods

1.5.1 Physical and chemical methods

Treatment of PAH contaminated soils has been conducted by means of different techniques including physical and chemical processes. The physical methods intend to remove the PAHs from soil by an individual solvent or mixtures of solvents. Various solvents, including water and organic solvents, surfactants, cyclodextrins, vegetable oil have been used to extract PAHs (Gan et al., 2009). Solvent extraction method could rapidly and efficiently remove PAHs from contaminated soil, however, it is only a separation process which transfers PAHs into the extract phase hence secondary treatment of the extracts is necessary. Besides, this two-step

PAH remediation method could hardly be widely applied owing to its high financial requirement.

Chemical treatments could mineralize PAHs by one-step oxidation. Different types of oxidants have been investigated ranging from the classic thermal technologies to new ones such as Fenton's reagent and ozone oxidation (Flotron et al., 2005; Renoldi et al., 2003). Oxidation is considered to be a valuable option due to some advantages such as low residence time required for remediation of soils, possibility of modifying the operating conditions to reach unbioavailable contaminant molecules, possibility of using small mobile units, insensitivity to external disturbances and/or possibility of combination with an extraction stage to conduct the PAH degradation in the extracting fluid. The disadvantage of this treatment is the severe disturbance of the soil ecosystem. In addition, several oxidation methods such as thermal ones require a lot of energy consumption.

Engineering-based physical and chemical techniques are proved to be efficient but very expensive and the environment is disturbed. In consideration of the economic and environment adaptability, bioremediation is gaining wider approval as a feasible alternative treatment technology for the PAH contaminated soil remediation.

1.5.2 Bioremediation

Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition (http://en.wikipedia.org/wiki/Bioremediation). It has advantages over thermal and some physico-chemical techniques in terms of relative low cost and disturbance. Since the 1970s, researches on the biological degradation of PAHs have demonstrated that bacteria, fungi and algae possess catabolic abilities that may be utilized for the remediation of PAH-

contaminated soil and water (Wilson and Jones, 1993). The possible fate of PAHs in the environment includes volatilization, photo-oxidation, stabilization, chemical oxidation, bioaccumulation and adsorption to soil particles. The principal process for the removal of PAHs from the soil is microbial transformation and degradation (Gibson et al., 1975; Garcia-Blanco et al. 2007). Phytoremediation is a form of bioremediation, which uses plants and associated microorganisms to remove pollutants from the environment or render them harmless. This technique have been investigated for PAH contaminated soils since the 1990s (Aprill and Sims, 1990; Reilley et al., 1996). It preserves the natural structure and texture of the soil; energy is derived primarily from sunlight; high levels of microbial biomass in the soil can be achieved; it is low in cost and has the potential to be rapid (Huang et al. 2004).

Biodegradation using microorganisms, the stimulation of biodegradation by biostimulation or bioaugmentation, and by using plants (phytoremediation) will be developed in the following chapters.

1.6 Biodegradation of PAHs

Biodegradation of PAHs is mainly an aerobic process. The first step in aerobic PAH catabolism mainly involves the action of dioxygenase / monoxygenase (bacteria / fungi), which incorporates atoms of molecular oxygen into the aromatic nucleus, resulting in the oxidation of the aromatic ring. Depending on the substituents on the original molecule, the two hydroxyl groups may be positioned either in ortho- (as in catechol and protocatechuate) or in para- position to each other (as in gentisate and homogentisate). The cis-dihydrodiols formed are further oxidized, first to the aromatic dihydroxy compounds (catechols), and then through the ortho- or meta- cleavage pathways. Further reactions lead to the precursors of

tricarboxylic acid cycle (TCA) intermediates (Johnsen et al., 2005). The factors influencing the PAH biodegradation includes the molecular weight of PAHs, the aging, and the presence or expression of PAH degrading genes existing in the system.

1.6.1 Parameters influencing the PAH biodegradation in soil

1.6.1.1 PAH molecular weight

LMW PAHs biodegradation

The biodegradation pathways of LMW PAHs, which could act as sole carbon source for microorganisms, have been described based on pure bacterial cultures. The pathways and biodegradation rates depend upon the species, strains, and even the culture conditions (Stingley et al., 2004).

The catabolism of phenanthrene by bacteria has been extensively studied, and, in most cases so far, phenanthrene is metabolized by initial dioxygenation at the 3,4-position (Mallick and Dutta, 2008). After dioxygenation at the 3,4-position, phenanthrene is metabolized to 1-hydroxy-2-naphthoic acid. In one pathway, 1-hydroxy-2-naphthoic acid is oxidized to 1,2-dihydroxynaphthalene, which is further metabolized via salicylic acid (fig.1, Samanta et al., 1999, Tao et al., 2007). It has been demonstrated that a common set of enzymes is responsible for the conversion of phenanthrene to 1-hydroxy-2-naphthoic acid as well as that of naphthalene to salicylic acid (Tao et al., 2007). In another pathway, 1-hydroxy-2-naphthoic acid undergoes ring-cleavage and is further metabolized via o-phthalic acid and protocatechuic acid (Stingley et al., 2004; fig. 2).

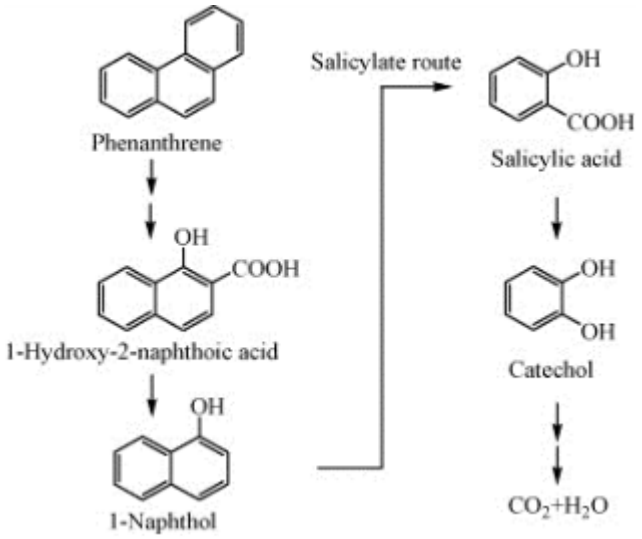


Fig. 1. Metabolic pathway for the degradation of phenanthrene by *Sphingomonas* sp. GY2B. (salicylic acid route). (Tao et al., 2007)

In addition to bacteria, algae and fungi also have the ability to degrade LMW PAHs. Prokaryotic and eukaryotic photoautotrophic marine algae (i.e. cyanobacteria, green algae, and diatoms) are known to metabolize naphthalene to a series of metabolites (Narro et al., 1992; Warshawsky et al., 1995). Hong et al. (2008) observed 16% phenanthrene degradation (initial concentration was 1000 mg L^{-1}) after 168 h incubation by *Skeletonema costatum*, which was much higher in comparison to the control (3.7%).

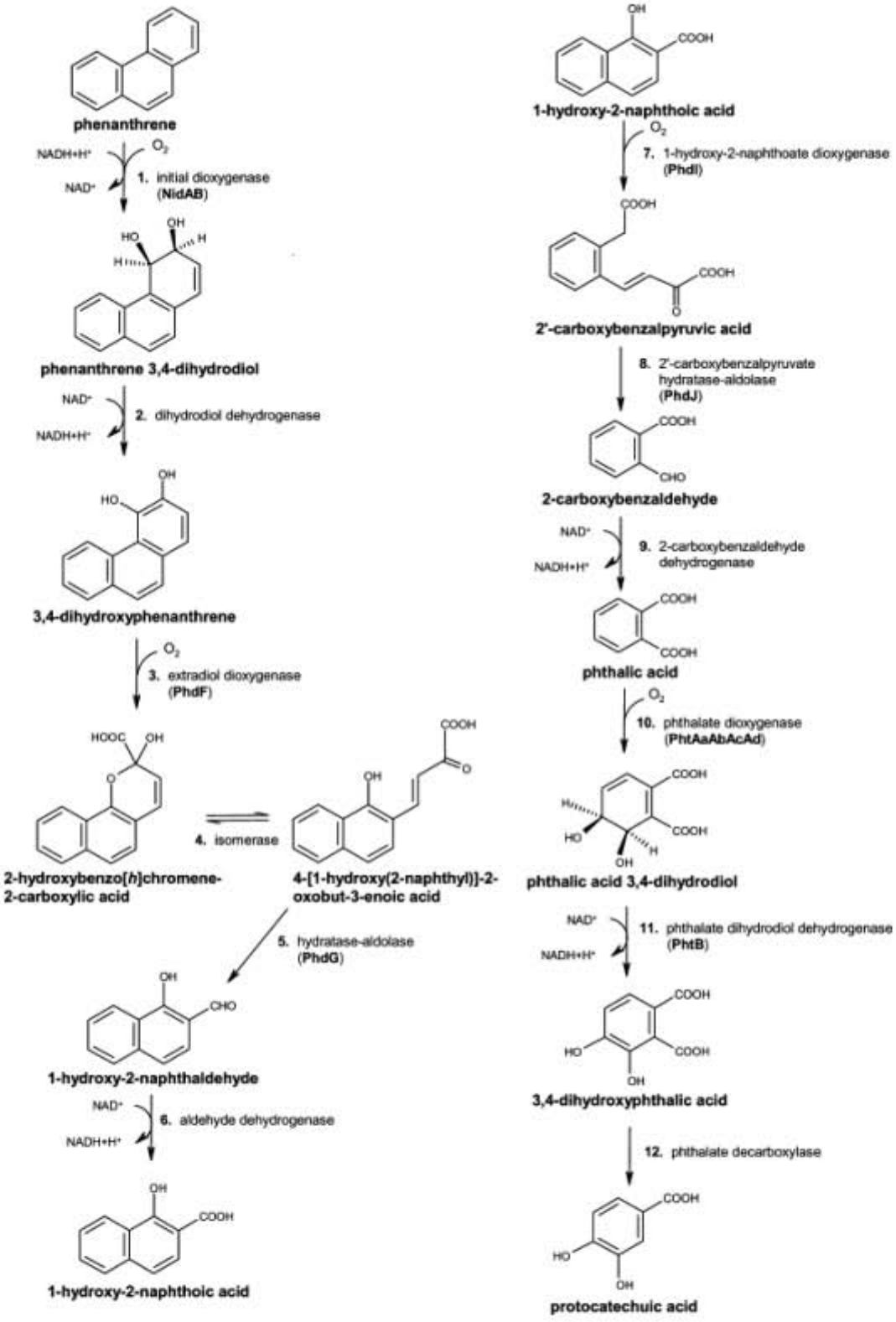


Fig.2 Proposed phenanthrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1. (o-phthalic acid and protocatechuic acid route).

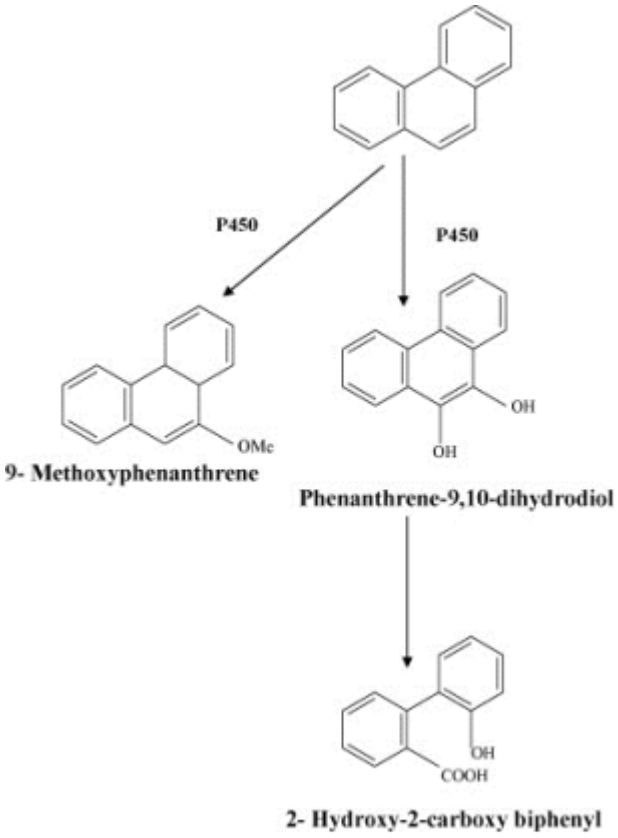


Fig. 3. Fungal degradation pathway of Phenanthrene using *Irpex lacteus*. (Cajthaml et al., 2002)

Several fungi are very efficient for PAH degradation, for instance, more than 90% of naphthalene removal had been obtained with the fungus *Phanerochaete chrysosporium* in agitated vessels within 24 h, in which only 20% of that was adsorbed on the fungal mycelia (Liao and Tseng, 1996). Although ^{14}C-labeled compounds were used and proved that ligninolytic fungi are able to mineralize PAHs completely to carbon dioxide, the intermediates arisen from ring-cleavage reactions by fungi deserve further investigation (Haritash and Kaushik, 2009). Some researches suggest that fungi degrade PAHs by producing extracellular enzymes such as lignin peroxidase, manganese dependent peroxidase, phenoloxidases (laccases, tyrosinases), and H_2O_2-producing enzymes (Haritash and Kaushik, 2009; Hofrichter et al., 1998). Fungal monooxygenase of cytochrom P-450 may be also involved in degradation of PAHs transforming them to hydroxyderivates or diols (Bezalel et al., 1997).

One of the possible pathways of phenanthrene degradation by fungi was proposed by Cajthaml et al. (2002), who found that the major degradation product by the lignolytic fungus *Irpex lacteus* was phenanthrene-9,10-dihydrodiol (Fig. 3).

Moreover, other kinds of fungi, including AM fungi also displayed positive effect on PAH dissipation (Joner et al., 2001). However, ectomycorrhizas displayed no or even negative effect on PAHs dissipation (Genney et al., 2004; Joner et al.,2006). Besides, high levels of residual nutrients and enzymes, such as laccase and Mn-dependent peroxidase, are still left in dead fungi, e.g. spent mushroom compost (SMC), and could reduce the toxicity of PAHs, increase the microorganisms in soil and finally enhance the rate of PAH degradation (Lau et al., 2003).

HMW PAH degradation and co-metabolism

HMW PAHs have more hydrophobic property (Kow > 5), and exhibit relativity lower rate of biodegradation than LMW ones. Bacteria, fungi and algae biodegradation efficiency are all limited for HMW PAHs (Hong et al. 2008; Juhasz et al., 1997; Liao and Tseng, 1996).

Li et al. (1996) detected pyrene biodegradation through cis-dihydrodiol pyrene, an initial ring oxidation product. Rehmann et al. (1998) isolated a *Mycobacterium sp*. (strain KR2) which was able to utilize pyrene as sole source of carbon and energy, from a PAHs contaminated soil of a gaswork plant. The isolate metabolized up to 60% of the pyrene added (0.5 mg ml^{-1}) within 8 days at 20 °C. Cis-4,5-pyrene dihydrodiol, 4,5-phenanthrene dicarboxylic acid, 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, phthalic acid, and protocatechuic acid were identified as degradation products and a degradation pathway for pyrene was also proposed (Fig. 4). Dean-Ross et al. (2002) isolated *Mycobacterium flavescens* which could mineralize pyrene with the initial reaction rates of 0.044 mg L^{-1}.

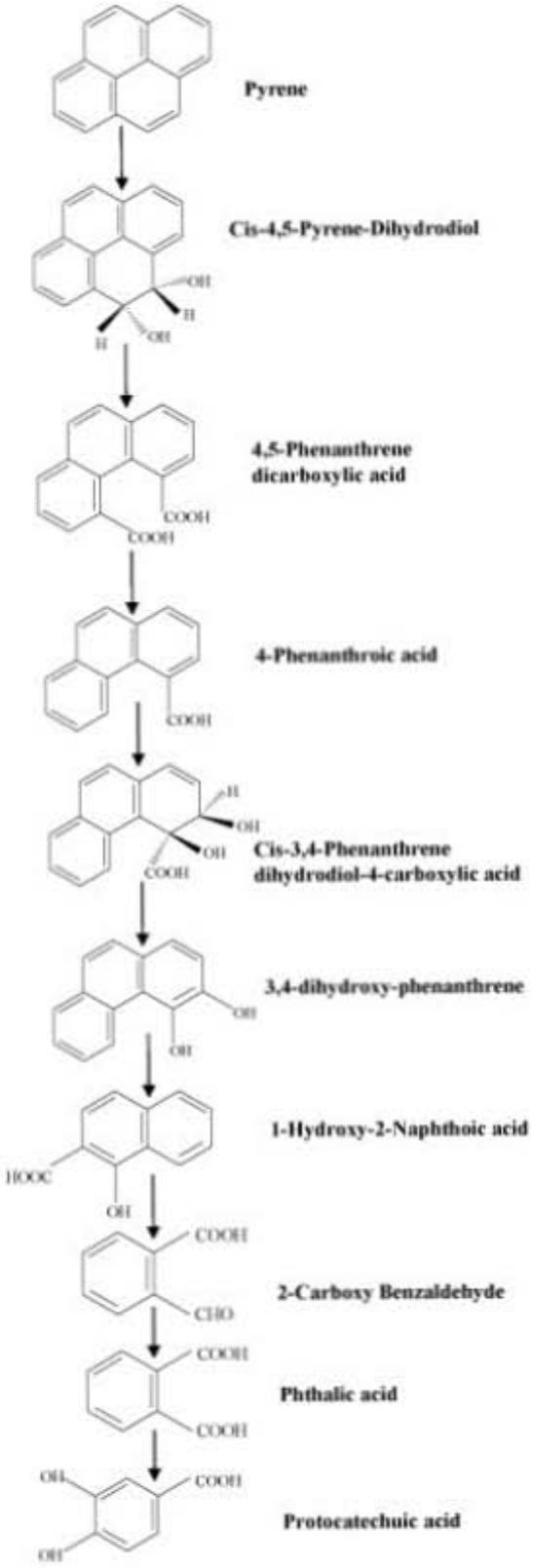


Fig.4 Proposed pyrene degradation pathway by *Mycobacterium* sp. Strain KR2.

The effect of fungi on biodegradation of HMW PAHs was significantly lower than LMW PAHs. For example, *Phanerochaete chrysosporium*, which significantly increased LMW PAHs biodegradation (i.e.increased the dissipation of acenaphthene, fluorene, phenanthrene, fluoranthene by up to 43%) in a soil-slurry, exhibited limited effect on degrading (oxidizing) HMW PAHs (i.e. chrysene, benzo[a]pyrene, dibenz[a,h]anthracene and benzo[g,h,i]perylene) (Zheng and Obbard, 2002). Cajthaml et al. (2006) showed that the ligninolytic fungus *Irpex lacteus* in a nutrient liquid medium degraded more than 70% of the initially applied benz[a]anthracene within 14 days. The product of the first step metabolization was identified as benz[a]anthracene-7,12-dione, and the possible degradation pathway was shown in Fig.5 .

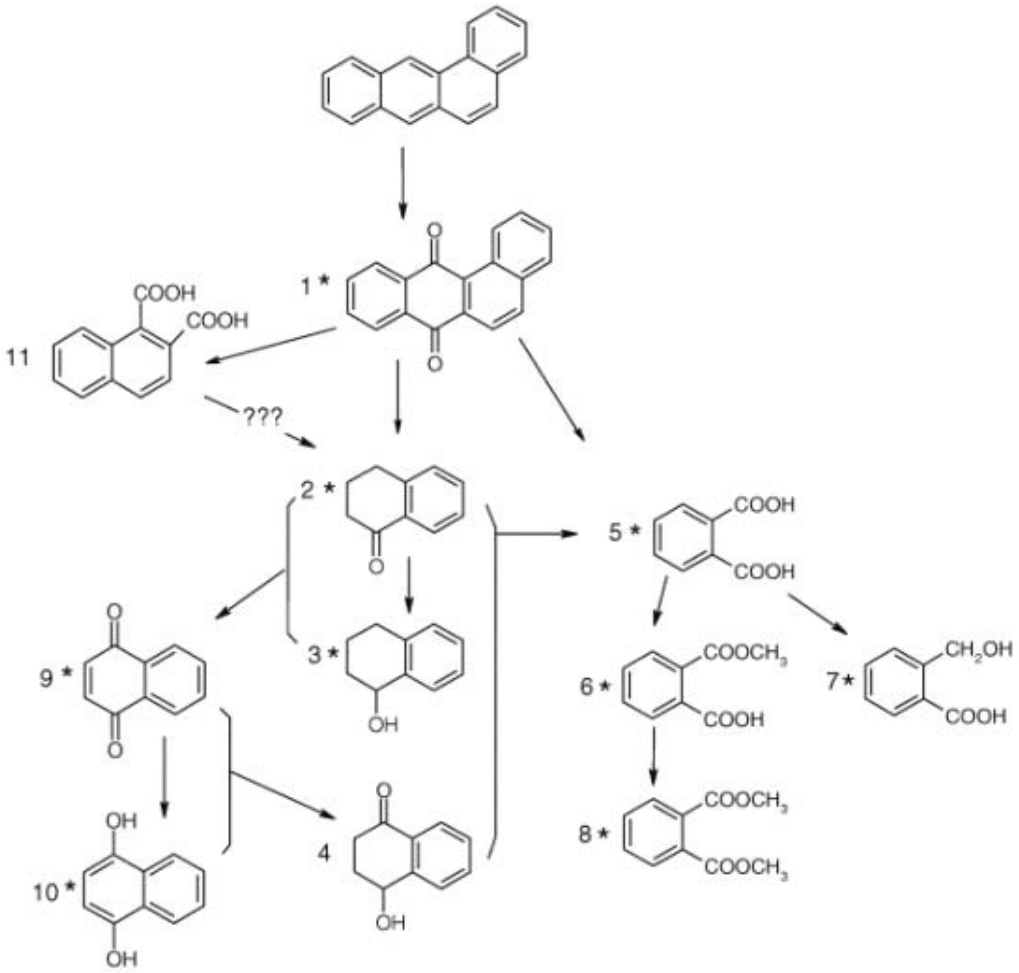


Fig 5 Proposed pathway of benzo[a]anthracene degradation by the ligninolytic fungus *I. Lacteus*.

The rate of PAH degradation by fungi is affected by many factors including the concentration of contaminant in soil, properties of PAHs and the enzyme type and quantity (Cutright, 1995; Hatakka, 1994; Liao and Tseng, 1996; Zheng and Obbard, 2002).

The bioremediation efficiency of algae also decreases with increased molecular weight of PAHs. Hong et al. (2008) compared the degradation of phenanthrene and fluoranthene by two species of algae enriched from a mangrove aquatic ecosystem *Skeletonema costatum* (Greville) Cleve and *Nitzschia sp.*, and found that the degradation of fluoranthene was much slower than that of phenanthrene, and that most of fluoranthene still existed in the cells after 168 h incubation.

Although numerous strains are able to use four ring PAHs as carbon resource (Boldrin et al., 1993; Müller et al., 1990), to date, no pure bacterial culture was shown to utilize any PAH compound containing more than five rings as the sole carbon and electron source (Cerniglia 1992). Co-metabolism, which is defined as the simultaneous metabolisms of two compounds, in which the degradation of the second compound (the secondary substrate) depends on the presence of the first compound (the primary substrate), is a way to expand the scope of PAH biodegradation with additional carbon and energy resource. In particular, initial ring attack with oxygenase enzymes is accomplished by bacteria that may or may not directly benefit from the reactions. Once hydroxylated, however, PAHs become increasingly soluble and can be attacked by enzymes from additional members of the bacterial community (Kanaly et al., 2000; Juhasz et al., 1997). Eggen and Majcherczyk (1998) applied *Pleurotus ostreatus* into aged creosote-contaminated soil, and 28% of benzo[a]pyrene was degraded by the white rot fungus during the first month. Both carbon source addition and plant cultivation could act as metabolite and benefit HMW PAH degradation (Joner et al., 2002; Rentz et al., 2005).

Co-existent LMW PAHs also could influence HMW PAH degradation (Juhasz et al., 1997).

The enzymes produced by bacteria for the degradation of PAH compounds can have a broad substrate range (Stringfellow and Aitken, 1995), thus the co-existence of LMW PAHs should benefit HMW PAH degradation. Bouchez et al. (1999) showed that the presence of phenanthrene allowed the degradation of fluorene, anthracene and fluoranthene up to 20%, 40% and 31% respectively by the *Pseudomonas sp. S Phe Na1* (this strain could not use fluorene, anthracene and fluoranthene as sole carbon resource). These results suggest that the presence of phenanthrene might enhance fluoranthene, anthracene and fluoranthene metabolism. Co-metabolic biodegradation of dibenz[a,h]anthracene and benzo[a]pyrene by *B. cepacia* was demonstrated when 100 mg L^{-1} of phenanthrene was added to cultures containing 50 mg L^{-1} of either compound (Juhasz et al., 1997). Chen and Aitken (1999) compared the benzo[a]pyrene biodegradation capability of *Pseudomonas saccharophilia* P15 pretreated with phenanthrene or not. After 48 h of incubation, the extent of ^{14}C benzo[a]pyrene mineralisation was 33 times higher in phenanthrene treated in comparison with uninduced *P. saccharophila* P15. The co-existence of PAHs not only benefits the HMW PAH dissipation, but also could increase the LMW PAH degradation. For example, Hong et al. (2008) showed that the degradation of PHE by *S. costatum*, reached 38% in a phenanthrene - fluoranthene mixture while it was only 16% with PHE as single substrate. The PAH degradation products, e.g. salicylic acid, could also act as co-metabolite or primary substrate and accelerate PAH (including LMW and HMW PAH) degradation (Chen and Aitken 1999). Thus, it appears that co-metabolism is an important feature of the degradation of PAHs, as it commonly occurs and widens the range of PAHs attacked by a defined strain. However, the mixture of PAHs do not always increase PAHs degradation. For example, some strain isolated from naphthalene substrate could not degrade other PAHs, and thus showed limited co-metabolism capacity for other PAHs (Bouchez et al., 1995). And as mentioned above (Bouchez et al. 1999), the addition of phenanthrene increased the anthracene and fluoranthene biodegradation by

Pseudomonas sp. S Phe Nal, however, the presence of fluorene and fluoranthene inhibited the phenanthrene degradation by 35% and 8% respectively. And in the same experiment, *Pseudomonas S Flu Aul* which possessed a high capability of fluorene degradation failed to degrade both fluorene and phenanthrene in presence of phenanthrene. The inhibition of co-existent PAHs probably resulted from the toxicity of the second PAH or the enzyme competition for degradation (Bouchez et al., 1995; Stringfellow and Aitken, 1995).

1.6.1.2 Aging of PAHs

In addition to the molecular weight of PAHs, the aging (or sequestration) of PAHs, which has been observed in soil, sediment, and aquifer materials (Alexander, 1995) is another factor that affects PAH biodegradation in soil. Behavior of aged compounds is different from that of freshly added chemicals (Loehr and Webster, 1996). The aging process begins since the moment PAHs are added. The mechanisms involved are poorly known, but involve the association of organic compounds with natural organic matter (Carroll et al., 1994) and the penetration of contaminants into small pores in soil (Wu and Gschwend, 1986).

1.6.1.3 Aromatic ring-hydroxylating dioxygenases (PAH-RHDα)

The density of PAH degraders (or PAH degrading genes) in the system is another important factor influencing the PAH degradation. In the soil environment, bacteria are generally considered as the predominant organic pollutant degrading element of microbial community (Song et al., 1986). Therefore, aromatic RHD, which is a family of bacterial enzymes that has an essential role in the recycling of carbon in nature (Jakoncic et al., 2007), is supposed to be the function gene related with PAH degradation. The initial step of the PAH metabolism commonly occurs via the incorporation of molecular oxygen into the aromatic nucleus by a multicomponent aromatic RHD enzyme system forming cis-dihydrodiol. Kauppi et al. (1998)

investigated the structure of dioxygenase, and showed that it is composed of large α and small β subunits. Alpha subunit (RHDα) contains two conserved regions: the [Fe2-S2] rieske centre and the mononuclear iron-containing catalytic domain. Most of the bacteria possess PAH-RHDα genes: for gram negative bacteria the PAH-RHDα genes (*nah*Ac, *nah*A3, *nag*Ac, *ndo*B, *ndo*C2, *pah*Ac, *pah*A3, *phn*Ac, *phn*A1, *bph*Ac, *bph*A1, *dnt*Ac and *arh*A1) form a tight monophyletic cluster; for gram positive bacteria, these genes were classified into four clusters including the *nar*A-like gene from *Rhodococcus* strains, the *nid*A*/pdo*A1-like genes from *Mycobacterium* strains, the *phd*A*/pdo*A2-like genes from *Nocardioides* and *Mycobacterium* strains and the *nid*A3/*fad*A1 genes from *Mycobacterium* and *Terr*abacter strains (Cébron et al., 2008). The detection of PAH-RHDα genes lead to the development of culture-independent approaches to quantify PAH degrading bacteria in soils. Cébron et al. (2008) showed that the PAH-RHDα gene copy number was related to the PAH concentration in soil, and may reflect the PAH degradation potential of the bacteria community.

1.6.1.4 Environmental parameters affecting PAH biodegradation

The structure stability and the hydrophobic property could limit the availability of PAHs for microorganisms. Besides, the environmental parameters could greatly influence the PAH biodegradation. The lack of essential nutrients (such as nitrogen, phosphorus, potassium), growth substrates, oxygen diffusion, and unsuitable soil temperature and pH value could inhibit PAH biodegradation (Hutchinson et al., 2001; Joner et al., 2002). All of these abiological factors indirectly influence PAH degradation by affecting the microorganism community, quantity and activity in the soil.

Nutrient availability is quite limited in PAH polluted sites because of the hydrophobic nature of the PAHs (Li et al, 1997). The lack of nutrients, especially macro-nutrients could reduce the multiplication and activity of the microorganisms, and finally decrease PAH microbial

degradation. The acceleration of the biodegradation of crude oil or gasoline in the soil by nitrogen and phosphorus fertilization was shown in several studies (Leahy and Colwell, 1990).

Most PAH contaminated sites suffer from water stress owing to their high hydrophobic property. The water stress not only increases the reluctance of PAHs, but also inhibits the nutrient transfer and results in poor growth and low activity of plants and associated microorganisms (Børresen and Rike, 2007; Johnsen et al., 2005; Li et al., 1997; Phillips et al., 1999). Enhancement of water regime could improve PAH mass transfer, but restrict oxygen diffusion and decrease oxidation activity, especially in the deep soil layer (Phillips et al., 1999). The initial ring oxidation process, in which atmospheric oxygen is incorporated into the substrate by bacteria to form cis-dihydrodiols, is considered to be the rate limiting step in most PAH biodegradation reactions (Cerniglia, 1992).

The optimal pH value and temperature are important factors for obtaining an efficient PAHs degradation (Ping et al., 2006). An increase in temperature can decrease the soil-water partition coefficient and as a result, increase dissolution of contaminants in water is observed. The partition-coefficient of PAHs decreases by 20-30 % for every 10 °C rise in temperature between 5 and 45 °C (Lüers and Hulscher, 1996). Most heterotrophic bacteria and fungi favor a pH near neutrality (Leahy and Colwell, 1990). The shift of pH from 5.2 to 7.0 can significantly enhance PAH degradation rate by *Sphingomonas paucimobilis* BA 2 strain (Kastner et al., 1998). Extremes pH values, as observed in some soils, would therefore be expected to have negative influence on PAH degradation.

Beside their hydrophobic property, PAHs could cause inhibition of biodegradation through the formation of toxic metabolites during biodegradation process. Girotti et al. (2008) indicated that although in a long run the toxicity tended to decrease, after a short time treatment (one month) the toxicity of the contaminated samples increased, since the

bioremediation made available long chain hydrocarbons before in a not-soluble form. The surfactants released by microorganisms and plants in soil could increase the concentration of soluble PAHs (Joner and Leyval., 2003a), and increase their availability for biodegradation, but could also increase the risk to humans and the environment.

1.6.2 Approaches to improve PAH biodegradation in contaminated sites

Due to the aforementioned limiting factors, the biodegradation of PAHs, especially HMW PAHs could be limited or even unsuccessful. A number of approaches have been investigated to overcome these factors and to improve microbial PAH degradation.

1.6.2.1 Bioaugmentation

Bioaugmentation is a technology which can increase PAH degradation rates by addition of microorganisms with PAH degradative capabilities. The bioaugmentation of recently oil-contaminated soils from Brazil with two crude oil degrading microorganisms (*Nocardia nova* and *Rhodotorula glutinis var. dairenesis*), doubled biodegradation efficiency (Trindade et al. 2005). Madsen and Kristensen (1997) applied isolated PHE degraders to an aged polluted soil, and the maximum $^{14}CO_2$ evolution rate from labeled phenanthrene increased from 0.57 (without bioaugmentation) to 0.87 nmol $g^{-1}d^{-1}$. Hamdi et al. (2007) added aged PAH-contaminated soil containing activated indigenous degraders to a fresh PAH spiked soil and increased anthracene degradation. The bioaugmentation could also benefit HMW PAH dissipation. Hamdi et al. (2007) reported that activated indigenous degraders increased pyrene and benzo[a]pyrene dissipation (after 120 d, the amendment 96% and 60% of pyrene and benzo[a]pyrene had disappeared respectively with amendment, and only 33% and 35% of them in the unamended controls). Garon et al. (2004) isolated *Absidia cylindrospora* from a fluorine contaminated soil and used it for bioaugmentation. Results showed that more than

90% of fluorene in soil slurry was degraded within 288 h when *A. cylindrospora* was added, while 576 h were necessary when only native microorganisms were used. Silva et al. (2009) compared the PAH biodegradation activity of native soil microorganisms in a PAH-impacted soil to bioremediation of the same soil bioaugmented with individual bacterial and fungal isolates and a fungal consortia. Although no difference has been detected on the LMW PAH dissipation owing to their fast degradation, the dissipation of benz[a]anthracene and to a lesser extent benzo[a]pyrene was improved in the microcosms bioaugmented with the *Asp*ergillus isolate alone by more than 10 mg kg^{-1} soil (initial concentration of benz[a]anthracene and benzo[a]pyrene was 50 mg kg^{-1}).

Bioaugmentation could also influence the bioavailability of pollutants. It may influence the bioavailability of pollutants when the application methods involve homogenization, slurrying, or intensive flushing of the system, or when the bacteria added differ from the indigenous population with respect to their specific affinity for the contaminant, maintenance requirements, ability to co-utilize natural substrates, active or passive mobility, adhesion behavior, or ability to produce biosurfactants and to ingest surfactant-solubilized chemicals (Johnsen et al., 2005).

However, in some cases, bioaugmentation efficiency decreased with the time going due to the die off of the laboratory adapted strains, limited substrate and nutrient availability, or lack of competitivity of introduced microorganism in the contaminated soil (Juhasz and Naidu, 2000).

1.6.2.2 Biostimulation

Biostimulation, by adding nutrients and/or a terminal electron acceptor, increases the activity of indigenous microbial populations. Easily degradable carbon sources and inorganic nutrients such as nitrogen, phosphorus are often used. Carmichael and Pfaender (1997) obtained a

significant increase of phenanthrene and pyrene degradation after 4 weeks incubation by adding pathway intermediate, such as salicylic acid, phthalic acid and gentisic acid. Joner et al. (2002) showed a positive effect of nitrogen and phosphorus addition on 3 and 4 rings PAH dissipation. Liebeg and Cutright (1999) showed an increasing bacterial activity when micro-nutrients were supplied. Biostimulation using air sparging and nutrient infiltration successfully enhanced the indigenous aerobic PAH biodegradation and the amount of PAH-catabolic genes (Nyyssönen, 2009). However, nutrient addition may not always increase the PAH degradation. In the field study of Garcia-Blanco et al. (2007) the degradation of resolved aromatics averaged 74% in the nutrient amended plots versus in the natural attenuation plots. Besides, sometimes nutrient have only a short-term influence on PAH degradation and may increase oxygen demand in contaminated soil (Liebeg and Cutright, 1999).

Bioaugmentation and biostimulation can occur simultaneously in case of amendments using active organic residues that contain microbial strains capable of metabolizing pollutants. For example, Hamdi et al. (2007) applied sewage sludge compost and decaying rice straw, which contained microbial strains capable of metabolizing pollutants, in a PAH (anthracene, pyrene and benzo[a]pyrene) spiked soil (3000 total PAH mg/ kg soil), and found that PAH dissipation rates were higher than in unamended control (anthracene, pyrene and benzo[a]pyrene dissipation were 63%, 33%, 35%), especially for decaying rice straw (all PAH dissipation > 96%).

1.7 Phytoremediation

Phytoremediation, or plant-assisted bioremediation, is commonly defined as the use of green or higher terrestrial plants and the associated rhizosphere microorganisms to transform,

degrade and stabilize the toxic pollutants in the soil, sediments, water and atmosphere (Susarla et al., 2002). Many research papers supported the observation that the addition of plants to a PAH contaminated medium (e.g. soil, wetland and hydroponics) could enhance the disappearance of the pollutant. Researches on phytoremediation of PAH-contaminated soils began in 1990s, and have increased over the last decade (Aprill and Sims, 1990; Reilley et al., 1996). Both positive and negative effects have been obtained. Recent studies mainly focused on the mechanisms of plant enhanced PAH dissipation, the fate of PAHs in rhizosphere, the controlling parameters and the potential way to increase the phytoremediation efficiency.

1.7.1 Fate of PAHs in plant rhizosphere

Different mechanisms affect the fate of PAHs in plant rhizosphere (Fig. 6). A number of studies have been conducted to study such mechanisms and determine the dominant ones (Corgié et al., 2006a, b; Joner et al., 2002; Leigh et al., 2002; Singer et al. , 2003).

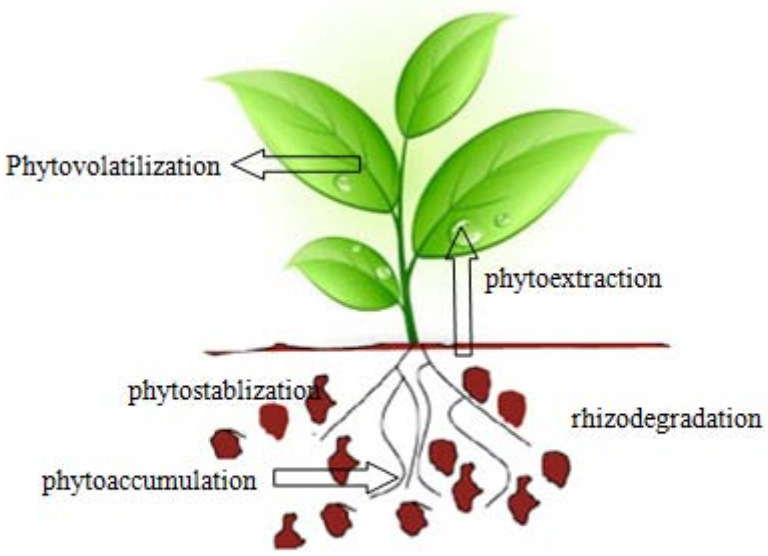


Fig 6. Different mechanisms of phytoremediation

1.7.1.1 Plant uptake of PAHs (phytoaccumulation, phytoextraction)

The uptake of organic chemicals by plants occurs via a number of pathways, and the main ones are the air-shoot and soil-root way. Plants are important sinks for atmospheric PAHs, playing a role in the annual cycling of PAHs. PAHs can be transferred to plants from a polluted atmosphere by particle-phase deposition on the waxy leaf cuticle or by uptake in the gas phase through stomata (Simonich and Hites, 1995). Vegetation has been successfully used as a quantitative indicator of exposure to both gaseous and solid phase PAHs in ambient air (Wild et al., 1992).

In soils, PAHs enter plants principally via root uptake, and the pathway is profoundly influenced by the transfer of contaminants from soil to soil solution (Collins et al., 2006; Li et al., 2005). Most experiments showed that PAH uptake and accumulation from soil into plant biomass is rather limited and could be negligeable in phytoremediation. Binet et al. (2000) investigated the fate of PAHs in rhizosphere, and found that PAHs adsorbed to roots, taken up by roots and in shoots represented below 0.11 ‰, 0.16 ‰ and 0.001 ‰ respectively of the extractable PAH concentration, while 36 - 66 % PAHs dissipated in soil. With a high hydrophobicity (log Kow higher than 3.5), the soil-bound PAHs are strongly associated with soil organic matter and as such, are not readily available for root uptake (Wild and Jones, 1992; Simonich and Hites, 1995). Watts et al. (2006) found up to 43 mg kg^{-1} of PAHs in root tissue in a marsh contaminated sediment with up to 900 mg kg^{-1} total PAHs. The concentrations in the roots are one to two orders of magnitude lower than the concentrations in the soil and are linearly related to the soil concentrations. Gao et al. (2006) observed 27 and 98 mg kg^{-1} PHE and PYR in the roots of rice (*Oryza sativa* L.) in a soil spiked with 200 mg kg^{-1} PHE and 200 mg kg^{-1} PYR after 80 days. Fismes et al. (2002) found PAH in all three plant species (lettuce, potato, and carrot) grown in contaminated soils, but their concentration

was low compared with the initial soil concentration. The bioconcentration factors of roots was lower than 1.5×10^{-2}, indicating that the concentration of PAHs in roots represented less than 1.5 % of the concentration in soil. The PAH root uptake may be influenced by plant species (Fismes et al., 2002), PAH property (Gao et al., 2006; Su and Zhu, 2008), the effective PAH concentration in soil solution (Chiou et al., 2001; Watts et al., 2006), soil property (Nam and Kim, 2002).

Furthermore, while PAH compounds are firmly adsorbed to the root surface, there is little transfer to the interior portions of the plant or transformed up to the shoots (Wang and Jones, 1994; Fismes et al., 2002). Most of investigations found relativity lower PAH concentration in shoot tissue, especially HMW PAHs, which were strongly absorbed on the root epidermis (Wild and jones, 1992; Kipopoulou et al., 1999). Watts et al. (2006) found that the concentrations in the leaves were only three to four orders of magnitude lower than in the soil. The low PAH mass transport within the plant tissue may be related to their high lipophily (Chaîneau et al., 1997; Gao et al., 2006; Su and Zhu, 2008; Watts et al., 2006).

1.7.1.2 PAH rhizodegradation

The mechanisms of plant promoting PAH biodegradation have not yet been well defined. Some studies found that plants could supply a variety of contaminant-degrading enzymes, such as laccase, nitrilase, peroxidase, nitroreductase, which could biodegrade the PAHs directly (Ndimele, 2010). However, other investigations suggest that the indirect effect of plants, stimulating microbial activity and altering the diversity of the root associated community involved in the dissipation of PAHs plays a major role in PAH biodegradation (Reilley et al., 1996). Roots could increase the contact surface between the microorganisms, soil particles and pollutants. Root exudates contain a large number of chemical compounds (Vancura, 1964) which could promote PAH dissipation (Singer et al., 2003).

Root exudates are commonly divided into low and high molecular weight compounds. Amino acids, carboxylic acids, sugars, phenolic compounds, and various secondary metabolites belong to the group of low molecular weight compounds, whereas high molecular weight exudates primarily include mucilage (high molecular weight polysaccharides) and proteins (Muratova et al., 2009; Walker et al., 2003).

Plant root exudates are a connecting link in the complex plant-microorganism-pollutant system, playing an important role in the degradation of PAHs. Corgié et al. (2004) used two-comparment devices to evaluate the effect of root exudates on phenanthrene biodegradation. These authors showed that root exudates increased phenanthrene biodegradation and that such effect declined with increasing distance from plant roots. The plant-assisted PAH degradation has been attributed to a general rhizosphere effect via a fortuitous enrichment of PAH degrading microorganisms, as well as possible induction of PAH degrading enzymes through secretion of compounds that cause PAH co-metabolism.

The density and the activity of PAH degraders are typically increased in the presence of root exudates. PAH degrader population and activity in the rhizosphere may be enhanced simply as a result of the general increase of microorganisms by root exudates. Many kinds of microorganisms have the ability to degrade PAHs, but their growth and PAH degradation efficiency in soil are limited owing to the nutrition stress and the low PAH bioavailablity. Root exudates could release carbon and nutrition stress in the soil. This rhizosphere stimulation of microorganisms was firstly observed by Hiltner in 1904. Plant roots modify favorably the life habitat of micro-organisms by exudates release, nutrient supply, e.g. nitrogen, sugars, aeration and water drainage. Around 30-60% of net carbon fixed during photosynthesis can be released into the rhizosphere, and some of the components may then serve as readily available substrate and contribute to the increase of microorganisms (Rentz et

al., 2004; Salt et al., 1998). The number and diversity of microorganisms are greatly increased by rhizosphere effect (Günther et al., 1996), and microbial community structure in rhizosphere differs from bulk soil (Steer and Harris, 2000). Plants could also increase the activity of PAH degraders by proliferation of PAH degrader group. In PAH polluted soils, the plant-derived chemicals, including those generated from root turnover, selectively foster the growth of PAH degrading microorganisms (Leigh et al., 2002). And many studies showed higher percentage of PAH-degrading bacteria and catabolic genes in rhizosphere than in bulk soils (Corgié et al. 2004; Miya and Firestone, 2000; Siciliano et al., 2003).

Plant exudates could also promote co-metabolism for PAHs, especially HMW ones. Ferro et al. (1997) reported that plant exudates may have served as co-metabolites during the biodegradation of (^{14}C) pyrene in the rhizosphere of crested wheatgrass. Many plant-derived chemicals, e.g. salicylate, linoleic acid, which induce systemic acquired resistance in plants, have the potential to act as co-metabolite and stimulate microbial degradation of PAHs (Chen and Aitken, 1999; Yi and Crowley, 2007). Besides, secondary plant metabolites play an important role in developing the myriad of organic pollutant-degrading enzymes found in nature (Singer et al., 2003)

What's more, root exudates might involve surfactants or phytochemicals increasing PAH solubility and availability to degradation (Harvey et al., 2002; Fava et al. 2004; Parrish et al. 2005).

1.7.1.3 PAH phytostabilization

Plants introduce organic matter into the soil through root decomposition. It has been estimated that approximately one-third of the carbon in plant residues remains in the soil after the first growing season in the form of labile and stable humus components (Chen et al., 2003). The

input of new organic matter may provide a source for contaminant adsorption. Detoxification of PAHs has been demonstrated after addition of humic acids due to the chemical binding of PAHs (Perminova et al., 2001). It has been hypothesized that organic matter and associated contaminants may be degraded during subsequent humus regeneration processes. Bound residues have been shown to degrade at a rate similar to humus turnover (Nieman et al., 1999). Therefore, given enough time, plants may be able to stabilize contaminants in the soil and this also provides an active long-term environment for microbial degradation.

1.7.1.4 PAH phytovolatilization

Phytovolatilization is the uptake and transpiration of a contaminant by a plant, with release of the contaminant or a modified form of the contaminant to the atmosphere from the plant. PAH phytovolatilization is limited since PAH compounds are hydrophobic, and only little proportion of them could be transfered to the shoots.

1.7.2 Parameters affecting PAH dissipation in rhizosphere

1.7.2.1 Soil parameters

The soil parameters affecting PAH degradation are mainly the soil texture, the content of soil organic matter (SOM), C/N, and pH (Chung and Alexander, 2002; Maliszewska-Kordybach, 2005; Chiapusio et al., 2007). Soils with high humic acid concentration may inhibit PAH degradation since humic substances have strong interactions with PAHs and decrease their bioavailability (Laor et al., 1999). Chiapusio et al. (2007) showed that the soil structure, especially clay concentration could greatly influence PAH dissipation efficiency. In addition to their influence on microorganism activity, pH value, humidity and soil toxicity could also influence plant fitness. PAH contaminated soils always suffer from water deficiency as well as limited nutrient availability, which will depress the growth, reproduction and activity of

plants and microorganisms (Li et al., 1997). For example, plants and microorganisms require macro-nutrients and micro-nutrients, such as nitrogen and phosphorus in order to develop substantial biomass to support microbial growth (Sylvia et al., 1998; Joner et al., 2002; Liebeg and Cutright, 1999). The addition of nitrogen and phosphorus fertilizer was proved to enhance PAH bioremediation (Lin and Mendelssohn,1998). Increased biomass of plants and pollutant degraders were reported as a result of inorganic nutrient amendments (Lindstrom et al., 1991; Margesin et al., 2000; Olson et al., 2008).

1.7.2.2 PAH property

The positive effect of plants on both LMW and HMW PAHs have been reported, as described previously for biodegradation (chapter 1.6). PAH dissipation in plant rhizosphere is higher for LMW than HMW PAHs. Joner et al. (2001) estimated the effect of a mixture of white clover and ryegrass on the degradation of three PAHs (anthracene,chrysene, and dibenzo[a,h]anthracene) in silty sand soil, and found that the extent of PAH disappearance was consistently greater in planted units compared to unplanted controls, and the PAHs dissipated in soil in the following order: anthracene (98%) > chrysene (65%) > dibenzo(a,h)anthracene (16%) (8 weeks). This ranking correlated with the molecular weight of the PAH compounds, i.e., the PAHs with higher molecular weight are more reluctant to the phytoremediation. Besides, plants are more effective in promoting LMW PAH biodegradation. Qiu et al. (1997) assessed the performance of 12 warm season grass species to remove various PAHs from contaminated soil. While prairie buffalo grass, common buffalo grass, Meyer zoysia grass and Verde Klein grass accelerated the loss of the LMW PAHs naphthalene, fluorene and phenanthrene compared to an unplanted control, only the Verde Kleingrass accelerated the loss of HMW PAHs, such as pyrene, benzo(a)anthracene and benzo(a)pyrene. Gao et al. (2006) reported that rice (*Oryza sativa* L.) could enhance

phenanthrene and pyrene dissipation in spiked soil, but the effect of plant on phenanthrene was significantly higher than pyrene. The addition of root exudates to a soil sample inhibited the 5 and 6 ring PAH dissipation, while it increased the 3 and 4 ring PAH dissipation by around 7% after 30 days (Joner et al., 2002). The major degradation mechanism for PAHs over five rings is co-metabolism, which should be promoted by root exudates, but it may be less efficient in comparison to PAH degrader degradation (Juhasz et al., 2000). Besides, the PAH degraders may prefer root exudates for their higher solubility and lower reluctance.

1.7.2.3 Plant species

Various plants have been identified for their potential to facilitate the bioremediation of soil contaminated with PAHs, but their tolerance to PAHs and capability of PAH dissipation varied among plants.

Tolerance to PAH toxicity in soil differs with plants. Liste and Prutz (2006) investigated 13 plant species for PAH dissipation in greenhouse pot experiments in a long-term contaminated soil from a former manufactured gas plant site. The germination rate ranged from 93% (ryegrass) to 0% (pansy). And the seedling survival rate of corn, oat, ryegrass and pea was 100%, while for lupine it was 8% in comparison to non-polluted control. Plant biomass reduction due to the presence of PAHs also differs with plant species. Sverdrup et al. (2003) found that red clover biomass was more sensitive to phenanthrene and pyrene than ryegrass.

The ability of plant species to promote PAH dissipation is highly variable ranging from negative or no effect to highly stimulatory effects (Yi and Crowley, 2007; Rentz et al., 2005). Among 9 plant species tested by Liste and Alexander (2000), the bioavailable pyrene concentration after 28 days cultivation varied from 22 (pepper, *Capsicum annuum*) to 60 (red

pine, *Pinus resinosa*) mg kg^{-1} soil (initial concentration was 100 mg kg^{-1}). Xu et al. (2006) studied the performance of three plant species (maize, ryegrass and white clover) for phenanthrene and pyrene dissipation at six different initial concentrations, and found that pyrene dissipation was higher with maize than with the two other plants. The difference in PAH dissipation by plants may relate to the different absorption and adsorption effect of plants (see 1.7.1.1), different root exudates (see 1.7.1.2), different plant size, and their different ability to form symbioses (see 1.7.2.5).

The lipids in cell membranes play an important role in the adaptation of plants to environmental stresses. Furthermore, the plant composition, lipid content in particular, also influences the uptake of organic contaminants. For example, Simonich and Hites (1994) observed higher concentrations of PAHs in vegetations with higher lipid contents, suggesting the primary role of plant lipids for the uptake of PAHs from atmosphere. Jiao et al. (2007) showed that PAHs adsorbed to root surfaces are closely related with root surface area, with accumulations typically in root lipids.

Plant size, and especially root growth and morphology may influence PAH dissipation. Groleau-Renaud (1998) found a positive correlation between carbon exudates and root surface area, root number and root dry weight. However, PAH dissipation was rarely estimated as a function of root biomass or root parameters.

Root exudates are important parameters in PAH phytoremediation and also differ quantitatively and qualitatively among plant species. Yi and Crowley (2007) examined the crushed tissue of 43 species, and only four of them, including celery (*Apium graveolens*), radish (*Raphanus sativus*), potato (*Solanum tuberosum*) and carrot (*Daucus carota*), which possessed high concentration of linoleic acid, could significantly increase the HMW PAH degradation within 40 days. They also showed that celery was more efficient than wheat

(*Triticum aestivum*) for PAH dissipation. However, root exudates of the other 39 species of plants showed no effect on HMW PAH dissipation. Certain compounds in exudates may also cause inhibitory effects by decreasing PAH degrader populations. Many terpenes have antibacterial properties which are the basis of their use as food preservatives and natural medicines. Besides, the same plant could secrete different exudates under different environmental conditions (Wittenmayer and Merbach, 2005), which could potentially affect PAH dissipation. Unfavourable environmental conditions caused an increase in plant root exudation (Neumann and Romheld, 2000).

The efficient plant species have been compiled on the basis of empirical data (http://www.phytopet.usask. ca/mainpg.php). Grasses are thought to make superior vehicles for phytoremediation because they have extensive, fibrous root systems. Grass root systems have the maximum root surface area and may penetrate the soil to a depth of up to 3 m (Aprill and Sims, 1990). Legumes are thought to have an advantage over non-leguminous plants in phytoremediation because of their ability to fix nitrogen, and reduced the nitrogen stress among plants and various rhizospheric microorganisms. Grasses and legumes, such as ryegrass (grass) , alfalfa (legume) and tall fescue (grass) have been recommended in PAH polluted soil owing to their high PAH and nutrient stress tolerance (Fan et al., 2008; Corgié et al., 2004). Other plants, such as celery root, which could excrete enzyme or co-metabolite, have also been considered (Yi and Crowley, 2007). However, in some special environments, e.g. coastal wetlands, the native plants such as mangrove and black needlerush were more suitable and efficient in PAH dissipation (Hong et al., 2008).

In addition to plant species selection, the combination of plants was also studied to enhance PAH bioremediation (Chen et al., 2008; Phillips et al., 2006). Xu et al. (2006) showed that the co-cultivation of maize and ryegrass increased the dissipation of phenanthrene and pyrene in

comparison to a single plant species, but there was no effect of ryegrass and maize co-cultivation.

1.7.2.4 Surfactant

As a result of the hydrophobic property, PAHs are inclined to adsorb on the surface of the soil particles, and the rate of PAH bioremediation is related to aqueous solubility rather than total PAH concentration (Cerniglia, 1992; Juhasz and Naidu, 2000). Providing appropriate surfactant could increase the solubility and bioavailalibity of PAHs, especially HMW PAHs to degraders. Wu et al. (2008a) obtained higher PHE transport to rhizosphere by using Triton X-100. However, some surfactants have biological toxicity, which may also threaten the ecological security. Thus, biosurfactants, which have environment friendly nature, should receive extensive attention (Joner and Leyval, 2001).

1.7.2.5 Symbiotic microorganisms

Ubiquitous symbiotic partners such as rhizobia and mycorrhiza fungi which facilitate plant nutrition and water uptake could affect PAH phytoremediation since PAH contaminated soil are often deficient in water and nutrients (Li et al., 1997). Johnson et al. (2004) reported improved remediation of chrysene in a spiked soil by employing mixed grass-legume, together with a symbiotic rhizobium. The symbiotic association improved plant vigor and growth thereby stimulating the rhizospheric microflora to degrade chrysene. Chiapusio et al. (2007) found that rhizobia could tolerate high concentrations (1000 mg kg^{-1}) of phenanthrene. Johnson et al. (2004) showed that rhizobium colonization increased root biomass in a PAH contaminated soil and the number of PAH degraders. Mycorrizha were used in phytoremediaiton since 1983 (Gildon and Tinker, 1983). Mycorrhizal fungi can play a role in phytoremediation by improving plant establishment and growth on PAH contaminated soil

(Joner et al., 2001; Leyval and Binet, 1998). Their effects on PAH dissipation are described in the following chapter.

1.8 Fate of PAHs in the rhizosphere of mycorrhizal plants

1.8.1. Mycorrhizal fungi

Mycorrhizal fungi are root symbiotic fungi associated to the roots of most plant species. There are two main types of mycorrhizal fungi: ectomycorrhizal (EM) fungi and arbuscular mycorrhizal (AM) fungi (Harley and Smith, 1983).

EM fungi are generally host specific and form a mycelial sheath around the plant root. The effect of EM fungi on the dissipation of PAHs in contaminated soil phytoremediation was investigated by Joner et al. (2006), and these authors showed that PAHs were degraded more slowly in soil where EM fungi were present.

AM fungi are ubiquitous soil fungi and constitute an important functional component of the rhizosphere. Contrary to N-fixing symbioses that concern a limited number of plant families, AM fungi form symbiotic relationships with roots of 80-90% terrestrial plants in natural, agricultural, and forest ecosystems (Smith and Read, 1997). AM symbiosis is 460 million years old and the most widespread type of mycorrhizal associations with plants possessing true roots, i.e. pteridophytes, gymnosperms and angiosperms (Khan, 2005). The benefit of AM fungi to plants is mainly attributed to increased uptake of nutrients, especially phosphorus and water. Such beneficial effect on nutrient uptake is attributed to increased surface area of soil contact, increased transport of nutrients into mycorrhizal hyphae, a modification of the root environment and increased storage (Bolan, 1991). For example, the

rate of inflow of phosphorus into mycorrhizae can be up to six times that of the root hairs (Bolan, 1991).

1.8.2 The effect of AM fungi on phytoremediation

The first investigation of AM fungi on phytoremediation was conducted in heavy metal contaminated soils (Gildon and Tinker, 1983). AM fungi were initially used to protect plants against toxicity and nutrient stress of heavy metal contaminated site (Heggo et al., 1990). For one thing, external fungal hyphae could exploit a larger volume of nutrient resources in the soil that are otherwise unavailable for uptake by roots. For another thing, AM colonization could increase the tolerance of plants to contamination by increasing the intracellular storage capacity of the roots and by binding the contaminant to the extraradical hyphae (Ducek et al., 1986; Joner and Leyval 1997; Joner et al., 2000; Verdin et al., 2006). AM fungi have been widely studied in inorganic polluted soils, and several studies are listed in table 2.

Various behaviors of AM fungi under metal stress were reported with different host plants, fungal isolates and substrates of culture.

Table 2. Recent studies on AM fungi in heavy metal contaminated soils.

Heavy metals	**Plant species**	**AM fungi species**	**Reference**
Cd	*Pisum sativum* L.	*G.intraradices* BEG 141	Rivera-Becerril et al., 2002
Cd	*Lolium* L.	*G. intraradices* *G. mosseae*	Feng et al., 2005
Zn	*Solanum nigrum*	*G. claroideum* *G. intraradices*	Marques et al., 2006
Zn	*Trifolium pratense*	*G. mosseae*	Li and Christie, 2001.
U	*Daucus carota*	*G. intraradices*	Rufyikiri et al., 2002
U	*Medicago truncatula* and *Lolium perenne.*	*G. intraradices*	Chen et al., 2008
Pb	*Kummerowia striata (Thunb.) Schindl, Ixeris denticulate L., Lolium perenne L., Trifolium repens L. Echinochloa crusgalli var.*	Mix AM fungi	Chen et al., 2005
Ni	*Trifolium repens*	*G. mosseae*	Vivas et al., 2006
Cu	*Coreopsis drummondii, Pteris vittata, Lolium perenne, Trifolium repens*	*G. mosseae*	Chen et al., 2007
As	*Helianthus annuus* L.	*G. aggregatum*	Ultra Jr et al., 2007
As	*Pteris vittata L.*	*G. mosseae,* *G. margarita*	Trotta et al., 2006
Zn, Cd	*Trifolium subterraneum, Lolium perenne*	*Glomus* spp.	Joner et al., 2000
Zn, Cd, Pb	*Medicago truncatula*	*G. intraradices,* *G. mosseae* BEG 69	Redon et al., 2009

AM fungi were not considered in organic pollutant contaminated soil until the end of last century, and only limited reports are available (Table 3). An increased dissipation of organic pollutants in the mycorrhizosphere has been observed with AM fungi e.g. *Glomus mosseae* (BEG 69), *Glomus etunicatum*, *Glomus intraradices*, *Glomus versiforme* (Joner et al., 2001; Wu et al., 2008a; Liu and Dalpé, 2009).

Table 3 Studies on AM fungi in soils contaminated with organic pollutants.

Organic pollutants	**Plant species**	**AM fungi species**	**Reference**
anthracene	*Allium porrum* L., *Zea mays* L., *Lolium perenne* L., *Trifolium subterraneum* L.	*G. mosseae* BEG 69	Leyval and Joner, 1998
anthracene	*Medicago sativa* L.	*G. mosseae* BEG 69	Criquet et al., 2000
anthracene or a mixture of 8 PAHs	*Lolium perenne* L.	*G. mosseae* BEG 69	Binet et al., 2000
anthracene, chrysene, dibenz[a,h]anthracene	*Lolium perenne* *Trifolium repens*	*G. mosseae* BEG 69	Joner et al., 2001
anthracene, chrysene, dibenz[a,h]anthracene	*Lolium perenne* *Trifolium repens*	*G. mosseae* BEG 69	Joner and Leyval, 2001
anthracene	*Lolium perenne* L.	*G. mosseae* BEG 69	Binet et al., 2001
aldrin	*Salix alaxensis* *Populus balsamifera*	Indigenous mycorrhizal	Schnabel and White, 2001
Two industrial soil with 12 PAHs	*Lolium perenne* L. *Trifolium repens*	*G. mosseae* BEG 69	Joner and Leyval, 2003a
Benzo[a]pyrene	*Medicago sativa*	*G. caledonium*	Liu et al., 2004
p,p-DDE	*Cucurbita pepo.*	mixed AM fungi	White et al., 2006 a
phenanthrene	*Lolium perenne* L.	*G. mosseae* BEG 69	Corgié et al., 2006 c
anthracene	*Cichorium Intybus* L.	*G. intraradices*	Verdin et al., 2006
p,p-DDE	*Cucurbita pepo.*	mixed AM fungi	White et al., 2006 b
atrazine	*Zea mays* L.	*G. caledonium*	Huang et al., 2007
phenanthrene	*Lolium perenne* L. *Trifolium pratense* L.	AM fungi	Chiapusio et al., 2007
phenanthrene	*Medicago sativa* L.	*G. etunicatum*	Wu et al., 2008a
DDT	*Medicago sativa* L.	*G. etunicatum*	Wu et al., 2008b
phenanthrene	*Medicago sativa* L.	*G. etunicatum*	Wu et al., 2009
anthracene phenanthrene	*Allium porrum* L.	*G. intraradices* *G. versiforme*	Liu and Dalpé, 2009
atrazine	*Zea mays* L.	G. *etunicatum*	Huang et al., 2009
anthracene	jute	*G. intraradices* *G. mosseae*	Cheung et al., 2008
fluorene phenanthrene	*Lolium perenne* L.	*G. etunicatum* *G. mosseae*	Gao et al.,2010

Wu et al. (2008b) showed an increased DDT dissipation in the rhizosphere of AM fungi colonized alfalfa. Huang et al. (2007) observed an enhancement of atrazine metabolism in mycorrhizophere. And PAHs, which are reluctant to biodegradation, showed an increasing dissipation in presence of AM fungi (Joner et al., 2001; Joner and Leyval, 2003a; Wu et al., 2008a).

1.8.3 Contribution of AM fungi to PAH phytoremediation

The first investigation showing a positive effect of AM fungi on PAH dissipation was conducted by Joner et al. (2001). In this experiment, clover and ryegrass grown on spiked (500 + 500 + 50 mg kg^{-1} of anthracene, chrysene and dibenz[a,h]anthracene) soil, and the presence of the AM fungus *Glomus mosseae* BEG 69 increased the dissipation of chrysene and dibenz[a,h]anthracene by 10 and 22% respectively. Wu et al. (2008a) showed that inoculation with *Glomus etunicatum* increased PHE dissipation. Liu and Dalpé (2009) inoculated two species of AM fungi (*Glomus intraradices* or *G. versiforme*) on leek (*Allium porrum* L.), and found that both of them increased the phenanthrene and anthracene dissipation in soil. Joner and Leyval (2003a) studied PAH dissipation in aged historical contaminated soils as a function of distance to roots and showed that PAH concentration was lower with mycorrhizal plants. The mechanisms involved in such AM effect are still under investigation.

Although no evidence of direct PAH catabolism by AM fungi has been reported yet (Criquet et al. 2000; Joner and Leyval, 2003b), Verdin et al (2006) showed some accumulation of PAHs in AM hyphae, Wu et al (2009) found an increased uptake in mycorrhizal roots and Binet et al. (2000) a decreased sorption to mycorrhizal roots.

The decrease of PAHs in soil was partly attributed to the enhanced nutrient uptake by AM fungi, leading to improved plant growth, which, in turn, may stimulate soil microbial activity. In the study of Joner and Leyval (2001), the shoot dry weight was 100% and 30% higher for mycorrhizal clover at 8 and 16 weeks, as compared to non-mycorrhizal clover, respectively, and the root length density of clover was higher in mycorrhizal treatments at both harvests. The AM fungus may modify the root exudation. This may again results in changes in the microbial community that may affect PAH degradation. Several studies have reported that the development of AM symbiosis could change bacterial community structure in the rhizosphere (Joner et al., 2001; Marschner et al., 2001; Marschner and Baumann, 2003), not only bacterial communities, but also their biodegradation activities. Using phospholipid fatty acid (PLFA) profiles, Joner et al. (2001) showed that AM fungus could alter microbial community structure in a PAH contaminated soils and suggested that the mycorrhiza-associated microflora may be responsible for the reduction in PAH concentration in the mycorrhizosphere. Mycorrhizal roots are known to contain and exude unique or enhanced levels of simple phenolic compounds (McArthur and Knowles, 1992), which could act as inducers and carbon resource to support PAH metabolism in a manner similar to that documented for salicylate and linoleic acid (Yi and Crowley, 2007). The extra-radical hyphal length can range between 1 and 30 m per gram of soil (Smith and Read, 1997), and are 2-3 times finer than even the finest root hairs (Bolan, 1991; Jakobsen, 1995). They could increase phytoremediation by hyphae penetrating in deep soil as well as very fine soil pores which are unavailable to roots, even the fine roots (Joner et al., 2001).

PAHs are toxic and inhibit the AM fungi. Mycorrhizal colonization of clover and leek decreased when industrial PAH polluted soil was added into unpolluted soil, while maize and ryegrass colonization was not affected (Leyval and Binet, 1998). Spiking of soil with a

mixture of three PAHs reduced colonization of clover by *Glomus mosseae* BEG69 to a half of that in non-spiked soil (Joner and Leyval, 2001). Liu et al. (2004) evaluated *G. caledonium* colonization on alfalfa and found that the colonization of *G. caledonium* was not significantly affected in soil spiked with benzo[a]pyrene up to 10 mg kg^{-1} but significantly decreased at 100 mg kg^{-1} (Liu et al., 2004).

However, AM fungi colonization did not always facilitate PAH dissipation. Wu et al. (2008a) found decreased root and shoot biomass with AM fungi colonization. No significant effect was detected on PAH dissipation by Binet et al. (2000). AM fungi effect on PAH dissipation may vary with AM fungi species (Liu and Dalpé, 2009), plant species, and soil texture (Chiapusio et al., 2007).

1.9 Objectives of the study

Many studies have been conducted on the fate of PAHs in plant rhizosphere, but it is still difficult to predict and control the results. Further, little research has been done on the contribution of AM fungi. In this study, pot studies were conducted with available PAHs spiked to a soil, to study some of parameters which could control PAH dissipation in plant rhizosphere and mycorrhizosphere and the overall objectives were:

(1) To compare different host plant species, single plants and a co-culture, and different AM fungi for PAH dissipation in plant rhizosphere. To address this objectives, four species of plants as well as two AM fungi species have been selected and combined in this study, and their efficiency on PAH dissipation and microorganism community was assessed.

(2) To evaluate some of the environmental parameters which could affect PAH dissipation in rhizosphere and mycorrhizosphere. The PAH contaminated soils often suffer from water and

nutrient deficiency, which could contribute to their slow dissipation. Thus, PAH dissipation in plant rhizosphere was investigated at different levels of phosphorus fertilization and watering regime.

(3) To take into account the fate of LMW and HMW PAHs and their interactions. Contaminated soils always contain a mixture of PAHs, which have different physico-chemical properties, toxicity, bioavailability. Therefore PAH dissipation not only differs with PAH molecular weight, but may also differ depending on the interactions between them.

Different experiments were performed with a PAH spiked soil in order to investigate these parameters. Three PAHs were considered: PHE, PYR and DBA, as representative of three, four and five-ring PAHs. Four plants (alfalfa (*Medicago sativa* cv. *Europe*), tall fescue (*Festuca arundinacea* cv. *Bariane*), annual ryegrass (*Lolium multiflorum* cv. *Barclay*) and celery roots (*Apium graveolen*)) and two AM fungi (*G. mosseae, G. intraradices*) were investigated. Plants were grown in growth chamber conditions for up to 6 weeks. Plant biomass, AM colonisatio*n*, PAH concentration in soil and in plants were analysed.

Microorganism community is a key component of PAH dissipation in plant rhizosphere. The root exudates and the AM fungi inoculation may change the microorganism community, including the quantity and activity of PAH degraders. The 16S rDNA, 18S rDNA and PAH-RHDα genes, which reflect the bacteria, fungi and PAH degrader population, were quantified, and their community structure was investigated.

Partie 2

Matériels et méthodes

Materials and methods

Partie 2: Matériels et méthodes - Materials and methods

2.1 Materials

2.1.1 Plant preparation

Four kinds of plants, alfalfa (*Medicago sativa cv. Europe*), tall fescue (*Festuca arundinacea cv. Bariane*), annual ryegrass (*Lolium multiflorum cv. Barclay*) and celery roots (*Apium graveolen*), were used in this study. The seeds were germinated on filter paper in Petri dishes and young seedlings were transplanted into the pots three days after germination.

2.1.2 AM fungi preparation

Two AM fungi were used in the experiments. They were both isolated from heavy metal polluted soil, but had been successfully used in experiments with PAH polluted soils (Joner et al 2001, Corgié et al., 2006c, Norini, 2007). *G. intraradices* was isolated by Pr Bothe's group (Hildebrandt et al, 1999) and was supplied by the Institüt für Pflanzenkultur (Solkau, Germany) as a mixture of propagules in lava substrate. The density of AM fungi propagules in this inoculum was unknown, thus a fixed quantity of inoculum was added in each pot (40 g kg^{-1}). *G. mosseae* P2*,* Gerdemann & Trappe (BEG 69) was isolated from a heavy metal contaminated soil, in the north of France (Weissenhorm et al., 1993) and was kept since then on a leek culture on the original soil at LIMOS. The inoculum of *G. mosseae* consisted of spores (200 spores per pot) isolated as follows from 6 months old leek cultures:

1) Preparation of the glucose solution (500 g L^{-1}): 500 g glucose was solved in 1 L hot water, and the solution was cooled to room temperature before use. The solution should be stored at 4 °C.

2) Extraction of spores: 10 g soil and root mixture were put into a centrifuge tube with 50 ml glucose solution, and shaken before centrifugation at 2500 g for 4 min. The fine roots and spores in the supernatant were filtered on a fine sieve (50 μm pore) and rinsed with tap water. The fine roots and spores on the sieve were then collected on filter paper and tissue paper, and then spores were counted under the binocular.

2.1.3 PAH polluted soil preparation

The polluted soil used in the experiments was an agriculture soil artificially spiked by high purity PAHs (PHE, PYR and DBA). The agriculture soil was a silty clay loam (Bouzule, North-East part of France, 2 mm sieved) with the following characteristics: pH 7.4; organic carbon, 16 g kg^{-1}; total nitrogen, 1.7 g kg^{-1}; Olsen P, 117 mg kg^{-1}; 16 PAHs: 2.2 mg kg^{-1} (Table 4). The soil was heated at 90 °C for 1 h to kill the potential indigenous AM fungi. The soil was mixed (1:1, wt:wt) with sand (washed, 2 mm sieved). The water retention capacity of the mixture of soil and sand was 0.26 kg kg^{-1} soil. The soil mixture was spiked with: PHE ($C_{14}H_{10}$, >97%) purchased from Fluka, PYR ($C_{16}H_{10}$, 98%) and DBA ($C_{22}H_{14}$, 97%) purchased from Acros Organics. PAHs solved in chloroform (the quantity differed among the experiments) were added into 1/10 of total sand, and left until chloroform evaporation under a fumehood. The spiked sand was then mixed and homogenized with the rest of the sand and soil mixture.

Table 4. The initial concentration of 16 PAHs in Bouzule soil

PAH	**Concentration (μg g^{-1} dry soil)**
Naphthalene	< L.D.*
Acenaphthylene	0.10
Acenaphthalene	< L.D.
Fluorene	< L.D.
Phenanthrene	< L.D.
Anthracene	0.27

Fluoranthene	0.29
Pyrene	0.17
Benzo[a]anthracene	0.20
Chrysene	< L.D.
Indeno[1, 2, 3-c,d]pyrene	0.28
Benzo[b]fluoranthene	0.25
Benzo[k]fluoranthene	0.19
Benzo[a]pyrene	0.27
Dibenz[a,h]anthracene	< L.D.
Benzo[g,h,i]perylene	0.22
Total	2.23

* L.D.: detection limit

In order to reinoculate the soil after the heating process with the microflora except AM fungi, a soil suspension was added to the soil and sand mixture (32 ml soil suspension kg^{-1}). The soil suspension was prepared with 40 g soil, 240 g sterile glass beads and 400 ml sterile NaCl (0.8%) shaken for 1h, and then filtered through a filter paper. The soil suspension was then filtered with 5 μm filter membrane with the help of the vacuum pump to remove the AM fungi spores in the soil suspension. The soil suspension was added in the soil with the first water addition.

2.1.4 Hewitt solution

A modified Hewitt solution (Table 5) was prepared for plant fertilization (Redon et al., 2009). 10 ml Hewitt solution / 100g soil was added to the soil every week.

Table 5 Composition of Hewitt nutrient solution

	Composition	**Molecular weight**	**Quantity in mother solution (g L^{-1})**	**Dosage in 10 L Hewitt**	**Nutrient in Hewitt (mg L^{-1})**
Solution 1	NH_4NO_3	80.04	32	25 ml	N: 56.03
	$Ca(NO_3)_2$ $4H_2O$	236.16	94.5		Ca: 40.10

Solution 2	Na_2HPO_4 $2H_2O$	177.99	7.12	25 ml	Na: 4.60 P:3.10
			28.48*	25 ml*	Na: 18.40* P:12.40*
Solution 3	K_2SO_4	174.26	70	25 ml	K: 78.53 S:32.20
Solution 4	$MgSO_4$ $7H_2O$	246.48	74	25 ml	Mg:18.25 S:24.08
Solution 5	H_3BO_3	61.83	0.27	5 ml	B: 0.14
	$MnSO_4$ $4H_2O$	223.07	0.27		Mn:0.27 S: 0.005
	$CuSO_4$ $5H_2O$	249.69	0.03		Cu: 0.02 S: 0.08
	$ZnSO_4$ $7H_2O$	287.54	0.13		Zn: 0.7 S: 0.01
	Na_2MoO_4 $2H_2O$	241.96	0.01		Mo:0.005 Na:0.002
	$CoCl_2$ $6H_2O$	237.93	0.02		Co:0.01 Cl:0.015
	$CoSO_4$ $7H_2O$	281.10	0.02		Co: 0.01 S: 0.03
Addition	Fe-EDTA	403.08		0.081 g	

Note: 1. In the first experiment two concentrations of phosphorus were used: “low” and “high”phosphorus concentration. The concentration of Na_2HPO_4 $2HO_2$ used at high phosphorus concentration is marked with *. 2. The Hewitt solution should be adjusted to pH = 6.0 with HCl and NaOH before use.

2.2 Methods

The roots of the plants mostly colonized all the pot volume, and that is was why all the soil in the pot was considered as rhizospheric soil. The soil in the pot was homogenized at harvest. 0.5 g soil was stored at -20 °C for molecular biology analysis, and the rest was stored in dark chamber at 4 °C for PAH analysis.

2.2.1 Plant assessment

2.2.1.1 Plant biomass

Plants were harvested 6 weeks after seeding. Shoots and roots were harvested separately. Root fragments were collected by sieving the soil and were added to the root samples. The shoots and roots were washed carefully with deionized water to remove any adhering soil particles,

and then dried with filter paper and weighed (wet weight). The roots were separated to evaluate the dry weight, AM fungi colonization, phosphorus concentration, PAH sorption according to the experiments. Shoot biomass was divided for PAH extraction and dry weight assessment. Part of root and shoot (weight 1) were dried at 70 °C in an oven for two days and weighed, the dry weight of the plant (root or shoot) was assessed by:

Dry weight of plant shoot or root = dry weight 1 (selected part) × total wet weight of shoot or root / wet weight 1 (selected part)

2.2.1.2 AM fungi colonization

Mycorrhizal colonization of roots was estimated after trypan blue staining (Koske and Gemma, 1989), according to the technique of Trouvelot et al (1986). Roots were cut in 1 cm segments before preservation in 50% ethanol solution. After clearing the root pieces in a bottle with 5% (w/v) KOH at room temperature for 15 min, the bottle was transferred to a 90 °C water base for another 15 min. After cooling, the roots were washed on the fine filter (50 µm pore) with tap water for 4-5 times. Then the root pieces were put into HCl (1%) overnight and stained with 0.5% trypan blue solution for 6 h at 4 °C. Finally, the roots were washed with distilled water and transferred to decoloration solution (acidified glycerol) until slide preparation. The composition of the 5% trypan blue solution and decoloration solution was shown in Table 6. For each pot, thirty stained root segments (three slides were made with 2 × 5 root fragments stained with trypan blue) were deposited on microscope slides and observed under the microscope (Fig. 7). The mycorrhizal fungi colonization rate was calculated by the following formulas (Trouvelot et al, 1986):

1) Frequency of mycorrhizal roots: F% = (nb of AM fragments / total nb) × 100

2) Intensity of the mycorrhizal colonization in the root system:

M% = (95 n5 + 70n4 + 30n3 + 5n2 +n1) / (nb total)

Where n5= number of fragments rated 5; n4= number of fragments 4 etc.

3) Intensity of the mycorrhizal colonization in the colonized root fragments

m% = M% × (nb total)/ (nb mycorrhizal fragments)

4) Arbuscule abundance in mycorrhizal parts of root fragments

a% = (100mA3 + 50 A2 + 10m A1) / 100

Where mA3, mA2, mA1 are the % of m, rated A3, A2, A1, respectively, with

mA3 = ((95n5A3 + 70n4A3 + 30n3A3 + 5n2A3 + n1A3) / nb myco) × 100 / m

5) Arbuscule abundance in the root system

A% = a × (M% / 100)

Table 6 Composition of (a) trypan blue and (b) decoloration solution.

(a) 0.5% trypan blue solution

Glycerol	500 ml
H_2O	450 ml
HCl (1%)	50 ml
trypan blue	0.5 g

(b) Decoloration solution

Glycerol	500 ml
HCl (1%)	50 ml
H_2O	450 ml

2.2.1.3 Phosphorus concentration in shoots

Plant shoots were cut in pieces and ground with a pestle in a mortar under liquid nitrogen and the powders were kept in plastic bottles. 200 mg (100 mg for celery roots) powders and 4 ml 65% HNO_3 + 2 mL 30% H_2O_2 (for 100 mg dry, 2 ml 65% HNO_3 + 1 mL 30% H_2O_2) were loaded in microwave vials and heated under high temperature and high pressure condition (170 ° C, 2 MPa, 1200 W microwave system MARS 5) for 1 h. After adjusting the volume of the solution with deionized water to 25 ml, the P concentration in the samples was analyzed by ICP optical emission spectrometer (Varian 720-ES).

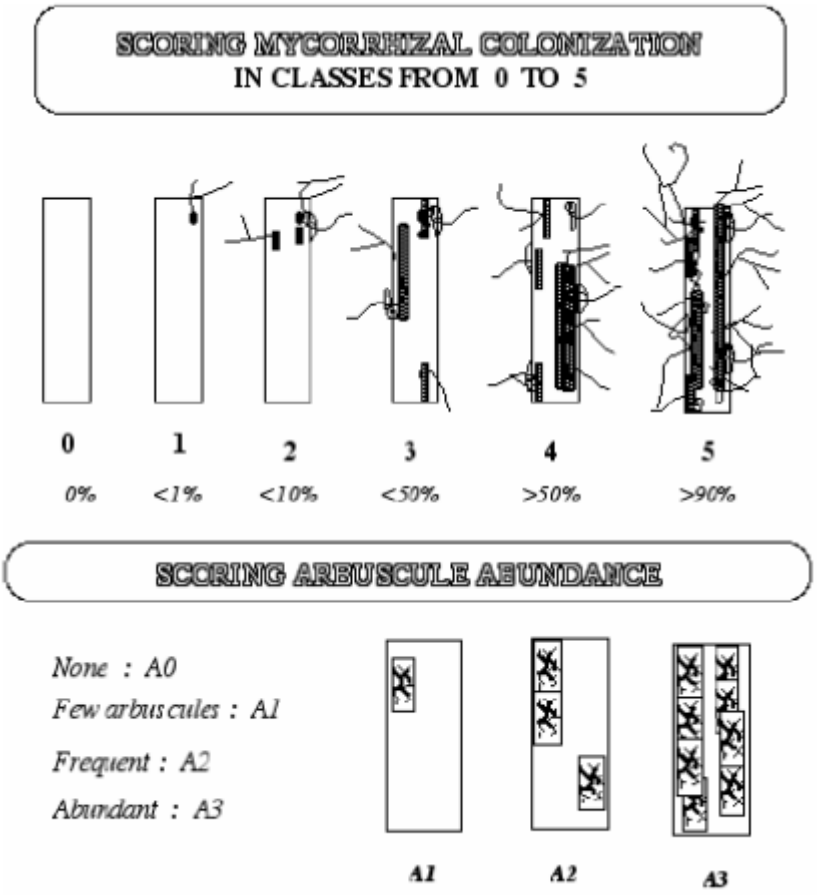


Fig. 7 Scoring mycorrhizal colonization and arbuscular abundance of AM fungi colonized roots

2.2.1.4 PAH adsorption and uptake by plants

Owing to the limited plant biomass, PAH sorption and uptake in plant tissue were only analyzed on a composite sample per treatment.

PAHs adsorbed on root surface and in root tissue.

PAHs adsorbed on the roots were extracted with chloroform at room temperature, according to Binet et al. (2000). 0.1 g root (dry weight) and 8 ml chloroform were put in a Teflon tube and shaken. At 25 min time internals, the chloroform extracts were replaced by a new equal volume of chloroform. The supernatant was transferred to a 50 ml flask, and 8 ml chloroform was added in the tube. The step was repeated 4 times. All the supernatants were collected in the flask, and chloroform added to reach a volume of 50 ml. After homogenizing the solution, 1 ml of filtered (0.22 μm) solvent solution was dried under nitrogen flow, and dissolved again by 1 ml acetonitrile. The sample was stored at 4 °C. The roots in the Teflon tube were collected and dried to analyze the PAHs contained in the tissue.

The roots collected in the first step were ground with a pestle in a mortar before soxhlet extraction with chloroform (soxhlet 150 ml chloroform, 4 h). The chloroform volume was adjusted to 50 ml. After homogenizing the solution, 1 ml of filtered (0.22 μm) solvent solution was dried under nitrogen flow, and dissolved again by 1 ml acetonitrile. The samples were stored at 4 °C.

PAHs in shoot tissue

0.1 g (dry weight) shoot was ground with a pestle in a mortar and was extracted with chloroform (soxhlet, 150 ml, 4 h). The chloroform volume was adjusted to 50 ml, and 1 ml of filtered (0.22 μm) solvent solution was dried under nitrogen flow, and dissolved again by 1 ml acetonitrile. The sample was stored at 4 °C.

2.2.2 Microbial analysis

According to previous studies, PAH dissipation in the rhizospheric soil mainly relies on microbial remediation (Binet et al., 2000). Thus, the density of the microorganisms, their

community structure, especially the amount and percentage of PAH degraders in rhizosphere were investigated using a combination of culture-based and culture-independent techniques.

2.2.2.1 Quantification of PAH degraders using culture based method - most probable number (MPN)

The MPN technique was used with 96-well microplates (Fig.8) containing a mineral medium and PAHs as carbon source (Binet et al., 2000). The PAHs (180 mg PHE, 90 mg PYR and 9 mg DBA) were weighed and solved in 80 ml hexane into three sterile wide-mouth glass reagent bottles separately. The bottles were covered with aluminum foil to prevent photolysis of the PAHs and shaken in a 40 ° C water bath for 4 h (DBA for one night). Six treatments were conducted including PHE, PYR, DBA, PHE+PYR, PHE+DBA, PHE+PYR+DBA. In all the treatments (3 replicates, 40 wells), PHE, PYR and DBA were added to the microplate wells to reach the final concentrations: PHE = 500 mg kg^{-1} (50 µl PHE solution); PYR = 500 mg kg^{-1} (100 µl PYR solution); DBA = 50 mg kg^{-1} (100 µl DBA solution). The solvent was evaporated in aseptic fume hood. 1 g soil was mixed with sterile NaCl solution (0.8%, soil: solution = 1:10 (W/W)) and 6 g of glass beads per gram of soil. The mixture was shaken for 1 h, and the suspension was filtered through filter paper. The solution obtained was then filtered to 5 µm using a vacuum pump. The suspension was then diluted with NaCl solution (0.8%) to a serial dilutions (10^{-1} to 10^{-5}): 2 ml soil suspension and 18 ml NaCl solution were mixed in sterile centrifuge tube. The tube was homogenized for 1 min, and the solution collected as 10^{-1} soil suspension. Serial dilutions were prepared with 10^{-1} soil suspension as described above. A mineral medium (Bushnel Haas; Difco, 200 µl per well) was used as inorganic substrate, and 25 µl soil suspension dilution (10^{-2} to 10^{-5}) was added to each well. The microplates were incubated at 28 °C for 2 or 4 weeks. The number of positive (brown colour, which was generated by incomplete degradation of PAHs) wells and negative (no colour changed) wells

were counted using a most probable number (MPN) procedure. Absorbance was measured at 450 and 620 nm with spectrophotometer (CERE UV 900C, Bio- tek instruments. Inc, Corgié et al., 2003). The microplates were also read visually to eliminate false positive results due to air bubbles at the bottom of the wells. A computer program with McCrady standards was used to calculate MPNs.

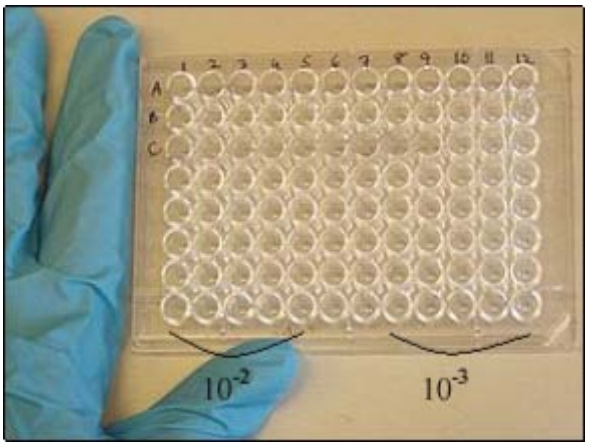


Fig. 8 The microplates for microorganism cultivation.

2.2.2.2 Quantification of bacteria and fungi and of PAH dioxygenase genes by culture independent method

Since culturable microorganisms represent a small component of the microbial community, the density and composition of microbial communities was also estimated using culture independent method. Molecular techniques, such as PCR (polymerase chain reaction) technique, have proven effective for characterizing complex microbial community in environmental samples. The PCR is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The density and community structure of bacteria, fungi and PAH

degrading bacteria were estimated by quantification of 16S rDNA, 18S rDNA and PAH-ring hydroxylating dioxygenase (RHDα) gene copies (Cébron et al., 2008).

DNA extraction from soil

Total nucleic acids isolation was performed by a modified protocol of Corgié et al. (2004). All the centrifuge tubes, tips of the micropipettes, buffers and glass beads were sterile. Soil samples (0.5 g) stored at -20 °C were loaded in 5 ml centrifuge tubes, with 800 µl of extraction buffer (100 mM Tris, 100 mM EDTA (ethylenediaminetetraacetic acid), 100 mM NaCl, 1% PVPP (polyvinylpolypyrrolidone) wt/vol, 2% SDS (sodium dodecyl sulphate) wt/vol, pH = 8), 40 µl buffer 2 (cetyltrimethylammonium bromide (CTAB) 6% and $CaCl_2$ 5 mM) and a mix of glass beads (86 mg of 3 mm diameter glass beads, 150 mg of 1 mm diameter glass beads and 160 mg of glass frit) and homogenized by mixing on a horizontal grinder at maximum speed (approx. 1 m s^{-1}) for 1 min at 4 °C. Then 800 µl phenol solution (phenol/ chloroform/ isoamyl alcohol, 25/24/1, vol/vol/vol) and 40 µl of Chelex (4%) were added, and mixed again on a horizontal grinder at maximum speed (approx. 1 m s^{-1}) for 30 s. The tube was centrifuged at 14000 g for 5 min at 4 °C, and the aqueous phase was transferred in a phase lock gel microcentrifuge tube. Chloroform solution (800 µl, chloroform/ isoamyl alcohol, 24/1, vol/vol) was added and the mixture was homogenized by mixing and then centrifuged at 14000 g for 5 min at 4 °C. The aqueous phase was transferred to a clean microcentrifuge tube. Chloroform solution (800 µl, chloroform/ isoamyl alcohol, 24/1, vol/vol) was added and the mixture was homogenized by mixing and centrifuged at 14000 g for 5 min at 4 °C. The aqueous phase was transferred to another clean microcentrifuge tube. Last step (rinsing-centrifuge-separation step) was repeated once. The remaining solution was evaluated, and an equal volume of isopropanol was added and mixed into the final microcentrifuge tube at 4 °C. The tube was incubated at 4 °C for 15 min before centrifugation at 14000 g at 4 °C

for 20 min. The supernatant was discarded, and the pellet DNA was rinsed again with 100 µl 70% ethanol. The tube was centrifuged at 14000 g for 1 min, and the supernatant discarded and the sample was air dried. The pellet DNA was resupended in 100 µl of Tris buffer (10 mM, pH = 8), and the sample stored at -20 °C for further analysis.

DNA purification

The PCR technique is sensitive, and the DNA amplification may be inhibited by soil components, including humic acids. Therefore, in order to quantify the DNA genes in rhizosphere, as well as investigate the microorganism community, Geneclean Turbo kit was employed to purify the extracted DNA according to the manufacturer instructions.

Agarose gel electrophoresis

The agarose gel electrophoresis was used to check the existence, and quantify the target DNA genes in extracts, and then to determine the volume of solution used in PCR amplification.

The steps of agarose gel electrophoresis were as follows: a 1% agarose solution was prepared (1 g agarose into 100 ml TBE (Tris/ Borate/ EDTA), dissolved in microwave), and cooled down to about 60 °C before pouring it into the gel rack. The comb was inserted at one side of the gel, about 5-10 mm from the end of the gel. The comb was carefully removed at the moment the gel formed, and the gel was put in the electronic field. The TAE buffer (a mixture of Tris base, acetic acid and EDTA, pH = 8) was added to the electronic field until it covered the gel. DNA ladder (molecular weight markers) and the samples were mixed with BET solution and loaded on the gel, and then the electricity field started (voltage: 100V, 2-10 V cm^{-1}). The DNA moved towards the positive anode due to its negative charges. The electric field was turned off when the blue dye bands had crossed the 2/3 of the gel, and the gels were

put in Ethidium bromide (0.5 mg l^{-1}, Biorad) solvent for 15 min. After washing the gel in water, it was illuminated with an ultraviolet (UV) lamp to view the DNA bands.

PCR technique

PCR method relies on thermal cyclying, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. It includes different steps: denaturation, annealing, extension/elongation and final elongation. In our study, both PCR and real time PCR were used, and the molecular analysis steps are shown in Fig 9.

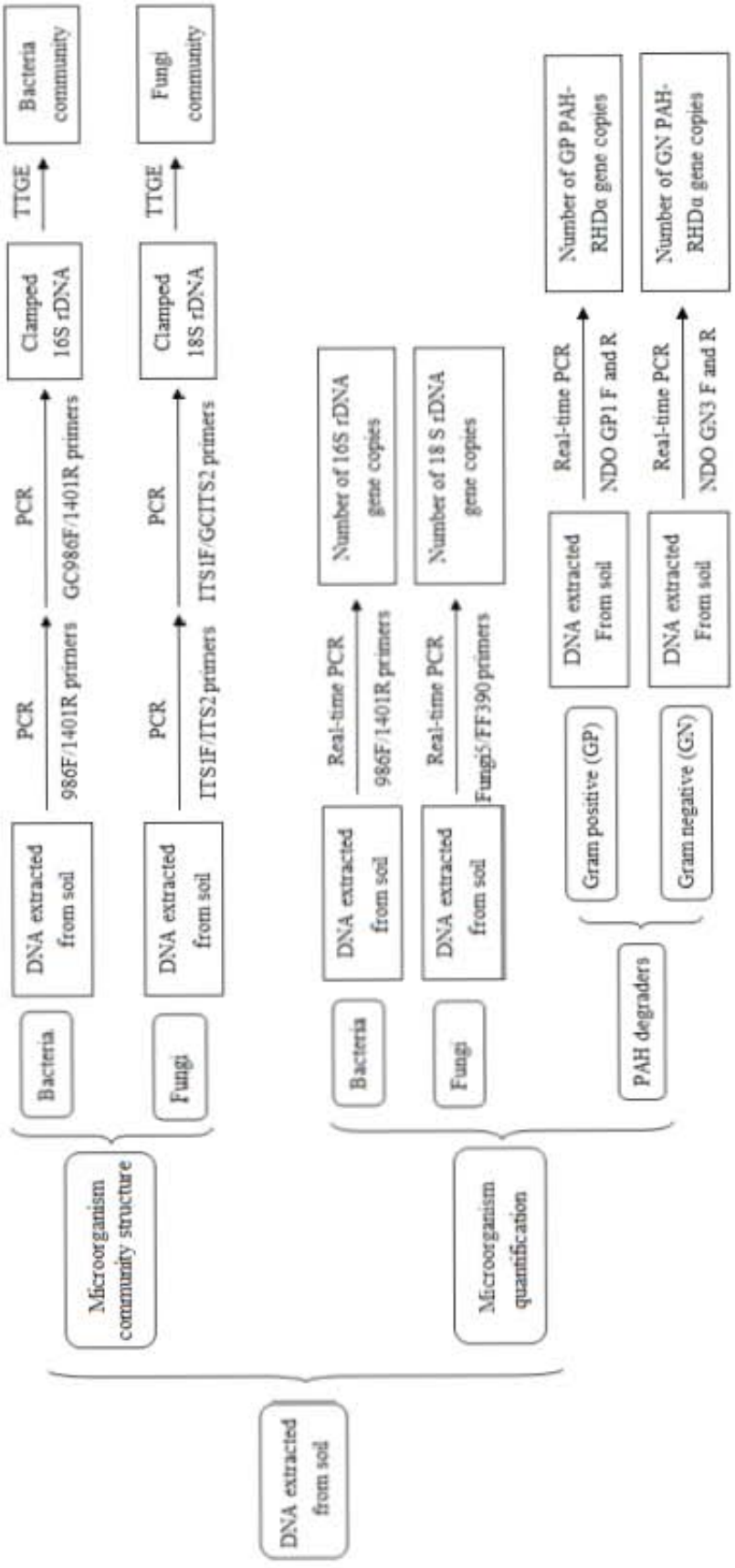


Fig. 9 The steps of molecular analysis of the soil microorganism community

Normal PCR technique was used to amplify DNA for microorganism community structure investigation with TTGE. The conditions to amplify 16S rDNA (bacteria) and 18S rDNA (fungi) are described in table 7 and 8.

Table 7 Conditions used to amplify 16S rDNA for bacteria. (a) primers (b) reagents used in each tube (c) protocol of PCR.

(a) primers

Primer	**Sequence (5'-3')**	**Amplicon size (bp)**	**Annealing temperature (°C)**
968F	AAC GCG AAG AAC CTT AC	433	58
1401R	CGG TGT GTA CAA GAC CC		
GC 968F	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG AAC GCG AAG AAC CTT AC	475	58
1401R	CGG TGT GTA CAA GAC CC		

(b) reagents in each tube

	Concentration	**Volume**
10 × Buffer (- $MgCl_2$)	Tris-HCl 50 mM KCl 10 mM	5 µl
$MgCl_2$	50 mM	1.5 µl
dNTP	10 Mm	1 µl
Primer F	20 µM	1 µl
Primer R	20 µM	1 µl
Taq	5U μl^{-1}	0.2 µl
H_2O (sterile)		39.3 µl
Samples		1 µl

(c) protocol of the PCR

Steps		**Cycles**	**Temperature**	**Time**
Denaturation		1	95	5 min
Amplification	Denaturation	35	95	30 s
	Annealing		58	30 s
	Elongation		72	40 s
Final elongation		1	72	7 min

Table 8 Conditions used to amplify 18S rDNA (a) primers (b) reagent used in each tube (c) protocol of PCR

(a) primers

Primer	**Sequence (5'-3')**	**Amplicon size (bp)**	**Annealing temperature (°C)**
ITS1F	CTT GGT CAT TTA GAG GAA GTA A	300	50
ITS2	GCT GCG TTC TTC ATC GAT GC		
ITS1F	CTT GGT CAT TTA GAG GAA GTA A	350	50
ITS2-GC	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGC TGC GCT GCG TTC TTC ATC GAT GC		

(b) reagents in each tube

	Concentration	**Volume**
10 × Buffer (- $MgCl_2$)	Tris-HCl 50 mM KCl 10 mM	5 µl
$MgCl_2$	50 mM	1.5 µl
dNTP	10 mM	1 µl
Primer F	20 µM	1 µl
Primer R	20 µM	1 µl
Taq	5U μl^{-1}	0.1 µl
H_2O (sterile)		38.4 µl
Samples		2 µl

(c) protocol of the PCR

Steps		**Cycles**	**Temperature**	**Time**
Denaturation		1	95	5 min
Amplification	Denaturation	35	95	30 s
	Annealing		50	30 s
	Elongation		72	90 s
Final elongation		1	72	10 min

Real-time PCR

Real-time PCR is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. This technique uses non-specific fluorescent dyes to bind to the target DNA, and then while the number of gene copies increases during the reaction, the fluorescence increases. In our study, the 16S rDNA gene, 18S rDNA gene, gram positive (GP) and gram negative (GN) PAH-RHDα genes were quantified, according to Cébron et al (2008). The fluorescent dye used was 1× iQ SYBR Green Supermix (Bio-Rad), and the primers to amplify 16S rDNA (bacteria), 18S rDNA (fungi) and gram positive (GP) and gram negative (GN) PAH-RHDα genes are given in table 9 (Cébron et al., 2008; Lueder et al., 2004).

The samples, as well as standards (ten times dilution series from 10^8 to 10^1 target gene copies μl^{-1} according to Cébron et al. (2008)), and two negative controls (sterile water) were prepared for the PCR amplification. Real-Time PCR were performed in 25 µl reaction volumes, with the composition in each tube described in table 10, and the protocols listed in table 11.

Table 9 Characteristics of PCR primer sets used in real time PCR

Primer	**Target gene**	**Sequence (5'-3')**	**Amplicon size (bp)**	**Annealing temperature (°C)**
968F	16 S rDNA	AAC GCG AAG AAC CTT AC	433	56
1401R		CGG TGT GTA CAA GAC CC		
Fung 5F	18S rDNA	GGG AAC CAG GAC TTT TAC	550	48
FF 390R		AGG TCT CGT TCG TTA TCG		
NDO GN3 F	Gram negative PAH-RHDα	GAG ATG CAT ACC ACG TKG GTT GGA	306	56
NDO GN3 R		AGC TGT TGT TCG GGA AGA YWG TGC MGT T		
NDO GP1 F	Gram positive PAH-RHDα	CGG CGC CGA CAA YTT YGT NGG	292	54
NDO GP1 R		GGG GAA CAC GGT GCC RTG DAT RAA		

Table 10 The reagents in each tube (real time PCR)

	Concentration	Volume (per PCR tube)
iQ SYBR GREEN SuperMix (BioRad)	2×	12.5µl
Primer F	10 µM	1µl
Primer R	10 µM	1µl
BSA	3%	0.75 µl
DMSO	100%	0.5 µl
T4gp32		0.1 µl
H_2O	Sterile	8.15 µl
Sample		1 µl

Table 11 Protocols of real time PCR of different target genes (a) 16S rDNA genes, (b) 18S rDNA genes, (c) PAH-RHDα GN, (d) PAH-RHDα GP.

(a) 16S rDNA

Steps		Cycles	Residence time	Temperature (°C)
Denaturation		1	5 min	95.0
Amplification	1	50	30 s	95.0
	2		30 s	56.0
	3		30 s	72.0
	4		10 s	82.0
Elongation		1	7 min	72.0
Melting curve		1	30 s	95.0
Analysis		88	10 s	95.0 ⟶ 51.0 decrease 0.5 °C per cycle

(b) 18S rDNA

Steps		Cycles	Residence time	Temperature (°C)
Denaturation		1	5 min	95.0
Amplification	1	50	30 s	95.0
	2		30 s	48.0
	3		1 min	72.0
Elongation		1	7 min	72.0
Melting curve		1	30 s	95.0
analysis		94	10 s	95.0 ⟶ 48.0 decrease 0.5 °C per cycle

(c) PAH-RHDα GN

Steps		Cycles	Residence time	Temperature (°C)
Denaturation		1	5 min	95.0
Amplification	1	50	30 s	95.0
	2		30 s	56.0
	3		30 s	72.0
	4		10 s	82.0
Elongation		1	7 min	72.0
Melting curve		1	30 s	95.0
analysis		88	10 s	95.0 ⟶ 51.0 decrease 0.5 °C per cycle

(d) PAH-RHDα GP

Steps	Repeat	Cycles	Residence time	Temperature (°C)
Denaturation		1	5 min	95.0
Amplification	1	50	30 s	95.0
	2		30 s	54.0
	3		30 s	72.0
	4		10 s	82.0
Elongation		1	7 min	72.0
Melting curve		1	30 s	95.0
analysis		88	10 s	95.0 ⟶ 51.0 decrease 0.5 °C per cycle

PCR could fail for various reasons, such as contamination causing amplification of spurious DNA products, the improper concentration of DNA extraction samples and so on. Thus, bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO), which could combine with inhibitors, were added into the reaction.

The output of the real time PCR reaction was a graph showing the number of PCR cycles against the increasing fluorescence. The threshold line was set up after amplification, the cycle number of each sample crossing the threshold was recorded, and the quantity of the target genes in the tested samples was evaluated based on the standard curve.

Temporal temperature gradient electrophoresis (TTGE)

TTGE was first introduced by Yoshino et al. (1991). It is based on the sequence-specific melting behavior of wild-type and mutant DNA in a temporal temperature gradient that increases gradually in a linear fashion over the length of the electrophoresis. This technique has been used to study bacterial and fungal community structure in soil (Gray et al. 2003; Corgié et al., 2004; Corgié et al. ,2006c; Cébron et al., 2009), and the presence-absence of bands is generally well suited for comparing profiles when they are really different with enough species not shared between the samples. In our experiment, this technique was used to compare the effects of different PAHs and plants on microorganism community structure in PAH contaminated soil. The protocol was as follows:

Acrylamide bisacrylamide gel preparation. Urea (7 M for bacteria, 5.5 M for fungi) (12.6g for bacteria, 9.9 g for fungi) was solved in 20 ml hot water. Add 0.75 ml 50 ×TAE (50 mM Tris-acetate, 2.5 mM EDTA, pH 8.0) buffer, 0.6 ml glycerol and 6 ml acrylamide-bisacrylamide were added into the tube. The mixture was homogenized and water added to obtain a final volume of 30 ml. The solution was filtered through 4.5 µm filter, and 300 µl APS (10%, Amnonium persulfate, Ammonium peroxodisulfalte, and ACS reagent) and 30 µl TEMED (N, N, N', N' - tetramethyl ethylenediamine) were added and the mixture was homogenized. The solution was added into the electrophoresis frame and left until the gel solidification. The gels were deposited in the tank of the Universal Dcode Mutation Detection System (Bio-Rad) filled with 1.25 × TAE running buffer.

TTGE process. Approximately 50 ng (around 10-15 µl volume) of GC- clamped DNA samples were deposited in wells of the polyacrylamide gels after mixing with loading buffer (loading buffer: bromophenol blue 0.5% xylene cyanol and 0.5% glycerol 60%). The conditions of DNA gene migration are shown in table 12.

Table 12. Conditions of 16S and 18S rDNA gene migration in TTGE

	16 S rDNA (bacteria)	**18 S rDNA (Fungi)**
Initial temperature (°C)	57	51.5
Final temperature (°C)	67	60
Increase (°C)	2	1.5
Voltage (V)	145	145

After electrophoresis, gels were washed in the tank with 1.25 × TAE buffer, and then stained with SYBR Gold nucleic acid gel stain (10000× concentrated in DMSO, Invitrogen) in 1.25 × TAE buffer for 30 min. The gels were washed in deionized water, and numerized under UV light (Geldoc). Quantification of band presence and band intensity was performed using Quality One software. Community similarities between TTGE profiles, based on the presence of the bands, were analyzed by principal component analysis (PCA).

2.2.3 PAH quantification

2.2.3.1 PAH extraction

PAH extraction in microplate experiment

After quantification of cultivable PAH degraders, the solution in microplates was collected, and the residual PAHs in each well were collected with three successive 100µl hexane extraction. The hexane solution of each extraction and the solution of the same sample (40 wells included) were mixed in a Teflon centrifuge. All the centrifuge tubes were homogenized and centrifuged at 14000g for 10 min, and then the supernatant hexane was collected and filtered (22 µm). 1 ml hexane solution was transferred into the injection vial, and dried under nitrogen flow. The PAHs in the vial were redissolved with 1 ml acetonitrile, and the PAH concentrations were analysed by HPLC.

PAH extraction in soil samples

Many investigations showed that bioavailability of organic pollutants in soil declines with time (Kesley et al., 1997; Reid et al., 2000). Thus, the exhausted extraction methods, such as Soxhlet and ASE (accelerated solvent extraction), may overestimate the accessibility of PAHs as well as their environmental (toxicity) impact. Thus, in our experiments, BuOH (1-butanol) extraction method, described by Kelsey et al. (1997) was used to estimate the bioavailable part of PAHs. The soil samples from the pot experiments were homogenized before 2 g was transferred to 50 ml Teflon centrifuge tubes. 25 ml BuOH solvent was added to each tube. The tubes were shaken for 2 h at room temperature, then centrifuged for 10 min at 7600 g, and the supernatant was filtered (22 μm). 1 ml BuOH solution was transferred into the injection vial, and dried under nitrogen flow. The PAHs in the vial were redissolved with 1 ml acetonitrile, and the sample was stored at 4°C before PAH concentration analysis by HPLC.

2.2.3.2 HPLC analysis

PAH concentration in the extracts was analyzed by reverse-phase chromatography using a Dionex HPLC system (Dionex pumps GP40) equipped with a UV-vis detector and a reverse-phase polymeric C-18 bonding column (250 mm, 4.6 mm, 5 μm). The mobile phase was a mixture of water / acetonitrile (20:80, v/v), with a flow rate of 2.0 ml min^{-1}. The wavelength used for detection was 254 nm. PAH concentrations were quantified with an external standard method.

2.2.4 Statistical analysis

Statistical analysis of the data was performed using one way (for plant biomass, AM fungi colonization and shoot phosphorus concentration) or two way (for PAH dissipation percentage and PAH-RHDα gene quantity and percentage) ANOVA followed by a Newman-

Keuls (SNK) test of xlstat 2009 to determine significant differences between treatments ($P < 0.05$). Percentage data were arcsine transformed prior to ANOVA analyses.

Partie 3

Résultats et discussion

Results and Discussion

Partie 3: Résultats et discussion – Results and Discussion

Chapitre 1: Les teneurs en eau et en phosphore influencent la dissipation des HAP dans un sol artificiellement contaminé planté de luzerne et de fétuque mycorhizées

Introduction/Objectif de l'expérience

Un certain nombre de travaux ont montré que la présence de plantes peut augmenter la biodégradation des HAP par l'influence des racines sur les communautés microbiennes dans la rhizosphère (Reilley et al., 1996). Par exemple, Nichols et al. (1997) ont constaté que les populations bactériennes en général, et les populations dégradant les hydrocarbures ont été stimulées par la croissance de la luzerne (*Medicago sativa L.*) et le pâturin alpin (*Poa alpina L.*) dans un sol contaminé par l'hexadécane, le phénanthrène, le pyrène, l'acide benzoïque, et cis-décahydronaphtalène. Cependant, dans certaines études, des effets négatifs ont été observés en présence de plantes. Par exemple, Liste et Prutz (2006) ont montré que la dissipation de HAP avait diminué en présence de pois, ou de cresson. L'échec de la phytoremédiation sur les HAP en fonction des plantes pourrait être en partie liée à la mauvaise croissance de la plante dans des sites contaminés par les HAP. Ainsi, Liste et Prutz (2006) ont obtenu la survie de 6 espèces sur 13 testées dans un sol contaminé par les HAP (2194 mg kg^{-1}). D'autre part, les HAP sont hydrophobes, et les sites contaminés sont souvent déficients en eau et en minéraux nutritifs (Li et al., 1997). Le manque d'eau peut accroître la persistance des HAP en diminuant leur transfert de masse (Phillips et al., 1999), et leur biodégradation, puisque les HAP peuvent être dégradés par les micro-organismes uniquement quand ils sont dissous dans la phase aqueuse (Johnsen et al., 2005). Une faible humidité peut également diminuer le transfert des éléments nutritifs comme le phosphore et l'azote, et

limiter la croissance et l'activité des organismes. L'addition de phosphore a permis d'augmenter la biodégradation de certains HAP (Liebeg et Cutright, 1999 ; Joner et al. 2002). Par conséquent, la teneur en eau et le phosphore biodisponible semblent être des paramètres importants pour la phytoremédiation des sols contaminés par les HAP.

La présence des champignons MA favorise la croissance des plantes dans un sol pollué par des HAP (Joner et al., 2001 ; Wu et al., 2008a). En plus de cet effet des mycorhizes sur la croissance et la nutrition minérale des plantes, ces études ont également révélé une augmentation de la dissipation des HAP en présence de champignons MA. Bien que l'implication directe des champignons MA dans le catabolisme des HAP ne soit pas clair, certains travaux ont montré que la colonisation de champignons MA pouvait augmenter l'activité oxydoréductase (Criquet et al., 2000), l'expression de dioxygénase (Corgié et al, 2006c) et modifier la structure des communautés microbiennes (Corgié et al., 2006c; Joner et Leyval, 2003).

L'objectif de cette expérience était d'étudier la dissipation de HAP dans un système plante - champignon MA dans un sol contaminé artificiellement en fonction du régime hydrique et de la concentration de phosphore. Deux plantes, préalablement décrites pour leur capacité à augmenter la dégradation des HAP (Cheema et al. 2009 ; Fan et al., 2008), la luzerne (*Medicago sativa cv. Europe*) et la fétuque (*Festuca arundinacea cv. Bariane*) ont été utilisées comme plante-hôte. Le sol a été artificiellement contaminé par un mélange de trois HAP représentatifs de composés à 3, 4 et 5 cycles aromatiques : le phénanthrène (PHE, 500 mg kg^{-1}), le pyrène (PYR, 500 mg kg^{-1}), et le dibenzo(a,h)anthracène (DBA, 65 mg kg^{-1}). Les deux plantes, cultivées en mélange, ont été inoculées par un champignon mycorhizien *Glomus intraradices*, disponible commercialement (Institüt für Pflanzenkultur, Solkau, Allemagne), et

choisi pour sa capacité à stimuler la croissance de plantes sur un sol fortement contaminé en HAP (Norini, 2007). La culture a été conduite pendant 6 semaines avec deux régimes d'arrosage et deux concentrations différentes en phosphore. Les principaux paramètres suivis ont été la colonisation mycorhizienne des racines, la croissance des végétaux, la densité des bactéries hétérotrophes et de celles dégradant les HAP, la biodégradation du PHE, du PYR et du DBA dans la rhizosphère.

Water and phosphorus content affect PAH dissipation in spiked soil planted with mycorrhizal alfalfa and tall fescue

Zhou X.B.[12], Cébron A. [1], Béguiristain, T. [1], Leyval C. [1]

[1]LIMOS, Nancy University, CNRS, BP 70239, 54506 Vandoeuvre-les-Nancy Cedex, France

[2]School of Environmental and Biological Science and Technology, Dalian University of Technology, Linggong Road, Dalian 116024, China

Published in Chemosphere, 2009, 79, 709-713.

Abstract:

Polycyclic aromatic hydrocarbon (PAH) dissipation efficiency can be increased in plant rhizosphere, but may be affected by various environmental factors. The effects of watering regime and phosphorus concentration on PAH dissipation in the rhizosphere of mycorrhizal plants were investigated in a pot experiment. Two plant species, alfalfa (*Medicago sativa*) and tall fescue (*Festuca arundinacea*), were co-cultured and inoculated with an arbuscular mycorrhizal (AM) fungus (*Glomus intraradices*) in PAH (phenanthrene (PHE) = 500 mg kg^{-1}, pyrene (PYR) = 500 mg kg^{-1}, dibenzo(a,h)anthracene (DBA) = 65 mg kg^{-1}) spiked agricultural soil for six weeks. Treatments with different phosphorus concentrations and watering regimes were compared. PHE dissipation reached 90% in all treatments and was not affected by the treatments. The major finding was the significant positive impact of mycorrhizal plants on the dissipation of high molecular weight PAH (DBA) in high water low phosphorus treatment. Such effect was not observed in high water high phosphorus and low water low phosphorus treatments where AM colonization was very low. A positive linear relationship was detected between PYR dissipation and the percentage of

gram positive PAH-ring hydroxylating dioxygenase genes in high water high phosphorus treatments, but not in the other two treatments with lower phosphorus concentration and water content. Such results indicated that phosphorus and water regime were important parameters for the dissipation of HMW-PAH.

Key words: Phytoremediation; phosphorus; water regime; PAH; arbuscular mycorrhizal fungi

1. Introduction

PAHs are recalcitrant organic pollutants consisting of two or more fused benzene rings in linear, angular, or cluster arrangements (Kanaly and Harayama, 2000). PAHs are among the most problematic substances as they could accumulate in the environment and threaten the development of living organisms because of their acute toxicity, mutagenicity or carcinogenity (Rezek et al., 2008). Among remediation techniques for PAH contaminated sites, phytoremediation has been recognized as one of the most promising methods owing to its economical and ecological benefits.

Phytoremediation uses plants and the associated rhizosphere microorganisms to transform, degrade and stabilize the toxic pollutants in the soil, sediments, water and atmosphere (Susarla et al., 2002). Promising phytoremediation results have been obtained from pots experiment and field trials (Liste and Alexander, 2000). However, due to the recalcitrant nature of PAH, multivariate and changeful environment factors, this technique is still limited in terms of effectiveness, especially when dealing with high molecular weight (HMW) PAHs (Juhasz et al., 1997; Reid et al., 2000).

Soil water potential, which has been widely recognized as a determinant factor controlling plant and soil microbial growth and activity (Børresen and Rike, 2007), is a complex factor affecting PAH degradation. Low moisture may reduce nutrient access, increase water stress and repellence, and finally compromise plant and associated microbial community growth and activity (Li et al.

1997). Low moisture may also increase PAH persistence by decreasing their mass transfer (Phillips et al., 1999), because PAHs can be degraded by microorganisms only when they are dissolved in the aqueous-phase (Johnsen et al., 2005). High soil water content may improve PAH mass transfer, but restrict oxygen diffusion and decrease the oxidation activity, especially in the deep soil layer (Phillips et al., 1999). Since the initial ring oxidation process, in which atmospheric oxygen is incorporated into the substrate by bacteria to form cis-dihydrodiols, is considered as the rate-limiting step in most of the PAH biodegradation reactions (Cerniglia, 1992), the increased water content may decrease PAH biodegradation efficiency.

Phosphorus is one of the most important inorganic nutrients limiting the bioremediation of hazardous organic compounds (Hutchinson et al., 2001). Nutrient availability is quite low in PAH polluted sites because of the hydrophobic nature of the PAHs. Increasing the nutrition level artificially in bioremediation process results in accelerated biodegradation: it is not only good for plant health but also for the stimulation of degraders in the soil (Joner et al., 2002). An increased level of phosphorus produced optimal results for cell maintenance and growth (Liebeg and Cutright, 1999) and also increased the biodegradation of some PAHs (Joner et al. 2002). Besides adjusting the nutrition level artificially, arbuscular mycorrhizal (AM) fungi inoculation is a more cost effective and environmental friendly way of improving the available phosphorus in the rhizosphere (Subramanian and Charest, 1997). AM fungi are proved beneficial for the growth of plants in PAH-polluted soils where they facilitate plant nutrition and water intake (Binet et al., 2001). They also contribute to increase PAH biodegradation in plant rhizosphere (Joner and Leyval, 2003a). The direct implication of AM fungi in PAH catabolism has not been clearly shown yet (Criquet et al., 2000), although using transformed roots, the results of Verdin et al. (2006) suggested a positive contribution of *Glomus intraradices* to anthracene dissipation in the absence of other microorganisms. Microbial community structure alteration as well as the mycorrhiza-associated

microflora could be involved in the reduction of PAH concentration in the mycorrhizosphere (Corgié et al., 2006c; Joner and Leyval, 2003).

The aim of the present study was to investigate the effect of water regime and phosphorus concentration on PAH dissipation in the rhizosphere of mycorrhizal plants in spiked soil. Phenanthrene (PHE), pyrene (PYR) and dibenzo(a,h)anthracene (DBA) were used to represent the three, four and five rings PAH. PAH dissipation, AM fungi colonization and PAH-ring hydroxylating dioxygenase (RHDα) genes were monitored.

2. Materials and methods

2.1 Soil

The soil used was a silty clay loam (Bouzule, collected in North-East part of France, 2 mm sieved) with the following characteristics: pH 7.4; organic carbon, 16 g kg^{-1}; total nitrogen, 1.7 g kg^{-1}; Olsen P, 117 mg kg^{-1}; 16 PAHs: 2.2 mg kg^{-1}). The soil was heated at 90 ºC for one hour to eliminate any indigenous AM fungi. The sand was 2 mm sieved, acid washed, rinsed and autoclaved. The soil and sand were mixed (1:1, wt/wt). The individual PAHs were dissolved in 300 ml acetone and added to 10% of the sand weight. After evaporation of the solvent, the spiked sand was mixed with the rest of the soil-sand mixture and homogenized, and reached final concentration of PHE (500 mg kg^{-1}), PYR (500 mg kg^{-1}) and DBA (65 mg kg^{-1}). Lower DBA concentration was added to reflect their relative concentrations in PAH contaminated industrial soils (Biache et al., 2008). After spiking, the soil was reinoculated with indigenous soil microflora, except mycorrhizal fungi, by adding 16 ml per pot of a non-sterile soil suspension filtered at 5 µm filter (Leyval and Binet, 1998).

2.2 Treatments

Three alfalfa (*Medicago sativa cv. Europe*) and tall fescue (*Festuca arundinacea cv. Bariane*) seeds were co-cultured in lighttight pots containing 500 g spiked soil and 20 g commercial AM fungi inoculum (*Glomus intraradices*) supplied by the Institüt für Pflanzenkultur (Solkau, Germany) as a mixture of propagules in lava substrate. A modified Hewitt nutrient solution (Redon et al., 2009) with two levels of phosphorus, named high phosphorus (0.4 mM) and low phosphorus (0.1 mM) concentration hereafter, was prepared. The experiment included 6 treatments with 5 replicates: planted pots watered up to 80% water holding capacity (WHC) when the soil dried up to 60% WHC and fertilized with high phosphorus nutrient solution (PHH); planted pots watered to 80% WHC when the soil dried up to 60% WHC and fertilized with low phosphorus nutrient solution (PHL); planted pots watered up to 80% WHC when the soil dried to 40% WHC (PLL) and fertilized with low phosphorus nutrient solution; and unplanted control of each treatment (CHH, CHL, CLL). The unplanted control pots received 20 g of autoclaved AM inoculum. Both plant seedlings were thinned to two in each pot on the 7th day after germination. The soil was covered with a layer of coarse sand to avoid PAH volatilization. Planted and unplanted pots were randomized in a growth chamber (24/20 ºC day/night, 16 h day, 80% RH, 200 - 300 μmol photons $s^{-1}m^{-2}$). The pots were fertilized with 50 ml per week of nutrient solution with the corresponding phosphorus concentration and watered by weight as described above. Six weeks after germination, pots were harvested. Alfalfa and tall fescue shoots were cut to estimate their dry weight and phosphorus concentration. Root systems were washed in 300 ml deionized water to remove the adherent particles, estimate root dry weight and AM colonization. Alfalfa and fescue shoot dry weights were measured separately, but not the roots because they were difficult to separate completely. All the soil obtained in planted treatments was considered to be rhizospheric because the roots colonized the whole volume of the pots. 0.5 g soil was kept at -20 ºC for DNA extraction,

and the rest was stored at 4 ºC for the residual PAHs concentration measurement.

2.3 Analysis

Shoot and root dry weights were determined after two days drying in an oven at 70 ºC. Frequency of mycorrhiza and arbuscular abundance in the alfalfa and fescue root systems were estimated using trypan blue staining (Koske and Gemma (1989) and Trouvelot et al. (1986)) notation method. Phosphorus concentrations in shoots were measured by ICP-OES after digestion of 0.2 g dry shoots in HNO_3 65% (4 ml) and H_2O_2 30% (2 ml) at high temperature and pressure (170 °C, 2 MPa) in a microwave digesting system (MARS 5).

The bioavailable PAHs in soil were extracted using butanol extraction method (Kelsey et al., 1997). PAH concentration in the extracts was analyzed by reverse-phase chromatography using a Dionex HPLC system (Dionex pumps GP40) equipped with a UV–vis detector and a reverse-phase polymeric C-18 bonding column (250 mm, 4.6 mm, 5 μm). The mobile phase was a mixture of water / acetonitrile (20:80, v/v), with a flow rate of 2.0 ml min^{-1}. The wavelength used for detection was 254 nm. PAH concentrations were quantified with an external standard method. Total DNA was extracted from soil-sand mixture samples using a bead beating based method as described in Cébron et al. (2008). The copy number of 16S rDNA genes as well as gram positive and gram negative PAH-ring hydroxylating dioxygenase (RHDα) genes were estimated by a SYBR Green based real-time PCR quantification using iCycler iQ (Bio-Rad) as described in Cébron et al. (2008).

2.4 Statistical analysis

Statistical analysis of the data was performed using one way (for plant biomass, AM fungi colonization and shoot phosphorus concentration) or two way (for PAH dissipation percentage and

PAH-RHDα) gene quantity and percentage) ANOVA followed by a Newman-Keuls (SNK) test on xlstat 2009 to determine significant differences between treatments ($P < 0.05$). Percentage data were arcsine transformed prior to ANOVA analyses.

3. Results

3.1 Plant biomass

No significant differences were observed in root and shoot biomass between treatments. The average shoot biomass was higher for tall fescue than for alfalfa (fig. 10).

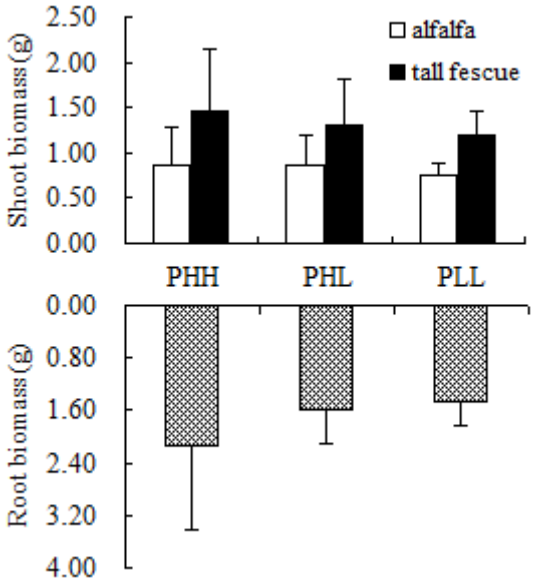


Fig. 10 Alfalfa and tall fescue shoot and root dry weight in planted (P) pots. HH: high water high P; HL: high water low P; LL: low water low P. Mean ±SD (n=5).

3.2 AM fungi colonization

For both plants, frequency of mycorrhizal roots (F%) and arbuscular abundance (A%) in the root system were significantly higher in PHL than in PHH and PLL treatments (fig. 11) ($P < 0.05$). The

arbuscule abundance (A% value) was very low in alfalfa and tall fescue root systems in PHH and PLL treatments (lower than 0.1%).

3.3 Shoot phosphorus concentration

Phosphorus concentration in alfalfa shoots significantly differed between treatments and decreased in the following order: PHH > PHL > PLL (fig. 12). The same tendency was observed for tall fescue shoots, but the differences were not significant. The phosphorus concentration in tall fescue shoots was significantly higher than in alfalfa.

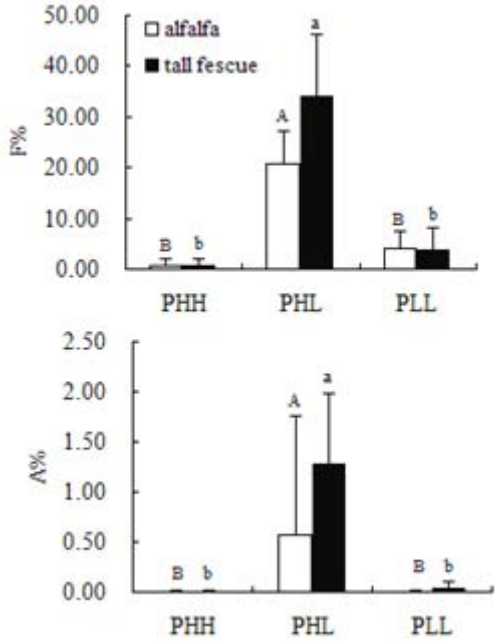


Fig. 11 Mycorrhizal fungi colonization of alfalfa and fescue inoculated roots, expressed as frequency of mycorrhizal roots (F%) and arbuscular abundance in the root system (A%). P: planted pots; C: unplanted control pots; HH: high water high P; HL: high water low P; LL: low water low P. Mean ±SD (n=5). Different letters indicate significant differences between treatments ($P<0.05$).

3.4 PAH dissipation in each treatment

After six weeks, PHE dissipation in all the treatments was above 90% (fig. 13). As to PYR, dissipation ranged between 50 and 80% depending on the treatments. No significant difference was

detected on PHE dissipation between treatments. However, ANOVA analysis indicated a negative effect of mycorrhizal plants on PYR dissipation ($P < 0.05$) in comparison to unplanted control. DBA dissipation greatly differed between treatments since it ranged between 6 and 72%. The DBA dissipation was significantly enhanced in high phosphorus treatments (HH) compared to low phosphorus ones ($P < 0.001$). Mycorrhizal plants significantly affected DBA dissipation, and a significant difference between planted and unplanted treatments was observed in high water low phosphorus treatment (HL), with 44% and 6% dissipation, respectively ($P < 0.01$).

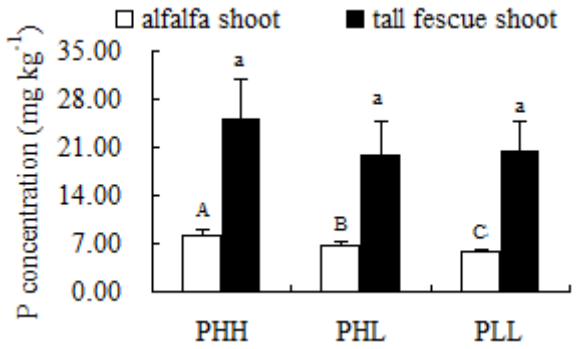
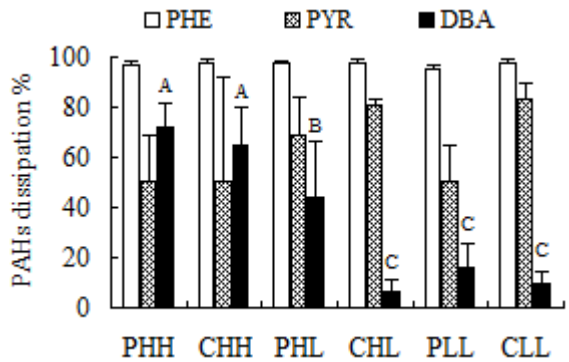


Fig.12 Shoot phosphorus concentration in different treatments. PHH: high water high P; PHL: high water low P, PLL: low water low P. (Mean and SD, n=5). Different letters indicate significant differences between treatments ($P<0.05$).



	Plant	Treatment	Plant × Treatment
PHE%	n.s.	n.s.	n.s.

PYR%	*	n.s.	n.s
DBA%	***	***	*

Fig.13 Phenanthrene (PHE), pyrene (PYR) and dibenzo-a,h-anthracene (DBA) dissipation after 6 weeks in planted (P) and control (C) pots. HH: high water high P; HL: high water low P, LL: low water low P. (Mean and SD, n=5). Different capital letters indicate significant differences between treatments for dibenzo-a,h-anthracene ($P<0.05$). ***Significant effect at $P < 0.001$; **Significant effect at $P < 0.01$; *Significant effect at $P < 0.05$.

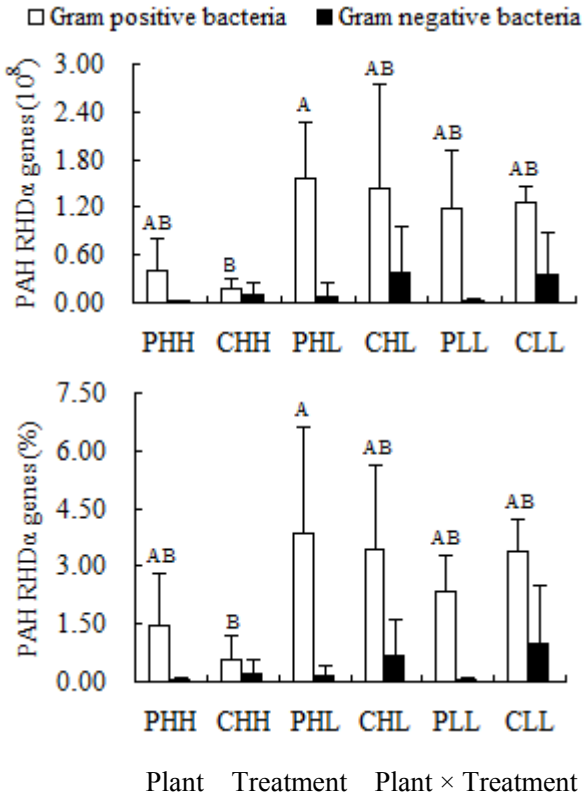
3.5 Quantification of 16S rDNA and PAH- RHDα genes

The 16S rDNA gene copy number was higher than 10^9 g^{-1} in all treatments (data not shown) with no significant difference between treatments. The number of PAH-RHDα genes of gram positive degraders overwhelmed that of gram negative degraders (fig. 14). The quantity and percentage of gram positive PAH-RHDα genes were significantly lower in high phosphorus high water treatment in comparison to low phosphorus treatments ($P < 0.05$). A positive correlation was observed between PYR dissipation and PAH-RHDα genes of gram positive bacteria in high water high phosphorus treatments ($R^2_{PHH} = 0.98$, $R^2_{CHH} = 0.94$). ANOVA analysis showed that there was no significant effect of the treatments on the gram negative PAH-RHDα gene percentage, while it was significantly lower in planted pots than in unplanted controls ($P < 0.05$).

4. Discussion

The major finding of the present study was the significant positive impact that the mycorrhizal plants had on the dissipation of high molecular weight PAH (DBA) in high water low phosphorus treatment. Such effect was not observed in high water high phosphorus (HH) and low water low phosphorus (LL) treatments where AM colonization was very low. Due to a high Kow (log Kow: 4.6; 5.2; 6.8 for PHE, PYR and DBA respectively), DBA tend to adsorb on soil particles and enter the micropores (Kelsey et al., 1997). AM fungi mycelium could enter the micro-pores of the soil,

and increase the HMW PAH dissipation by enhancing their bioavailability (Joner et al., 2001). AM fungi colonization induces various plant defense reactions, and enhances peroxidase activity which could contribute to the initial ring attack (Criquet et al., 2000), and further biodegradation of PAHs (Kraus et al., 1999). HMW-PAHs, especially those with more than five rings, hardly serve as carbon and energy sources for microbial populations during degradation (Juhasz and Naidu, 2000), and the major mechanism for their biodegradation is co-metabolism. AM fungi have been shown to enhance root exudation and change the composition of root exudates (Singer et al., 2003). The carbon sources, especially the phenolic compounds excreted by roots could induce degradation of HMW PAHs with comparable chemical structure (Singer et al., 2003; Singh et al., 2002). Finally, the physical and chemical improvement of soil structure and the alternative stimulation of microorganism community by plant and associated AM fungi could result in the increased dissipation of DBA (Corgié et al., 2006c; Joner and Leyval, 2003a).



Gram positive	n.s.	**	n.s.
Gram negative	*	n.s.	n.s.

Fig.14 PAH degrading gram positive and gram negative PAH-RHDα gene copy number g^{-1} and percentage relative to the total bacteria (16S rDNA gene copy number g^{-1}) in planted (P) and unplanted (C) pots. HH: high water high P; HL: high water low P, LL: low water low P. (Mean and SD, n=5). Different letters indicate significant differences between treatments for gram positive PAH-RHD_{α} gene copy number g^{-1} and its percentage ($P<0.05$). **Significant effect at $P < 0.01$; *Significant effect at $P < 0.05$.

The dissipation of DBA was significantly lower in LL than in HH treatment, and with high water low phosphorus (HL) differed between planted and control treatments. In unplanted treatments, DBA dissipation was lower in LL than HH and did not significantly differ between LL and HL treatment, suggesting that phosphorus concentration was a limiting factor. In planted treatments, the low water regime and low phosphorus treatment led to a reduced AM fungi colonization and to a lower phosphorus availability, as shown by the lower phosphorus concentration in alfalfa shoots in PLL than PHL treatments. This could finally contribute to the lower DBA dissipation. The lower water regime may also have reduced the DBA availability and limited the mass transfer of PAHs into the rhizosphere where the microorganisms are of higher density and motility (Masten and Davies, 1997). What's more, the lower soil water regime could decrease the availability of a number of compounds, e.g. macro and micro- mineral nutrients, and finally decrease the bacterial activity and PAH dissipation in soil (Liebeg and Cutright, 1999).

The supply of phosphorus was enhanced plant growth, microorganism community as well as the HMW PAH dissipation in some cases (Joner et al., 2002). The decreased mycorrhizal colonization resulting from higher phosphorus concentration (HH) observed in the present study was consistent with previous studies (Douds and Nagahashi, 2000). Although the phosphorus addition in this study didn't markedly increase the population of total and PAH degrading bacteria as previously reported by Carmichael and Pfaender (1997), it significantly increased DBA degradation in non-planted and planted treatments, which may be attributed to a nutritional effect on PAH degraders.

Improvement of PYR degradation in plant rhizosphere has been observed in previous studies (Liste and Alexander, 2000). However, in the present study, pyrene degradation was not increased in planted pots and was even 32% lower in planted than unplanted pots in LL treatment. This could result from the competition for nutrients and water between plants and soil microorganisms, including PYR degraders. This effect was not observed in HH treatment. A positive correlation was detected between PYR dissipation and the PAH- RHDα gram positive bacterial gene only in the HH treatment, indicating that gram positive PAH- RHDα bacteria dominated the PYR dissipation process. This was consistent with the hypothesis of Leys et al. (2005) that gram positive bacteria could outcompete for the biodegradation of most persistent HMW-PAHs. This result also confirmed that the PAH-RHDα gene copy number could well reflect PAH degradation potential of bacterial community (Cébron et al., 2008). However, no correlation was observed in the other treatments where the nutrient concentration and water content were lower. It indicated that in these treatments, water and phosphorus concentration may be limiting parameters controlling PYR dissipation.

The dissipation of PHE was not significantly affected by the presence of alfalfa and fescue or by watering regimes and phosphorus concentrations. Joner et al. (2001) also found no significant difference between mycorrhizal and non mycorrhizal clover and ryegrass treatment on anthracene (LMW PAH) dissipation, while chrysene and DBA dissipation significantly increased with mycorrhizal plants. This could be attributed to the higher availability and biodegradability of low than high molecular weight PAH. Indeed, more than 90% of PHE had disappeared after 6 weeks in unplanted controls. This high PHE dissipation already by unplanted controls might have prevented mycorrhizal plants from giving any further significant effect on biodegradation. Joner et al. (2001) showed that mycorrhizal plants contributed to the degradation of PAHs and suggested that the alteration of microbial community structure (estimated by PLFA) by mycorrhizal inoculation could

promote PAH dissipation. The mycorrhizal plants showed no effect on gram positive bacterial PAH-RHDα gene copy numbers, and reduced the PAH- RHDα gram negative ones in the present study. What's more, while the rhizosphere has been shown to benefit gram negative bacteria multiplication (Holding, 1960), PAH-RHDα genes in our soil was mainly gram positive PAH-RHDα genes. A higher percentage of gram positive than gram negative PAH degraders was also observed by Cébron et al. (accepted) on a long term field trial with PAH contaminated soil, especially in vegetated plots. In this field study, the number of PAH-RHDα genes was lower than in the present study, and the percentage of gram positive PAH-RHDα genes was lower than 1% while in our study it ranged from 1.1 to 3.8%, probably because the soil spiking favored the fast development of PAH degraders. Since the remaining PAHs were mainly HMW ones, the PAH degrader community was likely to be dominated by the gram positive PAH degraders which were efficient to degrade HMW PAHs (Leys et al., 2005).

Acknowledgements

The authors give special thanks to David Billet for PAH analysis.

Principaux résultats

Le principal résultat de cette première étude est que les plantes mycorhizées ont eu un effet positif sur la dissipation du HAP de haut poids moléculaire (DBA) dans les conditions où les teneurs en eau étaient élevées et celles en phosphore faibles. Dans cette situation, la dissipation du DBA dans les pots plantés mycorhizés a atteint 44% et seulement 6% pour les témoins sans plantes. Cet effet n'a pas été observé dans le traitement avec un niveau de phosphore et de teneur en eau élevés et dans le traitement où les teneurs en eau et en phosphore étaient faibles. Dans ces deux traitements, la colonisation des racines par le champignon MA a été très faible. Les champignons mycorhiziens sont connus pour améliorer la nutrition des plantes en éléments limitant et ainsi augmenter la concentration de ces éléments dans la plante. Ainsi, l'augmentation de la dissipation de DBA pourrait résulter en partie de l'augmentation de la croissante et l'activité des plantes, mais qui n'a pas pu être montrée dans cette expérience en absence de témoin non mycorhizés. Aucune différence significative n'a été constatée sur la densité des micro-organismes totaux ou dégradant les HAP entre le traitement planté mycorhizé et les témoins sans plantes. Une relation linéaire positive a été détectée entre la dissipation du PYR et le pourcentage de gène de HAP- dioxygénase de bactéries à Gram positif dans le traitement avec des teneurs en eau et en phosphore élevés, mais pas dans les deux autres traitements. Par ailleurs, la fertilisation en phosphore a également influencé la dissipation du DBA. Dans les traitements avec et sans plante, la dissipation du DBA était significativement plus élevée lorsque la concentration de phosphore ajoutée était élevée. Ces résultats indiquent que le phosphore et la teneur en eau, via le régime d'arrosage dans cette expérience, sont des paramètres importants pour la dissipation de HAP de haut poids moléculaire.

Chapitre 2: Capacité de dissipation des HAP de 4 plantes et influence de la mycorhization

Introduction/Objectif de l'expérience

L'expérience précédente a montré que la présence des plantes mycorhizées augmentait la dissipation du DBA dans le sol dans certaines conditions expérimentales. Cet effet bénéfique des plantes et des champignons MA associés sur la biodégradation des HAP dans le sol serait lié au moins en partie à l'amélioration de la croissance et de l'activité des microorganismes (Fan et al., 2008; Joner et al., 2001).

D'après la littérature, les plantes ont différentes capacités pour promouvoir la dégradation des HAP. Yi et Crowley (2007) ont utilisé les tissus des racines broyées de 43 plantes, et seulement quatre espèces, y compris le céleri, la pomme de terre, les radis, et les carottes, ont augmenté la dégradation de pyrène. D'une part, les racines produisent et sécrètent des enzymes et naturellement des composés polyaromatiques (flavonoïdes, coumarines, tanins …) (Bekkara et al., 1998; Yi et Crowley, 2007) qui peuvent initier la dégradation de composés aromatiques (Liste et Alexander, 2000). D'autre part, les racines reçoivent 30 à 60% du carbone photosynthétique et en libèrent 10 à 20% par rhizodéposition. La quantité et la qualité des exsudats, qui stimulent les microorganismes de la rhizosphère, est liée à de nombreux paramètres comme l'espèce, l'âge, et la taille des plantes. Les graminées ont été considérées comme des plantes efficaces pour la phytoremédiation de substances organiques des sols contaminés (Aprill et Sims, 1990; Schwab et Banks, 1994). Leur système racinaire fibreux conduit à une grande surface par unité de volume, à une plus grande surface pour la colonisation par les microorganismes qu'une racine pivotante (Anderson et al., 1993) et

permet une plus grande interaction entre la rhizosphère et les contaminants (Schwab et Banks, 1994). La luzerne est aussi considérée comme une plante efficace pour la dégradation des HAP (Fan et al., 2008). En plus de la ressource de carbone, en tant que légumineuse, la luzerne peut aussi faciliter la nutrition azotée en formant des nodules. Par ailleurs, Yi et Crowley (2007) ont montré que le céleri est une plante d'intérêt car elle contient de grandes quantités d'acide linoléique, qui en qualité d'agent surfactant ou de métabolite, peut améliorer la biodisponibilité des HAP et leur biodégradation. Toutefois, ces études sur les différentes plantes et la phytoremédiation des HAP sont effectuées dans des conditions expérimentales différentes, souvent des HAP différents, et ne prennent pas forcément en compte la mycorhization.

L'objectif de cette expérience était de comparer, dans les mêmes conditions expérimentales, l'effet de quatre plantes sur le devenir dans la rhizosphère de trois HAP représentatifs des 3, 4 et 5 cycles et d'évaluer l'effet de l'inoculation par un champignon MA. Quatre plantes, la luzerne (*Medicago sativa cv. Europe*), la fétuque (*Festuca arundinacea cv. Bariane*), le ray-grass (*Lolium multiflorum cv. Barclay*) et le céleri (*Apium graveolen*) ont été cultivées en pot dans un sol artificiellement contaminé avec du PHE (500 mg kg^{-1}), du PYR (500 mg kg^{-1}), et du DBA (50 mg kg^{-1}). Ces plantes ont été inoculées ou non par le champignon mycorhizien utilisé dans la première expérience, *Glomus intraradices*. La culture a été maintenue pendant 6 semaines à 80%-60% de la capacité de rétention en eau du sol par ajout d'eau distillée et fertilisée toutes les semaines avec une solution nutritive carencée en phosphore, c'est à dire dans les conditions expérimentales les plus favorables à mycorhization et à la dissipation des HAP selon les résultats de la première expérience. Dans cette expérience, la concentration résiduelle des HAP dans la rhizosphère, mais aussi les concentrations des HAP dans la plante et adsorbées aux racines ont été quantifiées. Les principaux paramètres suivis étaient la

colonisation mycorhizienne, la croissance des végétaux, la densité bactérienne des microflores hétérotrophes et de celle dégradant les HAP par la quantification des gènes 16S et HAP-dioxygénases, et enfin les concentrations en PHE, PYR et DBA dans la rhizosphère.

Polycyclic aromatic hydrocarbon (PAH) dissipation capacity of four plants and the impact of arbuscular mycorrhizal (AM) fungi colonization

Zhou X.B.[1,2], Cébron, A.[2], Béguiristain, T. [2], Xiang, X.M. [1], Zhou J.T. [1], and Leyval C. [2]

[1]School of Environmental and Biological Science and Technology, Dalian University of Technology, Linggong Road, Dalian 116024, China

[2]LIMOS, Nancy University, CNRS, BP 70239, 54506 Vandoeuvre-les-Nancy Cedex, France

Abstract: Alfalfa, tall fescue, ryegrass and celery, inoculated or not with the AM fungus *Glomus intraradices* were compared for their contribution to phenanthrene (PHE), pyrene (PYR), and dibenzo(a,h)anthracene (DBA) dissipation in a spiked soil. A pot experiment was conducted, where PAH concentration in soil, in plant shoots and roots, PAH sorbed to roots, and number of PAH degrading bacteria were evaluated. The results showed that PAH biotransformation was the dominant mechanism, while PAH accumulation in plant tissue was negligible and sorption was limited in comparison to biotransformation. PAH dissipation differed with plant species and decreased with the increase of PAH molecular weight. The four plant species displayed a positive effect on PHE dissipation, but only alfalfa improved PYR and DBA dissipation. The positive effect of alfalfa on PAH phytoremediation may be related to nitrogen nutrition. Although celery had been suggested as a good candidate for phytoremediation due to its content in phytochemicals, it inhibited DBA dissipation. Our results showed that plant biomass is a key parameter for evaluating plant contribution to PAH dissipation. AM inoculation increased plant biomass and phosphorus concentration, and

generally increased PHE dissipation but had no effect on the dissipation of PYR and DBA and on associated PAH degraders.

Key words: Arbuscular mycorrhizal fungi, phytoremediation, phenanthrene, pyrene, dibenzo(a,h)anthracene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread soil contaminants and typically are found as a mixture of low (< 4 rings) and high (≥ 4 rings) molecular weight chemicals (Juhasz and Naidu 2000). PAHs generates naturally and via human activities, and cause serious concern owing to their bioaccumulation property, toxicity and mutagenicity. A lot of research has been conducted to detoxify PAH contaminated soils, and phytoremediation is considered as an ecologically and economically attractive remediation technique.

Phytoremediation utilizes plants and rhizospheric microorganisms to enhance pollutant dissipation. The investigations on plant-assisted PAH bioremediation started in 1990s (Aprill and Sims, 1990). Many laboratory and greenhouse studies have been performed since then, and compelling evidence exists that plants can enhance the dissipation of PAHs. However, variable results were reported on the efficiency of plants to promote PAH dissipation, and the mechanisms of phytoremediation are still under investigation.

This rhizosphere effect may be attributed to the root exudates that contain carbon sources readily available for microorganisms and promote microbial activity (Günther et al., 1996; Rentz et al., 2004). Indeed, PAH dissipation in plant rhizosphere decreased, and bacterial density and community structure changed, with increasing distance from the roots (Joner et al., 2001; Joner and Leyval, 2003; Corgié et al. 2003; Corgié et al. 2004). Root growth and death

promote soil aeration, which can enhance oxidative degradation of recalcitrant organic compounds (Leigh et al., 2002). Several exuded compounds may act as surfactant and thus increase PAH solubility and availability (Harvey et al. 2002). Secondary plant metabolites, such as salicylate and linoleic acid could stimulate PAH-degrading enzymes or serve as co-metabolites for PAHs (Chen and Aitken 1999; Singer et al. 2003; Yi and Crowley 2007). PAH dissipation in plant rhizosphere could also be partially due to plant uptake or sorption to plant tissue. Although significant concentrations of PAHs were found in plant tissues in some experiments (Fismes et al., 2002; Watts et al., 2006; Gao et al., 2006), it seemed to contribute to a small part of PAH dissipation and PAH transport from the roots to the shoot tissue was limited (Binet et al., 2000).

The toxicity, high hydrophobicity of PAHs and the water and mineral nutrient limitations in PAH polluted sites were proved to restrain plant germination and growth (Li et al. 1997; Smith et al. 2006; Sverdrup et al. 2003). Differences between plants in tolerance to PAH toxicity, stimulation of PAH degrading bacteria or, alternatively PAH uptake, and PAH dissipation were reported (Binet et al. 2000; Liste and Prutz, 2006; Yi and Crowley 2007). However, other factors, such as PAH structure and solubility, soil physico-chemical properties, initial soil PAH concentrations and community structure and density of rhizosphere microorganisms (Chen et al. 2003; Chiapusio et al. 2007; Joner et al. 2004; Xu et al. 2006) interact with plant species and could influence the PAH dissipation in plant rhizosphere. For example, only the tissues of 4 plants, including celery, potato, radish, and carrot, out of 43 tested ones, significantly enhanced pyrene degradation in comparison to unamended control soil. This highlight the need to select PAH-tolerant and efficient plant species for PAH phytoremediation.

AM fungi help plants capturing nutrients such as phosphorus and micronutrients in soil, and play a crucial role in plant maintenance and healthiness in nutrient deficient soils (Boswell et al. 1998; Dashti et al. 2009). Positive effects of AM fungi inoculation on PAH dissipation in plant rhizosphere were recorded (Binet et al. 2000; Joner et al. 2001; Liu and Dalpé 2009; Liu et al. 2004; Wu et al. 2008a). These authors suggested that the increased PAH availability, the reduction of phosphorus and water stress, as well as the changes in the rhizosphere microorganism community and in root exudates composition could contribute to the increased PAH dissipation with AM colonized plants.

We compared the potential of four plant species, alfalfa, tall fescue, ryegrass and celery roots, which were suggested as good candidates for PAH phytoremediation in previous studies (Fang et al. 2001; Wu et al. 2008a; Yi and Crowley, 2007), to contribute to PAH dissipation and the effect of AM inoculation on plant growth and PAH dissipation. A pot experiment with PAH spiked soil was conducted for six weeks. Phenanthrene (PHE), pyrene (PYR) and dibenzo[a,h]anthracene (DBA) were used to represent the three, four and five rings PAH. The plants were inoculated or not with the AM fungus *Glomus intraradices*. Plant biomass and phosphorus concentration, PAH concentration in soil, in plant tissue and PAH sorption to roots, AM fungi colonization, 16S rDNA, 18S rDNA and PAH-ring hydroxylating dioxygenase (RHDα) genes (Cébron et al. 2008) were monitored.

2. Materials and methods

2.1 Soil

The soil used was a silty clay loam (Bouzule, collected in North-East part of France, 2 mm sieved) with the following characteristics: pH 7.4; organic carbon, 16 g kg^{-1}; total nitrogen,

1.7 g kg^{-1}; Olsen P, 117 mg kg^{-1}; 16 PAHs: 2.2 mg kg^{-1}. The soil was heated at 90 ºC for one hour to eliminate any indigenous AM fungi. The soil was mixed (1:1, wt/wt) with sand (2 mm sieved, acid washed, rinsed and autoclaved). The individual PAHs were dissolved in 300 ml acetone and added to 10% of the sand weight. After evaporation of the solvent, the spiked sand was mixed with the rest of the soil-sand mixture and homogenized, to reach final concentration of PHE (500 mg kg^{-1}), PYR (500 mg kg^{-1}) and DBA (50 mg kg^{-1}). Lower DBA concentration was added to reflect their relative concentrations in PAH contaminated industrial soils (Biache et al. 2008). After spiking, the soil/sand mixture was weighed and loaded in lighttight pots (250g/pot), and reinoculated with indigenous soil microflora, except mycorrhizal fungi, by adding 8 ml per pot of a 5 µm-filtered non-sterile soil suspension (Leyval and Binet 1998).

2.2 Treatments

The AM inoculated plants received 10 g of commercial *Glomus intraradices* inoculum supplied by the Institüt für Pflanzenkultur (Solkau, Germany) and consisting of mixed propagules in lava substrate (Redon et al. 2008). The non-AM fungi treatments and unplanted control pots received 10 g of autoclaved AM inoculum. Four planted treatments, with alfalfa (*Medicago sativa cv. Europe*), tall fescue (*Festuca arundinacea cv. Bariane*), annual ryegrass (*Lolium multiflorum cv. Barclay*) and celery roots (*Apium graveolen*), were used with or without mycorrhizal inoculation and unplanted pots were used as controls. Four seeds were sown per pot. The soil was covered with a layer of coarse sand to minimize PAH volatilization. Three replicate pots of each treatment were randomized in the growth chamber (Conviron, 24/20 °C day/night, 16 h day, 80% RH, 200-300 µmol photons $s^{-1}m^{-2}$) and re-randomized weekly. 25 ml P-deficient Hewitt nutrient solution (Redon et al. 2009) were

added weekly, and the humidity of the soil was maintained to 60-80% of the water holding capacity by regular weighing. The seedlings in each pot were thinned to 3 on the 7th day after germination.

Plants were harvested after 6 weeks. Pots were left unwatered for 2 days prior to harvest. The shoots and roots were washed carefully in 300 ml deionized water to remove any adhering soil particles. Shoots were analysed for dry weight, phosphorus concentration and PAH uptake measurements, and roots for dry weight, AM fungi colonization and PAH sorption and uptake. All the soil in the pots was considered as rhizosphere soil. 0.5 g soil was kept at -20 ºC for DNA extraction, the rest was stored at 4 ºC for the residual PAH concentration measurement.

2.3 Analyses

Frequency of mycorrhizal roots and arbuscular abundance in root systems were estimated using trypan blue staining (Koske and Gemma 1989) and Trouvelot et al. (1986) notation method. Phosphorus concentrations in shoots were measured by ICP-OES after digestion of 0.2 g (0.1 g for celery roots) dry shoots in HNO_3 65% (4 ml) and H_2O_2 30% (2 ml) at high temperature and pressure (170 °C, 2 MPa) in a microwave digesting system (MARS 5). The PAHs on the surface and in the tissue of plants were extracted following the method of Binet et al. (2000). Briefly, root samples (0.1 g dry weight) were successively extracted with 8 ml chloroform volumes at room temperature (at 25 min time intervals, the chloroform solution was replaced by a new one) for 100 min. The chloroform solutions were collected and adjusted to 50 ml. To estimate root and shoot absorption, the same root samples, as well as the shoot samples (0.1 g dry weight), were ground and extracted with 150 ml of chloroform in a Soxhlet for 4 h. The extracts were concentrated to a volume of 50 ml. Owing to the limited

plant biomass, PAH sorption and uptake in plant tissue were only analysed on a composite sample per treatment. The bioavailable PAH concentration was estimated using butanol extraction (Kelsey et al. 1997): 2 g homogenized soil and 25 ml 1-butanol (BuOH) were shaken in 50 ml Teflon centrifuge tubes at room temperature for 2 h, centrifuged at 10000 g for 10 min, and the supernatant was filtered at 22 μm. 1 ml BuOH solution was transfered into the injection vials, and dried under nitrogen flow. The PAHs in the vials were redissoved with 1 ml acetonitrile, and samples were analysed with reverse-phase chromatography using a Dionex HPLC system (Dionex pumps GP40) equipped with a UV-vis detector and a reverse-phase polymeric C-18 bonding column (250 mm, 4.6 mm, 5 μm). The mobile phase was a mixture of water / acetonitrile (20:80, v/v), with a flow rate of 2.0 ml min^{-1}. The wavelength used for detection was 254 nm. PAH concentrations were quantified with external standards. Total DNA was extracted from soil-sand mixture samples using a bead beating based method as described in Cébron et al. (2008). The copy number of 16S rDNA and 18S rDNA genes (Lueders et al. 2004) as well as gram positive and gram negative PAH-ring hydroxylating dioxygenase (RHDα) genes (Cébron et al., 2008) were estimated by a SYBR Green based real-time PCR quantification using iCycler iQ (Bio-Rad) as described in Cébron et al. (2008).

2.4 Statistical analysis

Statistical analysis of the data was performed using one way and two way ANOVA followed by a Newman-Keuls (SNK) test on xlstat 2009 to determine significant differences between means ($P < 0.05$). Percentage data were arcsine transformed prior to ANOVA analyses.

3. Results

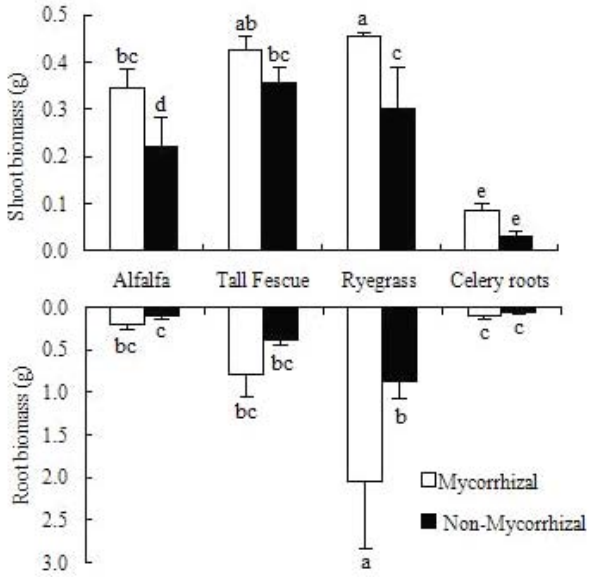
All inoculated plants were colonized by AM fungi while no mycorrhizal colonization was observed in the roots of uninoculated plants. No significant differences in colonization frequency and arbuscular abundance were observed between plants, although lower values were found with tall fescue (table 13). Plant species and AM fungi inoculation significantly influenced shoot and root biomass (Fig. 15). Celery root biomass was significantly lower than the other plants (Fig. 15). AM inoculation generally increased shoot and root biomass, and the difference was significant ($P<0.05$) for ryegrass and alfalfa shoots, and for ryegrass roots (Fig. 15). Shoot and root biomass were 1.5-2.6 and 1.9-2.4 fold higher in AM colonized than in nonmycorhizal treatments (Fig. 15). Phosphorus concentration in plant shoots was significantly higher for grasses than for the other plants (table 13). The AM fungus inoculation had no significant effect on shoot phosphorus concentration, but it significantly increased the total phosphorus quantity in shoots due to the increased shoot biomass (table 13).

Table 13. AM fungi colonization, shoot phosphorus concentration in planted treatments. Mycorrizal colonization is expressed as frequency of mycorrhizal roots (F%) and arbuscular abundance in the root systems (A%). Mean ± SD (n=3). Different letters indicate significant differences between treatments with: $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*). "n.s." no significant difference. "-" not evaluated.

Treatments		**AMF colonization**		**Shoot phosphorus**	
		F%	A%	Concentration (mg kg^{-1})	Quantity (μg/pot)
Alfalfa	AMF	21.42 ± 17.26	1.32 ± 2.10	8.36 ± 0.91 ^{b}	2.92 ± 0.61 ^{a}
	Non AMF	0.00 ± 0.00	0.00 ± 0.00	8.36 ± 0.90 ^{b}	1.87 ± 0.72 ^{a}
Tall fescue	AMF	7.77 ± 5.07	0.04 ± 0.05	18.71 ± 0.74 ^{cd}	7.97 ± 0.59 ^{bc}
	Non AMF	0.00 ± 0.00	0.00 ± 0.00	15.57 ± 0.68 ^{c}	5.57 ± 0.55 ^{b}
Ryegrass	AMF	22.22 ± 15.03	0.17 ± 0.16	21.81 ± 5.86 ^{cd}	9.97 ± 2.80 ^{c}
	Non AMF	0.00 ± 0.00	0.00 ± 0.00	24.75 ± 1.45 ^{d}	7.43 ± 1.90 ^{bc}
Celery	AMF	24.45 ± 13.47	1.05 ± 1.56	3.39 ± 0.36 ^{a}	0.29 ± 0.03 ^{a}
roots	Non AMF	0.00 ± 0.00	0.00 ± 0.00	3.10 ± 0.18 ^{a}	0.10 ± 0.02 ^{a}
ANOVA	Plant	-	-	***	***
	AMF	-	-	n.s.	**

Plant × AMF	-	-	n.s.	n.s.

After 6 weeks, the remaining concentration of PHE was generally lower in planted pots than unplanted controls, but the difference was significantly different only with alfalfa, due to large variations between replicates (Fig. 16). The ANOVA analysis showed that mycorrhizal inoculation of plants significantly increased PHE dissipation. On the contrary, there was no significant effect of plants and mycorrhizal colonization on residual PYR concentration.



ANOVA	Shoot biomass	Root biomass
Plant	***	***
AMF	***	**
Plant × AMF	n.s.	*

Fig. 15. Shoot and root dry weight in AM inoculated (+M) and uninoculated (-M) planted pots. (Mean and SD, n=3). Significant differences (P<0.05) between the AM inoculated and uninoculated groups are indicated by different letters. P < 0.001 (***); P < 0.01 (**); P < 0.05 (*).

Table 14. PHE, PYE and DBA dissipation per g of root and sorption to roots with the different plant species. PAH dissipation (mg/g root) = PAH content (mg) in unplanted pots-PAH content (mg) in planted pots) / g of roots per pot. (Mean and SD, n=3 Different lowercase letters indicate significant differences between AM and nonAM plants. Different capital letters indicate significant differences between plant species (P<0.05). Significant effect at ***P < 0.001; ** P < 0.01; * P < 0.05. "n.s." no significant difference. "-" not evaluated.

Treatments		PAH dissipation (mg PAH / g root)						PAH sorption (mg PAH / g root) (10^{-1})		
		PHE		PYR		DBA		PHE	PYR	DBA
Alfalfa	AM	129.45 ± 26.10^{ab}	A	55.35 ± 26.40^{b}	A	5.33 ± 10.50^{ab}	A	0.02	0.91	1.02
	Non AM	242.38 ± 21.40^{ab}		269.60 ± 79.49^{a}		20.96 ± 22.06^{a}		0.03	3.65	1.79
Tall fescue	AM	34.96 ± 10.19^{b}	B	6.47 ± 14.94^{bc}	B	-1.06 ± 2.03^{ab}	A	0.00	0.08	0.91
	Non AM	42.41 ± 31.01^{b}		-8.71 ± 53.06^{bc}		-1.07 ± 6.32^{ab}		0.00	0.17	0.70
Ryegrass	AM	12.20 ± 5.46^{b}	B	-4.33 ± 7.63^{bc}	B	0.30 ± 0.58^{ab}	A	0.01	0.62	0.69
	Non AM	20.65 ± 26.77^{b}		-1.49 ± 46.12^{bc}		1.43 ± 2.87^{ab}		0.00	0.75	0.72
Celery roots	AMF	227.84 ± 57.76^{ab}	A	-49.11 ± 108.07^{bc}	B	-17.99 ± 5.52^{bc}	B	0.12	6.33	0.81
	Non A	394.49 ± 311.47^{a}		-171.19 ± 152.77^{c}		-28.91 ± 17.85^{c}		0.09	1.02	0.92
ANOVA	Plant	***		***		***		-	-	-
	AM	n.s.		n.s.		n.s.		-	-	-
	Plant × AMF	n.s.		*		n.s.		-	-	-

DBA concentration did not significantly differ between mycorrhizal and nonmycorrhizal plants, but was significantly lower with alfalfa than with celery roots (Fig. 16). However, to take into account the different plant sizes, PAH dissipation in comparison to unplanted controls was expressed per gram of root ((PAH content (mg) in unplanted pots-PAH content (mg) in planted pots) / g of roots per pot) (table 14).

All plants displayed positive effects on PHE dissipation in comparison with unplanted controls, but celery roots and alfalfa were significantly more efficient than tall fescue and ryegrass. Plant effects on PYR and DBA ranged from positive to negative. A higher PYR dissipation efficiency was obtained with alfalfa in comparison to the other plants, while celery roots inhibited DBA dissipation. Among all species, only alfalfa displayed positive effects on the dissipation of the three PAHs. AM fungi inoculation significantly reduced PYR dissipation efficiency for alfalfa but had no significant effect on PAH dissipation efficiency with the other plants and PAHs. The PAH concentration in shoot tissue of the four plants was too low to be detected, while higher concentrations were detected in root tissue (table 14). Although there was no replicate, the data suggested that PYR and DBA sorption to roots was higher than PHE sorption.

The 16S rDNA gene copy number was much higher than 18S rDNA gene ones, indicating that the bacteria were dominant in the soil (table 15). The copy number of PAH-RHDα gene, expressed as a percentage of 16S rDNA genes (PAH-RHDα genes relative to 16S rDNA genes) tended to be higher in planted (0.9-1.6%) than in control treatment (0.7%). Among the PAH-RHDα genes, most of them belonged to Gram positive bacteria.

Table 15. Number of 16S, 18S rDNA and PAH- RHDα bacterial gene copies in AM inoculated, non inoculated planted and unplanted (Control) pots. Mean ± SD (n=3). ANOVA analyses showed no significant effects of plants and AM inoculation.

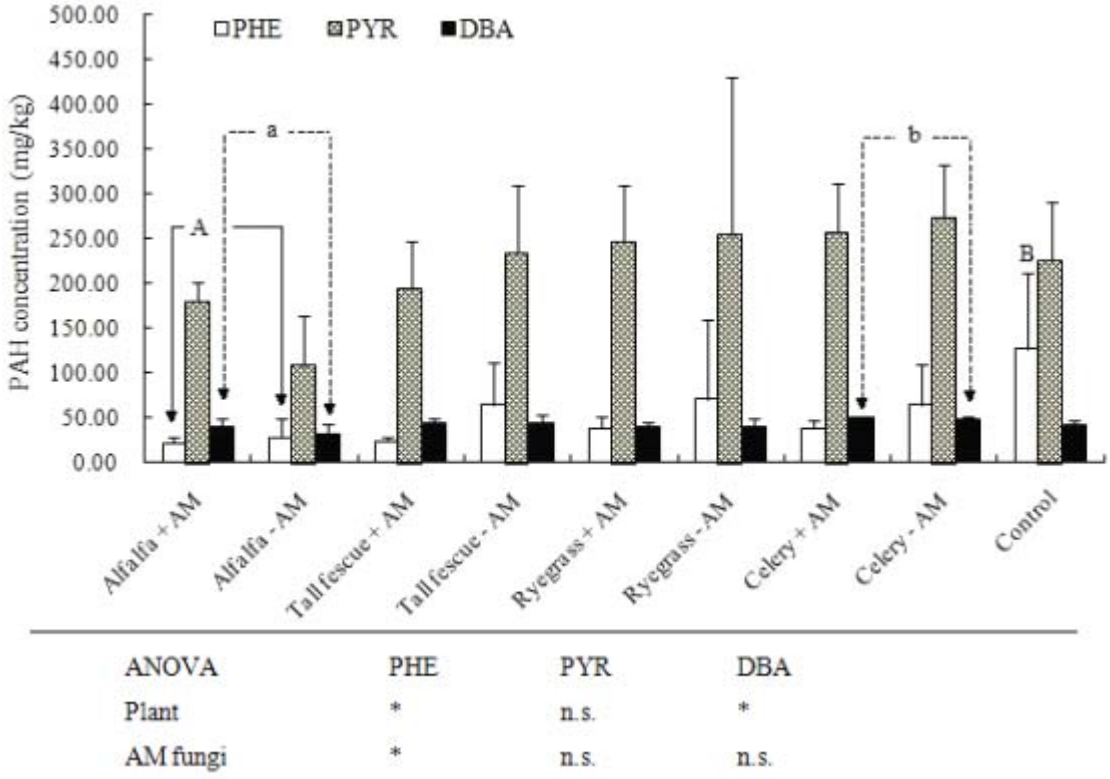
		Rhizosphere microorganisms		**PAH-RHDα bacterial genes**	
		16S rDNA (10^9 g^{-1})	18S rDNA (10^5 g^{-1})	Gram positive (10^7 g^{-1})	Gram negative (10^5 g^{-1})
Alfalfa	AMF	3.64 ± 1.30	2.95 ± 1.20	5.19 ± 3.33	0.15 ± 0.08
	Non AMF	3.97 ± 0.65	2.30 ± 0.29	4.48 ± 2.75	0.20 ± 0.20
Tall fescue	AMF	4.85 ± 0.26	5.23 ± 3.13	7.65 ± 2.44	3.91 ± 6.05
	Non AMF	3.85 ± 1.11	3.86 ± 1.25	4.28 ± 3.24	1.52 ± 1.49
Ryegrass	AMF	4.15 ± 0.58	8.56 ± 6.23	5.00 ± 2.31	0.18 ± 0.07
	Non AMF	2.99 ± 3.07	2.56 ± 1.73	2.71 ± 3.11	4.97 ± 3.91
Celery Root	AMF	2.96 ± 1.11	4.47 ± 3.44	3.03 ± 1.24	3.56 ± 4.85
	Non AMF	3.02 ± 1.99	8.94 ± 8.18	4.74 ± 5.83	1.65 ± 2.59
Control		1.77 ± 1.02	0.91 ± 0.29	1.18 ± 0.82	0.21 ± 0.20
ANOVA Plant		n.s.	n.s.	n.s.	n.s.
AMF		n.s.	n.s.	n.s.	n.s.
Plant × AMF		n.s.	n.s.	n.s.	n.s.

4. Discussion

4.1 PAH dissipation

The PAH concentration in or on plant tissue in all treatments only reached 0.003 to 0.372% of total PAH dissipation (table 14). The absence of significant uptake of PAHs by these plants was consistent with previous studies and confirmed that highly hydrophobic organic compounds (with log Kow > 3.5, such as 4.6, 5.2, 6.8 for PHE, PYR and DBA respectively) are strongly bound to the soil or root surfaces, making accumulation within plants difficult

(Wild and Jones 1992; Simonich and Hites 1995). Root sorption of PHE, PYR and DBA only accounted for 0.01%, 0.12% and 0.96% in comparison to total PAH dissipation in planted treatments. This is consistent with the study of Gao and Zhu (2004) in which the root concentration factor (PAH concentration in root relative to PAH concentration in soil) was 0.05-0.67 and 0.23-4.44 for PHE and PYR with plants grown in contaminated soils with initial concentrations of 133 and 172 mg kg^{-1}, respectively. The higher PAH sorption of the HMW PAH may be due to its higher lipophilic property and thus binding with lipids of roots (Gao et al. 2006; Su and Zhu 2008). The low PAH sorption and plant uptake confirmed that biotransformation is the dominant mechanism in PAH dissipation in plant rhizosphere. A significant decrease in PAH sorption to ryegrass roots due to AM colonization was reported by Binet et al. (2000), but such effect was not observed in the present study.



ANOVA	PHE	PYR	DBA
Plant	*	n.s.	*
AM fungi	*	n.s.	n.s.

Fig. 16 Remaining concentration of PHE, PYR and DBA in spiked soil after 6 weeks' treatment. +AM: mycorrhizal; -AM: non-mycorrhizal. (Mean and SD, n=3). The table shows plant and AM fungus effect

analysed by two-way ANOVA. Significant differences ($P<0.05$) between treatments are indicated by different capital and lowercase letters. $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*).

The percentage of dissipation decreased with PAH molecular weight increase and followed the order: PHE (mean value of all samples, 89%) > PYR (55%) > DBA (15%) (Fig. 16). As in previous investigations (Joner et al. 2001), the dissipation was higher for LMW than for HMW PAHs. Indeed LMW PAHs composed of two or three benzene rings are easily degraded through biological processes, while HMW PAHs with more than four rings hardly serve as carbon and energy source for microorganisms and are more resistant to microbial degradation (Juhasz and Naidu 2000). The major PAH-RHDα genes detected belonged to Gram positive bacteria. This result was consistent with our previous study (Zhou et al. 2009), and could be attributed to the high remaining concentration of HMW PAHs (Cébron et al. 2008) at sampling time, Gram positive bacteria being mainly involved in the degradation of HMW PAHs (Kanaly and Harayama 2000; Leys et al. 2005). The lower concentration of Gram negative bacteria genes, which could be involved in the degradation of LMW PAHs (Leys et al. 2005), could be related to the low remaining PHE concentration in all treatments. An earlier quantification of genes in the time course of the experiment would have been useful to confirm such hypothesis. In alfalfa planted pots, a linear relationship between PHE dissipation and the Gram negative PAH-RHDα genes was observed ($R^2>0.8$). Cébron et al. (2008) also detected a positive linear relationship between Gram negative PAH-RHDα genes and low molecular PAH concentration in an industrial polluted soil. However, no relationship was detected with the other plants and PAHs, indicating that the PAH degrader density was not the only factor controlling the phytoremediation efficiency.

4.2 Effect of plants on biodegradation

The ability of various plant species to stimulate PAH dissipation was highly variable ranging from negative or no effect to stimulatory effects. A positive effect of all four plants species was only obtained for PHE dissipation. Plants can generally facilitate the mobilization of LMW PAHs into the plant rhizosphere where they are likely to be more available for uptake and degradation by plant stimulated PAH degrading bacteria (Chiapusio et al. 2007). A higher efficiency of PHE dissipation was obtained with alfalfa and also with celery roots when root biomass was taken into account (table 14).

The results indicated a higher PYR dissipation efficiency with alfalfa in comparison to the other plants (table 14), however this plant effect could not be significantly detected in the pots (Fig 16). Alfalfa was considered to be an efficient plant for pyrene degradation. Fan et al. (2008) found that after 60 days of remediation, the pyrene dissipation in alfalfa rhizosphere was higher than in non-rhizosphere soils. Su et al. (2008) measured different plant effects with equal planted densities and showed that the efficiency of PYR degradation in alfalfa rhizosphere (28 %) was higher than that of tall fescue (9.9%). This effect was attributed to the higher density, and a greater activity of microorganisms in the rhizosphere than in the non-rhizosphere soil, and a possible increased bioavailability of the contaminants by root exudates (Nichols et al. 1997). In the present experiment, 16S rDNA, 18S rDNA and PAH-RHDα genes were 2.2, 2.5 and 3.4 fold higher in alfalfa planted than in control unplanted treatment respectively. PAH-RHDα gene copy numbers were also higher in alfalfa planted plots than in unplanted ones on a field trial with an industrial contaminated soil (Cébron et al. 2009). As a legume, the positive effect of alfalfa could be due to its ability to relieve the nitrogen deficiency. Nitrogen nutrient addition may increase microorganism activity and PAH dissipation (Hutchinson et al. 2001, Joner et al. 2002).

Rentz et al. (2004) showed that the PHE degrader *Pseudomonas putida* could use root products as carbon and energy source, and that the increase of PHE degraders was associated with the exudate addition. However, the presence of easily degradable carbon sources in plant rhizosphere could also prevent microorganisms from degrading the recalcitrant PAHs and partly explain the negative effect of some plant species (Corgié et al. 2006a).

The DBA dissipation showed the same tendency as PYR, with higher dissipation in presence of alfalfa than with other plants, and the celery roots displayed a negative effect on DBA dissipation. Yi and Crowley (2007) suggested celery root as an efficient plant improving HMW PAH bioavailability and biodegradation, for it contained large amounts of linoleic acid, which could act as inducer or surfactant. In their experiment, the celery planted treatment could degrade over 90% of 100 mg kg^{-1} PYR and benzo(a)pyrene within 40 and 60 days respectively.. Differences in plant size (celery roots were younger and their biomass smaller in our experiment than in the one of Yi and Crowley, 2007), growth phase and experimental conditions could affect the root exudates quantity and composition (Aulakh et al. 2001), including the release of compounds such as linoleic acid. The same plant may display different effects when employed in PAH contaminated sites with different soil properties (Chiapusio et al 2007). Besides, in stress conditions, plants may secret plant protection chemicals, such as terpenes, which have antibacterial properties and may thus reduce the PAH degradation (Yi and Crowley 2007). The different results with celery underlines the complexity of the processes controlling the fate of PAHs in plant rhizosphere.

4.3 Effect of AM inoculation on biodegradation

AM fungi colonization in all species was rather low. It was in the same order of magnitude as in a previous experiment with alfalfa and tall fescue using the same spiked soil (Zhou et al.

2009). AM fungi colonization could be affected by the toxicity and water repellence of PAHs (Cabello 1997; Chiapusio et al. 2007; Coleman et al. 1997; Debiane et al. 2008). However, in spite of the low colonization, AM inoculation significantly increased plant biomass and the quantity of phosphorous in shoots. The AM fungi improved plant fitness in PAH contaminated soil has been observed in previous studies (Leyval and Binet 1998; Joner and Leyval 2001) and could be attributed to their improvement of plant nutrition and water uptake (Binet et al. 2000).

AM colonization significantly increased PHE dissipation, but had no effect on PYR and DBA concentration. In a previous experiment with the same PAH spiked soil, the mixture of mycorrhizal alfalfa and tall fescue improved DBA dissipation in comparison to unplanted controls (Zhou et al. 2009). This was not observed here with alfalfa and fescue grown separately. PYR dissipation was 32% lower in planted than unplanted pots when watering regime and phosphorus fertilisation were low (Zhou et al. 2009). A competition for nutrients and water between plants and soil microorganisms, including PYR degraders could contribute to explain such result. In an experiment using compartment devices and sand spiked with PHE, AM inoculation increased the expression of PAH degrading genes (Corgié et al. 2006c). Joner et al. (2001) showed that AM inoculation changed bacterial community structure in a PAH contaminated soil. These results suggested that AM improved PAH dissipation could be due to changes of microbial community structure. In this experiment, we did not investigate bacterial community structure, but AM inoculation did not significantly increase the number of PAH-RHDα genes. The colonized plants were supposed to increase the PAH degrading microorganism number in rhizosphere by offering soluble carbon and phosphorus, however, the growth and activity of the PAH degraders may also be related to other nutrient, e.g. nitrogen and other macro- and micro- nutrient deficiency which may increase with the growth

of plants and the AM fungi presence, and finally became the rate-limiting factor for PAH biodegradation.

In conclusion, the PAH dissipation efficiency differed with plant species and PAH molecular weight. All the four plant species displayed a positive effect on LMW PAH (PHE), but only alfalfa showed a HMW PAH (DBA) dissipation potential. This results suggest that alfalfa may be a potential PAH phytoremediation plant. For celery roots, although this plant was suggested as a good candidate for phytoremediation due to its content in phytochemicals, opposite results were obtained. Our results show that plant biomass is a key parameter for evaluating plant contribution to PAH dissipation, and suggest that the relationship between phytochemicals and PAH dissipation merit further investigation. The *G.intraradices* inoculation increased plant biomass and phosphorus concentration, and generally increased PHE dissipation but had no effect on the dissipation of PYR and DBA and on associated PAH-RHDα genes. Further studies should focus on selecting plant-AM fungi symbionts for PAH contaminated soil phytoremediation.

Principaux résultats

Pour les quatre plantes étudiées, le champignon MA inoculé a augmenté la biomasse des parties aériennes et des racines, mais cet effet était significatif pour le ray-grass et la luzerne, et a globalement augmenté la quantité de phosphore dans les parties aériennes.

La dissipation des HAP a diminué avec l'augmentation de leur poids moléculaire, ainsi la dissipation moyenne après 6 semaines était de 89% pour le PHE, 55% pour le PYR et 15% pour le DBA. Les résultats ont montré que le mécanisme principal de dissipation (diminution de la concentration extractible) des HAP était la biodégradation ou biotransformation. L'adsorption et l'absorption des HAP dans les tissus de la plante ont représenté moins de 1% de la concentration totale dissipée des HAP. La concentration en PHE après 6 semaines était généralement plus faible dans les pots plantés que dans les témoins non plantés, mais la différence n'était significative que pour la luzerne, et était globalement plus faible dans les pots mycorhizés que les non mycorhizés. Aucun effet significatif des plantes n'a été observé sur le pyrène, et pour le DBA une différence entre les plantes a été observée avec une concentration résiduelle significativement plus faible pour la luzerne que pour le céleri. Afin de prendre en compte les différences de biomasse entre les plantes, et l'effet de la mycorhization sur la biomasse, la dissipation des HAP a été exprimée par gramme de sol d'une part, mais aussi par gramme de racine. Ce mode d'expression par gramme de racines a montré que la luzerne et le céleri étaient plus efficaces que les autres plantes pour la dissipation du PHE, mais seule la luzerne avait un effet sur le PYR et le DBA. L'effet de la mycorhization sur la dissipation des HAP n'est pas dépendant de l'effet sur la biomasse de plantes, il est donc plutôt influencé par le type de plantes et par la structure et les propriétés des HAP eux-mêmes. En conclusion, cette expérience a montré que pour les plantes testées,

un effet global a été observé sur la dissipation du PHE, mais la luzerne avait un effet plus significatif et, ramené à la biomasse produite, semble avoir un potentiel de dissipation pour le PYR et le DBA plus élevé que les autres plantes. La mycorhization par *G.intraradices* a augmenté la croissance des plantes et la quantité de P exportée dans les parties aériennes, et la dissipation du phénanthrène, mais n'a pas eu d'effet sur les HAP plus lourds.

Chapitre 3: Dégradation du phénanthrène, du pyrène et du dibenzo-a,h-antracène utilisés seuls ou en mélange comme source de carbone par les bactéries des sols plantés et non plantés

Introduction/Objectif de l'expérience

De nombreuses espèces et souches bactériennes du sol ont été décrites comme dégradant les HAP de faible poids moléculaire (≤ 3 cycles). Des genres comme *Pseudomonas*, *Burkholderia*, *Rhodoccocus*, *Mycobacterium* (Juhasz et al., 1997), *Alcaligines*, *Sphingomonas* ont été largement étudiés pour leur efficacité à dégrader dans les sols (Cutright et Lee, 1994) et même minéraliser totalement les HAP (Williams et Sayers, 1994). Ce métabolisme est inductible, et les voies métaboliques sont ainsi bien connues pour certains microorganismes et accessibles sur des bases de données. A l'opposé, les HAP de haut poids moléculaire (≥ 4 cycles) sont plus réfractaires à la biodégradation, ne sont pas dégradés directement, mais par co-métabolisme, et ce processus est lent et les voies métaboliques restent peu décrites. Le co-métabolisme, qui est défini comme la transformation d'un substrat ne servant pas à la division cellulaire en présence obligatoire d'un substrat de croissance ou d'un autre composé assimilable (Dalton et Stirling, 1982), a été observé pour des HAP de haut poids moléculaire avec des composés exsudés par les racines comme le salicylate, et un HAP de faible poids moléculaire (Chen et Aitken, 1999; Juhasz et al.,1997).

La présence des racines et les champignons MA affectent directement et indirectement les communautés bactériennes du sol et la dégradation des HAP. Les plantes et champignons MA agissent via les composés riches en carbone formés par la plante et qui sont transportés dans le sol via les racines et les hyphes des champignons MA (Corgié et al., 2006c). Les exsudats

racinaires contiennent une usine de métabolites secondaires tels que l'acide linoléique et des terpènes, qui jouent un rôle important dans le développement biologique des enzymes dégradant les polluants (Singer et al., 2003). Corgié et al. (2006c) et Joner et al. (2001) ont montré que les plantes (ray-grass) et les champignons MA (*Glomus mosseae*) augmentent le nombre de bactéries dégradantes dans la rhizosphère. Toutefois, bien que les plantes (luzerne et fétuque) inoculées par *Glomus intraradices* aient augmenté la dissipation du DBA dans notre première expérience (Chapitre 1), le nombre de bactéries dégradantes, estimé par quantification des gènes de dégradation HAP-dioxygénase n'était pas significativement plus élevé en présence de plante.

L'objectif de ces expériences d'incubation était 1) d'étudier et de comparer les communautés microbiennes des terres plantées et mycorhizées et non plantées de la première expérience, vis à vis de leur capacité à dégrader les 3 HAP, PHE, PYR et DBA et 2) d'étudier les interactions entre les HAP utilisés comme co-substrats. Pour cela, des microplaques de 96 puits ont été utilisées, contenant un ou plusieurs HAP (PHE, PYR et DBA) et un milieu minéral (Bushnel Haas; Difco), et ont été inoculées avec une suspension de sol (dilutions 10^{-2} à 10^{-5}) provenant des différents traitements de la première expérience. L'absorbance a été mesurée à 450 et à 620 nm, et la technique du nombre le plus probable (NPP) a été utilisée pour compter les bactéries cultivables dégradant les HAP. La structure des communautés a été analysée par extraction de l'ADN et par TTGE. Les HAP résiduels dans chaque puits ont été extraits à l'hexane et analysés par HPLC.

Degradation of phenanthrene, pyrene and dibenzo(a,h)anthracene used as single or combined carbon substrates by cultivable bacteria from planted and unplanted PAH spiked soil

Abstract

In a previous experiment, we studied the contribution of mycorrhizal alfalfa and tall fescue in the dissipation of phenanthrene (PHE), pyrene (PYR) and dibenzo(a,h)anthracene (DBA) in a spiked soil. Results showed that phosphorus concentration and watering regime affected the dissipation of PAHs, especially DBA. Dissipation with low phosphorus concentration and high watering regime was significantly higher with the mycorrhizal plant than with unplanted controls. The objective of the present experiment was to investigate in microplates the interactions between the co-substrate PAHs and the cultivable PAH bacteria and bacterial community structure of the soil samples from the previous experiment. The results showed that co-existence of different PAH compounds could lead to synergistic co-metabolism (PHE and DBA), no co-metabolism (PYR and DBA) and inhibition of PAH degradation (addition of PYR in PHE+DBA substrate), depending on the PAH combination used. The presence of PHE increased DBA degradation. The bacterial community structure was not significantly different when PHE and PHE+DBA were used as carbon source after four weeks of incubation, but differed when DBA alone was used as carbon source. PYR did not stimulate DBA degradation, and PYR also decreased the PHE and DBA degradation when added in PHE+DBA substrate. This experiment indicated that the soil samples from the planted pots (PHH and PHL) had a higher density of cultivable PAH degrading bacteria than the ones of unplanted pots, but no significant difference was detected in the bacterial community structure of the different soil samples.

Key words: high molecular weight PAHs; co-metabolism; rhizosphere; biodegradation; dibenzo(a,h)anthracene; phenanthrene; pyrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants highly persistent and hydrophobic in soils and sediments. There are increasing investigations on PAH contaminated site environmental impact and remediation as PAHs have been found to exhibit toxic, mutagenic and carcinogenic properties (Juhasz and Naidu, 2000).

Many bacteria have the ability to degrade low molecular weight PAHs, while a limited number of them have been shown to degrade HMW PAHs (Juhasz and Naidu, 2000). The high retention of HWM PAHs by solid soil phase results in too low mass-transfer rates to the bacterial cells to match the basic metabolic requirements of cells. Both co-metabolism and inhibition interactions were obtained with different PAHs added as co-substrate, which indicated that the degradation of a PAH could be either increased or decreased in presence of other PAHs. PAH biodegradation is also affected by environmental parameters, e.g. nutrient concentration, pH value, moisture, temperature (Yuan et al., 2002).

Plants, such as alfalfa, ryegrass and tall fescue have been proved to improve PAH dissipation by stimulating microorganisms and PAH degraders in the rhizosphere through the release of organic compounds providing carbon source and energy for microbial growth (Fan et al., 2008; Liste and Alexander, 2000; Cheema et al., 2009). Symbiosis of plants with arbuscular mycorrhizal (AM) fungi may also benefit PAH dissipation (Joner et al., 2001; Liu et al., 2004; Wu et al., 2008a). In a previous experiment, we showed that phosphorus concentration and watering regime affected the dissipation of DBA in the rhizosphere of mycorrhizal plants in a PHE, PYR and DBA spiked soil. A higher DBA dissipation was obtained in mycorrhizal planted treatment (44% dissipation) than in bulk soil with high water and low phosphorus conditions (6% dissipation). In the present manuscript, we performed microplate incubations

to study the bacterial community of the soil samples from the previous experiment, their ability to degrade LMW and HMW PAHs and the interactions between the co-substrate PAHs. Bacterial inoculum extracted from planted and unplanted soil of the previous experiment were incubated in microplates with different PAHs, single or in combination, as carbon sources. The density and community structure of cultivable PAH degrading bacteria and the PAH dissipation were measured, and the interactions between co-substrates were analysed.

2. Materials and methods

Two microplate incubations were performed to investigate the PAH degradation ability of the soil microbial community in the different experimental treatments of Zhou et al. (2009). In that experiment, alfalfa and tall fescue were cultivated on PHE, PYR and DBA spiked soil (500, 500 and 65 mg kg^{-1}respectively) and inoculated with the arbuscular mycorrhizal fungus *Glomus intraradices*. The planted pots and control unplanted pots were fertilized with Hewitt nutrient solution with "high" (0.4 mM) and "low" phosphorus (0.1 mM) nutrient solution and were watered with high (watering up to 80% water holding capacity (WHC) when the soil dried up to 60% WHC) and low watering regime (watered up to 80% WHC when the soil dried to 40% WHC). The different treatments were referred to as three successive letters: C or P for unplanted control and planted, H or L for high and low water regime, and a second H or L for high and low phosphorus concentration.

(1) In the first incubation, the bacterial community of the PHH treatment was incubated in microplates with six different carbon sources. PHE, PYR, DBA, PHE+PYR, PHE+DBA, PHE+PYR+DBA were added with the following concentrations: PHE = 500 mg kg^{-1}; PYR = 500 mg kg^{-1}; DBA = 50 mg kg^{-1}, in 96-well sterile microplates with a mineral medium (Bushnel Haas; Difco). The PAHs were dissolved in hexane solution then added to each well,

and the microplates were dried under sterile fume hood. Dilutions (10^{-2} to 10^{-5}) of 25 µl soil suspension (soil: glass beads: 0.85% NaCl solution = 1: 6: 10, w/w/w) were prepared and inoculated into 40 wells per dilution. Three replicates were made with soil samples taken from three PHH pots. Plates were incubated for 2 weeks at 28°C. PAH degraders were counted using a most probable number (MPN) procedure. Absorbance was measured at 450 and 620 nm for PAH degraders (Corgié et al., 2004). A computer program with McCrady tables was used to calculate MPNs. At the end of the incubation, the suspension was collected, and the residual PAHs in each well were collected with three successive 100µl hexane extraction. The hexane solution of each extraction was mixed with the suspension, centrifuged at 14000g for 10 min, and the supernatant hexane was collected. 1 ml hexane solution was transferred into an injection vial, and dried under nitrogen flow. The PAHs in the vial were redissolved with 1 ml acetonitrile, and the PAH concentrations were analyzed by reverse-phase chromatography using a Dionex HPLC system (Dionex pumps GP40) equipped with a UV-vis detector and a reverse-phase C-18 column (250 mm, 4.6 mm, 5 µm). The mobile phase was a mixture of water / acetonitrile (20:80, v/v), with a flow rate of 2.0 ml min^{-1}. The wavelength used for detection was 254 nm. The recovery of PAHs was 95.1%.

(2) The second incubation experiment intended to investigate the interaction between PHE and DBA, and the co-substrate effect on PAH degrading community structure. Three kinds of carbon substrates (PHE, DBA, PHE+DBA; PHE = 500 mg kg^{-1}; DBA = 50 mg kg^{-1}) were used, and soil samples of PHH, PHL and CHL treatments were incubated. The incubation was performed in 96-well microplates as described in the first incubation. Half of the microplates were harvested after two weeks, and the other half after four weeks. PAH concentration and degrader quantity were measured as described above, and the PAH degrading community

structure was analysed by DNA extraction and PCR - TTGE technology as described in Zhou et al (2009).

Statistical analysis of the data was performed using one, two and three way ANOVA followed by a Newman-Keuls (SNK) test on xlstat 2009 to determine significant differences between treatments ($P < 0.05$). The Principal Components Analysis (PCA) was performed on TTGE data to examine the similarity of PAH degrading community between treatments and carbon substrates.

3. Results

In the incubated PHH soil sample the number of cultivable PAH degraders was significantly higher when PHE had been added as carbon substrate than when PYR and DBA were used. In comparison to single PAHs, the number of cultivable PAH degrading bacteria was not significantly affected when two or three of them were added (table 16).

Table 16. Numbers of cultivable PAH degraders (MPN g^{-1} soil) in soil samples in PHH (mycorrhizal planted, high water, high phosphorus) treatment and PAH concentration in microplates after two weeks. (Mean ± SD, n=3). Different letters indicate significant differences between carbon sources ($P<0.05$).

Carbon sources in microplates	Bacterial number (MPN g^{-1} soil)	PHE (mg kg^{-1})	PYR (mg kg^{-1})	DBA (mg kg^{-1})
PHE	$(9.88\pm7.53)\times10^5$ a	181.1±20.9 b	-	-
PYR	$(6.88\pm1.98)\times10^3$ b	-	352.7±21.1 a	-
DBA	$(2.85\pm0.93)\times10^3$ b	-	-	43.4±1.5 a
PHE+DBA	$(1.14\pm0.84)\times10^6$ a	124.8±8.6 a	-	20.1±1.1 c
PYR+DBA	$(9.23\pm3.53)\times10^3$ b	-	360.2±52.8 a	42.9±2.6 a
PHE+PYR+DBA	$(7.73\pm5.00)\times10^5$ a	228±12.1 c	340.2±22.5 a	36.2±2.4 b

PAH dissipation differed with the PAHs and ranged between 54 and 75% for PHE and between 10 and 60% for DBA and reached 30% for PYR. It was significantly affected by the presence of other PAHs except PYR. The PHE residual concentration at the end of the incubation was significant lower in presence of DBA than with PHE alone (table 16). On the contrary, the residual PHE concentration was higher when the three PAHs were mixed than with PHE alone. PYR concentration at the end of the incubation did not significantly differ when PHE and DBA were present. The combination of PHE and DBA substrates significantly increased the dissipation of DBA. However, this effect was significantly reduced when PYR was present. And no significant difference in DBA dissipation was observed between the DBA alone and DBA+PYR substrates.

As observed in the first incubation experiment, the number of cultivable PAH degrading bacteria was higher with PHE than with DBA as carbon source, and the number of cultivable bacteria was even significantly higher in DBA+PHE than with PHE (table 17). The number of cultivable bacteria significantly differed with the soil samples: it was lower in CHL than PHH and PHL soil samples.

Table 17. Numbers of cultivable PAH degraders (cfu g^{-1} soil) in the soil samples of unplanted and mycorrhizal planted pots (Mean ± S.D., n=3) estimated in microplates with PHE, DBA and PHE+DBA as carbon sources. PHH: mycorrhizal planted, high water, high phosphorus; PHL: mycorrhizal planted, high water, low phosphorus, CHL: unplanted, high water, low phosphorus. Different letters indicate significant differences between treatments. Significant effect: ***: P< 0.001 **; P< 0.01 ; *: P<0.05; n.s. not significant.

Cultivable PAH degraders	**PHE**	**DBA**	**PHE+DBA**
PHH	$(2.54\pm2.89)\times10^{5}$	$(7.50\pm1.49)\times10^{3}$	$(4.40\pm2.91)\times10^{6}$
PHL	$(2.37\pm2.25)\times10^{5}$	$(5.45\pm3.34)\times10^{3}$	$(2.46\pm3.32)\times10^{6}$

CHL	(1.86±0.67) ×10^4	(1.16±0.08) ×10^3	(5.10±3.77) ×10^4

	Sig	PHH	PHL	CHL
Soil samples	***	A	A	B
	Sig	PHE	DBA	PHE+DBA
Carbon source	***	B	C	A

Note: the data were log10 transformed prior to ANOVA analyses to meet the normal distribution test.

Table 18. Phenanthrene (PHE) and dibenzo-a,h-anthracene (DBA) concentration (mg kg^{-1}) after two and four weeks of incubation in microplates with soil inoculum from PHH, PHL and CHL treatments (PHH: mycorrhizal planted, high water, high phosphorus; PHL: mycorrhizal planted, high water, low phosphorus, CHL: unplanted, high water, low phosphorus. Mean ± SD, n=3). Significant effect :***: P< 0.001 **; P< 0.01 ; *: P<0.05; n.s. no significant effect.

		After 2 weeks			**After 4 weeks**		
	Carbon source	**PHH**	**PHL**	**CHL**	**PHH**	**PHL**	**CHL**
PHE	PHE	212.4±64.4	187.6±116.0	258.0±160.4	81.2±38.4	34.0±44.7	99.5±85.3
	PHE+DBA	235.2±30.2	178.8±79.4	267.9±96.4	51.0±19.3	37.8±35.2	60.4±48.7
DBA	DBA	47.9±5.1	52.0±3.1	49.2±1.7	49.7±0.5	49.0±1.0	49.5±0.7
	PHE+DBA	32.8±2.7	23.5±9.6	30.1±10.6	30.5±1.5	18.1±10.8	25.7±11.7

ANOVA	Soil	Carbon	Time	Soil*carbon	Soil*time	Carbon*time
PHE	n.s.	n.s.	***	n.s.	n.s.	n.s.
DBA	n.s.	***	n.s.	n.s.	n.s.	n.s.

PCA analysis of TTGE data showed no significant difference of bacterial community structure between the PHE and PHE+DBA substrates, and between the soil samples after two

and four weeks of incubation (fig.17, only the data at four weeks are shown). But the bacterial community structure with DBA carbon source differed from the other sources after four weeks.

PHE concentration in the microplates significantly decreased over time with PHE and PHE+DBA carbon sources (table 18). There was no significant decrease of DBA concentration in the microplates with DBA only after two and four weeks. However, DBA concentration was significantly lower when PHE was present, as observed in the first experiment. Although the difference was not significant, the PHE and DBA degradation tended to be higher in PHL than in PHH and CHL soil samples.

4. Discussion

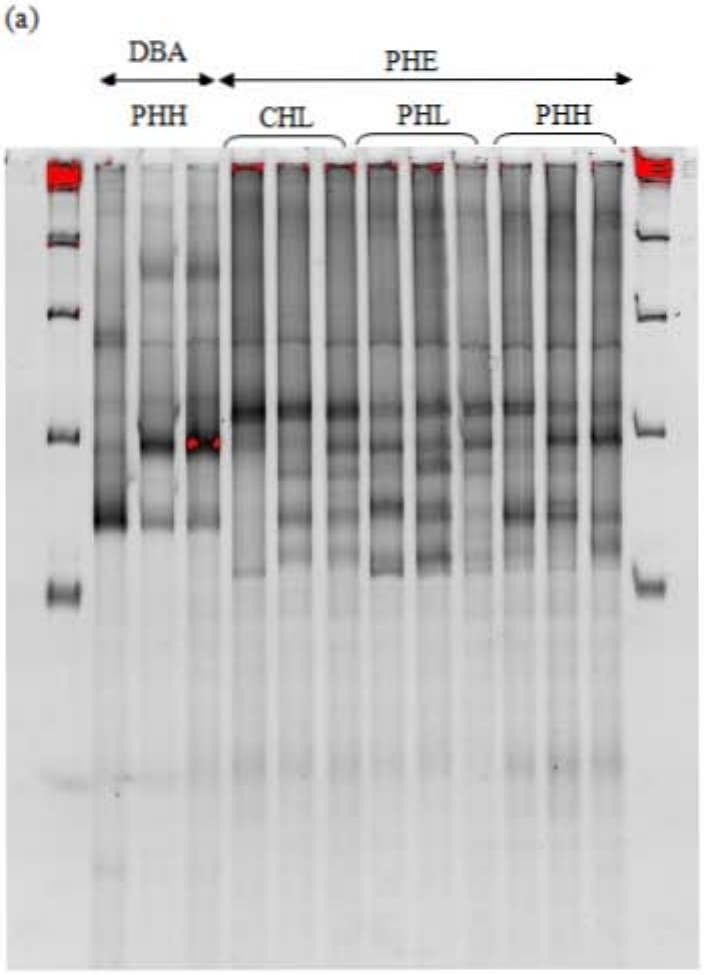
Co-metabolism is an important feature of the degradation of HMW PAHs, as it commonly occurs and widens the range of PAHs attacked by certain strains (Chen and Aitken, 1999; Juhasz et al., 1997). Microorganisms can only degrade PAHs that are dissolved in the aqueous-phase and PAHs absorbed onto or partitioned into a solid phase are not readily degraded by microorganisms (Luthy et al., 1994). The HMW PAHs, especially the ones with five rings or more, such as DBA, being highly hydrophobic, could hardly support bacteria growth and multiplication (Cerniglia, 1992). Indeed, the number of cultivable bacteria was lower when DBA and PYR were used as carbon substrate than PHE. LMW PAH-aided HMW PAH dissipation was observed by Juhasz et al. (1997) who added 100 mg kg^{-1} PHE in 50 mg kg^{-1} DBA substrate and has been observed in a number of other studies (Dean-Ross et al., 2002; Weissenfels et al., 1991). In the present study, the DBA degradation was significantly increased when PHE was added. PAH degrading bacteria could degrade but not support themselves in HMW PAHs, and could be augmented by co-metabolism (Juhasz et al., 1997).

The enzymes produced by bacteria for the degradation of PAH compounds could have a broad substrate range (Stringfellow and Aitken, 1995). Therefore, the enzymes generated for LMW PAHs could benefit the HMW PAH metabolism by accomplishment of the initial ring hydroxylation (Lopez et al., 2008; Rentz et al., 2005), which controls the rate of PAH degradation (Juhasz and Naidu, 2000). Once hydroxylated, the HMW PAHs become increasingly soluble and could be easily attacked by bacterial community (Lopez et al., 2008; Rentz et al., 2005).

PHE concentration significantly decreased between 2 and 4 weeks, indicating that PHE was degraded during the whole incubation. However, DBA concentration was significantly reduced for the first two weeks in presence of PHE but no significant difference between 2 and 4 weeks was observed. Juhasz et al. (1997) also reported that DBA degradation increased slowly over time when co-metabolised with PHE. The reduction of DBA dissipation rate with time could be due to the accumulation of intermediates, or to the low residual concentration of PHE.

The PCA analysis of TTGE data showed different bacterial community structures when PHE and DBA were brought as sole carbon substrate. This suggests that LMW and HMW PAHs were degraded by different bacterial groups as previously shown by Zhou et al. (2008) on cultivable PAH degraders from sediment in liquid culture (MSM) amended with HMW or LMW PAHs. PCR-TTGE showed no significant difference in bacterial community structure between the PHE and PHE+DBA substrates (fig.18). We can hypothesize that since PHE solubility and degradability was much higher in comparison to DBA, even if there were specific DBA degrading bacteria, they could hardly dominate the bacterial community and be observed on TTGE gels. The cultivable bacterial population in PHE+DBA substrate was

higher than with single substrates. This was associated with the increased PHE and DBA dissipation (fig.17, table 16).



(b)

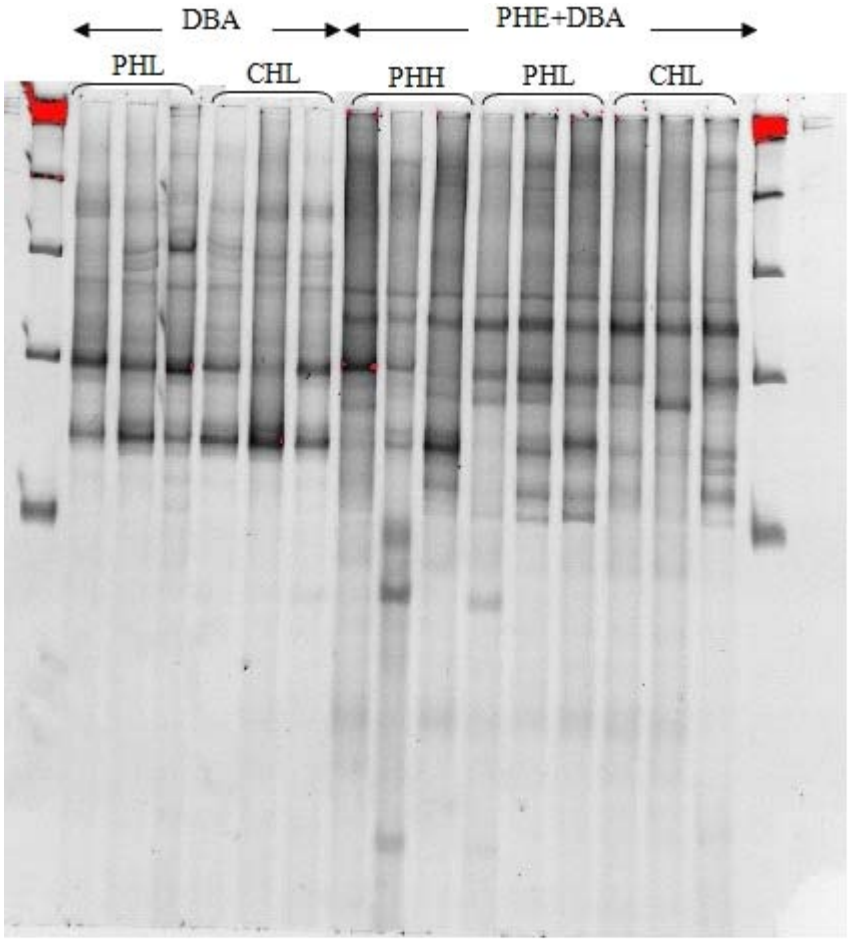


Fig. 17. TTGE gels of DNA from samples incubated for four weeks in microplates with a) DBA and PHE, b) DBA and PHE+DBA as carbons substrates. PHH: mycorrhizal planted, high water, high phosphorus; PHL: mycorrhizal planted, high water, low phosphorus, CHL: unplanted, high water, low phosphorus.

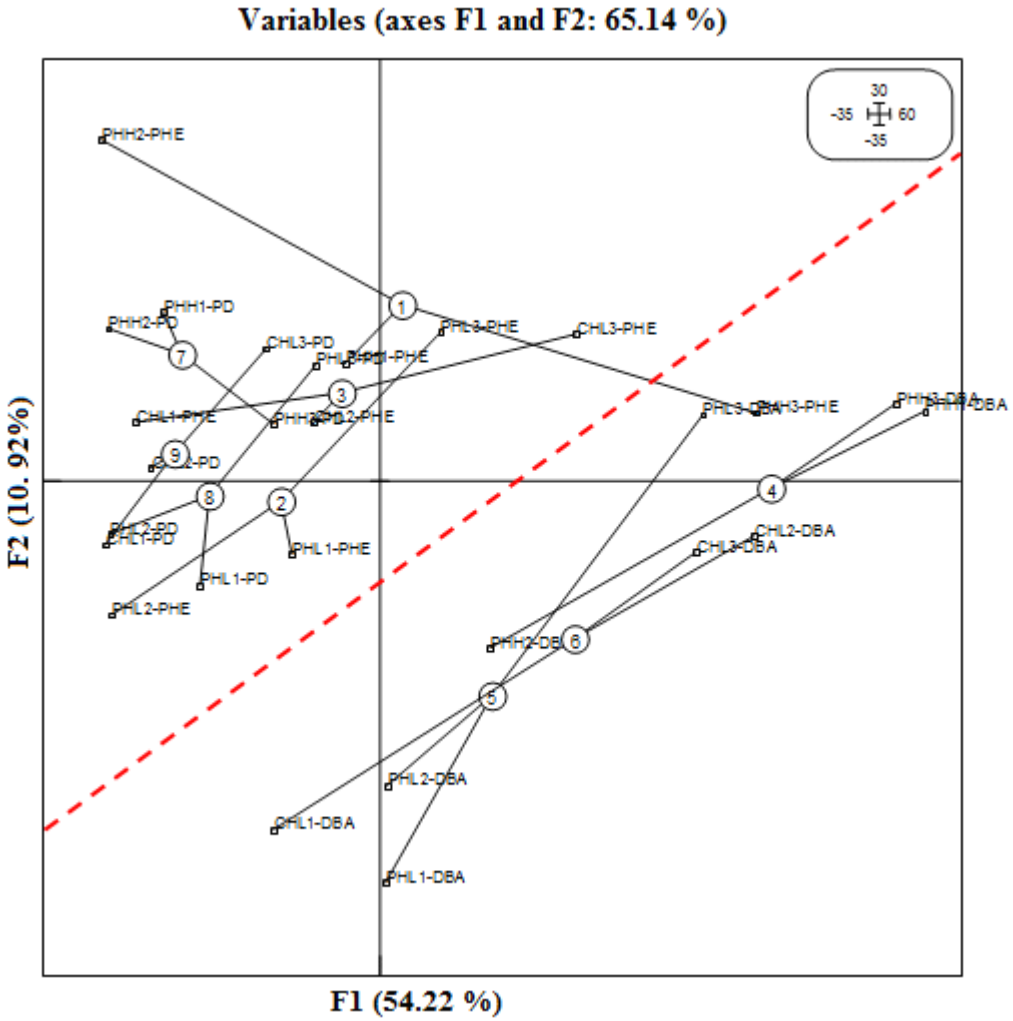


Fig. 18 Principal component analysis (PCA) of bacterial community structures based on TTGE data from samples incubated for four weeks in microplates with different PAHs . ①PHH soil, PHE substrate; ② PHL soil, PHE substrate; ③CHL soil, PHE substrate; ④PHH soil, DBA substrate ⑤ PHL soil, DBA substrate;⑥ PHH soil, DBA substrate; ⑦PHH soil, PHE+DBA substrate; ⑧PHL soil, PHE + DBA substrate; ⑨ CHL soil, PHE+DBA substrate. PHH: mycorrhizal planted, high water, high phosphorus; PHL: mycorrhizal planted, high water, low phosphorus, CHL: unplanted, high water, low phosphorus.

PAH interactions were not always favorable for degradation. The addition of PYR had no effect on DBA dissipation when PYR and DBA were combined, and even decreased PHE and DBA dissipation when it was added into PHE+DBA substrate (fig.17). An inhibition of the PAH co-substrate degradation was reported earlier (Bouchez et al., 1995), and attributed to

competition of active site of enzymes and to toxicity of additional PAHs and their metabolites (Bouchez et al., 1995; Dean-Ross et al., 2002). The inhibition of PHE and DBA degradation in presence of PYR may result from the toxicity of PYR or its intermediates, for the density of cultivable bacteria was lower and no enhancement of PYR dissipation was detected.

This experiment indicated that the soil samples from the planted pots (PHH and PHL) had a higher density of cultivable PAH degrading bacteria than the ones of unplanted pots. The higher bacterial density in planted treatment than in unplanted controls was shown in previous studies (Fan et al., 2008), and total bacteria and the PAH degraders were also stimulated in mycorrhizal rhizosphere (Corgié et al., 2006c). However, PHE and DBA degradation was not significantly higher in these incubation experiments with the microflora of PHL than CHL soil samples, which is not consistent with the results of the pot experiment (Zhou et al., 2009). According to the PCR-TTGE results, there was no significant difference in the community structure between the samples from CHL, PHH and PHL pots. In these microplate incubations, the bacterial community structure and activity seemed to depend more on the carbon substrate than on the origin of the soil samples. The difference in DBA dissipation between the mycorrhizal planted (PHL) and unplanted (CHL) soil in the pot experiment (Zhou et al., 2009) might be the result of the higher number of PAH degrading bacteria.

This experiment was performed to investigate the interactions between PAHs and to compare the bacterial community structure and PAH degrading activity of mycorrhizal planted and unplanted. Result showed that co-existence of different PAH compounds could lead to synergistic co-metabolism (PHE and DBA), no co-metabolism (PYR and DBA) and inhibition of PAH degradation (addition of PYR in PHE+DBA substrate), depending on the PAH combination used. No difference of the microorganism community was observed between the PHE and PHE+DBA substrates. The number of cultivable PHE and DBA

degraders was higher in the mycorrhizal planted than the unplanted soil samples, but no significant difference was detected in the bacterial community structure of the different soil samples. This higher number of PAH degrading bacteria could contribute to explain the results of the Zhou et al (2009), but other rhizosphere processes, which did not occur in the microplate incubations, could also be investigated.

ACKNOWLEDGEMENTS

The authors are grateful to David Billet for his assistance during PAH analysis.

Principaux résultats

Dans une première incubation, le nombre de bactéries cultivables a été quantifié dans le même échantillon de sol, en fonction de différents substrats carbonés : PHE, PYR, DBA, PHE+PYR, PHE+DBA, PHE+PYR+DBA. Le nombre de bactéries était significativement plus élevé lorsque le PHE était présent comme substrat par rapport au PYR ou au DBA. Les résultats ont montré que la co-existence des différents HAP comme source de carbone conduisait à des résultats variables en termes de dégradation des HAP: ainsi la présence de PHE augmentait la dégradation du DBA, tandis que la présence de PYR n'avait pas d'effet sur le DBA et l'addition de PYR réduisait la dégradation du mélange PHE+DBA. Dans une deuxième incubation, les échantillons de terre des pots plantés mycorhizés et des pots témoins sans plante ont été comparés. Le nombre de bactéries cultivables et dégradant le PHE et le mélange PHE+DBA était significativement plus élevé dans l'échantillon provenant des pots plantés mycorhizés. En revanche, il n'y avait pas de différence significative dans la structure des communautés bactériennes entre les échantillons plantés mycorhizés ou non plantés. Après quatre semaines d'incubation, la structure de la communauté bactérienne n'était pas significativement différente lorsque PHE et PHE + DBA ont été utilisés comme source de carbone, mais elle était différente entre DBA et PHE+DBA. Ainsi, dans l'expérience précédente (Chapitre 3-1), les différences en termes de dissipation du DBA dans les échantillons plantés mycorhizés pourraient être plutôt liées à des différences de densité et d'activité microbiennes, qu'à des communautés différentes.

Chapitre 4-1: Influence de *Glomus intraradices* et *Glomus mosseae* sur la dissipation du phénanthrène et du dibenzo-a,h-antracène dans un sol artificiellement contaminé

Introduction/Objectif de l'expérience

La présence de plantes favorise la dissipation des HAP, y compris celle de HAP de haut poids moléculaire (Yi et Crowley, 2007), et l'inoculation par un champignon mycorhizien favorise aussi cette dissipation. L'isolat de *Glomus mosseae* P2 (BEG 69) a montré un effet significatif sur la diminution de la concentration en HAP dans différents sols contaminés (Joner et al., 2001, 2003). Ce même isolat n'a pas eu d'effet significatif sur la dissipation du PHE dans une expérience où celle-ci était déjà élevée en présence de plantes non mycorhizées., mais l'expression de gène de dioxygénase était augmentée (Corgié et al., 2006c). Verdin et al. (2006) a montré une accumulation d'anthracène dans des vésicules lipidiques du champignon mycorhizien *Glomus intraradices*, dans le cas d'une culture de racines transformées d'endive mycorhizée in vitro. Les expériences précédentes ont permis de mettre en évidence une dissipation accrue du DBA en présence de luzerne et fétuque mycorhizées (Chapitre 1), et l'augmentation de la croissance des quatre plantes testées, de l'absorption du phosphore dans la plante et de la dissipation du phénanthrène (Chapitre 2), par un isolat de *Glomus intraradices*. Dans les sols contaminés par les HAP, les champignons MA pourraient jouer un rôle important dans le cycle des nutriments et stimuler les bactéries responsables de la dégradation des HAP (Joner et al., 2003; Liu et Dalpé, 2009). Les différentes espèces de champignons MA présentent une diversité fonctionnelle et des capacités différentes à promouvoir la croissance des plantes. Cette diversité fonctionnelle a été mise en évidence dans des sols contaminés en éléments en traces métalliques (Redon et al., 2008), et pour la

phytoremédiation de benzène, le toluène, l'éthylbenzène (Volante et al., 2005), mais cette diversité a été peu étudiée dans un sol contaminé par des HAP (Liu et Dalpé, 2009).

L'objectif de cette expérience était de comparer l'effet de deux isolats de champignons MA (*Glomus intraradices* et *Glomus mosseae*) sur la dissipation du PHE et du DBA. Nous avons vu dans le chapitre précédent que des interactions entre HAP pouvaient conduire à une augmentation de la dissipation de ces HAP. Par ailleurs, dans les sols contaminés, les HAP sont le plus souvent en mélange. C'est pourquoi cette étude a été réalisée en présence de DBA ou de PHE+DBA. Comme dans certaines expériences précédentes (chapitre 1) un mélange de luzerne et fétuque a été utilisé dans une culture en pot avec un sol artificiellement contaminé en HAP. La contamination du sol a été effectuée avec le DBA (50 mg kg^{-1}) et le mélange PHE (500 mg kg^{-1}) + DBA (50 mg kg^{-1}). Les deux espèces de champignons MA ont été inoculées dans la rhizosphère de la luzerne et la fétuque. La culture a été maintenue pendant 6 semaines à 80%-60% de la capacité de rétention en eau du sol par ajout d'eau distillée et fertilisée toutes les semaines avec une solution nutritive carencée en phosphore, comme dans les expériences précédentes. Les principaux paramètres suivis étaient la colonisation mycorhizienne, la densité bactérienne des microflores hétérotrophes et dégradantes estimées par quantification des gènes d'ADNr16S et de HAP-dioxygénases, la croissance des végétaux et enfin la dissipation du PHE et DBA dans la rhizosphère.

Effect of *Glomus mosseae* and *Glomus intraradices* on phenanthrene and dibenzo[a,h]anthracene dissipation in a spiked soil

X.B. Zhou[12], X.M. Xiang[2], J.T. Zhou[2], C. Leyval[1]*

[1] LIMOS, Nancy University, CNRS, BP 70239, 54506 Vandoeuvre-les-Nancy Cedex, France

[2]School of Environmental and Biological Science and Technology, Dalian University of Technology, Linggong Road, Dalian 116024, China

Abstract:

A pot experiment was conducted to assess the impact of *Glomus intraradices*, *Glomus mosseae* and co-cultured alfalfa (*Medicago sativa*) and tall fescue (*Festuca arundinacea*) on the dissipation of phenanthrene (PHE) and dibenzo[a,h]anthracene (DBA) in the soil spiked with either DBA or DBA and PHE. The results indicated that DBA dissipation in the rhizosphere of alfalfa and tall fescue was higher when PHE was present and was increased by inoculation with *G. intraradices*. Although *G. mosseae* significantly increased root biomass and gram positive PAH-ring hydroxylating dioxygenase (RHDα) gene copies, it had no significant effect on DBA dissipation. The presence of PHE greatly decreased the plant biomass and AM fungi colonization of tall fescue, indicating a lower PHE tolerance of tall fescue compared to alfalfa. The results clearly showed differences among fungi in their ability to improve DBA dissipation, and that this effect was not linked to the plant biomass increase.

Key words: PAH, AM fungi, PHE, DBA, cometabolism, PAH degrading gene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), referring to hydrocarbons with two or more fused benzene rings, are considered as a potential health risk due to their intrinsic chemical stability,

high recalcitrance to different types of degradation and high toxicity to living organisms (Jones et al. 1989). PAHs are wide-spread pollutants in the environment, and they are inclined to accumulate in soil due to their high hydrophobicity. Various methods have been used for remediation of PAH polluted soil, and phytoremediation appeared to be an appealing technology for recovery of these soil owing to its economical and ecological benefits (Reilley et al. 1996).

Phytoremediation, namely plant assisted bioremediation, has been widely studied as a potential tool in the reclamation of sites contaminated by PAHs (Liste and Prutz 2006). Plants could stimulate microbial PAH degradation by secretion of root exudates, modification of the soil physico-chemical properties, increase of rhizosphere microbial density, optimization of microorganism community structure and production of plant-derived chemicals acting as enzyme and co-metabolites (Leigh et al. 2002; Yi and Crowley 2007).

Inoculation of plants with AM symbiotic fungi, which are ubiquitous in natural and most anthropogenically influenced soils, had positive effects on PAH bioremediation. Joner and Leyval (2003a) showed that *G.mosseae* P2 BEG69 inoculation significantly decreased the concentration of 12 PAHs in comparison to uninoculated plants. The same AM fungal isolate did not significantly affect PHE dissipation in the study of Corgié et al (2006c) where the concentration of PHE in nonmycorrhizal controls was already low, but increased the expression of PAH-dioxygenase. Wu et al. (2008a) found that AM fungi colonization of alfalfa increased the dissipation of a LMW PAH (2.5, 5.0, 10.0 mg kg^{-1}PHE). Joner et al. (2001) showed a positive effect of *G.mosseae* on HMW PAH (chrysene and DBA) dissipation. Most of the studies performed with AM fungi considered single AM isolates. Different mechanisms have been proposed to explain the effect of AM fungi on PAH dissipation. The

positive effect of AM fungi on plant growth, toxicity tolerance, water and nutrient uptake in PAH polluted soils has been postulated (Leyval and Binet 1998; Zhou et al. 2009). The decrease of phosphorus deficiency and water stress by AM fungi could improve plant biomass and root exudation in PAH contaminated soils (Li et al. 1997; Liebeg and Cutright 1999; Zhou et al. 2009). Verdin et al. (2006) reported that *G.intraradices* colonising transformed carrot roots in vitro could accumulate anthracene in hyphae, and Wu et al. (2009) observed an increased phenanthrene uptake in mycorrhizal roots, suggesting that AM could promote PAH sorption or uptake in plants, while Binet et al (2000) found the opposite. AM fungi could increase the bioavailability of PAHs by growth of hyphae into small micropores. This could modify the surface penetrability and increase oxidative enzyme production (Criquet et al. 2000). The presence of AM fungi and the associated mechanisms could lead to changes in microorganism quantity, activity as well as their community structure in PAH contaminated soils (Joner et al. 2001; Joner and Leyval 2003a; Corgié et al. 2006c).

However, the PAH dissipation by plants and AM inoculated plants was not always efficient, and AM fungi species, as well as their interaction with host plants might contribute to the different results (Joner and Leyval, 2003a; Liu and Dalpé, 2009). Some AM fungi species may not be suitable for phytoremediation if they present a reduced development of extraradical mycelium and a decrease in sporulation, root colonization, and spore germination when exposed to PAHs (Verdin et al. 2006; Wu et al. 2008a). In some cases, the inoculated AM fungi failed to enhance, or even decreased the plant biomass (Binet et al. 2000; Joner et al. 2001; Wu et al. 2008a). Different AM fungi may affect root exudation of compounds, e.g. organic acids, flavonoids, differently, and thus influence PAH co-metabolism (Singer et al. 2003). Volante et al. (2005) showed that three AM fungi species had different efficiencies in benzene, toluene, ethylbenzene and xylene phytoremediation. Therefore, the selection of

appropriate AM fungi that could adapt to the polluted soil and favor the PAH degrader multiplication may be crucial for the successful use of mycorrhizal plants for PAH dissipation.

Phytoremediation efficiency is also related to different PAH properties. LMW PAHs are more easily degraded than HMW ones (Reilley et al. 1996), due to their lower hydrophobicity. PAHs with more than five rings could hardly serve as carbon source for microbes, and their mechanism of biodegradation is mainly co-metabolic (Juhasz et al. 1997). Many plant-derived chemicals, e.g. salicylate, or linoleic acid, could increase HMW PAH degradation by providing oxidase and carbon source or increasing their bioavailability (Chen and Aitken 1999; Yi and Crowley 2007). Other carbon compounds, including co-existing LMW PAHs could also act as carbon source and stimulate the HMW PAH degradation (Juhasz et al. 1997), but they could also inhibit the HMW PAHs degradation owing to their acute toxicity to plants and rhizospheric microorganisms.

We have previously shown a positive impact of mycorrhizal plants on the dissipation of DBA in a soil spiked with PHE, PYR and DBA, in comparison to unplanted control (Zhou et al., 2009). In the present experiment, we investigated the effect of two AM fungi species (*G. intraradices* and *G. mosseae*) on the dissipation of PHE and DBA as well as on PAH degrading bacteria in a pot experiment. PAH dissipation, plant biomass, AM fungi colonization, 16S rDNA and PAH-RHDα gene quantity were monitored.

2. Material and methods

2.1 Soil

The soil used was a silty clay loam (Bouzule, collected in North-East part of France, 2 mm sieved) with the following characteristics: pH, 7.4; organic carbon, 16 g kg^{-1}; total nitrogen, 1.7 g kg^{-1}; Olsen P, 117 mg kg^{-1}; 16 PAHs, 2.2 mg kg^{-1}). The soil was heated at 90 ºC for one

hour to eliminate any indigenous AM fungi. The sand was 2 mm sieved, acid washed, rinsed and autoclaved. The soil and sand were mixed (1:1, wt/wt). 10% sand were spiked with high purity either DBA or a mixture of PHE and DBA, then mixed and homogenized with the rest of mixed sand and soil to reach final concentration of 500 and 50 mg kg^{-1} of PHE and DBA, respectively. After spiking, the soil was reinoculated with indigenous soil microflora, except mycorrhizal fungi, by adding 8 ml per pot of a non-sterile soil suspension filtered at 5 μm filter (Leyval and Binet 1998).

2.2 Experimental design

Both AM fungi were isolated from metal polluted soils (Redon et al. 2009), and played positive role in PAH phytoremediation (Joner et al., 2001; Zhou et al., 2009). *Glomus intraradices* inoculum was supplied by the Institüt für Pflanzenkultur (Solkau, Germany) as a mixture of propagules in lava substrate. *Glomus mosseae* (Gerdemann & Trappe) (P2, BEG 69) inoculum consisted in spores extracted from a leek culture on the original soil at LIMOS. *G. intraradices* was inoculated by adding 10 g of inoculum substrate into each pot, while *G. mosseae* was inoculated by adding 200 spores per pot. Unplanted control, AM uninoculated planted control and *G. mosseae* inoculated planted treatments received 10 g of autoclaved inoculum substrate.

Alfalfa and tall fescue seeds were germinated in Petri dishes and 2 seedlings of each species were transplanted to each pot filled with 250 g contaminated soil three days after germination. One week after transplantation, the plants were thinned to 1 for each species. Five replicates of each treatment were prepared, and were randomized in a growth chamber (24/20 ºC day/night, 16 h day, 80% RH, 200 - 300 μmol photons $s^{-1}m^{-2}$). The pots were fertilized with 25 ml low phosphorus (0.1 mM) Hewitt nutrient solution per week, and constant soil moisture

(80% water holding capacity) was maintained through weighing and watering three times per week (Redon et al. 2009). The plants and soil were harvested after six weeks. Alfalfa and tall fescue shoots were cut to estimate their dry weight. Root systems were washed in 300 ml deionized water to remove the adherent particles, estimate root dry weight and AM colonization. Owing to the high root density in all the planted pots, all the soil was considered as rhizosphere soil. The soil was carefully collected and homogenized. 0.5 g soil was kept at -20 ºC for DNA extraction and analysis, and the rest was stored at 4 ºC for the residual PAH concentration measurement.

2.3 Analysis

Shoot and root dry weights were determined after two days drying in an oven at 70 ºC. Frequency and arbuscular abundance of mycorrhiza in alfalfa and fescue root systems were estimated using trypan blue staining (Koske and Gemma 1989) and Trouvelot et al. (1986) notation method.

The bioavailable PAHs were extracted by butanol as described in Kelsey et al. (1997). PAH concentration in the extracts was analyzed by reverse-phase chromatography using a Dionex HPLC system (Dionex pumps GP40) equipped with a UV-vis detector and a reverse-phase polymeric C-18 bonding column (250 mm, 4.6 mm, 5 μm). The mobile phase was a mixture of water / acetonitrile (20:80, v/v), with a flow rate of 2.0 ml min^{-1}. The wavelength used for detection was 254 nm. PAH concentrations were quantified with an external standard method. Total DNA was extracted from soil-sand mixture samples using a bead beating based method as described in Cébron et al. (2008). The copy number of 16S rDNA genes as well as gram positive and gram negative PAH- RHDα genes were estimated by a SYBR Green based real-time PCR quantification using iCycler iQ (Bio-Rad) as described in Cébron et al. (2008).

2.4 Statistical analysis

Statistical analysis of the data was performed using two way ANOVA followed by Newman-keuls (SNK) test on xlstat 2009 to determine significant differences between treatments ($P < 0.05$).

3. Results

3.1 Plant biomass and AM fungi colonization

The results indicated that the nature of the spiked PAH significantly influenced the growth and the AM fungi colonization for tall fescue, but not for alfalfa (table 19). AM fungi colonization and shoot biomass were significantly lower with DBA and PHE than with DBA alone. *G. intraradices* and *G. mosseae* increased tall fescue shoot biomass, but had no significant effect on alfalfa shoot biomass. The root biomass, including alfalfa and tall fescue ones, was significantly increased by *G. mosseae*. All plants inoculated by *G. intraradices* and *G. mosseae* were colonized, without any significant difference in colonization rate between the fungi in DBA and DBA+PHE treatments. Uninoculated plants were not colonized.

3.2 PAH dissipation

More than 90% PHE had disappeared after six weeks in all treatments, while DBA dissipation percentage varied from 20 to 40% (Fig. 19). PHE dissipation was not significantly different between unplanted, planted and planted and inoculated treatments. DBA dissipation was not significantly different between unplanted and uninoculated planted treatments. However, DBA dissipation was significantly increased by *G. intraradices* inoculation when DBA alone was present, but this was not observed when PHE was also present. On the contrary, the plants inoculated with *G. mosseae* had no significant effect on DBA concentration when it was the only PAH added, and even significantly reduced DBA dissipation when PHE+DBA were present, in

comparison to uninoculated plants. DBA dissipation was significantly increased by the presence of PHE (Fig. 19).

Since plant biomass was affected by AM inoculation and the presence of PHE, PAH dissipation was also expressed per g of root (Fig. 20). DBA dissipation per g of root was significantly higher with *G. intraradices* than with *G. mosseae* and non mycorrhizal plants when DBA was added alone. No significant difference was observed among different treatments when DBA and PHE were added (Fig. 19, 20).

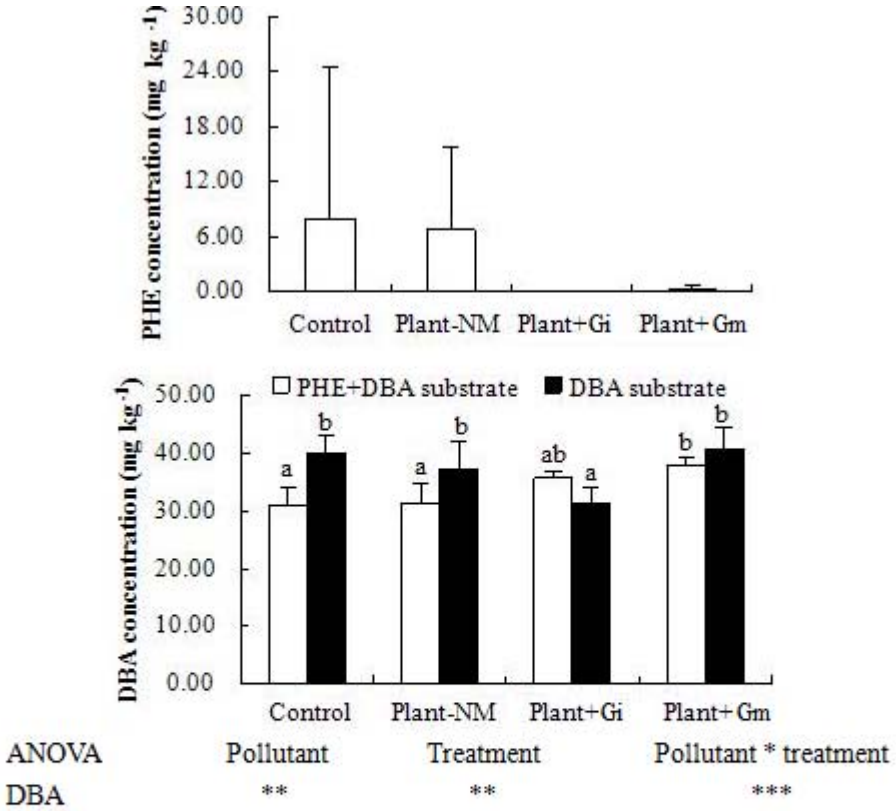


Fig. 19 Phenanthrene (PHE) and dibenzo[a,h]anthracene (DBA) concentration after 6 weeks in each treatment. NM= uninoculated plant; Gi = *G. intraradices* inoculation; Gm = *G. mosseae* inoculation. Mean ± SD (n=5). Different letters indicate significant differences of PAH concentration between groups (P<0.05). Significant effect at: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Table 19 Plant biomass and AM fungi colonization in different treatment. Mycorrizal colonization expressed as frequency of mycorrhizal roots (F %) and arbuscular abundance in the root systems (A%). NM= uninoculated plant; Gi = G. *intraradices*; Gm = *G. mosseae*. Data are presented as mean ± SD (n = 5). Different small letters indicate significant difference between groups, and different capital letters indicate significant differences between treatments (P<0.05). ANOVA analysis with significant effect at ***P < 0.001; ** P < 0.01; * P < 0.05. n.c. no colonization; n.s. not significant.

Treatments	Pollutant	Shoot biomass (g)			Root biomass (g)		Colonization intensity (M%)		Arbuscule abundance (A%)		
		Alfalfa	Tall fescue				Alfalfa	Tall fescue	Alfalfa	Tall fescue	
Plant – NM	PHE + DBA	0.25 ± 0.04	0.23 ± 0.12^{c}	B	0.35 ± 0.16^{b}	B	n.c.	n.c.	n.c.	n.c.	
	DBA	0.22 ± 0.03	0.41 ± 0.09^{abc}		0.43 ± 0.07^{b}		n.c.	n.c.	n.c.	n.c.	
Plant + Gm	PHE + DBA	0.21 ± 0.12	0.39 ± 0.11^{abc}	A	0.61 ± 0.20^{a}	A	44.31 ± 1.89^{b}	42.61 ± 2.74^{b}	37.33 ± 14.75^{a}	7.56 ± 2.91^{b}	A
	DBA	0.18 ± 0.05	0.57 ± 0.12^{a}		0.66 ± 0.12^{a}		67.97 ± 4.83^{a}	69.00 ± 5.40^{a}	52.80 ± 11.31^{a}	39.87 ± 20.37^{a}	
Plant + Gi	PHE + DBA	0.20 ± 0.04	0.35 ± 0.06^{bc}	A	0.37 ± 0.13^{b}	B	59.53 ± 15.00^{ab}	38.69 ± 8.91^{b}	25.12 ± 3.42^{a}	2.63 ± 0.66^{b}	B
	DBA	0.16 ± 0.06	0.52 ± 0.13^{ab}		0.36 ± 0.09^{b}		53.33 ± 17.68^{ab}	67.71 ± 4.65^{a}	38.44 ± 34.84^{a}	15.82 ± 7.57^{b}	
Pollutant		n.s.	***		n.s.		n.s.	***	n.s.	***	
Treatment		n.s.	**		***		n.s.	n.s.	n.s.	*	
Pollutant × treatment		n.s.	n.s.		n.s.		*	n.s.	n.s.	n.s.	

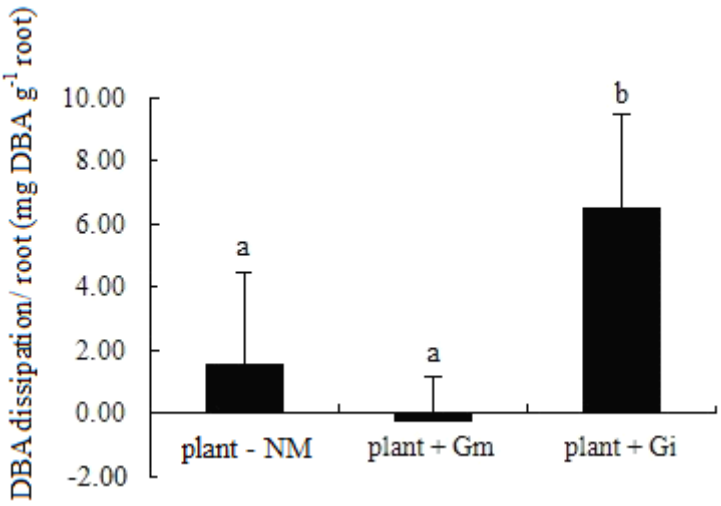


Fig.20 Dissipation of dibenzo[a,h]anthracene (DBA) per gram of root in DBA spiked soil. DBA dissipation (mg/g root) = DBA content (mg) in unplanted pots - DBA content (mg) in planted pots) / g of roots per pot. NM= uninoculated plant; Gi = *G. intraradices* inoculation; Gm = *G. mosseae* inoculation. Mean ± SD (n=5). Different letters indicate significant differences between planted treatments in DBA spiked soil ($P<0.05$). Significant effect at *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

3.3 Rhizosphere microorganisms

The copy number of 16S rDNA gene was significantly influenced by the pollutant source and the mycorrhizal treatment (table 20). The presence of PHE increased 16S rDNA gene copy number. The 16S rDNA gene copy number in the rhizosphere of nonmycorrhizal plants and plants inoculated with *G. intraradices* was significantly lower in comparison to plants inoculated with *G. mosseae* whatever the pollutant was. The copy number of 18S rDNA gene displayed the same tendency as 16S rDNA gene, but the differences were not significant. The gram positive PAH-RHDα gene copy number was higher in presence of PHE+DBA, and the quantity was significantly increased for *G. mosseae* inoculated plants. The average number of gram negative PAH-RHDα genes was lower than gram positive ones and no significant difference was observed between treatments.

Table 20 Microorganism numbers in rhizosphere soil after 6 weeks. NM= uninoculated plant; Gi = *G. intraradices* inoculation; Gm = *G. mosseae* inoculation. Data are presented as mean ± SD (n = 5). Different small letters indicate significant differences between groups, and different capital letters indicate significant difference between treatments (P<0.05). Anova analysis with significant effect at ***P < 0.001; ** P < 0.01; * P < 0.05.

Treatments	PAHs	16S rDNA gene copy (10^9/ g soil)		18S rDNA gene copy (10^8/ g soil)	Gram positive PAH -RHDα gene (10^6)		Gram positive PAH-RHDα% (10^{-1})	
Control	PHE + DBA	4.82 ± 1.07 ab	A	2.25 ± 1.98	1.68 ± 0.79 b	B	0.35 ± 0.11 b	B
	DBA	3.91 ± 0.57 ab		2.82 ± 1.90	0.82 ± 0.25 b		0.21 ± 0.07 b	
Plant - NM	PHE + DBA	2.17 ± 0.83 cd	B	1.39 ±0.89	1.22 ± 1.07 b	B	0.57 ± 0.55 b	B
	DBA	1.46 ± 1.14 d		0.96 ± 0.52	1.23 ± 1.76 b		0.61 ± 0.45 b	
Plant + Gm	PHE + DBA	5.36 ± 1.80 a	A	3.66 ± 2.93	6.94 ± 2.75 a	A	1.33 ± 0.39 a	A
	DBA	3.68 ± 1.12 abc		2.16 ± 1.32	1.86 ± 1.29 b		0.47 ± 0.25 b	
Plant + Gi	PHE + DBA	2.98 ± 1.36 bcd	B	1.40 ± 0.53	1.24 ± 0.47 b	B	0.48 ± 0.21 b	AB
	DBA	2.44 ± 1.02 cd		1.82 ± 0.97	0.57 ± 0.26 b		0.30 ± 0.27 b	
Pollutant		*		n.s.	***		**	
Treatment		***		n.s.	***		**	
Pollutant × treatment		n.s.		n.s.	n.s.		n.s.	

4. Discussion

4.1 Effect of AM fungi on PAH dissipation

According to this experiment, *G. mosseae* was more efficient to increase shoot biomass of tall fescue and root biomass of both plants than *G. intraradices*. Besides, *G. mosseae* increased 16S rDNA and PAH-RHDα gene copy numbers. However, the PAH dissipation in *G. mosseae* inoculated treatment was not increased in comparison to uninoculated one. In the PHE+DBA polluted treatment, the *G. mosseae* colonized treatment significantly restrained the DBA dissipation. The same *G. mosseae* P2 (BEG 69) isolate was previously shown to increase chrysene and dibenz[a,h]anthracene dissipation in ryegrass and clover rhizosphere of a spiked soil (Joner et al. 2001). The different results of these two experiments may be attributed to the following aspects: Firstly, although both studies used a combination of legume and grass, the plant species differed between the two experiments. Helgason et al. (2002) reported that different plants respond differently when colonized by the same fungal species. Joner et al. (2001) showed that AM inoculation with *G. mosseae* altered the microbial community structure and suggested that the mycorrhiza-associated microflora was responsible for the PAH dissipation in the presence of mycorrhiza. In our experiment, *G. mosseae* increased both bacteria and PAH degraders, which suggests that the lack of effect on PAH dissipation was not due to a lack of degrading microorganisms. Competition for water and mineral nutrients between roots and soil microorganisms may decrease the activity of PAH degraders (Zhou et al. 2009) and result in lower PAH dissipation efficiency than in unplanted controls. This would be coherent with biomass data since *G. mosseae* inoculated plants had the highest root biomass. Differences in soil physicochemical characteristics can also affect mycorrhizal plant effect on PAH dissipation (Chiapusio et al. 2007). Different plant sizes and growth stages could also influence PAH

dissipation (Aulakh et al. 2001). Indeed, the improved dissipation of DBA in clover and ryegrass rhizosphere due to *G. mosseae* inoculation was significant after 16 weeks but not after 8 weeks (Joner et al. 2001).

On the contrary, *G. intraradices* increased the dissipation of DBA in the DBA spiked soil although no enhancement of PAH degraders was detected. The benefit of *G. intraradices* on DBA dissipation was previously observed by Zhou et al. (2009) when the same plant combination and AM fungus were utilized in PHE+PYR+DBA spiked soil. The increase of DBA dissipation could result from a modification of root exudates, in which oxidoreductases as well as root exudate chemicals, such as linoleic acid and plant-derived phenolics could enhance PAH dissipation (Corgié et al. 2006c; Criquet et al. 2000). The increase of phosphorus uptake and water availability in hydrophobic soil could also enhance DBA dissipation (Zhou et al. 2009). The increase of DBA dissipation may also be caused by other mechanisms, such as the hyphae increased DBA bioavailability by penetrating into the soil micropores. However, this AM effect on DBA dissipation cannot be merely explained by a plant growth effect, since root biomass was lower with *G. intraradices* than with *G. mosseae*, and since the dissipation per g of root was higher with *G. intraradices* than with uninoculated and *G. mosseae* inoculated plants.

4.2 PAH toxicity and dissipation

A negative PHE effect was observed on tall fescue biomass and its AM fungal colonization, while it was not observed for alfalfa. The toxic effect of PAHs to AM fungi colonization and plant biomass was shown in several studies (Cheema et al. 2009; Chiapusio et al. 2007; Liu et al. 2004). The different responses indicated a lower tolerance of tall fescue than alfalfa. Both tall fescue and alfalfa have been commonly and successfully used for PAH phytoremediation

(Cheema et al. 2009; Fan et al. 2008). Considering its sensitivity towards PAHs, tall fescue may be less applicable for phytoremediation of highly contaminated sites. However, the toxic effect of PHE was certainly overestimated in our experiment by the use of freshly spiked and highly available PHE in comparison to its availability in ancient contaminated soils.

The addition of PHE significantly increased the number of bacteria and gram positive PAH degraders, and it also tended to increase the number of gram negative PAH-RHDα genes, which may be attributed to microbial use of PHE as carbon source. Although the addition of PHE slightly increased gram positive PAH-RHDα gene copies, the average percentage of this gene related to 16S rDNA gene (Cébron et al. 2008) in this experiment was lower than 0.08%, while it reached 2.7 % in a previous study in comparable conditions with PHE+PYR+DBA (500, 500, 65 mg kg^{-1}) spiked soil (Zhou et al. 2009). Dissipation of DBA was also higher in Zhou et al. (2009) experiment (36 mg kg^{-1} within 6 weeks) than in the present one (10 to 20 mg kg^{-1}).

The dissipation of PHE in all treatments was above 90%, which was much higher than the dissipation of DBA (lower than 40%). This is due to the higher availability and biodegradation of low than high molecular weight PAHs (Johnsen et al. 2005). The presence of PHE increased the DBA dissipation in non-planted control and non-mycorrhizal planted treatment. Co-metabolism of LMW and HMW PAHs has already been observed in flask experiment (Bouchez et al. 1995; Juhasz et al. 1997), where PHE stimulated DBA dissipation probably due to PHE providing a carbon source and stimulating the production of enzyme for DBA degradation (Juhasz et al. 1997). The enzymes, e.g. oxidoreductases produced by bacteria during the PHE degradation process have a broad substrate range and could contribute to the first step in aerobic PAH catabolism for the HMW PAH (Chauhan et al. 2008). Since the initial ring attack with oxygenase enzymes is supposed to be the rate limiting

step of PAH degradation (Chauhan et al. 2008; Juhasz and Naidu 2000), the increase of non-specific oxidase activity could enhance DBA dissipation.

However, in AM fungi colonized treatments, the presence of PHE did not enhance DBA dissipation, and the effect of *G. intraradices* on DBA dissipation was not observed when PHE was present. The reduction of AM fungi colonization in presence of PHE could partly explain the decrease of DBA dissipation. Another hypothesis was that the enzyme or other chemical compounds produced by the AM fungus or the mycorrhizal plant, which could be involved in DBA dissipation in DBA polluted soil, was inhibited or consumed by PHE. Finally, although not observed in this experiment where PHE degradation rate was rapid even without plants, a possible effect of AM inoculation on PHE dissipation (Wu et al. 2008a) could be considered.

In conclusion, DBA dissipation was higher in PHE+DBA than DBA spiked soil, suggesting co-metabolism interactions between PAHs. *G. mosseae* was more efficient than *G. intraradices* in promoting plant and bacterial growth, and it also increased the density and percentage of gram positive PAH-RHDα gene copies. However, only *G. intraradices* increased DBA dissipation in DBA spiked soil. The mechanisms of *G. intraradices* assisted DBA dissipation must be further investigated. However, our results indicated that this positive effect was not only dependant on a plant biomass increase.

Principaux résultats

Les résultats montrent des différences dans les effets des deux champignons utilisés sur la dissipation du DBA. Les deux champignons MA, *Glomus intraradices* et *Glomus mosseae*, ont bien colonisé les racines et ont significativement augmenté la biomasse des parties aériennes de la fétuque sans toutefois affecter celle de la luzerne, mais seul *G.mosseae* a augmenté la biomasse racinaire. Cependant, la dissipation du DBA a été significativement plus importante avec les plantes inoculées par *G.intraradices* qu'avec les témoins plantés non mycorhizés. En revanche, *G. mosseae* a augmenté le nombre de bactéries totales et le nombre de copies de gène de HAP-dioxygénase de bactéries à Gram positif, mais n'a eu aucun effet significatif sur la dissipation du DBA. Comme dans les expériences précédentes, la dissipation du PHE a été rapide et quasiment totale dans tous les traitements, et il n'a pas été possible de voir de différences significatives entre les traitements. Cependant, la présence de PHE dans le sol a conduit à une dissipation plus importante du DBA. Toutefois, l'effet positif de PHE sur la dissipation DBA était plus faible dans le sol que dans le substrat liquide dans l'expérience en microplaques, probablement à cause de la plus faible biodisponibilité du DBA dans le sol. Il faut souligner qu'en présence de PHE+DBA la biomasse végétale et la colonisation mycorhizienne de la fétuque étaient plus faibles qu'en présence de DBA seul, indiquant une plus faible tolérance au PHE de la fétuque que la luzerne.

Chapitre 4-2: Effet de la luzerne et de la fétuque mycorhizées séparément ou en co-culture sur la dissipation du phénanthrène et du dibenzo(a,h)anthracène

Introduction/Objectif de l'expérience

L'efficacité de la phytoremédiation varie grandement avec les espèces et variétés de plantes (Liste & Prutz, 2006). Certaines graminées et légumineuses ont été montrées comme des plantes efficaces pour la phytoremédiation des HAP (Anderson et al., 1993; Singer et al., 2003) et la co-culture de ces plantes permet d'associer des architectures racinaires différentes, une meilleure nutrition azotée, et modifie aussi les communautés microbiennes. Certaines études ont été réalisées sur la combinaison de plantes, et les effets sur la dissipation des HAP varient du positif au négatif (Phillips et al., 2006; Xu et al., 2006). Nos expériences antérieures ont montré que l'inoculation par *G. intraradices* de la co-culture de luzerne et fétuque conduisait à une dissipation du DBA plus importante que les témoins plantés non mycorhizés et les témoins non plantés (chapitre 1).

L'objectif de ce chapitre était d'évaluer si ce dernier résultat était lié à la co-culture des deux plantes ou pouvait être spécifiquement dû à l'une des deux. Cette expérience a donc été réalisée parallèlement et dans les mêmes conditions expérimentales que la précédente pour suivre l'effet de la luzerne, la fétuque et leur co-culture, toutes inoculées par *G.intraradices* sur la dissipation du mélange de PHE+DBA. La dissipation du PHE et du DBA, la colonisation mycorhizienne des racines, les bactéries totales et dégradantes, quantifiées par PCR en temps réel des gènes ADNr 16S et de HAP-dioxygénases, ont été mesurées.

Effect of mycorrhizal alfalfa and tall fescue separately or in combination on PAH dissipation in a spiked soil

Abstract:

This study was conducted to compare the ability of AM fungi colonized alfalfa and tall fescue and their combination in promoting the dissipation of phenanthrene (PHE) and dibenzo(a,h)anthracene (DBA) in spiked soil. The same experimental conditions as in the previous chapter were used. When the two plants were co-cultivated, the arbuscular mycorrhizal colonization of alfalfa was higher while it was lower for tall fescue, and the root biomass of alfalfa was lower, than when the corresponding plants were alone. Over 90% of PHE was dissipated, but only 20-40% of DBA dissipated after six weeks. The gram positive PAH-RHDα gene copy number was higher in alfalfa rhizosphere than in the other treatments, but DBA dissipation was lower with alfalfa alone. The co-cultivation of alfalfa and tall fescue had no synergistic effect on PHE and DBA dissipation.

Key words: co-cultivation, phytoremediation, PHE, DBA, PAH-RHDα gene

1. Introduction

Many studies have been conducted on the use of plants to improve PAH remediation (Reilley et al., 1996; Liste & Alexander, 2000; Binet et al., 2001). The phytoremediation efficacy varies greatly among plant species and varieties (Liste & Prutz, 2006). The dominant mechanism in phytoremediation is biodegradation. Plants provide carbon sources by fine root turn over and root exudates, which increase rhizosphere-inhabiting microbial numbers beyond those of the bulk soil. For example, Nichols et al. (1997) found that bacterial populations in general and hydrocarbon-degrader populations specifically, were stimulated by the growth of alfalfa and alpine bluegrass in soil. Besides, some of the plant-derived chemicals, e.g. salicylate, and linoleic acids, were proved to assist PAH degradation (Jones, 1998; Leigh et al., 2002; Singer et al., 2003). Therefore, root density, exudate quality and quantity which could influence the microorganism structure and activity, are important factors for the PAH dissipation in contaminated soils (Parrish et al., 2005). The plants with higher root biomass, e.g. ryegrass and tall fescue, and legumes which could release nitrogen stress in plants by rhizobia colonization, are often considered. In addition, several experiments have focused on the plant combination. Co-cultivated plants can favor the growth of rhizosphere microorgansims, and allow great infiltration and stimulation of microbial community with a net positive stimulation of metabolic potential (Chen et al., 2008). However, opposite results have been also obtained when combining different plants to improve PAH dissipation (Xu et al., 2006; Phillips et al.,2006; Cheema et al. 2010).

In our previous experiment (chapter 1 and 4-1), we investigated the effect of the combination of mycorrhizal alfalfa and tall fescue on the dissipation of PAHs. A higher DBA dissipation was observed in comparison to unplanted control. The present study was conducted to

investigate whether this effect would be obtained with alfalfa and tall fescue alone, or only in combination. The same experimental design as described in chapter 4-1 was used, with a pot experiment with PHE and DBA spiked soil, and either alfalfa, tall fescue or the combination of both plants. All plants were inoculated with the mycorrhizal fungus *Glomus intraradices*. PAH dissipation, AM fungi colonization and 16S rDNA and PAH- RHDα genes were monitored, and the single and combined plant effects on PAH dissipation were compared.

2. Materials and methods

2.1 Soil

The soil used was a silty clay loam (Bouzule), prepared and spiked with PHE (500 mg kg^{-1}) and DBA (50 mg kg^{-1}) as described in chapter 4-1. After spiking, the soil was reinoculated with indigenous soil microflora, except mycorrhizal fungi, as described in chapter 4-1.

2.2 Treatments and sample analysis

Four treatments were designed: (1) unplanted control; (2) mycorrhizal alfalfa; (3) mycorrhizal tall fescue; (4) mycorrhizal alfalfa + tall fescue. Each pot contained 250 g spiked soil and 10 g commercial AM fungi inoculum (*Glomus intraradices*) supplied by the Institüt für Pflanzenkultur (Solkau, Germany) as a mixture of propagules in lava substrate. Three days-old pre-germinated alfalfa (*Medicago sativa cv. Europe*) and tall fescue (*Festuca arundinacea cv. Bariane*) seedlings were transplanted into each pot (one seedling of each species, or two seedlings of a single species). The pots were randomized in a growth chamber (24/20 ºC day/night, 16 h day, 80% RH, 200 - 300 µmol photons $s^{-1}m^{-2}$). The pots were fertilized with 25 ml low phosphorus (0.1 mM) Hewitt nutrient solution per week, and constant soil moisture

(80% water holding capability). Six weeks after transplantation, pots were harvested and sample analyses were performed as described in chapter 4-1.

2.3 Statistical analysis

Statistical analysis of the data was performed using one way ANOVA followed by Newman-keuls (SNK) test on xlstat 2009 to determine significant differences between treatments ($P < 0.05$).

3. Results and discussion

The frequency of mycorrhizal roots did not significantly differ between alfalfa, tall fescue or their combination. However, when both plants were cultivated together, the arbuscule frequency in mycorrhizal colonization was significantly higher for alfalfa and lower for tall fescue than when each plant was alone (Table 1). Joner and Leyval (2001) also observed that the legume (clover) had a higher mycorrhizal colonization than the grass (ryegrass) when cultivated together in a pot experiment. This could be due to the lower mycorrhizal dependency of plant species with fine and dense root systems (e.g. tall fescue) than plants with short and thick roots or fewer or shorter root hairs like alfalfa (Baon et al., 1994; Declerck et al., 1995). However, in spite of the higher AM fungi colonization, the alfalfa shoot biomass was not significantly affected by the presence of tall fescue, as well as the one of tall fescue was not affected by the presence of alfalfa. Shoot biomass was smaller for alfalfa than tall fescue. This is consistent with the study of Joner and Leyval (2001) where shoot biomass was significantly lower for clover than ryegrass. When alfalfa and tall fescue were co-cultivated, root biomass was significantly lower than with tall fescue alone.

No significant difference was detected in 16S rDNA and 18S rDNA gene copy numbers between the different treatments (table 21). The gram positive PAH-RHDα gene copy numbers were significantly higher in the rhizosphere of alfalfa than in the other treatments (table 22). The percentage of gram positive PAH-RHDα gene copies ranged from 0.04% to 0.13% in this experiment, which is much lower than in the first experiment (chapter 1:1.1-3.8%), which could be related to the lower concentration of HMW PAHs used in this experiment.

As observed in the previous experiments, dissipation of PHE (more than 90%) was much higher than DBA (20%-40%), due to high resistance of the high molecular PAHs to biodegradation or low bioavailability related to their hydrophobic and structure stability (Juhasz et al., 2000). In this experiment, although large variations among replicates were observed, alfalfa seemed to contribute to PHE dissipation, while tall fescue did not, and when both plants were used, no residual PHE was found (Table 22). In our previous experiment (chapter1), alfalfa was also more efficient in improving PHE dissipation than tall fescue.

Although several experiments have claimed alfalfa as an effective host plant in high molecular weight PAH phytoremediation (Fan et al., 2008; Liu et al., 2004), in our experiment, it reduced the DBA dissipation in the spiked soil in comparison to the unplanted control. The same negative effect of alfalfa on PAH dissipation was obtained by Phillips et al. (2006), and the mechanisms deserve further investigation.

In our experiment, although the co-cultivated alfalfa and tall fescue resulted in a higher dissipation of PHE than tall fescue alone, no significant difference was detected between the co-cultivated plants and alfalfa treatments on PHE dissipation. In addition, no significant effect was observed on DBA dissipation when alfalfa and tall fescue were co-cultivated in

comparison to single cultivated alfalfa or tall fescue. The co-cultivation of clover and maize enhanced phenanthrene and pyrene dissipation efficiency in the study of Xu et al. (2006).

Table 21. Plant biomass and AM fungi colonization of each pot. Mycorrizal colonization expressed as frequency of mycorrhizal roots (F%) and arbuscular abundance in the root systems (A%). Different letters indicate significant differences between treatments (P<0.05). * alfalfa & tall fescue.

Treatment		Shoot biomass (g)	Root biomass (g)	Colonization frequency (F%)	Arbuscule abundance (A%)	16S rDNA genes (10^9)	18S rDNA genes (10^8)
Non plant control		-	-	-	-	4.82 ± 1.07	2.25 ± 1.98
Alfalfa - AM fungi		0.26 ± 0.08 ^{ab}	0.32 ± 0.12 ^{b}	54.67 ± 3.80	12.99 ± 3.78 ^{b}	3.75 ± 1.20	3.07 ± 1.42
Tall fescue - AM fungi		0.34 ± 0.07 ^{a}	0.66 ± 0.24 ^{a}	48.00 ± 16.26	10.91 ± 6.79 ^{b}	4.06 ± 1.33	1.58 ± 0.92
Mixture - AM fungi	Alfalfa	0.20 ± 0.04 ^{b}	0.37 ± 0.13 ^{b*}	58.67 ± 17.73	25.12 ± 3.42 ^{a}	2.95 ± 1.31 *	1.40 ± 0.53 *
	Tall fescue	0.35 ± 0.06 ^{a}		39.33 ± 9.25	2.63 ± 0.66 ^{c}		

Table 22. Remaining PAH concentration and PAH-RHDα gene copies in the soil of different treatments. PAH dissipation (mg/g root) = PAH content (mg) in unplanted pots-PAH content (mg) in planted pots) / g of roots per pot. Mean ± SD (n=5). Different capital letters and small letter indicate significant differences of PHE and DBA concentration among different treatments. (P<0.05)

Treatment	Remaining PAH concentration (mg kg^{-1})		PAH dissipation (mg PAH / g root)		PAH-RHDα genes copies (10^6)		PAH-RHDα genes / 16S rDNA genes (%)	
	PHE	DBA	PHE	DBA	Gram positive	Gram negative	Gram positive	Gram negative
Non plant control	8.00 ± 16.53 ^{ab}	31.02 ± 2.98 ^{b}	-	-	1.68 ± 0.79 ^{a}	0.89 ± 1.23	0.04 ± 0.01 ^{a}	0.02 ± 0.04
Alfalfa - AM fungi	0.50 ± 1.12 ^{b}	38.30 ± 6.94 ^{a}	7.0 ± 3.5 ^{a}	-5.5 ± 5.1	4.92 ± 1.91 ^{b}	5.48 ± 11.80	0.13 ± 0.04 ^{b}	0.12 ± 0.27
Tall fescue - AM fungi	25.52 ± 21.56 ^{a}	33.02 ± 0.71 ^{ab}	-8.3 ± 10.2 ^{b}	-1.0 ± 0.7	2.10 ± 1.50 ^{a}	0.04 ± 0.04	0.05 ± 0.03 ^{a}	0.00 ± 0.00
Mixture - AM fungi	0.00 ± 0.00 ^{b}	35.76 ± 1.12 ^{ab}	5.8 ± 1.5 ^{a}	-3.5 ± 1.3	1.24 ± 0.47 ^{a}	0.01 ± 0.01	0.05 ± 0.02 ^{a}	0.002 ± 0.005

Cheema et al. (2010) showed a higher dissipation of PHE and pyrene by combining alfalfa-rape seed and tall fescue-rape seed than with single planted treatments. However, Phillips et al. (2006) found that PAH dissipation was higher with either creeping red fescue or perennial ryegrass than when these plants were co-cultivated with alfalfa. This indicated that the plant fitness and efficiency of phytoremediation were not always enhanced with co-cultivated plants, but might be closely related to the unique properties of the host plant, influence of co-cultivation on rhizosphere microbiota, soil nutrient status, water status and PAH properties (Anderson et al., 1993; Chiapusio et al., 2007; Yi and Crowley, 2007; Zhou et al., 2009).

Principaux résultats

Comme dans les expériences précédentes, plus de 90% du PHE, mais seulement 20 à 40% DBA ont disparu pendant la culture de six semaines. Dans le cas où la fétuque et la luzerne étaient en co-culture, la biomasse des racines a été plus faible que lorsque les plantes étaient cultivées séparément et l'abondance des arbuscules dans les racines de fétuque était aussi plus faible. La co-culture n'a pas amélioré l'efficacité de dissipation des deux HAP contrairement à l'étude de Xu et al. (2006). Toutefois, cette expérience a montré que la dissipation du PHE avec la co-culture était liée à la luzerne, car elle était beaucoup plus faible avec la fétuque seule. Toutefois, malgré un nombre réduit de plantes dans les pots dans le cas de la co-culture, la compétition entre plantes a pu avoir un effet négatif sur un éventuel effet synergique des plantes.

Partie 4

Discussion générale et perspectives

General discussion and perspectives

Partie 4: Discussion générale et perspectives- General discussion and perspectives

Polycyclic aromatic hydrocarbons are one of the most frequent soil contaminants (PAHs represent 13% of total contaminations) present at polluted sites investigated in Europe (EEA 2007). They could accumulate in the environment and threaten the development of living organisms. Phytoremediation is a promising technique for PAH contaminated site remediation owing to its ecological and economical benefits. However, the non consistent results and low efficiency of phytoremediation on HMW PAHs restrained this technique from wide application (Reilly et al., 1996). AM fungi interact with the developing root system, affecting both morphology and ability to absorb mineral nutrients (Wu et al., 2009). AM fungi could also quantitatively and qualitatively change root exudation and soil microbial community, and finally affect PAH degradation (Joner et al., 2001). AM fungi enhanced dissipation of PAHs has been obtained in several studies (Joner et al., 2001; Joner and Leyval., 2003a; Wu et al., 2008a), but not in others (Corgié et al., 2006c), and the mechanisms and the parameters influencing PAH phytoremediation are still under investigation.

The purpose of this study was to investigate the parameters associated with the efficiency of PAH phytoremediation. Different plant species, phosphorus concentration, water regime, PAH combination and AM fungi species were employed, and the growth of the plants, the density and community of the microbes in soil, as well as PAH dissipation efficiency were evaluated.

4.1 Plant biomass in the PAH spiked soil

A major limitation of phytoremediation could be the poor growth of plants owing to the toxicity of PAHs and the inorganic nutrient and water deficiency in polluted sites (Li et al., 1997). Methods which could increase PAH tolerance and root biomass are critically important. According to our results, plant biomass on PAH contaminated soils depends on plant species and plant combinations, AM fungi species, on the watering regime and phosphorus concentration, and was also affected by PAH toxicity.

Indeed the growth of the four different plant species in the same contaminated soil was different. Out of the 4 plants evaluated, the plants with fibrous roots obtained higher shoot, root biomass and shoot phosphorus concentration. After six weeks, the average root and shoot biomass of each tall fescue / ryegrass seedling were up to 0.385 / 0.869 g and 0.357 / 0.303 g separately. The lowest biomass was obtained with celery roots (0.085 and 0.105 g per seedling). Phosphorus concentration varied among plant species too, and the phosphorus concentration in shoots decreased as follows: ryegrass > tall fescue > alfalfa > celery roots. In comparison to the single plants alfalfa and tall fescue, alfalfa shoot biomass was lower when it was co-cultivated with tall fescue, while no significant difference was detected for tall fescue. The average root biomass of the co-culture was lower than the average value of tall fescue and alfalfa, suggesting that competition occurred and could have affected the dissipation results.

In the fourth chapter where the soil was spiked with DBA or PHE+DBA the average shoot biomass of tall fescue in non-inoculated treatment was 0.41 and 0.23 g per plant, respectively, confirming a negative effect of PHE on tall fescue growth. On the contrary, no significant difference was detected in alfalfa biomass, indicating a higher tolerance of alfalfa than tall fescue towards PHE.

Both AM fungi species used in this study increased the plant biomass, but their efficiency differed with plants and AM fungi species. *G. intraradices* increased the biomass of alfalfa, tall fescue, ryegrass and celery roots by 72%, 64%, 144% and 116% respectively (chapter 2). In the fourth chapter, the root biomass was significantly higher for *G. mosseae* than *G. intraradices* inoculated co-cultivated alfalfa and tall fescue.

The phosphorus concentration and the watering regime also influenced the plant growth. The average plant biomass and shoot phosphorus concentration were higher in high phosphorus and high watering treatments (chapter 1).

4.2 AM fungi colonization in the PAH spiked soil

Roots inoculated with *G. intraradices* and *G. mosseae* were well colonized, but their colonization rate was influenced by phosphorus fertilization, watering regime, host plant species and the toxicity of PHE. The well known effect of high phosphorus concentration on AM fungi (Jensen and Jakobsen, 1980) was observed in chapter 1 where *G. intraradices* colonization in alfalfa and tall fescue roots decreased by 90% when phosphorus concentration increased in Hewitt solution from 0.1 to 0.4 mM. *G. intraradices* colonization decreased by 63% (alfalfa) and 90% (tall fescue) when the pots were dried up to 40% of water holding capacity instead of 60%.

The addition of PHE significantly decreased the colonization of tall fescue with *G .intraradices* and *G. mosseae*. A negative effect of PAHs on spore germination and AM fungi colonization was also observed previously (Liu et al., 2004; Verdin et al., 2006), but seems to depend on the plant species since it was not observed for alfalfa, when these two species were cultivated together. Root colonization with *G. intraradices* was higher for alfalfa when it was

co-cultivated with tall fescue than when cultivated alone. In co-cultured treatment, the arbuscular abundance was much higher in the root system of alfalfa than tall fescue for both *G. intraradices* and *G. mosseae*.

4.3 Microbial global and functional community

In this study, both culture dependant and independent methods were utilized to evaluate the microbial community in the rhizosphere. Results of the culture dependant method showed that the numbers of culturable PHE and PHE+DBA degraders were higher in planted than in unplanted pots (chapter 3). However, the same result was not observed by culture independent method, i.e. DNA extraction and quantification of genes, which showed no significant difference between planted and unplanted pots. On the contrary, the number of gram negative PAH-RHDα gene copies was significantly lower in planted than unplanted pots (chapter 1). However, the presence of genes may not indicate that bacteria were active. The quantification of gene expression, by RNA extraction, would give useful information on that, but preliminary RNA extraction assays (data not shown) were not successful.

The average percentages of the PAH-RHDα gene copies were 2.7% and 1.2% for the first and second experiment, while for the fourth experiment the value was lower than 0.08%. According to the investigation of Cébron et al. (2008) PAH-RHDα gene copies (gram positive + gram negative) were positively correlated with the PAH-contamination in soil. Thus the lower percentage in the fourth experiment could possibly result from the lower PAH concentration in the fourth experiment, especially at the end of the experiment when the genes were quantified. The average percentages of PAH-RHDα genes were higher than the percentages found in historical PAH contaminated Homécourt and Flémalle soils (Cébron et

al, 2008), where the concentration of PAHs reached 3000 mg kg^{-1}. This was probably due to the lower PAH bioavailability in aged-contaminated soils.

In all the experiments the numbers of gram positive PAH-RHDα gene copies surpassed the gram negative ones. The opposite was observed in a PAH multi-contaminated soil (Cébron et al, 2009) and in experiments with sand and soil spiked with PHE (Louvel, 2010). This could differ between soils. In the unspiked Bouzule soil, the copy number of PAH-RHDα gene was very low for gram negative genes and below detection limit for gram positive ones (Cébron et al, 2008). It is very interesting to notice that six weeks after spiking the number of degradation genes could reach 10^8copies.

The addition of PHE in DBA substrate significantly increased the bacteria (expressed by 16S rDNA genes) and gram positive PAH degraders (expressed by gram positive PAH-RHDα gene) (chapter 4). Besides, the quantity and percentage of gram positive PAH-RHDα gene copies was also affected by the nutrient conditions and was lower in the high-phosphorus high-water treatments than in the low-phosphorus ones (chapter 2).

4.4 PAH dissipation

With low volatilization (the pots were covered with coarse sand) and no leakage (the pots were sealed), the fate of the PAHs in soil was either adsorption by plants, sorption to soil contituents or biotransformation or biodegradation by microorganisms. The quantity of PAH uptake and sorbed by roots was limited (chapter 2), and no significant difference in PAH absorption had been observed between *G. intraradices* colonized and non-mycorrhizal plants. This indicated that the major mechanism of PAH dissipation due to plants should be biodegradation or biotransformation.

As previously observed (Reilley et al., 1996) and as expected, HMW PAHs dissipated much slower than LMW PAHs in our study in both pot and microplate studies (chapter 1, 2, 3, 4). Indeed, PHE is more soluble, less hydrophobic (Kow PHE = 4.6; Kow PYR = 5.2; Kow DBA = 6.8), and more easily biodegraded than PYR and especially DBA. HMW PAHs, especially the ones with five rings could not serve as carbon source for microorganisms (Cerniglia et al., 1992) and are much reluctant towards bioremediation. Results showed that DBA dissipation efficiency differed with factors such as plant species, PAH co-existence, AM fungi species, P fertilization and water concentration.

No direct relationship was found between PAH dissipation and numbers of PAH-RHDα gene copies (except for PYR in experiment 1), suggesting that the presence of degradation genes was not the limiting factor for PAH dissipation.

4.4.1 Plant species

Two way ANOVA showed a general positive effect of plants and AM fungi on the dissipation of PHE (chapter 2). This positive effect of plants and AM fungi on PHE degradation was consistant with previous studies (Wu et al., 2008a; Xu et al., 2006). However, PHE dissipation was very efficient even in non-planted control treatment (around 90% of PHE dissipated during six weeks incubation), which could be due to the fact that the experiments were performed with a freshly spiked soil. Although the four tested species were reported earlier as able to increase PYR dissipation (Fan et al., 2008; Su et al., 2008; Corgié et al., 2003; Yi and Crowley, 2007), no significant difference was detected between the planted and unplanted treatments or among different plant species. In our experiment, no significant effect of plants was observed on the dissipation of PYR. Bioremediation efficiency of PAHs with more than five rings was limited owing to their low bioavailability and stable structure,

however, some plants could assist their degradation. The study of Liu et al. (2004) showed that alfalfa could increase BaP (benzo-a-pyrene) dissipation in a soil spiked with different concentration of BaP (1, 10, 100 mg kg^{-1}). In our study, alfalfa seemed to have a higher effect than the other plants when DBA dissipation was expressed per gram of root biomass. Besides, the mixture of alfalfa and fescue had a significant effect on DBA in chapter 1, in which the presence of both plants enhanced the total number of bacteria and PAH degraders, and in chapter 4-1. Although such parameter was not investigated in our study, the legume plant could fix nitrogen and relieve the nitrogen stress in PAH contaminated soil (Joner et al. 2002). Via the release of enzymes, such as ABTS peroxidase (2, 2'-Azinobis-(3-ethylbenzo -thiazoline-6-sulphonate)), which increased in the roots of alfalfa in presence of PHE (Muratova et al., 2009), of secondary metabolites, such as flavonoids (Singer et al., 2003), alfalfa could stimulate PAH dissipation.

The inefficiency of the celery roots on PYR and DBA dissipation was opposite to the previous investigation of Yi and Crowley (2007), which considered celery roots as an efficient plant for PYR and BaP dissipation, since celery roots contained large amounts of linoleic acid, acting as metabolite or surfactant. This could be due to the poor growth of the celery roots in our experiment (Aulakh et al. 2001; Yi and Crowley 2007). Besides, the same plant may display different effects when employed in PAH contaminated sites with different soil properties (Chiapusio et al., 2007).

4.4.2 AM fungi effect on PAH dissipation

G. intraradices increased PHE dissipation in chapter 2 and DBA dissipation in chapter 4, in spite of no significant change in microorganism number and community. Mycorrhizal colonization affects root surface properties or rhizosphere soil properties and could enhance the DBA bioavailability by entering into the micropores of the soil (Joner et al., 2001). AM

fungi colonization induces various plant defense reactions, and enhances peroxidase activity, which could contribute to the initial ring attack (Criquet et al., 2000) enhance root exudation and change the composition of root exudates (Singer et al., 2003). AM fungi could increase the phosphorus bioavailability in the soil. It was notable that the stimulation effect of *G. intraradices* on DBA dissipation was not observed when PHE was present, possibly due to the toxicity of PHE, i.e. decreased mycorrhizal colonization and shoot biomass of tall fescue.

Although *G. mosseae* greatly increased biomass of plants and microorganisms, and was previously shown to increase PAH dissipation (Joner et al., 2001; Joner and Leyval, 2003a), this fungus had no effect on PHE and DBA dissipation in our experiments, suggesting as the results of Liu and Dalpé (2009), that different fungi may have different effect on PAH dissipation. Our results also showed that the AM fungi effect on PAH dissipation cannot simply be explained by a plant growth effect.

4.4.3 Interactions between PAHs

Although PHE was slightly toxic to plants and associated AM fungi, both microplate and pot experiments showed that PHE promoted DBA dissipation. In the microplate experiment, DBA dissipation in PHE+DBA treatment was more than 42 times higher than that in DBA treatment after four weeks. And in pot experiment, the DBA dissipation in PHE+DBA treatment increased by 90% and 44% in non-planted control and non-mycorrhizal plant treatments. This effect was higher in microplates than in soil pots probably due to a lower availability of DBA, the deficiency of nutrients and the competition between PAH degraders and other microorganisms in the soil. PHE stimulated DBA dissipation was previously observed by Juhasz et al. (1997). Our results showed that PHE increased bacterial number, while lower numbers were obtained with DBA alone because of its

inability to support the growth of PAH degraders. Another hypothesis is that PHE provided the enzymes, e.g. oxidoreductase produced by bacteria during its degradation process, and contributed to incorporate atoms of molecular oxygen into the aromatic nucleus of DBA (Chauhan et al., 2008). Since the hydroxylation was the rate limiting step, oxydase with broad substrate range would be desirable for HMW degradation.

Not all kinds of PAH combination could accelerate HMW PAH dissipation, e.g. the DBA dissipation was not increased with the addition of PYR. However, the bacterial number was very low with PYR as only carbon source. And when added PYR into the PHE + DBA spiked soil, it inhibited both PHE and DBA from degradation.

4.4.4 Watering regime and phosphorus fertilization

Water and phosphorus, which have been widely recognized as determining factors controlling plant and soil microbial growth and activity (Børresen and Rike, 2007; Joner et al., 2002), are always limited in PAH contaminated sites owing to the hydrophobic property of PAHs (Hutchinson et al., 2001; Li et al., 1997). Our study showed that phosphorus addition increased the DBA dissipation in both planted and unplanted treatments when watering regime was not limiting. In planted pots DBA dissipation was significantly higher in high water low phosphorus (44%) than in low water low phosphorus (16%) treatment. The decrease of water regime significantly decreased the efficiency of phytoremediation

Other parameters, such as soil property (Chiapusio et al., 2007), culture time (Joner and Leyval, 2003a), microorganism community (Joner et al., 2001; Juhasz et al., 1997), and other inorganic nutrient concentration (Joner et al., 2002; Liebeg and Cutright, 1999) could also influence PAH dissipation. As shown in our study, differences in such parameters could strongly affect the PAH dissipation in plant rhizosphere.

4.5 Conclusions

Our experiments were performed with a PAH spiked soil, where the availability of PAHs may not be compared with historical contamination. However, our results pointed out parameters affecting PAH phytoremediation.

(1) Different plant species showed different effects on plant biomass and PAH phytoremediation. Among the four kinds of plants, the fine roots plant had the highest plant biomass, while alfalfa showed the highest efficiency in HMW PAH (DBA) dissipation. Combination of alfalfa and tall fescue didn't increase the PAH dissipation in comparison with the single plant species.

(2) AM fungi could increase phosphorus and water uptake and transport, and thus increase the plant biomass. PHE and DBA dissipation was also increased due to AM inoculation, and this effect was not only due to the plant biomass effect. However, such AM effects depended on plant and AM fungi species. In our experimental conditions, *G. mosseae* was more efficient in increasing plant biomass, while only *G. intraradices* showed an effect on PAH dissipation.

(3) Fertilization with phosphorus and water regime are key parameters controlling plant growth and mycorrhizal colonization, and their effect on PAH dissipation.

(4) Six weeks after spiking the noncontaminated soil, the number of PAH-RHDα genes reached a high number of copies (10^8). The culture independent method showed that the average PAH degraders represented 0.08 to 2.7% of total bacteria in polluted soil, and the quantity of gram positive PAH degraders overwhelmed that of the gram negative PAH degraders. However, in most treatments, there was no correlation between the number of PAH-RHDα genes and PAH dissipation.

(5) PHE stimulated the degradation of DBA, while PYR had no effect on DBA dissipation, and even displayed negative effect on PHE and DBA dissipation when added into PHE + DBA substrate. These results suggested that complex interactions between PAHs could also occur in soil and should be considered.

4.6 Future research

Further investigation should focus on following issues:

(1) A large variability among replicates was observed in our experiments, although the spiked soil was carefully homogenized. The whole soil of the pots was considered as rhizosphere soil, not only the soil adhering to roots, which may have underestimated a potential rhizosphere effect. Further research could focus on soil adhering to roots as done by Corgié et al (2006c), or could use different experimental design with sand or glass beads instead of soil, or use small pots with limited quantity of soil and root mats.

(2) Historical contaminated soils are more complex than spiked soil since they contain a large number of PAHs and often other pollutants (such as heavy metal). Organic pollutants, as they persist, or age, in soil, become progressively less available. This could not be reflected by artificially spiked soil. Thus AM fungi assisted phytoremediation, and especially the selection of plant and AM fungi, should be investigated with real polluted soils. Our results suggest that PAH dissipation in plant rhizosphere is affected by many parameters, and should be considered as case specific.

(3) In our experiments, we analysed PAH concentration with butanol extraction method, which is supposed to extract the bioavailable PAHs within the soil (Kelsey et al., 1997), instead of methods considered as more exhaustive, such as soxlhet method. Indeed, the latter

can overestimate exposure from toxic chemicals in contaminated sites. Some experiments suggested that PAH availability was not the same for the earthworm and the bacteria (Tang et al., 1998). In historical contaminated soils, PAH availability is lower than in spiked soils, thus, it would be necessary to further investigate the bioavailable PAH concentrations in plant rhizosphere.

(4) No relationship was detected between the bacteria or PAH degraders and PAH dissipation. However, only the presence of degradation genes was investigated, not their expression. To precise the interactions between the bacteria and PAH degradation, the 16S rRNA which provide insights into the activity of bacteria should be measured during a similar trial, with particular emphasis placed on the PAH degraders within the soil.

(5) To date, limited information is available regarding the mechanisms of AM fungi interaction with PAH phytoremediation. The AM fungi were observed to increase the biomass of plants and affect bacterial community in plant rhizosphere. However, their precise effect on PAH dissipation still needs further investigation.

(6) Two strategies could be developed to increase the HMW PAH biodegradation. One is to increase their solubility, and help them to stimulate their own degraders. Several experiments found that surfactant could increase the solubility of LMW PAHs, and finally increase the biodegradation of these PAHs in the soil. The effect of surfactant on HMW PAH could be measured during a similar trial. Another way is to increase their co-metabolism by providing co-metabolites. In this study, we showed that there may be a link between PHE and DBA degradation with regards to the regulation of enzyme synthesis, but PHE cannot be applied to a DBA-contaminated site because it is harmful to the environment. Alternative cometabolites or inducers, for example some secondary plant metabolites with similar ring structure

(terpenes, flavonoids…) which could stimulate oxydases should be tested to increase HMW PAH dissipation, and the result may shed light on the selection of plants for phytoremediation.

Partie 5

Conclusion générale

Partie 5: Conclusion générale

Les HAP sont des polluants majeurs de l'environnement en raison de leur toxicité aiguë et de leur génotoxicité, et le développement de techniques de remédiation efficaces, à faible coût et à faible perturbation de l'écosystème, comme, la phytorémédiation, est un enjeu important. Cependant, les sols contaminés par les HAP sont souvent peu favorables à la croissance des plantes (toxicité, faible fertilité). Les champignons mycorhiziens à arbuscules (MA) sont très fréquemment présents dans les sols, établissant une symbiose avec la majorité des plantes herbacées. Mais à notre connaissance, un nombre limité d'études ont été réalisées sur la phytoremédiation des HAP par des plantes mycorhizées, et leurs résultats sont variables.

Le présent travail repose sur des expériences originales, qui ont évalué la dissipation des HAP dans la rhizosphère en prenant en compte l'effet de différentes plantes et de leur co-culture, de différents champignons MA, des propriétés des HAP, des conditions nutritives et des micro-organismes (en particulier ceux dégradant les HAP) dans la rhizosphère.

5.1. Effet des champignons MA sur la dissipation des HAP

L'inoculation par un champignon mycorhizien peut favoriser la dissipation des HAP par les moyens suivants :

1) Par la stimulation de la croissance des plantes. Les racines modifient les propriétés physico-chimiques du sol tant au niveau de sa microporosité que de sa macroporosité (modification de pH, de potentiel redox, de température, d'aération, d'humidité, de salinité) et conduisent à des modifications des propriétés biologiques et microbiologiques. L'augmentation de la densité des racines peut ainsi profiter à la dégradation des HAP.

Cependant, dans notre expérience, les deux champignons MA ont augmenté la biomasse des plantes, mais seulement *G. intraradices* a augmenté la dissipation du DBA, ce qui suggère que l'augmentation de la dissipation du DBA n'était pas seulement causée par l'augmentation de la biomasse végétale.

2) Par l'augmentation de la biodisponibilité du phosphore dans le sol et de la nutrition minérale de la plante. La fertilisation en général, et des apports de phosphore en particulier, ont permis d'accroître la dissipation de HAP (Joner et al., 2002). Ainsi, la colonisation par des champignons MA, pourrait favoriser la dissipation de HAP en favorisant la nutrition en phosphore des plantes. Dans la première et la seconde expérience, les plantes colonisées par *G. intraradices* avaient ainsi plus de phosphore que les plantes non colonisées.

3) Par une modification de la structure des communautés microbiennes en présence de mycorhizes. Dans notre étude, le nombre de copies des gènes de HAP-dioxygénase bactériens, qui peuvent contribuer à la réduction des concentrations de HAP, était significativement augmenté en présence du champignon MA *G. mosseae*. Toutefois la dissipation du DBA a été significativement plus importante avec les plantes inoculées par *G. intraradices* qu'avec les témoins plantés non mycorhizés, bien qu'il n'y ait pas de différence significative dans la structure des communautés entre les échantillons plantés mycorhizés ou non mycorhizés (Partie 4, chapitre 1).

4) Par un effet direct possible sur la dissipation des HAP, qui n'a pas encore été clairement démontré et qui n'a pas pu être étudié dans ce travail.

Ainsi les deux espèces de champignons MA sembleraient présenter une diversité fonctionnelle et des capacités différentes à promouvoir la dissipation des HAP, et la sélection des champignons et des plantes mycorhizées pourrait influencer la phytoremédiation des HAP.

5.2. Dissipation des HAP en fonction du poids moléculaire des HAP

Un inconvénient de la bioremédiation en général, et de la phytoremédiation, est sa faible efficacité sur la dissipation de HAP de haut poids moléculaire. Les HAP de haut poids moléculaire (plus de quatre cycles) sont effectivement plus réfractaires à la biodégradation à cause de leur faible solubilité dans l'eau, leur faible biodisponibilité, et le fait qu'ils ne peuvent pas servir de source de carbone pour les bactéries. Dans ce travail, nous avons étudié la dissipation de trois HAP modèles dont le DBA, qui représentait un HAP à cinq cycles, fréquemment retrouvé dans les sols contaminés.

Dans notre étude, la dissipation des HAP était effectivement plus faible avec l'augmentation du poids moléculaire des HAP. Elle était plus faible pour le DBA, que pour le PYR et le PHE dans toutes les expériences. Dans le deuxième chapitre, la dissipation moyenne après 6 semaines était de 55% pour le PYR et 15% pour le DBA (chapitre 2). Lors des essais de cultures en microplaques des bactéries rhizosphériques, nous avons montré que la présence de HAP multiples conduisait à des résultats variables en fonction de la combinaison des HAP utilisés : à un effet synergique sur la dégradation, par co-métabolisme (PHE et DBA), aucun effet (PYR et DBA) et à une inhibition de la dégradation des HAP (ajout de PYR de PHE + DBA substrat). L'augmentation de la dissipation du DBA en présence de PHE a aussi été observée dans le sol artificiellement contaminé (chapitre 4), mais l'effet positif était plus faible dans le sol que dans le substrat liquide dans l'expérience en microplaques, probablement à cause de la plus faible biodisponibilité du DBA dans le sol (chapitre 3, 4).

Les quatre plantes utilisées pour la phytoremédiation ont augmenté la dissipation du PHE, mais seule la luzerne, et le mélange luzerne et fétuque, ont montré des effets positifs sur la dissipation du DBA. Parmi les 4 plantes étudiées, et lorsque le résultat était exprimé par gramme de racines, seule la luzerne montrait une effet potentiel sur le PYR et le DBA

(chapitre 2). En outre, la co-culture de luzerne et fétuque n'a pas amélioré l'efficacité de la dissipation du DBA par rapport à ces deux plantes cultivées seules (chapitre 4). Alors que le nombre de bactéries dégradant le PHE était plus élevé que 10^6 par gramme de sol, celui des bactéries dégradant le PYR et le DBA a été inférieur à 10^4 copies (chapitre 3). Les HAP de haut poids moléculaire peuvent être dégradés par co-métabolisme, mais ce processus est lent et les voies métaboliques restent peu décrites.

Les deux champignons MA, *Glomus intraradices* et *Glomus mosseae*, ont bien colonisé les racines et ont significativement augmenté la biomasse des plantes, mais seul *G. intraradices* a augmenté la dissipation du DBA, tandis que *G. mosseae* a eu un effet négatif sur celle-ci (chapitre 4).

5.3 Influence de la teneur en phosphore et en eau sur la dissipation des HAP

L'apport de phosphore et la teneur en eau, via le régime d'arrosage (chapitre 1), se sont révélés être des paramètres importants pour la dissipation de HAP de haut poids moléculaire. La fertilisation en phosphore a augmenté la dissipation du DBA dans les traitements plantés ou sans plantes. Dans les traitements avec plantes, l'augmentaiton de la dissipation du DBA était plus élevée que dans les témoins non plantés uniquement lorsque le niveau de phosphore était bas et la teneur en eau élevée (chapitre 1).

5.4 Limites de l'étude et approches complémentaires

1) Notre étude a utilisé quatre types de plantes et deux espèces de champignons MA, et les résultats obtenus ici indiquent que l'association plante-champignon MA est un facteur clef pour la dissipation des HAP. Parce que les capacités des différentes espèces de plantes à

stimuler la biodégradation des HAP sont différentes, il serait important de tester d'autres espèces végétales, légumineuses, herbacées, en prenant en compte la qualité de leurs exsudats. L'inoculation avec deux champignons MA différents a montré des effets différents sur la dissipation des HAP. Les deux espèces des champignons MA utilisées dans cette étude ont été isolées de sols contaminés par des éléments en traces métalliques (métaux lourds). Bien qu'ils aient montré dans des travaux antérieurs, et dans nos travaux, leur capacité à croître en présence de HAP et à favoriser la dissipation des HAP, l'utilisation d'autres champignons MA, isolés par exemple de sols contaminés avec des HAP, pourrait peut-être permettre une meilleure stimulation des plantes ou une meilleure biodégradation des polluants.

2) Les résultats expérimentaux ont montré que les plantes et les champignons MA peuvent augmenter la dissipation des HAP dans le sol étudié. Mais ces résultats ont été obtenus avec un sol artificiellement contaminé en HAP. Ce type de contamination a été utilisé pour simplifier le système et étudier l'effet potentiel des champignons MA et des plantes sur biodégradation des HAP. Toutefois, l'utilisation d'un sol présentant une contamination historique et un vieillissement de la contamination en HAP, pourrait conduire à des résultats différents en raison d'une diminution de la biodisponibilité des contaminants, d'une toxicité des autres contaminants existants, et de l'eau et les éléments nutritifs déficients. Ainsi, des expériences complémentaires avec le sol des sites contaminés réels sont nécessaires. Les sols contaminés sur les sites industriels présentent souvent des contaminations multiples et anciennes. La comparaison de la croissance des plantes, des champignons MA et des communautés bactériennes sur des sols avec HAP d'âge et de composition différentes mériterait ainsi d'être effectuée.

3) L'implication directe des champignons MA dans le catabolisme des HAP n'a pas été abordée dans notre étude, bien que les plantes sans champignons MA aient été utilisées

comme témoin. En effet, les expériences ont été réalisées dans des conditions non stériles, dans un sol, où les champignons MA ont plusieurs effets sur la dissipation. Par exemple, les champignons MA peuvent influencer indirectement la dissipation de HAP par action sur la croissance des plantes et des microorganismes dans le sol, les propriétés physiques et chimiques du sol, et les exsudats de plantes. Il est difficile de séparer l'effet des plantes, des micro-organismes et des champignons MA dans ces conditions expérimentales. Parce que les champignons AM ne peuvent pas être cultivés sans la présence de plantes hôtes, l'étude du champignon seul n'est pas réalisable. Des cultures monoxéniques avec des racines transformées peuvent être réalisées (Verdin et al, 2006), mais les résultats ne sont pas forcément extrapolables à ce qui se passe dans un sol. Ce problème limite la compréhension des mécanismes impliqués chez ces champignons MA dans la dégradation des HAP, et d'autres dispositifs expérimentaux mériteraient d'être développés afin de préciser ces mécanismes. Un dispositif de culture à deux compartiments, qui séparent les hyphes des racines avec une membrane de nylon, pourrait être utilisé pour évaluer les effets des champignons AM sur la dégradation des HAP. La culture de racines transformées d'endives mycorhizées in vitro peut éliminer l'influence des micro-organismes dans la rhizosphère. Les deux méthodes pourraient aider à évaluer l'effet des champignons MA sur la dissipation des HAP, et les mécanismes impliqués.

4) Différentes méthodes ont été utilisées pour étudier et quantifier les microflores totales et dégradantes, comme le dénombrement des microorganismes cultivables, la TTGE et la PCR en temps réel. Cependant, des résultats différents ont été obtenus avec des méthodes différentes : tandis que par la méthode de dénombrement des microorganismes cultivables une augmentation des bactéries dégradant le PHE a été montrée dans le traitement planté (chapitre 3), la méthode de PCR en temps réel n'a pas montré de différence significative entre les

traitements plantés et non plantés. Ces différences pourraient être liées au fait que les deux méthodes ne quantifient pas le même compartiment de la microflore. La quantification des ADN (gènes 16S et HAP-dioxygénases) peut ne pas refléter l'expression des gènes et les bactéries actives. Ainsi, l'utilisation de l'ARN pourrait être un atout essentiel dans la compréhension de la dégradation bactérienne des HAP dans les sols étudiés. L'étude des communautés bactériennes d'un point de vue fonctionnel devrait être approfondie dans de futures expériences afin de connaître la structure des communautés effectivement actives et leur part dans la population totale. Cette technique pourrait aider à évaluer la relation entre l'activité des dégradeurs de HAP et la biodégradation des HAP. Par ailleurs, d'autres gènes impliqués dans la dégradation des HAP que les HAP-dioxygénases pourraient être ciblés. L'analyse des profils lipidiques des bactéries (phospholipid fatty acid, PLFA) pourrait mettre en évidence les organismes viables (Wu et al., 2008). Des marqueurs stables (^{13}C-HAP) pourraient également être utilisés afin de suivre les variations métaboliques ayant lieu durant la biodégradation (Johnson et al., 2002).

(5) La biodisponibilité des polluants est un des facteurs essentiels intervenant sur leur biodégradation. Cependant, bien que de nombreuses méthodes d'extraction aient été proposées pour accéder au compartiment biodisponible des HAP, la notion de HAP biodisponible reste mal définie, comme l'atteste le nombre de techniques d'extraction utilisées dans la littérature, et leur interprétation reste sujet à controverse (Kelsey et al., 1997 ; Parrish et al., 2005 ; Reid et al., 2000). Au lieu d'analyser les 16 HAP par une extraction exhaustive, nous avons choisi d'analyser les HAP disponibles. Le butanol-1 (Kelsey et al., 1997) a été utilisé dans ce travail pour mesurer les HAP biodisponibles dans le sol. Toutefois, dans les conditions expérimentales adoptées, avec un sol artificiellement contaminé, et avec une contamination très récente, la biodisponibilité est forte. Ainsi, des analyses préliminaires,

ont montré que la fraction extractible au butanol dans le sol artificiellement contaminé représentait 98% % des HAP extractibles par une méthode plus exhaustive (ASE et hexane), ce qui ne serait pas le cas dans un sol historiquement contaminé.

6) Deux stratégies peuvent être développées pour augmenter la biodégradation des HAP de haut poids moléculaire. La première consiste à augmenter leur solubilité, et ainsi à stimuler leur dégradation. Plusieurs expériences ont révélé que l'utilisation de surfactants pouvait augmenter la solubilité des HAP, et augmenter leur biodégradation dans le sol (Wu et al, 2008). La seconde consiste à augmenter leur co-métabolisme par l'ajout de co-métabolites. Notre étude a montré qu'en présence de PHE la dissipation du DBA était augmentée. En effet, les substances organiques qui ont des analogies de structure avec les contaminants du sol permettent de limiter le stress occasionné par la pollution ou d'augmenter la survie des bactéries dégradant les HAP (Singer et al., 2003). Il est possible de rechercher une substance non toxique qui pourrait stimuler la dégradation de HAP tels que le DBA. En outre, notre étude a montré que les différentes espèces de plantes ont des effets différents sur la dissipation du DBA, en particulier la luzerne et le céleri. L'étude des métabolites secondaires des plantes pourrait aider à comprendre les différents résultats obtenus et à apporter des informations sur la dynamique bactérienne de dégradation des HAP de haut moléculaire poids (Singer et al., 2003). Elle permettrait de déterminer les plantes secrétant les métabolites secondaires les plus propices à la dégradation des HAP par voie bactérienne.

Partie 6

Références bibliographiques

Partie 6: Références bibliographiques

Alexander, M., 1995. How toxic are toxic chemicals in the soil? Environmental Science and Technology 29, 2713-2717.

Alexander, M., 2000. Aging, bioavailability, and overestimation of risk from environmental pollutants. Environmental Science and Technology 34, 4259-4265.

Anderson, T.A., Guthrie, E.A. Walton, B.T., 1993. Bioremediation in the rhizosphere: Plant roots and associated microbes clean contaminated soil, Environmental Science and Technology 27, 2630-2636.

Aprill, W., Sims, R.C., 1990. Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. Chemosphere 20, 253-265.

Aulakh, M.S., Wassmann, R., Buenol, C., Rennenberg, B., 2001. Impact of root exudates of different cultivars and plant development stages of rice (*Oryza sativa* L.) on methane production in a paddy soil. Plant and Soil 230, 77-86.

Aziza, Chulan H., Martin, K., 1992. The vesicular-arbuscular (VA) mycorrhiza and its effects on growth of vegetatively propagated Theobroma cacao L. Plant and Soil 144, 227-233.

Baon, J.B., Smith, S.E., Alston, A.M., 1994. Growth response and phosphorus uptake of rye with long and short root hairs: interactions with mycorrhizal infection. Plant and Soil 167, 247-254.

Basheer, C., Obbard, J.P., Lee, H.K., 2003. Persistent organic pollutants in Singapore's coastal marine environment. Water, Air and Soil Pollution 149, 315-325.

Bezalel, L., Hadar, Y., Cerniglia, C.E., 1997. Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. Applied and Environmental Microbiology 63, 2495-2501.

Biache, C., Mansuy-Huault, L., Faure, P., Munier-Lamy, C., Leyval, C., 2008. Effects of thermal desorption on the composition of two coking plant soils: Impact on solvent extractable organic compounds and metal bioavailability. Environmental Pollution 156, 671-677.

Binet, P., Portal, J.M., Leyval, C., 2000. Fate of polycyclic aromatic hydrocarbons (PAH) in the rhizosphere and mycorrhizosphere of ryegrass. Plant and Soil 227, 207-213.

Binet, P., Portal, J.M., Leyval, C., 2001. Application of GC-MS to the study of anthracene disappearance in the rhizosphere of ryegrass. Organic Geochemistry 32, 217-222.

Bolan, N.S., 1991. A critical review of the role of mycorrhizae fungi in the uptake of phosphorus by plants. Plant and Soil 134, 189-207.

Boldrin, B., Thiem, A., Fritsche, C., 1993. Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium sp.*. Applied and Environment Microbiology 59, 1927-1930.

Børresen, M.H., Rike, A.G., 2007. Effects of nutrient content, moisture content and salinity on mineralization of hexadecane in an Aritic soil. Cold Regions Science and Technology 48, 129-138.

Boswell, E.P., Koide, R.T., Shumway, D.L., Addy, H.D., 1998. Winter Wheat cover cropping, VA mycorrhizal fungi and maize growth and yield. Agriculture, Ecosystems and Environment 67, 55-65.

Bouchez, M., Blanchet, D., Bardin, V., Haeseler, F., Vandecasteele, J.P., 1999. Efficiency of defined strains and of soil consortia in the biodegradation of polycyclic aromatic hydrocarbon (PAH) mixtures. Biodegradation 10, 429-435.

Bouchez, M., Blanchet, D., Vandecasteele, J-P., 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. Applied Microbiology and Biotechnology 43, 156-164.

Cabello, M.N., 1997. Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). FEMS Microbiology Ecology 22, 233-236.

Cajthaml, T., Moder, M., Kacer, P., Sasek, V., Popp, P., 2002. Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection, Journal of Chromatography A 974, 213-222.

Cajthaml, T., Erbanová, P., Šašek, V., Moeder, M., 2006. Breakdown products on metabolic pathway of degradation of benz[a]anthracene by a ligninolytic fungus. Chemosphere 64, 560-564.

Carmichael, L.M., Pfaender, F.K., 1997. The effect of inorganic and organic supplements on the microbial degradation of phenanthrene and pyrene in soil. Biodegradation 8, 1-13.

Carroll, K.M., Harkness, M.R., Bracco, A.A., Balcarcel, R.R., 1994. Application of a permeant/polymer diffusional model to the desorption of polychlorinated biphenyls from Hudson river sediments. Environmental Science and Technology 28, 253-258.

Cébron, A., Norini, M-P., Béguiristain, T., Leyval, C., 2008. Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHDα) genes from gram positive and gram negative bacteria in soil and sediment samples. Journal of Microbiological Methods 73, 148-159.

Cébron, A., Beguiristain, T., Faure, P., Norini, M-P., Masfaraud J-F., Leyval, C., 2009. Influence of vegetation on the in situ bacterial community and polycyclic aromatic hydrocarbon (PAH) degraders in aged PAH- contaminated or thermal-desorption-treated soil. Applied and Environmental Microbiology 75, 6322-6330.

Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351-368.

Chaîneau, C.H., Morel, J.L., Oudot, J., 1997. Phytotoxicity and plant uptake of fuel oil hydrocarbons. Journal of Environmental Quality 26, 1478-1483.

Chang, Y.Y., Corapcioglu, M.Y., 1998 . Plant-enhanced subsurface bioremediation of nonvolatile hydrocarbons. Journal of Environmental Engineering 124, 162-169.

Chang, Y.T., Lee, J.F., Hung, C.H., 2007. PAH biodegradation in surfactant-water systems based on the theory of cohesive energy density (CED). Journal of Chemical Technology and Biotechnology 82, 442-452.

Chauhan, A., Fazlurrahman, Oakeshott, J. G., Jain R.K., 2008. Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. Indian Journal of Microbiology 48, 95-113.

Cheema, S.A., Khan, M.I., Shen, C.F., Tang, X.J., Farooq, M., Chen, L., Zhang, C.K., Chen, Y.X., 2010. Degradation of phenanthrene and pyrene in spiked soils by single and combined plants cultivation. Journal of Hazardous Materials 177, 384-389.

Cheema, S.A., Khan, M.I., Tang, X.J., Zhang, C.K., Shen, C.F., Malik, Z., Ail, S., Yang, J.J., Shen, K.L., Chen, X.C., 2009. Enhancement of phenanthrene and pyrene degradation in rhizosphere of tall fescue (*Festuca arundinacea*). Journal of Hazardous Materials 166, 1226-1231.

Chen, B.D., Zhu, Y.G., Duan, J., Xiao, X.Y., Smith, S.E., 2007. Effects of the arbuscular mycorrhizal fungus *Glomus mosseae* on growth and metal uptake by four plant species in copper mine tailings. Environmental Pollution 147, 374-380.

Chen, M.M., Chen, B.D., Marschner, P., 2008. Plant growth and soil microbial community structure of legumes and grasses grown in monoculture or mixture. Journal of Environmental Sciences - China 20, 1231-1237.

Chen, S-H., Aitken, M.D., 1999. Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophilia* P15. Environmental Science and Technology 33, 435-439.

Chen, X., Wu, C.H., Tang, J.J., Hu, S.J., 2005. Arbuscular mycorrhizae enhance metal lead uptake and growth of host plants under a sand culture experiment. Chemosphere 60, 665-671.

Chen, Y-C., Banks, M.K., Schwab, A.P., 2003. Pyrene Degradation in the Rhizosphere of Tall Fescue (*Festuca arundinacea*) and Switchgrass (*Panicum virgatum L.*). Environmental Science and Technology 37, 5778-5782.

Chetwittayachan, T., Shimazaki, D., Yamamoto, K., 2002. A comparison of temporal variation of particle-bound polycyclic aromatic hydrocarbons (pPAHs) concentration in different urban environments: Tokyo, Japan, and Bangkok, Thailand. Atmospheric Environment 36, 2027-2037.

Chiapusio, G., Pujol, S., Toussaint, M.L., Badot, P.M., Binet, P., 2007. Phenanthrene toxicity and dissipation in rhizosphere of grassland plants (*Lolium perenne* L. and *Trifolium pratense* L.) in three spiked soils. Plant and Soil 294, 103-112.

Chiou, C.T., Sheng, G., Manes, M., 2001. A partition-limited model for the plant uptake of organic contaminants from soil and water. Environmental Science and Technology 35, 1437-1444.

Chung, N., Alexander, M., 2002. Effect of soils properties on bioavailability and extractability of phenanthrene and atrazine sequestred soil. Chemosphere 48, 109-115.

Coleman, J.O.D., Blake-Kalff, M.M.A., Davies, T.G.E., 1997. Detoxification of xenobiotics by plants : chemical modification and vacuolar compartmentation. Trends in Plant Science 2, 144-151.

Collins, C., Fryer, M., Grosso, A., 2006. Plant uptake of non-ionic organic chemicals. Environmental Science and Technology 40, 45-52.

Corgié, S.C., Joner, E., Leyval, C., 2003. Rhizospheric degradation of phenanthrene is a function of proximity to roots. Plant and Soil 257, 143-150.

Corgié S.C., Béguiristain, T., Leyval, C., 2004. Spatial distribution of bacterial communities and phenanthrene (PHE) degradation in the rhizosphere of *Lolium perenne* L. Applied and Environmental Microbiology 70, 3552-3557.

Corgié S.C., Béguiristain, T., Leyval, C., 2006a. Differential composition of bacterial communities as influenced by phenanthrene and dibenzo[a,h]anthracene in the rhizosphere of ryegrass (*Lolium perenne* L.). Biodegradation 17, 511-521.

Corgié, S.C., Béguiristain, T., Leyval, C., 2006b. Profiling 16S bacterial DNA and RNA: difference between community structure and transcriptional activity in phenanthrene polluted sand in the vicinity of plant roots. Soil Biology and Biochemistry 38, 1545–1553.

Corgié, S.C., Fons, F., Béguiristain, T., Leyval, C., 2006c. Biodegradation of phenanthrene, spatial distribution of bacteria populations and dioxygenase expression in the mycorrhizosphere of *Lolium perenne* inoculated with *Glomus mosseae*. Mycorrhiza 16, 207-212.

Criquet, S., Joner, E., Leglize, P., Leyval, C., 2000. Anthracene and mycorrhiza affect the activity of oxidoreductases in the roots and the rhizosphere of lucerne (*Medicago sativa* L.). Biotechnology Letters 22, 1733-1737.

Cutright, T.J., 1995. Polycyclic aromatic hydrocarbon biodegradation and kinetics using *Cunninghamella echinulata var. elegans.* International Biodeterioration and Biodegradation 35, 397-408.

Cutright, T.J., Lee, S., 1994. Microorganisms and metabolic pathways for remediation of PAH in contaminated soil. Fresenius Environmental Bulletin 3, 413-421.

Cuypers, C., Grotenhuis, T., Joziasse, J., Rulkens, W., 2000. Rapid persulfate oxidation predicts PAH bioavailability in soils and sediments, Environmental Science and Technology 34, 2057-2063.

Cuypers, C., Pancras, T., Grotenhuis, T., Rulkens, W., 2002. The estimation of PAH bioavailability in contaminated sediments using hydroxypropyl-beta-cyclodextrin and triton x-100 extraction techniques, Chemosphere 46, 1235-1245.

Dalton, H. Stirling, D.I., 1982. Co metabolism. philadelphia transactions of royal society of London B., 297, 481-486.

Dashti, N., Khanafer, M., El-Nemr, I., Sorkhoh, N., Ali, N., Radwan, S., 2009. The potential of oil-utilizing bacterial consortia associated with legume root nodules for cleaning oily soils. Chemosphere 74, 1354-1359.

Dean-Ross, D., Moody, J., Cerniglia, C.E., 2002. Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. FEMS Microbiology Ecology 41, 1-7.

Debiane, D., Garçon, G., Verdin, A., Fontaine, J., Durand, R., Grandmougin-Ferjani, A., Shirali, P., Sahraoui, Lounès-Hadj Sahraoui, A., 2008. In vitro evaluation of the oxidative stress and genotoxic potentials of anthracene on mycorrhizal chicory roots. Environmental and Experimental Botany 64, 120-127.

Declerck, S., Plenchette, C., Strullu, D.G., 1995. Mycorrhizal dependency of banana (*Musa acuminata*, AAA group) cultivar. Plant and Soil 176,183-187.

Douds, D.D., Nagahashi, G., 2000. Signalling and recognition events prior to colonisation of roots by arbuscular mycorrhizal fungi. In Current Advances in Mycorrhizae Research. Ed. Podila, G., Douds, D.D. Minnesota: APS Press. pp 11-18.

Dueck, T.A., Visser, P., Ernst, W.H.O., Schat, H., 1986. Vesicular-arbuscular mycorrhizae decrease zinc-toxicity to grasses growing in zinc-polluted soil. Soil Biology and Biochemistry 18, 331-334.

Eggen, T., Majcherczyk, A., 1998. Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus *Pleurotus ostreatus*. International Biodeterioration and Biodegradation 41, 111-117.

Erickson, M., Dalhammar, G., Borg-karlson, A.K., 2000. Biological degradation of selected hydrocarbons in an old PAH/creosote contaminated soil from a gas work site. Applied Microbiology and Biotechnology 53, 619-626.

Fan, S.X., Li, P.J., Gong, Z.Q., Ren, W.X., He, N., 2008. Promotion of pyrene degradation in rhizosphere of alfalfa (M*edicago sativa* L.). Chemosphere 71, 1593-1598.

Fang, C.W., Radosevich, M., Fuhrmann J.J., 2001. Atrazine and phenanthrene degradation in grass rhizosphere soil. Soil Biology and Biochemistry 33, 671-678.

Fava, F., Berselli, S., Conte, P., Piccolo, A., Marchetti, L., 2004. Effects of humic substances and soya lecithin on the aerobic bioremediation of a soil historically contaminated by polycyclic aromatic hydrocarbons (PAHs). Biotechnology and Bioengineering 88, 214-223.

Fernández, P., Vilanova, R.M., Martínez, C., Appleby, P., Grimalt, J.O., 2000. The historical record of atmospheric pyrolytic pollution over Europe registered in the sedimentary PAH from remote mountain lakes. Environmental Science and Technology 34, 1906-1913.

Ferro, A., Kennedy, J., Doucette, W., Nelson, S., Jaurgui, G., McFarland, B., Bugee, B., 1997. Fate of benzene in soils planted with alfalfa: uptake, volatilization and degradation. In: phytoremediation of soil and water contiminants. Kuger, E.L., Anderson, T.A. and Coats, J.R., Eds. American chemical society washington D.C., 223-237.

Fiala, Z., Cyskocil, A., Kraják, V., Masin, V., Masin, V., Emminger, S., Srb, V., Tejral, J., 1999. Polycyclic aromatic hydrocarbons I. Environmental contamination and environmental exposure. Acta Medica 42, 77-89.

Finlayson-Pitts, B.J., Pitts Jr., J.N., 1997. Tropospheric air pollution: ozone, airborne toxics, polycyclic aromatic hydrocarbons and particles. Science 276, 1045-1052.

Fismes, J., Perrin-Ganier, C., Empereur-Bissonnet P., Morel, J.L., 2002. Soil-to-root transfer and translocation of polycyclic aromatic hydrocarbons by vegetables grown on industrial contaminated soils. Journal of Environmental Quality 31, 1649-1656.

Flotron, V., Delteil, C., Padellec, Y., Camel, V., 2005. Removal of sorbed polycyclic aromatic hydrocarbons from soil, sludge and sediment samples using the Fenton's reagent process, Chemosphere 59, 1427-1437.

Gan, S., Lau, E.V., Ng, H.K., 2009. Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). Journal of Hazardous Materials 172, 532-549.

Gao, Y., Yu, X.Z., Wu, S.C., Cheung, K.C., Tam, N.F.Y., Qian, P.Y., Wong, M.H., 2006. Interactions of rice (*Oryza sativa* L.) and PAH-degrading bacteria (*Acinetobacter* sp.) on enhanced dissipation of spiked phenanthrene and pyrene in waterlogged soil. Science of Total Environment 372, 1-11.

Gao, Y.Z., Cheng, Z.X., Ling, W.T., Huang, J., 2010. Arbuscular mycorrhizal fungal hyphae contribute to the uptake of polycyclic aromatic hydrocarbons by plant roots. Bioresource Technology 101, 6895-6901.

Gao, Y.Z., Zhu, L.Z., 2004. Plant uptake, accumulation and translocation of phenanthrene and pyrene in soils. Chemosphere 55, 1169-1178.

Garcia-Blanco, S., Venosa, A.D., Suidan, M.T., Lee, K., Cobanli, S., Haines, J.R., 2007. Biostimulation for the treatment of an oil-contaminated coastal salt marsh. Biodegradation 18, 1-15.

Garon, D., Sage, L., Seigle-Murandi, F., 2004. Effects of fungal bioaugmentation and cyclodextrin amendment on fluorene degradation in soil slurry. Biodegradation 15, 1-8.

Genney, D.R., Alexander, I.J., Killham, K., Meharg, A.A, 2004. Degradation of the polycyclic aromatic hydrocarbon (PAH) fluorene is retarded in a Scots pine ectomycorrhizosphere. New Phytologist 163, 641-649.

Gibson, D.T., Mahadevan, V., Jerina, R.M., Yagi, H., Yeh, H.J.C., 1975. Oxidation of the carcinogens benzo[a]pyrene and dibenz[a,h]anthracene to dihydrodiols by a bacterium. Science 189, 295-297.

Gildon, A., Tinker, P.B., 1983. Interaction of vesicular-arbuscular mycorrhizal infections and heavy metals in plants. New Phytologist 95, 263-268.

Girotti, S., Maiolini, E., Bolelli, L., Ferri, E., Pompei, A., Matteuzzi, D., Medvedeva, S., Fonti, P., 2008. Bioremediation of contaminated soil by hydrocarbons degrading bacteria and

decontamination control. NATO Science for Peace and Security Series C: Environmental Security, Soil Chemical Pollution, Risk Assessment, Remediation and Security. Springer Netherlands, pp 369-383.

Gray, N.D., Hasting, R.C., Sheppard, S.K., Loughnane, P., Lloyd, D., McCarthy, A.J., Head, I.M., 2003. Effects of soil improvement treatments on bacterial community structure and soil processes in an upland grassland soil. FEMS Microbiology Ecology 46, 11-22.

Groleau-Renaud, V., Plantureux, S., Guckert, A., 1998. Influence of plant morphology on root exudation of maize subjected to mechanical impedance in hydroponic conditions. Plant and Soil 201, 231-239.

Günther, T., Dornberger, U., Fritsche, W., 1996. Effects of ryegrass on biodegradation of hydrocarbons. Chemosphere 33, 203-215.

Hamdi, H., Benzarti, S., Manusadžianas, L., Aoyama, I., Jedidi, N., 2007. Bioaugmentation and biostimulation effects on PAH dissipation and soil ecotoxicity under controlled conditions. Soil Biology and Biochemistry 39, 1926-1935.

Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. Journal of Hazardous Materials 169, 1-15.

Hatakka, A., 1994. Lignin-modifying enzymes from selected white rot fungi: production and role in lignin degradation. FEMS Microbiology Reviews 13, 125-135.

Harley, J.L., Smith, S.E., 1983. Mycorrhizal symbiosis. Academic Press, New York.

Harvey, P.J., Campanella, B.F., Castro, P.M.L., Harms, H., Lichtfouse, E., Schäffner, A.R., Smrcek, S., Werck-Reichhart, D., 2002. Phytoremediation of polyaromatic hydrocarbons, anilines, and phenols. Environmental Science and Pollution Research 9, 29-47.

Hawthorne, S.B., Grabanski, C.B., 2000. Correlating selective supercritical fluid extraction with bioremediation behavior of PAHs in a field treatment plot, Environmental Science and Technology 34, 4103-4110.

Heggo, A., Angle, J.S., Chaney, R.L., 1990. Effects of vesicular-arbuscular mycorrhizal fungi on heavy metal uptake by soybeans. Soil Biology and Biochemistry 22, 865-869.

Hildebrandt, U., Kaldorf, M., Bothe, H., 1999. The zinc violet and its colonization by arbuscular mycorrhizal fungi. Journal of Plant Physiology 154, 709-717.

Hiltner, L., 1904. Über neuere erfahrungen und probleme auf dem gebiete der bodenbakteriologie unter besonderer berücksichtigung der gründüngung und brache. Arbeiten der Deutschen Landwirtschaftlichen Gesellschaft 98, 59-78.

Hofrichter, M., Schneibner, K., Schneegab, I., Fritzche, W., 1998. Enzymatic combustion of aromatic and aliphatic compounds by manganese peroxidase from *Nematoloma frowardii*. Applied and Environmental Micorbiology 64, 399-404.

Holding, A.J., 1960. The properties and classification of the predominant gram-negative bacteria occurring in soil. Journal of Applied Bactoriology 23, 515-525.

Hong, Y.W., Yuan, D.X., Lin, Q.M., Yang, T.L., 2008. Accumulation and biodegradation of phenanthrene and fluoranthene by the algae enriched from a mangrove aquatic ecosystem. Marine Pollution Bulletin 56, 1400-1405.

Huang, X., Alawi, Y.E., Penrose, D.M., Glick, B.R., Greenberg, B.M., 2004. A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environmental Pollution 130, 465-476.

Huang, H.L., Zhang, S.Z., Shan, X.Q., Chen, B.D., Zhu, Y.G., Bell, J. N.B., 2007. Effect of arbuscular mycorrhizal fungus (*Glomus caledonium*) on the accumulation and metabolism of atrazine in maize (*Zea mays* L.) and atrazine dissipation in soil. Environmental Pollution 146, 452-457.

Huang, H.L., Zhang, S.Z., Wu, N.Y., Luo, L., Christie, P., 2009. Influence of *Glomus etunicatum/Zea mays* mycorrhiza on atrazine degradation, soil phosphatase and dehydrogenase activities, and soil microbial community structure. Soil Biology and Biochemistry 41, 726-734.

Hutchinson, S.L., Banks, M.K., Schwab, A.P., 2001. Phytoremediation of aged petroleum sludge: effect of inorganic fertilizer. Journal of Environmental Quality 30, 395-403.

Jakoncic, J., Jouanneau, Y., Meyer, C., Stojanoff, V., 2007. The catalytic pocket of the ring-hydroxylating dioxygenase from *Sphingomonas* CHY-1. Biochemical and. Biophysical Research Communication 352, 861-866.

Jakobsen, I., 1995. Transport of phosphorous and carbon in VA mycorrhiza. In: Varma, A., Hock, B. (Eds.), Mycorrhiza Structure, Function, Molecular Biology and Biotechnology. Springer, Berlin, pp. 297-324.

Jensen, A., Jokobsen, I., 1980. The occurrence of vesicular arbuscular mycorrhiza in barley and wheat grown in some Danish soils with different fertilizer treatments. Plant and Soil 55, 403-414.

Jiao, X.C., Xu, F.L., Dawson, R., Chen, S.H., Tao, S., 2007. Adsorption and absorption of polycyclic aromatic hydrocarbons to rice roots. Environmental Pollution148, 230-235.

Johnsen, A.R., Wick, L.Y., Harms, H., 2005. Principles of microbial PAH-degradation in soil. Environmental Pollution 133, 71-84.

Johnson, D.L., Leake, J.R., Ostle, N., Ineson, P., Read, D.J., 2002. In situ (CO_2) - ^{13}C pulse labeling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. New Phytologist, 153, 327-334.

Johnson, D.L., Maguire, K.L., Anderson, D.R., McGrath, S.P., 2004. Enhanced dissipation of chrysene in planted soil: the impact of a rhizobial inoculum. Soil Biology and Biochemistry 3, 33-38.

Joner, E.J., Briones, R., Leyval, C., 2000. Metal-binding capacity of arbuscular mycorrhizal mycelium. Plant and Soil 226, 227-234.

Joner, E.J., Corgié, S.C., Amellal, N., Leyval, C., 2002. Nutritional constraints to degradation of polycyclic aromatic hydrocarbons in a simulated rhizosphere. Soil Biology and Biochemistry 34, 859-864.

Joner, E.J., Hirmann, D., Szolar, O.J.H., Todorovic, D., Leyval, C., Loibner A., 2004, Priming effects on PAH degradation and ecotoxicity during a phytoremediation experiment, Environmental Pollution 128, 429-435.

Joner, E.J., Johansen, A., Loibner, A.P., Cruz, M.A.D., Szolar, O.H.J., Portal, J-M., Leyval, C., 2001. Rhizosphere effects on microbial community structure and dissipation and toxicity of polycyclic aromatic hydrocarbons (PAH) in spiked soil. Environmental Science and Technology 35, 2773-2777.

Joner, E.J., Leyval, C., 1997. Uptake of ^{109}Cd by roots and hyphae of a *Glomus mosseae/Trifolium subterraneum* mycorrhiza from soil amended with high and low concentrations of cadmium. New Phytologist 135, 353-360.

Joner, E.J., Leyval, C., 2001. Influence of arbuscular mycorrhiza on clover and ryegrass grown together in a soil spiked with polycyclic aromatic hydrocarbons. Mycorrhiza 10,155-159.

Joner, E.J., Leyval, C., 2003a. Rhizosphere gradients of polycyclic aromatic hydrocarbon (PAH) dissipation in two industrial soils and the impact of arbuscular mycorrhiza. Environmental Science and Technology 37, 2371-2375.

Joner, E., Leyval, C., 2003b. Phytoremediation of organic pollutants using mycorrizal plants: a new aspect of rhizosphere interactions. Agronomie 23, 495-502.

Joner, E.J., Leyval, C., Colpaert, J.V., 2006. Ectomycorrhizas impede phytoremediation of polycyclic aromatic hydrocarbons (PAHs) both within and beyond the rhizosphere. Environmental Pollution 142, 34-38.

Jones, D.L., 1998. Organic acids in the rhizosphere - a critical review. Plant and Soil 205, 25-44.

Jones, K.C., Stratford, J.A., Waterhouse, K.S., Vogt, N.B., 1989. Organic contaminants in Welsh soils: polynuclear aromatic hydrocarbon. Environmental Science and Technology 23, 540-550.

Juhasz, A.L., Britz, M.L., Stanley, G.A., 1997. Degradation of fluoranthene, pyrene, benz[a]anthracene and dibenz[a,h]anthracene by Burkholderia cepacia. Journal of Applied Microbiology 83, 189-198.

Juhasz, A.L., Naidu, R., 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. International Biodeterioration and Biodegradation 45, 57-88.

Kanaly, R.A., Bartha, R., Watanabe, K., Harayama, S., 2000. Rapid mineralization of benzo[a]pyrene by a microbial consortium growing on diesel fuel. Applied and Environmental Microbiology 66, 4205-4211.

Kanaly, R.A., Harayama, S., 2000. Biodegradation of high-molecular weight polycyclic aromatic hydrocarbons by bacteria. The Journal of Bacteriology 182, 2059-2067.

Kastner, M., Breuer-Jammali, M., Mahro, B., 1998. Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. Applied and Environmental Microbiology 64, 359-362.

Kauppi, B., Lee, K., Carredano, E., Parales, R., Gibson, D., Eklund, H., Ramaswamy, S., 1998. Structure of an aromatic-ring-hydroxylating dioxygenasenaphthalene 1,2-dioxygenase. Structure 6, 571-586.

Keith, L.H., Teilliard, W.A., 1979. Priority pollutants. I. A perspective view. Environmental Science and Technology 13, 416-423.

Kelsey, J.W., Kottler, B.D., Alexander, M., 1997. Selective chemical extractants to predict bioavailability of soil-aged organic chemicals. Environmental Science and Technology 31, 214-217.

Khan, A.G., 2005. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. Journal of Trace Elements in Medicine and Biology 18, 355-364.

Kipopoulou, A.M., Manoli, E., Samara, C., 1999. Bioconcentration of polycyclic aromatic hydrocarbons in vegetables grown in an industrial area. Environmental Pollution 106, 369-380.

Koske, R.E., Gemma, J.N., 1989. A modified procedure for staining roots to detect VA mycorrhizas. Mycological Research 92, 486-488.

Kraus, J., Munir, I., McEldoon, J., Clark, D., Dordick, J., 1999. Oxidation of polycyclic aromatic hydrocarbons catalyzed by soybean peroxidase. Applied Biochemistry and Biotechnology 80, 221-230.

Laor, Y., Strom, P.F., Farmer, W.J., 1999. Bioavailability of phenanthrene sorbed to mineral-associated humic acid. Water Research 33, 1719-1729.

Lau, K.L., Tsang, Y.Y., Chiu, S.W., 2003. Use of spent mushroom compost to bioremediate PAH-contaminated samples. Chemosphere 52, 1539-1546.

Law, R.J., Dawes, V.J., Woodhead, R.J., Matthiessen, P., 1997. Polycyclic aromatic hydrocarbons (PAH) in seawater around England and Wales. Marine Pollution Bulletin 34, 306-322.

Leahy, J.G., Colwell, R.R., 1990. Microbial degradation of hydrocarbons in the environment. Microbiological Reviews 54, 305-315.

Leigh, M.B., Fletcher, J.S., Fu, X., Schmitz, F.J., 2002. Root turnover: an important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants. Environmental Science and Technology 36, 1579-1583.

Leys, N.M., Ryngaert, A., Bastiaens, L., Wattiau, P., Top, E.M., Verstraete, W., Springael, D., 2005. Occurrence and community composition of fast growing Mycobacterium in soils contaminated with polycyclic aromatic hydrocarbons. FEMS Microbiology Ecology 51, 375-388.

Leyval, C., Binet, P., 1998. Effect of polyaromatic hydrocarbons in soil on arbuscular mycorrhizal plants. Journal of Environmental Quality 27, 402-407.

Li, H., Sheng, G., Chiou, C.T., Xu, O., 2005. Relation of organic contaminant equilibrium sorption and kinetic uptake in plants. Environmental Science and Technology 39, 4864-4870.

Li, X., Feng, Y., Sawatsky, N., 1997. Importance of soil-water relations in assessing the endpoint of bioremediated soil. Plant and Soil 192, 219-236.

Li, X.F., Cullen, W.R., Reimer, K.J., Le, X.C., 1996. Microbial degradation of pyrene and characterization of a metabolite. Science of the Total Environment 77,17-29.

Li, X.L., Christie, P., 2001. Changes in soil solution Zn and pH and uptake of Zn by arbuscular mycorrhizal red clover in Zn-contaminated soil. Chemosphere 42, 201-207.

Liao, W.L., Tseng, D.H., 1996. Biotreatment of naphthalene by PAH-acclimated pure culture with white-rot fungus *Phanerochaete chrysosporium.* Water Science and Technology 34, 73-79.

Liebeg, E.W., Cutright, T.J., 1999. Investigation of enhanced bioremediation through the addition of macro and micro nutrients in a PAH contaminated soil. International Biodeterioration and Biodegradation 44, 55-64.

Lin, Q.X., Mendelssohn, I.A., 1998. The combined effects of phytoremediation and biostimulation in enhancing habitat restoration and oil degradation of petroleum contaminated wetlands. Ecological Engineering 10, 263-274.

Lindstrom, J.E., Prince, R.C., Clark, J.C., Grossman, M.J., Yeager, T.R., Braddock, J.F., Brown, E.J., 1991. Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the T/V Exxon Valdez oil spill. Applied and Environmental Microbiology 57, 2514-2522.

Liste, H-H., Alexander, M., 2000. Plant-promoted pyrene degradation in soil. Chemosphere 40, 7-10.

Liste, H-H., Alexander, M., 2002. Butanol extraction to predict bioavailability of PAHs in soil. Chemosphere 46, 1011-1017.

Liste, H-H., Prutz, I., 2006. Plant performance, dioxygenase-expressing rhizosphere bacteria, and biodegradation of weathered hydrocarbons in contaminated soil. Chemosphere 62, 1411-1420.

Liu, A.G., Dalpé, Y., 2009. Reduction in soil polycyclic aromatic hydrocarbons by arbuscular mycorrhizal leek plants. International Journal of Phytoremediation 11, 39-52.

Liu, S.L., Luo, Y.M., Cao, Z.H., Wu, L.H., Ding, K.Q., Christie, P., 2004. Degradation of benzopyrene in soil with arbuscular mycorrhizal alfalfa. Environmental Health 26, 285-293.

Loehr, R.C., Webster, M.T., 1996. Behavior of fresh vs. aged chemicals in soil. Journal of Soil Contamination 5, 361-383.

Lopez, Z., Vila, J., Ortega-Calvo, J-J., Grifoll, M., 2008. Simultaneous biodegradation of creosote-polycyclic aromatic hydrocarbons by a pyrene-degrading Mycobacterium. Applied Microbiology and Biotechnology 78, 165-172.

Louvel, B., 2010. Etude en microcosmes de l'effet du ray-grass et de ses exsudats racinaires sur la dissipation des HAP et les communautés bactériennes dégradantes. Thèse de docteur de l'Université Henri Poincaré, Nancy I en Géosciences.

Luch, A. Nature and nurture - lessons from chemical carcinogenesis, 2005. Nature Reviews Cancer 5, 113-125.

Lueders, T., Wagner, B., Claus, P., Friedrich, M.W., 2004. Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. Environmental Microbiology 6, 60-72.

Lüers, F., Hulscher, Th.E.M., 1996. Temperature effect on the partitioning of polycyclic aromatic hydrocarbons between natural organic carbon and water. Chemosphere 33, 643-657.

Luthy, R.G., Dzombak, D.A., Peters, C.A., Roy, S.B., Ramaswami, A., Nakles, D.V., Nott, B.R., 1994. Remediating tar-contaminated soils at manufactured gas plant sites. Environmental Science and Technology 28, 266-275.

Mackay, D., Shiu, W.Y., Ma, K.C., 1992. Illustrated handbook of physical-chemical properties and environmental fate for organic chemicals. Volume I. Lewis Publishers. Chelsea, pp 697.

Mackay, D., Bentzen E., 1997. The role of the atmosphere in great lakes contamination. Atmospheric Environment 31, 4045-4047.

Madsen, T., Kristensen, P., 1997. Effects of bacterial inoculation and nonionic surfactants on degradation of polycyclic aromatic hydrocarbons in soil. Environmental Toxicology and Chemistry 16, 631-637.

Mallick, S., Dutta, T.K., 2008. Kinetics of phenanthrene degradation by *Staphylococcus* sp. strain PN/Y involving 2-hydroxy-1-naphthoic acid in a novel metabolic pathway. Process Biochemistry 43. 1004-1008.

Maliszewska-Kordybach B., 2005. Dissipation of polycyclic aromatic hydrocarbons in freshly contaminated soils-the effect of soil physicochemical properties and aging. Water, Air, and Soil Pollution 168, 113-128.

Margesin, R., Zimmerbauer, A., Schinner, F., 2000. Monitoring of bioremediation by soil biological activities. Chemosphere 40, 339-346.

Masten, S.J., Davies, S.H.R., 1997. Efficacy of in-situ ozonation for the remediation of PAH contaminated soils. Journal of Contaminant Hydrology 28, 327-335.

Marques, A.P. G.C., Oliveira, R.S., Rangel, A.O.S.S., Castro, P.M.L., 2006. Zinc accumulation in *Solanum nigrum* is enhanced by different arbuscular mycorrhizal fungi. Chemosphere 65, 1256-1263.

Marschner, P., Baumann, K., 2003. Changes in bacterial community structure induced by mycorrhizal colonisation in split-root maize. Plant and Soil 251, 279-289.

Marschner, P., Crowley, D.E., Lieberei, R., 2001. Arbuscular mycorrhizal interaction changes the bacterial 16S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11, 297-302.

Maxin, C.R., Kogelknabner, I., 1995. Partitioning of polycyclic aromatic hydrocarbons (PAH) to water-soluble soil organic matter. European Journal of Soil Science 46, 193-204.

McArthur, D.A.J., Knowles, N.R., 1992. Resistance responses of potato to vesicular-arbuscular mycorrhizal fungi under varying abiotic phosphorus levels. Plant Physiology 100, 341-351.

Mielke, H. W., Wang, G., Gonzales, C. R., Le, B., Quach, V. N., Mielke,P. W., 2001. PAH and metal mixtures in New Orleans soils and sediments. Science of the Total Environment 281, 217-227.

Miya, R.K., Firestone, M.K., 2000. Phenanthrene degrader community dynamics in rhizosphere soil from a common annual grass. Journal of Environmental Quality 29, 584-592.

Müller, J.G., Chapman, P.J., Blattmann, B.O., Pritchard, P.H., 1990. Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. Applied and Environmental Microbiology 56, 1079-1086.

Muratova, A., Golubev, S., Wittenmayer, L., Dmitrieva, T., Bondarenkova, A., Hirche, F., Merbach, W., Turkovskaya, O., 2009. Effect of the polycyclic aromatic hydrocarbon phenanthrene on root exudation of *Sorghum bicolor* (L.) Moench. Environmental and Experimental Botany 66, 514-521.

Muratova, A., Hubner, Th., Tischer, S., Turlcovskaya, O., Moder, M., Kuschk, P., 2003. Plant rhizosphere-microflora association during phytoremediation of PAH-contaminated soil. International Journal of Phytoremediation 5, 137-151.

Nam, K., Kim, J.Y., 2002. Role of loosely bound humic substances and humin in the bioavailability of phenanthrene aged in soil. Environmental Pollution 118, 427-433.

Narro, M.L., Cerniglia, C.E., Baalen, C.V., Gibson, D.T., 1992. Metabolism of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* strain PR-6. Applied and Environmental Microbiology 58, 1351-1359.

Ndimele, P.E., 2010. A review on the phytoremediation of petroleum hydrocarbons. Pakistan Journal of Biological Science 13, 715-722.

Neumann, G., Romheld, V., 2000. The release of root exudates as affected by the plants' physiological status. In: Pinton R, Varanini Z, Nannipieri P (eds) The rhizosphere-biochemistry and organic substances at the soil plant interface. Dekker, New York, pp 41-93.

Nichols, T.D., D.C. Wolf, H.B. Rogers, C.A. Beyrouty, and C.M. Reynolds. 1997. Rhizosphere microbial populations in contaminated soils. Water Air and Soil Pollution 95,165-178.

Nielsen, T., Jorgensen, H.E., Larsen, J.C., Poulsen, M., 1996. City air pollution of polycyclic aromatic hydrocarbons and other mutagens: occurrence, source and health effects. Science of Total Environment 189-190, 41-49.

Nieman, J.K.C., Sims, R.C., Sims, J.L., Mclean, J.E., Rice, J.A., 1999. [^{14}C]Pyrene bound residue evaluation using MIBK fractionation method for creosote-contaminated soil. Environmental Science and Technology 33, 776-781.

Norini, M.P., 2007. Ecodynamique des hydrocarbures aromaticques polycycliques (HAP) et des communautés microbiennes dans des sols à pollution mixte (HAP, métaux) avant et après traitement par biopile et par désorption thermique: Influence de la rhizosphère et de la mycorhization. Thèse de docteur de l'Université Henri Poincaré, Nancy I en Géosciences.

Nyyssönen, M., Kapanen, A., Piskonen, R., Lukkari, T., Itävaara, M., 2009. Functional genes reveal the intrinsic PAH biodegradation potential in creosote-contaminated groundwater following in situ biostimulation. Applied Microbiology and Biotechnology 84, 169-182.

Olson, P.E., Castro, A., Joern, M., Du Fau, M.D., Pilon-smits, E., Reardon, K.F. 2008. Effects of agronomic practices on phytoremediation of an aged PAH contaminated soil. Journal of Environmental Quality 37, 1439-1446.

Parrish, Z.D., Banks, M. K., Schwab, A. P., 2005. Effect of root death and decay on dissipation of polycyclic aromatic hydrocarbons in the rhizosphere of yellow sweet clover and tall fescue. Journal of Environmental Quality 34, 207-216.

Perminova, I.V., Grechishcheva, N.Yu., Kovalevskii, D.V., Kudryavtsev, A.V., Petrosyan, V.S., Matorin, D.N., 2001. Quantification and prediction of the detoxifying properties of humic substances related to their chemical binding to polycyclic aromatic hydrocarbons. Environmental Science and Technology 35, 3841-3848.

Phillips, L.A., Greer, C.W., Germida, J.J., 2006. Culture-based and culture-independent assessment of the impact of mixed and single plant treatments on rhizosphere microbial communities in hydrocarbon contaminated flare-pit soil. Soil Biology and Biochemistry 38, 2823-2833.

Phillips, T.M., Seech, A.G., Liu, D., Lee, H., Trevors, J.T., 1999. Monitoring bioremediation in creosote-contaminated soils using chemical analysis and toxicity tests. Environmental Toxicology 15, 99-106.

Ping, L., Luo, Y., Wu, L., Qian, W., Song, J. Christie, P., 2006. Phenanthrene adsorption by soils treated with humic substances under different pH and treatment. conditions, Environ. Geochem. Health 28, 189-195.

Pignatello, J.J., Xing, B., 1996. Mechanisms of slow sorption of organic chemicals to natural particles. Environmental Science and Technology 30, 1-11.

Pothuluri, J.V. and Cerniglia, C.E., 1994. Microbial metabolism of polycyclic aromatic hydrocarbons. In Biological degradation and Bioremediation of Toxic Chemicals (Chaudhry, G.R., ed.), pp. 92-124, Chapman and Hall.

Qiu, X., Leland, T.W., Shah, S.I., Sorensen, D.L., Kendan, E.W., 1997. Field study: grass remediation for clay soil contaminated with polycyclic aromatic hydrocarbons. In: phytoremediation of soil and water contiminants. Kuger, E.L., Anderson, T.A. and Coats, J.R., Eds. American chemical society washington D.C., 186-199.

Ramos, L., Kristenson, E.M., Brinkman, U.A.Th., 2002. Current use of pressurised liquid extraction and subcritical water extraction in environmental analysis. Journal of Chromatography A 975, 3-29.

Redon, P-O., Béguiristain, T., Leyval, C., 2008. Influence of *Glomus intraradices* on Cd partitioning in a pot experiment with *Medicago truncatula* in four contaminated soils. Soil Biology and Biochemistry 40, 2710-2712.

Redon, P-O. Béguiristain, T., Leyval, C., 2009. Differential effects of AM fungal isolates on *Medicago truncatula* growth and metal uptake in a multimetallic (Cd, Zn, Pb) contaminated agricultural soil. Mycorrhiza 19, 187-195.

Rehmann, K., Noll, H.P., Steiberg, C.E.W., Kettrup, A.A., 1998. Pyrene degradation by *Mycobacterium* sp. Strain KR2, Chemosphere 36, 2977-2992.

Reid, B.J., Jones, K.C., Semple, K.T., 2000. Bioavailability of persistent organic pollutants in soils and sediments - a perspective on mechanisms, consequences and assessment. Environmental Pollution 108, 103-112.

Reilley, K.A., Banks, M.K., Schwab, A.P., 1996. Dissipation of polycyclic aromatic hydrocarbons in the rhizosphere. Journal of Environmental Quality 25, 212-219.

Renoldi, F., Lietti, L., Saponaro, S., Bonomo, L., Forzatti, P., 2003. Thermal desorption of a PAH-contaminated soil: a case study. In: C.A. Brebbia, Editor, Ecosystems and Sustainable Development IV Volume 2, WIT Press, UK. pp. 1123-1132.

Rentz, J.A., Alvarez P.J.J., Schnoor, J.L., 2004. Repression of *Pseudomonas putida* phenanthrene degrading activity by plant root extracts and exudates. Environmental Microbiology 6, 574-583.

Rentz, J.A., Alvarez, P.J.J., Schnoor, J.L., 2005. Benzo[a]pyrene co-metabolism in the presence of plant root extracts and exudates: Implications for phytoremediation. Environmental Pollutiion 136, 477-484.

Rezek, J, in der Wiesche, C., Mackova, M., Zadrazil, F., Macek, T., 2008. The effect of ryegrass (*Lolium perenne*) on decrease of PAH content in long term contaminated soil. Chemosphere 70, 1603-1608.

Rivera-Becerril, F., Calantzis, C., Turnau, K., Caussanel, J.P., Belimov, A.A., Gianinazzi, S., Strasser, R.J., Gianinazzi-Pearson, V., 2002. Cadmium accumulation and buffering of cadmium-induced stress by arbuscular mycorrhiza in three *Pisum sativum* L. Genotypes. Journal of Experimental Botany 53, 1177-1185.

Rufyikiri, G., Thiry, Y., Wang, L., Delvaux, B., Declerck, S., 2002. Uranium uptake and translocation by the arbuscular mycorrhizal fungus, *Glomus intraradices*, under root-organ culture conditions. New Phytologist 156, 275-281.

Salt, D.E., Smith, R.D., Raskin, I., 1998. Phytoremediation. Annual Review of Plant Physiology 49, 643-668.

Samanta, S.K., Chakraborti, A.K., Jain, R.K., 1999. Degradation of phenanthrene by different bacteria: evidence for novel transformation sequences involving the formation of 1-naphthol. Applied Microbiology and Biotechnology 53, 98-107.

Schnabel, W.E., White, D.M., 2001. The effect of mycorrhizal fungi on the fate of aldrin: Phytoremediation potential. International Journal of Phytoremediation 3, 221-241.

Siciliano, S.D., Germida, J.J., Banks, K., and Greer, C.W., 2003. Changes in microbial community composition and function during a polycyclic aromatic hydrocarbon phytoremediation field trial. Applied and Environmental Microbiology 69, 483-489.

Sieverding, E., 1991. Vesicular-Arbuscular Mycorrhiza Managemment, Technical Cooperation-Federal Republik of German, Eschborn.

Silva, I.S., Santos, E.C., Menezes, C.R., Faria, A.F., Franciscon, E., Grossman, M., Durrant, L.R., 2009. Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia. Bioresource Technology 100, 4669-4675.

Sims, R.C., Overcash, M.R., 1983. Fate of polynuclear aromatic compounds (PNAs) in soil-plant systems. Residue Reviews 88, 1-68.

Simonich, S.L., Hites, R.A., 1994. Vegetation and atmosphere partitioning of polycyclic aromatic hydrocarbons. Environmental Science and Technology 28, 939-943.

Simonich, S.L., Hites, R.A. 1995. Organic pollutant accumulation in vegetation. Environmental Science and Technology 29, 2095-2103.

Sicbaldi, F., Sacchi, G.A., Trevisan, M., DelRe, A.A.M., 1997. Root uptake and xylem translocation of pesticides from different chemical classes. Pesticide Science 50, 111-119.

Singer, A. C., Crowley, D. E., Thompson, I. P., 2003. Secondary plant metabolites in phytoremediation and biotransformation. Trends in Biotechnology 21, 123-130.

Singh, U.P., Sarma, B.K., Singh, D.P., Bahadur, A., 2002. Plant Growth-Promoting Rhizobacteria - Mediated Induction of Phenolics in Pea (*Pisum sativum*) After Infection with *Erysiphe pisi.* Current Microbiology 44, 396-400.

Smith, M.J., Flowers, T.H., Duncan, H.J., Alder, J., 2006. Effects of polycyclic aromatic hydrocarbons on germination and subsequent growth of grasses and legumes in freshly contaminated soil and soil with aged PAH residues. Environmental Pollution 141, 519-525.

Smith, S.E., Read, D.J., 1997. Mycorrhizal symbiosis, second ed. Academic Press, London, pp605.

Song, H.G., Pedersen, T.A., Bartha, R., 1986. Hydrocarbon minerazation in soil relative bacteria and fungal contribution. Soil biology and biochemistry 18, 109-111.

Steer, J., Harris, J.A., 2000. Shifts in the microbial community in rhizosphere and non-rhizosphere soils during the growth of *Agrostis stolonifera.* Soil Biology Biochemistry 32, 869-878.

Stringfellow, W.T., Aitken, M.D., 1995. Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading pseudomonads. Applied and Environmental Microbiology 61, 357-362.

Stingley, R.L., Khan, A.A., Cerniglia, C.E., 2004. Molecular characterization of a phenanthrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1. Biochemical and Biophysical Research Communications 322, 133-146.

Su, Y.H., Yang, X.Y., Chiou, C.T., 2008. Effect of rhizosphere on soil microbial community and in-situ pyrene biodegradation. Frontiers of Environmental Science and Engineering in China 2, 468-474.

Su, Y.H., Zhu, Y.G., 2008. Uptake of selected PAHs from contaminated soils by rice seedlings (*O*ryza *sativa*) and influence of rhizosphere on PAH distribution. Environmental Pollution 155, 359-365.

Subramanian, K.S., Charest, C., 1997. Nutritional, growth, and reproductive responses of maize (*Zea mays* L.) to arbuscular mycorrhizal inoculation during and after drought stress at tasselling. Mycorrhiza 7, 25-32.

Susarla, S., Medina, V.F., McCutcheon, S.C., 2002. Phytoremediation: An ecological solution to organic chemical contamination. Ecological Engineering 18, 647-658.

Sverdrup, L.E., Krogh, P.H., Nielsen, T., Kjaer, C., Stenersen, J., 2003. Toxicity of eight polycyclic aromatic compounds to red clover (*Trifolium repens*) ryegrass (*Lolium perenne*) and mustard (*Sinapsis alba*). Chemosphere 53, 993-1003.

Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A., 1998. Principle and application of soil microbiology. Prentice-Hall, Inc. Upper Saddle River, New Jersey.

Tang, J. Carroguino, M. J., Robertson, B. K., Alexander, M., 1998. Combined effect of sequestration and bioremedication in reducing the bioavailability of polycyclic aromatic hydrocarbons in soil. Environmental Science and Technology 32, 3586-3590.

Tao, X.Q., Lu, G.N., Dang, Z., Yang, C., Yi. X.Y., 2007. A phenanthrene-degrading strain *Sphingomonas* sp. GY2B isolated from contaminated soils. Process Biochemistry 42, 401-408.

Trindade, P.V.O., Sobral, L.G., Rizzo, A.C.L., Leite , S.G.F., Soriano, A.U., 2005. Bioremediation of a weathered and a recently oil-contaminated soils from Brazil: a comparison study. Chemosphere 58, 515-522.

Trotta, A., Falaschi, P., Cornara, L., Minganti, V., Fusconi, A., Drava, G., Berta, G., 2006. Arbuscular mycorrhizae increase the arsenic translocation factor in the As hyperaccumulating fern *Pteris vittata* L.. Chemosphere 65, 74-81.

Trouvelot, A., Kough, J.L., Gianinazzi-Pearson, V., 1986. Mesure du taux de mycorhization VA d'un systéme radiculaire. In: Recherche de méthodes d'estimation ayant une signification fonctionnelle, Paris, pp. 217-221.

Ultra Jr, V.U., Tanaka, S., Sakurai, K., 2007. Effects of arbuscular mycorrhiza and phosphorus application on arsenic toxicity in sunflower (*Helianthus annuus* L.) and on the transformation of arsenic in the rhizosphere. Plant and Soil 290, 29-41.

Vancura, V., 1964. Root exudates of plants. I. Analysis of root exudates of barley and wheat in their initial phases of growth. Plant and Soil 21, 231-248.

Verdin, A., Sahraoui, AL-H., Fontaine, J., Grandmougin-Ferjani, A., Durand, R., 2006. Effects of anthracene on development of an arbuscular mycorrhizal fungus and contribution of the symbiotic association to pollutant dissipation. Mycorrhiza 16, 397-405.

Vivas, A., Biró, B., Németh, T., Barea, J.M., Azcón, R., 2006. Nickel-tolerant *Brevibacillus brevis* and arbuscular mycorrhizal fungus can reduce metal acquisition and nickel toxicity effects in plant growing in nickel supplemented soil. Soil Biology and Biochemistry 38, 2694-2704.

Volante, A., Lingua, G., Cesaro, P., Cresta, A., Puppo, M., Ariati, L., Berta, G., 2005. Influence of three species of arbuscular mycorrhizal fungi on the persistence of aromatic hydrocarbons in contaminated substrates. Mycorrhiza 16, 43-50.

Volkering, F., Quist, J.J., van Velsen, A.F.M., Thomassen, P.H.G., Olijve, M., 1998. A rapid method for predicting the residual concentration after biological treatment of oil-polluted soil. In: ConSoil'98, pp. 251-259.

Walker, T.S., Bais, H.P., Grotewold, E., Vivanco, J.M., 2003. Root exudation and rhizosphere biology. Plant Physiology 132, 44-51.

Wang, M.J., Jones, K.C., 1994. Uptake of chlorobenzenes by carrots from spiked and sewage sludge amended soil. Environmental Science and Technology 28, 1260-1267.

Warshawsky, D., Cody, T., Radike, M., Reilman, R., Schumann, B., LaDow, K., Schneider, J., 1995. Biotransformation of Benzo[a]pyrene and other polycyclic aromatic hydrocarbons and heterocyclic analogs by several green algae and other algal species under gold and white light, Chemico-Biological Interacaction. 97, 131-148.

Watts, A.W., Ballestero, T.P., Gardner, K.H., 2006. Uptake of polycyclic aromatic hydrocarbons (PAHs) in salt marsh plants *Spartina alterniflora* grown in contaminated sediments. Chemosphere 62, 1253-1260.

Weber, W.J., Huang, W., 1996. A distributed reactivity model for sorption by soils and sediments. 4. Intraparticle heterogeneity and phase-distribution relationships under nonequilibrium conditions. Environmental Science and Technology 30, 881-888.

Weissenfels, W.D., Beyer, M., Klein, J., Rehm, H.J., 1991. Microbial metabolism of fluoranthene isolation and identification of ring fission products. Applied Microbiology and Biotechnology 34, 528-535.

White, J.C., Ross, D.W., Gent, M.P.N., Eitzer, B.D., Mattina, M.J.I., 2006a. Effect of mycorrhizal fungi on the phytoextraction of weathered p,p-DDE by Cucurbita pepo. Journal of Hazardous Materials B 137, 1750-1757.

White, J.C., Parrish, Z.D., Gent, M.P.N., Iannucci-Berger, W., Eitzer, B.D., Isleyen, M., Mattina, M.I., 2006b. Soil Amendments, Plant Age, and Intercropping Impact p,p'-DDE Bioavailability to Cucurbita pepo. Journal of Environmental Quality 35, 992-1000.

Wild, S.R., Jones, K.C., 1992. Organic chemicals entering agricultural soils in sewage sludges: screening for their potential to transfer to crop plants and livestock. Environmental Science and Technology 119, 85-119.

Wild, S.R., Joner, K.C., 1995. Polynuclear aromatic hydrocarbons in the United Kingdom environment: a prelimary source inventory and budget. Environmental Pollution 88, 91-108.

Williams, P.A., Sayers, J.R., 1994. The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation 5, 195-217.

Wilson, S.C., Jones, K.C., 1993. Bioremediation of soil contaminated with ploynuclear aromatic hydrocarbons (PAHs): a review. Environmental Pollution 81, 229-249.

Wingfors, H., Sjodin, A., Haglund, P., Brorstrom-Lunden, E., 2001. Characterisation and determination of profiles of polycyclic aromatic hydrocarbons in a traffic tunnel in Gothenburg, Sweden. Atmospheric Environment 36, 6361-6369.

Wittenmayer, L., Merbach, W., 2005. Plant responses to drought and phosphorus deficiency: contribution of phytohormones in root-related processes. Journal of Plant Nutrition and Soil Science 168, 531-540.

Wu, N.Y., Huang, H.L., Zhang, S.Z., Zhu, Y.G., Christie, P., Zhang, Y., 2009. Phenanthrene uptake by *Medicago sativa* L. under the influence of an arbuscular mycorrhizal fungus. Environmental Pollution 157, 1613-1618.

Wu, N. Y., Zhang, S. Z., Huang, H. L., Christie, P., 2008a. Enhanced dissipation of phenanthrene in spiked soil by arbuscular mycorrhizal alfalfa combined with a non-ionic surfactant amendment. Science of the Total Environment 394, 230-236.

Wu, N. Y., Zhang, S. Z., Huang, H. L., Shan, X.Q., Christie, P., Wang, Y.S., 2008b. DDT uptake by arbuscular mycorrhizal alfalfa and depletion in soil as influenced by soil application of a non-ionic surfactant. Environmental Pollution 151, 569-575.

Wu, S., Gschwend, P.M., 1986. Sorption kinetics of hydrophobic organic compounds to natural sediments and soil. Environment Science and Technology 20, 717-725.

Xu, S.Y., Chen, Y.X., Wu, W.X., Wang, K.X., Lin, Q., Liang, X.Q., 2006. Enhanced dissipation of phenanthrene and pyrene in spiked soils by combined plants cultivation. Science of the Total Environment 363, 206-215.

Yi, H., Crowley, D.E., 2007. Biostimulation of PAH degradation with plants containing high concentrations of linoleic acid. Environmental Science and Technology 41, 4382-4388.

Yuan, S.Y., Shiung L.C., Chang, B.V., 2002. Biodegradation of polycyclic aromatic hydrocarbons by inoculated microorganisms in soil. Bulletin of Environmental Contamination and Toxicology 69, 66-73.

Yoshino, K., Nishigaki, K., Husimi, Y., 1991. Temperature sweep gel electrophoresis: a simple method to detect point mutations. Nucleic Acids Research 19, 3153.

Zheng, Z.M., Obbard, J.P., 2002. Oxidation of polycyclic aromatic hydrocarbons (PAH) by the white rot fungus, *Phanerochaete chrysosporium*. Enzyme and Microbial Technology 31, 3-9.

Zhou, H.W., Luan, T.G., Zou, F., Tam, N.F.Y., 2008. Different bacterial groups for biodegradation of three- and four-ring PAHs isolated from a Hong Kong mangrove sediment. Journal of Hazardous Materials 152, 1179-1185.

Zhou, X.B., Cébron, A., Béguiristain, T., Leyval, C., 2009. Water and phosphorus content affect PAH dissipation in spiked soil planted with mycorrhizal alfalfa and tall fescue. Chemosphere 77, 709-713.

Abstract

PAHs are among the most problematic substances as they could accumulate in the environment and threaten the development of living organisms because of their acute toxicity, mutagenicity or carcinogenity. Among remediation techniques for PAH contaminated sites, phytoremediation has been recognized as a promising method owing to its economical and ecological benefits. However, due to the recalcitrant nature of PAH, multivariate and changeful environment factors, this technique is still limited in terms of effectiveness, especially when dealing with high molecular weight PAHs. Inoculation of plants with arbuscular mycorrhizal (AM) fungi, which are ubiquitous in natural and most anthropogenically influenced soils, is known to benefit PAH phytoremediation. However, diverging results were reported on PAH dissipation in plant rhizosphere and the parameters affecting the AM fungi assisted PAH phytoremediation needed more investigation. Some of these parameters were considered in the present work: plant species, AM fungi species, phosphorus nutrition and watering regimes, PAH type, availability and interactions between PAHs. Experiments were performed in pot cultures and in microplates, with different plant species (including alfalfa, tall fescue, ryegrass and celery roots), two AM fungi (*Glomus intraradices* and *Glomus mosseae*) and three kinds of PAHs (phenanthrene (PHE), pyrene (PYR) and dibenzo[a,h]anthracene (DBA)), spiked to a soil. PAH molecular weight was a major parameter influencing PAH phytoremediation. With the increase of PAH molecular weight, the culturable PAH degraders decreased, so did the efficiency of phytoremediation. PHE decreased the plant biomass and AM fungi colonization, but it increased the DBA dissipation in both pot and microplate experiments. PYR did not increase DBA dissipation, and addition of PYR into PHE+DBA substrate decreased both PHE and DBA dissipation. PAH phytoremediation efficiency varied with the plant and AM fungi species. Although all four species of plants increased the disappearance of PHE, only alfalfa showed a positive effect on high molecular weight (HMW) PAHs. *Glomus intraradices* increased the plant biomass and phosphorus uptake of plants, and it also increased DBA dissipation in DBA or PHE+PYR+DBA spiked soil. Co-planted alfalfa and tall fescue colonized with *Glomus mosseae* obtained higher biomass and the concentration of the PAH-ring hydroxylating dioxygenase genes were significant higher, than with *Glomus intraradices*, but *Glomus mosseae* showed no or negative effect on DBA phytoremediation. The phosphorus concentration and water regime also influenced the AM fungus colonization and PAH dissipation. The highest AM colonization and a significant positive impact of mycorrhizal plants on the dissipation of DBA was detected in high-water and low-phosphorus treatment. Results indicated that complex interactions between plants, microorganisms and soil control the fate on PAHs. All the studied parameters significantly affected PAH dissipation in plant rhizosphere, and should be considered for controlling and improving phytoremediation efficiency.

Key words: PAH, phytoremediation, AM fungi, phosphorus, PAH degrading genes, cometabolism

Résumé

Les HAP sont parmi les substances les plus problématiques parce qu'ils ont un fort pouvoir cancérigène, mutagène et ont, par conséquent, des effets nocifs pour la santé humaine. Parmi les techniques de remédiation des sols contaminés par des HAP, la phytoremédiation a été reconnue comme une méthode prometteuse en raison de ses avantages économiques et écologiques. Toutefois, en raison de la nature récalcitrante des HAP, et de facteurs environnementaux difficile à maîtriser, cette technique est encore limitée en termes d'efficacité, en particulier lorsqu'il s'agit de HAP de poids moléculaires élevés. L'inoculation des plantes avec des champignons mycorhiziens à arbuscules (MA), qui sont omniprésents dans la plupart des sols naturels et anthropiques, est connue pour favoriser l'élimination des HAP. Cependant, des résultats variables ont été rapportés quant à l'effet des plantes et des microorganisms associés notamment les champignons MA, sur la phytoremédiation des HAP et nécessite des études complémentaires. Des expériences été réalisées dans des cultures en pot et en microplaques, pour étudier l'influence de certains paramètres sur la dissipation des HAP dans la rhizosphère: l'espèce végétale, l'espèce fongique, la nutrition minérale, la nature des HAP, leur disponibilité et les interactions entre HAP. Quatre espèces de plantes (luzerne, fétuque élevée, ray-grass et céleri) et deux espèces de champignons MA (*Glomus intraradices* et *Glomus mosseae*) ont été testées dans un sol artificiellement contaminé avec trois HAP: le phénanthrène (PHE), le pyrène (PYR) et le dibenzo[a, h] anthracène (DBA). Le poids moléculaire des HAP est un facteur majeur influencant leur élimination. Lorsque le poids moléculaire des HAP était plus élevé, le nombre de bactéries dégradantes cultivables était plus faible, et l'efficacité de la phytoremédiation des HAP limitée. La présence de PHE a diminué la biomasse végétale et la colonisation mycorhizienne, mais il a augmenté la dissipation du DBA par co-métabolisme dans les expériences en pots et en microplaques. En revanche, cet effet n'a pas été observé entre le PYR et le DBA . La dissipation des HAP a varié avec les espèces de plantes et de champignons MA. Bien que les quatre espèces de plantes aient augmenté l'élimination du PHE, seule la luzerne a montré des effets positifs sur la phytoremédiation du DBA. *Glomus intraradices* a augmenté la biomasse végétale et l'absorption du phosphore par des plantes, et il a également augmenté la dissipation du DBA. Lorsque la co-culture de luzerne et fétuque était colonisée par *Glomus mosseae*, la biomasse obtenue était plus élevée, et la concentration des gènes de HAP-dioxygénase était significativement plus élevée qu'avec *Glomus intraradices*. Mais *Glomus mosseae* n'a montré aucun effet significatif sur la phytoremédiation du DBA. Ainsi cet effet des champignons MA sur la dissipation des HAP n'est pas seulement un effet biomasse. La concentration de phosphore et le régime d'alimentation en eau ont également influencé la colonisation mycorhizienne et la dissipation des HAP. Ainsi la dissipation du DBA en présence de plantes était significativement plus élevée que dans les témoins non plantés lorsque la teneur en eau était élevée et celle en phosphore plus faible, ce qui correspondait à la situation où le taux de mycorhization des plantes était le plus élevé. L'ensemble de ces résultats souligne la complexité des interactions entre plantes, microorganismes et polluants dans les sols. Ils montrent que tous les paramètres considérés affectent significativement la dissipation des HAP dans la rhizosphère des plantes, et méritent d'être pris en compte pour contrôler et améliorer la phytoremédiation.

Mots clés: HAP, phytoremédiation, champignons MA, phosphore, gène de dioxygénase, co-métabolisme