Impact du phytomanagement de sites pollués par les éléments traces métalliques sur les micro-organismes du sol

Julie Foulon

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Impact du phytomanagement de sites pollués par les éléments traces métalliques sur les microorganismes du sol

Présentée et soutenue publiquement par

Julie FOULON

Le 2 Décembre 2016, devant le jury composé de :

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Résumé

Les activités anthropiques et la pollution qu'elles engendrent peuvent entraîner des changements drastiques dans les structures des écosystèmes et de leurs services écologiques. Le phytomanagement basé sur l'utilisation d'espèces ligneuses peut contribuer à la restauration des sols et de la diversité microbienne, tout en permettant la production de biomasses d'intérêt. Dans le cadre des projets PROLIPHYT (2013-2018, ADEME), BIOFILTREE (2010-2014, ANR) et PHYTOPOP (2007-2011, ANR) gérés par le laboratoire Chrono-environnement, des parcelles de phytomanagement ont été installées sur sites pollués par les éléments traces métalliques (ETMs), pour déterminer le potentiel de croissance d'espèces ligneuses, ainsi que les voies de valorisation des biomasses produites. Dans le cadre de mon projet de thèse, différentes approches ont été mises en œuvre afin d'identifier et de caractériser les microorganismes inféodés aux végétaux ligneux implantés, avec pour finalité la production de ressources microbiennes permettant d'améliorer la reprise et la croissance des arbres sur sols pollués.

La première approche a consisté à étudier les communautés microbiennes en association avec les espèces ligneuses par une approche innovante de séquençage à haut débit associée à l'analyse physico-chimique des sols. Cette méthode a été mise au point et testée sur trois sites expérimentaux contaminés par des ETMs. Elle a permis de révéler les changements dans la structure et la composition des communautés microbiennes dus à la nature du couvert végétal et aux caractéristiques du sol (Zappelini *et al.*, 2015; Foulon *et al.*, 2016a; b).

La deuxième approche a consisté à isoler et caractériser les champignons mycorhiziens et endophytes associés à des peupliers (Berthelot *et al.*, 2016; Lacercat-Didier *et al.*, 2016). Ces champignons sont connus pour favoriser la croissance des végétaux qu'ils colonisent mais également pour conférer une meilleure tolérance face à un stress métallique. Plusieurs campagnes de prélèvements de racines et de champignons sur différents sites pollués ont permis l'isolement de souches fongiques tolérantes aux ETMs (comme *Serendipita vermifera*) et améliorant la croissance (*Phialophora* sp. et *Leptodontidium* sp.).

Dans une troisième approche, les mécanismes impliqués dans la résistance aux ETMs ont été étudiés i) chez *Paxillus involutus* pour le Hg (Foulon *et al*. manuscrit en préparation) ii) par métatranscriptomique fonctionnelle pour le Zn et Cd (Lehembre *et al.*, 2013).

L'ensemble de ces travaux a permis i) d'améliorer nos connaissances sur l'impact du couvert ligneux sur la structure et la composition des communautés microbiennes, et sur la compréhension des mécanismes physiologiques mis en jeu ii) de constituer une collection de souches fongiques d'intérêt pour le phytomanagement de sols contaminés par les ETMs.

Mots-clés : phytomanagement, peuplier, éléments traces métalliques, communautés microbiennes, champignons mycorhiziens, champignons endophytes, bactéries, mercure, tolérance

Abstract

Anthropogenic activities can cause dramatic changes in ecosystem structures and their ecological services. Phytomanagement based on the use of woody species can contribute to soil and microbial diversity restoration, while allowing the production of biomass of interest. As part of PROLIPHYT (2013-2018, ADEME), BIOFILTREE (2010-2014, ANR) and PHYTOPOP (2007-2011, ANR) projects, managed by the Chrono-environment laboratory, phytomanagement plots were installed on trace elements (TE) polluted sites to determine the growth potential of woody species and the recovery methods of biomass produced. In my PhD project, different approaches have been implemented to identify and characterize microorganisms subservient to implanted woody plants, with the aim of producing microbial resources to improve the recovery and growth of trees on polluted soil.

The first approach was to study microbial communities in association with woody species by innovative high throughput sequencing approach associated with the physical and chemical analysis of soils. This method was developed and tested on three experimental sites contaminated by TE. It revealed changes in the structure and composition of microbial communities due to the nature of the vegetation cover and the soil characteristics (Zappelini *et al.*, 2015; Foulon *et al.*, 2016a; b).

The second approach was to isolate and characterize mycorrhizal fungi and endophytes associated with poplars (Berthelot *et al.*, 2016; Lacercat-Didier *et al.*, 2016). These fungi are known to promote the growth of the plants they colonize but also to increase their TE tolerance. Several sampling campaigns of roots and sporocarps on different polluted sites have allowed the isolation of fungal strains tolerant to TE (like *Serendipita vermifera*) and enhancing growth (*Phialophora sp*. and *Leptodontidium sp*.).

In a third approach, the mechanisms involved in TE resistance were studied i) in *Paxillus involutus* for Hg (Foulon *et al*. In prep) ii) by functional metatranscriptomics for Zn and Cd (Lehembre *et al.*, 2013).

In conclusion, this work allowed to i) improve our understanding of the impact of a woody cover on the structure and composition of microbial communities, and on the understanding of the physiological mechanisms involved ii) to constitute a collection of fungal strains that will be of great interest for future phytomanagement projects.

Keywords: phytomanagement, poplar, metal trace elements, microbial community, mycorrhizal fungi, fungal endophytes, bacteria, mercury, metal tolerance

Table des matières

1 Introduction 2

1.1 Les éléments traces métalliques 6
1.1.1 Définitions 6
1.1.2 Mobilité et biodisponibilité des ETMs dans les sols 8
1.1.3 Pollution métallique 12
1.1.3.1 Origines des ETMs 12
1.1.3.1.1 Origine naturelle 12
1.1.3.1.2 Origines anthropiques 14
1.1.3.2 Les normes et l'identification de pollution 14
1.1.4 Impact des ETMs 18
1.1.4.1 Au niveau des écosystèmes 18
1.1.4.2 Au niveau des organismes 20
1.1.4.2.1 Toxicité pour l'Homme 20
1.1.4.2.2 Toxicité pour les plantes 22
1.1.4.3 Toxicité des ETMs au niveau cellulaire 22
1.1.5 Mécanismes de tolérance 24
1.1.5.1 Mécanismes extracellulaires 26
1.1.5.1.1 Chélation extracellulaire 26
1.1.5.1.2 Liaison à la paroi 28
1.1.5.2 Mécanismes intracellulaires 30
1.1.5.2.1 Exclusion 30
1.1.5.2.2 Limitation de l'entrée 32
1.1.5.2.3 Chélation intracellulaire 32
1.1.5.2.4 Compartimentation 32
1.1.5.2.5 Transformation des métaux 34
1.1.5.2.6 Système antioxydant 34
1.1.5.2.7 Autres mécanismes 34

1.2 Réhabilitation et remédiation de sols pollués 36
1.2.1 Le sol 36
1.2.2 Services écosystémiques 36
1.2.3 Les différentes techniques de réhabilitation et de remédiation des sols pollués 38
1.2.3.1 Les techniques physico-chimiques 40
1.2.3.2 Les techniques biologiques 40
1.2.3.2.1 La bioremédiation 40
1.2.3.2.2 La phytoremédiation 42
1.2.3.2.3 Le phytomanagement 44

1.3 Les microorganismes de la rhizosphère 44
1.3.1 Les champignons 46
1.3.1.1 Saprophytes 46
1.3.1.2 Pathogènes et parasites 46
1.3.1.3 Symbiotiques et mutualistes 48
1.3.1.3.1 Les endomycorhizes 50
1.3.1.3.2 Les ectomycorhizes 52
1.3.1.4 Les endophytes 54
1.3.2 Les bactéries 56
1.3.2.1 Les rhizobactéries 56
1.3.2.2 Les MHB 58
1.3.3 Facteurs impactant la composition des communautés microbiennes 60
1.3.3.1 Impact du sol 60
1.3.3.2 Impact du couvert végétal 60

1.4 Techniques d'analyse des communautés microbiennes 62
1.4.1 Les profils électrophorétiques : empreintes moléculaires 64
1.4.2 La génomique environnementale 66

1.4.2.1 Historique 66
1.4.2.2 L'approche de clonage et séquençage 68
1.4.2.3 Les NGS 68
1.4.2.3.1 Roche 454 70
1.4.2.3.2 Illumina MiSeq 72
1.4.2.3.3 Ion Torrent PGM 72

1.5 Cadre général et objectifs du projet de thèse 74

2 Matériels & méthodes 78

2.1 Matériels 78
2.1.1 Les sites expérimentaux 78
2.1.1.1 Le site de Leforest 78
2.1.1.2 Le site de Pierrelaye 80
2.1.1.3 Le site de Fresnes-sur-Escaut 82
2.1.1.4 Le site de Tavaux 82
2.1.2 Matériels biologiques 86
2.1.2.1 Les champignons en collection 86
2.1.2.2 Espèces ligneuses des dispositifs expérimentaux 86

2.2 Méthodes 86
2.2.1 Prélèvements et préparation des échantillons 86
2.2.1.1 Prélèvements de sol et de racines 86
2.2.1.2 Prélèvements de fructifications de champignons 88
2.2.2 Techniques de biologie moléculaire 88
2.2.2.1 Extraction d'ADN 88
2.2.2.1.1 Echantillons de sol 88
2.2.2.1.2 Echantillons de racines 88
2.2.2.1.3 Echantillons de champignons 88
2.2.2.2 Amplification par PCR 90
2.2.2.3 Séparation et visualisation des ADNs sur gel d'agarose 92
2.2.2.4 Purification des ADNs 92
2.2.2.5 Dosage des ADNs 92
2.2.3 Séquençage 94
2.2.3.1 Sanger 94
2.2.3.2 Ion Torrent 96
2.2.3.3 Miseq 96
2.2.4 Quantification du taux d'ectomycorhization 98
2.2.5 Techniques de microbiologie 98
2.2.5.1 Isolement de souches fongiques ectomycorhiziennes 98
2.2.5.1.1 A partir de carpophores 98
2.2.5.1.2 A partir de racines d'espèces ligneuses 100
2.2.5.2 Entretien des souches fongiques 100
2.2.5.3 Tests de tolérance des souches fongiques aux ETMs 100
2.2.5.3.1 Test en plaques 12 puits 100
2.2.5.3.2 Test sur boîte de Pétri 102
2.2.5.3.3 Tests en milieu liquide 102
2.2.5.3.4 Tests de désorption à l'EDTA 102
2.2.5.3.5 Tests d'efflux de Hg 104
2.2.6 Lyophilisation et broyage des souches 104
2.2.7 Techniques biochimiques 104
2.2.7.1 Extraction des protéines 104
2.2.7.2 Dosage des protéines solubles 106
2.2.7.3 Séparation et visualisation des protéines sur gel de polyacrylamide 106
2.2.7.4 Dosage des capacités antioxydants 106
2.2.7.4.1 Dosage de la peroxydation lipidique (MDA) 106
2.2.7.4.2 Dosage de la capacité antioxydante totale 108
2.2.7.4.3 Dosage de l'activité catalase 108
2.2.7.4.4 Dosage de l'activité superoxide dismutase 110

2.2.7.5 Dosage des ETMs 110
2.2.7.5.1 Dosage du Hg par l'AMA 254 110
2.2.7.5.2 Dosage des ETMs par ICP-AES 112
2.2.7.5.3 Analyse physico-chimique des sols 112
2.2.8 Analyses bioinformatiques 114
2.2.9 Analyses statistiques 116

3 Résultats & discussions 118

3.1 Caractérisation par métabarcoding des communautés microbiennes de sols pollués par les ETMs 118
3.1.1 Diversité et complexité des communautés microbiennes d'une lagune de stockage de sédiments issus de l'activité industrielle chlore-alcali 120
3.1.2 Le métabarcoding environnemental appliqué à deux sites de phytomanagement basé sur le peuplier révèle des communautés microbiennes contrastées 135
3.1.3 Impact du phytomanagement utilisant le peuplier sur les propriétés du sol et les communautés microbiennes d'un site contaminé par des ETMs 155
3.1.4 Discussion 180

3.2 Production de ressources microbiennes 184
3.2.1 Isolement et identification de souches fongiques issues de sols pollués 186
3.2.1.1 Isolement à partir de fragments racinaires 186
3.2.1.2 Isolement à partir de carpophores 190
3.2.2 Promotion de la croissance des plantes, production de métabolites et tolérance vis à vis des ETMs des DSEs isolés de sols pollués 191
3.2.3 Caractérisation de champignons endophytes mutualistes isolés à partir de racines de peupliers et présentant une tolérance élevée aux ETMs 208
3.2.4 Discussion 224

3.3 Caractérisation fonctionnelle de mécanismes de tolérance aux ETMs 227
3.3.1 Criblage du métatranscriptome du sol pour sonder les gènes de résistance aux ETMs chez les eucaryotes 228
3.3.2 Absorption et internalisation du Hg par les champignons supérieurs 252
3.3.3 Discussion 281

4 Conclusions générales et perspectives 284

5 Références Bibliographiques 289

6 Annexes 307

Liste des tableaux

Tableau I : Spéciation chimique de quelques ETMs dans la solution du sol en fonction du pH (Tremel-Schaub & Feix, 2005). *9*
Tableau II : Différentes références françaises de teneurs totales en ETMs dans les boues d'épuration et les sols exprimées en mg.kg^{-1} MS (d'après Baize & Tercé, 2002). *13*
Tableau III : Teneurs totales en ETMs dans des sols français (Baize, 2000). *15*
Tableau IV : Génomes séquencés avec la méthode Sanger. *67*
Tableau V : Composition du milieu Malt. *85*
Tableau VI : Composition du milieu MMN. *85*
Tableau VII : Composition du milieu N2P2. ..... *85*
Tableau VIII : Composition du milieu Pachlewski. *87*
Tableau IX : Liste et principales caractéristiques des cultivars utilisés pour les essais de terrain dans le cadre du projet PHYTOPOP. *87*
Tableau X : Liste des différentes espèces ligneuses plantées à Leforest et à Tavaux dans le cadre du projet PROLIPHYT. *87*
Tableau XI : Exemple de mélange réactionnel et de programme PCR utilisant l'enzyme Phire Hot Start II. *89*
Tableau XII : Composition du tampon de charge (6X). *91*
Tableau XIII : Colonisation des racines de peuplier (clone Skado) par les champignons sur les sites de Fresnes-sur-Escaut (F), Leforest (M) et Tavaux (T). *185*

Liste des figures

Figure 1 : Répartition des sites et sols pollués en France selon la base de données BASOL (2016) et principaux polluants retrouvés dans les sols ou les nappes d'eaux souterraines (infoterre.brgm.fr et basol.developpement-durable.gouv.fr). 1
Figure 2 : Tableau périodique des éléments de Mendeleïev (Scaler, Michka B ; commons.wikimedia.org). 5
Figure 3 : Eléments impliqués comme cofacteurs d'enzymes. (Waldron et al., 2009). Chaque valeur (%) représente la proportion d'enzymes utilisant un élément donné comme cofacteur. 7
Figure 4 : Spéciation des ETMs dans les sols (ADEME & APCA, 2005). 7
Figure 5 : Observation microscopique de sol et distribution des éléments. 9
Figure 6 : Origines des ETMs dans les sols (Mathieu et al., 2008). 11
Figure 7 : Lignes d'isoconcentration prédites pour les concentrations totales en Cd, Pb et Zn dans les sols environnant l'usine de Metaleurop Nord (Fritsch et al., 2011). 13
Figure 8 : Réponse de l'écosystème sol face aux perturbations (Griffiths & Philippot, 2013). 17
Figure 9 : Proportion des taxa dominants au niveau des phyla bactériens entre un sol témoin (Cu 0) et un sol contaminé par du Cu (Cu 9) (Wakelin et al., 2010b). 17
Figure 10 : Représentation schématique de la résilience et de la résistance (Griffiths & Philippot, 2013). 19
Figure 11 : Cycle du Hg (www.prc.cnrs-gif.fr). 21
Figure 12 : Effets généraux des concentrations en ETMs sur la production de biomasse et sur la concentration en ETMs des tissus de la plante (Tremel-Schaub & Feix, 2005). 23
Figure 13 : Formation de radicaux libres après exposition aux ETMs et impact sur les biomolécules (Jomova et al., 2012). 25
Figure 14 : Les différents mécanismes de tolérance vis à vis des ETMs, mis en oeuvre chez les champignons (Bellion et al., 2006). 27
Figure 15 : Complexation des ETMs par l'acide oxalique. 27
Figure 16 : Augmentation de la biodisponibilité du Cd (a) et du Pb (b) dans des sols enrichis en ETMs après addition de di-rhamnolipide (Juwarkar et al., 2007). 29
Figure 17 : Les groupements de liaison aux ETMs les plus fréquemment impliqués dans la biosorption fongique (A) et exemple de biosorption du Hg (points noirs) sur la paroi de Saccharomyces cerevisiae (B) (Whittaker et al., 1990; Gadd et al., 2012). 31
Figure 18 : Transporteurs impliqués dans l'homéostasie des ETMs chez S. cerevisiae activé par Aft1 (vert), Mac1 (bleu), Zap1 (rouge), et Ace1 (violets) (Rutherford & Bird, 2004). 31
Figure 19 : Induction de l'expression du gène GSH1 codant une γ-glutamylcystéine synthétase chez Saccharomyces cerevisiae (A)(Westwater et al., 2002) et induction de Glutathione S-transférases chez Proteus mirabilis (B)(Zhang et al., 2013) en présence de différents ETMs. 33
Figure 20 : Les différents constituants du sol. 35
Figure 21 : Relations hypothétiques entre la biodiversité et les fonctions de l'écosystème ((Schwartz et al., 2000). 37
Figure 22 : Schéma représentant les deux modèles hypothétiques de la diversité microbienne en présence de concentrations croissantes d'ETMs (a) et la diversité des bactéries Rhizobium bv. trifolii (b) et Rhizobium bv. viciae (c) (Giller et al., 2009). 39
Figure 23 : Les différentes techniques de phytoremédiation (Favas et al., 2014). 41
Figure 24 : Produits possibles (gauche) et nouvelles aires de recherche (droite) dans le domaine du phytomanagement (Evangelou & Deram, 2014). 43
Figure 25 : La rhizosphère (Philippot et al., 2013). 43
Figure 26 : Les différents habitats des champignons et leurs fonctions écologiques (Harms et al., 2011). 45
Figure 27 : Fréquences relatives des OTUs fongiques classées dans les catégories écologiques et fonctionnelles majeures parmi les différents biomes dans le monde ((Tedersoo et al., 2014). 47
Figure 28 : Structures mises en place durant la symbiose ectomycorhizienne (en bleu) et endomycorhizienne (en rose) (Bonfante & Genre, 2010). 49

Figure 29 : Quelques illustrations d'endomycorhizes à l'état de spore (a) et en interaction avec les racines sous forme de vésicules (b) et d'arbuscules (c et d) 51
Figure 30 : Quelques illustrations d'ectomycorhizes sous forme de carpophores (a, d, et g), en culture pure sur boîte de Pétri (b, e et h) et en interaction avec les racines (c, f et i) pour Paxillus involutus (a, b et c), Laccaria sp. (d, e et f) et Hebeloma sp. (g,h et i) (Ważny, 2014 ; www.deemy.de). 53
Figure 31 : Pins mycorhizés avec une souche tolérante (T) ou non tolérante (NT) au Cu de S. luteus ou non mycorhizés (NM) et traités avec différentes concentrations en Cu : concentration normale (A) ou subléthale (B) (Adriaensen et al., 2005). 55
Figure 32 : Colonisation racinaire par des champignons endophytes de type DSE à l'intérieur de racines (a) (Lukešová et al., 2015) ainsi qu'en culture pure sur boîte de Pétri (b). 55
Figure 33 : Principaux impacts des PGPR sur la plante hôte (www.ecologiemicrobiennelyon.fr). 57
Figure 34 : Mécanismes des MHB pour promouvoir la mise en place de la symbiose ectomycorhizienne (Frey-Klett et al., 2007). 59
Figure 35 : Pourcentage de racines colonisées par les champignons ectomycorhiziens (ECM), endomycorhiziens (AM) et endophytes (EF) de quatre clones de peupliers sur trois sites (Karliński et al., 2010). 61
Figure 36 : Abondances relatives des phyla bactériens dominants dans quatre sites (S1 à S4) le long des horizons de sol A, B, C et D représentant les profondeurs 0-20, 20-40, 40-60 et 60-80 cm, respectivement (Liu et al., 2014). 63
Figure 37 : Schéma du site d'étude (A) et pourcentage des différents morphotypes ectomycorhiziens (B) de peupliers en système de rotation de 3 et 6 ans, et prélevés à l'automne 2008 (A_rot3 ; A_rot6) et au printemps 2009 (S_rot3 ; S_rot6) (Hrynkiewicz et al., 2010). 63
Figure 38 : Les différentes méthodes d'analyse de la diversité (Ma et al., 2016). 65
Figure 39 : Profils DGGE des fragments du gène codant l'ARNr 16S pour le sol témoin (control), et pour les sols contaminés par l'As et le Cd (Lorenz et al., 2006). 65
Figure 40 : Comparaison des différentes techniques de séquençage (http://www.biorigami.com). 69
Figure 41 : Le pyroséquençage 454 (Mardis, 2008). 71
Figure 42 : Abondance relative des genres bactériens (A) et fongiques (B) dominant dans les sols sous une culture de Miscanthus (P1) et de blé (P6).(Bourgeois et al., 2015). 71
Figure 43 : Relation entre la structure de la communauté bactérienne et les propriétés du sol (a) ou les contenus en éléments traces (b).(Hong et al., 2015). 73
Figure 44 : Résultats du séquençage par Ion Torrent PGM des communautés fongiques isolées de la rhizosphère de saules implantés sur sites contaminés par des ETMs (d'après (Bell et al., 2015). 73
Figure 45 : Dispositif expérimental de Leforest. 77
Figure 46 : Dispositifs expérimentaux de Pierrelaye. 79
Figure 47 : Dispositif expérimental de Fresnes-sur-Escaut. 81
Figure 48 : Dispositif expérimental de Tavaux. 83
Figure 49 : Localisation sur les ADNs ribosomiques nucléaires bactériens (A) et fongiques (B) des régions variables (V1 à V9 pour le 16S bactérien et ITS pour les champignons) amplifiées par PCR. 89
Figure 50 : Exemple de gel d'électrophorèse pour les ADNs totaux extraits de sol et de racines. 91
Figure 51 : Principe du séquençage Sanger avec un marqueur radioactif (A) ou un marqueur fluorescent (B) (www.atdbio.com). 93
Figure 52 : Principe du séquençage Ion Torrent PGM (Goodwin et al., 2016). 95
Figure 53 : Principe du séquençage Illumina MiSeq (Illumina.com). 95
Figure 54 : Principales étapes de l'isolement de souches fongiques à partir de fructifications ou de racines mycorhizées (Lacercat-Didier, 2013). 97
Figure 55 : Exemple d'un résultat de test de tolérance au Hg en plaque 12 puits. 99
Figure 56 : Exemple d'un résultat de test de tolérance au Hg en boite de Pétri. 99
Figure 57 : Aspect du milieu malt après une semaine d'incubation en présence ou non du champignon Paxillus involutus He01 et en présence de différentes concentrations de Hg. 101
Figure 58 : Réaction entre le malondialdéhyde et l'acide thiobarbiturique. 107
Figure 59 : Molécule de Trolox. 107

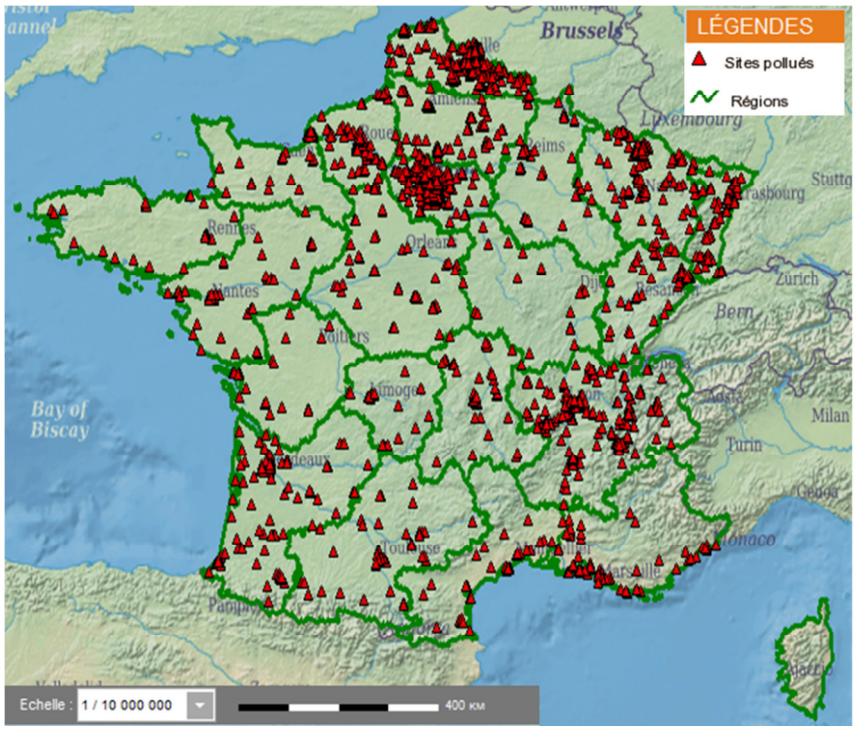
Figure 60 : Réactions entre les radicaux libres et les enzymes antioxydantes Superoxyde dismutase (SOD) et Catalase (CAT). *109*
Figure 61 : Principe du dosage de l'activité SOD. *109*
Figure 62 : Analyseur de Hg de type AMA 254 ouvert (à gauche) ou fermé et accompagné de son passeur automatique (à droite). *111*
Figure 63 : Les différents facteurs impactant la composition des communautés microbiennes *179*
Figure 64 : Arbre phylogénétique basée sur les séquences ITS des souches fongiques isolées sur les sites de Fresnes-sur-Escaut (F), Leforest (M) et Tavaux (T). *187*
Figure 65 : Carpophores collectés sur les différents sites expérimentaux. *189*
Figure 66 : Résume des différents objectifs *283*

Liste des annexes

Annexe 1 : Extraction d'ADNg avec le kit MoBio Powersoil *307*
Annexe 2 : Extraction d'ADNg avec le kit Qiagen..... *308*
Annexe 3 : Liste des amorces utilisées pour le séquençage par Ion Torrent des 16S bactériens (A) et des ITS fongiques (B), MiSeq et Sanger (C)...... *309*
Annexe 4 : Souches de champignons utilisées au cours de cette thèse et leurs origines *312*
Annexe 5 : Tolérance, milieu de prédilection et mode de conservation des souches de champignons utilisées au cours de cette thèse. *319*

Abréviations

ADEME :	Agence De l'Environnement et de la Maîtrise de l'Energie
AIA:	Acide Indole Acétique
AM:	Endomycorhizien
AMA:	Advanced Mercury Analyzer (en)
ANOSIM:	Analysis of Similarity (en)
ARDRA:	Amplified Ribosomal DNA Restriction Analysis (en)
ARISA/RISA:	Automated Ribosomal Intergenic Spacer Analysis (en)
BET:	Bromure d'Ethidium
BLAST:	Basic Local Alignment Search Tool (en)
BRGM :	Bureau de Recherches Géologiques et Minières
BSA/ASB :	Bovin serum albumin/Albumine de sérum bovin
CEC :	Capacité d'échange cationique
CMI /MIC :	Concentration minimale inhibitrice/Minimal inhibitory concentration
DGGE:	Denaturing Gradient Gel Electrophoresis (en)
DSE:	Dark septate endophyte (en)
ECM :	Ectomycorhizien
EPS :	Extracellular Polymeric Substances(en)
ETM :	Elément Trace Métallique
FPGL :	Fond Pédo-Géochimique Local
FPGN :	Fond Pédo-Géochimique Naturel
HAP :	Hydrocarbure Aromatique Polycyclique
ICP AES:	Inductively Coupled Plasma - Atomic Emission Spectrometry (en)
ITS :	Internal Transcribed Spacer (en)
LCE :	Laboratoire Chrono-Environnement
LH-PCR:	Length-Heterogeneity PCR (en)
LIEC :	Laboratoire Interdisciplinaire des Environnements Continentaux
MDA :	Malondialdéhyde
MES :	2-(N-morpholino) ethanesulfonic acid
MMN :	Modified Melin-Norkrans(en)
NCBI :	National Center for Biotechnology Information (en)
NGS :	Next Generation Sequencing (en)
NMDS :	Non-metric multidimensional scaling (en)
NRCC:	National Research Council Canada (en)
OTU:	Operational taxonomic unit /unité taxonomique opérationnelle
PGM :	Personal Genome Machine (en)
PMSF :	Fluorure de phénylméthylsulfonyle
RAPD:	Random Amplified Polymorphic DNA (en)
Rpm:	Rotation per minute
SOD :	SuperOxyde Dismutase
SSCP:	Single-Strand Conformation Polymorphism (en)
T(très)CR :	Taillis à (très) courte rotation
TAH :	Teneurs Agricoles Habituelles
TGGE:	Temperature Gradient Gel Electrophoresis (en)
T-RFLP:	Terminal Restriction Fragment Length Polymorphism Fingerprinting (en)



Polluants	Occurrence
Hydrocarbures	16.39 %
Plomb (Pb)	8.20 %
H.A.P.	7.47 %
Chrome (Cr)	6.26 %
Cuivre (Cu)	6.11 %
Solvants halogénés	6.01 %
Arsenic (As)	5.92 %
Zinc (Zn)	4.63 %
Nickel (Ni)	4.48 %
Cadmium (Cd)	2.80 %
Mercure (Hg)	2.49 %
Cyanures	2.41 %
PCB-PCT	2.41 %
Solvants non halogénés	1.58 %
BTEX	1.46 %
Baryum (Ba)	1.17 %
Pesticides	0.63 %
TCE	0.45 %
Ammonium	0.33 %
Cobalt (Co)	0.27 %
Molybdène (Mo)	0.27 %
Sélénium (Se)	0.20 %
Sulfates	0.14 %
Chlorures	0.08 %

Figure 1 : Répartition des sites et sols pollués en France selon la base de données BASOL (2016) et principaux polluants retrouvés dans les sols ou les nappes d'eaux souterraines (infoterre.brgm.fr et basol.developpement-durable.gouv.fr).

1 Introduction

Les activités anthropiques peuvent causer des changements drastiques dans les structures des écosystèmes et de leurs services écologiques (Dawson *et al.*, 2011). En effet, les activités anthropiques et l'industrialisation en développement libèrent de nombreux polluants dans le sol, l'eau et l'air qui sont nocifs pour l'environnement et les organismes vivants (Doble & Kumar, 2005). Cependant, la stabilité et le maintien des écosystèmes dépendent de la diversité biologique et la dynamique fonctionnelle des organismes (Johnson *et al.*, 1996).

Environ 3,5 milliards d'hectares de terres, ce qui équivaut à près de 30% de la terre ferme totale mondiale, ont été dégradés par les activités humaines (Hüttermann *et al.*, 2009). Un récent rapport européen estime un nombre total de 2,5 millions de sites européens potentiellement contaminés dont 340 000 sont susceptibles de nécessiter un assainissement (Van Liedekerke *et al.*, 2014). En France, en 2016, ce sont 6395 sites et sols pollués ou potentiellement pollués qui sont répertoriés sur l'ensemble du territoire (Figure 1 ; http://basol.environnement.gouv.fr).

Les contaminants les plus fréquents sont les éléments traces métalliques (ETMs) affectant 35% des sols européens et les hydrocarbures (pétrole et hydrocarbures aromatiques polycycliques ou HAP) qui affectent 24% des sols européens (Van Liedekerke *et al.*, 2014). En France, 50% des sols contaminés le sont par des ETMs et le Pb est le plus fréquemment retrouvé après les hydrocarbures (Figure 1). Les ETMs tel que l'As, le Pb et le Hg sont les trois premiers éléments sur la liste des substances dangereuses prioritaires recensées par Agence pour les substances toxiques et les maladies (https://www.atsdr.cdc.gov/spl).

Les ETMs sont les polluants les plus communs et les plus répandus, et en raison de leur toxicité, la pollution métallique représente un risque potentiel pour les organismes vivant dans le sol, comme les communautés microbiennes (Singh *et al.*, 2014). De plus, l'activité microbienne du sol a un impact majeur sur le fonctionnement des écosystèmes entiers, car les microorganismes sont des acteurs clés dans les fonctions centrales des écosystèmes tels que les cycles biogéochimiques et la décomposition de la matière organique (Azarbad *et al.*, 2016).

Les changements causés par la pollution métallique peuvent donc affecter la composition de la communauté microbienne, leurs fonctions mais également les services que procure l'écosystème comme la production de nourriture via l'agriculture. Pour remédier à cela, des techniques biologiques respectueuses de l'environnement regroupées sous le terme de phytomanagement peuvent être mises en œuvre. Ces techniques utilisent les plantes et leurs microorganismes associés pour assainir les sols contaminés et permettent la production de revenus économiques avec des sols impropres à la production agricole.

Le premier objectif de mon projet de thèse a été d'étudier **l'impact de cultures d'espèces ligneuses et des ETMs sur les communautés microbiennes du sol** et des racines dans le cadre de procédés de phytomanagement. Pour cela, la mise au point de techniques innovantes de métabarcoding a permis l'analyse des communautés fongiques et bactériennes sur différents sites expérimentaux contaminés par des ETMs.

Un second objectif a été **d'isoler et de caractériser des champignons résistants aux ETMs,** et conférant une meilleure croissance à la plante. Des souches fongiques présentes sur les sites ont été isolées puis identifiées. Les souches potentiellement intéressantes ont ensuite été caractérisées pour leur tolérance aux ETMs et leur capacité de promouvoir la croissance des plantes hôtes.

Un troisième objectif a été **d'identifier les mécanismes impliqués dans la résistance aux ETMs.** Pour cela deux approches ont été mises en œuvre, la première utilisant la métatranscriptomique et le seconde se basant sur deux souches de l'espèce *Paxillus involutus* pour étudier la tolérance au Hg.

Dans un premier temps, il sera présenté un bilan de nos connaissances concernant les ETMs, leur mobilité dans les sols, leurs impacts ainsi que les mécanismes de tolérance mis en place par les microorganismes du sol. Une seconde partie s'intéressera à l'écosystème sol, les services qu'il procure et les techniques de réhabilitation et de remédiation possibles. Une troisième partie est consacrée aux microorganismes rhizosphériques, leurs avantages pour les plantes ainsi que les facteurs influençant leur composition. Pour finir, les différentes techniques d'analyses des communautés microbiennes dans les sols seront présentées ainsi que leurs avantages et leurs utilisations.

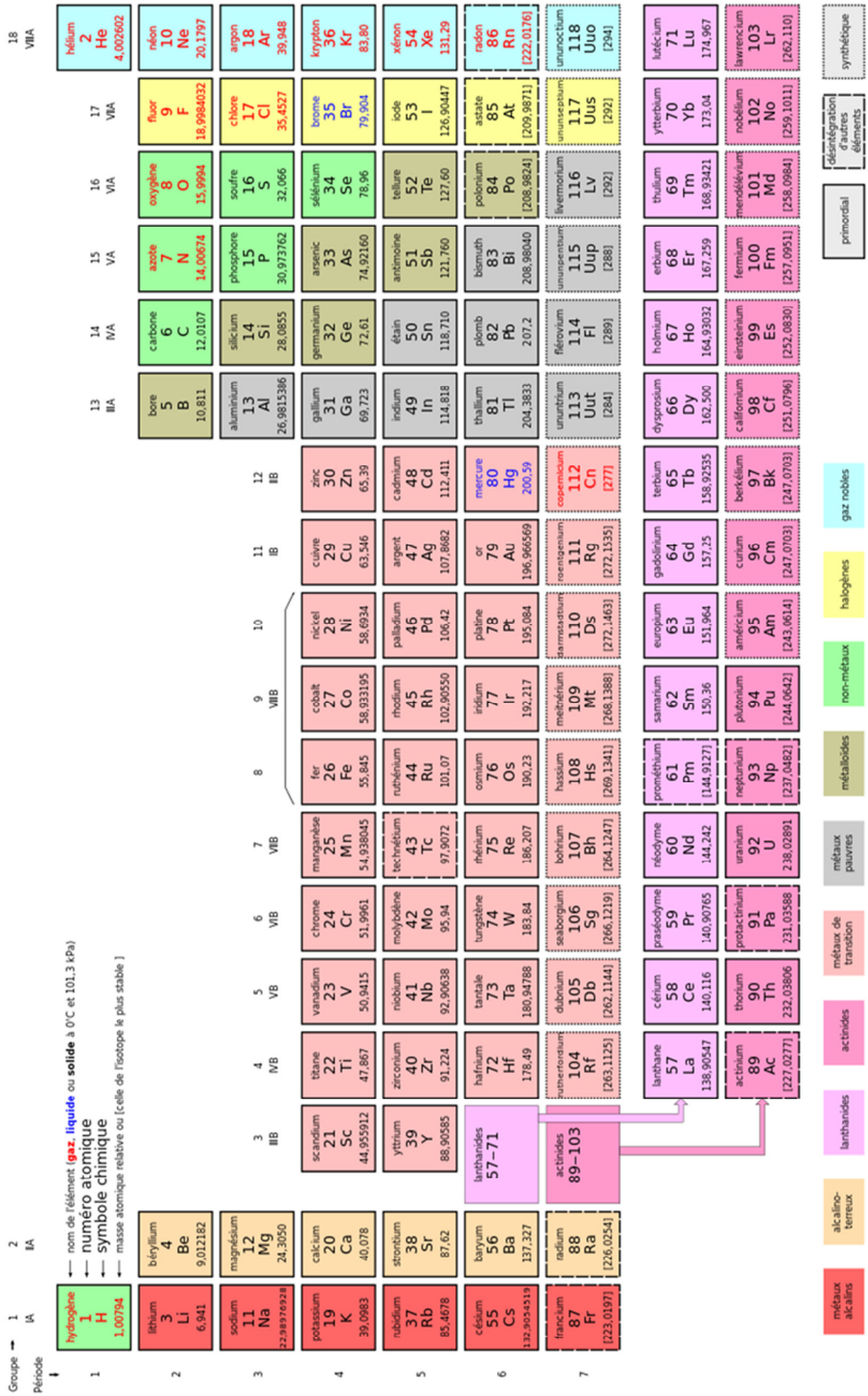


Figure 2 : Tableau périodique des éléments de Mendeleïev (Scaler, Michka B ; commons.wikimedia.org).

1.1 Les éléments traces métalliques

1.1.1 Définitions

Les métaux sont des éléments chimiques qui participent aux réactions sous forme de cations. Ils ont la propriété commune d'être d'excellents conducteurs électriques et thermiques et d'avoir une haute densité, malléabilité et ductilité (Bargagli, 1998). Les métalloïdes (Si, Ge, As, Sb, Se et Te) ont un caractère métallique partiel ou peuvent exister sous des formes métalliques ou non métalliques (Figure 2).

Le terme de « métaux lourds » est souvent utilisé dans la littérature. Les métaux « lourds » sont des éléments chimiques toxiques dont la masse volumique dépasse 5 g/cm^3, de numéro atomique supérieur à 20 et formant des précipités insolubles avec les sulfures (Wang & Chen, 2006). C'est le cas par exemple du Zn, Cu, Pb, Hg, Cr, Mn, Co, Ag, Ni, Fe, Mo ou Cd. Mais cette appellation est discutée car imprécise et mal définie (Nieboer & Richardson, 1980; Hodson, 2004).

En effet, sous ce terme sont également regroupés d'autres éléments toxiques appartenant aux metalloïdes tels que l'As et le Se ou encore des éléments qui ne sont pas « lourds » tels que l'Al ou le Be. Dans ce cas, l'appellation « éléments traces » est utilisée par de nombreux auteurs. Ce terme regroupe les 80 éléments chimiques trouvés à l'état de traces dans l'environnement, constituants la croûte terrestre et dont la concentration est pour chacun d'entre eux inférieure à 1 pour mille. Lorsque les éléments traces sont des métaux (par exemple Cd, Cr, Cu, Ni, Pb, Zn), ils sont nommés « éléments traces métalliques » (ETMs).

De plus, le terme « métal lourd » évoque une certaine toxicité. Cependant, certains de ces éléments sont indispensables à de nombreux processus biologiques et sont alors appelés oligoéléments, c'est le cas par exemple du Zn ou du Fe. D'autres n'ont aucun rôle biologique et sont donc toxiques, comme le Cd, le Pb ou le Hg (Bert & Deram, 1999). Dans les deux cas, en concentration trop importante, ceux-ci deviennent toxiques pour la biosphère (Alloway, 1995; Bliefert & Perraud, 2008).

Les oligoéléments peuvent par exemple être des constituants importants des pigments ou jouer le rôle de cofacteur d'enzyme, et la protéine formée sera appelée métallo-enzyme. Parmi les ETM, le Zn, le Fe et le Mn sont les trois principaux éléments impliqués comme cofacteurs enzymatiques (Figure 3 ;Waldron *et al.*, 2009). Il existerait environ 300 métallo-enzymes ayant comme cofacteur le Zn (Guerinot, 2000), avec par exemple la superoxyde dismutase (SOD) cytosolique (Wu *et al.*, 2009). Le Fe peut aussi être un cofacteur et former des centres fer-soufre ou des hèmes indispensables à la fonction de nombreuses protéines. Le centre fer-soufre, contenu dans les protéines, joue un rôle direct dans les voies essentielles pour la viabilité des cellules. Il participe à des réactions biologiques

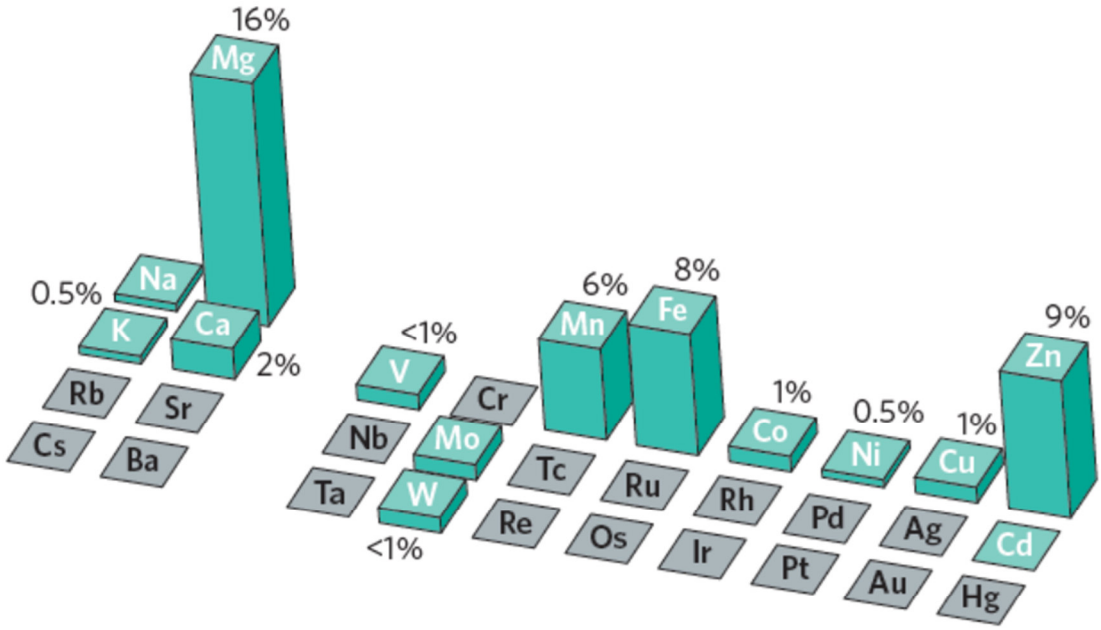


Figure 3 : Eléments impliqués comme cofacteurs d'enzymes. (Waldron *et al.*, 2009). Chaque valeur (%) représente la proportion d'enzymes utilisant un élément donné comme cofacteur.

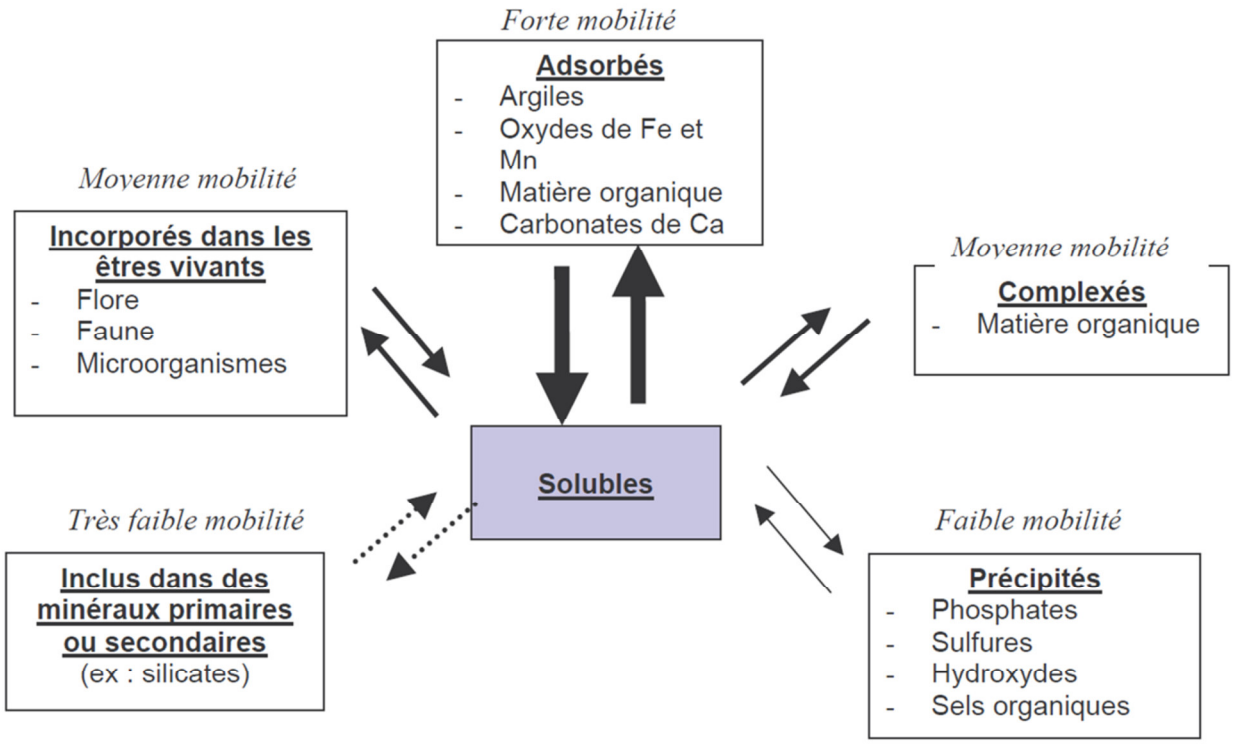


Figure 4 : Spéciation des ETMs dans les sols (ADEME & APCA, 2005).

comprenant la synthèse nucléotidique, la stabilité des protéines, leur translocation, la catalyse enzymatique et le métabolisme mitochondrial (Rawat & Stemmler, 2011).

1.1.2 Mobilité et biodisponibilité des ETMs dans les sols

Dans le sol, les ETMs sont distribués entre la phase solide et la phase liquide qui le constituent. Cette distribution dépend de la spéciation des ETMs c'est-à-dire d'une part, de la forme chimique sous laquelle ils ont été apportés au sol et d'autre part, des propriétés physico-chimiques ainsi que des espèces présentes dans le sol.

Le plus souvent, la proportion d'ETMs présents dans la solution du sol ne représente qu'un infime pourcentage de la quantité totale de ces éléments. Les ETMs se concentrent donc dans la fraction solide du sol, où ils se répartissent entre fraction organique (matière organique) et minérale (argiles, oxydes). En fonction de leur spéciation, les ETMs sont plus ou moins mobiles (Figure 4).

Les ETMs peuvent être inclus dans les minéraux primaires (minéraux non altérés, hérités de la roche-mère) et des constituants secondaires (minéraux résultant de l'altération pédogénétique) ce qui les rend très peu mobiles.

Les ETMs sont aussi adsorbés sur les phases hydroxylées ou oxydées du Fe, de l'Al, du Mn et associés aux surfaces des minéraux argileux, des carbonates et à la matière organique. L'adsorption des ETMs peut être plus ou moins réversible selon la liaison qu'ils forment avec les composés du sol. L'adsorption par échanges d'ions forme des liaisons faibles, facilement réversibles, notamment avec les argiles ou la matière organique. Au contraire, l'adsorption par complexation de surface, plus stable, a lieu avec les constituants du sol possédant des groupements oxygénés (-OH) et se forme avec les argiles, les carbonates, la matière organique, les oxyhydroxydes de Fe et de Mn (Citeau *et al.*, 2008).

Les ETMs peuvent être incorporés dans les êtres vivants et seront libérés ensuite au fur et à mesure de la minéralisation des résidus de matières organiques. Ils peuvent également être complexés ou inclus dans les macromolécules organiques présentes dans les sols. Dans les deux cas la mobilité est moyenne.

Les ETMs forment des précipités par agrégation avec de petites particules organiques (sels organiques) ou minérales (phosphates, sulfures et hydroxydes). La cohésion de ces agrégats est très forte et rend la réversibilité de la réaction plus difficile (Deneux-Mustin *et al.*, 2003).

Enfin, dans la solution du sol (phase liquide), les ETMs sont sous une forme soluble, colloïdale ou particulaire. Ils peuvent être présents sous forme de cations, d'hydroxydes ou d'oxyanions selon leur charge, leur électronégativité et/ou leur rayon ionique. Les ETMs sont souvent présents sous forme de complexes solubles inorganiques (sulfites, carbonates, nitrites, nitrates,

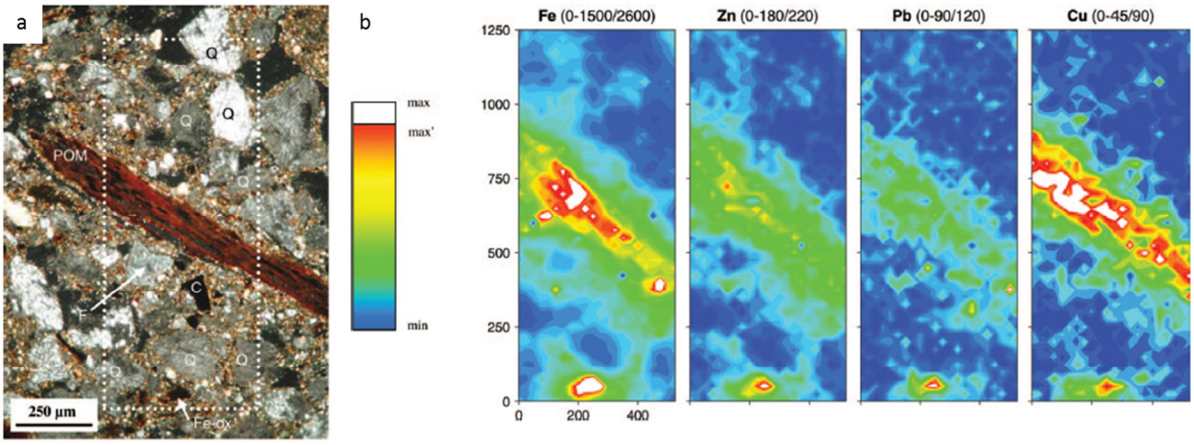


Figure 5 : Observation microscopique de sol et distribution des éléments.

Image de microscopie optique en lumière polarisée croisée d'un sol caractéristique de l'horizon supérieur (a) et cartes de distribution élémentaire obtenues par µ-SXRF (b). Q, quartz; F, feldspath; C, charbon de bois; POM, matière organique particulaire, Fe-ox., particule d'oxyde de fer (Van Oort *et al.*, 2008).

Tableau I : Spéciation chimique de quelques ETMs dans la solution du sol en fonction du pH (Tremel-Schaub & Feix, 2005).

Métal	Sol (pH modéré)	Additionnel dans	
		Sol acide	Sol alcalin
Cd(II)	Cd^{2+}		$CdHCO_3^+$
	$CdSO_4$		
	$CdCl^+$		
Cu(II)	Cu-org	Cu^{2+}	$CuCO_3$
			$CuB(OH)_4^+$
			$Cu[B(OH)_4]_4$
Mn(II)	Mn^{2+}	Mn-org	$MnCO_3$
	$MnSO_4$		$MnHCO_3^+$
			$MnB(OH)_4^+$
Ni(II)	Ni^{2+}	$NiSO_4$	$NiCO_3$
	$NiHCO_3^+$	Ni-org	$NiB(OH)_4^+$
Pb(II)	Pb-org	Pb^{2+}	$PbCO_3$
	$PbHCO_3^+$	$PbSO_4$	$Pb(CO_3)_2^{2-}$
			PbOH+
Zn(II)	Zn^{2+}		$ZnHCO_3^+$
	$ZnSO_4$		$ZnCO_3$
	Zn-org		$ZnB(OH)_4^+$

chlorures ou phosphates) ou organiques (acides organiques, peptides ou protéines liés à l'activité microbienne) dans la solution du sol (Deneux-Mustin *et al.*, 2003). Par exemple un sol sableux irrigué pendant 100 ans avec des eaux urbaines résiduaires affiche une forte accumulation de matière organique, de sels dissous et de polluants métalliques dans les horizons de surface. Dans ces horizons, les ETMs sont principalement liés à la matière organique et aux composants dissous ajoutés par l'eau d'irrigation (Figure 5 ; Van Oort *et al.*, 2008).

La répartition des ETMs dans les différents compartiments du sol ne demeure pas figée au cours du temps et les éléments passent d'une forme à une autre sous l'influence de facteurs externes (changement de température, d'humidité, acidification, diminution de la quantité de matière organique, taille des particules du sol, changement du potentiel redox, de la quantité de nutriments, ou présence d'autres ions ; Ernst, 1996; Lebeau *et al.*, 2008). Le pH est le facteur qui influence le plus la spéciation des ETMs (Tableau I). Les différences de composition des sols et de leurs caractéristiques physiques, chimiques et biologiques propres, entrainent donc des concentrations en ETMs très variables d'un sol à un autre, mais également d'un horizon de sol à l'autre (Baize, 1997).

La fraction mobile ou mobilisable des ETMs dans la solution du sol correspond à la fraction disponible pour les organismes vivants. Un élément est biodisponible quand il peut être assimilé par un organisme vivant (bactérie, champignon, plante ou animal) au cours de sa vie (Bourrelier & Berthelin, 1998). Par exemple le Pb peut être présent en grande quantité dans les sols, mais il sera quasiment inaccessible aux plantes et aux microorganismes en raison de sa faible solubilité et des interactions fortes avec les particules du sol (Nriagu & Pacyna, 1988). Afin d'évaluer la fraction biodisponible des ETMs, il était couramment admis que les extractions au DTPA (Acide Diéthylène Triamine Penta Acétique) ou à l'EDTA (Acide Ethylène Diamine Tétra Acétique) étaient une bonne alternative (Lindsay & Norvell, 1978; Echevarria *et al.*, 1998; Bakircioglu *et al.*, 2011). Récemment, plusieurs études ont montré que l'extraction au $CaCl_2$ (Chlorure de Calcium) était plus représentative de la fraction biodisponible pour la plante (Meers *et al.*, 2007; Lopareva-Pohu *et al.*, 2011; Anjos *et al.*, 2012).

En plus du dosage des ETMs, une caractérisation approfondie des sols (pH, capacité d'échange cationique ou CEC, carbone, calcaire, granulométrie, quantité de matière organique, abondance des oxydes de Fe ou de Mn) est également nécessaire pour estimer les risques de transferts (Baize, 1997).

La persistance de contaminants dans le sol est beaucoup plus longue que dans les autres compartiments de la biosphère, et la contamination du sol, en particulier par les ETMs, semble être quasi permanente. Les ETMs accumulés dans les sols sont épuisés lentement par lessivage, absorption par les plantes, ou l'érosion.

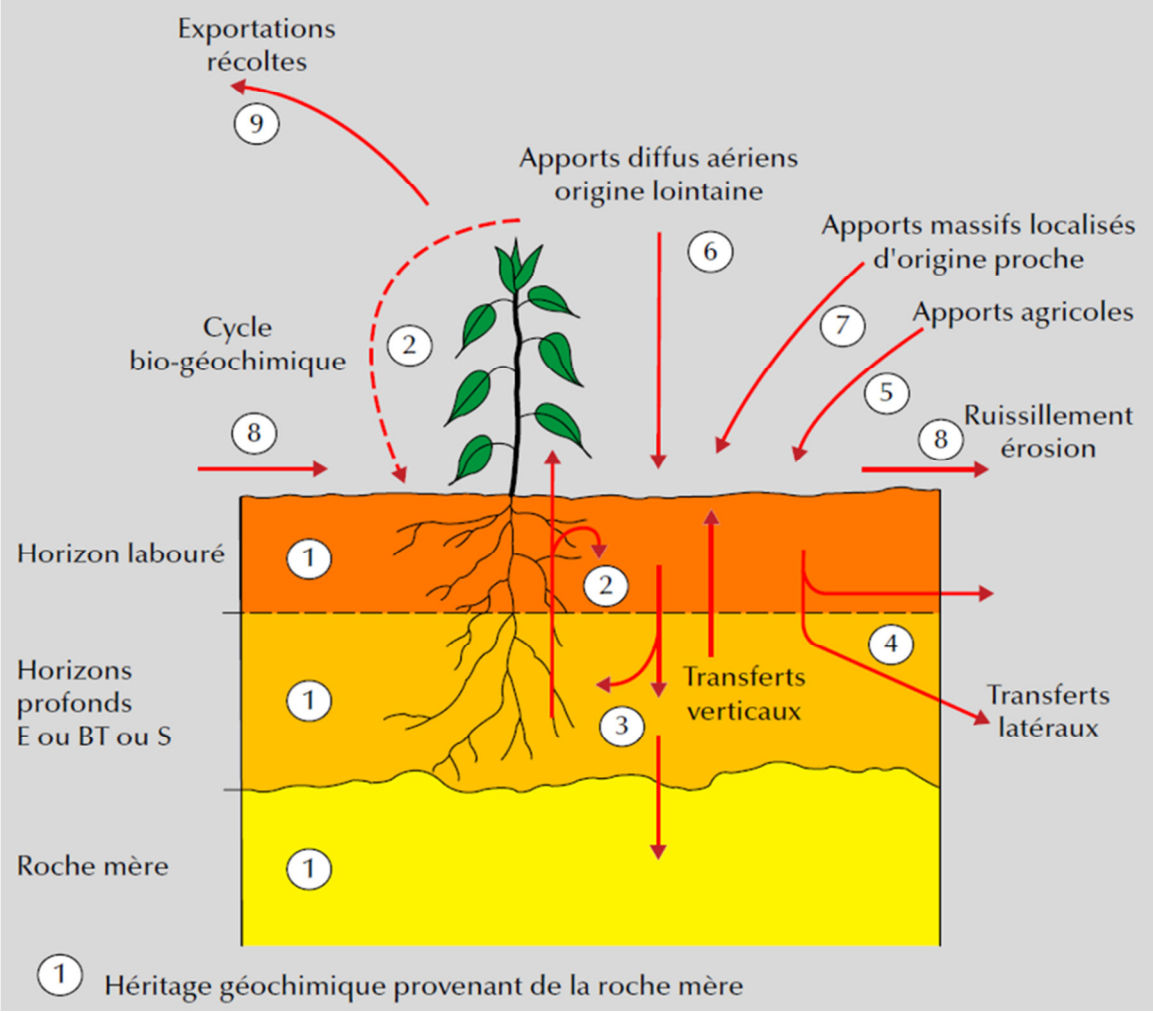


Figure 6 : Origines des ETMs dans les sols (Mathieu *et al.*, 2008).

1.1.3 Pollution métallique

La pollution est l'introduction directe ou indirecte, par l'activité humaine, de substances dans l'air, l'eau ou le sol, susceptibles de porter atteinte à la santé humaine ou à la qualité des écosystèmes aquatiques ou terrestres, qui entrainent des détériorations aux biens matériels, une détérioration ou une entrave à l'agrément de l'environnement ou à d'autres utilisations légitimes de ce dernier (Directive Européenne 2000/60/CE du 23 octobre 2000). Le terme contamination est également utilisé lorsque l'état chimique du sol dévie de la composition normale, mais n'entraine pas d'effet préjudiciable sur les organismes (Knox *et al.*, 1999).

1.1.3.1 Origines des ETMs

Les ETMs peuvent provenir de sources naturelles, telles que l'érosion des roches ou le volcanisme, et majoritairement d'apports anthropiques dus aux activités industrielles, actuelles ou anciennes, urbaines, minières, agricoles ou domestiques (Baize, 1997).

1.1.3.1.1 Origine naturelle

Les ETMs présents naturellement dans les sols sont hérités de la roche mère dont ces sols sont issus par altération biogéochimique (Figure 6①). Selon la composition chimique initiale de la roche, un sol sera plus ou moins enrichi en oligo-éléments et/ou en ETMs. En France par exemple, le Cr, le Ni et le Zn sont en concentration plus importante en Charentes et dans les montagnes du Jura et des Vosges dus à un sol volcanique (Saby *et al.*, 2011).

Les ETMs vont plus ou moins se concentrer dans les différents horizons des sols en fonction des zones climatiques, de la durée et des caractéristiques de la pédogenèse mais d'autres processus interviennent ensuite comme la libération, la lixiviation et les transferts des ETMs (Figure 6③et④). En effet, les eaux de ruissellement et l'érosion du sol en amont apportent des particules chargées en ETMs aux autres horizons et certains passent sous une forme soluble à des pH acides. Les divers horizons des sols peuvent alors se différencier en ce qui concerne non seulement leur aspect et leur propriété, mais aussi leur concentration en ETMs.

En plus des transferts des ETMs, les horizons de surface des sols sont touchés par des apports d'ETMs par le vent ou la pluie sous forme de poussières ou d'aérosols provenant d'activités volcaniques, d'embruns maritimes ou de la volatilisation de météorites lorsqu'elles pénètrent dans l'atmosphère (Figure 6⑥).

En présence de plantes, un cycle biogéochimique se met en place entre la plante et le sol (Figure 6②). Les ETMs sont absorbés par les racines et redistribués dans les différents organes. Ils retourneront dans les sols lors de la décomposition des racines ou des parties aériennes sous forme plus biodisponibles et plus mobiles. L'exportation des récoltes rompt ce cycle par l'export de la biomasse

Tableau II : Différentes références françaises de teneurs totales en ETMs dans les boues d'épuration et les sols exprimées en mg.kg^{-1} MS (d'après Baize & Tercé, 2002).

		Cd	Cr	Cu	Ni	Pb	Zn	Hg	Se
Norme NF U 44-041 (1985)	Boues	20	1000	1000	200	800	3000	10	100
	Sols	2	150	100	50	100	300	1	10

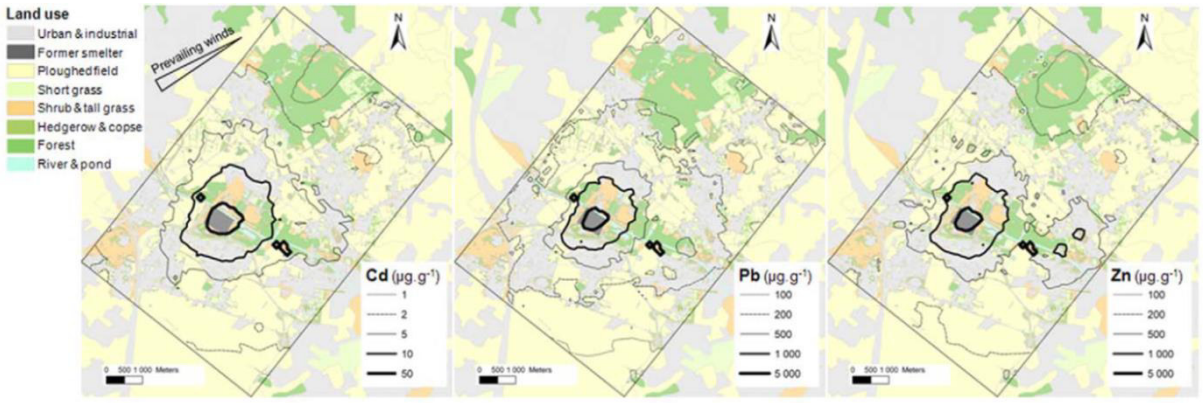


Figure 7 : Lignes d'isoconcentration prédites pour les concentrations totales en Cd, Pb et Zn dans les sols environnant l'usine de Metaleurop Nord (Fritsch *et al.*, 2011).

(feuilles, tubercules et/ou graines), et provoque le départ des ETMs qui entrent ainsi dans la chaine alimentaire humaine.

L'action de ces processus naturels pendant des milliers d'années conduit à la constitution du fond pédo-géochimique naturel (FPGN), lequel peut parfois être assez différent du fond géochimique initial lié à la roche mère. Les activités humaines peuvent également modifier cet équilibre directement ou indirectement.

1.1.3.1.2 Origines anthropiques

Les pratiques agricoles sont une des premières causes de pollution d'origine anthropique d'un sol par les ETMs (Figure 6⑤). En effet, certains engrais (notamment phosphatés), amendements calcaires, fumiers, lisiers, épandages divers, boues de stations d'épuration, composts urbains et produits de traitements phytosanitaires peuvent contenir des quantités non négligeables d'ETMs. Des études menées sur l'impact de l'épandage de boues d'épuration sur des sols ont montré une nette augmentation des ETMs dans ces sols (Walter & Cuevas, 1999; Baize & Tercé, 2002). Par ailleurs, une norme française mise en place en 1985, la norme AFNOR NF U 44-041, réglemente les teneurs en ETMs autorisées dans les boues d'épuration destinées à l'épandage agricole ainsi que les quantités possibles à épandre par hectare (Tableau II ; Baize & Tercé, 2002).

La pollution peut être également véhiculée par le vent lorsqu'il s'agit de particules, de poussières ou d'aérosols provenant des chauffages, des activités industrielles ou des automobiles (Sezgin *et al.*, 2004) et par l'eau de pluie qui ruisselle en surface ou qui percole dans les nappes phréatiques (Figure 6⑥et⑧).

La pollution qui est dispersée constitue des apports diffus d'origine lointaine ou des apports massifs d'origine proche lorsque la source de pollution se situe à proximité (Figure 6⑥et⑦). Par exemple, l'un des sites expérimentaux localisé dans le nord de la France, utilisé dans le cadre de cette thèse, est situé à côté d'une usine métallurgique désaffectée dont les fumées ont déposé de grandes quantités d'ETMs sur les parcelles avoisinantes (Figure 7 ; Fritsch *et al.*, 2011).

Lorsque les sols contiennent des matériaux technologiques ou anthropologiques en grande quantité, ils sont appelés technosols ou anthroposols. Ces sols anthropiques sont souvent contaminés en ETMs et comme c'est le cas par exemple des lagunes de dépôt de sédiments sur le site expérimental de Tavaux (Jura) présentant de forte quantité de Hg.

1.1.3.2 Les normes et l'identification de pollution

En France, l'article L110-1, II, 3° du code de l'environnement défini que « les frais résultant des mesures de prévention, de réduction de la pollution et de lutte contre celle-ci doivent être supportés par le pollueur. ». C'est le principe dit de pollueur payeur.

Tableau III : Teneurs totales en ETMs dans des sols français (Baize, 2000).

	Cd	Co	Cr	Cu	Ni	Pb	Zn	Mn	Fe
nb valeurs	768	695	791	787	797	790	804	735	767
minimum	<0,02	<1	<2	<2	<2	2,2	<5	<10	0,05
1er décile	0,03	3,1	27,5	5	8,7	17,2	31	149	1,27
1er quartile	0,07	7,6	45,1	8,2	18	23,1	49	408	2,16
médiane	0,16	14	66,3	12,8	31	34,1	80	754	3,24
moyenne	0,42	17,1	75	14,9	41,3	64,8	149	7585	3,65
3e quartile	0,47	21,3	90,7	18,1	48,6	48,5	132	1473	4,57
9e décile	1,06	32,4	118	27,2	78,9	91,5	275	3332	6,47
maximum	6,99	148	691	107	478	3088	3820	24975	19,16
Seuils[a]	2	30[b]	150	100	50	100	300	-	-

a = seuils de l'arrêté du 8 janvier 1998 sur l'épandage des boues d'épuration.

b = ce seuil, proposé par Godin en 1984 pour le Co, n'a pas été retenu dans la norme française.

Cependant, il est très difficile, voire impossible, de définir une valeur seuil pour un élément à partir de laquelle un sol présente un risque et/ou nécessite une intervention. En effet, ce risque dépend principalement du type de sol considéré, de la biodisponibilité des ETMs et de l'usage du site. C'est pourquoi, il existe peu de normes de pollution des sols et celles-ci varient d'un pays à un autre.

En France, il n'existe pas réellement de valeurs seuils à partir desquelles un sol est considéré comme pollué. Seule, la norme NF U 44-041 de juillet 1985 a pour but de réglementer l'épandage de boues de station d'épuration sur les sols à vocation agricole. Elle précise des teneurs maximales en ETMs à ne pas dépasser dans les boues et les sols (Tableau II). Cette norme française reprend à l'identique les proportions de Godin faites en 1984. Ce dernier s'est inspiré des valeurs émanant des travaux d'une commission AFNOR et de tableaux provisoires analogues établis dans d'autres pays européens (notamment aux Pays-Bas) définissant 3 types d'usages (la production maraîchère, les autres productions agricoles liées à l'alimentation et les autres usages).

Cette norme tient compte de quatre concentrations de référence : le seuil d'anomalie (concentration maximale rencontrée dans les sols courants) ; le seuil d'investigation (concentration du sol au-delà de laquelle une étude approfondie du site est nécessaire) ; le seuil de traitement (concentration du sol au-delà de laquelle un traitement est nécessaire) et le seuil d'urgence (concentration du sol au-delà de laquelle un traitement s'impose dans un délai de 3 mois). Le seuil d'investigation correspond à 2 fois le seuil d'anomalie, le seuil de traitement correspond à 5 fois le seuil d'anomalie et le seuil d'urgence correspond à 10 fois le seuil d'anomalie. Dans le texte de cette norme, il est aussi prévu d'effectuer une analyse de la teneur en ETMs avant le 1er épandage, puis tous les 10 ans. De plus, le pH du sol après épandage doit impérativement être maintenu supérieur ou égal à 6. En effet, les ETMs sont d'autant moins mobiles et biodisponibles dans les sols que le pH est neutre ou alcalin (Baize & Tercé, 2002). Une réglementation plus récente, l'article 11 de l'arrêté du 8 janvier 1998 reprend les mêmes valeurs seuils de pollution (Tableau II), mis à part la suppression des valeurs de référence pour le Hg et le Se. Les teneurs des ETMs mesurées sur différents échantillons de sol français considérés comme non contaminés ont été comparés à cette nouvelle réglementation et montrent une forte variabilité (Tableau III).

Afin d'identifier les sols susceptibles d'être pollués et faisant donc l'objet de mesures de gestion appropriées, il est essentiel d'avoir des données relatives aux concentrations usuelles en éléments traces mesurées dans les sols afin de servir de valeurs de référence locales. Ces valeurs de référence peuvent être le fond pédo-géochimique local (FPGL) ou encore les teneurs agricoles habituelles (TAH). Le fond pédo-géochimique local (FPGL) correspond aux teneurs totales en éléments chimiques retrouvés dans un sol, en absence de contaminations liées aux activités humaines. Les teneurs agricoles habituelles correspondent au fond pédo-géochimique local auquel se sont ajoutés les apports standards liés aux activités agricoles (apports de fertilisants/pesticides) et aux dépôts atmosphériques (Baize, 1997). Les horizons supérieurs des sols étant généralement contaminés par des

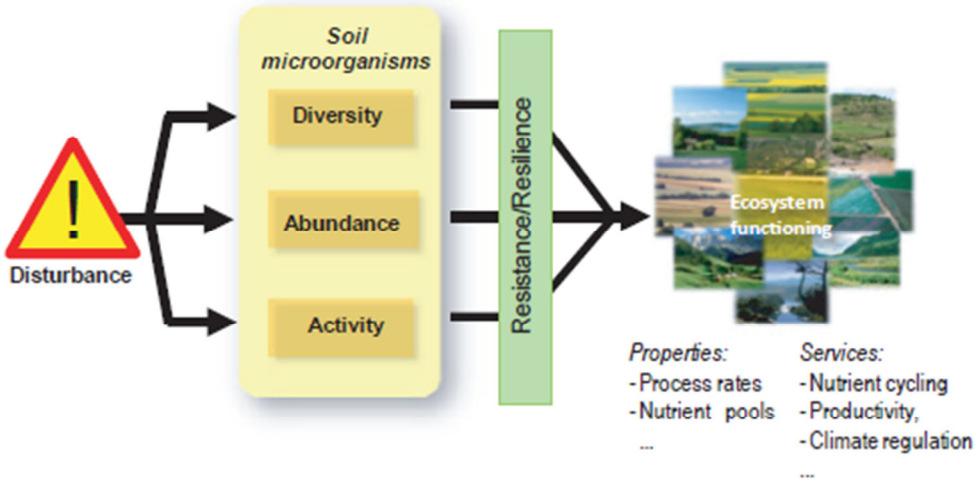


Figure 8 : Réponse de l'écosystème sol face aux perturbations (Griffiths & Philippot, 2013).

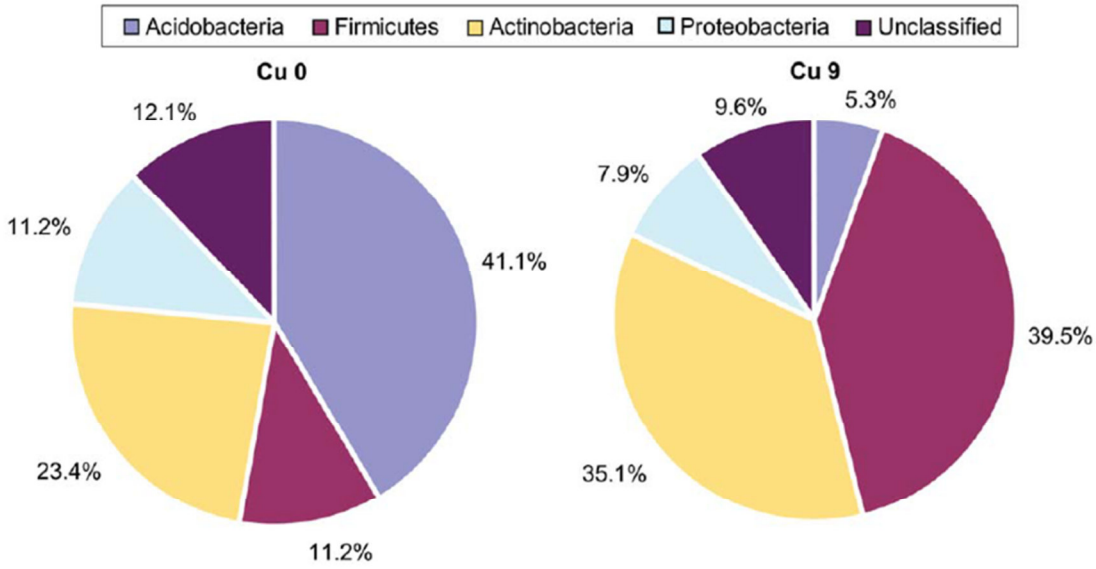


Figure 9 : Proportion des taxa dominants au niveau des phyla bactériens entre un sol témoin (Cu 0) et un sol contaminé par du Cu (Cu 9) (Wakelin *et al.*, 2010b).

apports d'origine diffuse, le fond pédo-géochimique local est évalué à partir des horizons profonds (Citeau *et al.*, 2008).

En France, chaque site potentiellement pollué est évalué au cas par cas en se basant principalement sur la comparaison entre le fond pédo-géochimique local et les données du site étudié. Quatre éléments majeurs sont pris en compte dans l'évaluation des risques liés à la contamination d'un site par les ETMs : la source (activités agricoles, industrielles…), le vecteur (l'air, l'eau ou les végétaux), la cible de la pollution (l'Homme ou l'écosystème) et l'usage du site (production maraîchère, habitation...).

1.1.4 Impact des ETMs

Les notions de dose et de temps d'exposition sont fondamentales pour l'étude des ETMs. L'exposition aigüe des organismes vivants à une dose élevée d'ETMs (qu'ils soient oligo-éléments ou contaminants stricts) ou bien l'accumulation dans les tissus suite à une exposition chronique, entraine des effets de toxicité. Le passage de la fonction d'oligo-élément à celle d'élément toxique dépend de la nature, de la teneur et de la disponibilité de l'élément et du milieu considéré (Tremel-Schaub & Feix, 2005).

1.1.4.1 Au niveau des écosystèmes

La perturbation entrainée par la présence d'ETMs a un impact sur la biodiversité des organismes mais également sur leur abondance et leur fonction ou activité (Figure 8 ;Griffiths & Philippot, 2013).

Les premières conséquences de la pollution sur un écosystème sont une modification des espèces et des populations présentes car seules celles qui se seront adaptées pourront survivre. Ce phénomène a par exemple été montré lors d'une pollution métallique sur les communautés de microorganismes (Wakelin *et al.*, 2010a; Macdonald *et al.*, 2011; Rieder & Frey, 2013). En plus du stress dû à la présence d'ETMs, les organismes sont en compétition et certaines espèces ou populations seront favorisées par rapport à d'autres et seront donc plus abondantes. Par exemple, le Cu semble avoir un effet positif sur les *Actinobactéries* et un effet négatif sur les *Acidobacteries* (Figure 9 **;** Lejon *et al.*, 2008; Wakelin *et al.*, 2010b; Macdonald *et al.*, 2011). Cette perte de biodiversité et d'abondance peut entrainer une perte ou une diminution de certaines fonctions spécifiques (Singh *et al.*, 2014).

Après une perturbation, l'écosystème va tendre vers un état stable. La stabilité de l'écosystème, face à une perturbation est déterminée par la résistance, la résilience et la redondance (Figure 10). Résistance et résilience peuvent se référer à la composition des communautés (la diversité des espèces, l'abondance relative ou absolue) ou une fonction (par exemple le taux des processus biogéochimiques, l'apparition de maladies) et peuvent résulter de la croissance, l'adaptation physiologique ou génétique. La résistance est le degré où un système change après la perturbation

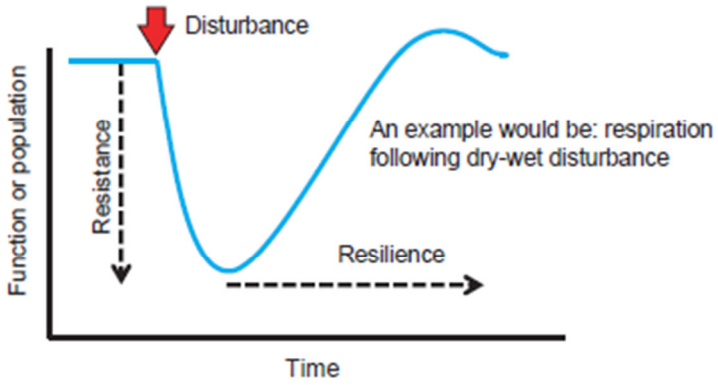


Figure 10 : Représentation schématique de la résilience et de la résistance (Griffiths & Philippot, 2013).

(Pimm, 1984) et est parfois considéré comme une composante de la résilience (Walker *et al.*, 2004). La résilience décrit la rapidité avec laquelle un système retourne à son état initial après une perturbation (Bissett *et al.*, 2013; Griffiths & Philippot, 2013; Pimm, 1984).

La redondance fonctionnelle se réfère à la capacité d'un groupe d'organismes pour effectuer les mêmes processus au même rythme dans un éventail de conditions. Les communautés microbiennes sont fonctionnellement redondantes et donc en grande partie résistantes et résilientes à la perturbation (Allison & Martiny, 2008). Les microorganismes ont une biomasse et un potentiel de croissance élevé, ils sont universellement dispersés, ils ont un potentiel d'extinction faible et une incidence élevée de transfert horizontal de gènes (Bissett *et al.*, 2013).

1.1.4.2 Au niveau des organismes

Au niveau des organismes, les ETMs ont différents modes d'entrée et de transfert selon l'organisme entrainant ainsi des symptômes caractéristiques.

1.1.4.2.1 Toxicité pour l'Homme

Chez l'Homme, les voies d'entrées des ETMs dans l'organisme sont le contact cutané, l'inhalation ou l'ingestion. En fonction de l'élément et de sa spéciation, des irritations ou des réactions allergiques localisées sont possibles, ainsi qu'une pénétration dans les différentes couches de l'épiderme pouvant aller jusqu'à une diffusion systémique (Hostynek, 2003). Les poussières contenant des ETMs peuvent être inhalées par la population proche et entrainer des maladies respiratoires ou cardiaques, des cancers du poumon ou l'atteinte d'autres organes (Caboche *et al.*, 2011).

Les ETMs peuvent être ingérés directement par absorption d'eau ou de particules de terre contaminée, particulièrement chez les enfants (Guney *et al.*, 2010) ou indirectement par la consommation de végétaux ou d'animaux contaminés (Silvera & Rohan, 2007). Les effets de l'ingestion d'ETMs sont très variables et dépendent de l'élément étudié, de sa spéciation, de la dose et de sa bioaccessibilité (Pelfrene *et al.*, 2012).

L'effet toxique le plus répandu lié à l'exposition aux ETMs chez l'Homme est la formation de cancers. Cet effet cancérogène est principalement dû à la génération d'espèces réactives de l'oxygène par les ETMs qui vont provoquer des dommages à l'ADN et aux biomolécules permettant la réparation de l'ADN (Galanis *et al.*, 2009). De plus, certains ETMs peuvent prendre la place d'éléments indispensables au bon fonctionnement des enzymes de réparation de l'ADN (Koedrith & Seo, 2011).

Certains ETMs entrainent des maladies caractéristiques comme le saturnisme, l'intoxication par le Pb, ou l'hydrargyrisme, l'intoxication par le Hg. La maladie de Minamata est l'exemple le plus connu d'hydrargyrisme. Elle a touché durant des décennies des milliers d'habitants du pourtour de la

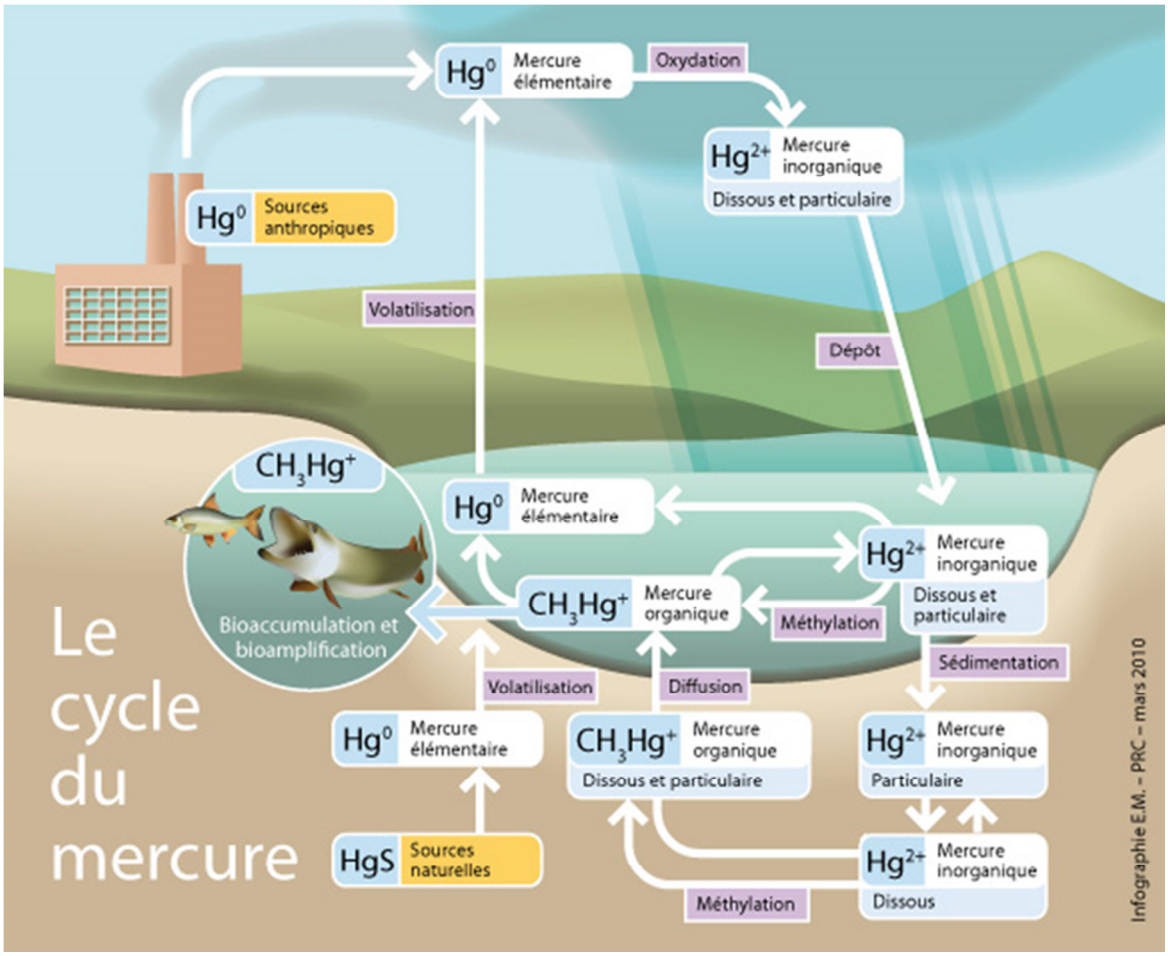


Figure 11 : Cycle du Hg (www.prc.cnrs-gif.fr).

baie de Minamata au Japon. En 1907 une usine pétrochimique s'est implantée sur cette baie et utilisait le Hg comme catalyseur puis rejetait les résidus dans la mer. Les symptômes de cette maladie sont physiques et neurologiques (perte de motricité, malformations congénitales…) et sont induits par l'intoxication *in utero* (principalement par du monométhylmercure) et par la consommation de poissons contaminés (Harada, 1995). En effet, la consommation de poissons est la principale source d'exposition au Hg (Lauwerys & Lauwerys, 2007) car il s'accumule dans les tissus des organismes vivants (bioaccumulation) et se concentrer le long des différents maillons de la chaîne alimentaire (bioamplification) (Figure 11).

1.1.4.2.2 Toxicité pour les plantes

Chez les plantes, l'entrée des ETMs peut se faire par les parties aériennes (via des particules en suspension dans l'air, des composés gazeux tels le Hg et le Se ou des composés dissous dans l'eau de pluie ou d'irrigation) ou par les racines via la solution du sol. Une fois prélevés par la plante, les éléments traces peuvent être piégés et ne pas circuler dans la plante, ou alors être transportés du lieu de l'absorption vers un autre organe végétal (Tremel-Schaub & Feix, 2005).

Les symptômes les plus souvent observés chez les plantes sont un ralentissement de la croissance racinaire et/ou aérienne, une perturbation des fonctions physiologiques, une nécrose ou une chlorose des feuilles. Les symptômes sont liés à la nature de l'élément en cause et à sa spéciation (Babula *et al.*, 2008; Nagajyoti *et al.*, 2010). En général, la toxicité des ETMs est soit liée à une carence en fer causée par l'excès d'un autre élément, soit liée à la génération d'espèces réactives de l'oxygène dans la cellule végétale (Nagajyoti *et al.*, 2010).

En réponse aux ETMs, la plante répond différemment selon l'élément (essentiel ou non) et selon sa concentration (Figure 12). Si l'élément est essentiel, la biomasse de la plante suivra une courbe en cloche qui commence par une carence à faible concentration, une tolérance et enfin une toxicité à forte concentration. Si l'élément n'est pas essentiel (comme le Hg, le Pb ou le Cd), la courbe sera identique à l'exception de la carence.

1.1.4.3 Toxicité des ETMs au niveau cellulaire

Les effets toxiques causés par les ETMs sont divers. Ils comprennent le blocage de groupes fonctionnels de molécules à fonctions biologiquement importantes (par exemple: les enzymes et les systèmes de transports de nutriments essentiels et d'ions), le déplacement et/ou la substitution des ions métalliques essentiels dans des biomolécules, les modifications conformationnelles, les perturbations de membranes cellulaires et organellaires Ils peuvent être également la cause de la production de radicaux libres qui s'attaquent ensuite aux membranes et/ou aux acides nucléiques induisant alors un stress oxydatif (Figure 13 ; Leonard *et al.*, 2004; Liu *et al.*, 2009).

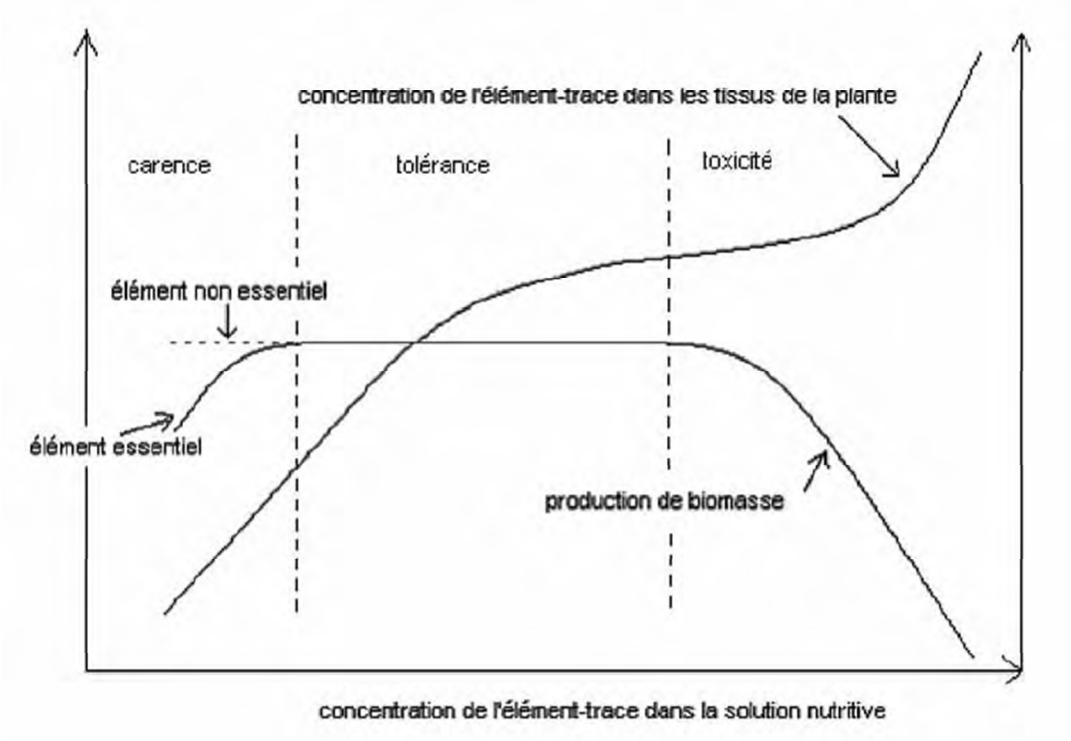


Figure 12 : Effets généraux des concentrations en ETMs sur la production de biomasse et sur la concentration en ETMs des tissus de la plante (Tremel-Schaub & Feix, 2005).

Cette altération des membranes cellulaires modifie ainsi leur perméabilité causant un efflux d'ions (Belagyi *et al.*, 1999; Valko *et al.*, 2005; Sanz *et al.*, 2009). Les radicaux libres générés par les ETMs peuvent induire des cassures dans les brins d'ADN ainsi que leur dégradation (Figure 13 ; Blokhina *et al.*, 2003). Les ETMs interagissent directement ou indirectement avec les acides nucléiques entrainant une réduction des appariements entre les deux brins de l'ADN, ou des cassures des ADN simple brin, ainsi que la formation de complexes avec l'ADN (Chiaverini & De Ley, 2010). Les ETMs peuvent induire une dépurination de l'ADN ayant pour conséquence des effets mutagènes (Bregadze *et al.*, 2008).

Les ETMs peuvent aussi agir sur les acides nucléiques de manière indirecte en interférant avec le fonctionnement d'enzymes impliquées dans la synthèse de ceux-ci. Ce qui peut ainsi conduire à une perturbation de l'information génétique en influant sur la fidélité de la réplication. Les ETMs peuvent interagir directement avec les groupes fonctionnels des enzymes, par exemple avec les groupements thiols des enzymes. Enfin, ils peuvent induire une carence en ETMs nécessaires aux métallo-enzymes ou les substituer au niveau du complexe enzymatique.

En conclusion, à trop forte concentration, les ETMs peuvent avoir des effets délétères sur les cellules et sont donc perçus comme un stress. Pour se défendre, la cellule met en place différents mécanismes spécifiques ou non du métal présent. Un stress peut par ailleurs être généré par le manque d'un oligoélément essentiel dans la cellule. Pour cela, il est vital que les cellules régulent finement la concentration en ETMs, afin de maintenir une homéostasie (Rutherford & Bird, 2004).

1.1.5 Mécanismes de tolérance

Les organismes présents sur des sols pollués par des ETMs ont dû développer des mécanismes de tolérance pour survivre. Chez les bactéries, la résistance est souvent spécifique d'un élément trace ou d'un petit nombre d'ETMs apparentés grâce à des plasmides bactériens portant des gènes de résistance aux ions métalliques comme Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+},Hg^{2+}, Ni^{2+}, Sb^{3+}, TeO_3^{2-}, Tl^{+} and Zn^{2+} (Gadd, 2010). Chez les organismes pluricellulaires compartimentés, comme les plantes ou les champignons, des mécanismes à large spectre confèrent une résistance à un certain nombre d'éléments traces différents.

Les organismes symbiotiques ou en interaction avec les plantes, telles que les bactéries endophytes, les champignons mycorhiziens ou endophytes, peuvent réduire la quantité d'ETMs disponibles pour leur plante hôte, diminuant ainsi le stress pour cette dernière (Jentschke & Godbold, 2001).

Chez tous ces organismes, il existe différents mécanismes de tolérance qui peuvent être classés dans les catégories suivantes : (Figure 14 ; Bellion *et al.*, 2006)

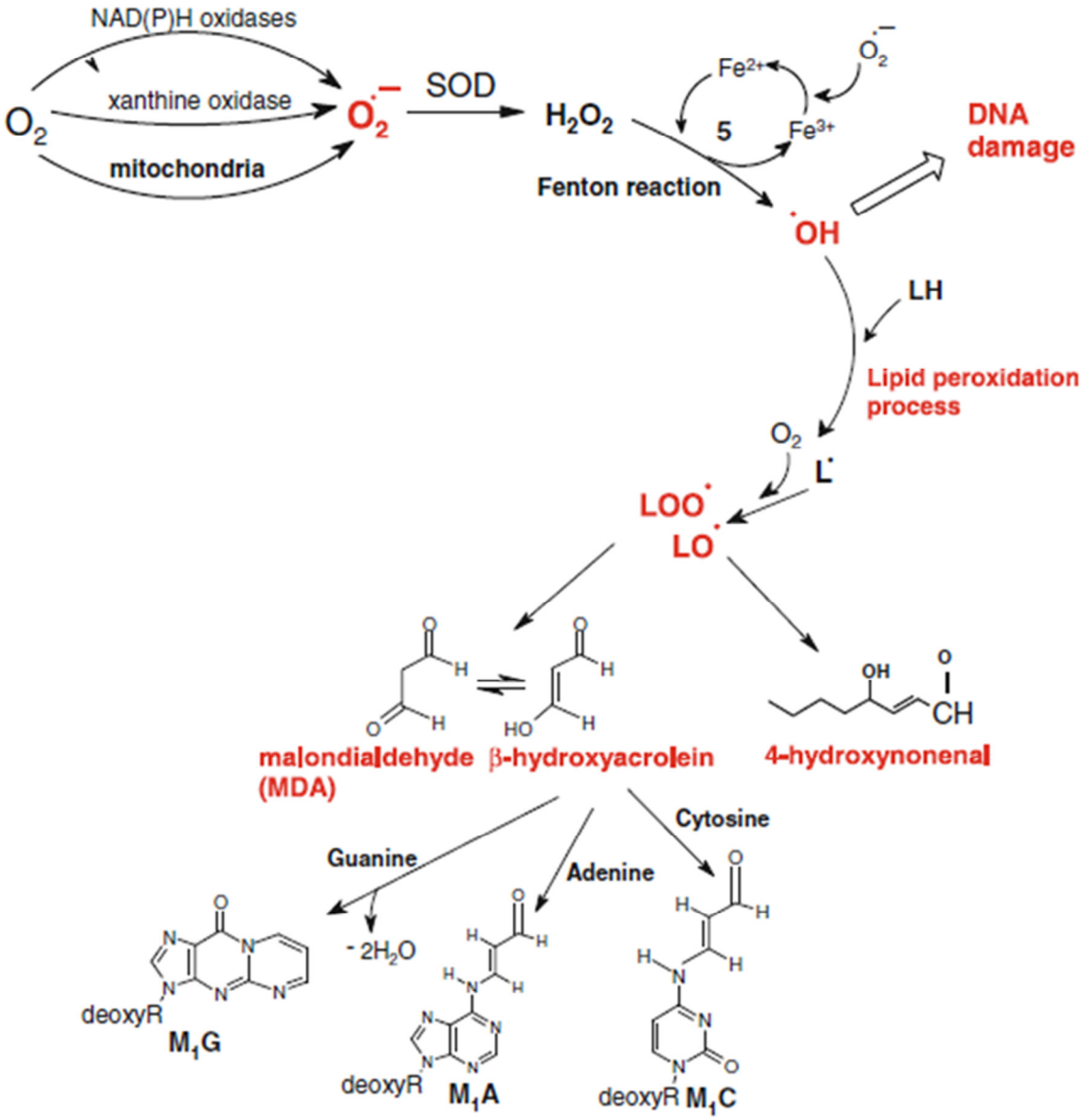


Figure 13 : Formation de radicaux libres après exposition aux ETMs et impact sur les biomolécules (Jomova *et al.*, 2012).

- la chélation extracellulaire par un ligand excrété ①
- la liaison à la paroi du métal ②
- l'exclusion par un système d'efflux ③
- la chélation intracellulaire ④ et ⑤
- la compartimentation intracellulaire du métal seul ⑥ ou du complexe chélateur-métal⑦
- et la lutte contre le stress oxydatif.

1.1.5.1 Mécanismes extracellulaires

Ce sont des mécanismes d'évitement qui consistent à freiner ou limiter l'absorption des ETMs dans les cellules.

1.1.5.1.1 Chélation extracellulaire

Pour limiter l'entrée des ETMs, les cellules peuvent libérer des molécules qui modifient leur biodisponibilité. Ces molécules peuvent être des agents chélatants tels que des acides organiques, des sidérophores, des minéraux secondaires, des substances polymériques extracellulaires ou des biosurfactants ; des agents de précipitation ou des pigments solubles ou membranaires.

Les molécules chélatrices sont des ligands capables de former un complexe stable avec un ion métallique pour le séquestrer. Toutes ces molécules rendent le métal insoluble et donc non assimilable. Ce sont le plus souvent des acides organiques qui sont excrétés pour chélater les ions métalliques (Gadd, 1999; Saber *et al.*, 1999; López-Bucio *et al.*, 2000; Haferburg & Kothe, 2007). Les acides di- et tricarboxyliques comme le citrate ou l'oxalate (Figure 15), sont bien connus pour jouer ce rôle entre autres fonctions chez les champignons (Gadd, 1999) ou chez les bactéries (Chen *et al.*, 2014). Par exemple chez *P. involutus*, l'acide oxalique réduit la quantité de Cd absorbée (Bellion *et al.*, 2006).

L'excrétion d'acides organiques est une source de protons pour la solubilisation des ETMs à partir de minerais contenant des métaux, aboutissant souvent à l'acidification du sol (Devêvre *et al.*, 1996; Fomina *et al.*, 2005). La libération de ces acides permet par exemple la solubilisation du phosphore pour les champignons (Gadd, 1999) et les bactéries (Chen *et al.*, 2014). Chez les champignons, l'immobilisation extracellulaire des ETMs peut également se produire à travers la formation de minéraux secondaire, par exemple en transformant la pyromorphite ($Pb_5(PO_4)_3Cl$) en un intermédiaire immobilisé (l'oxalate de Pb) après l'utilisation d'acides organiques (Figure 15 ; Sayer *et al.*, 1999).

Les sidérophores sont des chélateurs excrétés pour l'acquisition du fer qui peuvent réagir de façon croisée avec d'autres ETMs. Ces molécules sont présentes chez les bactéries (Złoch *et al.*, 2016), les champignons (Gadd *et al.*, 2012) ainsi que chez certaines plantes (Römheld, 1991).

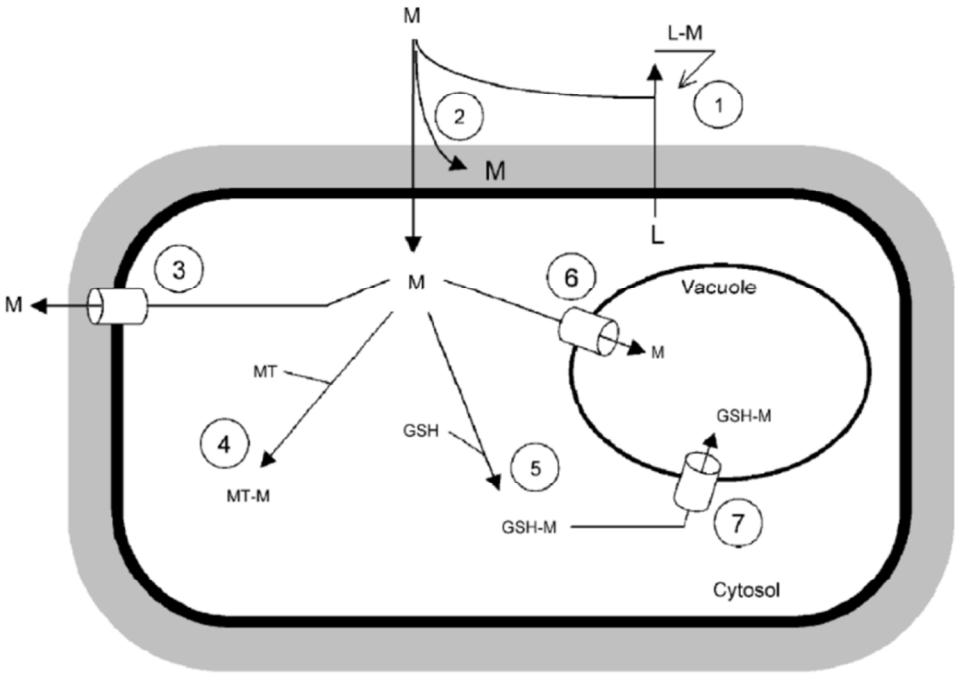


Figure 14 : Les différents mécanismes de tolérance vis à vis des ETMs, mis en oeuvre chez les champignons (Bellion *et al.*, 2006).

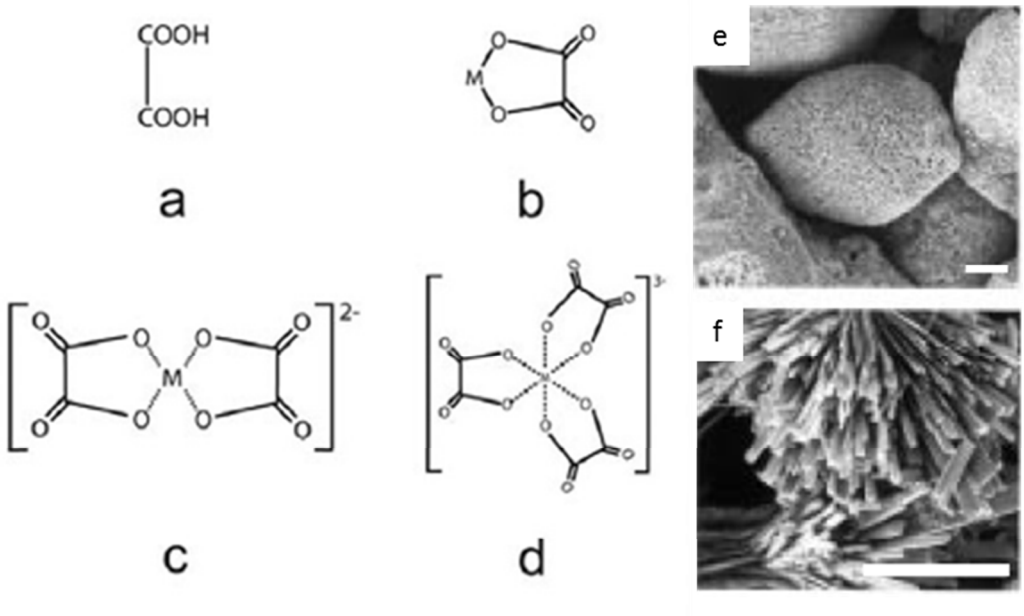


Figure 15 : Complexation des ETMs par l'acide oxalique.

Structure chimique de l'acide oxalique (a) complexé avec un ETM (M) (b, c et d) (Gadd *et al.*, 2012) et observation en microscopie électronique à balayage de pyromorphite (e) et d'oxalate de Pb dihydraté formé par *Aspergillus niger* (f) (Sayer *et al.*, 1999).

Les substances polymériques extracellulaires (EPS en anglais) peuvent également jouer un rôle dans la complexation des ETMs. Les bactéries peuvent sécréter des exopolysaccharides leur permettant de se fixer aux surfaces (par exemple les racines), de former un biofilm leur fournissant une protection mais également de fixer les ETMs (Gadd, 2010). Les champignons endomycorhiziens ont la particularité de synthétiser et d'excréter une glycoprotéine, appelée glomaline, capable de séquestrer les ETMs, en particulier le Cu, le Pb, et le Cd (Gonzalez-Chavez *et al.*, 2004). La glomaline est un facteur important dans la stabilité des sols par la formation d'agrégats et elle est retrouvée à des concentrations élevées dans les sols contaminés par des ETMs (Bronick & Lal, 2005).

Les biosurfactants, produits par les bactéries, augmentent la biodisponibilité des ETMs peu solubles, facilitant ainsi leur absorption (Bacon & Hinton, 2011). Ce sont des molécules amphiphiles de faible poids moléculaire constituées d'un fragment hydrophile et d'une partie hydrophobe qui comprennent un large éventail de structures chimiques (acides mycoliques, glycolipides, lipopeptides, complexes de polysaccharides-protéines, phospholipides, ou acides gras ; Banat *et al.*, 2010). Il a été montré que *Pseudomonas aeruginosa* produit un biosurfactant, le di-rhamnolipide, qui est capable d'augmenter la biodisponibilité du Cd et du Pb (Figure 16 ; Juwarkar *et al.*, 2007).

Les champignons et les bactéries excrètent des enzymes hydrolytiques qui découpent une grosse molécule, augmentant ainsi le nombre de molécules chélatrices. Par exemple, le champignon éricoïde *Oidiodendron maius* excrète une polygalacturonase qui hydrolyse la pectine, un constituant des parois des plantes, et l'activité de cette enzyme augmente en présence de Cd ou de Zn (Martino *et al.*, 2000a). Les champignons peuvent également excréter des composés polyphénoliques (pigments solubles), et des laccases en présence de Cd (Jacob *et al.*, 2004).

Les bactéries possèdent également d'autres mécanismes de détoxification via la sécrétion de ligands inorganiques permettant la précipitation des ETMs ou encore via des activités redox qui entrainent la méthylation des ETMs (Ma *et al.*, 2016).

1.1.5.1.2 Liaison à la paroi

L'ensemble des interactions (échange ionique, complexation, précipitation,...) entre un organisme et les éléments métalliques est nommé « biosorption » (Gadd, 1993). La biosorption sur la paroi est un mécanisme passif, indépendant du métabolisme et qui peut se faire sur des cellules vivantes ou mortes.

Chez les champignons, la paroi cellulaire est un site de liaison majeur pour les ETMs (Strandberg *et al.*, 1981). Les ETMs se fixent sur les groupements carboxyle, amino, hydroxyle, phosphate et thiol (Figure 17) et peuvent former un complexe avec la chitine comme cela a été démontré pour le Cd (Bhanoori & Venkateswerlu, 2000). L'adsorption, sur la paroi fongique de ces éléments, est un mécanisme important qui réduit la quantité d'ETMs disponibles pour l'hôte lors de la symbiose. Il a été démontré que la liaison du Cd aux parois cellulaires représente une fraction

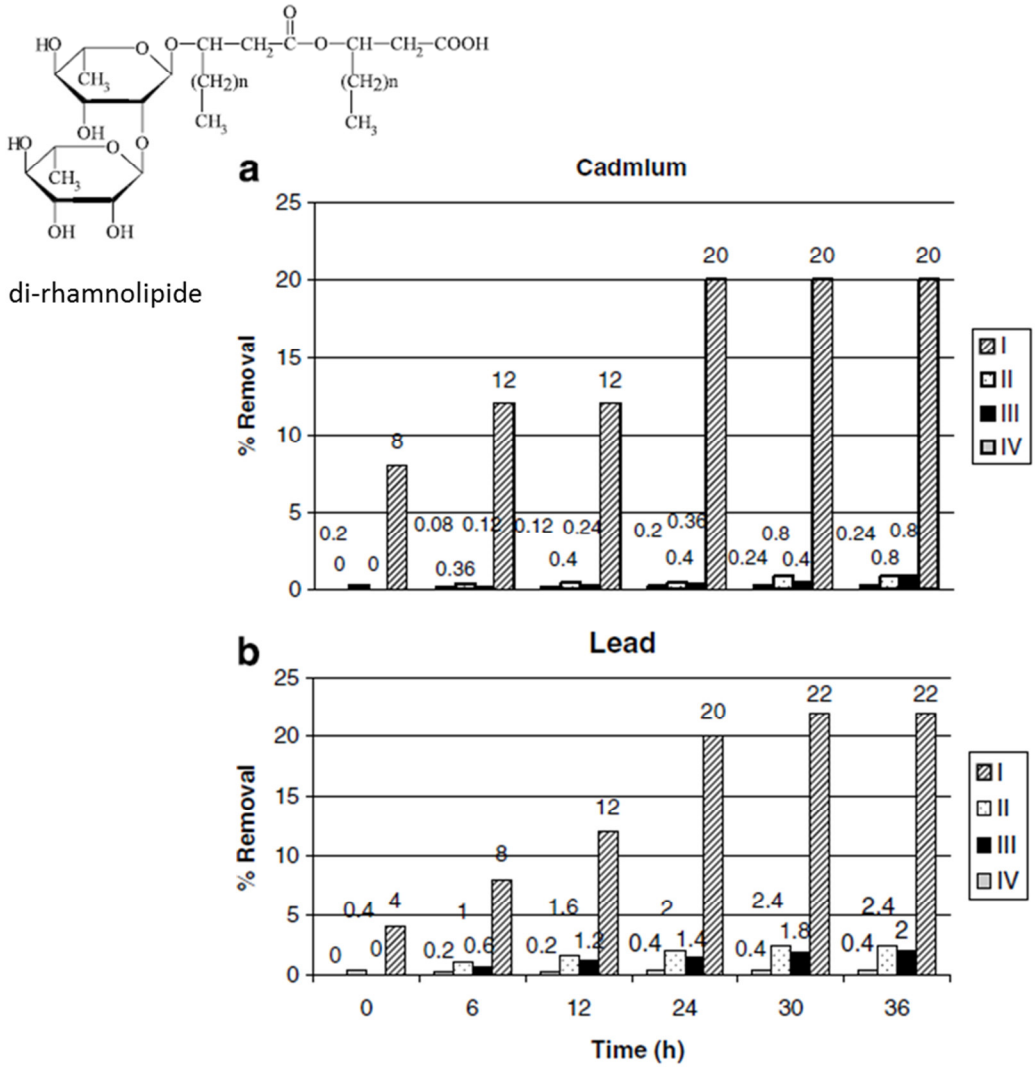


Figure 16 : Augmentation de la biodisponibilité du Cd (a) et du Pb (b) dans des sols enrichis en ETMs après addition de di-rhamnolipide (Juwarkar *et al.*, 2007).

I : sol naturel traité avec de l'eau, II : sol contaminé (Cd ou Pb) traité avec de l'eau, III : sol naturel traité avec du di-rhamnolipide et IV : sol contaminé (Cd ou Pb) traité avec du di-rhamnolipide

importante du métal accumulé par les hyphes de *P. involutus* (Blaudez *et al.*, 2000a; Frey *et al.*, 2000). Pour le Hg, il en est de même chez *Saccharomyces cerevisiae* (Figure 17 ; Whittaker *et al.*, 1990).

Chez les bactéries, la biosorption se fait par des réactions entre les ETMs et les groupements hydroxyle, carbonyle, carboxyle, sulfhydrile, thioether, sulfonate, amine, amide, et phosphonate (Ma *et al.*, 2011). Chez *Rhodobacter sphaeroides* par exemple, la biosorption est un mécanisme majeur en présence de Cd (Bai *et al.*, 2008).

La présence de mélanines parmi les composants de la paroi cellulaire des champignons peut encore accroître la capacité de biosorption des ETMs (Fogarty & Tobin, 1996). Par exemple chez les champignons endophytes, il a été démontré que la quantité de mélanine augmente en présence d'une concentration importante de Pb (Ban *et al.*, 2012).

Une seconde étape de biosorption active ou bioaccumulation se fait ensuite par l'absorption des ETMs via des transporteurs spécifiques ou non permettant l'entrée des ETMs à l'intérieur de la cellule.

1.1.5.2 Mécanismes intracellulaires

Les concentrations en Co, Fe, K, Mg, P, S, Se et Zn mesurées dans les cellules sont généralement de 3 à 30 fois plus élevées que les concentrations dans l'environnement extérieur. Cette observation est cohérente avec la capacité des cellules à concentrer ces éléments de façon intracellulaire (Eide *et al.*, 2005).

1.1.5.2.1 Exclusion

L'entrée des ions métalliques peut être limitée grâce à des systèmes d'exclusion qui prennent en charge l'élément dans le cytoplasme et l'expulse de la cellule. Ces systèmes d'exclusion peuvent être spécifiques ou non de l'élément.

Par exemple, l'As entre dans la cellule par l'intermédiaire du transporteur de phosphate mais les champignons éricoïdes sont capables de l'exclure par des transporteurs actifs spécifiques (Sharples *et al.*, 2000). Autre exemple décrit dans la littérature, chez le champignon ectomycorhizien *Suillus bovinus*, l'efflux renforcé de Zn agit comme un mécanisme de tolérance (Adriaensen *et al.*, 2005).

Chez les bactéries, *Bacillus sp*. a développé un mécanisme résistant aux ETMs via un système d'export actif lié à l'activité d'une pompe d'efflux ATPase de type P, qui peut transporter des ions métalliques à travers les membranes biologiques contre le gradient de concentration en utilisant l'énergie libérée par l'hydrolyse de l'ATP (Shin *et al.*, 2012).

L'efflux des ETMs se produit également par volatilisation pour le Hg et le Se. Certaines bactéries, principalement les bactéries gram négatives, possèdent l'opéron de résistance au Hg (Mer)

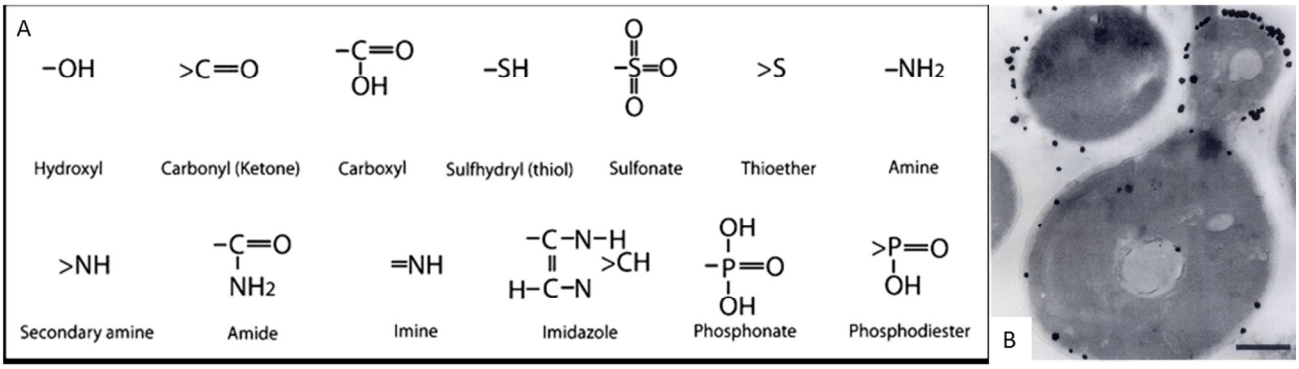


Figure 17 : Les groupements de liaison aux ETMs les plus fréquemment impliqués dans la biosorption fongique (A) et exemple de biosorption du Hg (points noirs) sur la paroi de *Saccharomyces cerevisiae* (B) (Whittaker *et al.*, 1990; Gadd *et al.*, 2012).

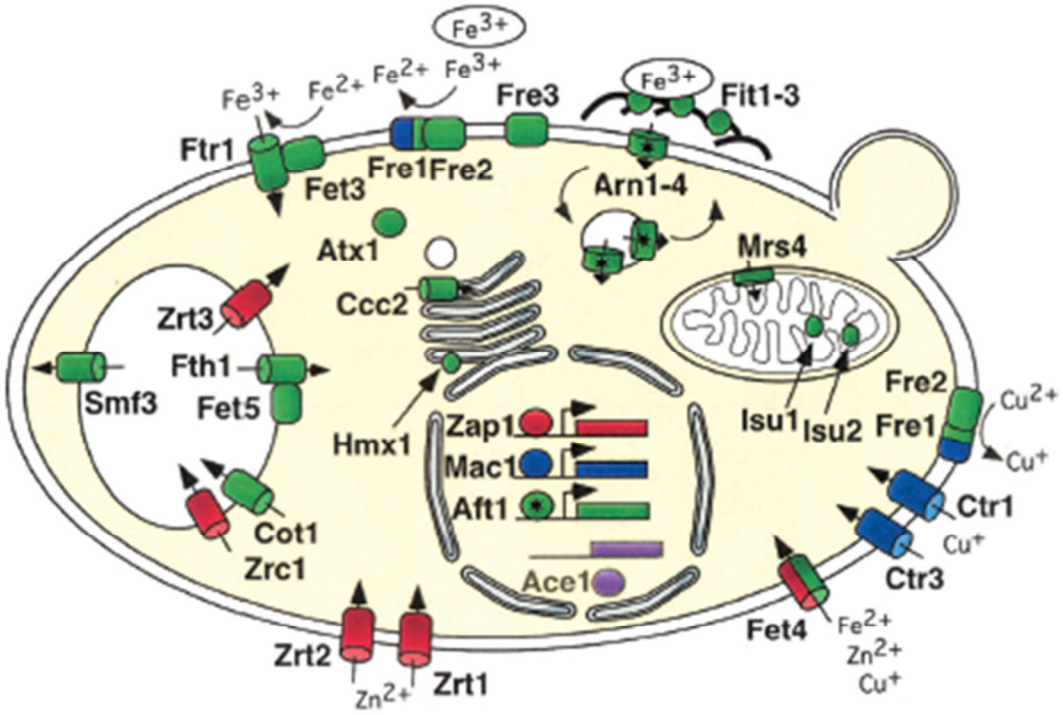


Figure 18 : Transporteurs impliqués dans l'homéostasie des ETMs chez *S. cerevisiae* activé par Aft1 (vert), Mac1 (bleu), Zap1 (rouge), et Ace1 (violets) (Rutherford & Bird, 2004).

codant pour une organomercurique lyase (codé par MerB), qui transforme les composés organomercuriques (CH_3Hg) en ions Hg (Hg^{2+}), et une mercurique réductase (codé par MerA), qui convertit l'ion Hg^{2+} très toxique en Hg volatile (Hg^0) moins toxique.

1.1.5.2.2 Limitation de l'entrée

Un autre mécanisme pour limiter l'entrée des ETMs consiste à réprimer les gènes codant les transporteurs impliqués dans l'absorption au niveau de la membrane plasmique de ces éléments (Eide, 2003).

Chez *S. cerevisiae*, le facteur de transcription Zap1 active la transcription des gènes impliqués dans l'homéostasie au Zn (Herbig *et al.*, 2005; Wang & Chen, 2006). En cas d'excès de Zn, Zap1 diminue l'expression des gènes codants les transporteurs ZRT1 et ZRT2 responsables de l'absorption du Zn présent dans le milieu extérieur (Figure 18).

1.1.5.2.3 Chélation intracellulaire

Malgré tous les moyens décrits précédemment, une quantité importante d'ETMs peut être présente dans les cellules, ce qui implique la mise en place de systèmes de défense intracellulaires.

Un des mécanismes est la chélation intracellulaire qui réduit la concentration des ETMs libres dans la cellule. Cette dernière utilise par exemple des peptides riches en cystéine tels que des métallothionéines (Morselt *et al.*, 1986; Clemens & Simm, 2003). Ces peptides de faible poids moléculaire sont impliqués dans l'homéostasie d'oligoéléments (le Zn et/ou le Cu) et la séquestration d'ETMs toxiques comme le Cd (Klaassen *et al.*, 2009; Waldron *et al.*, 2009; Chiaverini & De Ley, 2010; Fang *et al.*, 2010).

Les phytochélatines ont aussi ce rôle mais ne sont pas présentes chez tous les organismes (Zhou & Goldsbrough, 1994, 1995; Clemens & Simm, 2003). Les phytochélatines ont la capacité de séquestrer différents ETMs (Sneller *et al.*, 2000), comme le Cd (DalCorso *et al.*, 2008), le Zn (Gasic & Korban, 2006), l'As (Gasic & Korban, 2007). Ces petits peptides synthétisés par l'enzyme gamma-glutamylcystéine dipeptidyl transpeptidase (ou phytochélatine synthase), sont présents chez les végétaux et chez quelques rares autres organismes eucaryotes (*C. elegans, S. pombe*).

Le glutathion peut jouer les mêmes rôles en séquestrant les ions métalliques empêchant ainsi leurs actions toxiques chez les champignons (Courbot *et al.*, 2004; Pócsi *et al.*, 2004) et les bactéries (Zhang *et al.*, 2013). La présence de glutathion chez *S. cerevisiae* et *Proteus mirabilis* semblerait fortement liée à la présence de Hg (Figure 19 ;Westwater *et al.*, 2002 ; Zhang *et al.*, 2013).

1.1.5.2.4 Compartimentation

Un autre mécanisme de défense intracellulaire est la compartimentation dans la vacuole ou le réticulum endoplasmique (Hall, 2002). Ceci se fait grâce à des transporteurs spécifiques qui prennent

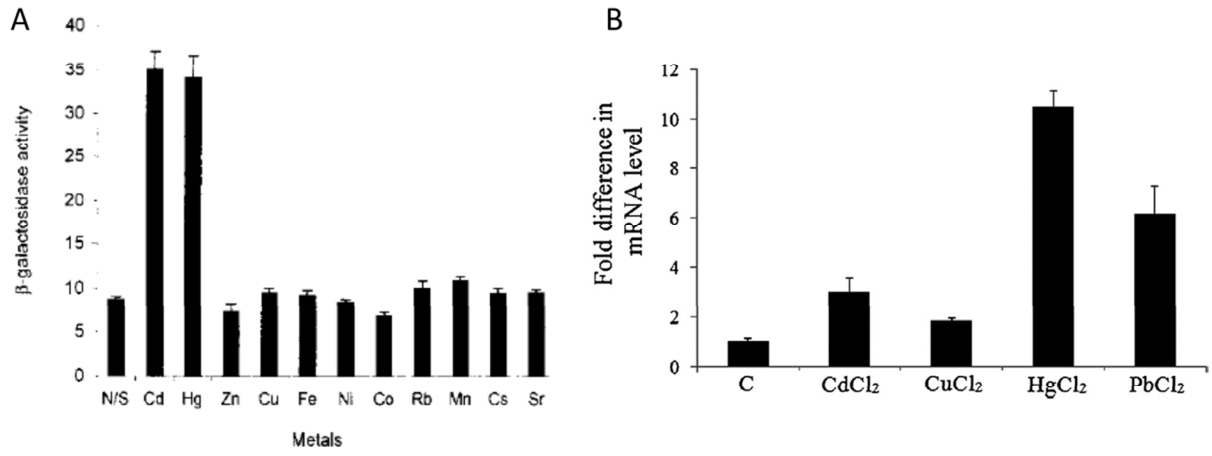


Figure 19 : Induction de l'expression du gène GSH1 codant une γ-glutamylcystéine synthétase chez *Saccharomyces cerevisiae* (A)(Westwater *et al.*, 2002) et induction de Glutathione S-transférases chez *Proteus mirabilis* (B)(Zhang *et al.*, 2013) en présence de différents ETMs.

en charge le métal seul ou chélaté par les molécules citées précédemment. Chez la levure, le transporteur ZRC1 permet de stocker le Zn dans la vacuole (MacDiarmid *et al.*, 2000) alors que COT1 le réalise pour le Co (Conklin *et al.*, 1992 ; Figure 18). De plus, le Zn entraine la transcription de chaperonnes qui le conduiraient dans les mitochondries et les vacuoles (Pagani *et al.*, 2007). *S. cerevisiae* et d'autres levures peuvent, elles aussi, accumuler des complexes glutathion-Cd, phytochélatine-Cd (Li *et al.*, 1997) ou glutathion-Hg (Gueldry *et al.*, 2003) dans la vacuole grâce au transporteur vacuolaire YCF1.

1.1.5.2.5 Transformation des métaux

Afin de réduire ou neutraliser leur toxicité, certains ETMs peuvent être transformés par des réactions de méthylation, d'oxydation, ou de réduction (Gadd, 1993). C'est le cas des métalloïdes avec, par exemple, l'As(III) qui peut être oxydé en As(V) (Bruneel *et al.*, 2003; Battaglia-Brunet *et al.*, 2006). La réduction du Hg^{2+} en Hg^{0} est bien connue chez les bactéries mais les systèmes analogues chez les champignons filamenteux et les levures sont plus rarement étudié (Gadd, 1993).

1.1.5.2.6 Système antioxydant

Les radicaux libres, induits par la présence d'ETMs, s'attaquent aux différents composants cellulaires et peuvent créer de graves dommages, c'est pourquoi des mécanismes antioxydants sont mis en place par les cellules. Ces mécanismes peuvent impliquer des molécules comme le glutathion ou les flavonoïdes ; ou des enzymes telles que les superoxydes dismutases (SOD), les peroxydases et les catalases qui servent à éliminer les formes réactives de l'oxygène. Par exemple *P. involutus*, en réponse au Cd, présente une induction de la superoxyde dismutase cytosolique, une accumulation de glutathion et d'autres systèmes liés au glutathion sont également activés (Ott *et al.*, 2002). *O. maius*, quant à lui, sécrète deux types de superoxydes dismutases extracellulaires en présence d'ETMs (Martino *et al.*, 2002).

Les thiorédoxines peuvent aussi être nécessaires au maintien de l'homéostasie redox dans des conditions de stress (Trotter & Grant, 2002). Ces molécules ont des rôles variés dans différents processus cellulaires comme le repliement et la régulation des protéines, la réparation des protéines endommagées par oxydation et plus généralement dans le métabolisme du soufre.

1.1.5.2.7 Autres mécanismes

D'autres mécanismes existent pour lutter contre l'effet toxique conféré par les ETMs tels que la production de protéines de stress comme les HSPs (Heat Shock Proteins). Les protéines de choc thermique ou HSP sont des chaperonnes, qui se lient à d'autres protéines pour leur assurer un repliement tridimensionnel correct lors d'un stress (Flora *et al.*, 2008; Gupta *et al.*, 2010). Par exemple, chez *Boletus edulis* l'expression d'une protéine de choc thermique de 70KDa (HSP70) semble corrélée positivement avec la concentration en Hg (Collin-Hansen *et al.*, 2005).

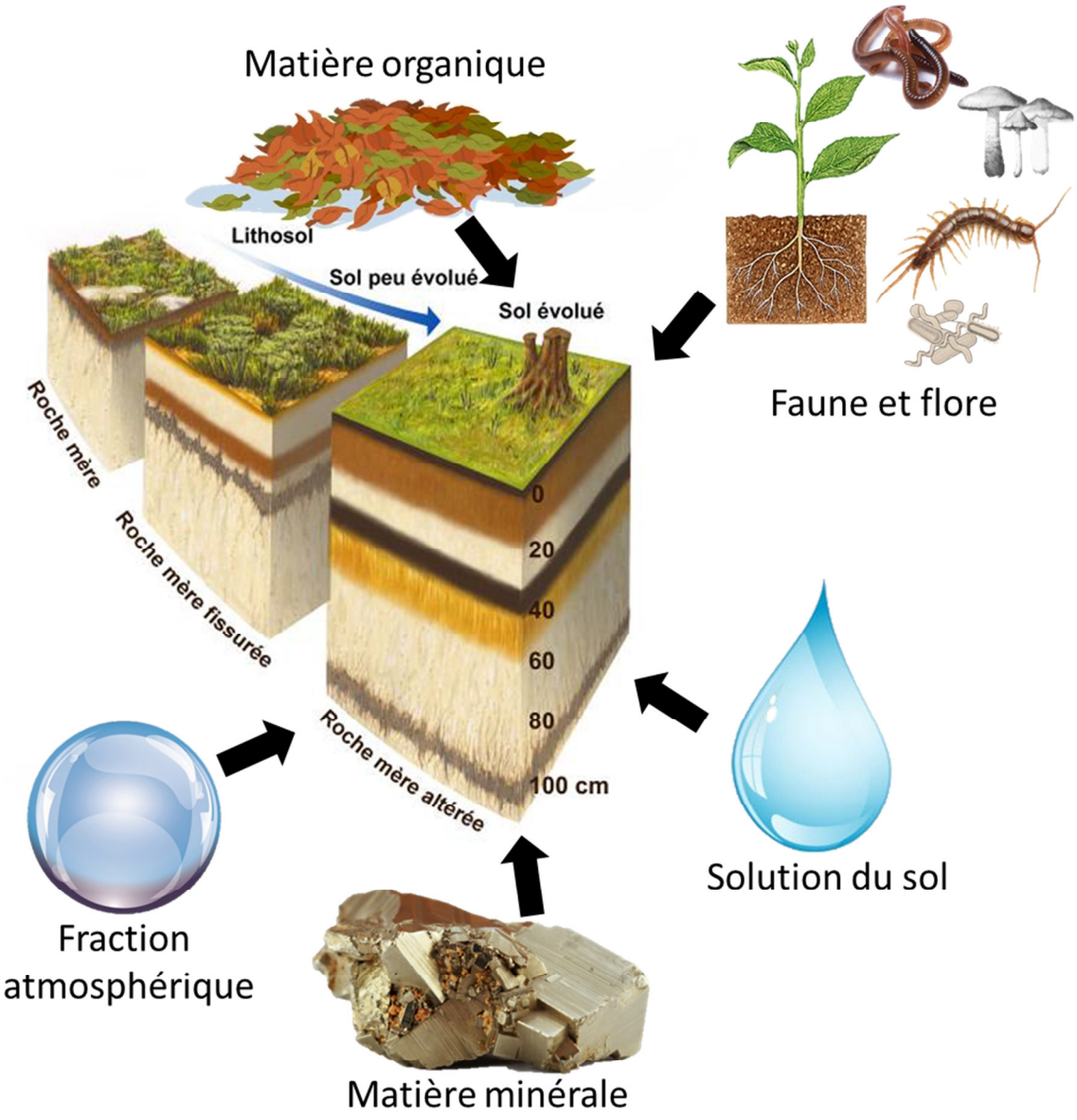


Figure 20 : Les différents constituants du sol.

1.2 Réhabilitation et remédiation de sols pollués

La pollution par des ETMs a donc des effets potentiels néfastes sur les organismes mais également sur un écosystème dans sa globalité, tel que le sol qui fournit de nombreux services d'où la nécessité de les réhabiliter.

1.2.1 Le sol

Le sol est essentiel à la vie; c'est le siège du fonctionnement des cycles biogéochimiques des éléments nutritifs terrestres, il fournit des substrats pour la production agro-alimentaire et il agit comme filtre pour la fourniture d'eau souterraine propre (Bissett *et al.*, 2013). Le sol est la couche externe de la croute terrestre qui est composée de différentes couches appelées horizons et se forme à partir de la roche mère (Figure 20).

Le sol est un environnement naturel complexe comprenant une grande abondance et une grande diversité d'espèces. Un gramme de sol contient jusqu'à 10^9 cellules (Gans *et al.*, 2005) et 10^4 espèces (Curtis *et al.*, 2002). L'extrême hétérogénéité spatiale, la nature polyphasique (incluant les gaz, l'eau et le matériel solide), et les propriétés chimiques et biologiques complexes de l'environnement sol contribuent à la diversité microbienne présente dans un échantillon de sol (Figure 20).

Ces micro-organismes, contenant des communautés de niveaux trophiques multiples, sont les principaux acteurs d'un grand nombre de fonctions des sols telles que la productivité des plantes, la décomposition de la matière organique, la minéralisation des éléments nutritifs ou l'élimination des polluants et sont essentiels pour l'intégrité des écosystèmes terrestres (Griffiths & Philippot, 2013). Le sol, qui est une ressource non-renouvelable, est de plus en plus sous la pression de l'environnement, le plus souvent liée à l'intensification des activités humaines (Creamer *et al.*, 2010). Ainsi, toute perturbation du sol (pollution, changement d'usage des sols…) peut perturber l'activité microbienne, modifier ces processus et avoir un impact sur les performances de l'écosystème (Bissett *et al.*, 2013).

1.2.2 Services écosystémiques

Les écosystèmes, et plus généralement la biodiversité, soutiennent et procurent de nombreux services dits services écologiques ou services écosystémiques.

Le rapport sur l'évaluation des écosystèmes pour le millénaire (Millennium Ecosystem Assessment (MEA), 2005) désigne les biens et services écologiques, ou services écosystémiques, comme des « biens et services que les hommes peuvent tirer des écosystèmes, directement ou indirectement, pour assurer leur bien-être ».

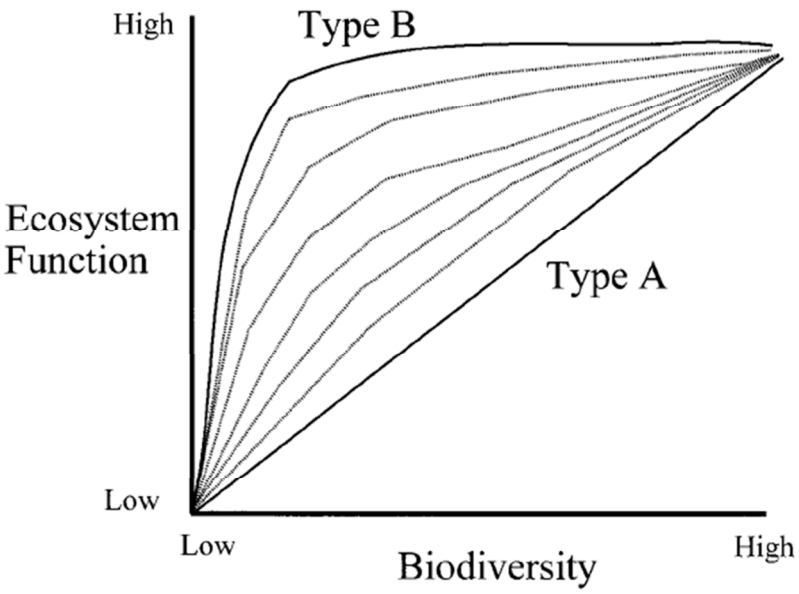


Figure 21 : Relations hypothétiques entre la biodiversité et les fonctions de l'écosystème ((Schwartz *et al.*, 2000).

Une relation de type A est linéaire et correspond à une espèce relativement rares au sein d'un écosystème qui contribue à une fonction. Dans une relation de type B, la fonction de l'écosystème est maximisée par une proportion relativement faible de la diversité totale et la plupart des espèces rares ne contribuent pas de façon importante au maintien d'une fonction. Les lignes interrompues représentent de nombreuses relations fonctionnelles qui sont intermédiaires entre le type A et de type B.

Les biens écologiques regroupent tout ce que la nature met à notre disposition : la nourriture (plantes, fruits, gibier, champignons, miel…), les matières premières et matériaux de construction (bois, fibres…), l'eau douce, l'air, les substances médicinales et pharmaceutiques naturelles, divers composés utilisés par l'industrie et trouvés chez des centaines d'espèces : gommes, exsudats, huiles essentielles, résines, graisses végétales, cires, insecticides...

Schématiquement, il est possible de distinguer quatre grands types de services écologiques : les services de support, les services de régulation, les services d'approvisionnement et les services culturels. Par rapport aux fonctions écologiques, les services de support constituent les fonctions écologiques de base (la photosynthèse par exemple), ceux de régulation correspondent aux services directs des fonctions écologiques (la détoxication et la dégradation des déchets). Les services d'approvisionnement et culturels correspondent aux services indirects des fonctions écologiques (approvisionnement d'eau douce ou tourisme).

De nombreux exemples montrent que la production de services écologiques et l'état de la biodiversité sont très souvent liés. Les principaux critères de biodiversité influant sur les services écologiques ne sont pas la quantité ou la variabilité, mais plutôt la composition fonctionnelle. Ainsi, Schwartz *et al.*, (2000) a émis l'hypothèse que la qualité et la quantité des services écologiques augmentait avec l'apparition de nouvelles interactions entre les espèces, créant de nouvelles ou de meilleures connexions entre les processus écologiques (Figure 21).

Les activités anthropiques peuvent causer des changements drastiques dans les structures des écosystèmes et leurs services écologiques (Dawson *et al.*, 2011). La stabilité et le maintien des écosystèmes dépendent de la diversité biologique et fonctionnelle dynamique des organismes (Johnson *et al.*, 1996). Cependant, de nombreuses études montrent une diminution et une sélection des microorganismes en présence de polluants (Figure 22 ; Gans *et al.*, 2005; Giller *et al.*, 2009; Glick, 2010). C'est pourquoi il existe un enjeu fort pour la réhabilitation des sols contaminés.

1.2.3 Les différentes techniques de réhabilitation et de remédiation des sols pollués.

La réhabilitation écologique d'un milieu, d'un sol ou d'une friche industrielle consiste à y restaurer l'écosystème présent antérieurement, ou un environnement proche. La remédiation est un processus d'assainissement qui utilise des méthodes pour restaurer un sol pollué. Le terme dépollution des sols est également utilisé et consiste principalement à rendre le sol et le sous-sol d'une zone apte à un nouvel usage industriel ou un usage résidentiel, voire apte à un retour à la nature ou à un usage agricole, après avoir été pollué par une activité ou un accident industriel.

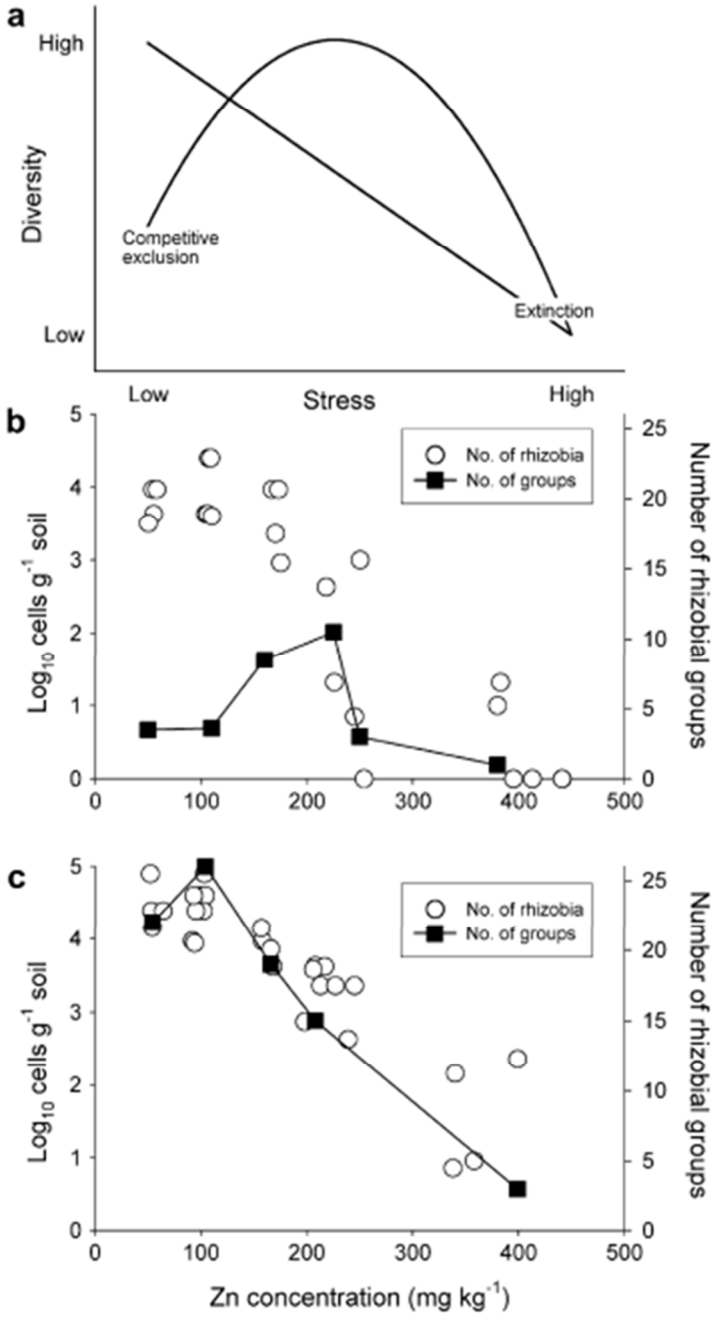


Figure 22 : Schéma représentant les deux modèles hypothétiques de la diversité microbienne en présence de concentrations croissantes d'ETMs (a) et la diversité des bactéries *Rhizobium* bv. *trifolii* (b) et *Rhizobium* bv. *viciae* (c) (Giller *et al.*, 2009).

D'une manière générale, l'assainissement des sols contaminés par des ETMs peut être effectué par des techniques "*ex situ*" c'est-à-dire après excavation des terres (ex : extraction) puis traitement sur site ou hors site et des techniques "*in situ*" directement sur le site pollué (ex : stabilisation) (Mora *et al.*, 2005).

Pour cela différentes techniques peuvent être mises en place. Il existe des techniques dites physico-chimiques et des techniques biologiques. Le choix de la méthode à utiliser dépend du type de contaminant, de la nature des sols, du niveau de pollution et de son usage futur (zone habitable, industrie...). C'est pourquoi, l'Agence de l'Environnement et de la Maîtrise de l'Energie (ADEME) et le Bureau de Recherches Géologiques et Minières (BRGM) ont élaboré « selecDEPOL », un outil interactif d'aide à la décision et à la gestion des sites pollués (www.selecdepol.fr).

1.2.3.1 Les techniques physico-chimiques

Les techniques physico-chimiques comprennent entre autres la désorption thermique, le lavage par des agents chimiques ou tensio-actifs, la stabilisation physico-chimique, l'électroremédiation ou le tri physique ou physico-chimique. En cas de pollution métallique dans un sol, les techniques d'oxydation chimique *in situ*, de confinement par couverture et étanchéification, ou encore d'encapsulation sur site et élimination en centre de stockage des déchets sont les plus utilisées.

Les méthodes physico-chimiques sont actuellement les plus utilisées, mais elles sont souvent très onéreuses, peu respectueuses de l'environnement et de la biodiversité (Ali *et al.*, 2013). Face à toutes ces contraintes, des techniques biologiques sont en plein essor (Bert & Deram, 1999).

1.2.3.2 Les techniques biologiques

Les techniques biologiques comprennent la phytoremédiation utilisant les plantes et la bioremédiation utilisant les microorganismes, ou encore un mix des deux techniques. Ces technologies prometteuses sont très efficaces, respectueuses de l'environnement et rentables (Singh *et al.*, 2015).

1.2.3.2.1 La bioremédiation

La bioremédiation utilise les bactéries, les algues (cyanoremédiation) ou les champignons (mycoremédiation) pour éliminer, atténuer ou transformer des substances polluantes ou contaminantes par l'utilisation de procédés biologiques.

La bioremédiation inclut différentes techniques comme la biominéralisation (synthèse de minéraux par des organismes vivants), la biosorption (sur la biomasse microbienne morte ou vivante), la biostimulation (stimulation des populations microbiennes vivantes), la bioaugmentation (introduction de microorganismes ou de consortia microbiens qui possèdent des capacités de remédiation), ou la bio-immobilisation (diminution de la mobilité des polluants) (Abhilash *et al.*, 2012; Ullah *et al.*, 2015).

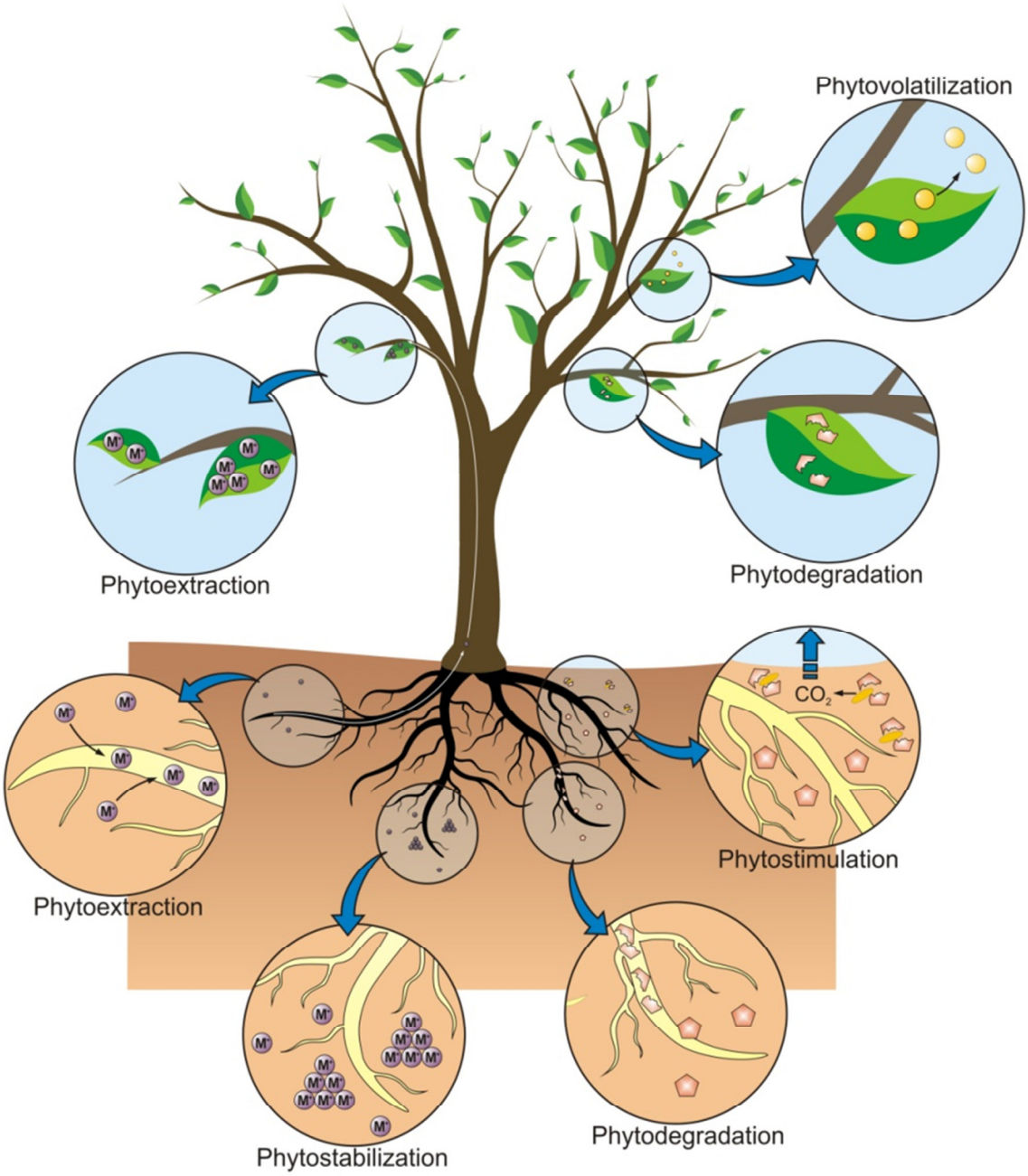


Figure 23 : Les différentes techniques de phytoremédiation (Favas *et al.*, 2014).

Parmi les souches bactériennes utilisées pour la bioremédiation, *Pseudomonas aeruginosa* est particulièrement intéressante car elle peut améliorer l'assainissement des polluants. En effet, *P. aeruginosa* a été testée pour la bioremédiation des ETMs (Singh *et al.*, 2013) et des hydrocarbures (Das & Mukherjee, 2007).

Les champignons peuvent aussi être utilisés comme agents de décontamination de diverses substances dangereuses dans le sol et les sédiments en raison de leur grande capacité d'absorption des polluants inorganiques tels que les ETMs (Urban *et al.*, 2005) et leur capacité de dégradation des molécules persistantes telles que les polluants organiques (Hadibarata *et al.*, 2009). Les champignons utilisés pour la remédiation des ETMs sont par exemple *Agaricus bisporus*, *Boletus edulis*, ou *Coprinus comatus* (Dulay, 2015).

1.2.3.2.2 La phytoremédiation

La phytoremédiation est définie par l'utilisation de plantes et des microorganismes associés pour contenir, séquestrer, éliminer ou dégrader les contaminants inorganiques ou organiques dans les sols, les sédiments, les eaux de surface ou souterraines (Tsao & Banks, 2003). La phytoremédiation contient elle aussi différentes méthodes comme la phytovolatilisation (volatilisation des polluants par les plantes), la phytoextraction (accumulation des polluants dans la biomasse récoltable), la phytostabilisation (diminution de la mobilité des polluants par les racines et la rhizosphère), la dendroremédiation (utilisation d'arbres), ou la rhizoremédiation qui utilise des plantes et des microorganismes associés à la rhizosphère (Figure 23 ; Abhilash *et al.*, 2012).

Les plantes utilisées peuvent être des espèces hyper-accumulatrices qui stockent de grandes quantités d'ETMs (plus de 100 mg/kg de poids sec de Cd, ou plus de 1000 mg/kg de poids sec de Ni, Cu, ou Pb, ou plus de 10 000 mg/kg de poids sec de Zn ou de Mn ; Baker & Brooks, 1989) mais ces plantes ne produisent pas suffisamment de biomasse pour rendre le processus de phytoremédiation efficace sur le terrain.

Les arbres sont réputés être plus appropriés pour la phytoremédiation en raison de leur forte biomasse et leur vaste système racinaire. Cependant, l'accumulation d'ETMs par les arbres est généralement faible et prend beaucoup de temps. Ce processus peut devenir efficace par l'utilisation d'espèces ligneuses à croissance rapide comme le peuplier ou le saule (Glick, 2010). Par exemple, même si la concentration de Cd dans les rosettes de la plante hyperaccumulatrice *Thlaspi caerulescens* est dix fois supérieure à celle mesurée dans les feuilles de *Salix* sp., la quantité de Cd extraite par hectare est deux fois plus faible dans le cas de *T. caerulescens* que dans le cas d'une sauleraie (Hammer & Keller, 2002). De plus, le peuplier et le saule sont des arbres pionniers qui sont parmi les premiers colonisateurs des sites contaminés et forment un couvert végétal qui diminue la dispersion des ETMs par lixiviation.

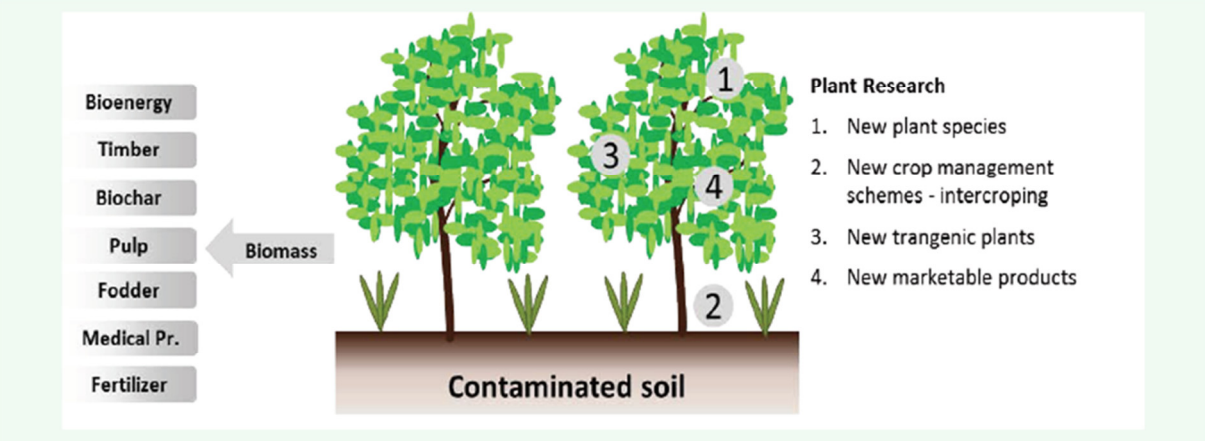


Figure 24 : Produits possibles (gauche) et nouvelles aires de recherche (droite) dans le domaine du phytomanagement (Evangelou & Deram, 2014).

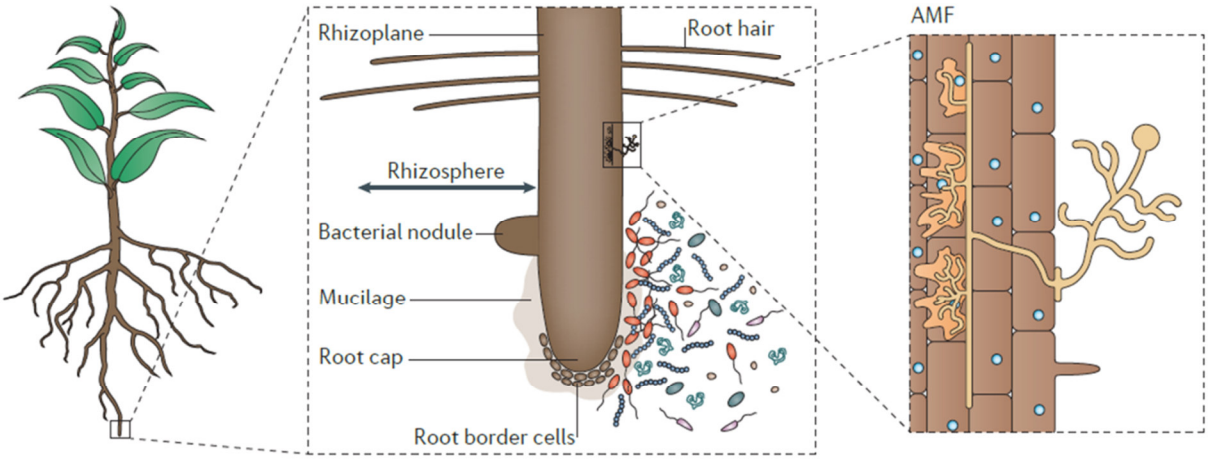


Figure 25 : La rhizosphère (Philippot *et al.*, 2013).

1.2.3.2.3 Le phytomanagement

La phytoremédiation (et notamment la phytoextraction) n'est jamais parvenue à la hauteur de ses attentes, en raison notamment des durées de traitement qui induisent une immobilisation des terres sur le long terme. Les approches plus récentes de phytomanagement contournent cet inconvénient en incorporant la production de revenus économiques (Figure 24). Le phytomanagement ne considère pas les sols contaminés comme un problème mais comme une opportunité économique et de précieuses ressources qui devraient être utilisées de manière durable. La biomasse végétale récoltée peut être utilisée (bois, pâte, fourrage) ou convertie en bioénergie par la formation de biodiesel (Van Ginneken *et al.*, 2007), la pyrolyse, la production de charbon ou de gaz combustible (Lievens *et al.*, 2008). Le phytomanagement pourrait donc non seulement offrir des avantages écologiques, mais aussi offrir une aide économique pour les collectivités à proximité des sites contaminés (Evangelou & Deram, 2014). Plusieurs études ont d'ailleurs montré que le phytomanagement pourrait générer un avantage économique pour les agriculteurs possédant des terres contaminées par des ETMs (Sas-Nowosielska *et al.*, 2004; Lewandowski *et al.*, 2006).

La recherche de nouvelles espèces de plantes commercialisables ainsi que les microorganismes auxquels elles sont associées pourrait aider à atteindre cet objectif de phytomanagement. En effet, l'efficacité de la phytoremédiation peut être renforcée par les bactéries (Agnello *et al.*, 2016) et les champignons (Baum *et al.*, 2006; Khan, 2006) associés aux plantes de par leur capacité de bioaccumulation, de mobilisation et de dégradation des contaminants ainsi que par l'amélioration de la croissance des plantes (Ullah *et al.*, 2015). L'alliance des techniques de phytoremédiation et de bioremédiation au niveau de la rhizosphère est alors appelé rhizoremédiation (Kuiper *et al.*, 2004; Wu *et al.*, 2006) et même mycorhizoremédiation pour les champignons mycorhiziens (Khan, 2006). La phytoextraction couplée à la bioaugmentation a par exemple été utilisée pour l'assainissement de sols contaminés par des ETMs (Lebeau *et al.*, 2008; Sessitsch *et al.*, 2013).

1.3 Les microorganismes de la rhizosphère

La rhizosphère comprend la surface des racines et également le sol autour des racines où les exsudats racinaires des plantes maintiennent une activité microbienne élevée et une importante densité microbienne (Figure 25 ; Yergeau *et al.*, 2014).

Les microorganismes associés aux plantes offrent de nombreux avantages comme la stimulation de la croissance des plantes, la mobilisation d'éléments nutritifs, la minéralisation de la matière organique (cycle biogéochimique), l'augmentation de la zone de prospection des racines, la contribution à la protection des plantes contre les pathogènes, l'augmentation de la résistance aux stress, l'amélioration de la nutrition minérale et hydrique, ainsi que celle de la reprise de croissance. De plus, le plan Ecophyto 2018 « former à la réduction et à la sécurisation des pesticides » prévoit la

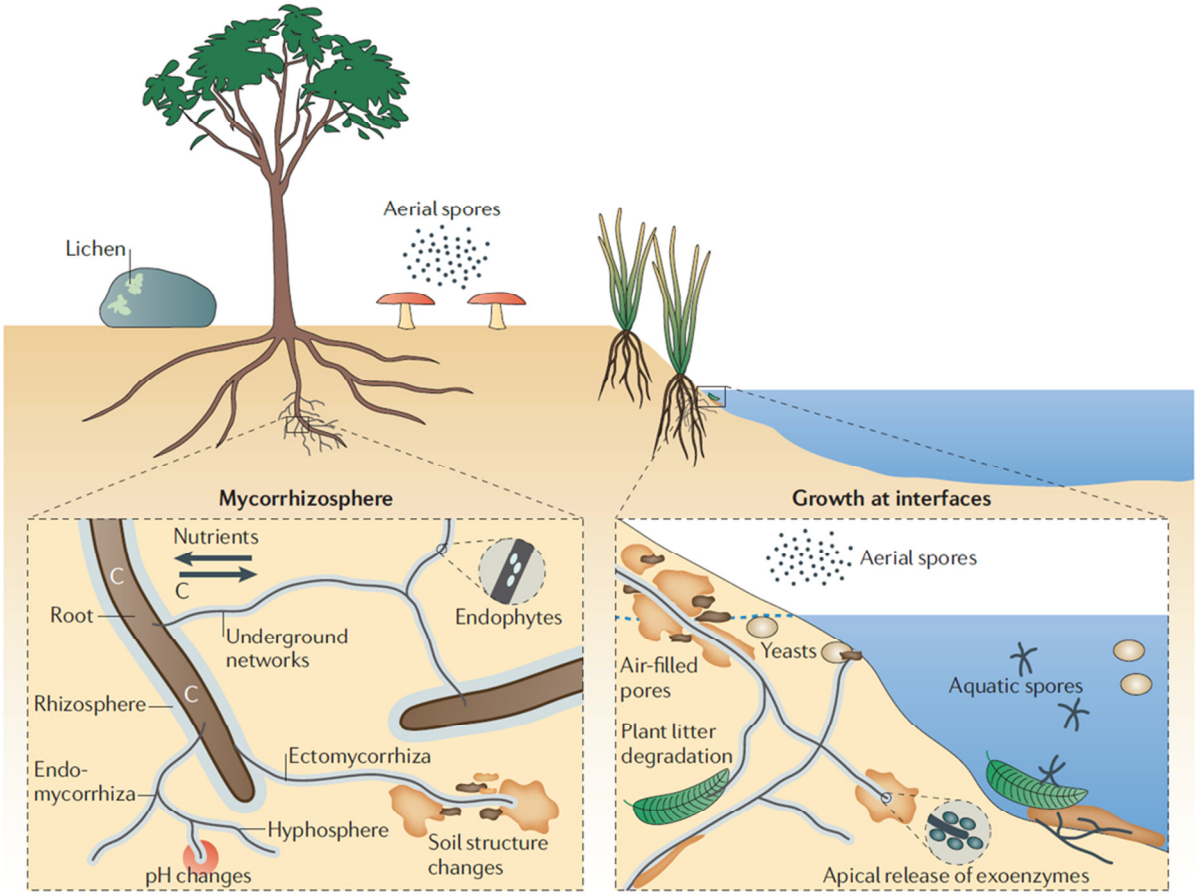


Figure 26 : Les différents habitats des champignons et leurs fonctions écologiques (Harms *et al.*, 2011).

réduction de l'utilisation des produits phytosanitaires en France. Celui-ci ouvre la voie à l'utilisation de microorganismes comme biofertilisants.

1.3.1 Les champignons

Les champignons sont la biomasse vivante dominante dans les sols (Hoshino & Morimoto, 2008). Ce sont des microorganismes eucaryotes ayant la particularité d'avoir une croissance sous forme d'hyphes et de posséder une paroi composée de chitine. Ils appartiennent au règne des *Fungi* et parmi eux sont retrouvés les quatre principaux phyla suivants : les *Zygomycètes*, les *Glomeromycètes*, les *Ascomycètes* et les *Basidiomycètes*.

Les champignons comprennent quelque 100 000 espèces décrites, mais l'étendue réelle de la diversité fongique globale est estimée entre 0,8 et 5,1 millions d'espèces (Tedersoo *et al.*, 2014). Ils sont répartis dans toutes les zones climatiques, et colonisent différents habitats (Figure 26) dans les écosystèmes tels que le sol (Bridge & Spooner, 2001), les tissus végétaux (Arnold *et al.*, 2000), l'eau (Jones, 2011), ou des roches (Gadd, 2007).

Les champignons sont un groupe d'une importance capitale car, jouant des rôles clés dans le cycle du carbone et de l'azote, ils améliorent la disponibilité des nutriments pour les autres organismes. Ils ont des rôles écologiques fondamentaux comme décomposeurs, mutualistes et agents pathogènes de plantes et d'animaux. Les associations mutualistes et symbiotiques sont des interactions favorables pour les deux partenaires, alors que les interactions saprophytes sont généralement neutres, contrairement aux pathogènes chez qui les interactions sont délétères pour l'hôte pouvant aller jusqu'à la mort (Adomas *et al.*, 2008).

1.3.1.1 Saprophytes

Les champignons saprophytes ont une importance fondamentale dans la décomposition de la litière, le flux d'énergie et le recyclage des éléments nutritifs dans les écosystèmes forestiers (Boddy, 1999). En plus de leur rôle clé dans la décomposition, ils sont aussi de bons candidats pour la mycoremédiation (Gadd, 1993) de par leur capacité à dégrader des molécules complexes (comme les HAP, les pesticides…) et de fixer les ETMs. Cela explique sans doute pourquoi ces champignons sont les plus répandus dans la rhizosphère et les sols (44% en moyenne ; Figure 27).

1.3.1.2 Pathogènes et parasites

Les pathogènes et les parasites sont des organismes qui vivent au sein d'un hôte. Contrairement aux pathogènes, les parasites n'entraînent pas d'effets délétères ni de symptômes.

Dans les sols et la rhizosphère, les champignons parasites et pathogènes peuvent être phytopathogènes, nématophages, mycoparasites ou encore des parasites d'animaux, mais ils ne représentent qu'une faible proportion (au total 6% en moyenne dans les sols ; Figure 27). Parmi ces

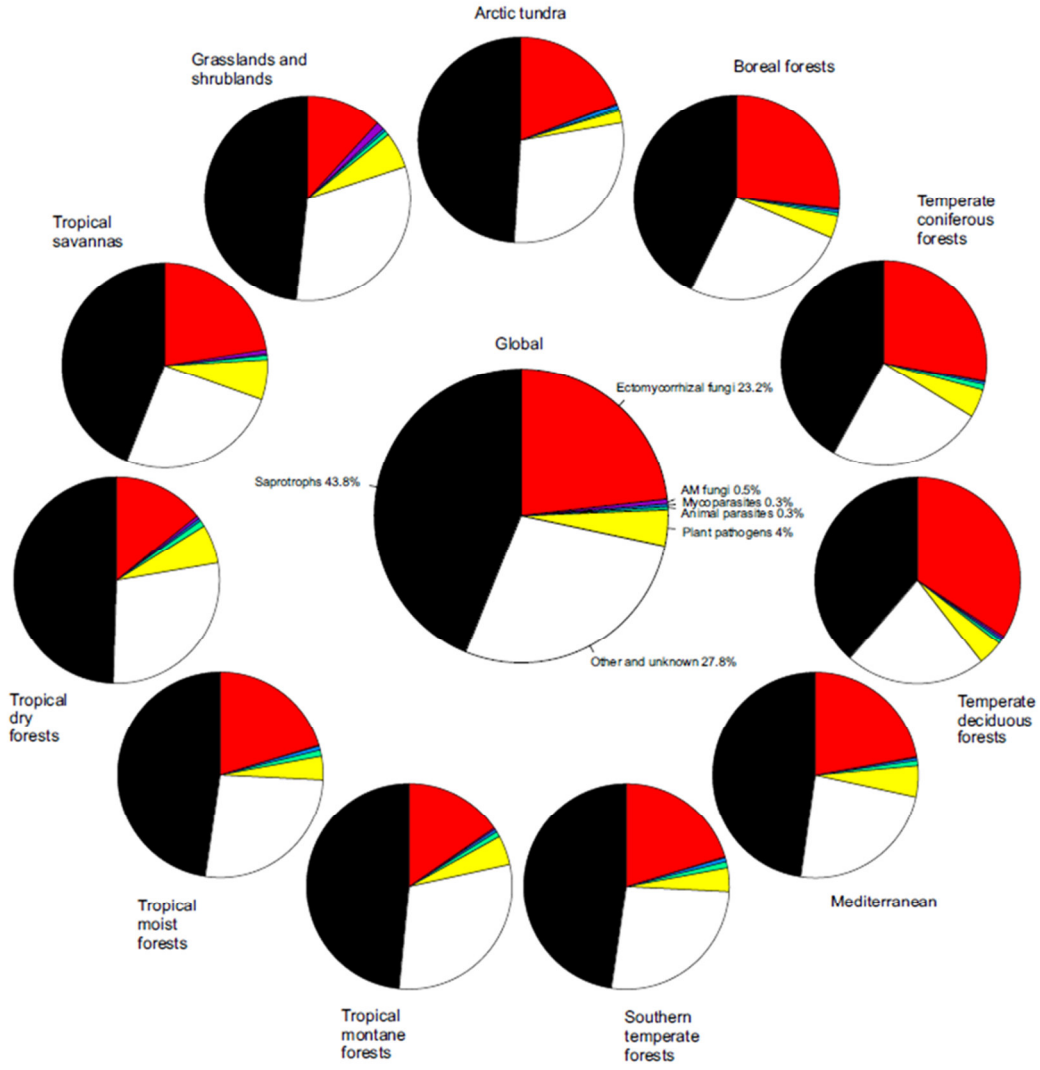


Figure 27 : Fréquences relatives des OTUs fongiques classées dans les catégories écologiques et fonctionnelles majeures parmi les différents biomes dans le monde ((Tedersoo *et al.*, 2014).

champignons, les phytopathogènes sont les plus abondants et peuvent donc causer des maladies à leur hôte. Cependant, les mutualistes et symbiotiques peuvent augmenter la protection de la plante contre certains phytopathogènes.

1.3.1.3 Symbiotiques et mutualistes

Les champignons mutualistes bénéfiques jouent un rôle important et économiquement significatif dans la croissance des plantes, le cycle des éléments nutritifs (Smith *et al.*, 1994; Smith & Read, 1997) ainsi que dans la tolérance au stress métallique (Jacob *et al.*, 2004).

Les champignons symbiotiques forment une association mutualiste directement en contact avec leur hôte. Les deux grands types de symbiose fongique sont la symbiose mycorhizienne entre un champignon et une plante au niveau des racines, et les lichens entre une algue (ou une cyanobactérie) et un champignon.

Dans un contexte de symbiose mycorhizienne, les deux partenaires retirent un avantage, les plantes fournissent des éléments organiques qu'elles synthétisent (grâce à la photosynthèse) et les champignons apportent les minéraux, comme le phosphore ou l'azote, qu'ils prélèvent dans le sol. La symbiose a un coût élevé en carbone pour la plante et doit donc être indispensable. La symbiose mycorhizienne a permis le passage de la vie aquatique à la vie terrestre et donc la colonisation du milieu terrestre par les plantes. D'ailleurs, 80% des plantes vasculaires sont mycorhizées (Wang & Qiu, 2006).

La mycorhization par certains champignons apporte de nombreux avantages comme une amélioration de la croissance et de la nutrition de la plante, une extension du système racinaire de la plante qui permet une prospection du sol plus grande et une optimisation de son approvisionnement en eau et en éléments minéraux peu mobiles dans le sol, tel que le phosphore. Cette symbiose entraine une augmentation de la protection de la plante contre certains parasites, une amélioration de la résistance des plantes à certains stress et une meilleure tolérance de la plante hôte vis à vis de la pollution et de contaminants divers comme les molécules organiques qu'ils peuvent dégrader. Les champignons mycorhiziens peuvent également protéger la plante hôte de la toxicité engendrée par les ETMs (Martino *et al.*, 2000b; Adriaensen *et al.*, 2006) à l'aide de différents mécanismes qui sont la synthèse de composés chélatant les ETMs, l'adsorption sur les parois ou l'absorption dans la biomasse (bioconcentration). Tout cela permet une immobilisation des ETMs, afin de limiter leur absorption par la plante.

Il existe deux grands types de champignons mycorhiziens, les endomycorhizienss qui forment des structures caractéristiques à l'intérieur des cellules, appelées vésicules et arbuscules et les ectomycorhiziens qui recouvrent les racines de mycélium afin de former un manteau et forment un réseau de Hartig entre les premières couches des cellules corticales (Figure 28).

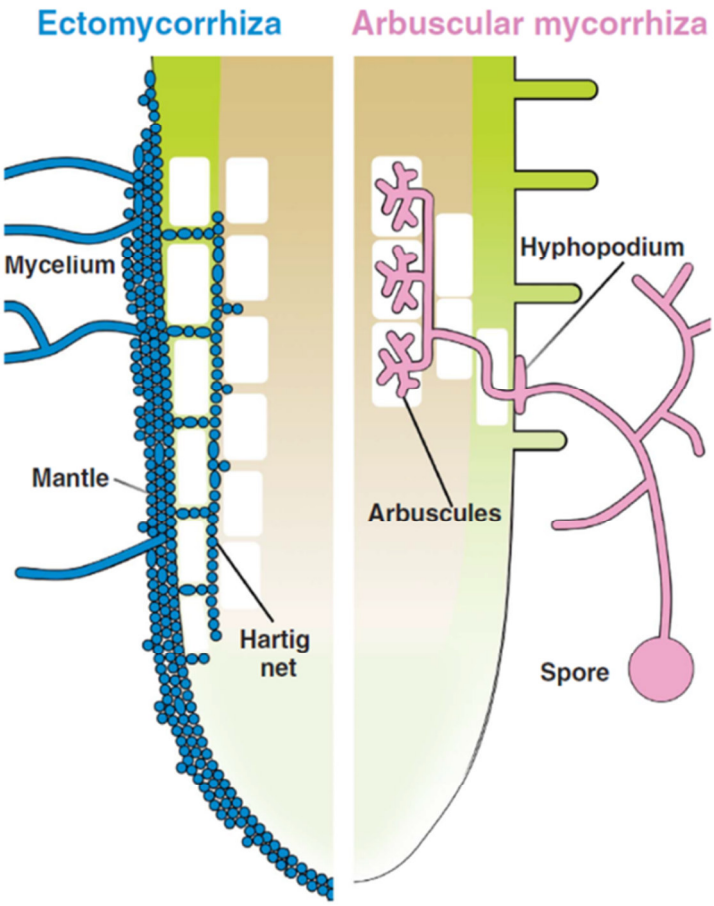


Figure 28 : Structures mises en place durant la symbiose ectomycorhizienne (en bleu) et endomycorhizienne (en rose) (Bonfante & Genre, 2010).

1.3.1.3.1 Les endomycorhizes

Les endomycorhizes à vésicules et arbuscules (AM) constituent le type de mycorhizes le plus ancien, sont les plus répandues et auraient co-évolué avec les plantes terrestres depuis au moins 400 millions d'années et seraient à l'origine évolutive des autres types de mycorhizes (Wang & Qiu, 2006). Les champignons endomycorhiziens sont en symbiose obligatoire (ils ne peuvent pas survivre sans la plante hôte) et appartiennent au phylum des Gloméromycètes. Ils sont capables de coloniser un large spectre d'espèces végétales, principalement herbacées, mais aussi certaines plantes ligneuses (peuplier, saule, eucalyptus…).

Les hyphes des champignons endomycorhiziens pénètrent dans les cellules corticales des racines où ils forment des structures intracellulaires ramifiées appelées « arbuscules », principal lieu d'échanges entre les deux partenaires. Les « vésicules » sont quant à elles les organes de réserves intracellulaires ou intercellulaires du champignon (Figure 29). Des hyphes extraradiculaires se développent sur plusieurs centimètres à l'extérieur de la racine, explorant le sol alentour, et pouvant porter une multitude de spores. Ces spores constituent l'organe de reproduction et de dissémination typique des champignons endomycorhiziens, elles peuvent contenir des centaines de noyaux qui ne possèdent pas forcément le même matériel génétique (Smith & Read, 1997).

La génétique des *Gloméromycètes* est néanmoins très mal connue. La diversité de structures et de formes des spores, isolées, sous formes de grappes ou dans des sporocarpes, a souvent été la base de la classification des champignons endomycorhiziens qui a été quelque peu remise en question ces dernières années. Une taxonomie plus récente basée sur l'analyse de la sous-unité 18S de l'ADNr a permis de construire l'arbre phylogénétique des *Gloméromycètes* qui nécessite encore d'être amélioré (Wang & Qiu, 2006). En 2013, une étude mêlant phylogénie et morphologie a clarifié la classification des *Gloméromycètes* et certains noms de champignons ont été changés (Redecker *et al.*, 2013).

Les champignons endomycorhiziens, rencontrés dans la plupart des sols en proportion très faible (1% ;Figure 27), sont symbiotiques des plantes dans 97% des cas (Abbott & Robson, 1991). Ils peuvent augmenter la surface d'échange entre la plante et le sol, améliorer l'absorption du phosphore (Smith & Read, 1997), protéger la plante contre les pathogènes (Abdel-Fattah & Shabana, 2002), limiter l'accumulation des ETMs dans la plante (Andrade *et al.*, 2010) et améliorer la croissance des plantes en condition de stress métallique (Khan *et al.*, 2000; Gaur & Adholeya, 2004; Göhre & Paszkowski, 2006; Hildebrandt *et al.*, 2007). Cependant, sur des sols contaminés, l'amélioration de la croissance des plantes inoculées avec des champignons endomycorhiziens varie selon les espèces végétales, la partie de la plante (racine et tige) et la concentration des ETMs dans le sol (Chen *et al.*, 2003, 2004).

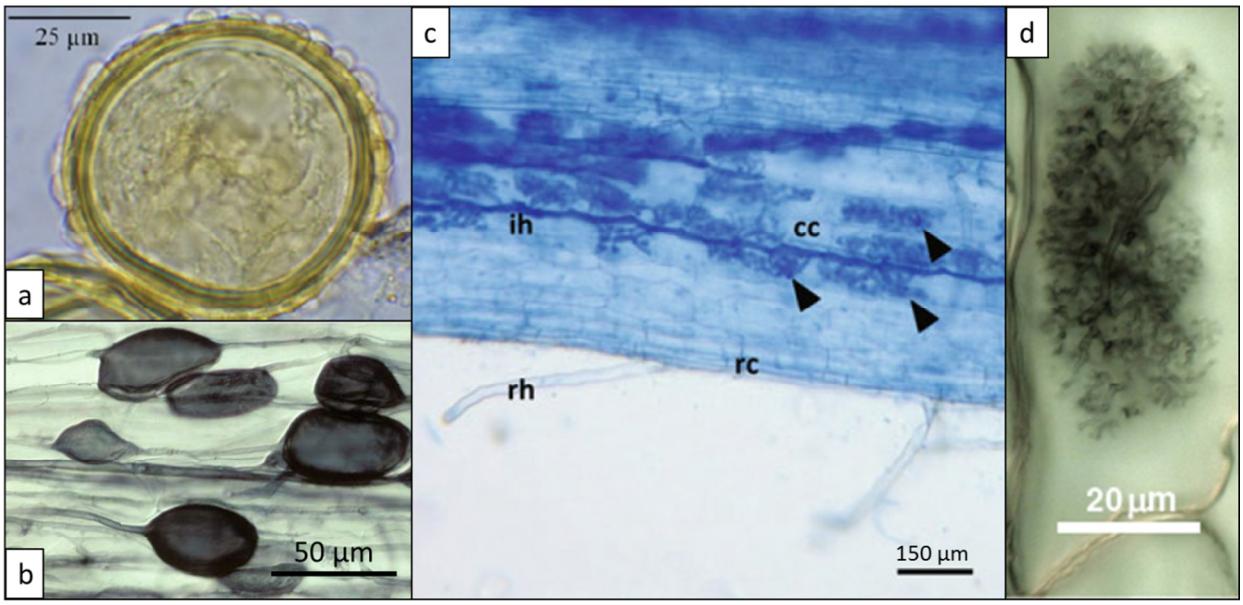


Figure 29 : Quelques illustrations d'endomycorhizes à l'état de spore (a) et en interaction avec les racines sous forme de vésicules (b) et d'arbuscules (c et d)

a : spore de *Acaulospora brasiliensis* (Redecker *et al.*, 2013) ; b : vésicules de *Glomus* sur des racines de poireau (http://mycorrhizas.info) ; c : arbuscules ramifiées (flèche) formées par *Funneliformis mosseae* dans les cellules corticales (cc) d'une racine de vigne, poils absorbants (rh), cellule de rhizoderme (rc) et hyphe intracellulaire (ih) (Bonfante & Desirò, 2015) ; d : Arbuscule dans une racine de *Smilacina racemosa* (Vierheilig *et al.*, 2005).

1.3.1.3.2 Les ectomycorhizes

Les champignons ectomycorhiziens (ECM) sont surtout connus pour former des fructifications (ou carpophores) qui se développent en milieu forestier. En effet, ces champignons ectomycorhiziens colonisent exclusivement les arbres et arbustes, qu'ils soient les Angiospermes ou Gymnospermes. Ce type de symbiose concerne au total environ 8000 espèces de plantes (Martin *et al.*, 2007). Les plantes associées aux champignons ectomycorhiziens poussent généralement dans des environnements pauvres en éléments nutritifs, dans les régions boréales, tempérées et montagneuses. Ces champignons appartiennent aux phyla des Ascomycètes (truffes, pézizes…) ou des Basidiomycètes (paxilles, hébélomes, lactaires, bolets…). Les premières traces fossiles de cette symbiose datent d'approximativement 50 millions d'années et seraient le résultat de plusieurs évolutions vers ce mode de vie à partir de champignons saprophytes (Wang & Qiu, 2006).

Les champignons ectomycorhiziens se développent de manière intercellulaire, en effet le mycélium progresse entre les cellules du cortex racinaire mais ne pénètre pas dans les cellules vivantes. Les ectomycorhizes se caractérisent par la formation de structures spécialisées. Les hyphes extramatriciels se propagent dans le milieu extérieur et assurent le rôle d'exploration et d'absorption. Les hyphes agglomérés autour de la racine forment un manchon fongique appelé manteau, et les hyphes qui s'insinuent entre les cellules du cortex forment un réseau appelé réseau de Hartig. C'est au niveau de ce réseau que se font les échanges d'éléments nutritifs entre la plante et le champignon (Figure 30).

Les champignons ectomycorhiziens représentent une proportion importante des champignons dans les sols (23% en moyenne ; Figure 27). Ce taux est plus élevé dans un sol forestier et principalement lors d'une densité importante de plantes. Les champignons ectomycorhiziens peuvent accroître significativement la surface d'échange entre la plante et le sol et fournissent à la plante de l'eau et des nutriments tels que l'azote et le phosphore. Ces champignons sont des symbiotes strictes ou des semi-saprophytes pouvant vivre indépendamment de la plante (Högberg *et al.*, 1999).

Ces champignons symbiotiques sont capables de produire une large gamme d'enzymes extracellulaires ou liées à la paroi cellulaire, qui hydrolysent les composées contenant de l'azote et du phosphore compris dans la matière organique du sol (acides aminés, protéines et chitine ; Leake & Read, 1990; Hodge *et al.*, 1996; Tibbett *et al.*, 1999; Leake *et al.*, 2003). Les champignons ectomycorhiziens produisent également des enzymes extracellulaires impliquées dans la dégradation de la cellulose et de la lignine (Gramss *et al.*, 1998; Courty *et al.*, 2005, 2006).

Les champignons ectomycorhiziens peuvent aussi améliorer la croissance des plantes en condition de stress métallique (Baum *et al.*, 2006) et protéger les plantes des effets néfastes générés par les ETMs (Figure 31 ; Krupa & Kozdrój, 2004; Adriaensen *et al.*, 2005). Par exemple l'inoculation

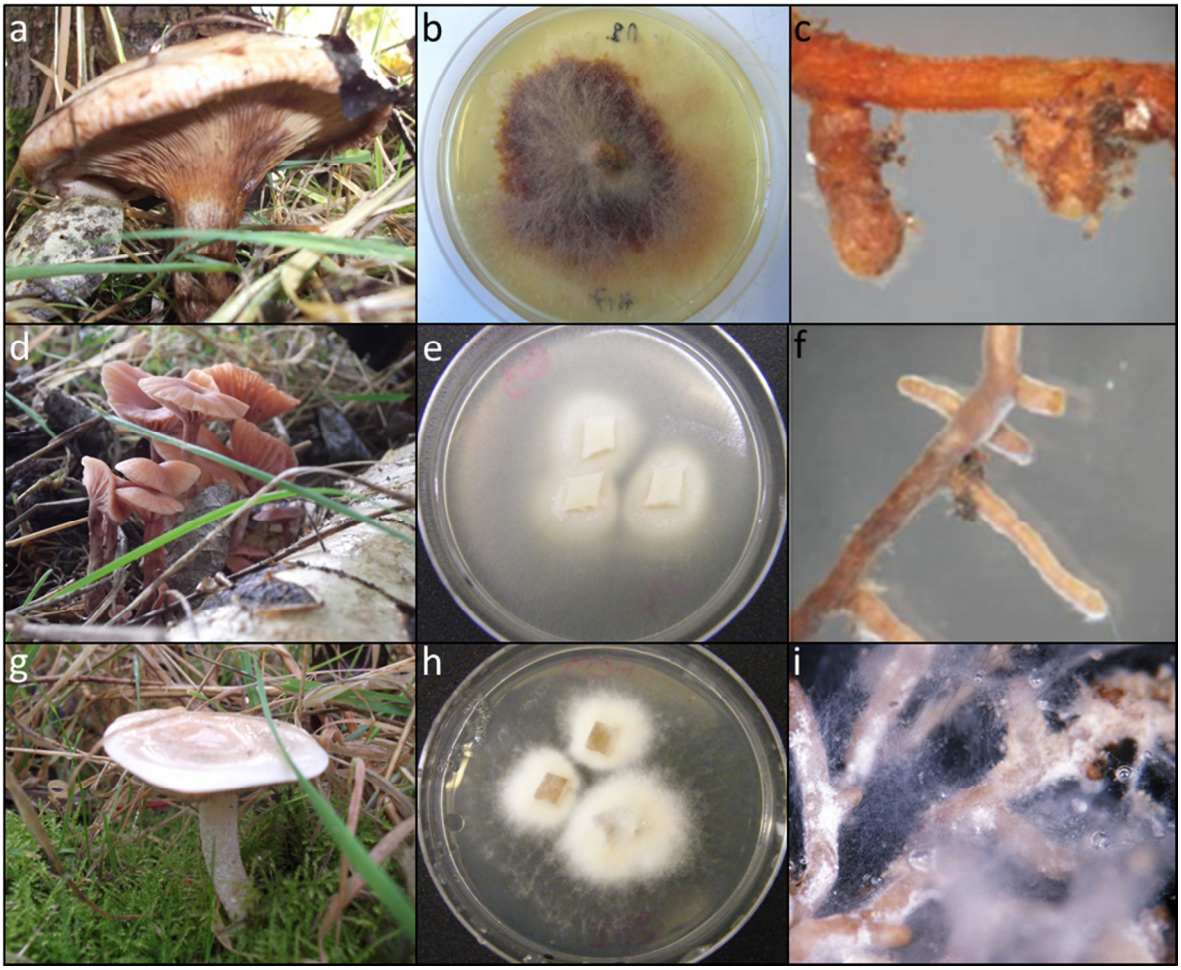


Figure 30 : Quelques illustrations d'ectomycorhizes sous forme de carpophores (a, d, et g), en culture pure sur boîte de Pétri (b, e et h) et en interaction avec les racines (c, f et i) pour *Paxillus involutus* (a, b et c), *Laccaria sp.* (d, e et f) et *Hebeloma sp.* (g,h et i) (Ważny, 2014 *; www.deemy.de*).

a, d et g : carpophores retrouvés sur le site de Leforest en octobre 2012 ; b : *Paxillus involutus* Bo02 ; c : *Paxillus involutus* en interaction avec *Abies alba* ; e : *Laccaria laccata* Pr03 ; f : *Laccaria maritima* en interaction avec *Abies alba* ; h : *Hebeloma crustuliniforme* Fe03 ; i : *Hebeloma sacchariolens* en interaction avec *Picea*.

du saule avec *Paxillus involutus* augmente la biomasse des racines et des tiges sur un sol contaminé par plusieurs ETMs (Baum *et al.*, 2006).

Les arbres tels que le peuplier ou le saule forment les deux types de mycorhizes décrits ci-dessus. Les endomycorhiziens apparaissent plutôt dans les phases précoces du développement puis les ectomycorhiziens deviennent majoritaires avec l'âge.

1.3.1.4 Les endophytes

Comme les champignons endomycorhiziens, les champignons endophytes sont des champignons qui effectuent leur cycle de vie à l'intérieur des tissus végétaux. L'association entre le champignon endophyte et la plante peut être parasite (seul un des partenaires en tire bénéfice au détriment de l'autre), symbiotique (les deux partenaires en tirent avantage, c'est la symbiose mycorhizienne) ou commensale (seul un des partenaires en tire bénéfice). Toutes les plantes sont naturellement colonisées par des endophytes (Rodriguez *et al.*, 2009).

Les communautés de champignons endophytes peuvent varier en fonction de l'espèce hôte, de la localisation, du climat, des saisons et de l'environnement (Carroll, 1995; Wilson, 2000; Lingfei *et al.*, 2005). Certains endophytes pourraient être des pathogènes latents (Redman *et al.*, 1999) et un changement d'hôte ou d'environnement peut déclencher leur pathogénicité (Johnson & Oelmüller, 2009).

Parmi les champignons endophytes, les plus souvent retrouvés dans les racines sont les « Dark Septate Endophyte » ou DSE. Ces champignons, appartenant au phylum des *Ascomycètes*, forment des structures mélanisées typiques inter- ou intracellulaires appelées microsclérotes (Figure 32 ; Mandyam & Jumpponen, 2005). Le rôle de ces champignons est encore mal connu, mais ils sont des partenaires principalement retrouvés dans les environnements stressants (Vrålstad *et al.*, 2002a), et certains pourraient être bénéfiques à la plante (Mandyam & Jumpponen, 2005). En effet, les DSE augmentent la teneur en éléments nutritifs et améliorent la croissance des plantes (Haselwandter & Read, 1982; Jumpponen *et al.*, 1998; Newsham, 1999; Brundrett, 2006; Schulz *et al.*, 2006; Usuki & Narisawa, 2007) ; ils ont la capacité de décomposer la matière organique complexe (Caldwell *et al.*, 2000) ; permettent de résister aux insectes (Faeth & Fagan, 2002; Gimenez *et al.*, 2007; Sikora *et al.*, 2008; Hartley & Gange, 2009; Gurulingappa *et al.*, 2010; Sumarah *et al.*, 2010) et aux champignons pathogènes (Maciá-Vicente *et al.*, 2008; Lee *et al.*, 2009) et sont couramment observés dans les racines des plantes dans des environnements stressants (Fujimura *et al.*, 2008; Soleimani *et al.*, 2010), contaminés par des ETMs (Bonnet *et al.*, 2000) ou carencés en éléments nutritifs (Haselwandter & Read, 1982; Barrow, 2003; Li & Guan, 2006; Porras-Alfaro *et al.*, 2008; Khidir *et al.*, 2010).

La composition fongique dans les sols peut dépendre de plusieurs facteurs, dont la plante hôte, la densité des plantes, la disponibilité des nutriments, les conditions environnementales, et les

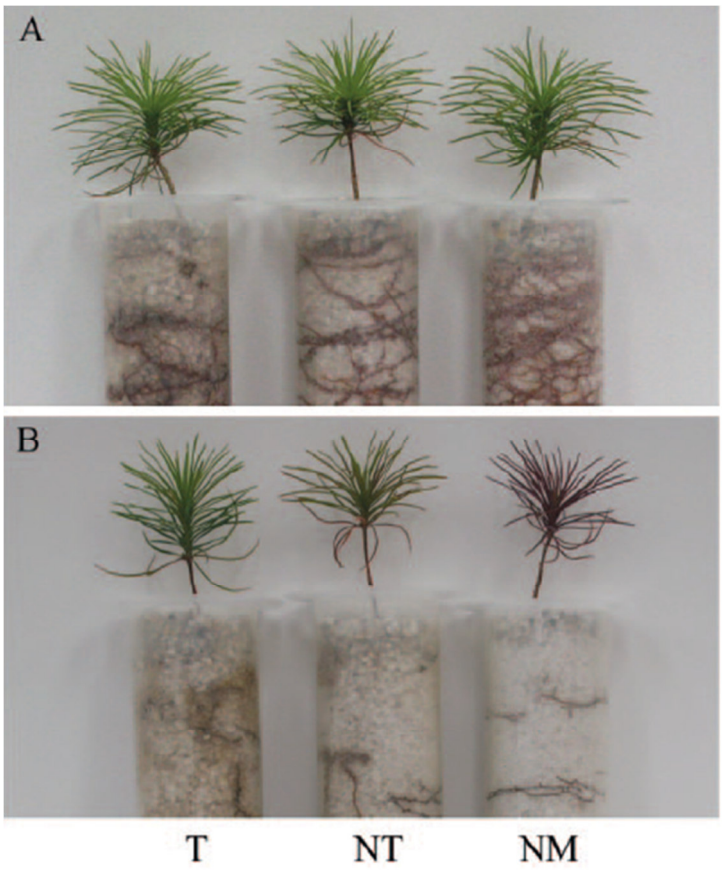


Figure 31 : Pins mycorhizés avec une souche tolérante (T) ou non tolérante (NT) au Cu de *S. luteus* ou non mycorhizés (NM) et traités avec différentes concentrations en Cu : concentration normale (A) ou subléthale (B) (Adriaensen *et al.*, 2005).

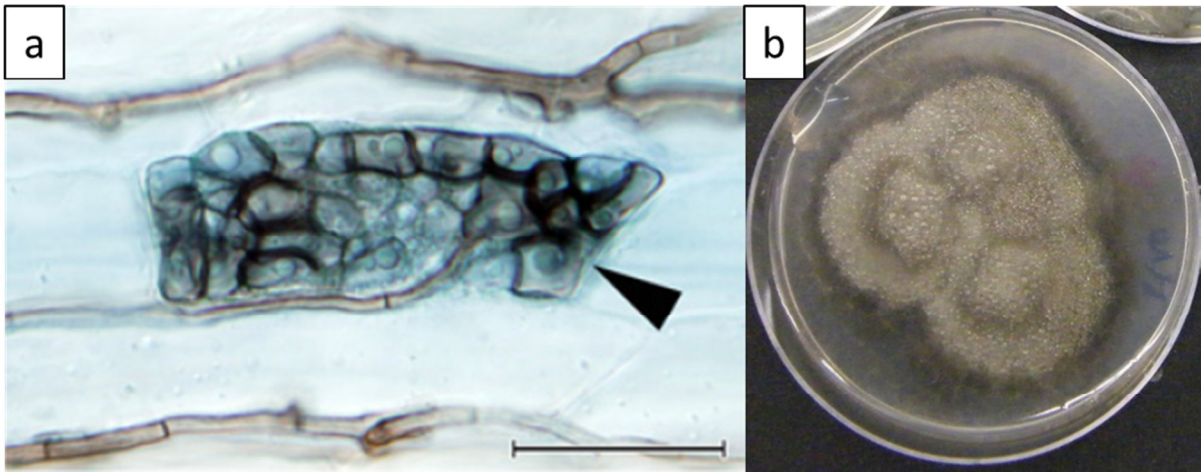


Figure 32 : Colonisation racinaire par des champignons endophytes de type DSE à l'intérieur de racines (a) (Lukešová *et al.*, 2015) ainsi qu'en culture pure sur boîte de Pétri (b).

a : *Vaccinium myrtillus* en interaction avec *Picea abies* (Barre = 25 µm) ; b: *Leptodontidium sp.* M10

interactions avec les autres microorganismes comme par exemple les autres champignons du sol et les bactéries (Porras-Alfaro & Bayman, 2011).

1.3.2 Les bactéries

Le compartiment rhizosphérique abrite une communauté microbienne riche, comprenant jusqu'à 10^{10} bactéries par gramme de sol (Gans *et al.*, 2005; Roesch *et al.*, 2008) et qui englobe une grande diversité de taxa (Kyselková *et al.*, 2009; Gomes *et al.*, 2010).

1.3.2.1 Les rhizobactéries

Certaines bactéries rhizosphériques peuvent être bénéfiques à la plante et sont appelées PGPR pour « Plant Growth-Promoting Rhizobacteria ». Ces bactéries PGPR peuvent vivre en symbiose (exemple des nodules formés entre *Rhizobia* et *Fabaceae* (Masson-Boivin *et al.*, 2009) ou entre *Frankia* et les plantes actinorhiziennes), être endophytes ou libres (Glick, 2012).

Les bactéries PGPR peuvent appartenir à différents genres (*Serratia, Bacillus, Pseudomonas, Burkholderia, Enterobacter, Erwinia, Klebsiella, Beijerinckia, Flavobacterium, Gluconacetobacter, Agrobacterium, Alcaligenes* (*Ralstonia*)*, Arthrobacter, Azospirillum, Azotobacter et Rhizobium* ; (Lebeau *et al.*, 2008; Ullah *et al.*, 2015) et ont pour point commun la stimulation de la croissance par différents mécanismes.

Les bactéries PGPR colonisent la rhizosphère en utilisant les exsudats racinaires comme substrats nutritifs, mais à la différence des autres bactéries rhizosphériques elles ont, en retour, un effet bénéfique sur la plante via une multitude de mécanismes (Vacheron *et al.*, 2013).

En effet, ces bactéries PGPR sont connues pour permettre une meilleure fixation de l'azote, une meilleure solubilisation du phosphore, une production d'acides organiques et de sidérophores entrainant ainsi une meilleure mobilisation d'éléments essentiels (Figure 33 ; Richardson *et al.*, 2009). Les bactéries PGPR sont également capables de stimuler la croissance et le développement des racines directement à l'aide de phytohormones (acide indole acétique (AIA), gibbérellines ou cytokinines) ou d'activités enzymatiques comme l'ACC déaminase qui diminue le niveau de stress dû à l'éthylène (Ullah *et al.*, 2015).

La compétition pour l'espace et les nutriments (via la production de sidérophores par exemple), la production d'antibiotiques, d'antifongiques, d'enzyme hydrolytiques (activités glucanolytiques, chitinolytiques, pectinolytiques, cellulolytiques et protéolytiques ; Krechel *et al.*, 2011) et d'éliciteurs de défense (Résistance Systémique Induite) par ces bactéries PGPR permet ainsi de lutter contre les pathogènes de la plante (Figure 33 ; Lugtenberg & Kamilova, 2009).

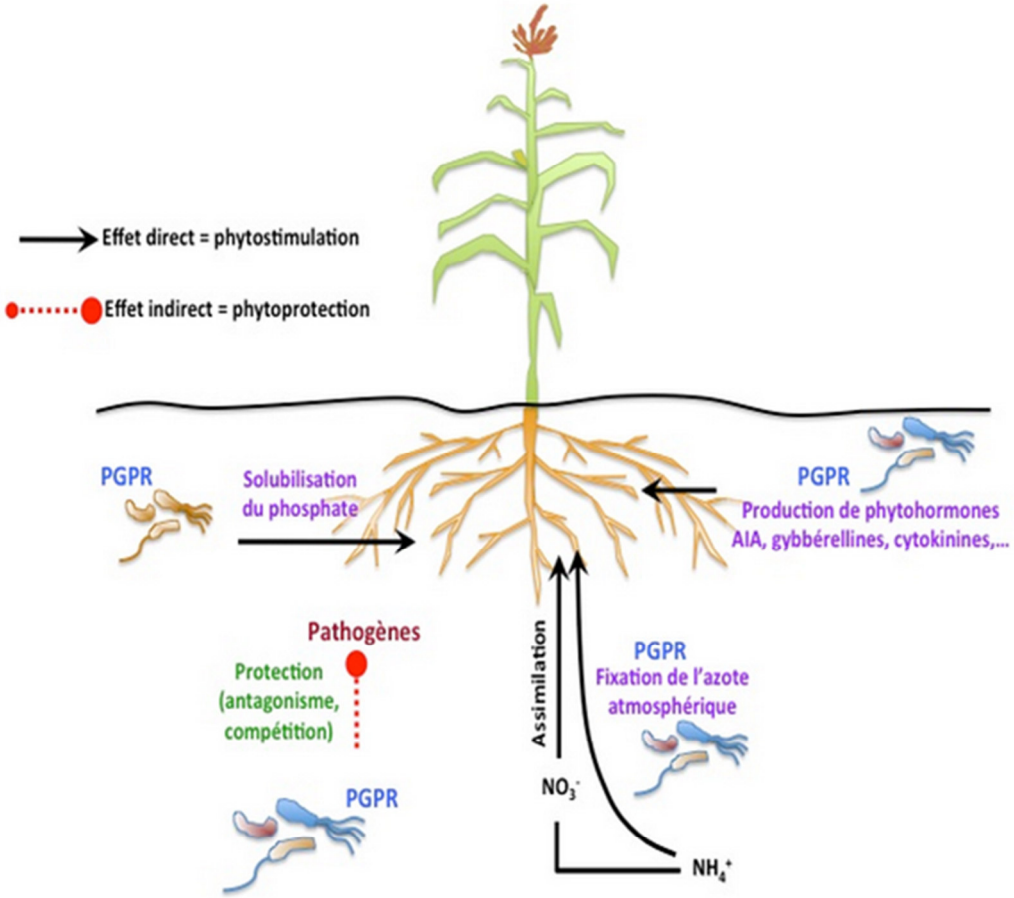


Figure 33 : Principaux impacts des PGPR sur la plante hôte (www.ecologiemicrobiennelyon.fr).

Les PGPR peuvent également transformer les ETMs sous forme biodisponible et soluble via l'action de sidérophores, d'acides organiques, de tensioactifs, de réactions de biométhylation, et de procédés d'oxydo-réduction pouvant ainsi participer à la remédiation des sols (Ullah *et al.*, 2015).

1.3.2.2 Les MHB

Les bactéries PGPR ont un rôle d'agent de biocontrôle qui limite les phytopathogènes mais elles peuvent également favoriser la croissance de champignons mycorhiziens et sont alors appelés MHB pour « Mycorrhiza Helper Bacteria ». Il se crée alors une relation tripartite entre les bactéries, les champignons mycorhiziens et la plante hôte qui contribue alors à favoriser la croissance de cette dernière.

De nombreuses souches bactériennes ont été rapportées pour être en mesure de promouvoir la croissance des champignons endomycorhiziens ou ectomycorhiziens. Les MHB peuvent être isolées de la mycorhizosphère, des fructifications des champignons ectomycorhiziens, et des spores des champignons endomycorhiziens. Les MHB peuvent également former un biofilm à la surface des hyphes fongiques ou coloniser l'intérieur des hyphes (Frey-Klett *et al.*, 2007).

Les bactéries MHB identifiées à ce jour appartiennent à de nombreux groupes et genres bactériens, tels que les Proteobactéries gram-négatives (*Agrobacterium , Azospirillum, Azotobacter, Burkholderia, Bradyrhizobium, Enterobacter, Pseudomonas, Klebsiella* et *Rhizobium*), les Firmicutes gram-positives (*Bacillus*, *Brevibacillus*, et *Paenibacillus*) et les Actinomycètes gram-positives (*Rhodococcus*, *Streptomyces* et *Arthrobacter*) (Frey-Klett *et al.*, 2007).

Les MHB stimulent la formation de mycorhizes initiée par certains champignons, mais inhibent la mise en place de cette symbiose chez d'autres espèces. Cela pourrait s'expliquer par la résistance de certains champignons symbiotique aux antibiotiques produits par les MHB. De plus, certains champignons peuvent moduler la production de ces antibiotiques par l'excrétion d'acides organiques ou de molécules volatiles (Figure 34 ; Garbaye & Duponnois, 1993).

Les MHB peuvent promouvoir la mise en place de la symbiose par de nombreux mécanismes, c'est-à-dire en stimulant l'extension du mycélium, la multiplication des contacts racine-champignon et la colonisation, mais aussi en réduisant l'impact des effets environnementaux sur le mycélium des champignons mycorhiziens (Frey-Klett *et al.*, 2007).

Les MHB peuvent également protéger indirectement les plantes contre un stress métallique via une amélioration de croissance du partenaire mycorhizien. Par exemple, l'inoculation par *Brevibacillus brevis*, isolé à partir des sols contaminés en Cd ou en Zn, améliore la germination des spores et la croissance de *Glomus mosseae* à de fortes concentrations en Cd ou en Zn (Vivas *et al.*, 2005).

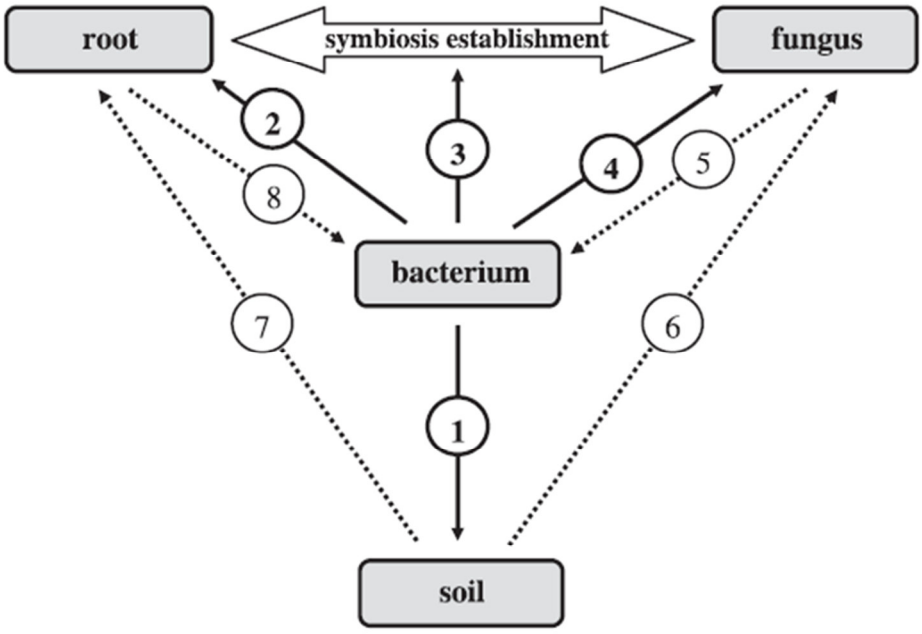


Figure 34 : Mécanismes des MHB pour promouvoir la mise en place de la symbiose ectomycorhizienne (Frey-Klett *et al.*, 2007).

Les flèches pleines (① à④) représentent les fonctions d'aide spécifique au MHB. En effet, la bactérie MHB rend le sol favorable à la colonisation fongique①, améliore la réceptivité des racines pour le champignon②, intervient dans la reconnaissance de la plantes et du champignon lors de la mise en place de la symbiose ③ et favorise la germination des propagules fongiques, la survie et la croissance du mycélium④. En parallèle, le champignon sélectionne les populations bactériennes dans la rhizosphère⑤, le sol soutient la croissance fongique presymbiotic ⑥ et détermine la réceptivité de la racine vis-à-vis du champignon ⑦ et les racines sélectionnent la population bactérienne dans la rhizosphère⑧.

1.3.3 Facteurs impactant la composition des communautés microbiennes

Les microorganismes rhizosphériques ont donc des rôles clés pour les sols et les plantes s'y développant, mais l'équilibre de ces communautés peut être impacté par de nombreux facteurs.

1.3.3.1 Impact du sol

Le sol joue un rôle important dans la structure des communautés et une modification de ses paramètres (pH, texture, compaction, quantité d'ETMs) peut entrainer des déséquilibres dans les communautés.

Les ETMs ont un impact sur la diversité et les fonctions des microorganismes (cf §1.1.4.1), les fonctions des écosystèmes (cf §1.2.2) et également sur la composition des communautés. Les proportions relatives des champignons ectomycorhiziens (ECM), endomycorhiziens (AM) et endophytes (EF) ont été évaluées dans trois sols présentant des taux de contaminations variables (site 1 < site 2 < site 3), et avec lesquels ont été cultivés différents clones de peupliers. Le sol le plus contaminé par les ETMs est caractérisé par une moindre biomasse fongique (Karliński *et al.*, 2010). Les proportions relatives des ECM et AM sont majoritairement impactées par la nature du sol, mais aussi par le clone considéré (Figure 35). Chez les bactéries, la présence d'ETMs dans le sol, et notamment de Hg, ne met pas en évidence de changements drastiques dans la diversité bactérienne (Figure 36 ; Liu *et al.*, 2014).Cependant, la contamination métallique peut également entrainer une diminution de la biomasse microbienne et une modification de la structure des communautés bactériennes (Wakelin *et al.*, 2010b; Gomez-Balderas *et al.*, 2014; Zhang *et al.*, 2016a)

La compaction du sol modifie la porosité du sol et la conductivité de l'eau qui influence les communautés. En effet, la compaction du sol réduit de manière significative l'abondance, la diversité et modifie la structure de la flore microbienne (Hartmann *et al.*, 2014).

Les communautés microbiennes du sol rhizosphérique semblent différentes de celles des racines des végétaux (Griffiths *et al.*, 1998; Gottel *et al.*, 2011). Alors que la microflore fongique tellurique est très diversifiée et les *Ascomycètes* sont prépondérants, les racines de peuplier sont majoritairement colonisées par des *Agaricomycètes* (Gottel *et al.*, 2011) et plus précisément des ectomycorhiziens (Danielsen *et al.*, 2012), ce qui montre également un impact du couvert végétal qui joue un rôle dans la sélection des communautés.

1.3.3.2 Impact du couvert végétal

Le couvert végétal joue aussi un rôle dans la structure des communautés, et plus particulièrement le mode de culture, l'espèce, voire le cultivar et l'occupation antérieure des sols peuvent influencer fortement sur ce paramètre.

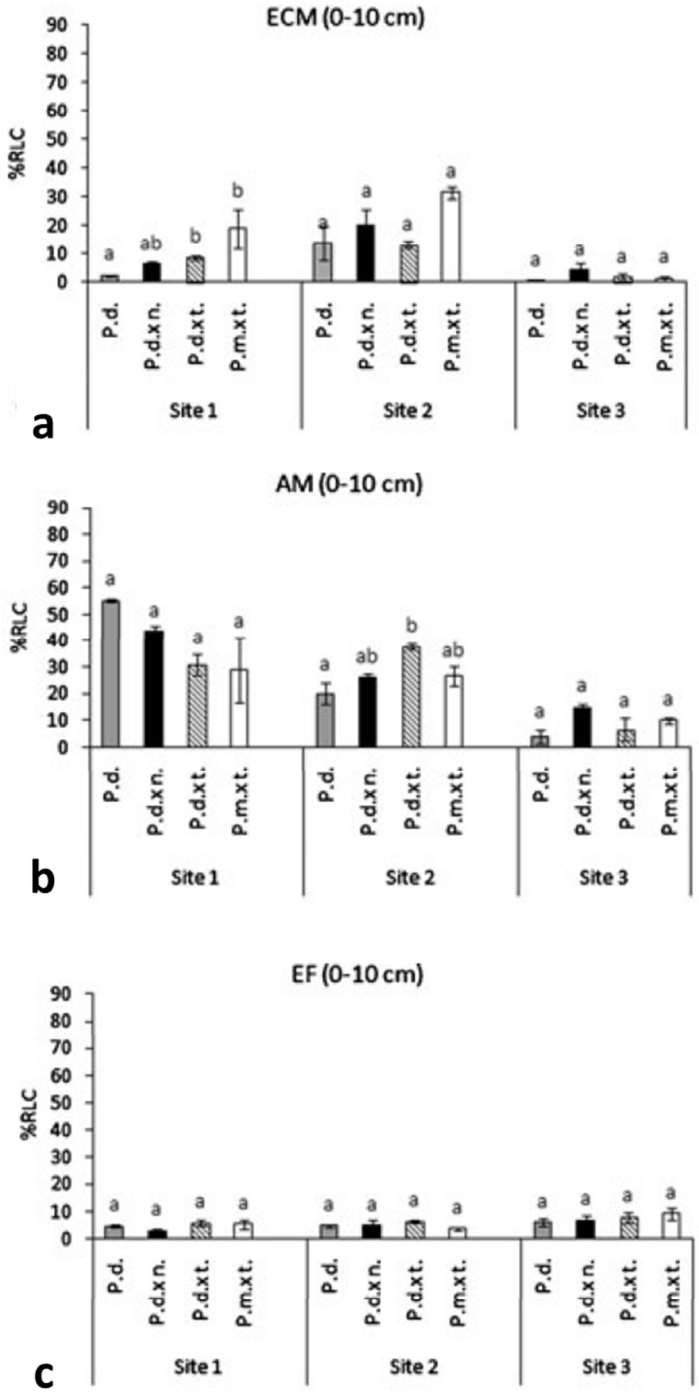


Figure 35 : Pourcentage de racines colonisées par les champignons ectomycorhiziens (ECM), endomycorhiziens (AM) et endophytes (EF) de quatre clones de peupliers sur trois sites (Karliński *et al.*, 2010).

P.d. : *P. deltoides* ; P.d.x n : *P. deltoides* × *P. nigra* ; P.d.x t : *P. deltoides* × *P. trichocarpa* ; P.m.x t. : *P. maximowiczii* × *P. trichocarpa* ; % RLC (Root Length Colonization) : pourcentage de longueur de racine colonisé

L'occupation antérieure des sols par différents couverts végétaux (forêt d'épinettes, peupliers, prairies et champs de maïs) est un facteur déterminant dans l'élaboration des communautés fongiques sous des cultures de type TCR (taillis à courte rotation) de peupliers (Gherghel *et al.*, 2014). Pour les communautés bactériennes, le couvert végétal joue également un rôle par exemple dans différents type de forêts (Li *et al.*, 2014) ainsi que le mode de culture (Lienhard *et al.*, 2014)

Il a par exemple été démontré que le mode de culture d'espèce d'intérêt pour les phytotechnologies (les salicacées) a un impact majeur sur les genres mycorhiziens dominants (Hrynkiewicz *et al.*, 2010). Dans un dispositif d'étude proche de celui utilisé dans ce travail de thèse (Figure 37A), il a été montré qu'une rotation de courte durée voit l'émergence des *Thelephoraceae* et du genre *Tuber*, alors qu'une rotation plus longue se caractérise par la dominance des genres *Laccaria* et *Inocybe* (Figure 37B).

1.4 Techniques d'analyse des communautés microbiennes

Afin d'étudier les communautés de microorganismes présents dans un échantillon, l'approche directe par isolement peut être utilisée mais également des techniques indirectes s'appuyant sur la biologie moléculaire (Figure 38).

L'approche directe d'isolement de microorganismes en utilisant des milieux de culture appropriés est la méthode traditionnelle mais seule une faible proportion de microorganismes du sol est cultivable (0,1% à 1% des bactéries du sol ; Torsvik & Øvreås, 2002). En effet, certains microorganismes endophytes ne peuvent vivre sans leur hôte et ne pourront donc pas être isolés. Cependant, cette approche permet de caractériser et d'étudier les microorganismes (identification moléculaire, analyses morphologiques par des colorations de gram ou au bleu trypan et des observations microscopiques, caractérisation biochimique (BIOLOG, galerie API,…), tests de tolérance aux ETMs, profil protéique)

Les approches de biologie moléculaire consistent à extraire les ADN environnementaux, appelés métagénomes, puis à les amplifier par PCR à l'aide d'amorces qui ciblent une région spécifique (comme la région qui code pour la sous unité ribosomique 16S pour les bactéries ou pour les régions inter-ribosomiques ITS pour les champignons). Les fragments d'ADN sont ensuite comparés entre eux par différentes techniques (ARISA/RISA, T-RFLP, DGGE, TGGE, SSCP, LH-PCR, RAPD, ANDRA), quantifiés pour leur biomasse (Q-PCR) ou assignés à une espèce (clonage puis séquençage ou séquençage de dernière génération (NGS)).

D'autres méthodes non basées sur l'amplification par PCR, permettent également de quantifier la biomasse (Chloroform Fumigation-Extraction (CFE), PhosphoLipid Fatty Acid (PLFA)) l'activité

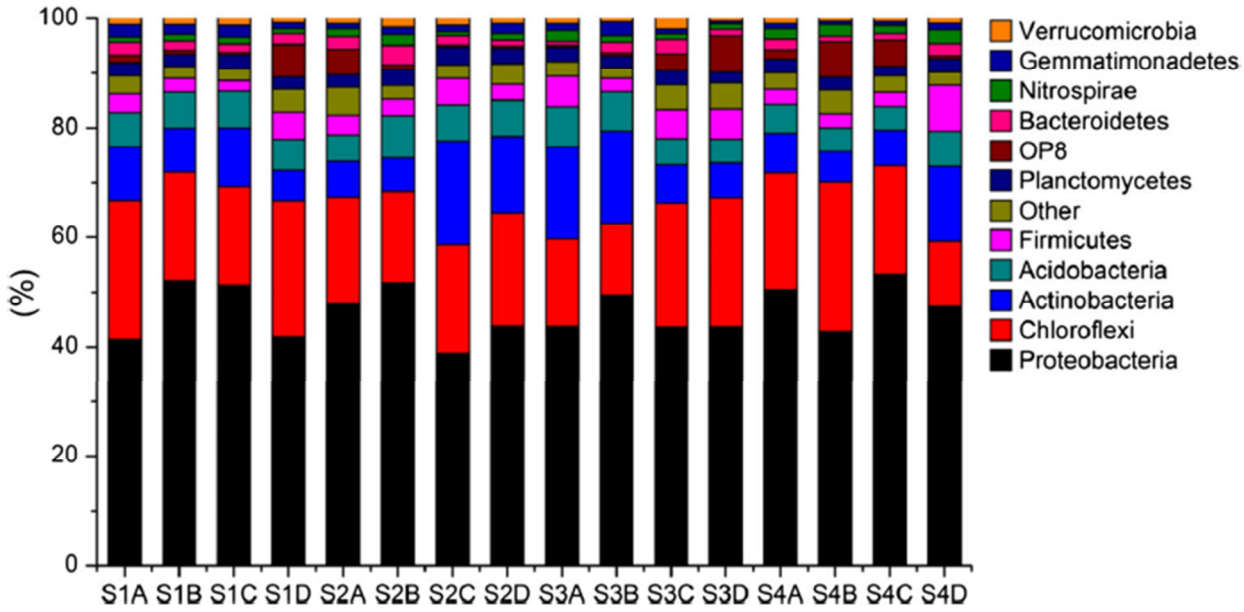


Figure 36 : Abondances relatives des phyla bactériens dominants dans quatre sites (S1 à S4) le long des horizons de sol A, B, C et D représentant les profondeurs 0-20, 20-40, 40-60 et 60-80 cm, respectivement (Liu *et al.*, 2014).

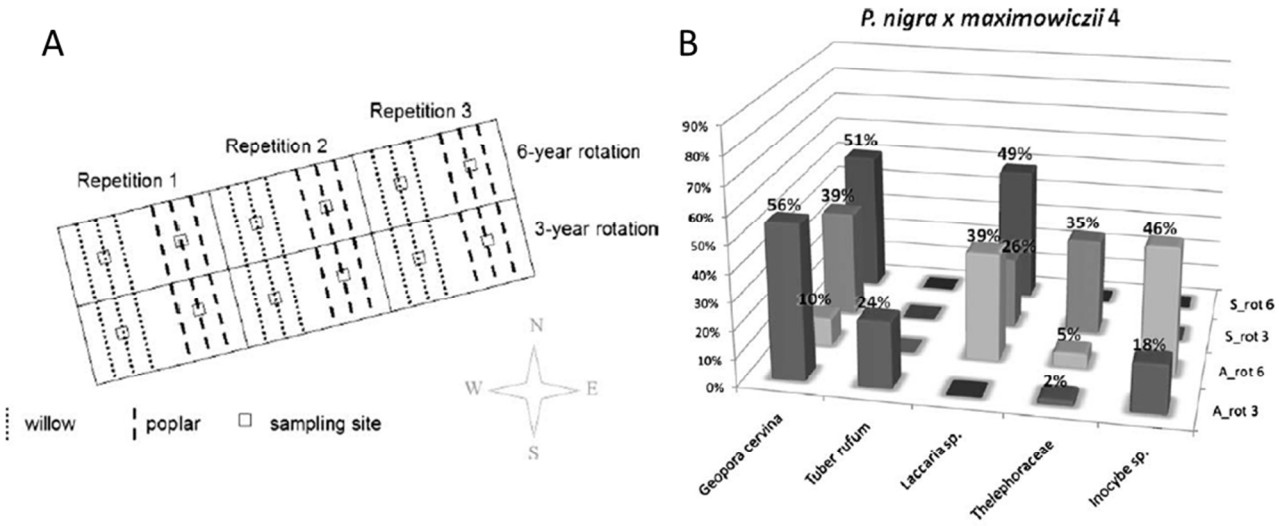


Figure 37 : Schéma du site d'étude (A) et pourcentage des différents morphotypes ectomycorhiziens (B) de peupliers en système de rotation de 3 et 6 ans, et prélevés à l'automne 2008 (A_rot3 ; A_rot6) et au printemps 2009 (S_rot3 ; S_rot6) (Hrynkiewicz *et al.*, 2010).

(Fluorescein DiAcetate (FDA), Stable-Isotope Probing (SIP)) ou la diversité (Micro-Array ou Fluorescence In Situ hybridization (FISH)) (Rincon-Florez *et al.*, 2013). Elles apportent des informations supplémentaires et sont parfois même utilisées en association avec les techniques précédentes (Chodak *et al.*, 2013).

Des techniques se basant sur les ARNm, comme le RNA-array, le RNA-seq ou la métatranscriptomique, apportent également des informations sur l'activité des microorganismes. La métatranscriptomique a par exemple été utilisée pour obtenir simultanément des informations sur la structure et la fonction d'une communauté du sol (Urich *et al.*, 2008).

1.4.1 Les profils électrophorétiques : empreintes moléculaires

Ces techniques d'empreintes génétiques sont basées sur l'amplification par PCR suivie d'une électrophorèse qui fournit des informations sur la structure génétique d'une communauté.

Elles peuvent être divisées en deux groupes en fonction de la migration électrophorétique différentielle sur agarose ou des gels de polyacrylamide. La migration se fait en fonction de la taille pour la DGGE (Denaturing Gradient Gel Electrophoresis) et la TGGE (Temperature Gradient Gel Electrophoresis) ; ou en fonction de la séquence pour la T-RFLP (Terminal Restriction Fragment Length Polymorphism Fingerprinting), l'ARISA/RISA (Automated Ribosomal Intergenic Spacer Analysis), la RAPD (Random Amplified Polymorphic DNA), le SSCP (Single-Strand Conformation Polymorphism), la LH-PCR (Length-Heterogeneity PCR) et l'ARDRA (Amplified Ribosomal DNA Restriction Analysis).

Ces techniques sont assez résolutives car elles révèlent les taxa spécifiques et abondants dans certaines conditions (Moon-van der Staay *et al.*, 2006) qui peuvent être identifiés par excision et séquençage des fragments séparés par électrophorèse. Cependant, les taxa rares peuvent être masqués, dans la mesure où la bande correspondante peut se confondre avec d'autres bandes plus intenses.

Ces approches sont particulièrement appropriées à l'étude de modifications de la diversité des communautés en fonction de variables environnementales. De nombreuses études ont rapporté l'utilisation de ces techniques pour détecter les changements de la communauté microbienne dans les sols contaminés par les ETMs (Li *et al.*, 2006; Long *et al.*, 2010). La technique de DGGE a par exemple permis de montrer une modification de la composition des communautés bactériennes par l'As et le Cd, plus marqué pour le Cd où le nombre de bandes DGGE est réduit surtout dans la partie supérieure du gel (Figure 39 ; Lorenz *et al.*, 2006).

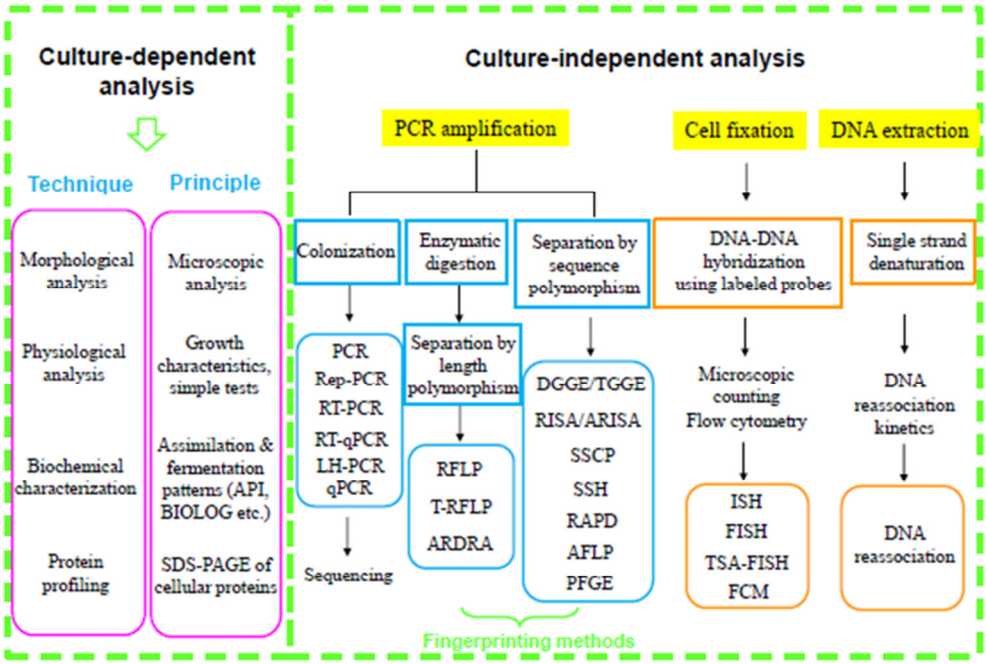


Figure 38 : Les différentes méthodes d'analyse de la diversité (Ma *et al.*, 2016).

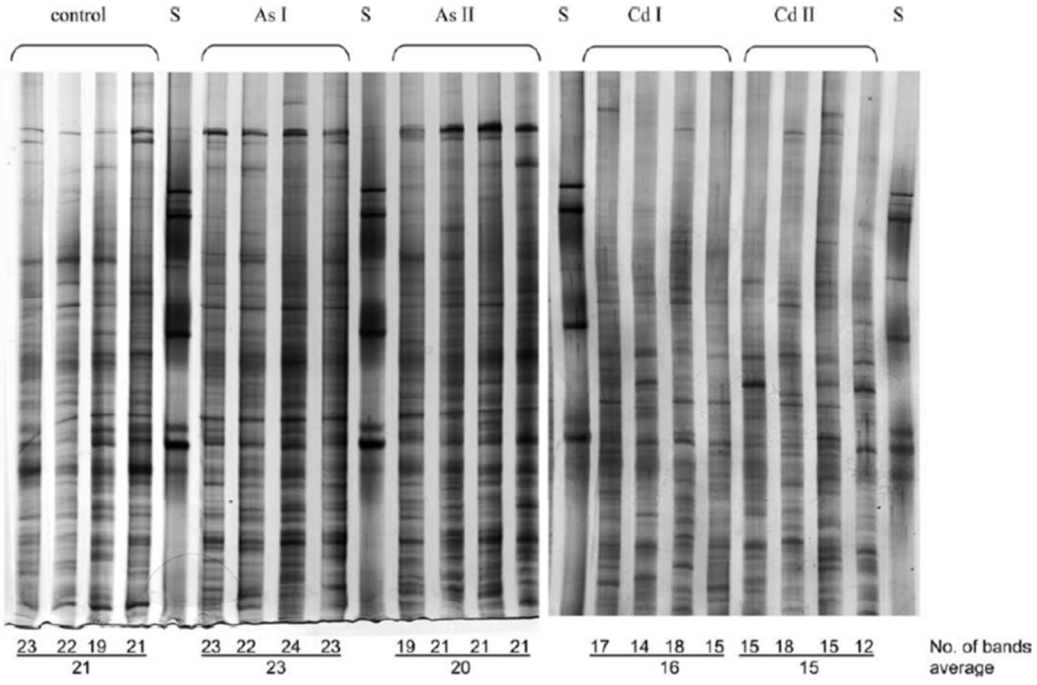


Figure 39 : Profils DGGE des fragments du gène codant l'ARNr 16S pour le sol témoin (control), et pour les sols contaminés par l'As et le Cd (Lorenz *et al.*, 2006).

S : standard

1.4.2 La génomique environnementale

La génomique environnementale est l'étude des acides nucléiques extraits des échantillons d'ADN environnementaux incluant tous les organismes présents à un instant donné (Porras-Alfaro & Bayman, 2011). Le séquençage direct du métagénome par NGS (Next Generation Sequencing) est de plus en plus utilisé pour les estimations de la diversité microbienne dans des environnements complexes tels que les sols. La métagénomique désigne l'exploration de la diversité microbienne globale d'un environnement, sans *a priori*, par séquençage de l'ADN de tous les génomes présents dans cet environnement. Si l'objectif est d'étudier la richesse taxonomique d'un environnement ou de réaliser une analyse différentielle de la structure des communautés microbiennes entre deux échantillons, une approche de type séquençage d'un fragment particulier d'ADN peut être réalisée en ciblant des séquences spécifiques. On parle alors de métabarcoding. En général, les séquences cibles sont les petites sous-unités de l'ADN ribosomique 16S pour les procaryotes ou 18S pour les eucaryotes.

1.4.2.1 Historique

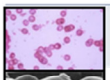
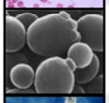
Le séquençage permettant d'obtenir la succession de nucléotides d'un fragment d'ADN ou d'un génome entier est une technique qui a beaucoup évolué au cours de ces dernières années.

En 1953, la structure en double hélice de l'ADN est découverte par Watson et Crick et il faudra attendre 1975 pour l'invention des premières techniques de séquençage d'ADN par Sanger et Gilbert. Il s'en suit en 1976 et 1977 les premiers génomes séquencés des virus bactériophages ϕX1741 (ADN ; 5,4kb) et MS2 (ARN ; 3,5kb). Le génome du premier organisme vivant (Haemophilus influenzae ; 1,8Mb ; Fleischmann et al., 1995) a été séquencé en 1995, puis celui du premier organisme multicellulaire (Caenorhabditis elegans ; 98Mb) en 1998 et enfin le génome humain (2,9Gb) en 2001 (Tableau IV).

La méthode de séquençage Sanger est basée sur une synthèse enzymatique sélective qui est stoppée à chaque nucléotide, fournissant ainsi la séquence nucléotidique. En 1987, le premier automate pour le séquençage Sanger est créé, puis le projet de séquençage du génome humain a stimulé le développement de cette technologie en passant par l'automatisation à différents niveaux (système d'électrophorèse capillaire piloté par ordinateur, robot passeur d'échantillons, marqueurs fluorescents et suites logicielles permettant l'analyse des signaux sortant des séquenceurs et leur transformation sous forme de fichiers analysables : électrophorégrammes et séquences).

Grâce aux progrès de la nanotechnologie et de la bioinformatique, de nouvelles technologies appelées NGS sont apparues afin d'accroitre le débit de séquençage d'ADN. En 2005, le premier séquenceur nouvelle génération est créé par Roche basé sur le pyroséquençage et est nommé 454. En 2006, le SOLiD de Life technologies est mis au point. En 2006, le Solexa GA de Illumina est créé, puis va évoluer en 2010 sous le nom de HiSeq. Les technologies NGS, contrairement à la méthode

Tableau IV : Génomes séquencés avec la méthode Sanger.

	Organismes	Année de séquençage	Taille du génome (Mpb)	Nombre de gènes
	Haemophilus influenzae	1995	1,8	1 800
	Saccharomyces cerevisiae	1996	12,5	5 800
	Escherichia coli K-12	1997	4,6	4 300
	Caenorhabditis elegans	1998	98	19 400
	Arabidopsis thaliana	2000	115	28 000
	Drosophila melanogaster	2000	160	14 000
	Homo sapiens	2001	2 900	23 000
	Mus musculus	2002	2 600	22 000
	Rattus norvegicus	2004	2 750	-

Sanger, ne nécessitent pas de clonage, permettent des analyses massives en parallèle, en haut débit, un coût réduit et une meilleure précision. Les séquenceurs compacts font ensuite leur apparition avec le GS Junior (Roche) en 2009, l'Ion Torrent PGM (Life Technologies) en 2010 et le Miseq (Illumina) en 2011. Ces séquenceurs de paillasse sont de petite taille, ont un turnover rapide mais un débit de données limité ; ils sont donc idéaux pour des applications cliniques et pour les petits laboratoires (Liu *et al.*, 2012).

Grâce à cette évolution des technologies, le nombre de génomes entièrement ou partiellement séquencés augmente de façon exponentielle enrichissant ainsi les bases de données. En 2016, 22 143 génomes ont été complètement séquencés dont 13 536 bactéries et 1028 champignons (http://www.genomesonline.org). Le coût du séquençage a, quant à lui, diminué passant de 2400 $/Mb pour du séquençage Sanger à 0.07 $/Mb pour du séquençage HiSeq (Liu *et al.*, 2012).

1.4.2.2 L'approche de clonage et séquençage

Le séquençage du génome entier d'un organisme n'est pas la seule finalité. Cette technique peut également permettre d'étudier les communautés présentes dans une niche écologique. Pour cela, les ADNs de toute la communauté sont extraits puis amplifiés par PCR à l'aide d'amorces qui ciblent une région spécifique (ARNr 16S ou ITS). Les fragments d'ADN obtenus sont ensuite clonés dans un vecteur, puis multipliés par transformation bactérienne (dans *E. coli* par exemple). Cela permet la création de banques d'ADNs environnementaux, afin de réaliser des criblages par séquençage. Les séquences obtenues sont confrontées à celles référencées dans les bases de données (GenBank), afin de leur assigner une appartenance à une espèce ou à un groupe taxonomique.

1.4.2.3 Les NGS

Les NGS sont parallélisés c'est-à-dire que des centaines de milliers de réactions de séquençage ont lieu en même temps dans des barrettes qui contiennent des puits minuscules en fibre optique. Ces nouvelles technologies de séquençage permettent d'amplifier spécifiquement un fragment d'ADN isolé (métabarcoding), en évitant les étapes de clonage bactérien particulièrement longues, cependant les fragments séquencés sont courts.

Comme pour l'approche par clonage puis séquençage, les NGS pour l'étude des communautés se basent sur des amorces qui ciblent une région spécifique. Cette région ciblée doit être faiblement variable en intra-espèce, fortement variable en inter-espèce et séquençable en une seule fois par la technique utilisée. Cette méthode taxonomique permettant d'identifier une espèce sur la base d'une courte séquence génétique est appelée barcoding.

Les régions utilisées, appelées barcodes, sont des régions variables bordées par des régions très conservées, par exemples l'ARNr 16S pour les bactéries, l'ITS pour les champignons

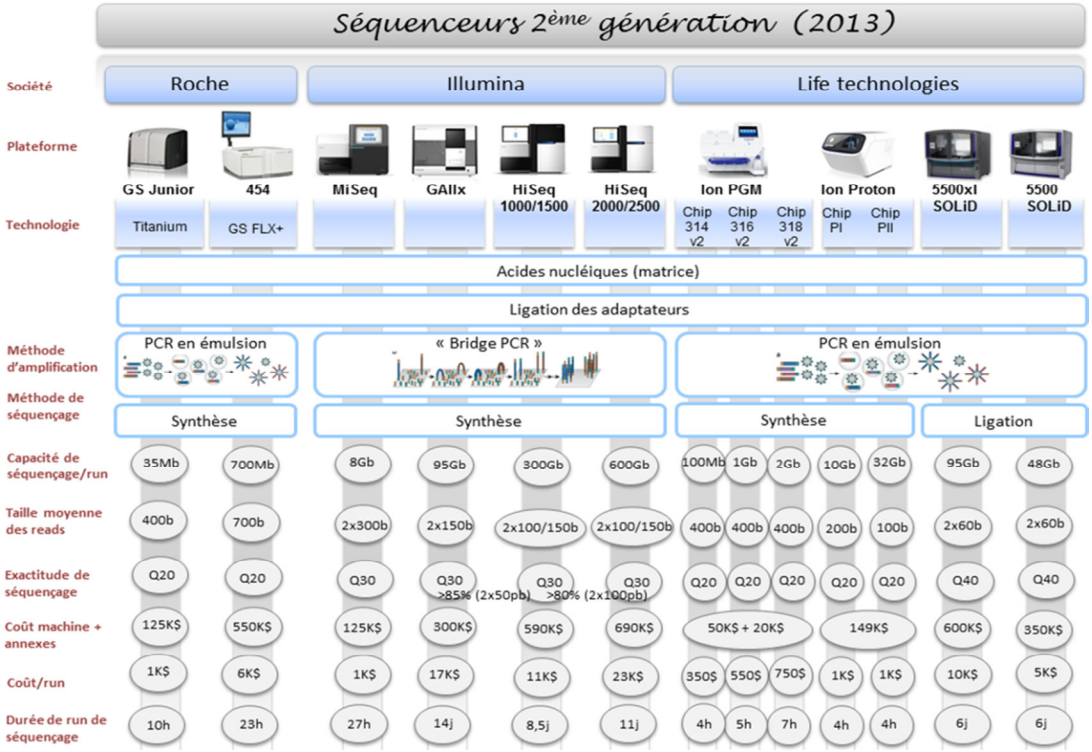


Figure 40 : Comparaison des différentes techniques de séquençage (http://www.biorigami.com)

(Schoch *et al.*, 2012) ou le trnL pour les plantes. Selon la technique de séquençage utilisée la région du barcode peut varier en fonction de la taille des séquences.

Les plates-formes de séquençage les plus utilisées pour évaluer la diversité microbienne du sol sont Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, USA), HiSeq 2000 (Illumina Inc., San Diego, CA, USA), et AB SOLiD™ System (Life Technologies Corp., Carlsbad, CA, USA). Le séquenceur HiSeq 2000 possède la plus grande production et le coût de réactif le plus bas, SOLiD a la plus grande précision et Roche 454 a la taille de séquences la plus longue (Rincon-Florez *et al.*, 2013). Depuis quelques années les séquenceurs de paillasse MiSeq et Ion Torrent PGM sont également utilisés (Figure 40).

1.4.2.3.1 Roche 454

La technologie développée par Roche 454 est basée sur l'amplification de l'ADN lié à une bille en émulsion et au pyroséquençage. Lors de la synthèse d'un nouveau brin d'ADN, la fluorescence portée par le nouveau nucléotide est lue. Ce système est basé sur la détection de pyrophosphate (PPi) qui est libéré lors de la synthèse de l'ADN. L'intensité de la lumière visible qu'il génère est proportionnelle au nombre de nucléotides incorporés (Figure 41).

Cette méthode a été largement utilisée pour caractériser la composition et la diversité des communautés microbiennes du sol (Buée *et al.*, 2009; Eilers *et al.*, 2012; Lundberg *et al.*, 2012; Yang *et al.*, 2012; Carvalhais *et al.*, 2013; Dohrmann *et al.*, 2013) et a également été appliquée pour comprendre l'effet des ETMs (Lami *et al.*, 2013) et des perturbations (Suleiman *et al.*, 2013; Sutton *et al.*, 2013) sur les communautés microbiennes du sol.

A titre d'illustration, l'implantation de *Miscanthus* sur le site de Pierrelaye contaminé par les ETMs induit des changements dans la composition des communautés bactériennes et fongiques, comme le montrent les résultats de séquençage par 454 (Figure 42). Les abondances relatives des *Glomeromycètes* et des *Nitrospira* augmentent ainsi de 3 et 2 fois, respectivement (Bourgeois *et al.*, 2015).

Cette approche a également contribué à l'identification des facteurs clés (climatiques, pédologiques, géographiques, floristiques) déterminant la composition des communautés fongiques et leur diversité (Tedersoo *et al.*, 2014). Les facteurs climatiques et dans une moindre mesure les facteurs pédologiques semblent en être les meilleurs prédicteurs.

Cette technologie 454 n'est désormais plus utilisée, car moins compétitive financièrement et techniquement par rapport aux autres techniques NGS.

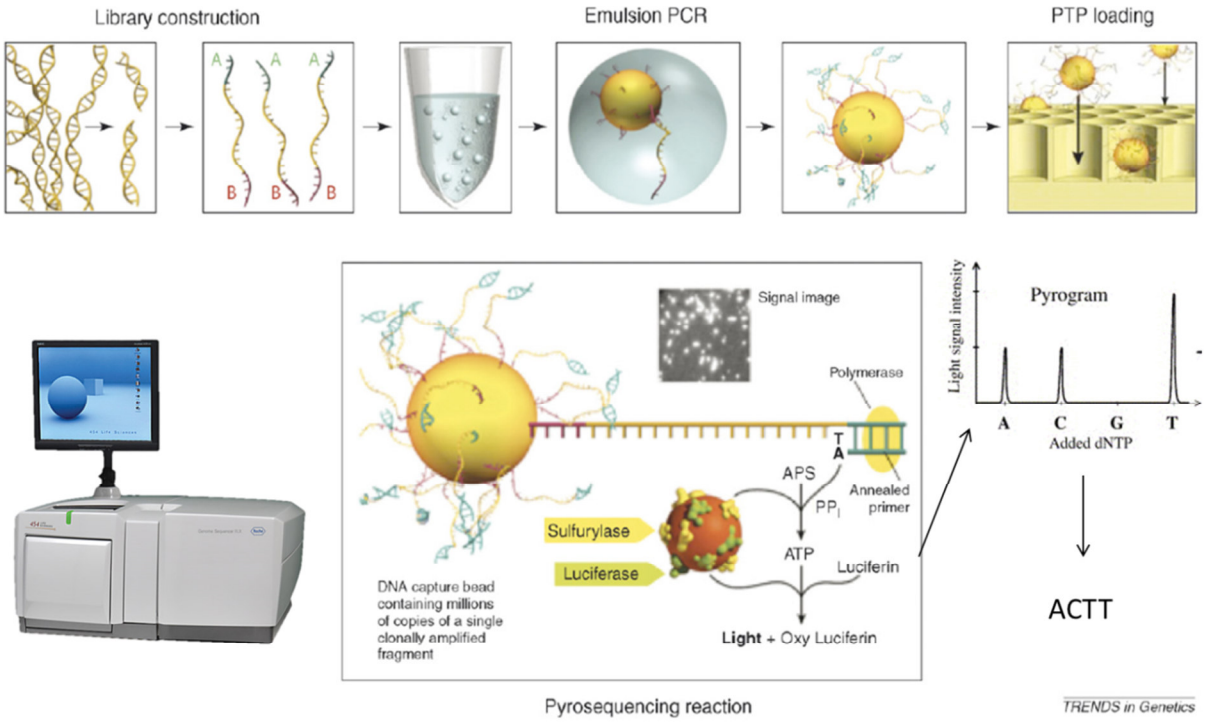


Figure 41 : Le pyroséquençage 454 (Mardis, 2008).

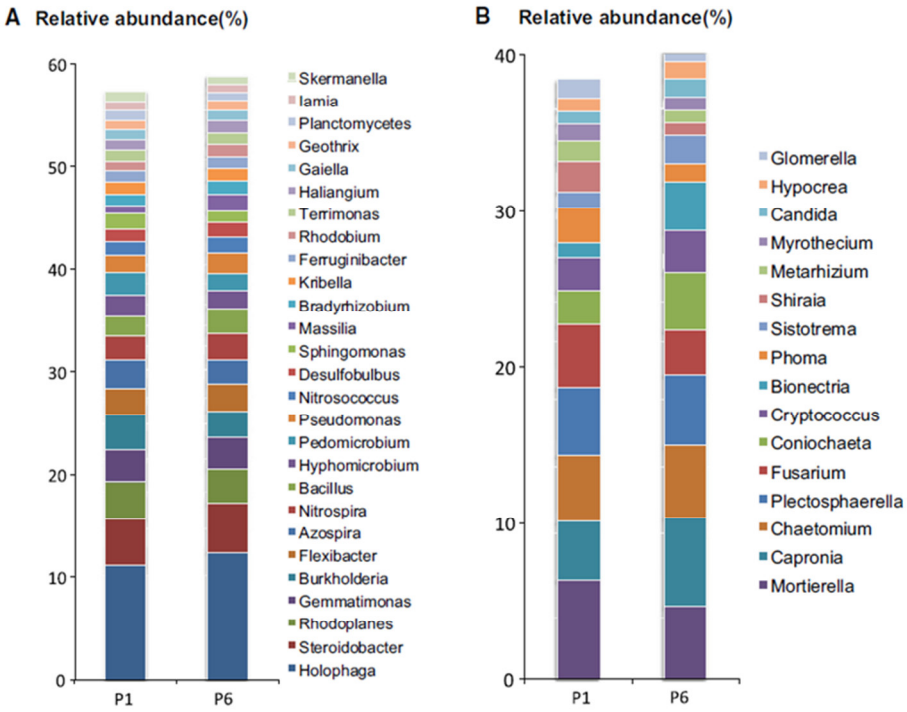


Figure 42 : Abondance relative des genres bactériens (A) et fongiques (B) dominant dans les sols sous une culture de *Miscanthus* (P1) et de blé (P6).(Bourgeois *et al.*, 2015).

1.4.2.3.2 Illumina MiSeq

La technologie développée par Illumina basée sur l'amplification, la liaison sur une puce et l'utilisation de terminateurs de chaîne réversibles marqués par des fluorochromes, permet un séquençage dans les deux sens de lecture diminuant ainsi les erreurs de séquençage.

Le séquenceur Miseq est le deuxième plus utilisé depuis 2012. Il a par exemple permis de caractériser la composition et la diversité des communautés microbiennes du sol (Ding *et al.*, 2015) contaminé par des ETMs (Azarbad *et al.*, 2015; Hong *et al.*, 2015; Chen *et al.*, 2016; Zhang *et al.*, 2016b), des HAPs (Thomas & Cébron, 2016) ou du pétrole (Hou *et al.*, 2015; Yergeau *et al.*, 2015). Il a également été utilisé pour caractériser les enzymes lignocellulolytiques des champignons décomposeurs (Barbi *et al.*, 2015).

Cette approche a par exemple permis de montrer que l'abondance relative des genres bactériens est modifiée sur des sites avec différents degrés de pollution et que les facteurs qui influencent le plus la variation de la structure des communautés bactériennes sur ces sites sont le pH et la quantité d'ETMs (Figure 43).

1.4.2.3.3 Ion Torrent PGM

La technologie Ion Torrent PGM est basée sur des puces semi-conductrices remplies de puits. Un proton est relargué quand un nucléotide est incorporé par la polymérase dans l'ADN. Il en résulte un changement de pH local détecté par cette technologie sensible à la variation d'ions.

Le séquenceur Ion Torrent PGM a été utilisé pour caractériser la composition et la diversité des communautés microbiennes de sols (Urbina *et al.*, 2016) contaminé par des ETMs (Bell *et al.*, 2015), par du pétrole (Bell *et al.*, 2013a; b) ou subissant des perturbations (Miura *et al.*, 2015; Morgado *et al.*, 2015; Detheridge *et al.*, 2016; Semenova *et al.*, 2016). Il a également été utilisé pour caractériser les communautés bactériennes fixatrices d'azote à l'aide du gêne cible *nif* (Zhang *et al.*, 2015).

Cette technologie a ainsi permis de démontrer que la plantation de saules sur sols contaminés par des ETMs induisait des changements importants dans les communautés microbiennes inféodées aux racines. Par exemple, les proportions d'*Agaricomycètes* et de *Pezizomycètes* augmentent de façon significative 4 mois après plantation (Figure 44 **;**Bell *et al.*, 2015).

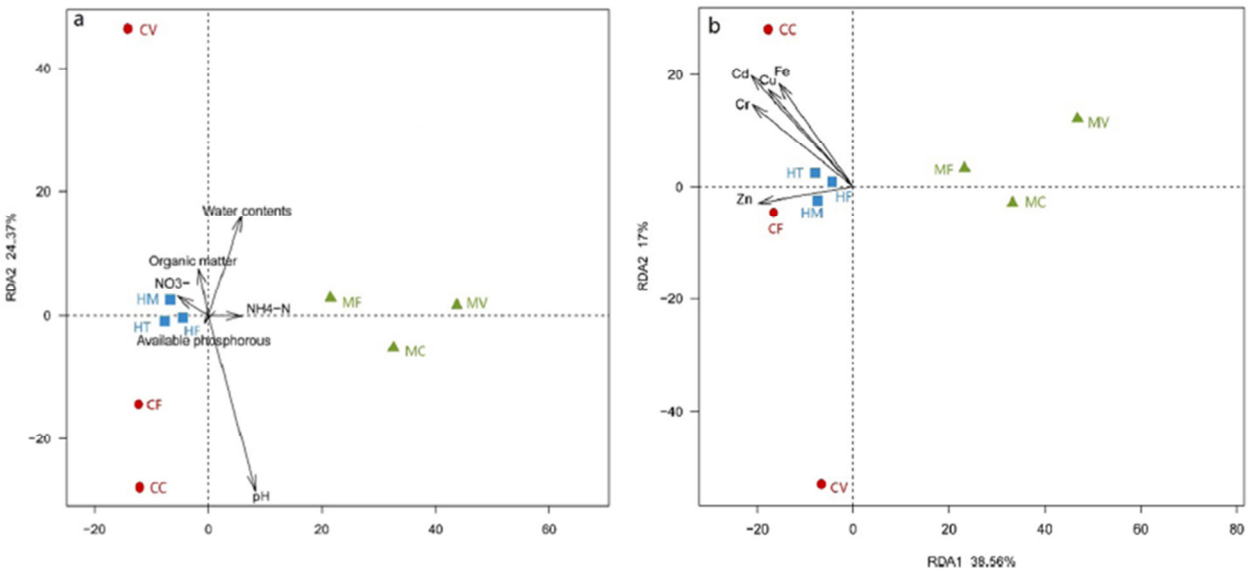


Figure 43 : Relation entre la structure de la communauté bactérienne et les propriétés du sol (a) ou les contenus en éléments traces (b).(Hong *et al.*, 2015).

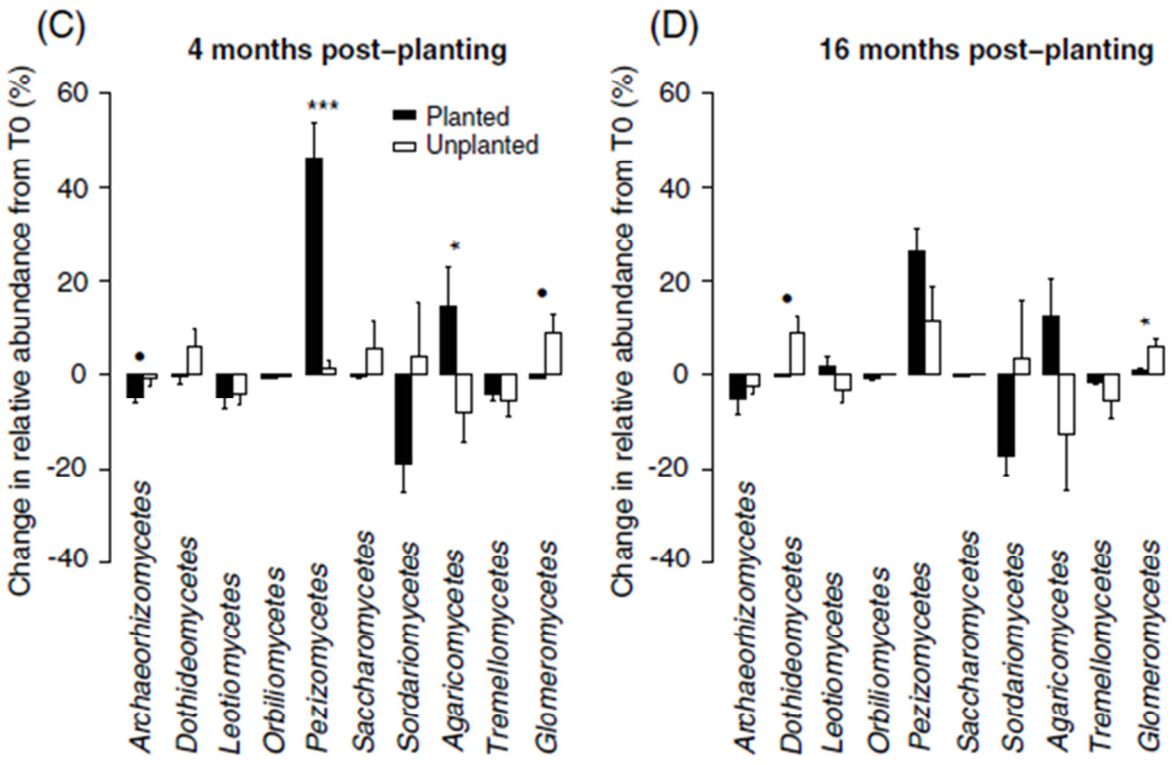


Figure 44 : Résultats du séquençage par Ion Torrent PGM des communautés fongiques isolées de la rhizosphère de saules implantés sur sites contaminés par des ETMs (d'après (Bell *et al.*, 2015).

1.5 Cadre général et objectifs du projet de thèse

Mon travail de thèse s'inscrit dans le cadre de différents programmes de phytomanagement, mis en place depuis 2006 par l'équipe dans laquelle j'effectue ce projet de thèse. Il s'agit des projets ANR-PHYTOPOP (2007-2011), ANR-BIOFILTREE (2010-2014) et ADEME-PROLIPHYT (2013-2018).

Le projet PHYTOPOP avait pour objectif général de mieux exploiter les potentialités offertes par le peuplier dans le domaine de la phytoremédiation par la mise en place et le suivi de parcelles expérimentales *in situ* de peupliers sur sites contaminés par les ETMs. Il a intégré de grandes parcelles de production de plusieurs hectares pour mettre en œuvre des chantiers de collecte et estimer des performances à une échelle réaliste. L'installation de dispositifs de terrain impliquant une palette large de cultivars (14) plantés à fortes densités, susceptibles d'augmenter la quantité de biomasse produite, leur évaluation en terme d'efficacité dépolluante, constituent une étape nécessaire dans la mise en place d'une filière alternative de phytoremédiation. Une seconde étape est l'étude des conditions de récolte de cette biomasse, avec pour objectif global pour les différents partenaires du projet, de préciser la localisation des polluants dans les différents tissus des peupliers et de réaliser une analyse technico-économique des méthodes de récolte, dans l'optique d'une valorisation énergétique. Ce projet visait aussi à étudier le potentiel d'exploitation énergétique que présente la ligniculture du peuplier sur sols pollués.

⇒ La productivité de certains cultivars de peupliers (Skado et I214) sur sols contaminés s'est avérée aussi élevée que sur d'autres parcelles non contaminées, ce qui a ouvert des perspectives intéressantes dans le domaine de la production de biomasse sur sols pollués. Les essais de combustion de la biomasse produite ont démontré qu'il est techniquement possible, en utilisant un dispositif de filtration efficace, de contenir les polluants métalliques extraits du sol, dans les cendres.

PHYTOPOP s'est poursuivi par le projet BIOFILTREE, basé sur l'utilisation de deux des 14 cultivars de peupliers (Skado et I214) en mode TCR, avec inoculation préalable par des champignons mycorhiziens. Les principaux défis ont été 1) la mise en œuvre de 3 ha d'essais de peupliers pour démontrer que l'inoculation mycorhizienne pouvait améliorer significativement les rendements en biomasse; 2) la caractérisation des communautés microbiennes des sols contaminés; 3) l'isolement des microbes endophytes qui représentent des ressources potentielles pour les technologies de biorestauration à base de ligneux, 4) la réalisation en pilote d'essais de la filière torréfaction et pyrolyse pour valoriser la phytomasse et 5) l'évaluation économique du concept.

⇒ L'analyse du transfert de contaminants aux parties aériennes des arbres a été effectuée et les mécanismes impliqués dans leur séquestration par les cellules fongiques ont été étudiés aux niveaux physiologiques et moléculaires. Des essais de torréfaction et de pyrolyse ont été effectués afin de tester le devenir des contaminants dans un processus dynamique, permettant la pleine valorisation de la phytomasse. L'étude de la viabilité technico-économique du concept a consisté à évaluer les coûts totaux de la phytostabilisation et l'efficacité de la technique (production de biomasse) tout en évaluant également les avantages de la biomasse produite et des services générés par la technique.

Le projet PROLIPHYT a pour objectif d'identifier une plus large palette de végétaux ligneux dont la combinaison sur site pollué devrait permettre une plus grande efficacité tout en assurant la biodiversité nécessaire à la restauration d'un écosystème. Les travaux portent également sur l'identification de souches mycorhiziennes qui, associées aux végétaux ligneux sélectionnés, permettront une meilleure reprise et une croissance plus rapide (Smith & Read, 1997), et éventuellement garante d'une meilleure capacité d'extraction ou de stabilisation des polluants.

L'objectif général de ma thèse est l'étude des communautés microbiennes en association avec les espèces ligneuses dans un contexte de contamination par les ETMs. Il repose principalement sur les dispositifs mis en place dans les projets PHYTOPOP et BIOFILTREE. Trois objectifs principaux se sont dessinés au démarrage du projet de thèse:

- **La caractérisation des communautés microbiennes sur sols pollués**

Les communautés microbiennes jouent un rôle majeur dans les sols et il est donc primordial de comprendre quels facteurs influencent le plus les communautés. A l'écriture du projet PROLIPHYT, les approches par isolement étaient privilégiées et l'objectif initial de PROLIPHYT était d'isoler et de caractériser des champignons mycorhiziens issus de sols pollués. Cependant, l'émergence de techniques modernes de génomique environnementale, et en particulier du métabarcoding a donné une nouvelle orientation à mon travail de thèse. Il s'agissait alors de mettre au point et de tester ces nouvelles approches sur des matrices peu étudiées jusqu'alors, les sols pollués. Dès lors, et au-delà de la caractérisation des communautés mycorhiziennes, nous nous sommes aussi intéressés aux champignons endophytes non mycorhiziens, dont l'intérêt dans la communauté scientifique est grandissant. Les communautés bactériennes ont également retenu notre attention, est notamment par exemple les bactéries du genre *Pseudomonas* qui s'avèrent être dominantes sur certains sites. En parallèle de cette approche, le dosage des ETMs ainsi que des analyses pédologiques ont été menées afin d'identifier les facteurs impactant les communautés (pollution, texture du sol, mode de culture, espèce végétale).

- **La génération de ressources microbiennes**

La génération de ressources microbiennes visait à isoler des souches fongiques résistantes aux ETMs, pouvant conférer une meilleure tolérance à leur plante hôte et une meilleure croissance. Pour ce faire, des souches ectomycorhiziennes et endophytiques issues de racines ou de carpophores récoltés sur sites contaminés ont été isolées. Ces souches ont ensuite été identifiées via l'utilisation d'outils moléculaires puis caractérisées pour leur niveau de tolérance à différents ETMs.

- **La caractérisation fonctionnelle de souches isolées**

Afin d'identifier les mécanismes impliqués dans la résistance aux ETMs, différentes méthodes ont été utilisées. Dans une première approche, une étude des mécanismes de tolérance au Hg a été réalisée chez le champignon ectomycorhizien *Paxillus involutus*. En effet, les champignons sont des organismes qui accumulent généralement de fortes quantités de Hg dans leur environnement mais les mécanismes impliqués dans la tolérance au Hg sont encore mal connus. Une seconde approche de métatranscriptomique fonctionnelle a permis d'identifier de nouveaux gènes impliqués dans la tolérance au Zn et au Cd de microorganismes eucaryotes du sol.

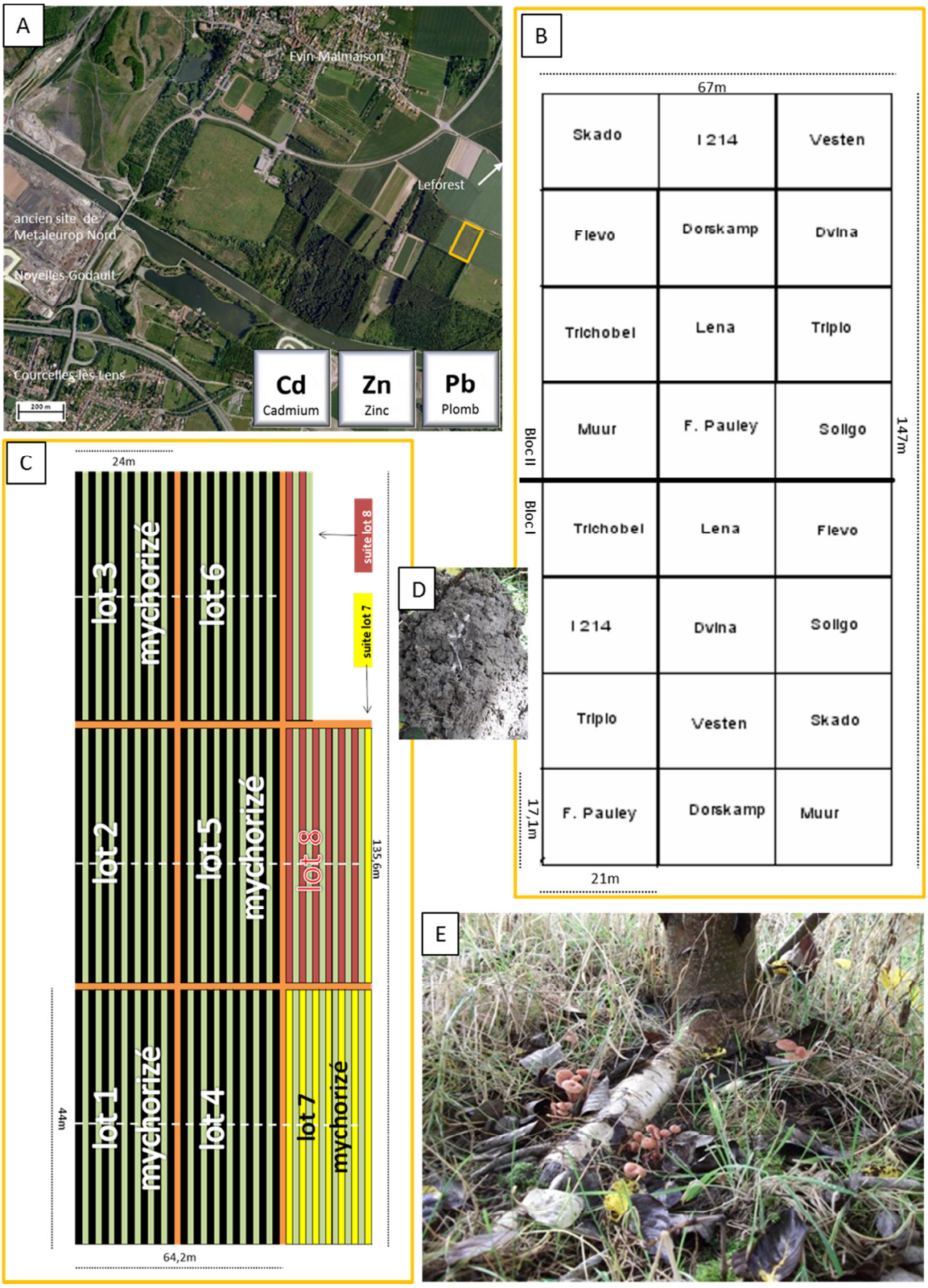


Figure 45 : Dispositif expérimental de Leforest.

Localisation (A) et plans des dispositifs expérimentaux de Leforest des projets PHYTOPOP (B) et PROLIPHYT (C). Illustrations du sol (D) et de fructifications de *Laccaria* sp. au pied d'un peuplier du dispositif (octobre 2012) (E).

2 Matériels & méthodes

2.1 Matériels

2.1.1 Les sites expérimentaux

Au travers des différents projets PHYTOPOP (ANR PRECODD, 2007-2011) BIOFILTREE (ANR Blanc, 2011-2014) et PROLIPHYT (ADEME, 2012-2015), plusieurs parcelles de phytomanagement ont été installées. Seules celles qui ont servi à mes travaux de thèse seront présentées ci-après.

2.1.1.1 Le site de Leforest

Le site de Leforest se situe dans la commune de Leforest dans le département du Pas-de-Calais (62) (Latitude : 50° 25' 48" Nord, Longitude : 3° 2' 51" Est). Ce site est proche de l'ancienne usine de Metaleurop Nord qui raffinait principalement le Zn, le Pb et d'autres métaux (dont Cu, Sb, In, Ge, Au, Ag, Cd). Ces ETMs (principalement du Zn, du Pb et du Cd) contenus dans les fumées, ont contaminé les sols sur plusieurs kilomètres autour de l'usine (Sterckeman *et al.*, 2002; Douay *et al.*, 2009).

L'usine, fondée en 1894 par la société anonyme des mines de Malfidano, était une fonderie qui raffinait principalement du Zn et du Pb. A partir de 1970, les filtres à gaz de l'usine sont progressivement modernisés ou remplacés. Les rejets atmosphériques ont alors été réduits de manière drastique de 350 tonnes de Pb en 1970 à une quinzaine de tonnes en 2002. A sa fermeture en 2003, l'usine raffinait le Zn et d'autres métaux (Pb, Cu, Sb, In, Ge, Au, Ag, Cd). En 2003, la société Metaleurop Nord SA, en liquidation judiciaire, dépose le bilan. Le site sera alors repris en 2003 par la société SITA en charge du démantèlement à partir de 2004 et de la réhabilitation du site. Malgré la fermeture de l'usine Metaleurop Nord, les sols sont toujours fortement contaminés en ETMs, sur une surface de 120 km^2 sur laquelle vivent 55000 habitants. Des lignes d'isoconcentration ont été définies pour les principaux polluants (Figure 7 ; Fritsch *et al.*, 2011) dont la répartition est liée au sens du vent dominant dans la région.

Les teneurs en ETMs ont été mesurées dans les sols aux alentours de Metaleurop Nord à Noyelles-Godault et sur un site témoin de référence. Les teneurs moyennes en Cd sont 23 fois plus importantes à Noyelles-Godault, celles de Pb 13 fois plus importantes et celles de Zn 9 fois plus importantes que sur le site loessique de référence. Les sols sont contaminés en moyenne sur une profondeur de 40 cm, et ces éléments restent confinés dans les couches superficielles du sol à l'exception du Zn, plus mobile (Sterckeman *et al.*, 2002; Douay *et al.*, 2009).

En 2007, un dispositif expérimental a été mis en place sur le site de Leforest dans le cadre du projet PHYTOPOP (Figure 45). Cette parcelle expérimentale représente une surface de 1 ha et le sol

Figure 46 : Dispositifs expérimentaux de Pierrelaye.

Localisation (A) et plans des dispositifs expérimentaux de Pierrelaye des projets PHYTOPOP (B) et BIOFILTREE (C). Fosse pédologique (D) et illustration du TCR de peupliers du dispositif PHYTOPOP (novembre 2012) (D) avec au premier plan des fructifications de Hebeloma sp.

est de texture argilo-limoneuse. Sur ce site, douze clones de peuplier (Fritzi Pauley, Muur, Skado, Trichobel, Vesten, Dvina, I-214, Lena, Soligo, Triplo, Dorskamp et Flevo) ont été plantés (boutures) au mois de mai 2007 à une densité de 1000 tiges par hectare. En 2014, les peupliers ont été récoltés pour des essais de combustion. Après dessouchage, le site expérimental a été replanté en avril 2014 par 16 espèces de ligneux, inoculés ou non, dans le cadre du projet PROLIPHYT.

2.1.1.2 Le site de Pierrelaye

Ce site expérimental est situé sur la plaine de Pierrelaye-Bessancourt dans le département du Val-d'Oise (95) (Latitude : 49° 1' 21" Nord ; Longitude : 2° 9' 1" Est). Cette plaine agricole fut polluée par l'épandage des eaux usées non traitées de la ville de Paris. Cette pratique a entrainé une contamination des sols par plusieurs ETMs comme le Zn, le Cd ou le Pb.

En 1899, furent inaugurés 2150 hectares de champs d'épandage avec la mise en service d'une usine de relevage à Colombes permettant d'assurer l'épandage d'eaux usées sur les plaines d'une douzaine de communes des départements du Val d'Oise et des Yvelines, dont celles de Pierrelaye et de Méry-sur-Oise notamment. La pollution du sol de la plaine par les ETMs fut détectée en 1997. Un arrêté préfectoral interdit alors, en 1999, la mise sur le marché des plantes légumières et aromatiques produites dans ce secteur. A partir de cette date les cultures maraîchères ont été remplacées par de la monoculture de maïs à destination animale (Baize & Tercé, 2002).

La réalisation de profils de sols révèle deux horizons distincts. En surface, un horizon avec une texture de sol sablonneux et calcaire, de couleur très foncée et riche en matière organique varie de 20 à 60 cm selon la zone, la profondeur fluctuant en fonction de la pente du terrain (Figure 46D). Cette couche est particulièrement contaminée par divers ETMs tels que le Zn, le Cd, le Pb et le Cu. L'horizon profond (20 cm à 1 m) est argileux et présente de faibles niveaux de contamination, décroissant avec la profondeur (Lamy *et al.*, 2004).

En 2007, un premier dispositif expérimental a été mis en place sur ce site dans le cadre du projet PHYTOPOP (Figure 46). Cette parcelle expérimentale a une surface de 3 ha. Quatorze clones de peuplier (Bakan, Koster, Fritzi Pauley, Muur, Skado, Trichobel, Vesten, Dvina, I-214, Lena, Soligo, Triplo, Dorskamp et Flevo) ont été plantés (boutures) en mars 2007 à deux densités de plantations différentes : TCR (Taillis à Courte Rotation ; 1000 tiges/ha, rotations de 7 à 8 ans) et TTCR (Taillis à Très Courte Rotation ; 10000 tiges/ha ; rotations de 2 à 3 ans). En 2011, dans le cadre du projet BIOFILTREE, un deuxième dispositif expérimental a été mis en place à côté du premier (Figure 46). Sur cette parcelle expérimentale de 1 ha deux clones de peuplier (Skado et I214), inoculés ou non, ont été plantés en mars 2011 (tiges âgées d'un an). Les placettes unitaires ont été réalisées, soit en TCR pur de plançons d'un an des deux clones de peuplier (Skado et I214), soit en co-culture avec des plants enracinés d'aulne (*Alnus glutinosa*) (1 rang sur 2 « I214 »- aulne ou « Skado »-aulne).

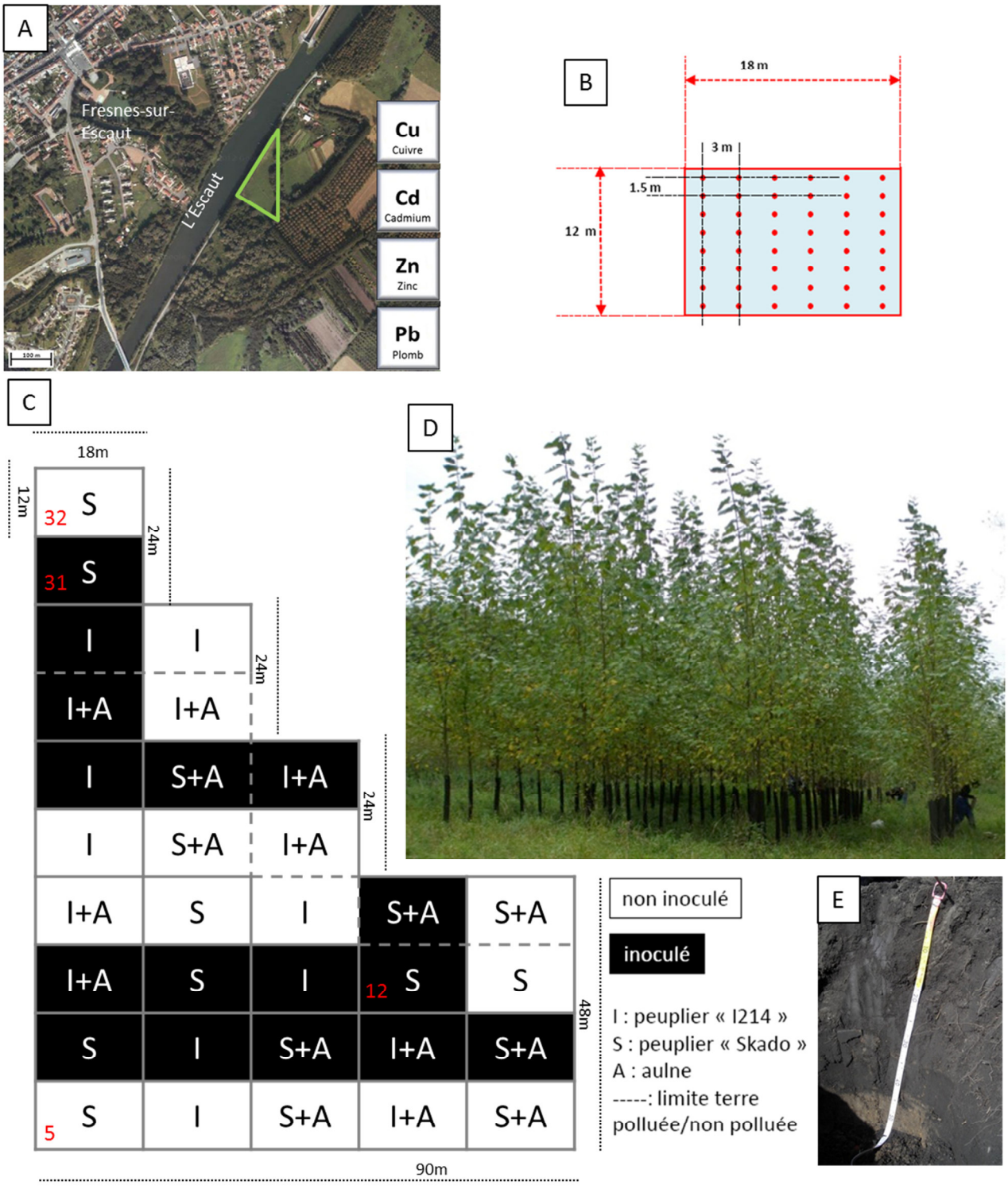


Figure 47 : Dispositif expérimental de Fresnes-sur-Escaut.

Localisation du dispositif (A), détail d'une placette (B), plan du dispositif expérimental (C), photographie d'une placette (octobre 2012) (D) et fosse pédologique (E).

Ces placettes unitaires ont été inoculées ou non avec un mélange commercial de champignons endo- et ectomycorhiziens fourni par les sociétés Symbiom et Agronutrition. L'inoculum fongique appliqué dans les trous de plantation se composait d'un mélange de champignons endomycorhiziens (*Glomus intraradices* BEG140, *G. mosseae* BEG95, *G. claroideum* BEG96, *G. microaggregatum* BEG56, *G. etunicatum* BEG92, *G. geosporum* BEG199, *Rhizophagus irregulare/intradices* DAOM 197198) et ectomycorhiziens (*Hebeloma mesophaeum*, *Amanita rubescens*, *Laccaria proxima* et *Paxillus involutus*).

2.1.1.3 Le site de Fresnes-sur-Escaut

Ce site expérimental est situé sur la commune de Fresnes-sur-Escaut dans le département du Nord (59) (Latitude : 50° 25' 46" Nord, Longitude : 3° 35' 4" Est). Ce site est localisé en bordure de la rivière Escaut et a reçu plusieurs fois des sédiments issus du curage de cette rivière. Les polluants majeurs identifiés sont le Zn, le Cd, le Pb et le Cu.

Le terrain, qui est géré par l'organisme publique VNF (Voies Navigables de France), a été utilisé pendant plusieurs années pour le dépôt de sédiments issus du curage de l'Escaut. Aucune donnée n'est disponible quant à la fréquence et la quantité de sédiments déposés sur ce site. Plusieurs couches de sédiments successives ont ainsi été apportées dont l'épaisseur totale varie de quelques centimètres (20 à 40 cm) (Figure 47E) à plusieurs mètres (2 à 3 m). Les différents polluants, organiques ou métalliques, sont ainsi concentrés dans ces différentes couches de sédiments. Le site a été exploité comme pâturage avant l'installation de notre dispositif expérimental.

En 2011, un dispositif expérimental a été mis en place dans le cadre du projet BIOFILTREE (Figure 47). Cette parcelle expérimentale a une surface de 1 ha. Le sol est de texture limoneuse. Comme à Pierrelaye, des plançons d'un an des deux clones de peuplier (Skado et I214), inoculés ou non, ainsi que des plants enracinés d'aulne en co-culture ont été plantés en mars 2011. Ce dispositif présente la particularité de supporter des parcelles contaminées (en dessous de la ligne en pointillé) et des parcelles très peu contaminées (en dessous de la ligne en pointillé ; Figure 47C**).** Ces parcelles peu contaminées se situent en bordure des zones où ont été effectués les dépôts de sédiments.

2.1.1.4 Le site de Tavaux

Ce dispositif expérimental est situé sur la commune de Saint-Symphorien-sur-Saône dans le département de la Côte-d'Or (21) (Latitude : 47° 4' 52 " Nord, longitude : 5° 20' 14" Est). Il s'agit d'un ancien bassin de décantation des eaux usées provenant de l'usine chimique Solvay de Tavaux (département du Jura, 39). Les polluants majeurs sont le Hg, le Ba et l'As ainsi que des composés organo-chlorés.

L'usine Solvay de Tavaux est une plate-forme chimique implantée depuis les années 1920 et son activité a débuté en 1930. L'installation fait actuellement 200 hectares. Elle a été installée à

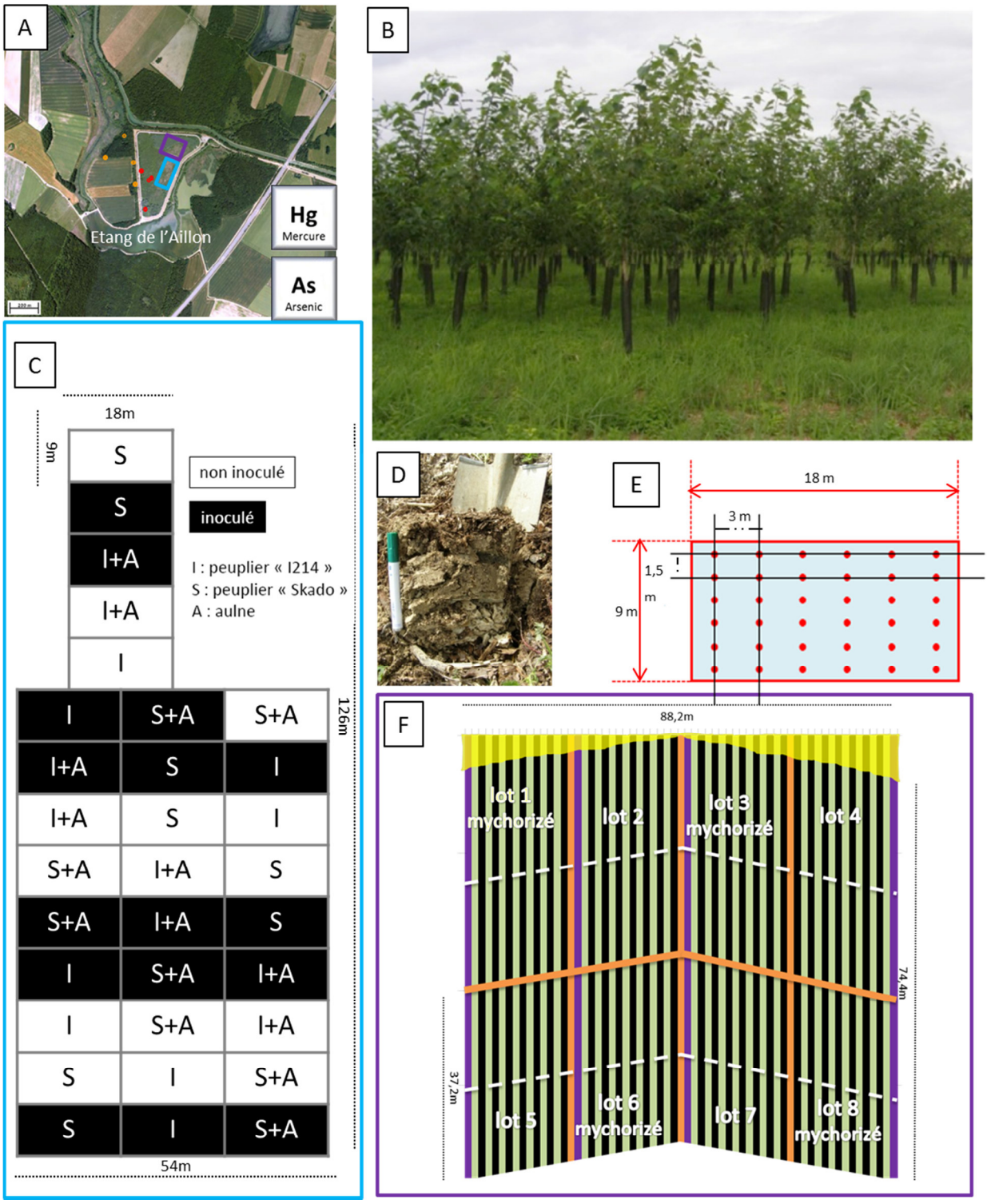


Figure 48 : Dispositif expérimental de Tavaux.

Localisation du dispositif (A), photographie du sol (D) et d'une placette de peupliers (juillet 2012) (B), plan des dispositifs expérimentaux de Tavaux des projets BIOFILTREE (C) et PROLIPHYT (F) et détail d'une placette (E). Les points rouges (lagune) et orange (zone contrôle) de la figure A localisent les zones d'échantillonnage des sols prélevées dans le cadre de la publication de Zappelini *et al*, (2015).

Tavaux du fait de la présence de sel et de calcaire à proximité ; ces deux éléments permettant la formation de carbonate de soude utile pour les réactions chimiques. L'usine s'est aussi implantée à Tavaux pour le faible coût du terrain et la présence du canal Rhin-Rhône. Le sel, matière première pour faire de la soude caustique et des molécules chlorées, est importé sur le site sous forme de saumure et qui est ensuite électrolysée pour donner du NaOH, H et Cl. Jusqu'en 2012, l'électrolyse était réalisée avec une technologie basée sur des cathodes en Hg, ce métal était alors facilement mis en solution et également volatilisé dans l'atmosphère, ce qui entrainait la production d'effluents contaminés au Hg. Aujourd'hui l'électrolyse se fait sur membrane et n'utilise plus de Hg.

A partir de l'électrolyse du sel, on obtient ainsi du chlore qui est la molécule d'intérêt industriel pour SOLVAY, car elle est ensuite fixée sur d'autres molécules carbonées pour former des polymères comme le chlorométhane ou le trichlorométhane (=chloroforme). Les molécules carbonées utilisées sont le méthane, le propane, la glycérine ou l'éthylène. Cette dernière molécule est aussi utilisée pour former du chlorure de vinyle qui est un intermédiaire de production du PVC. Les déchets de production sont réutilisés pour faire d'autres produits comme l'HCl, ou l'eau de javel. Les effluents industriels sont déversés dans différents bassins et ils contiennent de la chaux, des déchets salins, et des traces de Hg, de Ba et d'As. En 2016, seuls deux bassins de décantation sur quatre sont encore utilisés. Le premier bassin non utilisé, représente 12 ha, est composé d'un sol très salin et a été colonisé naturellement par des bouleaux et des tamarix. Le deuxième bassin, utilisé jusqu'en 2002, est situé à l'extrémité d'un petit canal convoyant les effluents, à environ 6 km du site industriel. En plus de ces effluents, ce bassin a reçu les sédiments de curage de l'étang de l'Aillon, situé en contrebas du bassin. Cet étang servait à sédimenter les particules en suspension provenant du canal.

Ce dernier bassin a une superficie de 14 ha et accueille les plantations de BIOFILTREE et de PROLIPHYT (Figure 48). Par endroits, ce bassin a été recolonisé spontanément par du saule, du robinier, du frêne ou encore du peuplier depuis l'arrêt de l'utilisation de cette lagune. Le sol est constitué d'un mélange alcalin contaminé majoritairement par du Hg. En avril 2011, un dispositif expérimental a été mis en place sur ce site dans le cadre du projet BIOFILTREE. Cette parcelle expérimentale a une surface de 1 ha et, comme à Pierrelaye et à Fresnes-sur-Escaut, deux clones de peuplier (Skado et I214), inoculés ou non, ainsi que de l'aulne en co-culture ont été plantés sous forme de plançons ou plants enracinés âgés d'un an.

Ce site a également été utilisé dans le cadre du projet PROLIPHYT où 16 espèces de ligneux, inoculés ou non, ont été plantées en avril 2014. La sécheresse de l'été 2014 et des conditions de sol difficiles ont provoqué un taux de mortalité supérieur à 75%. Le dispositif a alors été replanté à l'automne 2014.

Tableau V : Composition du milieu Malt.

Composés	Concentration finale (g/L)
Extrait de malt	**12**
Ajuster le pH à 5,5	
Agar	**15**
Autoclaver 20 min à 120°C	
Si besoin d'antibiotique :	
Ampicilline (100g/L soit 2g dans 20mL)	**1mL**
Chloramphénicol (100g/L)(dissoudre dans EtOH)	**1mL**

Tableau VI : Composition du milieu MMN.

Composés	Concentration de la solution mère (g/L)	Volume pour 1L	Concentration finale
KH_2PO_4	50	10 mL	500mg/L
$(NH_4)_2HPO_4$	25	10 mL	250mg/L
$CaCl_2$, $2H_2O$	5	10 mL	50mg/L
NaCl	2,5	10 mL	25mg/L
$MgSO_4$, $7H_2O$	15	10 mL	150mg/L
Thiamine	1	100 µL	100µg/L
$FeCl_3$, $6H_2O$	10	100 µL	1mg/L
Glucose	-	10 g	10g/L
Ajuster le pH à 5,5			
Agar	-	10 g	10 g/L
Autoclaver 20 min à 120°C			

Tableau VII : Composition du milieu N2P2.

Composés	Concentration de la solution mère (g/L)	Volume pour 1L	Concentration finale
$CaCl_2$, $2H_2O$	5	10 mL	50 mg/L
$MgSO_4$, $7H_2O$	15	10 mL	150 mg/L
Na_2HPO_4, $12H_2O$	16	10 mL	160 mg/L
$(NH_4)_2SO_4$	25	10 mL	250 mg/L
KH_2PO_4	50	5 mL	250 mg/L
NaH_2PO_4	45	100 µL	4,5 mg/L
Citrate ferrique	12	1 mL	12 mg/L
Thiamine	1	40 µL	40 µg/L
Kanieltra	-	100 µL	100 µl/L
Glucose	-	2,5 g	2,5 g/L
H_2O	-	Qsp 950 mL	
Ajuster le pH à 5,7			
Agar (Difco)	-	15 g	15 g/L
Autoclaver 20min à 120°C			
MES pH 5,7 filtré à 0,22 µm	100 g/L	50 mL	5 g/L

2.1.2 Matériels biologiques

2.1.2.1 Les champignons en collection

Plusieurs souches de champignons ectomycorhiziens et endophytes, issues de la collection du laboratoire et de plusieurs campagnes de prélèvements, sont conservées au laboratoire à 25 °C à l'obscurité (Annexe 4). L'entretien de ces différentes souches se fait par repiquages successifs sur les milieux de culture malt (Tableau V), Modified Melin-Norkrans (MMN) (Tableau VI ; Brundrett *et al.*, 1996), N2P2 (Tableau VII ; Gay, 1990; Benjdia *et al.*, 2006) ou Pachlewski (Tableau VIII **;** Pachlewski & Pachlewska, 1974) solides et par conservation à plus long terme dans du glycérol 10% à -80°C (§2.2.5.2).

2.1.2.2 Espèces ligneuses des dispositifs expérimentaux

Les dispositifs expérimentaux PHYTOPOP comptent 14 clones de peuplier (Tableau IX) sous deux modes de culture (TCR et TTCR). Les dispositifs expérimentaux BIOFILTREE utilisent les clones de peuplier « Skado » et « I214 » (en TCR) et de l'aulne glutineux. L'aulne glutineux (*Alnus glutinosa*) est l'espèce d'aulne la plus répandue en France, il permet d'enrichir le sol en azote grâce aux nodules fixateurs de N_2 qu'il forme avec les bactéries *Frankia* et fait partie des espèces qui accumulent peu les ETMs dans ses parties aériennes. Enfin, les dispositifs expérimentaux PROLIPHYT comptent différentes espèces ligneuses choisies en fonction de l'inventaire floristique et des conditions pédologiques locales (Tableau X).

2.2 Méthodes

2.2.1 Prélèvements et préparation des échantillons

Afin de caractériser les propriétés des sites d'étude et des ligneux qui s'y développent, de nombreux prélèvements sont effectués. Ceux-ci peuvent être de différentes natures et concerner le matériel biologique (végétal et fongique) ou le sol.

2.2.1.1 Prélèvements de sol et de racines

Afin d'isoler et de caractériser les microorganismes s'associant aux arbres étudiés, divers prélèvements de racines et de sol ont été effectués tous les ans en automne. D'une manière générale, lors de l'échantillonnage sur chaque site d'étude, la litière recouvrant le sol est d'abord ôtée avant de prélever les racines et du sol adhérant aux racines. Les différents prélèvements ont été réalisés dans une zone allant de 0 à 20 cm sous la surface du sol. Ces derniers ont toujours été effectués sous le couvert des arbres et seules les racines encore connectées à l'arbre ont été sélectionnées. Pour chaque parcelle, cinq arbres individuels ont été échantillonnés de manière aléatoire et, pour chaque arbre, trois prélèvements ont été effectués et mélangés afin d'obtenir (par arbre) des échantillons composites de

Tableau VIII : Composition du milieu Pachlewski.

Composés	Concentration de la solution mère	Volume/Concentration pour 1L
Glucose	-	20 g/L
Maltose	-	5 g/L
Tartrate d'ammonium	-	2,3 g/L
KH_2PO_4	-	0,5 g/L
$MgSO_4$, $7H_2O$	-	1 g/L
Thiamine	solution à 1 g/L	10µl
Kanieltra :		100µL
$ZnSO_4$, $7H_2O$	0,63 mg/L (3,2 µM)	1mL
$MnSO_4$, H_2O	1,54 mg/L (11,7 µM)	1mL
$CuSO_4$, $5H_2O$	0,25 mg/L (0,47 µM)	100µl
$Mo_7O_2NH_4$, $4H_2O$	0,05 mg/L (0,28 µM)	100µl
H_3BO_3	1,28 mg/L (20,6 µM)	1mL
Fe-EDTA	3,44 mg/L (10,7 µM)	1mL
Ajuster le pH à 5,5		
Agar	-	20g/L
Autoclaver 20 min à 120°C		

Tableau IX : Liste et principales caractéristiques des cultivars utilisés pour les essais de terrain dans le cadre du projet PHYTOPOP.

Nom du clone	Espèce	Pays	Nom botanique	Sexe
Bakan	TM	B	*P. maximowiczii* x *P. trichocarpa*	mâle
Fritzi Pauley	T	B	*P. trichocarpa*	femelle
Muur	DN	B	*P.* x *euramericana*	mâle
Skado	TM	B	*P. maximowiczii* x *P. trichocarpa*	femelle
Trichobel	T	B	*P. trichocarpa*	mâle
Vesten	DN	B	*P.* x *euramericana*	femelle
Dvina	D	I	*P. deltoides*	mâle
I-214	DN	I	*P.* x *euramericana*	femelle
Lena	D	I	*P. deltoides*	mâle
Soligo	DN	I	*P.* x *euramericana*	mâle
Triplo	DN	I	*P.* x *euramericana*	mâle
Dorskamp	DN	NL	*P.* x *euramericana*	mâle
Flevo	DN	NL	*P.* x *euramericana*	mâle
Koster	DN	NL	*P.* x *euramericana*	mâle

D : *Populus deltoides*, N : *P. nigra*, T : *P. trichocarpa* et M : *P. maximowiczii* ; *P. euramericana* est un hybride *P. deltoides* x *P. nigra* ; B : Belgique, I : Italie, NL : Pays-Bas.

Tableau X : Liste des différentes espèces ligneuses plantées à Leforest et à Tavaux dans le cadre du projet PROLIPHYT.

Leforest				Tavaux			
1	*Alnus subcordata*	9	*Quercus pedonculata*	1	*Cornus sanguineum*	9	*Salix alba*
2	*Betula pubescens*	10	*Quercus sessiliflora*	2	*Euonymus europaeus*	10	*Salix alba chermesina*
3	*Capinus betulus*	11	*Salix acutifolia*	3	*Fraxinus excelsior*	11	*Salix aquatica grandis*
4	*Ostrya carpinifolia*	12	*Salix alba*	4	*Ostrya carpinifolia*	12	*Salix balsamifere*
5	*Platanus orientalis*	13	*Salix aquatica grandis*	5	*Platanus orientalis*	13	*Salix daphnoides*
6	*Populus oxford*	14	*Salix caprea bouliata*	6	*Prunus spinosa*	14	*Salix rosmarinifolia*
7	*Pterocarya stenoptera*	15	*Salix fragilis*	7	*Pterocarya stenoptera*	15	*Salix triandra*
8	*Quercus cerris*	16	*Salix phyticipholia*	8	*Robinia pseudo acacia*	16	*Salix viminalis jaune*

racines et de sol. Pour les parcelles témoins (parcelle agricole cultivée en maïs et parcelle non plantée), cinq échantillons de sol ont également été prélevés.

Les échantillons sont ensuite conservés à 4°C jusqu'à leur conditionnement final. Les racines sont manuellement agitées afin de les séparer du sol rhizosphérique. Les échantillons de sols sont soit directement séchés en vue du dosage des ETMs ou du Hg (§2.2.7.5) ou séchés, broyés, tamisés à 2 mm puis conservés à -20°C en vue des analyses moléculaires. Les racines sont soigneusement lavées à l'eau du robinet, puis rincées trois fois à l'eau distillée stérile. Les racines fines sont ensuite récoltées puis congelées à -20 °C en vue des analyses de biologie moléculaire (§2.2.2).

2.2.1.2 Prélèvements de fructifications de champignons

Des carpophores de champignons ont également été récoltés sur les différents sites expérimentaux. Ces échantillons sont conservés à 4°C jusqu'à leur utilisation au laboratoire : ils seront utilisés pour obtenir des cultures pures sur boîte de Pétri (§2.2.5.1.1), identifiés (§2.2.2) et/ou analysés pour leur contenu en ETMs (§2.2.7.5).

2.2.2 Techniques de biologie moléculaire

2.2.2.1 Extraction d'ADN

2.2.2.1.1 Echantillons de sol

Afin d'isoler l'ADN des microorganismes telluriques présents sur les différentes parcelles des sites expérimentaux, les sols conditionnés à -20°C sont utilisés (§2.2.1.1). L'extraction d'ADN de ces derniers est réalisée à partir de 10 g de sol en utilisant le kit « PowerMax® Soil DNA Isolation » (MO-BIO) (travaux liés à l'article (Zappelini *et al.*, 2015) ou avec 0,5g de sol (travaux liés à l'article (Foulon *et al.*, 2016a; b) à l'aide du kit « PowerSoil® DNA Isolation » (MO-BIO) selon les recommandations du fournisseur (Annexe 1).

2.2.2.1.2 Echantillons de racines

Les racines préalablement congelées à -20°C (§2.2.1.1) (100 mg) sont broyées à l'aide de billes de tungstène (Qiagen) dans un vibrobroyeur (Model MM400; Retsch) pendant trois fois 1 min à une fréquence de 30Hz. L'ADN est ensuite extrait en utilisant le kit « PowerSoil® DNA Isolation » (MO-BIO) selon les recommandations du fournisseur (Annexe 1).

2.2.2.1.3 Echantillons de champignons

Ce paragraphe concerne la démarche entreprise à la fois pour les analyses des champignons récoltés sur les dispositifs expérimentaux sous forme de carpophores et des souches en culture pure qui ont été isolées. L'extraction d'ADN est réalisée à l'aide des kits « REDextract-N-AmpTM Plant

Tableau XI : Exemple de mélange réactionnel et de programme PCR utilisant l'enzyme Phire Hot Start II.

Mélange réactionnel	Volume (µL)	Programme			
Eau ultra pure	16		Température	Temps	Nombre de cycles
Tampon 5x	5	Dénaturation initiale	98 °C	3 min	1
dNTP (10mM)	0,5	Dénaturation	98 °C	5 sec	
Amorce forward (10 µM)	0,5	Hybridation	55 °C	5 sec	30
Amorce reverse (10 µM)	0,5	Elongation	72 °C	15 sec	
ADNg (100 ng/µl)	2	Elongation finale	72 °C	1 min	1
Phire Hot Start II	0,5	Conservation	4 °C	∞	1
Total	25				

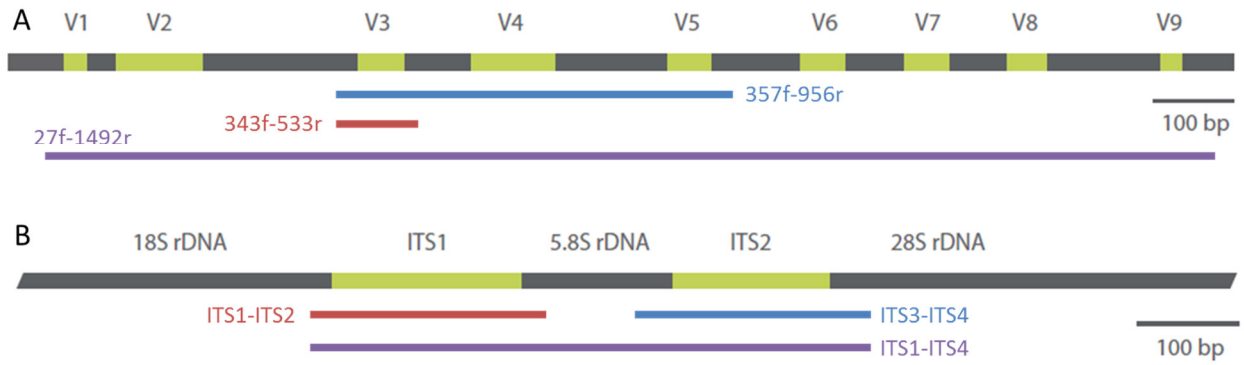


Figure 49 : Localisation sur les ADNs ribosomiques nucléaires bactériens (A) et fongiques (B) des régions variables (V1 à V9 pour le 16S bactérien et ITS pour les champignons) amplifiées par PCR.

En rouge les régions amplifiées pour le séquençage par Ion Torrent, en bleu par Illumina MiSeq et en violet par Sanger.

PCR » (Sigma-Aldrich) ou « DNeasy® Plant Mini Kit » (QIAGEN) selon les protocoles des fournisseurs. Pour les champignons très pigmentés, dix à vingt milligrammes de PVPP (PolyVinylPolyPyrrolidone) sont ajoutés avant le broyage dans les tubes du kit « DNeasy® Plant Mini Kit » (QIAGEN) (Annexe 2).

2.2.2.2 Amplification par PCR

Afin d'accroître la quantité d'ADN d'une région spécifique, la technique d'amplification par PCR (Polymerase Chain Reaction) est utilisée. Cette technique repose sur la synthèse d'un brin d'ADN complémentaire à l'aide d'une polymérase thermostable : la Taq polymérase. Pour réaliser sa synthèse, l'enzyme a besoin d'un fragment oligonucléotidique complémentaire à une région de l'ADN à amplifier appelé amorce et des quatre désoxyribonucléotides (dATP, dCTP, dGTP, dTTP). Un cycle d'amplification est composé de trois étapes : une dénaturation thermique qui ouvre le double brin d'ADN matriciel, une hybridation des amorces à leur séquence cible et une élongation à partir des amorces par l'action de la Taq polymérase.

L'enzyme que nous avons couramment utilisée pour les PCR de type standard est la Taq polymérase Paq 5000TM (Stratagene). Pour l'amplification d'ADN issu de matrices complexes, telles que les sols, les racines ou certains champignons pigmentés, la Taq polymérase « Phire Hot Start II » (Thermo Scientific) (Tableau XI) a été préférée pour sa moindre sensibilité aux inhibiteurs de PCR.

Les couples d'amorces utilisés varient selon les techniques de séquençage. En vue des séquençages Sanger, les amorces ITS1-ITS4 (White *et al.*, 1990) et 27f-1492r ont été utilisées. Pour les séquençages NGS (Ion Torrent et Illumina MiSeq), les séquences obtenues devant être plus courtes, les amorces ne ciblent pas l'ensemble des portions ribosomiques mais des régions plus courtes comme l'ITS2 pour les champignons ou V345 pour les 16S bactériens dans le cas du séquençage MiSeq (Figure 49).

Pour la technique de séquençage par Ion Torrent, les amorces utilisées sont spécifiques et comprennent 3 éléments. Le premier élément est constitué de l'amorce classique c'est-à-dire la région complémentaire à l'ITS1 pour les champignons ou à la région V3 pour les bactéries. Le second élément est un adaptateur qui permet la fixation sur une bille pour le séquençage. Enfin, le troisième élément est une étiquette (appelé MID) variable pour chaque échantillon et qui permet d'assigner une séquence donnée à un échantillon donné (les différents échantillons étant en effet poolés avant le séquençage). Cette étiquette est présente uniquement sur l'amorce sens (forward) et non sur l'amorce antisens (reverse) qui lui est associée. Pour chaque échantillon, le couple d'amorces choisi comprend l'amorce sens spécifique à chaque échantillon et l'amorce antisens commune à tous les échantillons (Annexe 3).

Tableau XII : Composition du tampon de charge (6X).

Composé	Concentration
Tris pH 8	10 mM
EDTA pH 8	1 mM
Bleu de bromophénol	0,25 % w/v
Bleu de xylène cyanol	0,25 % w/v
Glycérol	30 % v/v

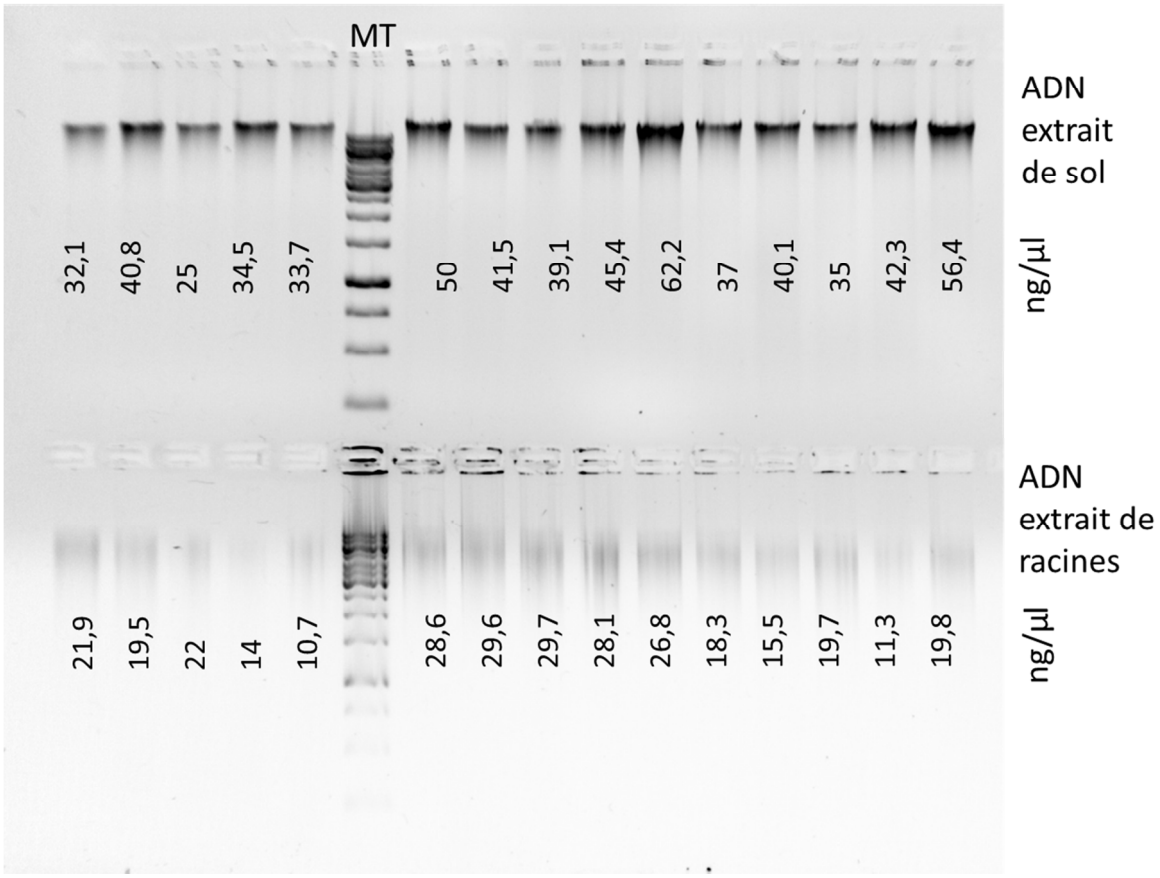


Figure 50 : Exemple de gel d'électrophorèse pour les ADNs totaux extraits de sol et de racines.

Les valeurs indiquent la quantité d'ADN en ng/µl après dosage avec le kit Quant-iT^{TM} PicoGreen. MT= marqueur de taille 1Kb (Euromedex).

2.2.2.3 Séparation et visualisation des ADNs sur gel d'agarose

Les ADNs extraits et amplifiés, mélangés à un tampon de charge (Tableau XII), ainsi qu'un marqueur de taille (DNA Ladder 1 Kb d'Euromedex à 0,5 µg/µl), sont déposés sur un gel à 1 % (m/v) d'agarose et sont mis à migrer à 90 V. Pour la préparation du gel, l'agarose est dissout dans du tampon TBE (107,8 g/l de Tris-HCl pH 6,5 ; 55 g/l de Borate ; 5,8 g/l d'EDTA). La visualisation de l'ADN s'effectue via une révélation au BET (Bromure d'Ethidium ajouté à 2 µl / 100ml à partir d'une solution stock à 10 mg/ml) sous illumination UV.

Après migration, l'ADN est visualisé à l'aide d'un appareil « Gel Doc » (Bio-Rad) qui expose le gel aux UV et permet l'acquisition de l'image. Le gel ainsi révélé permet de visualiser les bandes d'ADN, d'évaluer leur taille, et leur concentration à l'aide du logiciel Image Lab associé au Gel Doc (Figure 50).

2.2.2.4 Purification des ADNs

Afin d'éliminer d'éventuels composés inhibiteurs pour les réactions en amont utilisant les ADN extraits, ces derniers sont ensuite purifiés. La purification peut se faire directement à partir des produits PCR ou à partir du gel d'électrophorèse à l'aide du kit «Geneclean turbo kit » de GE Healthcare (selon les recommandations du fournisseur). Dans le second cas, la zone du gel contenant le fragment d'ADN d'intérêt (en connaissant par exemple la taille attendue issue de l'amplification PCR) est spécifiquement excisée du gel d'électrophorèse, puis purifiée.

2.2.2.5 Dosage des ADNs

Dans le but de déterminer la quantité d'ADN obtenue après extraction et dans les produits PCR, les ADN sont dosés à l'aide de différentes techniques.

L'ADN peut être dosé à l'aide d'un spectrophotomètre, fournissant une concentration en ng/µL à partir d'une absorbance à 260 nm. L'appareil indique en plus de l'absorbance à 260nm, celles à 230 et à 280nm ainsi que les rapports 260/230nm et 260/280nm, indiquant indirectement la pureté de l'ADN. Le ratio 260/280nm informe d'une contamination en protéines (si inférieur à 1.7) ou en ARN (si supérieur à 2), tandis que le rapport 260/230nm révèle une contamination en molécules organiques. Cependant, la présence d'impuretés ou de pigments dans l'échantillon peut fausser la valeur obtenue.

Pour les techniques de séquençage NGS, les ADNs sont dosés de façon plus précise et fiable via l'utilisation du kit Quant-iT™ PicoGreen® (Invitrogen) ou du Fluorimètre Qubit®.

Le kit Quant-iT™ PicoGreen® contient un intercalent de l'ADN qui permet le dosage de l'ADN en se référant à une gamme étalon. Le dosage se fait dans des microplaques noires de 96 puits (fournissant un arrière-plan neutre pour les dosages fluorescents) placées dans un spectrofluorimètre

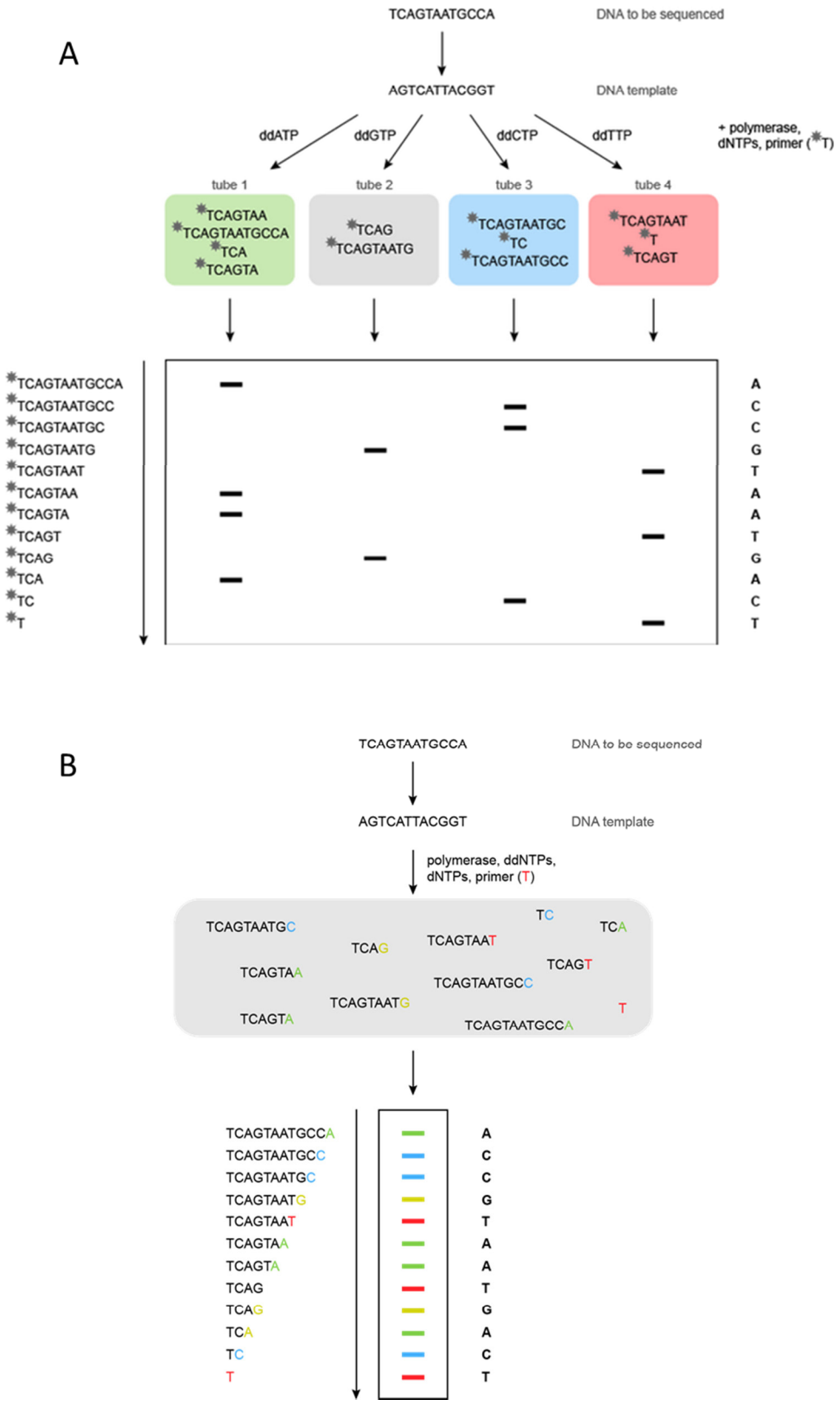


Figure 51 : Principe du séquençage Sanger avec un marqueur radioactif (A) ou un marqueur fluorescent (B) (www.atdbio.com).

dNTPs : désoxyribonucléotides ; ddNTPs (ddATP, ddCTP, ddGTP ou ddTTP) : didésoxyribonucléotides

FLX-Xenius (SAFAS, Monaco). Pour chaque échantillon, 2 µl d'ADN sont mélangés à 98 µL de tampon tris-EDTA (TE 20x ; 200 mM Tris-HCl, 20 mM EDTA, pH 7.5) puis 100 µl de réactif (Quant-iT™ PicoGreen® dsDNA reagent dilué 200 fois dans du TE 20x) sont ajoutés. Le mélange est ensuite incubé cinq min à température ambiante, puis le réactif est excité à 420 nm et le signal lié à l'émission de fluorescence est mesuré à 530 nm. Pour la gamme étalon, 100 µl d'ADN de phage Lambda plus ou moins dilué avec du tampon est mis en présence de 100 µl de réactif selon les recommandations du fournisseur. Pour chaque échantillon, 2 réplicats techniques sont réalisés. De plus, trois lectures sont réalisées lors de chaque dosage et une moyenne de ces valeurs est calculée.

Le Qubit® (Thermo Fisher Scientific) est un fluorimètre de paillasse conçu pour évaluer la quantité d'ADN d'un plus petit nombre d'échantillons. Il est utilisé en association avec un kit contenant des tubes de dosage spécifique et un colorant fluorescent qui est un intercalent de l'ADN. Après un étalonnage de la machine avec des standards, 1 à 20 µl d'échantillon est mélangé au réactif (qsp 200 µl) dans un tube spécifique. L'échantillon est alors vortexé puis incubé 2 min à température ambiante. La lecture se fait pour chaque échantillon un par un dans le fluorimètre qui affiche la quantité en ng/µl directement. Le Qubit® permet également de doser les ARNs et les protéines à l'aide de colorants spécifiques.

Les ADNs dosés sont ensuite mélangés de façon équimolaire (à une concentration finale de 1 µg dans 100 µl) pour les produits PCR séquencés par Ion Torrent et par Illumina MiSeq.

2.2.3 Séquençage

2.2.3.1 Sanger

Le séquençage de produits d'amplification PCR pour l'identification des champignons a été réalisé par la méthode de séquençage Sanger par la société Beckman Coulter Genomics ; La méthode de séquençage Sanger est basée sur une synthèse enzymatique sélective qui est stoppée à chaque nucléotide fournissant ainsi la séquence nucléotidique. Elle consiste à réaliser quatre réactions de polymérisation de l'ADN en parallèle à l'aide d'amorces, d'une polymérase, des quatre désoxyribonucléotides (dATP, dCTP, dGTP, dTTP) et une faible concentration de l'un des quatre didésoxyribonucléotides (ddATP, ddCTP, ddGTP ou ddTTP). Ces didésoxyribonucléotides (ddNTP), ayant un groupement OH supplémentaire, stoppent la réaction de polymérisation et empêchent l'élongation quand ils sont intégrés. Pour les quatre réactions, ayant chacune un ddNTP différent, des fragments d'ADN stoppés à différentes tailles seront présents. Ces fragments sont ensuite séparés par électrophorèse sur un gel de polyacrylamide, ce qui permet ainsi de repérer dans la séquence la position des A grâce au 1er tube, des T grâce au 2ème, des C grâce au 3^{éme} et des G grâce au 4^{éme} tube (Figure 51A). La détection des fragments ainsi synthétisés se fait en incorporant un traceur qui était initialement radioactif et qui est maintenant fluorescent (Figure 51B).

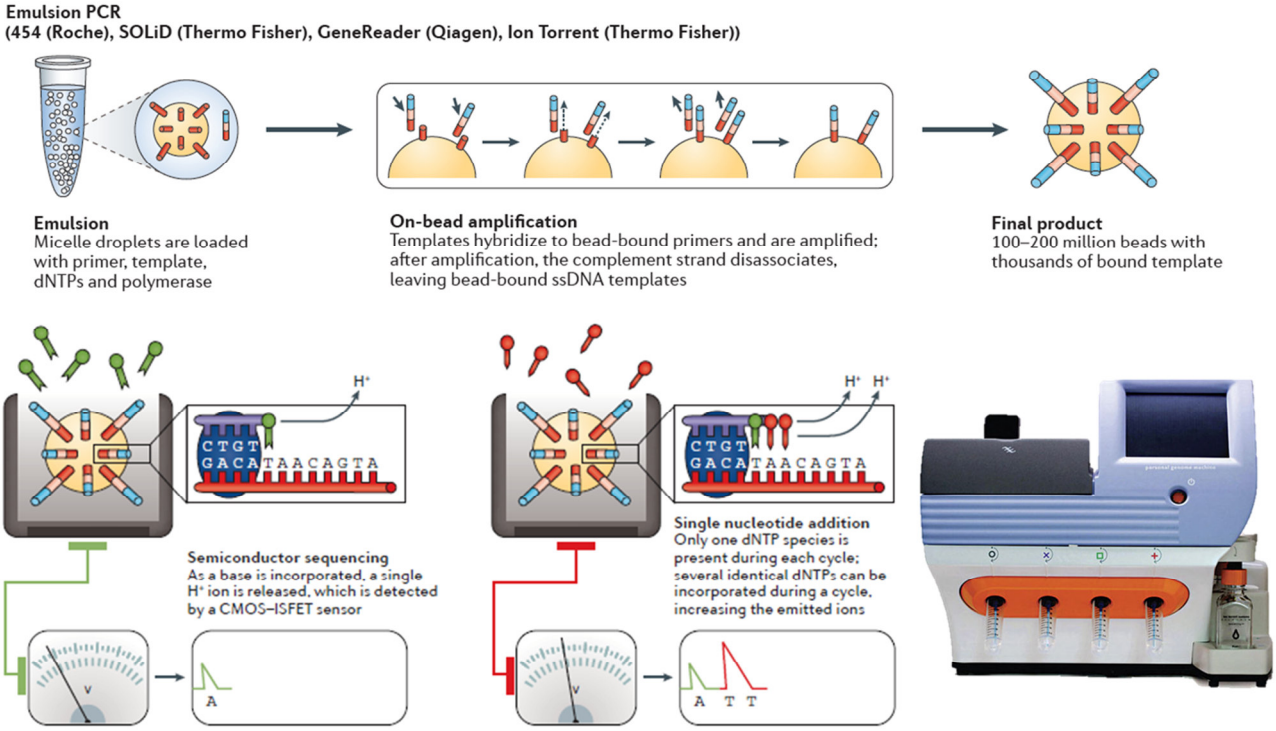


Figure 52 : Principe du séquençage Ion Torrent PGM (Goodwin *et al.*, 2016).

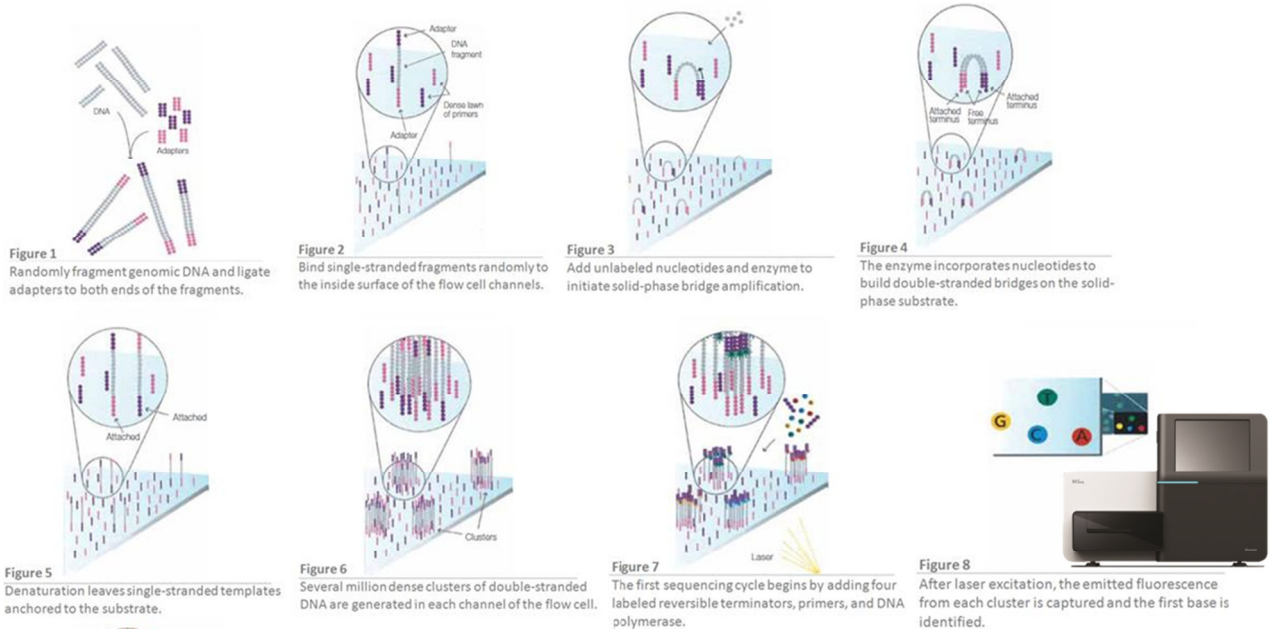


Figure 53 : Principe du séquençage Illumina MiSeq (Illumina.com).

2.2.3.2 Ion Torrent

Le séquençage des produits d'amplification PCR pour l'analyse des communautés microbiennes des parcelles du site de Tavaux a été réalisé par Ion Torrent PGM (Personal Genome Machine) au National Research Council Canada (NRCC) de Montréal, en collaboration avec le Dr E Yergeau et le Prof. C Greer. Après extraction et amplification des ADN à l'aide d'amorces comportant une étiquette spécifique pour chaque échantillon, les produits PCR sont mélangés de façon équimolaire.

La technologie Ion Torrent PGM repose sur l'utilisation d'une puce semi-conductrice comprenant plusieurs puits. L'incorporation d'un nucléotide par la polymérase dans la molécule d'ADN libère un proton, ce qui entraine une variation du pH local qui est alors détectable. Le séquenceur PGM délivre les nucléotides les uns après les autres afin d'établir la séquence nucléotidique. En effet, si le nucléotide injecté s'intègre dans la séquence d'ADN, il sera incorporé et un ion hydrogène sera libéré. Le pH de la solution présente dans le puits va donc changer et cette variation sera détectée par le détecteur d'ions qui transforme l'information chimique en donnée numérique. S'il y a deux bases identiques sur le fragment d'ADN, le voltage sera doublé et la puce enregistre deux bases identiques à la suite. Si le nucléotide injecté n'est pas le suivant sur la molécule d'ADN, il n'y a pas de changement de voltage enregistré et la machine passe au nucléotide suivant. C'est donc une détection directe et chaque incorporation de nucléotides est réalisée en quelques secondes permettant des temps d'exécution très courts (Figure 52).

La première étape de ce séquençage consiste à générer une bibliothèque de fragments d'ADN flanqués par les adaptateurs de l'Ion Torrent. Ceci a été réalisé lors des PCR précédentes avec les amorces spécifiques (§2.2.2.2). Dans un second temps, les fragments de la bibliothèque sont amplifiés par clonage sur les billes Ion Sphere™ et l'amplification clonale est accomplie en émulsion PCR (emPCR). En respectant un ratio nombre de molécules d'ADN/nombre de billes, un seul fragment d'ADN sera fixé sur chaque bille. Les billes sont ensuite placées dans une émulsion contenant les composés nécessaires à la réaction de polymérisation en chaîne et permettant d'individualiser chaque bille. Les billes revêtues des fragments d'ADN sont mises en contact avec la puce et ensuite déposées dans le micro-puits de la puce par une étape de centrifugation courte. La puce est placée sur la machine PGM afin d'effectuer le séquençage.

2.2.3.3 Miseq

Le séquençage des ADNs pour l'analyse des communautés microbiennes des parcelles des sites de Leforest et de Pierrelaye a été réalisé avec la plateforme de séquençage Illumina MiSeq de la société Microsynth (Suisse). Les ADNs extraits, après vérification de leur qualité et de leur concentration, ont été envoyés à Microsynth pour la réalisation de toutes les étapes aboutissant au séquençage.

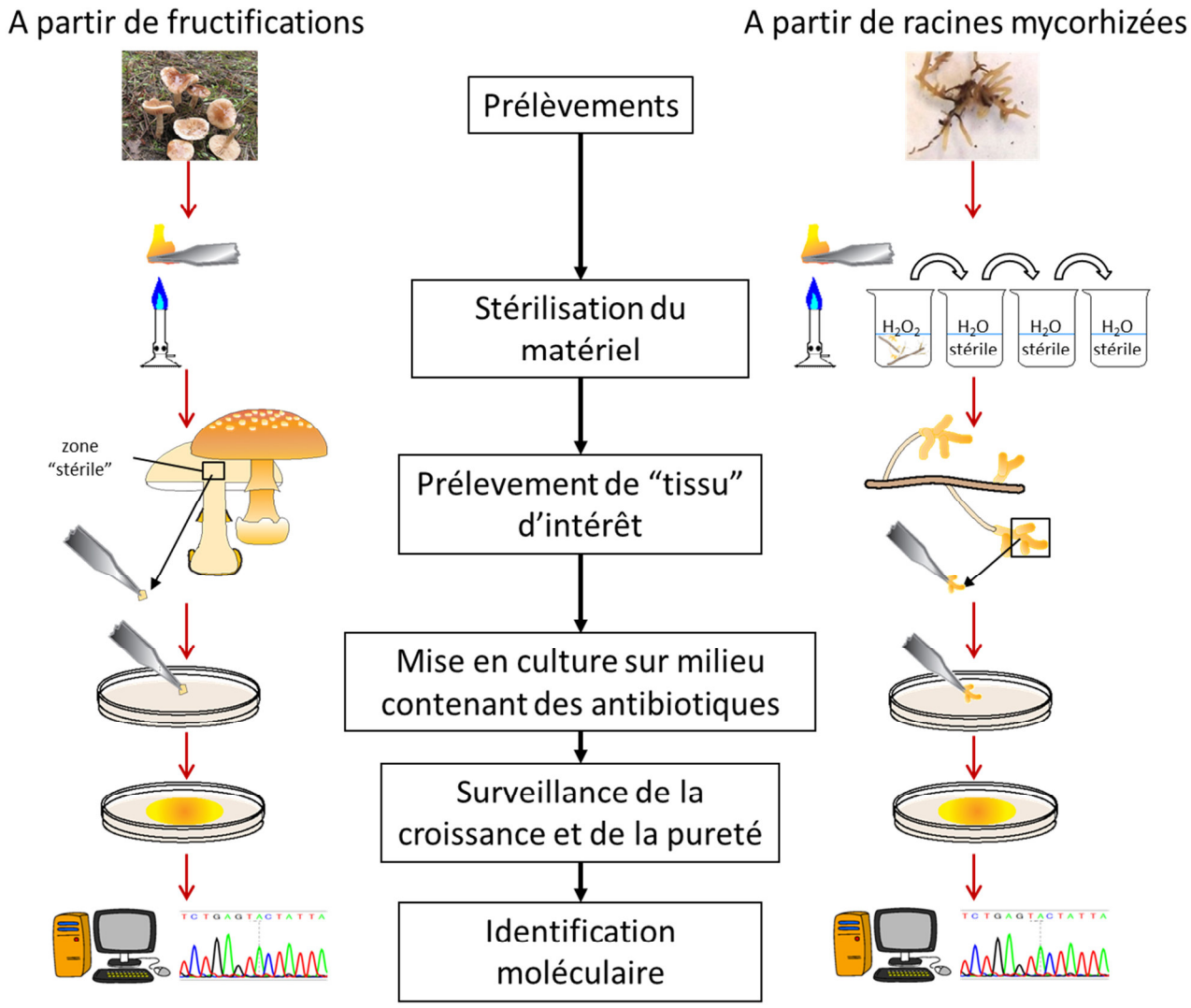


Figure 54 : Principales étapes de l'isolement de souches fongiques à partir de fructifications ou de racines mycorhizées (Lacercat-Didier, 2013).

La technologie développée par Illumina repose sur une amplification en ponts des fragments d'ADN sur un support solide (flow cell) suivie d'un séquençage par synthèse chimique des ADN préalablement amplifiés. La première étape est l'ajout d'un adaptateur aux ADNs qui permet l'ancrage sur le support solide. Une fois fixés, les ADNs sont amplifiés par l'ajout d'enzyme et de dNTP entrainant ainsi la formation d'ADN double brin. Ces ADNs double brin sont séparés par une étape de dénaturation, puis une seconde étape d'amplification permet la création de plusieurs fragments d'ADN double brin dans chaque canal du support solide. Le premier cycle de séquençage débute par l'ajout de quatre terminateurs de chaîne réversibles marqués par des fluorochromes, d'amorces et d'ADN polymérase. Après une excitation par un laser, la fluorescence émise par chaque groupe est capturée et la première base est identifiée (Figure 53). Pour les bases suivantes, la première étape de séquençage et l'excitation sont répétées autant de fois que nécessaire. Pour l'analyse des communautés microbiennes de nos échantillons, les ADNs extraits ont été amplifiés une première fois à l'aide d'une amorce ciblant la région d'intérêt puis après purification, une deuxième PCR vient ajouter les adaptateurs et les étiquettes. Comme pour l'Ion Torrent PGM, cette analyse nécessite le mélange équimolaire des produits PCR (multiplexage) avant séquençage des échantillons. Il est donc essentiel de garder l'information nécessaire pour attribuer chaque séquence obtenue à son échantillon originel. Cette information est contenue dans un oligonucléotide, différent pour chaque échantillon, qui joue le rôle d'une étiquette (tag).

Le séquenceur MiSeq est le seul séquenceur de paillasse qui peut produire des lectures de 2 x 300 paires de bases (pb) en une seule réaction de séquençage. Cette méthode permettant un séquençage bidirectionnel diminue ainsi les erreurs de séquençage.

2.2.4 Quantification du taux d'ectomycorhization

Afin d'évaluer le taux d'ectomycorhization des racines de peuplier des différents sites expérimentaux étudiés, les racines lavées sont analysées à l'aide d'une loupe binoculaire. L'évaluation du taux d'ectomycorhization est effectuée en comptant le nombre d'apex mycorhizés et en le rapportant au nombre total d'apex. Un minimum de 300 apex a été analysé par échantillon. Après comptage, des mycorhizes, représentatives de la diversité observée, sont prélevées afin de réaliser des isolements de souches ectomycorhiziennes en culture pure sur milieu gélosé.

2.2.5 Techniques de microbiologie

2.2.5.1 Isolement de souches fongiques ectomycorhiziennes

2.2.5.1.1 A partir de carpophores

En conditions stériles, les carpophores sont sectionnés en deux, et un fragment d'environ 1 cm^3 est découpé à l'aide d'un scalpel dans la zone interne du chapeau (Figure 54). Les fragments sont

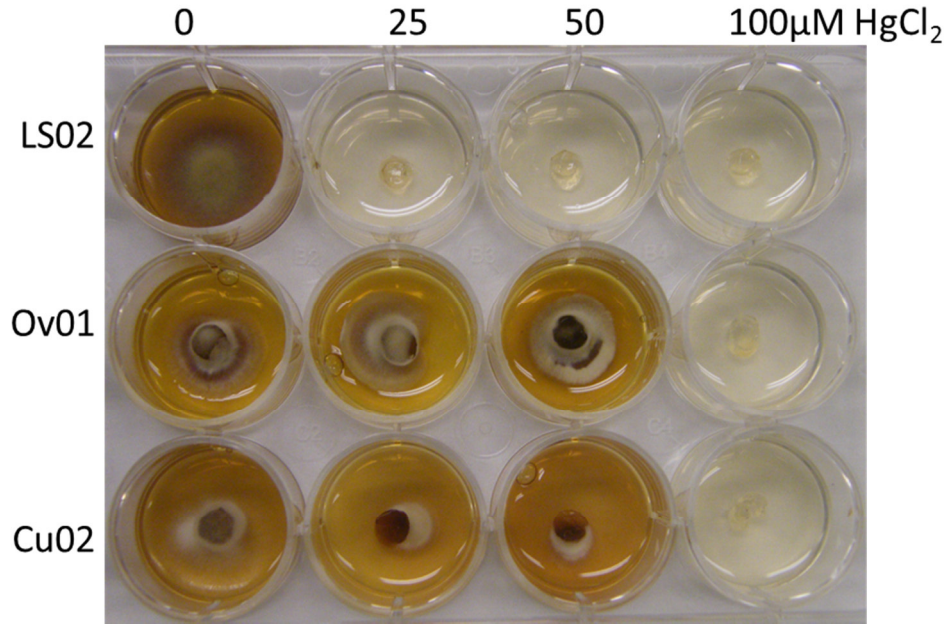


Figure 55 : Exemple d'un résultat de test de tolérance au Hg en plaque 12 puits.

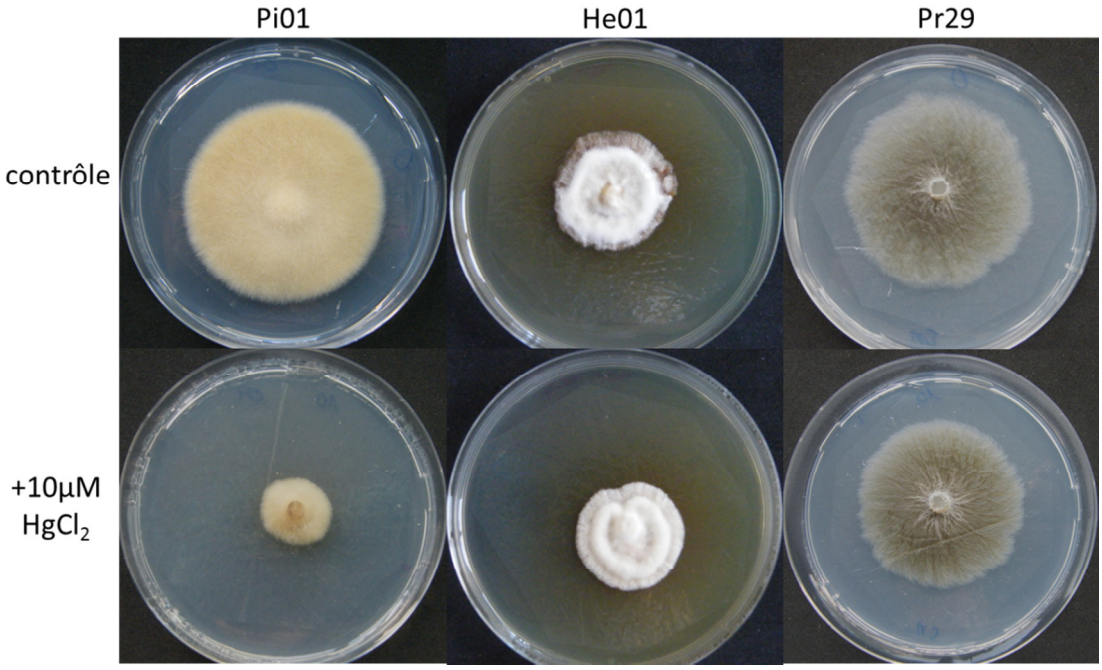


Figure 56 : Exemple d'un résultat de test de tolérance au Hg en boite de Pétri.

La croissance de la souche de *Paxillus involutus* Pi01 montre une diminution dès la première concentration en Hg (10µM) contrairement aux souches *Paxillus involutus* He01 et *Cadophora sp.* Pr29.

ensuite déposés sur différents milieux gélosés supplémentés avec 100 mg/l d'ampicilline et 100 mg/l de chloramphénicol (Tableau V ; Tableau VI ; Tableau VII ; Tableau VIII).

2.2.5.1.2 A partir de racines d'espèces ligneuses

Les parties contenant plusieurs ectomycorhizes provenant des racines précédemment lavées (§2.2.3.2) sont prélevées. Une étape de désinfection des racines est ensuite nécessaire afin d'éliminer un maximum d'organismes saprophytes (bactéries ou champignons). Une immersion de 30 sec des échantillons est ainsi réalisée dans de l'eau oxygénée (H_2O_2) à 35 % (v/v) (Menkis *et al.*, 2005; Menkis & Vasaitis, 2011). Les échantillons sont ensuite rincés trois fois à l'eau distillée stérile (Figure 54).

Enfin, chaque mycorhize sélectionnée est déposée sur une boîte de Pétri (Ø 55 mm) contenant un milieu gélosé supplémenté de 100 mg/l d'ampicilline et 100 mg/l de chloramphénicol. Plusieurs milieux sont testés car les différentes espèces fongiques requièrent différents éléments nutritifs (Tableau V ; Tableau VI ; Tableau VII ; Tableau VIII).

2.2.5.2 Entretien des souches fongiques

Les souches fongiques en collection au laboratoire sont conservées par repiquages successifs sur un nouveau milieu correspondant au milieu de prédilection de la souche et les cultures sont placées à 25 °C à l'obscurité (§2.1.2.1 et Annexe 5).

Pour certains espèces fongiques, une conservation à long terme est possible (Annexe 5). Les souches sont cultivées sur un milieu gélosé additionné de glycérol (5% v/v) durant 2 à 4 semaines à 25°C, puis placé à 4°C pendant 4 jours. Le mycélium est alors découpé en petits morceaux et placé dans un tube contenant une solution de glycérol à 10% (v/v). Ce dernier est ensuite congelé lentement à -80°C et peut être conservé plusieurs mois/années au congélateur (Gorfer *et al.*, 2007).

2.2.5.3 Tests de tolérance des souches fongiques aux ETMs

2.2.5.3.1 Test en plaques 12 puits

Afin de sélectionner les champignons en collection les plus tolérants aux ETMs, des tests préliminaires ont été réalisés en plaques de 12 puits contenant 1 mL de milieu malt gélosé ou liquide supplémenté avec différentes concentrations de divers ETMs : Hg (25, 50, 100 et 120 µM $HgCl_2$), Zn (1, 2, 3, 5, 10 mM $ZnCl_2$), Cd (10, 25, 50, 100 µM $CdCl_2$), Pb (100, 250, 500, 1000 µM $Pb(NO_3)_2$), Ni (20, 100, 200, 350, 500 µM $NiCl_2$), Cu (100, 250, 500, 1000 µM $CuSO_4$), et Mn (4, 10, 20, 100 mM $MnCl_2$). Chaque puits est ensemencé avec un implant de 0,5 cm de diamètre provenant d'une culture-mère fongique. Les cultures sont incubées à 25°C à l'obscurité pendant 2 à 4 semaines (Figure 55). Ce test est utilisé pour déterminer la concentration minimale inhibitrice (MIC, en anglais) qui est la plus petite concentration capable d'inhiber totalement la croissance d'une souche donnée.

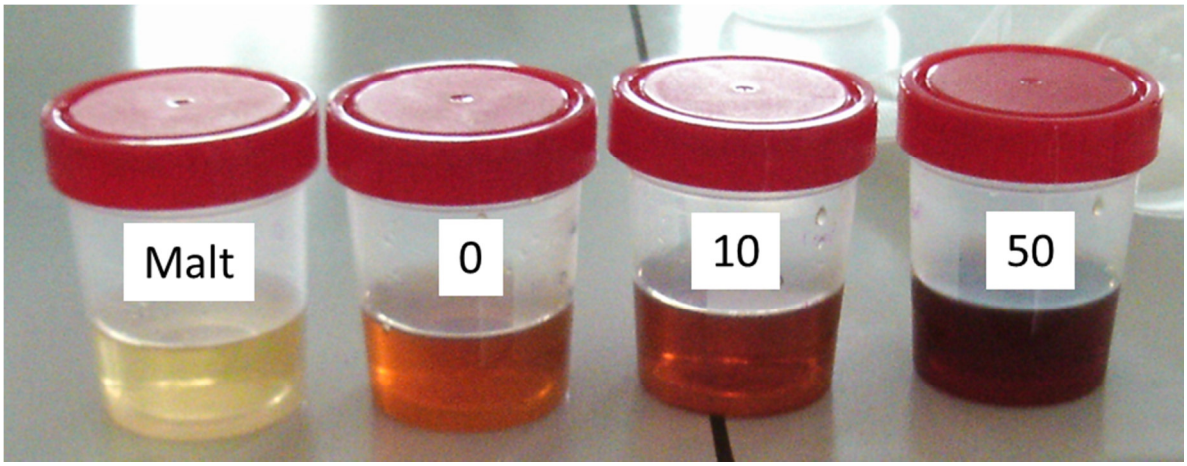


Figure 57 : Aspect du milieu malt après une semaine d'incubation en présence ou non du champignon *Paxillus involutus* He01 et en présence de différentes concentrations de Hg.

Malt : milieu de culture sans champignon ; 0, 10 et 50 : milieux auxquels des concentrations en $HgCl_2$ (µM) ont été ajoutées pour la culture du champignon.

2.2.5.3.2 Test sur boîte de Pétri

Ces tests ont été réalisés pour affiner les données concernant le niveau de tolérance au Hg de certaines souches. Les souches sélectionnées ont été cultivées en présence de différentes concentrations de Hg (10, 25, 50, 75 et 100 µM $HgCl_2$) sur milieu malt gélosé. Le milieu sans ajout de Hg constitue le milieu témoin. Afin de permettre une récolte plus aisée des champignons, les différentes souches fongiques ont été cultivées sur une feuille de cellophane déposée sur le milieu gélosé. Les membranes de cellophane ont été préalablement bouillies pendant 10 min dans une solution d'EDTA à 10 mM.

Chaque boîte est ensemencée avec un implant de 0,5 cm de diamètre. Pour chaque concentration de Hg, 5 réplicats biologiques sont réalisés. Les incubations sont également réalisées à 25°C et à l'obscurité. Les souches ont été récoltées lorsque la colonie avait atteint un diamètre de 6 cm sur le milieu soit de 12 à 42 jours de croissance (Figure 56). La croissance radiale de chacune des colonies a été mesurée hebdomadairement, puis les masses sèches ont été mesurées après récolte et lyophilisation des échantillons pendant 2 jours.

2.2.5.3.3 Tests en milieu liquide

Dans le but de mesurer la quantité de Hg restant dans le milieu à la fin de la culture, des tests de tolérance au Hg ont ainsi été réalisés en milieu liquide dans des flacons pour deux souches (Pi01 et He01) de *P. involutus*.

Des souches âgées d'une semaine sont préalablement repiquées (implants de 0,5 cm) sur du milieu N2P2 gélosé recouvert d'une membrane de cellophane. Les boites de Pétri sont incubées à 25 °C pendant 7 et 14 jours pour *P. involutus* Pi01 et He01, respectivement. Après ces temps d'incubation, les colonies sont alors mises à flotter dans des flacons de 125 ml contenant 40 mL de milieu malt liquide. Ce transfert est facilité grâce à la membrane de cellophane qui permet de récupérer la colonie intacte. Les souches sont incubées 4 jours (adaptation au milieu liquide) avant d'ajouter ou non différentes concentrations de Hg (10 et 50 µM) (Figure 57). Les colonies sont récoltées à différents temps de croissance : 30 min, 12 h, et 1 semaine. En parallèle, le milieu de culture est également prélevé. Des témoins consistent en des flacons ne contenant que du milieu de culture (Figure 57). Après récolte, les colonies et les milieux sont conservés à -80 °C avant analyse.

2.2.5.3.4 Tests de désorption à l'EDTA

Afin d'évaluer la quantité de Hg présente dans les hyphes et la fraction adsorbée au niveau des parois cellulaires, des tests de désorption ont été réalisés sur une partie des souches cultivées en milieu liquide. Les souches récoltées sont ainsi divisées en deux à l'aide d'une lame de scalpel. Une moitié est plongée dans 5 mL d'EDTA (10 mM à pH 5,35) pendant 2 min puis rincée trois fois à l'eau distillée. L'autre moitié est seulement rincée à l'eau distillée. Les échantillons sont ensuite lyophilisés.

2.2.5.3.5 Tests d'efflux de Hg

Une expérience visant à suivre la cinétique d'efflux de ce métal hors des cellules a été réalisé. Cette expérience a été réalisée avec les deux souches de *P. involutus* (Pi01 et He01), à raison de trois réplicats par souche et condition. Des implants de 15 mm sont réalisés à partir des colonies fongiques. Les implants sont ensuite mis à flotter dans 40ml d'une solution d'absorption (0,5 mM $CaCl_2$; 2 mM MES à pH 4,5 et 10 µM $HgCl_2$) pendant 30 min ou 12 h à température ambiante. A l'aide d'un appareil de filtration, les implants sont récupérés et rincés 3 fois à l'aide de la solution de lavage (0,5 mM $CaCl_2$ et 2 mM MES à pH 4,5). Les implants sont ensuite incubés à 4°C dans 40 mL d'une solution de désorption (5 mM $CaCl_2$ et 2 mM MES à pH 4,5). Des prélèvements de 200 µl de solution de désorption sont réalisés à différents temps sur une période de 10 h et selon les séquences suivantes : toutes les 2 min jusqu'à 20 min, toutes les 5 min jusqu'à 40 min, toutes les 10 min jusqu'à 90 min, toutes les 30 min jusqu'à 6 h, toutes les 90 min jusqu'à 8 h et au temps final de 10 h pour le dernier prélèvement. La quantité de Hg est ensuite déterminée dans les solutions prélevées et les implants fongiques (§2.2.7.5.1).

2.2.6 Lyophilisation et broyage des souches

Pour une meilleure conservation dans le temps, les échantillons sont séchés, broyés et lyophilisés. Les souches fongiques sont lyophilisées pendant 2 jours et sont broyées à l'aide de deux billes en tungstène de 3mm dans un broyeur (model MM400; Retsch) pendant 1 min à 30 Hertz. La masse sèche est alors mesurée avant d'être analysée pour différents paramètres : quantité de Hg (§2.2.7.5.1), dosage des protéines (§2.2.7.2) et mesure des activités antioxydantes (§2.2.7.4).

2.2.7 Techniques biochimiques

Les analyses suivantes ont été réalisées avec les souches de *P. involutus* Pi01 et He01.

2.2.7.1 Extraction des protéines

A partir de la poudre lyophilisée des colonies fongiques, les protéines et les autres constituants cellulaires sont séparés. Pour cela, une solution d'extraction a été préparée et conservée à 4°C. Cette dernière contient du Tris-HCl (10 mM) qui sert de tampon, du fluorure de phénylméthylsulfonyle (PMSF ; 0,1 mM) qui est un inhibiteur des protéases et du tween 80 (0.05 % (v/v)) qui permet de solubiliser les protéines. Un ml de cette solution est ajouté à 10 mg de poudre lyophilisée, le mélange est ensuite vortexé quelques secondes. 190 µl de la suspension sont prélevés pour passage à l'analyseur de Hg (AMA) après ajout de 10 µl de HNO3 (65% v/v). Ensuite, une centrifugation à 10 000 rpm à 4°C pendant 45 min est appliquée et permet d'obtenir un surnageant contenant les protéines solubles.

2.2.7.2 Dosage des protéines solubles

La concentration des protéines solubles totales du surnageant et des milieux de culture est analysée par la méthode de Bradford à l'aide d'un kit commercial (Coomassie Bradford ProteinAssay Kit, Thermo Scientific). La méthode de Bradford est un dosage colorimétrique qui utilise comme réactif le bleu de coomassie. Le réactif de bleu de coomassie, en se liant avec les protéines, produit un composé quantifiable à 595 nm. Pour nos analyses, 15 µl d'échantillon (surnageant ou milieu de culture) sont mélangés à 750 µl de réactif de Coomassie. Les concentrations en protéines sont calculées en se référant à une courbe étalon réalisée avec une protéine standard (albumine de sérum bovin, BSA).

2.2.7.3 Séparation et visualisation des protéines sur gel de polyacrylamide

Les protéines extraites sont ensuite déposées sur un gel de polyacrylamide natif 4-20% (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad). Cette technique permet la séparation selon leur poids moléculaire sous l'effet d'un champ électrique. Les protéines extraites sont ajustées à 5 µg/ml et 25 µl d'extrait sont mélangés à 5 µL de tampon de charge (4x Laemmli, Bio-Rad). Le poids moléculaire est estimé en utilisant un standard (Precision Plus Protein™ Dual Color Standards, Bio-Rad). L'électrophorèse est réalisée pendant 1 h à 100V dans une cuve Mini-Protean II contenant un tampon de migration (1xTris/Glycine/Méthanol, Bio-Rad). Les gels sont révélés dans une solution de coloration qui est composée de bleu de Coomassie (bleu brillant 0.25% w/v, méthanol 45.5% v/v et de l'acide acétique 9% v/v) pendant 45 min. Cette solution de coloration permet de mettre en évidence les différentes bandes présentes sur le gel et de fixer les protéines. Après la coloration, le gel est placé deux jours dans une solution de décoloration qui contient de l'acide acétique (7.5% v/v) et du méthanol (5% v/v).

Les bandes obtenues sont découpées à l'aide d'un papier millimétré tous les 0,5 cm. Ces morceaux de gel sont placés dans des tubes Eppendorf et dosés à l'AMA.

2.2.7.4 Dosage des capacités antioxydants

Le dosage des capacités antioxydantes permet de déterminer si la colonie fongique est soumise à un stress oxydant et si elle y répond en mettant en place des mécanismes antioxydants. Ainsi la poudre lyophilisée a été utilisée pour déterminer différents paramètres : peroxydation des lipides, activité antioxydante totale, activité superoxyde dismutase et activité catalase.

2.2.7.4.1 Dosage de la peroxydation lipidique (MDA)

La peroxydation des lipides des échantillons est évaluée à l'aide d'un kit (Lipid Peroxidation Assay Kit, Sigma-Aldrich) selon les recommandations du fournisseur. Pour ce kit 10 mg de poudre de champignons sont utilisés.

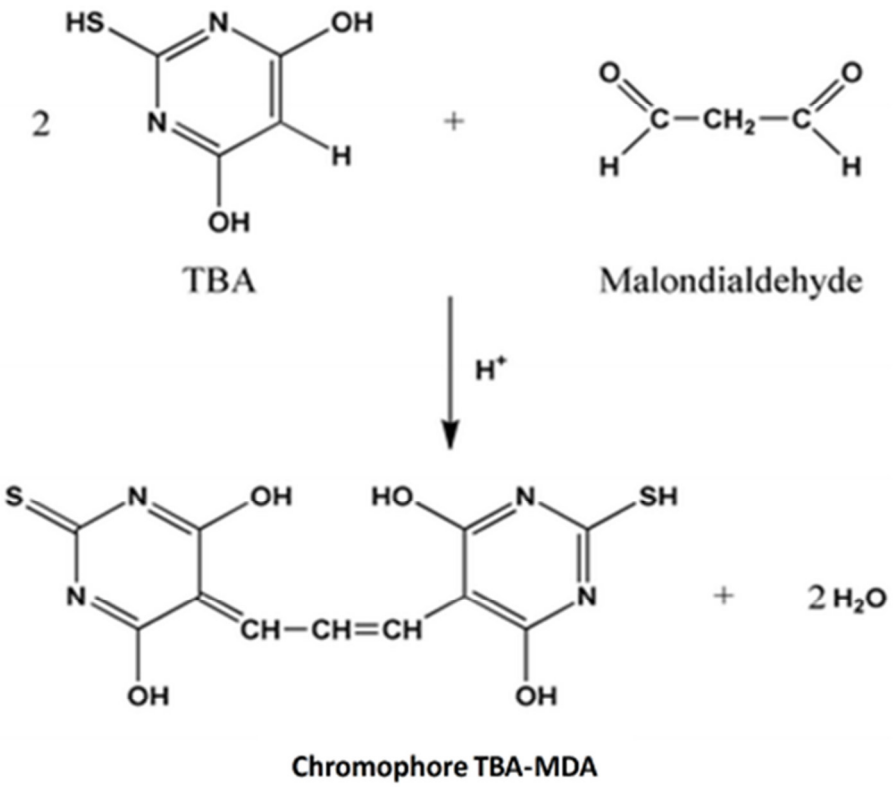


Figure 58 : Réaction entre le malondialdéhyde et l'acide thiobarbiturique.

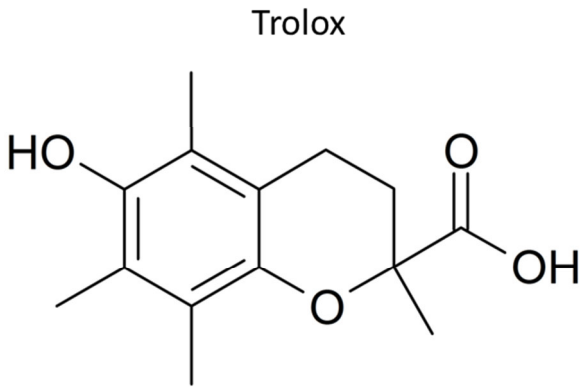


Figure 59 : Molécule de Trolox.

La peroxydation lipidique correspond à la dégradation des lipides se produisant à la suite de dommages oxydatifs et est un marqueur utile pour quantifier le stress oxydatif présent. Les lipides polyinsaturés sont sensibles à une attaque par oxydation, généralement en espèces réactives de l'oxygène, ce qui entraîne une réaction en chaîne bien définie avec la production de produits finis tels que le malondialdéhyde (MDA).

Dans ce kit, la peroxydation des lipides est déterminée par la réaction de MDA avec l'acide thiobarbiturique (TBA) pour former un produit coloré (mesuré à 532 nm) dont la quantité sera proportionnelle à celle du MDA présent (Figure 58).

2.2.7.4.2 Dosage de la capacité antioxydante totale

Les oxydants, tels que les espèces réactives de l'oxygène et les espèces réactives de l'azote, peuvent causer des dommages oxydatifs aux lipides cellulaires, membranaires, aux protéines et à l'ADN. Les antioxydants peuvent piéger ces radicaux libres et prévenir le stress oxydatif cellulaire par des mécanismes enzymatiques et non-enzymatiques. Le système enzymatique antioxydant comprend la catalase, la superoxyde dismutase et les peroxydases. Les tocophérols, les carotènes, la vitamine A, et ubiquinols fonctionnent comme des antioxydants solubles dans les lipides, tandis que, le glutathion et l'ascorbate font partie des antioxydants solubles dans l'eau. La mesure de la capacité antioxydante non enzymatique totale d'échantillons biologiques est une indication de leur capacité à lutter contre les dommages oxydatifs induits par le stress dans les cellules. Ce kit permet de déterminer aussi bien la concentration en petites molécules antioxydantes (non enzymatiques) que la combinaison de petites molécules et de protéines antioxydantes (telles que la catalase) par la transformation de l'ion Cu^{2+} en Cu^{+}. Les ions réduits Cu^{+} chélatent une sonde colorimétrique, ce qui donne un large pic d'absorbance à 570 nm, qui est proportionnel à la capacité anti-oxydante totale. Le kit donne la capacité antioxydante en équivalents Trolox. Le Trolox, un analogue de la vitamine E soluble dans l'eau, sert de norme antioxydante (Figure 59).

La capacité antioxydante totale des échantillons est évaluée à l'aide d'un kit (Total AntioxidantCapacityAssay Kit, Sigma-Aldrich) selon les recommandations du fournisseur. Un microlitre d'extrait protéique a été utilisé lors de chaque dosage.

2.2.7.4.3 Dosage de l'activité catalase

La catalase est une enzyme antioxydante ubiquitaire dans les cellules aérobies contenant un système de cytochrome. La catalase permet la décomposition du peroxyde d'hydrogène en eau et oxygène (Figure 60). Le peroxyde d'hydrogène est formé, dans la cellule eucaryote, en tant que sous-produit de diverses oxydases et des réactions de la superoxyde dismutase. Le peroxyde d'hydrogène est très nuisible à la cellule et son accumulation provoque l'oxydation de cibles cellulaires telles que l'ADN, les protéines et les lipides conduisant à une mutagenèse et la mort cellulaire. L'élimination du peroxyde d'hydrogène à partir de la cellule par la catalase fournit une protection contre les dommages

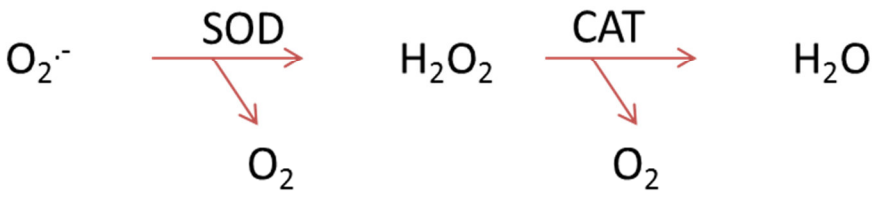


Figure 60 : Réactions entre les radicaux libres et les enzymes antioxydantes Superoxyde dismutase (SOD) et Catalase (CAT).

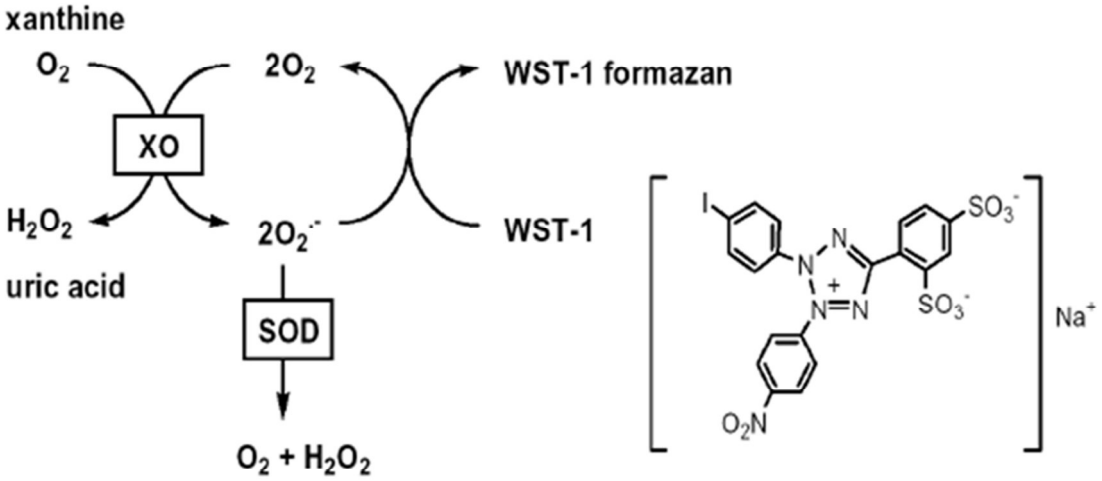


Figure 61 : Principe du dosage de l'activité SOD.

XO : xanthine oxydase ; WST-1 : sel de tétrazolium monosodium soluble

oxydatifs de la cellule. Tout d'abord, la catalase transforme le peroxyde hydrogène en eau et oxygène (voie catalytique), puis cette réaction enzymatique est arrêtée avec un azide de sodium. La quantité de peroxyde d'hydrogène restant est déterminée par une méthode colorimétrique. La méthode colorimétrique utilise un phénol substitué qui, en présence de peroxyde d'hydrogène, donne un colorant rouge qui absorbe à 520 nm.

L'activité de la catalase est évaluée à l'aide d'un kit (Catalase Assay Kit, Sigma-Aldrich) selon les recommandations du fournisseur. Pour ce kit 10 µl d'extrait protéique non dilué sont utilisés pour chaque dosage.

2.2.7.4.4 Dosage de l'activité superoxide dismutase

La superoxide dismutase (SOD) qui catalyse la dismutation de l'anion superoxyde ($O2^{\cdot-}$) en peroxyde d'hydrogène et oxygène, est l'une des enzymes antioxydantes les plus importantes. Afin de déterminer l'activité de la SOD, une méthode indirecte à l'aide d'un sel de tétrazolium soluble dans l'eau (WST-1) est ici utilisée. Le sel de tétrazolium WST-1 produit un colorant soluble dans l'eau, le formazan, lors de la réduction d'un anion superoxyde. La réduction d'un anion superoxyde est proportionnelle à l'activité xanthine oxydase (XO) qui est inhibée par la SOD. L'activité de la SOD est alors mesurée comme une activité d'inhibition et peut être quantifiée en mesurant la diminution de l'apparition de la couleur à 440 nm (Figure 61).

L'activité de la SuperOxyde Dismutase (SOD) est évaluée à l'aide d'un kit (SOD Assay Kit, Sigma-Aldrich) selon les recommandations du fournisseur. Pour ce kit 20 µl d'extrait protéique sont utilisés lors de chaque dosage.

2.2.7.5 Dosage des ETMs

2.2.7.5.1 Dosage du Hg par l'AMA 254

L'analyseur de Hg AMA 254 (Altec) a été utilisé pour doser la quantité de Hg dans tous les échantillons aussi bien liquides que solides. AMA 254 signifie Advanced Mercury Analyzer, il est basé sur le principe de l'absorption atomique à 254nm. Il est constitué d'un passeur automatique pour échantillons solides (ASS 254) qui permet l'insertion de nacelles (contenant 500 µl ou 500 mg maximum) dans l'analyseur (Figure 62). Dans ce dernier, l'échantillon placé dans la nacelle est ensuite introduit dans un tube catalytique où il est séché puis décomposé sous oxygène à 850°C. Le Hg s'évapore et est piégé sur un amalgameur en or. L'amalgameur est ensuite chauffé pendant un temps très court, pour libérer les vapeurs de Hg qui sont dirigées vers les cellules de mesure. Les atomes de Hg absorbent alors la radiation émise par la lampe du système photométrique, permettant ainsi de doser le Hg à l'aide d'une gamme étalon fourni avec chaque appareil (http://www.onlinecas.com). Les résultats sont transmis dans l'ordinateur qui donne la valeur de l'absorbance, la concentration en

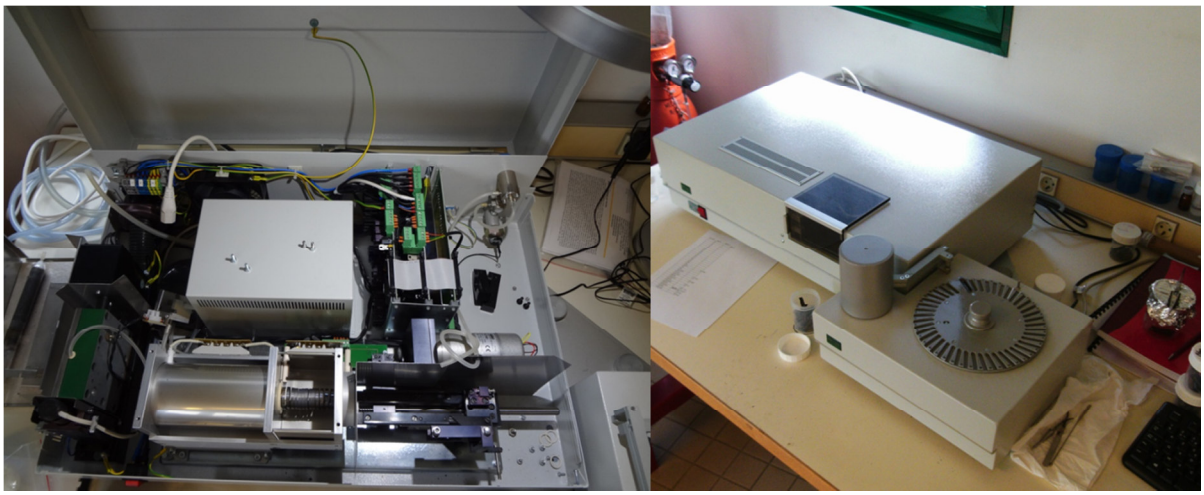


Figure 62 : Analyseur de Hg de type AMA 254 ouvert (à gauche) ou fermé et accompagné de son passeur automatique (à droite).

ng/mg ou ng/µL calculée à partir du poids ou du volume d'échantillon indiqué au préalable. Les échantillons sont mélangés à de l'acide nitrique (3% v/v) qui permet une meilleure solubilisation du Hg. Pour chaque série d'analyse, 3 nacelles vides (blanc) et 2 nacelles contenant du standard (oriental basma tobacco leaves, INCT-OBTL-5, LGC Promochem) sont utilisées afin de vérifier la justesse et la répétabilité des analyses. Les échantillons sont dilués si besoin, afin d'être dosés dans la même gamme que le standard qui est à 20,9 ppb (±1,3).

2.2.7.5.2 Dosage des ETMs par ICP-AES

L'ICP-AES (Inductively Coupled Plasma - Atomic Emission Spectrometry) est une méthode basée sur le principe que le plasma d'argon échange son énergie avec les atomes présents en solution. En retournant à un état moins énergétique, ces atomes émettent des radiations de longueurs d'ondes caractéristiques de l'élément dosé. La spectrométrie d'émission plasma permet de doser la plupart des éléments à des concentrations de l'ordre de 1 µg/l, soit de l'ordre de la partie par milliard en masse (ou ppb, « part per billion »).

Les analyses ICP-AES ont été réalisées au LCE pour les sols de Tavaux et les champignons et au LIEC pour les sols de Pierrelaye et de Leforest.

Pour les échantillons de sol de Pierrelaye et Leforest, 5 g de sol tamisé à 2 mm ont été séchés à 60 °C pendant 48 h, puis mis en incubation avec 50 ml de chlorure de calcium (10 mM) sous agitation (40 rpm) pendant 2 h à température ambiante. Le mélange a d'abord été filtré à l'aide de filtres sans cendres, puis filtré à 0,45 micromètre et finalement acidifié avec 2% (v/v) de HNO_3 avant d'être analysé par ICP-AES (Thermo Fischer Scientific).

Pour les échantillons de sol de Tavaux, 500 mg de sol sont minéralisés dans 2 ml de HCl, 5 ml d'HNO_3 et 33 ml d'eau déminéralisée (protocole modifié à partir de la norme ISO 11466 norme française) pendant 260 min à 100 °C dans une unité Digiprep (SCP sciences Siège social, États-Unis). Les concentrations en ETMs (Al, As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn) ont été déterminées par ICP-AES (Thermo Fischer Scientific) et tous les échantillons ont été analysés avec des matériaux de référence certifiés (Loamy Clay 1, CRM052-050, Sigma Aldrich).

2.2.7.5.3 Analyse physico-chimique des sols

Les caractéristiques physico-chimiques des sols ont été mesurées par le laboratoire SADEF et comprenaient la taille des particules (français Norm X 31e107), le pH (ISO 10390 NF), les carbonates totaux (ISO 10693 NF), le carbone organique total et de la matière organique (MO, ISO 14235 NF), l'azote total (méthode Ntot, DUMAS ISO 13878 NF), le rapport C/N (ISO 13878 NF), le phosphore total disponible (Joret Hebert NF Norm X 31e161), la capacité d'échange cationique (CEC, NF X 31e130) et plusieurs oligo-éléments (NF X 31e108).

2.2.8 Analyses bioinformatiques

Les analyses bioinformatiques des séquences nucléotidiques issues de l'identification des souches fongiques en collection ont été réalisées par différents programmes hébergés sur le serveur http://www.expasy.ch/. Les comparaisons de séquences ont été réalisées à l'aide de l'algorithme BLAST (Basic Alignment Search Tool) (Altschul *et al.*, 1997) hébergé sur le site internet du NCBI (National Center for Biotechnology Information ; http://www.ncbi.nlm.nih.gov/). Les alignements des séquences ont été réalisés en utilisant la méthode ClustalW. Les analyses phylogénétiques et les arbres qui en découlent ont été construits à l'aide du logiciel MEGA version 7 (Kumar *et al.*, 2016). Les analyses phylogénétiques ont été réalisées en utilisant la méthode du maximum de vraisemblance (Maximum Likelihood) mis en œuvre dans MEGA avec l'option « pairwise deletion » pour traiter les lacunes d'alignement, et les distances évolutives ont été calculées en utilisant la méthode « Kimura 2-parameter ». Des tests bootstrap ont été effectués à l'aide de 1000 répétitions.

Les données des séquençages Ion Torrent ont été traitées à l'aide du logiciel Mothur (Schloss *et al.*, 2009). Le fichier « fastq » a été transformé en « .fasta » et les fichiers « .qual » ont été lus en utilisant la commande « fastq.info ». Ensuite, les séquences ont été coupées à l'aide de la commande « trim.seqs » avec les paramètres suivants: longueur minimal = 100 pb, taille de la fenêtre de qualité = 10, fenêtre de qualité moyenne = 20 (Liu *et al.*, 2007, 2008). Les données de qualité filtrées ont ensuite été classées en fonction de la base de données GreenGenes en utilisant la commande « classify.seqs » avec la méthode « wang » et un cutoff de 50. Les séquences de qualité filtrées ont été regroupées à l'aide de CD-HIT (Li & Godzik, 2006) avec un seuil de cutoff de 97%. Les groupes ont ensuite été importés dans Mothur et les indices de diversité ont été calculés. Les distances normalisées pondérées Unifrac entre chaque paire d'échantillons ont été calculées en utilisant le site Web FastUnifrac (Hamady *et al.*, 2010) basé sur la version d'août 2013.

Pour les séquençages issus du Miseq, le logiciel Mothur est utilisé pour toutes les étapes (Schloss *et al.*, 2009). Les séquences obtenues sont tout d'abord assignées à leur échantillon à l'aide de leur étiquette spécifique, puis les séquences de chaque sens de lecture sont regroupées. Les séquences sont ensuite filtrées selon leur taille et leur qualité. Les séquences 16S ont été alignées en utilisant Sumaclust (Mercier *et al.*, 2013) à une identité de 0,99. Les séquences ITS sont simplement regroupées en séquences uniques. Seuls les groupes de séquences ayant au moins 5 séquences pour les bactéries et 10 séquences pour les champignons sont conservés. Les séquences 16S sont ensuite alignées à la base de données Silva pour éliminer les séquences non-16S. La région conservée 28S des séquences ITS sont recherchées par HMM (Hartmann *et al.*, 2010) et les contaminations par des séquences végétales sont éliminées par l'identification des ITS chloroplastiques du peuplier.

Les assignements taxonomiques ont été effectués en utilisant une approche bayésienne (Wang *et al.*, 2007) dans la base de données Greengene (DeSantis *et al.*, 2006) pour les bactéries et la base de données UNITE (Kõljalg *et al.*, 2005) pour les champignons. Enfin, les OTUs ont été construites en utilisant la distance de Needleman et un regroupement moyen d'une distance de 0,03. Les paramètres de diversité sont calculés sur la base d'un ensemble de données dans lesquelles les nombres de lectures par échantillon ont été raréfiés respectivement à 2140 et 1000 séquences par échantillon pour les champignons et les bactéries.

2.2.9 Analyses statistiques

Les analyses statistiques de ce travail de thèse ont été réalisées à l'aide du logiciel R v. 3.0.2 (R Core Team, 2013). Pour chaque jeu de données, les tests de Shapiro et de Bartlett sont utilisés pour vérifier la normalité et l'homoscédasticité des données ainsi que pour définir l'utilisation d'ANOVA (données paramétriques) ou du test de Kruskal-Wallis (données non-paramétriques). Les indices de richesse et de diversité sont calculés en utilisant les packages Vegan et pgirmess. Les courbes de raréfaction sont créées avec la fonction "rarecurve" du package Vegan. La NMDS a été calculée selon la méthode Bray Curtis (k = 3) en utilisant la fonction « metaMDS » du package Vegan. La PERMANOVA a été exécutée en utilisant la fonction « adonis » du package Vegan. L'analyse de similarité (ANOSIM) a été réalisée à l'aide du package Vegan. Les « heatmap », qui associent des arbres de clustering et des corrélations de Spearman entre les abondances relatives, sont créées avec « heatmap.2 » du package gplots. Les numéros d'OTUs qui sont partagés entre les échantillons ont été visualisés en utilisant les diagrammes de Venn par la fonction VennDiagram dans le package Vegan. Enfin, les graphiques représentant l'abondance des différents OTUs en fonction de leur phylum ont été créés avec la fonction « riverplot » dans le package riverplot.

3 Résultats & discussions

3.1 Caractérisation par métabarcoding des communautés microbiennes de sols pollués par les ETMs

Le premier objectif de mon travail de thèse a été de **caractériser les communautés microbiennes inféodées aux espèces ligneuses implantées sur sols pollués aux ETMs**, et de déterminer **les facteurs qui contribuent à leur structuration et composition**. Pour cela des approches de métabarcoding ont été mises en œuvre sur trois sites et ont fait l'objet de trois publications :

1) La mise au point de la méthode de métabarcoding a été réalisée à partir d'échantillons du site de Tavaux. La problématique abordée sur ce site concerne l'étude des communautés microbiennes se développant sur une lagune de stockage de sédiments industriels. Les échantillons ont été prélevés sur la lagune qui a été naturellement recolonisée par des peupliers et des saules, ainsi que sur une zone témoin située à quelques centaines de mètres, et n'ayant pas reçu de sédiments.

⇒ Zappelini C, Karimi B, **Foulon J**, Lacercat-Didier L, Maillard F, Valot B, Blaudez D, Cazaux D, Gilbert D, Yergeau E, Greer C, Chalot M (2015) Diversity and complexity of a microbial communities from a chlor-alkali tailings dump. Soil Biology and Biochemistry 90: 101-110. DOI: http://dx.doi.org/10.1016/j.soilbio.2015.08.008

2) La méthode de métabarcoding a ensuite été appliquée à la comparaison des communautés microbiennes des deux sites expérimentaux de Leforest et de Pierrelaye (PHYTOPOP). Il s'agissait dans cette étude de comparer la structure et la composition des communautés microbiennes des sols et des racines de peupliers, en lien avec les caractéristiques physico-chimiques des sols dans le but de déterminer les facteurs les plus structurants.

⇒ **Foulon J**, Zappelini C, Durand A, Valot B, Girardclos O, Blaudez D, Chalot M (2016b) Environmental metabarcoding reveals contrasting microbial communities at two poplar phytomanagement sites. Science of the Total Environment 571 : 1230-1240. DOI: http://dx.doi.org/10.1016/j.scitotenv.2016.07.151.

3) Enfin, le dispositif expérimental du site de Pierrelaye (PHYTOPOP) a servi de base pour la comparaison des communautés microbiennes inféodées à huit clones de peupliers, de déterminer l'importance de l'itinéraire cultural (TCR ou TTCR), et de souligner l'impact d'une couverture ligneuse sur les communautés microbiennes du sol et des racines, en comparaison avec des sols adjacents, non plantés ou supportant une culture de maïs. Cette production agronomique est par ailleurs celle qui avait précédé la culture de peuplier.

⇒ **Foulon J**, Zappelini C, Durand A, Valot B, Blaudez D, Chalot M (2016a) Impact of poplar-based phytomanagement on soil properties and microbial communities in a metal-contaminated site. FEMS Microbiology Ecology 92 (10) : fiw163. DOI: http://dx.doi.org/10.1093/femsec/fiw163.

3.1.1 Diversité et complexité des communautés microbiennes d'une lagune de stockage de sédiments issus de l'activité industrielle chlore-alcali

La revégétalisation des lagunes de stockage produites par diverses activités industrielles est nécessaire pour prévenir l'érosion et la dispersion de poussières et polluants par voie atmosphérique, et représente un grand défi pour la restauration écologique. La colonisation microbienne et la structure des communautés des lagunes revégétalisées sont peu étudiées à ce jour. Dans cet article, le séquençage des amplicons d'ARNr 16S bactériens et ITS fongiques a été réalisé sur des anthroposols provenant d'une lagune utilisée pour stocker les déchets liés aux activités d'une production industrielle de molécules chlorées et sur un sol adjacent non anthropisé, afin de définir la composition et l'assemblage des communautés microbiennes de la rhizosphère de ces deux environnements contrastés. Après les étapes de filtration des séquences, un total de 72373 et 89929 séquences bactériennes et de 122618 et 111209 séquences fongiques a été utilisé pour l'analyse des communautés du sol non anthropisé et de la lagune, respectivement. Ces séquences sont affiliées à 45 phyla et 113 classes bactériennes ainsi qu'à 9 phyla et 35 classes fongiques. Nous observons une nette dominance des *Gammaprotéobactéries* sur la lagune (24% du total des séquences), représentées en majorité par le genre *Pseudomonas* (72% de séquences de *Gammaprotéobactéries*) ainsi que la prédominance de quelques taxa fongiques, comme *Hebeloma* et *Geopora*. Cependant, nous pouvons également remarquer que le microbiome commun comprend 64,4% et 62,4% des genres bactériens et fongiques, respectivement, en dépit des différences marquées dans les propriétés physico-chimiques des sols. Une représentation des corrélations entre les paramètres et les taxa du sol a confirmé qu'environ 50% des 33 taxa dominants colonisent les deux types de sol. Nous avons aussi démontré que la topologie du réseau bactérien et fongique global de l'anthroposol avoisine celle du sol non perturbé. Notre approche souligne l'importance d'étudier plusieurs composantes de la communauté microbienne et représente une avancée dans le domaine de l'écologie microbienne des milieux perturbés. Notre étude fournit également de nouvelles informations sur la communauté globale qui nous amène à conclure que la sélection de l'environnement est plus susceptible de se produire à travers l'activité des racines des arbres plutôt que comme un résultat de caractéristiques spécifiques du sol et pourraient être un moteur important dans l'assemblage d'au moins une partie des communautés microbiennes.

- ***Pour ce travail, j'ai participé à l'échantillonnage sur le terrain, réalisé les dosages d'ADNs et les mélanges équimolaires préliminaires au séquençage. J'ai également contribué à l'encadrement de Cyril Zappelini qui a réalisé son stage de Master 2 au laboratoire et à la rédaction de l'article. J'ai aussi eu l'occasion de présenter ces résultats sous forme d'une communication orale au congrès de l'IPS (International Phytotechnology Society) en octobre 2014 à Héraklion (Grèce).***

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Diversity and complexity of microbial communities from a chlor-alkali tailings dump

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ABSTRACT

Revegetation of the tailings dumps produced by various industrial activities is necessary to prevent dust storms and erosion and represents a great challenge for ecological restoration. Little is known about the microbial colonisation and community structure of revegetated tailings following site exploitation. Here, we report the sequencing of 16S rRNA and internal transcribed spacer (ITS) fungal RNA gene amplicons from chlor-alkali residue and from an adjacent undisturbed soil to define the composition and assembly of the rhizosphere microbial communities. After quality filtering, a total of 72,373 and 89,929 bacterial sequences and 122,618 and 111,209 fungal sequences remained for community analysis from undisturbed soil and tailings dump samples, respectively. These reads were affiliated with 45 bacterial and 9 fungal phyla and 113 bacterial and 35 fungal classes. We observed a clear dominance of *Gammaproteobacteria* at our study site (24% of total sequences), especially of the *Pseudomonas* genera (72% of *Gammaproteobacteria* sequences), together with the dominance of a few fungal taxa, such as *Hebeloma* and *Geopora*. However, we also noticed that the core microbiome comprised 64.4% and 62.4% of the bacterial and fungal genera, respectively, despite marked differences in soil physico-chemical properties. A heatmap of correlations between soil parameters and taxa confirmed that approximately 50% of the 33 dominant taxa colonised both types of soil. We further demonstrated that the global bacterial-fungal network topology of the dump approximated that of the undisturbed soil. Our approach illuminates the importance of studying more than just a single component of the microbial community and represents a step forward in uncovering the microbial ecology of disturbed environments beyond what is generally found in conventional studies. Our study also provides novel global community proxies that have led us to conclude that environmental filtering is more likely to occur through the activity of tree roots rather than as a result of specific soil characteristics and could be an important force in the assembly of at least some microbial communities.

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1. Introduction

More than 300 million tons of mining and quarrying waste is estimated to be generated annually in the European Union according to a reference document produced by the European Commission (European Commission, 2009) as a follow-up to the tailings dam bursts that occurred at *Baia Mare* in Romania and *Aznalcóllar* in

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Spain. Large dumps that are used to store industrial tailings can generate significant unvegetated surfaces after they are abandoned, suggesting that these regions become biologically infertile. Microorganisms play crucial roles in energy transfer, the mobilisation and cycling of nutrients, the establishment of plants, and ecosystem perennity (Paul, 2007). They can act as a selective force behind plant installation success and growth (Morris et al., 2007) because of the high level of interaction that occurs between plants and microbes in the rhizosphere region.

Successful revegetation of tailings has been observed following site exploitation, and insights into the microbial communities associated have been provided in previous papers (Epelde et al., 2010; Sułowicz et al., 2011). In addition to estimating the microbial fertility of contaminated tailings, knowledge of the associated microbial compartment could be used to help predicting the potential recovery of disturbed lands (Kozdroj, 2000). Most of the existing data on the microbial populations that are associated with unvegetated soils have described these populations using molecular tools such as terminal restriction fragment length polymorphism (T-RFLP) and targeted sequencing of major operational taxonomic units (OTU). However, these methods provide an approximated estimate of OTU diversity and only identify the dominant taxa (Rieder and Frey, 2013). Thus, a greater understanding of the composition of the whole community (including both dominant and rare taxa) is necessary for identifying both resistant and sensitive organisms in contaminated and disturbed soils. Interactions between bacteria, fungi and plants have been shown to contribute to microbial community stability (Bonfante and Anca, 2009; Bell et al., 2014) and some, although scarce studies have investigated the bacterial and fungal communities that arise in unvegetated environments (Li et al., 2015).

However, the nature of microbial community structure is greatly determined by various characteristics of the soil, including pH, moisture levels (Ansola et al., 2014) and/or contamination (Müller et al., 2001; Rasmussen and Sørensen, 2001; Turpeinen et al., 2004; Lorenz et al., 2006; Yergeau et al., 2014), each of which can impose constraints on the establishment of plant–microbe interactions. It may therefore be hypothesized that the composition and structure of microbial community will be dependent upon the soil characteristics and presence of contaminants. In order to check this hypothesis, we compared fungal and bacterial communities from a chlor-alkali tailings dump to an adjacent undisturbed forest soil. We obtained amplicon sequencing data to provide information about these two major microbial compartments to determine how they were associated with plant establishment and soil characteristics. The data were then analysed in terms of diversity, composition, and co-occurrence network structure to quantify various properties of the communities, an approach that has never been applied to anthropogenic soils.

2. Materials and methods

2.1. Site and sampling

Tailings dumps are facilities designed to receive and store tailings, which is the refuse material produced from the sedimentation of minerals. The site used in the current study was exploited as a storage area from the 1950s to 2003 to contain sediments from the adjacent sedimentation basin and contained a total surface area of 12 ha. This region is characterised by an annual average temperature of 11 °C and an average 75% humidity. The sediments originated from effluents produced during electrolytic processes that were associated with a mercury cell chlor-alkali process that was used to produce chlorine until 2012. The tailings dump was confined by 5 m high dikes to preserve the surrounding environment and contained a multi-contaminated calcareous and alkaline anthropogenic soil. The soil from an adjacent undisturbed forest was not affected by anthropogenic activity and was therefore used as the undisturbed soil control in this study. Each region was dominated by slightly different plant covers. In the tailings dump, the plant cover was primarily comprised of woody species such as *Salicaceae* (poplar and willow), *Fabaceae* (black locust), and *Caprifoliaceae* (black elder), in addition to herbaceous genera such as *Poaceae* (phragmites). The undisturbed area was also covered by *Salicaceae* and *Fabaceae* species, and comprised additional tree species from the *Fagaceae and Betulaceae families.* Phragmites were absent at the control location, whereas other species from i.e. the *Rubus*, *Senecio*, *Sambucus*, *Eupatorium*, *Urtica* genera constituted the forest floor vegetation. Both areas experienced similar climatic conditions.

Rhizosphere soil, the thin layer of soil where roots and soil organisms interact in myriad ways (Richter et al., 2007), was sampled from seven Salicaceous trees in each area (tailings dumps and undisturbed soil) in April of 2013. All samples were obtained over a one-day period to reduce any heterogeneity imparted by climatic conditions. After the removal of litter, soils that were attached to roots were collected from the upper 20 cm layer of soil from under the canopy of the trees. A total of 3 pseudo-replicates were sampled from each tree and mixed to obtain a soil composite. Samples were dried for 24 h at 24 °C under airflow and hand-crushed at 2 mm for homogenisation. Subsamples were stored at either 4 °C for molecular analysis or at ambient temperature (24 °C ± 1) for physical and chemical analyses.

2.2. Soil physico-chemical properties

The extraction of metal trace elements (TE) was performed on a 0.5 g aliquot of each soil sample via mineralisation in 2 mL HCl, 5 mL HNO_3 and 33 mL deionised water (modified from the ISO 11466 French Norm) for 260 min at 100 °C in a Digiprep unit (SCP Science Corporate Headquarters, USA). TE (Al, As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn) concentrations were determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Thermo Fischer Scientific, Inc., USA) and all samples were analysed in triplicate and run together with certified reference material (a loamy clay soil).

The physico-chemical characteristics that were measured included particle size (French Norm X 31–107), pH (ISO 10 390 French Norm), total carbonates (ISO 10 693 French Norm), total organic carbon and organic matter (OM, ISO 14 235 French Norm), total nitrogen (Ntot, DUMAS method ISO 13 878 French Norm), C/N ratios (ISO 13 878 French Norm), total available phosphorus (Joret Hebert method French Norm X 31–161), cationic exchangeable capacity (CEC, French Norm X 31–130) and several oligo-elements (French Norm X 31–108).

2.3. DNA extraction and amplification

DNA was extracted from a 10 g soil sample using the PowerMax® Soil DNA Isolation Kit (MO-BIO Laboratories, Inc., Carlsbad, CA USA; (Yergeau et al., 2014). DNA quality was assessed using a 1% (w/v) agarose gel and by measuring 260/280 nm and 260/230 nm ratios using a BioPhotometer (Eppendorf, AG, Hamburg). DNA concentrations were quantified by measuring the absorbance at 260 nm using the BioPhotometer.

PCR amplification of the partial 16S rRNA and ITS genes was performed using the bacterial 520F (5′-AGC AGC CGC GGT AAT-3′) and 799R (5′-CAG GGT ATC TAA TCC TGT T-3′) primers and the fungal ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and 58A2R (5′-CTG CGT TCT TCA TCG AT-3′) primers, respectively. The

amplification of 16S rRNA was carried out using 100 ng of template DNA, 0.5 μM of each primer, 200 μM dNTPs (Euromedex, France), 1 μL of Phire Hot Start II DNA Polymerase II, and 10 μL of 5X Phire reaction buffer (Thermo Fisher Scientific, Inc., USA) in a final volume of 50 μl. The cycling conditions involved an initial 3 min denaturing step at 98 °C followed by 25 cycles of 5 s at 98 °C, 5 s at 55 °C, and 5 s at 72 °C, with a final elongation step of 1 min at 72 °C. The same protocol was performed to amplify ITS genes, using a 15 ng of template DNA in this case. The cycling conditions for these genes included an initial 3 min denaturing step at 98 °C followed by 30 cycles of 5 s at 98 °C, 5 s at 60 °C, and 15 s at 72 °C, with a final elongation step of 1 min at 72 °C (Yergeau et al., 2012).

2.4. Amplicon sequencing

Both primer pairs contained the 10 bp multiplex identifiers and adaptor sequences necessary for Ion Torrent sequencing, as previously described (Yergeau et al., 2012). Sequencing of the pooled library was performed using the Ion Torrent Personal Genome Machine system (Life Technologies, Burlington, ON, Canada). High-throughput sequencing of the taxonomically informative 16S rRNA gene provides a powerful approach for exploring microbial diversity (Salipante et al., 2014). The sequence data were processed using Mothur (Schloss et al., 2009). The fastq file was transformed into .fasta and .qual files using the fastq.info() command, after which the sequences were trimmed using the trim.seqs() command with the following parameters: minimum length = 100 bp, quality window size = 10, quality window average = 20 (Liu et al., 2007, 2008). The quality-filtered data were then classified according to the GreenGenes taxonomy using the classify.seqs() command with the method = wang and cutoff = 50 options. The quality-filtered sequences were also clustered in CD-HIT (Li and Godzik, 2006) with a cutoff of 97%. The groups were then imported into Mothur and the diversity indices were calculated. The weighted, normalised Unifrac distances between each sample pair were calculated using the FastUnifrac website (Hamady et al., 2010) and based on the August 2013 release of the GreenGenes core data set. The procedure described above can be considered comparable to 454 pyrosequencing and has been fully validated (Yergeau et al., 2012).

2.5. Real-time PCR quantification

The quantity of total bacteria and the quantity of bacteria belonging to the *Pseudomonas* genus were both quantified by targeting 16S rDNA (bacteria) via real-time PCR; gene copy numbers were quantified with the primer sets 968F/1401R (Felske et al., 1998), Pse435F/Pse686R (Bergmark et al., 2012) and FF390R/Fung5F (Lueders et al., 2004), respectively. The real-time PCR experiments were conducted using an iCycler iQ (Bio-Rad) outfitted with iCycler Optical System Interface software (version 2.3). The final volume used (20 μl) contained 10 μl of 2X iQ SYBR Green SuperMix (Biorad), 0.4 μM of each primer, 0.06% (w/v) of bovine serum albumin (BSA), 0.2 μl of DMSO, 40 ng of T4 gp32 (MP Biomedicals) and 1 μl of DNA. The qPCR program consisted of 5 min at 95 °C followed by four steps of 40 cycles of 20 s at 95 °C, 20 s at the primer-specific annealing temperature (56 °C, 60 °C and 50 °C for the 968F/1401R, Pse435F/Pse686R and FF390R/Fung5F primer sets, respectively), 30 s at 72 °C, and 10 s at 80 °C to dissociate primer dimers and capture the fluorescence intensity of the SYBR green. The procedure concluded with a final elongation step of 5 min at 72 °C. After the procedure was complete a melting curve analysis was performed from 60 °C to 95 °C, with a temperature increase of 0.5 °C every 5 s. A negative control was included in each of the qPCR assays and all were performed in triplicate. The linearized plasmids were serially diluted (from 10^8 to 10^1 target gene copies/μl) and the relevant target gene inserts were used to create the standard curves. The presence of PCR inhibitors was evaluated by mixing 1 μl of environmental DNA with 1 μl of 10^6 copies of lambda standard plasmid and compared to the lambda standard curve (Cébron et al., 2008); no inhibitory effect was observed. Real-time PCR quantification was also used to provide information about molecular biomass, which was expressed as copy number per nanogram of DNA.

2.6. Statistical procedures

All statistical analyses were performed using R software v. 3.0.2 (R Development Core Team, 2013). Physico-chemical characterisation and TE data (Al, As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn) were compared between undisturbed soil and tailings dump samples using Principal Component Analyses and an intergroup comparison test validated by a Monte Carlo permutation test (*ade4* R package). The Shapiro test and the Bartlett test were used to respectively verify the normality and homoscedasticity of the data, and we compared each trace element using either ANOVA or Kruskal–Wallis tests.

The numerical analysis of the biological component of the soil was based on the number of sequences that were obtained for each genus of bacterial or fungal lineage. Various aspects of the microbial communities were compared between the undisturbed soil and tailings dump samples. Four diversity indices were initially calculated (*pgirmess* R package; see Supplementary Table 1 for detailed calculation methods), including the following: i) the genus richness, which represented the total number of the observed genera; ii) the Shannon and inverse Simpson diversities; iii) the genus evenness computed as Hill's ratios between the Shannon or inverse Simpson indices and the richness, wherein the inverse Simpson evenness index represents the proportion of the dominant genus within the community irrespective of genus richness and the Shannon evenness index highlights the abundance of genera; and iv) the Pielou evenness, which is a ratio between the Shannon entropy and the maximal entropy that can be used to measure equitability independently of richness, thereby indicating potential dominance. All indices were compared between the tailings dump and the undisturbed soil via the Kruskal–Wallis test for non-parametric data. The detailed structures of the communities that were present in each of the areas were then compared using a Kruskal–Wallis test, with a Bonferroni correction of the p-value for each genus. Finally, community composition was determined via Correspondence Analysis, followed by an intergroup comparison test that was validated by a Monte Carlo permutation test (*ade4* R package). Redundancy analysis of the physico-chemical characteristics and TE was conducted using the *vegan* R package (Dray et al., 2007) to link TE contaminants to other soil parameters according to a method that has been previously described (Braak and Smilauer, 1998).

The number of fungal and bacterial sequences was correlated to soil parameters using Spearman correlation for the dominant taxa (>2% of total sequences). The sensitivity to soil parameters of major fungal and bacterial taxa was compared using the UPGMA clustering. The resulting clustering trees were paired a heatmap of correlation data created with the heatmap.2 function from the *gplots* R package.

Semi-quantitative data obtained from the amplicon sequencing of the bacterial and fungal communities that were present in the soil samples were used to model the microbial co-occurrence networks of the undisturbed soil and tailings dump. A total of seven replicates per area were used to calculate the Spearman coefficient correlations for each pair of genera (*Hmisc* R package) (Barberán et al., 2012). The matrix of statistically significant

coefficients (p-value $\leq$ 5%, r > 0.80) was then transformed into an adjacency matrix based on the presence or absence of links between pairs. This new matrix was used to build the network (*statnet* R package) and to calculate the following network indices (as defined in Supplementary Table 1): node number (N), link number (L), average degree (aD), maximal degree (mD), average betweenness (aB), maximal betweenness (mB), connectance (C), connectedness (Cd), transitivity (T) and clusters (G). To compare the microbial communities of the two areas a total of seven network replicates were created using only six of seven field replicates for each; indices were calculated for all network replicates. The difference in the calculated distributions (n = 7) of the indices was quantified using the Kruskal–Wallis test for non-parametric data for the few replicates that required such analysis or ANOVA when the data followed a normal distribution.

3. Results

3.1. Two contrasting environments were depicted following physico-chemical analysis

To provide geochemical and mineralogical context for our microbial community analysis we compared the physico-chemical characteristics of topsoil (0–20 cm layer) taken from the tailings dump to the adjacent undisturbed soil. Physical analysis (see Supplementary Table 2 for detailed soil analyses) showed that tailings dumps contained a significantly greater proportion of thin silt, whereas undisturbed soils contained more clay and coarse sand (N = 14; p-values = 0.045, 0.003 and 0.020, respectively, for clay, thin silt and coarse sand). The above detailed physical parameters were further investigated using multivariate analysis (Fig. 1a). Chemical analysis revealed that pH, calcium carbonate ($CaCO_3$) concentration and exchangeable calcium oxide (CaOex) were all significantly higher in the soil of the tailings dump, whereas CEC was significantly enhanced in undisturbed soil (ANOVA or Kruskal–Wallis for non-parametric data, p-value < 0.0001; Supplementary Table 2). Interestingly, no significant differences were found between the two soils with respect to the total quantities of organic carbon and nitrogen, the total organic matter (OM), or the C/N ratios; therefore, these variables could not be used to discriminate between samples taken from the tailings dump versus the adjacent soil (Fig. 1a). ICP-AES analyses revealed that the quantities of total Hg and total As (p-value < 0.0001) were significantly increased (see Supplementary Table 3 for detailed TE soil analyses) in the tailings dump. Other TE were weakly represented in the tailings dump compared to the adjacent soil (Fig. 1b). A redundancy analysis showed that silt, $CaCO_3$, pH and exchangeable CaO, and total Hg and As were all positively correlated in the tailings dump (p-value = 0.04, adjusted R^2 = 0.88; Supplementary Fig. 1).

3.2. Analysis of bacterial and fungi community diversity

A total of 186,558 16S bacterial and 398,578 ITS fungal raw Ion Torrent reads were obtained from 14 DNA soil samples. After quality filtering and target extraction (demultiplexing, trimming and denoising) a total of 72,373 and 89,929 bacterial sequences and 122,618 and 111,209 fungal sequences remained for community analysis of undisturbed soil and tailings dump samples, respectively. These reads were found to be associated with 45 bacterial and 9 fungal phyla and 113 bacterial and 35 fungal classes (Fig. 2). To further compare the bacterial and fungal communities residing within the tailings dump and undisturbed soil samples we calculated richness and diversity indices (Supplementary Table 1) and found that there was a significant site-specific effect on the bacterial variables that were examined, particularly with respect to richness (P = 0.035) and inverse Simpson evenness (P = 0.048; Fig. 3a), including a trend of increasing soil bacteria richness in the tailings dump of approximately 20%. In contrast, neither the calculations of soil bacteria Pielou evenness nor those representing Shannon evenness were found to be significantly different between the soils. There were also no remarkable differences found between

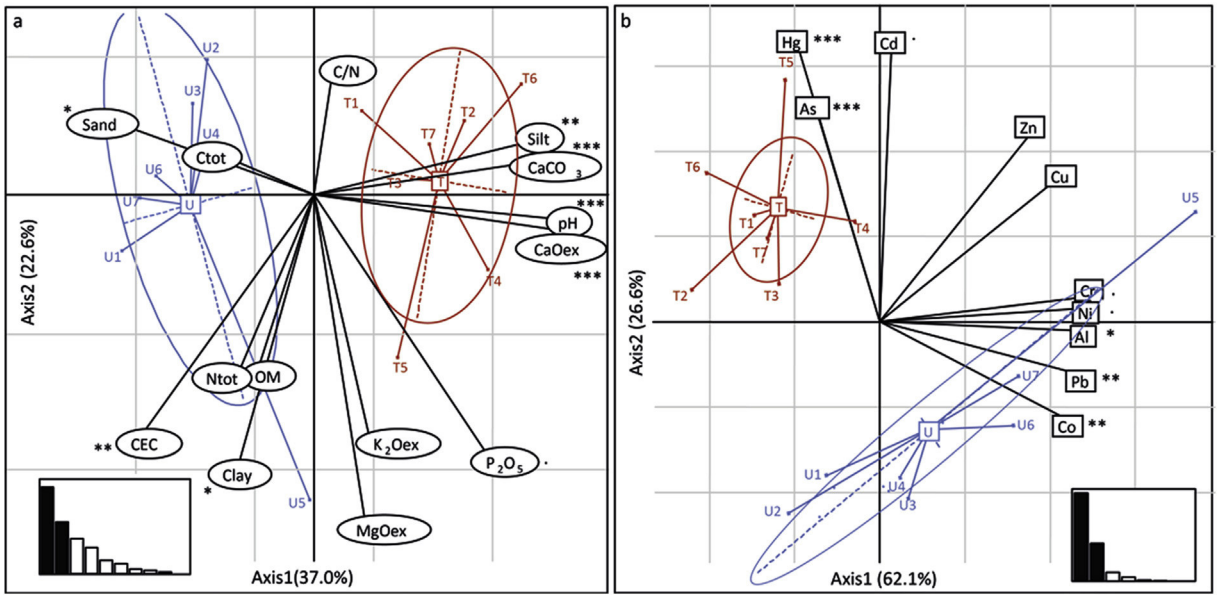

Fig. 1. Principal component analysis showing the positions of undisturbed soil (U) and tailings dump (T) samples according to site environmental characteristics. **a**, Results from the between test followed by the Monte Carlo permutation test showed that samples taken from undisturbed soil and an adjacent tailings dump had significantly different physico-chemical characteristics (33.2% variance explained, P = 0.001). The detailed results of soil parameter analyses are provided in Supplementary Table 2. **b,** Results from the between test followed by the Monte Carlo permutation test demonstrated that samples taken from undisturbed soil and an adjacent tailings dump had significantly different chemical characteristics (35.4% variance explained, P = 0.001). The detailed results of ICP-AES analyses are provided in Supplementary Table 3.

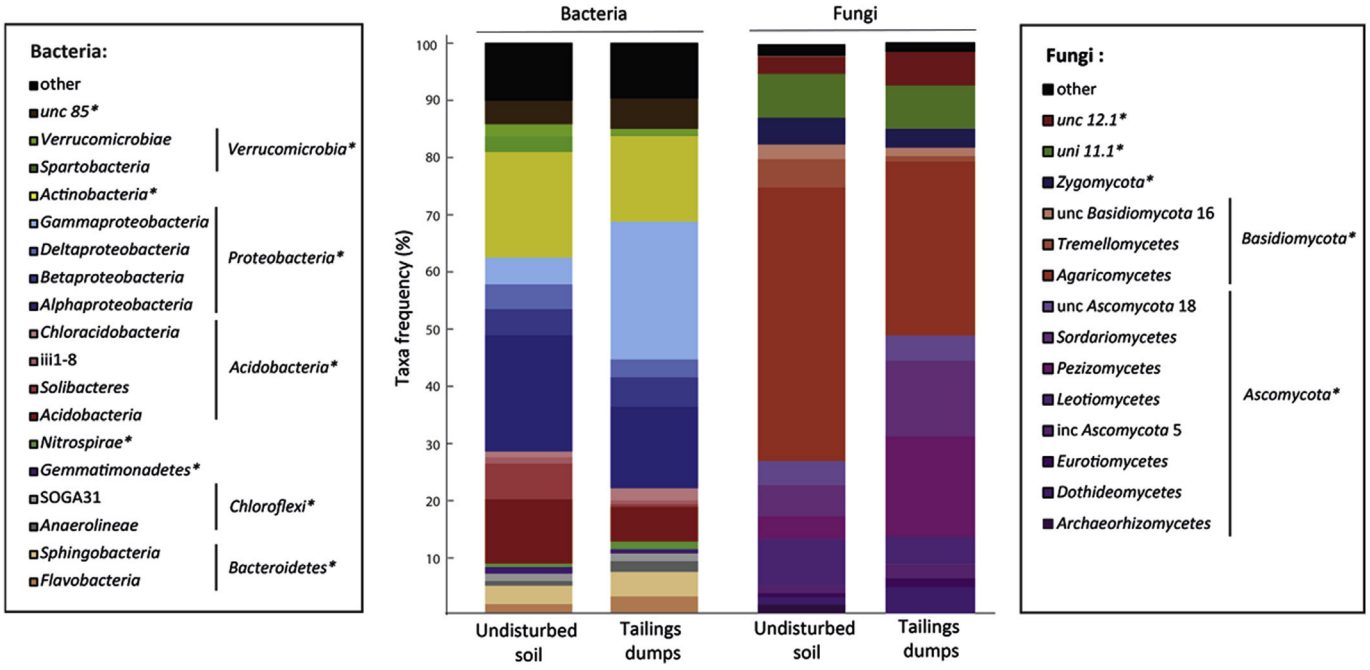

Fig. 2. Microbial community composition at the phylum/class level averaged for each site (N = 7), based on 16S rRNA and ITS gene sequencing of samples taken from undisturbed soil and an adjacent tailings dump.

a

Area		Richness	Shannon eveness	Inverse Simpson eveness	Pielou eveness
Undistiurbed soil	mean (N=7)	333.43	0.19	0.07	0.72
	SD	57.71	0.05	0.03	0.04
Tailings dump	mean (N=7)	394.71	0.22	0.10	0.74
	SD	15.90	0.03	0.01	0.02
	P value	0.035	0.142	0.048	0.406

b

Area		Richness	Shannon eveness	Inverse Simpson eveness	Pielou eveness
Undistubed soil	mean (N=7)	248.71	0.13	0.06	0.62
	SD	41.55	0.03	0.02	0.04
Tailings dump	mean (N=7)	221.43	0.15	0.08	0.65
	SD	27.97	0.04	0.03	0.04
	P value	0.159	0.180	0.848	0.277

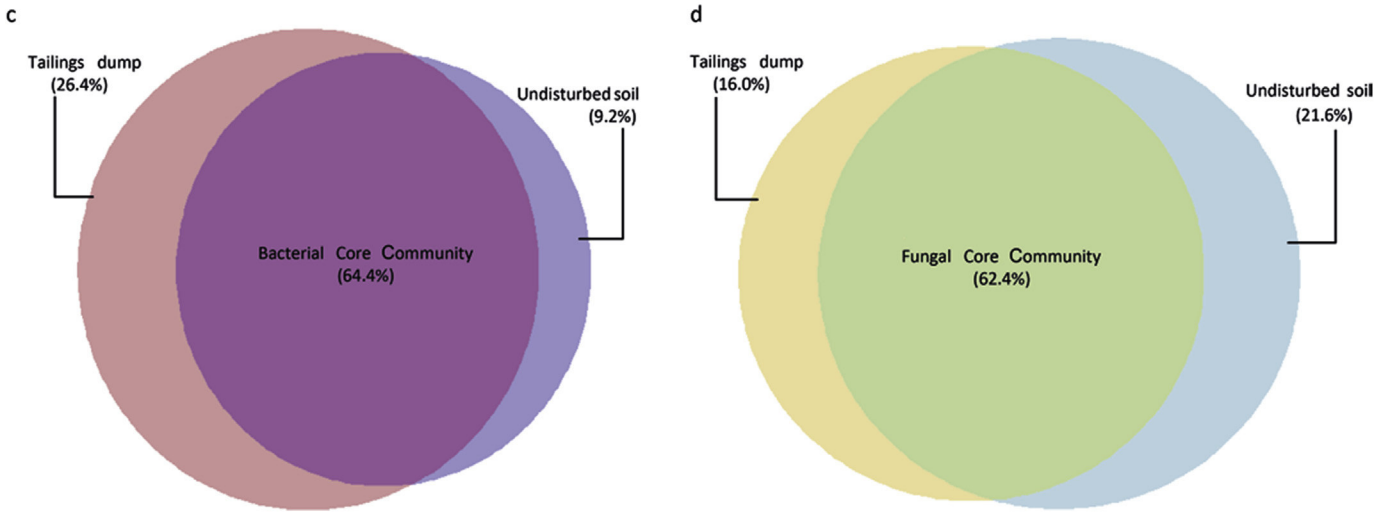

Fig. 3. Diversity indices and composition of bacterial and fungal communities found within samples taken from undisturbed soil and an adjacent tailings dump. **a**, Diversity indices of the bacterial community. **b,** Diversity indices of the fungal community. The mean values and standard deviations of the diversity indices (as defined in Supplementary Table 1) are provided in addition to the P-value of the Kruskal–Wallis comparison test. **c,** Weighted Venn diagram of the percentage of bacterial genera that were either shared between (not significantly different at P = 0.05) or specific (significantly different at P = 0.05) to the two sites. **d,** Weighted Venn diagram of the percentage of fungal genera that were either shared between (not significantly different at P = 0.05) or specific (significantly different at P = 0.05) to the two sites.

the fungal communities that were residing in the two study areas for any of the four calculated diversity indices that were used in our study (Fig. 3b). We found that 599 bacterial and 500 fungal genera, and 386 bacterial genera (i.e., 64.4%, Fig. 3c) and 312 fungal genera (i.e., 62.4%, Fig. 3d) were shared between the tailings dump and undisturbed soil samples, respectively. Thus, on a global scale 63.4% genera were shared. We used qPCR to further quantify 16S and 18S to lend additional support to the conclusions derived from these data, which allowed us to estimate bacterial/fungal molecular biomass ratios. The ratios were 43.3 in the tailings dump and 40.3 in the undisturbed soil, respectively, and the difference between the two was not found to be significant.

3.3. Changes in the compositions of the bacterial and fungal communities

We used correspondence analysis followed by an intergroup comparison to establish that the bacterial and fungal communities residing within the tailings dump and the undisturbed soil samples each had significantly different compositions (Monte Carlo permutation test, P = 0.001; Supplementary Fig. 2), which were primarily influenced by the presence of a few specific taxa within each soil. The majority of the emergent bacterial genera that were found within the tailings dump represented less than 2% of the total sequences; a noticeable exception was the *Pseudomonas* genus, which represented 17.0% of the sequences in the tailings dump and only 0.9% of the sequences in undisturbed soil (Fig. 4). We further confirmed the large-scale *Pseudomonas* colonisation of the tailings dump by using qPCR. The results indicated an average molecular biomass of 26,818 copies/ng DNA in the tailings dump compared with 1820 copies/ng DNA in undisturbed soil. In contrast, the bacterial genus MC18 was dominant in the undisturbed soil (2.3% of total DNA sequences) and was not significantly represented in the tailings dump (0.007% of total DNA sequences; Fig. 4). The bacterial genus *Acidobacteria* g.1 was also highly present in the undisturbed soil (3.1% of total DNA sequences) and barely represented in the tailings dump (0.0004% of total DNA sequences). Most of the fungal genera that were present in the undisturbed soil were also detected in significant proportions in the tailings dump (Fig. 3d); however, fungi of the Agaricomycete family represented 6.6% of the DNA sequences that were found in the tailings dump and only 1.5% of the sequences found in the undisturbed soil (Fig. 4).

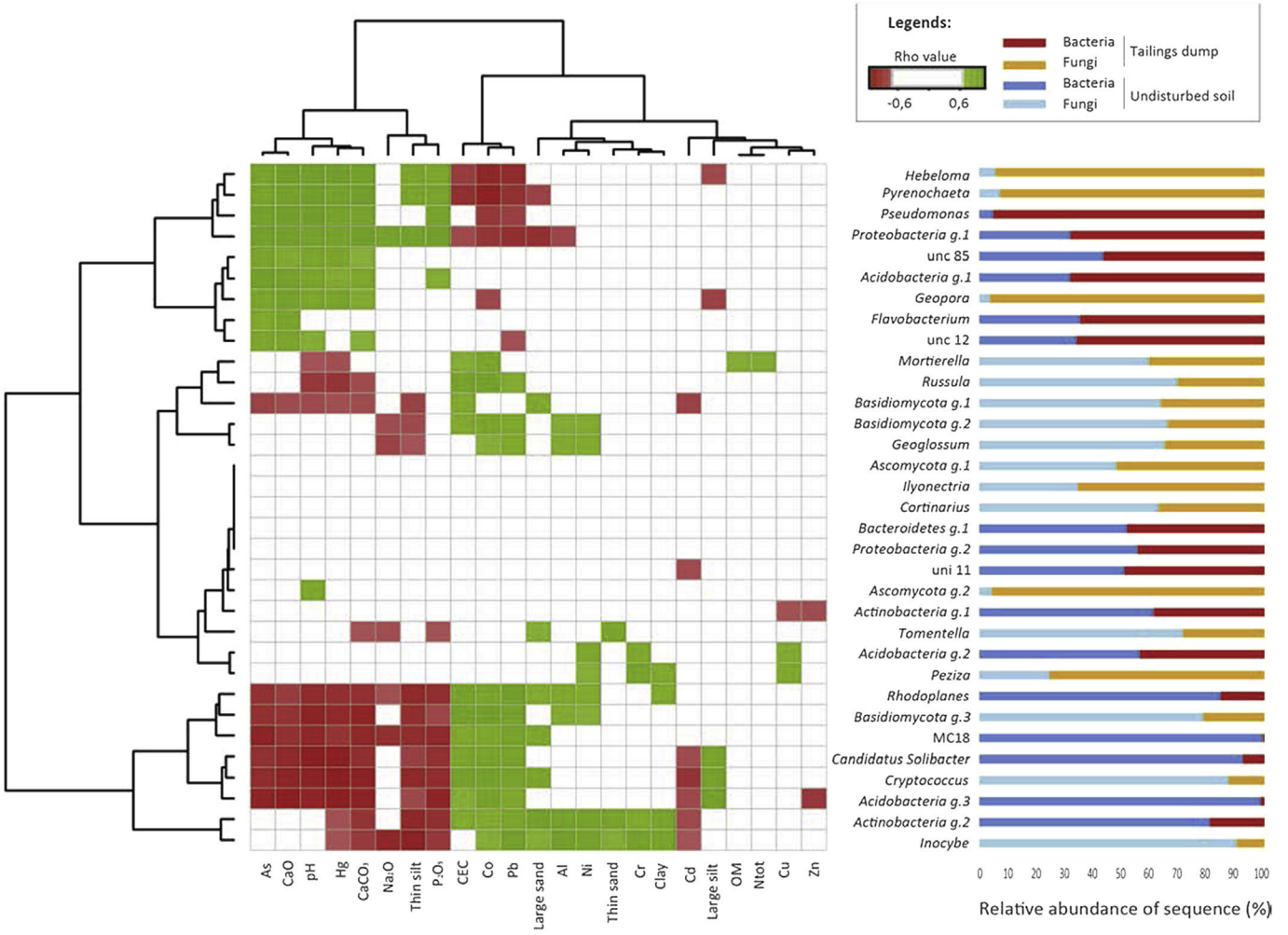

Fig. 4. Soil-microbe relationships. Left. Heatmap of the Spearman correlation coefficient values computed for bacterial or fungal genera and soil parameters using relative microbial abundance data >2%. The colour key for the correlation values is shown on the top right of the figure; non-significant correlations are in grey, positive correlations are in green, negative correlations are in red. Dendrograms of hierarchical cluster analysis grouping genera and soil parameters are also shown on the left and at the top, respectively. b. Relative abundance of dominant microbial genera (>2%) in tailings dump and undisturbed soil samples. The colour key for the relative abundance is shown on the top right of the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Microbial co-occurrence networks

We built co-occurrence networks to further assess links both within and between the bacterial and fungal communities in addition to the community complexity. To maintain consistency across the analyses we used sequencing data at the genus taxonomic level and calculated indicators of the network topology (Table 1). However, network visualization was found to be more appreciable at the class level (Fig. 5) and was highlighted with phylum details (Supplementary Fig. 3). The node that was calculated to have the greatest maximal betweenness, which represented the bacterial genera with the most connections (8105 connections), was an unidentified environmental bacterium (GN07) found to be residing in the tailings dump. The most highly represented bacteria (*Pseudomonas*, Fig. 4) were found to be poorly connected to other bacteria, as they did not exhibit the highest number of either direct or indirect links (Betweenness = 1291 connections). The key bacterial genus discovered within the undisturbed soil network was an unclassified clostridia (with 8106 total connections). Both the bacterial and the fungal correlation networks exhibited less complexity in the tailings dump compared to undisturbed soil, as illustrated by the values calculated for the connectance (i.e., the ratio between the number of realised links and the number of potential links) and the degree, which were approximately 25% and 24% lower for bacteria and fungi, respectively ($P < 0.0001$, Fig. 5, Table 1, Supplementary Fig.3). A corresponding 20% reduction in the complexity of the overall network (merging bacteria and fungi) was calculated. In parallel, calculation of the connectedness and betweenness indices of the tailings dump revealed that bacterial connectedness and betweenness increased by 23 and 21%, respectively, whereas fungal connectedness and betweenness decreased by 17 and 24%, respectively. Most importantly, no differences were observed in either connectedness or betweenness between the two areas with regard to the merged bacterial and fungal communities.

3.5. Soil microbe relationships

We calculated the Spearman correlation coefficient for each pairwise combination of parameters (i.e., taxa versus physicochemical parameters) and the results were displayed as heatmaps (Fig. 4). Calculations were made on the dominant genera (>2% of

Table 1
Network indices of microbial communities in undisturbed soil and tailings dump. Calculation of network indices as defined in Supplementary Table S1, of bacterial, fungal and the merged (bacterial plus fungal) network built at the genus taxonomic level.

Microbial community	Links direction	Area		Links	Connectance	Connectedness	Average degree	Average betweenness
Bacteria	Positive	Undisturbed soil	mean (N = 7)	9619.43	0.05	0.53	32.12	743.38
			SD	999.89	0.01	0.03	3.34	59.25
		Tailings dump	mean (N = 7)	5801.57	0.03	0.63	19.37	999.53
			SD	435.49	0.00	0.01	1.45	34.58
			P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Negative	Undisturbed soil	mean (N = 7)	2070.43	0.01	0.46	6.91	815.26
			SD	511.85	0.00	0.05	1.71	74.99
		Tailings dump	mean (N = 7)	2957.57	0.02	0.63	9.88	955.14
			SD	382.19	0.00	0.01	1.28	21.31
			P value	0.002	0.002	<0.0001	0.002	0.001
	Global	Undisturbed soil	mean (N = 7)	11689.86	0.07	0.54	39.03	511.01
			SD	1275.99	0.01	0.02	4.26	33.73
		Tailings dump	mean (N = 7)	8759.14	0.05	0.63	29.25	631.78
			SD	222.41	0.00	0.01	0.74	9.67
			P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Fungi	Positive	Undisturbed soil	mean (N = 7)	4786.71	0.0384	0.4579	19.15	631.60
			SD	453.09	0.0036	0.0226	1.81	42.92
		Tailings dump	mean (N = 7)	3460.43	0.0277	0.3530	13.84	552.47
			SD	261.74	0.0021	0.0158	1.05	32.40
			P value	<0.0001	<0.0001	<0.0001	<0.0001	0.003
	Negative	Undisturbed soil	mean (N = 7)	1388.00	0.0111	0.4425	5.55	658.69
			SD	179.34	0.0014	0.0289	0.72	48.11
		Tailings dump	mean (N = 7)	1259.14	0.0101	0.3431	5.04	489.40
			SD	163.81	0.0013	0.0171	0.66	19.46
			P value	0.234	0.222	<0.0001	0.235	<0.0001
	Global	Undisturbed soil	mean (N = 7)	6174.71	0.0495	0.4630	24.70	394.28
			SD	434.30	0.0035	0.0241	1.74	20.70
		Tailings dump	mean (N = 7)	4719.57	0.0378	0.3546	18.88	310.13
			SD	352.23	0.0028	0.0163	1.41	10.36
			P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Global	Positive	Undisturbed soil	mean (N = 7)	23473.00	0.04	0.50	42.69	1312.84
			SD	2106.85	0.00	0.02	3.83	69.50
		Tailings dump	mean (N = 7)	15859.86	0.03	0.50	28.86	1377.77
			SD	786.58	0.00	0.01	1.43	39.71
			P value	0.002	0.002	0.338	0.002	0.025
	Negative	Undisturbed soil	mean (N = 7)	7040.86	0.01	0.50	12.81	1357.28
			SD	962.68	0.00	0.02	1.75	41.42
		Tailings dump	mean (N = 7)	8644.29	0.01	0.49	15.73	1235.99
			SD	559.74	0.00	0.01	1.02	23.40
			P value	0.006	0.007	0.654	0.006	0.003
	Global	Undisturbed soil	mean (N = 7)	30499.57	0.05	0.50	55.50	862.11
			SD	2065.45	0.00	0.02	3.76	36.81
		Tailings dump	mean (N = 7)	24504.14	0.04	0.50	44.59	864.73
			SD	834.86	0.00	0.01	1.52	15.35
			P value	0.002	0.002	0.338	0.002	0.406

a

Area		Links	Connectance	Connectedness	Average Degree	Average Betweenness
Undisturbed soil	mean (N=7)	30499.57	0.05	0.50	55.50	862.11
	SD	2065.45	0.00	0.02	3.76	36.81
Tailings dump	mean (N=7)	24504.14	0.04	0.50	44.59	864.73
	SD	834.86	0.00	0.01	1.52	15.35
	P value	0.002	0.002	0.338	0.002	0.406

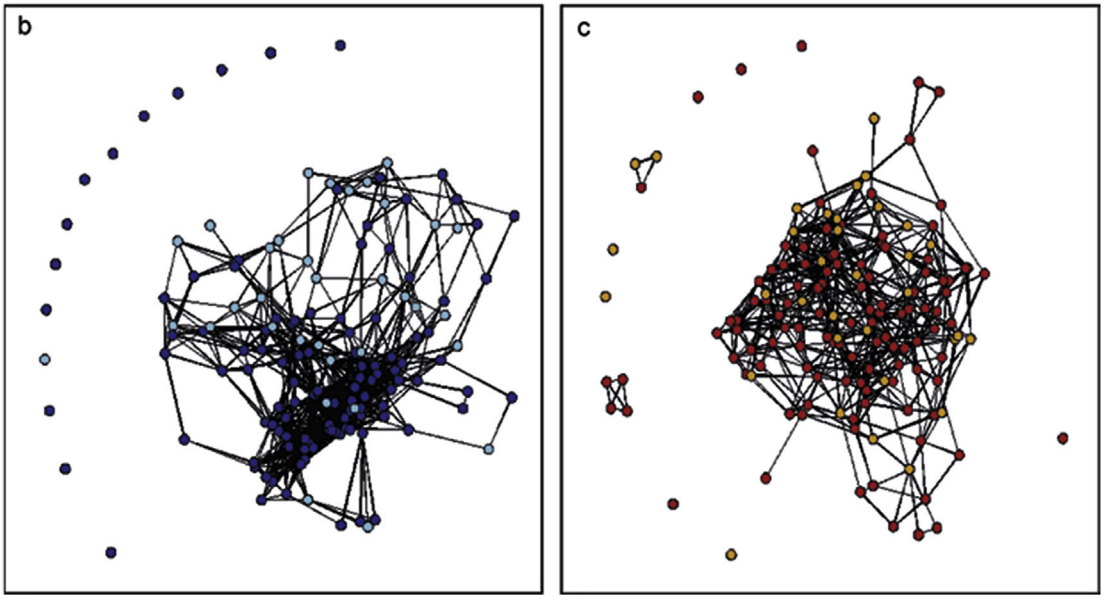

Fig. 5. Co-occurrence network analysis of bacterial and fungal communities found within a tailings dump and the adjacent undisturbed soil. a, Calculation of network indices of merged bacterial and fungal genera. **b, c,** Correlation networks are best illustrated at the class level for (**b**) the undisturbed soil and (**c**) the tailings dump. In these graphical representations nodes represent classes and edges represent interactions. Dark blue and dark red dots represent bacterial nodes; cyan and orange dots represent fungal nodes. For clarity, node identities were omitted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequences). A total of nine fungal and bacteria taxa (i.e., *Hebeloma, Pyrenochaeta*, *Pseudomonas* and *Geopora*) grouped together into a single cluster and were highly correlated with As, Hg, exchangeable CaO, total $CaCO_3$ and pH conditions in the tailings dump. In contrast, a total of eight microbial taxa (primarily *Basidiomycota* fungal class and MC18 bacteria) clustered together and were primarily correlated with CEC, Co and Pb. When considering the bacterial communities residing within the soil samples we found that the *Proteobacteria* dominance was primarily *Alphaproteobacteria* in the undisturbed soil (*Rhodoplanes* for example) and *Gammaproteobacteria* in the tailings dump (primarily *Pseudomonas* and *Proteobacteria g1.*). In addition to these taxa specific features we noticed that approximately 50% of the 33 dominant taxa were equally represented in both soils and that few were sensitive to soil parameters (Fig. 4).

4. Discussion

With respect to diversity indices, major microbial taxa, and soil-taxa relationships the tailings dump might be considered to represent a habitat for a relatively small number of taxa (e.g., *Pseudomonas* or *Hebeloma*). In agreement with our work, previous studies have indicated that among structuring factors, anthropogenic Hg has a broad-spectrum effect and can alter the structure of both the culturable and total bacterial communities (Müller et al., 2001). For example, some studies have shown anthropogenic Hg to impart a negative effect on the culturable diazotrophic community (Lauber et al., 2008; Boer et al., 2012) and a predominance of *Gammaproteobacteria Pseudomonas* isolates among Hg tolerant bacteria (Oliveira et al., 2010). We observed a dominance of *Gammaproteobacteria* in our study site (24% of total number of sequences) and found that *Pseudomonas* genera (72% of *Gammaproteobacteria* sequences) were especially well represented within the tailings dump community. These findings were confirmed by quantitative PCR and revealed the specialization of the bacterial community. Mercury conditions within the dump had likely facilitated the dominance of *Pseudomonas* within the associated bacteria community; this genus may have rapidly adapted to its environment, for instance by acquiring Hg resistant genes via horizontal gene transfer (Barkay et al., 2003). The other metallic contaminant that was detected at the dumpsite was arsenic. Previous work has revealed that dominant As-resistant isolates within As-contaminated soils can be identified by their fatty acid methyl ester (FAME) profiles and include *Pseudomonas* (Turpeinen et al., 2004) in addition to other bacterial species. Similarly, fungi and *Proteobacteria* were shown to be able to evolve tolerance to As contamination, whereas all other groups of bacteria were reduced (Lorenz et al., 2006). Thus, the cumulative presence of Hg and As could explain the abundance of *Pseudomonas* species that were found within the tailings dump.

In interpreting our data it is important to take into account the potential effects that can be imparted by other confounding factors, as has been recently highlighted in the literature (Azarbad et al., 2013; Chodak et al., 2013). Among the edaphic characteristics that

were measured here, pH is often regarded as capable of shaping the bacterial community (Lauber et al., 2008; Boer et al., 2012) and the presence of *Acidobacteria* can be indicative of acidic soils. Here, we found the abundance of *Acidobacteria* to be consistently and significantly lower in tailings dump samples, wherein pH was the highest (pH 8.1 versus 5.7 in undisturbed soil). High levels of $CaCO_3$ and exchangeable CaO within the dumpsite may also have been a key determinant in shaping the associated bacterial community. Indeed, calcite-rich sediments have often been found to be dominated by *Gammaproteobacteria* (Nercessian et al., 2005), *as was observed in our study.*

There were only minor differences found between the fungal communities residing in the tailings dump and undisturbed soil samples; differences between genus richness, diversity and biomass were all insignificant. It was also demonstrated that fungal communities did not robustly respond to changes in environmental conditions (Müller et al., 2001), whereas other studies reported the responses of fungi to specific physico-chemical conditions from a metallurgical slug compared with a natural soil (Kozdroj, 2000). In details, our study did find that the fungal community structure changed with respect to a few genera; for example, increased numbers of *Hebeloma* and *Geopora* sequences were found at the tailings dump. Correspondingly, members of the *Hebeloma* mycorrhizal genus (notably *H. mesophaeum* species) have been frequently found within unvegetated soils (Hrynkiewicz et al., 2008; Krpata et al., 2008) and have been known to promote the growth of host trees in soils contaminated with metal (Hrynkiewicz et al., 2012). Our data therefore indicated that the fungal colonization of the Salicaceous rhizosphere within the tailings dump by indigenous taxa of the adjacent undisturbed soil had occurred during the decade after substrate deposition had ceased. The early colonisation of deposited substrates by woody plants can substantially increase the input of carbon into the soil through both litter and root exudates, which in turn can induce root colonisation by mycorrhizal symbionts (i.e., *Hebeloma* species).

The strong evolutionary relationships between plants and microbes that have been previously described might offer an explanation for the weak divergence that was observed amongst the communities that we assayed, as the samples used in this study were collected from rhizospheric environments that were present under similar tree species. Indeed, recent research suggests that plant species in natural ecosystems are likely to be a more important determinant of the rhizosphere microbial community than soil type (Westover et al., 1997; Chaparro et al., 2012; Bell et al., 2014). This viewpoint might well apply to our study, as we found that both Salicaceous trees and a complex associated microbial community had fully colonised the dump. Environmental filtering, as was discussed earlier, is more likely to occur through the activity of tree roots than in response to soil characteristics and could be an important force in the assembly of at least some microbial communities. Overall, plant–microbe interactions are known to influence soil properties through a number of plant and microbial processes (Chaparro et al., 2012).

In addition to some of the more specific community characteristics that were found, we also calculated a core microbiome of 63.4% among the whole community and of 50% among the 33 more dominant genera. A core microbiome consists of a suite of members that are shared among the microbial consortia of similar habitats; the estimation that was calculated for the core microbiome here is reasonable in light of the additional community information that was observed. Regardless of the calculation method that was employed, however, to our knowledge this study represents the first description of a core microbiome that possesses similarities between anthropogenic and unvegetated soils. One explanation for this finding might relate to the fact that previous studies have focused only the groups that were found to be different among dominant taxa (Rieder and Frey, 2013). Specifically, previous studies have described individual microbial populations at the class or genus level using molecular tools such as terminal restriction fragment length polymorphism and targeted sequencing of major OTUs (Rieder and Frey, 2013).

The network dataset generated in this study indicates that detailing the extensive fungal–bacterial relationships that exist within soil provides complementary information on the complexity of the associated community (Rousk et al., 2008). To our knowledge, our work represents the first published field study of a microbial co-occurrence network within samples taken from anthropogenic, contaminated soils. Even if the network topology was more simplistic in the tailings dump than in the undisturbed soil we were still able to demonstrate that a complex structure could be achieved in this highly constrained environment. It has been previously highlighted (Shade and Handelsman, 2012) that the number of interactions within a microbial community may be more informative than the identity of its OTUs for predicting and managing core microbiomes across systems. Indeed, the slightly similar network structures that we observed between the two soils is consistent with the size of the core microbiome and suggests that a large number of interactions may stabilise the overall community of microbes by providing them with greater resistance to harsh conditions (Pimm, 1984; Neutel et al., 2007; Shade et al., 2012).

In conclusion, the multidisciplinary approach (physico-chemical analysis, Ion Torrent sequencing and related bioinformatics tools) used in this study facilitated in-depth exploration of the characteristics of the soil microbial communities that were found to reside within a tailings dump and those found within adjacent undisturbed soil and let us to conclude that environmental filtering is more likely to occur through the activity of tree roots rather than as a result of specific soil characteristics. Accordingly, the multidisciplinary approach that was used in this study might offer the most appropriate method of studying the impact of anthropogenic activities on soil biology and ecology.

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B.K. and C.Z. contributed equally well to the work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.08.008.

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Table S1: Calculation methods and definitions of diversity and network indices, community composition and structure.

Type	Calculation	Interpretation
Diversity indices		
Genera richness	*No*	Number of different genera
Shannon eveness		Index sensitive to rare genera
Inverse Simpson diversity		Index sensitive to dominant genera
Pielou eveness		Index given equitability in the community
Community composition and structure		
Correspondance analysis	Multivariate analysis	Qualitative change in the community composition
Abundance		Appearance or disappearance of dominant genera Quantitative change in the community structure
Pseudomonas / 16S copy		*Pseudomonas* proportion
Network indices		
Nodes	*Nc*	Number of connected taxa (nodes) in the ecological network
Unconnected vertices	*1-Nc*	Number of unconnected taxa in the ecological network
Links	*L*	Number of edges between taxa within the network
Cluster	*G*	Index of compartmentalization. The set of unique connected part of a network containing n taxa Can be thought of as the fundamental building blocks of complex networks
Connectance	$C=L/S^2$	Proportion of potential links that do occur. giving an estimation of the degree of inter-connectivity and of complexity reached by the network
Transitivity	*T*	Probability that the node C is connected to both nodes B and A if A is connected to B
Connectedness	*Cd*	Probability at least one chain between any pair of units Quantify the fragmentation within the network
Average degree	*aD*	Average number of links per taxon. the direct linkage density is a index of complexity More complexity is often interpreted as instability
Maximum degree	*mD=max (Li)*	Maximal number of links per taxon. Indicate the node with the most of direct links
Average betweenness	*aB*	Average number of shorter chains going through the nodes (including direct and indirect links)
Maximum betweenness	*mB*	Maximal number of shorter chains going through one node Indicate eventual keystone or central taxa in the network

Table S2: Physico-chemical parameters of natural soil and tailings dump. The mean and standard deviation of all parameters for the natural soil and the tailings dump are shown with the P-value of the variance analysis.

Area		Physical characterization					pH and carbonates		Assimilable phosphorus (g/kg)
		Clay (‰)	Thin Silt (‰)	Large Silt (‰)	Thin Sand (‰)	Coarse Sand (‰)	pH	$CaCO_3$ (‰)	
Undisturbed soil	mean (N=7)	238.57	308.43	266.86	112.71	73.43	5.67	0.00	0.16
	SD	113.68	89.19	62.42	64.44	40.27	0.71	0.00	0.29
Tailings dump	mean (N=7)	151.86	566.43	169.43	87.99	24.30	8.13	438.96	0.38
	SD	19.63	157.46	96.48	72.48	27.37	0.15	237.11	0.07
P value		**0.044**	**0.002**	0.054	0.513	**0.020**	**<0.0001**	**0.0003**	0.068

Area		Organic status				CEC (meq/kg)	Exchangeable oligos		
		Corg (g/kg)	OM (g/kg)	Ntot (g/kg)	C/N		K_2Oex (g/kg)	MgOex (g/kg)	CaOex (g/kg)
Undisturbed soil	mean (N=7)	28.43	49.16	2.60	10.97	171.29	0.33	0.16	3.33
	SD	5.17	8.90	0.92	1.69	51.32	0.10	0.04	2.11
Tailings dump	mean (N=7)	21.51	47.10	2.32	11.14	90.57	0.40	0.16	11.80
	SD	11.17	15.20	0.73	0.38	24.64	0.14	0.03	0.98
P value		0.163	0.763	0.551	0.798	**0.002**	0.362	0.947	**<0.0001**

Table S3: Metal trace element content of undisturbed soil and tailings dump. Mean and standard deviation of the measured elements for the undisturbed soil and the tailings dump and the P-value of the variance analysis.

Area		Al	As	Cd	Co	Cr	Cu	Hg	Ni	Pb	Zn
		mg/kg dry soil									
Undisturbed soil	mean (N=7)	20850.22	8.06	0.18	6.93	31.30	11.12	0.57	17.98	26.41	52.40
	SD	11483.27	2.24	0.14	2.11	15.83	4.95	0.49	8.63	6.91	24.36
Tailings dump	mean (N=7)	10477.53	20.42	0.32	3.51	19.71	10.01	3.83	11.53	16.85	54.60
	SD	1962.72	5.45	0.12	0.85	2.51	1.78	1.61	2.39	2.47	8.53
P value		0.011	<0.0001	0.067	0.002	0.066	0.814	<0.0001	0.060	0.001	0.532

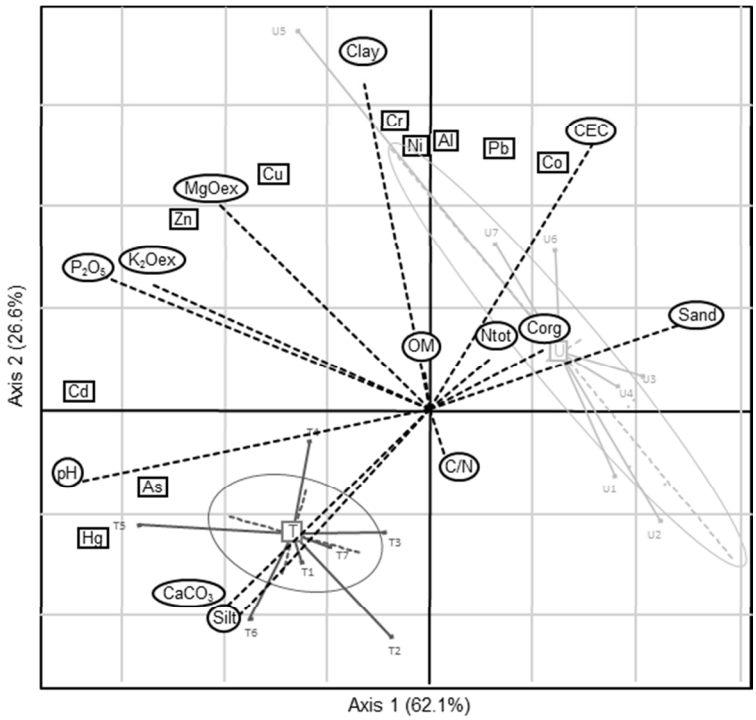

Figure S1: Redundant analysis between TE and physico-chemical characteristics of natural, undisturbed soil (U) and tailings dump (T) samples.

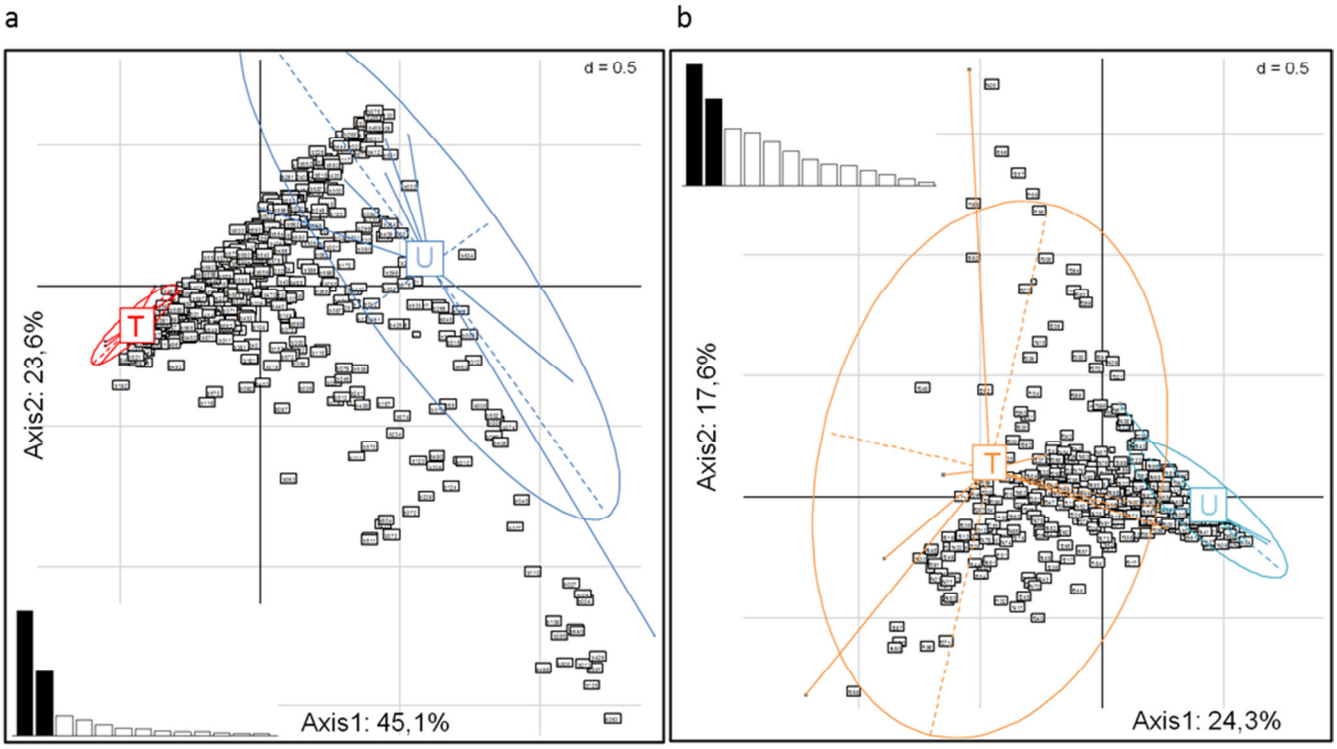

Figure S2: Constrained correspondence analysis followed by the Monte Carlo permutation test comparing the composition of a, bacterial and b, fungal communities in natural, undisturbed soil (U) and tailings dump (T). The dark blue and cyan ellipses show the undisturbed soil data and the dark red and orange ellipses indicate the tailings dump data on the two first axes of each analysis.

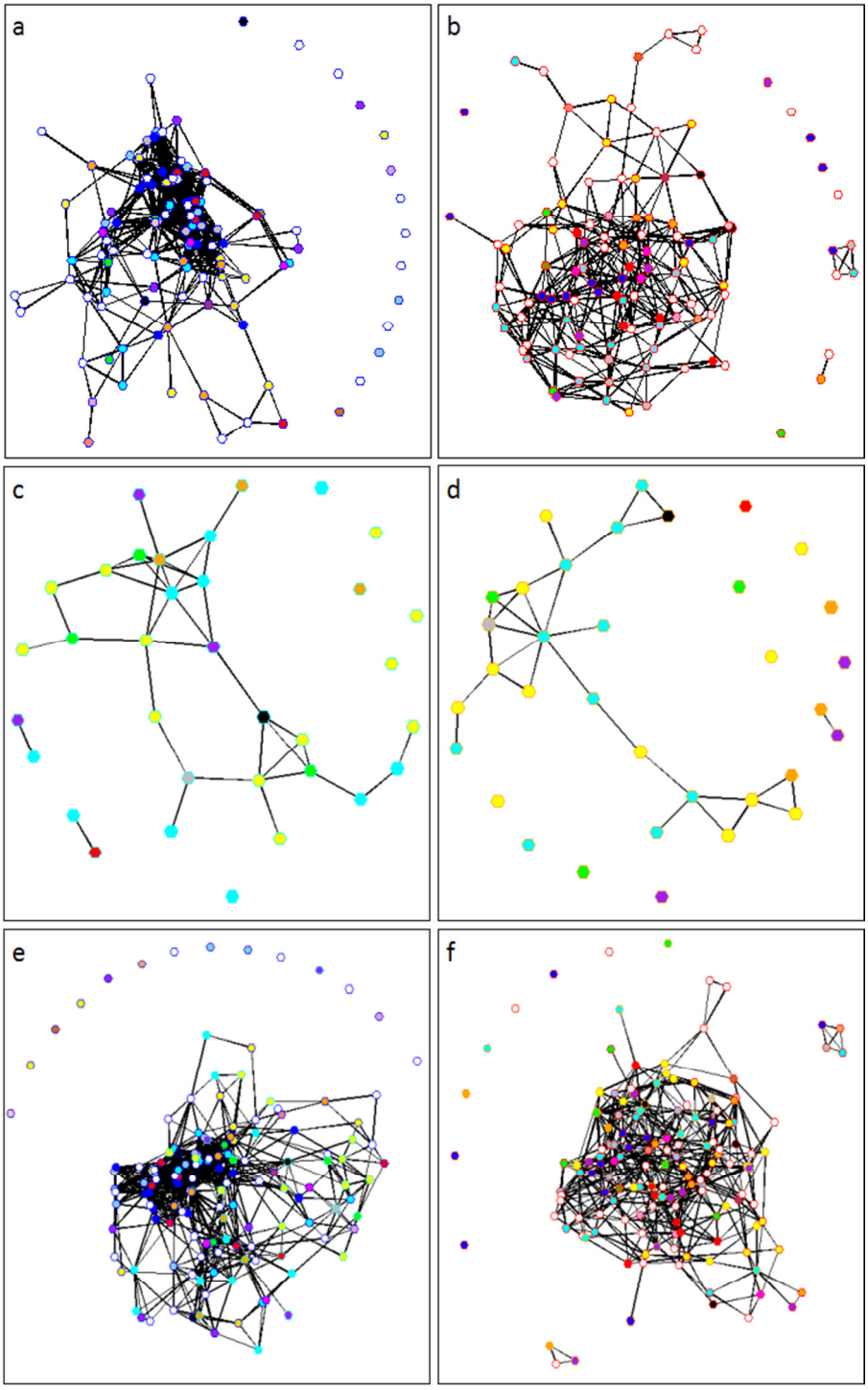

Figure S3: Correlation network analysis at the class level of microbial communities in undisturbed soil and tailings dump. a, and b, bacterial classes; **c, and d,** fungal classes; **e, and f,** merged fungal + bacterial classes; with **a, c and e,** for the natural soil and **b, d and f,** for the tailings dump. In these graphical representations, nodes are classes, and edges represent interactions. Dots circled in dark blue and dark red represent bacteria, and dots circled in cyan and orange represent fungi. The inside colours of dots represent different phyla.

3.1.2 Le métabarcoding environnemental appliqué à deux sites de phytomanagement basé sur le peuplier révèle des communautés microbiennes contrastées

Le but de cette étude est d'améliorer les connaissances des communautés microbiennes de sites pollués gérés par phytomanagement, et utilisant du peuplier, pour révéler les facteurs environnementaux qui favorisent l'abondance, la diversité et la composition des communautés microbiennes. Une analyse du sol a révélé que les deux sols affichent des caractéristiques physico-chimiques contrastées, avec un pH significativement plus faible et une fraction extractible au $CaCl_2$ de Cd, de Zn et de Mn supérieure pour le site de Leforest, par rapport au site de Pierrelaye. Les profils des communautés fongiques et bactériennes dans le sol et des racines de peuplier sont évalués par séquençage Illumina MiSeq. Les indices de diversité et les mesures de β-diversité illustrent le fait que les communautés microbiennes des racines étaient effectivement distinctes des communautés microbiennes du sol, et ce sur les deux sites. Une étude détaillée de la composition fongique a montré que les *Ascomycètes* dominent l'ensemble de la communauté fongique pour les échantillons de sols et des racines de peuplier de Pierrelaye, et dans les sols non plantés sur les deux sites expérimentaux. A l'inverse, les *Basidiomycètes* représentent un pourcentage beaucoup plus élevé de la communauté fongique dans les échantillons de sols et de racines de peuplier issus du site de Leforest. Les communautés bactériennes des racines sont dominées par les *Alphaproteobacteria* et les *Actinobacteria*, et les échantillons de sol sont dominés par les *Alphaproteobacteria* et les *Acidobacteria*. Le résultat majeur de cette étude est l'apparition et la dominance de la communauté ectomycorhizienne à Leforest, contrairement à Pierrelaye. Dans l'ensemble, les symbiotes ectomycorhiziens des racines semblent être fortement contraints par les caractéristiques du sol sur les sites de phytomanagement. Nos données soutiennent l'idée que l'inoculation mycorhizienne est nécessaire dans des environnements fortement perturbés et pauvres en éléments nutritifs comme celui de Leforest.

- ***Pour cet article, j'ai réalisé toutes les étapes allant de l'échantillonnage sur le terrain à l'écriture de la publication avec l'aide des co-auteurs.***

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Environmental metabarcoding reveals contrasting microbial communities at two poplar phytomanagement sites

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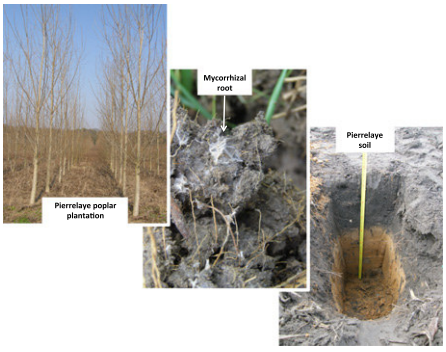
HIGHLIGHTS

- Two anthropospheric soils displayed contrasting physico-chemical characteristics.
- Fungal and bacterial communities were assessed through Illumina Miseq sequencing.
- Root microbial communities were well separated from soil microbial communities.
- *Ascomycota* dominated the fungal community in unplanted soils and Pierrelaye site.
- The ectomycorrhizal (mostly Basidiomycota) community dominated at Leforest.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the present study is to deepen the current understanding of the microbial communities at two poplar phytomanagement sites to reveal the environmental factors that drive the abundance, diversity and composition of microbial communities. A soil analysis revealed that the two soils displayed contrasting physico-chemical characteristics, with significant lower pH and higher Cd, Zn and Mn $CaCl_2$-extractable fractions at Leforest site, compared with Pierrelaye site. The fungal and bacterial community profiles in the poplar roots and soils were assessed through Illumina MiSeq sequencing. Diversity indices and β-diversity measures illustrated that the root microbial communities were well separated from the soil microbial communities at both sites. A detailed study of the fungal composition showed that *Ascomycota* dominated the overall fungal communities on poplar soil, the root samples at Pierrelaye, and the unplanted soil at the experimental sites. Conversely, *Basidiomycota* accounted for a much higher percentage of the fungal community in poplar root samples from the Leforest site. The root bacterial communities were dominated by *Alphaproteobacteria* and *Actinobacteria*, and the soil samples were dominated by *Alphaproteobacteria* and *Acidobacteria*. The occurrence and dominance of the ectomycorrhizal community at Leforest but not at Pierrelaye is the major feature of our data set. Overall, ectomycorrhizal root symbionts appeared to be highly constrained by soil characteristics at the phytomanagement sites. Our data support the view that mycorrhizal inoculation is needed in highly stressed and nutrient-poor environments.

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1. Introduction

Plants that are able to tolerate degraded, contaminated, and often nutrient-poor soils and those that can improve soil quality are essential initiators of ecological restoration in terrestrial environments. Poplars (*Salicaceae*) are now increasingly chosen for the reforestation of post-agricultural lands and the revegetation of areas that have been degraded by industrial or environmental pollution as well as renewable sources of biomass for bioenergy production (Chalot et al., 2012; Sabatti et al., 2014; Tognetti et al., 2013). Little is known about the entirety of the microbial communities that are associated with tree roots in derelict soils. In addition to estimating the microbial fertility of contaminated soils, knowledge of the associated microbial compartment could be used to help predict the potential recovery of disturbed lands (Kozdrój, 2000). Recent data suggest that the soil microbiome may indeed have the greatest impact on plant function during the early stages of revegetation (Bell et al., 2015). The possible structural changes of indigenous microbial communities by crops constitute a major ecological concern because of the important role that microorganisms have in regulating soil conditions (Conrad, 1996; Jeffries et al., 2003). The re-establishment of belowground interactions is required to ensure a successful restoration and the creation of sustainable plant cover.

To study soil and root microbial communities, researchers have witnessed the emergence of a high-throughput metabarcoding tool based on 454 (Danielsen et al., 2012; Gottel et al., 2011; Op De Beeck et al., 2015; Tedersoo et al., 2014), Ion Torrent (Zappelini et al., 2015) or Illumina MiSeq (Schmidt et al., 2013; Wu et al., 2015) metabarcoding technologies. When applied to contaminated soils in particular (Bell et al., 2014), these technologies have been used to reveal changes in microbial communities in the rhizosphere soils and plant roots and to uncover dominant species in the rhizosphere and the endosphere of tree species under contaminant stress (Azarbad et al., 2015; Bell et al., 2015; Yergeau et al., 2015; Zappelini et al., 2015). However, detailed information on the affected microbial groups under revegetation in derelict areas is still missing, although some attention has been focused on the isolation and characterization of culturable endophytes (Van der Lelie et al., 2009).

Thus, a greater understanding of the composition of the microbial community (including both dominant and rare taxa) is necessary for identifying both resistant and sensitive organisms in contaminated and disturbed soils. However, the structure of microbial communities may be largely determined by various soil characteristics, including the pH, moisture levels (Ansola et al., 2014; Gherghel et al., 2014; Shakya et al., 2013; Tedersoo et al., 2014) and/or contamination (Lorenz et al., 2006; Op De Beeck et al., 2015; Turpeinen et al., 2004; Yergeau et al., 2014). The plant cover itself, including the plant species, the age of the plantation and the physiological (seasonal) activity, may also greatly impact microbial communities (Hrynkiewicz et al., 2010; Labbé et al., 2011; Shakya et al., 2013; Tagu et al., 2004; Vargas-Gastélum et al., 2015; Xue and Huang, 2013).

As a first step towards tree-based phytoremediation that is assisted by symbiotic fungi, we have adopted a metabarcoding approach that employs the high-throughput Illumina MiSeq platform to investigate whether the soil structure and composition and the contaminant levels have the largest effects on the diversity of bacterial and fungal phyla. To investigate potential variance caused by the site's history, we compare two contrasting contaminated areas in France. We hypothesized that the implementation of poplar stands would significantly and differentially shape the fungal and bacterial communities. We believe that this study is relevant to the field of environmental microbiology because it reveals the contrasting consequences of poplar implementation on the microbial communities at two phytomanaged trace metal-contaminated sites.

2. Materials and methods

2.1. Study sites

Poplars were planted in April 2007 as a short-rotation coppice at a density of 1000 stems ha^{-1} for bioenergy production as part of the Phytopop project. Poplar genotypes were chosen on the basis of their capacity to grow on the two sites and to cover the preferred poplar genetic diversity (several species and hybrids from different sections) within the limits imposed by French regulations. Each of the 14 genotypes was growing on an independent plot of 24.5 × 20 m, and each plot contained 49 trees. Details on the genotypes are given in (Pottier et al., 2015). The present work was performed on 2 of the 14 poplar clones, namely Skado (*Populus trichocarpa* × *Populus maximowiczii* section Tacahamaca) and Vesten (*Populus deltoides x Populus nigra* section Aigeiros) clones, which were planted at the two demonstration sites.

The experimental site at Pierrelaye is located northwest of Paris in Pierrelaye (49°01′47.6″N 2°10′34.2″E). Initially dedicated to vegetable gardening, the soils at Pierrelaye were irrigated with raw wastewater from 1899 to 2002 for fertilization purposes. Consequently, TE accumulated in the soil, leading to polymetallic pollution characterized by Pb, Cu, Zn and Cd concentrations that were 10 times higher than those of a non-irrigated reference soil (Lamy et al., 2006; Pottier et al., 2015). Crop production for human consumption was prohibited in the late 1990s (Lamy et al., 2006) and had to be replaced by alternative uses, providing other types of ecosystem services. The experimental site at Leforest is located northwest of France in Leforest (49°01′22.3″N 2°09′12.9″E), in the Metaleurop area. Metaleurop Nord, which was one of the biggest zinc and lead production plants in Europe, was located in northern France (Noyelles-Godault 59) (Douay et al., 2009; Migeon et al., 2009). It was created in 1894 and produced approximately 170,000 tons of lead and 105,000 tons of zinc per year until it was closed in 2003. This plant generated a significant quantity of dust, and its fallout contaminated the surrounding area to a distance of several kilometers, with high amounts of Cd, Zn and Pb. Studies have been undertaken to investigate the nature of the contamination, the contaminant transfer mechanisms and their impact on the local populations (Migeon et al., 2009). The two sites have very similar climatic conditions, with average annual precipitation amounts of 662 mm and 630 mm and mean annual temperatures of 10.1 °C and 10.9 °C at Leforest and Pierrelaye, respectively.

2.2. Sampling procedure

All soil and root samplings were performed in October 2013. After litter removal, the roots were collected from the upper 20 cm layer of soil from under the tree canopies. The roots were traced to their origin to ensure that they were indeed connected to the selected tree, and they were sampled at a distance of 1 m from the trunk. For each poplar clone, five individual trees were randomly sampled, and for each individual tree, 3 pseudo-replicates were sampled and mixed to obtain root and soil composites. Within the planted or unplanted control plots, five bulk soil samples were collected directly below the root system of the poplars (planted) or natural vegetation (unplanted) from the top 20 cm of soil. Subsamples were stored at 4 °C prior to DNA extraction. The roots were carefully washed with tap water to remove visible soil, washed with sterile deionized water tree times, and separated from the roots of other plant species by shape and color. The fine roots were then cut off and transferred to Eppendorf tubes and frozen at −20 °C. The letter codes are as follows: LRS, Leforest root Skado; LRV, Leforest root Vesten; LSS, Leforest soil Skado; LSV Leforest soil Vesten; LSU, Leforest soil unplanted; PRS, Pierrelaye root Skado; PRV, Pierrelaye root Vesten; PSS, Pierrelaye soil Skado; PSV Pierrelaye soil Vesten; and PSU, Pierrelaye soil unplanted.

2.3. Soil physico-chemical properties

To determine the TE extractable fractions, 5 g of 2-mm sieved soil was dried at 60 °C for 48 h and incubated with 50 ml of 10 mM calcium chloride under agitation (40 rpm) for 2 h at room temperature. The mixture was first filtered with ash-free filters and subsequently passed through a 0.45-micrometer mesh and acidified at 2% (v/v) with HNO_3 prior to inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fischer Scientific, Inc., Pittsburg, USA) analysis. The methods for measuring physico-chemical characteristics are those described in Zappelini et al. (2015).

2.4. Molecular methods

The total DNA was extracted from 500 mg of soil with a PowerSoil® DNA Isolation Kit (MO-BIO laboratories, Inc., Carlsbad, CA USA) according to the manufacturer's instructions. To isolate microbial DNA from the roots, plant cell lysis was first performed using 100 mg of frozen root samples that were pulverized with 3-mm tungsten carbide beads (Qiagen S.A.S., Courtaboeuf, France) in a Mixer Mill for 3 min at 30 Hz (model MM400; Retsch Inc., Newtown, PA). Root DNA was then extracted with a PowerSoil® DNA Isolation Kit according to the manufacturer's instructions. The DNA quality and quantity were assessed by agarose gel electrophoresis and with a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) in 96-well microplates using a spectrofluorometer FLX-Xenius (SAFAS, Monaco). Equimolar DNA pools were created and adjusted to 10 ng/μl. The sequencing of the V3-V5 domains of 16S rRNA genes and the fungal ITS2 was performed with an Illumina MiSeq platform (Microsynth AG, Switzerland). PCR amplifications were performed with the bacterial primers 341F (5′- CCT ACG GGR SGC AGC AG -3′) and 909R (5′- TTT CAG YCT TGC GRC CGT AC -3′) and the fungal primers ITS3 (5′- GCA TCG ATG AAG AAC GCA GC -3′) and ITS4 (5′- TCC GCT TAT TGA TAT GC -3′).

2.5. Data analysis

Reads were assigned to each sample according to a unique barcode, and contigs were then assigned using the Mothur pipeline (Schloss et al., 2009). Raw reads were filtered by length and quality. The 16S reads were pre-clustered using sumaclust (Mercier et al., 2013) at 0.99 identity. ITS reads were simply clustered at unique sequences. Only sequences with at least 5 reads for bacteria and 10 reads for fungi were conserved. The 16S sequences were aligned with those in the Silva database to remove non-16S sequences. The 28S conserved regions of ITS sequences were searched using HMM (Hartmann et al., 2010), and plant contamination was removed by identifying *Populus* chloroplastic ITS.

Taxonomic assignments were performed using a Bayesian approach (Wang et al., 2007) in the Greengene database (DeSantis et al., 2006) for bacteria and the UNITE database (Kõljalg et al., 2005) for fungi. Finally, the OTUs were constructed using the Needleman distance and average neighbor clustering at a distance of 0.03. Diversity parameters were calculated on the basis of a data set in which the numbers of reads per sample were rarefied to 2140 and 1000 reads per sample for fungi and bacteria, respectively. We calculated the rarefaction curves at a 97% similarity level to verify whether the amount of sequencing reflected the diversity of the original microbial communities. No differences in fungal or bacterial diversity patterns were observed between the rarefied and non-rarefied data sets; therefore, we opted to keep the rarefied data set to control for unequal sampling depths across samples.

2.6. Statistical analysis

All statistical analyses were performed using R software v. 3.0.2 (R Core Team, 2013). The Shapiro and Bartlett tests were used to verify the normality and homoscedasticity of the data, respectively, and we compared each TE and measured soil parameters using either an ANOVA or a Kruskal-Wallis test (with the "kruskal.test" function in R). Richness and diversity indices were calculated using pgirmess and Vegan package in R. Rarefaction curves were created with the "rarecurve" function in the Vegan package in R. NMDS was calculated using the Bray Curtis method ($k = 3$) using the "metaMDS" function in the Vegan package in R. PERMANOVA was run with 1000 permutations using the "adonis" function in the Vegan package in R. The resulting clustering trees were paired with a heatmap of Spearman's correlations between the relative abundances created with "heatmap.2" from the gplots package. The numbers of OTUs that were shared between crops were visualized using Venn diagrams implemented by VennDiagram in the Vegan package in R. Graphs were created with the "riverplot" function in the riverplot R package.

3. Results and discussion

3.1. Massive Illumina MiSeq sequencing reveals the microbial species richness and diversity across two contrasting environments

Although they had similar former land use (i.e., agricultural) types, the two Pierrelaye and Leforest sites represented two contrasting environments with different pollution histories (Table 1). These soils are consequently heavily polluted with Zn, Cd and Pb. A physical analysis revealed that the Leforest soil contained significantly greater proportions of silt and clay, whereas the Pierrelaye soil contained more sand. The pH was approximately one unit lower at Leforest, and the Leforest soil was also characterized by higher Cd, Zn and Mn $CaCl_2$-extractable fractions. Although the Pierrelaye soil was significantly enriched in phosphorus, organic C and total N, no significant differences were found between the two soils with respect to the C/N ratio and total K (Table 1).

A total of 3,917,236 16S bacterial and 10,404,129 internal transcribed spacer (ITS) fungal raw MiSeq reads were obtained from 30 DNA soil samples and 20 DNA root samples. After quality filtering and target extraction but before subsampling, 163,932 and 231,162 bacterial sequences and 640,643 and 822,740 fungal sequences remained for the community analyses of Leforest and Pierrelaye, respectively. A detailed breakdown of the recovered sequence numbers that was performed sample-by-sample is presented in Table 2. A rarefaction analysis revealed that estimates of the operational taxonomic units (OTUs) sharply increased before approaching a plateau. Our results showed that all of the fungal and bacterial libraries from our samples represented the fungal (Supplementary material Fig. S1A) and bacterial (Supplementary material Fig. S1B) communities well, and the rarefaction curves tended towards saturation. These rarefaction curves indicated the presence of large variations in the total numbers of OTUs in different samples (Supplementary material Fig. S1). In comparison with the soil samples, all of the poplar root samples had lower bacterial and fungal OTU densities.

An OTU-based approach was used to calculate the richness (as indicated by the OTU number and Chao1) and diversity (as indicated by the Shannon and Simpson indices and Pielou evenness). The OTU richness, Chao1, Shannon and inverse Simpson indices were significantly higher ($P < 0.05$) in poplar soil samples in comparison with the root samples for both study sites and both fungal and bacterial communities, findings that were consistent with the rarefaction curves (Table 2). The same trends were observed in the Pielou evenness, although they were less significant. In comparing the two sites, the fungal and bacterial richness and diversity indices were higher in most cases in Pierrelaye than in Leforest. Spearman's correlation coefficients revealed statistically negative correlations between the fungal richness, Shannon and Simpson indices, Pielou evenness and Cd and Zn contents at Leforest (Supplementary material Table S1). No significant differences were observed for the fungal richness and diversity indices and the soil characteristics at Pierrelaye ($P > 0.05$) (Supplementary material Table S1). The Cd, Fe, Mn, Na, Ni, Pb, and Zn contents were negatively correlated with

Table 1
Physico-chemical parameters of Leforest and Pierrelaye soils. Mean values and standard deviations are provided (n = 8 for physico-chemical parameters and n = 15 for $CaCl_2$-extractable elements).

Site	Physical characterization					pH and carbonates		Phosphorus	
	Clay[a] (%)	Thin silt (%)	Large silt (%)	Thin sand (%)	Coarse sand (%)	pH	$CaCO_3$ (%)	Assimilable (g/kg)	$CaCl_2$ extractable (mg/kg)
Leforest (L)	26.5 ± 2.0	20.4 ± 1.3	36.8 ± 2.0	13.4 ± 0.8	2.9 ± 0.4	6.5 ± 0.2	0.05 ± 0.09	0.18 ± 0.04	2.2 ± 1.2
Pierrelaye (P)	3.8 ± 1.3	4.4 ± 1.6	4.5 ± 1.6	39.4 ± 2.5	47.9 ± 4.6	7.4 ± 0.1	0.78 ± 0.27	1.10 ± 0.23	8.0 ± 0.8
Level of significance	***,[b]	***	***	***	***	***	***	***	***

Site	Organic status				CEC ($cmol_c$/kg)	Exchangeable oligos			
	Corg (g/kg)	OM (g/kg)	Ntot (g/kg)	C/N		K_2O (g/kg)	MgO (g/kg)	CaO (g/kg)	Na_2O (g/kg)
Leforest (L)	21 ± 3	37 ± 5	1.9 ± 0.1	11 ± 1	18 ± 3	0.23 ± 0.01	0.32 ± 0.03	5.0 ± 0.7	0.01 ± 0.01
Pierrelaye (P)	31 ± 8	53 ± 13	2.9 ± 0.7	10 ± 1	8 ± 2	0.24 ± 0.07	0.19 ± 0.05	3.8 ± 1.0	0.00 ± 0.00
Level of significance	**	**	**	–	***	–	***	*	***

Site	$CaCl_2$ extractable metals								
	Cd (μg/kg)	Cu (μg/kg)	Fe (μg/kg)	Mg (mg/kg)	Mn (mg/kg)	Na (mg/kg)	Ni (μg/kg)	Pb (μg/kg)	Zn (μg/kg)
Leforest (L)	0.66 ± 0.53	<LQ	0.17 ± 0.34	127 ± 10	12.8 ± 6.3	17.9 ± 2.9	0.17 ± 0.16	0.06 ± 0.11	11.4 ± 7.8
Pierrelaye (P)	0.06 ± 0.03	0.06 ± 0.06	<LQ	66 ± 10	0.7 ± 0.2	5.4 ± 1.7	<LQ	<LQ	2.8 ± 1.2
Level of significance	***	**	***	***	***	***	***	*	***

Notes: –, not significant.
[a] Soil samples were separated each into five particle-size fractions: coarse sand (2000–200 μm), thin sand (200–50 μm), large silt (50–20 μm), thin silt (20–2 μm), and clay (<2 μm).
[b] Asterisks indicate significant differences between Leforest and Pierrelaye soils.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

the bacterial richness, Shannon and Simpson indices, and Pielou evenness (Supplementary material Table S2). Conversely, K was the only soil parameter that was positively correlated with the bacterial richness and/or fungal richness at Leforest. The K, Mg, Mn, and Na contents were significantly and positively correlated with the bacterial richness and/or richness indices at Pierrelaye, while Cd and Zn were significantly but negatively correlated (Supplementary material Table S2).

The fungal OTU richness and diversity data compared well with previous data, with Shannon indices of approximately 2 and 4 for the roots and soil habitats, respectively (Buée et al., 2009; Danielsen et al., 2012; Vargas-Gastélum et al., 2015). Our MiSeq sequencing results (total OTUs) and Chao diversity estimates for bacteria were slightly lower than those of a previous study, although the Shannon indices were comparable (Hong et al., 2015). Regardless of the site, the fungal and bacterial species richness and diversity were always higher in the soils in comparison with the roots (Table 2), as previously shown in Danielsen et al. (2012) and Shakya et al. (2013). Although our data indicated that the fungal and bacterial species richness and diversity in soil habitats were not different between planted and unplanted plots, the roots may offer a different habitat when stronger competition appears, especially for C nutrition, as suggested earlier (Danielsen et al., 2012; Druebert et al., 2009; Pena et al., 2010). Therefore, the priority concept developed for ectomycorrhizal (ECM) colonization (e.g., Kennedy et al. (2009)) might be expanded to the whole microbial community.

Table 2
Richness and diversity indices of Pierrelaye and Leforest microbial communities.

	Total no. of sequence reads	No. of OTUs[a]		Diversity estimates[b]		
		Observed	*Chao*	*Shannon*	*Simpson*	*Pielou*
Fungi						
LRS[c]	80,532	84 ± 21 (f)[d]	134 ± 41 (e)	2.3 ± 0.6 (e)	0.79 ± 0.09 (e)	0.53 ± 0.10 (f)
LRV	37,982	135 ± 35 (e)	217 ± 52 (d)	3.0 ± 0.6 (de)	0.86 ± 0.09 (cde)	0.61 ± 0.09 (ef)
LSS	212,540	191 ± 19 (d)	315 ± 29 (c)	3.5 ± 0.4 (cd)	0.9 ± 0.06 (bcde)	0.66 ± 0.07 (de)
LSV	100,885	211 ± 42 (cd)	331 ± 65 (c)	3.6 ± 0.6 (bc)	0.91 ± 0.06 (abcd)	0.67 ± 0.10 (bcde)
LSU	208,704	229 ± 31 (cd)	342 ± 42 (c)	4.0 ± 0.4 (ab)	0.95 ± 0.03 (a)	0.73 ± 0.06 (abc)
PRS	60,557	156 ± 27 (e)	241 ± 72 (d)	3.4 ± 0.4 (cd)	0.90 ± 0.06 (bcd)	0.67 ± 0.06 (cde)
PRV	27,681	137 ± 21 (e)	199 ± 37 (de)	3.0 ± 0.4 (de)	0.86 ± 0.04 (de)	0.60 ± 0.06 (ef)
PSS	253,929	284 ± 20 (a)	459 ± 34 (a)	4.4 ± 0.1 (a)	0.96 ± 0.01 (a)	0.77 ± 0.02 (abc)
PSV	379,262	248 ± 21 (bc)	368 ± 43 (bc)	4.0 ± 0.3 (ab)	0.93 ± 0.03 (abc)	0.73 ± 0.04 (abcd)
PSU	101,311	265 ± 30 (ab)	409 ± 38 (ab)	4.1 ± 0.5 (a)	0.93 ± 0.06 (ab)	0.73 ± 0.08 (ab)
Bacteria						
LRS	38,482	185 ± 15 (g)	324 ± 11 (g)	3.8 ± 0.2 (f)	0.92 ± 0.01 (f)	0.72 ± 0.03 (f)
LRV	40,236	223 ± 33 (f)	382 ± 56 (fg)	4.1 ± 0.4 (f)	0.93 ± 0.02 (f)	0.76 ± 0.05 (f)
LSS	25,672	308 ± 12 (d)	538 ± 69 (de)	4.9 ± 0.1 (d)	0.98 ± 0.01 (d)	0.86 ± 0.02 (d)
LSV	25,314	353 ± 27 (bc)	642 ± 64 (c)	5.2 ± 0.2 (c)	0.99 ± 0.01 (c)	0.89 ± 0.01 (c)
LSU	34,228	328 ± 16 (cd)	594 ± 61 (cd)	5.1 ± 0.1 (cd)	0.99 ± 0.01 (c)	0.88 ± 0.02 (cd)
PRS	44,392	262 ± 23 (e)	538 ± 109 (de)	4.6 ± 0.2 (e)	0.97 ± 0.01 (d)	0.83 ± 0.02 (e)
PRV	55,487	245 ± 15 (ef)	474 ± 61 (ef)	4.5 ± 0.2 (e)	0.96 ± 0.01 (e)	0.81 ± 0.02 (e)
PSS	30,525	395 ± 10 (a)	822 ± 21 (a)	5.5 ± 0.1 (a)	0.99 ± 0.01 (a)	0.91 ± 0.01 (ab)
PSV	54,094	379 ± 6 (a)	773 ± 94 (ab)	5.5 ± 0.1 (a)	0.99 ± 0.01 (a)	0.92 ± 0.01 (a)
PSU	46,664	396 ± 12 (b)	726 ± 55 (b)	5.4 ± 0.1 (b)	0.99 ± 0.01 (b)	0.91 ± 0.01 (b)

[a] The richness is calculated using the OTU number and Chao's species richness estimator (*Chao*).
[b] The diversity is estimated from the Shannon-Wiener H′ (*Shannon*) and Simpson's D indices (*Simpson*), and the equitability is estimated using Pielou's evenness J (*Pielou*).
[c] LRS, Leforest root Skado; LRV, Leforest root Vesten; LSS, Leforest soil Skado; LSV Leforest soil Vesten; LSU, Leforest soil unplanted; PRS, Pierrelaye root Skado; PRV, Pierrelaye root Vesten; PSS, Pierrelaye soil Skado; PSV Pierrelaye soil Vesten; and PSU, Pierrelaye soil unplanted.
[d] Values designated with the same letters are not significantly different ($P < 0.05$) according to a Kruskal-Wallis test.

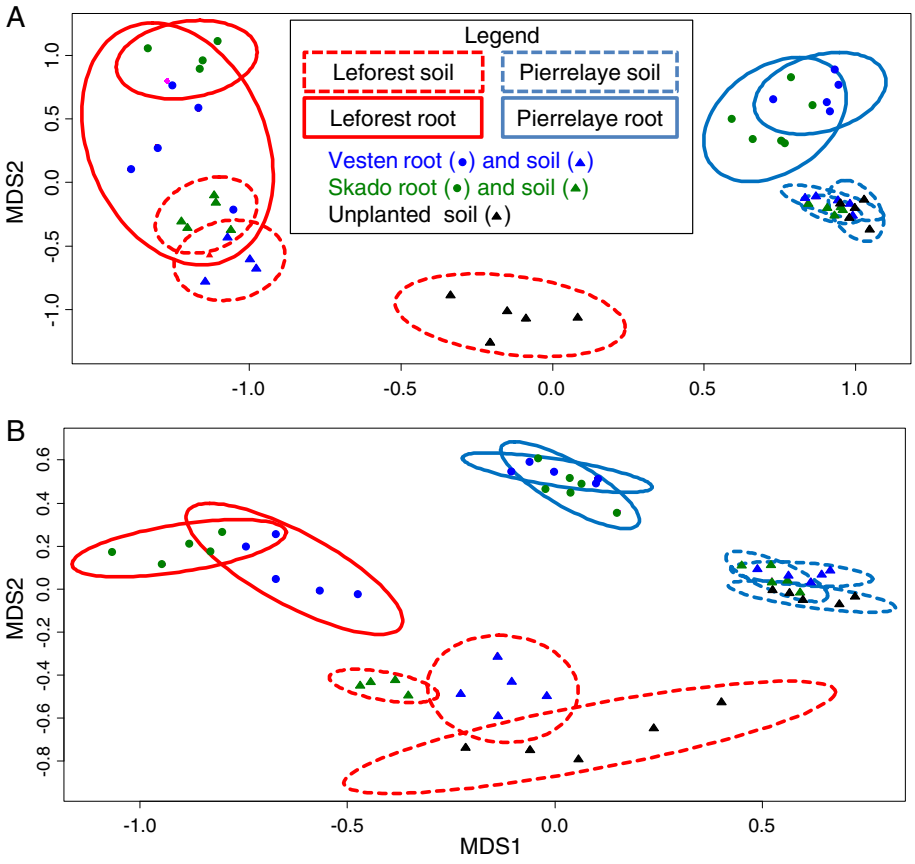

Fig. 1. NMDS plots of the fungal (A) or bacterial (B) community structures using the Bray Curtis dissimilarity measure. Confidence area of ellipses = 0.95.

We used a β-diversity measure based on OTUs (Bray-Curtis) to compare the microbial communities that quantifies the taxa abundances that are shared between samples in comparison with those that are unique to one sample. In the 2-dimensional non-metric multi-dimensional scaling (NMDS) plot visualized here, the Bray-Curtis method indicated that the fungal communities of Leforest (red ellipses) and Pierrelaye (blue ellipses) were well separated (Fig. 1A), which was also the case for the bacterial communities (Fig. 1B). A permutation test confirmed that the site (Pierrelaye in comparison with Leforest, $R^2 = 0.26$) explained most of the variance in the fungal community composition and made a major contribution to the variance in the bacterial community composition (Supplementary material Table S3). Most of the variance in the bacterial community composition was explained by habitats (roots in comparison with soil at Leforest, $R^2 = 0.29$, Supplementary material Table S3). Clear separations of microbiomes have also been reported for the soil and roots of mature poplar sites (Gottel et al., 2011) and 2 year-old poplars (Danielsen et al., 2012). The scattering of soil and root data was always lower at Pierrelaye than at Leforest (see sizes of ellipses in Fig. 1). These data denoted the higher homogeneity of species distribution in soil or root samples at Pierrelaye, whereas the soil or root samples at Leforest were less homogeneous.

3.2. Microbial compositions at two phytomanagement sites

To identify the phylogenetic diversity of microbial communities at the two sites, effective reads were assigned to the phyla, classes and genera. These reads were associated with 27 bacterial and 7 fungal phyla and 83 bacterial and 26 fungal classes (Fig. 2). At Leforest, the classifiable soil and root fungal ITS sequences from the Vesten and Skado poplar plots were primarily dominated by the *Agaricomycetes* (*Basidiomycota*; Fig. 2A), whereas the unplanted soil was dominated by the *Sordariomycetes* (*Ascomycota*; Fig. 2A). In contrast, the fungal community profiles at Pierrelaye between planted and unplanted soils were very similar (Fig. 2A), with the *Eurotiomycetes* (*Ascomycota*) and the *Sordariomycetes* (*Ascomycota*) dominating the root and soil samples, respectively. As further depicted in Fig. 3A, the three most dominant OTUs in the Skado poplar clone soil samples from Leforest (LSS) were *Cortinarius diasemospermus* (*Basidiomycota*; 25%), *Inocybe curvipes* (*Basidiomycota*; 8.7%) and *Exophiala* sp. *KL 2011f* (*Ascomycota*; 5.7%). Conversely, these dominant Basidiomycota were not detected in unplanted soil samples at Leforest (LSU; Fig. 3B), whereas the *Ascomycota Verticillium nigrescens* (10.1%), *Podospora appendiculata* (6.3%) and *Lasiosphaeriaceae* (5.8%) were dominant. At Pierrelaye, the dominant OTUs were the *Ascomycota Exophiala* sp. *KL 2011f* and *Penicillium canescens* in the Skado and Vesten root and soil samples and in unplanted soil samples (Supplementary material Table S4). In the Pierrelaye samples, the dominant *Basidiomycota* OTU represented <3% of all OTUs (Supplementary material Table S4). A hierarchical cluster analysis of fungal communities at the class level (Fig. 4A) showed that *Orbiliomycetes*, *Leotiomycetes*, and *Agaricomycetes* were positively correlated with root samples and planted soil samples at Leforest (LRS, LRV, LSS and LSV) and were negatively correlated with all of the other root or soil samples (LSU, PSU, PRS, PRV, PSS and PSV). *Sordariomycetes* and *Dothideomycetes* were positively correlated with soil samples from unplanted plots at both sites (LSU and PSU) and in planted soils at Pierrelaye (PSS and PSV).

Thus, *Ascomycota* dominated the overall fungal communities in poplar soil and root samples at Pierrelaye and the unplanted soil at the Leforest and Pierrelaye sites, representing at least 2/3 of the overall fungi in these 6 habitats. The *Basidiomycota* accounted for much smaller percentages of the communities in these samples (usually <20%). The dominance of *Ascomycota* was also observed in roots and soil collected under *Populus*

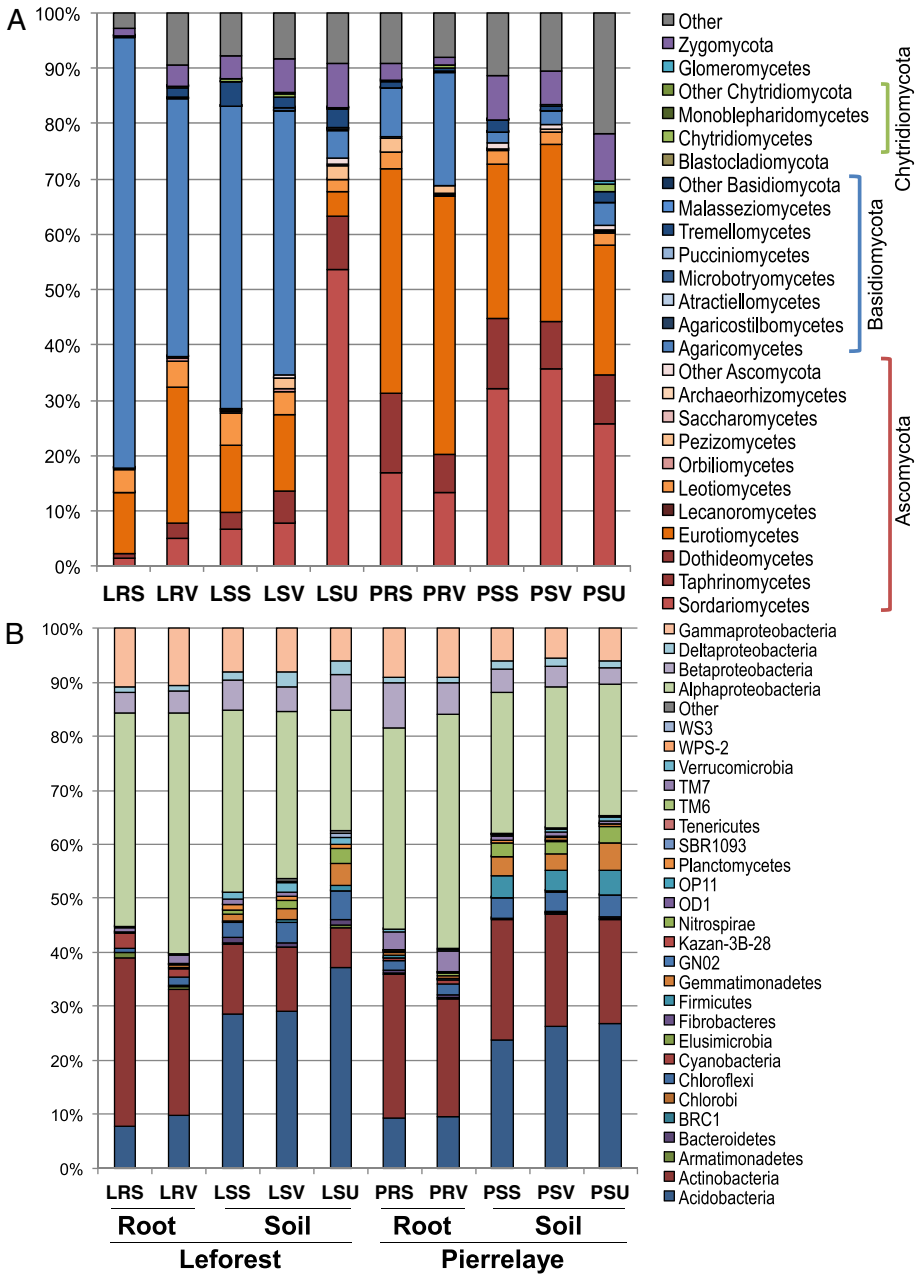

Fig. 2. Compositions of the fungal (A) and bacterial (B) communities at the Leforest and Pierrelaye sites. The data were derived from MiSeq sequencing for soil and root samples from contaminated sites. Refer to the legend in Table 2 for sample abbreviations.

deltoides trees that were grown in unpolluted soil (Shakya et al., 2013). Schadt et al. (2003) also found a large proportion of *Ascomycota* in 125 cloned fungal sequences from tundra soils. However, in four of our studied habitats, which corresponded to the poplar soil and root samples at Leforest, the *Basidiomycota* was the most abundant phylum (> 49%). *Basidiomycota* dominance is often observed in forest soils (Buée et al., 2009; O'Brien et al., 2005) and more generally in various biomes, including boreal, temperate coniferous and deciduous forests, tropical montane and moist forests (Tedersoo et al., 2014; Zappelini et al., 2015).

All of the root samples were dominated by *Alphaproteobacteria* (>37%), *Actinobacteria* (>18%) and *Gammaproteobacteria* (>9%), which corresponds with previous reports on *Populus* (Gottel et al., 2011; Shakya et al., 2013). The *Acidobacteria* (>6%) increased notably in unplanted and planted soil samples at both sites, while the *Actinobacteria* decreased (<14%) (Fig. 2B). A hierarchical cluster analysis of bacterial communities at the class or phylum level confirmed the clear separation of root samples relative to soil samples (Fig. 4B). *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria* and *Cyanobacteria* were positively correlated with all of the root samples at both sites (LRS, LRV, PRS, and PRV). We also observed a second cluster of bacterial phyla and classes (*Nitrospirae*, *Gemmatimonadetes*, *Chloroflexi*, and *Acidobacteria*) associated with Pierrelaye (PSS, PSV and PSU) and some Leforest (LSU, LSV) soil samples (Fig. 4B). The prevalence of *Alphaproteobacteria* in root samples was highlighted by the dominance of *Rhizobiaceae* and *Bradyrhizobiaceae* family members in most root samples from both sites and both poplar clones (Supplementary material Table S5). The co-occurrence of *Micromonosporaceae* and *Rhizobiaceae*/*Bradyrhizobiaceae members* in our root samples was in line with recent works reporting that *Micromonosporae* contain major components of nitrogen-fixing root nodules in both leguminous and actinorhizal plants (for a review see Trujillo et al. (2015)). Although the planted trees are not nitrogen fixers, the *Rhizobiaceae* and

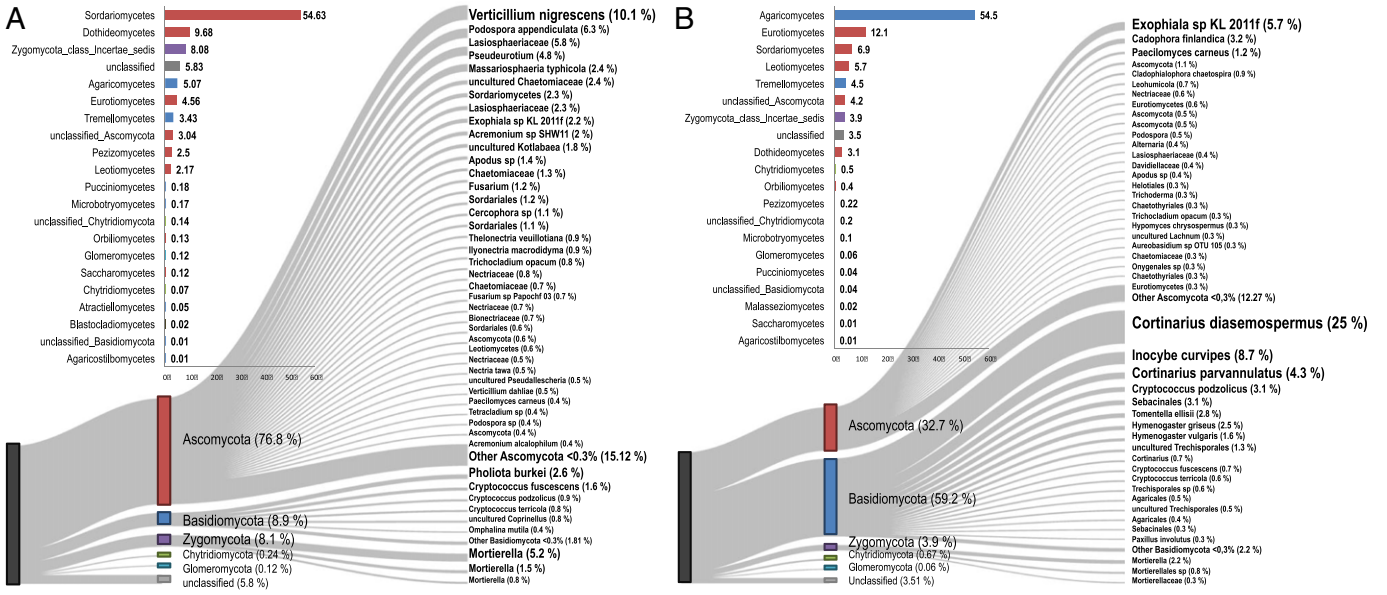

Fig. 3. Proportions of most abundant fungal OTUs at the Leforest unplanted (A) and poplar (*Populus* cv Skado) planted (B) soils. Samples were assigned to phyla, classes or species when possible. OTUs were assigned at the class, order, family, genus or species level when possible. The relative proportions are given in parentheses. Inserts indicate the proportions of fungal classes.

Bradyrhizobiaceae genera are frequently encountered in non-fixer tree microbiomes (Bonito et al., 2014; Shakya et al., 2013).

3.3. The ectomycorrhizal community became dominant at Leforest

Each fungal species, genus, family, or class was further assigned to functional groups of fungi as described in Tedersoo et al. (2014) or after literature research. To examine the distributions of functional categories in the soil and root samples, the abundances of the various OTU groups were set to 100%, and the OTUs were classified as ectomycorrhizal (ECM), arbuscular mycorrhizal (AM), endophytic, saprotrophic or biotrophic (Fig. 5 and Supplementary material Fig. S2 and Tables S6 and S7). In unplanted soil samples from both sites, saprophytic fungi formed the largest group (65% and 56% at Leforest and Pierrelaye, respectively), followed by biotrophs (17% and 13% at Leforest and Pierrelaye, respectively). In the Skado planted plots at Pierrelaye, this distribution remained similar, with saprophytic fungi representing 60% in both root and soil samples. In contrast, ECM fungi were the dominant group at Leforest, comprising 23% and 50% of the total abundance in Skado root and soil samples, respectively (Fig. 5). OTUs that could not be assigned to functional groups represented between 16 and 30% of the total fungal OTUs (Fig. 5 and Supplementary material Table S6). Very similar trends were obtained for the Vesten poplar clone at both sites (Supplementary material Fig. S2 and Table S7).

Correlations between fungal OTUs of the various identified functional groups are depicted in Supplementary material Table S8. Negative correlations between ECM and saprotrophic/biotrophic communities were identified. These data highlighted that the dominance of ECM fungi occurred at the expense of biotrophs for the most part and at the secondary expense of saprotrophs at Leforest. This finding is exemplified by the dominance of the biotrophic fungus *Verticillium nigrescens* (10.1% of total OTUs) in the unplanted soil at Leforest, whereas the ECM fungus *Cortinarius diasemospermus* (25% of total OTUs) dominates in the poplar soil and root samples at Leforest. Among the 5 ECM species that dominated the fungal community at Leforest, the genera *Cortinarius*, *Inocybe*, *Hymenogaster*, *Cadophora* and *Tomentella* made up most of the OTUs. *Cortinarius* species are ubiquitously detected in forest soils (Buée et al., 2009). Interestingly, the species *Cadophora finlandica* is exclusively detected in poplar-planted soil and roots at Leforest, where ECM fungi dominated. This fungus is an *Ascomycete* species belonging to the *R. ericae* aggregates that are believed to be able to form ECM and ericoid associations with different plant species (Vrålstad, 2004); they are also frequently found in TE-polluted soils (Gorfer et al., 2009; Krpata et al., 2008). Some reports have also indicated the presence of *Inocybe* species on TE-polluted soils (Huang et al., 2012). The major ECM genera at Pierrelaye were *Tuber*, *Tomentella* and *Geopora*, which were not present at Leforest. Fungi from the *Thelephoraceae* and *Cortinariaceae* families were previously the most common ECM fungi on Salix species grown on polluted soils (Hrynkiewicz et al., 2008). Interestingly, a Spearman's rank calculation showed that, among all fungal OTUs, the Otu00010 (*Cortinarius diasemospermus*) gave the strongest positive correlation between fungal OTUs and Cd and Mn soil concentrations. Although many type collections have yet to be sequenced, the infrequency of matches to database entries indicates that a majority of soil-inhabiting fungal taxa remain to be described (Taylor et al., 2014). The anamorphic *Basidiomycota* species *Cryptococcus podzolicus*, *Cryptococcus terricola* and *Cryptococcus fuscescens* occur on the root surfaces and in the rhizosphere (Golubtsova et al., 2007). These species represented a large number of reads in our study, as depicted in temperate forest soils (Buée et al., 2009).

As is the case for most trees in temperate and boreal forests, the establishment, growth, and survival of poplar trees in the wild and in large plantations depend strongly on colonization by mycorrhizal fungi (Smith and Read, 2008). Mycorrhizal fungi are important for the recovery of polluted sites via phytoremediation because they play crucial roles in soil fertility, plant nutrition and colonization and they can both enhance plant mineral uptake and restrict plant TE accumulation. Shifts in the relative dominance of ECM fungi at the expense of other functional groups of fungi, namely biotrophs and, secondarily, saprotrophs, are important in view of the finding that saprotrophs and mycorrhizal fungi play opposite roles in soil organic matter cycling (Tedersoo et al., 2014). A large amount of research has been conducted on the potential effects of mycorrhizal colonization on root pathogens, and it has long been accepted that ECM inoculation might reduce the incidence and severity of root diseases (Smith and Read, 2008).

Factors that may be taken into consideration when interpreting our data include the climatic parameters, the age of the trees, host genotypes, soil properties and former land use (Gherghel et al., 2014; Labbé et al., 2011; Tagu et al., 2004; Tedersoo et al., 2014). In our study, the soil origin (Leforest versus Pierrelaye) had a stronger

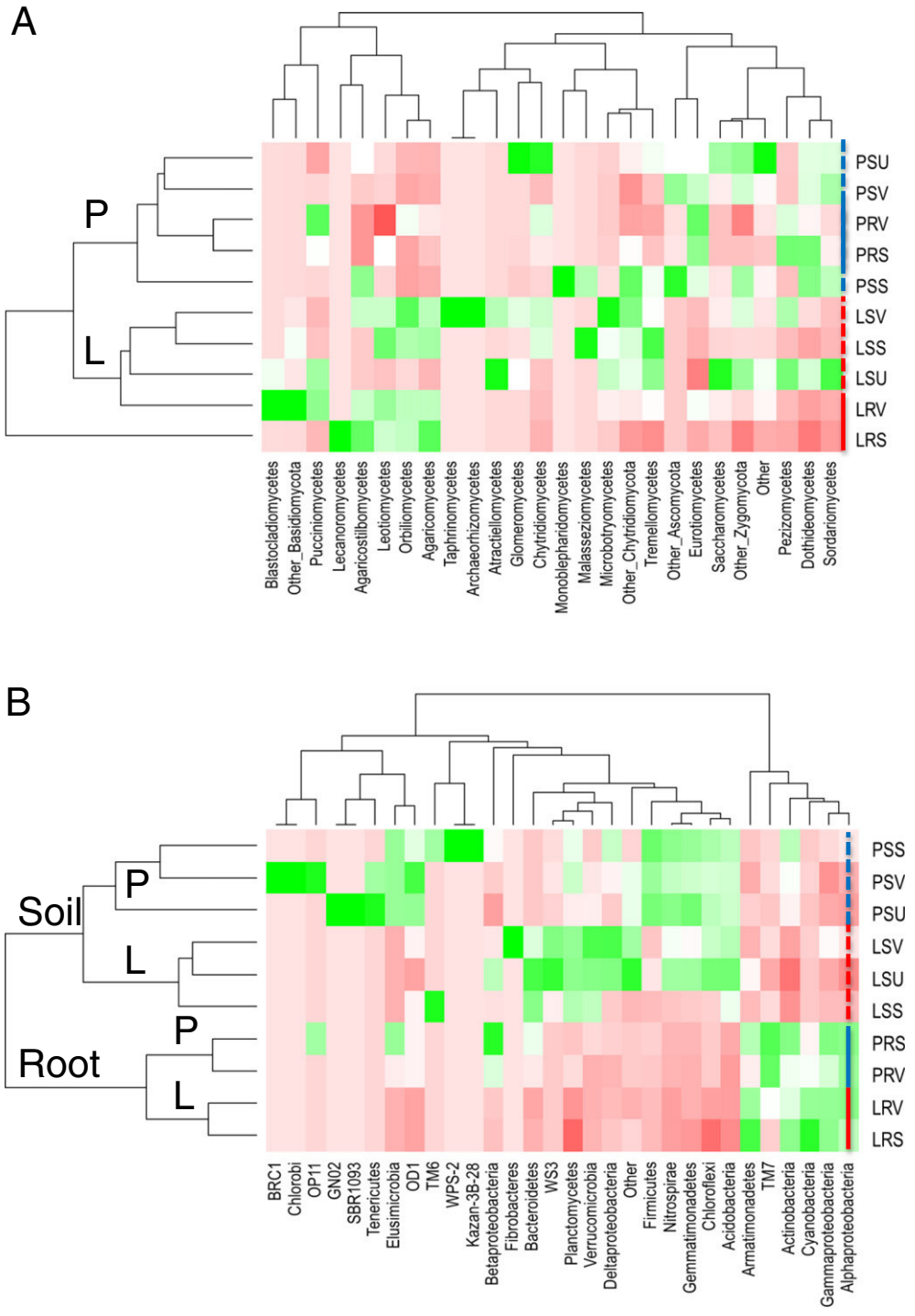

Fig. 4. Heat map and hierarchical cluster analysis based on Spearman's correlation between the relative abundances of fungal classes (A) and bacterial phylum/classes (B) at the Leforest (L) and Pierrelaye (P) sites. Red, strong negative correlation; green, strong positive correlation. Refer to the legend in Table 2 for sample abbreviations.

influence on the fungal community assemblage than the poplar clone or habitat (soil in comparison with root samples). Thus, our findings corroborate previous reports indicating that soil properties are major drivers of differences in the distributions and compositions of microbial communities (Buyer et al., 2002; Jesus et al., 2010). To substantiate our findings further, the growth of the poplar "Skado" clone was estimated at the two sites in 2013 and was calculated to be 53.0 ± 23.8 kg DW/tree and 88.8 ± 43.1 kg DW/tree at Leforest and Pierrelaye, respectively. More complete datasets, including tree biomasses and mineralomasses at these two experimental sites, which cannot be detailed in the present manuscript, will be analyzed in further manuscripts. We think however that these data illustrate the fact that, despite the lack of ECM colonization, poplar growth was substantially and significantly ($P < 0.001$) higher at Pierrelaye than at Leforest. These data reinforce our findings that the Pierrelaye soil is sufficiently fertile and less toxic than the Leforest soil to support tree growth without the presence of ECM fungi. The lower toxicity and increased fertility features of the Pierrelaye site refer to the higher pH and lower Cd, Zn and Mn $CaCl_2$-extractable fractions at the Pierrelaye site, together with higher phosphorus, organic C and total N. Although mycorrhizal fungi were able to spontaneously

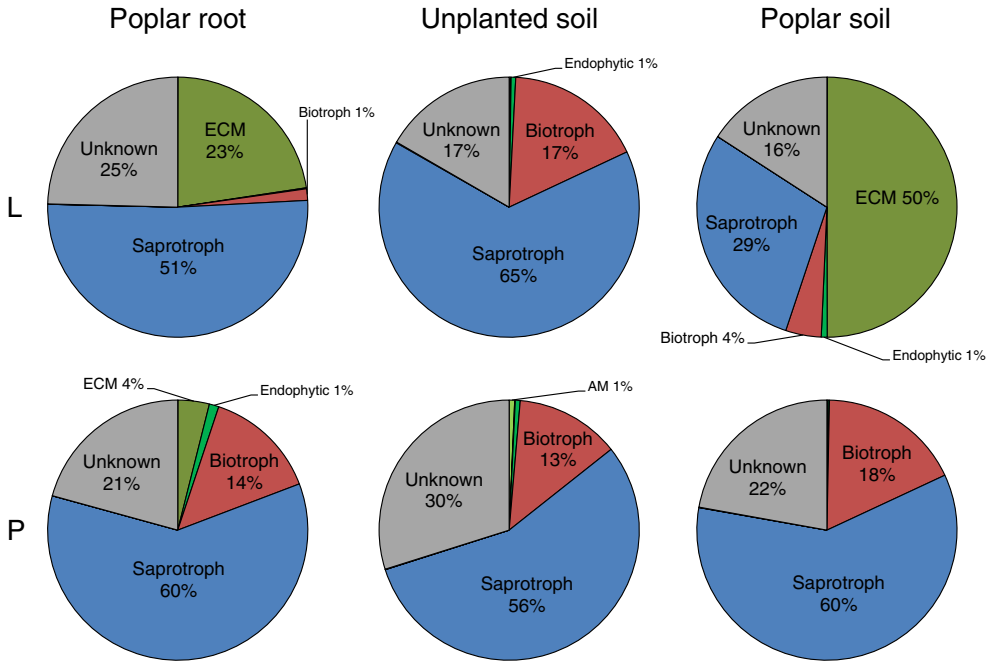

Fig. 5. Relative proportions of fungal sequences assigned to major functional groups. The abundances of the various OTU groups from soil and root samples that were collected under *Populus* cv Skado or from the unplanted soil at the Leforest (L) and Pierrelaye (P) experimental sites were set to 100%, and the OTUs were classified as ectomycorrhizal (ECM), arbuscular mycorrhizal (AM), endophytic, saprotrophic or biotrophic.

colonize poplar roots from Leforest, a tailored inoculation of planted trees with more appropriate mycorrhizal species might be suited for improving biomass production under such stressing environment. Two ongoing research programs include the poplar Skado clone used in the present study and an alder species planted in 2011 (BIOFILTREE), as well as 16 other tree species planted in 2014 (PROLIPHYT), at both sites. All trees have received (or not) a tailored mycorrhizal inoculum before plantation. These large-scale programs should allow to fully understand the impact of tailored inoculation on microbial communities and, hence on biomass production at these two contrasting sites.

4. Conclusions

The occurrence and dominance of the ECM community at Leforest but not at Pierrelaye is the major feature of our data set, together with the dominance of *Rhizobiaceae* and *Bradyrhizobiaceae* family members in most root samples from both sites and both poplar clones. The relatively low pH and, hence, the high $CaCl_2$ extractable fraction of TE, in addition to the low P and N contents at Leforest, may have induced a stress situation in the biotic community. This selection might have favored microorganisms with resistance traits over those with strong competitive capacity because microbial communities tend towards facilitation, as demonstrated in other stress situations (Bertness and Callaway, 1994; Wagner and Jones, 2004). Selective uptake within the mycorrhizal assemblages may indeed facilitate primary production in spite of the high TE loads (Krpata et al., 2008). Bell et al. (2015) concluded that, although the plants that are introduced for site restoration are pre-selected for specific traits (e.g., TE bioaccumulation or rapid growth in poor soils), the in situ success of these plants likely depends on the recruitment of appropriate rhizosphere microorganisms from their new environment. Our data from the Pierrelaye site revealed a clear deficiency in the ECM colonization of poplar roots and soil that may be related to soil resource availability. Johnson et al. (2010) concluded that soil resource availability might be a key determinant in driving the plants to minimize AM colonization as mycorrhizal trading partnerships became unlikely. Our data suggest that this theory could be applied equally to ECM colonization. Our study is in line with recent findings on the impact of engineering the plant-associated microbiome on improved plant survival and performance in stressful environments such as contaminated soils (Yergeau et al., 2015). Our data support the view that mycorrhizal and tailored inoculation might be needed in highly stressed and nutrient-poor environments, such as the Leforest soil.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.07.151.

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Table S1. Spearman's rank correlation coefficients between richness and diversity fungal indices and measured $CaCl_2$ extractible metals at the Leforest (L) and Pierrelaye (P) experimental sites. Ns: non-significant.

	Cd	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
Leforest										
No. of OTUs	**-0.56**	ns	ns	ns	ns	ns	ns	ns	ns	**-0.56**
Chao1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Shannon (H')	**-0.62**	ns	ns	ns	ns	**-0.56**	ns	ns	ns	**-0.61**
Simpson's (D)	**-0.52**	ns	ns	ns	ns	ns	ns	ns	ns	**-0.54**
Pielou (J)	**-0.55**	ns	ns	ns	ns	ns	ns	ns	ns	**-0.58**
Pierrelaye										
No. of OTUs	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Chao1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Shannon (H')	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Simpson's (D)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Pielou (J)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table S2. Spearman's rank correlation coefficients between richness and diversity bacterial indices and measured $CaCl_2$ extractible metals at the Leforest (L) and Pierrelaye (P) experimental sites. Ns: non-significant.

	Cd	Cu	Fe	K	Mg	Mn	Na	Ni	Pb	Zn
Leforest										
No. of OTUs	ns	ns	**-0.57**	**0.65**	ns	ns	**-0.73**	ns	**-0.54**	**-0.54**
Chao1	ns	ns	ns	ns	ns	ns	**-0.61**	ns	ns	ns
Shannon (H')	ns	ns	**-0.66**	**0.71**	ns	ns	**-0.74**	**-0.59**	**-0.55**	**-0.60**
Simpson's (D)	**-0.55**	ns	**-0.79**	**0.76**	ns	**-0.67**	**-0.73**	**-0.75**	**-0.64**	**-0.71**
Pielou (J)	ns	ns	**-0.70**	**0.73**	ns	**-0.60**	**-0.75**	**-0.61**	ns	**-0.61**
Pierrelaye										
No. of OTUs	ns	ns	ns	**0.60**	ns	ns	ns	ns	ns	ns
Chao1	**-0.52**	ns	ns	**0.65**	**0.69**	**0.55**	**0.65**	ns	ns	**-0.63**
Shannon (H')	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Simpson's (D)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Pielou (J)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table S3. Effects of the primary factors and their interactions as assessed by PERMANOVA. Levels of significance are indicated by asterisks: *P ≤ 0.05;
**P ≤ 0.01; ***P ≤ 0.001.

	Fungi		Bacteria	
	Pr(>F)	R^2	Pr(>F)	R^2
site	31.51***	0.262	25.92***	0.203
habitat	13.26***	0.110	36.77***	0.288
clone	5.50***	0.091	3.34**	0.052
site x habitat	5.99***	0.049	8.46***	0.066
site x clone	5.60***	0.093	2.87***	0.045
habitat x clone	2.66**	0.022	1.50	0.011
site x habitat x clone	2.40*	0.020	1.24	0.009

Table S4. The 10 most abundant fungal operational taxonomic units in poplar (*Populus* clones Skado and Vesten) planted and unplanted soils. The OTUs were assigned to kingdom (k), phyla (p), classes (c), orders (o), families (f), genera (g) or species (s), when possible. The relative proportions are given in parenthesis. Refer to the legend in Table 2 for sample abbreviations.

LRS	PRS
Otu00053_s__uncultured_Trechisporales (30.2)	Otu00002_s__Exophiala_sp_KL_2011f (23.3)
Otu00010_s__Cortinarius_diasemospermus_var (13.1)	Otu00015_o__Chaetothyriales (8.1)
Otu00078_o__Agaricales (10)	Otu00023_s__Pyrenochaeta_sp_IZ_1543 (5.9)
Otu00002_s__Exophiala_sp_KL_2011f (5.1)	Otu00001_s__Penicillium_canescens (5.8)
Otu00295_s__uncultured_Trechisporales (4.9)	Otu00008_s__Ilyonectria_macrodidyma (3.9)
Otu00084_o__Sebacinales (4.8)	Otu00031_s__Pleosporales_sp_BESC803s (3.1)
Otu00324_o__Agaricales (3.7)	Otu00007_s__Fusarium_chlamydosporum (3)
Otu00038_s__Inocybe_curvipes (2.9)	Otu00005_g__Fusarium (2.1)
Otu00088_s__Cortinarius_parvannulatus (2.9)	Otu02191_s__Hemimycena_cucullata (2)
Otu00092_g__Exophiala (1.6)	Otu00101_g__Tuber (1.7)
LRV	PRV
Otu00010_s__Cortinarius_diasemospermus_var (18.1)	Otu00002_s__Exophiala_sp_KL_2011f (25.6)
Otu00002_s__Exophiala_sp_KL_2011f (15.9)	Otu00001_s__Penicillium_canescens (16.7)
Otu00243_o__Sebacinales (7)	Otu00054_o__Agaricales (7.4)
Otu00069_s__Paecilomyces_carneus (5.7)	Otu00141_g__Scleroderma (5)
Otu00409_s__Hemimycena_gracilis (4.1)	Otu00079_s__uncultured_Laccaria (3.5)
Otu00038_s__Inocybe_curvipes (2.9)	Otu00023_s__Pyrenochaeta_sp_IZ_1543 (3)
Otu00110_s__Cadophora_finlandica (2.6)	Otu00005_g__Fusarium (2.3)
Otu01065_o__Agaricales (2.4)	Otu00015_o__Chaetothyriales (2)
Otu00828_f__Sebacinaceae (1.9)	Otu00008_s__Ilyonectria_macrodidyma (1.6)
Otu00290_o__Helotiales (1.6)	Otu00014_s__Microdochium_sp_E9023a (1.5)
LSS	PSS
Otu00010_s__Cortinarius_diasemospermus_var (25)	Otu00001_s__Penicillium_canescens (16.4)
Otu00038_s__Inocybe_curvipes (8.7)	Otu00002_s__Exophiala_sp_KL_2011f (5.8)
Otu00002_s__Exophiala_sp_KL_2011f (5.7)	Otu00007_s__Fusarium_chlamydosporum (3.8)
Otu00088_s__Cortinarius_parvannulatus (4.3)	Otu00006_s__Verticillium_nigrescens (3)
Otu00110_s__Cadophora_finlandica (3.2)	Otu00005_g__Fusarium (2.5)
Otu00099_s__Cryptococcus_podzolicus (3.1)	Otu00016_g__Preussia (2.5)
Otu00084_o__Sebacinales (3.1)	Otu00034_f__Davidiellaceae (1.9)
Otu00133_s__Tomentella_ellisii (2.8)	Otu00061_s__Aureobasidium_sp_OTU_105 (1.8)
Otu00044_s__Hymenogaster_griseus (2.5)	Otu00043_g__Mortierella (1.7)
Otu00004_g__Mortierella (2.2)	Otu00004_g__Mortierella (1.5)
LSU	PSU
Otu00006_s__Verticillium_nigrescens (10.1)	Otu00001_s__Penicillium_canescens (15)
Otu00083_s__Podospora_appendiculata (6.3)	Otu00175_k__Fungi (8.1)
Otu00049_f__Lasiosphaeriaceae (5.8)	Otu00042_s__Metarhizium_anisopliae (4.7)
Otu00004_g__Mortierella (5.2)	Otu00002_s__Exophiala_sp_KL_2011f (3.8)
Otu00051_g__Pseudeurotium (4.8)	Otu00003_s__uncultured_Mortierellaceae (3.3)
Otu00155_s__Pholiota_burkei (2.6)	Otu00029_s__Minimedusa_sp (2.6)
Otu00019_s__uncultured_Chaetomiaceae (2.4)	Otu00005_g__Fusarium (2.1)
Otu00230_s__Massariosphaeria_typhicola (2.4)	Otu00004_g__Mortierella (1.6)
Otu00018_c__Sordariomycetes (2.3)	Otu00071_p__Ascomycota (1.6)
Otu00036_f__Lasiosphaeriaceae (2.3)	Otu00009_s__Cryptococcus_fuscescens (1.5)
LSV	PSV
Otu00044_s__Hymenogaster_griseus (17.2)	Otu00001_s__Penicillium_canescens (21.9)
Otu00038_s__Inocybe_curvipes (9.4)	Otu00002_s__Exophiala_sp_KL_2011f (5.1)
Otu00002_s__Exophiala_sp_KL_2011f (4.2)	Otu00005_g__Fusarium (5)
Otu00010_s__Cortinarius_diasemospermus_var (4.1)	Otu00007_s__Fusarium_chlamydosporum (3)
Otu00069_s__Paecilomyces_carneus (3)	Otu00003_s__uncultured_Mortierellaceae (2.1)
Otu00051_g__Pseudeurotium (2.9)	Otu00006_s__Verticillium_nigrescens (1.9)
Otu00276_s__Entoloma_sericeum (2.8)	Otu00125_s__Podospora_curvicolla (1.9)
Otu00013_f__Mortierellaceae (2.6)	Otu00004_g__Mortierella (1.7)
Otu00004_g__Mortierella (2.4)	Otu00021_p__Ascomycota (1.6)
Otu00339_s__Naucoria_salicis (2.4)	Otu00022_f__Chaetomiaceae (1.5)

Table S5. The 10 most abundant bacterial operational taxonomic units in poplar (*Populus* clones Skado and Vesten) planted and unplanted soils. The OTUs were assigned to classes (c), orders (o), families (f), genera (g) or species (s), when possible. The relative proportions are given in parenthesis. Refer to the legend in Table 2 for sample abbreviations.

LRS	PRS
Otu00001_f__Bradyrhizobiaceae (21.5)	Otu00001_f__Bradyrhizobiaceae (9.9)
Otu00002_f__Micromonosporaceae (16.4)	Otu00002_f__Micromonosporaceae (6.7)
Otu00156_f__Sinobacteraceae (4.8)	Otu00007_g__Rhizobium (3.9)
Otu00210_o__Streptophyta (2.9)	Otu00006_s__Variovorax_paradoxus (3.1)
Otu00007_g__Rhizobium (2.2)	Otu00008_g__Streptomyces (2.9)
Otu00028_f__Microbacteriaceae (1.4)	Otu00016_s__Kineosporia_rhamnosa (2.9)
Otu00097_g__Actinoplanes (1.4)	Otu00003_f__Sinobacteraceae (2.4)
Otu00021_g__Mesorhizobium (1.3)	Otu00011_f__Sinobacteraceae (1.9)
Otu00059_g__Dokdonella (1.1)	Otu00020_f__Sphingomonadaceae (1.6)
Otu00256_f__Xanthomonadaceae (1.1)	Otu00012_g__Devosia (1.5)
LRV	PRV
Otu00001_f__Bradyrhizobiaceae (22)	Otu00001_f__Bradyrhizobiaceae (14.4)
Otu00002_f__Micromonosporaceae (10)	Otu00002_f__Micromonosporaceae (7.5)
Otu00007_g__Rhizobium (2.1)	Otu00007_g__Rhizobium (3.1)
Otu00059_g__Dokdonella (2)	Otu00003_f__Sinobacteraceae (2.8)
Otu00011_f__Sinobacteraceae (2)	Otu00124_o__EW055 (2.4)
Otu00025_g__Kaistobacter (1.8)	Otu00006_s__Variovorax_paradoxus (2.1)
Otu00210_o__Streptophyta (1.7)	Otu00008_g__Streptomyces (2.1)
Otu00016_s__Kineosporia_rhamnosa (1.5)	Otu00016_s__Kineosporia_rhamnosa (1.9)
Otu00156_f__Sinobacteraceae (1.4)	Otu00012_g__Devosia (1.7)
Otu00021_g__Mesorhizobium (1.1)	Otu00021_g__Mesorhizobium (1.6)
LSS	PSS
Otu00001_f__Bradyrhizobiaceae (9.3)	Otu00001_f__Bradyrhizobiaceae (4)
Otu00069_o__Solibacterales (5)	Otu00004_c__Gemm-1 (2.2)
Otu00031_o__RB41 (2.5)	Otu00009_o__iii1-15 (1.9)
Otu00025_g__Kaistobacter (2.1)	Otu00005_o__iii1-15 (1.8)
Otu00039_s__Reyranella_massiliensis (2)	Otu00010_f__Sphingomonadaceae (1.6)
Otu00014_o__RB41 (1.8)	Otu00043_g__Blastococcus (1.5)
Otu00084_g__Rhodoplanes (1.5)	Otu00044_g__Pedomicrobium (1.4)
Otu00011_f__Sinobacteraceae (1.4)	Otu00025_g__Kaistobacter (1.3)
Otu00071_o__Ellin329 (1.4)	Otu00011_f__Sinobacteraceae (1.2)
Otu00026_o__iii1-15 (1.4)	Otu00018_s__Arthrobacter_oxydans (1.2)
LSU	PSU
Otu00025_g__Kaistobacter (4.4)	Otu00004_c__Gemm-1 (3.6)
Otu00001_f__Bradyrhizobiaceae (3.5)	Otu00001_f__Bradyrhizobiaceae (3)
Otu00014_o__RB41 (3.5)	Otu00009_o__iii1-15 (2.2)
Otu00069_o__Solibacterales (3.4)	Otu00003_f__Sinobacteraceae (2)
Otu00031_o__RB41 (2.3)	Otu00010_f__Sphingomonadaceae (1.6)
Otu00005_o__iii1-15 (2.1)	Otu00047_g__Pedomicrobium (1.5)
Otu00116_o__envOPS12 (1.8)	Otu00005_o__iii1-15 (1.4)
Otu00131_g__Rhodoplanes (1.6)	Otu00018_s__Arthrobacter_oxydans (1.4)
Otu00082_g__Candidatus_Solibacter (1.5)	Otu00017_g__Rhodoplanes (1.4)
Otu00009_o__iii1-15 (1.2)	Otu00044_g__Pedomicrobium (1.2)
LSV	PSV
Otu00001_f__Bradyrhizobiaceae (6.3)	Otu00001_f__Bradyrhizobiaceae (3.1)
Otu00031_o__RB41 (2.9)	Otu00009_o__iii1-15 (2.6)
Otu00069_o__Solibacterales (2.6)	Otu00004_c__Gemm-1 (1.9)
Otu00039_s__Reyranella_massiliensis (2.1)	Otu00010_f__Sphingomonadaceae (1.8)
Otu00011_f__Sinobacteraceae (2)	Otu00005_o__iii1-15 (1.8)
Otu00014_o__RB41 (1.6)	Otu00018_s__Arthrobacter_oxydans (1.6)
Otu00084_g__Rhodoplanes (1.5)	Otu00013_c__Ellin6529 (1.4)
Otu00131_g__Rhodoplanes (1.4)	Otu00240_s__Bacillus_flexus (1.3)
Otu00009_o__iii1-15 (1.4)	Otu00047_g__Pedomicrobium (1.2)
Otu00025_g__Kaistobacter (1.3)	Otu00014_o__RB41 (1.2)

Table S6. Relative proportions of fungal sequences that were assigned to major functional groups. The abundances of the various OTU groups from soil and root samples that were collected from the Skado *Populus* clone or from the unplanted soil at the Leforest (L) and Pierrelaye (P) experimental sites were set to 100% and the OTUs were classified as either ectomycorrhizal (ECM), endophytic, saprotrophic or biotrophic. Refer to the legend in Table 2 for sample abbreviations. Values designated with the same letters are not significantly different (P<0.05) according to a Kruskal-Wallis test.

	AM (%)	1	Biotrophic (%)	1	ECM (%)	1	Endophytic (%)	1	Saprotrophic (%)	1	Unknown (%)	1
LRS	0.04 ± 0.04	bcd	1.43 ± 0.56	b	22.58 ± 16.66	a	0.12 ± 0.10	c	51.20 ± 12.23	b	24.60 ± 22.41	a
LSS	0.06 ± 0.04	bc	4.43 ± 1.16	b	49.91 ± 14.93	a	0.73 ± 0.56	a	29.01 ± 6.63	c	15.86 ± 8.61	a
LSU	0.12 ± 0.09	b	17.21 ± 7.35	a	0.12 ± 0.12	c	0.57 ± 0.48	ab	65.23 ± 11.64	a	16.66 ± 4.69	a
PRS	0.03 ± 0.06	cd	14.11 ± 7.80	a	3.89 ± 3.47	b	1.19 ± 1.29	a	60.04 ± 13.23	ab	20.73 ± 5.32	a
PSS	0.01 ± 0.02	d	17.75 ± 1.67	a	0.13 ± 0.08	c	0.17 ± 0.21	bc	59.72 ± 1.90	ab	22.16 ± 1.46	a
PSU	0.68 ± 0.38	a	12.97 ± 4.69	a	0.08 ± 0.14	c	0.61 ± 0.79	abc	55.75 ± 10.82	ab	29.86 ± 14.14	a

Table S7. Relative proportions of fungal sequences that were assigned to major functional groups. The abundances of the various OTU groups from soil and root samples that were collected from the Vesten *Populus* clone or from the unplanted soil at the Leforest (L) and Pierrelaye (P) experimental sites were set to 100% and the OTUs were classified as either ectomycorrhizal (ECM), endophytic, saprotrophic or biotrophic. Refer to the legend in Table 2 for sample abbreviations. Values designated with the same letters are not significantly different (P<0.05) according to a Kruskal-Wallis test.

	AM (%)	1	Biotrophic (%)	1	ECM (%)	1	Endophytic (%)	1	Saprotrophic (%)	1	Unknown (%)	1
LRV	0.04 ± 0.04	c	7.66 ± 5.99	bc	31.70 ± 25.10	ab	0.37 ± 0.24	b	33.22 ± 14.71	b	27.01 ± 13.54	ab
LSV	0.19 ± 0.11	ab	6.20 ± 2.27	c	45.29 ± 22.59	a	0.93 ± 0.32	a	32.08 ± 15.65	b	15.31 ± 6.07	b
LSU	0.12 ± 0.09	bc	17.21 ± 7.35	a	0.12 ± 0.12	d	0.57 ± 0.48	ab	65.23 ± 11.64	a	16.66 ± 4.69	b
PRV	0.04 ± 0.04	c	10.51 ± 6.79	abc	11.16 ± 5.63	b	0.06 ± 0.10	c	56.65 ± 7.98	a	21.57 ± 6.99	ab
PSV	0.04 ± 0.05	c	17.10 ± 2.81	a	1.45 ± 1.50	c	0.09 ± 0.07	c	61.36 ± 7.26	a	19.91 ± 4.00	ab
PSU	0.68 ± 0.38	a	12.97 ± 4.69	ab	0.08 ± 0.14	d	0.61 ± 0.79	ab	55.75 ± 10.82	a	29.86 ± 14.14	a

Table S8. Spearman's rank correlation coefficients between the fungal functional groups were assigned as follows: ectomycorrhizal (ECM), endophytic, saprotrophic or biotrophic. Calculations were made for either soil or root samples from both sites and both poplar clones. Ns: not significant.

	Biotrophic	ECM	Endophytic	Saprotrophic
Soil				
AM	ns	ns	**0.41**	ns
Biotrophic		**-0.56**	ns	**0.45**
ECM			ns	**-0.67**
Endophytic				**-0.37**
Roots				
AM	ns	ns	ns	ns
Biotrophic		**-0.63**	ns	ns
ECM			ns	**-0.47**
Endophytic				ns

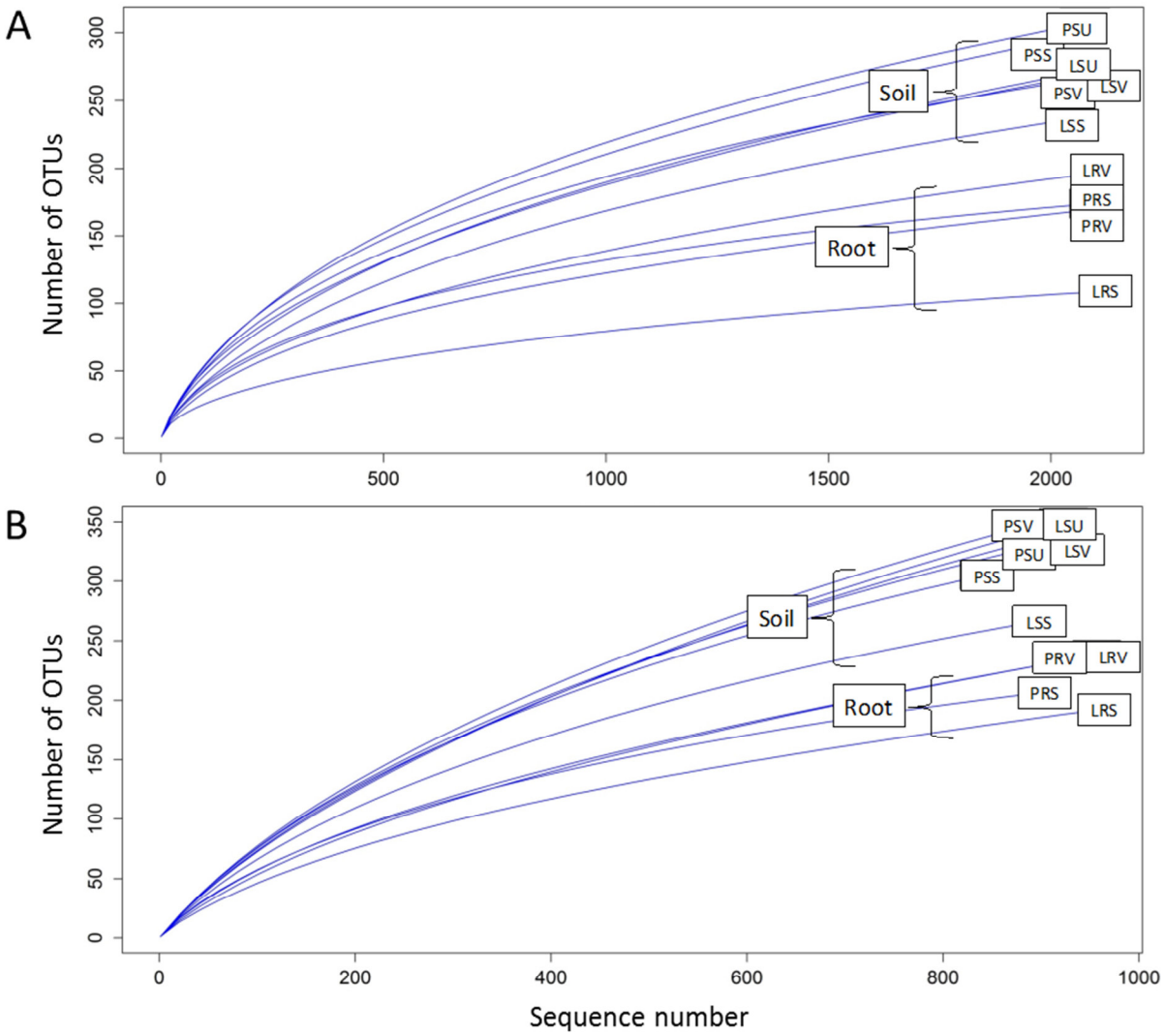

Fig. S1. Rarefaction curves for fungal (A) and bacterial (B) OTUs. Refer to the legend in Table 2 for sample abbreviations. The rarefaction curves are based on the average number of OTUs of five samples that were collected from the same plot.

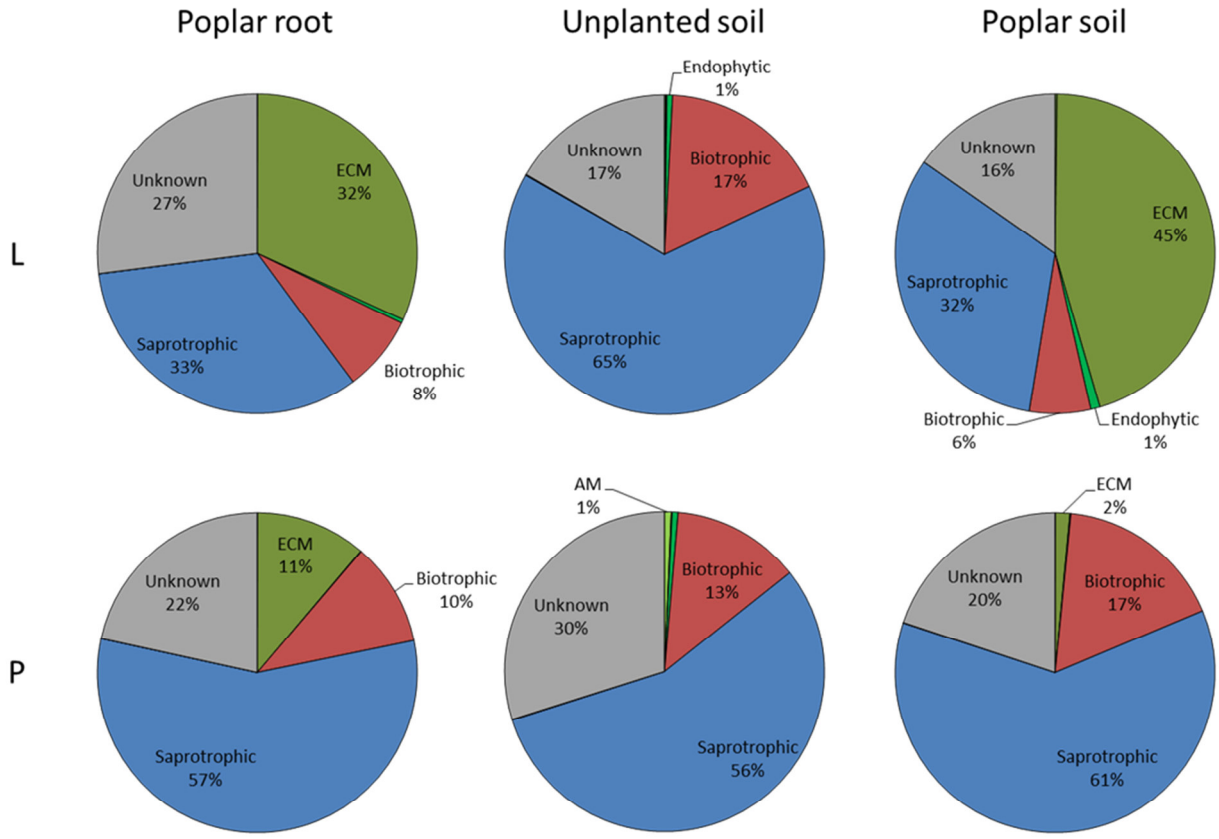

Fig. S2. Relative proportions of fungal sequences assigned to major functional groups. The abundances of the various OTU groups from soil and root samples that were collected under *Populus* cv Vesten or from the unplanted soil at the Leforest (L) and Pierrelaye (P) experimental sites were set to 100% and the OTUs were classified either as ectomycorrhizal (ECM), endomycorrhizal (AM), endophytic, saprotrophic or biotrophic.

3.1.3 Impact du phytomanagement utilisant le peuplier sur les propriétés du sol et les communautés microbiennes d'un site contaminé par des ETMs

En dépit d'une longue histoire de l'utilisation du peuplier dans les stratégies de phytomanagement, les impacts de cette espèce ligneuse sur la structure et la fonction des communautés microbiennes qui résident dans le sol, demeurent largement inconnus. L'étude décrite ici combine l'analyse des communautés bactériennes et fongiques provenant de différents itinéraires culturaux via le séquençage par Illumina MiSeq aux analyses de sol. Les itinéraires culturaux de peupliers dans le cadre du phytomanagement ont conduit à une augmentation significative de la fertilité des sols et une diminution de la biodisponibilité du Zn et du Cd, de concert avec des changements dans les communautés microbiennes. Les changements les plus notables dans l'abondance relative des taxa et des unités taxonomiques opérationnelles (OTUs) ont indiqué sans surprise que les racines et le sol constituent des habitats écologiques distincts pour les microorganismes, comme le montre la dominance de *Laccaria* dans des échantillons de racines. Le cultivar de peuplier a également été un facteur important, expliquant 12% et 6% de la variance dans les données fongiques et bactériennes, respectivement. La dominance globale des champignons saprophytes, par exemple *Penicillium canescens*, pourrait être liée aux activités de décomposition nécessaires sur le site expérimental. Nos données ont également mis en évidence que la colonisation mycorhizienne des cultivars de peuplier varie fortement selon les espèces et les génotypes considérés, ce qui est, par exemple, illustré par la dominance de *Scleroderma* dans les échantillons du cultivar Vesten. D'autres interactions entre les champignons et les groupes fonctionnels bactériens ont souligné le potentiel des technologies de séquençage à haut débit dans le domaine de l'écologie microbienne des milieux perturbés.

- ***Pour cet article, j'ai réalisé toutes les étapes allant de l'échantillonnage sur le terrain à l'écriture de la publication avec l'aide des co-auteurs.***

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RESEARCH ARTICLE

Impact of poplar-based phytomanagement on soil properties and microbial communities in a metal-contaminated site

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One sentence summary: *Laccaria* dominates the poplar root fungal community on a contaminated soil.
Editor: Petr Baldrian

ABSTRACT

Despite a long history of use in phytomanagement strategies, the impacts of poplar trees on the structure and function of microbial communities that live in the soil remain largely unknown. The current study combined fungal and bacterial community analyses from different management regimes using Illumina-based sequencing with soil analysis. The poplar phytomanagement regimes led to a significant increase in soil fertility and a decreased bioavailability of Zn and Cd, in concert with changes in the microbial communities. The most notable changes in the relative abundance of taxa and operational taxonomic units unsurprisingly indicated that root and soil constitute distinct ecological microbial habitats, as exemplified by the dominance of *Laccaria* in root samples. The poplar cultivar was also an important driver, explaining 12% and 6% of the variance in the fungal and bacterial data sets, respectively. The overall dominance of saprophytic fungi, e.g. *Penicillium canescens*, might be related to the decomposition activities needed at the experimental site. Our data further highlighted that the mycorrhizal colonization of poplar cultivars varies greatly between the species and genotypes, which is exemplified by the dominance of *Scleroderma* under Vesten samples. Further interactions between fungal and bacterial functional groups stressed the potential of high-throughput sequencing technologies in uncovering the microbial ecology of disturbed environments.

Keywords: bacterial 16S; fungal ITS; Illumina-based sequencing; metal-contaminated soils; phytomanagement, poplar cultivars

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INTRODUCTION

Since the 1980s, there has been increasing demand for fast-growing, stress-tolerant trees that can be used for the afforestation of postagricultural lands, the recultivation of areas that have been degraded by industry and the production of bioenergy. A short rotation coppice (SRC) is defined as a production system in which fast-growing recoppicing tree and bush species are intensively managed to yield large quantities of woody biomass in rotations of approximately 3–10 years (Šlapokas and Granhall 1991). Phytomanagement of metal-contaminated sites strongly relies on the capacity of the vegetation cover to tolerate high concentrations of metals. Salicaceae species respond to that constraint by accumulating trace elements (TEs) in their aboveground biomass (Migeon *et al.* 2009). The impact of land management intensity on soil microbial diversity (i.e. richness and evenness) has been recently explored in agricultural soils (Tardy *et al.* 2015), but the effects of planting trees in former agricultural soils on the diversity of soil microbial communities remain largely unknown. The impact of tree planting needs to be addressed in phytomanaged soils, because the changes in land use induced by fast-growing recoppicing trees may have a strong impact on the structure and function of organism communities living in the system, in addition to modifying soil characteristics. This impact may be particularly true for microorganisms, which are the primary agents responsible for key ecosystem processes (Jeffries *et al.* 2003; Philippot *et al.* 2013). Differences between fungal communities in forested and tree-less ecosystems have been recently revealed using a metabarcoding analysis of global soils (Tedersoo *et al.* 2014), although contaminated soils were not included in that work. As for most trees in temperate and boreal forests, the establishment, growth and survival of trees in large plantations depend strongly on colonization by mycorrhizal fungi and associated bacteria (Smith and Read 2008) and mycorrhizal fungi are crucial for tolerance to various abiotic and biotic stresses (Smith and Read 2008).

Fungal endophytes also represent key microorganisms, as highlighted by our recent study (Lacercat-Didier *et al.* 2016), where three isolates belonging to the order Helotiales and the species *Serendipita vermifera* were highly tolerant to metals (Cd, Zn, Pb and Cu). Further interactions between bacteria, fungi and plants have been shown to contribute to microbial community stability (Bonfante and Anca 2009; Bell *et al.* 2014) and many bacterial strains have indeed been reported to promote mycorrhizal symbioses (Frey-Klett, Garbaye and Tarkka 2007; Hrynkiewicz *et al.* 2010). Other key soil microorganisms such as saprotrophic fungi are central in forest soils due to their capacity to degrade recalcitrant organic compounds and may strongly interact with soil bacteria (Baldrian 2008). Saprotrophic fungi may also have the potential to mobilize metals from contaminated soils, due to their ability to exude chelating agents (Arwidsson *et al.* 2009). In addition to estimating the microbial fertility of contaminated soils, knowledge of the associated microbial compartment could be used to help predict the potential recovery of disturbed lands (Kozdrój 2000).

Only recently, high-throughput sequencing technologies have been used to reveal microbial communities' structure and composition in the rhizosphere and endosphere of tree roots in contaminated soils. For instance, metal pollution level significantly impacted bacterial community structure in two metal-polluted areas in Poland (Azarbad *et al.* 2015). The fungal community composition in a young pine plantation in Zn- and Cd-polluted soil was strongly correlated with Zn, Cd and soil organic matter content (Op De Beeck *et al.* 2015). We recently employed physico-chemical analysis, ion torrent sequencing and related bioinformatics tools to explore the characteristics of the soil microbial communities that were found to reside within a tailings dump and adjacent undisturbed soil (Zappelini *et al.* 2015). This multidisciplinary approach led us to conclude that environmental filtering is more likely to occur through the activity of tree roots than as a result of specific soil characteristics. Some studies based on high-throughput sequencing technologies have recently assessed the microbial communities associated with willows growing, for example, on hydrocarbon-contaminated (Bell *et al.* 2014; Yergeau *et al.* 2014; Pagé, Yergeau and Greer 2015; Leewis *et al.* 2016) and Zn-contaminated (Bell *et al.* 2015) soils. Among the fast-growing trees, *Populus* is also increasingly employed for the reforestation of post-agricultural lands, the recultivation of areas degraded by industry or environmental pollution, and as a renewable source of biomass for bioenergy production (Capuana 2011). Little is known about the whole microbial communities that are associated with poplar SRC or very short rotation coppice (vSRC), although some attention has been focused on the isolation and characterization of culturable bacterial and fungal endophytes from poplar roots (Van der Lelie *et al.* 2009; Gottel *et al.* 2011; Gamalero *et al.* 2012; Lacercat-Didier *et al.* 2016).

In this study, we combined highly novel high-throughput sequencing technologies, data analysis approaches and soil parameter analyses to (1) clarify the microbial diversity under different management regimes for contaminated soils, (2) characterize the taxonomic distribution of root and soil microbiomes, and (3) reveal the relations between microbiomes, soils and poplar cultivars.

MATERIALS AND METHODS

Site description, planting, sampling and chemical analysis

The experimental site was located northwest of Paris at Pierrelaye (latitude: 49°1′21′ N; longitude: 2°9′1′ E), on a plain that was initially dedicated to vegetable gardening. The soil has been irrigated with wastewater from Paris for more than 100 years, which has resulted in the accumulation of trace elements (TEs), mostly Zn, Cd, Pb and Cu. Detailed concentration data are given by Pottier *et al.* (2015). Crop production for human consumption was prohibited here in the late 1990s (Lamy *et al.* 2006), and this land had to be devoted to alternative uses, providing other types of ecosystem services. The Pierrelaye region has a continental climate with annual precipitation varying between 43.6 and 62.7 mm and an average mean annual temperature of 10.8°C (the minimum and maximum temperature averages were 6.5 and 15.2°C, respectively).

As part of the Phytopop project (ANR PRECODD), a 5-ha field demonstration trial was implemented in April 2007, with 14 poplar cultivars grown under two management regimes. The regimes consisted in an SRC (at a planting density of 1000 trees/ha) and a vSRC (at a planting density of 10 000 trees/ha). The present work was performed on 8 of the 14 poplar cultivars, which were selected to represent the three poplar families. These families were *P. trichocarpa* × *P. maximowiczii* (Bakan and Skado), *P. deltoides* × *P. nigra* (Muur, I214, Soligo and Vesten), and *P. trichocarpa* (Fritzi Pauley and Trichobel). More details on these genotypes are given in Pottier *et al.* (2015). The stems in the vSRC plots were cut back in 2010 to elicit the coppice response, which is characterized by the vigorous growth of multiple new stems

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in the spring. There was no application of N fertilizer, pesticide or herbicide. Mechanical weed control and irrigation were performed during the first two years. After litter removal, the roots were collected from the upper 20 cm layer of soil from under the tree canopies. The roots were traced to their origin to ensure that they were indeed connected to the selected tree, and they were sampled at a distance of 1 m from the trunk. For each poplar cultivar, five individual trees were randomly sampled, and for each individual tree, three pseudo-replicates were sampled and mixed to obtain root and soil composites. In addition to poplar plots, soil samples were collected from an unplanted plot, which has been naturally recolonized by endogenous herbaceous plant species, and from a maize plot located near the poplar plot. Five bulk soil samples from the poplar plot, the maize plot and the unplanted plots were collected directly below the root system of the poplars and the maize or natural vegetation from the top 20 cm of soil. The poplar roots were carefully washed with tap water to remove visible soil, washed with sterile deionized water three times, and separated from the roots of other plant species by shape and color. The fine roots were then cut off and transferred to Eppendorf tubes and frozen at –20°C.

To determine the TE-extractable fractions, 5 g of 2 mm sieved soil was dried at 60°C for 48 h and incubated with 50 ml of 10 mM calcium chloride under agitation (40 r.p.m.) for 2 h at room temperature. The mixture was first filtered using ash-free filters and subsequently passed through a 0.45-μm mesh and acidified at 2% (v/v) with HNO_3 prior to inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fischer Scientific, Inc., Pittsburg, PA, USA) analysis. The methods for measuring the physico-chemical characteristics are described in Zappelini *et al.* (2015).

DNA extraction and sequencing methods

Total DNA was extracted from 500 mg of soil with a PowerSoil® DNA Isolation Kit (MO-BIO laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. To isolate microbial DNA from the roots, plant cell lysis was first performed using 100 mg of frozen root samples that were pulverized with 3-mm tungsten carbide beads (Qiagen S.A.S., Courtaboeuf, France) in a Mixer Mill for 3 min at 30 Hz (model MM400; Retsch Inc., Newtown, PA, USA). Root DNA was then extracted with a PowerSoil® DNA Isolation Kit according to the manufacturer's instructions. The DNA quality and quantity were assessed by agarose gel electrophoresis and with a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) in 96-well microplates using an FLX-Xenius spectrofluorometer (SAFAS, Monaco).

The sequencing of the V3–V5 domains of 16S rRNA genes and the fungal internal transcribed spacer (ITS) 2 was performed on an Illumina MiSeq platform (Microsynth AG, Balgach, Switzerland) according to the standard protocols. PCR amplifications were performed with bacterial primers 341F (5′-CCT ACG GGR SGC AGC AG-3′) and 909R (5′-TTT CAG YCT TGC GRC CGT AC-3′) and fungal primers ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′) and ITS4 (5′-TCC GCT TAT TGA TAT GC-3′), using a 600-cycles Miseq Reagent Kit v3.

Data analysis

Reads were assigned to each sample according to a unique barcode, and contigs were then assigned using the Mothur pipeline (Schloss *et al.* 2009). Raw reads were filtered by length and quality. The reads were pre-clustered at 100% identity for ITS and 99% identity for 16S using sumaclust (Mercier *et al.* 2013). Possible artifactual sequences observed with only a few reads (5 for ITS and 10 for 16S) were removed from the following analysis. The 16S sequences were aligned with those in the Silva database to remove non-16S sequences (www.arb-silva.de) (Quast *et al.* 2013). The 28S conserved regions of ITS sequences were searched using a hidden Markov model (Hartmann *et al.* 2010), and plant contamination was removed by identifying *Populus* chloroplastic ITS. Taxonomic assignments were performed using a Bayesian approach (Wang *et al.* 2007) in the Greengene database for bacteria (DeSantis *et al.* 2006) and the UNITE database for fungi (Kõljalg *et al.* 2005). Finally, operational taxonomic units (OTUs) were constructed using the Needleman distance and average neighbor clustering at a distance of 0.03. Diversity parameters were calculated on the basis of a data set in which the numbers of reads per sample were rarefied to 2140 and 1000 reads per sample for fungi and bacteria, respectively. We calculated the rarefaction curves at a 97% similarity level to verify that the amount of sequencing reflected the diversity of the original microbial communities. No differences in fungal or bacterial diversity patterns were observed between the rarefied and non-rarefied data sets; therefore, we opted to keep the rarefied data set to control for unequal sampling depths across samples.

Statistical analysis

All statistical analyses were performed using R software v. 3.0.2 (R Development Core Team, Vienna, Austria, 2013). The Shapiro and Bartlett tests were used to verify the normality and homoscedasticity of the data, respectively, and we compared each TE and measured the soil parameters using either ANOVA or a Kruskal–Wallis test (with the 'kruskal.test' function in R). The richness and diversity indices were calculated using the pgirmess and Vegan packages in R. Rarefaction curves were created with the 'rarecurve' function of the Vegan package in R. The 2-dimensional non-metric multi-dimensional scaling (NMDS) was calculated using the Bray-Curtis method ($k = 3$) with the 'metaMDS' function of the Vegan package in R. Analysis of similarity (ANOSIM) was performed using the Vegan package in R. PERMANOVA was run with 10 000 permutations using the 'adonis' function of the Vegan package in R. The resulting clustering trees were paired with a heatmap of Spearman's correlations between the relative abundances created with 'heatmap.2' from the gplots package. The numbers of OTUs that were shared between crops were visualized using Venn diagrams implemented by VennDiagram of the Vegan package in R.

RESULTS AND DISCUSSION

The management regimes greatly impacted the soil properties

The soil at our experimental site contained large amounts of sand (>80%), whereas silt and clay represented less than 10% (Table 1). After 7 years of phytomanagement, the thin sand, $CaCO_3$, content, and C/N ratio decreased and the $CaCl_2$-extractible K and Mg increased significantly in poplar soils in comparison with the initial 2007 values (Table 1). Conversely, the thin silt, total C content, organic matter, cationic exchangeable capacity (CEC) and CaO decreased only in unplanted soil in comparison with the initial soil values. The pH and coarse sand increased slightly only in planted soils (maize and poplar), whereas large silt and K_2O decreased only in the maize and unplanted plots in comparison with the initial soil. The $CaCl_2$-

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Table 1. Physico-chemical parameters of Pierrelaye soils. Mean values ± standard deviation are provided ($n = 5$ for maize, unplanted and initial soil; $n = 8$ for SRC and vSRC physico-chemical parameters; $n = 40$ for SRC and vSRC $CaCl_2$-extractible elements). Values designated with the same letters were not significantly different ($P < 0.05$) according to a Kruskal–Wallis test.

	Physical characterization					pH and carbonates			
	Clay (%)	Thin silt (%)	Large silt (%)	Thin sand (%)	Coarse sand (%)	pH	$CaCO_3$ (%)	Assimilable phosphorus (g/kg)	B (g/kg)
SRC	3.77 ± 1.26 (a)	4.4 ± 1.65 (a)	4.55 ± 1.59 (a)	39.36 ± 2.49 (b)	47.94 ± 4.65 (a)	7.40 ± 0.00(a)	0.78 ± 0.27 (c)	1.10 ± 0.23 (a)	0.83 ± 0.2 (ab)
vSRC	3.71 ± 1.23 (ab)	4.55 ± 1.89 (a)	4.44 ± 1.65 (ab)	36.24 ± 4.01 (b)	51.05 ± 6.38 (a)	7.41 ± 0.06 (a)	1.07 ± 0.30 (b)	1.09 ± 0.23 (a)	0.74 ± 0.19 (b)
Maize	3.03 ± 0.54 (ab)	3.30 ± 0.52 (a)	3.05 ± 0.53 (bc)	44.62 ± 4.80 (a)	46.00 ± 4.20 (ab)	7.37 ± 0.05 (a)	1.62 ± 0.39 (a)	0.96 ± 0.06 (a)	0.95 ± 0.15 (a)
Unplanted	2.60 ± 0.42 (b)	2.13 ± 0.30 (b)	2.85 ± 0.24 (c)	49.62 ± 5.18 (a)	42.80 ± 5.08 (bc)	7.22 ± 0.08 (b)	1.28 ± 0.31 (ab)	1.03 ± 0.08 (a)	0.40 ± 0.05 (c)
Initial	2.98 ± 0.77 (ab)	4.12 ± 2.17 (a)	4.92 ± 1.37 (a)	48.78 ± 4.68 (a)	39.20 ± 3.90 (c)	7.28 ± 0.04 (b)	1.48 ± 0.21 (a)	1.16 ± 0.21 (a)	0.85 ± 0.22 (ab)

	Organic status					Exchangeable oligos			
	C org (g/kg)	OM (g/kg)	N tot (g/kg)	C/N ratio	CEC (meq/kg)	K_2O (g/kg)	MgO (g/kg)	CaO (g/kg)	K/Mg ratio
SRC	30.95 ± 7.75 (a)	53.55 ± 13.39 (a)	2.94 ± 0.72 (a)	10.54 ± 1.16 (c)	81.00 ± 19.92 (a)	0.24 ± 0.07 (a)	0.19 ± 0.05 (a)	3.83 ± 1.05 (a)	1.24 ± 0.09 (b)
vSRC	31.68 ± 9.28 (a)	54.81 ± 16.03 (a)	2.61 ± 0.83 (ab)	12.30 ± 1.97 (b)	84.88 ± 30.00 (a)	0.23 ± 0.05 (a)	0.17 ± 0.04 (ab)	4.06 ± 1.02 (a)	1.40 ± 0.16 (a)
Maize	31.52 ± 3.71 (a)	54.53 ± 6.44 (a)	2.26 ± 0.15 (ab)	13.88 ± 1.63 (a)	86.50 ± 7.29 (a)	0.12 ± 0.02 (b)	0.14 ± 0.01 (b)	4.37 ± 0.35 (a)	0.87 ± 0.10 (c)
Unplanted	18.63 ± 1.84 (b)	32.22 ± 3.20 (b)	1.23 ± 0.18 (c)	15.33 ± 2.22 (a)	55.17 ± 4.71 (b)	0.10 ± 0.02 (b)	0.10 ± 0.01 (c)	2.38 ± 0.20 (b)	0.97 ± 0.08 (c)
Initial	32.43 ± 9.23 (a)	56.12 ± 15.97 (a)	2.06 ± 0.48 (b)	15.53 ± 1.06 (a)	80.50 ± 24.98 (a)	0.19 ± 0.05 (a)	0.14 ± 0.03 (b)	3.98 ± 0.82 (a)	1.38 ± 0.17 (a)

	$CaCl_2$ extractable elements								
	Cd (μg/kg)	Cu (μg/kg)	K (μg/kg)	Mg (mg/kg)	Mn (mg/kg)	Na (mg/kg)	P (mg/kg)	Pb (μg/kg)	Zn (μg/kg)
SRC	0.05 ± 0.01 (b)	0.21 ± 0.21 (a)	143.15 ± 34.74 (a)	85.73 ± 17.67 (a)	0.94 ± 0.55 (b)	7.53 ± 2.38 (b)	8.70 ± 1.56 (a)	<LQ	2.18 ± 0.54 (d)
vSRC	0.04 ± 0.01 (c)	0.26 ± 0.21 (a)	147.22 ± 26.95 (a)	77.83 ± 16.71 (ab)	0.66 ± 0.40 (c)	8.71 ± 4.48 (ab)	7.24 ± 1.30 (bc)	<LQ	2.42 ± 0.54 (c)
Maize	0.03 ± 0.01 (d)	0.15 ± 0.04 (a)	68.16 ± 12.23 (b)	65.9 ± 5.58 (bc)	0.68 ± 0.40 (bc)	10.48 ± 2.79 (a)	6.27 ± 0.41 (c)	<LQ	2.82 ± 0.47 (d)
Unplanted	0.09 ± 0.02 (a)	0.12 ± 0.02 (a)	62.40 ± 10.15 (b)	55.60 ± 2.59 (c)	0.60 ± 0.07 (c)	4.14 ± 0.23 (c)	8.25 ± 0.79 (ab)	<LQ	4.39 ± 0.56 (a)
Initial	0.06 ± 0.01 (a)	0.21 ± 0.12 (a)	100.36 ± 26.57 (b)	60.21 ± 14.84 (c)	1.4 ± 0.45 (a)	9.49 ± 3.25 (a)	6.95 ± 1.07 (c)	0.01 ± 0.03	2.08 ± 0.31 (b)

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extractible Mn fraction decreased in all the plots in comparison with the initial 2007 values. The most notable changes in TEs were the significant decrease of the $CaCl_2$-extractible Cd fraction in planted (with poplar and maize) soils, in comparison with the 2007 values or the unplanted soil. The $CaCl_2$-extractible Zn fraction increased significantly in unplanted soils but decreased in all planted soils, compared with the initial soil (Table 1). The total N content and the MgO increased in the SRC plot and decreased in unplanted soil in comparison with the initial 2007 values. The $CaCl_2$-extractible P fractions increased, whereas the $CaCl_2$-extractible Na decreased in the SRC plot and in unplanted soil compared with the initial soil values. Moreover, the SRC plot contained significantly less $CaCO_3$, a lower C/N ratio and lower $CaCl_2$-extractible Zn but more $CaCl_2$-extractible Cd, Mg, Mn and P relative to the vSRC plot. The total concentrations for all the elements remained unchanged (data not shown). No significant differences in the Cu concentrations and assimilable phosphorus were found between plots or from the initial values.

At our experimental site, there was an earlier suggestion that the clay destruction initiated by excessively large irrigation rates would imply a reduction in the filtering capacity of the horizon, and an increased risk of metal leaching into the groundwater (VanOort *et al.* 2008). The phytomanagement regime that we chose in 2007 resulted in a slightly but significantly higher pH in comparison with the initial pH or the unplanted option. An acidification risk that was observed in a previous study on poplars (Mertens *et al.* 2007), which led the authors to conclude that poplar was not a desirable option for their experimental sites, did not occur at our site. This difference is likely related to the different nature of the two soils; our soil contains a large amount of sand (>80%), whereas the soil in Mertens *et al.* (2007) contained approximately 30% clay. A higher soil fertility (in terms of K, Mg, N and P) was likely caused by the mineralization of organic matter and the higher release of minerals from soil particles, which was also measured at our site. An increase in the bioavailable P content was also found by Gamalero *et al.* (2012) under poplar plots grown on contaminated soils, which were correlated with an increased in phosphomonoesterase activity. Similarly, an increase in phosphatase activity was found in soil under poplars grown in an SRC plot (Lagomarsino *et al.* 2008). This mineralization may have also induced increased concentrations of metal-chelating substances in the soil that led to decreased bioavailability and thereby decreased the phytotoxicity of the TEs (notably Zn and Cd) compared with a 'no action (unplanted)' option, which might have led to a decreased risk of metal leaching into the ground water (Baum *et al.* 2009). Mann and Tolbert (2000) noticed a number of soil ecological benefits from poplar coppices that can be caused by continuous plant cover. Among these benefits, our study confirmed that a reduction in leaching losses and an increase of organic carbon are observed under poplar SRC or vSRC.

Roots were less enriched and less diverse in fungal and bacterial OTUs

A total of 13 088 974 16S bacterial and 38 843 584 ITS fungal raw MiSeq reads were obtained from 90 DNA soil samples and 80 DNA root samples. After quality filtering and target extraction, 1 402 648 bacterial effective sequences and 5 370 652 fungal effective sequences remained for the community analyses. A detailed breakdown of the recovered sequence numbers that was performed sample-by-sample is presented in Supplementary Tables S1 and S2. To determine the rarefaction curves, richness and diversity, 2140 and 1000 reads were randomly selected from each sample for fungi and bacteria, respectively. Rarefaction analyses were performed to evaluate the depth of sequencing and to compare the relative fungal and bacterial communities in different samples. Our results showed that all the fungal and bacterial libraries from our samples represented the fungal (Supplementary Fig. S1A) and bacterial (Supplementary Fig. S1B) communities well, and the rarefaction curves tended towards saturation. In comparison with the soil samples, all the poplar root samples had lower bacterial and fungal OTU densities.

The OTU richness, Chao1 and ACE estimators, Pielou evenness, and Shannon and Simpson diversity indices for the fungal (Supplementary Table S1) and bacterial (Supplementary Table S2) communities were always significantly higher ($P < 0.05$) in the soil samples (SRC, vSRC, unplanted and maize) relative to the root samples, which is consistent with the rarefaction curves (Supplementary Fig. S1). There was no significant difference for the richness and diversity indices between the poplar SRC and vSRC (soil or root) samples except for the soil fungal communities, which were slightly more diverse under vSRC plots. Bacterial communities in maize and unplanted soils were slightly but significantly less enriched and less diverse than those from poplar-planted soils, whereas the fungal communities were not different. Spearman's correlation coefficients revealed significant, although few, statistical correlations between the richness and diversity indices and soil parameters (Supplementary Table S3). In brief, the fungal diversity was negatively correlated with Cd, and the fungal richness was positively correlated with phosphorus. By contrast, the bacterial richness and diversity indices were more strongly correlated with soil parameters such as the physical (sand) and chemical (C/N ratio, K and Mg, pH and $CaCO_3$) composition.

We used a β-diversity measure based on OTUs (Bray–Curtis) to compare the fungal (Fig. 1A) and bacterial (Fig. 1B) communities, which quantifies the taxa abundances shared between samples. In the visualized two-dimensional non-metric multidimensional scaling (NMDS) plot, the Bray–Curtis method indicated that the fungal and bacterial communities from SRC or vSRC poplar roots were well separated from those of the different planted and unplanted soils. The NMDS further indicated that maize and unplanted soils were clearly separated from the SRC or vSRC poplar soils for the fungal community, which also showed high fungal heterogeneity in root samples. The scattering of the fungal data was always higher for root samples than for soil samples, which denoted a higher homogeneity of species distribution in the soil samples (see sizes of ellipses in Fig. 1). A permutation test confirmed the separation of the microbial soil and root communities and showed that the habitat (soil in comparison with roots) explained 38% and 49% of the variance in the fungal and bacterial data sets, respectively (Table 2). The vegetation type (eight poplar cultivars, maize and unplanted soil samples) was also an important driver, explaining 12% and 6% of the variance in the fungal and bacterial data sets, respectively. The management regime (vegetation cover by poplars as SRC or vSRC, maize or unplanted soils) had a significant, although limited, impact on the fungal community, whereas bacterial communities were not affected (Table 2). A clear separation of microbiomes was previously reported for the soil and roots of mature poplar sites (Gottel *et al.* 2011; Shakya *et al.* 2013) and in an early phase of stand development (Danielsen *et al.* 2012). In this previous study, the richness and diversity were lower in the roots, and clear separations were observed between the soil and roots, explaining 40 and 50% of the variation for the fungal and bacterial communities, respectively.

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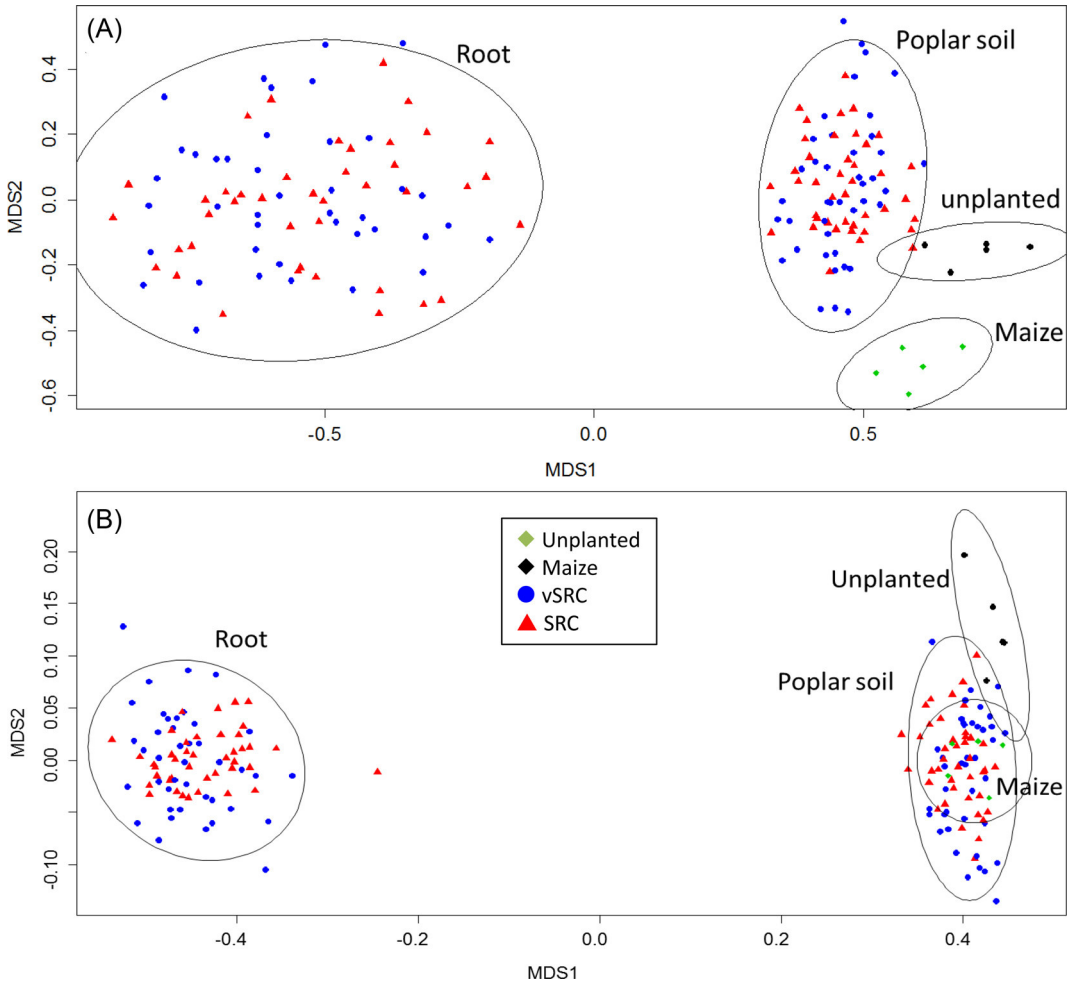

Figure 1. NMDS plot of the fungal (**A**) and bacterial (**B**) community structure using the Bray–Curtis dissimilarity measure. Each point represents the fungal or bacterial community of a given sample (vSRC, blue; SRC, red; unplanted, green; maize black). The ellipses clearly separated the sample into two groups as follow: left ellipse, root samples; and right ellipse, soil samples. Confidence area of ellipses = 0.95.

Table 2. PERMANOVA and ANOSIM data set for the bacterial and fungal community structures based on Bray–Curtis distances. Levels of significance are indicated by asterisks: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. *P*-values based on 10 000 permutations. Results are shown for analyses performed with poplar root samples versus all soil samples (habitat); with the eight poplar cultivars, maize and unplanted control samples (vegetation); with SRC, vSRC, maize and unplanted control samples (management regime).

	Fungi			Bacteria		
	PERMANOVA		ANOSIM	PERMANOVA		ANOSIM
	F(*p*)	R^2	*R*	*F*(*p*)	R^2	*R*
Habitat	167.9***	0.382	0.945***	198.9***	0.491	1.000***
Vegetation	6.1***	0.124	0.111***	2.9***	0.064	0.071***
Management regime (MR)	9.1***	0.021	0.100***	2.4	0.006	0.053**
Habitat × vegetation	3.2***	0.052		2.1**	0.037	
Habitat × MR	4.2**	0.010		1.6	0.004	
Cultivars × MR	3.6***	0.057		1.8*	0.031	
Habitat × vegetation × MR	2.3***	0.037		1.4	0.025	

In a previous study that was performed at the same experimental site, a *Miscanthus* plantation was shown to stimulate bacterial diversity by approximately 20% (Bourgeois *et al.* 2015), which is in agreement with the higher bacterial diversity and richness indices in all poplar soils in comparison with the unplanted soils. These authors also found higher fungal diversity and richness indices under *Miscanthus*, compared with maize plots, which they explained by the intensive use of fungicides (Bourgeois *et al.* 2015) and soil tillage that may have led to the physical breakdown of fungal mycelia (Lienhard *et al.* 2014). We did not observe significant effects of poplar plantation on fungal diversity and richness indices. The overall significant decrease

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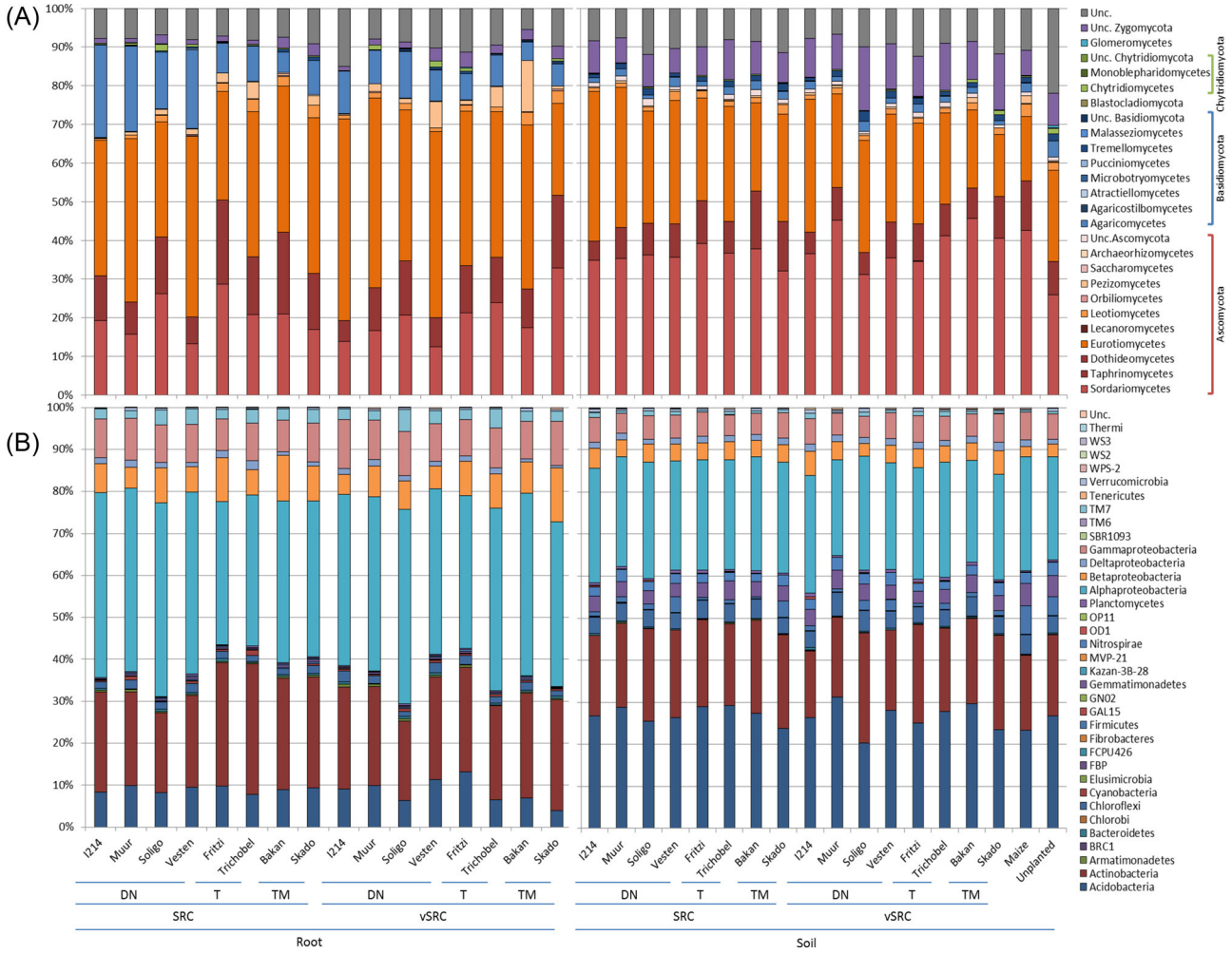

Figure 2. Relative abundance of fungal class (**A**) and bacterial phylum/class (**B**) identified in soils (right panels) or roots (left panels) for different poplar cultivars and families (TM, *P. trichocarpa* × *P. maximowiczii*; DN, *P. deltoides* × *P. nigra*; T, *P. trichocarpa*) grown as SRC or vSRC, and maize or unplanted soils. Unc., unclassified.

of fungal diversity and richness in poplar roots may well have impacted the surrounding soil area.

We used a Mantel test to compare the fungal and bacterial community Bray–Curtis dissimilarity matrices within all the experimental plots (Supplementary Fig. S2). The Mantel correlation coefficients showed that significant relations ($P < 0.05$) were found between the fungal and bacterial communities for all the plots. However, the fungal and bacterial communities from the unplanted plot were the highest correlated ones ($R^2 = 0.70$), whereas less correlation was found between fungal and bacterial communities from the roots of the SRC plots ($R^2 = 0.22$).

Root and soil constitute distinct ecological microbial habitats

To identify the phylogenetic diversity of the microbial communities, effective sequences were assigned to phyla, classes or genera. The overall set of reads was associated with seven fungal phyla and 26 fungal classes (Fig. 2A). Across all the root and soil samples, *Ascomycota* (83 and 77%, respectively) dominated the fungal communities, and in addition, *Basidiomycota* (4–11%) occurred in the root samples and *Zygomycota* (2–8%) occurred in the soil samples (Fig. 2A). Within the *Ascomycota* phylum, the *Sordariomycetes* (20–35%) dominated all the soil samples, whereas *Eurotiomycetes* (23–40%) dominated the root samples. Within the *Basidiomycota* phyla, the *Agaricomycetes* (3–11%) were predominant in the roots but accounted for much lower proportions in the soil samples. In more detail, the relative abundance of *Agaricomycetes* was significantly higher in the roots of the SRC compared with that of the vSRC, whereas unclassified *Zygomycota* and *Pezizomycetes* accounted for larger proportions in the roots of vSRC poplars in comparison with the SRC (Supplementary Table S4). In poplar soils, only *Eurotiomycetes* and unclassified *Zygomycota* showed significant differences between SRC and vSRC. The unplanted soils contained larger amounts of *Glomeromycetes* and unclassified fungal OTUs in comparison with other samples (Supplementary Table S4).

The dominating genera in the poplar root and soil samples were the two *Eurotiomycetes*, that is, *Exophiala* and *Penicillium*, respectively (Supplementary Table S5). In the maize and unplanted plots, in addition to these two genera, a *Monodictys* species and a *Metarhizium* species also dominated, respectively. The fungal classes showed variations between poplar cultivars, independent of the management regime. For example, *Agaricomycetes* represented 20% of the root species of the *P. deltoides* × *P. nigra* poplar hybrids (I214, Muur, Soligo and Vesten) but only 8% of the root species of the other cultivars (Bakan, Skado, Fritzi Pauley and Trichobel) (Fig. 2A). A hierarchical

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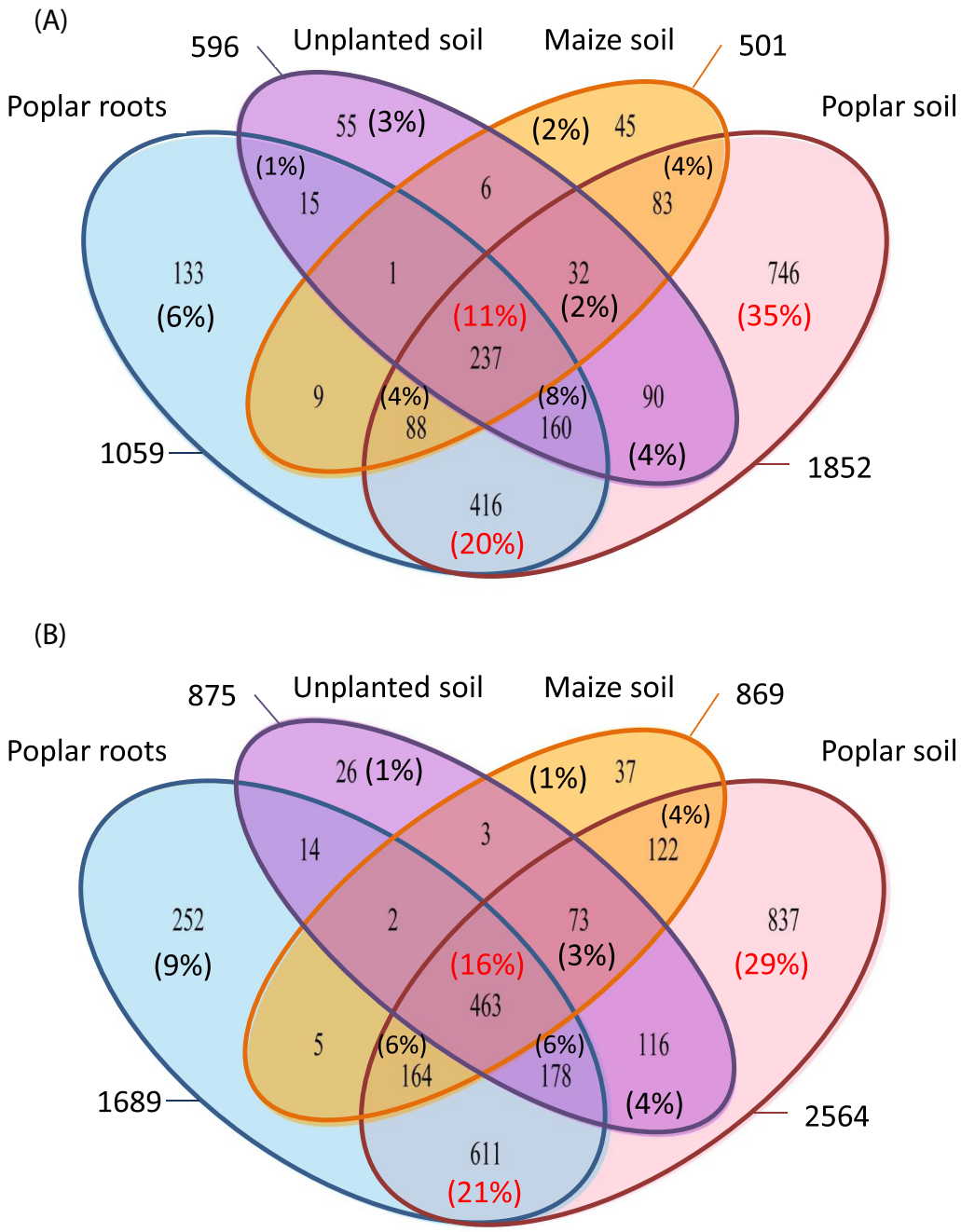

Figure 3. Overlap of the different fungal (**A**) and bacterial (**B**) communities from poplar roots, poplar soil, unplanted and maize soils based on OTUs (97% similarity). The numbers of OTUs shared by all the communities were 2116 for fungi and 2903 for bacteria.

cluster analysis of fungal communities at the class level confirmed that root and soil samples have different compositions (Supplementary Fig. S3). In detail, *Sordariomycetes*, unclassified *Zygomycota*, *Tremellomycetes* and some unclassified *Ascomycota* were positively correlated with soil samples, and *Agaricomycetes* and *Eurotiomycetes* were positively correlated with root samples. The total fungal-observed OTUs in the four habitats was 2116, whereas only 237 (11% of the total OTUs) were shared between them (Fig. 3A). Soil and root samples from the poplar plots shared the highest number of fungal OTUs (416 OTUs, that is, 20% of the total OTUs) (Fig. 3A). OTUs that were unique to each community are highlighted, and they represented 746 OTUs for poplar soil (35% of total OTUs), 133 OTUs for poplar roots (6%), 55 for unplanted soil (3%) and 45 for maize soil (2%).

Each fungal species, genus, family, or class was further assigned to functional groups of fungi. This functional assignment is fully described in Tedersoo *et al.* (2014), and was performed by a group of taxonomic experts. Taxa were for instance considered to be ectomycorrhizal (ECM) if they best matched any sequences of known ECM lineages. If different functional categories were present within a specific genus, the dominant group was chosen (>75% of species assigned to a specific category) or its ecology considered unknown (<75% of species assignable to a single category). All Glomeromycota were considered to be

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Table 3. Relative abundances of fungal OTUs that were assigned to major functional groups. The various OTU groups from soil and root samples that were collected from the SRC, vSRC, maize or unplanted plots were classified as either biotrophic, ectomycorrhizal (ECM), endomycorrhizal (AM), endophytic or saprotrophic. Mean values ± standard deviation are provided soil or root samples for SRC ($n = 40$) and vSRC ($n = 40$) poplars and control soils (unplanted $n = 5$ and maize $n = 5$). Values designated with the same letters were not significantly different ($P < 0.05$) according to a Kruskal–Wallis test.

	SRC root	vSRC root	SRC soil	vSRC soil	Maize	Unplanted
AM	1.32 ± 1.79 (b)	1.49 ± 2.68 (b)	0.66 ± 1.11 (b)	0.59 ± 1.18 (b)	1.60 ± 2.07 (b)	14.60 ± 8.20 (a)
Biotrophic	381.41 ± 185.70 (ab)	382.10 ± 207.19 (b)	399.88 ± 70.80 (a)	388.49 ± 83.85 (ab)	366.80 ± 91.72 (ab)	277.60 ± 100.31 (b)
ECM	126.54 ± 124.68 (a)	120.10 ± 140.76 (a)	8.54 ± 15.07 (c)	11.56 ± 12.26 (b)	4.40 ± 9.29 (c)	1.60 ± 3.05 (c)
Endophytic	20.61 ± 22.25 (a)	18.73 ± 17.24 (a)	3.15 ± 3.43 (c)	4.80 ± 4.06 (b)	3.40 ± 2.07 (bc)	13.00 ± 17.01 (ab)
Saprotrophic	1155.02 ± 218.47 (b)	1169.68 ± 194.41 (b)	1306.85 ± 117.02 (a)	1295.05 ± 103.53 (a)	1285.00 ± 97.78 (ab)	1193.00 ± 231.56 (ab)
Unknown	455.02 ± 113.06 (ab)	447.90 ± 96.89 (ab)	419.76 ± 71.41 (b)	439.10 ± 60.27 (ab)	478.00 ± 108.77 (ab)	639.00 ± 302.74 (a)

arbuscular mycorrhizal (AM). The OTUs were finally classified as ECM, AM, endophytic, saprotrophic or biotrophic (Table 3). In all of the samples, saprotrophic fungi dominated (54–61%), whereas endophytic and ECM fungi were significantly more abundant in the root samples. The saprotrophic species *Penicillium canescens* that dominated in our samples is known to produce xylanase (Delvigne *et al.* 2008) and to solubilize phosphate (Mendes *et al.* 2012) and could therefore have a key role in litter degradation. The other *Penicillium* species present in all the poplar clones (but not in the maize or unplanted soils) of *P. brevicompactum*, although less frequent, can produce plant growth regulators (Kusano *et al.* 1998). *Penicillium* species have been shown to produce organic acids in the presence of trace elements that can lead to immobilization of metals (Arwidsson *et al.* 2009).

Although the endophytic OTUs were less abundant, a *Tetracladium maxilliforme* OTU dominated the endophytic OTUs in all the root samples (Supplementary Table S6). *Exophiala* OTUs that dominated in our samples are potential root endophytes, and they help plants to overcome many biotic and abiotic stresses (Zhan *et al.* 2014; Maciá-Vicente, Glynou and Piepenbring 2016). The *Cyphellophora vermispora* species detected in the roots of all the poplar clones was previously detected in living plant material such as stems, roots and leaves, and it could behave as a root endophyte (Gao *et al.* 2015).

Tuber and *Laccaria* OTUs (and to a lesser extent an unclassified *Sebacinaceae* OTU, a *Geopora*, *Tomentella* and a *Hebeloma*) were significantly more frequently represented in root samples than in soil samples. *Tuber* OTUs also dominated in vSRC soil and root samples but were less frequently represented in SRC soil samples, from which *T. rufum*, *T. scruposum* and *T. rapaeodorum* were missing. The *Laccaria* OTUs largely dominated in SRC root samples and to a lesser extent in vSRC root samples, whereas they were virtually absent from the soil samples (Fig. 4A and Supplementary Table S6). Interestingly, a *Scleroderma* OTU dominated in all Vesten poplar samples, and it was almost absent from all the other (root, soil, SRC or vSRC) poplar samples (Fig. 4A), which also explained the high variability observed in the SRC root samples (Supplementary Table S6). Conversely, *Geopora* OTUs were detected in almost all soil and root poplar samples, but they were absent from maize and unplanted soil samples. Our results confirmed those of a previous study (Hrynkiewicz *et al.* 2010) that determined that selective promotion occurred for some *Pezizales* (*Geopora*, *Tuber* and *Peziza* spp.) and some *Agaricales* (*Laccaria* spp.) under short rotation regimes, and a selective promotion of other *Agaricales* (*Inocybe* sp.) and *Boletales* (*Scleroderma* spp.) because of the longer rotations. The clear dominance of *Tuber* species in vSRC in comparison with SRC is consistent with this observation. Using an ITS sequencing approach, we also detected *Tuber*, *Geopora* and *Hebeloma* species at our experimental site (Lacercat-Didier *et al.* 2016). The *Geopora* species found in the present work and in previous studies (Hrynkiewicz *et al.* 2008) formed ECM in various early and late successional woodlands in acidic to alkaline forest soils as well as in nutrient-poor volcanic cinder soils (Gehring *et al.* 1998; Fujimura *et al.* 2004). The high adaptation ability of these organisms to diverse soil conditions and their long survival as spores might explain the successful spread of *Geopora* (Hrynkiewicz *et al.* 2010). *Laccaria* spp. were found on other *Populus* species (*P. trichocarpa* and *P. nigra* × *maximowiczii*) during pot and field experiments (Baum and Makeschin 2000; Hrynkiewicz *et al.* 2010). Our data confirmed that the mycorrhizal colonization of salicaceous cultivars varies greatly between the species and genotypes (Khasa *et al.* 2002; Puttsepp, Rosling and Taylor 2004), which is exemplified specifically by the detection of *Scleroderma* on Vesten roots, *Laccaria* on all but Skado cultivars, and *Geopora* on all cultivars.

Bacterial composition and interactions with fungal communities

The overall set of reads was associated with 27 bacterial phyla and 83 bacterial classes (Fig. 2B). *Proteobacteria* (mostly *Alphaproteobacteria* followed by *Beta-* and *Gammaproteobacteria*) and *Actinobacteria* dominated the root microbiome, whereas *Alphaproteobacteria*, *Acidobacteria* and *Actinobacteria* were the three major bacterial groups in the soil samples (Fig. 2B and Supplementary Table S7). *Nitrospirae* and *Chloroflexi*, which were also less abundant than the 3 former groups, accounted for significantly much larger proportions of the soil samples compared with the root samples (Supplementary Table S7). *Firmicutes* and *Gemmatimonadetes* were highly represented in maize and unplanted soils, whereas they accounted for significantly much lower proportions of poplar root or soil samples. Conversely, *Cyanobacteria* were highly represented in root samples in comparison with soil samples (Supplementary Table S7). A hierarchical cluster analysis of bacterial communities at the phylum and class levels confirmed that the root and soil samples have different compositions (Supplementary Fig. S4). The soil samples contained mostly *Acidobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae* and *Firmicutes* for the most abundant phylum, whereas the root samples were enriched in *Alphaproteobacteria*, *Actinobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *TM7*, *Cyanobacteria*, *Armatimonadetes* and *Bacteroidetes*. The total for observed bacterial OTUs in the four habitats was 2903, whereas only 463 (16% of the total OTU) were shared between them (Fig. 3B). Soil and root samples from the poplar plots shared 611 bacterial OTUs, that is, 21% of the total OTUs (Fig. 3B). OTUs that were unique to each community are highlighted, and they represented 29% of the total OTUs for poplar soil, 9% for poplar roots, and 1% for unplanted and maize soils.

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Figure 4. Relative proportions of (**A**) fungal genera that were assigned to ECM fungal and (**B**) bacterial genera in which PGPBs are currently recognized. The abundances of the various OTU groups are showed for soils (right panels) or roots (left panels) for different poplar cultivars and families (TM, *P. trichocarpa* × *P. maximowiczii*; DN, *P. deltoides* × *P. nigra*; T, *P. trichocarpa*) that were grown in SRC or vSRC, and maize or unplanted soils. Unc., unclassified.

Table 4. Relative abundances of bacterial OTUs that were assigned to bacterial genera, in which plant growth promoting-bacteria (PGPB) are currently recognized. Soil and root samples were collected from the SRC, vSRC, maize or unplanted plots. Mean values ± standard deviation are provided for soil or root samples for SRC ($n = 40$) and vSRC ($n = 40$) poplars and unplanted ($n = 5$) and maize ($n = 5$) soils. Values designated with the same letters were not significantly different ($P < 0.05$) according to a Kruskal–Wallis test.

	SRC root	vSRC root	SRC soil	vSRC soil	Maize	Unplanted
Bacillus	1.00 ± 0.92 (c)	0.93 ± 1.27 (c)	8.29 ± 8.08 (b)	6.33 ± 5.63 (b)	17.6 ± 7.99 (a)	18.00 ± 8.72 (a)
Flavobacterium	1.20 ± 1.40 (a)	1.20 ± 1.65 (a)	0.27 ± 0.45 (b)	0.20 ± 0.52 (b)	0	0.40 ± 0.89 (b)
Mesorhizobium	11.61 ± 4.57 (a)	14.68 ± 10.61 (a)	3.54 ± 1.52 (b)	3.92 ± 2.27 (b)	1.60 ± 1.14 (c)	1.60 ± 1.14 (c)
Microbacterium	1.85 ± 1.82 (bc)	2.32 ± 2.35 (b)	3.98 ± 2.53 (a)	4.40 ± 4.18 (a)	0.40 ± 0.55 (c)	0.80 ± 0.84 (bc)
Pseudomonas	6.80 ± 9.27 (a)	11.71 ± 15.27 (a)	2.00 ± 1.55 (b)	1.65 ± 1.58 (bc)	0.20 ± 0.45 (c)	2.60 ± 3.21 (bc)
Rhizobium	43.22 ± 16.00 (a)	53.20 ± 23.83 (a)	1.95 ± 1.79 (b)	2.10 ± 1.89 (b)	1.80 ± 0.84 (b)	2.80 ± 1.30 (b)
Rhodococcus	0.51 ± 0.78 (b)	0.44 ± 0.63 (b)	1.32 ± 1.08 (a)	1.30 ± 1.26 (a)	0.60 ± 0.89 (ab)	1.00 ± 0.71 (ab)
Sphingomonas	6.07 ± 2.85 (a)	7.41 ± 3.97 (a)	3.29 ± 2.78 (b)	2.95 ± 2.31 (b)	1.60 ± 1.34 (b)	1.60 ± 1.52 (b)
Streptomyces	29.49 ± 15.87 (a)	27.02 ± 11.04 (a)	5.02 ± 2.83 (c)	4.90 ± 3.18 (c)	8.00 ± 2.12 (b)	8.00 ± 3.16 (b)
Variovorax	27.93 ± 13.26 (a)	27.98 ± 15.24 (a)	8.24 ± 3.83 (b)	8.05 ± 4.65 (b)	1.00 ± 0.71 (c)	3.20 ± 2.28 (c)

As described for fungal OTUs, bacterial OTUs that could be classified to phylum/class or genera were further assigned to categories in which plant growth-promoting bacteria (PGPB) are currently recognized, i.e. symbiotic nitrogen-fixing bacteria and mycorrhiza helper bacteria solely based on a literature search (Frey-Klett, Garbaye and Tarkka 2007; Hrynkiewicz *et al.* 2008; Singh, Pandey and Singh 2011; Trujillo *et al.* 2015; Ullah *et al.* 2015). Relative abundances of bacterial OTUs that were classified at the genus or at the phylum/class level, in which PGPB are currently recognized, are provided in Table 4 and Supplementary Table S7, respectively. Overall, 56 OTUs were assigned to a hypothetical PGPB, and they were associated with 14 bacterial genera, representing 14% of the total OTUs in the root samples but only 4% of the total OTU in the soil samples. The *Rhizobium*, *Streptomyces*, *Mesorhizobium* and *Variovorax* OTUs were most abundant in root samples, whereas *Microbacterium*, *Rhodococcus* and *Bacillus* OTUs dominated the soil samples (Fig. 4B and Table 4). The relative abundance of a *Bradyrhizobiaceae* OTU was significantly higher in root samples compared with soil samples (Supplementary Table S5). Overall, the OTUs from nitrogen-fixing

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bacteria in the *Bradyrhizobiaceae* and *Rhizobiaceae* families clearly dominated (>15%) the root samples (Table 4 and Supplementary Table S5). There was otherwise much less variation in the OTU profiles compared with the fungal OTUs between management regimes and cultivars (Fig. 4A and B). Although the planted trees are not nitrogen fixers, the *Rhizobium* and *Mesorhizobium* genera were frequently encountered in non-nitrogen-fixer tree microbiomes (Shakya *et al.* 2013; Bonito *et al.* 2014) and can promote symbiosis (Frey-Klett, Garbaye and Tarkka 2007). Many soil bacteria affiliated with the dominating genus *Variovorax* have shown resistance to various TEs and are able to adapt to a wide variety of soil environments (Gamalero *et al.* 2012; Durand *et al.* 2015). The *Variovorax paradoxus* species also had PGPB properties (Jiang *et al.* 2012). Our results also showed that *Sphingomonas wittichii* organisms were present in the roots of all the poplar cultivars, but not in the maize or unplanted soils. *Sphingomonas wittichii* was found in a variety of habitats including soil, sediment, oceans and lakes, where it had a profound role in the degradation of large, complex aromatic compounds associated with decaying plant matter and chemical pollution such as dioxin (Miller *et al.* 2010).

Spearman's correlation coefficients revealed statistically significant and positive correlations between PGPB and ECM OTUs ($P < 0.001$, $\rho = 0.50$), between PGPB and fungal endophytic ones ($P < 0.001$, $\rho = 0.50$) and between ECM and fungal endophytic OTUs ($P < 0.001$, $\rho = 0.41$). Conversely, saprotrophic OTUs were negatively correlated with PGPB OTUs ($P < 0.001$, $\rho = -0.41$). In more detail, the bacterial *Rhizobium* and *Streptomyces* OTUs were positively correlated with one another ($\rho = 0.79$) and with the bacterial OTUs *Variovorax paradoxus* ($\rho = 0.74$ and 0.69, respectively), *Mesorhizobium* sp. ($\rho = 0.74$ and 0.70, respectively), *Flavobacterium succinicans* ($\rho = 0.48$ and 0.41, respectively), *Sphingomonas* sp. ($\rho = 0.60$ and 0.58, respectively), and *Pseudomonas* sp. ($\rho = 0.53$ and 0.46, respectively), and the fungal OTUs *Tetracladium maxilliforme* ($\rho = 0.59$ and 0.57, respectively), *Sebacina* ($\rho = 0.41$ and 0.47, respectively) and *Laccaria* ($\rho = 0.42$ and 0.44, respectively). Consistent with our data, many bacterial strains have indeed been reported to be able to promote ECM symbioses (Frey-Klett, Garbaye and Tarkka 2007). Hrynkiewicz *et al.* (2008) showed that the inoculation of willows with *Sphingomonas* sp. improved nitrogen uptake by plants, increased plant growth and stimulated the formation of *Geopora* sp. ectomycorrhizae. *Pseudomonas* and *Bacillus* species were found to have a helper effect on *Tuber*, *Pisolithus*, *Laccaria* and *Suillus* species (Frey-Klett, Garbaye and Tarkka 2007). Because many *Streptomyces* sp. produce secondary metabolites with antifungal properties or because they secrete chitinolytic enzymes, they are generally considered antagonists of both plant pathogenic and saprophytic soil fungi (Reddy and Satyanarayana 2006; Lehr *et al.* 2007; Tarkka and Hampp 2008). However, some cases have been described in which *Streptomyces* promoted the growth of rhizospheric fungi, notably those that are involved in forming ectomycorrhizae with plants (Seipke, Kaltenpoth and Hutchings 2012; Bontemps *et al.* 2013). Similarly, *Streptomyces* and *Pseudomonas* species that were positively correlated in our study with *Laccaria* sp., *Sebacina* sp. and *Tetracladium maxilliforme* could be considered as helper bacteria.

Bacteria can be photoautotrophs, heterotrophs or chemoautotrophs, and many can fix atmospheric nitrogen (Nemergut *et al.* 2007; Duc *et al.* 2008; Schmidt *et al.* 2008), whereas all fungi are heterotrophs. However, fungi are the primary decomposers of recalcitrant components in plant litter because they can produce lignin-modifying enzymes (Lundell, Mäkelä and Hildén 2010). These two microbial populations may therefore have complementary functions.

CONCLUSIONS

From the analysis of rRNA gene amplicon MiSeq sequencing data from 7-year-old poplars, we may conclude that the implementation of poplar SRC or vSRC led to a significant increase in soil fertility and a decrease in the bioavailability of Zn and Cd that accompanied changes in microbial communities. Planting poplars increased the proportion of symbiotic/endophytic fungi in the rhizosphere, although at lower proportions than those frequently observed in forest stands. This finding might be explained by the overall decomposition activities needed at the Pierrelaye site, where the accumulation of large amounts of organic carbon from raw wastewater occurred for more than one century, which is revealed by the dominance of saprotrophic fungi in all the samples. We further showed that roots and soil constitute distinct habitats for fungal and bacterial communities, independent of the poplar management regimes (SRC and vSRC) and of the cultivars in use.

Finally, we reveal that planting poplar crops on contaminated soils that have been irrigated with raw but contaminated wastewaters can be considered as a relevant strategy to stabilize the metal elements and produce biomass on an otherwise unused agricultural soil. Poplar biomass production has not been discussed here, and it will be fully detailed in a further paper. We may, however, reveal that the biomass yield was indeed rather high in comparison with the production of the same poplar cultivars in non-contaminated areas, producing between 12 and 16 t DW/ha/year. We have addressed this question in a previous study based on a decision tool, in which we did conclude that poplar plantations may reduce the risk of dusting and leaching (Andersson-Sköld *et al.* 2014).

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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Table S1. Richness and diversity indices of fungal communities. The richness was calculated using the OTU number, Chao1 and ACE estimators. The diversity was estimated from the Shannon-Wiener (H') and Simpson's (D) indices and the equitability was estimated using Pielou's evenness (J). Mean values and standard deviations (mean ± SD) are provided in soil or root samples for SRC (n=40) and vSRC (n=40) poplars and in unplanted (n=5) and maize (n=5) soils. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test.

	Sequences			Richness			Diversity		
	raw sequence	effective sequences	coverage	OTU Richness	Chao1	ACE	Shannon's index (H')	Inverse Simpson index (D)	Pielou evenness (J)
SRC root	11,491,100	10,744 ± 6,534 (d)	0.98 ± 0.01 (a)	127.34 ± 26.43 (c)	199.46 ± 53.22 (c)	197.38 ± 46.36 (c)	3.17 ± 0.33 (c)	0.90 ± 0.04 (c)	0.66 ± 0.05 (c)
vSRC root	10,904,106	7,294 ± 4,686 (e)	0.98 ± 0.01 (a)	124.56 ± 21.46 (c)	186.32 ± 43.50 (c)	185.17 ± 42.68 (c)	3.10 ± 0.30 (c)	0.89 ± 0.05 (c)	0.64 ± 0.05 (c)
SRC soil	8,156,750	56,425 ± 20,041 (a)	0.95 ± 0.01 (bc)	248.54 ± 26.11 (ab)	372.29 ± 55.94 (ab)	379.25 ± 57.48 (ab)	4.09 ± 0.30 (b)	0.94 ± 0.03 (b)	0.74 ± 0.04 (b)
vSRC soil	7,120,082	48,726 ± 27,035 (b)	0.96 ± 0.01 (b)	244.56 ± 17.67 (ab)	349.65 ± 43.97 (b)	358.70 ± 39.67 (b)	4.25 ± 0.20 (a)	0.96 ± 0.02 (a)	0.77 ± 0.03 (a)
Maize	768,860	43,724± 17,550 (b)	0.96 ± 0.01 (b)	227.60 ± 24.59 (b)	348.05 ± 49.18 (b)	343.79 ± 49.17 (b)	4.19 ± 0.27 (ab)	0.97 ± 0.01 (a)	0.77 ± 0.04 (ab)
Unplanted	1,225,826	20,262 ± 7,807 (c)	0.95 ± 0.01 (c)	265.4 ± 29.69 (a)	408.62 ± 38.34 (a)	409.91 ± 53.95 (a)	4.09 ± 0.53 (b)	0.93 ± 0.06 (b)	0.73 ± 0.08 (b)

Table S2. Richness and diversity indices of bacterial communities. The richness was calculated using the OTU number, Chao1 and ACE estimators. The diversity was estimated from the Shannon-Wiener (H') and Simpson's (D) indices and the equitability was estimated using Pielou's evenness (J). Mean values and standard deviations (mean ± SD) are provided in soil or root samples for SRC (n=40) and vSRC (n=40) poplars and in unplanted (n=5) and maize (n=5) soils. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test.

	Sequences			Richness			Diversity		
	raw sequence	effective sequences	coverage	OTU Richness	Chao1	ACE	Shannon's index (H')	Inverse Simpson index (D)	Pielou evenness (J)
SRC root	3,428,450	9,394 ± 2,706 (a)	0.86 ± 0.02 (b)	240.15 ± 22.52 (c)	475.59 ± 82.54 (c)	480.24 ± 72.78 (b)	4.44 ± 0.19 (c)	0.97 ± 0.01 (c)	0.81 ± 0.02 (c)
VSRC root	3,258,276	8,871 ± 3,571 (ab)	0.87 ± 0.02 (a)	235.10 ± 25.99 (c)	447.90 ± 67.87 (c)	468.01 ± 73.99 (b)	4.42 ± 0.18 (c)	0.97 ± 0.01 (c)	0.81 ± 0.02 (c)
SRC soil	2,892,628	7,472 ± 3,440 (ab)	0.77 ± 0.01 (d)	391.83 ± 12.04 (a)	794.02 ± 70.74 (a)	826.36 ± 71.44 (a)	5.47 ± 0.04 (a)	0.99 ± 0.00 (a)	0.92 ± 0.00 (a)
VSRC soil	2,840,402	6,337 ± 2,664 (b)	0.77 ± 0.01 (d)	394.68 ± 16.45 (a)	794.37 ± 82.82 (a)	830.93 ± 82.60 (a)	5.47 ± 0.07 (a)	0.99 ± 0.00 (a)	0.92 ± 0.01 (a)
Maize	437,418	9,265 ± 3,677 (ab)	0.78 ± 0.01 (c)	374.40 ± 13.65 (b)	717.05 ± 73.82 (b)	807.33 ± 56.69 (a)	5.37 ± 0.07 (b)	0.99 ± 0.00 (b)	0.91 ± 0.01 (b)
Unplanted	867,862	9,333 ± 2,099 (a)	0.77 ± 0.01 (cd)	379.40 ± 6.15 (b)	726.31 ± 55.05 (b)	771.74 ± 65.12 (a)	5.41 ± 0.03 (b)	0.99 ± 0.00 (b)	0.91 ± 0.00 (b)

Table S3. Spearman's rank correlation coefficients between richness (OTU, Chao1, ACE) and diversity (H', D, J) indices and soil parameters (physico-chemical parameters and $CaCl_2$-extractible elements). ns: non-significant.

		Fungi						Bacteria					
		OTU	chao1	ACE	H'	D	J	OTU	chao1	ACE	H'	D	J
physico-chemical parameters	Clay	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Thin Silt	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.43	ns
	Large Silt	ns	ns	ns	0.40	ns	ns	ns	ns	ns	ns	0.44	0.45
	Thin Sand	ns	0.45	ns	ns	ns	ns	-0.68	ns	ns	-0.77	-0.78	-0.75
	Coarse Sand	ns	ns	ns	ns	ns	ns	0.68	ns	0.41	0.66	0.54	0.53
	$CaCO_3$	ns	ns	ns	ns	ns	ns	-0.64	-0.48	ns	-0.68	-0.54	-0.50
	pH	ns	ns	ns	ns	ns	ns	ns	0.53	ns	0.46	0.51	0.44
	OM	ns	-0.43	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Corg	ns	-0.43	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Ntot	ns	-0.42	ns	ns	ns	ns	ns	ns	ns	ns	0.40	ns
	C/N	ns	ns	ns	ns	ns	ns	-0.45	ns	ns	-0.51	-0.50	-0.47
	CEC	ns	ns	ns	ns	0.45	0.44	ns	ns	ns	ns	ns	ns
	P_2O_5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	$K0_2$	ns	ns	ns	ns	ns	ns	0.50	ns	ns	0.62	0.69	0.58
	Mg0	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.49	0.61	0.52
	Ca0	ns	-0.44	ns	ns	0.41	0.43	ns	ns	ns	ns	ns	ns
	k/Mg	ns	ns	ns	ns	ns	ns	0.63	0.49	0.42	0.68	0.64	0.54
	B	ns	-0.45	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CaCl2-extractible elements	Cd	ns	ns	ns	-0.36	-0.39	-0.36	ns	ns	ns	ns	ns	ns
	Cu	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	K	ns	ns	ns	ns	ns	ns	0.50	ns	ns	0.62	0.69	0.57
	Mg	ns	ns	ns	ns	ns	ns	0.44	ns	ns	0.54	0.62	0.53
	Mn	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Na	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	P	0.38	ns	0.34	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Sb	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Zn	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table S4. Relative abundances of fungal classes identified in soil or root samples for SRC and vSRC poplars and in unplanted and maize soils. Mean values and standard deviations (mean ± SD) are provided in soil or root samples for SRC (n=40) and vSRC (n=40) poplars and in unplanted (n=5) and maize (n=5) soils. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test. Unc.: unclassified.

sample	SRC root	vSRC root	SRC soil	vSRC soil	Maize	Unplanted
Agaricomycetes	296.39 ± 234.86 (a)	173.71 ± 99.99 (b)	37.29 ± 21.01 (d)	36.34 ± 17.14 (d)	49.60 ± 40.31 (cd)	87.80 ± 51.51 (c)
Microbotryomycetes	0.05 ± 0.22 (ab)	1.00 ± 3.61 (ab)	0.34 ± 0.57 (a)	0.44 ± 0.71 (a)	1.20 ± 2.17 (a)	0.20 ± 0.45 (ab)
Pucciniomycetes	1.32 ± 3.17 (a)	0.88 ± 2.69 (ab)	0.41 ± 0.89 (ab)	0.07 ± 0.26 (b)	0	0.20 ± 0.45 (ab)
Tremellomycetes	8.46 ± 8.29 (c)	7.78 ± 6.78 (c)	30.37 ± 13.01 (b)	32.71 ± 17.93 (b)	28.80 ± 23.17 (b)	41.60 ± 7.44 (a)
Dothideomycetes	306.27 ± 154.48 (a)	247.61 ± 114.79 (a)	223.90 ± 87.19 (ab)	187.07 ± 63.04 (b)	278.80 ± 79.96 (a)	192.40 ± 71.07 (b)
Eurotiomycetes	797.32 ± 216.03 (a)	879.54 ± 253.74 (a)	648.24 ± 164.77 (b)	533.73 ± 159.28 (c)	355.40 ± 79.46 (d)	502.40 ± 138.50 (cd)
Leotiomycetes	39.93 ± 41.98 (b)	39.05 ± 28.36 (b)	37.22 ± 21.06 (b)	34.29 ± 12.01 (b)	80.00 ± 25.19 (a)	55.20 ± 30.90 (ab)
Orbiliomycetes	2.29 ± 2.72 (a)	2.76 ± 5.14 (a)	0.63 ± 1.02 (b)	0.63 ± 0.99 (b)	1.00 ± 1.73 (ab)	1.20 ± 1.30 (ab)
Pezizomycetes	36.88 ± 69.51 (b)	80.32 ± 119.01 (a)	7.83 ± 6.17 (b)	11.32 ± 10.25 (b)	43.40 ± 44.29 (a)	8.00 ± 8.77 (b)
Saccharomycetes	0	0	0.27 ± 0.50 (a)	0.20 ± 0.56 (a)	0.60 ± 0.89 (a)	1.40 ± 2.61 (a)
Sordariomycetes	431.56 ± 191.97 (c)	432.39 ± 201.66 (c)	770.02 ± 85.58 (b)	833.98 ± 137.81 (ab)	917.40 ± 133.22 (a)	553.80 ± 165.75 (c)
Unc. Ascomycota	117.00 ± 55.46 (ab)	135.46 ± 83.82 (a)	99.12 ± 31.66 (ab)	97.12 ± 32.08 (b)	106.40 ± 16.71 (ab)	96.60 ± 23.07 (ab)
Unc. Zygomycota	38.46 ± 26.15 (d)	52.68 ± 32.16 (c)	169.71 ± 59.64 (b)	254.29 ± 107.98 (a)	137.80 ± 91.4 (b)	178.80 ± 72.28 (ab)
Glomeromycetes	1.32 ± 1.79 (b)	1.49 ± 2.68 (b)	0.66 ± 1.11 (b)	0.59 ± 1.18 (b)	1.60 ± 2.07 (b)	14.60 ± 8.20 (a)
Chytridiomycetes	10.71 ± 12.95 (a)	11.85 ± 16.18 (a)	4.15 ± 4.03 (ab)	8.78 ± 9.97 (a)	8.40 ± 4.72 (a)	29.60 ± 51.74 (a)
Unc. Chytridiomycota	0.73 ± 1.55 (b)	0.93 ± 4.83 (b)	2.37 ± 3.10 (a)	3.98 ± 7.13 (a)	1.60 ± 0.89 (a)	2.20 ± 1.79 (a)
Unc.	50.51 ± 26.03 (d)	71.93 ± 62.48 (c)	104.27 ± 39.60 (b)	101.68 ± 28.95 (b)	127.00 ± 53.33 (ab)	372.60 ± 345.60 (a)

Table S5. The 10 most abundant fungal and bacterial OTUs in soil or root samples for SRC and vSRC poplars and in unplanted and maize soils. The OTUs were assigned to kingdoms (k), phyla (p), classes (c), orders (o), families (f), genera (g) or species (s), when possible. The relative proportions are given in parenthesis.

	Fungi	Bacteria
SRC root	Otu00002_s__Exophiala_sp_KL_2011f (19.6)	Otu00001_f__Bradyrhizobiaceae (11.4)
	Otu00001_s__Penicillium_canescens (9.0)	Otu00002_f__Micromonosporaceae (8.4)
	Otu00015_o__Chaetothyriales (6.3)	Otu00007_g__Rhizobium (4.3)
	Otu00023_s__Pyrenochaeta_sp_IZ_1543 (5.6)	Otu00008_g__Streptomyces (2.9)
	Otu00008_s__Ilyonectria_macrodidyma (5.4)	Otu00006_s__Variovorax_paradoxus (2.8)
	Otu00031_s__Pleosporales_sp_BESC803s (3.5)	Otu00016_s__Kineosporia_rhamnosa (2.7)
	Otu00054_o__Agaricales (2.9)	Otu00003_f__Sinobacteraceae (2.6)
	Otu00079_s__uncultured_Laccaria (2.9)	Otu00020_f__Sphingomonadaceae (1.9)
	Otu00048_g__Cylindrocarpon (1.9)	Otu00012_g__Devosia (1.7)
	Otu00094_g__Phomopsis (1.8)	Otu00023_f__Rhizobiaceae (1.7)
vSRC root	Otu00002_s__Exophiala_sp_KL_2011f (24.0)	Otu00001_f__Bradyrhizobiaceae (10.8)
	Otu00015_o__Chaetothyriales (8.6)	Otu00002_f__Micromonosporaceae (8.1)
	Otu00008_s__Ilyonectria_macrodidyma (8.6)	Otu00007_g__Rhizobium (5.3)
	Otu00001_s__Penicillium_canescens (5.7)	Otu00006_s__Variovorax_paradoxus (2.8)
	Otu00023_s__Pyrenochaeta_sp_IZ_1543 (4.1)	Otu00008_g__Streptomyces (2.6)
	Otu00031_s__Pleosporales_sp_BESC803s (2.9)	Otu00003_f__Sinobacteraceae (2.6)
	Otu00066_p__Ascomycota (2.0)	Otu00016_s__Kineosporia_rhamnosa (2.2)
	Otu00054_o__Agaricales (1.9)	Otu00020_f__Sphingomonadaceae (2.0)
	Otu00079_s__uncultured_Laccaria (1.6)	Otu00023_f__Rhizobiaceae (2.0)
	Otu00101_g__Tuber (1.6)	Otu00012_g__Devosia (1.9)
SRC soil	Otu00001_s__Penicillium_canescens (20.7)	Otu00001_f__Bradyrhizobiaceae (2.9)
	Otu00002_s__Exophiala_sp_KL_2011f (4.3)	Otu00010_f__Sphingomonadaceae (2.1)
	Otu00007_s__Fusarium_chlamydosporum (3.5)	Otu00005_o__iii1-15 (2.1)
	Otu00005_g__Fusarium (3.3)	Otu00009_o__iii1-15 (2.1)
	Otu00006_s__Verticillium_nigrescens (2.9)	Otu00004_c__Gemm-1 (1.9)
	Otu00016_g__Preussia (2.5)	Otu00018_s__Arthrobacter_oxydans (1.5)
	Otu00003_s__uncultured_Mortierellaceae (2.1)	Otu00044_g__Pedomicrobium (1.3)
	Otu00008_s__Ilyonectria_macrodidyma (1.7)	Otu00047_g__Pedomicrobium (1.2)
	Otu00022_f__Chaetomiaceae (1.7)	Otu00025_g__Kaistobacter (1.2)
	Otu00004_g__Mortierella (1.5)	Otu00013_c__Ellin6529 (1.2)
vSRC soil	Otu00001_s__Penicillium_canescens (14.6)	Otu00001_f__Bradyrhizobiaceae (2.9)
	Otu00002_s__Exophiala_sp_KL_2011f (4.7)	Otu00010_f__Sphingomonadaceae (2.1)
	Otu00003_s__uncultured_Mortierellaceae (3.8)	Otu00005_o__iii1-15 (2.1)
	Otu00006_s__Verticillium_nigrescens (3.4)	Otu00004_c__Gemm-1 (2.1)
	Otu00005_g__Fusarium (2.4)	Otu00009_o__iii1-15 (1.9)
	Otu00007_s__Fusarium_chlamydosporum (1.9)	Otu00044_g__Pedomicrobium (1.6)
	Otu00008_s__Ilyonectria_macrodidyma (1.9)	Otu00018_s__Arthrobacter_oxydans (1.4)
	Otu00018_c__Sordariomycetes (1.8)	Otu00013_c__Ellin6529 (1.4)
	Otu00004_g__Mortierella (1.7)	Otu00011_f__Sinobacteraceae (1.3)
	Otu00019_s__uncultured_Chaetomiaceae (1.7)	Otu00047_g__Pedomicrobium (1.2)
Maize	Otu00050_s__Monodictys_sp_3_JAS_2013 (8.3)	Otu00004_c__Gemm-1 (3.4)
	Otu00001_s__Penicillium_canescens (7.6)	Otu00025_g__Kaistobacter (3.4)
	Otu00036_f__Lasiosphaeriaceae (5.5)	Otu00005_o__iii1-15 (2.1)
	Otu00017_g__Podospora (5.2)	Otu00001_f__Bradyrhizobiaceae (2.0)
	Otu00002_s__Exophiala_sp_KL_2011f (5.1)	Otu00009_o__iii1-15 (1.7)
	Otu00025_c__Sordariomycetes (3.7)	Otu00003_f__Sinobacteraceae (1.7)
	Otu00045_s__Cladorrhinum_foecundissimum (3.6)	Otu00010_f__Sphingomonadaceae (1.4)
	Otu00006_s__Verticillium_nigrescens (1.9)	Otu00044_g__Pedomicrobium (1.4)
	Otu00062_k__Fungi (1.9)	Otu00032_g__Hyphomicrobium (1.3)
	Otu00004_g__Mortierella (1.6)	Otu00018_s__Arthrobacter_oxydans (1.2)
Unplanted	Otu00001_s__Penicillium_canescens (15.0)	Otu00004_c__Gemm-1 (3.6)
	Otu00175_k__Fungi (8.1)	Otu00001_f__Bradyrhizobiaceae (3.0)
	Otu00042_s__Metarhizium_anisopliae (4.7)	Otu00009_o__iii1-15 (2.2)
	Otu00002_s__Exophiala_sp_KL_2011f (3.8)	Otu00003_f__Sinobacteraceae (2.0)
	Otu00003_s__uncultured_Mortierellaceae (3.3)	Otu00010_f__Sphingomonadaceae (1.6)
	Otu00029_s__Minimedusa_sp (2.6)	Otu00047_g__Pedomicrobium (1.5)
	Otu00005_g__Fusarium (2.1)	Otu00005_o__iii1-15 (1.4)
	Otu00004_g__Mortierella (1.6)	Otu00018_s__Arthrobacter_oxydans (1.4)
	Otu00071_p__Ascomycota (1.6)	Otu00017_g__Rhodoplanes (1.4)
	Otu00009_s__Cryptococcus_fuscescens (1.5)	Otu00025_g__Kaistobacter (1.2)

Table S6. Relative abundances of fungal genera that were assigned to ectomycorrhizal (ECM), arbuscular mycorrhizal (AM) and endophytic (ENDO) fungi and identified in soil or root samples for SRC and vSRC poplars and in unplanted and maize soils. Mean values and standard deviations (mean ± SD) are provided in soil or root samples for SRC (n=40) and vSRC (n=40) poplars and in unplanted (n=5) and maize (n=5) soils. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test. Unc.: unclassified.

genus	group	SRC root	vSRC root	SRC soil	vSRC soil	Maize	Unplanted
Cadophora	ECM/ENDO	0.20 ± 1.10 (a)	0.56 ± 2.34 (a)	0.05 ± 0.22 (a)	0.39 ± 1.32 (a)	0.40 ± 0.89 (a)	0
Claroideoglomus	AM	0	0.02 ± 0.16 (b)	0.12 ± 0.46 (b)	0.22 ± 0.57 (b)	0.60 ± 1.34 (b)	3.20 ± 4.15 (a)
Cortinarius	ECM	5.49 ± 19.59 (ab)	1.85 ± 3.71 (ab)	0.59 ± 0.89 (b)	2.88 ± 5.33 (a)	0.40 ± 0.89 (b)	0
Entrophospora	AM	0.51 ± 0.90 (ab)	0.44 ± 0.84 (ab)	0.24 ± 0.54 (b)	0.22 ± 0.65 (b)	0.60 ± 0.89 (ab)	1.00 ± 1.22 (a)
Geopora	ECM	9.05 ± 35.53 (ab)	3.68 ± 7.63 (a)	1.24 ± 1.74 (bc)	2.51 ± 5.92 (bc)	0	0
Glomus	AM	0.73 ± 1.38 (a)	0.88 ± 1.85 (a)	0.07 ± 0.26 (ab)	0.07 ± 0.26 (ab)	0.40 ± 0.89 (ab)	0.60 ± 0.89 (a)
Hebeloma	ECM	1.29 ± 5.09 (a)	0.46 ± 1.94 (ab)	0	0.02 ± 0.16 (b)	0	0
Hymenogaster	ECM	0.07 ± 0.26 (b)	0.20 ± 0.75 (b)	0.78 ± 4.84 (b)	0.56 ± 1.43 (a)	1.80 ± 4.02 (ab)	0
Laccaria	ECM	61.10 ± 103.32 (a)	34.49 ± 64.57 (a)	0.15 ± 0.48 (b)	0.51 ± 1.47 (b)	0	1.60 ± 3.05 (ab)
Paxillus	ECM	0.22 ± 0.88 (ab)	0	0.56 ± 2.38 (a)	0.07 ± 0.26 (ab)	0	0
Periconia	ENDO	0.12 ± 0.40 (c)	0.85 ± 1.88 (b)	0.37 ± 0.66 (bc)	2.51 ± 3.86 (a)	1.80 ± 0.84 (a)	1.40 ± 2.07 (ab)
Scleroderma	ECM	12.98 ± 50.93 (ab)	0.07 ± 0.47 (b)	3.15 ± 13.11 (a)	0.27 ± 1.41 (ab)	0	0
Terfezia	ECM	1.76 ± 3.92 (a)	0.78 ± 3.60 (ab)	0.10 ± 0.30 (b)	0.05 ± 0.22 (b)	0	0
Tetracladium	ENDO	18.61 ± 21.61 (a)	15.51 ± 16.78 (a)	2.00 ± 2.86 (b)	1.54 ± 2.18 (b)	1.20 ± 2.17 (b)	10.80 ± 15.42 (a)
Tomentella	ECM	6.10 ± 18.34 (a)	1.93 ± 6.00 (ab)	1.02 ± 3.80 (b)	0.46 ± 1.40 (b)	0	0
Tuber	ECM	22.71 ± 63.32 (b)	71.10 ± 119.49 (a)	0.63 ± 3.00 (c)	3.51 ± 6.29 (b)	0	0
Unc. fungal endo	ENDO	1.88 ± 6.18 (a)	2.27 ± 5.34 (a)	0.76 ± 1.36 (ab)	0.71 ± 2.00 (b)	0.40 ± 0.55 (ab)	0
Unc. Glomeraceae	AM	0.05 ± 0.22 (b)	0.10 ± 0.44 (b)	0.02 ± 0.16 (b)	0.02 ± 0.16 (b)	0	9.80 ± 5.07 (a)
Unc. Sebacinaceae	ECM/ENDO	5.15 ± 7.51 (a)	3.80 ± 10.99 (b)	0.10 ± 0.44 (c)	0	0	0

Table S7. Relative abundances of bacterial phylum/class in which the PGPB are currently recognized, in soil or root samples for SRC and vSRC poplars and in unplanted and maize soils. Mean values and standard deviations (mean ± SD) are provided in soil or root samples for SRC (n=40) and vSRC (n=40) poplars and in unplanted (n=5) and maize (n=5) soils. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test. Unc.: unclassified.

	SRC root	vSRC root	SRC soil	vSRC soil	Maize	Unplanted
Acidobacteria	89.90 ± 18.41 (b)	82.90 ± 33.69 (b)	270.12 ± 28.04 (a)	265.52 ± 39.49 (a)	233.80 ± 47.67 (a)	267.40 ± 33.10 (a)
Actinobacteria	251.32 ± 55.18 (a)	238.20 ± 40.82 (a)	208.49 ± 27.21 (b)	206.80 ± 41.92 (bc)	177.80 ± 20.18 (c)	193.40 ± 29.14 (bc)
Armatimonadetes	3.51 ± 2.08 (a)	4.44 ± 3.02 (a)	1.39 ± 1.24 (b)	1.32 ± 1.31 (b)	1.40 ± 0.55 (b)	1.60 ± 1.34 (b)
Bacteroidetes	4.34 ± 2.46 (a)	4.34 ± 2.24 (a)	2.39 ± 1.59 (c)	3.25 ± 2.06 (b)	1.80 ± 1.92 (c)	2.40 ± 1.67 (c)
Chloroflexi	17.37 ± 5.61 (b)	18.37 ± 7.42 (b)	40.78 ± 8.78 (a)	43.08 ± 9.85 (a)	46.20 ± 5.12 (a)	40.60 ± 6.88 (a)
Cyanobacteria	5.12 ± 4.75 (a)	4.73 ± 2.83 (a)	1.05 ± 0.95 (b)	0.82 ± 1.03 (bc)	1.00 ± 1.00 (bc)	0
Firmicutes	2.07 ± 1.66 (c)	1.93 ± 1.75 (c)	16.90 ± 16.95 (b)	13.28 ± 9.96 (b)	66.40 ± 35.90 (a)	45.40 ± 9.48 (a)
Gemmatimonadetes	4.39 ± 3.14 (c)	3.37 ± 2.37 (c)	35.78 ± 6.82 (b)	36.83 ± 10.12 (b)	53.00 ± 8.94 (a)	50.00 ± 6.44 (a)
Nitrospirae	2.66 ± 2.38 (c)	1.93 ± 2.13 (c)	22.68 ± 6.14 (b)	24.77 ± 7.26 (b)	26.60 ± 3.29 (ab)	31.20 ± 5.63 (a)
OD1	0.17 ± 0.38 (b)	0.22 ± 0.47 (b)	1.41 ± 1.45 (a)	1.88 ± 2.31 (a)	0.40 ± 0.55 (ab)	1.00 ± 1.00 (a)
Planctomycetes	2.95 ± 1.48 (bc)	2.37 ± 1.74 (c)	5.44 ± 2.50 (a)	6.22 ± 2.71 (a)	3.00 ± 1.00 (bc)	4.60 ± 2.07 (ab)
TM7	29.07 ± 11.21 (a)	30.85 ± 15.26 (a)	7.44 ± 4.07 (b)	7.50 ± 4.11 (b)	3.80 ± 2.28 (c)	5.40 ± 0.89 (bc)
Verrucomicrobia	3.80 ± 2.34 (b)	4.32 ± 2.63 (ab)	5.63 ± 3.41 (a)	5.97 ± 3.25 (a)	2.80 ± 0.84 (b)	6.20 ± 2.28 (a)
Unc.	1.02 ± 1.11 (b)	2.00 ± 2.71 (b)	1.93 ± 1.65 (ab)	3.25 ± 1.90 (a)	3.40 ± 1.82 (a)	2.40 ± 2.70 (ab)
Alphaproteobacteria	403.00 ± 54.48 (a)	413.10 ± 44.70 (a)	264.90 ± 19.06 (b)	256.80 ± 24.41 (b)	270.20 ± 18.32 (b)	244.40 ± 27.02 (b)
Betaproteobacteria	76.95 ± 27.49 (a)	76.32 ± 34.55 (a)	41.85 ± 8.50 (b)	44.65 ± 12.01 (b)	25.40 ± 3.85 (c)	29.80 ± 4.76 (c)
Deltaproteobacteria	13.80 ± 6.38 (b)	11.98 ± 5.03 (b)	14.85 ± 3.45 (ab)	16.32 ± 3.96 (a)	15.20 ± 1.64 (ab)	11.80 ± 2.77 (b)
Gammaproteobacteria	87.95 ± 17.37 (b)	98.12 ± 20.92 (a)	54.37 ± 9.86 (d)	58.62 ± 10.70 (cd)	66.40 ± 8.29 (c)	60.80 ± 9.15 (cd)

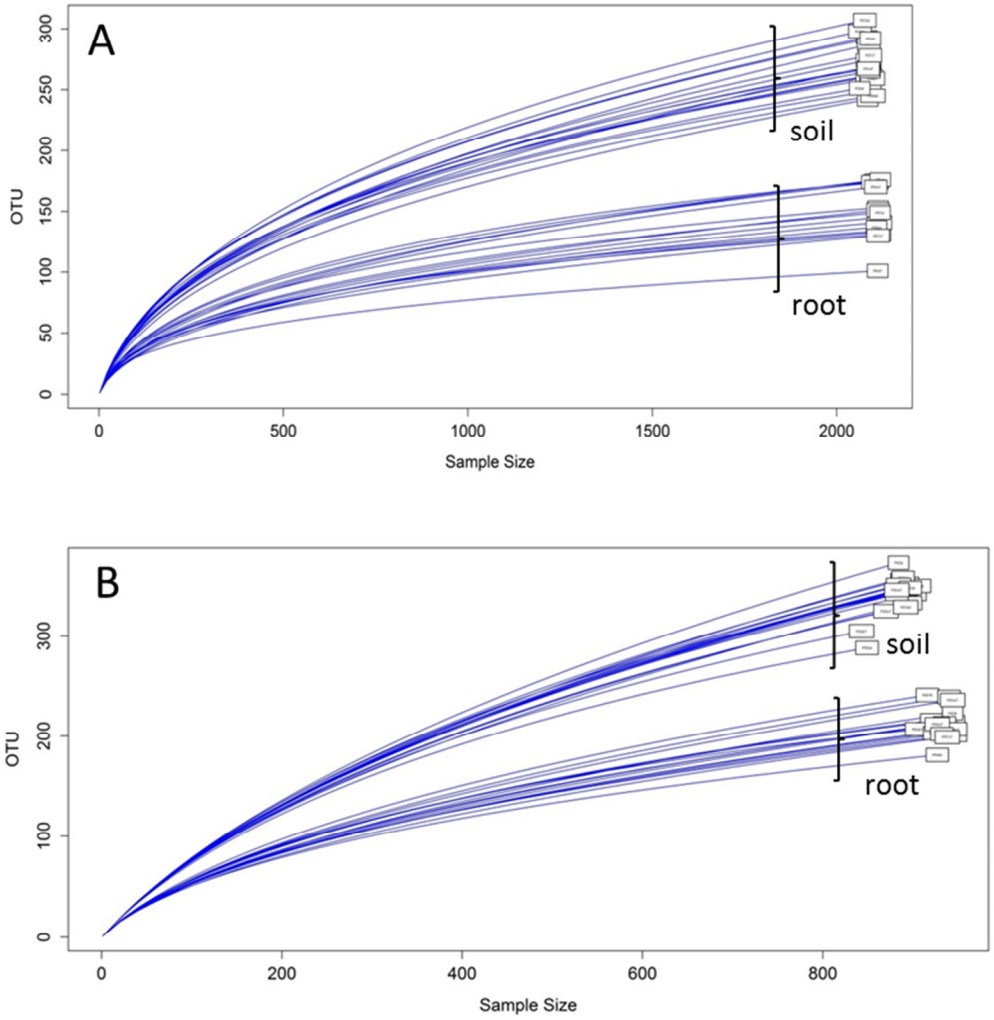


Fig. S1. Rarefaction curves for fungal (A) and bacterial (B) OTUs. The rarefaction curves are based on the average number of OTUs of five samples that were collected from the same plot.

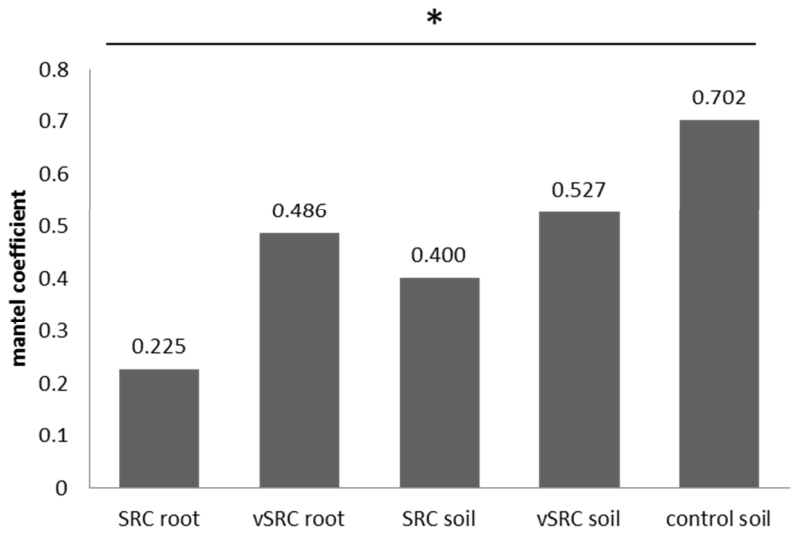


Fig. S2. Comparison of bacterial 16S rRNA and fungal ITS Bray-Curtis community dissimilarity matrices across management regimes using Mantel correlation tests. Columns indicated by a star and a horizontal line represented significant Mantel coefficients ($P<0.05$).

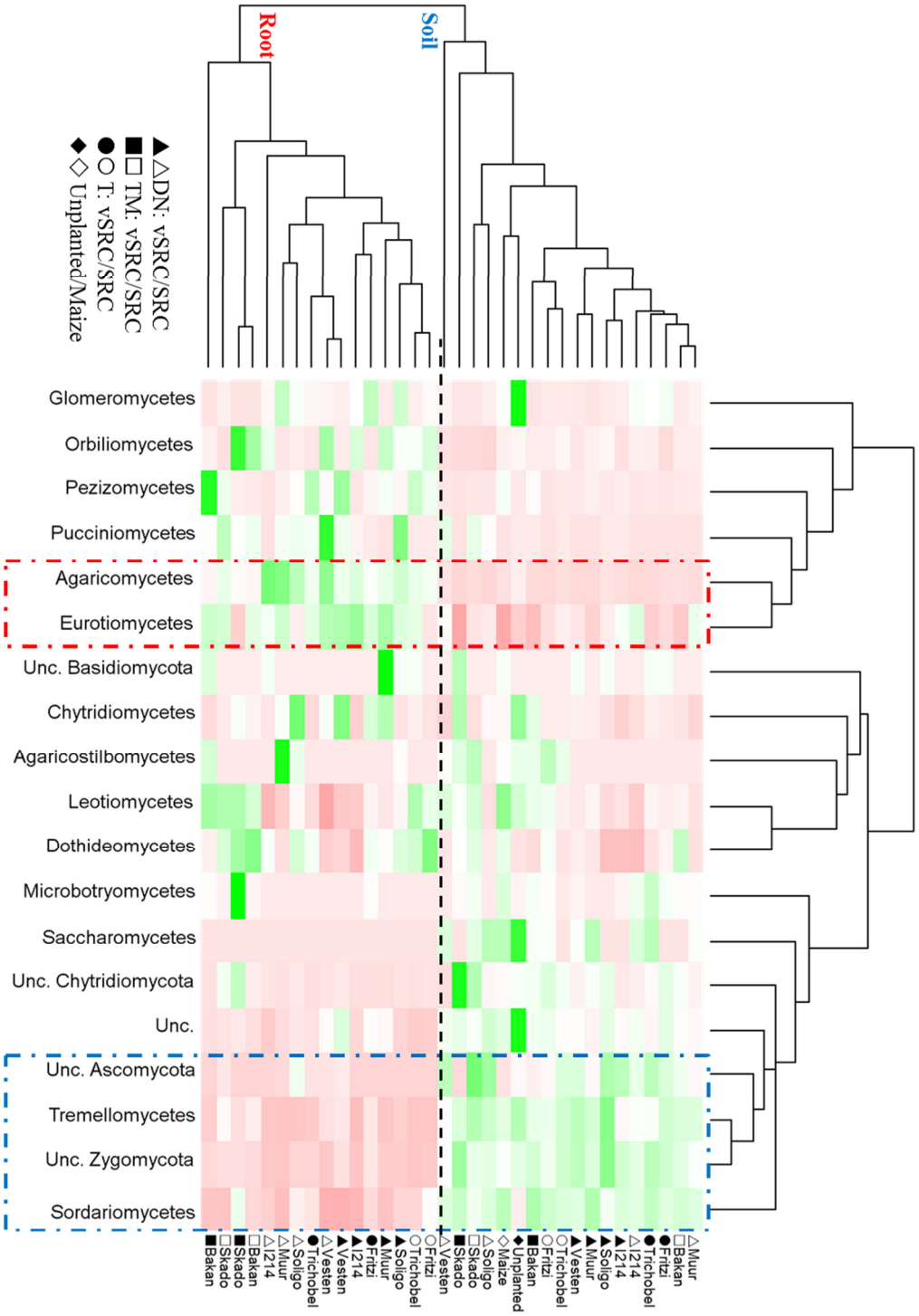


Fig. S3. Heat map and hierarchical cluster analysis based on Spearman's correlation between the relative abundances of fungal classes in soil or root samples for SRC and vSRC poplars and in unplanted and maize soils. Red, strong negative correlation; and green, strong positive correlation. Unc.= unclassified ; TM= *P. trichocarpa* x *P. maximowiczii* ; DN=*P. deltoides* x *P. nigra*; and T= *P. trichocarpa.*

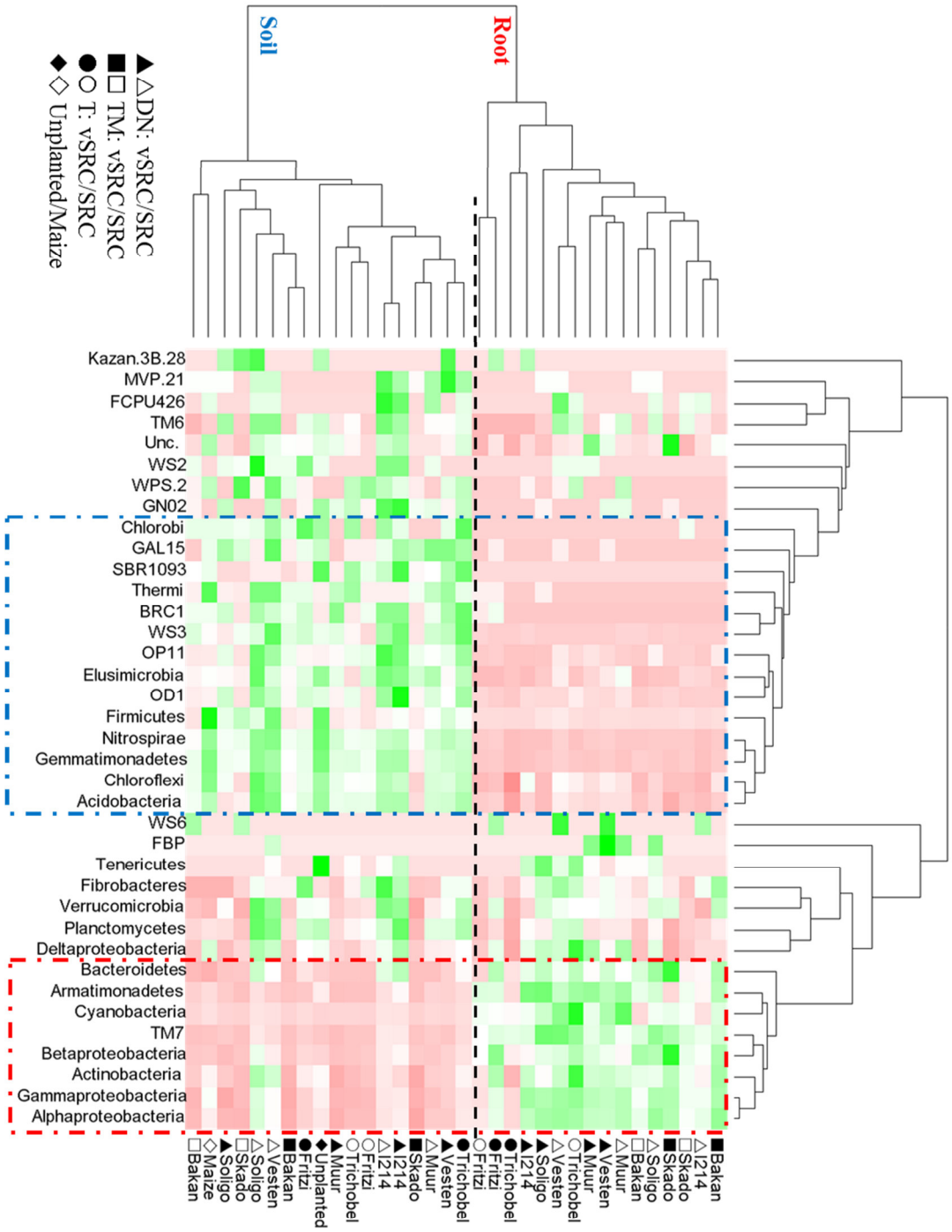


Fig. S4. Heat map and hierarchical cluster analysis based on Spearman's correlation between the relative abundances of bacterial phylum/classes in soil or root samples for SRC and vSRC poplars and in unplanted and maize soils. Red, strong negative correlation; and green, strong positive correlation. Unc.= unclassified ; TM= *P. trichocarpa* x *P. maximowiczii* ; DN=*P. deltoides* x *P. nigra*; and T= *P. trichocarpa*.

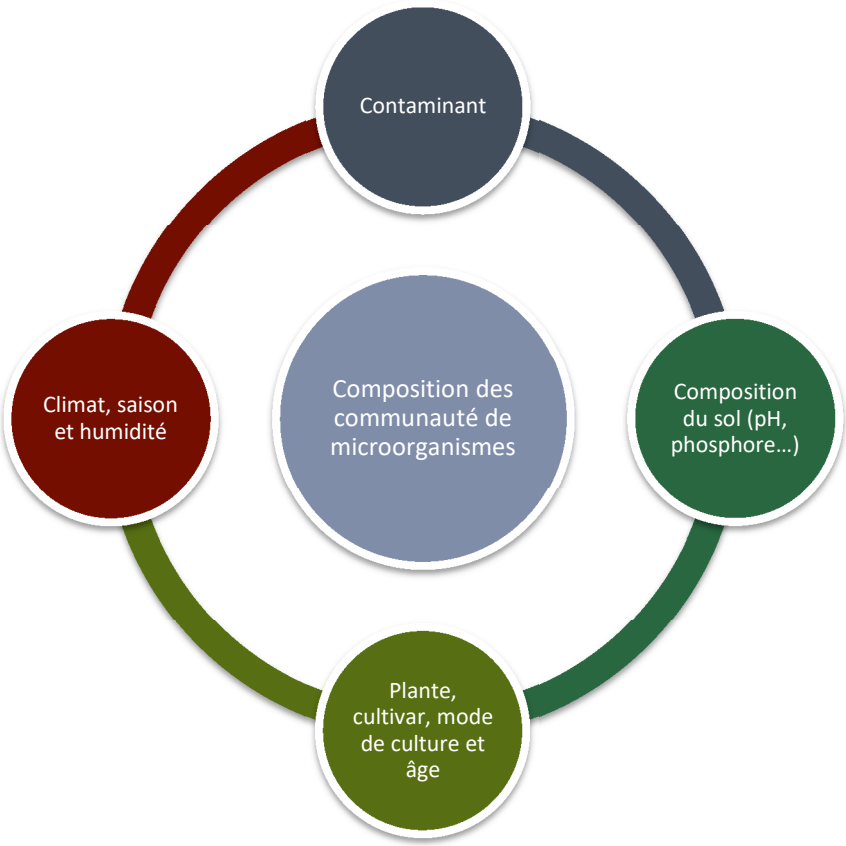


Figure 63 : Les différents facteurs impactant la composition des communautés microbiennes

3.1.4 Discussion

En conclusion, les facteurs majeurs impactant la communauté microbienne du sol sont les propriétés physico-chimiques du sol (ETMs, pH, matière organique, …), la présence d'un couvert végétal et le cultivar ligneux (Figure 63).

Cependant les communautés bactériennes et fongiques ne réagissent pas de la même manière aux variations de l'environnement. En effet, la richesse et la diversité bactériennes sont plus impactées par les paramètres du sol que celles des champignons, comme démontré dans nos trois publications. De plus, les bactéries montrent moins de variations de leur communauté que les champignons sur les sites de Leforest et Pierrelaye. Ceci a également été montré pour des cultures de saules sur des sols contaminés par du Zn (Bell *et al.*, 2015). Inversement, sur le site de Tavaux, les bactéries montrent plus de variation entre l'anthroposol et le sol forestier. Des résultats similaires ont été constatés sur des sols contenant du Hg où la communauté bactérienne semblait plus affectée que la communauté fongique (Müller *et al.*, 2001; Rieder & Frey, 2013). Les communautés microbiennes semblent donc être différemment affectées selon le polluant.

Les communautés microbiennes des racines sont très différentes de celles du sol, comme démontré sur les sites de Pierrelaye et Leforest mais également lors de plusieurs études précédentes (Gottel *et al.*, 2011; Danielsen *et al.*, 2012; Shakya *et al.*, 2013). Ceci est dû au fait que les racines constituent une niche écologique différente du sol. De plus, sur le site de Leforest un impact des racines sur le sol est observé, puisque les communautés des sols plantés sont différentes de celles du sol non planté, ce qui est surtout marqué pour les communautés fongiques. Les plants de peuplier permettent l'émergence de microorganismes spécifiques comme des symbiotes ectomycorhiziens ou des saprotrophes pouvant dégrader la matière organique (Yannikos *et al.*, 2014).

Dans nos différentes études, nous avons montré que l'origine du sol et sa composition impactaient fortement les communautés comme cela a déjà été montré dans la littérature (Buyer *et al.*, 2002; Jesus *et al.*, 2010; Azarbad *et al.*, 2013; Chodak *et al.*, 2013). L'utilisation antérieure du sol influence aussi les communautés, et la culture de peuplier semble favoriser certains champignons mycorhiziens (Gherghel *et al.*, 2014). Le site de Leforest, après avoir accueilli pendant 7 ans des peupliers, a été replanté avec différentes espèces de ligneux (projet PROLIPHYT) et il sera donc intéressant d'observer l'impact de cette culture antérieure sur les microorganismes présents car les peupliers hébergeaient une proportion importante de champignons ectomycorhiziens.

La conversion des terres agricoles en culture de peuplier est une pratique de plus en plus courante pour la production de biomasse pour l'industrie des fibres (Yannikos *et al.*, 2014). Cependant ce changement d'utilisation des sols affecte les communautés microbiennes (Suleiman *et al.*, 2013), ce que confirme nos travaux.

Le peuplier est une espèce à croissance rapide qui produit également de la biomasse pour la filière énergétique (Monclus *et al.*, 2006). Il a également une large distribution géographique, une capacité à croître sur des terres marginales (Rooney *et al.*, 2009), contaminées par des ETMs (Pulford & Watson, 2003), c'est pourquoi il est très utilisé en phytomanagement. En effet, notre étude sur le site de Pierrelaye a montré une capacité de phytostabilisation des ETMs par le peuplier, en accord avec une autre étude (Sebastiani *et al.*, 2004), mais aussi un apport de biomasse. Cependant, le peuplier est parfois déconseillé en phytostabilisation en raison de sa tendance à diminuer le pH rendant ainsi les ETMs plus disponibles (Mertens *et al.*, 2007), ce qui n'est pas le cas sur le site de Pierrelaye. Le pH a au contraire légèrement augmenté après 7 ans de populiculture. En plus de ses capacités de phytostabilisation, le peuplier apporte un couvert végétal continu qui diminue la perte des ETMs par lessivage et augmente l'apport de carbone organique (Mann & Tolbert, 2000).

Le cultivar et la densité de plantation (TCR et TTCR) de peupliers impactent la structure des communautés microbiennes et notamment celle des communautés fongiques. Dans nos études, certains champignons comme *Scleroderma* semblent être favorisés par le clone Vesten et d'autres comme *Tuber* sont majoritaires dans le mode de plantation le plus dense (TTCR). Plusieurs études ont également montré l'influence du cultivar de peuplier (Tagu *et al.*, 2001; Khasa *et al.*, 2002; Gehring *et al.*, 2006; Karliński *et al.*, 2010, 2013; Gherghel *et al.*, 2014) et de la densité de plantation (Gilbert *et al.*, 2002), mais l'impact de ses facteurs est toujours plus faible que l'impact des facteurs pédologiques.

D'autres paramètres peuvent également influencer la structure des communautés comme l'âge de la plante ou les paramètres climatiques. Ainsi, le peuplier, à l'état juvénile, est majoritairement colonisé par des champignons endomycorhiziens, puis la colonisation par des champignons ectomycorhiziens augmente avec l'âge de l'arbre (Smith & Read, 2008). En effet, la colonisation par les champignons *Basidiomycètes* et plus particulièrement les *Agaricomycètes* (qui regroupent un grand nombre de champignons ectomycorhiziens) semble être plus faible chez les jeunes peupliers puis augmente avec l'âge et décroît ensuite (Hur *et al.*, 2012).

Avec l'âge, la communauté ectomycorhizienne des racines de peupliers montre une augmentation de son taux de colonisation et de sa diversité (Dhillion, 1994; Chen *et al.*, 2000; Egerton-Warburton & Allen, 2001; Danielsen *et al.*, 2012). Un autre exemple montre une augmentation du taux de mycorhization sur un peuplier âgé de 6 ans par rapport à un peuplier âgé de 3 ans et également un changement d'abondance des champignons. *Peziza* et *Laccaria* sont dominants au niveau du peuplier de 3 ans alors que *Inocybe* et *Scleroderma* le sont sur les racines des peupliers, et cette différence pourrait être due à la densité des racines de peuplier qui augmente avec l'âge (Hrynkiewicz *et al.*, 2010).

Les paramètres climatiques ont un impact sur les communautés du sol (Shakya *et al.*, 2013) et un sol trop sec ou trop humide peut avoir un effet délétère sur les champignons mycorhiziens (Gehring *et*

al., 2006; Karliński *et al.*, 2010). La saison et le mois de prélèvement influencent les communautés, par exemple les champignons potentiellement saprophytes et antagonistes majoritairement associés aux racines de chêne au mois de mai ont laissé leur place à des champignons ectomycorhiziens au mois de septembre (Jumpponen *et al.*, 2010). En ce qui concerne les bactéries, les *Protéobactéries* dominent au printemps alors que les *Actinobactéries* dominent en automne (Shakya *et al.*, 2013).

En plus des paramètres environnementaux, les techniques liées au séquençage peuvent entrainer des biais. Les amorces utilisées pour l'amplification de l'ADN environnemental peuvent expliquer certaines variations entre les études. En effet, pour les communautés fongiques, les amorces peuvent expliquer jusqu'à 38% de variation (Tedersoo *et al.*, 2015). Pour les champignons, les auteurs recommandent donc d'utiliser les couples d'amorces amplifiant la région inter-ribosomique ITS2 (Bazzicalupo *et al.*, 2013; Tedersoo *et al.*, 2015). Pour les ARN 16S bactériens, certains couples d'amorces amplifient spécifiquement les bactéries en interaction avec les plantes, et non les chloroplastes et mitochondries qui possèdent également des séquences 16S, permettant ainsi de limiter le nombre de séquences contaminantes issues des cellules végétales au détriment des séquences bactériennes (Beckers *et al.*, 2016). C'est le cas des amorces 799F-1391R qui couvrent les régions V5-V6-V7 et qui permettent la meilleure élimination des séquences contaminantes de plantes, tout en favorisant l'amplification d'OTUs bactériennes. Le séquenceur peut également jouer un rôle et le Miseq est le séquenceur compact qui présente les taux d'erreurs les plus faibles (Loman *et al.*, 2012). L'amplification par PCR est une étape clé entrainant aussi des variations et la comparaison de trois amplifications PCR a montré que 58,5% des OTUs fongiques n'étaient pas retrouvées en se limitant à une seule réaction PCR (Schmidt *et al.*, 2013).

Enfin les interactions positives et négatives entre les microorganismes jouent un rôle clé dans la structure des communautés microbiennes, comme montré par les réseaux et les corrélations dans nos études ainsi que dans d'autres études (Jeong *et al.*, 2013; Gehring *et al.*, 2014).

Nos travaux ont également permis de mettre en évidence l'émergence de microorganismes qui pourraient être adaptés à la pollution et au site expérimental considéré. Ainsi, les bactéries du genre *Pseudomonas* (sur le site de Tavaux), *Variovorax* (sur le site de Pierrelaye) ou de la famille *Rhizobiaceae* et *Bradyrhizobiaceae* (sur les sites de Pierrelaye et Leforest) et les champignons du genre *Hebeloma* (sur le site de Tavaux), *Cortinarius, Inocybe* (sur le site de Leforest), *Laccaria* (sur le site de Pierrelaye) ou *Exophiala* (sur le site de Pierrelaye et Leforest) pourraient être des candidats pertinents pour un usage dans les filières de bioremédiation. Ils pourraient potentiellement permettre une meilleure reprise des plants après transplantation, une croissance améliorée et une résistance aux ETMs plus élevée.

Cependant les approches de métabarcoding, si elles permettent de dresser un inventaire des espèces microbiennes qui composent les communautés microbiennes, ne comportent pas d'étape d'isolement. Afin d'étudier plus précisément ces microorganismes cultivables et leur potentiel en bioremédiation, différentes campagnes d'isolement ont été réalisées, permettant ainsi de constituer un catalogue de ressources microbiennes d'intérêt. Au cours de ce travail de thèse, nous nous sommes focalisés plus particulièrement sur les espèces fongiques. Ces travaux sont présentés dans le chapitre suivant.

3.2 Production de ressources microbiennes

Le second objectif de ce travail de thèse concerne en effet la production de ressources microbiennes au travers de **l'isolement et de la caractérisation de souches fongiques résistantes aux ETMs, pouvant conférer une meilleure tolérance à leur plante hôte et une meilleure croissance**. Pour cela, des isolements de champignons issus de racines ou de carpophores provenant des sites expérimentaux ont été réalisés. Ces travaux ont fait l'objet de deux publications qui seront préalablement introduites dans la suite du mémoire par les résultats obtenus à l'issue d'une campagne de prélèvements.

1) Un premier chapitre présentera donc les résultats d'une campagne de prélèvements de racines de peuplier clone Skado qui a été réalisée en octobre 2012 sur trois sites expérimentaux : Fresnes-sur-Escaut, Tavaux et Leforest.

2) Un second chapitre sera consacré à l'étude de certains champignons issus de cette campagne, qui ont été caractérisés pour leur tolérance aux ETMs et leur capacité à améliorer la croissance des plantes.

⇒ Berthelot C., Leyval C., **Foulon J.**, Chalot M., Blaudez D., (2016) Plant growth promotion, metabolite production and metal tolerance of dark septate endophytes isolated from metal-polluted poplar phytomanagement sites. FEMS Microbiology Ecology 92 (10): fiw144. DOI: http://dx.doi.org/10.1093/femsec/fiw144.

3) Le troisième chapitre sera consacré à l'étude des communautés fongiques couplée à l'isolement et à la caractérisation de souches issues du site de Pierrelaye.

⇒ Lacercat-Didier L., Berthelot C., **Foulon J.**, Errard A., Martino E., Chalot M., Blaudez D., (2016) New mutualistic fungal endophytes isolated from poplar roots display high metal tolerance. Mycorrhiza. 26: 657–671. DOI : http://dx.doi.org/10.1007/s00572-016-0699-y.

Tableau XIII : Colonisation des racines de peuplier (clone Skado) par les champignons sur les sites de Fresnes-sur-Escaut (F), Leforest (M) et Tavaux (T).

Parcelles		F05SN	F12SI	F31SI	F32SN	M2SN	T1SI	T4SN		
Colonisation fongique										
	%ECM	67,3 ± 4,66 (ab)	55,7 ± 3,78 (bc)	74,81 ± 2,67 (a)	69,5 ± 3,4 (a)	60,78 ± 2,95 (bc)	47,52 ± 4,81 (c)	66,31 ± 5,32 (ab)		
Identification et abondance										
Classes	Genres								Total	%
Dothideomycetes	*Paramicrothyrium*				1				1	0,9
	Phoma				1				1	0,9
	Clavariopsis					1			1	0,9
	Massariosphaeria						1		1	0,9
	Leptosphaeria					2			2	1,8
	Pyrenochaeta	1						10	11	9,6
	Tumularia					1			1	0,9
	Epicoccum			1					1	0,9
	Ochroconis					1			1	0,9
Eurotiomycetes	*Exophiala*	2	1		5				8	7,0
Leotiomycetes	*Cadophora*			1					1	0,9
	Leptodontidium			1		2			3	2,6
	Meliniomyces				1	3			4	3,5
	Cistella	1		1	1				3	2,6
	Lachnum					1			1	0,9
	Geomyces					1			1	0,9
	Tetracladium			3	1				4	3,5
	Uncultured Helotiales				2				2	1,8
Saccharomycetes	*Meyerozyma*		1	2					3	2,6
Sordariomycetes	*Colletotrichum*							1	1	0,9
	Myrothecium	1			1		1	2	5	4,4
	Cylindrocarpon	4		3	4				11	9,6
	Dactylonectria	1							1	0,9
	Fusarium		1	1				1	3	2,6
	Ilyonectria	5	4	1	2				12	10,5
	Nectria						2		2	1,8
	Neonectria	2	3	3	2				10	8,8
	Acremonium						2	1	3	2,6
	Acrostalagmus	2							2	1,8
	Plectosphaerella	2						2	4	3,5
	Podospora	1							1	0,9
	Arthrinium						2		2	1,8
	Nemania						1		1	0,9
Agaricomycetes	*Hypholoma*			1					1	0,9
	Pholiota						1	1	2	1,8
Zygomycota	*Ramicandelaber*	1				1			2	1,8
	Mortierella				1				1	0,9
Total		23	10	18	22	13	10	18	114	
Endophyte?		2	1	5	10	6	0	0	24	21,1

F5SN= Fresnes-sur-Escaut Parcelle 5 peuplier Skado non inoculé ; **F12SI**= Fresnes-sur-Escaut Parcelle 12 peuplier Skado inoculé ; **F31SI**= Fresnes-sur-Escaut Parcelle 31 peuplier Skado inoculé ; **F32SN**= Fresnes-sur-Escaut Parcelle 32 peuplier Skado non inoculé ; **M2SN**= Leforest (usine MetalEurop) Bloc II peuplier Skado non inoculé ; **T1SI=** Tavaux parcelle 1 peuplier Skado inoculé et **T4SN**= Tavaux parcelle 4 peuplier Skado non inoculé

3.2.1 Isolement et identification de souches fongiques issues de sols pollués

3.2.1.1 Isolement à partir de fragments racinaires

En octobre 2012, des prélèvements de racines de peuplier clone Skado ont été réalisés sur les sites de Fresnes-sur-Escaut, Tavaux et Leforest, comme décrit au chapitre 0. A partir de ces racines, des isolements de souches fongiques ont été effectués (§2.2.5.1) ainsi que l'évaluation du taux d'ectomycorhization (§2.2.4).

De ces prélèvements, 660 fragments de racines mycorhizées ont été mis en culture et l'isolement a permis d'obtenir 114 isolats (17,3% des fragments totaux de racines), qui ont été identifiés par des outils moléculaires (Tableau XIII). Parmi ces champignons, 24 isolats étaient des champignons endophytes hypothétiques (3,6% des fragments totaux de racines ou 21,1% des champignons identifiés). Afin de confirmer l'identification des champignons, un arbre phylogénétique a été réalisé (Figure 64).

Le taux d'ectomycorhization des racines de peuplier a montré une forte colonisation mais aucun champignon ectomycorhizien n'a pu être isolé des différents sites (Tableau XIII). Les deux morphotypes ectomycorhiziens les plus fréquemment retrouvés sur les échantillons racinaires ont été identifiés par des outils moléculaires. Ils appartiennent aux genres *Peziza* et *Geopora*. Dans cette campagne d'échantillonnage, de nombreux champignons pathogènes de la famille *Nectriaceae* (*Cylindrocarpon, Dactylonectria, Fusarium, Ilyonectria*, *Nectria* et *Neonectria*) représentent plus de 30% des champignons isolés (Tableau XIII). En effet, les méthodes basées sur la culture favorisent les champignons à croissance rapide, les saprophytes, les endophytes et les biotrophes. Les biotrophes mutualistes tels que les espèces ectomycorhiziennes sont pour cette raison plus difficiles à isoler (Bonito *et al.*, 2016) car ils ont généralement une croissance plus lente. Les racines ayant été stérilisées, la forte proportion de saprophytes, endophytes et biotrophes pourrait montrer que les ectomycorhizes du peuplier contiennent un certain nombre de taxa étroitement liés avec de larges fonctions écologiques comme c'est le cas pour les agrégats d'*Hymenoscyphus ericae* (Vrålstad *et al.*, 2002b).

Sur le site de Tavaux, aucun champignon ectomycorhizien ou endophytique n'a pu être isolé mais le pourcentage d'ectomycorhization montre des différences significatives entre les deux parcelles (Tableau XIII). A l'inverse, le site de Leforest présente la plus grande proportion de champignons endophytes par rapport au nombre total de champignons isolés (6 champignons endophytes sur 13 soit 46,2% des champignons isolés sur le site Leforest). Cette forte proportion peut s'expliquer par l'absence de *Sordariomycètes* isolés qui seraient en plus faible nombre sur ce site. Sur le site de Leforest, les peupliers plantés en 2007 dans le cadre du projet PHYTOPOP étaient âgés de 5 ans lors du prélèvement contrairement aux deux autres sites où les peupliers plantés dans le cadre du projet

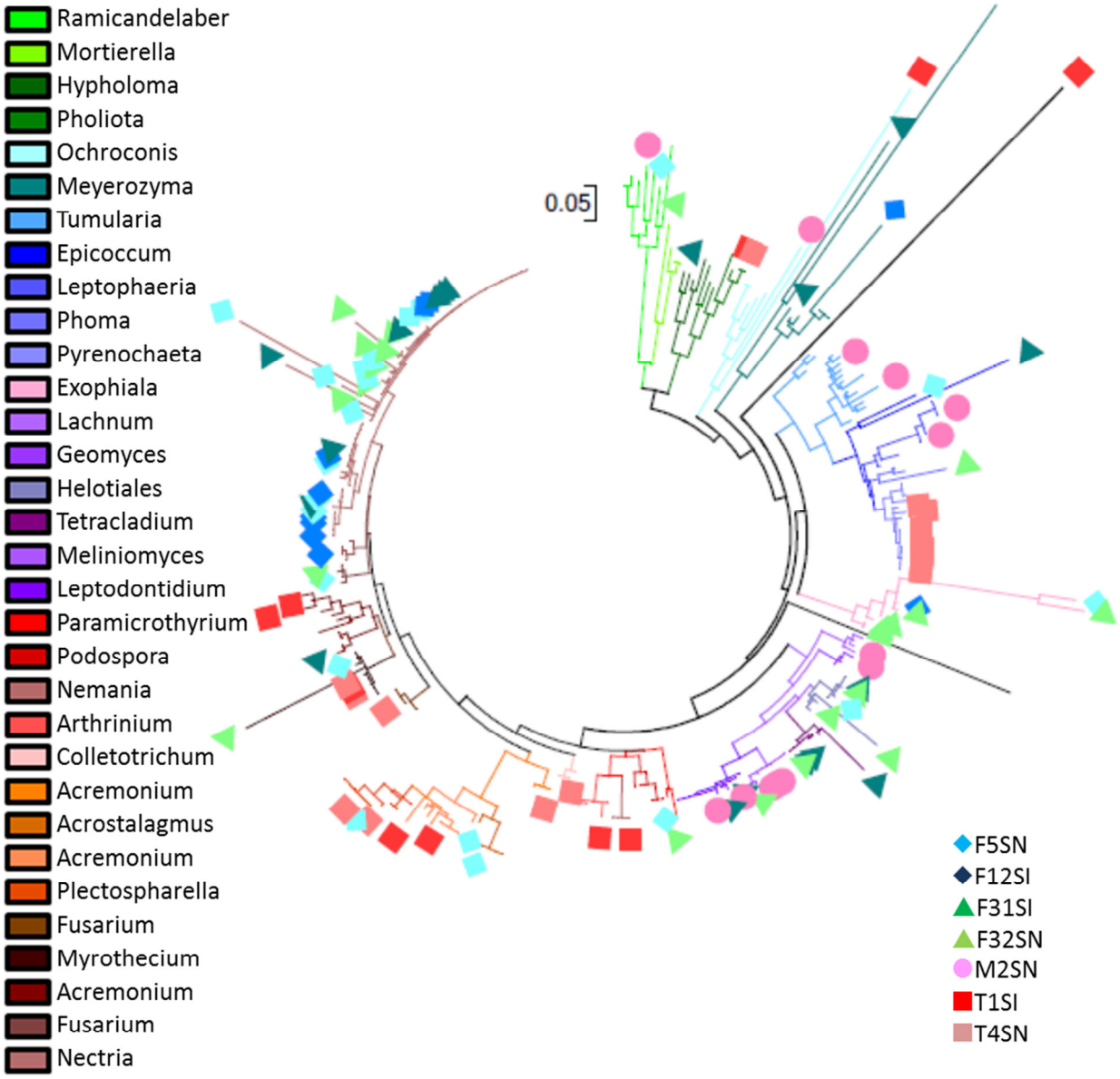


Figure 64 : Arbre phylogénétique basée sur les séquences ITS des souches fongiques isolées sur les sites de Fresnes-sur-Escaut (F), Leforest (M) et Tavaux (T).

Analyse phylogénétique réalisé en utilisant la méthode du maximum de vraisemblance basé sur les paramètres du modèle Kimura 2 (Kimura, 1980). L'arbre a été obtenu en appliquant l'algorithme de « Neighbor-Joining » à une matrice de distances appariées estimées en utilisant l'approche de maximum de vraisemblance. L'analyse, constituée de 292 séquences de nucléotides, a été menée en utilisant MEGA7 (Kumar *et al.*, 2016).

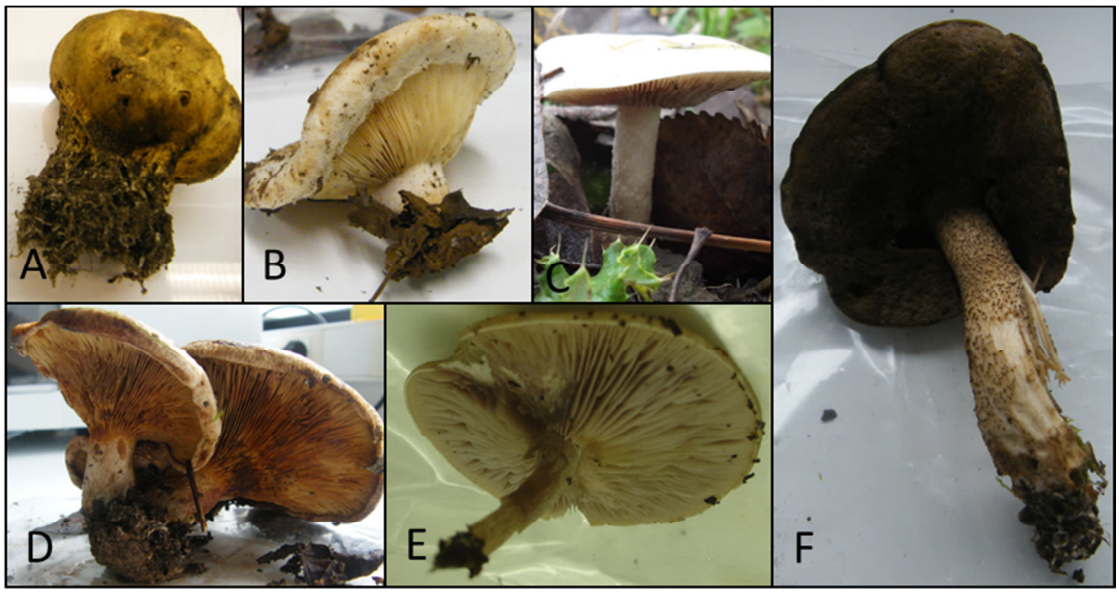
BIOFILTREE avaient 2 ans. L'âge du peuplier pourrait donc expliquer le plus faible nombre de champignons pathogènes sur le site de Leforest. Il semble donc que les isolements soient plus efficaces sur des sites où les ligneux sont plus âgés. En effet, les parcelles de PHYTOPOP sont plus propices à l'apparition de carpophores que celles de BIOFILTREE. Cette observation est nettement évidente sur le site de Pierrelaye où les deux plantations sont directement juxtaposées. Le site de Fresnes-sur-Escaut a aussi permis l'isolement d'un grand nombre de champignons endophytes, surtout sur deux de ses parcelles (10 isolats sur la parcelle F32SN et 5 isolats sur la parcelle F31SI). Ces deux parcelles à l'extrémité du dispositif sont plus faiblement contaminées que les parcelles 5 et 12 et présentent les pourcentages d'ectomycorhization les plus élevés (69% sur F31SI et 75% sur F32SN). Le niveau de contamination plus faible semble favoriser certains champignons endophytes et augmenter la colonisation ectomycorhizienne.

L'isolement à partir de fragments racinaires a permis d'obtenir des champignons appartenant principalement aux *Ascomycètes* (Tableau XIII ; Figure 64), en particulier aux *Sordariomycètes* (*Nectriaceae* ou *Myrothecium*), *Leotiomycètes* à Leforest et Fresnes-sur-Escaut (avec des champignons endophytes *Cadophora, Lachnum, Leptodontidium* ou *Tetracladium*) et *Eurotiomycètes* à Fresnes-sur-Escaut (*Exophiala*). Les *Dothideomycètes* dominent à Tavaux avec les champignons pathogènes *Pyrenochaeta* surtout sur la parcelle 4. Seuls trois *Basidiomycètes* ont été isolés: un *Hypholoma* (sur le site Leforest) et deux *Pholiota* (site de Tavaux) qui sont tous deux des champignons saprophytes.

Il est difficile de déterminer si ces résultats sont dus à un biais méthodologique ou d'autres facteurs comme le sol, l'environnement ou l'échantillon. Les agents phytopathogènes comme *Fusarium, Ilyonectria*, et d'autres espèces fongiques appartenant aux *Nectriaceae* et *Pleosporaceae* ont fréquemment été isolés dans d'autres études (Baum & Hrynkiewicz, 2006; Higgins *et al.*, 2011; Tan *et al.*, 2012; Bonito *et al.*, 2016). *Leptodontidium* et *Meliniomyces* ont également été isolés à partir des racines de peuplier (Bonito *et al.*, 2016).

Les endophytes sont des champignons qui vivent à l'intérieur des tissus de plantes tout en restant asymptomatiques pendant au moins une partie de leur cycle de vie (Wilson, 1995). Ainsi, les endophytes, qui colonisent les racines, englobent un large éventail de champignons, y compris des agents pathogènes latents et des saprophytes dormants comme *Fusarium sp.* ou *Pyrenochaeta sp*.

Les conditions de culture et les vitesses de croissance étant différentes entre les champignons, quatre milieux de culture ont été utilisés pour ces isolements : N2P2, Malt, MMN et Pachlewski. Par exemple, les *Basidiomycètes* ont été isolés sur les milieux N2P2 pour *Hypholoma* et Pachlewski pour *Pholiota.* Les endophytes se sont développés sur tous les milieux de culture mais de préférence sur le milieu N2P2 (9 sur 24 endophytes). Pour les prochains prélèvements, l'efficacité d'isolement pourrait être accrue en utilisant un milieu de culture supplémenté en ETMs à des concentrations similaires au site expérimental d'origine.



	Pierrelaye	Thann	Fresnes-sur-Escaut	Tavaux
***Scleroderma* (A)**	2 (2013)			
***Lactarius* (B)**		5 (2014)		
***Hebeloma* (C)**			3 (2012)	
***Paxillus* (D)**	1 (2013)	2 (2014)		
***Tricholoma* (E)**				1 (2013)
***Boletus* (F)**		2 (2014)		

Figure 65 : Carpophores collectés sur les différents sites expérimentaux.

Aucun champignon ectomycorhizien n'a pu être isolé à partir de fragments racinaires, en dépit de l'abondance de certains genres (comme par exemple *Hebeloma, Inocybe, Laccaria, Tomentella, Tuber*, etc.) mise en évidence par les outils moléculaires (§2.2.2), notamment à Leforest. Pour pallier à cette limite de la méthode d'isolement à partir de fragments racinaires, nous avons échantillonné et mis en culture des carpophores collectés sur les différents sites.

3.2.1.2 Isolement à partir de carpophores

Entre 2012 et 2014, des carpophores ont été prélevés sur les sites de Pierrelaye, Thann, Fresnes-sur-Escaut et Tavaux et parmi eux 16 isolats ont pu être obtenus. Ces champignons mycorhiziens ont été identifiés par des outils moléculaires et appartenant aux genres *Hebeloma, Paxillus, Scleroderma, Boletus, Tricholoma* et *Lactarius* (Figure 65).

L'isolement de souches ectomycorhiziennes à partir de carpophores est une méthode aisée et rapide à mettre en œuvre. En général, la zone de prélèvement privilégiée se situe dans la partie interne du chapeau, car elle est moins exposée à l'environnement (particules du sol) et souvent exempte de contaminants (bactériens ou fongiques). Cependant, les espèces fongiques produisant des carpophores de petite taille (< à 5 cm de haut) ou à chapeau peu épais sont souvent plus difficiles à isoler car ils contiennent peu de zones « axéniques ». De plus, si le fragment prélevé est trop petit le mycélium ne se développe pas. Par exemple, de nombreux carpophores du genre *Hebeloma* ont été retrouvés sur les sites de Leforest, Tavaux ou Thann mais des souches n'ont pas pu être isolées en culture pure. Des carpophores plus volumineux tels que ceux de *P. involutus* ou de *Boletus edulis* sont plus faciles à isoler mais selon leur état (vieillissants, véreux ou contenant des insectes) les chances d'avoir du mycélium pur peuvent aussi s'amoindrir.

Les conditions climatiques et la période de l'année influencent beaucoup le développement des carpophores, il n'est donc pas toujours aisé d'en récolter (Egli, 2011). Enfin, de nombreuses espèces de champignons ectomycorhiziens appartenant aux *Ascomycètes* ne produisent pas de fructifications. Ainsi, la présence de carpophores ne reflètent que partiellement les communautés fongiques des espèces de champignons mycorhiziens associés aux racines des arbres (Guidot *et al.*, 2001; Yamada & Katsuya, 2001).

En conclusion, nous avons pu isoler avec succès 24 souches endophytiques et 16 souches ectomycorhiziennes provenant de sites contaminés par des ETMs. Ces isolats fongiques endophytes et ectomycorhiziens pourraient être des candidats prometteurs pour la revégétalisation d'environnements contaminés. Cependant, des expériences complémentaires sont nécessaires pour caractériser la tolérance aux ETMs et prouver l'intérêt de ces souches dans des expériences de ré-inoculation. C'est précisément l'objet des deux chapitres suivants.

3.2.2 Promotion de la croissance des plantes, production de métabolites et tolérance vis à vis des ETMs des DSEs isolés de sols pollués

De nombreuses études portent sur la distribution et la diversité des « Dark Septate Endophytes » (DSEs) dans la littérature, mais peu d'informations sont disponibles concernant leur rôle écologique et leurs effets sur les plantes hôtes, en particulier dans les sols pollués par les ETMs. Sept souches de type DSEs appartenant à *Cadophora, Leptodontidium, Phialophora* et *Phialocephala* ont été isolées à partir des racines de peuplier collectées sur des sites pollués par les ETMs. Toutes les souches se sont développées en présence d'une large gamme de composés carbonés, y compris en présence de composés constituant la paroi cellulaire végétale. Les souches ont colonisé uniformément les racines de bouleaux, d'eucalyptus et de ray-grass dans les expériences de re-inoculation. Une amélioration de la croissance des racines et des tiges a été observée et était à la fois dépendante de la plante et de la souche utilisée. Deux souches de *Phialophora* et de *Leptodontidium* ont particulièrement amélioré la croissance des plantes. Cependant, nous n'avons pas mis en évidence de corrélation entre le niveau de colonisation des racines par les DSEs et l'intensité de stimulation de croissance. Toutes les souches produisent de l'auxine et six souches ont également stimulé la croissance des plants de tabac par la libération de composés organiques volatiles (COVs). Des analyses par SPME-GC/MS ont révélé quatre principaux COVs émis par *Cadophora* et *Leptodontidium*. Les souches ont montré une croissance à des concentrations élevées pour plusieurs ETMs. La capacité des souches DSEs résistantes aux ETMs à produire à la fois des composés solubles et volatiles pour la promotion de la croissance des plantes démontre l'intérêt de cette ressource microbienne à fort potentiel pour soutenir la production durable des cultures bioénergétiques dans le contexte de phytomanagement des sites contaminés par les ETMs.

- ***Pour ce travail, j'ai réalisé l'échantillonnage sur le terrain (Pierrelaye, Fresnes-sur-Escaut et Leforest), l'isolement, l'identification et la caractérisation préliminaire des souches (extractions d'ADNs et séquençage, tests de croissance), et contribué à la constitution et à l'entretien de la mycothèque.***

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Research Article

RESEARCH ARTICLE

Plant growth promotion, metabolite production and metal tolerance of dark septate endophytes isolated from metal-polluted poplar phytomanagement sites

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One sentence summary: Metal-tolerant dark septate endophytic fungi can use complex carbon sources and promote plant growth through the release of auxin and volatile organic compounds.

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ABSTRACT

Numerous studies address the distribution and the diversity of dark septate endophytes (DSEs) in the literature, but little is known about their ecological role and their effect on host plants, especially in metal-polluted soils. Seven DSE strains belonging to *Cadophora*, *Leptodontidium*, *Phialophora* and *Phialocephala* were isolated from roots of poplar trees from metal-polluted sites. All strains developed on a wide range of carbohydrates, including cell-wall-related compounds. The strains evenly colonized birch, eucalyptus and ryegrass roots in re-synthesis experiments. Root and shoot growth promotion was observed and was both plant and strain dependent. Two *Phialophora* and *Leptodontidium* strains particularly improved plant growth. However, there was no correlation between the level of root colonization by DSEs and the intensity of growth promotion. All strains produced auxin and six also stimulated plant growth through the release of volatile organic compounds (VOCs). SPME-GC/MS analyses revealed four major VOCs emitted by *Cadophora* and *Leptodontidium*. The strains exhibited growth at high concentrations of several metals. The ability of metal-resistant DSE strains to produce both soluble and volatile compounds for plant growth promotion indicates interesting microbial resources with high potential to support sustainable production of bioenergy crops within the context of the phytomanagement of metal-contaminated sites.

Keywords: dark septate endophytes; plant growth promotion; metal tolerance; auxin; volatile organic compounds

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INTRODUCTION

Dark septate endophytes (DSEs) are polyphyletic ascomycetes mainly characterized by brown to blackish mycelium. Due to their singular morphology, they were initially described as 'mycelium radicis atrovirens' or 'pseudomycorrhizal' fungi (Melin 1921), and later introduced as fungi with sterile and dark septate—DS hyphae—that occur in roots of various plants (Read and Haselwandter 1981).

DSEs are widespread: they have been observed in more than 600 plant species spanning 100 plant families. They colonize both herbaceous and woody plants, and more particularly roots of metal hyperaccumulators, halophytes, orchids, or marine macrophytes (Mandyam and Jumpponen 2005). They occur in diverse habitats such as arid, arctic, boreal, alpine, temperate forest or tropical ecosystems (Jumpponen and Trappe 1998a; Mandyam and Jumpponen 2005).

Pathogenic or beneficial interactions between DSEs and host plants have been discussed in a number of studies (Mandyam and Jumpponen 2005; Newsham 2011; Mayerhofer, Kernaghan and Harper 2013). These studies concluded that responses to DSEs were variable and depended on the host-symbiont combination. Several studies suggest that DSEs produce a variety of extracellular enzymes that break down complex forms of carbon, nitrogen and phosphorus commonly present in soils, and thus potentially provide originally unavailable nutrients for plants (Jumpponen and Trappe 1998a; Jumpponen, Trappe and Mattson 1998; Caldwell, Jumpponen and Trappe 2000; Usuki and Narisawa 2007; Upson, Read and Newsham 2009; Mandyam, Loughin and Jumpponen 2010).

DSEs have received much attention in the recent years because they colonize plants growing in stressed environments such as elevated CO_2, poor nutrient and high salinity (Alberton, Kuyper and Summerbell 2009; Sonjak *et al.* 2009). Moreover, abundant and diverse DSEs have been found associated to the roots of a large range of plant species growing on metal-contaminated soils (Vrålstad, Schumacher and Taylor 2002; Ruotsalainen, Markkola and Kozlov 2007; Regvar *et al.* 2010; Deram, Languereau and Haluwyn 2011; Zhang, Li and Zhao 2013). Interestingly, mycorrhizal colonization by *Salix caprea* decreased under metal pollution, whereas DSE colonization was not affected (Likar and Regvar 2009). Similarly, mycorrhizal colonization of herbaceous species drastically decreased in highly polluted soils, whereas infection by DSEs remained unchanged (Ruotsalainen, Markkola and Kozlov 2007; Deram, Languereau and Haluwyn 2011). Despite these numerous indications about the worldwide distribution and diversity of DSEs in the literature, little is known about their ecological role and their effect on host plants in polluted soils.

DSEs could be considered for the phytomanagement of metal-contaminated soils by trees and their associated microorganisms. For that purpose, there is a need to take into account (i) the wide range of plants potentially colonized by DSEs, especially trees with high biomass productivity, (ii) their growth promotion capabilities and (iii) their tolerance to metal pollution. As compared to mycorrhizal fungi or clavicipitaceous endophytes, only a limited number of DSEs have been characterized. Among the few studies about the mechanisms involved in plant growth promotion and tolerance to stress, very few address DSEs. Thus, there is a need to better characterize the diversity, metabolism and interactions of DSEs with host plants to investigate their potential use in phytomanagement studies.

In the present work, we first isolated fungal endophytic strains including non-DSEs (Lacercat-Didier *et al.* 2016), and DSEs from poplar trees grown on different phytomanagement plots of metal-contaminated sites. Here, based on their taxonomy, we focused our work on DSE *sensus stricto* (Jumpponen and Trappe 1998a) belonging to four genera (*Phialophora*, *Cadophora*, *Leptodontidium* and *Phialocephala*) of the Helotiales. The main objectives of this study were (i) to study their ability to metabolize various carbon sources, and to grow in the presence of different metals, (ii) to investigate their ability to colonize different plant species, (iii) to demonstrate the production of plant-growth-promoting (PGP) metabolites such as auxin and volatile organic compounds (VOCs) and (iv) to determine their overall impact on plant growth in mesocosm experiments.

MATERIALS AND METHODS

Isolation and culture of root-colonizing DSE fungi

Root samples were collected from poplar trees growing in three metal-polluted sites in France: Fresnes-sur-Escaut (Nord; N 50°25′46″ E 3°35′4″), MetalEurop (Pas-de-Calais; N 50°26′17″ E 3°03′54″) and Pierrelaye (Val d'Oise; N 49°1′21″ E 2°9′1″). Sediments from the dredging of the Escaut River have been dumped at the Fresnes-sur-Escaut site for several decades. The major pollutants of this site are Cd, Pb and Zn (1.0, 57 and 191 mg of total element per kilogram of sediment, respectively, pH 7.8). The MetalEurop site is a former agricultural area that was strongly contaminated by Cd, Pb and Zn (3.1, 176 and 294 mg of total element per kilogram of soil, respectively, pH 6.5) from atmospheric particles generated by the activity of the MetalEurop smelter (Sterckeman *et al.* 2002). The experimental site of Pierrelaye is an agricultural plain of ca 1200 ha that was irrigated for almost one century by the non-treated waste waters from Paris, hence contamination of top soils by Cd, Pb and Zn (1.5, 139 and 318 mg of total element per kilogram of soil, respectively, pH 7.4) (Lamy *et al.* 2006).

Roots were first washed with tap water, surface-sterilized by immersion in hydrogen peroxide (35% v/v) for 30 s and finally rinsed three times with sterile distilled water. Root segments (5 mm) were placed on malt extract agar (MEA, malt extract: 12 g l^{-1}; agar: 15 g l^{-1}, pH 5.5) at 24°C in the dark. Ampicillin (100 μg ml^{-1}) and chloramphenicol (100 μg ml^{-1}) were also added to the medium to avoid bacterial contamination. After 2 weeks of growth, mycelium growing out of the roots was isolated and subcultured in new MEA plates.

Two other endophytic strains from unpolluted soils (*Piriformospora indica DSM 11827* and *Leptodontidium* sp. PMI_412) were also included in our study. They were supplied by P. Franken (Humboldt University of Berlin, Germany) and by G. Bonito (Michigan State University, USA), respectively.

Molecular identification of DSEs

Fungal DNA was extracted using a REDextract-N-AmpTM Plant PCR kit (Sigma-Aldrich, Saint-Quentin Fallavier, France) according to the manufacturer's protocol. The internal transcribed spacer (ITS) region was amplified using the primers ITS1 and ITS4 (White *et al.* 1990). Twenty microliters of a mixture containing 0.2 μM of each primer, 4 μl of genomic DNA, 5.2 μl of water and 10 μl of REDExtract-N-Amp PCR ready mix were used for PCR amplification. The following PCR program was used: 3 min denaturation at 94°C, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and 10 min final extension at 72°C. PCR products were cloned into the pGEM-T® vector system I (Promega, Charbonnières-les-Bains, France), and the corresponding plasmids were sequenced. The ITS sequences were used to retrieve similar sequences from the GenBank

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using the NCBI BLAST program. Sequence alignment was carried out using Clustal X. Bootstrap analysis was performed with 1000 replications. The maximum-likelihood (ML) tree was drawn with the kimura 2-parameter model, and the neighbor-joining (NJ) tree was drawn with the p differences model and gamma for rate heterogeneity using MEGA 6 (Tamura *et al.* 2013).

Metal tolerance tests

The sensitivity of the strains to different metals was evaluated by investigating the minimum inhibitory concentration (MIC) of As, Cd, Mn and Zn on fungal growth. Pachlewski medium (Pachlewski 1974) was selected as the growth medium to have maximal metal availability and avoid metal precipitation. Plugs (4 mm) were cut from the edge of actively growing 2-week-old colonies and placed on solid Pachlewski medium (pH 5.5) amended with metals at the following concentrations: 0–12 mM $ZnCl_2$ and $MnCl_2$, 0–30 mM Na_2HAsO_4 and 0–1.5 mM $CdCl_2$. Growth was observed after 2 weeks of incubation at 24°C.

Growth of DSE isolates on different carbon sources

The hydrolytic capacities of the fungi were determined in modified Pachlewski medium (NH_4Cl 1.3 g l^{-1}; KH_2PO_4 1 g l^{-1}; $MgSO_4$, $7H_2O$ 0.5 g l^{-1}; thiamine hydrochloride 10 μg l^{-1}; pH 5.5) supplemented with the appropriate target substrates as the sole carbon source (8 g l^{-1} C). The monosaccharides glucose, fructose, xylose and mannitol, the disaccharides sucrose, maltose and cellobiose, and the polymers lignin, cellulose, starch and xylan were tested. Triplicate flasks were shaken at 140 rpm and incubated for 2 weeks at 24°C. Mycelia were harvested and their dry biomass was determined. Data were expressed relatively to growth values on glucose. To determine cellulase activity, fungal plugs (4 mm) were placed on minimal medium containing 5 g l^{-1} of carboxymethyl-cellulose (CMC) (Sigma-Aldrich) as the sole carbon source. After 2 weeks, the area of CMC hydrolysis was evaluated by flooding the plate with 3 ml of Congo red solution (0.1% w/v) for 5 min. Plates were subsequently washed three times with 15 ml of phosphate buffer (50 mM phosphate, 1 M NaCl; pH 7.0). Cellulase activity was estimated by calculating the 'diameter of the hydrolytic zone' over 'diameter of the colony' ratio.

Determination of auxin secretion by DSEs

To estimate the ability of the strains to produce auxin (IAA), seven mycelial plugs (4 mm) were first grown in 50 ml of complete minimal medium (CM) (Hilbert *et al.* 2012). After 1 week of growth, the medium was supplemented with 2.5-mM tryptophan or not for 3 extra days, as previously described (Waqas *et al.* 2012). IAA concentrations were determined following the method of Salkowski (1885). Briefly, 3 ml of the culture were centrifuged, and 1-ml aliquots were mixed with 3 ml of Salkowski reagent (12 g $FeCl_3$ in 7.9 M H_2SO_4). The mixture was incubated for 20 min in the dark, and then a colorimetric assay was performed at 540 nm. A standard curve was generated with commercial IAA (Sigma-Aldrich) in CM minimal medium with or without tryptophan. The experiment was repeated with three independent cultures.

Emission of VOCs by DSEs and SPME-GC/MS analyses

To study the emission of VOCs by DSEs, a *Nicotiana tabacum* assay was used according to a previously described test (Paul and Park 2013). *Nicotiana tabacum* seeds were surface-sterilized for 4 h under a gaseous atmosphere produced by mixing NaClO 9% (w/v) and HCl 12% (v/v). After 1 week of growth on water agar (1%), tobacco seedlings were placed on MS agar (Murashige and Skoog 1962) amended with 3% sucrose in 24-well plates (one seedling per well). Twelve wells on the longer side of the plate were dedicated to the culture of the DSE strains. These wells were filled with 1 ml of MEA medium and inoculated with 4-mm-diameter fungal plugs. Plates were prepared in triplicate per fungal strain. Controls consisted of plates inoculated with fungus-free MEA plugs. The plates were hermetically sealed and placed in a growth chamber with a photoperiod of 14 h light (660 μmol m^{-2} s^{-1}) and 10 h dark, and a temperature of 23°C/18°C (day/night). After 1 month of growth, shoot fresh weights were measured.

Twenty-milliliter glass vials filled with 7 ml of solid MEA medium were used to study VOC production by two selected DSE strains (Pr30 and Fe06). Each vial was inoculated with one 8-mm fungal plug, whereas control vials were inoculated with fungus-free MEA plugs. After inoculation, each vial was hermetically sealed and incubated for 1 month at 24°C in the dark. Each treatment was performed in six biological replicates. The volatile metabolites were extracted by headspace SPME using a 100-μM polydimethylsiloxane fiber (SUPELCO) for 15 min at 25°C, followed by 2 min desorption in the GC injector. GC/MS analyses were performed with an Agilent 6890 GC Plus coupled to a quadrupole mass spectrometer 5973 MSD (Agilent, Santa Clara, USA), and equipped with an HP-5 MS capillary column (30 m × 250 μm i.d., 0.5-μm thick film). Working conditions were as follows: injector at 250°C and transfer line to MS system at 250°C. The oven temperature was programmed as follows: initial temperature at 38°C for 4 min, then from 38°C to 100°C at a rate of 7°C min^{-1}, from 100°C to 200°C at a rate of 23°C min^{-1} and 200°C for 2 min. The carrier gas was He at 1.0 ml min^{-1}. Injection was performed in splitless mode and scan mode. Electron impact ionization was set to 70 eV, and the acquisition parameters (scanned *m/z*) were 50/300 with a threshold of 150. The fungal metabolites were identified by comparing their mass spectra and Kováts retention indices with the US National Institute of Standards and Technology (NIST) Mass Spectral Library.

Plant growth promotion experiment

Fungal inocula were prepared by aseptically growing DSE strains in flasks containing 500 ml of perlite moistened with 250 ml of MEA medium, according to Likar and Regvar (2013). The flasks were incubated at 24°C in the dark for 2 weeks. They were shaken manually twice a week to allow for homogenous fungal growth. Controls contained fungus-free perlite. *Betula pendula*, *Eucalyptus globulus* and *Lolium perenne* were used as model plants. Seeds were surface-sterilized (5 min in 70% ethanol; 20 min in 5% NaClO) and carefully rinsed five times with sterile distilled water. Birch and eucalyptus seedlings were initially aseptically pregrown in sterilized perlite for 2 weeks, whereas ryegrass seeds were directly sown in pots. Plants were grown in a sterilized mixture of 90%/10% (v/v) perlite/fungal inoculum for 2 weeks for ryegrass and 8 weeks for birch and eucalyptus. Plants were irrigated alternatively with water and half-strength Ingestad solution. Fourteen independent replicates were performed for each plant/fungus combination. Plants were placed in a growth chamber with a 16/8 h photoperiod (260 μmol m^{-2} s^{-1}) and a temperature of 23°C/18°C (day/night). At the end of the experiment, the plants were harvested, and roots were analyzed for

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fungal colonization. Finally shoots and roots were dried at 50°C for 2 days and weighed.

Microscopic observation of root colonization

Root colonization by the DSE strains was assessed by using two protocols. Sub-samples of root systems were cleared in 5% KOH at 90°C for 15 min, neutralized in 1% HCl at 20°C overnight and stained with trypan blue. The frequency of root colonization (F%), the intensity of root colonization by intraradical hyphae (H%) and intracellular microsclerotia (MS%) were determined under a microscope by examining 30 1-cm long root fragments per sample, according to Trouvelot, Kough and Gianinazzi-Pearson (1986) and Likar and Regvar (2013).

Roots were also analyzed by confocal laser microscopy. Selected root segments were first cut into pieces of ∼5–10 mm in length and were labeled with 5 μg ml^{-1} WGA-AF®488 (Invitrogen, Cergy pontoise, France) for 10 min to visualize fungal structures. The autofluorescence of root cell walls was detected between 420 and 470 nm after UV laser irradiation at 405 nm. An argon laser was used to excite Alexa Fluor® 488, and fluorescence was detected between 500 and 550 nm. Image stacks were obtained using a Nikon eclipse TE2000U equipped with a confocal scanning head (AGR-3Q-BLD, Rainbow, Radiance Biorad) and a Plan APO x20/0.75 objective. All samples were scanned at an x/y scanning resolution of 512 × 512 pixels; the z direction step size was 2.2 μm. The image stacks were visualized and processed using the software program Image J (v.1.43u). The two detection channels of plant and fungal cell fluorescence were assigned different colors using RGB color tables to discriminate between the two organisms. Mycelia from 2-week-old pure cultures were also stained with WGA-AF488(R) and similarly studied by fluorescence microscopy.

Statistical analyses

Statistical analyses were performed with R 3.1.3 software (R Core Team 2013). Normality of the data was tested using a Shapiro–Wilk test. Data were then analyzed using one-way analysis of variance (ANOVA) or a one-way non-parametric Kruskal–Wallis test with an alpha level of 0.05. The effect of plants, strains and the interaction between these two factors on root colonization by DSEs was tested with a two-way ANOVA. Pearson correlation coefficients were calculated to determine the relationship between root colonization by DSEs and plant growth. Percentage values were arcsin-transformed before statistical analyses.

RESULTS

Isolation and identification of DSE strains

Seven DSE strains were isolated from the roots of poplar trees grown on metal-polluted sites: Me07 and Me10 from the MetalEurop site, Fe06 from the Fresnes-sur-Escaut site and Pr27, Pr28, Pr29, Pr30 from the Pierrelaye site. The prefixes Me, Fe and Pr were respectively assigned to MetalEurop, Fresnes-sur-Escaut and Pierrelaye. All strains produced round-shaped, fast-growing colonies on MEA plates. Pr27 and Pr29 produced yellow to orange colonies, whereas the other colonies were brown to black (Table 1). All strains had typical dark septate hyphae (data not shown). Moreover, looping and twisting hyphae were observed for Pr27, Pr28, Pr30 and Fe06 (Fig. 1f). Me10 sporulated *in vitro* after 1 week of growth.

Table 1. Colonization of *B. pendula*, *E. globulus* and *L. perenne* roots by DSEs.

Strains	*B. pendula* Colonization				*E. globulus* Colonization				*L. perenne* Colonization			
	F%	H%	MS%	MS type	F%	H%	MS%	MS type	F%	H%	MS%	MS type
Fe06 (dark brown)	44.4 ± 8.0^{a,b}	7.5 ± 4.5^{b,c}	3.4 ± 1.0^{a,b}	Lp, Fp	68.9 ± 5.9^{c}	2.7 ± 0.3^{b,c}	1.4 ± 1.0^{a,b}	Fp	70.0 ± 8.2^{a}	5.0 ± 3.1^{a,b}	11.3 ± 9.7^{a}	Bl
Me07 (light gray)	44.4 ± 14.7^{a,b}	2.7 ± 1.2^{a,b}	1.5 ± 1.0^{a}	Lp	56.7 ± 3.3^{a,b,c}	1.2 ± 0.2^{a}	1.4 ± 1.0^{a}	Lp	49.3 ± 20.3^{a}	5.0 ± 3.1^{a,b}	11.3 ± 9.7^{a}	Lp
Me10 (dark gray)	60.0 ± 5.9^{b}	4.5 ± 1.7^{a,b,c}	4.3 ± 1.0^{a,b}	Fp	60.0 ± 7.2^{a,b,c}	2.7 ± 0.4^{b,c}	3.6 ± 1.1^{a,b}	Bl	62.2 ± 3.9^{a}	7.2 ± 1.9^{a,b}	10.0 ± 4.2^{a}	Lp, Fp
Pr30 (dark gray)	56.7 ± 8.8^{a,b}	2.0 ± 0.2^{a,b}	2.2 ± 1.1^{a}	Lp	56.7 ± 8.8^{b,c}	6.1 ± 3.9^{b,c}	2.1 ± 2.1^{a}	Bl	55.0 ± 12.2^{a}	1.3 ± 0.2^{a}	3.1 ± 0.5^{a}	Lp
PMI.412 (dark gray)	53.3 ± 6.7^{a,b}	12.1 ± 3.0^{c}	18.3 ± 11^{b}	Lp	66.7 ± 6.7^{b,c}	1.3 ± 0.3^{a,b}	4.6 ± 2.5^{a,b}	Lp	66.7 ± 3.3^{a}	7.3 ± 2.5^{b}	7.0 ± 2.6^{a}	Lp
Pr28 (black)	66.7 ± 6.7^{b}	2.8 ± 0.2^{a,b,c}	4.8 ± 1.8^{a,b}	Lp	55.0 ± 4.1c	4.5 ± 0.4^{a,b,c}	9.7 ± 0.3^{b}	Lp, Fp	56.7 ± 8.8^{a}	7.1 ± 2.7^{b}	4.7 ± 2.5^{a}	Lp, Fp
Pr27 (yellow-red)	33.3 ± 9.6^{a}	1.7 ± 0.7^{a}	5.6 ± 2.4^{a,b}	Lp	55.0 ± 4.1^{a,b,c}	1.3 ± 0.3^{a}	0.5 ± 0.4^{a}	Lp, Bl	43.3 ± 16.7^{a}	1.4 ± 1^{a}	1.2 ± 0.2^{a}	Lp
Pr29 (yellow- gray)	36.7 ± 12.6^{a}	2.3 ± 0.7^{a,b}	5.0 ± 2.5^{a,b}	Lp	53.3 ± 3.3^{a,b}	1.2 ± 0.2^{a}	1.4 ± 1.0^{a}	Lp	40.0 ± 5.8^{a}	1.6 ± 0.3^{a}	4.2 ± 1.3^{a}	Lp
DSM 11827 (beige)	41.3 ± 7.9^{a}	4.1 ± 3.1^{a,b}	NA	Ch	25.0 ± 4.1^{a}	1.0 ± 0.0^{a}	NA	Ch	nd	nd	nd	nd

Inoculated plants were grown for 2 weeks (ryegrass) or 2 months (birch and eucalyptus) on perlite substrate. Colonization frequency (F%), colonization intensity by hyphae (H%) and microsclerotia (MS%) are given as means (±SE) of 30 root fragments. Between brackets: color of fungal colonies growing on MEA plates. Bl: Brain-like microsclerotia; Fp: Fully packed microsclerotia; Lp: Loosely packed microsclerotia; Ch: Chlamydospores; NA: not applicable; nd: not determined. Statistically significant differences between DSE strains ($P < 0.05$, Kruskal-Wallis test) are indicated by different letters.

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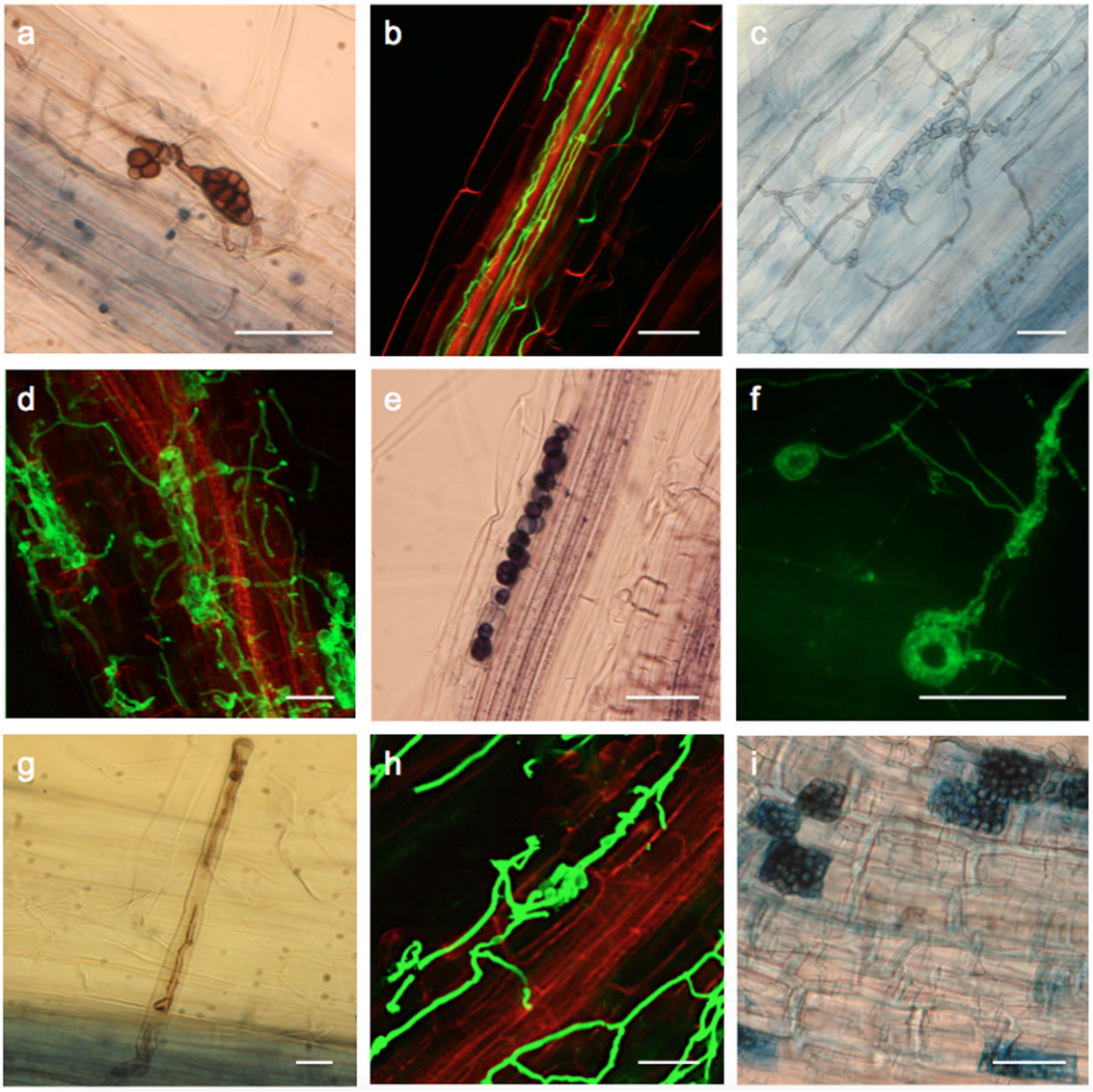

Figure 1. Morphological characteristics of DSEs in roots or in pure cultures. Fungal structures presented in **c**-**d**-**h**, **a**-**e**-**i** and **b**-**g** were observed in roots of *E. globulus*, *B. pendula* and *L. perenne*, respectively. Trypan blue-stained roots were observed by optical microscopy (a-c-e-g-i), whereas WGA-AF488-stained roots and mycelia were observed by confocal microscopy. Green: fungal material; red: autofluorescence of root cells. Typical brain-like (a), loosely packed (c and h) and fully packed (d and i) microsclerotia were observed in the cortical cells of plants inoculated with Fe06, PMI_412 and Pr27, Pr27 and Me10, respectively. Vascular cylinder colonized by hyphae of Pr30 (b). Chlamydospores (e) of *P. indica* DSM 11827 localized in cortical cells. Root hair colonized by Pr28 (g). Asexual looping structures (**f**) observed in Pr27 colonies growing on MEA plates. Scale bars = 25 μm.

The taxonomic identification of the strains was performed by analyzing their ITS1-5.8S rDNA-ITS2 regions. A ML phylogenetic tree was drawn, including sequences from previously reported DSE strains as well as other endophytic strains (Fig. 2). Me07, Me10, Pr30 clustered together with sequences of the previously described *Leptodontidium orchidicola* and the reference *Leptodontidium* sp. PMI_412 strain. Pr27 and Pr29 had a very close evolutionary relationship with *Phialophora mustea* (JN123359). Pr28 matched with *Phialocephala fortinii* (AY078144) with 98% identity of their respective ITS sequences. Fe06 was assigned to the genus *Cadophora* (99% identity with *Cadophora* sp. JN859254). Additional phylogenetic analysis using the NJ method was performed and gave congruent conclusions that confirmed the confidence level of the clustering (data not shown). Sequence data were deposited in GenBank under the following accession numbers: KU886589 (Fe06), KU886584 (Me07), KU886585 (Me10), KU886587 (PMI_412), KU886586 (Pr27), KU886588 (Pr28), KU886582 (Pr29) and KU886583 (Pr30).

Impacts of the carbon source and metal on the growth of DSE isolates

DSEs colonize root tissues, so they have access to a variety of carbon sources. We therefore investigated the use of various carbon compounds by the different strains. All strains produced measurable biomass on the soluble carbohydrates (Table 2). However, their degradation ability was both carbon source- and strain-dependent. We used the *Piriformospora indica* DSM 11827 strain as a reference of endophytic fungi. Its growth was the highest on glucose and sucrose, but lower on the other carbon sources, especially on mannitol. The DSE strains displayed three distinct patterns. First, the carbon source did not significantly affect the

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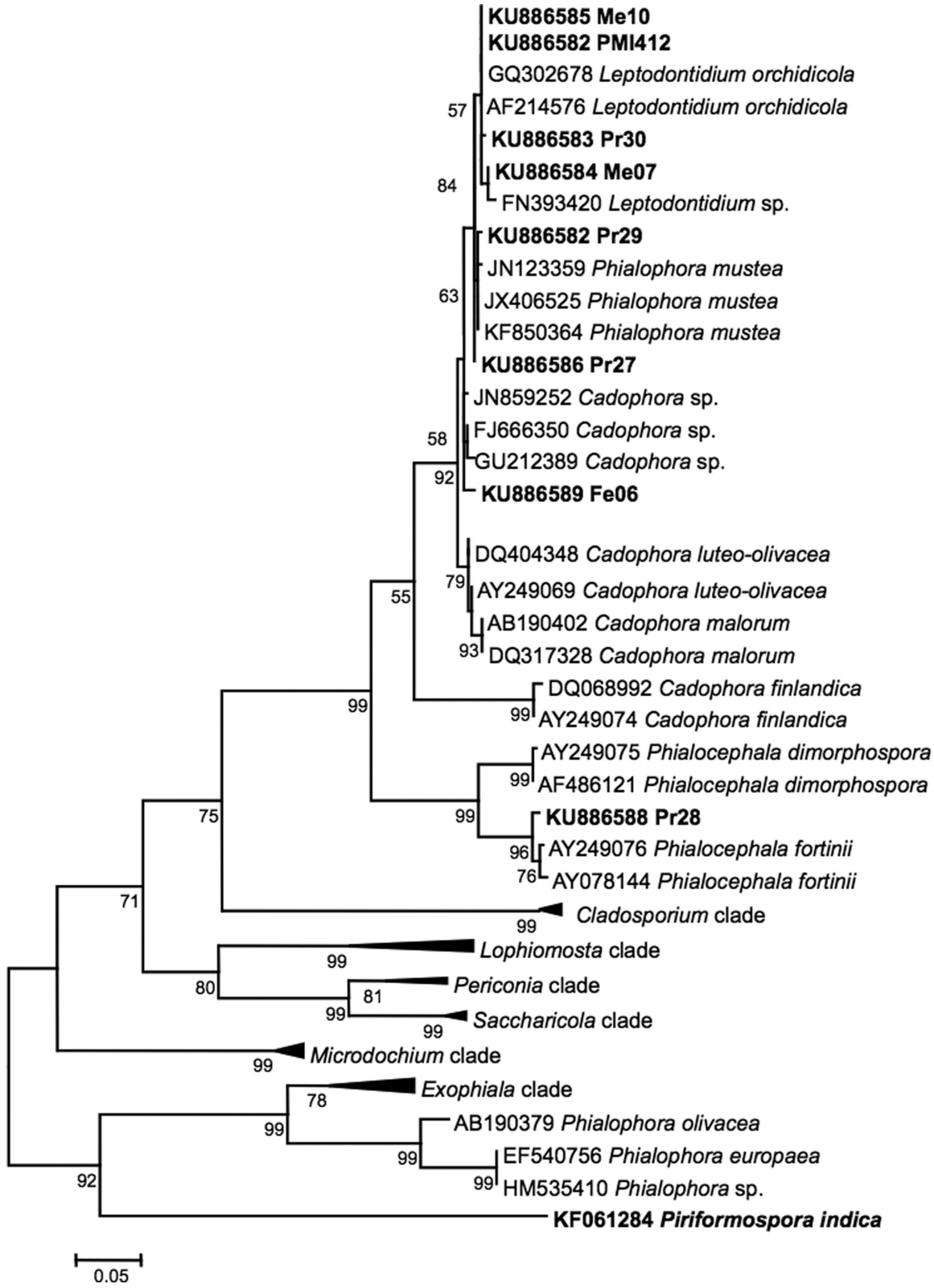

Figure 2. ML phylogram showing the position of strains based on ITS1-5.8S-ITS2 sequences. The evolutionary history was inferred using the ML method based on the Tamura-Nei model. The percentage of trees (>50%) in which the associated taxa clustered together is shown below the branches. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.* 2013). The strains used in the present study (in bold) were compared with known and selected fungal species from GenBank with high sequence similarities. The different clades are represented and are compressed for the sake of simplicity. The sequences contained within each genus are provided in Table S1, Supporting Information.

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Table 2. Utilization of different carbon substrates by the fungal strains.

Carbon sources		Strains								
		Fe06	Me07	Me10	PMI_412	Pr30	Pr28	Pr27	Pr29	DSM 11827
Cellulose[1]	+		−	+	+	+	+	+	+	+
Lignin[1]	−		−	−	−	−	−	−	−	−
Starch[1]	+		+	+	+	+	+	+	+	+
Xylan		231 ± 34^{e}	100 ± 9^{a,b}	108 ± 11^{a,b}	96 ± 3^{a}	95 ± 8^{a}	49 ± 8^{a}	73 ± 14^{a,b,c}	75 ± 12^{a,b}	73 ± 13^{a,b}
Cellobiose		107 ± 5^{b,c}	75 ± 15^{a}	69 ± 10^{a}	102 ± 17^{a}	81 ± 5^{a}	74 ± 5^{b}	73 ± 19^{a,b,c}	73 ± 17^{a,b}	60 ± 4^{b,c}
Maltose		116 ± 5^{a,b}	109 ± 9^{a,b}	166 ± 25^{c}	124 ± 9^{a}	112 ± 9^{a}	85 ± 2^{b,c}	89 ± 18^{b,c}	86 ± 2^{a,b}	91 ± 4^{d}
Sucrose		131 ± 36^{b}	190 ± 30^{c}	99 ± 15^{a,b}	110 ± 11^{a}	112 ± 11^{a}	114 ± 7^{c}	100 ± 9^{c}	114 ± 15^{b}	103 ± 5^{e}
Fructose		74 ± 2^{a}	125 ± 4^{b,c}	96 ± 22^{a,b}	103 ± 12^{a}	95 ± 5^{a}	82 ± 8^{b}	47 ± 2^{a}	69 ± 10^{a,b}	84 ± 4^{d}
Glucose		100 ± 3^{a,b}	100 ± 19^{a,b}	100 ± 15^{a,b}	100 ± 3^{a}	100 ± 26^{a}	100 ± 19^{b,c}	100 ± 11^{c}	100 ± 21^{a,b}	100 ± 4.8^{e}
		(55.5)	(79.3)	(101.3)	(92.3)	(67.1)	(86.9)	(111.1)	(130.6)	(71.5)
Mannitol		188 ± 19^{d,e}	91 ± 2^{a,b}	121 ± 1^{b,c}	110 ± 11^{a}	90 ± 9^{a}	46 ± 5^{a}	55 ± 7^{a,b}	64 ± 2^{a}	25 ± 2^{a}
Xylose		188 ± 24^{d,e}	101 ± 9^{a,b}	81 ± 3^{a,b}	106 ± 6^{a}	93 ± 5^{a}	73 ± 5^{b}	94 ± 6^{c}	90 ± 11^{a,b}	65 ± 2^{c}
CMC[2]	Growth	3.1 ± 0.12	3.1 ± 0.05	3.0 ± 0.10	4.8 ± 0.10	2.1 ± 0.47	3.6 ± 0.03	3.7 ± 0.1	3.5 ± 0.5	4.1 ± 0.27
	Halo	4.9 ± 0.20	3.0 ± 0.03	3.4 ± 0.15	5.6 ± 0.03	2.2 ± 0.49	5.0 ± 0.13	4.3 ± 0.19	5.2 ± 0.29	5.3 ± 0.18
	Ratio	1.6 ± 0.05^{f}	1.0 ± 0.01^{a}	1.1 ± 0.01^{a,b,c}	1.3 ± 0.02^{b,c}	1.1 ± 0.05^{a,b}	1.4 ± 0.02^{d,e}	1.2 ± 0.06^{b,c}	1.5 ± 0.15^{e,f}	1.3 ± 0.05^{c,d}

Values (expressed as %) represent relative fungal growth after 2 weeks of culture on a specific carbon source as compared to growth on glucose used as a control. Values between brackets represent fungal dry biomass (mg) in minimum liquid medium supplemented with glucose. Statistically significant differences between carbon sources are indicated by different letters ($P < 0.05$, Kruskal–Wallis test). [1]Due to the non-solubility of the compounds, the degradation ability was considered as positive when the strain displayed more than 5 mm mycelial development around the initial plugs and formation of new small fungal pellets as compared to the negative control (glucose) (+: growth, −: absence of growth).

[2]CMC solubilization by the endophytic strains was recorded on agar plates after 2 weeks of growth. The solubilization index was calculated as the 'diameter of the solubilization halo' over 'diameter of the colony' ratio. Values are the means of three replicates ±SE. Statistically significant differences ($P < 0.05$, Kruskal–Wallis test) between strains are indicated by different letters.

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Table 3. Metal tolerance of the strains.

Strains	MIC (mM)			
	As	Cd	Mn	Zn
Fe06	10	0.6	9	9
Me07	25	1.5	9	7
Me10	6	0.5	6	5
PMI_412	4	0.2	12	>12
Pr30	6	0.3	12	10
Pr28	14	0.5	12	12
Pr27	16	0.4	5	10
Pr29	14	0.5	3	3
DSM 11827	>30	0.3	9	9

MIC is the minimum inhibitory concentration of metal that completely inhibited fungal growth.

growth of PMI_412, Pr30 and Pr29. Second, growth of Pr28 on xylan and mannitol, as well as of Pr27 on fructose and mannitol was reduced by ~50% as compared to glucose. Finally, growth of Fe06, Me07 and Me10 was increased 1.7–2.3-fold by different carbon compounds. Fe06 growth was the highest on xylan, xylose and mannitol, whereas sucrose and maltose treatments significantly enhanced biomass production by Me07 and Me10, respectively.

The cellulose (Avicel), starch and lignin substrates were insoluble and adhered to mycelial pellets, artificially increasing mycelium weight at harvest. Therefore, qualitative growth on cellulose, starch and lignin was solely evaluated, and was not compared to growth on other carbon sources (Table 2). Whatever the strain, no growth was noted on lignin. Mycelial growth was evident for all strains on cellulose and starch, except Me07 on cellulose. To confirm the cellulolytic activity of the strains, an agar test was performed on plates containing CMC. We measured the ratio of the diameter of the hydrolytic zone (degradation halo) over the diameter of the colony (Table 2). Fe06 exhibited the highest cellulolytic ability with a ratio of 1.6, followed by Pr29, Pr28, PMI_412 and DSM 11827 (1.3–1.5). No degradation halo was noted for Me07. Very low ratios (1.1) were recorded for Me10 and Pr30.

Since the DSE fungi were isolated from metal-contaminated soils, their metal tolerance was further assessed by determining MIC values for As, Cd, Mn and Zn. Metal tolerance varied among strains and metal species (Table 3). Me10, PMI_412 and Pr30 were the least tolerant to As, with MICs ranging between 4 and 6 mM, whereas Me07 and DSM 11827 were the most tolerant with MICs of 25 and >30 mM, respectively. Me07 was also the most tolerant strain to Cd (MIC = 1.5 mM), and PMI_412, Pr30 and DSM 11827 were the least tolerant ones. PMI_412, Pr30, Pr28 were the most tolerant to Mn, and PMI_412, Pr30, Pr28 and Pr27 were the most tolerant to Zn. Conversely, Pr27 and Pr29 were the least tolerant strains to Mn, and Pr29 the least tolerant to Zn.

PGP properties

IAA production by DSEs grown in liquid CM medium with or without tryptophan, the IAA precursor, was examined by colorimetric assays (Table 4). No IAA was recovered in the tryptophan-free treatments. Conversely, all strains produced IAA in medium supplemented with tryptophan. Most of the strains produced similar concentrations of IAA. IAA production ranged between 106 μg (g dry wt.)$^{-1}$ for Pr27 and 581 μg (g dry wt.)$^{-1}$ for PMI_412. However, statistically significant differences were only found for Pr30 and PMI_412 relative to Pr27.

Table 4. PGP properties of DSEs.

Strains	IAA production[1] (μM g^{-1} dry wt.)	Tobacco SFW[2] (mg)
Control	–	75 ± 2^{a}
Fe06	$277 \pm 35^{a,b}$	95 ± 3^{f}
Me07	$245 \pm 9^{a,b}$	$80 \pm 5^{a,b}$
Me10	$262 \pm 34^{a,b}$	$87 \pm 2^{d–f}$
PMI_412	581 ± 266^{b}	$90 \pm 3^{e,f}$
Pr30	401 ± 64^{b}	$91 \pm 2^{e,f}$
Pr28	$296 \pm 166^{a,b}$	78 ± 2^{a}
Pr27	106 ± 35^{a}	$81 \pm 2^{a–c}$
Pr29	$271 \pm 23^{a,b}$	$88 \pm 5^{c–e}$
DSM 11827	nd	$91 \pm 4^{e,f}$

[1]IAA production was measured in liquid growth medium after 2 weeks of culture in minimal medium supplemented with tryptophan ($n = 3$).
[2]Production of VOCs was determined by measuring the shoot fresh weight (SFW) of 1-month-old tobacco seedlings in the absence or presence of DSEs ($n = 36$). Values are the means (±SE). Statistically significant differences ($P < 0.05$. Kruskal–Wallis test) between strains are indicated by different letters. nd: not determined.

Nine strains were screened for their growth promotion effect on *Nicotiana tabacum* seedlings (Table 4). The partners were grown for 1 month in 24-well microplates without any physical contact. No strain showed deleterious effects on tobacco growth. Tobacco biomass was not affected by Pr28, Pr27 or Me07, but was significantly increased by the other strains (Table 4). The highest growth promotion was recorded with Fe06 co-cultures, with a 21% increase in shoot biomass as compared to the fungus-free treatment.

To determine the nature of the VOCs emitted by Pr30 and Fe06, SPME-GC/MS analyses were performed with 1 month-old DSE cultures. The major VOCs (i.e. those with a relative proportion >1%) are given in Table 5. These compounds were absent from mock-inoculated vials. Altogether, seven major VOCs were identified by comparison with the NIST database. Ethyl acetate, 3-methyl-1-butanol, 2-methyl-1-butanol and styrene were emitted by both strains. The relative proportion of each compound was similar between the two strains. Among these four compounds, ethyl acetate was the most produced. Pr30 emitted two other compounds. 1,3-Cyclopentadiene, 1-3 bis was produced in the same proportion as ethyl acetate, whereas β-chamigrene was present in a similar proportion to styrene. These two compounds were not synthesized by Fe06, but Fe06 emitted a fifth compound (cyclohexene, 1-ethyl) in a similar proportion to *n*-methyl-1-butanol ($n = 2$ or 3).

Effect of DSEs on plant growth

To assess the effect of DSEs on plant growth, *Betula pendula*, *Eucalyptus globulus* and *Lolium perenne* were inoculated with each of the eight DSE strains and harvested after 2 (*L. perenne*) or 8 weeks (*B. pendula*, *E. globulus*). *Piriformospora indica* was used as an endophytic reference. Microscopic observations of roots revealed the presence of fungal structures (Fig. 1; Table 1), whereas the non-inoculated plants were fungus free. All strains colonized the roots of the different plants (Table 1), formed microsclerotia (Fig. 1; Table 1) and spread in both the root epidermal and cortical cells. Three types of microsclerotia (Table 1), namely brain-like (Fig. 1a), loosely packed (Fig. 1c and h) and fully packed microsclerotia (Fig. 1d and i) were identified in the roots of the different plants. Several strains produced different types of microsclerotia according to the plant species they colonized (Table 1). Hyaline and septate hyphae were also observed in the

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Table 5. Identification of VOCs synthesized by strains Fe06 and Pr30 by SPME-GC/MS.

	Pr30			Fe06			
Compounds	Retention time	Relative %	Quality	Retention time	Relative %	Quality	Molecular weight
Ethyl acetate ($C_4H_8O_2$)	1.9	33.6 ± 4.7^c	43	1.9	40.0 ± 1.0^c	78	88
3-methyl-1-butanol ($C_5H_{12}O$)	3.6	12.1 ± 2.0^b	64	3.6	18.4 ± 5.0^b	64	88
2-methyl-1-butanol ($C_5H_{12}O$)	3.7	13.3 ± 2.2^b	83	3.7	19.8 ± 3.7^b	83	88
Styrene (C_8H_8)	7.4	4.1 ± 1.2^a	22	7.7	5.1 ± 1.1^a	64	104
β-Chamigrene ($C_{15}H_{24}$)	9.7	3.0 ± 0.3^a	80	–	–	–	150
1,3-cyclopentadiene 1,3bis ($C_{13}H_{22}$)	10.2	33.9 ± 3.1^c	58	–	–	–	150
Cyclohexene, 1-ethyl (C_8H_{14})	–	–	–	12.0	15.4 ± 3.6^b	72	110

Fungi were grown for 2 weeks in glass vials containing MEA medium. The compounds corresponding to the major peaks (area > 1%) are presented as relative percentages. Values are means ±SE ($n = 9$). Statistically significant differences ($P < 0.05$; Kruskal–Wallis test) between compounds are indicated by different letters.

root hairs of ryegrass inoculated with Pr28 (Fig. 1g) and Me07 (data not shown). Ryegrass roots inoculated with Me07 (data not shown) and Pr30 (Fig. 1b) were also colonized by hyphae in vascular parenchyma tissues, but the cells were not degraded. Moreover, microsclerotia formed by Me07 were also detected in these tissues. *Piriformospora indica* DSM 11827 formed typical chlamydospores in birch and eucalyptus roots (Fig. 1e). Two-way ANOVA results revealed no significant differences among plant species or for the interaction between plant species and fungal strains for any of the three root-colonization parameters (F%, H% and MS%) (Table S2, Supporting Information). However, there were significant differences among fungal strains for H% and MS% (Table S2, Supporting Information). All plants taken together, Pr28 and PMI_412 were the best colonizing strains, while Pr27 was the least effective (Kruskal–Wallis test, *P*-value < 0.05). Pr28- and Me10-inoculated plants displayed the highest colonization frequencies in birch roots (F% = 66.7% and 60%, respectively), whereas eucalyptus roots displayed the highest colonization frequencies when inoculated with Fe06 (Table 1). Generally, DSE-inoculated plants were more intensively colonized than *P. indica*-inoculated plants. The colonization frequency of ryegrass was similar with all DSEs. Results for microsclerotia indices (MS%) were similar. PMI_412 significantly produced more microsclerotia than Me07 and Pr30 in birch roots. Similarly, more microsclerotia were observed in Pr28-inoculated eucalyptus roots than in Me07-, Pr30-, Pr27- and Pr29-inoculated ones.

Shoot and root dry weights of birch and eucalyptus inoculated with Pr30 and Pr27 were 3–5-fold higher than those of the non-inoculated controls (Fig. 3). PMI_412 also promoted birch and eucalyptus shoot growth, but to a lesser extent. The endophytic reference strain DSM 11827 promoted both shoot and root growth of birch, but did not affect eucalyptus growth. The other strains did not significantly alter tree seedling growth. In *L. perenne*, four strains had a positive effect on root growth, and Pr30 also significantly increased shoot growth. Root dry weight of ryegrass inoculated with Me07 and Me10 reached 160% of the non-inoculated control value set to 100%. Root growth of Fe06-inoculated seedlings was doubled as compared to the control. Conversely, inoculation with PMI_412 significantly decreased root biomass of *L. perenne*. There was no correlation between plant growth and root colonization intensity (Pearson test, *P*-value = 0.5 for F%; *P*-value = 0.9 for MS% and *P*-value = 0.9 for H%).

DISCUSSION

Isolation of metal-tolerant DSEs from poplar

DSEs represent a polyphyletic group of ascomycetes, with members distributed in the Helotiales, Pleosporales, Sordariales or Pezizales orders (Grünig, Queloz and Sieber 2011). In the present study, the seven DSE strains isolated from poplar all belonged to the Helotiales order, but to two different groups. *Phialocephala* Pr28 belonged to the *Mollisia*, *Phaeomollisia* and *Vibrissea* group, whereas the other six strains (as well as PMI_412) belonged to another group clustering with the well-known *Leptodontidium orchidicola* and several *Cadophora* species. Whereas strains belonging to the *Leptodontidium*, *Cadophora* and *Phialocephala* genera had a typical DSE gray to black phenotype on agar medium, the *Phialophora* strains had orange to red colors. However, the distinction based on the degree of pigmentation is often arbitrary because pigment strength varies greatly in some species (Hambleton and Sigler 2005), especially in mycelium of colonized roots (Yu, Nassuth and Peterson 2001; Barrow 2003). These four genera are known colonizers of tree roots, and to a lesser extent of herbaceous plants (Jumpponen and Trappe 1998b; Yu, Nassuth and Peterson 2001; Grünig *et al.* 2008; Peterson, Wagg and Pautler 2008; Likar and Regvar 2009; Knapp, Pintye and Kovács 2012; Zhang, Li and Zhao 2013; Op De Beeck *et al.* 2015). However, few studies have evidenced the presence of DSEs in poplar roots by microscopy (Beauchamp, Stromberg and Stutz 2005; Krpata *et al.* 2008; Gehring *et al.* 2014). Except *Cadophora*, *Exophiala* and *Leptodontidium* isolated from poplar roots (Bonito *et al.* 2016), molecular identification is still lacking, and DSE strains have not yet been isolated.

DSEs are abundant on highly metal-enriched locations. For example, *Phialophora* sp., *Phialocephala* sp. and *Leptodontidium* sp. were identified and/or isolated from Chinese and European metal-polluted sites (Ban *et al.* 2012; Likar and Regvar 2013; Zhang, Li and Zhao 2013). Similarly, *Cadophora* sp. was also found in Zn/Cd-polluted soils, in pine roots from a Zn/Cd-polluted site in Belgium and in a metal-polluted soil in Austria (Utmazian *et al.* 2007; Op De Beeck *et al.* 2015). We also isolated DSEs from Cd-, Zn- and Pb-contaminated soils and tested their tolerance to these metals as well as to As and Mn. Although our work focused on only a few strains, we noted that metal tolerance was strain- and metal-dependent and did not seem to be related to the metal concentration in the soil of origin. Such variations between DSE strains have been reported in different studies (Zhang *et al.* 2008; Ban *et al.* 2012; Likar and Regvar 2013). These differences could be explained by the presence/absence of different tolerance mechanisms, e.g. efflux systems or vacuolar compartmentalization, or by various levels of expression of genes/proteins involved in these mechanisms (Ban *et al.* 2012; Zhan *et al.* 2014, 2015; Shen *et al.* 2015). Several studies report that under severe metal contamination, the rate of root colonization by arbuscular mycorrhizal (AM) fungi decreases but remains unaffected for DSEs (Ruotsalainen, Markkola and Kozlov 2007; Likar and Regvar 2009; Deram, Languereau and Haluwyn 2011).

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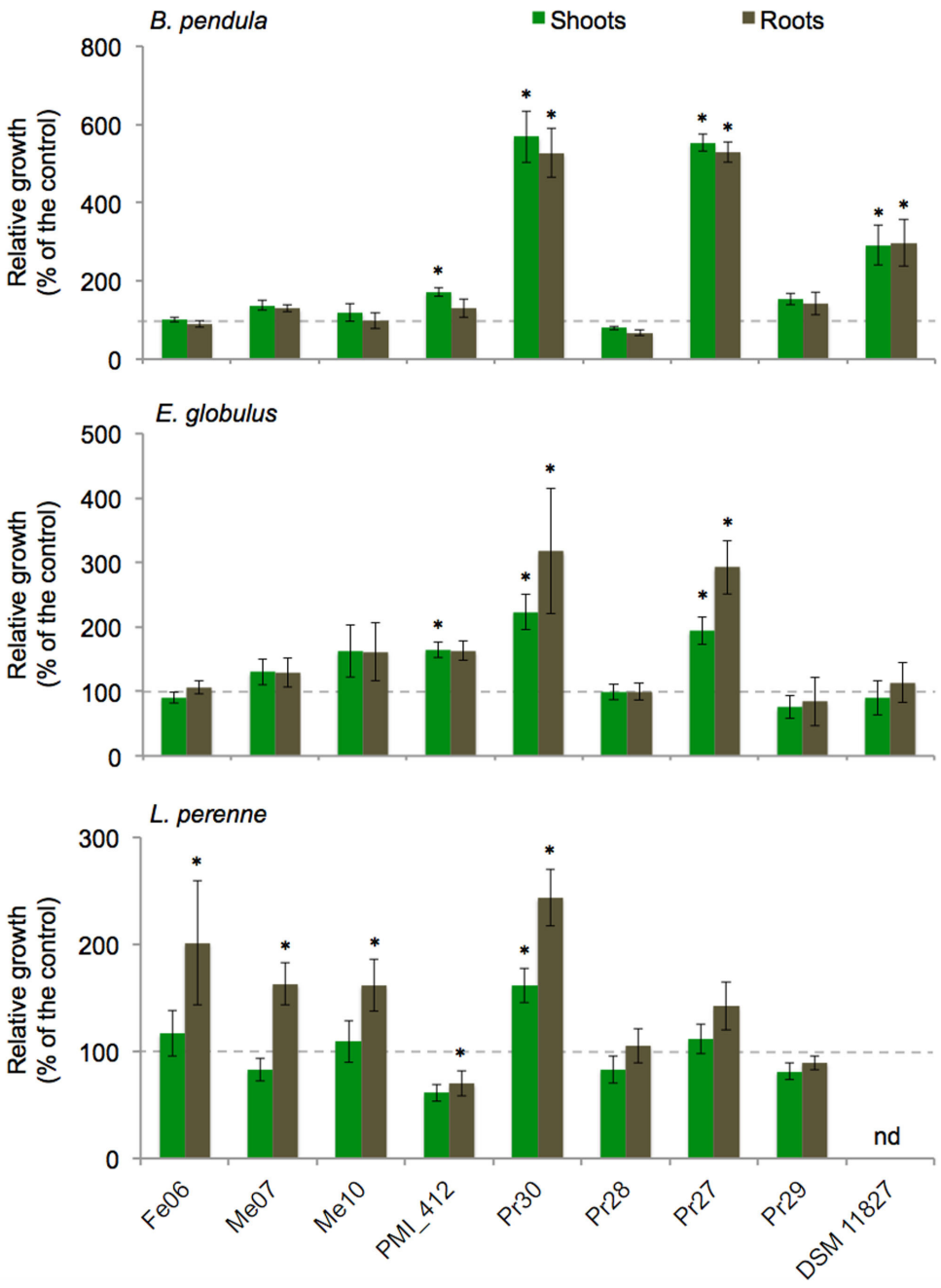

Figure 3. Effect of fungal inoculation on plant growth. Root biomass (brown bars) and shoot biomass (green bars) were measured after 2 weeks (*L. perenne*) or 2 months (*B. pendula* and *E. globulus*) of growth. Values of the controls (non-inoculated plants) were set to 100% (dotted lines). Data of the inoculated plants are given as relative values of the controls. Data are given as means ±SE ($n = 14$). Asterisks indicate significant differences as compared to the control ($P \leq 0.05$. Kruskal–Wallis test). nd: not determined.

The relatively high metal tolerance of DSEs could be related to the high melanin content in their cell walls. Melanin contains carboxyl, phenolic, hydroxyl and amine groups that provide many potential binding or biosorption sites for metal ions, ensuing immobilization of metals on cell walls, and decreased metal toxicity (Fogarty and Tobin 1996; Bellion *et al.* 2006; Zhan *et al.* 2011). However, DSEs and other endophytic strains forming white/cream colonies have comparable MIC values. This was the case for *Piriformospora indica* (this study) and endophytic Helotiales (Lacercat-Didier *et al.* 2016). Therefore, the role of melanin in the high metal tolerance of dark-pigmented strains still requires further genetic evidence. Moreover, experiments are also needed to determine whether root colonization by DSEs is less affected by metal stress than AM or ectomycorrhizal (ECM) symbiosis in the same soils.

Degradation of cell-wall-related compounds

The isolated DSEs usually grew better on a larger set of C compounds than the endophytic model *P. indica*. The best evidence was with mannitol, where *P. indica* growth was strongly limited as compared to DSEs. We also investigated the ability of the

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strains to degrade complex carbon compounds. Laccases are involved in lignin degradation. DSEs, especially *Periconia* and *Microdochium* can display high laccase activity (Mandyam, Loughin and Jumpponen 2010). However, we found no evidence for lignin degradation in any of our strains. This inability could be explained by the lack of one or several enzymes of the lignin degradation pathway. Xylan is a hemicellulose compound that is degraded into xylose by xylanase activity and subsequently into glucose by xylosidase activity. All strains grew on xylan, xylose and glucose as sole carbon sources, suggesting that these strains can entirely degrade xylan. All strains but Me07 degraded Avicel cellulose, CMC and cellobiose. These results suggest the production of cellobiohydrolases, endoglucanases and β-glucosidases enabling the complete hydrolysis of cellulose into glucose. Interestingly, the capacity of *Cadophora*, *Leptodontidium* PMI_412, *Phialophora* and *Phialocephala* to grow on Avicel cellulose or to degrade CMC was similar to that of *P. indica*. However, complete hydrolysis of cellulose is expected, as these enzymes are presumably required for root cell penetration. *Leptodontidium* Me07 did not grow on Avicel cellulose and produced a minute CMC degradation halo. This result may reflect a difference in cellobiohydrolase or cellulase activity as compared to the other DSEs. Another possibility is that the forms of cellulose (Avicel, CMC) were not optimal for this strain. These fungi exclusively colonize root tissues, so their lifestyle requires several enzymes to degrade cell-wall components (Rodriguez *et al.* 2009). But, as detailed below, re-synthesis experiments can best demonstrate the fungal capacity to enter root cells.

Root colonization by DSEs and impact on plant growth

Three types of microsclerotia were observed in roots: fully packed, loosely packed and brain-like microsclerotia. Loosely packed microsclerotia were the most abundant structure across all treatments. We therefore report for the first time that a given DSE strain can form various types of microsclerotia when associated to different plant species, but also within a same plant species. Little information is available about the diversity and function of the different types of microsclerotia (Yu, Nassuth and Peterson 2001). Therefore, more experiments are required to state which of the following factors—DSE strain, plant species or environment—is uppermost in shaping microsclerotium type.

Root colonization by DSEs has been reported to cause a variety of host responses ranging from pathogenic to mutualistic interactions (Jumpponen and Trappe 1998a). A few studies report a positive impact of *Leptodontidium, Cadophora* and *Phialocephala* on the growth of woody and herbaceous plants (Andrade-Linares *et al.* 2011; Newsham 2011; Khastini *et al.* 2014). In our study, several *Leptodontidium* and *Cadophora* strains also enhanced plant growth. Additionally, *Phialophora mustea* Pr27 promoted birch and eucalyptus growth. To our knowledge, this is the first report about plant growth promotion by *Phialophora mustea*.

Surprisingly, two *Leptodontidium* strains (Me07 and Pr30) colonized the vascular stele of ryegrass, as in roots of *Salix glauca* and *Asparagus officinalis* colonized by *Leptodontidium orchidicola* and *Phialocephala fortinii*, respectively (Fernando and Currah 1996; Yu, Nassuth and Peterson 2001). Necrosis of the stele tissues of *Salix* roots was evident, so the authors suggested a parasitic lifestyle for *Leptodontidium* in that association. However, in the present study, we did not notice any cell damage in ryegrass roots, and both strains enhanced ryegrass growth. Cumulatively, these results suggest that, under our experimental conditions, *Leptodontidium* acted more as a mutualistic fungus than as a parasite.

Plant growth promotion was strain-dependent since different host responses were observed as a result of inoculation by different *Leptodontidium* and *Phialophora* strains. Fernando and Currah (1996) also showed that inoculation by four strains of *L. orchidicola* induced variable effects on *Salix* and *Picea* growth. Inoculation of *Arabidopsis thaliana* with different strains of *Periconia* or *Microdochium* yielded similar results (Mandyam, Roe and Jumpponen 2013). We hypothesized that the different strains could colonize roots in quantitatively different manners and consequently produce different responses. This hypothesis prompted us to investigate the levels of root colonization by DSEs in the different plants. The three plant species were colonized whatever the DSE strain used in the inoculum. This experiment therefore demonstrates that DSEs have a broad host specificity spanning monocots and dicots, including woody species. Additionally, as observed previously on AM fungi, we found no correlation between plant growth and the intensity of root colonization by DSEs (Hildebrandt, Regvar and Bothe 2007). This result suggests that growth promotion was more probably strain-dependent. However, additional experiments under different experimental conditions are needed to fully demonstrate this statement. More particularly, soil nutrient levels could influence plant responses when plants are associated to DSEs. DSEs could promote plant growth by enhancing nitrogen, carbon and/or phosphorus nutrition (Caldwell, Jumpponen and Trappe 2000; Barrow and Osuna 2002; Mandyam and Jumpponen 2005; Usuki and Narisawa 2007; Upson, Read and Newsham 2009). For example, in response to N fertilization, *Pinus contorta* growth response to *Phialocephala fortinii* inoculation switched from neutral to positive (Jumpponen, Trappe and Mattson 1998).

PGP abilities

To gain further insight into DSE-related growth promotion, we analyzed the ability of the strains to secrete IAA and to release VOCs. IAA production was monitored in the culture medium. The results suggested the existence of IAA biosynthesis pathways in all strains. Several studies report that endophytic fungi produce IAA (e.g. *Chaetomium globosum*, *Phoma glomerata*, *Penicillium* sp. and *P. indica*) (Sirrenberg *et al.* 2007; Khan *et al.* 2012; Waqas *et al.* 2012). Secretion of auxin by hyphae could contribute to increasing plant biomass (Khan *et al.* 2012; Waqas *et al.* 2012; Sukumar *et al.* 2013). Synthesis of fungal-derived IAA could play key roles in root and shoot development, increase the root surface absorption area with resulting better water and nutrient uptake, and promote root colonization by other fungi (Hilbert *et al.* 2012; Sukumar *et al.* 2013).

VOCs are low-molecular-weight compounds that can easily diffuse through the soil and the atmosphere. Their functions and exact mechanisms of action are poorly understood (Kramer and Abraham 2012; Lemfack *et al.* 2014). However, several authors suggest a major role of VOCs emitted by rhizospheric and endophytic fungi in plant growth promotion (Hung, Lee and Bennett 2013; Naznin *et al.* 2013; Paul and Park 2013). That is why we further tested whether DSEs could also promote plant growth by releasing VOCs. A microplate culture system was used to restrict physical contact between fungi and seedlings and only allow for gaseous exchanges. Interestingly, five strains enhanced the growth of tobacco seedlings, demonstrating that DSEs can also promote plant growth without any physical contact. This promoting effect could be explained by the release of phytostimulant VOCs and/or by the presence of additional respiration-derived CO_2 increasing photosynthesis (Jumpponen and Trappe 1998b; Naznin *et al.* 2013). Although we did not

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measure the final CO_2 concentration, it is unlikely that CO_2 concentrations alone accounted for the total increase in biomass accumulation. A more probable explanation is that growth promotion resulted from a combination between the VOCs released by the fungi and the elevated CO_2 concentration (Naznin *et al.* 2013). VOC nature was characterized for the two strains that best promoted tobacco growth. To our knowledge, this is the first detailed analysis of VOCs produced by DSEs. Both strains produced hydrocarbon-like molecules with C_4 to C_{15} skeletons, and with alcohol derivatives such as 2-methyl-1-butanol and 3-methyl-1-butanol. These compounds were identified among the major VOCs emitted by different fungi and likely play key roles in plant growth promotion (Strobel *et al.* 2001; Naznin *et al.* 2013; Paul and Park 2013). However, the complete set of VOCs differed between the two strains, suggesting that VOC emission is species- or strain-dependent, as demonstrated before for saprophytic, ECM and endophytic fungi (Bäck *et al.* 2010).

CONCLUSIONS

In the context of fungus-assisted phytomanagement of polluted sites, we successfully isolated six PGP DSE strains from metal-contaminated sites. Based on their ability to colonize different plants, to promote plant growth and on their metal tolerance, we suggest that DSE inoculants could be used to sustainably improve growth of trees or other bioenergy crops on rehabilitated brownfields. Further studies using DSE inoculation of birch and poplar in metal-contaminated soils are currently under way. More generally, and due to the broad host specificity of these fungi, they could also be used as plant growth promoting fungi in horticulture or agriculture.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Supplemental Table S1 Species used in the phylogenetic analysis (ITS1-5.8S-ITS2), and corresponding GenBank accession numbers.

Accession numbers	Species
AF214576	*Leptodontidium orchidicola*
GQ302678	*Leptodontidium orchidicola*
FN393420	*Leptodontidium* sp.
KF850364	*Phialophora mustea*
JN123359	*Phialophora mustea*
JN859252	*Cadophora* sp.
FJ666350	*Cadophora* sp.
GU212389	*Cadophora* sp.
AY249069	*Cadophora luteo-olivacea*
DQ404348	*Cadophora luteo-olivacea*
AB190402	*Cadophora malorum*
DQ317328	*Cadophora malorum*
JX406525	*Phialophora mustea*
DQ068992	*Cadophora finlindica*
AY249074	*Cadophora finlindica*
AY249075	*Phialocephala dimorphosa*
AF486212	*Phialocephala dimorphosa*
AY249076	*Phialocephala fortinii*
AY078144	*Phialocephala fortinii*
AM502264	*Microdochium bolleyi*
AJ279454	*Microdochium bolleyi*
FJ439588	*Microdochium* sp.
AB272124	*Microdochium nivale*
AF187912	*Microdochium nivale*
HM596871	*Cladosporium* sp.
HM148218	*Cladosporium tenuissimum*
FJ210539	*Cladosporium* sp.
HM852082	*Cladosporium cladosporioides*
FJ904921	*Cladosporium cladosporioides*
FJ372389	*Lophiomosta* sp.
AJ972793	*Lophiomosta* sp.
AB433275	*Lophiomosta macrostomum*
AB369268	*Lophiomosta sagittiforme*
KP183999	*Periconia macrospinosa*
KP128003	*Periconia* sp.
GQ370379	*Saccharicola bicolor*
AF439462	*Saccharicola taiwanensis*
HM535410	*Phialophora* sp.
EF540756	*Phialophora europea*
AB190379	*Phialophora olivacea*
AF147492	*Exophiala dermatidis*
DQ826738	*Exophiala dermatidis*
DQ826739	*Exophiala pisciphila*
AM176667	*Exophiala salmonis*
AY213652	*Exophiala salmonis*
KF061284	*Piriformospora indica*

Supplemental Table S2 Two-way ANOVA results comparing the effects of plant, strain, plant x strain interactions on root colonization by DSEs (colonization frequency (F%), colonization intensity by hyphae (H%), and microsclerotia (MS%)).

	F%		**H%**		**MS%**	
	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Plant	1.54	0.227	2.13	0.131	2.56	0.089
Strain	2.11	0.063	4.17	0.001*	4.24	0.001*
Plant x Strain	0.53	0.892	1.53	0.148	0.92	0.542

Significant differences are indicated by *, p.value<0.01.

3.2.3 Caractérisation de champignons endophytes mutualistes isolés à partir de racines de peupliers et présentant une tolérance élevée aux ETMs

Cette étude visait à isoler, identifier et caractériser les champignons tolérants aux ETMs qui colonisent les racines de peuplier sur un site de phytoremédiation contaminé par des ETMs. Les racines de peuplier sont colonisées par des champignons endomycorhiziens (AM), ectomycorhiziens (ECM) et endophytes ; ces espèces ont été identifiées par des analyses moléculaires. Huit isolats différents ont été cultivés avec succès en culture pure. Trois isolats, appartenant aux *Hélotiales* (P02, P06) et à l'espèce *Serendipita vermifera* (P04), ont montré une très forte tolérance aux ETMs (Cd, Zn, Pb et Cu), comparée à celles des souches mycorhiziennes isolées (*Hebeloma*). Les trois isolats dégradent les hydrates de carbone complexes tels que la cellulose et le xylane, ce qui indique qu'ils pourraient dégrader partiellement les parois des cellules de racines et y pénétrer. Cette hypothèse a été confirmée par d'autres expériences *in vitro* de re-inoculation, qui ont montré que les trois isolats ont colonisé les tissus des racines de plantules de peupliers et deux d'entre eux ont également formé des structures de type microsclérotes. Ces résultats suggèrent un mode de vie endophytique de ces isolats. Ceci est la première preuve que *S. vermifera* serait un endophyte des racines de peuplier. Une autre espèce potentiellement endophytique, appartenant aux *Hélotiales* et étroitement liée à *Leohumicola* (P06), a également été identifiée. Il est intéressant de constater que, par rapport aux plantes non inoculées, les deux isolats P06 et P04 ont augmenté le nombre d'extrémités racinaires des plantules de peuplier inoculées *in vitro*. De plus, l'isolat *S. vermifera* P04 a également permis l'augmentation de la masse fraiche des parties aériennes. Ces résultats semblent prometteurs en relation avec l'utilisation potentielle de ces souches pour le phytomanagement des sites contaminés par les ETMs basé sur l'utilisation de ligneux et donc des microorganismes endophytiques associés.

- ***Pour ce travail, j'ai réalisé l'échantillonnage sur le terrain (Pierrelaye), l'isolement, la caractérisation et l'identification des souches (extractions d'ADNs, RFLP, et séquençage), et contribué à la constitution et à l'entretien de la mycothèque.***

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ORIGINAL ARTICLE

New mutualistic fungal endophytes isolated from poplar roots display high metal tolerance

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Abstract This study aimed to isolate, identify, and characterise metal-tolerant fungi colonising poplar roots at a metal-contaminated phytoremediation site. Poplar roots were colonised by arbuscular mycorrhizal, ectomycorrhizal, and endophytic fungi, and the species were determined by ITS molecular analyses. Eight different isolates were successfully isolated into pure culture. Three isolates belonging to the Helotiales (P02, P06) and the *Serendipita vermifera* species (P04) were highly tolerant to metals (Cd, Zn, Pb, and Cu) compared to the mycorrhizal *Hebeloma* isolates. The three isolates degraded complex carbohydrates, such as xylan and cellulose, indicating that they could partially degrade root cell walls and penetrate into cells. This hypothesis was confirmed by further in vitro re-synthesis experiments, which showed that the three isolates colonised root tissues of poplar plantlets whereas two of them formed microsclerotia-like structures. Taken together, these results suggest an endophytic lifestyle of these isolates. This is the first evidence of *S. vermifera* as a root endophyte of poplar. A new endophytic putative species belonging to the Helotiales and closely related to *Leohumicola* is also reported. Interestingly, and when compared to mock-inoculated plants, both P06 and P04 isolates increased the number of root tips of inoculated poplar plantlets in vitro. Moreover, the *S. vermifera* P04 isolate also increased the shoot biomass. The results are discussed in relation to the potential use of endophytic strains for tree-based phytoremediation of metal-contaminated sites.

Keywords Helotiales · Mycorrhizal fungi · Endophytic fungi · Poplar · Metals · *Serendipita vermifera*

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Introduction

Poplars (Salicaceae) are widely distributed in the northern hemisphere in different habitats and comprise approximately 30 species (Hamzeh and Dayanandan 2004). *Populus* is now increasingly utilised for reforestation of post-agricultural land, recultivation of areas degraded by industry or environmental pollution, and as a renewable source of biomass for bioenergy production (Capuana 2011). Poplars are ideal candidates for use in phytoremediation of metal-contaminated sites, as they meet several requirements: high biomass production usable for renewable energy production, fast growth, a deep and widespread root system, and excellent metal tolerance and accumulation capacities (Migeon et al. 2009; Capuana 2011; Migeon et al. 2012). Moreover, poplar is a model species for molecular studies of woody plants (Tuskan et al. 2006). Researchers have mainly focused their studies on the above-ground parts of poplar trees, analysing their responses to environmental factors (Migeon et al. 2009; Capuana 2011; Migeon et al. 2012).

Tree roots are naturally associated to various soil microorganisms, and these associations are crucial because they can directly or indirectly affect the mobility, bioavailability, and

accumulation of elements by host plants. As for most trees in temperate and boreal forests, the establishment, growth, and survival of poplar trees in the wild and in large plantations depend strongly on colonisation by mycorrhizal fungi (Smith and Read 2008). Mycorrhizal fungi are therefore important for the recovery of polluted sites via phytoremediation, as they play crucial roles in soil fertility, plant nutrition, and colonisation and they can both enhance plant mineral uptake and restrict metal accumulation in plants (Likar 2011; Singh et al. 2011; Meier et al. 2012). Poplars are among the few cultivated trees in the temperate climate zone that together with willows and alders form tripartite symbiotic associations with ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi (Khasa et al. 2002; Gehring et al. 2006). Endophytic fungi can also influence the distribution, ecology, and biology of plants (Rodriguez et al. 2009). Endophytic fungi form putative symbiotic interactions with plants and live within plant tissues without producing noticeable symptoms (Rodriguez et al. 2009). Unlike mycorrhizal fungi that colonise plant roots and grow into the rhizosphere, fungal endophytes reside entirely within plant tissues. Dark septate endophytes (DSE) are fungi colonising plant roots and are distinguished as a functional group based on the presence of darkly melanised inter- and intracellular septate hyphae and microsclerotia (Rodriguez et al. 2009). Recent studies reported a high diversity of DSE associated with the roots of willows or poplars and suggested that root colonisation by these fungi was not affected in highly metal-contaminated locations (Likar and Regvar 2009; Karliński et al. 2010; Regvar et al. 2010). These authors therefore suggested that DSE could improve metal tolerance of trees in highly contaminated areas.

The results obtained regarding tree interactions with those beneficial soil microorganisms could open up perspectives for their use in phytoremediation. Fungi isolated from contaminated soils develop adaptative mechanisms to tolerate metals (Colpaert et al., 2000; Colpaert et al., 2004; Gonçalves et al. 2009; Jourand et al. 2010; Colpaert et al. 2011). Some authors suggested that mycorrhizal fungi isolated from metal-polluted sites presented a higher tolerance to metals than fungi isolated from non-polluted sites (Gonçalves et al. 2009; Jourand et al. 2010; Colpaert et al. 2011). Moreover, plants inoculated with metal-tolerant mycorrhizal (Adriaensen et al. 2005; Redon et al. 2009; Colpaert et al. 2011) or endophytic (Shahabivand et al. 2012; Likar and Regvar 2013) fungi showed enhanced tolerance when compared to plants inoculated with non-tolerant fungi.

The present work was part of a large-scale project whose objective was to study the impact of genotypic variability on phytoremediation capacities of poplar (very) short-rotation coppice (SRC). As a first step toward tree-based phytoremediation assisted by symbiotic fungi, our objectives were: (1) to identify the main symbiotic fungi associated with poplar roots collected from a contaminated site, (2) to isolate metal-tolerant fungal endophytes, and (3) to provide some initial characterisation of the most metal-tolerant isolates.

Materials and methods

Description of the phytoremediation site and sampling

The experimental site was located northwest of Paris in Pierrelaye (latitude: 49°029 north; longitude: 2°176 east). The raw wastewaters from Paris have been used for more than 100 years for the irrigation of the Pierrelaye-Bessancourt plain, which was used for market gardening. As a result of these inputs, this area of more than 1200 ha has been strongly enriched in metals in the surface horizons compared to the natural pedogeochemical background values and to the usual local agricultural contents (Table 1). Zn, Cd, Pb, and Cu are the major pollutants, and the site is also slightly contaminated by Co, Cr, and Ni. Other soil characteristics are reported in Table 1. Crop production for human consumption was prohibited in the late 1990s (Lamy et al. 2006) and had to be replaced by alternative uses (e.g. production of non-accumulating cereals for animals, culture of miscanthus or woody species for fibre or energy production) providing other types of ecosystem services.

Within the Phytopop project (ANR PRECODD), a 3-ha field demonstration trial was implemented in 2007, with poplars grown as SRC for bioenergy production. Our major goal was to select the most efficient poplar hybrids for high biomass production in this contaminated zone. For the present study, the two most promising poplar hybrids (Chalot et al., unpublished data) were selected, namely *P. trichocarpa* x *P. maximowiczii* and *P. deltoides* x *P. nigra*. Fine roots (5–20 cm depth) of both poplar hybrids were collected in autumn from ten trees (3 years old) from four different areas of 400 m^2, giving a total of 40 samples per hybrid. The roots were traced to their origin to ensure that they were indeed connected to the selected tree and were sampled at a distance of 1 m from the trunk.

Evaluation of root colonisation by fungi

To evaluate the rate of root colonisation by ECM fungi, the entire root portions were first carefully washed with distilled water, and 300 randomly selected root tips per sample were subsequently examined and assessed as ECM or non-mycorrhizal under a stereomicroscope (×10 magnification). To evaluate the rate of root colonisation by AM fungi and DSE, the fungal structures within the roots were stained with methyl blue and observed under a microscope, as previously described (Trouvelot et al. 1986). Overall, 75 root fragments were observed per sample. The intensity of cortex colonisation by AM fungi (%AM) and the arbuscular density (%A)

Table 1 Metal contents and soil characteristics at the phytoremediation site of Pierrelaye

	Units	Phytoremediation site	NPBV	UAC
Cd total	mg/kg	2.2–4.0	0.02–0.14	0.2–0.4
Co total	mg/kg	5.4–13	2–4	3–8
Cr total	mg/kg	43–89	14–21	15–29
Cu total	mg/kg	99–230	2–6	8–19
Pb total	mg/kg	184–254	4–8	18–43
Ni total	mg/kg	19–42	4–8	6–20
Zn total	mg/kg	394–712	9–19	34–63
Cd (NH_4NO_3-extracted)	µg/kg	21–29	nd	nd
Cu (NH_4NO_3-extracted)	µg/kg	530–812	nd	nd
Pb (NH_4NO_3-extracted)	µg/kg	14–29	nd	nd
Zn (NH_4NO_3-extracted)	µg/kg	1400–2010	nd	nd
pH	–	6.8–7.4	nd	7.7–8.2
C total	g/kg	28–66	nd	10–11
N total	g/kg	1.6–2.7	nd	nd
P_2O_5 (Olsen)	g/kg	2.4–5.1	nd	nd

NPBV natural pedogeochemical background values from five deep horizons (from Lamy et al. 2006), *UAC* usual agricultural contents in surface horizons in the non-irrigated surrounding area (from Lamy et al. 2006), *nd* not determined

were calculated, as previously described (Trouvelot et al. 1986). Similarly, the rate of root colonisation by DSE was estimated by determining the frequency of brown/dark microsclerotia within cells (%MS). These latter fungi were hardly stained with the dye but appeared brown to black after root discoloration by KOH. It should be noted that this measure likely underestimates the colonisation rate by DSE; however, it was solely used here as a metric that allows comparisons among the samples.

Molecular identification of root-colonising fungi

The identification of fungi living on and/or inside poplar roots was performed as followed. Total DNA was extracted from approximately 100 mg of fine roots per sample using the DNeasy Plant Mini Kit (Qiagen S.A.S., Courtabœuf, France). DNAs extracted from 10 root samples of the same area were pooled (equimolar concentrations), and the composite sample was diluted 1:25 for PCR amplification. PCR was performed with the ITS1-ITS4 primer set (White et al. 1990) and the Phire® Hot Start II DNA Polymerase (Fisher Scientific, Illkirch, France). PCR products were purified and cloned into the pGEM-T® vector system I (Promega, Charbonnières-les-Bains, France). Amplification products were independently digested with MboI and TaqI (New England Biolabs, Ipswich, UK) according to the manufacturer's instructions. Representative RFLP patterns were further selected, and the corresponding plasmid DNAs were sequenced. At least two clones per RFLP pattern were sequenced; the other sequences were classified by RFLP typing.

Sequence similarities were determined using the BLASTN sequence similarity search tool (Altschul et al. 1997). Phylogenetic analyses were carried out using the sequences obtained in this study and those corresponding to the closest matches from GenBank. Sequences were aligned by ClustalW and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 6 (Tamura et al. 2013). Phylogenetic analyses were conducted using the Maximum Likelihood (ML) method implemented in MEGA with the pairwise deletion option for handling alignment gaps, and the evolutionary distances were computed using the Kimura 2-parameter method. Bootstrap tests were conducted using 1000 replicates. For the determination of operational taxonomic units (OTUs), we used both blastclust (http://toolkit.tuebingen.mpg.de/blastclust) and the rapidOTU pipeline (Legrand et al. 2008) with a 97 % identity threshold. The results were compared with the phylogenetic analyses to determine OTUs. DNA sequences obtained in this study were submitted to GenBank and corresponding accession numbers are given in Table 2.

Isolation of root-colonising fungi into pure culture and identification

To isolate root-inhabiting fungal strains into pure culture, roots were first washed with water, sterilised in 35 % hydrogen peroxide for 30 s, and rinsed three times in sterile deionised water. About 600 root fragments of approximately 1 cm were incubated on modified Melin-Norkrans medium (Brundrett et al. 1996), malt extract agar medium (malt: 12 g/l; agar: 15 g/l), NP2/2 medium (Benjdia et al. 2006), or Pachlewski medium (Pachlewski and Pachlewska 1974). The NP2/2 medium (pH 5.5) contained (mg/l): $CaCl_2$ (50), $MgSO_4$ (150), KH_2PO_4 (250), NaH_2PO_4 (4.5), Na_2HPO_4 (160), $(NH_4)_2SO_4$ (250), thiamine hydrochloride (0.04), and glucose (2500). Ampicillin (100 mg/l) and chloramphenicol (100 mg/l) were added to the media to avoid bacterial contaminations. Dishes were incubated at 25 °C in the dark from 1 week up to 3 months. Putative endophytic and mycorrhizal isolates were selected and identified via ITS sequencing, and corresponding sequences were aligned with fungal sequences obtained from root samples.

Table 2 Colonisation of poplar roots by fungi at the phytoremediation site of Pierrelaye

			P. trichocarpa × *P. maximowiczii*	*P. deltoides* × *P. nigra*
Fungal colonisation				
%AM			13.9 ± 1.4 a	22.3 ± 2.4 b
%A			2.1 ± 0.5 a	5.6 ± 0.8 b
%ECM			21.1 ± 3.5 a	21.1 ± 2.8 a
%MS			1.1 ± 0.3 a	3.7 ± 1.1 b
Identification and abundance of fungal OTUs				
OTU affiliation	Acc. number	Lifestyle	Relative abundance (%)	
Peziza ostracoderma	JX135041	ECM	0.93	12.82
Peziza sp.	JX135042	ECM	1.87	7.69
Geopora sp.	JX135043	ECM	0.93	0.00
Tuber rufum	JX135044	ECM	1.87	0.00
Geopora cervina	JX135045	ECM	0.93	0.00
Helotiales sp.	JX135046	ECM/E	0.00	5.13
H. mesophaeum	JX135047	ECM	0.93	0.00
H. populinum	JX135048	ECM	19.63	0.00
H. mesophaeum	JX135049	ECM	20.56	0.00
Pyrenochaeta sp.	JX135050	P/E	15.89	6.41
Pythium sp.	JX135051	P/E	0.00	11.54
Thanatephorus sp.	JX135052	P/E	1.87	1.28
Ilyonectria sp.	JX135053	P/E	0.93	0.00
Phoma exigua	JX135054	P/E	6.54	0.00
Paraphoma chrysanthemicola	JX135055	E	5.61	0.00
Unc. basal lineage fungus	JX135056	unkn	6.54	6.41
Mortierella hyalina	JX135057	S	0.93	0.00
Tetracladium sp.	JX135058	P/E	10.28	28.21
Rhizoctonia sp.	JX135059	P/E	0.93	7.69
Ceratobasidium sp.	JX135060	P/E	1.87	6.41
Psathyrella sp.	JX135061	S	0.00	1.28
Glomus sp.	JX135062	AM	0.93	5.13

Values are expressed as the means ($n = 40$) ± SE, and within a row, significant differences ($P < 0.05$, ANOVA, followed by Tukey's test) are indicated by different letters

%AM rate of arbuscular mycorrhizal colonisation, *%A* arbuscular density, *%ECM* rate of ectomycorrhizal colonisation, *%MS* microsclerotia frequency, *Unc.* uncultured, *E* endophytic, *P* parasitic, *S* saprotrophic, *unkn* unknown

Metal tolerance tests

The responses of isolates to various concentrations of cadmium, copper, lead, or zinc were assessed by growing the isolates on MP medium (NP2/2 medium supplemented with 5 g/l malt extract, pH 4.3). MP medium, which allowed the growth of all isolates used in this study, was chosen for consistency. Plugs (5 mm) were cut from the actively growing edge of 20-day-old colonies and placed on a sterile EDTA-treated cellophane membrane, on agar (Fluka, product #05039) MP medium amended with six concentrations of each metal. The concentrations used were 0, 10, 50, 100, 250, and 500 μM $CdCl_2$; 0, 0.05, 0.1, 0.5, 1, and 2 mM $CuCl_2$; 0, 0.5, 1, 2, 5, and 10 mM $Pb(NO_3)_2$; and 0, 1, 2, 5, 10, and 20 mM $ZnCl_2$. Colonies were harvested in the late exponential phase of growth, dried at 60 °C, and weighed. Five replicates were performed for each treatment, except for the controls ($n = 10$). For each isolate/metal combination, the minimum inhibitory concentration (MIC) was determined, and growth data were curve-fitted to obtain the effective concentration that inhibited 50 % of mycelial growth (EC_{50}).

Growth of the isolates on different carbon sources

The isolates were tested for their capacity to use different carbon sources. Growth assays were performed in a minimal medium (0.5 g/l KH_2PO_4; 1 g/l $MgSO_4 \cdot 7H_2O$; 2.3 g/l NH_4Cl; 100 μl/l Kanieltra solution (5 mg/l $MnSO_4 \cdot 4H_2O$; 8.5 mg/l

H_3BO_3; 0.3 mg/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$; 6 mg/l $FeCl_3$; 0.6 mg/l $CuSO_4{\cdot}5H_2O$; 2.7 mg/l $ZnSO_4{\cdot}7H_2O$), pH 5.5) containing 8 g of one of various compounds as sole carbon source (Table 6). Liquid cultures were shaken at 150 rpm for 3 weeks at 23 °C. For lignin, cellulose, and starch, degradation abilities were considered positive when the isolate showed mycelial development (>5 mm) around the initial plugs and formation of new small fungal pellets in comparison with the negative control (no carbon). Cellulolytic activities of the isolates were also estimated on agar plates. Isolates were grown for 2 weeks on agar minimal medium containing 5 g/l carboxymethyl cellulose, and plates were further stained by Congo red (0.1 % *m*/*v*) and washed three times with 1 M NaCl in 50 mM phosphate buffer (pH 7.0). Cellulase activity was indexed as the diameter of the clear zone (degradation halo) divided by the diameter of the colony.

Re-synthesis experiments between *Serenpidita vermifera* or Helotiales isolates and poplar clones

The poplar clone INRA 717-1B4 was used in the present experiment. For in vitro micropropagation, 2-cm-long cuttings from 4-week-old plantlets were grown on Murashige and Skoog medium (pH 5.8) (Murashige and Skoog 1962) in a growth room under controlled conditions (24 °C/18 °C, 14 h/10 h light/dark cycle, light intensity of 660 μmol/m^2/s). Petri dishes containing Murashige and Skoog medium were prepared, and after solidification, one half of the medium was removed. Three-week-old plantlets were transferred to the middle of the plates. The fungal treatments (isolates P02, P04, and P06) contained seven 8 mm, 15-day-old fungal plugs, whereas fungus-free controls were mock-inoculated with agar medium plugs. All experiments were repeated six times. After 2 months of incubation, the plants were harvested, and both shoot and root biomasses were measured. The total number of root tips was measured using the ImageJ software (version 1.47) (Schneider et al. 2012).

Fluorescence microscopy analyses

Roots from the re-synthesis experiment were analysed for fungal colonisation by epifluorescence microscopy. Selected 1-cm-long root segments were incubated for 10 min with 5 μg/mL WGA-AF488 (Wheat Germ Agglutinin - Alexa Fluor® 488 conjugate, Molecular Probes) to specifically stain fungal structures. A filter was used to select the wavelength of the excitation light to the 475–500 nm band, and the fluorescence emission was recorded above 520 nm. Root autofluorescence was observed by using a 405-nm UV lamp and by recording the fluorescence emission in the 420–470 nm band. A Nikon D1 digital camera was used to document results, and the images were visualised and processed with the ImageJ software. Mycelia from pure cultures were also stained with WGA-AF488 and similarly processed by fluorescence microscopy.

Statistical analyses

Data were analysed with XLSTAT 2011 (Addinsoft, Inc.). To evaluate the EC_{50}, a nonlinear regression analysis was conducted with the best-fitted sigmoid equation model. A one-way ANOVA with Tukey's post hoc test ($P < 0.05$) was used to evaluate significant differences between data in the following experiments: (i) fungal root colonisation assessment, (ii) EC_{50} determination (ii) carbon utilisation tests, and (iii) growth responses in the re-synthesis experiments.

Results

Fungal colonisation of poplar roots at the phytoremediation site

Because poplar is able to form symbiotic structures with both AM and ECM fungi, both types of mycorrhizal fungi were investigated. Both poplar genotypes were very similarly colonised by ECM fungi (Table 2). Both poplar genotypes were also colonised by AM fungi producing typical arbuscules and vesicles, and colonisation rate and arbuscular density were significantly higher for *P. deltoides* × *P. nigra* compared to *P. trichocarpa* × *P. maximowiczii* (Table 2). The DSE fungal structures microsclerotia were also observed on both root hybrids. The intensity of root colonisation by microsclerotia was higher for *P. deltoides* × *P. nigra* when compared to the other clone (Table 2).

The ITS1-ITS4 primer set (White et al. 1990) was used to assess the fungal colonisers of poplar roots from the two hybrids (Table 2). Overall, a total of 22 fungal operational taxonomic units (OTUs) were recovered from poplar roots. Nineteen and 12 OTUs were identified in *P. tricho carpa* × *P. maximowiczii* and in *P. deltoides* × *P. nigra* roots, respectively. Ten were identified in samples from both hybrids. Ten OTUs were assigned to mycorrhizal fungi by BLAST analyses. Six OTUs belonged to Ascomycota (*Peziza*, *Geopora*, *Tuber*, Helotiales sp.), three to Basidiomycota (*Hebeloma*) and one to Glomeromycota (*Glomus*). Among the ascomycetous OTUs, five belonged to the Pezizales. The *Peziza* genus was the most highly represented and was associated with both poplar genotypes. The OTU belonging to the Helotiales was ambiguously assigned by BLAST analyses either to ericoid mycorrhizal or to ECM fungi from environmental samples, but the lifestyle of this species remains unknown. *Hebeloma* was the only ECM basidiomycetous genus recovered from this analysis. Non-mycorrhizal fungal taxa, such as saprotrophic or endophytic/parasitic fungi, were also identified from poplar roots

(Table 2). These fungi belonged either to Ascomycota or Basidiomycota. The most represented saprotrophic, parasitic or endophytic fungal OTUs were *Ceratobasidium* sp., *Tetracladium* sp., *Rhizoctonia* sp., and *Pyrenochaeta* sp..

Isolation and taxonomic assignment of fungal strains

The isolation protocol allowed us to obtain a final set of 38 fungal colonies corresponding to putative mycorrhizal species. After redundancy checking (comparison of ITS sequences), 8 fungal isolates were definitively selected for subsequent experiments (Table 3). Five isolates belonged to the *Hebeloma* genus (P13, P14, P17, P23, P33), two to an unknown species of the Helotiales (P02, P06), and one to the Sebacinales (P04).

ITS sequences were further compared with those obtained through the OTU analysis together with those previously published. Phylogenetic trees were then constructed based on phylogenies of the *Hebeloma* genus (Boyle et al. 2006) (Fig. 1), the Sebacinales (Riess et al. 2014) (Fig. 2) and the Helotiales (Wang et al. 2006) (Fig. 3).

The ITS sequences of the five *Hebeloma* isolates P13, P14, P17, P23 and P33 that originated from the Pierrelaye site matched with the OTUs previously identified from roots (JX135047, JX135073, JX135048, JX135072 and JX135068, respectively) (Fig. 1, Table 3). The phylogenetic analysis divided the five isolates within two major clades in which several *Hebeloma* species clustered together (Fig. 1). More particularly, these results suggested that P14, P17 and P33 belonged to the *Hebeloma denudata* clade, whereas P13 and P23 belonged to the *Hebeloma indusiata* clade.

The ITS sequence of the P04 isolate was closely related to the OTU JX135065, previously identified from poplar roots by using a basidiomycetous-specific primer set (ITS1F-ITS4B) in a preliminary experiment (data not shown). Both sequences clustered into clade B of the Sebacinales phylogenetic tree and were closely related to the *S. vermifera* species (Fig. 2, Table 3). Other environmental sequences of Sebacinales retrieved from GenBank, previously identified from poplar roots, were also used to construct the phylogenetic tree, but all clustered within clade A (Fig. 2). When grown on MP medium, *S. vermifera* P04 appeared white and slightly glossy with regular margins. Hyphae were cylindrical, thin-walled and transparent. With age, monilioid cells, smooth and colourless, appeared (Fig. 4a, d).

Table 3 Pure culture mycorrhizal and endophytic isolates from poplar roots

Isolates	Taxonomic affiliation	Lifestyle	Nearest blast hit	
			Acc. number	Identity (%)
P14	*Hebeloma populinum*	ECM	JX135073	99
P17	*Hebeloma populinum*	ECM	JX135048	99
P33	*Hebeloma populinum*	ECM	JX135068	99
P13	*Hebeloma mesophaeum*	ECM	JX135048	99
P23	*Hebeloma mesophaeum*	ECM	JX135072	99
P06	Helotiales new clade	unkn	JX135046	98
P02	Helotiales new clade	unkn	JX135046	96
P04	*Serendipita vermifera*	unkn	JX135065	99

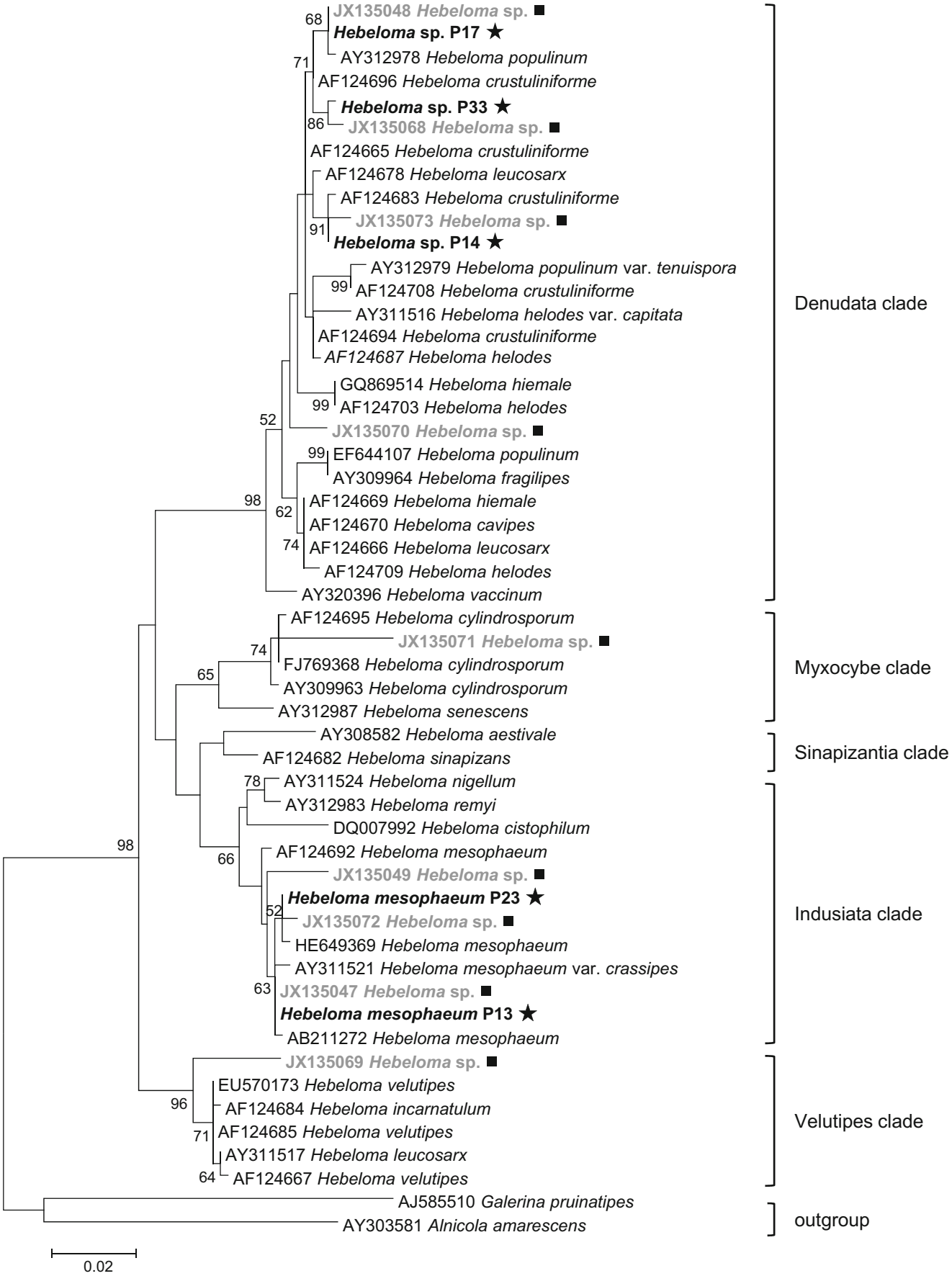
ECM ectomycorrhizal, *unkn* unknown

Fig. 1 Maximum Likelihood phylogenetic tree based on rDNA ITS sequence data from *Hebeloma* OTUs identified by ITS-RLFP analysis (*squares*) or pure culture strains isolated from poplar roots (*stars*) with known and selected fungal species from GenBank with high sequence similarities. Bootstrap values over 50 % (1000 replicates) are indicated above the branches. The different clades described by Boyle et al. (2006) are represented

The ITS sequences of the isolates P02 and P06 shared 97 % identity and were closely related to the fungal OTU JX135046, previously identified from poplar roots. The phylogenetic analysis of Helotiales revealed that P02 and P06 clustered in a new clade, not described by Wang et al. (2006). This new clade was closely related to the *Leohumicola* clade (Fig. 3). BLAST searches allowed us to retrieve other ITS sequences belonging to this new clade. These sequences arose from either soil or root environmental samples, but none from isolated strains. These taxa were identified from a variety of plants (ligneous or herbaceous) from different continents (America, Asia, Europa and Oceania) (Fig. 3). On MP medium, colonies of the Helotiales P06 and P02 were white to cream-coloured with an irregular surface. Aging hyphae of Helotiales P02 and P06 turned green after 7 and 21 days of growth, respectively (Fig. 4b, c). Hyphae were cylindrical and thin-walled. With aging, the cells and hyphae of Helotiales P02 and P06 became swollen and formed a chain of spherical spore-like structures (Fig. 4e, f).

Metal tolerance assays

The EC_{50} and MIC values were determined for the isolates on a set of four metals (Table 4). Metal tolerance of the fungal isolates varied between isolates and metal species. The Helotiales P02 and P06 isolates exhibited the highest tolerance to Cd, Cu, Pb and Zn compared to the other isolates, whereas the isolates P13 and P23 (belonging to the *H. denudata* clade) exhibited a weak tolerance to the metals tested. The EC_{50} of the isolates of the *H. indusiata* clade (P14, P17 and P33) were similar to those of P13 and P23 but were slightly higher. The


JX135048 Hebeloma sp. ■
Hebeloma sp. P17 ★
AY312978 Hebeloma populinum
AF124696 Hebeloma crustuliniforme
Hebeloma sp. P33 ★
JX135068 Hebeloma sp. ■
AF124665 Hebeloma crustuliniforme
AF124678 Hebeloma leucosarx
AF124683 Hebeloma crustuliniforme
JX135073 Hebeloma sp. ■
Hebeloma sp. P14 ★
AY312979 Hebeloma populinum var. tenuispora
AF124708 Hebeloma crustuliniforme
AY311516 Hebeloma helodes var. capitata
AF124694 Hebeloma crustuliniforme
AF124687 Hebeloma helodes
GQ869514 Hebeloma hiemale
AF124703 Hebeloma helodes
JX135070 Hebeloma sp. ■
EF644107 Hebeloma populinum
AY309964 Hebeloma fragilipes
AF124669 Hebeloma hiemale
AF124670 Hebeloma cavipes
AF124666 Hebeloma leucosarx
AF124709 Hebeloma helodes
AY320396 Hebeloma vaccinum
Denudata clade
AF124695 Hebeloma cylindrosporum
JX135071 Hebeloma sp. ■
FJ769368 Hebeloma cylindrosporum
AY309963 Hebeloma cylindrosporum
AY312987 Hebeloma senescens
Myxocybe clade
AY308582 Hebeloma aestivale
AF124682 Hebeloma sinapizans
Sinapizantia clade
AY311524 Hebeloma nigellum
AY312983 Hebeloma remyi
DQ007992 Hebeloma cistophilum
AF124692 Hebeloma mesophaeum
JX135049 Hebeloma sp. ■
Hebeloma mesophaeum P23 ★
JX135072 Hebeloma sp. ■
HE649369 Hebeloma mesophaeum
AY311521 Hebeloma mesophaeum var. crassipes
JX135047 Hebeloma sp. ■
Hebeloma mesophaeum P13 ★
AB211272 Hebeloma mesophaeum
Indusiata clade
JX135069 Hebeloma sp. ■
EU570173 Hebeloma velutipes
AF124684 Hebeloma incarnatulum
AF124685 Hebeloma velutipes
AY311517 Hebeloma leucosarx
AF124667 Hebeloma velutipes
Velutipes clade
AJ585510 Galerina pruinatipes
AY303581 Alnicola amarescens
outgroup
68
71
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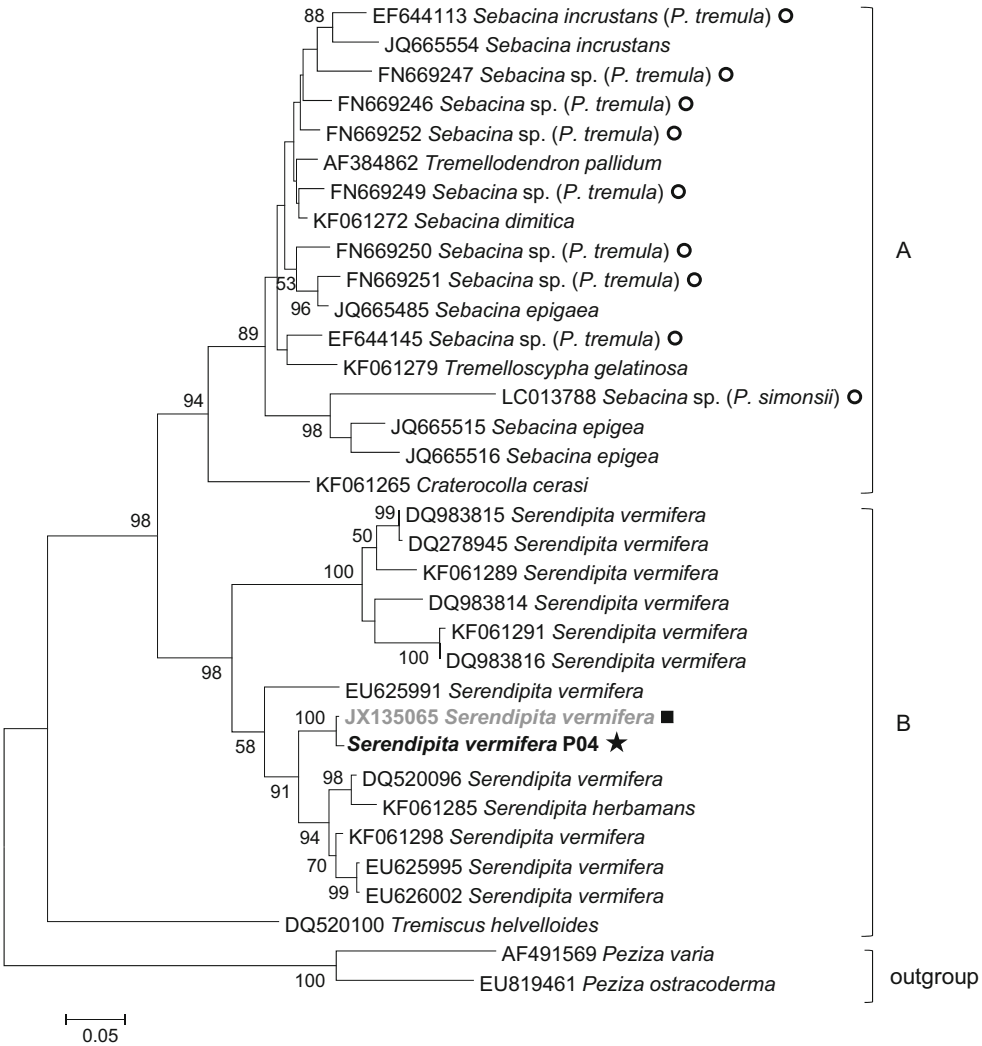

Fig. 2 Maximum Likelihood phylogenetic tree based on rDNA ITS sequence data from Sebacinales identified by ITS-RLFP analysis (*square*) or from pure-cultured isolated strains (*star*) with known and selected fungal species from GenBank with high sequence similarities. GenBank Sebacinales sequences arising from ECM poplar roots are denoted by open circles. Bootstrap values over 50 % (1000 replicates) are indicated above the branches. The two Sebacinales clades (A & B) described by Riess et al. (2014) are represented

S. vermifera P04 isolate presented a moderate tolerance to Cd and Cu.

Lifestyles of the Helotiales P02 and P06 isolates and the *S. vermifera* P04 isolate

A re-synthesis experiment with the *S. vermifera* P04 isolate and the Helotiales P02 and P06 isolates was conducted to obtain structural and functional evidence of the mycorrhizal or endophytic status of these isolates. Our experimental interaction assays demonstrated that two isolates had positive effects on poplar (Table 5). Poplar cuttings inoculated by P02 and P04 showed a more extensive root branching pattern when compared to the mock-inoculated plants. The number of root tips indeed doubled in the presence of the fungal inocula. When compared to the control plants, both root and shoot fresh weights of poplar inoculated by the Helotiales isolates were not affected. Inoculation by *S. vermifera* P04 did not modify root fresh weight. However, the shoot fresh weight significantly increased by 15 % in the presence of *S. vermifera* P04 compared to mock-inoculated plants (Table 5).

Roots were labelled with WGA-AF488, a fungus-specific dye, and further analysed by epifluorescence microscopy. Our observations confirmed the lack of fungal hyphae/structures in roots of the mock-inoculated plants. When inoculated by Helotiales P02, roots were covered by hyphae but no fungal structures could be observed within roots (data not shown). In contrast, numerous microsclerotia-like structures were observed within roots of plants inoculated by *S. vermifera* P04 (Fig. 5a, b) or Helotiales P06 (Fig. 5c, d). These structures were only detected in the cortical cell layer and not in the endodermis nor in the central cylinder (Fig. 5). The hyphae of the microsclerotia were thin-walled, non-coloured, septate and usually tightly packed within root cells. In some cases, microsclerotia formed by *S. vermifera* P04 were sparsely packed (Fig. 5a).

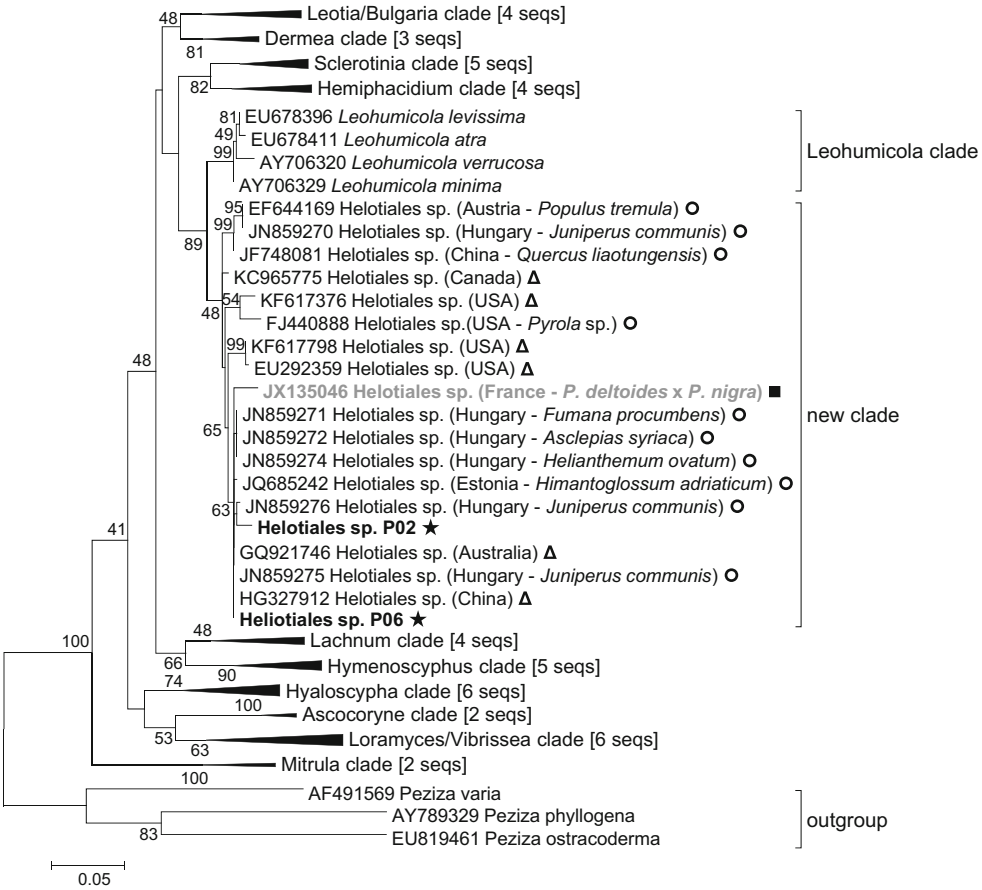

Fig. 3 Maximum Likelihood phylogenetic tree based on rDNA ITS sequence data from Helotiales identified by ITS-RLFP analysis (*square*) or from pure-cultured isolated strains (*stars*) with known and selected fungal species from GenBank with high sequence similarities. Sequences retrieved from GenBank derived from either soil samples (*open triangles*) or roots of diverse plants (*open circles*), and both the origin and the name of the host plant are given in brackets. Bootstrap values over 40 % (1000 replicates) are indicated above the branches. The different clades described by Wang et al. (2006) are represented and are compressed for simplicity. For each grouping, the number of sequences contained within is provided in *square brackets*, and a representative genus is indicated

Growth of the isolates on different carbon sources

The ability of P02, P04 and P06 to use various simple and complex carbon substrates was investigated by growing them in liquid cultures with various substrates as sole carbon source. *Piriformospora indica* was used as an endophytic reference. Cellulose, starch and lignin substrates were insoluble and adhered to the hyphae at the time of harvesting, increasing

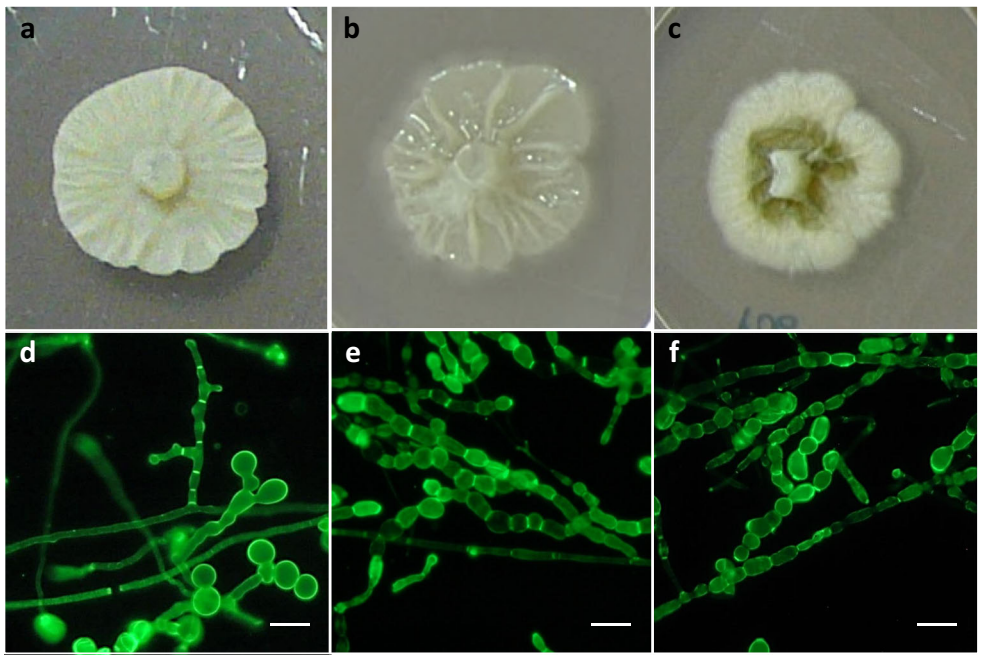

Fig. 4 Morphological characteristics of the fungal isolates *Serendipita vermifera* P04, Helotiales P02 and Helotiales P06. **a** Colony of *S. vermifera* P04 at day 30; **b** colony of Helotiales P06 at day 15; **c** colony of Helotiales P02 at day 15; **d** cylindrical and monilioid hyphae without clamps of *S. vermifera* P04; **e** hyphal branching and monilioid cells of Helotiales P06; **f** hyphal branching and monilioid cells of Helotiales P02. The hyphae were stained with WGA-AF488 and observed under a fluorescence microscope. Scale bars = 15 μm

Table 4 Metal tolerance of the isolates

Isolates	EC_{50}[a] (mM)				MIC[b] (mM)			
	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
P14	0.08 ± 0.02 bc	0.15 ± 0.00 c	0.81 ± 0.03 de	0.85 ± 0.07 d	0.25	0.5	2.0	5.0
P17	0.09 ± 0.03 bc	0.14 ± 0.04 c	1.08 ± 0.14 c	1.07 ± 0.01 d	0.25	1.0	2.0	2.0
P33	0.24 ± 0.02 bc	0.24 ± 0.03 c	0.96 ± 0.13 cd	1.34 ± 0.11 d	>0.5	1.0	2.0	5.0
P13	0.02 ± 0.01 c	0.07 ± 0.01 c	0.56 ± 0.01 f	1.40 ± 0.42 cd	0.25	0.5	2.0	5.0
P23	0.01 ± 0.00 c	0.10 ± 0.03 c	0.61 ± 0.23 ef	0.85 ± 0.12 d	0.05	0.5	2.0	2.0
P06	>0.5 a	1.96 ± 0.26 a	3.82 ± 0.15 b	6.19 ± 0.60 b	>0.5	>2.0	10	20
P02	>0.5 a	2.00 ± 0.25 a	5.07 ± 0.05 a	9.50 ± 0.48 a	>0.5	>2.0	10	20
P04	0.33 ± 0.01 b	0.70 ± 0.02 b	1.06 ± 0.01 c	1.94 ± 0.15 c	>0.5	2.0	2.0	5.0

[a] EC_{50} is the effective concentration of metals that inhibited mycelial growth by 50 %; sigmoid curves were fitted to the growth data (dry weight), and EC_{50} were deduced. Values are expressed as the means of 3 biological replicates ± SD. Statistically significant differences ($P < 0.05$, ANOVA, followed by Tukey's test) are indicated by different letters

[b] MIC is defined as the minimum inhibitory concentration of metals that completely inhibited fungal growth

the apparent biomass yield of all isolates on these substrates. For this reason, growth on these three carbon sources was not compared to other substrates. From visual observations of these treatments, however, it was possible to qualitatively determine whether mycelial growth was higher than in the non-carbon treatment.

Growth of *P. indica* was optimal on glucose, sucrose and xylan, but poor on CMC, xylose and especially mannitol (Table 6). The response pattern of *S. vermifera* P04 to the different carbon substrates was similar to that of *P. indica*. The only exception was the absence of growth on cellulose for *S. vermifera* P04. Growth of Helotiales P06 was higher than that of Helotiales P02 on most carbon sources. Growth of both isolates was optimal on glucose-amended media but poor on CMC- or xylose-supplemented media. However, Helotiales P02 and P06 slightly differed in their utilisation patterns of the other carbon sources. When compared to Helotiales P02, Helotiales P06 grew better on cellobiose, maltose and xylan. Surprisingly, moderate growth of Helotiales P02 was observed on lignin-containing medium (Table 6). To confirm the cellulose degradation ability of the different isolates, another experiment was set up on agar medium to confirm the degradation of CMC. A CMC solubilisation halo was observed after Congo red staining of the plates. *S. vermifera* P04 produced a small halo below the centre of the fungal colony (Fig. 6). Large halos were observed in the presence of Helotiales P02 and P06, and to a lesser extent with *P. indica*. The largest degradation halo was induced by Helotiales P02 (Fig. 6).

Table 5 Effect of fungal inoculation on in vitro growth of poplar cuttings

Treatment	SFW (mg/plant)	RFW (mg/plant)	Number of root tips
Control	137.7 ± 15.3 a	15.9 ± 2.6 a	14.2 ± 2.9 a
P02	117.8 ± 17.6 a	21.7 ± 4.9 a	28.0 ± 4.9 ab
P04	158.4 ± 17.7 b	16.6 ± 4.9 a	28.3 ± 2.9 b
P06	124.6 ± 11.5 a	21.1 ± 2.5 a	33.3 ± 7.2 b

Cuttings were grown for 2 months on agar Murashige & Skoog medium. Values are the means of six replicates ± SE. Statistically significant differences ($P < 0.05$, ANOVA, followed by Tukey's test) are indicated by different letters

SFW shoot fresh weight, *RFW* root fresh weight, *control* mock-inoculated poplar cuttings

Discussion

Fungi associated with poplar roots from a metal-contaminated site

We identified both endophytic and mycorrhizal (AM and ECM) fungi from poplar roots. Similar colonisation rates were reported for several poplar clones from contaminated soils, although higher ECM colonisation rates are often reported (Khasa et al. 2002; Gehring et al. 2006; Karliński et al. 2010). However, those data were derived from studies with older trees, and poplar preferentially associates with AM species during the first years of growth. This statement was confirmed by the analysis of poplar root colonisation over time from our experimental site (Chalot et al., unpublished data).

In the present study, the number of fungal OTUs was consistent with that reported in a study focusing on the fungal diversity of poplar roots from a metal-polluted soil using the same molecular method (Krpata et al. 2008). The AM fungal taxa we identified belonged to the *Glomus* genus. This genus is widespread in metal-contaminated environments (Turnau et al. 2001; Vallino et al. 2006; Zarei et al. 2008), suggesting that these *Glomus* species could be metal tolerant. We also identified ECM OTUs belonging to *Peziza*, *Tuber*, *Geopora*

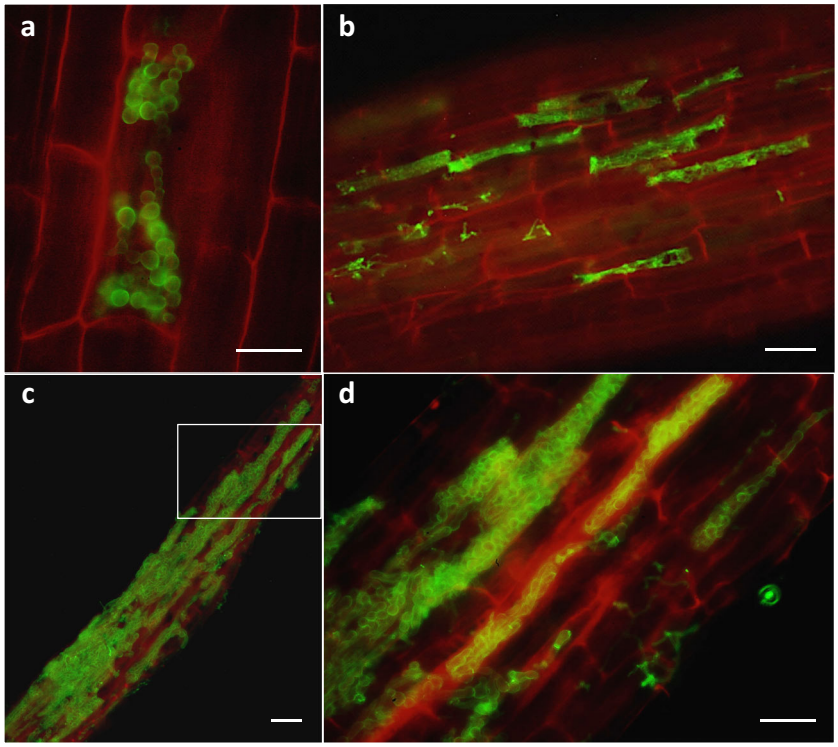

Fig. 5 Fungal structures of *Serendipita vermifera* P04 (**a**, **b**) and Helotiales P06 (**c**, **d**) within poplar roots. Hyphae were stained with WGA-AF488. Plant tissues are displayed in *red* and fungal tissues in *green*. The *white rectangle* indicates the location of the enlarged zone displayed in **d**. Scale bars = 20 μm

and *Hebeloma*, as reported in different studies investigating fungal communities from poplar roots (Krpata et al. 2008; Stefani et al. 2009; Hrynkiewicz et al. 2010; Danielsen et al. 2013; Karliński et al. 2013; Bonito et al. 2014). Notably, Krpata et al. (2008) found species belonging to the genera *Peziza*, *Laccaria*, *Tuber*, *Sebacina* and *Hebeloma* along with several ericoid mycorrhizal fungi in the roots of *Populus tremula* sampled from a metal-polluted (Pb, Zn and Cd) site.

Endophytic fungi are involved in the phytostabilisation processes of the plants that they are associated with. The plant growth-promoting DSE isolate DB146 has been used to enhance the establishment and growth of willow (*Salix caprea*) in a metal-contaminated soil (Likar and Regvar 2013). Interestingly, one undetermined fungus belonging to the Helotiales was also identified in our study and was assigned to either ECM or endophytic fungi according to the different

Table 6 Growth of the isolates on different carbon sources

Substrate	Isolate			
	P02	P04	P06	*P. indica*
CMC	44.4 ± 1.9 g	38.6 ± 1.2 d	47.5 ± 1.6 e	41.9 ± 3.0 fg
Cellobiose	79.4 ± 5.9 abc	40.2 ± 3.2 c	120.4 ± 11.3 ab	57.5 ± 0.6 de
Fructose	72.0 ± 1.6 cde	67.4 ± 0.5 b	104.7 ± 1.88 abc	60.1 ± 2.6 cd
Glucose	111.1 ± 15.8 a	68.2 ± 8.0 b	147.1 ± 15.8 a	71.5 ± 3.4 ab
Maltose	76.5 ± 1.7 bcd	78.2 ± 5.5 b	102.7 ± 28.6 abcd	64.8 ± 0.2 ab
Mannitol	67.1 ± 7.3 def	23.4 ± 2.5 d	93.6 ± 2.3 abcd	18.2 ± 1.3 g
Sucrose	85.6 ± 1.8 ab	78.6 ± 6.9 b	82.6 ± 19.0 cd	73.6 ± 3.6 a
Xylan	66.8 ± 2.6 ef	103.2 ± 2.0 a	134.0 ± 26.8 ab	71.8 ± 7.4 ab
Xylose	60.0 ± 6.2 fg	38.9 ± 1.0 d	64.4 ± 5.7 de	46.4 ± 1.7 ef
Cellulose[a]	+	−	+	+
Lignin[a]	+	−	−	−
Starch[a]	+	+	+	+

Values are expressed as the means (mg dry weight) of 3 biological replicates ± SD. Liquid cultures were harvested after 3 weeks of growth. Statistically significant differences ($P < 0.05$, ANOVA, followed by Tukey's test) are indicated by different letters

CMC carboxymethyl-cellulose

[a] Due to the insolubility of these compounds, only qualitative growth of the fungi was recorded (+: growth, −: absence of growth)

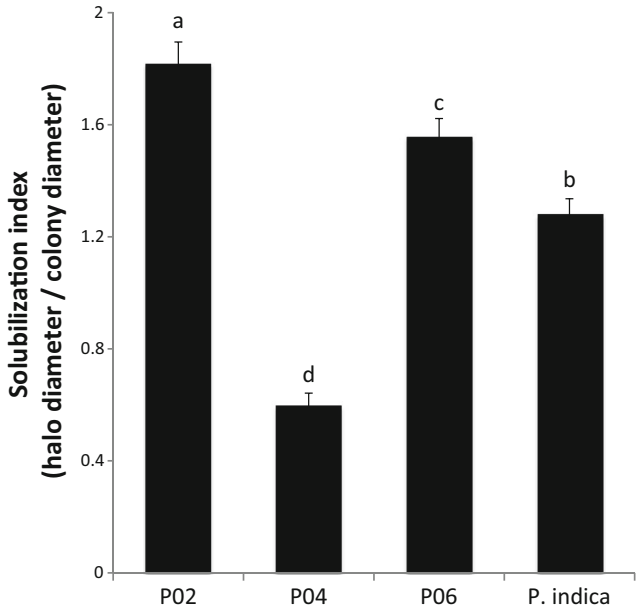

Fig. 6 Carboxy-methyl cellulose solubilisation by the endophytic isolates. The solubilisation index was calculated as the ratio between the diameter of the solubilisation halo and the diameter of the colony. *Piriformospora indica* was used as reference. Values are the means of three replicates ± SE. Statistically significant differences ($P < 0.05$, ANOVA, followed by Tukey's test) are indicated by *different letters*

blast results. Moreover, three putative endophytic isolates were established in pure culture and were further studied.

Metal tolerance of the isolates

The identification of fungi that could form symbiotic association with poplar roots is a prerequisite to any fungal-based bioaugmentation trial. One of our objectives was therefore to isolate some of the major mutualistic species associated with poplar from this metal-enriched environment. Metal-tolerant strains better protect host plants against metal toxicity, whether they are mycorrhizal (Adriaensen et al. 2005; Redon et al. 2009; Colpaert et al. 2011) or endophytic fungi (Shahabivand et al. 2012; Likar and Regvar 2013). Therefore, the different isolates were screened for their tolerance levels on a set of metals.

The Helotiales P02 and P06 isolates were the most tolerant to metals, while *S. vermifera* P04 was moderately tolerant and *Hebeloma* isolates were the least tolerant. Interspecific variations have been demonstrated in a number of studies of axenically cultured ECM or endophytic fungi (Blaudez et al. 2000; Ray et al. 2005; Fomina et al. 2005; Ban et al. 2012), which may be due to the presence or absence of different tolerance mechanisms within the different isolates, or to differential expression levels of genes/proteins involved in these mechanisms (Bellion et al. 2006). Interestingly, when metal contamination increases at an experimental site, mycorrhizal colonisation of *S. caprea* roots decreases while colonisation by DSE fungi was not affected (Likar and Regvar 2009; Regvar et al. 2010). Thus, considering the high metal tolerance of the endophytic isolates compared to the mycorrhizal isolates, we focused on the former to determine their lifestyle and capacity to improve plant growth.

Characterisation of new endophytic isolates of poplar roots

The nearest BLAST matches poorly assigned the fungal isolates P02, P04 and P06 as "uncultured mycorrhizal fungus from environmental samples". However, in literature, members of the Helotiales or Sebacinales have been described as endophytes, mycorrhiza-forming fungi or even plant pathogens (Wang et al. 2006; Selosse et al. 2009). A set of experiments including phylogenetic analyses, morphological observations, capacity to use various carbon substrates and a re-synthesis experiment with poplar cuttings were conducted to address their mycorrhizal or endophytic status.

Sebacinales can be endophytic, ECM, ericoid mycorrhizal, ectendomycorrhizal, or orchid mycorrhizal (Selosse et al. 2009; Oberwinkler et al. 2013). To our knowledge, only ECM associations between Sebacinales and Salicaceae have been reported (Oberwinkler et al. 2013). Moreover, ECM Sebacinales only clustered into clade A (Fig. 2); while *S. vermifera* P04 clustered into clade B, whose members are either endomycorrhizal fungi of autotrophic orchids and Ericaceae, or endophytic of liverworts (Selosse et al. 2009). Morphologically, hyphae and pure culture colonies of *S. vermifera* P04 resemble those of *Serendipita herbamans*, an endophytic fungus, also clustered into clade B (Riess et al. 2014). Growth of *S. vermifera* P04 on the various carbon sources was similar to that of *P. indica*, a well-known biotrophic fungus from Sebacinales. *S. vermifera* P04 was able to use cell wall-related carbohydrates, indicating the production of cellulolytic enzymes, suggesting that this fungus could also act as an endophyte colonising root cells. In our re-synthesis experiments, *S. vermifera* P04 formed microsclerotia-like structures within poplar roots. Moreover, poplar root development and shoot biomass were stimulated in inoculated plants compared to mock-inoculated controls. We therefore provide the first evidence that *S. vermifera* is a growth-promoting endophyte of poplar.

Members of the Helotiales have a wide range of lifestyles and have been described as plant pathogens, endophytes, nematode-trapping fungi, mycorrhiza-forming fungi, fungal or ECM parasites, saprobes and wood rot fungi (Wang et al. 2006). In this study, two isolates (P02 and P06) belonging to the Helotiales were grown in pure culture. Their ITS sequences presented 97 % identity. This result suggests that P02 and P06 might belong to the same species or to two closely related species. These two isolates were closely related to fungi isolated from various plants, woody or herbaceous

and from various continents. In this regard, this taxon seems to be widely distributed. The ITS sequences of the P02 and P06 isolates clustered into a new clade within the Helotiales, but diverged from the accession sequences of *Leohumicola* species included in the analysis. Therefore, Helotiales P02 and P06 could belong to either a new species or a previously morphologically described species for which no molecular data exists. The DSE isolate DB146, closely related to the *Leohumicola* genus, was highly tolerant to Zn and Pb and improved the growth and the tolerance of *S. caprea* cuttings in a metal-enriched soil (Likar and Regvar 2013). The isolate P06 stimulated root development of poplar in vitro, even if root biomass was not affected. These isolates were highly tolerant in vitro and could potentially protect host plants against metal-induced toxic effects, as earlier demonstrated for endophytic (Shahabivand et al. 2012; Likar and Regvar 2013) and mycorrhizal (Adriaensen et al. 2005; Redon et al. 2009; Colpaert et al. 2011) fungi. However, further plant experiments, including metal-contaminated soils and dose response analyses, will be required to demonstrate whether the symbiosis with these new endophytes would have a stronger effect on plant health than a symbiosis with ECM fungi such as *Hebeloma* sp. under heavy metal exposure. Particularly, endophytic and mycorrhizal fungi should be included, in single and mixed inoculations. The P02 and P06 isolates also degraded different cell wall-related carbohydrates, suggesting that production of fungal cellobiohydrolases, endoglucanases and β-glucosidases may enable complete hydrolysis of cellulose to glucose. The complete use of cellulose is unsurprising, as these enzymes are presumably required for host cell penetration (Smith and Read 2008). Moreover, from our resynthesis experiments, P06 seemed to be an endophyte of poplar roots, as demonstrated by the presence of hyphae and microsclerotia within root cells. Such structures were not identified within roots colonised by Helotiales P02 and this strain could: (i) be incompatible with the poplar hybrid we have used, (ii) be a casual rhizosphere inhabitant, or (iii) request different experimental conditions to associate with poplar roots. However, further experiments will be necessary to demonstrate the lifestyle of this isolate.

Conclusions and perspectives

Our results advance the understanding of fungal diversity associated with poplar in metal-polluted sites. The metal-tolerant isolates may be considered novel root endophytes of poplar. *S. vermifera* P04 could be a promising candidate to assist poplar growth in contaminated environments. However, further inoculation experiments in metal-polluted soils should be tested prior to its use in phytoremediation field trials. We already demonstrated that poplar growth, in large-scale plantations, was enhanced through inoculation with AM fungi at the same metal-polluted site (Chalot et al. unpublished data). The inoculation of poplar by endophytic consortia could be used as a new strategy for tree-based phytoremediation projects. Further experiments will be needed to assess whether *S. vermifera* P04 also has the potential to promote growth of a wide range of terrestrial plants, including food crops, as demonstrated for *P. indica*.

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3.2.4 Discussion

En conclusion, les isolements ont permis d'obtenir de nombreux champignons endophytes et ectomycorhiziens issus de sols contaminés. **Au total, 117 souches de champignons composent la collection du laboratoire débutée en 2007** (Annexe 4).

En comparaison avec les analyses moléculaires, les techniques basées sur la culture de champignons montre des résultats comparables. Par exemple sur le site de Tavaux, un grand nombre de *Pyrenochaeta* ont été isolés et sont également retrouvés par la technique de séquençage Ion Torrent (Zappelini *et al.*, 2015). De même, les isolats *Ilyonectria sp*., *Fusarium sp*., *Phoma sp*., *Bionectria sp*., *Leptodontidium sp*., *Exophiala sp., Mortierella sp., Lachnum sp., Cadophora. sp., Pyrenochaeta*. *sp.,* et *Tetracladium sp.* ont déjà été identifiés dans la rhizosphère de peuplier (Stefani *et al.*, 2009; Gottel *et al.*, 2011; Danielsen *et al.*, 2012; Shakya *et al.*, 2013; Bonito *et al.*, 2014) y compris sur sols contaminés (Zappelini *et al.*, 2015; Foulon *et al.*, 2016b; Lacercat-Didier *et al.*, 2016). Les genres *Geopora* et *Peziza*, morphotypes majoritaires sur les racines, n'ont pas pu être isolés mais semblent être des champignons ubiquistes retrouvés dans plusieurs études sur le peuplier (Hrynkiewicz *et al.*, 2010; Zappelini *et al.*, 2015; Foulon *et al.*, 2016b; Lacercat-Didier *et al.*, 2016). *Geopora* était d'ailleurs le seul genre présent dans tous les sols et les racines des différents clones de peuplier, mais absent des sols témoins (parcelle maïs et sol non planté) à Pierrelaye (Foulon *et al.*, 2016a). La technique basée sur la culture, tout comme la technique de métabarcoding, ont montré que des facteurs, liés à l'âge du peuplier, au site d'étude ou au niveau de contamination, semblent fortement impacter les espèces isolées.

Les techniques basées sur la culture pour l'étude de la colonisation fongique des racines décrit un spectre d'espèces détectables largement inférieur aux méthodes moléculaires, puisque seulement 17% des espèces fongiques connues peuvent être cultivées avec succès (Bridge & Spooner, 2001). De plus, seuls 30 à 40 % des isolements réalisés permettent d'obtenir un mycélium pur ; le reste correspondant aux isolements contaminés par des bactéries, ou présentant un développement de plusieurs mycélia à la fois, ou une absence de croissance (Kwasna *et al.*, 2008; Menkis & Vasaitis, 2011; Chen *et al.*, 2012). Pour les champignons mycorhiziens, le taux d'isolement de ces souches est encore plus faible, de l'ordre de 0,1 à 1 % (Menkis *et al.*, 2005; Kwasna *et al.*, 2008; Chen *et al.*, 2012). Cette technique, basée sur la culture, a l'avantage de faire la distinction entre les microorganismes morts et ceux qui sont actifs (Gams, 1992).

De nombreux champignons uniques et rarement cultivés ont été identifiés à partir de racines de peuplier. En effet, les isolats appartenant aux *Sordariomycètes* et aux *Hélotiales* sont difficiles à identifier en raison du faible nombre de séquences dans GenBank ou de l'absence de séquences de référence pour les comparaisons phylogénétiques. Les meilleurs résultats de BLAST de ces isolats

correspondent à des séquences environnementales de champignons non cultivés et identifiés de façon insuffisante (Bonito *et al.*, 2016). Cependant, les bases de données évoluent et doivent être corrigées. A titre d'exemple, deux champignons isolés dans nos études, et initialement identifiés comme *Entrophospora* (un endomycorhizien, Glomeromycètes) via les résultats de BLAST, ont récemment été ré-affiliés aux *Pleosporales* (Ascomycètes).

La technique d'identification des isolats fongiques, associés aux racines de peuplier prélevées sur le site de Pierrelaye en 2010, est basée sur l'approche d'un clonage/typage moléculaire puis séquençage (Lacercat-Didier *et al.*, 2016). Cette technique permet d'obtenir des séquences relativement longues par ce séquençage de type Sanger, mais exige des étapes de clonage particulièrement longues et redondantes, limitant de ce fait le nombre d'espèces identifiées. Le séquençage direct par NGS réalisé à partir d'échantillons prélevés à Pierrelaye en 2013 conforte les résultats de 2010 à savoir l'importance du cultivar de peuplier sur les communautés fongiques associées aux racines (Foulon *et al.*, 2016a). Le séquençage MiSeq a ainsi permis l'identification d'un plus grand nombre d'espèces et révèle la domination du genre *Exophiala* qui a remplacé le genre *Hebeloma* présent en 2010. Des genres comme *Laccaria*, *Hebeloma* et *Tricholoma* sont fréquemment retrouvés sur des sites perturbés ou pollués par des ETMs (Nara *et al.*, 2003; Krpata *et al.*, 2008; Regvar *et al.*, 2010; Zappelini *et al.*, 2015). La disparition des Hébélomes sur le site de Pierrelaye est peut-être liée à l'augmentation de la matière organique et à une diminution des ETMs biodisponibles. A l'inverse, il est également possible que les typages de PCR-RFLP effectués sur un nombre relativement faible d'échantillons (quelques dizaines *versus* plusieurs milliers par NGS) aient contribué à surreprésenter le genre *Hebeloma* dans nos analyses.

Dans nos différentes études, la variabilité des taux d'ectomycorhization, de 10 à 75 %, est proche de ceux relevés dans d'autres études s'intéressant aux communautés ectomycorhiziennes du peuplier (Khasa *et al.*, 2002; Gehring *et al.*, 2006; Karliński *et al.*, 2010). Ces variations peuvent s'expliquer par le cultivar de peuplier utilisé ou les conditions environnementales (humidité du sol, type de sol, pollution, profondeur) conformément aux résultats sur la structure des communautés étudiées par métabarcoding.

Les travaux d'isolement et de criblage ont permis de sélectionner des souches potentiellement intéressantes et en particulier les tests de tolérance aux ETMs et de ré-inoculation ont permis d'identifier les meilleurs candidats dans une optique de phytomanagement assisté par les champignons. En effet, les souches fongiques natives de sites pollués par des ETMs semblent plus tolérantes et plus adaptées à ces conditions particulières que des souches issues de sites non pollués (Colpaert *et al.*, 2004, 2011; Goncalves *et al.*, 2009; Jourand *et al.*, 2010a). Par ailleurs, des souches fongiques tolérantes aux ETMs semblent apporter une meilleure protection à leur plante hôte sur un substrat contaminé par rapport à des souches non tolérantes (Adriaensen *et al.*, 2004, 2005; Jourand *et al.*, 2010b).

Cependant, les mécanismes de tolérance mis en place par ces souches restent mal connus. La tolérance observée peut être due à des mécanismes d'adsorption sur la paroi fongique ou d'accumulation dans les organites intracellulaires, d'exclusion des ETMs hors de la cellule fongique ou de mise en place de la synthèse accrue de molécules antioxydantes. Dans le cadre du phytomanagement couplant arbres et champignons associés, il serait alors intéressant de sélectionner les souches fongiques capables d'accumuler une forte quantité d'ETMs dans leurs hyphes ou d'immobiliser les ETMs dans le sol par l'excrétion/sécrétion de composés chélatants, limitant ainsi la quantité d'ETMs absorbée par la plante. Afin d'étudier certains de ces mécanismes, deux approches ont été mises en place et sont détaillées dans la section suivante.

3.3 Caractérisation fonctionnelle de mécanismes de tolérance aux ETMs

Le dernier objectif de ce travail de thèse est la **caractérisation des mécanismes de tolérance face à un stress métallique**. Pour cela deux approches différentes ont été utilisées et ont fait l'objet de deux manuscrits dont un est déjà publié (Lehembre et al., 2013). Le second sera soumis en novembre prochain.

1) La première approche utilise la métatranscriptomique fonctionnelle afin d'isoler et d'étudier les gènes provenant de sols contaminés et conférant une résistance au Zn ou au Cd. Des banques d'ADNc environnementales ont été réalisées à partir d'ARNm extraits d'échantillons de sols du site de Pierrelaye. Ces banques ont été criblées dans des souches de levures mutantes sensibles au Zn ou au Cd.

⇒ Lehembre F., Doillon D., David E., Perrotto S., Baude J., **Foulon J.**, Blaudez D., Chalot M. (2013). Soil metatranscriptomics for mining eukaryotic heavy metal resistance genes. Environmental Microbiology 15: 2829-2840. DOI: 10.1111/1462-2920.12143.

2) La seconde approche repose sur des tests de tolérance de champignons provenant des campagnes d'isolement, afin de déterminer les mécanismes mis en place en présence de Hg. Les souches fongiques isolées et identifiées, sont testées pour leur tolérance au Hg. Après le criblage de l'ensemble de la collection, deux souches de *P. involutus* montrant des niveaux contrastés de tolérance à cet élément ont été sélectionnées afin d'étudier de façon plus approfondie les mécanismes de tolérance au Hg.

⇒ **Foulon J.**, Gimbert F., Chalot M. (-). Mercury uptake and internalization by higher fungi. Microbiological Research. A soumettre.

3.3.1 Criblage du métatranscriptome du sol pour sonder les gènes de résistance aux ETMs chez les eucaryotes

Les éléments traces métalliques sont des polluants qui affectent tous les organismes. Etant donné qu'un petit nombre d'eucaryotes ont été étudiés en ce qui concerne leur résistance aux ETMs, l'hypothèse est faite que de nombreux gènes qui contrôlent ce phénomène restent à identifier. Cela a été testé par le criblage du métatranscriptome eucaryote du sol qui englobe l'ARN d'organismes appartenant aux principaux phyla eucaryotes. Les ARNm polyadénylés extraits du sol ont été convertis en ADNc et 35 d'entre eux, introduits dans des mutants de levure présentant un phénotype sensible, ont été sélectionnés pour leur capacité à séquestrer l'ETM (Cd ou Zn). Quelques-uns de ces gènes appartiennent à des familles connues pour conférer une résistance aux métaux lorsqu'ils sont surexprimés dans la levure. A l'inverse, certains d'entre eux étaient homologues à des gènes qui n'ont pas été étudiés dans le contexte de la résistance aux ETMs. Par exemple, les gènes BolA, qui conféraient une résistance croisée aux ETMs (Zn, Co, Cd, Mn) peuvent agir en interférant avec l'homéostasie du Fe. D'autres gènes, tels que ceux codant pour des polypeptides riches en cystéine, n'ont pas d'homologues dans les bases de données. Cette étude confirme que la métatranscriptomique fonctionnelle représente une approche puissante pour traiter les processus biologiques fondamentaux chez les eucaryotes. Les gènes sélectionnés peuvent être utilisés pour sonder de nouvelles voies impliquées dans l'homéostasie aux métaux et pour manipuler le niveau de résistance des organismes sélectionnés.

- ***Pour ce travail collaboratif réalisé dans le cadre d'un programme ANR (EUMETATOX), et impliquant les équipes de Lyon et Nancy, j'ai réalisé, pendant mon stage de master 2 (à Nancy) et finalisé lors du début de la thèse, les tests fonctionnels concernant les gènes* BolA *et la préparation des échantillons pour les analyses d'ETM par ICP-AES (Fig. 2). J'ai également contribué à la relecture du manuscrit.***

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Soil metatranscriptomics for mining eukaryotic heavy metal resistance genes

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Summary

Heavy metals are pollutants which affect all organisms. Since a small number of eukaryotes have been investigated with respect to metal resistance, we hypothesize that many genes that control this phenomenon remain to be identified. This was tested by screening soil eukaryotic metatranscriptomes which encompass RNA from organisms belonging to the main eukaryotic phyla. Soil-extracted polyadenylated mRNAs were converted into cDNAs and 35 of them were selected for their ability to rescue the metal (Cd or Zn) sensitive phenotype of yeast mutants. Few of the genes belonged to families known to confer metal resistance when overexpressed in yeast. Several of them were homologous to genes that had not been studied in the context of metal resistance. For instance, the *BOLA* ones, which conferred cross metal (Zn, Co, Cd, Mn) resistance may act by interfering with Fe homeostasis. Other genes, such as those encoding 110- to 130-amino-acid-long, cysteine-rich polypeptides, had no homologues in databases. This study confirms that functional metatranscriptomics represents a powerful approach to address basic biological processes in eukaryotes. The selected genes can be used to probe new pathways involved in metal homeostasis and to manipulate the resistance level of selected organisms.

Introduction

Ecosystem contamination by heavy metals resulting from human, industrial and agricultural practices is a widespread concern. These contaminants represent a health hazard (Schell *et al.*, 2006; Wasserman *et al.*, 2008; Mitchell *et al.*, 2011; Esteban-Vasallo *et al.*, 2012) and result in vast land areas that are considered unsuitable for agriculture and other activities (Pan *et al.*, 2010; http://www.eea.europa.eu/data-and-maps/indicators/progress-in-management-of-contaminated-sites/progress-in-management-of-contaminated-1). The cellular and molecular aspects of heavy metal toxicity, homeostasis, tolerance and resistance have been investigated in a number of metal-sensitive model eukaryotic organisms and several naturally resistant or tolerant fungi, plants and animals. These studies have identified potential cellular targets that are at the origin of metal toxicities as well as general cellular responses and detoxification mechanisms including chelation, cellular compartmentalization or the excretion of the metals (e.g. Clemens, 2006; Blindauer and Leszczyszyn, 2010; Gadd, 2010; Wysocki and Tamás, 2010).

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In eukaryotes, many of these processes are largely distributed, as illustrated by intracellular metal chelation mediated by the gene-encoded cysteine-rich metallothioneins. These low-molecular-weight proteins, classified in different subfamilies, could however have distinct phylogenetic origins (Blindauer and Leszczyszyn, 2010; Palacios *et al*., 2011). Other mechanisms have a more restricted or patchy phylogenetic distribution. Still taking metal chelation as an example, this is the case of metal-binding phytochelatins, small peptides synthesized by phytochelatin synthase. Originally thought to be plant-specific, phytochelatins and phytochelatin synthases have now been reported in a few fungal taxa, such as the yeast *Schizosaccharomyces* sp. and the mycorrhizal ascomycete *Tuber melanosporum* (Clemens *et al*., 1999; Bolchi *et al*., 2011), and invertebrates belonging to the nematodes (Vatamaniuk *et al*., 2001), annelids (Brulle *et al*., 2008) or plathyhelminths (Ray and Williams, 2011). These observations suggest that additional, yet non-described, resistance mechanisms and genes are likely to exist in the eukaryotes.

Experimental strategies followed to uncover the basis of metal sensitivity or resistance and the associated genes include both top-down and bottom-up ones. In the field of genomics, the most common bottom-up approach is the screening of genomes for sequences homologous to known resistance genes followed by their experimental validation (Peiter *et al*., 2007; Bolchi *et al*., 2011). One major drawback of this approach, based on sequence homology, is that it is unlikely to uncover novel genes and resistance mechanisms. A more recent approach is the search for genomic signatures, which distinguish metal-tolerant individuals collected in metal-rich habitats from sensitive ones isolated from non-contaminated environments. This latter approach has indeed led to the identification of novel genes not identified in other studies (Baxter *et al*., 2010; Chang and Leu, 2011). An additional bottom-up strategy is the functional screening of genomic or complementary DNA (cDNA) libraries for genes that restore metal tolerance when expressed in a metal-sensitive model organism, generally the yeast *Saccharomyces cerevisiae* for which several metal-hypersensitive mutants are available. This latter approach has largely contributed to the recognition that several resistance genes are widely distributed in the eukaryotes, but has also uncovered novel gene families participating to heavy metal homeostasis (Kim *et al*., 2008; Blanvillain *et al*., 2009; Matsuda *et al*., 2009).

One of the bottlenecks in the elucidation of the diversity of cellular responses to heavy metal damage and in the identification of additional resistance genes could be the limited number of biological species that have currently been investigated in this specific context. In the present study we tested this hypothesis by implementing a multigenome screening approach based on the exploration of the soil eukaryotic metatranscriptome. A soil metatranscriptome encompasses all RNA present in the cells of all the different soil-inhabiting microorganisms. It therefore represents a largely unexplored reservoir of genes and unique cellular functions. Indeed, soils are home to numerous uni- and multicellular eukaryotes (O'Brien *et al*., 2005; Lesaulnier *et al*., 2008; Damon *et al*., 2012) that are representative of almost all of the major eukaryotic lineages (Parfrey *et al*., 2010), including many currently undescribed and uncultivable species (e.g. Lejzerowicz *et al*., 2010; Rosling *et al*., 2011). From a practical point of view, as specifically regards eukaryotes, polyadenylated messenger RNA directly purified from soil samples are converted into cDNA which, upon cloning in an adequate plasmid vector constitute soil cDNA libraries representative of the diversity of the genes expressed by the different soil eukaryotic species (Bailly *et al*., 2007; Kellner *et al*., 2010). As for a cDNA library prepared from a single species, a metatranscriptomic library can be screened by expression in an appropriate microbial host such as *S. cerevisiae*. This functional environmental genomics approach dedicated to the study of the functional diversity of eukaryotes has, thus far, led to the identification of full-length genes involved in biomass degradation in the bovine rumen (Qi *et al*., 2011), a phosphatase and a novel family of fungal oligopeptide membrane transporters from soil (Kellner *et al*., 2010; Damon *et al*., 2011). Functional metatranscriptomics represents the counterpart of functional metagenomics where environmental DNA is screened in an appropriate host for specific categories of bacterial genes. This latter approach has been followed to identify genes conferring stress resistance in bacteria, such as heavy metal (Mirete *et al*., 2007) or more recently low pH resistance (Guazzaroni *et al*., 2012).

Here we report the construction in a yeast expression plasmid of eukaryotic cDNA libraries made from RNA extracted from three soil samples collected in heavy metal polluted or unpolluted sites. These libraries were screened for complementation of the metal (either Zn or Cd) sensitive phenotype of three *S. cerevisiae* mutants. A selected set of the cloned genes was further characterized.

Results and discussion

Diversity of the studied metatranscriptomes

The three sampling sites of Paal, Lommel and Balen (Table S1) have been extensively studied in the context of heavy metal pollution (Colpaert *et al*., 2004). The Paal site has no history of heavy metal pollution, while aerial deposits of mainly Zn, Cd and Pb, contaminated Lommel and Balen until the late 1970s (Table S1). At the time of

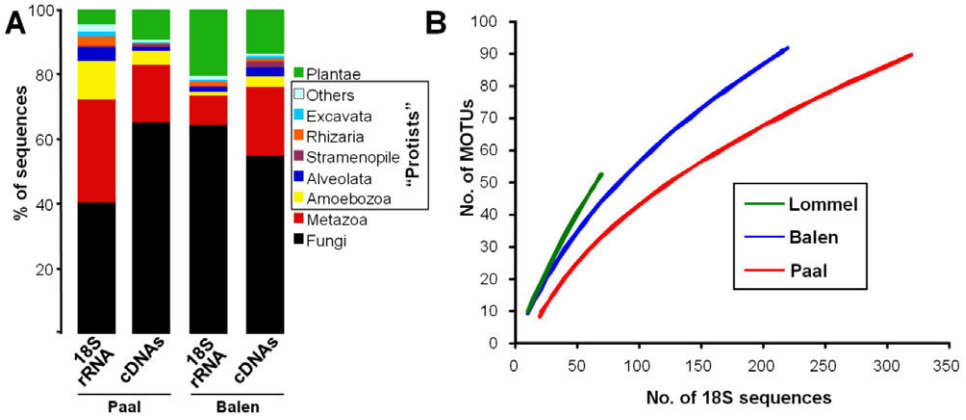

Fig. 1. Taxonomic breadth and diversity of the studied soil metatranscriptomes.
A. Taxonomic annotation of both 18S rDNA and cDNA sequences obtained from the Paal (non-polluted site) and Balen (polluted) soil-extracted RNA indicates that all of the major eukaryotic phyla are represented in both soils and that the *Fungi* and *Metazoa* phyla are dominant.
B. Evaluation of the 18S rRNA phylotype richness of the three studied soil eukaryotic communities. MOTUs (= phylotypes) were defined using a 97% identity threshold between rRNA sequences (Table S6).

sampling, the Lommel soil had lost most of its heavy metal load, whereas the Balen soil contained toxic levels of bioavailable Zn and Cd (Table S1). Total RNA was extracted from each soil and poly-A mRNAs were converted into cDNAs that were cloned into a yeast expression plasmid to produce environmental cDNA libraries with more than $3{\cdot}10^5$ clones each (Table S2). A total of 30 000 cDNAs from Balen (the contaminated site) and 30 000 from Paal (the non-contaminated control site) were subjected to systematic Sanger sequencing (Table S3). For each soil, amplified 18S ribosomal cDNAs were also cloned and sequenced (Table S4).

The taxonomic affiliations of both 18S rRNAs and cDNAs from Balen and Paal indicated that their soil eukaryotic communities were diverse and dominated by fungi and *Metazoa*, altogether representing up to 73% of the 18S rRNA sequences in Balen (Fig. 1A, Table S5). 18S rRNA and cDNA sequences were, nevertheless, also attributed to species belonging to other, mostly unicellular, eukaryotic phyla, the *Amoebozoa*, *Alveolata*, *Excavata*, *Stramenopiles* and *Rhizaria*. Under-representation of these latter groups among the cDNA data sets is likely an annotation artefact reflecting the under-representation of homologous sequences from these taxonomic groups in protein databases. Based on the calculation of richness estimators and the drawing of collector's curves, the different studied soil eukaryotic communities encompassed a minimum of 100 species (Fig. 1B, Table S6). These figures are certainly conservative estimates (i) as the use of a single primer pair for the amplification of environmental 18S rRNA sequences is likely to miss part of the actual diversity (Jeon *et al*., 2008), but also (ii) because a single 18S rRNA sequence can encompass several related species. Most MOTUs seemed to be site-specific and only seven of them were identified in all three sites.

Library screening

For the three cDNA libraries, cDNAs were selected based on their capacity to rescue either the Zn-hypersensitivity phenotype of the *zrc1Δ S. cerevisiae* mutant or the Cd-hypersensitivity phenotype of the *yap1Δ* and *ycf1Δ* yeast mutants. For each of the 3 libraries × 3 yeast mutant combinations, a number of transformants that were similar or greater to the number of plasmids present in each library were screened on lethal concentrations of either Zn or Cd (Table S2) yielding a total of 20 and 16 distinct cDNA sequences restoring tolerance to Zn and Cd respectively. Genes were selected from all three soils that were either contaminated or not by heavy metals and for each of the three yeast mutants used.

The genes were grouped based on sequence homologies (Table 1) and at least one member of each of the 19 resulting gene categories (Table S7) was studied for (i) its capacity to restore tolerance to additional toxic metals (Co, Cu and Mn) to metal-sensitive yeast cells and (ii) its effect on 12 different metallic and non-metallic element concentrations in yeast cells growing in the absence or in the presence of Cd (in *yap1Δ* or *ycf1Δ* mutants for the Cd resistance genes) or Zn (in the *zrc1Δ* mutant for the Zn resistance genes; Table S7).

Based on sequence analysis and functional annotation, the genes grouped into two major categories. Category one encompassed genes with homologues in databases while category two included genes encoding proteins with no known homologues (Table 1).

Table 1. Main characteristics of the different 'environmental genes' restoring metal tolerance to the metal-sensitive yeast mutants.

Gene categories	Clades	Representative clones	Yeast mutant/metal	Origin (site)	Protein sizes (aa)	Taxonomic origin	Putative functional role
Metallothionein	–	Ycf06L1-108	*ycf1Δ*/Cd	L	36	*Fungi/Basidiomycota*	Metal chelation
Cysteine-rich protein (CRP) family 1	–	YCFC1	*ycf1Δ*/Cd	B	77	Unknown	Metal chelation
Cysteine-rich protein (CRP) family 2	I	Ycf06L1-57/70/84/112	*ycf1Δ*/Cd	L	110–112	Unknown	Metal chelation
Cysteine-rich protein (CRP) family 2	II	Ycf06L1-94	*ycf1Δ*/Cd	L	124	Unknown	Metal chelation
Cysteine-rich protein (CRP) family 2	IV	Ycf06Lp-67	*ycf1Δ*/Cd	L	128	Unknown	Metal chelation
Cysteine-rich protein (CRP) family 2	V	Ycf06L1-69/96/85	*ycf1Δ*/Cd	L	132	Unknown	Metal chelation
Cysteine-rich protein (CRP) family 2	IX	YCF-P-49	*ycf1Δ*/Cd	P	133	Unknown	Metal chelation
C-terminal domain of a Zn Alcohol dehydrogenase	–	YCF-B-87	*ycf1Δ*/Cd	B	160	Unknown	Unknown
Saccharopine dehydrogenase	–	YAP-C-115	*yap1Δ*/Cd & *zrc1Δ*/Zn	B	342	Unknown	Unknown
Hypothetical protein	–	YAP-L-123	*yap1Δ*/Cd	L	185	Unknown	Unknown
Similar to interaction site E2 of the ubiquitin domain of a ubiquitin-ribosomal protein L40	–	YAP-L93	*yap1Δ*/Cd	L	36	Unknown	Protein degradation
Polyubiquitin	–	ZRC-J280	*zrc1Δ*/Zn	L	304	*Fungi*	Protein degradation
BolA (pfam PF01722)	I	ZRC A52	*zrc1Δ*/Zn	B/L	93	*Plantae/Pinus sylvestris*	Fe homeostasis
BolA (pfam PF01722)	II	ZRC B2	*zrc1Δ*/Zn	B/L	113	Unknown	Fe homeostasis
SKP1, SCF ubiquitin ligase	I	ZRC A40/B13	*zrc1Δ*/Zn	L/P/B	158	*Plantae/Pinus sylvestris*	Protein degradation
SKP1, SCF ubiquitin ligase	II	ZRC A19/L197/A47/A36/B9	*zrc1Δ*/Zn	L/P/B	138–162	*Amoebozoa/Ichthyosporea?*	Protein degradation
SKP1, SCF ubiquitin ligase	III	ZRC I99	*zrc1Δ*/Zn	B/P	164	*Amoebozoa/Ichthyosporea?*	Protein degradation
SKP1, SCF ubiquitin ligase	IV	ZRC J281	*zrc1Δ*/Zn	B/L	163	*Amoebozoa/Ichthyosporea?*	Protein degradation
SKP1, SCF ubiquitin ligase	V	ZRC A18/B8/I148/A4/A15/B26	*zrc1Δ*/Zn	B/L/P	159–164	*Amoebozoa/Ichthyosporea?*	Protein degradation

Sites: L, Lommel; P, Paal; B, Balen.

Genes with known homologues in databases

Several genes from category one encode for proteins previously implicated in heavy metal response and/or homeostasis. One of these genes was homologous to metallothioneins and belongs to a family found in the *Agaricomycetes* class of fungi. These short (*c*. 35 amino acids) cysteine-rich cytosolic proteins efficiently chelate metals intracellularly (Blindauer and Leszczyszyn, 2010; Palacios *et al*., 2011).

In addition to metal inactivation by chelation, the degradation of misfolded proteins or the proteolytic inactivation of regulatory proteins represents a major cellular response to heavy metals (Jungmann *et al*., 1993; Hwang *et al*., 2007; Medicherla and Goldberg, 2008). Within category one, we also identified two ubiquitin-encoding genes, whose overexpression in yeast conferred tolerance either to Zn alone or to Cd and Zn. The ubiquitination of proteins targeted to the 26S proteasome proceeds through a multistep process involving several enzyme complexes, including the SCF (Skp1–Cdc53/cullin–F-box protein) ubiquitin ligase (Hershko and Ciechanover, 1998). We independently selected 15 distinct *SKP1* genes (pfam Nos. PF03931 and PF01466) for zinc resistance from the Balen and Lommel and/or Paal cDNA libraries (Table 1). These genes have at least two distinct phylogenetic origins. One is 99% identical at the nucleotide level to a *Pinus sylvestris* EST while all the others are related to sequences from the phyla *Amoebozoa* and *Ichthyosporea* (*Opisthokonta*). However, the limited number of homologous sequences from these groups in databases, combined with the low number of phylogenetically informative characters precluded rigorous evaluation of their exact phylogenetic positions (Fig. S1). The SCF complex has been shown to be central in the response to Cd stress in yeast (Yen *et al*., 2005; Baudouin-Cornu and Labarre, 2006) but not to Zn. Accordingly, although no *SKP1* genes were selected in the screen for Cd tolerance, overexpression of the different environmental *SKP1* genes restored Cd tolerance in the Cd-sensitive *yap1Δ* and/or *ycf1Δ* yeast mutants (Table S7).

Other genes from category one encoded for proteins that had seemingly not been previously studied in the context of heavy metal resistance (Table 1). One of these genes, which restored Cd tolerance, encoded the C-terminal part of an alcohol dehydrogenase (pfam PF00107). The other genes, selected for Zn tolerance, encoded for a saccharopine dehydrogenase (pfam PF03435) and two different BolA proteins (pfam PF01722). These latter BolA protein-coding genes were studied in further detail (Fig. 2). As for the *SKP1* genes, *BOLA* genes were selected independently from the Balen and Lommel sites and had two phylogenetic origins. One gene likely originated from the tree *P. sylvestris*, while the other did not show a clear association with known sequences in phylogenetic analyses (Supplementary text, Fig. S2). Furthermore, overexpression of environmental BolAp in the wt BY4741 metal tolerant yeast strain resulted in a unique gain-of-function phenotype conferring Zn 'hyper-resistance' to the wt yeast (Fig. 2E).

FRA2, the *S. cerevisiae BOLA* homologue, encodes a protein that has been shown to form, in an iron-independent manner, a cytosolic complex with the Grx3p and Grx4p glutaredoxins and with Fra1p, a protein involved in the negative regulation of the iron regulon (Kumánovics *et al*., 2008). Li and Kaplan (1998) showed that deletion of *FRA2* leads to an increase of the *AFT1*-dependent iron regulon, resulting in increased iron uptake and metabolism. In line with these data, we observed that the overexpression of environmental *BOLA* genes resulted in a specific increase in the yeast intracellular iron concentration, up to twofold the concentration assayed in control yeast cells (Fig. 2D). Intracellular concentrations of other elements were not affected. Of the different selected environmental genes, *BOLA* overexpression was also one of the few cases where the phenotype of yeast mutants sensitive to different metals (Cd, Co, Mn and Zn) was rescued (Fig. 2A). Interestingly, interplay between Zn and Fe, as well as Co and Fe, and Cd and Fe homeostasis has been documented in several organisms including yeast (Li and Kaplan, 1998; Stadler and Schweyen, 2002; Pagani *et al*., 2007; Ruotolo *et al*., 2008) and filamentous fungi (Yasmin *et al*., 2009). For example, the loss of high-affinity iron uptake by inactivation of the reductive iron assimilation pathway results in an increased sensitivity to Zn and other transition metals (Li and Kaplan, 1998). We therefore hypothesized that a direct link could exist between (i) the overexpression of environmental *BOLA*, (ii) the associated heavy metal tolerance phenotypes and (iii) increased intracellular Fe concentrations. In support of this assumption, we showed that the Zn tolerance phenotype was abolished in a *fet3Δ fet4Δ* yeast double mutant defective in iron uptake (Pedas *et al*., 2008), thus confirming that BolAp-induced Zn tolerance is dependent on the cell's iron status (Fig. 2E).

Genes with no known homologues in databases

Genes from category two with no homologues in the databases were all identified in screening for Cd tolerance (Fig. 4, Table 1). One of these genes (clone YAP-L-123, Accession No. HE716987), which was selected in the *yap1Δ* mutant, encodes for a putative peptide that is 185 amino acids long and lacks specific structural features (e.g. a predicted signal peptide, nuclear localization or transmembrane domains, enrichment in specific amino acids), which might suggest its functional role. This observation is in contrast with the other cDNAs selected by the

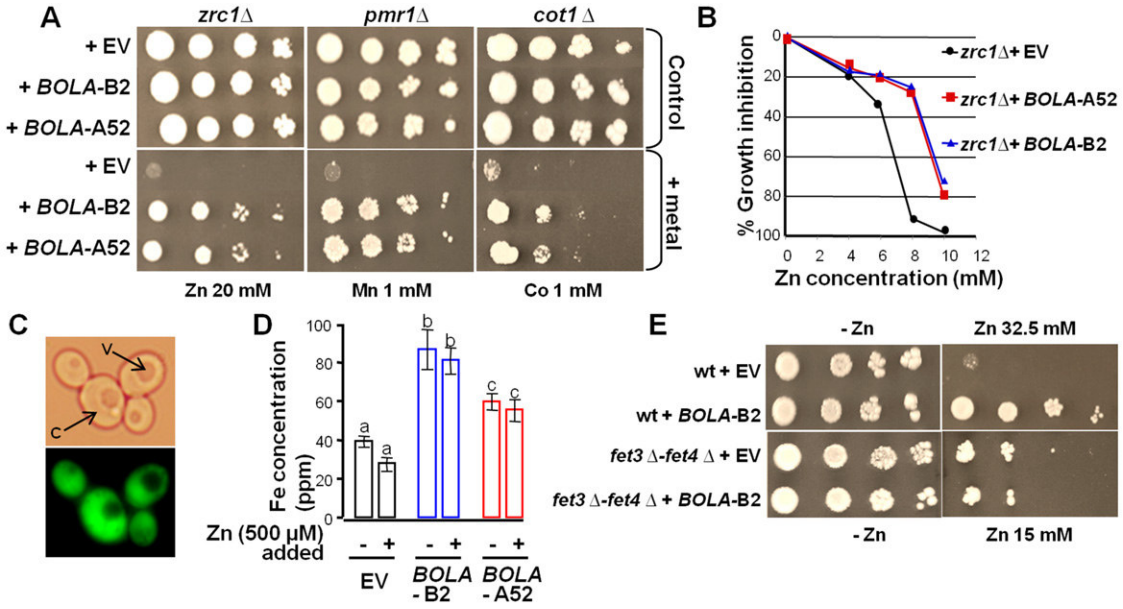

Fig. 2. Characterization of *BOLA* genes selected for Zn tolerance.
A. The expression of two environmental *BOLA* genes restores tolerance to toxic concentrations of Zn, Mn and Co when expressed in yeast mutants sensitive to one of these metals (respectively *zrc1Δ*, *pmr1Δ* and *cot1Δ*); EV: empty vector. A serial 10-fold dilution series of liquid cultures were spotted on agar plates.
B. Zn tolerance levels conferred by environmental *BOLA* genes expressed in the *zrc1Δ* Zn-yeast-sensitive mutant grown in liquid medium.
C. Expression of the *BOLA-B2*::*GFP* fusion gene suggests a cytoplasmic localization for the BolA-B2 protein (C, cytoplasm; V, vacuole).
D. The expression of environmental *BOLA* genes in the *zrc1Δ* yeast mutant results in increased intracellular Fe concentrations irrespective of the culture medium's Zn status. Different letters above the bars indicate significantly different mean values ($P < 0.05$).
E. Expression of the environmental *BOLA-B2* gene in the wt Zn-tolerant BY4741 yeast strain results in a 'hypertolerant' phenotype. Tolerance is abolished upon expression in a *fet3Δ*, *fet4Δ* yeast mutant defective in iron uptake, thus highlighting the interplay between *BOLA* overexpression, Zn resistance and iron homeostasis.

functional complementation of the *ycf1Δ* mutant, which all encode for cysteine-rich polypeptides (CRP, Table 1). These polypeptides did not however share any other sequence similarities to known metallothioneins, which are other well-known cysteine-rich polypeptides. Among these environmental sequences, we identified two subsets. One subset, represented by a single sequence from Lommel, encodes for a 77-amino-acid-long peptide containing 25% of cysteine residues (clone YCFC1, Accession No. HE717000). The other subset was represented by 11 homologous cDNAs, identified from either the Lommel (10 sequences) or the Paal (one sequence) libraries. These cDNAs encode for 110- to 133-amino-acid-long, cysteine-rich (20–24%) polypeptides, whose positions are conserved between sequences (Fig. 3B). These sequences, referred to as *CRP2* sequences, constitute, along with the metallothioneins of the ciliates *Tetrahymena* spp. (Gutiérrez *et al.*, 2011), the longest cysteine-rich polypeptides ever characterized so far whose expression confers Cd tolerance. Besides their lengths, few other structural features are shared between the environmental Crp2p proteins and metallothioneins of *Tetrahymena*. They include the presence of unusual CCC motifs; one of them at N-terminal positions in both environmental and *Tetrahymena* proteins and CXCC motifs (X being any amino acid) (Fig. 3B). They also include a conserved histidine residue which could be implicated in metal chelation as shown for some *Tetrahymena* metallothioneins (Gutiérrez *et al.*, 2011). Nevertheless, the lack of obvious conserved amino acid positions between sequences of these two protein families suggest that they may not share a common ancestral sequence and that *CRP2* environmental sequences do not necessarily originate from soil ciliates.

Homologues of these environmental *CRP2* sequences were also present among systematically sequenced cDNAs from Paal and Balen. This observation suggests that a group of organisms expressing these genes might be abundant and widespread in the environment. This hypothesis was tested using PCR on environmental metagenomic DNAs along with degenerate primers targeting a 240- to 258-nt-long internal segment of these genes. The 12 different soil and water samples tested were collected under diverse natural and agricultural vegetation types in different parts of the world from the tropics to the Mediterranean and in cold temperate regions of Europe (Table S8). In total, 34 distinct partial nucleotide sequences were obtained from the different 12

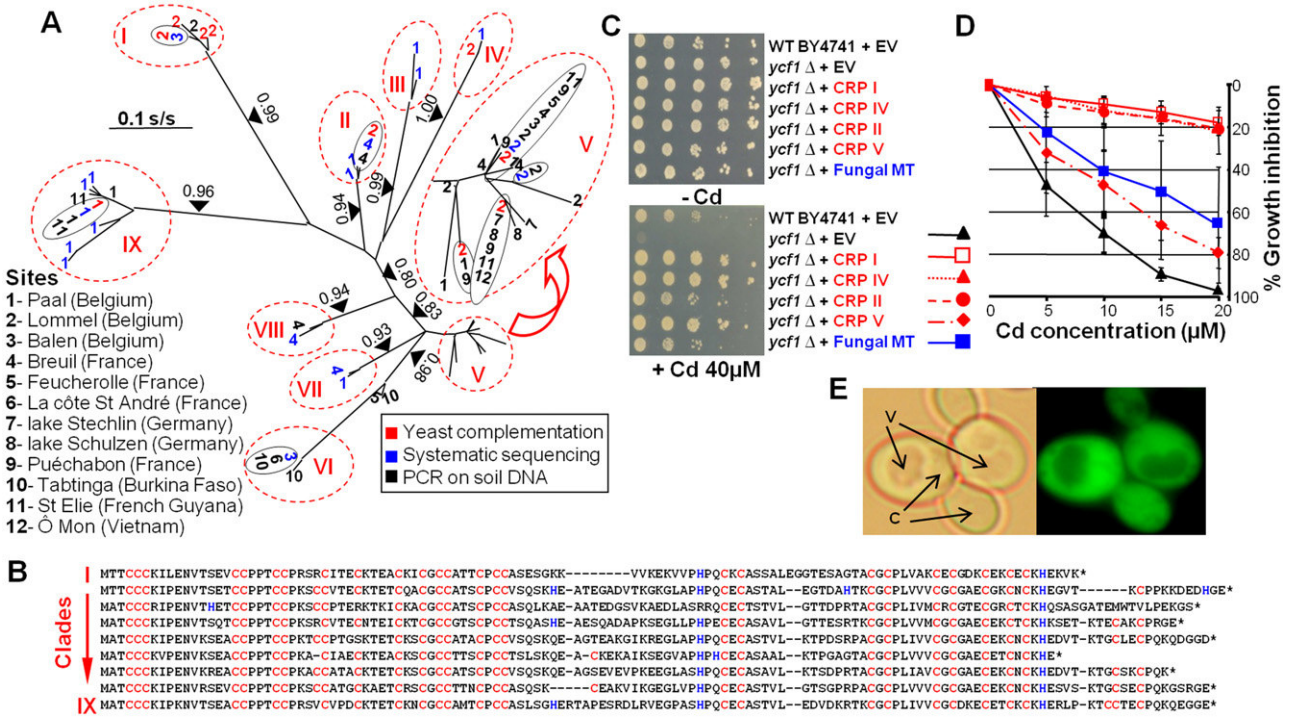

Fig. 3. Characterization of cysteine-rich proteins (CRP2) selected for Cd resistance.
A. Members of the CRP2 gene family were identified in different sites (1–12) by either complementation of Cd-sensitive yeast mutants (sequences in red), systematic sequencing of soil cDNA libraries (in blue) or by PCR on soil- or water-extracted DNA (in black). Phylogenetic (PhyML) analysis of the amino acid sequences defined nine clades (I to IX) and indicated that identical sequences might be present in distinct geographic sites and might be retrieved using different screening methods. Branch posterior probabilities above 0.8 are indicated.
B. Amino acid alignment of representative sequences of the nine clades shows the positions of conserved cysteine motifs (in red) as well as the presence of histidine residues (in blue) which might also participate in metal chelation.
C. Expression of four full-length CRPs from clades I, II, IV and V in the Cd-sensitive *ycf1*Δ yeast mutant results in a Cd-tolerant phenotype similar to the phenotype observed when expressing an environmental fungal metallothionein. Serial 10-fold dilution series of liquid cultures were spotted on agar plates. EV, empty vector.
D. Cd-induced growth inhibitions measured in liquid cultures illustrate differences between the tolerance levels conferred by the different CRPs.
E. The expression in yeast of a *CRP2-I*::*GFP* fusion gene in yeast suggests a cytoplasmic localization of the Crp2-I protein (C, cytoplasm; V, vacuole).

samples thus demonstrating the widespread distribution of the corresponding organisms in a diversity of habitats. Maximum likelihood (PhyML) phylogenetic analyses identified nine different sequence subfamilies (Fig. 3A), characterized by specific arrangements of cysteine residues in the full-length sequences (Fig. 3B). Several of these subfamilies included sequences from different sites and the sequences from one site (e.g. Paal) could belong to different subfamilies (Fig. 3A). Isolation of the corresponding organisms will be required to clarify their taxonomic positions as well as the genomic organization of this gene family, and to determine whether members of different sequence subfamilies can coexist in a single genome or occur in different related species.

Conclusions

The present work, based on the functional screening of soil eukaryotic metatranscriptomic libraries, revealed high degrees of diversity and novelty among the recovered sequences, demonstrating that this methodology is suitable and powerful for characterizing novel genes whose overexpression restored tolerance or even conferred heavy metal resistance in yeast. A similar approach carried out with bacteria using metagenomic soil DNA instead of RNA also led to the identification of novel gene bacterial families conferring nickel resistance in *Escherichia coli* (Mirete *et al.*, 2007). In this respect functional environmental genomics represents a universal approach to study fundamental aspects of the biology of not only bacteria, but also eukaryotes.

As anticipated, our preliminary functional analyses of the recovered genes and their protein products suggest that several of them (i) originated from unknown taxonomic groups and (ii) could act by interfering with cellular processes that have been poorly studied in the context of heavy metal homeostasis (Fig. 4). In this respect, these genes could be used to probe these processes and to

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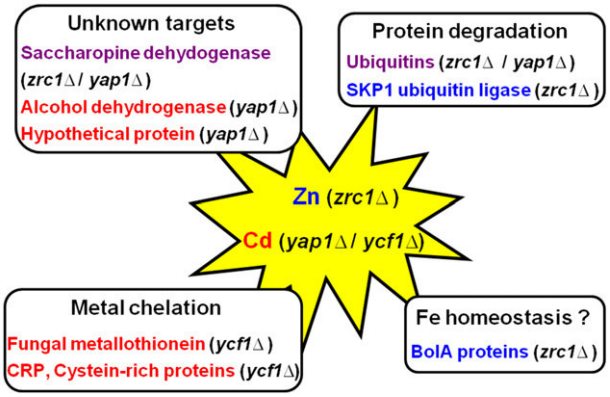

Fig. 4. Overview of the selected genes and of the putatively altered cellular functions. The functional screening of these taxonomically diverse metatranscriptomes for genes that restore a tolerant phenotype in Zn (*zrc1Δ*, in blue), Cd (*yap1Δ*, *ycf1Δ*, in red) or both (in purple) sensitive yeast mutants resulted in the selection of genes that participated in diverse cellular processes (protein degradation, metal chelation, Fe homeostasis or unknown mechanisms).

evaluate their contribution to the overall capacity of organisms to cope with heavy metals. These genes could find applications as biomarkers either to detect heavy metals in the environment or to manipulate the tolerance/resistance levels of species of interest for the bioremediation or revegetation of polluted lands.

Experimental procedures

Study sites and sampling

The study sites of Balen, Lommel and Paal are situated in NE Belgium (Table S1). Sampling plots in Balen and Lommel occurred 1.5 and 1.7 km, respectively, away from a metal smelter. Until the mid-seventies, and for more than 70–80 years, the Balen and Lommel smelters emitted large quantities of Zn and several other heavy metals (Pb, Cd) into the atmosphere. At the time of sampling (2006–2008), the Lommel soil had lost most of its heavy metal load through natural leaching processes, whereas the Balen soil contained toxic levels of bioavailable Zn, which was found at high levels in *Pinus* needles (Table S1). The reference site in Paal, 17 km away from the Balen smelter, was only poorly exposed to aerial metal depositions.

Twenty soil cores were collected per site. Each soil core was sieved (2 mm mesh size) and an aliquot was taken for metal content measurements. For each site equal volumes of each sample were mixed together to form composite samples which were frozen and kept at −70°C until RNA extraction. The heavy metal contents (Cd, Pb and Zn) were assayed from oven-dried soil samples (total soil metal content), soil extracts (potentially bioavailable soil metal content) and oven-dried *P. sylvestris* needles collected at each of the three sites by inductively coupled plasma emission (Supplementary text).

Soil nucleic acid extraction and cDNA library construction

Total RNA (~ 100 μg) was extracted from 200 g (Balen) to 450 g (Lommel and Paal) of each of the soil samples, as previously described (Bailly *et al.*, 2007; Damon *et al.*, 2010) but with some minor modifications. The final concentrations of guanidine isothiocyanate and SDS in the extraction buffer varied from 100 to 600 μM and 1.6% to 1.85%, respectively, which was dependent on the soil. Poly-A mRNA purification and cDNA synthesis and amplification were performed as described in Bailly and colleagues (2007). cDNAs were digested with Sfi1 and ligated downstream of the *S. cerevisiae PGK1* promoter in the pFL61 yeast-*E. coli* shuttle vector (Minet *et al.*, 1992) into which Sfi1A&B cloning sites had been introduced. The cDNAs were also cloned into the Sfi1-digested pDNR-LIB plasmid vector (Clontech) for the Balen and Paal soils. Each cDNA-pFL61 and cDNA-pDNR-LIB library was introduced into electro-competent *E. coli* cells (DH10B, Invitrogen).

Screening of cDNA libraries for heavy metal resistance genes

The *S. cerevisiae* strains used in this study are listed in Table S9. Synthetic yeast nitrogen base (YNB) medium (Sigma Aldrich, St Quentin-Fallavier, France) with or without uracil and supplemented with 2% glucose and drop out solution (Sigma Aldrich) was used for all screening experiments. For the metal tolerance assays, yeast cells were grown in liquid YNB until $OD_{600} = 1$. These cultures were subsequently used to inoculate YNB control plates or YNB plates containing toxic concentrations of Zn, Cu, Cd, Mn or Co. Yeast cells expressing *GFP*-fused cDNAs under the control of the GAL1-inducible promoter were grown in liquid YNB medium containing 2% (w/v) galactose instead of glucose.

To identify the cDNAs that were able to increase cadmium or zinc tolerance, the *ycf1Δ*, *yap1Δ* and *zrc1Δ* yeast strains were transformed with the three environmental pFL61 cDNA libraries described above. *YCF1* encodes for an ABC transporter that confers Cd tolerance through the transport of Cd conjugates into vacuoles (Szczypka *et al.*, 1994; Li *et al.*, 1997). *YAP1* encodes for a transcription factor regulating stress-induced genes and its mutant is sensitive to Cd (Wu *et al.*, 1993). Deletion of the *ZRC1* gene, which encodes for a transporter that sequesters Zn into vacuoles, renders the mutant highly sensitive to Zn (Kamizono *et al.*, 1989; Li and Kaplan, 1998). Transformations were performed using the standard lithium acetate method (Gietz *et al.*, 1992). Cd^{2+}- and Zn^{2+}-tolerant ura^{+} transformants were selected after plating the transformants on YNB medium supplemented with the appropriate amino acids and containing 20 or 40 μM $CdCl_2$ or 20 mM $ZnCl_2$ respectively.

All plasmids conferring Cd or Zn tolerance were used to transform other yeast mutants that are sensitive to other metals (Zn or Cd, Co, Mn and Cu). The *cot1Δ* mutant is hypersensitive to both Zn and Co because *COT1* mediates the efflux of both ions into the vacuole (Conklin *et al.*, 1992; Li and Kaplan, 1998; Lyons *et al.*, 2000). *PMR1* is responsible for the high-affinity transport of Mn and Ca into the Golgi and confers Mn tolerance (Ton *et al.*, 2002). The DTY4 strain is

highly sensitive to copper due to a natural truncation of *CRS5* and the deletion of *CUP1*, which encode the two yeast metallothioneins (Longo *et al*., 1996). Tolerance tests were performed on YNB media containing either 15 or 40 μM Cu, 1 or 2 mM Co, or 1 or 2 mM Mn (Fig. 2A, Table S7). In parallel experiments, culture tubes containing 4 ml of YNB-ura medium supplemented with a range of Cd (1–20 μM) or Zn (4–10 mM) concentrations were inoculated with log-phase pre-cultures of *ycf1Δ*, *yap1Δ* and *zrc1Δ* cells. Cultures were grown at 30°C at 220 r.p.m. and cell density was measured after 48 h.

GFP fusions

To construct the different gene of interest–*GFP* fusions, the coding sequences were amplified with the primers shown in Table S10. Digested PCR products were cloned into a BamHI + EcoRI-digested pYES2-GFP plasmid (Blaudez *et al*., 2003). Yeast transformants were grown in YNB-galactose medium. A Nikon Optiphot-2 fluorescence microscope equipped with a Nikon D1 camera was used to document the fluorescence (excitation 475–500 nm emission above 520 nm).

Yeast element content

To determine the total yeast cell metallic (Ca, Cd, Co, Cu, Fe, K, Mg, Mn, Na, Zn) and non-metallic (P, S) element concentrations, yeasts were grown in YNB-ura media that were supplemented, or not, with either 2 μM Cd or 500 μM Zn at 30°C at 220 r.p.m. Sample processing and inductively coupled plasma emission assay are described in Supplementary text.

Amplification of cystein-rich protein (CRP) coding sequences from environmental DNA samples

DNA extracted from either freshwater environments (the Stechlin and Schulzen lakes in Germany) or soils collected in temperate or tropical regions were kindly provided to us by several Colleagues (Table S8). Based on the sequence alignments of full-length CRP2 coding sequences we designed a set of three PCR primers (Table S10) that were used to amplify a 240- to 258-bp-long internal segment of CRP2 ORFs. Primers NMTF and NMTR were used in a first PCR reaction to which environmental DNA was added. PCR reaction mixes (25 μl) contained 6.25 μg of bovine serum albumin, 1.5 mM of $MgCl_2$, 200 μM of each dNTPs, 1 μM of each primer, 0.5 U of *Taq* DNA polymerase (Invitrogen), 10–100 ng of DNA and the appropriate reaction buffer (Invitrogen). The amplification proceeded through 35 cycles (hybridization temperature, 55°C). Two microlitres of this first PCR mix was added to a new identical mix that lacked primer NMTR which was replaced with primer NMTR3. After a second amplification reaction identical to the first one, DNA fragments of the expected size were gel-purified, cloned into plasmid PCR4-TOPO (TOPO TA Cloning kit for sequencing Invitrogen) and sequenced.

Sequence and phylogenetic analyses

For each gene sequence whose expression in yeast conferred metal resistance, systematic BLASTN, TBLASN and/or BLASTX searches through the NCBI web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) were performed against the GenBank nr, EST, GSS, HTG and ENV databases to identify potential homologous sequences. Similar searches were also performed against whole eukaryote genome sequences accessible through the Joint Genome (http://genome.jgi-psf.org/) and Broad Institutes (http://www.broadinstitute.org/scientific-community/data?) web servers. Analyses were last performed in September 2012.

To infer the most likely phylogenetic origin of the environmental sequences, homologous protein sequences from organisms representative of the largest number of major eukaryotic phyla (Parfrey *et al*., 2010) were collected and aligned to the environmental sequence using MUSCLE (Edgar, 2004). Preliminary phylogenetic trees were then produced using PhyML (Guindon and Gascuel, 2003) with and without curing the alignment with GBlocks (Castresana, 2000). These preliminary alignments and trees were inspected to identify (i) the most divergent and fastest evolving sequences, which were eliminated, and (ii) the protein sequences most phylogenetically related to the environmental ones. Additional protein sequences from the candidate taxonomic groups and their 'sister groups' were then added to the sequence alignments, and the phylogenetic analyses were performed again. The whole process was performed using the phylogeny.fr (Dereeper *et al*., 2008) and Seaview (Gouy *et al*., 2010) platforms. Default parameters were used for the different software with the exception of the use of less stringent parameters for GBlocks and the WAG substitution model for PhyML.

cDNA systematic sequencing and annotation

Randomly selected cDNA clones from the Balen and Paal pDNR-LIB libraries (30 000) were plated, arrayed robotically and amplified using phi29 polymerase. The plasmids were 5′ end-sequenced by the Genoscope Sequencing Center (Evry, France) using BigDye Termination kits on Applied Biosystems 3730xl DNA Analysers. An additional 382 sequences were also obtained for the Lommel cDNA library. Sequence data sets were processed and annotated as described in Damon and colleagues (2012); see Supplementary text.

18S ribosomal cDNA amplification, sequencing and annotation

Soil non-polyadenylated RNA (500 ng) was reverse transcribed using 0.2 μg of random hexamers or 0.04 μg of the primer Euk516R (Table S4) and 200 U of M-MuLV Reverse Transcriptase according to the manufacturer's instructions (MBI Fermentas). A *c.* 520-bp-long fragment located at the 5′ end of the 18S ribosomal cDNA sequence was amplified by PCR using the primers Euk1A (Table S10) and Euk516R, as previously described (Diez *et al*., 2001). Cloned fragments were sequenced and the sequences analysed as described in Supplementary text.

Sequences were submitted to the GenBank/EMBL/DDJB database and are available under the Accession Nos

FO181451–181538 (Balen), FO181361–181450 (Paal) and FN396422–6515 (Lommel).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary text. Supplementary experimental procedures and results.
Fig. S1. Putative phylogenetic origins of five environmental *SKP1* genes (red) representative of clades II.
Fig. S2. Putative phylogenetic origins of environmental *BOLA* gene clone ZRC B2 (red) which belongs to clade II
Table S1. Main characteristics of the three sampling sites.
Table S2. Characteristics of the different metatranscriptomic cDNA libraries and outcome of their screening for the phenotypic rescue of metal-sensitive yeast mutants.
Table S3. Characteristics of the Paal and Lommel cDNA sequence data sets.
Table S4. Characteristics of the PCR-amplified 18S rRNA sequence data sets.
Table S5. Taxonomic affiliation of the 18S rRNA and cDNA sequence data sets from the Paal (first figures), Balen (second figures) and Lommel (third figures) sites.
Table S6. Predicted eukaryotic community species richness based on the identification of 18S rRNA molecular taxonomic units (MOTUs) and the calculation of richness estimators.
Table S7. Phenotypes associated with the expression of 'environmental resistance genes' in different metal-sensitive yeast mutants.
Table S8. Origin and characteristics of the environmental samples from which the metagenomic DNAs, used to amplify sequences coding for Cysteine Rich Proteins (CRP2), were isolated.
Table S9. Yeast strains used in this study.
Table S10. Oligonucleotides used in the present study.

Table S1. Main characteristics of the three sampling sites.

Sites	Paal	Lommel	Balen
Coordinates	51°03'33"N	51°14'49"N	51°12'45"N
Climate	temperate / oceanic		
Vegetation	pioneer *Pinus sylvestris* forest		
Mean annual Temperature	9.0	9.0	9.0
Mean annual rainfall (mm)*	799	799	799
Soil type	nutrient-poor, well-drained sand soil without profile		
Soil pH (water)	4.7	4.7	5.8
Organic matter content (%)	0.5	1.6	3.1
C/N	24	20	19
Heavy metal status	Non-polluted	formerly polluted	polluted
Total soil Zn content (ppm)	16.9 ± 0.6	29.2 ± 3.9	197.0 ± 6.3
Total soil Cd content (ppm)	ND	ND	4.4 ± 0.1
Total soil Pb content (ppm)	7.9 ± 0.4	87.8 ± 13.9	152.9 ± 2.3
Soil $CaCl_2$-extractable Zn	nd	0.2 ± 0.0	35.2 ± 3.4
Soil $CaCl_2$-extractable Cd	ND	ND	0.7 ± 0.1
Soil $CaCl_2$-extractable Pb	ND	ND	0.2 ± 0.0
Zn in *P. sylvestris* needles	40.5 ± 9.2	40.0 ± 8.1	105.0 ± 9.4
Cd in *P. sylvestris* needles	1.8 ± 0.6	0.9 ± 0.2	2.4 ± 0.3
Pb in *P. sylvestris* needles	3.4 ± 0.7	ND	6.9 ± 0.3
Sampling date	11 Nov. 2006	11 Nov. 2006	16 June 2008
Nos. of soil cores	20	20	20
volume of cores (cm^3)	2000	2000	2000
Soil temperature (°C)#	11	11	12
Soil water content (%)#	4	4.8	8.7

* according to the weather station of Kleine Brogel (some 20 km from the sites); # on the sampling date; ND, not detected

Table S2. Characteristics of the different metatranscriptomic cDNA libraries and outcome of their screening for the phenotypic rescue of metal-sensitive yeast mutants.

			No. of cDNA screened per			Total unique cDNAs		
Site	Status	cDNA libraries	*ycf1*Δ (Cd 40	*yap1*Δ (Cd 20	*zrc1*Δ (Zn 20	*ycf1*Δ	*yap1*Δ	*zrc1*Δ
Paal	Non-	$3\ 10^5$	$8.1\ 10^5$	$1.4\ 10^6$	$3.8\ 10^5$	1	0	5
Lom	Formerly	$5\ 10^6$	$6.1\ 10^6$	$2\ 10^6$	$4.9\ 10^6$	10	2	16
Balen	Polluted	$3.5\ 10^6$	$1.3\ 10^8$	$8\ 10^7$	$1.2\ 10^7$	1	1	12

Table S3. Characteristics of the Paal and Lommel cDNA sequence datasets.

	Paal	Balen
No. of sequenced clones	30,000	30,000
No. of contaminating rRNA sequences	2314	1287
No. of "good quality cDNAs"	22444	19467
Average length of cDNA sequences (nucleotides)	466	409
cDNA GenBank/EMBL/DDJB Accession Nos.	FO137100-159543	FO117633-137099
% cDNA with BLASTX hits (GenBank nr, $e<^{-6}$)	48	39
% cDNA with taxonomic annotation (MEGAN, eukaryotes only)	32	25.5

Table S4. Characteristics of the PCR-amplified 18S rRNA sequence datasets.

Putative chimeric sequences were identified using the CHECK_CHIMERA program

	Paal	Balen	Lommel
Total no. of 18S rRNA sequences	371	275	95
Putative chimeric sequences	6	1	1
"good quality" 18S rRNA sequences	319	229	80
No. of MOTUs (97% nt identity threshold)	91	94	58
Accession Nos.	FO181361-181450	FO181451-181538	FN396422-6515

Table S5. Taxonomic affiliation of the 18S rRNA and cDNA sequence datasets from the Paal (first figures), Balen (second figures) and Lommel (third figures) sites.

	18S rRNA (PCR-amplified)	cDNA
Fungi	129 / 147 / 15	4712 / 2718 / 63
Metazoa	101 / 21 / 28	1285 / 1061 / 15
Amoebozoa	38 / 3 / 6	312 / 157 / 1
Alveolata	14 / 3 / 8	97 / 142 / 0
Rhizaria	9 / 3 / 9	10 / 36 / 0
Heterokonta	1 / 1 / 1	59 / 91 / 0
Excavata	5 / 1 / 6	30 / 45 / 0
Plantae	15 / 47 / 6	691 / 677 / 33
Bacteria	0 / 0 / 0	1618 / 995 / 13
Other Eukaryotes	7 / 3 / 1	41 / 38 / 1
Multiple affiliations	0 / 0 / 0	1306 / 1341 / 9

Table S6. Predicted eukaryotic community species richness based on the identification of 18S rRNA molecular taxonomic units (MOTUs) and the calculation of richness estimators. MOTUs were defined based on a 97% of identity threshold between nucleotide sequences.

Site	Paal	Balen	Lommel
No. of 18S rDNA sequences	319	229	80
Number of observed MOTUs	91	88	58
Predicted value of S_{ACE}	194	185	189
Predicted value of S_{Chao1}	174	184	144
Observed MOTUs / S_{ACE}	0.5	0.5	0.3
Observed MOTUs / S_{Chao1}	0.5	0.5	0.4

Gene category	Clades*	Studied clones	Metal resistance (metal / yeast mutant / metal concentration)						Element accumulation
			Cd / *ycf1*Δ / 40µM	**Cd / *yap1*Δ / 20µM**	**Zn / *zrc1*Δ / 20mM**	**Cu / *cup1*Δ / 15 µM**	**Co / *cot1*Δ / 1mM**	**Mn / *pmr1*Δ / 1 mM**	
	-	Mutant + empty vector	no	no	no	no	no	no	none
Metallothionein	-	Ycf06L1-108	yes	yes	no	no	no	no	none
Cysteine rich protein (CRP) family 1	-	YCFC1	yes	yes	no	no	no	no	none
Cysteine rich protein (CRP) family 2	IV	Ycf06L1-69/96/4	yes	yes	no	no	no	no	none
Cysteine rich protein (CRP) family 2	V	YCF-P-49	yes	yes	no	no	no	no	none
Cysteine rich protein (CRP) family 2	VI	Ycf06L1- 67	yes	no	no	no	no	no	none
Cysteine rich protein (CRP) family 2	VII	Ycf06L1-94	yes	yes	no	no	no	no	none
Cysteine rich protein (CRP) family 2	VIII	Ycf06L1-84/57/113/70	yes	yes	no	no	no	no	none
C-terminal domain of a Zn Alcohol dehydrogenase	-	YCF-B-87	yes	yes	no	no	no	no	none
Saccharopine dehydrognase	-	YAP-C-115	yes	yes	yes	no	no	no	none
Hypothetical protein	-	YAP-L-123	yes	yes	no	no	no	no	none
Similar to interaction site E2 of the ubiquitin domain of a ubiquitin-ribosomal protein L40	-	YAP-L-93	yes	yes	yes	no	no	no	none
Polyubiquitin	-	ZRC-J280	no	yes	yes	no	no	no	none
BolA (pfam PF01722)	I	ZRC A52	no	yes	yes	no	yes	yes	Fe
BolA (pfam PF01722)	II	ZRC B2	yes	yes	yes	no	yes	yes	Fe
SKP1, SCF ubiquitin ligase	I	ZRC A40/B13	no	yes	yes	no	no	no	none
SKP1, SCF ubiquitin ligase	II	ZRC A19/L197/A47/A36/B9	no	yes	yes	no	no	no	none
SKP1, SCF ubiquitin ligase	II	ZRC I99	no	yes	yes	no	no	no	none
SKP1, SCF ubiquitin ligase	II	ZRC J281	yes	yes	yes	no	no	no	none
SKP1, SCF ubiquitin ligase	II	ZRC A18/B8/I148/A4/A15/BZ6	yes	no	yes	no	no	no	none

Table S7. Phenotypes associated with the expression of "environmental resistance genes" in different metal-sensitive yeast mutants. Clades nos. refer to the phylogenetic affiliations of the different sequences (see Fig. 3A for the CRP family 2).

Table S8. Origin and characteristics of the environmental samples from which the metagenomic DNAs used to amplify sequences coding for Cysteine rich proteins (CRP2) were isolated.

Sites	Coordinates	Climate	Land use	Soil texture	% C	pH (H2O)	Reference
1 - Paal (Belgium)	51°04'N, 5°10'E	Temperate - Oceanic	Pine forest (*P. sylvestris*)	Sandy	0.59	4.7	(a)
2 - Lommel (Belgium)	51°14'N, 5°15'E	Temperate - Oceanic	Pine forest (*P. sylvestris*)	Sandy	1.58	4.7	(a)
3 - Balen (Belgium)	51°10'N, 5°10'E	Temperate - Oceanic	Pine forest (*P. sylvestris*)	Sandy	3.09	5.0	(a)
4 - Breuil (France)	47°18 N, 4°4'E	Temperate - Continental	Beech and Spruce forests	Sandy loam (upper organic horizons)	13.4	3.9	(c)
5 - Feucherolles (France)	48°52'N, 1°58'E	Temperate - Oceanic	Maize/cereal rotation	NC	ND	ND	(b)
6 - La Côte Saint-André (France)	45°23'N, 5°15'E	Temperate - Continental	Maize culture	Clay loam	2.0	7.0	Ranjard *et al*., 2001
7 - lake Stechlin (Germany)	53°9′N, 13°1′E	Temperate - Continental	Fresh water oligotrophic lake / artificial biofilm	NA	NC	8.7	(e)
8 - lake Schulzen (Germany)	53°15'N13°16'E	Temperate - Continental	Fresh water lake	NA	NC	7.3	(e)
9 - Puéchabon (France)	43°44'N, 3°35'E	Mediterranean	*Quercus ilex* Mediterranean forest	Clay	ND		(d)
10 - Saint-Elie (French Guyana)	4°49'N, 53°16'W	Tropical moist	Tropical forest	Clay	14.4	4.4	(b)
11 - Tabtenga (Burkina Faso)	12°22'N, 1°27'W	Dry tropical (Sahelian)	Sorgho culture	Sandy	ND	6.0	(b)
12 - Ô Mon (Vietnam)	10°7'N, 105°38'E	Tropical moist	Paddy field	Silty-clay	NC	NC	Ranjard *et al*., 2001

NA, not applicable; NC, not communicated; ND, not determined; (a) to (e), soil DNA and data kindly provided by F. Lehembre (a), S. Nazaret (b), Coralie Damon (c) and P. Luis (d) from the laboratoire d'Ecologie Microbienne, University Lyon 1 and I Salka, C Wurzbacher, IGB Leibniz Institute of Freshwater Ecology and Inland Fisheries,Stechlin, Germany (e).

Table S9. Yeast strains used in this study.

Euroscarf, (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/)

Strain	Genotype	Relevant phenotype	Source
BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	heavy metal tolerant	Euroscarf
*zrc1*Δ	BY4741 *zrc1*Δ	zinc sensitive	Euroscarf
*cot1*Δ	BY4741 *cot1*Δ	cobalt sensitive	Euroscarf
*ycf1*Δ	BY4741 *ycf1*Δ	cadmium sensitive	Euroscarf
*yap1*Δ	BY4741 *yap1*Δ	cadmium sensitive	Euroscarf
*pmr1*Δ	BY4741 *pmr1*Δ	manganese sensitive	Euroscarf
DTY4	MAT*α*, *leu2-3, 112his3Δ1, trp1-1, ura3-50, gal1, cup1::URA3*	copper sensitive	Longo *et al.*, 1996
fet3Δfet4Δ	BY4741, *YMR058w::kanMX4, YMR319c::kanMX4*	deficient in Fe transport	Pedas *et al.*, 2008

Table 10. Oligonucleotides used in the present study.

Primer name	Sequence (5’ to 3’)
Eum.B2(gfp)f	CCCAAGCTTATGAGTGGTATTGTTCAAACT
Eum.B2(gfp)r	CCCGGATCCCCCTCATCTTCTTTCTTTTTTTC
Eum.YCF67(gfp)f	CCCAAGCTTATGGCTACTTGTTGTTGTAAGAT
Eum.YCF67(gfp)r	CCCGGATCCCCCTCACCTTTGGGACACTTGG
NMTF	AATGRCTACTTGTTGYTGC
NMTR	YTCATGYTTRCASTYRCAC
NMTR3	CYAYCARRGGGACATCCACA
Euk516R	ACCAGACTTGCCCTCC
Euk1A	CTGGTTGATCCTGCCAG

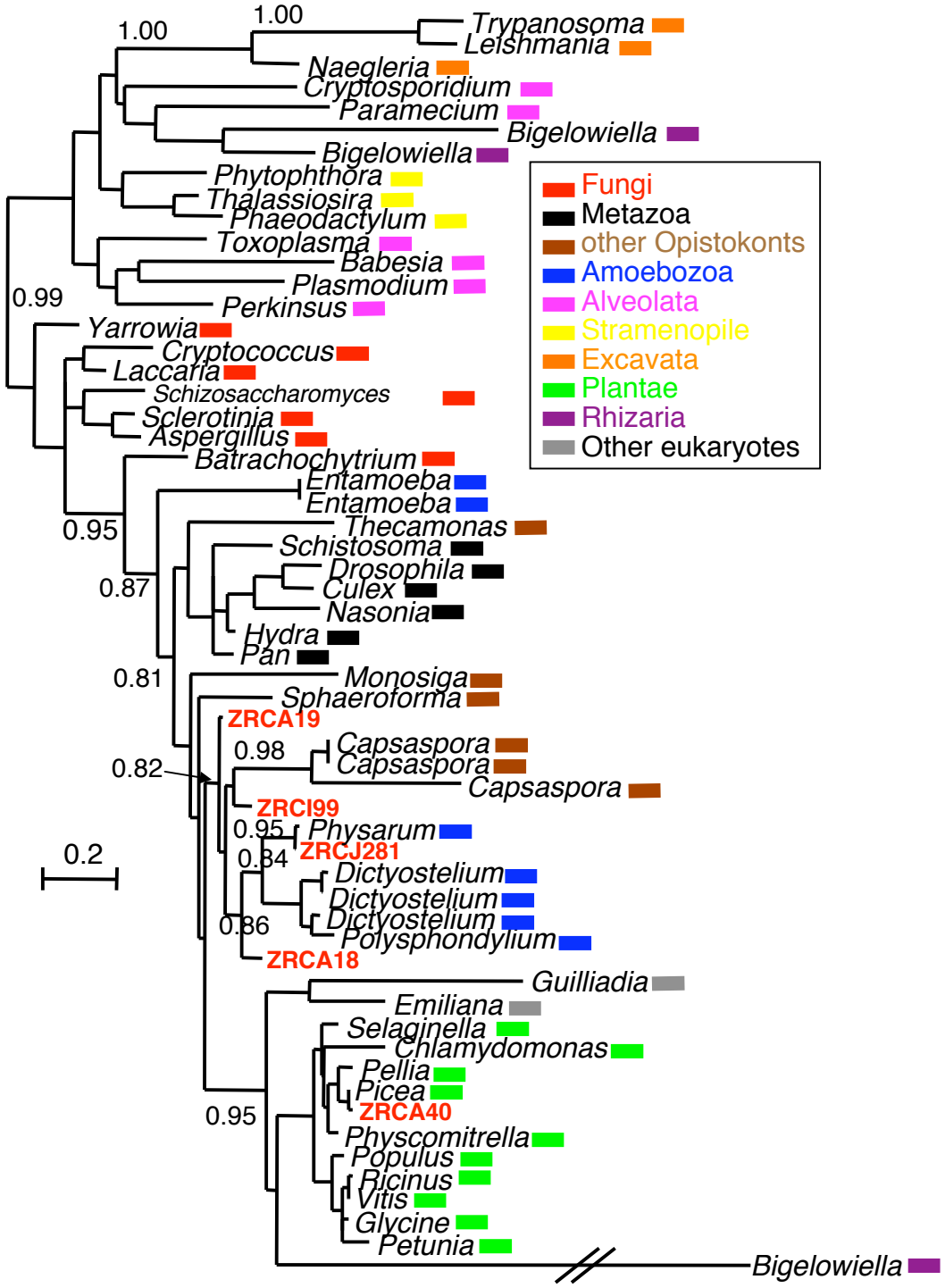

Figure S1. Putative phylogenetic origins of five environmental *SKP1* genes (red) representative of clades II (Table S7). The maximun likelihood (PhyML) phylogenetic tree includes protein sequences from different taxonomic groups, each identified by a specific color. Branch posterior probabilities above 0.8 are indicated.

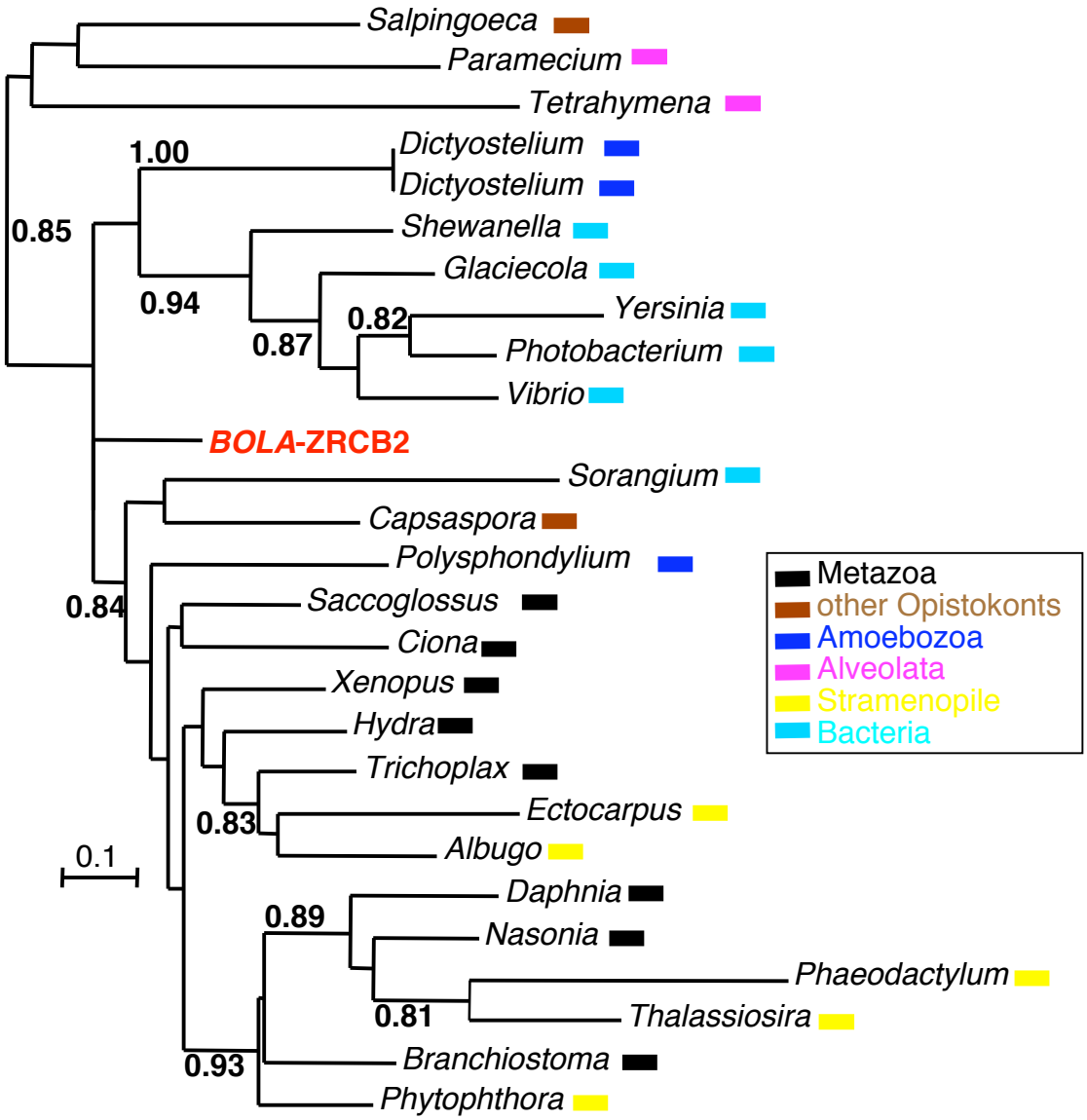

Figure S2. Putative phylogenetic origins of environmental *BOLA* gene clone ZRC B2 (red) which belongs to clade II (Table S7). The maximun likelihood (PhyML) phylogenetic tree includes protein sequences from different taxonomic groups, each identified by a specific color. Branch posterior probabilities above 0.8 are indicated.

Supplementary Text

Supplementary Experimental Procedures

Soil sampling

To maximize the number of organisms present in the soil samples, and therefore the taxonomic diversity of the genes present in the cDNA libraries, at each sampling sites we collected several soil cores which were mixed to constitute composite soil samples. After removing surface vegetation (lichens, mosses) and litter, 20 soil cores (5 cm in diameter, ca 20 cm in depth) were taken at regular intervals along transects. In Balen and Paal, cores were collected at one meter intervals along two 11 m-long transects. In Lommel, cores were collected at one meter intervals along two transects of 6 and one of 11 meters. Soil cores were transported in ice boxes to the laboratory and processed with the 12 hours following sampling.

The sampled sandy soil cores appeared homogeneous along their entire depth and were therefore entirely sieved (2mm mesh size). Equal volumes of each of them were mixed together to constitute the composite samples which were frozen and stored at) -70°C before RNA extraction.

Screening of cDNA libraries for heavy metal resistance genes.

Following transformation, as a first step to eliminate false positives, clones were first cleared of the incorporated plasmids after growth in presence of 0.1% w/v 5-fluoro-orotic acid and their growth on metal-supplemented medium was subsequently compared in the absence or presence of the plasmid. Positive recombinant pFL61 plasmids that conferred metal tolerance were isolated using the Zymoprep Yeast Plasmid Miniprep kit (Proteigene, Saint Marcel, France). The inserts of the selected plasmids were sequenced.

cDNA annotation.

Sequences were trimmed using the TIGR SEQCLEAN tool (http://www.tigr.org/tdb/tgi/software) with default parameters to eliminate 3'-end poly-A sequences, vector, adaptor and primer sequences and undetermined nucleotides. Sequences shorter than 100 nucleotides were eliminated. Contaminant ribosomal RNA sequences were identified by BLASTN analysis (cut-off threshold of E-value ≤ 10-10, word-size=11) of all sequences against the LSUrdb and SSUrdb_SSURef100 libraries as previously described (Urich et al., 2008; available at http://services.cbu.uib.no/supplementary/community-profiling/) as well as against the NCBI-nr nucleotide database (looking for "ribosomal RNA" or "rRNA" in the title).

For taxonomic annotation, cDNA sequences were queried (BLASTX) against NCBI nr protein database (http://www.ncbi.nlm.nih.gov/) using Q-BLAST via BLAST2GO (Conesa et al., 2005) with the parameters E-value ≤ 10-6 and an overlapping length > 33% to the corresponding best hit. Using BLASTX output files, cDNAs were taxonomically assigned using MEGAN v 3.5 (Metagenome analyzer, www-ab.informatik.uni-tuebingen.de/software/megan, (Huson et al., 2009). This program uses the result of a BLAST comparison and assigns each read to a taxon at a specific taxonomic level. All parameters of MEGAN were kept at default values.

18S ribosomal cDNA sequencing and annotation.

Amplification products of the expected size from 15 PCR tubes were pooled, isolated from an agarose gel (QIAquick kit, Qiagen) and ligated into the plasmid pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning kit, Invitrogen), which was then used to transform electrocompetent DH10B E. coli cells (Invitrogen).

For each ribosomal cDNA library, the inserts of more than 92 clones were entirely sequenced (Agowa GmbH, Berlin, Germany and Genoscope Sequencing Centre, Evry, France) using the universal primer M13-20. BLASTn searches were performed against the GenBank nr nucleotide database at NCBI (http://www.ncbi.nlm.nih.gov/) using default parameters except for the word size, which was set to 7. Potential chimeras were detected using the CHECK_CHIMERA program (http://rdp8.cme.msu.edu/). Potential chimeras were further analyzed by separately blasting the two dissimilar segments of the sequences against GenBank. Confirmed chimeras and artifacts were eliminated from the sequence datasets.

Element content

To determine the concentrations of bioavailable heavy metals in the soils, 10 g of soil was dried and incubated with 100 ml of 10 mM calcium chloride under agitation (Menzies et al., 2007). This mixture was filtered and the filtrate was diluted and finally acidified with HNO3 before the inductively coupled plasma emission (ICP-OES Vista MPX, Varian) analysis.

To determine element content in yeast cells growing in liquid medium, cultures were inoculated at OD600=0.05 and harvested at OD600=1.8. Cells were washed three times with 50 mM Tris-HCl pH 6.5, 10 mM EDTA and once with ultrapure water. Cell pellets were dried for 48 h at 50°C and mineralized with 5 ml HNO3 70% (v/w) in a microwave oven. After dilution to a final concentration of 3.5% HNO3, the metal content was determined using inductively coupled plasma emission (ICP-OES Vista MPX, Varian). Quality control was based on the use of internal control samples and certified samples (Astasol-Mix from Analytica Ltd., Prague, Czech Rep.). The experiment was performed independently twice and, for each experiment, the measurements were performed with two different independent transformants for each gene x medium combination.

Supplementary Results

Putative phylogenetic origin of the genes conferring metal resistance in yeast

The taxonomic/phylogenetic origins of genes with no homologues in databases could obviously not be predicted. However, for two of the sequences, which encoded for a BolA protein (clone ZRC A52) and a Skp1 ubiquitin ligase (clone ZRC A40), their taxonomic origins could be traced to the species level. Indeed, these sequences were, 99 and 98% identical, respectively, at the nucleotide level to EST sequences from Pinus sp. Therefore these two sequences certainly originated from Pinus sylvestris, the only pine species present at the study sites.

Clone ycf06 L1-108 encodes for a cysteine-rich 36-amino acid long peptide and was 60-65% identical at the amino acid level to short metallothioneins from Basidiomycotina with a perfect conservation of the cysteine residues. Therefore, this gene certainly originated from a Basidiomycota species.

In the phylogenetic analyses, the 14 other Skp1 ubiquitin ligase protein sequences consistently clustered with sequences from the ameobae Dictyostelium, Physarum and Polysphondylium as well as from the Ichthyosporea Capsaspora (Fig. S1) and could, therefore, originate from one of these phyla whose diversity is grossly underrepresented in databases. Depending on the taxa included in the phylogenetic analysis, protein sequences from Entamoeba sp. That were clustered, or not, with sequences from the other Amoebozoa.

As illustrated in Fig. S2 for the second BolA protein, all other environmental proteins with homologs in databases could not be reliably affiliated to any specific taxonomic groups. This result can be explained, in part, by the low number of phylogenetically informative characters in these sequences.

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3.3.2 Absorption et internalisation du Hg par les champignons supérieurs

Le Hg est un métal hautement toxique, sans fonction biologique connue et qui est facilement biodisponible dans les écosystèmes terrestres. Bien que les champignons soient des contributeurs importants à un certain nombre de processus du sol, dont l'absorption des nutriments par les plantes ou de la décomposition de ces dernières, peu d'informations sont disponibles concernant les effets du Hg sur les champignons. Des tests préliminaires visant à évaluer la tolérance au Hg de champignons isolés de sols contaminés ont montré une grande variation de tolérance entre les isolats fongiques. En effet, la souche ectomycorhizienne *P. involutus* HE01 accumule jusqu'à 6000 ng/gMS de Hg lorsqu'il est cultivé sur un milieu malt gélosé supplémenté avec 100 µM de $HgCl_2$. Afin de déterminer les mécanismes de tolérance impliqués, nous avons sélectionné deux souches de *P. involutus* l'une tolérante au Hg (HE01), l'autre plus sensible (PI01). De grandes quantités de Hg ont été adsorbées au niveau des parois cellulaires, mais aussi internalisées, lorsque les mycélia ont été exposés à des concentrations de Hg modérées et élevées. A la concentration la plus élevée (50 µM $HgCl_2$), les deux souches produisent des protéines de haut poids moléculaire (entre 75 à 100 kDa), qui pourraient se lier au Hg, alors que les mécanismes connus de résistance aux métaux ont montré une faible contribution.

- ***Pour cet article, j'ai réalisé toutes les expériences en collaboration avec Hugo Moreau et Magali Lambert, stagiaires de BTS que j'ai encadrés au sein du laboratoire. J'ai également rédigé la publication avec l'aide des co-auteurs.***

Table S1. **The effects of $HgCl_2$ on radial growth of 104 fungal isolates.** Mycelia were grown on malt agar medium supplemented with 0 to 200 µM $HgCl_2$.

Isolates	Sites	Isolated from	MIC HgCl2 (µM)
Amanita muscaria S02	Mirecourt (France)	Sporocarps	>100
Boletus sp. Th03	Thann (France)	Sporocarps	>100
Cadophora sp. Fe06	Fresnes-sur-Escaut (France)	root	50
Cadophora sp. Pr29	Pierrelaye (France)	root	50
Entrophospora sp. Me11	Metaleurop (France)	root	>40
Entrophospora sp. Me08	Metaleurop (France)	root	100
Exophiala sp. Fe07	Fresnes-sur-Escaut (France)	root	50
Exophiala sp. Fe08	Fresnes-sur-Escaut (France)	root	50
Exophiala sp. Fe10	Fresnes-sur-Escaut (France)	root	100
Exophiala sp. Fe14	Fresnes-sur-Escaut (France)	root	100
Exophiala sp. Fe15	Fresnes-sur-Escaut (France)	root	100
Exophiala sp. Fe16	Fresnes-sur-Escaut (France)	root	50
Exophiala sp. Fe17	Fresnes-sur-Escaut (France)	root	100
Exophiala sp. Fe24	Fresnes-sur-Escaut (France)	root	100
Geomyces sp. Me09	Metaleurop (France)	root	>40
Hebeloma crustuliniforme Fe01	Fresnes-sur-Escaut (France)	Sporocarps	10
Hebeloma crustuliniforme Fe02	Fresnes-sur-Escaut (France)	Sporocarps	50
Hebeloma crustuliniforme Fe03	Fresnes-sur-Escaut (France)	Sporocarps	100
Hebeloma crustuliniforme M26DOM	collection	collection	>100
Hebeloma crustuliniforme Pr09	Pierrelaye (France)	Sporocarps	100
Hebeloma crustuliniforme Pr11	Pierrelaye (France)	Sporocarps	25
Hebeloma crustuliniforme Pr16	Pierrelaye (France)	Sporocarps	50
Hebeloma crustuliniforme Pr17	Pierrelaye (France)	Sporocarps	100
Hebeloma cylindrosporum HcD1	collection	collection	100
Hebeloma cylindrosporum Vi04	Micheville pins (France)	Sporocarps	50
Hebeloma cylindrosporum Vi07	Micheville pins (France)	Sporocarps	25
Hebeloma sp. Cu08	Custine (France)	Sporocarps	25
Hebeloma sp. Pr10	Pierrelaye (France)	Sporocarps	25
Hebeloma sp. Pr12	Pierrelaye (France)	Sporocarps	25
Hebeloma sp. Pr13	Pierrelaye (France)	Sporocarps	100
Hebeloma sp. Pr19	Pierrelaye (France)	Sporocarps	100
Hebeloma sp. Pr20	Pierrelaye (France)	Sporocarps	25
Hebeloma sp. Pr21	Pierrelaye (France)	Sporocarps	100
Hebeloma sp. Pr22	Pierrelaye (France)	Sporocarps	100
Helotiales sp. Fe04	Fresnes-sur-Escaut (France)	root	100
Helotiales sp. Fe05	Fresnes-sur-Escaut (France)	root	>100
Helotiales sp. Fe18	Fresnes-sur-Escaut (France)	root	100
Helotiales sp. Fe19	Fresnes-sur-Escaut (France)	root	25
Helotiales sp. Fe21	Fresnes-sur-Escaut (France)	root	25
Helotiales sp. Me01	Metaleurop (France)	root	50
Helotiales sp. Me03	Metaleurop (France)	root	100

Mercury uptake and internalization by higher fungi

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Abstract

Mercury (Hg) is a highly toxic metal with no known biological function and it can be highly bioavailable in terrestrial ecosystems. Although fungi are important contributors to a number of soil processes including plant nutrient uptake and decomposition, little is known about the effect of Hg on fungi. Preliminary tests aiming at evaluating the Hg tolerance of fungi isolated from contaminated soils, showed a large variation of tolerances between fungal isolates. Indeed, the mycorrhizal fungus *Paxillus involutus* HE01 accumulate up to 6000 ng/g DM of Hg when grown on malt agar medium supplemented with 100 µM $HgCl_2$. To determine the tolerance mechanisms involved, we used a tolerant (He01) and a more sensitive (Pi01) *Paxillus involutus* species. Large amounts of Hg was adsorbed on the cell wall but also further internalized, when the mycelia were exposed to moderate and high Hg concentrations. At the highest concentration (50 µM HgC_{l2}) both fungi produce high molecular weight proteins (between 75 to 100 kDa) that could bind Hg, whereas known mechanisms of resistance to metals showed little contribution to Hg tolerance.

Keywords: Ectomycorrhizae, Endophytes, Mercury, *Paxillus involutus*

Table S1 suite

Isolates	Sites	Isolated from	MIC HgCl2 (µM)
Helotiales sp. Me04	Metaleurop (France)	root	100
Helotiales sp. Pr01	Pierrelaye (France)	root	>100
Helotiales sp. Pr02	Pierrelaye (France)	root	100
Helotiales sp. Pr06	Pierrelaye (France)	root	100
Helotiales sp. Vi08	Micheville boues (France)	root	100
Helotiales sp. Vi09	Micheville boues (France)	root	100
Helotiales sp. Vi10	Micheville boues (France)	root	200
Helotiales sp. Vi11	Micheville boues (France)	root	50
Laccaria laccata Pr03	Pierrelaye (France)	root	100
Laccaria proxima PRAC	collection	collection	100
lachnum sp. Me06	Metaleurop (France)	root	100
Lactatrius sp. Th05	Thann (France)	Sporocarps	50
Lactatrius sp. Th07	Thann (France)	Sporocarps	100
Lactatrius sp. Th08	Thann (France)	Sporocarps	50
Lactatrius sp. Th09	Thann (France)	Sporocarps	25
Leccinum scabrum Th03	Thann (France)	Sporocarps	100
Lepista nuda Vi02	Micheville boues (France)	Sporocarps	50
Leptodontidium orchidicola Pr30	Pierrelaye (France)	root	40
Leptodontidium sp. Fe20	Fresnes-sur-Escaut (France)	root	100
Leptodontidium sp. Me10	Metaleurop (France)	root	50
Leptodontidium sp. Me12	Metaleurop (France)	root	100
Lyophyllum connatum Pr14	Pierrelaye (France)	Sporocarps	100
Meliniomyces sp. Fe22	Fresnes-sur-Escaut (France)	root	25
Meliniomyces sp. Me07	Metaleurop (France)	root	50
Meliniomyces sp. Me13	Metaleurop (France)	root	10
Meliniomyces sp. Me14	Metaleurop (France)	root	>40
Paxillus involutus Bo01	Bordeaux (France)	Sporocarps	100
Paxillus involutus Bo02	Bordeaux (France)	Sporocarps	25
Paxillus involutus Bo03	Bordeaux (France)	Sporocarps	50
Paxillus involutus Bo04	Bordeaux (France)	Sporocarps	50
Paxillus involutus Bo05	Bordeaux (France)	Sporocarps	100
Paxillus involutus Bo06	Bordeaux (France)	Sporocarps	50
Paxillus involutus Cu01	Custine (France)	Sporocarps	100
Paxillus involutus Cu02	Custine (France)	Sporocarps	100
Paxillus involutus HE01	Hechtel-Eksel (Belgium)	Sporocarps	100
Paxillus involutus LS01	Lommel-Sahara (Belgium)	Sporocarps	25
Paxillus involutus LS02	Lommel-Sahara (Belgium)	Sporocarps	25
Paxillus involutus LS03	Lommel-Sahara (Belgium)	Sporocarps	25
Paxillus involutus Ov01	Overpelt (Belgium)	Sporocarps	100
Paxillus involutus Ov02	Overpelt (Belgium)	Sporocarps	100
Paxillus involutus Pi01	Midlothian (Scotland)	Sporocarps	25
Paxillus involutus Pi04	Vedby (Sweden)	Sporocarps	100
Paxillus involutus S01	Mirecourt (France)	Sporocarps	25

Introduction

Mercury is a highly toxic metal with no known biological function. The presence of Hg in soil can be due to geological processes or environmental contamination from either point sources or atmospheric deposition (Fitzgerald et al., 1998). The major source of environmental contamination of Hg arises from various anthropogenic activities including chlor-alkali industries, recovery of gold, burning coal, natural gas, metal processing and petroleum products, use of mercurial fungicides. Elemental gaseous Hg (Hg0) is the major form of Hg in the atmosphere, where Hg0 is extremely volatile and can be oxidized photochemically to mercuric ion (Hg2+) and methylated by microorganisms (Bacci et al., 1994; Guimarães et al., 1998).

The toxicity of Hg arises mainly from its oxidizing properties. As a metal ion, it induces oxidative stress or predisposes cells to oxidative stress, with much subsequent damage to proteins, lipids and DNA. Mercuric cations have a high affinity for sulphydryl (-SH). Because almost all proteins contain sulphydryl groups or disulphide bridges, mercurials can disturb almost any function in which critical or nonprotected proteins are involved (Clarkson, 1972). In multicellular and compartmentalized organisms broad-spectrum mechanisms, which confer resistance to various trace elements (TE), have evolved (for example, metallothioneins in animals and phytochelatins in plants and fungi). In bacteria, resistance is often specific to one metal or a small number of related metals. Many bacteria reduce Hg2+ ions into volatile Hg coded by mer operon (Dash and Das, 2012) in cooperation with other biotic factors including glutathione-transferase activity (Zhang et al., 2013). Methylmercury is produced by anaerobic microorganisms from inorganic Hg by a pathway containing a two-gene cluster, consisting of hgcA and hgcB, that encode proteins essential for this activity (Smith et al., 2015).

Certain higher plants have an armory of metal-protection mechanisms, including metal sequestration by specially produced organic compounds, sequestration in certain cell compartments, metal ion efflux, and organic ligand exudation. In Elodea nuttallii, uptake of Hg into the cell sap of shoots seems linked to the metabolism and to copper transporters (Regier et al., 2013). Mycorrhizal fungi participate in crucial symbiotic relationships with plants that grow on contaminated sites, and alleviate metal toxicity for their host plants (Jentschke and Godbold, 2001; Schützendübel and Polle, 2002). Unlike mycorrhizal fungi that colonize plant roots and grow into the rhizosphere, fungal endophytes reside entirely within plant tissues. Recent studies suggested that root colonization by fungal endophytes was the highest in highly metal-contaminated locations and this fungi could improve metal tolerance of trees in highly contaminated areas (Karliński et al., 2010; Likar and Regvar, 2009; Regvar et al., 2010). We recently showed that endophytic isolates belonging to the Helotiales order were highly tolerant to TEs (Cd, Zn, Pb, and Cu) compared to some mycorrhizal isolates (Lacercat-Didier et al., 2016). We also demonstrated that DSE isolated from polluted soils could promote tree growth and protection against high TE concentrations (Berthelot et al., 2016).

The mechanisms that may be involved in the tolerance of fungi to excess metals in their environments include mechanisms that reduce uptake of metals into the cytosol by extracellular chelation through extruded ligands (for example, organic acids: citrate and oxalate, siderophores) and binding onto cell-wall components (such as chitin and chitosan). Metal uptake occurs, for example, through specific transporters for the acquisition of essential metals, and these transporters may cross-react with other metals. Intracellular chelation of metals in the cytosol by a range of ligands (glutathione, metallothioneins and phytochelatins), or increased efflux from the cytosol out of the cell (by transporters or by metal volatilization) or into sequestering compartments (storage in vacuoles) are also key mechanisms conferring tolerance. Free-radical scavenging capacities through the activity of superoxide dismutase or production of glutathione add another line of defense against the toxic effect of metals (Bellion et al., 2006; Harms et al., 2011). There is a significant amount of literature related to the accumulation of Hg in edible mushrooms, as recently reviewed in (Falandysz and Borovicka, 2013; Krasińska and Falandysz, 2016; Ostos et al., 2015). However, the mechanisms of Hg entry into fungal cells remain unclear.

Table S1 suite

Isolates	Sites	Isolated from	MIC HgCl2 (µM)
Paxillus involutus Vi01	Micheville boues (France)	Sporocarps	25
Paxilus sp. Th01	Thann (France)	Sporocarps	50
Paxilus sp. Th02	Thann (France)	Sporocarps	25
Phialocephala fortinii Pr28	Pierrelaye (France)	root	50
Phialophora Mustea Pr27	Pierrelaye (France)	root	50
Piriformospora indica Pind	Allemagne	root	100
Pisolithus tinctorius Pt01	collection	collection	50
Podospora sp. Podo	collection	collection	200
Scleroderma bovista Pr25	Pierrelaye (France)	Sporocarps	50
Scleroderma bovista Pr26	Pierrelaye (France)	Sporocarps	>100
Sebacina vermifera Pr04	Pierrelaye (France)	root	50
Suillus granulatus Vi06	Micheville boues (France)	root	50
Suillus luteus Vi03	Micheville Pins (France)	Sporocarps	100
Suillus luteus Vi05	Micheville Pins (France)	Sporocarps	50
Tetracladium sp. Fe09	Fresnes-sur-Escaut (France)	root	50
Tetracladium sp. Fe11	Fresnes-sur-Escaut (France)	root	100
Tricholoma mongolicum Tx01	Tavaux (France)	Sporocarps	50
Tricholoma ustale Pr05	Pierrelaye (France)	root	50
Trichopezizella nidulus Me02	Metaleurop (France)	root	200

In Saccharomyces cerevisiae, the major fraction of Hg is tightly bound to the cell wall (Murray and Kidby, 1975). Hg was associated with the cell wall and cytoplasmic membrane and was localized in dense bodies consisting of a core of Hg sulfide polymers (Whittaker et al., 1990). S. cerevisiae can also produce hydrogen sulfide (H2S) gas which co-precipitate Hg (Amin and Latif, 2011). Another defense mechanism involving an induction of the expression of Ycf1p and transport by this protein of Hg–glutathione adducts into the vacuole has been showed by (Gueldry et al., 2003). In contrast to Cd, metallothionein CUP1 does not conferred resistance to Hg in S. cerevisiae (Ecker et al., 1986). In other fungi, carboxyl groups of cell walls have been shown to be involved involved in the biosorption of Cd2+, Ag+, Hg+ and CH3Hg+, for example in Aspergillus niger (Karunasagar et al., 2003). The hydrophobins may well play a role in the sequestration of Hg into the cell wall leading to the accumulation of the metal outside the cell in Trichoderma harzianum (Puglisi et al., 2012). In cell, Hg interfered with biomolecules, such as enzymes and structural proteins. Studies of several enzymes and structural proteins containing Zn have demonstrated that substitution of Cd or Hg for the chemically related Zn may take place, often with devastating effects on the structure and/or biological function of the protein (Collin-Hansen et al., 2007). Hg exerts its toxic effect mainly by binding to SH groups present in the active or regulation sites of enzyme and causing their irreversible inactivation (Baldrian, 2003). Kojo and Lodenius (1989) found a strong correlation between the sulfhydryl and Hg contents in mycorrhizal fungi and a positive correlation between the catalase activity and the Hg content as well. Tuber melanosporum respond to Hg by synthesizing phytochelatins (PCs) that act as chelators (Bolchi et al., 2011).

In the present work, we hypothesized that Hg is actively captured by fungal cells and sequestrated in intracellular compartments. To test this hypothesis, we first determined the minimum inhibitory concentration (MIC) of fungal strains isolated from various TE-contaminated or non-contaminated sites by growing 104 isolates on agar plates with increasing HgCl2 concentrations. We further detailed the mechanisms involved in Hg accumulation in two Paxillus involutus isolates exposed to Hg by performing time-dependent kinetics of Hg desorption, and by evaluating the impact of Hg on known biochemical mechanisms.

Materials and methods

Isolation and identification of fungi

Root and sporocarp samples were collected from several experimental sites in Europe (Table S1), including a Hg-contaminated site previously described (Assad et al., 2016). In addition, snails from the Helicidae and Hygromiidae families, as well as slugs from the Arionidae et Agriolimacidae families were collected at this site on the same day. The biological samples were washed, freeze-dried, and grounded before Hg analysis.

Fungal mycelia were isolated either from sporocarps or from roots. To isolate root-inhabiting fungal strains into pure culture, roots were first washed with water, sterilised in 35 % hydrogen peroxide for 30 s, and rinsed three times in sterile deionised water. Sporocarps of fungi were cleaned using a scalpel and cut in two pieces in sterile condition. Then, fragments of about 1 cm² were cut with a scalpel in the inner area of the cap. Root fragments of approximately 1 cm or fruit-body fragments were incubated on modified Melin-Norkrans medium (Brundrett et al., 1996), malt extract agar medium (malt: 12 g/l; agar: 15 g/l), NP2/2 medium (Benjdia et al., 2006), or Pachlewski medium (Pachlewski and Pachlewska, 1974). Ampicillin (100 mg/l) and chloramphenicol (100 mg/l) were added to the media to avoid bacterial contaminations. Dishes were incubated at 24 °C in the dark from 1 week up to 3 months (Lacercat-Didier et al., 2016).

Putative endophytic and mycorrhizal isolates were selected and identified via ITS sequencing, and corresponding sequences were aligned with fungal sequences obtained from samples. Sequence similarities were determined using the BLASTN sequence similarity search tool (Altschul et al., 1997).

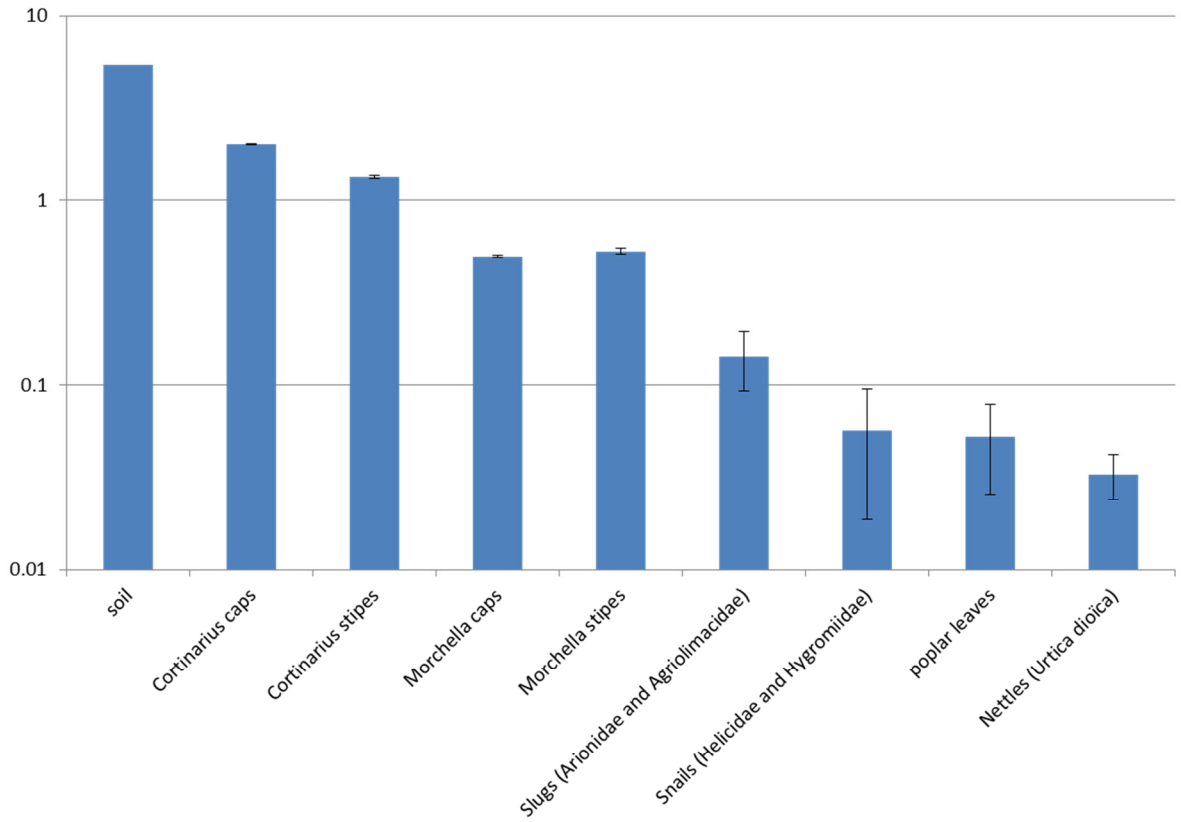


Figure 1. Total Hg concentrations (µg.g^{-1} DM) in various biological and soil samples. All samples were collected at the Tavaux site, within the same day.

Hg tolerance tests upon acute or chronic exposure

The sensitivity of the fungal isolates to Hg concentration was evaluated by investigating the minimum inhibitory concentration (MIC) of HgCl2 on fungal growth. Several cylindrical agar plugs (5 mm diameter) were removed from the edges of actively growing 1-week-old fungal colonies and incubated on a sterile EDTA-treated cellophane membrane, on malt extract agar medium amended with Hg from 0 to 100 µM HgCl2. Growth was observed after sufficient growth or after 2 weeks of incubation at 24°C for a preliminary screening. This incubation protocol might mimic a chronic exposure of mycelia to Hg.

To further mimic an acute exposure to Hg, agar plugs (5 mm diameter) were cut from the actively growing edge of 1-week-old colonies and incubated on a sterile EDTA-treated cellophane membrane, on malt extract agar medium. After sufficient growth (7 days of for Pi01 and 14 days for He01), mycelia were transferred in liquid malt medium and incubated 4 days at 24°C. Mycelia were further exposed to Hg at the following concentrations: 0, 10 (sub-lethal) and 50 (lethal) µM HgCl2. Growth was measured after 30 min, 12 hr and 1 week (168 h) of incubation at 24°C. To determine the proportion of cell wall-bound Hg, mycelia were incubated for 2 min on a desorption solution containing 10 mM EDTA at pH 5.35 and rinsed 3 times with sterile deionized water. To determine the total mycelium-bound Hg, mycelia were solely rinsed 3 times with sterile deionized water. The mycelia and desorption solution were analysis for Hg content.

Hg desorption kinetics

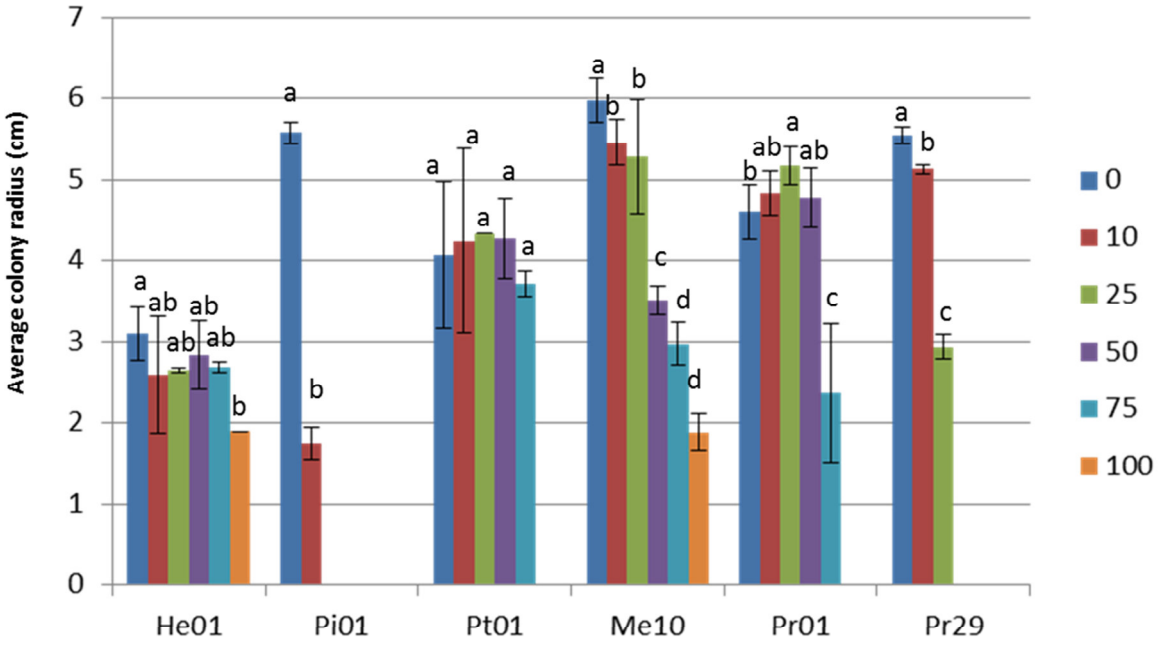
For the determination of desorption rates, discs of Paxillus involutus mycelia (Pi01 and He01) were cut from the actively growing edge of 10-days-old colonies using a 15 mm diameter cork borer. The discs were exposed for 30 min or 12 hr to the standard assay medium containing 0.5 mM CaCl2, 2 mM MES and 10 µM HgCl2 at pH 4,5 and 20 °C. Discs were briefly rinsed in the uptake solution from which HgCl2 was omitted, and finally floated on a desorption solution containing 5 mM CaCl2 in 2 mM MES (pH 4,5) at 4 °C to initiate Hg desorption. At various time intervals, 200 µl aliquots of the desorption solution were taken out and Hg content was determined. Desorption of Hg from mycelia in the external solution was monitored for 10 hr (Blaudez et al., 2000a).

Extraction and determination of soluble proteins and stress related compounds under Hg exposure

Freeze-dried mycelia (10 mg) were pulverized with 3-mm tungsten carbide beads (Qiagen S.A.S., Courtaboeuf, France) in a Mixer Mill for 1 min at 30 Hz (model MM400; Retsch Inc., Newtown, PA) and homogenized with 1 ml of extraction solution containing 10 mM Tris HCl, 0.1 mM PMSF and 0.05 % Tween 80 to extract the soluble proteins. The homogenate was centrifuged at 10,000 rpm for 45 min at 4°C. The supernatant was used to determine the total soluble protein content using a commercial kit (Coomassie Bradford Protein Assay Kit, Thermo Scientific) according to its manual instructions.

After protein extraction, enzyme activities of superoxide dismutase (SOD) and catalase (CAT), lipid peroxidation (MDA) and total antioxidant capacity (TAC) were measured with commercial kits (Sigma) according to their manual instructions. The SOD and CAT activities, MDA and total antioxidant activity were expressed as U.mg-1 DM, nmol.mg-1 DM and nM Trolox eq. mg-1 DM, respectively.

To determine the protein profiles on polyacrylamide gels, the soluble protein extracts were adjusted to 5 µg.ml-1 and 25 µl were mix with 5 µl 4x Laemmli sample buffer (Bio-Rad, Marnes la Coquette, France). Hundred and 25 µg of proteins were loaded onto 4-20 % native PAGE (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad). The electrophoresis was performed in a Mini-Protean II unit at 100 V for about 1 hr with 1x Tris/Glycine/Méthanol (Bio-Rad), and gels were stained with Coomassie Brilliant Blue according to standard procedures (Green et al., 2012). Molecular mass estimations were carried out using standards of known molecular mass (Precision Plus Protein™ Dual Color Standards, Bio-Rad, France).



	He01	Pi01	Pt01	Me10	Pr01	Pr29
days	42	12	42	21	35	20
EC_{50}	95.5	9.3	85.8	96.3	75.1	24.7
MIC	>100	25	100	>100	100	50
	ectomycorrhizal			endophyte		

Figure 2. The effects of $HgCl_2$ on radial growth of 6 selected fungal isolates. *Paxillus involutus* He01, *Paxillus involutus* Pi01, *Pisolithus tinctorius* Pt01, *Leptodontidium sp.* M10, *Helotiales* Pr01 and *Cadophora sp*. Pr29 were incubated on malt agar medium supplemented with 0 to 100 µM $HgCl_2$ for various lengths of times. Means and standard deviations of at least five replicates are presented, with the exception of *Paxillus involutus* He01 and *Pisolithus tinctorius* Pt01grown at 75 and 100 µM Hg (two replicates). Values designated with the same letters were not significantly different ($P<0.05$) according to a Kruskal-Wallis test.

Mercury analysis in biological materials and soils

The milled freeze-dried mycelia and liquid solution were analyzed using an advanced Hg analyser (AMA 254, Courtage Analyses Services, Mont Saint Aignan, France), as fully described in Assad et al. (2016) and Maillard et al. (2016). All samples were run together with adequate certified reference material (oriental basma tobacco leaves, INCT-OBTL-5, LGC Promochem, Molsheim, France, 20.9 ± 1.3 ng g_1 dry mass) + TORT-2 (lobster hepatopancreas) et DOLT-4 (fish liver). The detection limit was 0.145 ng g-1.

Statistical analyses

The experiments were set up with three to five replicates. Data were statistically analyzed using R software v. 3.0.2 (R Development Core Team 2013) and presented as means values ± standard deviations (SD). To evaluate the EC50, a nonlinear regression analysis was performed with the best-fitted sigmoid equation model. A Kruskal-Wallis test ($P< 0.05$) was used to evaluate significant differences between treatments.

Results and discussion

Hg in biological and soil samples from a Hg-contaminated experimental site

We performed Hg analysis in a number of compartments at our experimental site in Tavaux (France), which has been recently described (Maillard et al., 2016; Zappelini et al., 2015). We collected fungal fruit-bodies from the well-known edible Morchella species in April 2013, as well as in other biomasses such as snails, slugs and herbaceous plants (Fig. 1). The Hg contents measured in Morchella fruit-body were at least 1 order of magnitude greater than those from other living organisms. There are large amounts of data on the accumulation of Hg by fungi in the literature, and our data fall in the ranges measured in various fruiting fungi (Falandysz and Borovicka, 2013; Krasińska and Falandysz, 2016; Ostos et al., 2015). However, data on the accumulation of TEs, and hence of Hg by Morchella species are scare. Osturk et al (2010) reported a Hg concentration of 0.06 mg / kg DM in M. conica collected in a non contaminated area.

Some Morchella species are tasty, popular, and valuable wild-grown fungi used traditionally as gourmet in Western Europe, as well as sources of bioactive compounds (Vieira et al., 2016). Our THg data of Morchella species is close to the median THg value reported by (Krasińska and Falandysz, 2016), around 650 μg kg−1 DM. A meal made of 100 g of fresh caps of M. conica from the Hg-contaminated sites and containing an average of 500 μg / kg DM of THg, would provide around 5 μg of Hg. As calculated recently (Krasińska and Falandysz, 2016), considering a Hg reference dose (RfD) of 0.3 μg kg−1 body mass daily, amount corresponds to a dose of around 0.083 μg/kg bm for a person of 60 kg bm, assuming no Hg intake from other sources. Theoretically, if eaten daily, the Hg intake rate calculated for the contaminated Morchella is far lower (ca. 40-fold) than the RfD value. Additionally, given the relatively elevated price of morels or the rarity in its wild environments, fruiting bodies of Morchella are not eaten daily and this mass is probably highly overestimated.

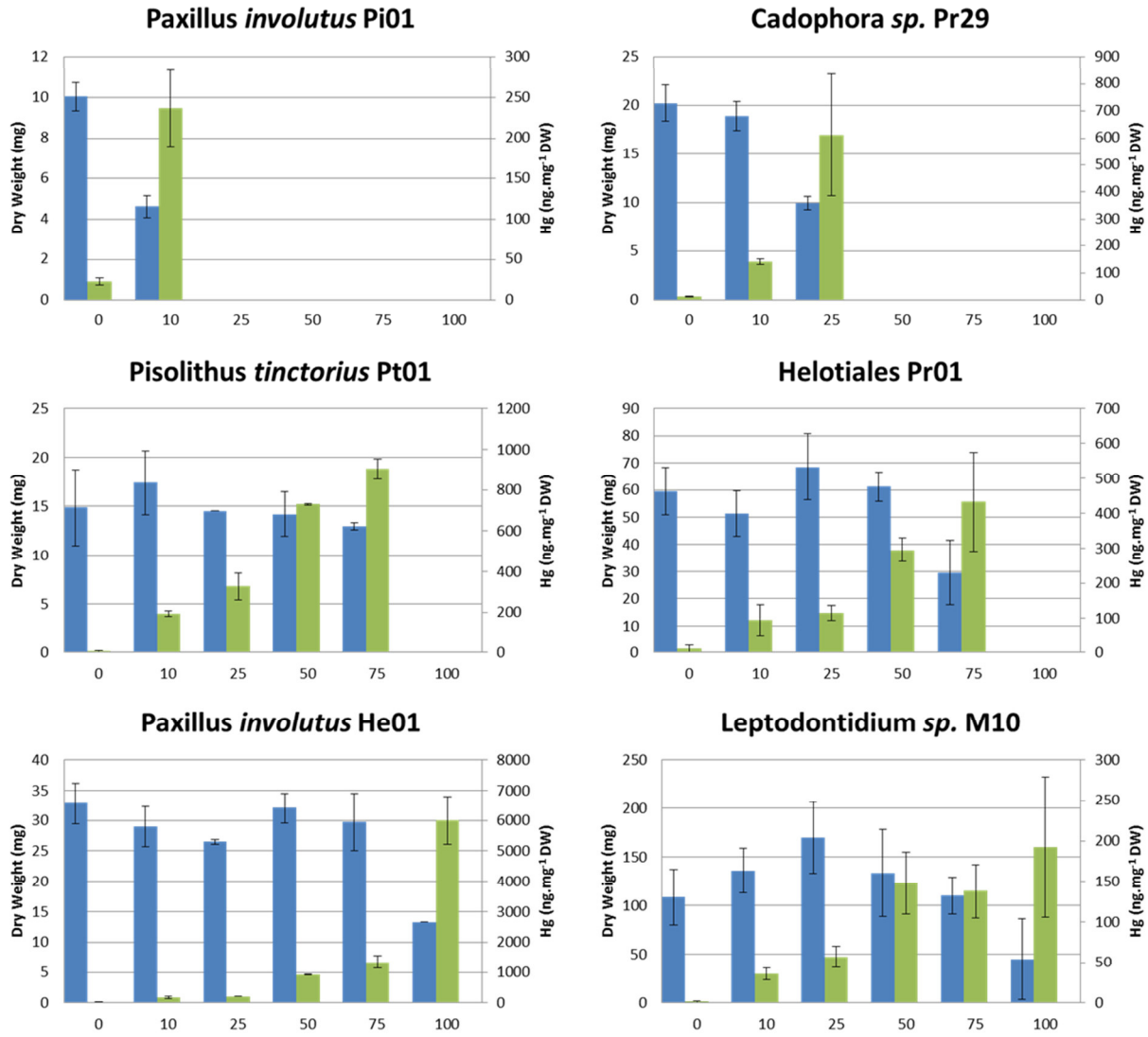


Figure 3. The effects of $HgCl_2$ on fungal biomass production (mg DM) and Hg accumulation (ng.mg^{-1} DM) under chronic Hg exposure on 6 selected fungal isolates. *Paxillus involutus* He01, *Paxillus involutus* Pi01, *Pisolithus tinctorius* Pt01, *Leptodontidium sp.* M10, , *Helotiales* Pr01 and *Cadophora sp*. Pr29 were incubated on malt agar medium supplemented with 0 to 100 µM $HgCl_2$ for various lengths of times. Means and standard deviation of at least five replicates are presented, with the exception of *Paxillus involutus* He01 and *Pisolithus tinctorius* Pt01grown at 75 and 100 µM Hg (two replicates). Values designated with the same letters were not significantly different ($P<0.05$) according to a Kruskal-Wallis test.

Growth rate and Hg accumulation by fungi

To further elucidate Hg accumulation mechanisms by higher fungi, we measured Hg tolerance of 104 isolates of ectomycorrhizal and endophytic fungi collected from TE- and Hg-polluted sites and 2 from unpolluted site by growing them on Hg-amended media to determine their in vitro tolerance, as measured by growth inhibition (Table S1). Testing fungi in axenic culture has been considered as a preliminary screening step in the selection of fungi inoculates for reclamation projects (Blaudez et al., 2000b). For most fungi, the minimum inhibitory concentrations (MIC) fallen within the 10 to 200 µM range. For saprophytic fungi, MIC was between 50 and 200 mM (Baldrian, 2003). The MIC for Hg and Cd are usually lower than those for Zn, Mn, Cu or Ni, as Hg and Cd have no known biological function in fungi (Frey et al., 2000). Possible mechanisms for passive binding, or metabolic detoxification which can lead to metal tolerance in fungi have been discussed by several authors and reviewed in (Bellion et al., 2006; Falandysz and Borovicka, 2013; Fomina and Gadd, 2014; Gadd et al., 2012).

Following this preliminary screening, three ectomycorrhizal and three endophytic isolates were further chosen to determine more precisely their response to Hg, measured by radial growth, biomass and Hg uptake. The final colony radii showed that sensitivity of the six fungi to Hg varied greatly, with MIC values greater than 100 µM Hg for the Helotiale Pr01 Leptodontidium sp. M10 and Paxillus involutus He01. Paxillus involutus Pi01 was highly sensitive (Fig. 2). This was not related to growth, as Paxillus involutus He01 and Pisolithus tinctorius Pt01 grew very slowly (42 days) in contrast to Paxillus involutus Pi01 (12 days). Ten µM Hg reduced growth of Pi01 by more than 50%, whereas growth of He01 was not significantly affected at this Hg concentration. The radius of Leptodontidium sp. M10 expanded exponentially whereas Paxillus involutus He01 displayed short lag phases followed by slow growth in the presence of Hg (not shown).

Hg concentrations lower than 100 µM also reduced biomass production (dry weight) for all fungi except for Pisolithus tinctorius Pt01 (Fig 3), which is consistent with the radial growth (Fig 2). Increased concentrations of intracellular Hg could be observed in all fungi upon exposure to increasing concentrations of Hg in the solid growth medium. However, mycelial Hg concentrations differed greatly between fungi, with the He01 mycelium accumulating up to 6000 ng /g Hg DM, compared to less than 250 ng/g Hg DM for Pi01 and around 1000 ng /g Hg DM for Pt01. There were also noticeable variations between tolerant fungi, M10 accumulating less than 200 ng/g Hg DM at 100 µM compared to the 6000 ng /g Hg DM for He01 grown at the same Hg concentration. Our mycelial Hg concentrations when fungi were exposed at 25 µM are similar to those measured by Crane et al. (2010), that fall within the range 50 to 700 µg.g-1 DM. The highest accumulation of Hg in He01 might probably be due to its slow growth rate and a longer exposure period to Hg (42 days). The decrease of fungal growth rate and the increase of the lag phase were also recorded in saprophytic fungi (Baldrian, 2003), wood-rotting Basidiomycetes (Mandal et al., 1998) and ectomycorrhizal fungi (Crane et al., 2010). An increase in lag phase followed by the resumption of a normal growth rate, for example in P. involutus He01 (Fig. S1), suggests the possible induction of an adaptive response to Hg (resistance), as already well documented for the bacterial response to Hg (Barkay et al., 2003).

Although metal tolerance is often linked to the origin of the fungal isolates (Colpaert et al., 2004), this is not the case for Hg tolerance. For instance, although the Hg sensitive P. involutus species originated from a non-contaminated site, many other P. involutus from Hg contaminated sites (Tavaux, Thann) were not tolerant to Hg (Table S1). Hg tolerance between ectomycorrhiza and saprophytic fungi did not differed, as e.g. sensitive (P. involutus Pi01 and Cadophora sp. Pr29) and tolerant (Leptodontidium sp. M10 and P. involutus He01) isolates were found in the two categories. We further use the sensitive (Pi01) and tolerant (He01) P. involutus isolates to study Hg tolerance mechanisms.

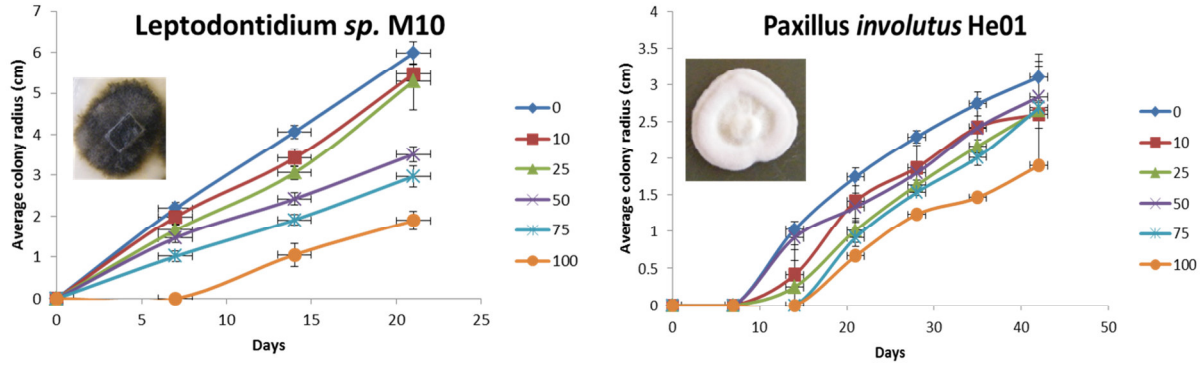


Figure S1: **The effect of $HgCl_2$ on radial growth of *Leptodontidium sp.* M10 and *Paxillus involutus* He01.** Mycelia were grown on malt agar medium supplemented with 0 to 100 µM $HgCl_2$ for various lengths of times. Means and standard deviation of at least five replicates are presented.

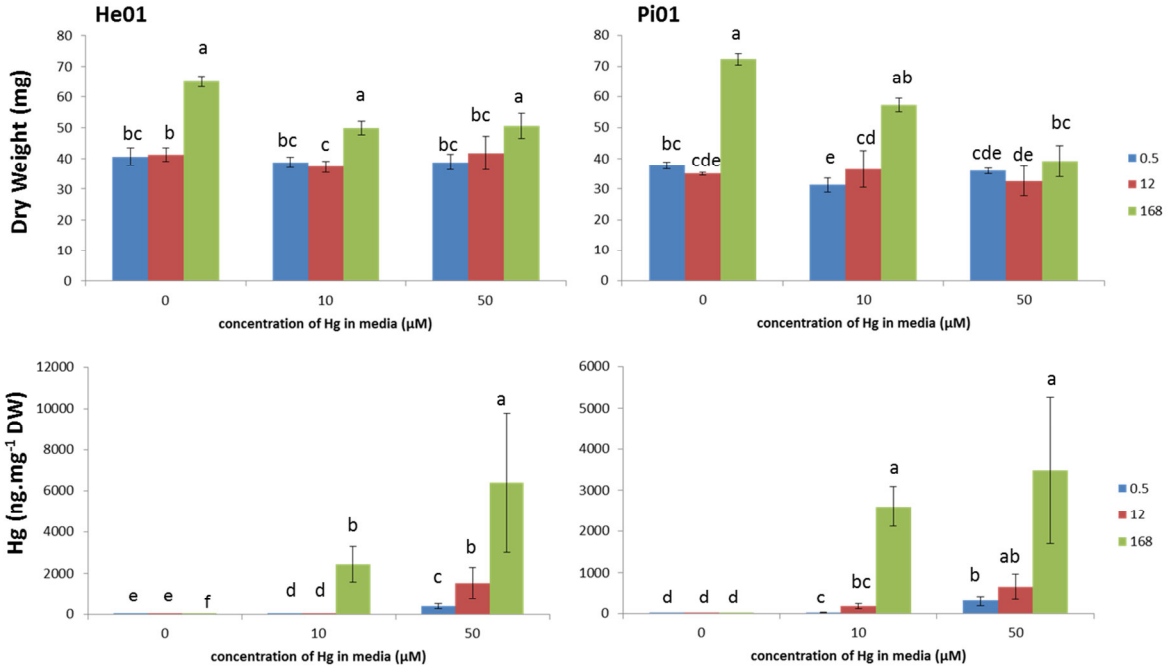


Figure 4. The effects of $HgCl_2$ on fungal biomass production (mg DM) and Hg accumulation (ng.mg^{-1} DM) under acute Hg exposure. *Paxillus involutus* He01 and *Paxillus involutus* Pi01 agar plugs (5 mm diameter) were cut from the actively growing edge of 1-week-old colonies and incubated on a sterile EDTA-treated cellophane membrane, on malt extract agar medium. After sufficient growth (7 days of for Pi01 and 14 days for He01), mycelia were transferred in liquid malt medium. After 4 days at 24°C, Hg was added at 0, 10 and 50 µM $HgCl_2$ for various lengths of times (0.5, 12 and 168 h). Means and standard deviations of at least tree replicates are presented. Values designated with the same letters were not significantly different ($P<0.05$) according to a Kruskal-Wallis test.

To overcome the differences in growth rate previously observed, we further grew these two isolates in liquid growth media without Hg, and added Hg after 4 days of growth, thus mimicking an acute exposure rather than a chronic exposure as experienced in Figures 2 and 3. Biomass and mycelial Hg concentrations were recorded after 0.5, 12 and 168 hr (Fig. 4). The two isolates showed similar biomass production, which significantly increased at the latest harvest for all treatments and both isolates. Unexpectedly, the Hg sensitive isolate Pi01 was not affected by the highest Hg concentration and accumulated up to 3000 ng/g Hg DM. It therefore seems that a chronic exposure of the Pi01 isolate to Hg (Fig. 3) prevented the fungal cells to initiate growth of the mycelium and thereof preventing expression of tolerance mechanisms. In contrast, once mycelial growth is established, Pi01 can fully grow and accumulate Hg, although at a lower concentration than the He01 isolate (Fig. 4).

Mercury compartmentation under acute exposure

Fungi were exposed to increasing Hg concentrations for various lengths of time as described for figure 4. Hg analysis were performed in the growth medium, in the EDTA desorption solution and in the remaining mycelia (Table 1). The EDTA solution contained the Hg slightly bind to the cell wall, whereas the remaining mycelia contained the Hg that has been internalized, as described in (Oyetibo et al., 2014).

At a sub-lethal Hg concentration (10 µM), Hg was quickly taken up by He01 and Pi01mycelia (14 and 13 ng.min-1, for Pi01 and He01, respectively) and bound to cell walls (4 and 2.6 ng.min-1, for Pi01 and He01, respectively) after 30 min of incubation. The rate of Hg uptake decreased after 12 hr of incubation (3 and 4 ng Hg.min-1) and after 1 week of incubation (1.8 and 2 ng Hg.min-1, for Pi01 and He01, respectively) but remained similar for both fungi. The proportions of Hg bound to the mycelia (e.g. in fungi + EDTA solution versus total Hg in the growth medium) represented 0.66 and 0.59% of THg after 30 min of incubation in He01 and Pi01, respectively (Table 1). After 1 week of incubation at 10 µM Hg, most (>95% for both fungi) of the Hg was internalized.

At a lethal Hg concentration (50 µM), the overall quantities of Hg taken up by the mycelia exposed to 50 µM Hg were 4- and 6-fold higher for Pi01 and He01 mycelia, respectively, after 12 hr of incubation compared with the lowest Hg concentration. At the highest incubation period, the differences were lowered. Hg was quickly taken up by both fungi (86 and 97 ng.min-1, for Pi01 and He01, respectively) and bound to cell walls (51 and 16 ng.min-1, for Pi01 and He01, respectively) after 30 min of incubation. The uptake rate decreased after 12 hr of incubation (17 and 11ng.min-1, for Pi01 and He01, respectively) and after 1 week of incubation (6 and 3.8ng.min-1, for Pi01 and He01, respectively) in He01 and Pi01, respectively, but remained higher in He01. Oyetibo et al. (2014) showed different desorption capacity using CaCl2 (68 %) and EDTA (48%) for Hg. However Hg might adhere more strongly to He01 mycelium after short-term (30 min) and at a lethal Hg concentration (50 µM). He01 could also excrete higher amounts of hydrophobins, involved in the sequestration of Hg onto the cell walls (Puglisi et al., 2012). In Suillus luteus, a hydrophobin was differentially expressed between a Zn-tolerant and a Zn-sensitive isolate exposed to increased external zinc concentrations (Muller et al., 2007).

Table 1. **Hg compartmentation in *Paxillus involutus* (Pi01 and He01) mycelia.** *Paxillus involutus* He01 and Pi01 agar plugs (5 mm diameter) were cut from the actively growing edge of 1-week-old colonies and incubated on a sterile EDTA-treated cellophane membrane, on malt extract agar medium. After sufficient growth (7 days of for Pi01 and 14 days for He01), mycelia were transferred in liquid malt medium. After 4 days at 24°C, Hg was added at 0, 10 and 50 µM $HgCl_2$ for various lengths of times (0.5, 12 and 168 h). The content (ng THg) and proportions (%) of cell wall-bound Hg and total mycelium-bound Hg were determined. Means and standard deviations of at least tree replicates are presented.

	Hg in media	10 µM or 2,7ppm			50 µM or 13,5ppm		
	Time (h)	0.5	12	168	0.5	12	168
He01	Hg in Malt (ng) = 0	85,188 ± 5,419.75	45,179.2 ± 41,846.25	22,871.8 ± 26,587.04	379,052 ± 89,796.90	403,501.33 ± 50,467.32	144,942 ± 10,302.67
	Hg on cell wall (ng) = Hg ds EDTA lavage =1	125.64 ± 25.5	750.16 ± 44.7	630.1 ± 345.97	1551.18 ± 412.78	6006 ± 3555.73	8431.11 ± 4945.54
	Hg in He01 (ng) = ds le champignon =2	444.85 ± 226.15	2,235.31 ± 640.51	18,855.49 ± 9,416.35	2,588.97 ± 346.79	12,762.63 ± 931.26	61,042.82 ± 42,024.86
	%Hg in He01 = 1+2 / 0+1+2	0.66 ± 0.21	26.66 ± 38.09	58.48 ± 43.23	1.03 ± 0.16	4.44 ± 0.23	30.59 ± 11.04
	%Hg absorbé = 2/2+1	74.77 ± 14.53	74.2 ± 4.94	96.76 ± 1.06	62.79 ± 11.05	69.31 ± 13.28	80.4 ± 20.81
	%Hg adsorbé = 1 /2+1	25.23 ± 14.53	25.8 ± 4.94	3.24 ± 1.06	37.21 ± 11.05	30.69 ± 13.28	19.6 ± 20.81
	Protein (µg.mg^{-1} DW)	7.84 ± 1.81	17.25 ± 4.67	15.07 ± 3.41	21.25 ± 0.12	12.89 ± 2.53	8.57 ± 1.46
Pi01	Hg in Malt (ng)	77,309.33 ± 8,025.71	85,529.47 ± 16,289.2	51,369.87 ± 3,922.39	438,089.33 ± 65,866.88	345,325.33 ± 51,406.71	150,353.33 ± 3,339.31
	Hg on cel wall (ng)	77.93 ± 46.64	186.24 ± 50.24	935.02 ± 134.78	501 ± 228.67	1446.23 ± 772.26	4484.55 ± 2269.16
	Hg in Pi01 (ng)	393.26 ± 208.77	2,879.55 ± 398.17	20,513.1 ± 11,641.66	2,927.03 ± 1214.75	8,093.52 ± 951.03	38,566.4 ± 16,971.14
	%Hg in Pi01	0.59 ± 0.28	3.57 ± 0.92	29.4 ± 11.62	0.78 ± 0.28	2.72 ± 0.49	21.82 ± 6.53
	%Hg absorbé	82.84 ± 5.24	93.78 ± 2.28	95.07 ± 3.16	85.4 ± 2.89	85.17 ± 7.38	88.78 ± 5.64
	%Hg adsorbé	17.16 ± 5.24	6.22 ± 2.28	4.92 ± 3.16	14.6 ± 2.89	14.83 ± 7.38	11.22 ± 5.64
	Protein (µg.mg^{-1} DW)	13.96 ± 2.13	15.66 ± 3.27	12.78 ± 2.02	19.92 ± 2.19	13.56 ± 2.25	8.31 ± 0.18

In a second series of experiments, and to further investigate the subcellular compartmentation of Hg in fungal hyphae, both short-term (30 min) and long-term (12 h) exposures were carried out on the time-dependent kinetics of Hg desorption for the sensitive (Pi01) and tolerant (He01) P. involutus isolates (Figs 5 and 6), in liquid media. As previously found, He01 accumulated more Hg than Pi01, 730 ppm after 30 min to 4080 ppm after 12h for He01 and 380 ppm after 30 min to 1680 ppm after 12h for Pi01. Plots representing a first-order kinetic transformation of Hg desorption (log of Hg remaining in the mycelium as a function of time) could be dissected into three compartments in series, the vacuole, cytoplasm and cell wall, as previously described for Cd2+ in P. involutus (Blaudez et al., 2000a). After a 30 min incubation of mycelia in Hg uptake solution, the cell wall compartment represented 47% of the total Hg accumulated for Pi01 and 57% for He01, the cytoplasm represented 30% of the total Hg accumulated for Pi01 and 8% for He01 and the vacuoles contained 23 and 35% of the THg accumulated for Pi01 and He01, respectively (Fig. 5). The proportions of vacuolar Hg slightly increased after a 12hr exposure in both fungi, whereas the cell wall fractions decreased (Fig. 6). Accumulations of Hg into the different compartments were 2- to 3-fold higher in the He01 isolate, compared to Pi01. The distribution of Hg in the three compartments would however unlikely explained the differences in tolerance between the two isolates observed in Fig. 2.

Under a short-term Hg exposure, desorption times from cell walls reached 16 min for Pi01 and 35 min for He01 (Fig. 5), whereas desorptions from cell walls under a long-term exposure reached 25 min for both fungi (Fig. 6) Hg was therefore removed more slowly from He01 mycelia compared with Pi01 at a 30 min incubation period, which can be explained by a stronger binding capacity as explained above. That might also explain the higher proportion of THg measured in the cell wall compartment (57% compared to 47%) at 30 min incubation. However after a 12 hr incubation of mycelia in Hg-uptake solution time to desorption of cell wall was similar for both fungi and the cell wall compartment represented a much lower proportion for He01.

Impact of Hg on known intracellular mechanisms

P. involutus is known to produce melanins, as a response to metal exposure (Jacob et al., 2004). We therefore quantified coloration of media by measuring the absorbance (DO600nm) (Fig. S2a). The absorbances of non-exposed mycelia were higher for He01 and remained stable overtime, whereas the absorbance significantly increased at the highest exposure time for Pi0. This might be due to melanin formation, as already observed in Gaeumannomyces cylindrosporus under Pb exposure (Ban et al., 2012) and in P. involutus under Cd exposure (Jacob et al., 2004). However tolerance of Pi isolates cannot clearly be linked to pigment formation. We also measured pH under Hg exposure and found little changes induced by Hg (Fig. S2b)

Changes in the soluble protein content in P. involutus under different concentrations of Hg and different exposure times are shown in Table 1. A short exposure (12 hr) to a moderate Hg concentration (10 µM) of either Pi01 or He01 did not impact the protein content. A longer incubation time at the same Hg concentration increased the protein content in He01 but not in Pi01, compared with non-exposed mycelia. Both fungi synthesized more proteins upon a very short exposure (30 min) to an elevated Hg concentration (50 µM). Higher incubation times decreased the protein contents of both fungi, compared with non-exposed mycelia. Hg also induced a decrease of protein contents in pure culture of Phanerochaete chrysosporium (Dhawale et al., 1996) or Gaeumannomyces cylindrosporus (Ban et al., 2012) and in sporocarps of Pleurotus sajor-caju (Purkayastha et al., 1994) or of Coprinus comatus (Cen et al., 2012).

As a first step toward the characterization of fungal proteins induced upon Hg exposure, we run PAGE gels to determine the profiles of Hg-binding proteins in both fungi (Fig. 7). The most striking differences observed for the Hg content of each protein fraction reside in the proteins of mass ranging from 25 to 50 kDa. That was more evidenced in the He01 mycelia that were exposed to 50 µM Hg for 1 week, were the Hg represented up to 40% of the total Hg of the protein extract. For the Pi01 mycelia, a second peak of Hg-binding proteins appeared at the lowest protein fraction of less than 10 kDa, under a short term Hg exposure. As a control, we run non-exposed proteins that showed only background Hg levels (not shown).

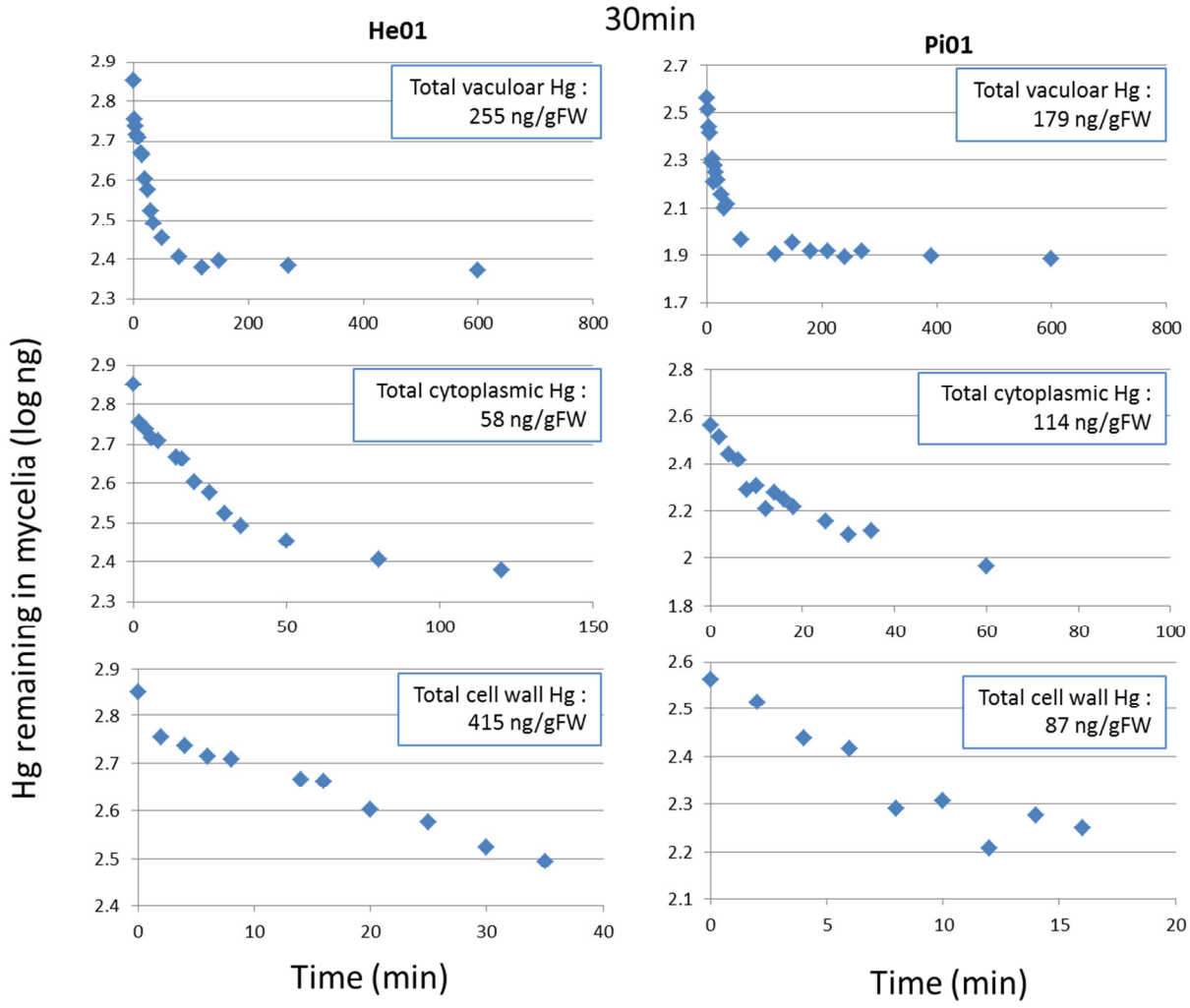


Figure 5. Desorption kinetics of Hg from fungal mycelia after a short-term exposure to Hg. Discs of *Paxillus involutus* mycelia (Pi01 and He01) were cut from the actively growing edge of 10-days-old colonies using a 15 mm diameter cork borer. The discs were exposed for 30 min to the standard assay medium containing 10 µM $HgCl_2$. Discs were briefly rinsed in the uptake solution from which $HgCl_2$ was omitted, and finally floated on a desorption solution containing 5 mM $CaCl_2$ in 2 mM MES (pH 4,5) at 4 °C to initiate Hg desorption. At various time intervals, 200 µl aliquots of the desorption solution were collected and Hg content was determined during a 10 hr period. The curves represent a typical experiment of three replicates.

Hg is known to be associated with high molecular-weight compounds on Boletus edulis (Collin-Hansen et al., 2007) and Agaricus bisporus (Byrne and Tušek-Žnidarič, 1990) but only a minor fraction is present as low molecular weight components (Murray and Kidby, 1975)(Murray and Kidby, 1975). In some of our previous studies, we identified a metallothionein that may act as a potential determinant in the response of mycorrhizal fungi to Cu and Cd stress (Bellion et al., 2007; Courbot et al., 2004). Silver is also known to be associated with low molecular weight proteins (8-10 kDa) possibly corresponding to metallothioneins (MT) or MT-like species (Byrne and Tušek-Žnidarič, 1990). However, metallothioneins did not conferred resistance to Hg in Saccharomyces cerevisiae (Ecker et al., 1986). In the present work, we did not attempt to characterize further the protein that may bind to Hg, but the proteins of low MM might correspond to a metallothionein. Phytochelatins, like metallothioneins, are small proteins enriched in SH groups (1.5-4 kDa) and Tuber melanosporum respond to Hg by synthesizing phytochelatins (PCs) that may act as chelators (Bolchi et al., 2011). In P. involutus in the absence of phytochelatins, GSH was reported as the major TE-responsive thiol (Courbot et al., 2004). The remarkable capability of P. involutus to overproduce GSH in TE-polluted environments might explain the high TE tolerance (Courbot et al., 2004; Ott et al., 2002). Again, these compounds of low molecular weight could not account for the proteins of high molecular weight that could bind Hg in our experimental conditions. Higher molecular-weight compounds could be represented by e.g. a glycoprotein like Pyranose-2-dehydrogenase (75 kDa), that was shown to be inhibited by Hg^{2+} (Ng, 2004). Arbuscular mycorrhizal fungi are known to excreted glomalin, which are metal-sorbing glycoproteins (Gonzalez-Chavez et al., 2004). Higher molecular-weight compounds could also be represented by heat shock proteins of 70 kDa, also known as stress proteins. Significant positive correlations were established between concentrations of Hg and expression of HSP70 in Boletus edulis (Collin-Hansen et al., 2005).

Effect of Hg on antioxydant properties

We measured a number of well-identified markers to identify potential mechanisms that might explain differences of tolerance in the two P. involutus isolates. The MDA contents were slightly higher in non-exposed Pi01 but did not change consistently when the mycelia were exposed to moderate or elevated Hg concentrations for short of longer exposure periods (Fig. S3). Malondialdehyde (MDA) is a common biochemical marker for ROS-mediated oxidative damage (Mittler, 2002; Zhan et al., 2014), which was however not clearly affected by Hg in P. involutus. Similarly, we measured the total antioxidant capacities (TAC) of P. involutus mycelia and found no obvious trends, except a slight decrease in Pi01 exposed to 50 µM Hg. The TAC protective system, which includes enzymatic reactions such as SOD, CAT and glutathione peroxidase (GSH-Px) and non-enzymatic reactions such as ascorbic acid, vitamin E and GSH (Dandan et al., 2011), provides a better estimate of the overall antioxidative state of the organism than the measurements of individual components (Razinger et al., 2008).

The activities of SOD and CAT in the two P. involutus isolates were measured to detect the responses of antioxidant substances to HgCl2 stress (Fig. S4). Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion (O2.-) into hydrogen peroxide and molecular oxygen and catalase (CAT) catalyses the decomposition of hydrogen peroxide (H2O2) to water and oxygen. For SOD, the two isolates showed similar activities in non-exposed mycelia, and we found no clear trends under Hg exposure, except a slight increase in Pi01 exposed to 50 µM Hg. The CAT activity of P. involutus He01 in non-exposed mycelia was significantly higher in He01 (on average 0.8 U.mg-1 DM) compared to Pi01 (on average 0.34 U.mg-1 DM). However, no significant changes upon Hg exposure could be detected in the two isolates. The higher CAT capacity of P. involutus He01 could be part of the tolerance mechanism under Hg stress. Oxidative stress produced by Hg exposure is similar to that produced by a H2O2 treatment with consistent with previous finding that the Yap1 protein is also involved in the response of yeast towards Hg (Westwater et al., 2002).

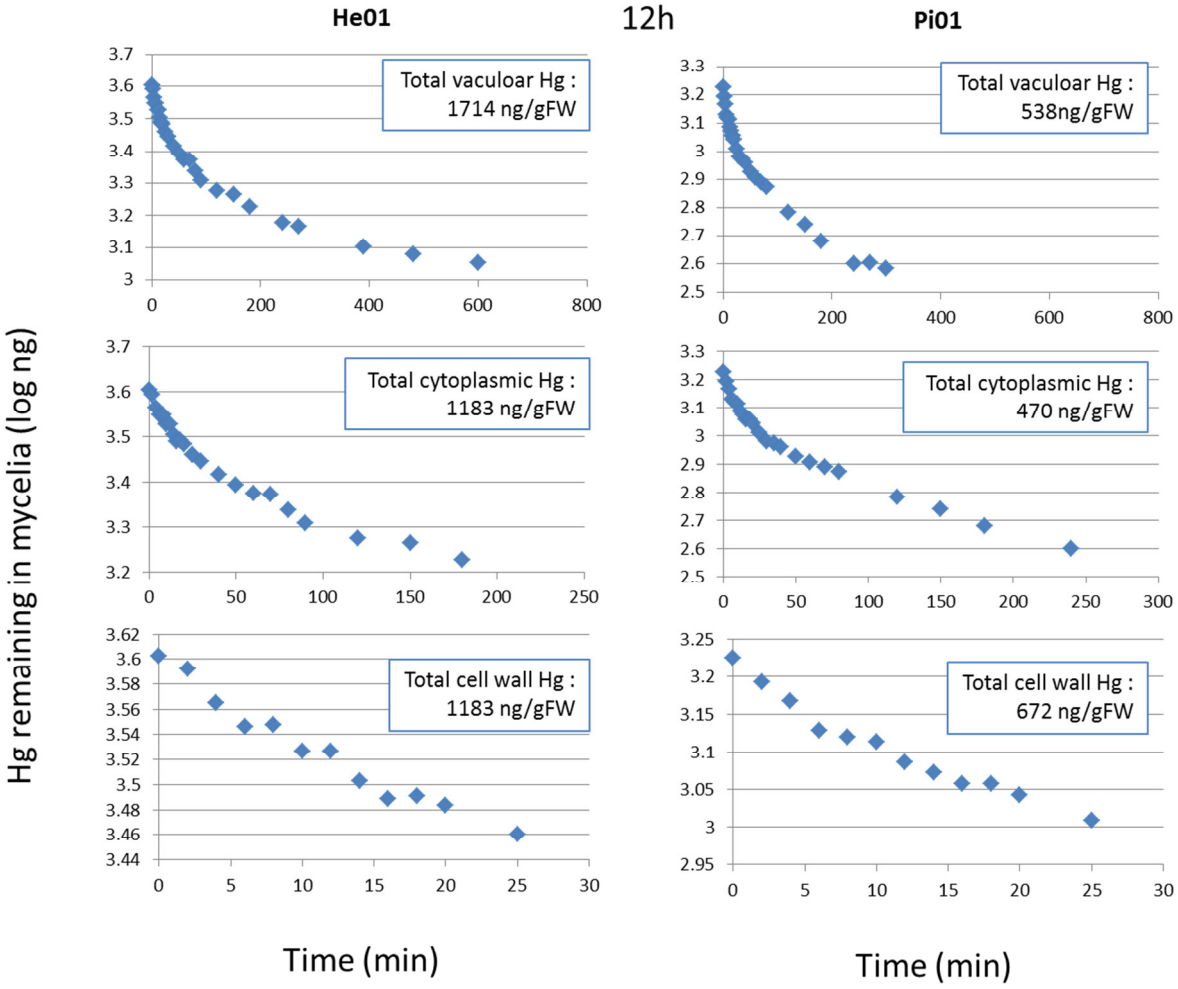


Figure 6. Desorption kinetics of Hg from fungal mycelia after a long-term exposure to Hg. Discs of *Paxillus involutus* mycelia (Pi01 and He01) were prepared as described in Fig. 5, except that the discs were exposed for 12 hr. The curves represent a typical experiment of three replicates.

Overall, the Hg stress had only little impact on antioxidant compounds in P. involutus, in contrast to earlier results reported elsewhere (Cen et al., 2012). The only relevant observation in our study is the decrease of antioxidant compounds (TAC) and ROS-sensitive compounds (MDA), correlated to a increase of the SOD activity, upon a long exposure to an elevated Hg concentration in the less tolerant isolate Pi01. Increased of lipid peroxidation, SOD, CAT and POD activities with Hg concentrations in sporocarps of C. comatus can be explained by much longer exposure periods (Cen et al., 2012).

Conclusions

THg concentrations measured in mycelial culture of a number of higher fungi (Fig. 2) are within the range measured in fruiting bodies of a number of wood decomposers and mycorrhizal fungi, that may accumulate up to 7 mg /kg DM of Hg. That corresponds to values of Hg BCF well above 1, and for some of the sites, the median values for caps were up to 65 (Krasińska and Falandysz, 2016). Hg clearly affected fungal growth rate and increased the lag phase of the fungal isolates tested in our study. However, we found large variations between fungal isolates, even within the same species. The possible tolerance mechanisms developed by fungi under Hg exposure are described in Fig. S8. As suggested by the present study, sequestration on cell walls, in the cytoplasm, and in the vacuolar compartment might allow fungi to cope with elevated concentrations of THg. Although we did not attempt to further characterize the molecular mechanisms involved, these data set represent a first step towards the understanding of the mechanisms underlying Hg tolerance and accumulation in fungal species. The mechanisms involved in the entry of Hg within fungal cells, as well as the identity of organic compounds produced by fungi to chelate Hg would deserve further research. In a previous study (Assad et al., 2016), we concluded that Hg entry into plant leaves was achieved primarily through the atmospheric pathway. It remains to be determined whether Hg entry into fungal cells is solely due to the active transport of the ionic Hg form (either ions or in chlorinated forms), or whether the Hg0 form might also contribute to the Hg accumulation observed in fungal cells. However, as noticed by (Krasińska and Falandysz, 2016), the quick emergence as well as the short life-time of fruiting bodies of macromycetes, as well as the low Hg content in he atmosphere, might render this transfer pathway negligible.

Acknowledgments

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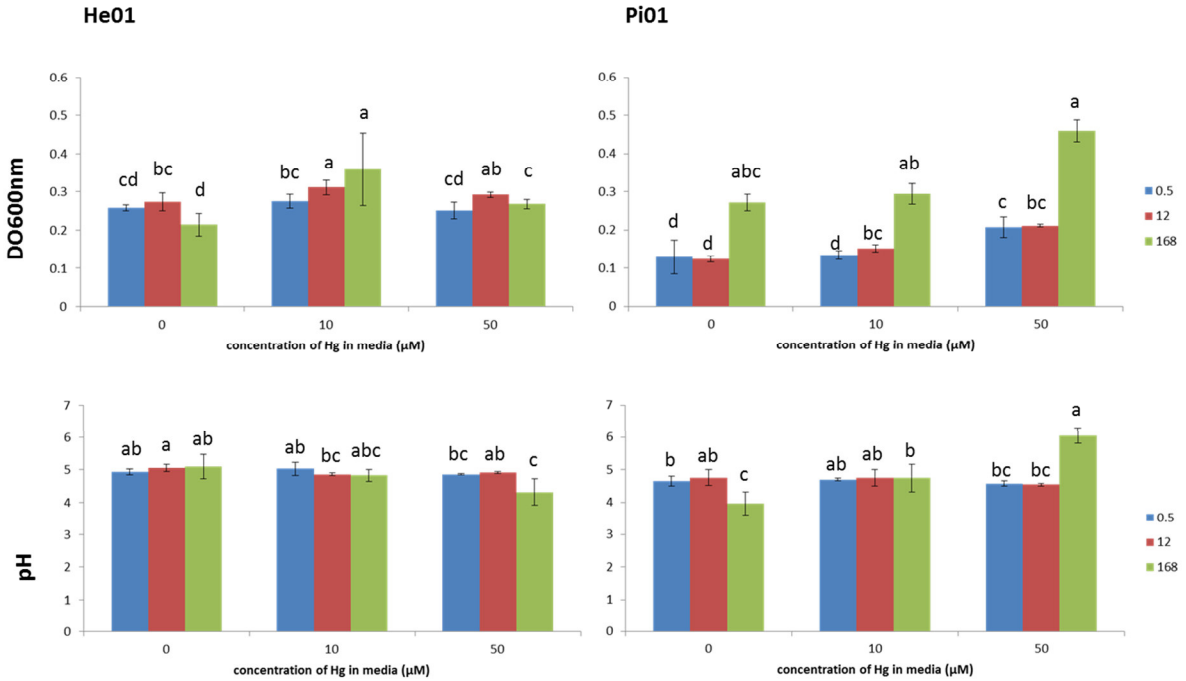

Figure S2. **DO 280 and pH changes in the liquid growth medium of *Paxillus involutus* isolates exposed to acute Hg exposure.** *Paxillus involutus* isolates He01 and Pi01 were grown as described in legend to figure 4. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test.

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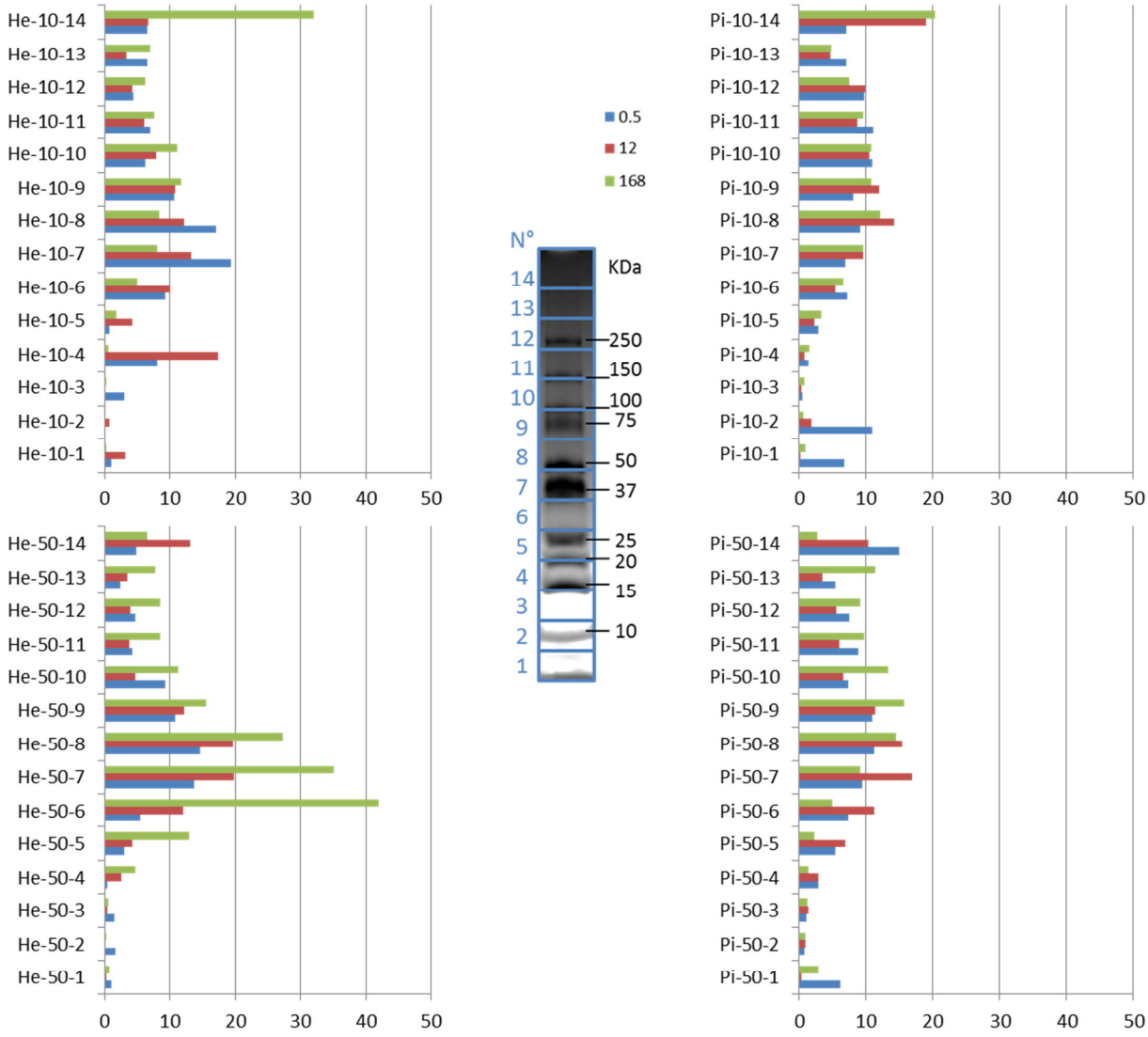

Figure 7. Hg-linked protein profiles in *Paxillus involutus* mycelia (Pi01 and He01) exposed to Hg. Mycelia were exposed to Hg as described in Fig. 4, washed and stored. Proteins were further extracted and loaded onto a 4-20 % native PAGE. After protein staining, bands were cut from the gels and directly analyzed for Hg content, generating 14 gel samples for *Paxillus involutus* He01 (left panel) and Pi01 (right panel). The graphic represent a typical experiment of two replicates.

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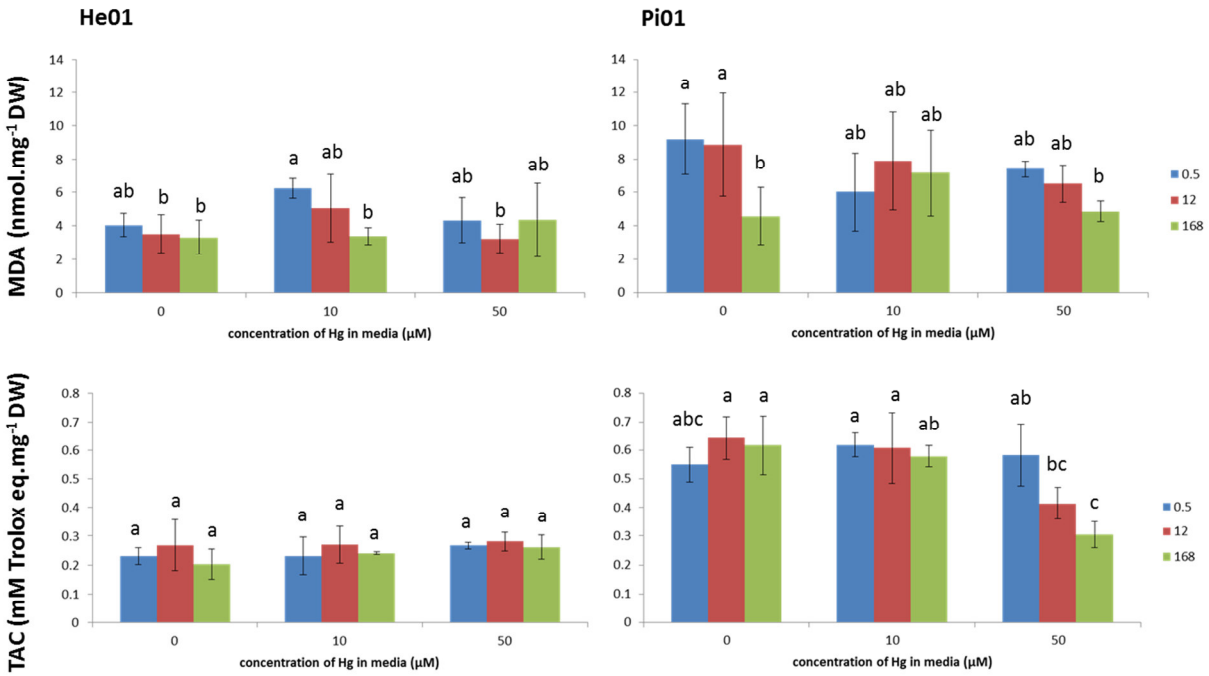

Figure S3. The effect of $HgCl_2$ on malondialdehyde (MDA) content and total antioxidant capacities (TAC) in mycelia of *Paxillus involutus* isolates exposed to acute Hg exposure. *Paxillus involutus* isolates He01 and Pi01 were grown as described in legend to figure 4. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test.

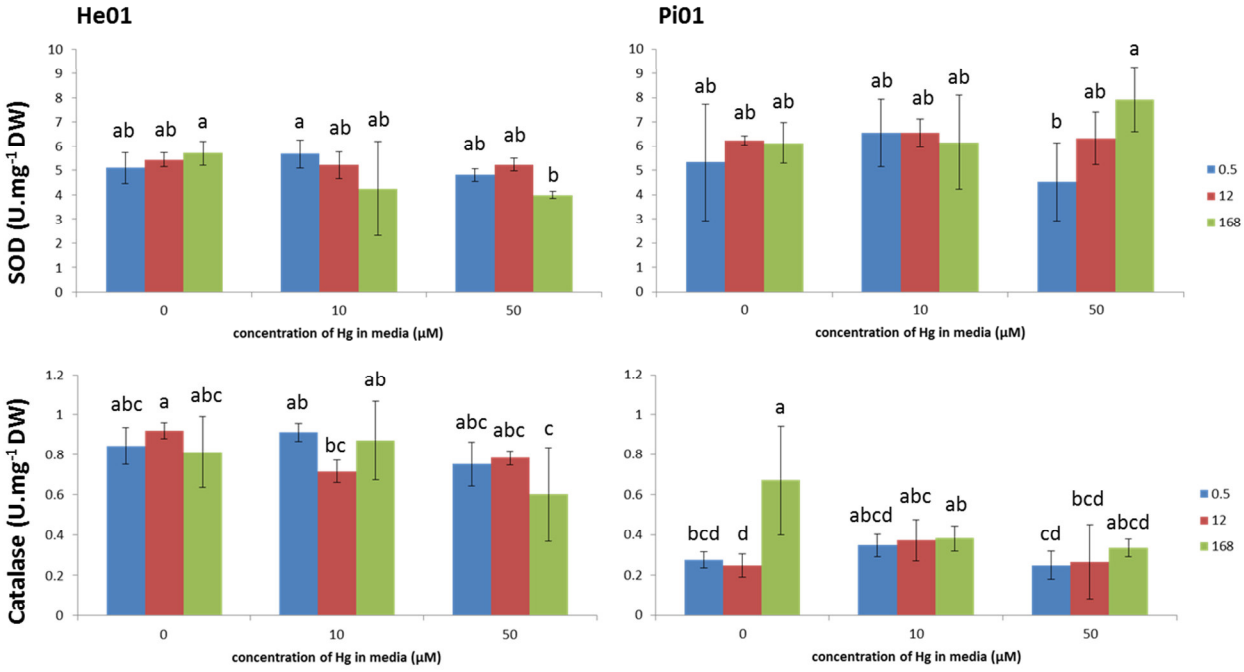

Figure S4. The effect of $HgCl_2$ on SOD and catalase activities in mycelia of *Paxillus involutus* isolates exposed to acute Hg exposure. *Paxillus involutus* isolates He01 and Pi01 were grown as described in legend to figure 4. Values designated with the same letters were not significantly different (P<0.05) according to a Kruskal-Wallis test.

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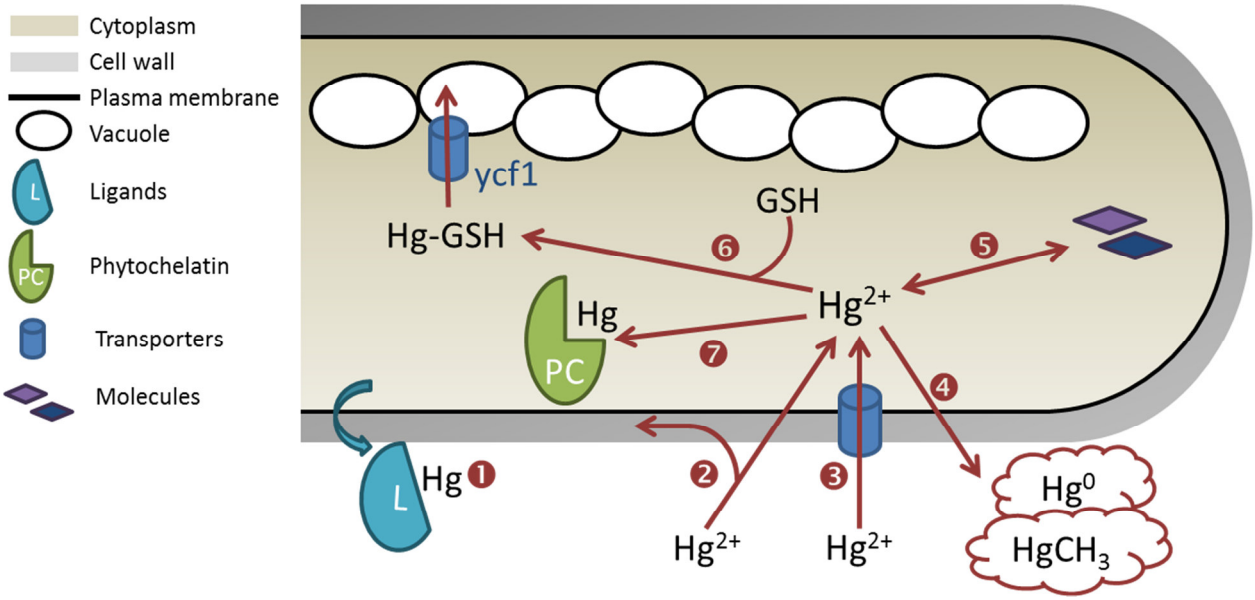

Figure 8. Schematic representation of cellular mechanisms potentially involved in Hg tolerance in ectomycorrhizal fungi. Extra-hyphal immobilization of Hg occurs through the formation of secondary minerals by excreted ligands❶ and biosorption to cell wall constituents such as chitin and chitosan❷. Hg uptake could occur, for example, through specific transporters (yet unknown) for the acquisition of other essential metals ❸. Hg transformations such as reactions involving organometals (for example, methylations) and redox reactions ❺ frequently result in metal volatilization❹. Intracellular Hg immobilization involves complexation by cytoplasmic phytochelatins (PC) ❼ or glutathion (GSH). Hg–glutathione complexes can be further transferred to the vacuolar compartment by i.e. ycf1 transporter❻.

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3.3.3 Discussion

En conclusion, les deux approches ont permis d'identifier plus précisément certains mécanismes de résistance impliqués en présence de Zn, Cd ou Hg.

La première approche par métatranscriptomique a permis d'isoler de nouvelles protéines impliquées dans la résistance au Cd et au Zn par différents processus cellulaires comme la dégradation de protéines (Ubiquitines, SKP1 ubiquitine ligase), la chélation des ETMs (métallothionéines et des protéines riches en cystéine), l'homéostasie du Fe (protéines BolA) ou par des mécanismes inconnus (saccharopine déshydrogénase et alcool déshydrogénase).

La deuxième approche a révélé une forte accumulation de Hg par les champignons. Cet élément peut se fixer sur les parois fongiques, mais également pénétrer dans la cellule où il semble être associé à des protéines de haut poids moléculaire. La variation de la tolérance entre deux souches de *P. involutus* semble d'ailleurs dépendre de la liaison du Hg à ces protéines de haut poids moléculaire.

Les tests de tolérance aux ETMs réalisés sur différentes souches fongiques ont souvent montré des variations interspécifiques (Blaudez *et al.*, 2000b; Fomina *et al.*, 2005; Ray *et al.*, 2005; Ban *et al.*, 2012; Berthelot *et al.*, 2016; Lacercat-Didier *et al.*, 2016), et intraspécifiques (Colpaert *et al.*, 2004; Goncalves *et al.*, 2009; Krznaric *et al.*, 2009; Jourand *et al.*, 2010a). Cependant, le niveau de contamination du site d'origine et la tolérance des souches ne sont pas toujours corrélés (Blaudez *et al.*, 2000b). Les deux souches de *P. involutus* étudiées dans le cadre de la tolérance au Hg sont issues de sites non pollués et pourtant *Paxillus involutus* He01 montre une tolérance accrue au Hg, mais également au Zn et au Cd (Annexe 5). La tolérance à la pollution serait alors espèce-spécifique, souche-spécifique et métal-spécifique, comme rapporté par Leyval *et al.*, (1997) pour les champignons endomycorhiziens. Cette variation dans la réponse aux ETMs pourrait s'expliquer par la spécificité des mécanismes de défense et de tolérance aux ETMs chez les espèces fongiques. *P. involutus* est connu pour excréter des polyphénols sous forme de gouttelettes à la surface du mycélium en présence de Cd dans le milieu de culture (Jacob *et al.*, 2004). Cette espèce est également capable de produire une thiorédoxine, induite par les ions Cu^{2+} et Cd^{2+}, qui contribuerait au maintien de l'homéostasie redox généralement perturbée lors d'un stress métallique (Bellion *et al.*, 2006). *P. involutus* serait ainsi plus tolérant que d'autres espèces, via ces mécanismes particuliers, et un niveau d'expression différent de ces protéines/molécules pourrait entrainer des variations du niveau de tolérance des différentes souches.

Les deux approches menées dans nos travaux montrent l'importance des protéines chélatantes comme les métallothionéines ou les protéines riches en cystéine chez les champignons face à un stress métallique. Les métallothionéines sont des protéines de faible poids moléculaire (6-10 kDa) riches en cystéine et qui ont une forte affinité pour les ions métalliques. Chez les champignons

ectomycorhiziens, les métallothionéines jouent un rôle déterminant en réponse aux ETMs (Bellion *et al.*, 2007; Sácký *et al.*, 2014). D'autres molécules riches en cystéine sont impliquées dans la résistance aux ETMs chez les champignons comme les phytochélatines (présentes uniquement chez *Candida glabrata, Schizosaccharomyces pombe*, *Heliscus lugdunensis* ou *Tuber melanosporum;* Bellion *et al.*, 2006; Bolchi *et al.*, 2011) ou encore le glutathion (Hegedűs *et al.*, 2007). Ces molécules peuvent être spécifiquement et ne peuvent être induites que par certains ETMs (Reddy *et al.*, 2014; Sácký *et al.*, 2014; Shen *et al.*, 2015). Par exemple, il a été montré que les métallothionéines ne confèrent pas de tolérance au Hg chez *S. cerevisiae* (Ecker *et al.*, 1986).

L'impact des protéines impliquées dans l'homéostasie du Fe sur la tolérance des levures face au Zn démontre la connexion entre la régulation de ces deux éléments. En effet, il a été montré qu'un excès ou une carence en Fe, n'affecte pas seulement les gènes impliqués dans la régulation de l'homéostasie au Fe, mais également ceux impliqués dans l'homéostasie au Zn (Santos *et al.*, 2003). De même, le Hg semble lié au métabolisme du S car il possède une forte affinité pour les groupements thiols. L'affinité du Hg pour les groupements thiols des acides aminés, peptides et protéines, entraine leur inactivation, puisque ces groupes sont souvent impliqués dans le maintien de la structure ou de la fonction des macromolécules (Collin-Hansen et al. 2005). Ceci souligne l'importance des molécules de chélation dans le maintien du bon fonctionnement cellulaire en présence d'ETMs.

Différentes études ont montré une forte accumulation des ETMs dans les champignons (Kalač, 2010; Falandysz & Borovicka, 2013), avertissant ainsi les consommateurs des risques pour la santé. Dans le cadre du phytomanagement, la présence de tels champignons non comestibles (*Hebeloma*, *Paxillus*) est un atout car ils peuvent limiter la quantité d'ETMs absorbée par la plante en les fixant sur leurs parois ou à l'intérieur de leurs hyphes, sans présenter de risques pour la santé humaine.

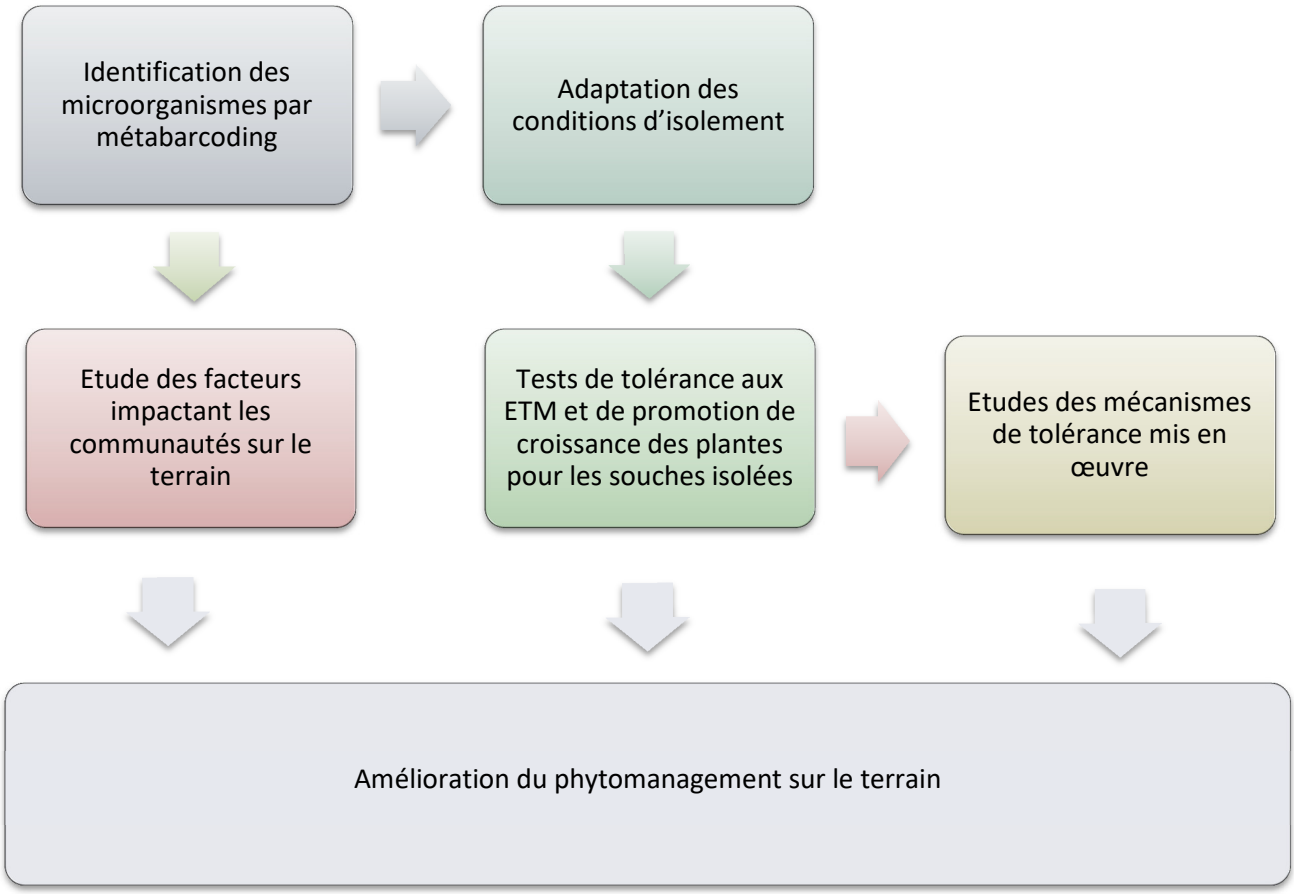

Figure 66 : Résume des différents objectifs

4 Conclusions générales et perspectives

L'objectif général de ma thèse visait à étudier les communautés microbiennes en association avec les espèces ligneuses implantées sur sites pollués aux ETMs, décliné en 3 objectifs spécifiques (Figure 66) :

Objectif 1 : Caractériser des communautés microbiennes sur sols pollués par métabarcoding

Au travers du premier objectif, nous avons pu :

1) **Mettre au point les méthodes innovantes de caractérisation des communautés par séquençage haut débit**, sur des échantillons issus de sols pollués, qui s'avèrent constituer des outils pertinents.
    a. Dans nos études, les deux méthodes de séquençage par MiSeq et Ion Torrent ont été utilisées. Le séquenceur Ion Torrent possède le débit le plus important et le prix le plus bas (Quail *et al.*, 2012). La technologie MiSeq présente les avantages d'un coût par base plus faible et d'un taux d'erreur inférieur grâce à son système de lecture dans les deux sens. Cependant l'Ion Torrent et le MiSeq ont des précisions inférieures au séquençage Sanger (erreur de substitution pour le Miseq ou d'homopolymère pour l'Ion Torrent) et des tailles de lecture limitée mais la parallélisation permet d'obtenir un grand nombre de séquences.

    b. Cette approche de métabarcoding nécessite la mise au point à plusieurs niveaux. L'extraction de la matrice initiale (sols ou racines) est une étape clé qu'il a fallu tester, et adapter pour les racines et les différents sols. Dans nos études, nous avons utilisé successivement un kit d'extraction MoBio utilisant 10 g, puis un second n'utilisant que 500 mg afin de diminuer le coût et le temps des extractions au vu du nombre important d'échantillons. Le choix des amorces et l'optimisation des conditions d'amplification par PCR ont aussi nécessité de nombreux tests.

    c. L'approche de métabarcoding permet d'identifier de nombreuses espèces mais elle ne fait pas la distinction entre les microorganismes actifs et les phases de repos de ces espèces. De plus, certains OTUs demeurent non identifiées car aucune séquence de référence n'existe dans les bases de données. D'où la nécessité d'isoler et d'identifier morphologiquement certaines espèces pour enrichir les bases de données.

2) Souligner **les facteurs prépondérants qui structurent les communautés** microbiennes
    a. Le site de Tavaux nous a permis de montrer que la recolonisation spontanée par des espèces ligneuses d'un milieu plutôt a priori hostile à la croissance végétale,

s'accompagne d'une recolonisation du milieu par les communautés microbiennes, dont environ les 2/3 sont identiques à celles du sol témoin. Le pH et la présence de Ca semblent fortement contribuer à cette structuration.

b. Les dispositifs expérimentaux de Pierrelaye et Leforest ont souligné l'importance des facteurs pédologiques (notamment du pH), des teneurs en éléments nutritifs (P et N) qui semblent impacter fortement l'émergence d'espèces ectomycorhiziennes au niveau des racines du peuplier. Un des faits marquants de mon travail est en effet la démonstration qu'un même clone de peuplier (Skado), implanté dans deux sites différents, présente des communautés ectomycorhiziennes très différentes.

c. Les bactéries montrent peu de variations de leur communauté sur les sites de Pierrelaye et Leforest mais davantage sur le site de Tavaux. Les sols semblent caractérisés par une dominance des *Alphaprotéobactérie*s et *Actinobactéries* à l'exception de la lagune de Tavaux où les *Gammaprotéobactéries* sont majoritaires par rapport aux *Alphaprotéobactéries*.

d. Ces trois études ont également souligné les interactions entre microorganismes. Ceci ouvre la voie à la production et l'utilisation d'inocula mixtes bactéries /champignons, pour des essais de laboratoire.

⇒ Un suivi des communautés microbiennes par métabarcoding à différentes périodes permettrait de mieux comprendre la succession des espèces au cours du temps et ainsi évaluer la meilleure période pour appliquer un inoculum. Des prélèvements avant plantation ont d'ailleurs été réalisés sur les parcelles de PROLIPHYT et permettront de révéler quels microorganismes étaient initialement présents et lesquels ont été favorisés par les plantations, quels microorganismes sont apportés par les plants issus de la pépinière et quels microorganismes issus de l'inoculum apporté se sont développés en interaction avec les végétaux implantés.

⇒ En plus de l'étude des communautés, des analyses fonctionnelles pourraient être menées afin de déterminer les fonctions clés dans ces sols pollués. L'étude des activités enzymatiques, la métatranscriptomique ou le métabarcoding utilisant des gènes codant pour des enzymes clé (comme les peroxydases ou les xylanases ; Barbi *et al.*, 2015) pourrait compléter les informations sur la structure et la fonction des communautés microbiennes.

⇒ Les couples d'amorces utilisées pour identifier les champignons et les bactéries ont permis d'obtenir de nombreuses séquences mais afin d'optimiser encore la technique, des amorces limitant les séquences appartenant aux plantes pourraient être utilisées comme ITS1f-ITS2

⇒ L'étude des communautés a donc permis de souligner l'abondance de certains genres bactériens et fongiques comme *Pseudomonas*, *Streptomyces*, *Variovorax*, *Laccaria*, *Hebeloma* ou *Cortinarius*. Il serait donc intéressant d'adapter les conditions de culture pour favoriser leur isolement. Les milieux de culture spécifiques à ces genres bactériens pourraient être utilisés (Milieu de King A ou B pour *Pseudomonas* ou un milieu sélectif glycérol-arginine pour *Streptomyces ;* Bontemps *et al.*, 2013). Pour les genres fongiques, les morphotypes d'ectomycorhizes présent sur les racines (deemy.de) correspondant à ces genres pourraient être visualisés, prélevés et placés dans des milieux de culture spécifique (milieu Pachlewski pour *Laccaria* ou N2P2 pour *Hebeloma*).

Objectif 2 : Générer des ressources microbiologiques.

Au travers du deuxième objectif, nous avons souligné :

3) L'émergence de **souches fongiques endophytes** qui constituent un potentiel nouveau en terme de biofertilisation. Ce travail se poursuit actuellement par la thèse de C. Berthelot (LIEC, Nancy).

4) L'émergence de bactéries inféodées aux sols contaminés. L'émergence de ***Pseudomonas*** illustre parfaitement l'intérêt de coupler les approches plutôt généralistes (séquençage haut débit) qui permettent d'identifier des genres/espèces candidats pour ensuite optimiser les conditions qui favorisent leur isolement. Ce travail se poursuit par la thèse de C. Zappelini et A. Durand (LCE, Montbéliard).

⇒ L'utilisation de milieux de culture contenant des concentrations en ETMs similaires à celles du sol d'étude et/ou des exsudats racinaire pourrait permettre d'optimiser les isolements fongiques et bactériens et serait à tester pour de futurs isolements.

⇒ Certaines souches fongiques doivent encore être étudiées (test de tolérance, potentiel PGP) et des tests de conservation en glycérol à -80°C sont à mettre au point pour limiter les nombreux et fastidieux repiquages successifs et diminuer la probabilité de contamination de ces souches pouvant entrainer leur perte.

⇒ Les souches les plus intéressantes pourraient ensuite être testées à une échelle plus large, en bioréacteur par exemple, pour étudier leur croissance et le mode de propagation le mieux adapté.

⇒ Dans le but de créer un inoculum contenant différentes souches fongiques voire bactériennes il serait intéressant de confronter ces souches endophytes, ectomycorhiziennes et bactériennes sur milieu gélosé et sur un sol d'intérêt stérilisé afin d'observer leurs interactions.

⇒ Enfin des tests dans les sols d'intérêts et sur le terrain pourraient être envisagés pour évaluer au mieux les capacités de phytomanagement des souches isolées. C'est le travail envisagé dans le cadre des projets BIOFILTREE et PROLIPHYT.

⇒ Les parcelles expérimentales mise en place dans le cadre du projet PROLIPHYT ont été plantées par différentes espèces ligneuses et les démarches identiques d'identification et d'isolement seront menées et permettront de confirmer les résultats obtenus sur la structure des communautés et d'isoler, le cas échéant, de nouveaux candidats spécifiques ou non de certaines plantes. L'utilisation de milieux de culture contenant des concentrations en ETMs similaires à celles du sol d'étude et/ou des exsudats racinaire pourrait permettre d'optimiser les isolements fongiques et bactériens et serait à tester pour de futurs isolements.

Objectif 3 : La caractérisation fonctionnelle

Au travers de ce dernier objectif, nous avons :

5) Mis en évidence que les mécanismes de tolérance aux ETMs sont variés, pouvant impliquer des **mécanismes spécifiques des ETMs (métallothionéines, fixation à la paroi, séquestration dans la vacuole) et d'autres encore méconnus.**

a. L'étude de la tolérance au Hg chez *P. involutus* a permis de mettre en évidence des **protéines de haut poids moléculaire pouvant complexer le Hg.** Celles-ci sont en cours d'identification

b. En parallèle de ces travaux, la thèse de M. ASSAD (LCE, Montbéliard) révèle les mécanismes de tolérance du peuplier (Assad *et al.*, 2016). L'étude des mécanismes de tolérance au Hg chez d'autres champignons se poursuit par la thèse de A. Durand (LCE, Montbéliard)

⇒ D'autres champignons, tels que les *Hebeloma* ou certain DSEs, devraient être testés pour leur tolérance au Hg pour permettre d'évaluer si les protéines complexant le Hg sont spécifiques de *P. involutus* ou si elles sont ubiquistes chez les champignons mycorhiziens/endophytes.

⇒ Des tests de transfert de Hg à la plante hôte sont à envisager entre *P. involutus* et le peuplier et pourraient permettre de mieux évaluer la quantité de Hg transféré.

⇒ Il serait intéressant d'isoler et d'identifier les protéines se liant au Hg par des gels bidirectionnels et des analyses des spots obtenus. Ces analyses sont en cours, en collaboration avec M. Le Jean (LIEC, Metz).

En conclusion, l'implantation (naturelle comme par exemple sur le site de Tavaux ; ou dans le cadre d'un procédé de phytomanagement sur les autres sites étudiés) d'un couvert ligneux sur sol contaminé par les ETMs impacte fortement les communautés microbiennes, comme le révèlent les approches de métabarcoding. Cependant les caractéristiques du sol ont aussi un impact majeur sur les communautés microbiennes, aboutissant à une sélection des microorganismes, qui seraient probablement les mieux adaptés aux conditions locales. Cette adaptation peut s'expliquer par la mise en œuvre de mécanismes spécifiques, comme par exemple la biosorption sur les parois ou la séquestration de Hg dans les vacuoles des champignons. Toutefois, l'isolement de souches constitue une approche toujours nécessaire et indispensable pour permettre de mieux cerner le potentiel qu'offrent les champignons endophytes dans le cadre de phytomanagement de sols contaminés par des ETMs

Un des faits marquants de mon travail de thèse réside sans doute dans la conclusion que les caractéristiques du sol peuvent à elles seules entraîner des modifications drastiques dans la composition des communautés microbiennes. Ainsi le déficit en éléments nutritifs et la présence de fortes teneurs en ETMs extractibles au $CaCl_2$, se sont traduits par l'émergence et la domination très nette de la communauté mycorhizienne sur le site de Leforest. Inversement, le couvert ligneux du site de Pierrelaye est caractérisé par l'absence notable d'un microbiome mycorhizien.

L'ensemble de ce travail de thèse souligne donc que le choix des approches mises en œuvre a été pertinent, et permet de conclure sur le fait que les stratégies de phytomanagement doivent impérativement prendre en considération les conditions locales de sol.

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6 Annexes

Annexe 1 : Extraction d'ADNg avec le kit MoBio Powersoil

Extraction à partir de racines

- Placer 100 mg de racines dans un tube de 2 ml
- Prélever 400 µl du tube powerbead et l'introduire dans le tube de 2 ml contenant les racines puis vortexer doucement
- Ajouter **60 µl de solution C1** puis inverser plusieurs fois le tube ou vortexer rapidement
- Placer 2 billes par tube
- Utiliser le vibrobroyeur à 30Hz pendant 3x1 min
- Verser le reste du tube powerbead

Extraction à partir de sol

- Placer 500 mg de sol dans un tube de 2 ml
- Verser le contenu du tube powerbead dans le tube de 2 ml puis vortexer doucement
- Ajouter **60 µl de solution C1** puis inverser plusieurs fois le tube ou vortexer rapidement

Suite du protocole (quelque soit le type d'échantillon) :

- Utiliser le vortex Mobio à vitesse maximale pendant 10 min
- Centrifuger 30 sec à 10 000 g (ou 9700 rpm) puis placer le surnageant dans un nouveau tube de 2 ml
- Ajouter **250 µl de solution C2** et vortexer 5 sec
- Incuber 5 min à 4°C
- Centrifuger 1 min à 10 000g
- Transférer 600 µl maximum de surnageant dans un nouveau tube de 2 ml
- Ajouter **200 µl de solution C3,** vortexer rapidement
- Incuber 5 min à 4°C
- Centrifuger 1 min à 10 000 g
- Transférer 750 µl maximum de surnageant dans un nouveau tube de 2 ml
- Agiter la **solution C4** puis ajouter **1200 µl** de cette solution et vortexer 5 sec
- Transférer 650 µl maximum de surnageant sur la colonne (spin filter). Centrifuger 1 min à 10 000 g puis éliminer le surnageant
- Répéter l'étape précédente jusqu'à filtration complète de la solution
- Ajouter **500 µl de solution C5**
- Centrifuger 30 sec à 10 000 g puis éliminer le surnageant
- Centrifuger 1 min à 10 000 g
- Placer la colonne sur un nouveau tube de 2 ml
- Eluer avec **100 µl de solution C6**
- Centrifuger 30 sec à 10 000 g
- Doser au spectrophotomètre et déposer un aliquot sur gel d'agarose
- Conserver l'ADN à -20°C

Annexe 2 : Extraction d'ADNg avec le kit Qiagen

- Placer 2 billes/tube
- Ajouter 400 µl de tampon AP1*, 4 µl de Rnase A, quelques mg de PVPP
- Broyer 2X 1 min à 30 Hz, répéter l'opération si nécessaire
- Vortexer soigneusement puis incuber 10 min à 65°C. Vortexer 2 à 3X pendant
- Ajouter 130 µl de tampon AP2, mélanger par inversion du tube et incuber 5 min dans de la glace
- Centrifuger 5 min à 14000 rpm
- Placer le surnageant sur une colonne violette et centrifuger 2 min à 14000 rpm
- Mélanger le surnageant (environ 450 µl) à 675 µl de tampon AP3/E*** et transférer 675 µl maximum du mélange sur la colonne blanche, centrifuger 1 min à 8500 rpm
- Répéter l'étape précédente si nécessaire
- Changer de tube collecteur
- Ajouter 500 µl de tampon AW, centrifuger 1 min à 8500 rpm, éliminer le surnageant
- Ajouter 500 µl de tampon AW, centrifuger 2 min à 14000 rpm
- Placer délicatement**** les colonnes dans un eppendorf de 1,5 ml
- Eluer avec 50 µl de tampon AE préchauffé à 65°C, incuber 5 min à 65°C, centrifuger 1 min à 8500 rpm
- Répéter l'étape précédente
- Doser au spectrophotomètre et déposer un aliquot sur gel d'agarose

* si présence d'un précipité, placer le flacon 5 min à 65°C

Annexe 3 : Liste des amorces utilisées pour le séquençage par Ion Torrent des 16S bactériens (A) et des ITS fongiques (B), MiSeq et Sanger (C).

A)

Nom	Séquence(5' ---> 3')	F / R	Taille	Tm (°C)
16S-F343_IonA_L01	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L02	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L03	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L04	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L05	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L06	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L07	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L08	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L09	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L10	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L13	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L14	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L15	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L17	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCACGATTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L18	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGTGTCGCATACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L19	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATGATTGCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L20	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGATAATCTTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L21	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACCATACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L22	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCCAAGTATACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGACATTACTTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L24	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCTTACCGTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L25	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCGAGGCATACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L26	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCAAGCCTTTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L27	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACATTACATTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L28	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAAGCACCGTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L29	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCTTACCGTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L30	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCATGATCAATACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L31	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGACCGCATCTACGGRAGGCAGCAG	F	55	55
16S-F343_IonA_L32	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGTGTAGCATACGGRAGGCAGCAG	F	55	55
16S-R533_Ion_P1	CCTCTCTATGGGCAGTCGGTGATATTACCGCGGCTGCTGGC	R	41	55

En vert la zone correspondante aux amorces 343f et 533r, en noir l'espaceur, en rouge l'étiquette (différente pour chacune des amorces) et en bleu l'adaptateur.

B)

Nom	Séquence(5' ---> 3')	F / R	Taille	Tm (°C)
ITS1F_IonA_L02	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L03	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L04	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L05	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L06	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L07	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L08	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L09	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L10	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L13	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L14	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L15	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L17	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCACGATCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L18	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGTGTCGCACTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L19	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATGATTGCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L20	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGATAATCTCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L21	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACCACTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L22	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCCAAGTACTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGACATTACTCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L24	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCTTACCGCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L25	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCGAGGCACTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L26	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCAAGCCTTCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L27	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACATTACATCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L28	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAAGCACCGCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L29	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCTTACCGCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L30	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCATGATCAACTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L31	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGACCGCATCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L32	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGTGTAGCACTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L33	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTCGTCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L34	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L35	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACCTTGGTCATTTAGAGGAAGTAA	F	62	55
ITS1F_IonA_L36	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCATCCTTGGTCATTTAGAGGAAGTAA	F	62	55
58A2R_Ion_P1	CCTCTCTATGGGCAGTCGGTGATCTGCGTTCTTCATCGAT	R	40	55

En vert la zone correspondante aux amorces ITS1f et 58A2r, en noir l'espaceur, en rouge l'étiquette (différente pour chacune des amorces) et en bleu l'adaptateur.

C)

Nom	Séquence(5' ---> 3')	F / R	Taille	Tm (°C)	Utilisation
27F	AGAGTTTGATCMTGGCTCAG	F	20	60,8	Sanger 16S
1492R	TACCTTGTTACGACTT	R	16	45,4	Sanger 16S
ITS1	TCCGTAGGTGAACCTGCGG	F	19	68,8	Sanger ITS
ITS4	TCCTCCGCTTATTGATATGC	R	20	62	Sanger ITS
357F_hmp	CCTACGGGAGGCAGCAG	F	17	55	MiSeq 16S
926R_hmp	CCGTCAATTCMTTTRAGT	R	18	55	MiSeq 16S
ITS3	GCATCGATGAAGAACGCAGC	F	20	55	MiSeq ITS
ITS4	TCCTCCGCTTATTGATATGC	R	20	55	MiSeq ITS

Annexe 4 : Souches de champignons utilisées au cours de cette thèse et leurs origines

nom définitif	lieu de prélèvement	détail du site	Pollution du site (mg/Kg)	isolé à partir de	essence hôte	date de prélèvement
Paxillus involutus LS01	Lommel-Sahara (Belgique)	fonderie de zinc désaffectée	Cd(39), Cu(1300), Zn(11425)	carpophores	Pin sylvestre	Automne 2007
Paxillus involutus LS02	Lommel-Sahara (Belgique)	fonderie de zinc désaffectée	Cd(39), Cu(1300), Zn(11425)	carpophores	Pin sylvestre	Automne 2007
Paxillus involutus LS03	Lommel-Sahara (Belgique)	fonderie de zinc désaffectée	Cd(39), Cu(1300), Zn(11425)	carpophores	Pin sylvestre	Automne 2007
Paxillus involutus Ov01	Overpelt (Belgique)	manipulation stockage de minerais Zn	Cd(36), Cu(97), Ni(7), Pb(548) and Zn (1307)	carpophores	Pin sylvestre	Automne 2007
Paxillus involutus Ov02	Overpelt (Belgique)	manipulation stockage de minerais Zn	Cd(36), Cu(97), Ni(7), Pb(548) and Zn (1307)	carpophores	Pin sylvestre	Automne 2007
Paxillus involutus HE01	Hechtel-Eksel (Belgique)	activité pyrometallurgique	Non pollué: Zn(3-13).	carpophores	Pin sylvestre	Automne 2007
Paxillus involutus Pi01	Midlothian (Ecosse)	déchets de charbon	Non pollué	carpophores	Bouleau	en collection
Paxillus involutus Pi04	Vedby (Suède)	en collection	en collection	carpophores	inconnu	en collection
Paxillus involutus S01	Mirecourt (France)	Non pollué	Non pollué	carpophores	Bouleau	30/11/2009
Paxillus involutus Vi01	Micheville boues (France)	fonderie de fer désaffectée	Cd(2.88), Cu(117), Ni(103), Zn(1648)	carpophores	Bouleau/Saule	30/11/2009
Paxillus involutus Bo01	Bordeaux (France)	site de préservation et conservation du bois et des poteaux électriques	Cu	carpophores	Peuplier/Saule	30/11/2009
Paxillus involutus Bo02	Bordeaux (France)	site de préservation et conservation du bois et des poteaux électriques	Cu	carpophores	Peuplier/Saule	30/11/2009
Paxillus involutus Bo03	Bordeaux (France)	site de préservation et conservation du bois et des poteaux électriques	Cu	carpophores	Peuplier/Saule	30/11/2009
Paxillus involutus Bo04	Bordeaux (France)	site de préservation et conservation du bois et des poteaux électriques	Cu	carpophores	Peuplier/Saule	30/11/2009
Paxillus involutus Bo05	Bordeaux (France)	site de préservation et conservation du bois et des poteaux électriques	Cu	carpophores	Peuplier/Saule	30/11/2009
Paxillus involutus Bo06	Bordeaux (France)	site de préservation et conservation du bois et des poteaux électriques	Cu	carpophores	Peuplier/Saule	30/11/2009

Paxillus involutus Cu01	Custine (France)	un ancien bassin de décantation d'une usine de fer et d'acier		carpophores	Bouleau et autre	25/10/2012
Paxillus involutus Cu02	Custine (France)	un ancien bassin de décantation d'une usine de fer et d'acier		carpophores	Bouleau et autre	25/10/2012
Paxilus sp. Cu07	Custine (France)	un ancien bassin de décantation d'une usine de fer et d'acier		carpophores	Bouleau et autre	25/10/2012
Pisolithus tinctorius Pt01	en collection	en collection	en collection	en collection	en collection	en collection
Paxilus sp. Pr24	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Bakan	01/10/2013
Scleroderma bovista Pr25	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Flévo	02/10/2013
Scleroderma bovista Pr26	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Flévo	02/10/2013
Paxilus sp. Th01	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014
Paxilus sp. Th02	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014
Leccinum scabrum Th03	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014
Laccaria bicolor S238N	Crater lake (US)	en collection	en collection	en collection	en collection	en collection
Laccaria tortilis PDA6	en collection	en collection	en collection	en collection	en collection	en collection
Laccaria proxima PRAC	en collection	en collection	en collection	en collection	en collection	en collection
Suillus luteus Vi03	Micheville Pins (France)	fonderie de fer désaffectée	Non pollué: Cd(0.04), Cu(2.1), Ni(74.9), Zn(249).	carpophores	Pin	30/11/2009
Suillus luteus Vi05	Micheville Pins (France)	fonderie de fer désaffectée	Non pollué: Cd(0.04), Cu(2.1), Ni(74.9), Zn(249).	carpophores	Pin	02/11/2011
Suillus granulatus Vi06	Micheville boues (France)	fonderie de fer désaffectée	Cd(2.88), Cu(117), Ni(103), Zn(1648)	racines	Pin/Bouleau	02/11/2011
Amanita muscaria S02	Mirecourt (France)	Non pollué	Non pollué	carpophores	Bouleau	30/11/2009
Boletus edulis S03	Mirecourt (France)	Non pollué	Non pollué	carpophores	inconnu	30/11/2009

Tricholoma mongolicum Tx01	Tavaux (France)	Dépôt de sédiments issus du curage de l'étang de l'Aillon et bassin de décantation d'un usine de produit chimique	Hg(5)	carpophores	Saule	18/10/2013
Lepista nuda Vi02	Micheville boues (France)	fonderie de fer désaffectée	Cd(2.88), Cu(117), Ni(103), Zn(1648)	carpophores	Bouleau/Saule	30/11/2009
Helotiales sp. Pr01	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier Triplo	06/06/2011
Helotiales sp. Pr06	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier Skado	06/06/2011
Helotiales sp. Me01	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Beaupré	06/06/2011
Helotiales sp. Me04	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Beaupré	06/06/2011
Helotiales sp. Pr02	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier Lena	06/06/2011
Laccaria laccata Pr03	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier Lena	06/06/2011
Sebacina vermifera Pr04	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier Skado	06/06/2011
Tricholoma ustale Pr05	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier Fritzi-Pauley	06/06/2011
Trichopezizella nidulus Me02	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Beaupré	06/06/2011
Helotiales sp. Me03	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Beaupré	06/06/2011
Hebeloma leucosarx Me05	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Fritzi Pauley	06/06/2011
Lactatrius sp. Th04	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014
Lactatrius sp. Th05	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014

Lactatrius sp. Th07	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014
Lactatrius sp. Th08	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014
Lactatrius sp. Th09	Thann (France)	Production de titane, Sous-produits (gypse rouge)	S, Fe, Mn	carpophores	Bouleau	09/10/2014
Hebeloma crustuliniforme Pr08	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Koster	15/11/2011
Hebeloma crustuliniforme Pr09	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Skado	15/11/2011
Hebeloma sp. Pr10	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Triplo	15/11/2011
Hebeloma crustuliniforme Pr11	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Dorkamp	15/11/2011
Hebeloma sp. Pr12	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Skado	15/11/2011
Hebeloma sp. Pr13	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Dorskamp	15/11/2011
Lyophyllum connatum Pr14	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Fritzi Pauley	15/11/2011
Hebeloma crustuliniforme Pr15	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Koster	15/11/2011
Hebeloma crustuliniforme Pr16	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Koster	15/11/2011
Hebeloma crustuliniforme Pr17	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Koster	15/11/2011
Hebeloma sp. Pr18	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Skado	15/11/2011
Hebeloma sp. Pr19	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Skado	15/11/2011
Hebeloma sp. Pr20	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Koster	15/11/2011

Hebeloma sp. Pr21	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Skado	15/11/2011
Hebeloma sp. Pr22	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier Dorskamp	15/11/2011
Hebeloma sp. Pr23	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	carpophores	Peuplier I214	15/11/2011
Hebeloma sp. Cu08	Custine (France)	un ancien bassin de décantation d'une usine de fer et d'acier		carpophores	Bouleau et autre	25/10/2012
Hebeloma crustuliniforme Fe01	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	carpophores	Peuplier Skado	18/10/2012
Hebeloma crustuliniforme Fe02	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	carpophores	Peuplier Skado	18/10/2012
Hebeloma crustuliniforme Fe03	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	carpophores	Peuplier Skado	18/10/2012
Hebeloma cylindrosporum Vi04	Micheville Pins (France)	fonderie de fer désaffectée	Non pollué: Cd(0.04), Cu(2.1), Ni(74.9), Zn(249).	carpophores	Pin	02/11/2011
Hebeloma cylindrosporum Vi07	Micheville Pins (France)	fonderie de fer désaffectée	Non pollué: Cd(0.04), Cu(2.1), Ni(74.9), Zn(249).	carpophores	Pin	02/11/2011
Helotiales sp. Vi08	Micheville boues (France)	fonderie de fer désaffectée	Cd(2.88), Cu(117), Ni(103), Zn(1648)	racines	Pin/Bouleau	02/11/2011
Helotiales sp. Vi09	Micheville boues (France)	fonderie de fer désaffectée	Cd(2.88), Cu(117), Ni(103), Zn(1648)	racines	Pin/Bouleau	02/11/2011
Helotiales sp. Vi10	Micheville boues (France)	fonderie de fer désaffectée	Cd(2.88), Cu(117), Ni(103), Zn(1648)	racines	Pin	02/11/2011
Hebeloma sp. Vi11	Micheville boues (France)	fonderie de fer désaffectée	Cd(2.88), Cu(117), Ni(103), Zn(1648)	racines	Pin/bouleau	02/11/2011
Hebeloma cylindrosporum HcD1	en collection	en collection	en collection	en collection	en collection	en collection
lachnum sp. Me06	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Meliniomyces sp. Me07	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Entrophospora sp. Me08	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Geomyces sp. Me09	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012

Leptodontidium sp. Me10	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Entrophospora sp. Me11	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Leptodontidium sp. Me12	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Meliniomyces sp. Me13	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Meliniomyces sp. Me14	Metaleurop (France)	rejets atmosphérique d'une fonderie de plomb	Cd, Pb, Zn	racines	Peuplier Skado	19/10/2012
Helotiales sp. Fe04	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Helotiales sp. Fe05	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Cadophora sp. Fe06	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe07	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe08	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Tetracladium sp. Fe09	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe10	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Tetracladium sp. Fe11	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe14	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe15	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe16	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe17	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Helotiales sp. Fe18	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012

Helotiales sp. Fe19	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Leptodontidium sp. Fe20	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Helotiales sp. Fe21	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Meliniomyces sp. Fe22	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Exophiala sp. Fe24	Fresnes sur Escaut (France)	Dépôt de sédiments issus du curage de l'Escaut	Zn, Cd, Pb et Cu	racines	Peuplier Skado	18/10/2012
Phialophora Mustea Pr27	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier	printemps 2012
Phialocephala fortinii Pr28	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier	printemps 2012
Cadophora sp. Pr29	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier	printemps 2012
Leptodontidium orchidicola Pr30	Pierrelaye (France)	Zone d'épandage des eaux usées de Paris	Zn, Cd et Pb	racines	Peuplier	printemps 2012
Piriformospora indica	Allemagne	en collection	en collection	racines	en collection	en collection
Podospora sp.	en collection	en collection	en collection	en collection	en collection	en collection

Annexe 5 : Tolérance, milieu de prédilection et mode de conservation des souches de champignons utilisées au cours de cette thèse.

nom définitif	MIC HgCl2 (µM)	MIC ZnCl2 (mM)	MIC CdCl2 (µM)	MIC Pb(NO3)2 (µM)	MIC NiCl2 (µM)	MIC CuSO4 (µM)	MIC MnCl2 (mM)	milieu de prédilection	Conservation Glycérol 10%
Paxillus involutus LS01	25							MMN	
Paxillus involutus LS02	25							MMN	
Paxillus involutus LS03	25							MMN	
Paxillus involutus Ov01	100							MMN	
Paxillus involutus Ov02	100							MMN	
Paxillus involutus HE01	100	5	150	>1000	500	>1000	20	MMN	
Paxillus involutus Pi01	25	3	50	>200	>500	>500	>20	MMN	
Paxillus involutus Pi04	100	5	150	>1000	350	>1000	100	MMN	
Paxillus involutus S01	25							MMN	
Paxillus involutus Vi01	25							MMN	
Paxillus involutus Bo01	100	5	150	>1000	500	>1000	20	MMN	
Paxillus involutus Bo02	25	2	25	500	500	500	>100	MMN	
Paxillus involutus Bo03	50							MMN	
Paxillus involutus Bo04	50							MMN	
Paxillus involutus Bo05	100	5	25	>5000	500	5000	>100	MMN	
Paxillus involutus Bo06	50							MMN	
Paxillus involutus Cu01	100	5	50	>1000	200	>1000	>100	MMN	
Paxillus involutus Cu02	100							MMN	
Paxilus sp. Cu07								malt ou MMN	
Pisolithus tinctorius Pt01	50		>100			250		MMN	
Paxilus sp. Pr24		2	25	>1000	200	1000	20	MMN	
Scleroderma bovista Pr25	50							MMN	
Scleroderma bovista Pr26	>100	5	50	>1000	200	>1000	>100	MMN	
Paxilus sp. Th01	50							MMN	
Paxilus sp. Th02	25							MMN	
Leccinum scabrum Th03	100							malt	
Laccaria bicolor S238N								Pachlewski	

Laccaria tortilis PDA6		2	25	500	500	250	4	Pachlewski	
Laccaria proxima PRAC	100	2	>150	>5000	200	5000	>100	Pachlewski	
Suillus luteus Vi03	100	5	150	5000	200	5000	>100	Pachlewski	
Suillus luteus Vi05	50	5	150	>5000	200	5000	100	Pachlewski	
Suillus granulatus Vi06	50	>10	>150	5000	3000	>5000	>100	N2P2	
Amanita muscaria S02	>100							Malt	
Boletus edulis S03								Pachlewski	
Tricholoma mongolicum Tx01	50	3	>100	100	>500	>500	20	Pachlewski	
Lepista nuda Vi02	50	2	50	>1000	200	>1000	20	Malt	
Helotiales sp. Pr01	>100	10	>150	5000	500	5000	100	malt ou N2P2	X
Helotiales sp. Pr06	100	20	>500	5000	200	5000	100	malt ou N2P2	X
Helotiales sp. Me01	50	>10	>150	5000	500	5000	>100	malt ou N2P2	X
Helotiales sp. Me04	100	>10	>150	5000	500	5000	>100	malt ou N2P2	X
Helotiales sp. Pr02	100	10	>500	5000	200	2000	>100	malt ou N2P2	X
Laccaria laccata Pr03	100	2	>150	2000	350	2000	4	malt ou N2P2	
Sebacina vermifera Pr04	50	2	>150	1000	500	2000	20	malt ou N2P2	
Tricholoma ustale Pr05	50	2	>150	2000	350	5000	4	malt ou N2P2	
Trichopezizella nidulus Me02	200	>10	>150	>1000	500	>1000	>100	malt ou N2P2	X
Helotiales sp. Me03	100							malt ou N2P2	X
Hebeloma leucosarx Me05								malt ou N2P2	
Lactatrius sp. Th04								N2P2	
Lactatrius sp. Th05	50							N2P2	
Lactatrius sp. Th07	100							N2P2	
Lactatrius sp. Th08	50							Pachlewski	
Lactatrius sp. Th09	25	2	25	>1000	500	1000	4	Pachlewski	
Hebeloma crustuliniforme Pr08								N2P2	
Hebeloma crustuliniforme Pr09	100							N2P2	
Hebeloma sp. Pr10	25	10	150	5000	>500	2000	4	N2P2	
Hebeloma crustuliniforme Pr11	25	>10	>150	>1000	500	>1000	>100	N2P2	
Hebeloma sp. Pr12	25	10	150	5000	>500	500	20	N2P2	

Hebeloma sp. Pr13	100	10	250	2000	500	500	20	N2P2	
Lyophyllum connatum Pr14	100	>10	>150	5000	350	5000	20	N2P2	
Hebeloma crustuliniforme Pr15		2	250	2000		1000		N2P2	
Hebeloma crustuliniforme Pr16	50	>10	50	5000	2000	1000	20	N2P2	
Hebeloma crustuliniforme Pr17	100							N2P2	
Hebeloma sp. Pr18		5	50	2000	500	>1000	4	N2P2	
Hebeloma sp. Pr19	100	>10	25	5000	200	500	4	N2P2	
Hebeloma sp. Pr20	25							N2P2	
Hebeloma sp. Pr21	100	>10	50					N2P2	
Hebeloma sp. Pr22	100	10	150	>1000	500	500	20	N2P2	
Hebeloma sp. Pr23		5	>500	2000		1000		N2P2	
Hebeloma sp. Cu08	25	10	>150	5000	350	1000	20	N2P2	
Hebeloma crustuliniforme Fe01	10	10	150	>1000	500	1000	20	N2P2	
Hebeloma crustuliniforme Fe02	50	>10	150	>1000	500	>1000	20	N2P2	
Hebeloma crustuliniforme Fe03	100	10	50	>1000	500	>1000	100	N2P2	
Hebeloma cylindrosporum Vi04	50	10	50	5000	350	2000	4	N2P2	
Hebeloma cylindrosporum Vi07	25	>10	50	5000	200	2000	4	N2P2	
Helotiales sp. Vi08	100	5	>150	5000	350	5000	20	N2P2	
Helotiales sp. Vi09	100	10	>150	>5000	350	>5000	100	N2P2	
Helotiales sp. Vi10	200	>10	>100	>5000	>500	5000	100	N2P2	
Hebeloma sp. Vi11	50	>10	>150	>5000	>500	5000	>100	N2P2	
Hebeloma cylindrosporum HcD1	100	10	>150	1000	500	>1000	100	N2P2	
lachnum sp. Me06	100							MMN	X
Meliniomyces sp. Me07	50	7	1500	>5000	>500	5000	>100	N2P2	X
Entrophospora sp. Me08	100							N2P2	X
Geomyces sp. Me09	>40							Pachlewski	X
Leptodontidium sp. Me10	50	5	500	>200	>500	>500	>20	Pachlewski	X
Entrophospora sp. Me11	>40							N2P2	X
Leptodontidium sp. Me12	100	>5	>100	>200	>500	>500	>20	N2P2	X
Meliniomyces sp. Me13	10							N2P2	X

Meliniomyces sp. Me14	>40							MMN	
Helotiales sp. Fe04	100							N2P2	X
Helotiales sp. Fe05	>100							Malt	
Cadophora sp. Fe06	50	9	600				9	N2P2	X
Exophiala sp. Fe07	50							N2P2	
Exophiala sp. Fe08	50							N2P2	X
Tetracladium sp. Fe09	50							Malt	X
Exophiala sp. Fe10	100							N2P2	
Tetracladium sp. Fe11	100							N2P2	X
Exophiala sp. Fe14	100							MMN	X
Exophiala sp. Fe15	100							MMN	X
Exophiala sp. Fe16	50							MMN	X
Exophiala sp. Fe17	100							N2P2	X
Helotiales sp. Fe18	100							N2P2	X
Helotiales sp. Fe19	25							N2P2	X
Leptodontidium sp. Fe20	100							N2P2	X
Helotiales sp. Fe21	25							Pachlewski	X
Meliniomyces sp. Fe22	25							Pachlewski	X
Exophiala sp. Fe24	100							N2P2	X
Phialophora Mustea Pr27	50	10	400					Malt ou Pachlewski	X
Phialocephala fortinii Pr28	50	12	500					Malt ou Pachlewski	X
Cadophora sp. Pr29	50	>5	500	>200	>500	>500	>20	Malt ou Pachlewski	
Leptodontidium orchidicola Pr30	40	5	300					Malt ou Pachlewski	
Piriformospora indica	100							Malt ou Pachlewski	X
Podospora sp.	200	2	>150	5000	>500	5000	20	Malt	

Résumé

Les activités anthropiques et la pollution qu'elles engendrent peuvent entraîner des changements drastiques dans les structures des écosystèmes et de leurs services écologiques. Le phytomanagement basé sur l'utilisation d'espèces ligneuses peut contribuer à la restauration des sols et de la diversité microbienne, tout en permettant la production de biomasses d'intérêt. Dans le cadre des projets PROLIPHYT (2013-2018, ADEME), BIOFILTREE (2010-2014, ANR) et PHYTOPOP (2007-2011, ANR) gérés par le laboratoire Chrono-environnement, des parcelles de phytomanagement ont été installées sur sites pollués par les éléments traces métalliques (ETMs), pour déterminer le potentiel de croissance d'espèces ligneuses, ainsi que les voies de valorisation des biomasses produites. Dans le cadre de mon projet de thèse, différentes approches ont été mises en œuvre afin d'identifier et de caractériser les microorganismes inféodés aux végétaux ligneux implantés, avec pour finalité la production de ressources microbiennes permettant d'améliorer la reprise et la croissance des arbres sur sols pollués. La première approche a consisté à étudier les communautés microbiennes en association avec les espèces ligneuses par une approche innovante de séquençage à haut débit associée à l'analyse physico-chimique des sols. Cette méthode a été mise au point et testée sur trois sites expérimentaux contaminés par des ETMs. Elle a permis de révéler les changements dans la structure et la composition des communautés microbiennes dus à la nature du couvert végétal et aux caractéristiques du sol (Zappelini *et al.*, 2015; Foulon *et al.*, 2016a; b). La deuxième approche a consisté à isoler et caractériser les champignons mycorhiziens et endophytes associés à des peupliers (Berthelot *et al.*, 2016; Lacercat-Didier *et al.*, 2016). Ces champignons sont connus pour favoriser la croissance des végétaux qu'ils colonisent mais également pour conférer une meilleure tolérance face à un stress métallique. Plusieurs campagnes de prélèvements de racines et de champignons sur différents sites pollués ont permis l'isolement de souches fongiques tolérantes aux ETMs (comme *Serendipita vermifera*) et améliorant la croissance (*Phialophora* sp. et *Leptodontidium* sp.). Dans une troisième approche, les mécanismes impliqués dans la résistance aux ETMs ont été étudiés i) chez *Paxillus involutus* pour le Hg (Foulon *et al*. manuscrit en préparation) ii) par métatranscriptomique fonctionnelle pour le Zn et Cd (Lehembre *et al.*, 2013). L'ensemble de ces travaux a permis i) d'améliorer nos connaissances sur l'impact du couvert ligneux sur la structure et la composition des communautés microbiennes, et sur la compréhension des mécanismes physiologiques mis en jeu ii) de constituer une collection de souches fongiques d'intérêt pour le phytomanagement de sols contaminés par les ETMs.

Mots-clés : phytomanagement, peuplier, éléments traces métalliques, communautés microbiennes, champignons mycorhiziens, champignons endophytes, bactéries, mercure, tolérance

Abstract

Anthropogenic activities can cause dramatic changes in ecosystem structures and their ecological services. Phytomanagement based on the use of woody species can contribute to soil and microbial diversity restoration, while allowing the production of biomass of interest. As part of PROLIPHYT (2013-2018, ADEME), BIOFILTREE (2010-2014, ANR) and PHYTOPOP (2007-2011, ANR) projects, managed by the Chrono-environment laboratory, phytomanagement plots were installed on trace elements (TE) polluted sites to determine the growth potential of woody species and the recovery methods of biomass produced. In my PhD project, different approaches have been implemented to identify and characterize microorganisms subservient to implanted woody plants, with the aim of producing microbial resources to improve the recovery and growth of trees on polluted soil. The first approach was to study microbial communities in association with woody species by innovative high throughput sequencing approach associated with the physical and chemical analysis of soils. This method was developed and tested on three experimental sites contaminated by TE. It revealed changes in the structure and composition of microbial communities due to the nature of the vegetation cover and the soil characteristics (Zappelini *et al.*, 2015; Foulon *et al.*, 2016a; b). The second approach was to isolate and characterize mycorrhizal fungi and endophytes associated with poplars (Berthelot *et al.*, 2016; Lacercat-Didier *et al.*, 2016). These fungi are known to promote the growth of the plants they colonize but also to increase their TE tolerance. Several sampling campaigns of roots and sporocarps on different polluted sites have allowed the isolation of fungal strains tolerant to TE (like *Serendipita vermifera*) and enhancing growth (*Phialophora sp*. and *Leptodontidium sp*.). In a third approach, the mechanisms involved in TE resistance were studied i) in *Paxillus involutus* for Hg (Foulon *et al*. In prep) ii) by functional metatranscriptomics for Zn and Cd (Lehembre *et al.*, 2013). In conclusion, this work allowed to i) improve our understanding of the impact of a woody cover on the structure and composition of microbial communities, and on the understanding of the physiological mechanisms involved ii) to constitute a collection of fungal strains that will be of great interest for future phytomanagement projects.

Keywords: phytomanagement, poplar, metal trace elements, microbial community, mycorrhizal fungi, fungal endophytes, bacteria, mercury, metal tolerance