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Cyril Zappelini

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UNIVERSITÉ DE BOURGOGNE FRANCHE-COMTÉ

École doctorale Environnement-Santé

Laboratoire Chrono-Environnement (UMR UFC/CNRS 6249)

THÈSE

Présentée en vue de l'obtention du titre de

Docteur de l'Université Bourgogne Franche-Comté

Spécialité « **Sciences de la Vie et de l'Environnement** »

**ÉTUDE DES COMMUNAUTÉS MICROBIENNES RHIZOSPHERIQUES
DE LIGNEUX INDIGÈNES DE SOLS ANTHROPOGÈNIQUES, ISSUS
D'EFFLUENTS INDUSTRIELS**

Présentée et soutenue publiquement par

Cyril ZAPPELINI

Le 3 juillet 2018, devant le jury composé de :

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Bertrand AIGLE (Professeur, Univ. de Lorraine)

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Examinatrice

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Co-directeur de thèse

Directeur de thèse

« En vérité, le chemin importe peu,
la volonté d'arriver suffit à tout. »
Albert Camus

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Résumé

Mon sujet de thèse intègre l'un des projets globaux de l'UMR UFC/CNRS 6249 Chrono-Environnement intitulé « stratégies de phytoremédiation basées sur l'utilisation d'arbres et de microorganismes associés », qui s'appuie, entre autre, sur 2 projets de recherche :

- le projet PROLIPHYT (Eco-Industrie, ADEME, 2013-2018) ou « PROduction de Ligneux PHYToremédiants »,
- le projet PHYTOCHEM (ANR CD2i, 2013-2018) ou « Développement de procédés chimiques éco-innovants pour valoriser les biomasses issues des phytotechnologies ».

Les objectifs généraux sont d'améliorer le potentiel de phytoremédiation d'un panel d'espèces ligneuses et de développer le potentiel microbien pour une phytoremédiation aidée sur sol contaminé. En plus de limiter l'impact des polluants, cette stratégie vise à promouvoir la production de biomasse sur sols délaissés et non exploitables par l'agriculture, tout en assurant la biodiversité nécessaire à la restauration d'un écosystème anthropogénique.

Mon travail de thèse est financé au travers un contrat doctoral ministériel handicap (dyslexie). Il s'appuie sur la réhabilitation de deux zones de stockage de sédiments industriels, utilisées jusque dans les années 2000. Ces deux sites expérimentaux (terril de l'Aillon, site INOVYN de Saint-Symphorien-sur-Saône en Côte-d'Or ; terril de l'Ochsenfeld, site CRISTAL de l'Ochsenfeld en Alsace) présentent des caractéristiques physico-chimiques très particulières qui en font des lieux d'étude privilégiés. Le premier est un ancien terril de décantation dont les sédiments enrichis en Hg, Ba et As proviennent du traitement des eaux usées issues du procédé d'électrolyse à Hg de l'entreprise SOLVAY. Le second est un terril constitué d'un remblai dans lequel ont été stockés depuis les années 1930, les résidus d'extraction du dioxyde de titane de l'usine CRISTAL de Thann. A l'inverse du premier site expérimental, on observe une flore peu abondante qui se traduit par un développement hétérogène d'une espèce ligneuse principale, le bouleau.

La recolonisation naturelle et spontanée de végétaux, plus particulièrement d'espèces ligneuses sur les deux sites est sans doute le résultat d'étroites collaborations avec des microorganismes telluriques situés aux abords de leur système racinaire. Nous avons ainsi choisi de travailler sur 3 espèces pionnières qui se sont naturellement réimplantées sur les deux sites d'études : le saule et le peuplier pour la friche industrielle du site de l'Aillon INOVYN et le bouleau pour l'unité de traitement des effluents du terril de l'Ochsenfeld.

Mots-clés :

Tailings (sol anthropogénique), milieu naturel (sol non-perturbé), Salicacées (peuplier, saule), Bétulacées (bouleau), rhizobactéries, métabarcoding, diversité, identification, caractérisation, *Plant Growth Promoting Rhizobacteria*, microbiologie environnementale, site INOVYN de Tavaux, site de l'Ochsenfeld.

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Abréviations

- ADN : Acide désoxyribonucléique
- ARNr : Acide ribonucléique ribosomique
- C/N : Rapport Carbone sur Azote
- CaCO₃ : Carbonate de calcium
- CaO : Chaux
- CCD : Charge-Coupled Device (eng)
- CEC : Capacité d'Echange Cationique
- Ctot : Carbone Organique Total
- DMA : Drainage Minier Acide
- Eh : Potentiel Redox
- ETM : Élément Trace Métallique
- FPGN : Fond Pédo-Géochimique Naturel
- HAP : Hydrocarbure Aromatique Polycyclique
- ITS : Internal Transcribed Spacer (eng)
- MgO : Magnésie
- MO : Matière Organique
- NGS : Next Generation Sequencing (eng)
- Ntot : Azote Total
- OTU : Operational Taxonomic Unit (eng)
- P₂O₅ : Pentoxyde de Phosphore
- PCR : Polymerase Chain Reaction (eng)
- PGM : Personal Genome Machine (eng)
- PGPR : Plant Growth Promoting Rhizobacteria (eng)
- PVC : PolyChlorure de Vinyle
- TiO₂ : Dioxyde de Titane

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Manuscrit et statut	Références
Manuscrit 1 Publié.	<ul style="list-style-type: none">• Zappellini 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. <i>Soil Biology and Biochemistry</i> 90: 101-110. DOI: http://dx.doi.org/10.1016/j.soilbio.2015.08.008. (IF 2015 : 4 ,2).
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Manuscrit 3 Publié.	<ul style="list-style-type: none">• Foulon J, Zappellini C, Durand A, Valot B, Blaudez D, Chalot M (2016) Impact of poplar-based phytomanagement on soil properties and microbial communities in a metal-contaminated site. <i>FEMS Microbiology Ecology</i>. 92 (10): fiw163. DOI: https://doi.org/10.1093/femsec/fiw163. (IF 2016 : 3 ,7).
Manuscrit 4 Publié.	<ul style="list-style-type: none">• Foulon J, Zappellini C, Durand A, Valot B, Girardclos O, Blaudez D, Chalot M (2016) Environmental metabarcoding reveals contrasting microbial communities at two poplar phytomanagement sites. <i>Science of the Total Environment</i> 571: 1230-1240. DOI /10.1016/j.scitotenv.2016.07.151. (IF 2016 : 4 ,9).
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Manuscrit 8 En préparation.	<ul style="list-style-type: none">• Zappellini C, Moulin S, Capelli N, Maillard F, Guyeux C, Hocquet D, Chalot M (-) Dominance and characterization of <i>Pseudomonas</i> at a chlor-alkali tailings dumps.

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DNA Watch Frasnes, France	<ul style="list-style-type: none"> • Zappelini C, Foulon J, Karimi B, Blaudez D, Cazaux D, Yergeau E, Greer C, Chalot M. Next-Generation Sequencing of Microbial Communities from a strongly anthropogenic soil. DNA Watch: 1st Emerging Topics meeting du réseau franco-suisse Environnement-Homme-Territoire. (4 - 5 Décembre 2013).
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ICOBTE Fukuoka, Japon	<ul style="list-style-type: none"> • Chalot M, Foulon J, Zappelini C, Karimi B, Valot B, Roy S, Yergeau E, Greer C, Bert V, Blaudez D. Phytomanagement of contaminated lands: considerations from above-and below-ground. 13th International Conference on the Biogeochemistry of Trace Elements. (12 - 16 Juillet 2015).
AQUACONSOIL Lyon	<ul style="list-style-type: none"> • Chalot M, Foulon J, Zappelini C, Durand A, Valot B, Blaudez D. Environmental metabarcoding as a relevant tool to reveal structure and composition of microbial communities at phytomanagement sites. 14th International Conference on Sustainable Use and Management of Soil, Sediment and Water Resources. (26 - 30 Juin 2017).
ICOBTE Zurich, Suisse	<ul style="list-style-type: none"> • Zappelini C, Assad M, Capelli N, Tatin-Froux F, Parelle J, Valot B, Maillard F, Blaudez D, Chalot M. Natural recolonization of tailings dumps by trees and microbes: a case study. • Álvarez-López V, Zappelini C, Blaudez B, Chalot M. Role of plant-associated microorganisms in the establishment and growth of <i>Betula</i> spp. in a Mn-contaminated mine dump. • Ciadamidaro L, Parelle J, Tatin-Foux F, Moyen C, Durand A, Zappelini C, Chalot M. Increasing the panel of tree species in phytomanagement strategies. 14th International Conference on the Biogeochemistry of Trace Elements. (16 - 20 Juillet 2017).
ICOM Prague, RepTchèque	<ul style="list-style-type: none"> • Blaudez D, Ciadamidaro L, Foulon J, Zappelini C, Durand A, Girardclos O, Valot B, Bert V, Roy S, Chalot M. Large-scale demonstration of increased production of poplar biomass by mycorrhizal inoculation at metal-contaminated phytomanagement sites, and investigation of associated fungal communities. 9th International Conference on Mycorrhizas. (30 Juillet - 4 Août 2017).

Communications par Poster

Communication	Références
SETAC Bâle, Suisse	<ul style="list-style-type: none"> • Zappellini C, Karimi B, Foulon J, Blaudez D, Cazaux D, Yergeau E, Greer C, Chalot M. Next-generation sequencing of microbial communities from a mercury polluted soil. 24th Annual Meeting of the Society of Environmental Toxicology and Chemistry. (11 - 15 Mai 2014).
DNA Watch Frasnes, France	<ul style="list-style-type: none"> • Foulon J, Zappellini C, Lacercat-Didier L, Karimi B, Blaudez D, Cazaux D, Yergeau E, Greer C, Chalot M. Next-Generation Sequencing of Microbial Communities from a poplar plantation in a phytoremediation perspective. DNA Watch: 1st Emerging Topics meeting du réseau franco-suisse Environnement-Homme-Territoire. (4 - 5 Décembre 2013).
ADEME Paris	<ul style="list-style-type: none"> • Foulon J, Assad M, Zappellini C, Blaudez D, Soupe N, Reynaud P, Cazaux D, Benbrahim M, Chalot M. Le projet PROLIPHYT : Accroître le potentiel de production de ligneux phytoremédians. 3^{ème} Journées sites et sol pollués de l'ADEME. (18 - 19 Novembre 2014).
GSBI Dijon	<ul style="list-style-type: none"> • Zappellini C, Maillard F, Foulon J, Karimi B, Lacercat-Didier L, Blaudez D, Cazaux D, Hocquet D, Chalot M. High bacterial diversity of a chlor-alkali and Hg-enriched tailing pond. • Foulon J, Zappellini C, Karimi B, Valot B, Blaudez D, Yergeau Y, Greer C, Chalot M. Impact of phytomanagement practices on microbial communities as revealed by next-generation sequencing technology. • Karimi B, Zappellini C, Foulon J, Maillard F, Blaudez D, Cazaux D, Gilbert D, Yergeau E, Greer C, Chalot M. Turning tailing pond into soil as revealed by metagenomic and ecological interaction. First Global Soil Biodiversity Conference. (2 - 5 Décembre 2014)
ICHMET Ghent, Belgique	<ul style="list-style-type: none"> • Chalot M, Foulon J, Zappellini C, Durand A, Valot B, Blaudez D. Environmental metabarcoding reveals contrasting microbial communities at two poplar phytomanagement sites. 18th International Conference on Heavy Metals in the Environment. (12 - 15 Septembre 2016).
AQUACONSOIL Brussels, Belgique	<ul style="list-style-type: none"> • Chalot M, Assad M, Durand A, Foulon J, Maillard F, Zappellini C. Trees and microbes at a Hg contaminated chlor alkali facility. SETAC EUROPE, 27th Annual Meeting. (7 - 11 Mai 2017).

Chapitre 1. Introduction générale

Depuis la révolution industrielle, de nombreux contaminants ont été introduits dans l'environnement. De nos jours, cette pollution est accentuée par une plus forte urbanisation et l'augmentation accrue et à grande échelle de mégalopoles fortement industrialisées. Dans ce contexte, le traitement des pollutions représente un potentiel économique auxquelles les méthodes conventionnelles du génie civil ne répondent pas toujours, principalement en raison de leur impact environnemental et de leur coût. Parallèlement, les mesures visant à accélérer le développement et la diffusion de technologies propres qui soient sûres et durables occupent une place majeure dans la panoplie générale de mesures visant à promouvoir des économies plus vertes.

1.1 Remédiation des sols et sites pollués

Le sol est un milieu extrêmement complexe, variable et vivant. Il représente une ressource naturelle non renouvelable à l'échelle humaine vitale pour l'humanité (Alexander, 1988). Une utilisation inadaptée des terres associée à des pratiques de gestion inappropriées peut entraîner une dégradation des sols (Figure 1.), phénomène de plus en plus amplifié par le changement climatique (Rhodes, 2014).

Les activités anthropiques qui accompagnent le développement économique des pays industrialisés sont sources de contaminations dans le sol, l'eau et l'air responsables de changements drastiques de la structure des écosystèmes et des services écosystémiques du sol (Dawson et al., 2011).

Selon le dernier rapport de l'Agence Européenne de l'Environnement (2017), la contamination des sols pourrait concerner près de 3 millions de sites en Europe. Les sites pollués sont définis comme « des sites dont le sol, le sous-sol ou les eaux souterraines ont été pollués, du fait d'anciens dépôts de déchets ou d'infiltration de substances polluantes, cette pollution étant susceptible de provoquer une nuisance ou un risque pérenne pour les personnes ou l'environnement » (Ministère de l'Environnement, 1994). Depuis les années 1990, 80 000 sites ont été réhabilités.

Tous les secteurs d'activités anthropiques sont susceptibles d'émettre des polluants (transport, activités domestiques, agriculture, sylviculture) mais les plus importants sont les activités commerciales et industrielles ainsi que le traitement et l'élimination des déchets (Figure 2.a.).

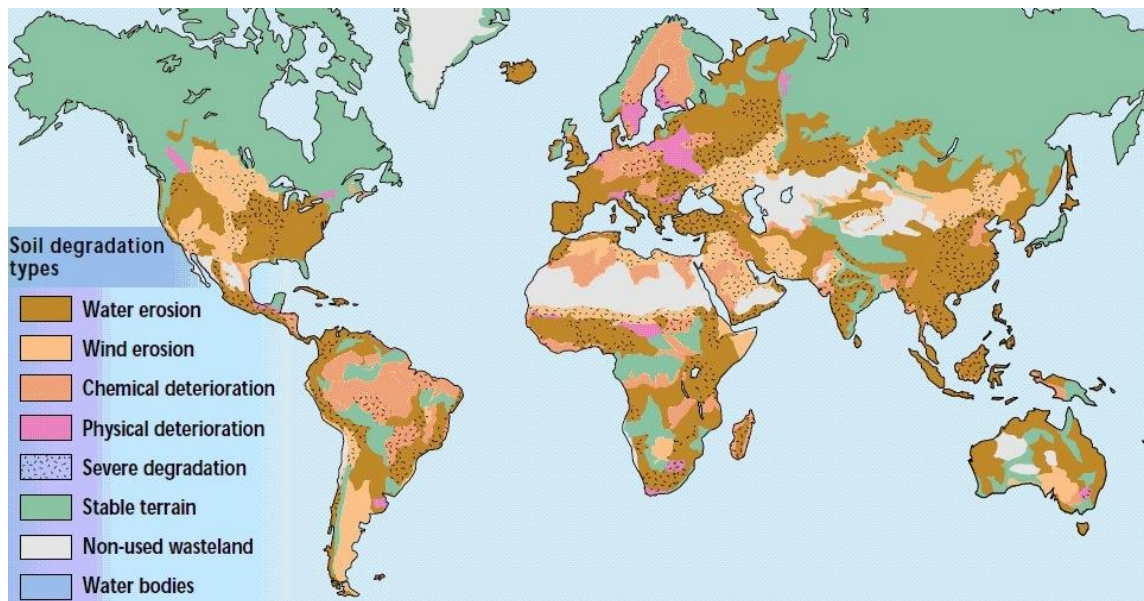


Figure 1. Carte du monde de l'état des sols dégradés par l'activité humaine (ISRIC et al., 1996).

Même si la nature des polluants que l'on retrouve dans le sol est très variée (huiles minérales, hydrocarbures aromatiques polycycliques (HAPs), pesticides, solvants...), les éléments traces métalliques (ETMs) sont les contaminants inorganiques les plus communs affectant 37% des sols pollués en Europe (Figure 2.b.).

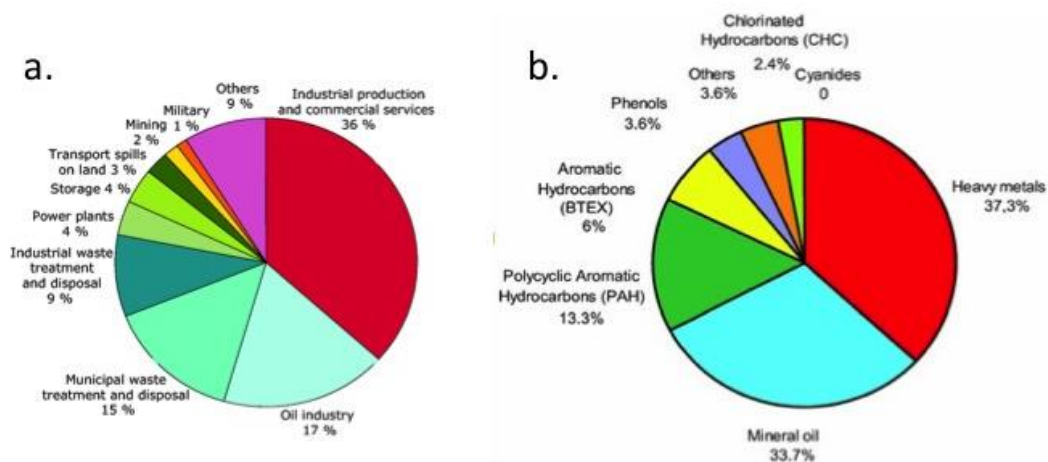


Figure 2. a. Les secteurs d'activités économiques responsables de la contamination des sols en Europe. b. Les contaminants affectant les sols et les eaux souterraines en Europe (European Environment Agency 2017).

En pédologie, le terme d'élément trace définit 68 minéraux, constituant la couche terrestre, présents chacun à une concentration inférieure à 0,1%, pour un total de 0,6% alors que seuls 12 éléments dits majeurs (O, Si, Al, Fe, Ca, Na, K, Mg, Ti, H, P, Mn) représentent 99,4% de la croûte terrestre (Baize, 1997). Un ETM répond à la fois à la définition précédente, mais doit également appartenir aux métaux qui sont des éléments cationiques, dotés de pouvoirs conducteurs électrique et thermique.

Les ETMs sont aussi communément désignés sous le terme « métaux lourds »,

terminologie peu à peu abandonnée, car on considère qu'un métal est lourd dès lors que sa masse volumique est supérieure à 5 g.cm^{-3} , que son numéro atomique est au-delà de 20 et qu'il forme avec des sulfures un précipité insoluble. De plus la désignation de métal lourd a une connotation réductrice aux métaux toxiques tels que Hg, Cr, Pb, Ni, Cd, Co (Wang et Chen, 2006). Inversement certains ETMs sont indispensables à de nombreux processus biologiques, il s'agit d'oligoéléments. Le Zn, le Cu et le Mn peuvent avoir le rôle de cofacteur enzymatique pour former des métallo-enzymes essentielles au fonctionnement du métabolisme (Waldron et al., 2009). Le Fe peut aussi former des centres Fe-S ou des hèmes indispensables à la fonction de nombreuses protéines : enzymes de la voie de synthèse des isoprénoïdes, ferrochélatase, hélicases, déshydratases (Py et Barras, 2014). Le terme « trace » est également connoté et sans définition scientifique, voire trompeur dans les contextes de forte contamination. Près de l'ancienne fonderie Métaleurop-Nord la teneur du sol ou de sédiments en métaux toxiques tels que le Pb peut largement dépasser 10 % (en poids), ce qui ne permet plus de parler de traces (ng à μg par kg). Néanmoins le terme d'ETM est désormais le plus couramment utilisé dans la littérature scientifique.

L'évaluation récente de la distribution des ETMs au niveau des sols arables dans l'Union Européenne montre une certaine diversité à la fois au niveau de la variabilité géographique et des différentes concentrations mesurées. Plus que cette dernière, c'est la spéciation de l'élément, sous forme d'ion libre donc biodisponible, ou liée à des particules de sol qui est déterminante pour évaluer sa mobilité et sa toxicité pour les organismes vivants. La spéciation est conditionnée par les paramètres physico-chimiques et minéralogiques du milieu (Baize, 1997) parmi lesquels : le potentiel redox (Eh), la capacité d'échange cationique (CEC), la teneur en phosphate disponible, la teneur en matière organique (MO) et les activités biologiques (solubilisation et complexation par les exsudats racinaires et les organismes telluriques). Le pH est un des facteurs qui joue un rôle majeur dans la spéciation des ETMs (Spurgeon et al., 2006).

Les ETMs sont présents naturellement dans les sols, ils peuvent provenir des flux entre la roche mère et la surface, d'apports d'éruptions volcaniques ou d'embruns marins, mais aussi sous l'effet des plantes qui les extraient, les accumulent et les relarguent sous des formes différentes (Figure 3. ; (Dixit et al., 2015)).

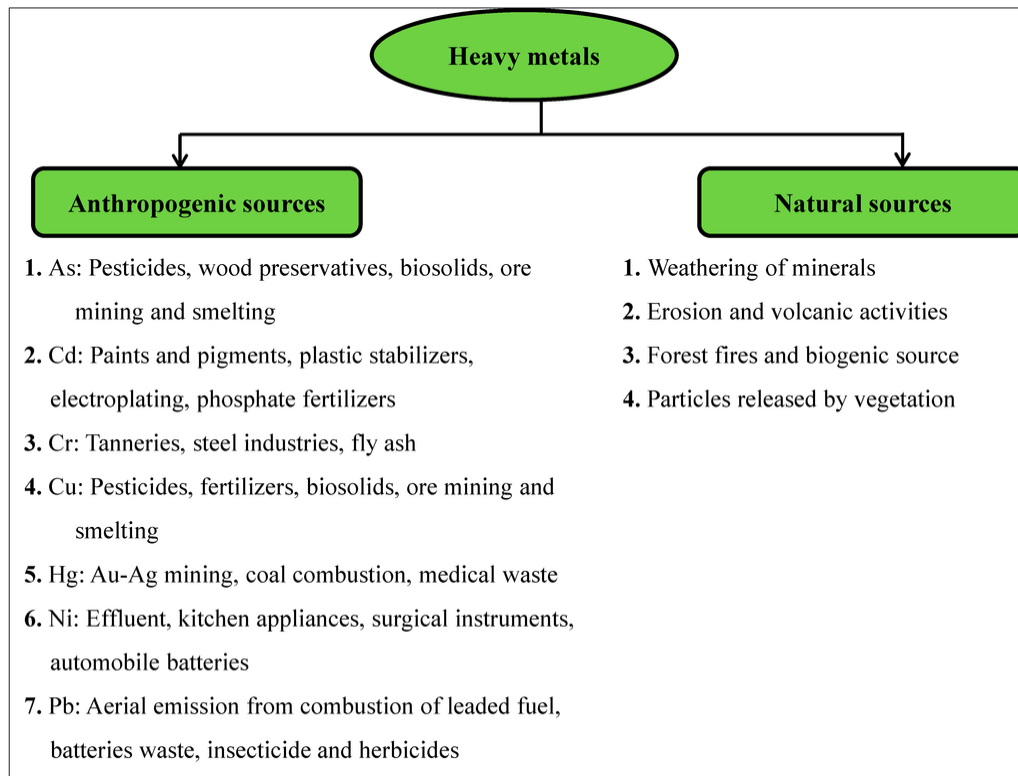


Figure 3. Origine des ETMs dans le sol (Dixit et al., 2015).

Le problème principal des ETMs comme le Pb, le Cd, le Cu et le Hg est qu'ils persistent de façon quasi permanente dans les sols, car ils ne peuvent pas être biodégradés. Leur accumulation dans les sols anthropiques comme les friches industrielles souvent localisés à proximité ou dans des zones urbaines développées (Prokop et al., 2000 ; Thornton et al., 2007) augmente leur transfert potentiel dans la chaîne alimentaire et constitue un risque accru non seulement pour la santé humaine mais aussi pour le milieu environnant (Dixit et al., 2015).

D'après le rapport de référence de la Commission européenne (CE, 2009), l'UE 27 produit chaque année plus de 300 millions de tonnes de déchets d'extraction provenant d'activités minières. La réhabilitation de l'ensemble des sites potentiellement contaminés représente une somme estimée à environ 17,3 milliards d'euros par an. Ces sites de tailles extrêmement variables peuvent être bâtis ou non et résultent le plus souvent de l'arrêt temporaire ou définitif d'une activité industrielle. Ils sont dus à d'anciennes pratiques sommaires d'élimination des déchets comme les terrils de dépôt de sédiments miniers ou les bassins de décantation, et restent des réservoirs de contaminants potentiellement dangereux. Les conséquences environnementales sont parfois dramatiques lors d'épandages fortuits ou accidentels de résidus chimiques. En avril 1998, la rupture d'une digue d'un bassin de stockage de la mine de métaux à ciel ouvert d'Aznalcollar dans la province de Séville en Espagne a provoqué le déversement de plus de sept millions de tonnes de boues toxiques sur quelque 5 000 ha. Plus récemment en janvier 2000, une cause similaire s'est traduite par le déversement des cyanures de l'exploitation de la mine d'or

d'Aurul aux alentours de Baia Mare en Roumanie. La pollution a totalement détruit la faune et la flore de la Tisa, un des affluents du Danube, sur une longueur de 30 à 40 km. Ces accidents ont attiré l'attention du public sur la gestion des bassins de résidus et des digues de retenue.

Les décharges à grandes échelles utilisées pour stocker les résidus industriels peuvent d'autre part générer d'immenses surfaces de sol non végétalisées avec d'importantes répercussions sur la résilience des écosystèmes.

En France, l'héritage de plus de 200 ans d'activité industrielle a fait du suivi détaillé et de la gestion des sols pollués un enjeu d'autant plus important que les questions des impacts sur la santé sont aujourd'hui au centre des préoccupations sociétales (Figure 4.).

Les résultats sont disponibles sur deux bases de données complémentaires :

- Basol (<http://basol.developpement-durable.gouv.fr>) gérée par le Ministère de la transition écologique et solidaire qui résume la localisation, la situation technique, la nature des polluants et l'impact des sites et sols pollués (ou potentiellement pollués) appelant une action des pouvoirs publics, à titre préventif ou curatif. En décembre 2017, sur les 6617 sites recensés, 47,26% sont traités avec surveillance et/ou restriction d'usage. La base de données identifie les hydrocarbures (13,95%), le Pb (6,79%), les HAPs (6,56%), le Cr (5,18%), le Cu (5,08%) et les solvants halogénés (5%) comme principaux contaminants des sols ou des nappes d'eau souterraine en France.
- Basias (<http://www.georisques.gouv.fr/dossiers/basias>) créée par un arrêté ministériel de 1998, gérée par le Bureau de Recherches Géologiques et Minières dont les principaux objectifs sont de répertorier, de façon large et systématique, tous les sites industriels abandonnés ou non, susceptibles d'engendrer une pollution de l'environnement, de conserver la mémoire de ces sites, de fournir des informations utiles aux acteurs de l'urbanisme, du foncier et de la protection de l'environnement.

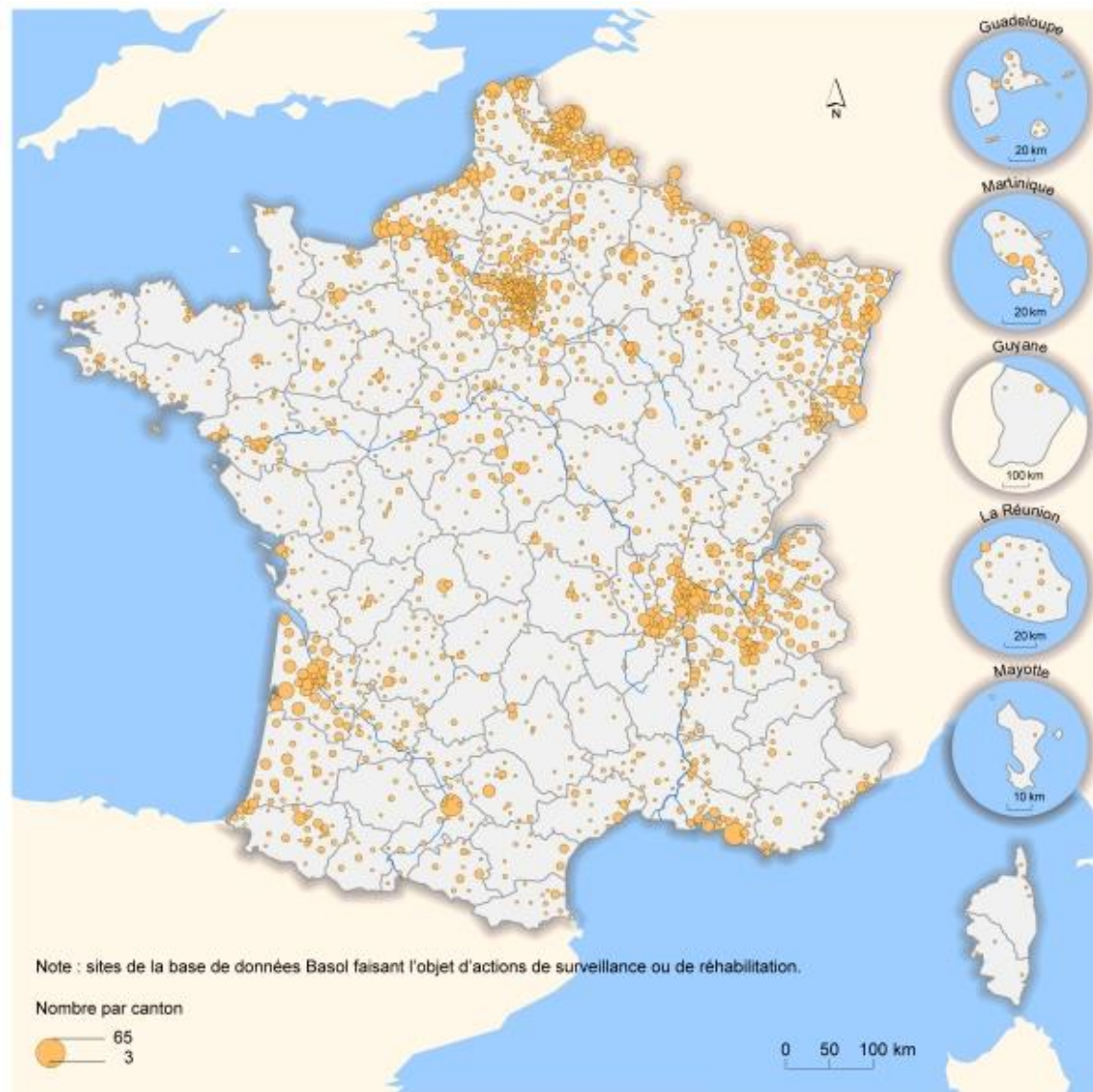


Figure 4. Les sites et sols pollués début 2015 (sites sur lesquels l'état a entrepris des actions de remédiation).

Note : sites de la base de données Basol faisant l'objet d'actions de surveillance ou de réhabilitation. Source : Medde, DGPR (Basol au 5 mars 2015), 2012. Traitements : SOEs, 2015.

Trois phénomènes socio-économiques se combinent pour rendre prioritaire la valorisation des sites pollués :

- les mutations du secteur industriel qui conduisent à l'arrêt de nombreuses exploitations,
- l'augmentation du nombre de friches industrielles,
- l'augmentation de la pression démographique et foncière en zones urbanisées.

Les deux dernières décennies ont été marquées par le développement de techniques douces de réhabilitation des sols par différentes espèces végétales et la combinaison de biotechnologies microbiennes. Ces phytotechnologies regroupent un

ensemble de techniques qui utilisent des végétaux pour extraire, contenir ou dégrader des polluants inorganiques ou organiques (Mench et al., 2010, 2009 ; Vangronsveld et al., 2009). Émergentes sur les marchés du traitement et de la gestion des sites pollués, ces techniques douces peuvent s'appliquer *in situ* sur une large variété de sols pollués (sols agricoles, sols délaissés, friches industrielles, sédiments excavés...) aussi bien en milieu rural que urbain. Jugées à priori plus conformes aux enjeux du développement durable que les techniques classiques de traitement sur site et hors site, elles impactent positivement les fonctions et la structure du sol. Les phytotechnologies constituent une alternative prometteuse sinon un complément aux techniques conventionnelles, dans le cas notamment de surfaces polluées importantes. De plus, afin de dégager des revenus de surfaces délaissées, et qui plus est polluées, les végétaux sélectionnés sont susceptibles d'être cultivés en taillis. La production de biomasse sur ces sites présente un double avantage. D'une part, la plantation de végétaux phytoremédians permet la dépollution des sols ; d'autre part, la valorisation de la biomasse permet de dégager des revenus pour des surfaces qui habituellement impactent négativement le budget des propriétaires (Bardos et al., 2011 ; Licht et Isebrands, 2005 ; Suer et Andersson-Sköld, 2011 ; Witters et al., 2012).

Cependant, si le principe de la revégétalisation des terrains affectés par l'activité industrielle est simple, son application est toutefois plus difficile en raison notamment des conditions biotiques précaires des sols anthropogéniques en général. Carencées en éléments nutritifs essentiels, certaines aires d'accumulation de rejets miniers sont composées de matières inorganiques, affichant parfois des concentrations élevées en ETMs et autres contaminants, peu propices à la croissance et à l'établissement d'un couvert végétal (Allaire et al., 2013).

1.2 Activités minières et enjeux environnementaux

Conséquence de la croissance démographique et de l'essor économique rapide de pays émergents, comme l'Inde et la Chine, la demande en matières premières minérales n'a cessé d'augmenter depuis la fin du 20^{ème} siècle. Même si l'année 2015 a été marquée par une baisse générale de 25% des prix des matières premières par rapport à 2014, les géants du secteur minier ont désormais renoué avec le profit. En 2017, l'entreprise d'exploitation minière suisse Glencore Xstrata s'est classée première avec des recettes d'environ 153 milliards de dollars. À l'heure actuelle, certaines régions nordiques du Québec et du Canada connaissent même une vague d'activités minières sans précédent. Si le développement de l'exploitation minière représente un vecteur positif au plan des retombées socio-économiques et financières, il reste critiqué pour ses impacts environnementaux potentiellement négatifs (Aubertin et al., 2002 ; Bridge, 2004 ; MMSD, 2002 ; Ripley et al., 1996). En France, suite au passé minier, on recense quelques 300 gisements exploités de manière significative ou ayant fait l'objet de travaux d'exploration avancés. En 2015, le sel concentrait l'essentiel de l'activité minière en métropole avec une vingtaine de concessions en activité et plus de 4,4 millions de tonnes de sel extraites du

sous-sol. Si la quasi-totalité des mines ont aujourd'hui cessé leur activité sur le territoire métropolitain, les stériles d'extraction et d'enrichissement sont restés en place et peuvent poser un problème sanitaire et environnemental. Par ailleurs, l'activité minière continue dans les DOM-TOM avec l'extraction d'Au et de Ni.

Une des principales conséquences du développement minier est donc l'impact sur les écosystèmes de la grande quantité de rejets produits lors des étapes d'extraction et de traitement du minerai. En effet, la très grande majorité des projets miniers génère et déplace des volumes considérables de terre, de roches et de résidus miniers (Bridge, 2004). Parmi les deux principales méthodes d'extraction, celle dite « à ciel ouvert » est généralement préférée par les exploitants car moins coûteuse et considérée comme plus sécuritaire. Cependant, le décapage et le retrait du mort-terrain, constitué de matériaux meubles (terre, sable, sédiment...) indispensable pour accéder au minerai exploité, génèrent 2 à 10 fois plus de rejets (dont les poussières relarguées dans l'atmosphère) que l'extraction « souterraine ». Elle induit également la destruction du couvert végétal originel et la destruction permanente d'habitats naturels avec des répercussions notables sur les espèces à mobilité réduite ou sédentaires (invertébrés, rongeurs, reptiles, petits mammifères...). L'impact visuel et esthétique pour l'environnement des mines à ciel ouvert est également plus dommageable et doit être prise en compte lors de la fermeture du site et sa réhabilitation. Les conséquences visuelles et esthétiques occasionnées peuvent même avoir un impact économique pour la région dans le cas où le tourisme est présent.

Parmi les autres déchets, on peut distinguer :

- les stériles miniers constitués par les sols et roches excavés lors de l'exploitation et dont la teneur en métal recherché est nulle ou très faible ;
- les différents produits générés lors du traitement et de la séparation du minerai au concentrateur comme, par exemple, le cyanure pour l'extraction du minerai d'or ou d'argent de sa gangue ou l'acide sulfurique dans le cas de l'uranium ;
- Enfin, les boues de traitement des résidus miniers avant leur stockage sur des sites d'entrepôts aménagés.

Le transport et l'entreposage en surface des rejets miniers (solide ou liquide) potentiellement contaminés constituent ainsi le principal défi environnemental auquel doit faire face l'industrie extractive en raison des effets directs sur les écosystèmes locaux. La composition et la stabilité chimique des rejets entreposés doivent être régulièrement contrôlées, de même que la mise en place systématique de mesures de sécurité autour des sites de stockages afin de limiter les accès non autorisés.

Concernant la gestion des effluents liquides, une attention particulière doit être accordée aux conditions de formation du drainage minier acide (DMA) associé à l'oxydation des minéraux sulfureux. Exposés aux conditions climatiques, ces minéraux (pyrite, pyrrhotite, sphalérite...) peuvent réagir avec l'eau et l'oxygène atmosphérique et

engendrer un lixiviat acide. En absence de matériaux neutralisants (carbonates, silicates), ce dernier est susceptible de favoriser la dissolution de métaux (Cu, Fe, Zn...) et autres contaminants résultant des procédés de traitement des minerais, ou bien encore d'être responsable de l'acidification des eaux de surface ou souterraine, avec des impacts à long terme qui peuvent être dévastateurs sur l'environnement. Outre le DMA, il est aussi possible d'observer un lessivage de certains métaux sulfureux exposés à l'air et à l'eau à pH neutre (STANTEC Consulting LTD, 2004).

D'autres enjeux environnementaux d'importance sont liés à l'activité extractive. Citons de façon non exhaustive :

- les risques de déversement accidentel (transport) d'additifs (cyanure, acide sulfurique, agents de flottation, xanthates...) utilisés pour l'extraction du minerai ou la rupture de bassins de rétention de rejets miniers (Amegbey et Adimado, 2003 ; Bridge, 2004 ; Moody, 2005).

- l'érosion hydrique par les eaux de ruissellement, source de sédiment en suspension, pouvant entraîner une dégradation de la qualité des cours d'eau en aval du site avec des impacts potentiels sur la flore, la faune naturelle et le réseau trophique. Les minéraux associés aux sédiments peuvent également abaisser le pH de l'eau et ainsi favoriser la mobilisation de ETMs.

- l'érosion éolienne susceptible d'émettre de nombreuses poussières potentiellement toxiques dans l'atmosphère, qui pourront se déposer et contaminer des terres sur de longues distances.

- les sites orphelins contaminés dont les exploitants n'existent plus ou ne sont plus solvables pour envisager la restauration d'un écosystème.

- une hausse des émissions de gaz à effet de serre liée aux pratiques d'exploitation et de transport sur mine. A titre d'exemple, BHP Billiton Ltd, l'une des plus grandes compagnies minières au monde, est au 20^{ème} rang des 100 entreprises les plus émettrices de gaz à effet de serre avec 317 Mégatonnes d'équivalent en dioxyde de carbone (éq. CO₂) rejetés dans l'atmosphère en 2015 (CDP Carbon Majors Report, 2017).

1.3 Les communautés bactériennes des sites miniers

Afin d'adapter au mieux mon propos au contexte de mes travaux, j'ai choisi de limiter l'étude bibliographique aux bactéries, à l'exclusion des autres catégories de microorganismes telles que les *Archea*, les espèces fongiques ou les algues unicellulaires, qui participent également à la biodiversité microbienne des sols miniers.

1.3.1 Diversité microbienne des sites miniers

Le sol abrite une multitude de microorganismes qui est associée à une grande complexité d'interactions écologiques. Parmi celles-ci, les communautés bactériennes telluriques sont de loin les plus abondantes en termes de biomasse (Kaymak, 2011). L'étude

de métagénomiques de sols contrastés en termes de compositions physicochimiques révèle la présence de 10^8 à 10^{11} bactéries par gramme de sol (Curtis et al., 2002 ; Roesch et al., 2007) dont la plupart ne sont pas encore connues, ni même cultivables en laboratoire. Les bactéries se caractérisent par leur extraordinaire plasticité génomique, qui leur permet de s'adapter aux variations de leur milieu beaucoup plus rapidement et efficacement que les autres organismes de notre planète. Cette diversité physiologique et génétique extrêmement importante est à l'origine de leurs potentialités à coloniser les environnements les plus divers. Elles constituent un réservoir énorme de biodiversité grâce à leurs métabolismes énergétiques très variés et une large palette de processus biochimiques. Elles jouent également un rôle primordial dans les cycles biogéochimiques du carbone, de l'azote et d'autres éléments.

En exploitant les ressources minérales depuis des millénaires, les activités anthropiques ont favorisé le contact des couches géologiques, ou les déchets miniers, avec l'eau et l'oxygène, induisant l'oxydation des sulfures métalliques par des bactéries acidophiles (Johnson et al., 2002 ; Ledin et Pedersen, 1996). Comme indiqué dans le paragraphe précédent, ce phénomène connu sous le terme de DMA a des conséquences environnementales importantes dans les régions où l'activité minière est, ou a été, importante.

Dans les sites miniers, l'activité microbienne est principalement localisée dans :

- l'environnement de la couche superficielle du sol (20 à 30 cm),
- les effluents acides issus de l'oxydation spontanée des minéraux sulfurés présents au fond des mines à ciel ouvert ou provenant de la percolation des piles de stockage des matériaux excavés,
- les sédiments et résidus d'extraction,
- la rhizosphère et la phyllosphère où les microorganismes résident principalement comme endophytes ou épiphytes.

La diversité microbienne des sites miniers varie selon les régions, en raison de la nature du processus d'extraction des minerais exploités. Comme détaillé dans la table 1, l'embranchement le plus représenté dans les communautés microbiennes des sols en contexte minier ou dans les DMA est celui des *Proteobacteria*. De par la nature du milieu dans lequel ils vivent, la plupart des espèces identifiées possèdent une résistance aux ETMs et sont des extrêmophiles tolérants un pH inhabituel.

En Espagne, la région minière du Rio Tinto représente la plus large formation géologique pyriteuse au monde. En raison des effets du DMA, les eaux du fleuve passant à proximité des mines présentent une forte concentration en ETMs, une teinte rougeâtre liée à la sédimentation ferrugineuse et un pH compris entre 2 et 4. Dans les eaux minières métallifères de Rio Tinto, l'identification taxonomique par séquençage du gène de l'ARNr 16S a permis de mettre en évidence deux principales espèces bactériennes, à savoir *Acidithiobacillus ferrooxidans* et *Leptospirillum ferrooxidans*, ainsi qu'une nouvelle bactérie

semblable au *Ferrovum*, "*F. myxofaciens*" (García-Moyano et al., 2012).

Isolés respectivement en 1951 (Temple et Colmer, 1951) et 1972 (Markosyan, 1972), *A. ferrooxidans* et *L. ferrooxidans* sont deux microorganismes chimolithoautotrophes qui puisent leur source d'énergie dans l'oxydation du fer ferreux. À ce titre, elles représentent des micro-organismes actifs au sein des réacteurs de biolixiviation, technologie utilisée pour traiter des stériles miniers ou des eaux du DMA pour l'extraction de nombreux métaux d'intérêts économiques, comme Au, Ag, Cu, Co, As et Mn.

À Iron Mountain dans la région aride du nord de la Californie, l'ancienne mine Richmond est également un site d'étude particulièrement intéressant. L'activité microbiologique combinée à l'évaporation naturelle génère des eaux de drainage très fortement chargées en métaux, dont la température dépasse 40°C avec des valeurs de pH les plus basses mesurées sur Terre. Malgré ces conditions extrêmes, des biofilms de couleur rose, comprenant notamment des bactéries oxydant le Fe du genre *Leptospirillum* sp., flottent à la surface et colonisent activement ces eaux dont le pH est compris entre 0,5 et 1 (Bond et al., 2000 ; Edwards et al., 1999).

Table 1. Aperçu de la diversité microbienne dans plusieurs anciens sites miniers (Thavamani et al., 2017).

Silver Bow creek mine tailings, Montana, USA	As, Cd, Cu, Fe, Mn, Pb and Zn	<i>Thermoplasma</i> sp., <i>Ferroplasma</i> sp., <i>Sulfolobus</i> sp., <i>Methanothermus</i> Predominantly iron- and sulphur-oxidizing, manganese-reducing bacterial species	Wielinga et al. (1999)
Iberian Pyrite belt acidic mine waters, Spain	SO ₄ (8.5 g L ⁻¹), Mg (1.0 g L ⁻¹), Metals: Fe (760 mg L ⁻¹), Al (190 mg L ⁻¹), Mn (84 mg L ⁻¹), Cu (29 mg L ⁻¹)	<i>Leptospirillum ferrooxidans</i> , <i>Acidithiobacillus ferrooxidans</i> , <i>Acidiphilium</i> sp., Actinobacteria, Acidimicrobiales, <i>Ferroplasma</i> sp., Acidobacteria, <i>Ferrovum</i> sp.	Santofimia et al. (2013)
La-Zarza-Perrunal Gold and silver mines, Iberian pyrite belt, Spain	Fe, Al, Na, Mg, Ca, Cd, Cu, Zn, Ni, Co, K, As, Pb	<i>Desulfosporosinus</i> sp., <i>Syntrophobacter</i> sp., <i>Acidiphilium</i> sp., <i>Acidobacterium</i> sp., <i>Acidosphaera</i> sp., <i>Leptospirillum</i> sp., <i>Acidithiobacillus ferrooxidans</i> , Thermoplasmata, <i>Chlorella</i> sp., Zygnematales	Gonzalez-Toril et al. (2011)
Lusatian acidic lignite mine, (Lake 77) in East-central Germany	Ni, Co, Cu, Zn, Pb, As, Cd, U	<i>Ferrovum myxofaciens</i> , <i>Sideroxydans</i> sp., <i>Albidiferax ferrireducens</i> , <i>Pelobacter propionicus</i> , <i>Geobacter chapellei</i> , <i>Geobacter psychrophilus</i> strain P35, Acidimicrobium <i>ferrooxidans</i> , <i>Chlorobium ferrooxidans</i>	Reiche et al. (2011)
Lead-Zinc abandoned mine site, Aravaipa Valley, Graham County, Arizona, USA	Zn, As, Cu, Fe, Pb	Firmicutes, Actinobacteria, α -, β - and γ -Proteobacteria, Nitrospira, Acidobacteria	Mendez et al. (2008)
Iron mountain, California, USA	Fe, S, Cu	<i>Thiobacillus ferrooxidans</i> , <i>Leptospirillum ferrooxidans</i>	Edwards (1999)
Zhong Tiaoshan copper mine, China	Fe, Cu, S, Co, Ni, Zn, Pb, As	α -, β - and γ -Proteobacteria, <i>Leptospirillum ferrooxidans</i> , <i>Leptospirillum ferriphilum</i> , Firmicutes, Bacteroidetes, <i>Herbaspirillum</i> sp., <i>Alcaligenes</i> sp., <i>Acidithiobacillus ferrooxidans</i> , <i>Pseudomonas synxantha</i> , <i>Sphingomonas</i> sp., <i>Brevundimonas</i> sp., <i>Sulfobacillus thermotolerans</i> , <i>Chimaerocella alkaliphila</i> , Nitrospira	He et al. (2007)
Mynydd Parys copper mines at Anglese, Wales	Fe, S, Mn, Cu and Zn	<i>Acidithiobacillus ferrooxidans</i> , <i>Ferrimicrobium acidiphilum</i> , <i>Acidobacterium capsulatum</i> , <i>Acidiphilium</i> sp., <i>Thiomonas</i> sp., <i>Acidiphilium acidophilum</i> , <i>Acidiphilium cryptum</i> , <i>Acidocella</i> sp.	Hallberg et al. (2006)
Rio Tinto acid mine drainage (AMD), Spain	Fe, Zn, Cu, Ni, Mn	<i>Acidiphilium</i> sp., <i>Acidocella</i> sp., <i>Acidisphaera</i> sp., <i>Betaproteobacteria</i> sp., Nitrosomonadales, <i>Ferrovum myxofaciens</i> , Acidobacteria, Firmicutes, Actinobacteria, Thermoplasmata, Cyanobacteria, <i>Planctomycetes</i> sp., <i>Chloroflex</i> sp.	García-Moyano and Gonzalez-Toril, 2012
Dyffryn Adda AMD, U K	Zn, Cu, Al, Fe, Mn	<i>Acidithiobacillus ferrivorans</i> , <i>Ferrovum myxofaciens</i> , <i>Acidithrix ferrooxidans</i> , <i>Acidocella</i> sp., <i>Acidithiobacillus ferrooxidans</i> , <i>Leptospirillum ferrooxidans</i>	Kay et al. (2013)

D'autres bactéries, dont la plupart appartiennent à la classe des *Betaproteobacteria*, ont également la capacité à produire des biofilms à la surface des eaux acides d'origine minière et ont été identifiées dans d'anciennes mines de Cu au nord du Pays de Galles et de la Chine (Kay et al., 2013). En Allemagne, Heinzl et collaborateurs (Heinzl et al., 2009) ont montré que la principale espèce bactérienne présente dans les effluents acides d'une usine de traitement des eaux de mine par les plantes était une *Betaproteobacteria* du genre *Ferribacter polymyxa*. Enfin en Andalousie, la diversité bactérienne des effluents acides de l'ancienne mine de La Zarza Perrunal, est principalement constituée par des bactéries anaérobies sulfato-réductrices du genre *Desulfosporosinus* sp., ainsi que par des bactéries réductrices du Fe du genre *Acidobacterium* sp. (González-Toril et al., 2011). Le développement continu des méthodes d'identification taxonomique haut débit permet désormais d'entrevoir l'extraordinaire diversité des communautés microbiennes dans les sites miniers (Table 1. et Figure 5.) et repousse ainsi les limites de la microbiologie environnementale.

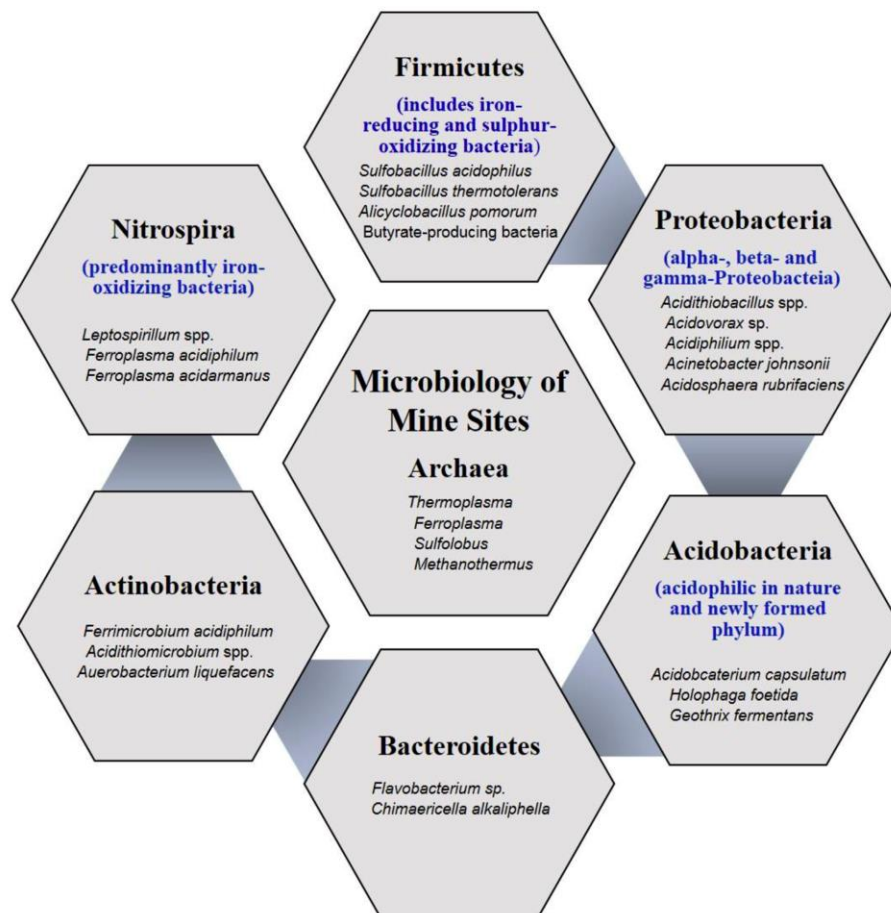


Figure 5. Diversité des communautés microbiennes, en termes de phyla, dans les sites miniers (Thavamani et al., 2017).

1.3.2 Rhizobactéries en contexte de sols miniers

La rhizosphère est un environnement écologique remarquable colonisé par de nombreux micro-organismes. Cette zone d'intense activité microbienne conditionne la croissance des végétaux sur sol pollués, que ce soit de manière directe ou indirecte (modifications des cycles du carbone et des nutriments, de la structure du sol, interactions trophiques et contrôle des pathogènes).

Les végétaux libèrent des exsudats racinaires, mettent ainsi à la disposition de la microflore tellurique des sucres, acides aminés, acides organiques, enzymes, isoflavonoïdes, régulateurs de croissance, polysaccharides du mucilage racinaire. Ces exsudats favorisent le développement des microorganismes, qu'ils soient pathogènes ou bénéfiques (Paul et Clark, 1989). Parmi ces derniers, environ 2 à 5% de rhizobactéries (Goswami et al., 2016) sont qualifiées de Plant Growth Promoting Rhizobacteria (PGPR) (Ahmad et al., 2008 ; Kloepper, 1980) car elles sont promotrices de la croissance des plantes et capables de s'opposer à l'activité d'agents pathogènes (Son et al., 2014). Les PGPR cultivables *in vitro* incluent des taxons bactériens très divers, qui appartiennent majoritairement aux quatre phyla suivants : *Proteobacteria*, *Firmicutes*, *Actinobacteria* et *Bacteroidetes* (Hugenholtz, 2002). Certaines PGPR du genre *Rhizobium*, *Bradyrhizobium* ou

Frankia (Glick, 2014 ; De Meyer et al., 2015), établissent une relation symbiotique avec la plante hôte. Un deuxième groupe de PGPR non symbiotique sont libres à proximité des racines (Vessey, 2003), les genres les plus étudiés sont : *Agrobacterium*, *Arthrobacter*, *Azospirillum*, *Burkholderia*, *Bacillus* et *Pseudomonas* (Goswami et al., 2016). Les PGPR sont des hétérotrophes qui utilisent des composés organiques comme source d'énergie. La densité et l'activité des PGPR évoluent donc tout au long du cycle de développement de la plante. Parmi les nombreux paramètres qui influencent la structure de communauté de PGPR, citons :

- la quantité et la composition des exsudats racinaires,
- le pH qui impose une contrainte physiologique directe sur les rhizobactéries et influence plusieurs facteurs de la rhizosphère comme la disponibilité en nutriments, la solubilisation des cations métalliques, la teneur en MO,
- la compaction du sol, la nature et l'abondance du couvert végétal,
- la présence de contaminants.

Les PGPR jouent un rôle essentiel dans l'implantation et la promotion de la croissance des végétaux, en particulier sur sols anthropogéniques (Figure 6.).

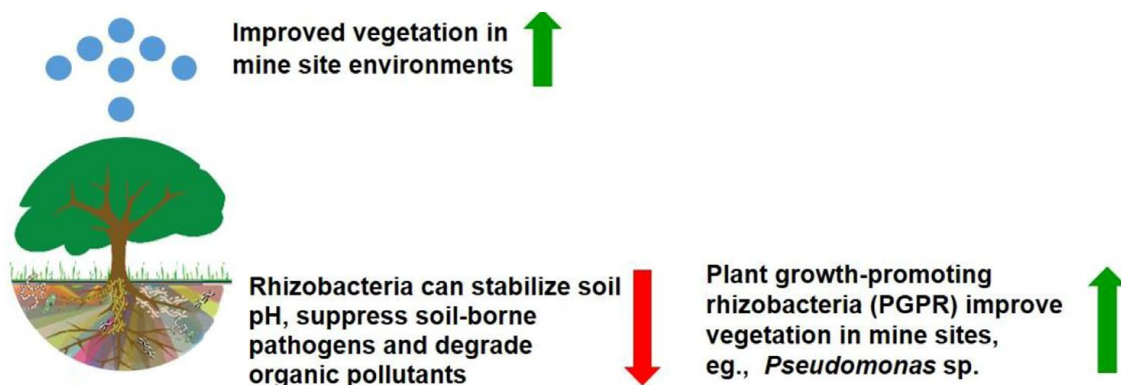


Figure 6. Mécanismes d'action des PGPR favorisant l'implantation d'un couvert végétal dans les sites miniers (Thavamani et al., 2017).

Les différents mécanismes d'action mis en jeu peuvent être actifs simultanément ou séquentiellement à différentes étapes du cycle de développement de la plante (Figure 7.).

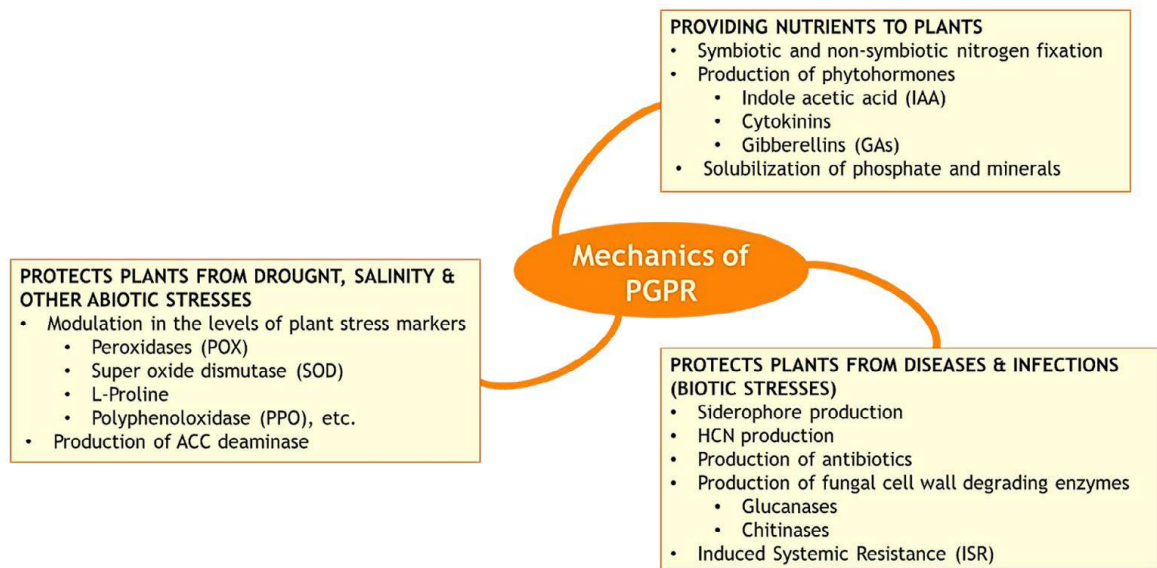


Figure 7. Diagramme schématique représentant les mécanismes d'actions des PGPR (Goswami et al., 2016).

Certains mécanismes ont une action qui affecte directement le métabolisme de la plante en absence d'agents pathogènes racinaires comme :

- l'amélioration de la nutrition (fixation de l'azote atmosphérique, solubilisation du phosphate inorganique, chélation du Fe par les sidérophores) (Figure 8.),
- la production de régulateurs de croissance (auxine, gibbérellines, cytokinines, acide abscissique, éthylène).

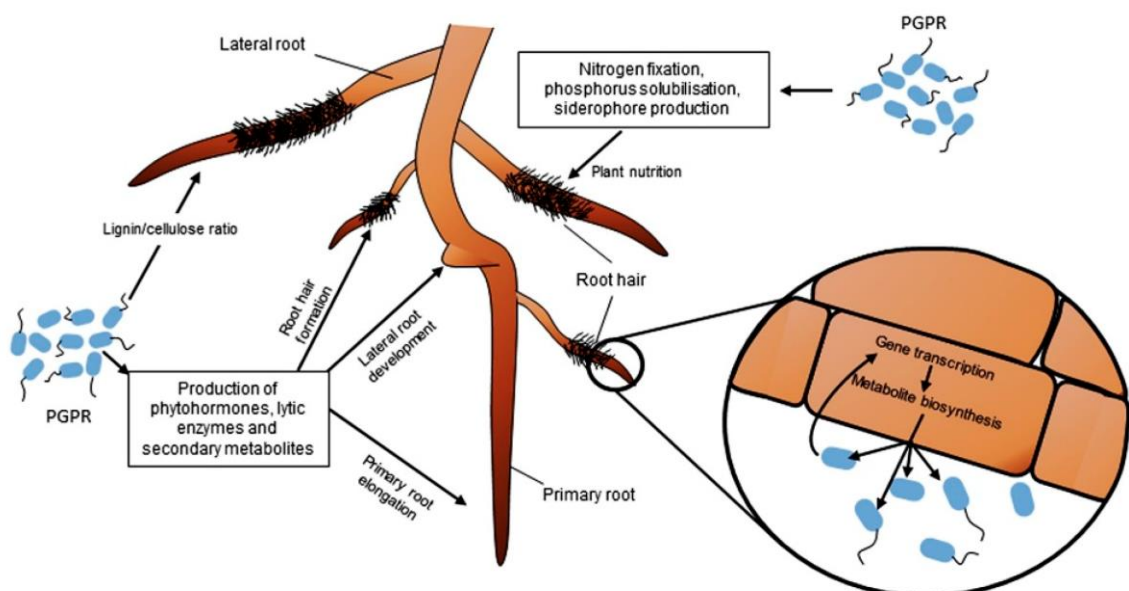


Figure 8. Mode d'action des PGPR pour promouvoir la croissance chez les plantes.

La localisation des zones impliquées dans la fixation de l'azote, la solubilisation du phosphore et la production de sidérophores est indiquée (Vacheron et al., 2013).

D'autres mécanismes mis en jeu stimulent indirectement la croissance des végétaux par :

- leur effet antagoniste sur les agents pathogènes de la microflore (production de cyanure d'hydrogène, de sidérophores, d'antibiotiques, d'enzymes hydrolytiques ou présentant une activité antioxydante ; Gupta et al., 2000),
- la dégradation de métabolites toxiques (effet rhizoremédiateur),
- l'induction de résistance/tolérance systémique (synthèse de protéines Pathogenesis Related et de phytoalexines, accumulation de composés phénoliques, dépôt de callose et lignification des parois cellulaires végétales),
- la diminution du stress éthylène (activité enzymatique de type ACC désaminase).

En contexte de sols pollués, les PGPR contribuent également à l'amélioration de la tolérance de la plante aux métaux en favorisant la solubilisation des ETMs par l'action de tensioactifs, de réactions d'oxydo-réduction et/ou de biométhylation (Ullah et al., 2015). Des souches de *Pseudomonas asplenii* AC, isolées à partir de sols présentant une forte concentration en HAP, augmentent de façon significative la croissance des racines et des pousses de canola sur des sols contaminés par du Cu et du crésote (Reed et al., 2005). Une autre étude (Hrynkiwicz et al., 2009) a démontré que l'utilisation de *Proteobacteria* de la famille des *Sphingomonadaceae* pouvaient promouvoir l'établissement et la croissance de saules (*Salix* sp.) sur un sol anthropogénique composé de cendres volantes. En 2012, une étude du microbiome de la rhizosphère d'un peuplier, *Populus deltoïdes*, un des genres les plus utilisés en phytoremédiation de sites pollués, a permis d'identifier 21 souches différentes de *Pseudomonas* ainsi que la présence de 19 autres espèces bactériennes particulièrement proches des genres *Acidovorax*, *Bradyrhizobium*, *Brevibacillus*, *Caulobacter*, *Chryseobacterium*, *Flavobacterium*, *Herbaspirillum*, *Novosphingobium*, *Pantoea*, *Phyllobacterium*, *Polaromonas*, *Rhizobium*, *Sphingobium* et *Variovorax* (Brown et al., 2012). Plus récemment des PGPR isolées de sols pollués ont été utilisées pour participer à la phytoremédiation de l'As et du Hg (Franchi et al., 2017). Néanmoins, la sélection et l'utilisation de PGPR en bioremédiation demeure un défi scientifique majeur (Richardson, 2001) qui doit tenir compte de l'adaptation des inoculants bactériens à une plante et à un écosystème particulier.

1.4 Analyse des communautés bactériennes

L'analyse de la diversité des communautés bactériennes associées à la rhizosphère se heurte aux difficultés techniques d'analyse, mais surtout à la nature même du sol qui est un environnement des plus complexes et des plus hétérogènes. La figure 9. résume les principales méthodes disponibles (Ma et al., 2016).

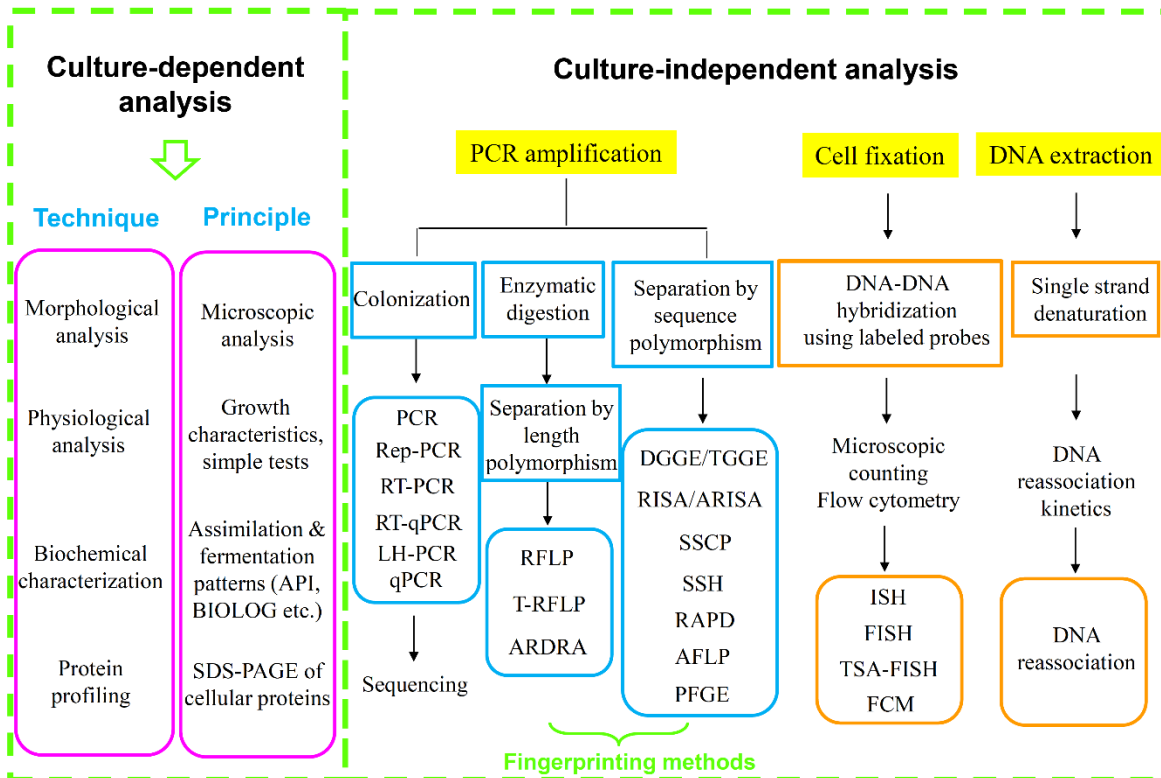


Figure 9. Les principales méthodes d'analyse de la diversité (Ma et al., 2016).

La diversité des bactéries rhizosphériques peut être appréhendée par des techniques microbiologiques traditionnelles ou plus innovantes :

- **Une approche culturale qui consiste à ensemencer un échantillon sur un milieu de culture** afin de permettre la croissance bactérienne et la multiplication clonale. Après dénombrement des bactéries par comptage des colonies sur milieu solide, une première caractérisation taxonomique est réalisée selon l'aspect macroscopique (forme, aspect, consistance et pigmentation des colonies isolées), la coloration de Gram, la caractérisation biochimique et les profils protéiques. Afin de déterminer avec fiabilité l'appartenance à une espèce donnée, l'identification moléculaire est principalement basée sur le séquençage et l'analyse du gène de l'ARNr 16S ou de génomes (Janssen, 2006). Cette approche présente cependant un biais certain car il est admis que seul 0,1 à 10% des bactéries de l'environnement est cultivable dans les conditions du laboratoire (Hugenholtz, 2002 ; Rappé et Giovannoni, 2003).
- **une approche moléculaire qui permet de comparer la diversité microbienne sur la base du génotype.** Ces techniques d'empreintes moléculaires (fingerprinting) basées sur l'amplification par PCR donnent des indications quantitatives, par la mise en œuvre de l'électrophorèse en gradient de gel dénaturant (Denaturing Gradient Gel Electrophoresis) ou du polymorphisme de longueur de fragment de restriction terminaux (Terminal Restriction Fragment Length Polymorphism). Cependant même si ces techniques assez résolutes ont notamment été utilisées pour analyser les variations des communautés microbiennes de sols contaminés par les ETMs (Li et al.,

2006 ; Long et al., 2010), elles ne permettent pas d'étudier de façon qualitative la diversité microbienne à une très large échelle.

- **une approche de génomique environnementale par métabarcoding** (Escobar-Zepeda et al., 2015). Depuis 2005, le développement des technologies de type Next Generation Sequencing (NGS) a révolutionné l'écologie microbienne en permettant d'explorer la diversité microbienne globale d'un environnement, sans *a priori*, par séquençage de l'ADN de tous les génomes présents dans cet environnement. Deux techniques récentes sont couramment appliquées. La technologie Ion Torrent (ThermoFischer Scientific) repose sur des puces semi-conductrices qui détectent le relargage d'ions H⁺ et une variation de pH dans des micro-puits lors de la polymérisation de l'ADN. Chaque micro-puits permet de séquencer un fragment d'ADN d'environ 200 bases. La technologie MiSeq (Illumina) s'appuie sur la détection de fluorescence de nucléotides ou de leurs résidus de polymérisation par un capteur optique Charge-Coupled Device (CCD). Elle permet un séquençage d'environ 300 bases dans les deux sens de lecture diminuant ainsi les risques d'erreurs. Outre la mise en parallèle des réactions de séquençage, ces deux technologies partagent quatre étapes constitutives de la méthode : (i) la préparation des bibliothèques qui contient une étape d'amplification par PCR, (ii) les cycles de réactions de séquençage, (iii) la prise d'image après chacun de ces cycles pour déterminer le nucléotide correspondant, (iv) l'analyse bio-informatique des données. La technologie Illumina MiSeq a notamment permis d'analyser la composition et la diversité des communautés microbiennes de sites perturbés lors d'une contamination par les ETMs (Azarbad et al., 2015 ; Belimov et al., 2005 ; Chao et al., 2016 ; Chen et al., 2016 ; Durand et al., 2017 ; Foulon et al., 2016a, 2016b ; Hong et al., 2015 ; Zhang et al., 2016), des HAPs (Thomas et Cébron, 2016), du pétrole (Hou et al., 2015 ; Yergeau et al., 2015 ; Zheng et al., 2018) ou encore par des effluents de DMA (Wang et al., 2018). Le séquenceur Ion Torrent a également été mis à contribution pour explorer la diversité des communautés microbiennes de sols contaminés par les ETMs (Bell et al., 2015 ; Zappelini et al., 2015).

Malgré leurs hautes résolutions, l'analyse de la diversité bactérienne par les approches moléculaires dépend largement de l'extraction des acides nucléiques du sol, ce qui reste une étape cruciale puisqu'elle est une source majeure de biais (Maarit Niemi et al., 2001). Les méthodes d'extraction et de purification utilisées doivent aussi garantir l'élimination de substances inhibitrices des ADN polymérases (Kirk et al., 2004) telles que les matières humiques et les composés phénoliques associés (Engel et al., 2012). Enfin, les approches NGS permettent désormais de découvrir un nombre considérable d'unités taxonomiques opérationnelles (OTUs), mal identifiées, qui ne peuvent être rattachées qu'à des niveaux de taxonomie élevés (famille, ordre, classes ou phylum) puisque inconnues jusqu'alors.

Chapitre 2. Objectifs

Ce travail doctoral se décline en deux volets :

- Le premier volet est fondamental, il a consisté à mieux appréhender la réponse adaptative de l'ensemble des microorganismes d'un anthroposol contaminé aux ETMs. Dans ce contexte, nous avons développé une approche innovante de séquençage à haut débit par Ion Torrent Personal Genome Machine (PGM) et Illumina MiSeq pour caractériser : (i) la diversité des populations microbiennes inféodées aux racines de peupliers et saules indigènes implantés sur le site INOVYN, (ii) la composition des communautés microbiennes rhizosphériques de bouleau, principale espèce pionnière du teruil de résidus de gypse rouge de Thann. Les résultats de l'étude par métabarcoding nous ont permis d'évaluer les changements potentiels d'un point de vue taxonomique mais aussi fonctionnel des communautés de rhizobactéries dans leurs activités de coopération en relation avec ces trois espèces ligneuses et les caractéristiques des sols contaminés (Zappelini et al., 2015, 2018 in prep ; Álvarez-López et al., 2018 in prep).
- Le second volet est plus appliqué, il a permis d'isoler et de cultiver des isolats microbiens à partir de sols contaminés du site de l'Ochsenfeld afin d'évaluer leurs propriétés de bioremédiation, en améliorant la reprise et la croissance du bouleau sur sols pollués (Zappelini et al., 2018 in prep).

Chapitre 3. Communautés microbiennes du terril de l'Aillon, site INOVYN

3.1 Présentation du terril de l'Aillon, INOVYN en Côte-d'Or

Le premier site expérimental (Latitude : 47° 5' 5.985" Nord, longitude : 5° 19' 44.0322" Est) se situe sur la commune de Saint-Symphorien-sur-Saône dans le département de la Côte-d'Or, en région Bourgogne Franche-Comté (Figure 10.).

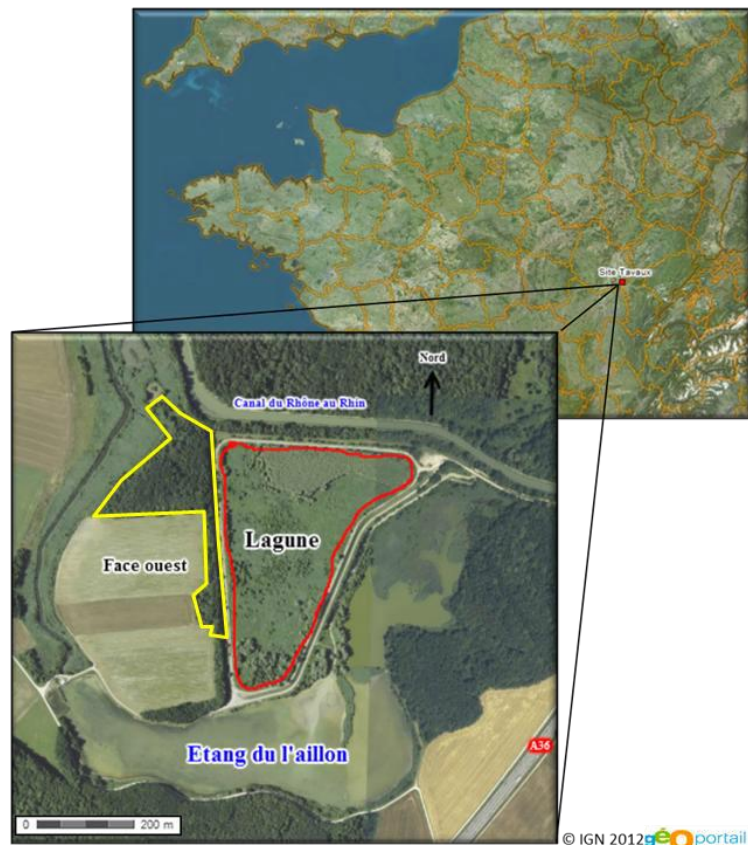


Figure 10. Vue aérienne et localisation friche industrielle INOVYN.

En 1925, l'entreprise Solvay décide d'implanter une usine dans le Jura à Tavaux, en raison de la proximité des matières premières, avec le sel de Poligny et le calcaire de Damparis, de la disponibilité et du prix avantageux de terrains spacieux (terres incultes et marécageuses) et de l'existence de voies de communication comme le canal Rhin-Rhône. Le complexe industriel chimique s'étend sur 200 ha et représente aujourd'hui l'un des plus importants employeurs privés du Jura avec environ 630 collaborateurs.

Le sel, matière première pour produire la soude caustique et des molécules chlorées, est importé sur le site sous forme de saumure qui est ensuite électrolysée pour donner de l'hydroxyde de sodium, du dihydrogène et du dichlore. Le chlore gazeux est ensuite fixé sur d'autres molécules carbonées pour former des polymères comme le chlorométhane ou le 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 PolyChlorure de Vinyle (PVC). Les déchets de production sont réutilisés pour créer d'autres produits comme l'acide chlorhydrique ou l'eau de javel. Jusqu'en 2012, l'électrolyse était réalisée avec une technologie basée sur des cathodes en Hg. L'électrolyse permet la production de dichlore pur et de soude caustique à 50% mais ce processus énergivore nécessite un apport de saumure pure, exempte de contaminants métalliques, pour éviter la production d'H₂ qui peut être explosif. D'autre part, bien que ce métal soit recyclé, sa nature volatile et sa manutention entraîne la production d'effluents contaminés au Hg. Aujourd'hui l'électrolyse se fait sur membrane et n'utilise plus de Hg. Depuis 2012, l'usine a remplacé ses deux salles d'électrolyse Hg par une salle d'électrolyse membrane de capacité équivalente, moins nocive pour l'environnement, l'utilisation de l'électrolyse à Hg sera d'ailleurs définitivement interdite à partir de 2020.

En raison des natures toxiques et inflammables de nombreux produits chimiques, l'usine de Tavaux est classée « Seveso » seuil haut, une directive qui impose aux états membres de l'Union Européenne d'identifier les industries présentant un risque d'accidents majeurs. La directive SEVESO 3 a été votée par l'UE le 4 juillet 2012 ; elle concerne environ 10 000 sites en Europe et concerne 1171 sites en France, dont 656 seuil haut¹. Depuis 2016, l'activité liée à la production de produits chlorés est passée sous l'enseigne du groupe INOVYN (co-entreprise entre INEOS et Solvay).

Les effluents industriels provenant de l'usine sont acheminés via un canal partiellement couvert et déversés dans des bassins de décantation sur la commune de Saint-Symphorien-sur-Saône. Cette étape élimine l'essentiel des polluants des eaux usées provenant de l'usine, cependant le Hg n'est pas éliminé à cette étape. Les sédiments ainsi décantés vont finalement être transférés par des pompes de relevage

¹ <http://archive.wikiwix.com/cache/?url=http%3A%2F%2Fwww.developpement-durable.gouv.fr%2FLa-directive-SEVESO-3-pour-une.html>).

sur le terril de l'Aillon qui est enrichi en Hg, en Ba, en As et en composés organo-chlorés et qui présente un pH alcalin.

Durant près de 40 ans, un curage régulier de ces bassins a conduit à l'accumulation de sédiments sur le terril de 12 ha constitué d'un remblai entouré par des digues de 5 m. Le dépôt de sédiments dragués a été stoppé en 2003. Malgré la présence d'un sol anthropogénique constitué d'un mélange alcalin majoritairement contaminé par du Hg et de l'As, une flore pionnière ligneuse et herbacée abondante s'est développée, principalement des salicacées (saules, peupliers) ainsi que des robiniers et des frênes. Les zones non boisées sont abondamment colonisées par des phragmites et secondairement par des orties.

La zone contrôle est une zone boisée située en dehors du terril, présente antérieurement à la mise en place de celui-ci, comme le révèlent les vues aériennes des années 1960.

Deux études complémentaires ont porté sur ce site :

- l'une basée sur l'utilisation d'une méthode de séquençage haut-débit (Ion Torrent) afin de caractériser les communautés bactériennes et fongiques sur le terril de stockage de déchets industriels de l'entreprise INOVYN (partie 3.2),
- l'autre qui a consisté à retravaillé ce jeu de données et à approfondir quelques aspects de l'écologie des *Pseudomonas* dominant sur le terril de l'Aillon (partie 3.3).

3.2 Diversité et complexité des communautés microbiennes d'un terri de stockage de sédiments issus de l'activité industrielle chlore-alkali

3.2.1 Résumé

*La revégétalisation des dépôts de résidus issus de diverses activités industrielles représente un grand défi pour la restauration écologique. Elle est nécessaire pour limiter l'érosion et la dispersion de poussières de ces sols anthropogéniques, sources potentielles de pollution. Il existe peu de données sur la structure des communautés de microorganismes des résidus revégétalisés après l'arrêt de l'exploitation d'un site industriel. L'étude métabarcoding a consisté à séquencer l'ARNr 16S et des amplicons d'ARN fongiques de l'espaceur transcrit interne (ITS) à partir d'échantillons de sol rhizosphérique de salicacées (peupliers, saules). Cette approche nous a permis de comparer les communautés microbiennes entre un sol rhizosphérique d'un terri, constitué de matériaux issus de la décantation d'effluents industriels, dont le polluant principal est le Hg et un sol rhizosphérique naturel prélevé à proximité immédiate. Après traitement informatique des données, nous avons pu obtenir un total de 162 302 séquences de bactéries et 233 827 séquences de champignons réparties de la façon suivante : 72 373 séquences bactériennes et 122 618 séquences fongiques au niveau du sol rhizosphérique non perturbé et 89 929 séquences bactériennes et 111 209 séquences fongiques pour le sol rhizosphérique contaminé. Les séquences se distribuent dans 45 lignées bactériennes et 9 lignées fongiques ainsi que dans 113 classes bactériennes et 35 classes fongiques. Nous avons observé une nette dominance des Gammaproteobacteria sur notre site d'étude (24% des séquences totales), notamment des genres *Pseudomonas* (72% des séquences), avec la prédominance de quelques taxons fongiques, tels que *Hebeloma* et *Geopora*. Le calcul des indices de Shannon révèle, tant pour les communautés bactériennes que fongiques, que malgré des différences marquées dans les propriétés physico-chimiques du sol, les diversités microbiennes sont proches entre le terri et en dehors du terri (respectivement 64,4% et 62,4% des genres bactérien et fongique). L'analyse des corrélations entre les paramètres du sol et les taxons a confirmé qu'environ 50% des 33 taxons dominants colonisaient les deux types de sol rhizosphérique. Nous avons également démontré que la structure globale des communautés bactérienne et fongique était proche entre le sol rhizosphérique prélevé sur le terri et celui prélevé hors terri. Notre approche souligne ainsi l'importance d'étudier plusieurs composantes de la communauté microbienne et représente un pas en avant dans l'étude de l'écologie microbienne des environnements perturbés. Notre étude fournit également de nouveaux indicateurs, transposables à une multitude de problématiques, permettant le suivi des communautés microbiennes dans le temps, dans l'espace, en fonction de contraintes biotiques ou abiotiques.*

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

3.2.2.1 Authors

Cyril Zappellini, Battle Karimi, Julie Foulon, Laurence Lacercat-Didier, François Maillard, Benoit Valot, Damien Blaudez, David Cazaux, Daniel Gilbert, Etienne Yergeau, Charles Greer, Michel Chalot

3.2.2.2 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.

3.2.2.3 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 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 (OTUs). 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 (Bell et al., 2014; Bonfante and Anca, 2009) 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 (Lorenz et al., 2006; Müller et al., 2001; Rasmussen and Sørensen, 2001; Turpeinen et al., 2004; 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

3.2.2.4 *Materials and methods*

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 *Salicaceae* 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.

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 HNO₃ 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 (N_{tot}, 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).

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).

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., 2008, 2007). 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).

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 μ L) contained 10 μ L of 2X iQ SYBR Green SuperMix (Biorad), 0.4 μ M of each primer, 0.06% (w/v) of bovine serum albumin (BSA), 0.2 μ L of DMSO, 40 ng of T4 gp32 (MP Biomedicals) and 1 μ L 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/ μ L) and the relevant target gene inserts were used to create the standard curves. The presence of PCR inhibitors was evaluated by mixing 1 μ L of environmental DNA with 1 μ L 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.

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.

3.2.2.5 **Results**

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. 11.a). Chemical analysis revealed that pH, calcium carbonate (CaCO₃) 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. 11.a). ICP-AES analyses revealed that the quantities of total Hg and total As (p-value < 0.0001) were significantly increased (Fig. 11.b) in the tailings dump. Other TE were weakly represented in the tailings dump compared to the adjacent soil (Fig. 11.b). A redundancy analysis showed that silt, CaCO₃, pH and exchangeable CaO, and total Hg and As were all positively correlated in the tailings dump (p-value=0.04, adjusted R²=0.88; Supplementary Fig. 1.).

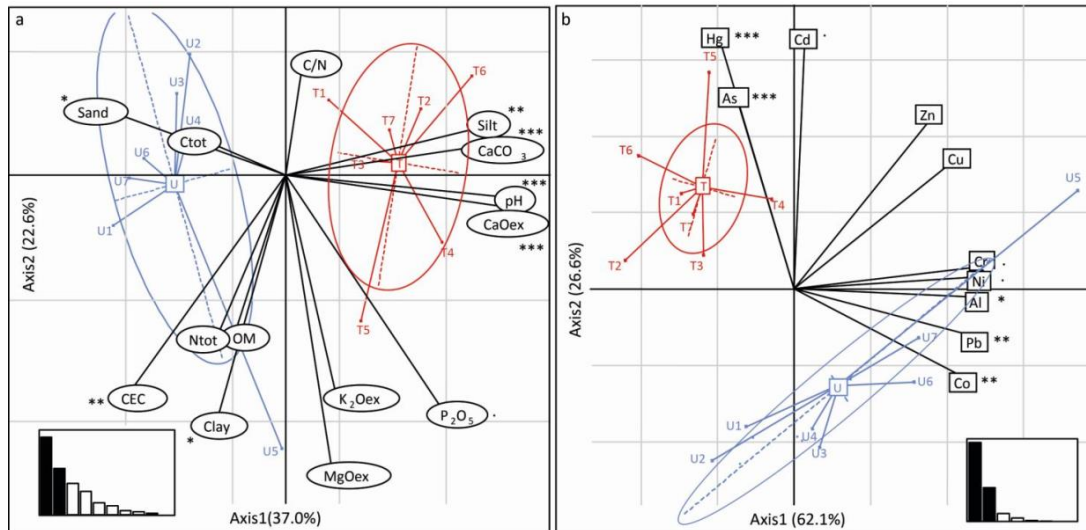
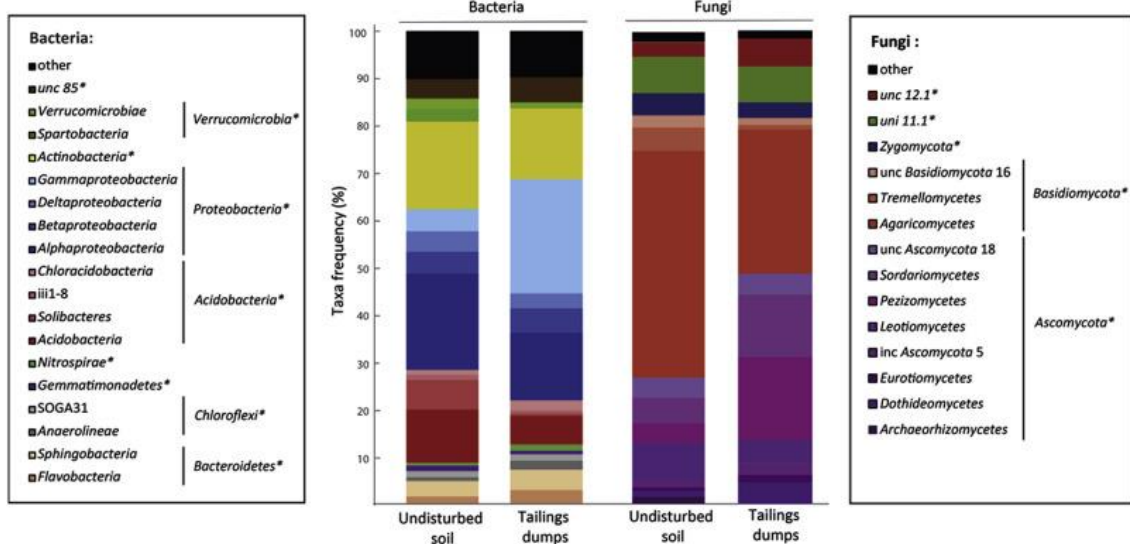


Figure 11. Principal component analysis showing the positions of undisturbed soil (U) and tailings dump (T) samples according to site environmental characteristics.

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.

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. 12.).



based on 16S rRNA and ITS gene sequencing of samples taken from undisturbed soil and an adjacent tailings dump.

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. 13.a), 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 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. 13.b). We found that 599 bacterial and 500 fungal genera, and 386 bacterial genera (i.e., 64.4%, Fig. 13.c) and 312 fungal genera (i.e., 62.4%, Fig. 13.d) 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.

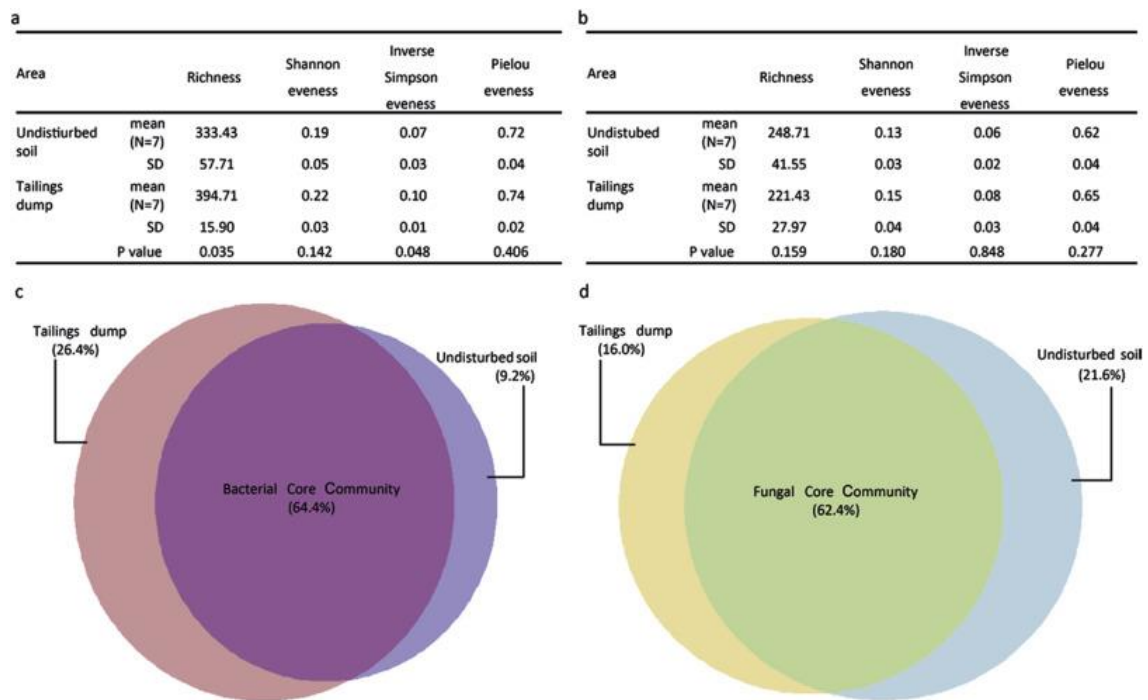


Figure 13. Diversity indices and composition of bacterial and fungal communities.

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.

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. 14.). 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 1,820 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. 14). 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. 13.d); 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. 14.).

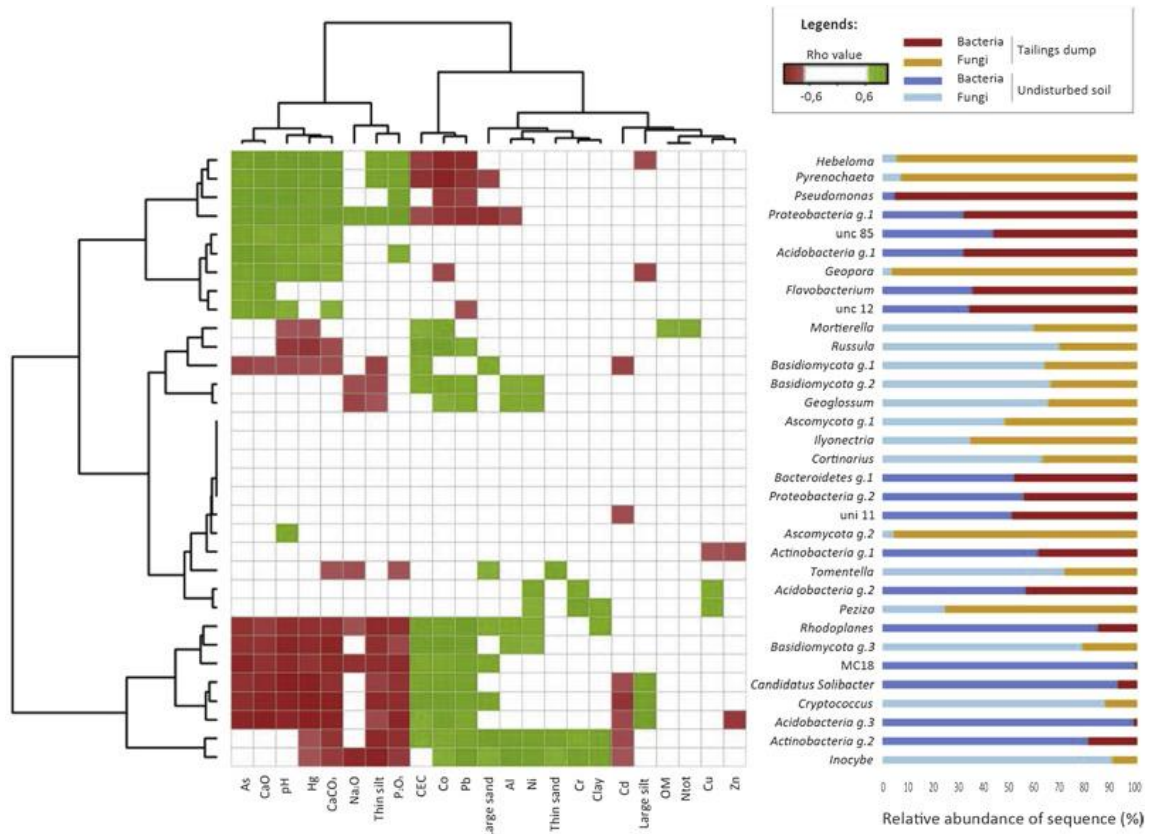


Figure 14. Soil-microbe relationships.

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.

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 2.). However, network visualization was found to be more appreciable at the class level (Fig. 15.) 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. 14.) 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. 15., Table 2., 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.

Table 2. Network indices of microbial communities in undisturbed soil and tailings dump.

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		

Calculation of network indices as defined in Supplementary Table 1, of bacterial, fungal and the merged (bacterial plus fungal) network built at the genus taxonomic level.

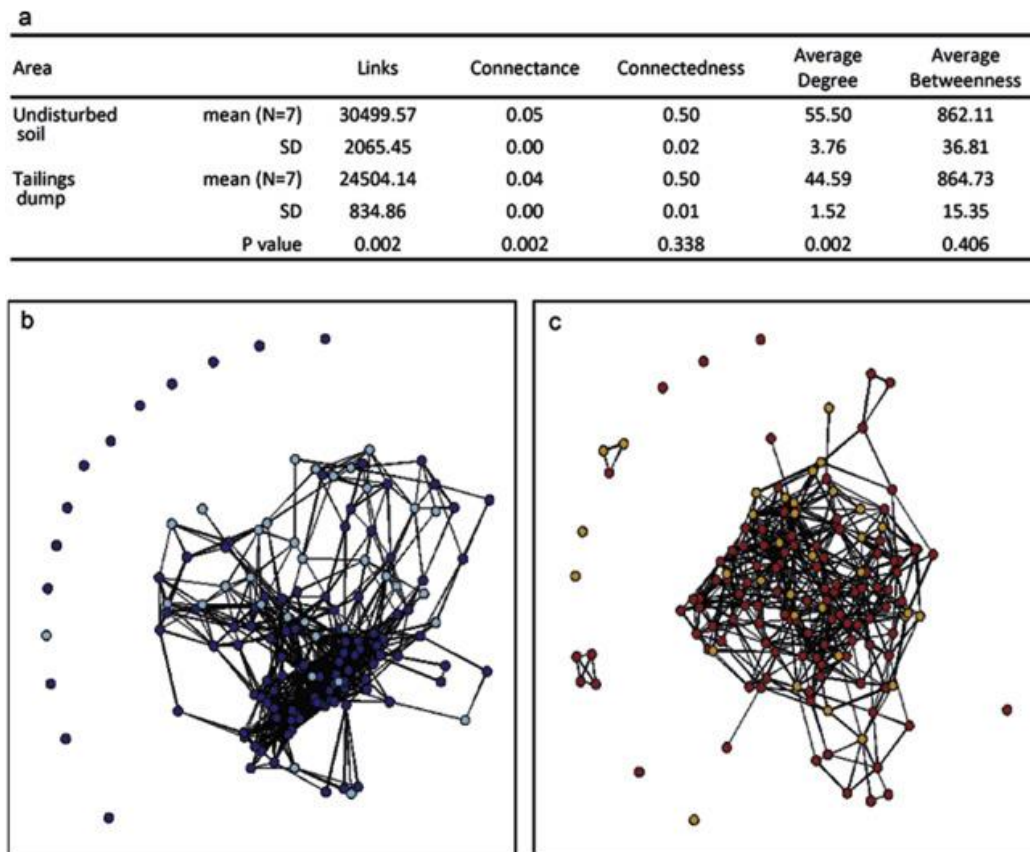


Figure 15. Co-occurrence network analysis of bacterial and fungal communities.

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.

Soil microbe relationships

We calculated the Spearman correlation coefficient for each pairwise combination of parameters (i.e., taxa versus physico-chemical parameters) and the results were displayed as heatmaps (Fig. 14.). Calculations were made on the dominant genera (>2% of 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 CaCO₃ 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. 14.).

3.2.2.6 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 (De Boer et al., 2012; Lauber et al., 2008) 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 (De Boer et al., 2012; Lauber et al., 2008) 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 CaCO₃ 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 (Hryniewicz et al., 2008; Krpata et al., 2008) and have been known to promote the growth of host trees in soils contaminated with metal (Hryniewicz 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 (Bell et al., 2014; Chaparro et al., 2012; Westover et al., 1997). 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).

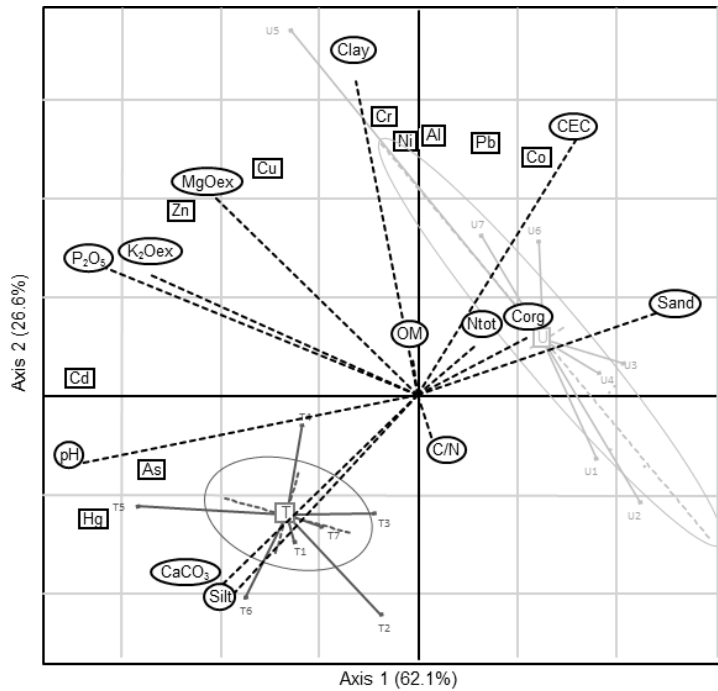
The network dataset generated in this study indicates that detailing the extensive fungal–bacterial relationships that exist within soil provides complementary information on the complexity of the associated community (Rousk et al., 2008). To our knowledge, our work represents the first published field 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. As has been previously highlighted (Shade and Handelsman, 2012), 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 (Neutel et al., 2007; Pimm, 1984; 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.

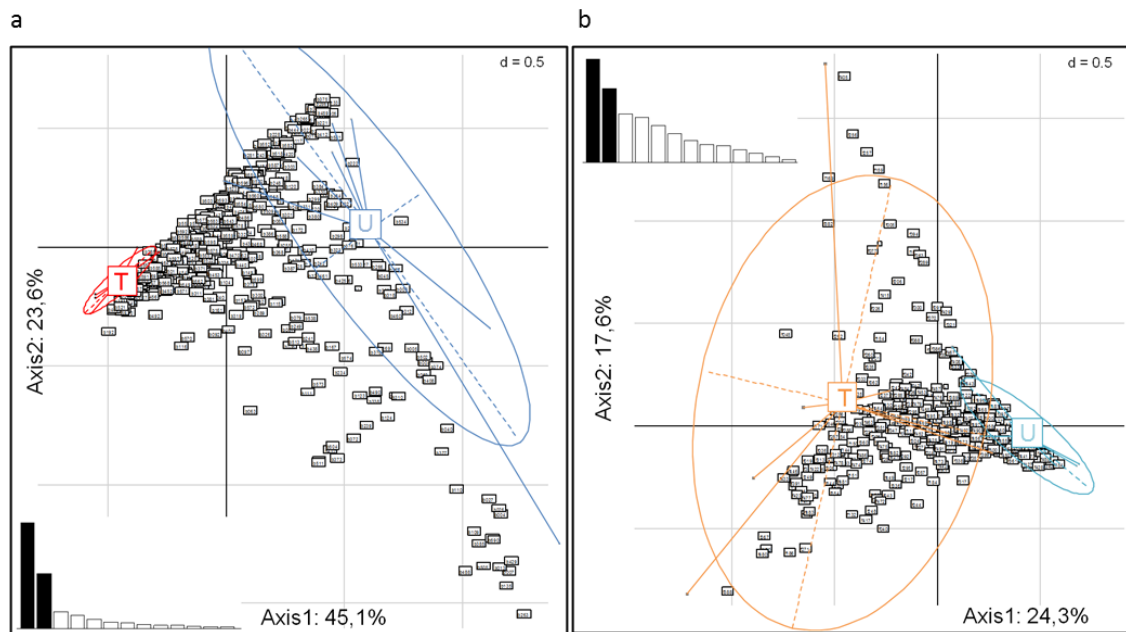
3.2.2.7 Acknowledgements

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3.2.2.8 **Supplementary data**



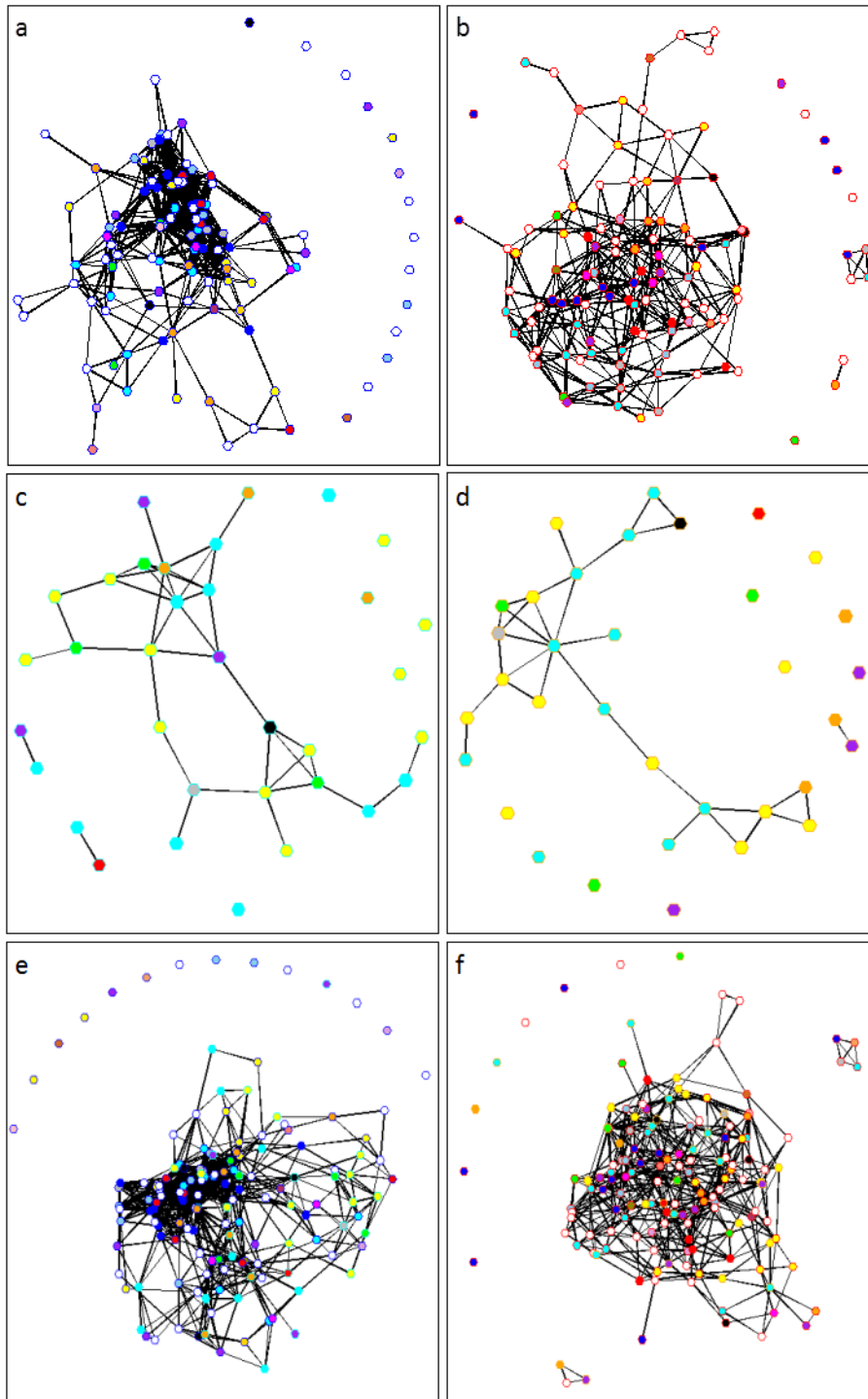
Supplementary Fig 2 Redundant analysis between TE and physico-chemical characteristics of natural, undisturbed soil (U) and tailings dump (T) samples



The dark blue and cyan ellipses show the undisturbed soil data and the dark red and orange ellipses

Supplementary Fig 1. 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).

indicate the tailings dump data on the two first axes of each analysis.



Supplementary Fig 3. 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.

Supplementary Table 1. Calculation methods and definitions of diversity and network indices, community composition and structure.

Type	Calculation	Interpretation
<i>Diversity indices</i>		
Genera richness	<i>No</i>	Number of different genera
Shannon evenness		Index sensitive to rare genera
Inverse Simpson diversity		Index sensitive to dominant genera
Pielou evenness		Index given equitability in the community
<i>Community composition and structure</i>		
Correspondance analysis	Multivariate analysis	Qualitative change in the community composition
Abundance		Appearance or disappearance of dominant genera Quantitative change in the community structure
<i>Pseudomonas</i> / 16S copy		<i>Pseudomonas</i> proportion
<i>Network indices</i>		
Nodes	<i>Nc</i>	Number of connected taxa (nodes) in the ecological network
Unconnected vertices	<i>1-Nc</i>	Number of unconnected taxa in the ecological network
Links	<i>L</i>	Number of edges between taxa within the network
Cluster	<i>G</i>	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	<i>T</i>	Probability that the node C is connected to both nodes B and A if A is connected to B
Connectedness	<i>Cd</i>	Probability at least one chain between any pair of units Quantify the fragmentation within the network
Average degree	<i>aD</i>	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	<i>aB</i>	Average number of shorter chains going through the nodes (including direct and indirect links)
Maximum betweenness	<i>mB</i>	Maximal number of shorter chains going through one node Indicate eventual keystone or central taxa in the network

Supplementary Table 2. Physico-chemical parameters of natural soil and tailings dump.

The mean and standard deviation of all parameters for the natural soil and the tailings

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 ₂ Oex (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

3.3 Dominance et caractérisation des *Pseudomonas* sur un site chlore-alkali

3.3.1 Résumé

*Le procédé chlore-alkali, basé sur la technologie des électrodes à Hg, est une source passée d'émissions de Hg dans l'atmosphère et de contamination connexe des sédiments et des sols. Cette étude a porté sur la composition et l'assemblage des communautés microbiennes de la rhizosphère sur un terril de stockage chlore-alkali, en retravaillant un ensemble de données de métabarcoding environnemental par une analyse de type ROC (caractéristique de fonctionnement du récepteur, de l'anglais « receiver operating characteristic ») et une Analyse en Composantes Principales. Nous avons également isolé les bactéries des arbres indigènes du terril et les avons caractérisées en recherchant diverses activités favorisant la croissance des plantes (PGPA), telles que la production d'auxine, la production de sidérophores et la tolérance au Hg. Le profilage a été réalisé en utilisant des approches MALDI-TOF, BOX-PCR et le séquençage des gènes rpoD. Parmi l'ensemble des OTUs (zone de contrôle et décharges de résidus), 2 OTUs étaient relativement abondantes (OTU # b'423 non classifiée et *Pseudomonas*). Cependant, les courbes ROC indiquent en outre que les deux sites peuvent également être bien discriminés par des OTUs moins représentées, telles que les OTUs *Bosea*, *Methylotenera*, *Agrobacterium*, *Aminobacter* et *Polaromonas* et d'autres OTUs non classifiées qui appartiennent aux *Proteobacteria*. La dominance de *Pseudomonas* a été confirmée par sa biomasse moléculaire relative, qui était 27 et 4 fois plus élevée pour les échantillons de peuplier et de salix récoltés sur les terrils de stockage respectivement, et par une analyse de type Analyse en Composantes Principales Sparse ou « Sparse PCA » en anglais. Compte tenu de leur abondance sur ce site chlore-alkali, nous avons en outre isolé au total 57 bactéries en utilisant un milieu de croissance spécifique des *Pseudomonas*, 11 d'entre eux étant attribués à l'espèce *Pseudomonas putida* par MALDI-TOF. Les 53 isolats de *Pseudomonas* restants ont ensuite été attribués à 13 génogroupes en utilisant des profils BOX-PCR. Dans l'ensemble, nous avons pu récupérer un plus grand nombre et une plus grande diversité de *Pseudomonas* sur le terril de stockage comparativement à la zone de contrôle. Certaines de ces bactéries isolées présentaient une capacité élevée à produire des auxines et des sidérophores et pourraient être des candidats pertinents pour la revégétalisation accélérée des terrils de stockage.*

3.3.2 Dominance and characterization of *Pseudomonas* at a chlor-alkali tailings dumps

3.3.2.1 Authors

Cyril Zappelini, Serge Moulin, Nicolas Capelli, François Maillard, Christophe Guyeux, Didier Hocquet, Michel Chalot

3.3.2.2 Abstract

The chlor-alkali process, based on Hg cell technology, is known to be past sources of Hg emissions to the atmosphere and related contamination of sediment and soils. This study addressed the composition and assembly of the rhizosphere microbial communities at a chlor-alkali site, refining a previous high-throughput metabarcoding set of data using a receiver operating characteristic (ROC) and a sparse-PCA analyses. We further isolated bacteria from the endogenous trees and characterized them by screening for various plant growth promoting activities (PGPAs), such as IAA production, siderophore production, and Hg tolerance. Profiling was performed using MALDI-TOF, BOX-PCR and sequencing of rpoD genes. Among the set of OTUs (control area and tailings dumps), 2 OTUs were relatively abundant (unclassified b'423 and Pseudomonas). However, the ROC curves further indicate that the two sites may also be well discriminated by less represented OTUs, such as Bosea, Methylothermus, Agrobacterium, Aminobacter, and Polaromonas OTUs and additional unclassified OTUs that belong to the Proteobacteria. The dominance of Pseudomonas was further confirmed by its relative molecular biomass, which was 27- and 4- fold higher for poplar and salix tree samples collected on the tailings dumps, respectively and by a Sparse PCA analysis. Given their abundance at this chlor-alkali site, we further isolated a total of 57 bacteria using a specific Pseudomonas growth medium, 11 of them being assigned to the Pseudomonas putida species by MALDI-TOF. The 53 remaining Pseudomonas isolates were further assigned to 13 genogroups using BOX-PCR profiles. Overall, we were able to recover a higher number and higher diversity of Pseudomonas in the tailings dump as compared with the control area. Some of these isolated bacteria showed elevated capacity to produce IAA and siderophores and might be relevant candidates for the accelerated revegetation of the dumps.

3.3.2.3 Introduction

Metal(loid) contamination due to anthropogenic activities, such as extractive activities, poses a threat to the surrounding environment and human health. In particular, tailings that are artificial soil-forming substrates that have not been created by the natural processes of soil formation and weathering (Cross et al., 2017), are susceptible to wind and water erosion, facilitating contaminant transport (Honeker et al., 2017). Mercury (Hg) is a contaminant of concern because of its potential impacts on human and ecological health. It is rather persistent in the environment and can be transported to large distances in the atmosphere. A recent paper summarized the natural and anthropogenic sources that have contributed to the increase of Hg concentration in soil and reviewed major remediation techniques and their applications

to control soil Hg contamination (Xu et al., 2015). The chlor-alkali process, based on Hg cell technology, is known to be a past source of Hg emissions to the atmosphere and related contamination of sediment and soils (Biester et al., 2002; Esbrí et al., 2015). Due to the process characteristics, Hg is released through air emissions, waste-water discharges, and solid waste disposal (OSPAR Commission, 2013). Vegetation is often promoted on such tailings, because it can effectively control the erosion of tailings fines by wind and water and can improve the landscape of these wastelands (Mendez and Maier, 2008).

There is an abundant literature on the characterization of microbial communities from forest or agricultural soils contaminated by trace element (TE) or polycyclic aromatic hydrocarbons (PAH) (Foulon et al., 2016a, 2016b; Tardy et al., 2015; Yergeau et al., 2015). Microbial populations in tailings of the mining industry have also attracted considerable interest in the past decades, especially in acid mine drainage dumps (Bruneel et al., 2017; Gupta et al., 2017; Méndez-García et al., 2015; Mesa et al., 2017a). However in other environments with different soil characteristics (Hg contamination), there are considerable fewer studies. Soil microbes are more than just indicators of ecological function, they are increasingly recognized as facilitators of the belowground metabolic recovery required for subsequent aboveground restoration. Despite acknowledgement of the link between above and belowground communities, there is still a lack of mechanistic understanding on how microbial communities facilitate restoration of highly degraded environments such as post-mining landscapes. However, a recent study reported that application of soil inocula can promote ecosystem restoration, whereas origins of soil inocula play a major role in the establishment of plant communities (Wubs et al., 2016). Therefore, efforts to characterize endogenous microbial communities from these soils are urgently needed, to achieve an optimal plant recovery.

We recently reported on 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. We concluded 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. However, 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*. Redundancy analysis (RDA), principal component analysis (PCA), or multi-dimensional scaling (MDS) are widely used in environmental metabarcoding analysis to reveal the relationship between soil and vegetation characteristics with microbial community structure, and to test the significance of each with a permutation test. In a previous paper, we used a permutation test to establish that the bacterial and fungal communities residing within

a tailings dump *versus* undisturbed soil samples had significantly different compositions (Zappelini et al., 2015). In another recent paper, we used a 2-dimensional non-metric multi-dimensional scaling (NMDS) analysis combined with a permutation test to demonstrate that the site characteristics explained most of the variance in the composition of a fungal community (Foulon et al., 2016a, 2016b). Based on PCA analysis, Hong et al. (2015) showed that dominant genera changed in mine sites with the degree of pollution. Lallias et al. (2015) used MDS analysis coupled with a permutation test to demonstrate that microbial taxa are likely to respond to different environmental drivers and in particular, the hydrodynamics, the salinity range, and the granulometry according to varied life-history characteristics. Based on a PCA analysis and permutation test, Yergeau et al. (2015) highlighted key factors that should be considered when engineering the plant rhizosphere microbiome, including the presence and abundance of keystone species, the diversity and evenness of the initial inoculum, the ecological differences between fungi and bacteria, the environmental conditions, and the plant growth stage from which the inoculum originates.

Detection of the most site-dependent OTUs is mainly done in the literature by the comparison of relative abundances. The aim of this work is to (i) implement a new bioinformatics tool, namely the ROC curve analysis, to easily achieve OTU detection and unambiguously demonstrate OTU dominance (ii) to isolate indigenous bacteria from the endogenous trees on the basis of morphological, and physiological characteristics as well as by rpoD gene sequence analysis, (iii) to screen bacteria for various plant growth promoting activities (PGPAs), such as IAA production, siderophore production, and Hg tolerance.

3.3.2.4 **Material and methods**

Study site Location

The polluted site was a tailings dump located at Saint Symphorien-sur-Saône in the Bourgogne Franche-Comté region in France (lat. 47° 5' 5.985" N – long. 5° 19' 44.0322" E), which has a total surface of 12 ha. The region is characterized by an annual average temperature of 11°C and 75% moisture. From the 1950's to 2003, this site was exploited as a storage area for sediments from the adjacent sedimentation basin. These sediments originated from effluents produced during electrolytic processes based on an Hg cell chlor-alkali process used to produce chlorine until 2012. The tailings dump was confined by 5-m high dikes to preserve the surrounding environment and is composed of a multi-contaminated calcareous and alkaline anthropogenic soil. The control site was located at Montbéliard-France (lat. 47° 29' 42.9" N – long. 6° 48' 9.684" E). Soil characteristics are those described in (Zappelini et al., 2015).

Sampling

The samples were collected under poplar trees on October, 2015 from the

tailings dump and an adjacent undisturbed forest that was not affected by anthropogenic activity and was therefore used as the undisturbed soil control in this study. These two zones have been fully described in Zappelini et al. (2015). Both areas experienced similar climatic conditions. Rhizosphere soil was sampled from 5 poplar trees in each area (tailings dumps and undisturbed soil). 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 stored at 4°C before microbe isolation.

Microbial isolation and characterization

Bacterial isolation from soil samples

Five g of soil was homogenized in 45 ml of 10 mM MgSO₄, stirred at 100 rpm for 15 min at room temperature. One ml was used to perform serial dilutions in 10-fold series, and 100 µL were plated onto NAA1.100-agar medium (Aagot et al., 2001) in duplicate dilution and kept for 7 days at 25°C.

DNA extraction, BOX-PCR and rpoD gene amplification

For DNA preparation, the isolates were grown in the 284 liquid medium at 25°C, 7 days at 250 rpm (Gallenkamp Orbital Incubator). After centrifugation, DNA was extracted from the pellets using an EZNA bacterial DNA isolation kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) according to the manufacturer's instructions. The BOX-PCR fingerprinting method was used to group genotypic profiles at a similarity level of 90 % as previously described (Cristina Becerra-Castro et al., 2011). One strain was selected for each BOX-group and R rDNA genes were amplified in a reaction volume of 25 µL, containing 12.5 µL of Ready mix PCR Master mix (Thermo Fisher Scientific, Carlsbad, California, USA), 2 µM of BOX A1R primer (5'- CTACGGCAAGGCGACGCTGACG-3', Eurofins Genomics, Paris, France), and 5 µL of bacterial DNA. DNA amplification was carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) under the following conditions: 1 cycle of 5 min at 95°C, 40 cycles of 25 sec at 95°C, 35 sec at 55°C and 1.05 min at 72°C with an additional 5-min cycle at 72°C. The amplicons obtained were separated by electrophoresis, on an agarose gel 1.8% at 45 V 3 h. Gel images were analyzed with the software Gel.J (Heras et al., 2015) by using Pearson correlation coefficient and UPGMA clustering algorithm.

Bacterial species identification and Pseudomonas phylogeny

Bacterial species were identified by MALDI-TOF MS with a log value ≥ 2 according to the manufacturer's recommendations (Bruker Daltonik GmbH, Bremen, Germany). The *rpoD* gene was PCR amplified from each DNA extract according to Mulet et al. (2009) and purified PCR products were further sequenced. The *rpoD* rRNA PCR products were sequenced by Sanger sequencing (Genewiz Beckman Coulter Genomics, United

Kingdom). DNA sequences were edited with BioEdit software v7.2.6. (Hall, 1999) screened against the GenBank database using the BLASTn tool at the NCBI website (<http://www.ncbi.nlm.nih.gov/>) and then aligned with reference sequences of *Pseudomonas* using Clustal W (Thompson et al., 1994) as implemented in Bioedit. Neighbour Joining (NJ) tree reconstructions were produced using MEGA 5 (Tamura et al., 2011). The most appropriate model of *rpoD* rRNA sequence evolution was done using the K2P model (Kimura, 1980).

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), 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 μ L) contained 10 μ L of 2X iQ SYBR Green SuperMix (Biorad), 0.4 μ M of each primer, 0.06% (w/v) of bovine serum albumin (BSA), 0.2 μ L of DMSO, 40 ng of T4 gp32 (MP Biomedicals) and 1 μ L 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, and Pse435F/Pse686R 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/ μ L) and the relevant target gene inserts were used to create the standard curves. The presence of PCR inhibitors was evaluated by mixing 1 μ L of environmental DNA with 1 μ L 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.

Functional traits

Isolates were screened for their ability to produce siderophores and indoleacetic acid (IAA) production. All those analysis were performed for one representative of each BOX group. The siderophore production of each bacterial strain was determined using chrome azurol sulfonate (CAS) agar medium (Alexander and Zuberer, 1991; Schwyn and Neilands, 1987). After 5 days of incubation on CAS medium at 30°C, a red-orange halo around colonies indicated the production of siderophores. For each strain, the ratio between the diameter of the halo and the diameter of colony was recorded. IAA

production was evaluated in liquid medium (5.0 g glucose, 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 2.0 g K_2HPO_4 , 0.5 g CaCO_3 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g NaCl, 0.1 g yeast extract adjusted to pH 7; modified from Sheng et al. (2008) supplemented with 0.5 mg/mL of tryptophan). After 5 days incubation at 28°C, cultures were centrifuged and the supernatant was incubated with Salkowski reagent for 25 min. The production of IAA was evaluated by measuring the OD at 540 nm. Hg resistance was tested using LB agar medium supplemented with increasing concentrations of HgCl_2 (0, 0.075 and 0.184 mM) and further incubated at 28°C for 7 days.

Statistical analyses

Normality was tested with the Shapiro-Wilk (all data sets), and homoscedasticity was tested with Bartlett's (abiotic dataset) and Levene (biomass dataset, PGP and metal resistance traits) tests using R. Data that were normally distributed were analyzed using a parametric test (Student's t-test) in R. Data that were not normally distributed were analyzed using a non-parametric Mann-Whitney-Wilcoxon (soil data) or Kruskal-Wallis (inoculation) tests using "R". The Principal Component Analysis (PCA) was performed using the R ade4 package. Data expressed as % (PGP and metal resistance traits) were analyzed using a Chi-2 test in R.

Sparse PCA

The sparse PCA (sPCA), (Zou et al., 2004) is a variant of the well-known principal component analysis (PCA) method. As in the PCA case, the sparse PCA allows to project a cloud of points from a high-dimensional space to a low dimensional one. Most of the time, points are projected in a 2-dimension space, in order to reach readability. Compared to a normal PCA, the sparse PCA introduces a penalization of vector coefficients that induces a space of small dimension. More precisely, to compute a PCA is to solve:

$$(U^*, V^*) = \arg \min_{U, V} \frac{1}{2} \|X - UV\|_2^2 + \alpha \|V\|_1$$

$$\text{subject to } \|U_k\|_2 = 1 \text{ for all } 0 \leq k < n_{\text{components}}$$

In the minimization problem formulated above, the first rows of U contain the vectors that will generate the space of small dimension while the first columns of V are the coordinates of the points projected in the space of small dimension. The main interest of a sparse PCA, when compared with a usual principal component analysis, is its robustness. A slight modification of the data will not cause a too important modification of the final representation. Moreover, the vectors that generate the small space contain more null components than in the normal PCA, which generally simplifies the interpretation of what these vectors mean in relation to the starting space. We use the «SparsePCA» function from module «sklearn.decomposition» (Pedregosa et al.,

2012) with a penalization of $\alpha=0.5^2$. Note that various formulations of the sPCA can be found in the literature. The one that has been used in sklearn, which implements the above optimization problem, can be found in (Mairal et al., 2009).

Gaussian mixture model analysis

OTUs were separated in clusters using the “GMM” function of the “sklearn.mixture” package (Pedregosa et al., 2012)³, that applies the Gaussian mixture model (GMM) to our set of points. This GMM algorithm (Day, 1969) is an unsupervised classification method. That is to say, GMM allows to separate a set of elements in clusters without any *a priori* hypothesis on the clusters meaning. The mathematical assumption of a GMM is that the point cloud follows a distribution of the form:

$$\sum_{i=1}^k \delta_i N(\mu_i, \sigma_i)$$

ROC curve analysis

The ROC curve analysis was developed in the early 1970s (Egan, 1975) and is currently used in medical statistics to determine the best threshold for a diagnostic test (Zou, 2002). For instance, ROC curves have been used to assess the value of diagnostic tests by providing a standard measure of the ability of a test to correctly classify subjects (Morrison et al., 2003). A ROC curve is a graphical representation that allows illustration of the performance of a binary classifier. This graph consists of a representation of the false positive rate in the abscissa and the true positive rate in the ordinate. The curve joins this pair of rates for each possible threshold. In addition to this ability to search the best threshold, ROC curves permit the assignment of a numerical value to the discriminatory power of the binary classifier due to their area under the curve (AUC). The more relevant the classifier is, the larger its ROC AUC is, with a value up to 1 for a perfect classifier. For a larger and more accurate explanation about ROC curve analysis, see Fawcett (2006).

3.3.2.5 Results and discussion

Dominance of Pseudomonas at the tailings dumps

We applied the ROC analysis described previously to our previous metabarcoding dataset (Zappellini et al., 2015) by defining two different groups of variables: (1) bacteria operational taxonomic units (OTUs), and (2) soil physico-chemical properties obtained from the two experimental locations (the tailings dump and undisturbed soils). ROC AUCs were computed for each group (*i.e.*, bacterial OTUs and soil physico-chemical

²<http://scikit-learn.org/stable/modules/generated/sklearn.decomposition.PCA.html>

³<http://scikit-learn.org/stable/modules/generated/sklearn.mixture.GaussianMixture.html>

variables), and the groups of variables were sorted by decreasing AUCs. Supplementary Table 4. Shows the results for all the soil physico-chemical variables, whereas Table 1 shows the top 30 most discriminating bacterial OTUs, respectively. The complete lists of sorted variables for bacterial OTUs are available as supplementary data (Supplementary Table 3.). In these tables, Delta_norm is used as a tiebreaker between the variables that have the same ROC AUC. This sorting method allowed us to rapidly detect the variables that best discriminate the experimental site.

For the physico-chemical variables, pH, calcium carbonate (CaCO_3) concentration and exchangeable calcium oxide (CaOex), Hg and As were found to be the most discriminating soil parameters (Supplementary Table 3.3-2.), which is in agreement with our previous study (Zappelini et al., 2015). However, ROC curves further indicated that Ca, Na, and Sr were the three additional most relevant parameters to discriminate the two sites with AUC values of 1, which were similar to the AUC for pH. For the bacterial community, 22 OTUs perfectly discriminated the two sites with an AUC of 1 (Supplementary Table 3.). Among these set of OTUs, 2 were relatively abundant (*unclassified b'423* and *Pseudomonas*). However, the ROC curves further indicate that the two sites may also be well discriminated by less represented OTUs, such as *Bosea*, *Methylothera*, *Agrobacterium*, *Aminobacter*, and *Polaromonas* OTUs and additional unclassified OTUs that belong to the *Proteobacteria*. Additional OTUs from other phyla (*Tsukamurella*, *Iamia*, *Aeromicrobium*, and *Marmoricola*) also showed AUC values of 1 and were also less represented. *Bosea* OTUs, corresponding to the most discriminating bacteria, was represented in each tree from the tailings dump by at least 3 sequences, whereas trees from the undisturbed soil owned, at the most, 1 sequence. If we consider the 30 most discriminating bacterial OTUs, all of them were more abundant on the tailings dump (Supplementary Table 3.). If we consider 74 OTUs that lead to a correct classification of 15 or 16 trees, only 11 of them were related to the undisturbed soil (Supplementary Table 3.). This ROC analysis was applied to a real metabarcoding dataset related to a Hg-enriched tailings dump. We also performed an extensive comparison with other available methods and with our previous analysis, and found that the proposed analytical tool demonstrates better performance for discriminating and highlighting keystone species in environmental analysis, independently of their abundance (not shown).

The dominance of *Pseudomonas* was further confirmed by measuring its relative molecular biomass under the collected poplar and willow trees (Fig. 16.). The ratio *Pseudomonas* 16S rDNA (as estimated by the 16S rDNA copy number quantified with the primer set Pse435F/Pse686R) / total bacteria 16S rDNA (as estimated by the 16S rDNA copy number quantified with the primer set 968F/1401R) was 27- and 4- fold higher for poplar and *Salix* tree samples collected on the tailings dumps; respectively.

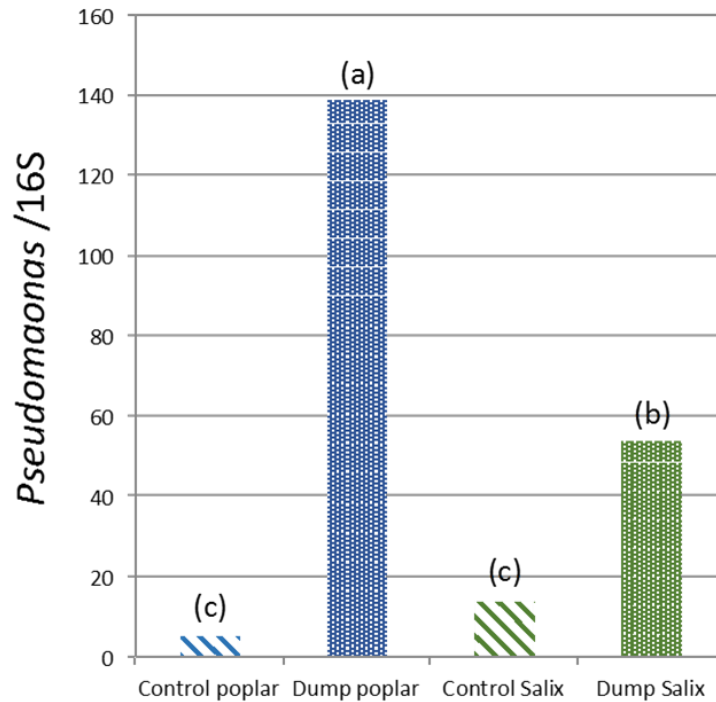


Figure 16. Box diagram showing the relative molecular biomass of *Pseudomonas* as a function of tree species and sampling area.

The ratio *Pseudomonas* 16S rDNA / total bacteria 16S was estimated by quantifying the 16S rDNA copy number using qPCR with the primer set Pse435F/Pse686R for *Pseudomonas* and the primer set 968F/1401R for total bacterial 16S rDNA.

We also applied a Sparse PCA analysis (Fig. 17.) that demonstrated the overall dominance of *Pseudomonas* OTUs over the other bacteria from the tailings dumps. The number of sparse atoms to extract, in order to find the set of sparse components that can optimally reconstruct the data, has been set to 2, as depicted in the plane of Fig. 17. In this sparse representation of OTUs projected in a space of small dimension, an obvious separation of the cloud of points appeared, in which the 2 sparse components extracted from the *Pseudomonas* OTU data was far more larger than the other ones. This separation has been post-validated using a Gaussian Mixture Model (GMM) approach coupled with a Bayesian Information Criterion (BIC) to find the optimal number of clusters. GMM has been applied to the sparse components provided by the sPCA, in which an optimal number of 7 clusters has been designated by the BIC criterion. All clusters are represented in Figure 17. using different colors and, as can be seen, the *Pseudomonas* OTU is isolated again in a single cluster.

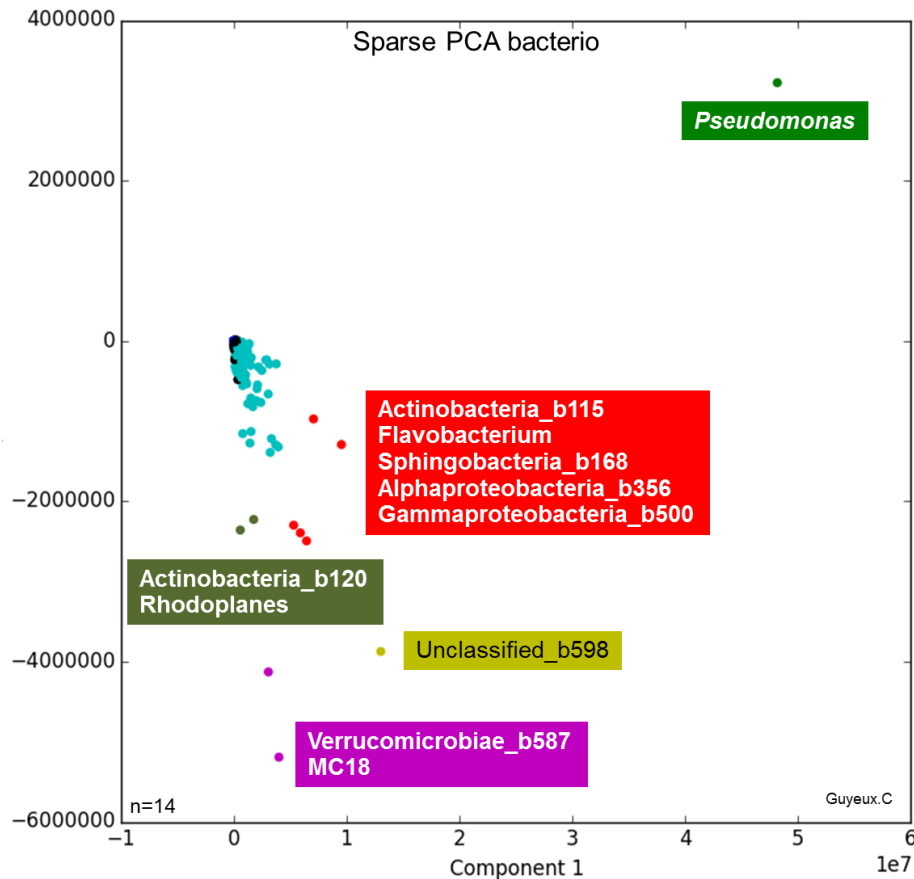


Figure 17. Sparse-PCA showing the dominance of *Pseudomonas* OTU at the tailings dumps.

Taxonomic affiliation

We isolated a total of 57 bacteria using the specific *Pseudomonas* growth medium. Only 11 of these 57 isolates could be assigned to a *Pseudomonas* species (*P. putida*) by MALDI-TOF, 10 from the dump and 1 from the control areas. The development of MALDI-TOF MS devices has revolutionized the routine identification of microorganisms in clinical microbiology laboratories by introducing an easy, rapid, high throughput, low-cost, and efficient identification technique. MALDI-TOF MS has been used successfully for microbial typing and identification at the subspecies level, demonstrating that this technology is a potential efficient tool for epidemiological studies and for taxonomical classification (Croxatto et al., 2012). However, in our study most of the other isolates could not be assigned to *Pseudomonas* species. This is probably due to the fact that the database used mostly contains *Pseudomonas* collected from the environment of hospital, personnel, and patients. Four of the 57 were assigned to other bacterial genera (1 *Serratia*, 1 *Paenibacillus* and 2 *Buttiauxella*) and were therefore discarded from further analysis. The 53 remaining *Pseudomonas* isolates were further assigned to taxonomic group using BOX-PCR profiles (Fig. 18.).

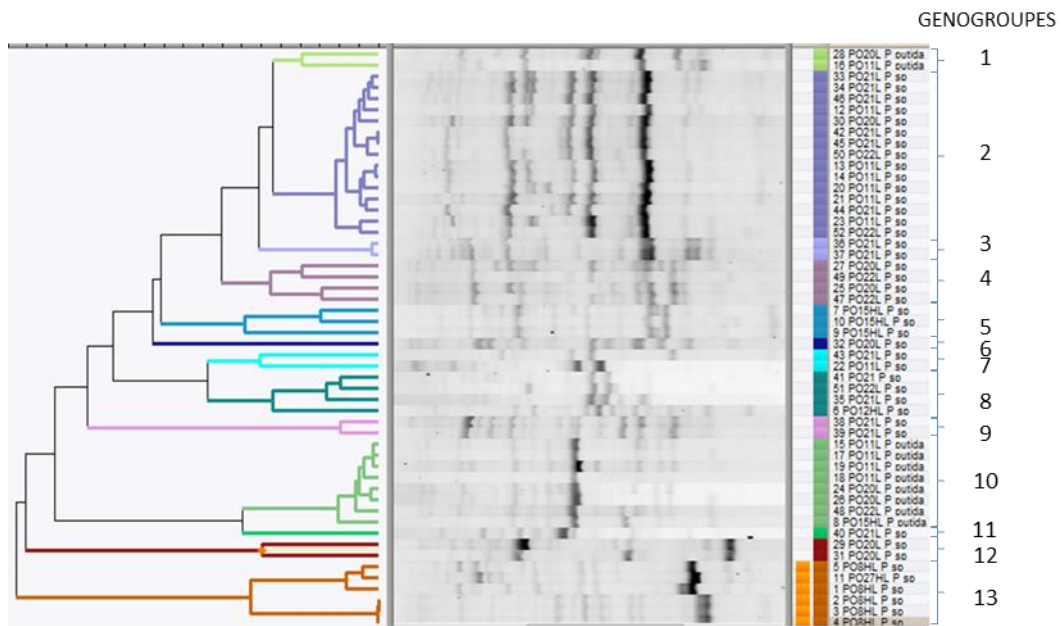


Figure 18. Genotyping of the *Pseudomonas* isolates using the BOX-PCR.

The BOX-PCR profiling allowed us to differentiate 13 genogroups. The *rpoD* gene of one representative of each group was further sequenced and included into a wider phylogenetic tree (Fig. 19.). In a previous study (Ghyselinck et al., 2013), the *rpoD* gene was shown to have a high phylogenetic content and the highest taxonomic resolution amongst other genes investigated, and it also had a gene phylogeny that related well with that of the *rpoD* gene. Using the NAA1/100 media, we were able to recover a higher number and higher diversity of *Pseudomonas* in the tailings dump as compared with the control area, which agreed with the difference with relative molecular biomass (Fig. 16.).

Soil, in particular, has an astounding number and diversity of microbes, and constitutes a fertile, easily accessible, and generally safe resource for the isolation of bacteria. *Pseudomonas* species are ubiquitous in soil and although some have long been recognized as plant pathogens (Höfte and De Vos, 2007), others are emerging as plant-associated growth promoters with potential roles in biocontrol (Santoyo et al., 2012; Zachow et al., 2013). The genus *Pseudomonas* described in 1894 is one of the most diverse and ubiquitous bacterial genera, which encompass species isolated worldwide. In the last years more than 70 new species have been described, which were isolated from different environments, including soil, water, sediments, air, animals, plants, fungi, algae, compost, human and animal related sources. Some of these species have been isolated in extreme environments, such as Antarctica or Atacama desert, and from contaminated water or soil (Peix et al., 2018). Atypical carbon and nitrogen sources in a minimal enrichment medium exploit the degradative capacity of *Pseudomonas* species and increase their abundance for facile isolation, as demonstrated by Mulet et al. (2011). Although culture-independent methods provide a wider outlook on microbial diversity and ecology, cultivation of new isolates is still important and routinely carried out in most laboratories of microbiology. This is particularly true for environmental

microorganisms, which represent an immense resource of new natural products for food and feed additives, drug development, and other industrial products (Stafsnes et al., 2013).

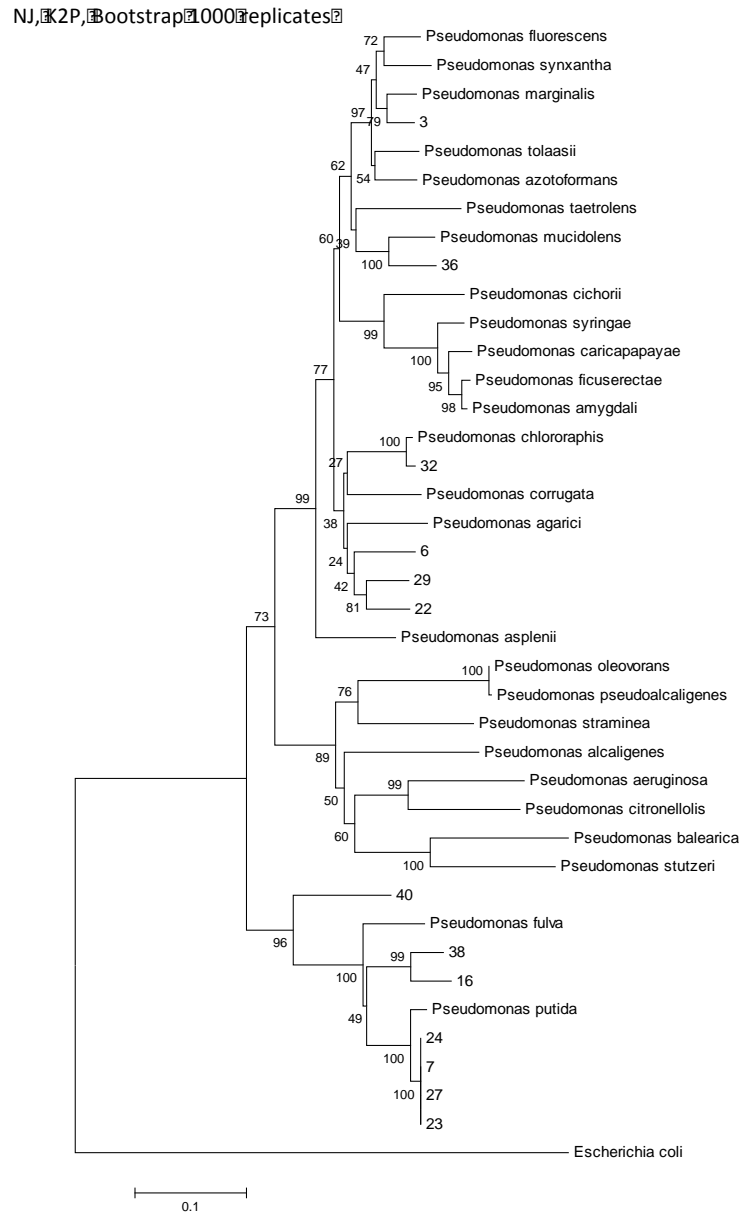


Figure 19. Phylogenetic tree of the 13 representative *Pseudomonas* genogroups, based on the *rpoD* gene.

The phylogenetic tree was constructed using a K2P Neighbor-joining model based on aligned DNA sequences with 1,000 iterations.

Overall, there was a poor concordance between the phyloproteomic MALDI-based results reported in this study with those obtained by the *rpoD* gene analysis. Concordance between the two methods was 100% at the genus level, while at the species level it was 13%. In our case, attribution concordance for *Pseudomonas* species was rather low (19%), compared with other studies regarding environmental bacteria,

confirming the problematic taxonomy of this genus, whereas that of strains from other genera was quite high in other studies (> 60%) (Timperio et al., 2017).

Functional traits

Plant growth promoting properties of *Pseudomonas* collection, comprising the 13 BOX-PCR groups, were tested *in vitro* for plant growth promotion properties such as phytohormone and siderophore production (Table 3.). Siderophore production varied between 1.3 and 5.0 (ratio), whereas the IAA production was in a much wider range (0-26.4 mg/L). The Hg resistance was evaluated and we found only one isolate that was able to grow at 0.075 mM Hg. The best IAA producers were the isolates 22 (from the *Pseudomonas fluorescens* group), 7 and 23 (from the *Pseudomonas putida* group) (Table 3.). The *P. fluorescens* species also showed a higher capacity to produce siderophores. Belowground and aboveground plant tissues provide habitats, which can be easily colonized by specific PGPB (Vessey, 2003; Vorholt, 2012). In the soil, free-living bacteria of various genera such as *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Curtobacterium*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, and *Serratia* exert beneficial effects on plants (Kloepper et al., 1989; Glick et al., 1999; Benizri et al., 2001) and are classified as rhizospheric PGPB. We previously demonstrated that *Alphaproteobacteria* and *Actinobacteria* dominated root bacterial communities, whereas soil samples were dominated by *Alphaproteobacteria* and *Acidobacteria* (Foulon et al., 2016b).

Table 3. Phenotypic characterizations of the 13 *Pseudomonas* isolates.

Isolates	IAA production mg/L	Siderophore production (ratio between the diameter of the halo and the diameter of colony)
3	0.00	5,53
6	7,77	4,32
7	9,37	1,32
22	26,41	5,03
23	9,74	1,89
24	0,28	2,12
27	0,00	1,65
29	6,92	3,71
32	1,94	2,74
36	0,09	3,04
38	6,44	3,53
40	0,00	2,56

3.3.2.6 **Conclusions**

The bacterial communities of the 2 habitats (tailings dumps and control area) associated with poplars growing at an Hg-contaminated site were previously characterized using metabarcoding tools (Zappelini et al., 2015) and traditional isolation-based techniques (this work). Detection of the most site-dependent OTUs is mainly done in the literature by the comparison of relative abundances using standardized methods, widespread among the scientific community (Azarbad et al., 2015; Bell et al., 2014, 2013, Foulon et al., 2016a, 2016b; Op De Beeck et al., 2015; Schmidt et al., 2013; Tedersoo et al., 2014; Wu et al., 2015; Yergeau et al., 2015; Zappelini et al., 2015) where the OTU selection stage was essential. One of the aims of the present work was to implement a new tool to easily achieve such detection. It is based on the so-called ROC curves, allowing to detect the most site-dependent OTUs and to determine, for each OTU, the ideal threshold under which one can assume to be in one site rather than in the other one. ROC curve analysis was developed in the early 1970s (Egan, 1975) and is currently used in medical statistics to determine the best threshold for a diagnostic test (Zou, 2002). For instance, ROC curves have been used to assess the value of diagnostic tests by providing a standard measure of the ability of a test to correctly classify subjects (Morrison et al., 2003). We hope our user-friendly tool will facilitate good practice in the analysis of metabarcoding datasets and help to deepen our understanding of microbial communities in natural environments. Together with Sparce-PCA and measurements of molecular biomass, *Pseudomonas* species were found to dominate the chlor-alkali tailings dumps.

3.3.2.7 **Funding**

This work was supported by the French Environment and Energy Management Agency [PROLIPHYT 1172C0053], the Région Franche-Comté [Environnement-Homme-Territoire 2014-069], the Pays de Montbéliard Agglomération [13/070-203-2015], and the French national programme EC2CO-MicrobiEen FREIDI-Hg. C.Z. and S.M. received a PhD grant from the French Ministry of Higher Education and Research.

Supplementary Table 3. ROC AUCs and related parameters of the top 30 most discriminating bacterial OTUs.

AUC, area under the curve; Delta norm, difference between the threshold inferior and the threshold superior; WCS, well-classified subjects; Pref, output preference; Inf Thres, inferior threshold; Sup Thres, superior threshold ; #T, nonzero subjects in the tailing dump samples; #U nonzero subjects in the undisturbed soil samples. For each variable, we computed a Wilcoxon test of rank p-value. In the column "Rel ab in U", the number without parenthesis indicates the percentage of the considered OTU in the undisturbed soil (i.e $\frac{100 \times \text{sequences of this OTU} \in \text{the undisturbed soil}}{\text{all sequences} \in \text{the undisturbed soil}}$) while the number in the parentheses indicates the percentage of the undisturbed soil for the considered OTU (i.e $\frac{100 \times \text{sequences of this OTU} \in \text{the undisturbed soil}}{\text{sequences of this OTU} \in \text{both sites}}$), for OTUs that satisfy $\frac{\text{sequences of this OTU} \in \text{the undisturbed soil}}{\text{all sequences} \in \text{the undisturbed soil}} \geq 0,02$ or $\frac{\text{sequences of this OTU} \in \text{the tailings dump}}{\text{all sequences} \in \text{the tailings dump}} \geq 0.02$ in Zappellini et al. (2015). Similar calculations for the tailings dump appear in column "Rel ab in T". Rank, ranking of the most abundant OTUs, as determined by the standard method. The full data set is provided in appendix S1.

OTU	ID	Threshold	Pref	WCS	AUC	Delta norm	Inf Thres	Sup Thres	Wilcoxon	#U	#T	Rel Ab in U	Rel Ab in T	Rank
<i>Bosea</i>	b'236	2	T	16	1.000	0.746	1	3	0.0008	3	8			
Unclassified	b'346	13	T	16	1.000	0.526	8	18	0.0008	5	8			
<i>Tsukamurella</i>	b'533	5.5	T	16	1.000	0.501	4	7	0.0008	4	8			
<i>Iamia</i>	b'458	26.5	T	16	1.000	0.497	21	32	0.0008	8	8			
Unclassified	b'423	339.5	T	16	1.000	0.293	311	368	0.0008	8	8	1.8 (25.0)	4.1 (75.0)	7
Unclassified	b'214	12.5	T	16	1.000	0.284	11	14	0.0008	6	8			
<i>Methylothera</i>	b'332	1.5	T	16	1.000	0.282	1	2	0.0008	2	8			
unclassified	b'076	14.5	T	16	1.000	0.279	12	17	0.0008	6	8			
<i>Aeromicrobium</i>	b'503	12	T	16	1.000	0.279	10	14	0.0008	6	8			
unclassified	b'539	2	T	16	1.000	0.277	1	3	0.0008	5	8			
<i>Agrobacterium</i>	b'221	4.5	T	16	1.000	0.271	4	5	0.0008	8	8			
unclassified	b'051	2.5	T	16	1.000	0.251	2	3	0.0008	5	8			
<i>Haliangium</i>	b'373	2.5	T	16	1.000	0.249	2	3	0.0008	4	8			
unclassified	b'081	5.5	T	16	1.000	0.225	5	6	0.0008	6	8			
unclassified	b'424	80.5	T	16	1.000	0.222	73	88	0.0008	8	8			
<i>Cytophaga</i>	b'024	89.5	T	16	1.000	0.205	81	98	0.0008	8	8			
<i>Stenotrophomonas</i>	b'404	1	T	16	1.000	0.198	0	2	0.0008	0	8			
<i>Marmoricola</i>	b'506	26	T	16	1.000	0.191	24	28	0.0008	7	8			
unclassified	b'074	1.5	T	16	1.000	0.174	1	2	0.0008	1	8			
<i>Aminobacter</i>	b'213	4.5	T	16	1.000	0.158	4	5	0.0008	2	8			
<i>Polaromonas</i>	b'311	6.5	T	16	1.000	0.124	5	8	0.0008	5	8			
<i>Pseudomonas</i>	b'395	310	T	16	1.000	0.039	284	336	0.0008	8	8	1.0 (5.0)	16.1 (95.0)	3
<i>Steroidobacter</i>	b'422	25.5	T	15	0.992	0.859	9	42	0.0009	6	8			
<i>Hyphomicrobium</i>	b'242	30	T	15	0.992	0.663	20	40	0.0009	8	8			
<i>Kaistobacter</i>	b'290	90	T	15	0.992	0.406	71	109	0.0009	8	8			
unclassified	b'576	4	T	15	0.984	0.889	2	6	0.0011	6	8			
<i>Lawsonia</i>	b'363	0.5	T	15	0.984	0.478	0	1	0.0011	1	8			
unclassified	b'080	4	T	15	0.984	0.439	3	5	0.0011	4	8			
<i>Pimelobacter</i>	b'508	6.5	T	15	0.984	0.424	3	10	0.0011	7	8			
unclassified	b'112	2.5	T	15	0.984	0.406	2	3	0.0011	7	8			

Supplementary Table 4. ROC AUCs and related parameters of all soil physico-chemical variables.

AUC, area under the curve; Delta norm, difference between the threshold inferior and the threshold superior; TPR, true positive rate; TNR, true negative rate; WCS, well-classified subjects; Pref, output preference; Inf Thres, inferior threshold; Sup Thres, superior threshold; #T, nonzero subjects in the tailing dump samples; #U nonzero subjects in the undisturbed soil samples. For each variable, we computed a Wilcoxon test of rank p-value

Variable	Threshold	Pref	TPR	TNR	Sum	WCS	AUC	Delta norm	Inf Thres	Sup Thres	Wilcoxon	#U	#T
pH	7.85	T	1	1	2	16	1.000	0.236	7.70	8.00	0.0008	8	8
Ca	41959	T	1	1	2	16	1.000	0.230	29683	54234	0.0008	8	8
Na	196	T	1	1	2	16	1.000	0.164	166	226	0.0008	8	8
Sr	53.0	T	1	1	2	16	1.000	0.161	44.5	61.5	0.0008	8	8
Carbonate	3.85	T	1	0.875	1.875	15	0.984	0.030	0.000	7.70	0.0011	1	8
Pb	20.6	U	1	0.875	1.875	15	0.969	0.091	20.2	21.1	0.0016	8	8
As	14.0	T	0.875	1	1.875	15	0.969	0.055	13.8	14.2	0.0016	8	8
Co	5.18	U	1	0.875	1.875	15	0.922	0.155	4.98	5.38	0.0046	8	8
Thin silt	447	T	0.75	1	1.75	14	0.906	0.072	441	453	0.0063	8	8
CEC	96.0	U	1	0.625	1.625	13	0.906	0.024	95.0	97.0	0.0063	8	8
P ₂ O ₅	0.185	T	1	0.875	1.875	15	0.883	0.862	0.110	0.260	0.0101	8	8
CaO	8.83	T	1	0.875	1.875	15	0.875	0.684	7.16	10.5	0.0117	8	8
Hg	1.49	T	1	0.875	1.875	15	0.875	0.242	1.23	1.75	0.0117	6	8
P	541	T	0.875	0.875	1.75	14	0.844	0.119	525	557	0.0209	8	8
Fe	10433	U	1	0.625	1.625	13	0.797	0.026	10348	10518	0.0460	8	8
Al	15498	U	0.75	0.875	1.625	13	0.781	0.416	13424	17572	0.0587	8	8
Sn	0.951	T	1	0.625	1.625	13	0.781	0.018	0.936	0.966	0.0587	8	8
Clay	195	U	0.75	0.875	1.625	13	0.766	0.191	184	205	0.0742	8	8
Coarse sand	14.0	U	0.875	0.625	1.5	12	0.766	0.156	11.0	17.0	0.0742	8	8
Mg	1905	U	0.75	0.875	1.625	13	0.750	0.080	1868	1942	0.0929	8	8
Na ₂ O	0.025	T	1	0.75	1.75	14	0.750	0.060	0.024	0.025	0.0929	8	8
Large silt	250	U	0.625	0.875	1.5	12	0.750	0.023	249	251	0.0929	8	8
Ni	14.1	U	0.75	0.75	1.5	12	0.734	0.321	12.6	15.5	0.1152	8	8
Cr	25.1	U	0.75	0.875	1.625	13	0.719	0.297	23.0	27.2	0.1415	8	8
Bo	0.780	T	0.625	0.875	1.5	12	0.719	0.137	0.760	0.800	0.1415	8	8
Cd	0.183	T	1	0.5	1.5	12	0.703	0.179	0.156	0.209	0.1722	7	8
K ₂ O	0.315	T	0.875	0.5	1.375	11	0.695	0.146	0.300	0.330	0.1893	8	8
K	1992	U	0.75	0.75	1.5	12	0.688	0.042	1968	2015	0.2076	8	8
OM	36.7	U	1	0.25	1.25	10	0.648	0.434	31.5	41.9	0.3184	8	8
Total C	21.2	U	1	0.25	1.25	10	0.648	0.432	18.2	24.2	0.3184	8	8
Se	0.490	U	0.25	1	1.25	10	0.625	1.478	0.000	0.979	0.4008	2	0
Total N	1.78	U	1	0.375	1.375	11	0.617	0.015	1.77	1.79	0.4309	8	8
Mn	135	T	1	0.5	1.5	12	0.594	0.304	116	155	0.5286	8	8
Thin sand	15.5	U	1	0.25	1.25	10	0.578	0.173	10.0	21.0	0.5995	8	8
Sb	0.288	T	0.5	0.75	1.25	10	0.563	0.663	0.000	0.576	0.6744	2	4
Si	281	U	0.5	0.75	1.25	10	0.563	0.138	277	284	0.6744	8	8
Cu	9.94	U	0.625	0.625	1.25	10	0.563	0.082	9.71	10.2	0.6744	8	8
MgO	0.150	T	0.75	0.5	1.25	10	0.531	0.410	0.140	0.160	0.8336	8	8
C/N	11.5	U	0.375	0.875	1.25	10	0.523	0.938	11.0	12.0	0.8748	8	8

Chapitre 4. Communautés microbiennes du terril de l'Ochsenfeld, site CRISTAL

4.1 Présentation de l'unité de traitement des effluents du terril de l'Ochsenfeld en Alsace

Le deuxième site d'étude est situé à Thann dans la vallée de la Thur dans le département du Haut Rhin (lat. 47° 47' 47.6124" N - long. 7° 8' 18.6498" E). Il appartient au groupe Cristal France SAS, le second plus gros producteur mondial de dioxyde de Ti (TiO_2). Cristal exploite huit sites de production de TiO_2 à travers le monde : aux États-Unis, au Brésil, en Australie, Arabie Saoudite, Royaume-Uni, Chine et à Thann. Le groupe exploite également des mines en Australie et au Brésil. Il emploie environ 4 100 salariés dans le monde entier dont 235 à 240 sur le site thannois. Cristal et le groupe américain Tronox ont récemment annoncé la signature d'un accord portant sur l'acquisition par Tronox de l'activité TiO_2 de Cristal. La combinaison des deux groupes devrait ainsi créer le premier producteur mondial de TiO_2 , représentant 20% du marché.

En 1922, l'usine de Thann a été la première dans le monde à produire du TiO_2 . Le TiO_2 est un pigment blanc utilisé pour donner de la blancheur, du brillant, de l'opacité et de la durabilité aux peintures et revêtements, aux plastiques, au papier et aux élastomères. Il est produit par un procédé chimique par voie humide à base de sulfate, qui utilise l'acide sulfurique pour l'extraire et le purifier sous la forme cristalline anatase. L'extraction du Ti par le procédé sulfurique conduit à la production de gypse rouge, produit par la neutralisation des eaux usées de l'usine de TiO_2 avec le calcaire.

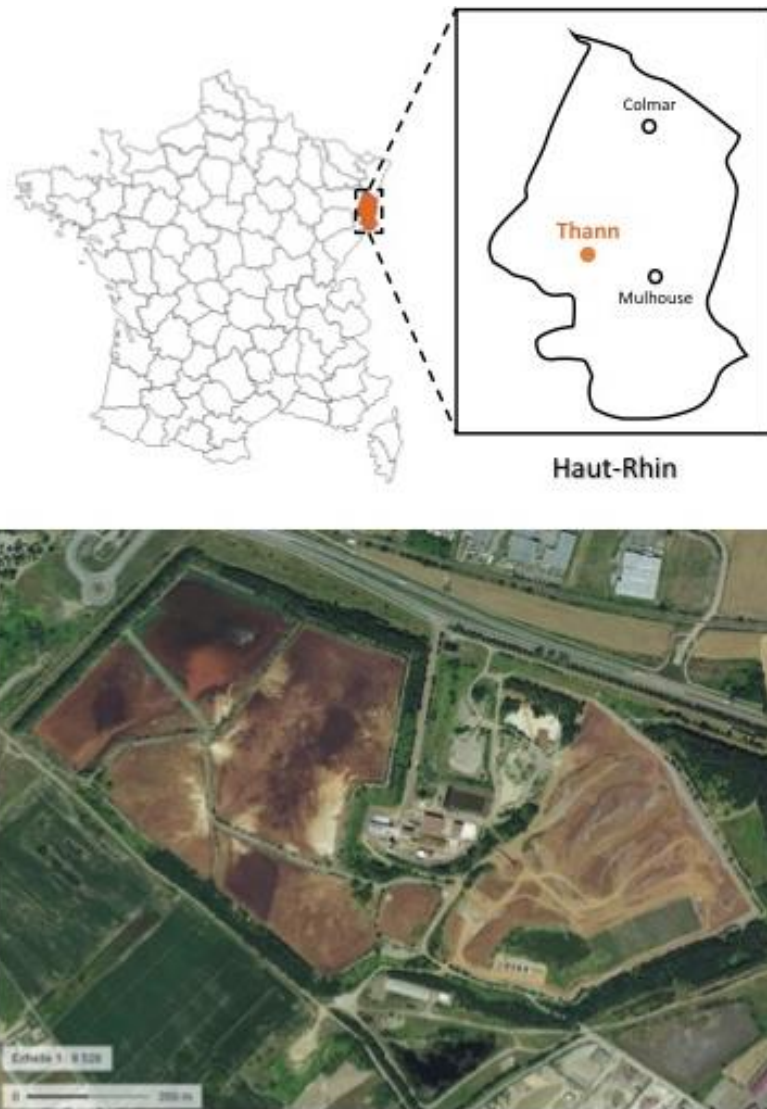


Figure 20. Vue aérienne de l'usine de traitement des effluents et du site de stockage des résidus (site de l'Ochsenfeld) issus de l'extraction du Ti (usine Cristal de Thann, Haut-Rhin).

Cristal Thann fabrique également des produits de spécialité et de haute performance tels que le tétrachlorure de Ti utilisé dans l'industrie automobile (pigments perlés pour les peintures automobiles) et également en tant que catalyseur dans l'industrie chimique. D'autres produits comme les TiO_2 ultrafins sont essentiellement utilisés pour améliorer l'environnement (dépollution de l'air et de l'eau) et pour la conception de surfaces autonettoyantes par photocatalyse. Le site étudié, site de l'Ochsenfeld, est une unité de traitement et de valorisation des effluents industriels issus de cette production de TiO_2 à partir de la roche de type ilménite. Cette unité, située sur une décharge de 80 ha, se trouve à environ 3 km de Thann dans la partie méridionale de la plaine d'Alsace (Figure 20.).

Le site de l'Ochsenfeld comprend des terrils de sédimentation constitués d'un remblai où ont été stockés depuis les années 1930, le titanogypse produit par la neutralisation des effluents d'extraction de TiO_2 . Une paroi moulée de 3,5 km de long a

été construite pour protéger les aquifères de la nappe phréatique de la région des pollutions industrielles du passé.

La zone d'étude est une surface de lagunage d'effluents traités qui n'a pas été utilisée depuis 2006. Ceci a donné naissance à un anthroposol qui a permis l'installation d'une flore peu abondante avec une répartition hétérogène. A l'inverse du site expérimental de Tavaux, on observe une zone peu végétalisée essentiellement dominée par une espèce ligneuse, le bouleau (*Betulaceae* sp.) et des zones dépourvues de toute végétation (Figure 21.a).



Figure 21. a) Vue d'ensemble du terril de stockage de l'usine Cristal (site de l'Ochsenfeld). b) Substrat du terril.

Deux études complémentaires ont porté sur ce site :

- l'une repose sur une approche traditionnelle par isolement afin de caractériser les communautés bactériennes des sols végétalisés du terril de l'Ochsenfeld de l'entreprise CRISTAL et les comparer à celles des sols non végétalisés (partie 4.2),
- l'autre est basée sur l'utilisation d'une méthode de séquençage haut-débit (MiSeq) afin de caractériser les communautés bactériennes et fongiques sur ce même terril (partie 4.3).

4.2 Les actinobactéries dominent la rhizosphère des bouleaux qui colonisent naturellement une décharge de gypse rouge

4.2.1 Résumé

La réhabilitation des sites de stockage de déchets industriels est un enjeu majeur pour limiter l'impact des résidus miniers sur l'environnement. Cette étude a caractérisé la communauté bactérienne de sols végétalisés et non végétalisés d'une décharge de gypse rouge résultant de l'extraction industrielle du titane. Un ensemble de 275 bactéries isolé à partir d'échantillons de sols végétalisés (VS) et non végétalisés (NVS) a été caractérisé au niveau taxonomique par BOX-PCR. L'étude a également évalué la capacité d'un sous-ensemble de 88 bactéries isolées à produire des caractères PGP (production d'auxine, solubilisation du phosphate, production de sidérophores) et de résistance aux métaux. Vingt souches ont également été sélectionnées pour réaliser des tests d'inoculation avec le bouleau. Une Analyse en Composantes Principales a montré que l'ensemble des paramètres pédologiques (pH, granulométrie, C, MO, teneur en Mg) explique à lui seul environ 40% des différences entre les deux sols. La plus forte densité de bactéries cultivables totales a été trouvée dans la fraction VS. Le phylum des Actinobacteria dominait la communauté des sols cultivables (70% dans VS, 95% dans NVS), alors que les représentants des phyla Firmicutes (incluant les genres Bacillus) et Bacteroides (incluant les genres Pedobacter et Olivibacter) n'étaient présents que dans la fraction VS. D'autres genres (Rhizobium, Variovorax et Ensifer) ont également été trouvés uniquement dans la fraction VS. Les bactéries VS hébergeaient les bactéries PGP les plus bénéfiques, avec 12% des isolats présentant au moins 3 caractères PGP. Les souches présentant la plus grande tolérance aux métaux étaient Phyllobacterium sp WR140 (R01.15), Phyllobacterium sp WR140 (R01.34) et Streptomyces sp (R04.15), toutes isolées de la fraction VS. Parmi les isolats testés, Phyllobacterium (R01.34) et Streptomyces sp (R05.33) présentent le plus grand potentiel PGPR et sont donc des candidats potentiels pour une restauration biologique des décharges de résidus.

4.2.2 Actinobacteria dominate the soil under *Betula* trees that naturally colonize a red gypsum landfill

4.2.2.1 Authors

Cyril Zappelini, Vanessa Álvarez-López, Nicolas Capelli, Christophe Guyeux, Michel Chalot

4.2.2.2 Abstract

The successful restoration of well-engineered tailings storage facilities is needed to avoid mine tailings troubles. This study characterized the bacterial community from the vegetated and non-vegetated soils from a red gypsum landfill resulting from the industrial extraction of titane. A set of 275 bacteria was isolated from a vegetated soil and non-vegetated soil areas and taxonomically characterized by BOX-PCR. The study also evaluated the ability of a subset of 88 isolated bacteria on producing plant growth promoting (PGP) traits (indol acetic acid production, phosphate solubilization, siderophore production) and resistance to potentially toxic elements (PTE). Twenty strains were further chosen to build an inoculum for birch-challenging experiments. A principal component analysis showed that the set of pedological parameters (pH, granulometry, carbon, organic matter, Mg content) alone explained around 40% of the differences between the two soils. The highest density of total culturable bacteria was found in the vegetated soil and it was much higher than in the non-vegetated soil. The Actinobacteria phyla dominated the culturable soil community (70% in vegetated soil, 95% in non-vegetated soil), whereas the phyla Firmicutes (including the genera Bacillus) and Bacteroides (including the genera Pedobacter and Olivibacter) representatives were found only in the vegetated soil fraction. Additional genera (Rhizobium, Variovorax, and Ensifer) were found in the vegetated soil only. The vegetated soil bacteria harbored the most beneficial PGP bacteria, with 12 % of the isolates showing 3 or more plant growth promoting traits. The strains with the higher metal tolerance in our study were the Phyllobacterium sp WR140 (R01.15), Phyllobacterium sp WR140 (R01.34) and Streptomyces sp (R04.15), all isolated from the vegetated soil. Among the isolates tested in challenging experiments, the Phyllobacterium (R01.34) and Streptomyces sp (R05.33) have the greatest potential to act as plant growth promoting rhizobacteria and therefore to be used in the biological restoration of tailings dumps.

4.2.2.3 Introduction

Metal(loid) contamination due to anthropogenic activities, such as extractive activities, poses a threat to the surrounding environment and human health. In particular, tailings that are artificial soil-forming substrates that have not been created by the natural processes of soil formation and weathering (Cross et al., 2017), are susceptible to wind and water erosion, facilitating contaminant transport (Honeker et al., 2017). Mine operations generate substantial volumes of waste substrates e.g. post extraction 'tailings', which may be crushed and/or chemically treated waste rock from which ores have been extracted (Kumaresan et al., 2017). They are often characterized by poor physical structure and hydrology, unstable geochemistry, and potentially toxic chemicals (Wang et al., 2017). Historically, tailings management plans have focused

largely on confinement and containment, with little regard to long-term alteration of the chemical, physical, and biological properties of tailings materials (Santini and Banning, 2016). They further represent an abundant by-product that could be used as a surrogate planting substrate that can be used in land farming, but clearly contain significant abiotic constraints to plant and microbial survival. Vegetation is often promoted on such tailings, because it can effectively control the erosion of tailings fines by wind and water and can improve the landscape of these wastelands (Mendez and Maier, 2008).

The successful restoration of well-engineered tailings storage facilities may be the best way to avoid future mine tailings disasters (Cross et al., 2017). Salisbury et al. (2017) demonstrated the effectiveness of a vegetation cover to retain some potentially toxic elements (PTE) in its upper soil horizons for several decades following plant community establishment. Indeed, natural vegetation plays a considerable role in reclaiming contaminated land by tolerating inorganic contamination through many mechanisms for growth and reproduction in harsh environments. However, *in situ* remediation of tailings is likely to require some form of substrate amendment or accelerated weathering (Santini and Banning, 2016). The addition of a topsoil to the rooting soil area represents an effective method of alleviating some of the abiotic constraints present in pure tailings, and is likely to accelerate the return of microbial functions (Huang et al., 2012). Another key step to successful revegetation is the selection of appropriate plant species, and generally those endemic to the tailings zone are the best choice (Jana et al., 2012; Wanat et al., 2014; Wang et al., 2008). Among plant species that are readily colonizing tailings, birches have a recognized ability to quickly colonize bare areas, and are characterized by a lack of affinity for any particular soil type and the ability to grow on nutrient-poor soils (Atkinson, 1992; Jana et al., 2012).

There is an abundant literature on the characterization of microbial communities from forest or agricultural soils contaminated by PTE or PAH (Foulon et al., 2016b, 2016a; Tardy et al., 2015; Yergeau et al., 2015). Microbial populations in mine tailings have also attracted considerable interest in the last decade, especially in acid mine drainage dumps (Bruneel et al., 2017; Gupta et al., 2017; Méndez-García et al., 2015; Mesa et al., 2017a). However in other environments with different soil characteristics (Bauxite, red gypsum), there are considerable fewer studies. Soil microbes are more than just indicators of ecological function, they are increasingly recognized as facilitators of the belowground metabolic recovery required for subsequent aboveground restoration. Despite acknowledgement of the link between above and belowground communities, there is still a lack of mechanistic understanding on how microbial communities facilitate restoration of highly degraded environments such as post-mining landscapes. However, a recent study reported that application of soil inocula can promote ecosystem restoration, whereas origins of soil inocula play a major role in the establishment of plant communities (Wubs et al., 2016). Therefore, efforts to

characterize endogenous microbial communities from these soils are urgently needed, to achieve an optimal plant recovery.

Among soil bacteria, *Actinobacteria* are a group of bacteria present in high concentrations in soils. They play an important role in recycling xenobiotic or natural substances, since they are able to metabolize complex organic matter (Kieser et al., 2000). The important ecological role played by *Actinobacteria* is demonstrated by their capability to remove xenobiotics compounds such as pesticides, and PTE, among others substances. Nowadays, its members are considered among the most successful colonizers of all environments in the extremobiosphere, in opposite to the traditional perception of *Actinobacteria* as autochthonous soil and freshwater organisms (Álvarez et al., 2017). Like other beneficial microorganisms, *Actinobacteria* can affect plant growth in two general ways, either directly or indirectly. Indirect promotion occurs when they prevent the harmful effects of one or more deleterious microorganisms. This is chiefly done through biocontrol or antagonism toward soil plant pathogens. Direct promotion of plant growth occurs when the plant is supplied with a compound that is synthesized by the bacteria, or when the latter otherwise facilitates plant uptake of soil nutrients (Barka et al., 2016). For instance, the potential of *Streptomyces* species as PGPR has not been widely studied. This is surprising because *Streptomyces*, which generally account for an abundant percentage of the soil microflora, are particularly effective colonizers of plant root systems, and they are able to endure unfavorable growth conditions by forming spores. *Actinomycetes* strains were isolated from the rhizosphere of birch, one of a few native tree forms capable of thriving on the upper level of a coal mine dump (Ostash et al., 2013).

Research focused on specific tree-root-microbe interactions during phytostabilization of Ti tailings is essential to advance our understanding of how plant-microbe interactions help to sustain plants under such stressful environmental conditions. The main objectives of the present study were i) to isolate indigenous bacteria from the birch (*Betula spp.*) on the basis of morphological, and physiological characteristics as well as by 16S rRNA gene sequence analysis, (ii) to screen bacteria for various plant growth promoting (PGP) traits, such as indoleacetic acid (IAA) production, phosphate solubilization, siderophore production, and metal tolerance and (iii) to study the PGP potential of selected isolates *in-vivo* under axenic conditions.

4.2.2.4 **Material and methods**

Study site Location

The study site belongs to a 80 ha titanium industry effluent treatment unit located in the North-East of France, in the southern part of the Alsace plain (47°47'47.7"N 7°08'18.5"E). The study was carried out in a tailing dump consisting of an embankment, where by-products produced during neutralization of titanium dioxide extraction effluents have been stored since the '30s. The dump surface studied here is

unused since the early 2000s, which has allowed its natural revegetation with a flora that is however not very abundant and distributed in a heterogeneous way. We may thus observe heavily vegetated areas, and on the contrary, areas completely bare of vegetation. The flora at this dump-site is almost exclusively dominated by the woody species *Betula* sp.

Sampling

The samples were collected on 27th of October 2015. They consisted in samples from two areas, a vegetated and a non-vegetated areas (Fig. 22.). Five birches distributed over the vegetated area were harvested, and the soil fraction adhering to the root system was collected (vegetated soil or VS). For the non-vegetated area, 5 samples (non-vegetated soil or NVS) were also taken using an auger, at a depth approximately close to that of the root system for the vegetated area. The whole samples were packed on site in plastic bags and transported to the laboratory at a temperature approaching 4°C.

Pedological characterization

The soils were dried at 40°C and then ground by hand at 2 mm. The soil analysis were carried out by a service provider in accordance with the following French standards for grinding (NF ISO 11464), residual humidity (NF ISO 11465), granulometry (5 fractions - NFX 31-107), pH water + KCl (NF ISO 10390), total organic carbon and organic matter (NF ISO 14235), total nitrogen (NF ISO 13878), CEC Metson (NFX 31-130), bore soluble boiling water (NFX 31-122), oligo-elements, K₂O, MgO, CaO, Na₂O (French Norm X 31-108), and total available phosphorus (Joret Hebert method French Norm X 31-161).

In addition, pseudo-total concentrations in the soils were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fischer Scientific, Inc., Pittsburg, USA) analysis after acid digestion of 500 mg of sample in a microwave digestion system (Mars Xpress, CEM), using a mix of 2 mL of 67% nitric acid, 6 mL of 34% hydrochloric acid, and 2 mL of 48% hydrofluoric acid. To assess the analytical quality, a standard reference material (Loamy Clay soil) was used. To determine the TE extractable fractions, 5 g of 2-mm sieved soil was dried at 60°C for 48 h (or air-dried) and incubated with 50 mL of 10mM CaCl₂ 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 HNO₃ prior to ICP-AES, analysis.

Microbial characterization

Vegetated and a non-vegetated soil fractions were homogenized in 45 ml of 10 mM MgSO₄, stirred at 100 rpm for 15 min at room temperature. One ml was used to perform serial dilutions in 10-fold series, and 100 µL were plated onto 284-agar medium (Becerra-Castro et al., 2011) in duplicate dilution and kept for 7 days at 25°C. The 284

medium contains (per liter): 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH₄Cl, 0.43 g Na₂SO₄, 0.2g MgCl₂.6H₂O, 0.03 g CaCl₂.2H₂O, 0.04 g Na₂HPO₄.2H₂O, 10 mL Fe(III)NH₄ citrate solution (containing 48 mg/100mL) plus oligoelements (1.5 mg FeSO₄.7H₂O, 0.3 mg H₃BO₄, 0.19 mg CoCl₂.H₂O, 0.1 mg MnCl₂.4H₂O, 0.08 mg ZnSO₄.7H₂O, 0.02 mg CuSO₄.5H₂O, 0.036 mg Na₂MoO₄.2H₂O) adjusted to pH 7. The medium was supplemented with a mixture of different carbon sources: lactate (0.7 g /L), glucose (0.5 g /L), gluconate (0.7 g /L), fructose (0.5 g /L), and succinate (0.8 g /L). Bacterial densities were calculated and expressed as CFU per g dry soil. Single morphotypes were isolated by plating them twice onto 284 medium-agar plates. The isolates were further stored in cryotubes in Brain-Heart-Infusion Broth (Roth, D) with 15% glycerol glucosate at -80°C.

Genotypic Characterization

DNA extraction and BOX-PCR

For DNA preparation, the isolates were grown in the 284 liquid medium at 25°C, 7 days at 250 rpm (Gallenkamp Orbital Incubator). After centrifugation, DNA was extracted from the pellets using an EZNA bacterial DNA isolation kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) according to the manufacturer's instructions. The BOX-PCR fingerprinting method was used to group genotypic profiles at a similarity level of 90 % as previously described (Becerra-Castro et al., 2011). Box reactions were performed in a reaction volume of 25 µL, containing 12.5 µL of Ready mix PCR Master mix (Thermo Fisher Scientific, Carlsbad, California, USA), 2 µM of BOX A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3', Eurofins Genomics, Paris, France), and 5 µL of bacterial DNA. DNA amplification was carried out in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) under the following conditions: 1 cycle of 5 min at 95°C, 40 cycles of 25 sec at 95°C, 35 sec at 55°C and 1.05 min at 72°C with an additional 5-min cycle at 72°C. The amplicons obtained were separated by electrophoresis, on an agarose gel 1.8% at 45 V 3 h. Gel images were analyzed with the software Gel.J (Heras et al., 2015) by using Pearson correlation coefficient and UPGMA clustering algorithm. VS and NVS bacteria were treated separately.

16S Taxonomic assignment

A PCR was performed on one representative of each BOX group, under the following conditions: volume of 50 µL containing: 25 µL AccuStart™ II PCR ToughMix® (2X) (quantas), 5 µM of 27f (*Escherichia coli* positions 8-27, 5'-AGAGTTTGAT CCTGGCTCAG-3') and 1492r (*E. coli* positions 1492-1510, 5'-ACGGTTACC TTGTTACGACTT-3') were used to amplify nearly full-length 16S rRNA genes (Mark Ibekwe et al., 2007), and 5 µL of cell lysate. Thermocycling conditions were: 1 cycle of 94°C for 3 min; 40 cycles of 25 sec at 94°C; 25 sec at 49.4°C and 1.30 min at 72°C. Alignments were performed using the SILVA website (<https://www.arb-silva.de/>).

Functional traits

Characterization of plant growth promoting traits

Isolates were screened for their ability to solubilize inorganic phosphate, siderophore production, organic acid production, and IAA production. All those analysis were realized for one representative of each BOX group. The ability to solubilize inorganic phosphate was assessed in a modified NBRIP agar medium (1.8%) supplied with 5 g L^{-1} of hydroxyapatite and incubated at 28°C for 5 days (10.0 g glucose, 5.0 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KCl, 0.1 g $(\text{NH}_4)_2\text{SO}_4$, 0.1 g yeast extract in 1 L deionized water adjusted to pH 7.0, modified from (Nautiyal, 1999). A clear halo around the bacterial colony indicated solubilisation of mineral phosphate. Siderophore production was detected in a modified 284 liquid medium (without Fe) using the Chrome Azurol S (CAS) method described by Schwyn and Neilands (1987). All glassware used in this assay was previously cleaned with 30% HNO_3 followed by washing in distilled water (Cox 1994).

The ability to produce organic acids was tested on agar medium containing 0.002% bromocresol purple (per liter medium): 10.0 g glucose, 1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.03 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Colonies forming a yellow halo after 1 day of growth at 28°C indicated a pH change in the medium and were considered acid producers. IAA production was evaluated in liquid medium (5.0 g glucose, 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 2.0 g K_2HPO_4 , 0.5 g CaCO_3 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g NaCl, 0.1 g yeast extract adjusted to pH 7; modified from Sheng et al. (2008) supplemented with 0.5 mg/mL of tryptophan). After 5 days incubation at 28°C , cultures were centrifuged and the supernatant was incubated with Salkowski reagent for 25 min. The production of IAA was recognized by the presence of red coloring and isolates were considered IAA-producers when the concentration of IAA determined was more than 4 mg/L culture.

Metal resistance

Metal resistance was tested for Cr, Mn and Zn using the 284 agar medium supplemented with increasing concentrations of Hg (0.1 mM, 0.25 mM, 0.5 mM, 1 mM, 2.5 mM and 5 mM; added as $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$), and further incubated at 28°C for 7 days. The Maximal Tolerable Concentration (MTC) of each metal was recorded for one selected isolate of each BOX-group.

Inoculation Tests

Birch seeds were germinated in commercial potting mixture. Three-month-old birch seedlings were transplanted into pots containing 200 g of soil collected from the study site. After one week of plant adaptation, bacterial inoculation was carried out. Fresh cultures of bacterial strains were grown in 869 liquid medium (Mergeay et al., 1985) for 24 h, harvested by centrifugation (6000 rpm, 15 min) and re-suspended in 10mM MgSO_4 to a dry mass weight of 0.5 mg/L. Each pot was inoculated with 10 mL of bacterial suspension. The same amount of sterile 10 mM MgSO_4 was added to non-inoculated pots. Six replicates of each plant species were prepared for each inoculation strain. Plants were watered regularly to maintain soil moisture and incubated in a

growth chamber in the following climatic conditions: day light for 16 h (250–300 $\mu\text{mol m}^{-2} \text{s}^{-1}$); day temperature of 22°C; night temperature of 18°C; day and night humidity of 30%.

After a 3 months growth period, plants were harvested and shoot and root dry weight (DW) yield determined. Plant material was washed in deionised water, oven-dried at 45°C, weighed and ground. Oven-dried plant material was digested in a 2:1 HNO_3 :HCl mixture and the concentrations of P, K, Ca, Mg, Fe, Cd, Pb and Zn were measured by ICP-AES.

Statistical analyses

Normality was tested with the Shapiro-Wilk (all data sets), and homoscedasticity was tested with Bartlett's (abiotic dataset) and Levene (biomass dataset, PGP and metal resistance traits) tests using R. Data that were normally distributed were analyzed using a parametric test (Student's t-test) in R. Data that were not normally distributed were analyzed using a non-parametric Mann-Whitney-Wilcoxon (soil data) or Kruskal-Wallis (inoculation) tests using "R". The Principal Component Analysis (PCA) was performed using the R ade4 package. Data expressed as % (PGP and metal resistance traits) were analyzed using a Chi-2 test in R.

4.2.2.5 **Results and discussion**

Pedological characterization of the two areas

The sampling zone where the vegetation was found (*Betula pendula*) is separated by about 60 m from the non-vegetated area (Fig 22.a). Investigations carried out at the physico-chemical level show that the VS fraction differed significantly from the NVS fraction for several pedological parameters (Table 4.) and elements (Table 5.). Physico-chemical analysis revealed that the VS contained significantly less silt and more sand (Table 4.), and was slightly more acidic than the NVS. It also contained more C and OM. Significant differences between NVS and VS samples were found for the following parameters: Ti (+25.40% in VS), Mn (+60.04% in NVS), K (+27.66% in NVS), Sb (+28.88% in NVS), As (+32.43% in NVS), and B (+33.31% in NVS). In the CaCl_2 extractable fraction, only B, Cr, Fe, K, Mg, Mn, Na, P, S, Si, Sr, Ti, and Zn were detected in significant amounts in this fraction (>0.01% from the total) (Table 5.). However, for Fe, Mn, Ti the extractable fraction accounted for less than 0.01%. Conversely, for Cr, K, Mg, Si, S and Sr, the extractable fraction accounted for around 2-10 %. Only total concentrations of As, B, Mg, Sr, Sb, and Ti differed significantly between VS and NVS samples, whereas only the CaCl_2 extractable fraction of Mg differed between the two soils.

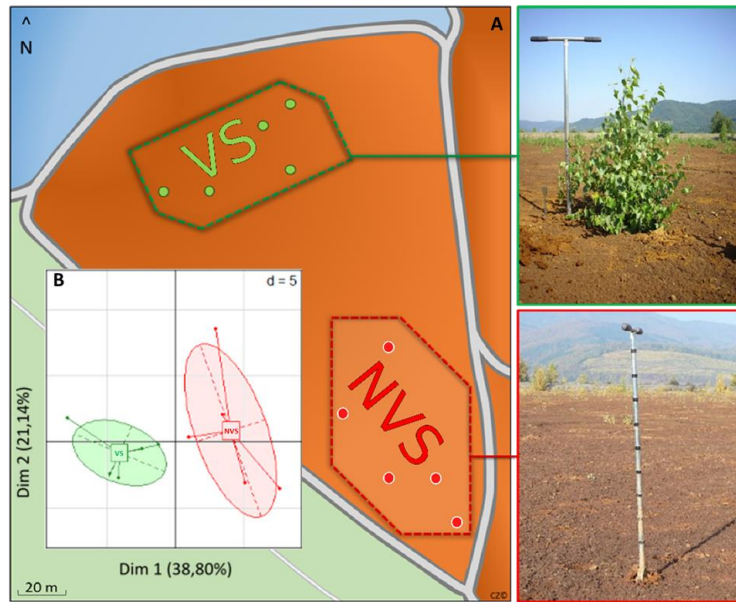


Figure 22. A. Diagram of the sampling site. B. PCA carried out with soil data and soil concentration of elements.

The sampling soils taken in a vegetated area under birch trees (VS, left top photo, green dotted line) or soils taken in a non-vegetated area (NVS, left bottom photo). n=5.

This set of data indicates that the soil of the tailings dumps is not suitable for revegetation, due to its low nutrient content, the very low N content (below the detection limit) and the slightly alkaline pH. These extreme conditions indeed often suppress root growth in trees and cause leaf chlorosis and lower biomass production (Wang et al., 2017) The large amount of Fe and Mn, mostly in oxide forms (Carbonell, unpublished data) may also limit the availability of nutrients at this whole area. Previous works on the relationships between plant abundance and the physicochemical properties of tailings dumps have identified some of the factors that limit plant establishment on these sites, namely pH, metal concentration and bioavailability (Santos et al., 2017).

Table 4. Physico-chemical parameters of vegetated soils (VS) and non-vegetated soils (NVS).

	Non-vegetated soils	Vegetated soils	P-v	Test value
Clay (‰)	100.40 ± 6.73	93.60 ± 5.37	0.1172	t = -1.7664
Thin Silt (‰)	228.60 ± 38.72	152.20 ± 23.97	0.00781 **	t = -3.7512
Large Silt (‰)	220.20 ± 17.91	138.80 ± 13.63	5.95E-05 ***	t = -8.0884
Thin Sand (‰)	169.20 ± 45.65	157.20 ± 29.99	0.6385	t = -0.49122
Coarse Sand (‰)	281.80 ± 57.22	458.40 ± 26.90	0.0009578 ***	t = 6.2457
pH	8.20 ± 0.35	7.84 ± 0.05	0.007495 **	W = 25
pH KCl	8.08 ± 0.36	7.84 ± 0.05	0.009467 **	W = 25
CaCO ₃ (g/kg)	280.40 ± 115.79	301.60 ± 51.23	0.7221	t = 0.37438
C.org (g/kg)	3.78 ± 0.87	6.88 ± 1.70	0.01124 *	t = 3.6238
O.M (g/kg)	6.54 ± 1.48	11.90 ± 2.95	0.01134 *	t = 3.6264
Ntot (g/kg)	<ddl	<ddl		
C/N	NC	NC		
CEC (meq/kg)	26.80 ± 6.06	45.20 ± 11.21	0.01732 *	t = 3.2286
K ₂ Oex (g/kg)	0.00 ± 0.00	0.01 ± 0.01	0.07201	W = 5
MgOex (g/kg)	0.76 ± 0.20	0.30 ± 0.16	0.004538 **	t = -3.9879
CaOex (g/kg)	76.84 ± 0.44	77.02 ± 0.36	0.5009	t = 0.70602
Na ₂ Oex (g/kg)	0.02 ± 0.00	0.02 ± 0.00	0.2042	t = -1.4072

Mean values and standard deviations are provided (n=5).

Disparities between the VS and NVS emerged, as illustrated by the principal component analysis (Fig. 22.). This PCA showed that the set of pedological parameters alone explains around 40% of the differences between the two soils. The VS also presented significantly lower pH and higher CEC (Table 4.). The differences between the VS and NVS might be due to the presence of the *Betula* trees. The lower pH in the vicinity of the birch roots, which could lead to an increase in the CEC in this area, is probably due to the root metabolic activity. Birch trees are indeed known to exude several acids in the mM range, especially as monocarboxylic acids (Sandnes et al., 2005). The birch litter may also slightly contribute to the observed enrichment of the vegetated soil in C and OM.

Table 5. Total and CaCl₂ extractable element concentrations in vegetated soils (VS) and non-vegetated soils (NVS).

	NVS		VS					
	Total (ppm)	CaCl ₂ extractable (ppm)	Total (ppm)	CaCl ₂ extractable (ppm)	Total		CaCl ₂ extractable	
					P-v	Test value	P-v	Test value
Al	2 708 ± 158		3 010 ± 469		0.309	W = 7		
As	6.11 ± 1.24		4.13 ± 0.89		0.022 *	t = -2.90		
B	5.13 ± 2.68	0.61 ± 0.21	3.42 ± 1.27	0.40 ± 0.12	0.015 *	t = -3.10	0.101	t = -1.91
Ca	194 267 ± 19 462		186 187 ± 8 115		0.341	t = -1.04		
Cd	0.21 ± 0.21		0.15 ± 0.21		0.824	W = 14		
Co	16.24 ± 2.53		21.54 ± 4.33		0.077	t = 2.11		
Cr	147.48 ± 43.06	8.26 ± 4.73	186.10 ± 27.67	3.79 ± 0.49	0.156	t = 1.60	0.151	W = 20
Cu	38.50 ± 2.78		45.78 ± 7.03		0.118	t = 1.87		=
Fe	41 559 ± 4 542	0.99 ± 0.24	52 198 ± 9 242	1.13 ± 0.37	0.088	t = 2.04	0.526	t = 0.667
K	156.23 ± 17.91	6.97 ± 1.53	113.02 ± 31.47	10.39 ± 4.03	0.041 *	t = -2.58	0.136	t = 1,774
Mg	4 063 ± 1 336	330.87 ± 67.62	1 623 ± 372.30	141.35 ± 71.13	0.001 **	t = -4.88	0.002 **	t = -4.318
Mn	3 167 ± 538.06	0.19 ± 0.05	4 228 ± 1 023	0.16 ± 0.05	0.112	t = 1.863	0.409	t = -0.872
Na	76.99 ± 24.88	8.70 ± 1.58	98.54 ± 26.59	7.99 ± 1.75	0.059	t = 2.397	0.521	t = -0.671
Ni	22.15 ± 16.81		24.61 ± 18.15		0.471	t = 0.770		
P	45.57 ± 22.63	0.14 ± 0.01	47.18 ± 24.95	0.17 ± 0.03	0.758	t = 0.322	0.104	t = 1.923
Pb	19.74 ± 3.06		21.19 ± 2.91		0.841	W = 11		
S	94 917 ± 32 445	4 995 ± 233.56	83 238 ± 32 415	4 808 ± 266.14	0.334	t = -1.065	0.272	t = -1.180
Sb	4.72 ± 0.62		5.99 ± 0.65		0.016 *	t = 3.157		
Si	276.27 ± 75.50	9.25 ± 3.07	287.06 ± 84.44	8.14 ± 0.60	0.594	t = 0.555	1	W = 12
Sr	97.70 ± 11.66	4.57 ± 0.88	107.94 ± 15.36	5.89 ± 0.76	0.151	w = 5	0.035 *	t = -2.540
Ti	5 234 ± 1 833	0.25 ± 0.07	6 564 ± 1 760	0.27 ± 0.05	0.012 *	t = 3.306	0.619	t = 3.306
Zn	83.70 ± 48.75	0.03 ± 0.02	98.21 ± 52.66	0.02 ± 0.00	0.233	t = 1.302	0.786	W = 9

Mean values and standard deviations are provided (n=5).

Microbial characteristics

The highest density of total culturable bacteria was found in the VS fraction and it was much higher than in the NVS fraction. In the latter samples, the CFUs were over 5 orders of magnitude lower than the CFU found in VS (Fig. 23.). A total of 170 (VS) and 105 (NVS) bacteria were isolated. According to their BOX-PCR profiles, isolates were allocated into 53 (VS) and 43 (NVS) distinct groups and were further identified through comparative sequence analysis of their 16S rDNA sequences. Isolates were affiliated to a total of 16 different genera belonging to 4 phyla. The Shannon diversity (H') index was calculated on the basis of the genera, similar values were found for VS (H'=1.16) and NVS (H'=1.17).

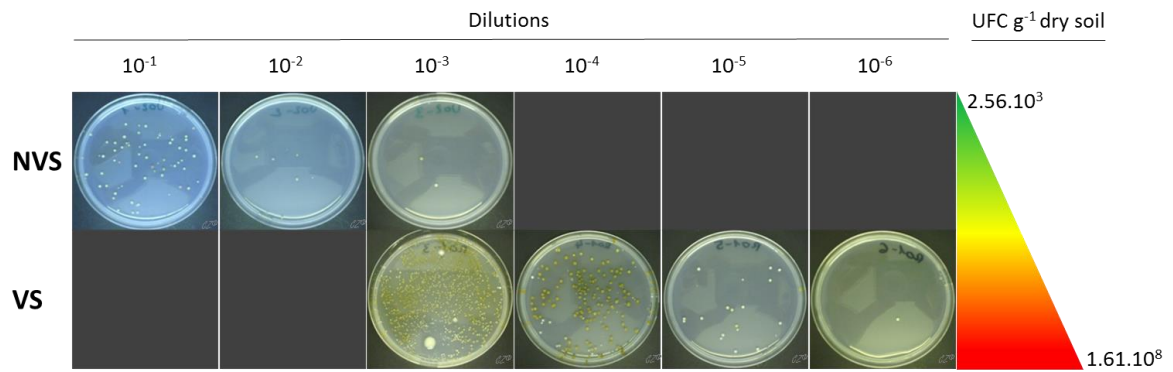


Figure 23. CFU culturable bacterial density, expressed as UFC g⁻¹ dry soil.

The dilution series used to determine density is represented by the Petri dishes after incubation, for the non-vegetalized soil (NVS) and the vegetalized soil (VS).

The phyla *Actinobacteria* and *Proteobacteria* were represented in the two soils, whereas the *Firmicutes* (including the genera *Bacillus*) and *Bacteroides* (including the genera *Pedobacter* and *Olivibacter*) representatives were found only in the VS fraction. The *Actinobacteria* accounted for more than 95% in the NVS fraction. Within that phylum, the abundance of each genus differed between the two bacterial populations (Fig. 24.). In both soils, the *Streptomyces* dominated (around 70% of the total isolates). Additional *Actinobacteria* were found in the NVS (*Amycolatopsis*, *Nocardia*, *Nocardioides*, *Paenartrobacter*) but were absent from the VS. The *Rhodococcus* isolates were found only in the VS. Within the *Actinobacteria* phylum, the two soils shared only the *Pseudoarthrobacter* and *Arthrobacter* (Fig. 24.). The two soils shared also *Proteobacteria* members, although less represented in the NVS. However the two soils shared only *Pseudomonas* and *Phyllobacterium* isolates. *Rhizobium*, *Variovorax*, and *Ensifer* isolates were detected only in the VS fraction.

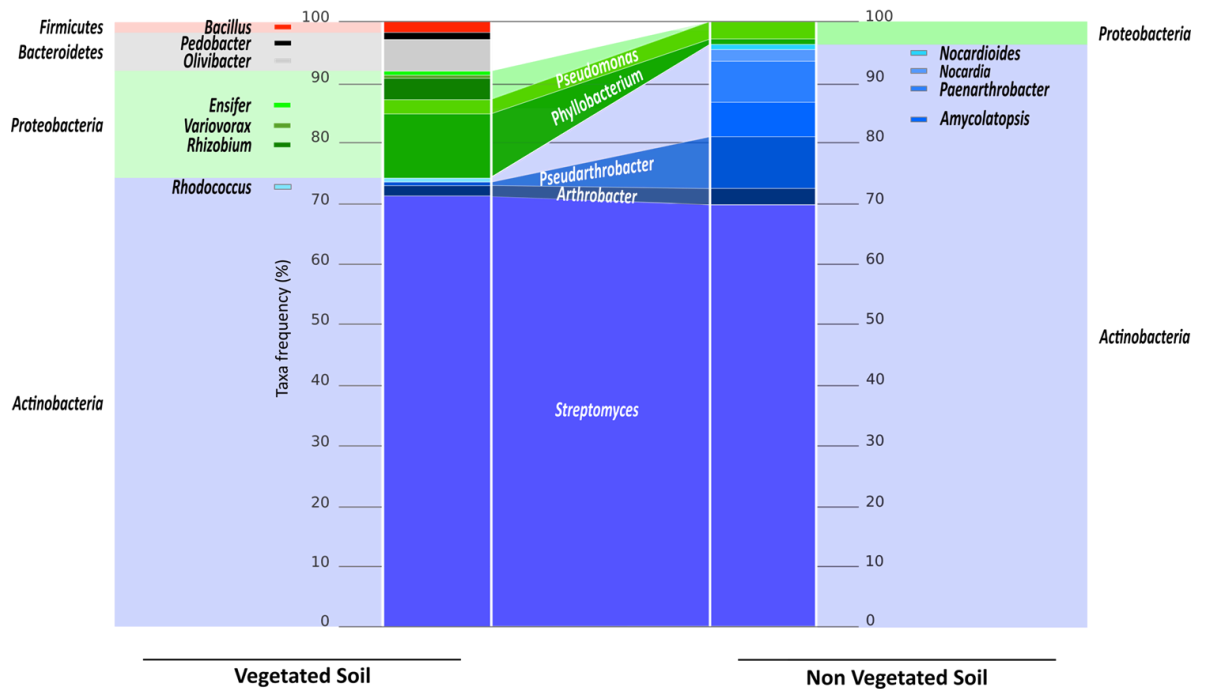


Figure 24. The cultivable bacterial communities from the VS and NVS fractions.

The light-colored histograms at the edge represent the abundance of bacterial phyla expressed as % of the total number of bacteria for the VS (left) and the NVS (right). The dark-colored central histogram represents the abundance of bacterial genera expressed as % of the total number of bacteria for the VS (left) and the NVS (right). The names in white in the centre indicate the shared bacteria between the two habitats.

The *Actinobacteria* phyla dominated the culturable soil community (70% in VS, 95% in NVS). These results are in agreement with other found in the literature. For example, bacterial diversity from trace metal-contaminated rhizosphere of the metal-hyperaccumulating plant *Thlaspi caerulescens* were analyzed and compared with that of contaminated bulk soil (Gremion et al., 2003). The most remarkable result was that sequences belonging to *Actinobacteria* dominated both bulk and rhizosphere soil, indicating that *Actinobacteria* might be a dominant part of the active bacteria in trace metal-polluted bulk and rhizosphere soils. *Actinobacteria* exhibit cosmopolitan distribution since their members are widely distributed in aquatic and terrestrial ecosystems (Álvarez et al., 2017). Isolates from our soils were restricted to the genera *Streptomyces*, *Arthrobacter* and *Rhodococcus*, as previously found (Álvarez-López et al., 2015). *Streptomyces* were also isolated from Mn-contaminated soils (Mo et al., 2017) and from the birch rhizosphere collected on coal mining dump (Ostash et al., 2013). Culture dependent methods have allowed the isolation and characterization of over 35 genera of *Actinobacteria* tolerant to trace elements (Álvarez et al., 2017). Members of the order *Actinomycetales*, notably the *Streptomyces* genus, remain the richest source of natural products, including clinically useful antibiotics, antimetabolites, and antitumour agents (Bérdy, 2005; Olano et al., 2009). *Actinomycetes* produce about 45% of all microbial bioactive secondary metabolites, with 80% of these compounds being

produced by the *Streptomyces* genus (Bérdy, 2005). Different life styles are encountered among *Actinobacteria*, saprophytic free-living aquatic and soil *Actinobacteria* as well as plant commensals, nitrogen-fixing symbionts. Within the class *Actinobacteria*, members tolerant to trace elements have been found in 10 of the 16 orders.

Margesin et al. (2011) determined that the physiologically active fraction in sites contaminated with trace elements was not only represented by *Proteobacteria* (as other research has indicated) but also by *Actinobacteria*. Similar results were found by Oliveira and Pampulha (2006). The quantitative analysis of soil microbial populations through total culturable numbers showed a marked decrease of the different microbial groups for contaminated soil samples, in comparison with uncontaminated samples. However, *Actinobacteria* showed less sensitivity than other culturable heterotrophic bacteria and symbiotic nitrogen fixers.

Within the *Actinobacteria*, *Arthrobacter* was the second most important genus, after *Streptomyces*, in relation to tolerance to trace element and its potential use in bioremediation. Because *Arthrobacter* tolerate alkaline conditions, which are common in soils contaminated with Cr, the authors propose the use of both, intact cells and cell-free extract for the bioremediation of alkaline soils contaminated with chromate (Elangovan et al., 2010). Becerra-Castro et al. (2011) isolated and characterized Ni-resistant rhizosphere bacteria from two subspecies of *Alyssum serpyllifolium*. The most Ni-resistant bacteria were mainly strains of the *Streptomyces* and *Arthrobacter* genera.

Functional traits of bacterial isolates

Plant growth promoting properties of bacterial collection, comprising 53(R) and 43 (S) BOX-PCR groups, were tested *in vitro* for plant growth promotion properties such as phytohormone production, nutrient acquisition, and the metabolism of plant-growth regulating compounds (Fig. 25.) the VS bacteria harbored the most beneficial PGP bacteria, with 12 % of the isolates showing 3 or more PGP-traits (Supplementary Table 5. and 6.). The ability to produce IAA was detected in the isolates both from the VS and the NVS fractions, although there were statistically more abundant in the VS. Conversely, the siderophore-producing capacity was higher in isolates from the NVS. Organic acid-producers and rare phosphate solubilizers were present in both populations, and not significantly different. The production of siderophores by *Streptomyces* isolates has already been found by Ostash (2013). The genetic and enzymatic bases of siderophore biosynthesis and their transport in model families of this phylum are well understood (Cruz-Morales et al., 2017). More generally, due to its strong antimicrobial potential, and soil dominant saprophytic nature, the *Actinomycetes* group gained much attention as plant growth promoters (PGP) (Franco-Correa et al., 2010).

In the present study, resistances to the three metals Zn, Cr, and Mn were investigated (Table 5.). As revealed in Figure 25., the relative order of bacterial toxicity

of the 3 metals was determined as follows: Mn > Zn > Cr. Resistance to Cr and Zn was considered to be reached at concentrations higher than 0.5 mM and 1 mM, respectively (Navarro-Noya et al., 2012). In our samples, isolates tolerated Cr concentrations below that threshold, much lower than that measured for *Cupriavidus metallidurans* (2.5 mM) (Zhao et al., 2012). Conversely, our isolates were more resistant to Zn, with MTC up to 10 mM for some VS isolates. Two % of our isolated have MTC of 10 mM, which is in the same order of magnitude as those determined for the metal resistant *Cupriavidus metallidurans* (Zhao et al., 2012). Kuffner et al. (2008) characterized Zn resistant endophytic and rhizospheric bacteria of Zn accumulating willows.

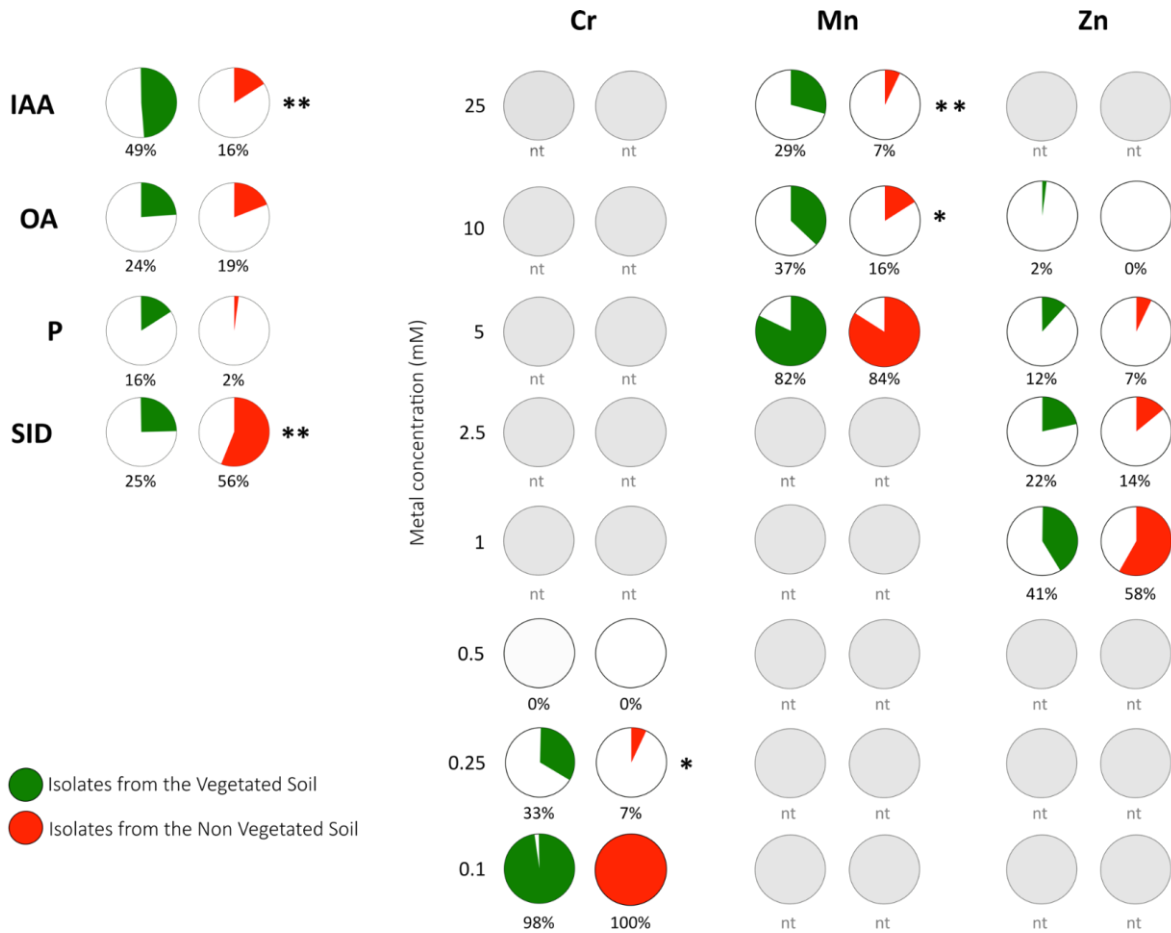


Figure 25 Functional traits of bacterial isolates from the Ochsenfeld site.

Plots representing the % of isolates harboring the positive functional trait (PGP characteristics), or showing metal resistance. PGP traits were auxin production (IAA), organic acid production (OA), phosphorus solubilization (P), and siderophore production (SID). Metal resistance was tested for Cr, Mn, and Zn at concentrations ranging from 0.1 to 25 mM. Grey plots indicate non tested concentrations (nt) for a given metal. Significant differences are indicated (P value < 0.05, *; P value < 0.01, **).

Comparing isolates both from the VS and the NVS samples, Mn and Cr resistance was higher for the VS bacteria at the highest concentrations. The strains with the higher metal tolerance in our study were the *Phyllobacterium* sp WR140 (R01.15), *Phyllobacterium* sp WR140 (R01.34) and *Streptomyces* sp (R04.15), all isolated from the VS fraction. The least tolerant species were isolated from the NVS fraction. The first

more tolerant soil bacterium was the *Streptomyces flavovirens* (U04.24). To our knowledge, Mn resistance in *Phyllobacterium*, or *Streptomyces* has been barely studied. The *Proteobacteria*, although less abundant species in our isolation experiment, appeared to be the most metal tolerant bacteria (4 over 5 more tolerant). There is an abundant literature on Mn-oxidation by bacteria (Adams and Ghiorse, 1985; Wang et al., 2009). A deep-sea Mn-oxidizing bacterium *Brachybacterium* strain could grow in liquid medium supplemented with up to 55 mM MnCl (Wang et al., 2009). Bacterial cells have mechanisms to sense excess metals (Chandrangsu et al., 2017). In general, efflux is the most expedient mechanism for bacteria to deal with excess metal ions. Recent results have identified analogous proteins that mediate the efflux of both and Mn. Some of our isolated bacteria (i.e., *Phyllobacterium* sp WR140) exhibited Mn MIC higher than the *Cupriavidus metallidurans* (6 mM) (Zhao et al., 2012). In a previous study, the proportions of metal-tolerant bacterial isolates were mostly represented by Gram-negatives, *Proteobacteria* (*Pseudomonas* and *Variovorax* species) dominated (Piotrowska-Seget et al., 2005).

One of the first systematic studies on the tolerance to trace element in *Streptomyces* was carried out by Abbes and Edwerds (1990) They evaluated the toxicity of Hg(II), Cd(II), Co(II), Zn(II), Ni(II), Cu(II), Cr(VI), and Mn(II), on 34 *Streptomyces* species representatives of different taxonomic cluster groups. Another study mentioned the isolation of several *Streptomyces* strains with resistance to different trace elements from contaminated areas, and some exhibit multiple tolerances against different metals (Álvarez et al., 2013). Due to the extreme abundance of the genus *Streptomyces* in our study, further characterization of tolerant *Streptomyces* from this red gypsum dump might led to a better assessment / new discovery of the physiological mechanisms involved in metal tolerance in this genus, and hence to ecological applications.

However, we dealt here with the resistance to Mn ionic form, whereas Mn in the Thann soil was mostly as Mn oxides (Zapata, Unpublished results). A recent review also pointed out the use of bacteria in Mn biomining process, mentioning diverse taxonomically dissimilar bacteria that have been reported to reduce manganese either enzymatically or non-enzymatically (Das et al., 2011). The Mn-reducing bacteria include aerobes and facultative anaerobes and Mn may be reduced to satisfy a nutritional need for soluble Mn(II).

Inoculation tests

A set of 20 bacterial strains isolated from the VS was chosen to carry out an inoculation pot experiment. After 3 months of growth, the biomass of birch plants inoculated was analysed (Fig. 26.). Among the 20 isolates, 5 isolates increased significantly the total biomass production (at $P < 0.01$), and an additional set of 6 isolates increased significantly the total biomass production (at $P < 0.05$), as compared with the non-inoculated control. This was mostly due to an increase root biomass, whereas shoot

biomass was only slightly affected (Fig. 26.). We also measured the element in birch leaves and found no significant effect of the inoculated bacteria (data not shown).

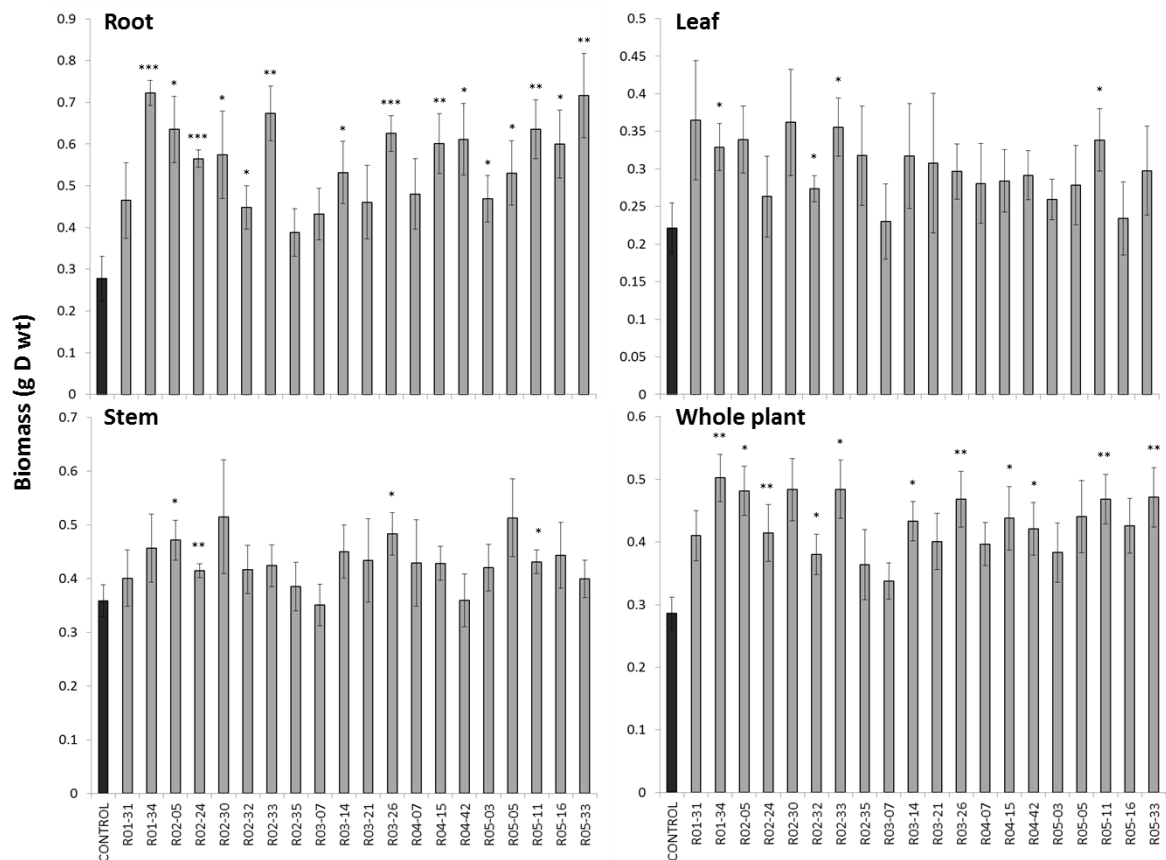


Figure 26. Impact of bacterial inoculation of birch growth.

Birches were grown for 3 months on the Thann soil inoculated with 20 bacterial isolates collected from the VS fraction. Significant differences are indicated (P value < 0.05 , *; P value < 0.01 , **).

Among the isolates tested, the *Phyllobacterium* sp isolate (R01.34) that showed the greatest performance on birch also exhibited multiple plant growth beneficial features including the production of IAA and solubilization of P. It has been widely reported (Chen et al., 2010; Luo et al., 2011; Xinxian et al., 2011) that plant growth-promoting rhizobacteria and bacterial endophytes have great potential to enhance phytoremediation of metal contaminated sites (Burgess et al., 2017). Ma et al. (2013) demonstrate similar performance of *Phyllobacterium myrsinacearumon* on *Sedum plumbizincicola* growth although this strain further increased metal transfer to the shoot part, which was not observed in our study. However another tested *Phyllobacterium* strain in our study did not demonstrate any phytoremediation relevant feature, which indicates intragenera variability. A *Pseudomonas* isolate also significantly increased birch biomass production. Similarly Huang et al. (2016) isolated a Cd-resistant *P. aeruginosa* from a Cd-contaminated oil field and the inoculation of Cd-polluted soil with that strain significantly elevated the shoot and root biomass. The *Variovorax* isolate (R05-11) also exhibited PGP trait in our study. Similarly, the inoculation of

hyperaccumulator plants by *Variovorax paradoxus* isolated from the rhizosphere of these plants resulted in a significant increase of the root biomass (Durand et al., 2018). However, growth promotion was not always linked to functional traits in our study. For instance, the *Streptomyces* (R05-33) showed significant growth promotion effect although without having any significant functional trait. In general, we found that *Streptomyces* strains did not show a great potential in the studied plant growth promoting traits. Other potential traits (such as for example, the release of Volatile Organic compounds (VOCs) which are very well known in the genus *Streptomyces* (Dias et al., 2017) could be responsible of this beneficial effect. The *Olivibacter soli* (R04-07) isolate exhibit all functional traits while having no growth promotion effect. In a greenhouse studies (Alekhya and Gopalakrishnan, 2017), isolates of *Streptomyces* enhanced the plant growth of chickpea by promoting root length and weight, nodule numbers, shoot weight, pod numbers and pod weight over the un-inoculated control, demonstrating the colonizing capability of bacteria belonging to this genera.

Besides *Arthrobacter* and *Rhodococcus* genera, microorganisms of the genus *Streptomyces* have received considerable attention as an effective biotechnological approach to clean up polluted environments. In addition to their metabolic diversity, strains of *Streptomyces* may be well suited for soil inoculation as a consequence of their mycelial growth habit, relatively rapid growth rates, colonization of semi-selective substrates, and their ability to be genetically manipulated (Álvarez et al., 2017). However, most of the studies mentioned in this review paper concerned pesticides-degrading *Actinobacteria*. In the study by Ali (2017), phytoremediation was practiced on TEs rich mines polluted soils, by inoculated with *Streptomyces pactum*. Metal extraction amount results confirmed that Act12 promoted the uptake of TEs in *Brassica juncea* shoot collected from different soils.

4.2.2.6 Conclusions

The present study demonstrates that the vegetated soils from the red gypsum tailings dumps exhibited a higher bacterial diversity as compared to non-vegetated soils. The number of bacterial isolates having the capacity to produce IAA was higher for bacteria from the vegetated soil, whereas siderophore production was higher in bacteria from the non-vegetated soil. Mn and Cr resistance was also higher for bacteria isolated from the VS samples. The potential of some bacterial isolates to promote birch growth was observed, although not always linked to PGP traits. However, the results of the inoculation tests and the dominance of some *Streptomyces* (within the VS Actinobacteria population) and *Phyllobacterium* (within the VS Proteobacteria population) associated to *Betula* growing on this soil could suggest that those bacteria are involved in the early establishment of woody species in the dump. They appear to be good candidates to find new approaches for the management of tailings dumps. *Phyllobacterium* sp isolate (R01.34) and *Streptomyces* (R05-33) appear to be promising alternatives for future inoculum formulation and application on the field.

4.2.2.7 Acknowledgments

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4.2.2.8 Author Contributions

M.C., C.Z. V.A.L. and N.C. planned and designed the research. V.A.L. and C.Z. performed experiments, conducted fieldwork. M.C., V.A.L., C.Z. N.C. and C.G. analysed data and wrote the manuscript.

4.2.2.9 Conflict of Interest Statement

The authors declare no conflicts of interest.

4.2.2.10 Funding

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4.2.2.11 **Supplementary data.**

Supplementary Table 5. Detailed functional traits of bacterial isolates.

Isolate	Code	Group box	IAA	OA	P	SID	Cr (mM)			Mn (mM)			Zn (mM)			
							0.1	0.25	0.5	5	10	25	1	2.5	5	10
Streptomyces sp. 13	R03.14	R01	-	-	-	+	+	+	-	+	-	-	+	+	+	-
Streptomyces bobili	R03.07	R02	+	-	-	+	+	-	-	-	-	-	+	-	-	-
<i>Streptomyces flavofungini</i>	R03.11	R03	-	-	-	-	+	-	-	-	-	-	+	+	-	-
Streptomyces flavofungini	R05.33	R04	-	-	-	-	+	-	-	-	-	-	+	+	-	-
Streptomyces sp. cpRA37	R02.05	R05	-	-	-	-	+	-	-	-	-	-	+	-	-	-
<i>Streptomyces flavofungini</i>	R01.18	R06	-	-	-	-	+	-	-	-	-	-	+	+	+	-
<i>Streptomyces ederenis</i>	R05.34	R07	-	-	-	-	+	-	-	-	-	-	+	-	-	-
<i>Streptomyces ederenis</i>	R05.10	R08	-	-	-	-	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces ederenis</i>	R05.08	R09	-	-	-	-	+	-	-	+	-	-	+	-	-	-
Streptomyces phaeochromogenes	R04.42	R10	-	-	-	-	+	+	-	+	-	-	+	-	-	-
Streptomyces ederenis	R03.21	R11	-	-	-	-	+	-	-	-	-	-	+	+	-	-
Streptomyces sp. SIB_Zn_R12	R05.16	R12	+	-	-	-	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces phaeochromogenes</i>	R05.29	R13	-	-	-	-	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces phaeochromogenes</i>	R02.21	R14	-	-	-	-	+	-	-	+	-	-	+	-	-	-
Streptomyces ederenis	R01.31	R15	-	-	-	-	+	-	-	-	-	-	+	-	-	-
<i>Streptomyces phaeochromogenes</i>	R01.19	R16	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Pedobacter sp	R05.03	R17	+	+	-	-	+	-	-	+	-	-	-	-	-	-
<i>Pseudomonas sp. SJZ</i>	R01.27	R18	+	-	-	-	+	-	-	+	+	-	-	-	-	-
<i>Phyllobacterium myrsinacearum</i>	R02.04	R19	+	+	-	-	+	+	-	+	+	+	-	-	-	-
<i>Olivibacter soli</i>	R04.13	R20	+	+	-	+	+	+	-	+	-	-	+	-	-	-
<i>Olivibacter soli</i>	R05.14	R22	+	+	-	+	+	-	-	+	-	-	-	-	-	-
<i>Phyllobacterium sp. sptzw02</i>	R05.17	R23	+	-	-	-	+	+	-	+	-	-	-	-	-	-
Phyllobacterium sp. sptzw02	R05.05	R24	+	-	-	-	+	+	-	+	-	-	-	-	-	-
<i>Phyllobacterium sp. CCB AU 83356</i>	R04.29	R25	+	-	+	-	+	-	-	+	+	+	+	+	-	-
Olivibacter soli	R04.07	R26	+	+	-	+	+	-	-	+	-	-	-	-	-	-
<i>Rhizobium radiobacter</i>	R05.22	R27	+	-	+	-	+	+	-	+	+	+	+	+	+	-
<i>Olivibacter soli</i>	R01.28	R28	+	+	-	+	+	-	-	+	-	-	-	-	-	-
<i>Phyllobacterium myrsinacearum</i>	R05.06	R29	+	-	-	-	+	-	-	+	+	+	-	-	-	-
<i>Phyllobacterium myrsinacearum</i>	R05.02	R30	+	-	-	-	+	-	-	+	+	+	-	-	-	-
<i>Pseudarthrobacter sp</i>	R04.26	R31	-	+	-	+	+	+	-	+	+	+	+	-	-	-
Pseudomonas sp. Q71576	R02.33	R32	-	-	+	-	+	-	-	+	+	+	-	-	-	-
<i>Phyllobacterium myrsinacearum</i>	R02.22	R33	+	-	+	+	+	+	-	+	+	+	-	-	-	-
Phyllobacterium sp. WR140	R01.34	R35	+	-	+	-	+	-	-	+	+	+	+	+	+	-
<i>Phyllobacterium sp. sptzw02</i>	R05.24	R36	+	-	-	-	+	+	-	+	-	-	-	-	-	-
<i>Arthrobacter sp. HBUM179104</i>	R02.34	R37	+	+	-	+	+	+	-	+	-	-	-	-	-	-
<i>Arthrobacter sp</i>	R02.14	R38	-	+	-	+	+	+	-	+	-	-	-	-	-	-
<i>Phyllobacterium sp. WR140</i>	R01.15	R39	-	-	+	+	+	+	-	+	+	+	+	+	+	+
<i>Olivibacter soli</i>	R05.15	R40	+	-	-	-	+	-	-	+	+	-	-	-	-	-
Variovorax sp. LZA10	R05.11	R41	+	+	-	-	+	-	-	+	-	-	-	-	-	-
Sinorhizobium sp. S242	R02.32	R42	-	-	-	-	+	-	-	+	+	+	-	-	-	-
<i>Pseudomonas reinekei</i>	R05.13	R43	-	-	-	-	+	+	-	+	+	-	-	-	-	-
Bacillus megaterium	R02.30	R44	-	-	-	+	+	+	-	+	+	+	-	-	-	-
Rhodococcus rhadochrous	R02.35	R45	-	-	-	-	+	+	-	+	-	-	+	+	-	-
Rhizobium sp. M20	R02.24	R46	+	-	-	-	+	-	-	+	-	-	-	-	-	-
<i>Pedobacter sp. V48</i>	R03.15	R47	-	+	-	-	+	-	-	+	-	-	-	-	-	-
<i>Phyllobacterium sp. WR140</i>	R01.24	R48	-	-	+	-	+	-	-	+	+	+	-	-	-	-

The bold isolates are those selected for the inoculation experiment (Figure 26.).

Supplementary Table 6. Detailed functional traits of bacterial isolates.

Isolate	Code	Group box	IAA	OA	P	SID	Cr (mM)			Mn (mM)			Zn (mM)			
							0.1	0.25	0.5	5	10	25	1	2.5	5	10
<i>Amycolatopsis roodepoortensis</i>	U04.23	U01	-	-	-	+	+	-	-	+	+	+	+	+	-	-
<i>Streptomyces sp. RE2</i>	U04.15	U02	-	-	-	-	+	-	-	-	-	-	+	+	-	-
<i>Streptomyces flavofungini</i>	U05.06	U03	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Streptomyces lomondensis</i>	U04.13	U04	-	-	-	-	+	-	-	-	-	-	+	+	+	-
<i>Streptomyces bobili</i>	U04.11	U05	-	-	-	+	+	-	-	+	-	-	+	-	-	-
<i>Streptomyces sp. AS34</i>	U01.09	U06	+	-	+	+	+	-	-	+	-	-	+	+	+	-
<i>Paenarthrobacter aurescens</i>	U04.31	U07	-	+	-	+	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces bobili</i>	U03.09	U08	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Paenarthrobacter aurescens</i>	U04.32	U09	-	+	-	+	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces sp. RE2</i>	U01.18	U10	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Nocardioides kribbensis</i>	U04.06	U11	-	-	-	+	+	-	-	+	+	-	-	-	-	-
<i>Streptomyces flavovirens</i>	U04.24	U12	-	-	-	+	+	-	-	+	+	-	+	+	+	-
<i>Pseudomonas moraviensis</i>	U04.16	U13	+	-	+	-	+	-	-	+	-	-	-	-	-	-
<i>Paenarthrobacter nitroguajacolicus</i>	U05.28	U14	-	+	-	+	+	-	-	+	-	-	+	-	-	-
<i>Arthrobacter sp</i>	U04.18	U15	-	+	-	+	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces sp. K56(2011)</i>	U04.01	U16	-	-	-	+	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces bobili</i>	U02.10	U17	-	-	-	+	+	-	-	+	-	-	+	-	-	-
<i>Pseudarthrobacter sp</i>	U02.14	U18	+	-	+	+	+	-	-	-	-	-	-	-	-	-
<i>Streptomyces sp. RE2</i>	U02.09	U19	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Pseudarthrobacter sp</i>	U05.25	U20	+	-	+	+	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces bobili</i>	U01.16	U21	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Arthrobacter sp. Tibet-YD4524-4</i>	U01.12	U22	+	+	+	+	+	+	-	-	-	-	-	-	-	-
<i>Streptomyces flavovindis</i>	U05.17	U23	-	-	-	-	+	-	-	-	-	-	+	-	-	-
<i>Streptomyces bobili</i>	U05.08	U24	-	+	-	+	+	-	-	-	-	-	-	-	-	-
<i>Streptomyces sp. Ds10</i>	U05.18	U25	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Paenarthrobacter aurescens</i>	U04.10	U26	-	+	-	+	+	+	-	+	-	-	-	-	-	-
<i>Pseudarthrobacter sp</i>	U03.11	U27	-	-	-	+	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces phaeochromogenes</i>	U05.22	U28	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Streptomyces bobili</i>	U03.07	U29	+	-	+	-	+	-	-	+	-	-	+	-	-	-
<i>Streptomyces bobili</i>	U02.16	U30	-	-	-	+	+	-	-	+	+	-	+	-	-	-
<i>Streptomyces sp. VTT E-052903</i>	U05.15	U31	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Nocardia sp.</i>	U02.06	U32	-	-	-	-	+	-	-	+	-	-	-	-	-	-
<i>Phyllobacterium sp. CCB AU 83356</i>	U05.34	U33	+	-	+	-	+	-	-	+	-	-	-	-	-	-
<i>Amycolatopsis coloradensis</i>	U05.11	U34	-	-	-	+	+	-	-	+	+	+	+	-	-	-
<i>Amycolatopsis sp. K6-08</i>	U05.24	U35	-	-	-	+	+	-	-	+	+	+	+	+	-	-
<i>Pseudarthrobacter sp</i>	U05.12	U36	-	-	-	+	+	-	-	-	-	-	-	-	-	-
<i>Streptomyces sp. RE2</i>	U02.17	U37	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Streptomyces bobili</i>	U05.09	U38	-	-	-	+	+	-	-	+	-	-	+	-	-	-
<i>Streptomyces bobili</i>	U02.05	U39	-	-	-	+	+	-	-	+	-	-	+	-	-	-
<i>Streptomyces bobili</i>	U01.03	U40	-	-	-	-	+	-	-	+	-	-	+	-	-	-
<i>Paenarthrobacter aurescens</i>	U04.17	U41	-	+	-	+	+	+	-	+	+	-	-	-	-	-
<i>Streptomyces lomondensis</i>	U05.33	U42	-	-	-	-	+	-	-	+	-	-	-	-	-	-
<i>Streptomyces sp. 19504</i>	U01.02	U43	-	-	-	-	+	-	-	+	-	-	-	-	-	-

4.3 Étude par métabarcoding environnemental des communautés microbiennes associées aux racines des ligneux d'un terril de déchets industriels issus de l'activité extractive du titane

4.3.1 Résumé

*Dans cette étude, nous avons caractérisé les communautés microbiennes associées aux racines de *Betula pendula*, qui constitue la végétation pionnière du terril de gypse rouge de Thann (NE France), issu de l'activité d'extraction du titane (Société Cristal France). Le séquençage du gène de l'ARNr 16S bactérien (régions V3-V4) et des gènes codant pour les ITS fongiques (région comprenant les espaceurs internes transcrits ITS1 et ITS2) ont été réalisés sur une plate-forme Illumina MiSeq. Les racines de *B. pendula* ont été récoltées dans deux endroits bien différenciés : au centre du terril (où poussent uniquement *B. pendula*) et en bordure des parcelles d'étude où d'autres espèces végétales mixtes cohabitent (*B. pendula*, *Populus nigra*, *P. tremula* et *Salix purpurea*) ont également été échantillonnées à des fins de comparaison. Les résultats ont clairement montré que les communautés bactériennes associées aux racines de *B. pendula* qui poussaient au centre du terril étaient significativement différentes de celles des autres échantillons récoltés sur les espèces ligneuses des parcelles mixtes. En revanche, les communautés bactériennes des différentes espèces ligneuses (y compris du bouleau) n'étaient pas différentes sur les parcelles mixtes. La communauté bactérienne était dominée par les phyla Proteobacteria (38%), Actinobacteria (35%) et Bacteroidetes (20%). Les trois familles les plus abondantes sont les Streptomycetaceae (16%), Cytophagaceae (19%) et Hyphomicrobiaceae (15%). Seules 33 OTU (sur les 1 322) présentaient une abondance plus élevée de 0,5%, mettant en évidence la présence d'OTU très rares dominant la communauté. Les OTUs les plus abondantes appartiennent à la famille des Streptomycetaceae (OTU00001, 14,9%). Cette OTU présentait l'abondance la plus élevée dans les racines de *B. pendula* et de *S. purpurea*, bien qu'elle ait été uniformément distribuée aux racines de toutes les espèces et de toutes les parcelles végétalisées. La communauté fongique était dominée par Ascomycota (60%) suivie de Basidiomycota (30%). La famille la plus abondante était Pyronemataceae (33%), suivie des Russulaceae (15,5%). La communauté fongique était clairement dominée par l'OTU 25_92637 appartenant à l'espèce *Geopora arenicola* (31,8% de l'ensemble des données). Cette OTU était présente en abondance dans les racines de toutes les plantes étudiées; son abondance était légèrement plus élevée dans les racines de *B. pendula* au centre du terril. Les OTUs bactériennes caractéristiques de la présence du bouleau au centre incluaient une OTU appartenant à la famille des Gammaprotéobactéries, une appartenant à la famille des Cytophagales, 3 à la famille des Rhizobiales, une appartenant à la famille des Streptomycetaceae et une appartenant à la famille des Hyphomicrobiacées, genre *Rhodoplanes* et une au genre *Catellatospora*. Dans le cas des champignons, les OTUs indicatrices appartenaient à l'espèce *Oidiodendron maius*, au genre *Russula* et à l'ordre Agaricales et Hypocreales.*

4.3.2 Study of the root-associated microbial communities of plants growing at a red gypsum dump using environmental metabarcoding

4.3.2.1 Authors

Vanessa Álvarez-López, Cyril Zappellini, Alexis Durand, Damien Blaudez, Michel Chalot

4.3.2.2 Abstract

Here, we characterised the root associated microbial communities of *Betula pendula*, (which is the pioneering vegetation at the red gypsum tailings dump of Thann (NE France), though sequencing of the V3-V4 domains of 16S rRNA genes and the fungal internal transcribed spacer 1-2 (ITS1-2) on an Illumina MiSeq platform. Roots of *B. pendula* were collected in two well differentiated locations: 1) at the centre of the dump (where are only plant species growing) and 2) around the dump edges where other plant species (*Populus nigra*, *P. tremula* and *Salix purpurea*) were also sampled for comparative purposes. Results showed clearly that the bacterial community associated to roots of the pioneer trees of *B. pendula* growing at the centre of the dump were significantly dissimilar to all the other plants. On the other hand, the root bacterial communities of different plants species growing at the same sampling points (at the edges) did not differ one from each other. The bacterial community was dominated by the Proteobacteria (38%), Actinobacteria (35%) and Bacteroidetes (20%) phylum. The three most abundant identified families were Streptomycetaceae (16%), Cytophagaceae (19%) and Hyphomicrobiaceae (15%). Only 33 OTUs (from the 1,322) presented a higher abundance of 0.5%, highlighting the presence of very rare OTUs dominating the community. The most abundant OTU belong to the family Streptomycetaceae (OTU00001, 14.9%). This OTU presented the highest abundance in roots of *B. pendula* and *S. purpurea*, although it was uniformly distributed at the roots of all the plant species and locations. The fungal community was dominated by Ascomycota (60%) followed by Basidiomycota (30%). The most abundant identified family was Pyronemataceae (33%), followed by Russulaceae (15.5%). Fungal community was clearly dominated by the OTU 25_92637 belonging to *Geopora arenicola* species (31.8% of the total dataset). This OTU was present in high abundance in roots of all the studied plants however; its abundance was slightly higher in the roots of *B. pendula* at the centre. Bacterial OTUs indicators of the presence of birch at the centre included one OTU belonging to the class Gammaproteobacteria, one belonging to the family Cytophagales, 3 to the family Rhizobiales, one belonging to the family Streptomycetaceae and one belonging to the family Hyphomicrobiaceae, one to the genus *Rhodoplanes* and one to the genus *Catellatospora*. In the case of fungi, indicator OTUs belonged to the species *Oidiodendron maius*, the genus *Russula*, and the order Agaricales and Hypocreales.

4.3.2.3 Introduction

Gentle Remediation Options (GRO) are focused on the use of plants in combination with their associated microorganisms for the remediation and revitalisation of contaminated sites. Soil toxicity can severely limit the performance and establishment of the plants in polluted areas. The selection of metal-tolerant plant species able to successfully establish and growth at each specific site is therefore of vital

importance for the success of GRO. Metal(loid) toxicity can severely limit the performance and establishment of the plants, and the selection of TE-tolerant plant species is therefore vital for the successful implementation of GRO in metalliferous sites. However, plants must also tolerate numerous additional abiotic and biotic stress factors, such as water and nutrient deficiency, soil acidity or salinity, soil erosion or compaction, flooding, herbivory or pests. TE-contaminated sites (such as mine tailings and spoils) are important sources of metal-tolerant plant genotypes. Despite the unfavourable growth conditions at these sites, several metallophyte plants have evolved mechanisms to tolerate these conditions and are able to colonise these types of substrates (Batty, 2005; Mendez and Maier, 2008; Whiting et al., 2004). Within the same plant species various ecotypes, cultivars, varieties or clones can differ greatly in their response to the presence of contaminants (Marmiroli et al., 2011; Ruttens et al., 2011; Vyslouzilova et al., 2003). For phytoextraction plants must be able to tolerate and accumulate high concentrations of TEs in their harvestable parts and have a reasonably high biomass production. Fast growing trees are ideal plant species due to their extensive root systems, rapid growth, large biomass production and easy harvesting with subsequent re-sprouting (Peuke and Rennenberg, 2005).

The understanding of the mechanisms which influence the response of the plants to the abiotic factor frequently found at these sites could help to the improvement of the efficiency of GRO techniques. Although an increasing number of studies have been addressed to understand the influence of the associated microbial community in plant metal tolerance, its role is still not completely unravelled. Metalliferous sites are an important source of metal-tolerant plant genotypes and microorganisms. Plants interact closely with microbes, and these can enhance plant growth and health by increasing nutrient uptake and improving plant resistance to pathogens and stress (Kidd et al., 2017; Weyens et al., 2009). Plant growth promoting bacteria (PGPB) include nitrogen-fixing bacteria, biocontrol microorganisms or mycorrhizal fungi, among others (Coninx et al., 2017; Kidd et al., 2017). Plant associated microorganisms can also improve nutrient availability thus the production of organic acids and siderophores or P solubilisation. Bacteria can also produce phytohormones, such as auxins, cytokinins and gibberellins, which stimulate plant growth and development (Taghavi et al., 2009; Tanimoto, 2005). Plant associated bacteria can also reduce plant stress through the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which reduces the level of ethylene in stressed plants (Glick, 2014). Other well-known mechanisms of PGPBs include increasing plant water uptake, alteration of root morphology, production of antibiotics and the induction of plant defence mechanisms (Kidd et al., 2009; van Loon and Bakker, 2003).

Fungal endophytes also represent key players, as highlighted by (Lacercat-Didier et al., 2016), where three isolates belonging to the Helotiales order and the *Serendipita vermifera* species were highly tolerant to metals (Cd, Zn, Pb, and Cu). Root-associated

arbuscular mycorrhizal fungi (AMF) usually improve the growth of plants in a non-fertile soil by globally increasing the exchange surface between plant and soil (Smith et al., 1996). AMF have been mostly studied for their role in plant growth, thanks to the higher phosphorus uptake in soils (Smith and Read, 2002). Other key soil microorganisms such as saprotrophic fungi are central in forest soils due their capacity to degrade recalcitrant organic compounds and may strongly interact with soil bacteria (Baldrian, 2008). Saprotrophic fungi may also have a potential in mobilizing metals from contaminated soils, due to their potential to exudate chelating agents (Arwidsson et al., 2009). Further interactions between bacteria, fungi and plants have been shown to contribute to microbial community stability (Bell et al., 2014; Bonfante and Anca, 2009) and many bacterial strains have indeed been reported to promote mycorrhizal symbioses (Frey-Klett et al., 2007; Hrynkiewicz et al., 2010).

Here, we characterised the soil-plant-system of *Betula* spp., which is the pioneering vegetation at the red gypsum tailings dump of Thann (NE France). The dump contains the by-products of the extraction of TiO₂, mainly enriched in S, Fe and Mn. One of the major ecological concerns around this site is the reduction of dust emission enriched in trace metals, and therefore an adequate vegetation cover is essential. At this site, *Betula* is the only species which is successfully established across the whole dump while other woody species showed only sparse growth at some specific spots. Moreover, *Betula* spp. trees growing at this site have been shown in previous studies to efficiently accumulate Mn and therefore this species is a promising candidate for their use as biocatalyst. The objective of this study is to analyse the structure of the root microbial communities associated to *Betula* spp. trees and to elucidate the possible plant-microbial community responsible for the high adaptability to the soil conditions of *B. pendula* trees at this site. For this purpose we combined highly novel high-throughput sequencing technologies (Illumina next-generation sequencing)

In this study, we combined highly novel high-throughput sequencing technologies, data analysis approaches and soil parameter analyses to characterize the taxonomic distribution of root and soil microbiomes, and reveal the relations between microbiomes, soils and plant species colonizing the mine dumps.

4.3.2.4 **Material and methods**

Study site

The study site of this proposal is located at Thann (NE of France). Cristal France is a major international producer of titanium dioxide TiO₂ (an important pigment) and TiCl₄ (an excellent Lewis acid catalyst). The Thann plant produces TiO₂ through a sulfate-based technology, a wet chemical process that uses sulfuric acid to extract and purify TiO₂ in its crystal form. Production of TiCl₄ is based on the use of chlorine process with production of acidic effluents with a high load in trace metals. The extractive industry led to the production of by-products, such as red gypsum that are stored on large and

open landfills.

Red gypsum is produced by the neutralization of waste-waters with limestone and is characterized by high Mn and Fe concentrations and elevated pH (Asad et al., 2017). *Betula pendula* was found to be the dominant (> 80%) plant species that had naturally recolonized the landfill after the deposit of red gypsum ceased in 2006.

Sampling procedure

All soil and root samplings were performed in October 2016. Roots and shoots of *Betula* spp. were collected across the whole mine dump. For comparative purposes other woody species (mainly *Populus* and *Salix* spp.) found at the site were sampled at three specific spots (Fig. 27). Briefly, 10 individual plants of *B. pendula* were sampled at the centre of the dump (named as *B. pendula*) where this is mainly the only plant growing, 10 individual plants of *Populus tremula* (named as *P. tremula*), *P. nigra* (named as *P. nigra*) and *Salix purpurea* (named as *S. purpurea*) were sampled at the edges of the dump. At the same sampling points 10 individual of *B. pendula* growing together with these plants at the edges were also collected and named as *B. pendula*-(Pt) for those growing with *P. tremula*, *B. pendula*-(Pn) for those growing with *P. nigra* and as *B. pendula*-(Sp) for those growing with *S. purpurea*. These plant combinations were growing always at the same point and only one soil sample was taken for each couple.

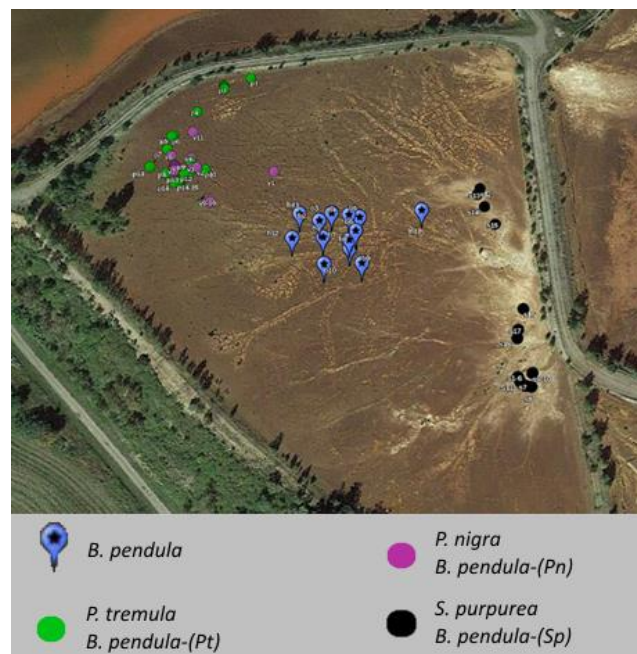


Figure 26. Plant species and locations sampled.

Betula pendula was sampled at the center of the dump and is named as *B. pendula*. Other plants of *B. pendula* growing at the edges were sampled at the same spots where plants of *P. tremula*, *P. nigra* and *S. purpurea* were found growing and were named respectively as *B. pendula* (Pt), *B. pendula* (Pn) and *B. pendula* (Sp). Only one soil sampling point was carried out for each plant pair.

DNA was extracted from roots of 10 single individual trees from each plant species at each sampling point. Sequencing of the V3-V4 domains of 16S rRNA genes and the fungal internal transcribed spacer 1-2 (ITS1-2) was performed on an Illumina MiSeq platform. 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 colour. The fine roots were then cut off and transferred to Eppendorf tubes and frozen at -20°C. The letter codes are as follows:

Soil and plant physicochemical properties

Supplementary Table 5 and Supplementary Table 6 present the main soil properties and leaf macro- and micro- nutrients used in later statistical analysis as environmental variables. It can be noted that in the case of plants growing at the edges a single soil sample was taken that plants of both species were always growing at the same sampling spot. Briefly, soils present a pH strongly alkaline (between 7.8 up to 9.9) due to the use of CaSO₄ as neutraliser of highly acidic metal-enriched effluents obtained after Ti extraction). The main CaCl₂-extractable elements found at the dump were Mg > Fe > K > Mn > P > Cu. A relation between soil pH and CaCl₂-extractable elements is clearly observed. Supplementary Table 6 evidences the good Mn accumulation capacity of *B. pendula*. These plants showed the highest levels of Mn leaf concentration (up to 570 mg kg⁻¹). Moreover, this accumulation was independent of the site of growing and even on soil pH and CaCl₂-extractable Mn. Plants of birch also tended to accumulate higher P concentration at their leaves than the other plant species growing at the same sampling points.

Molecular methods

To isolate microbial DNA from the roots, plant cell lysis was first performed using 50 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 IMM400; Retsch Inc., Newtown, PA). Root DNA was then extracted with a PowerSoil® DNA Isolation Kit (MO-BIO laboratories, Inc., Carlsbad, CA USA) according to the manufacturer's instructions. To improve DNA extraction, an additional step of 10 min at 60°C was realised after addition of C1 solution. The DNA quality and quantity were assessed by agarose gel electrophoresis and with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) using an FLX-Xenius spectrofluorometer (SAFAS, Monaco). The sequencing of the V3-V4 domains of 16S rRNA genes and the fungal ITS1-2 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 802R (5'- GAC TAC HVG GGT ATC TAA TCC -3') and the fungal primers ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A -3') and ITS2 (5'- GCT GCG TTC TTC ATC GAT GC -3').

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. 16S reads were pre-clustered using sumacust (Mercier et al., 2013) at 0.99 identity. Only sequences with at least 8 reads were retained. The retained 16S sequences were aligned with those present in the Silva database to remove non-16S sequences, and plant DNA contamination was removed by suppressing reads identified as k__Bacteria (100), p__Cyanobacteria (100), c__Chloroplast (100), o__Streptophyta (100), and unclassified (100). Taxonomic assignments were made using a Bayesian approach (Wang et al., 2007) with the Greengenes database (DeSantis et al., 2006). Finally, OTUs were derived using the Needleman distance and average neighbor clustering at a distance of 0.03.

Sequence de-multiplexing and bioinformatics processing of the datasets were performed using the PIPITS pipeline (Gweon et al., 2015). PIPITS is an automated bioinformatics pipeline dedicated for fungal ITS sequences which incorporates ITSx to extract subregions of ITS and exploits the latest RDP Classifier to classify sequences against the curated UNITE fungal dataset. Briefly, all raw read pairs were joined at the overlapping region and then quality filtered, chimera filtered, singleton filtered, contaminant filtered, merged, and clustered into OTUs, defined at 97% sequence similarity. We excluded singleton OTUs to avoid technical artefacts and overestimation of the number of species (Dickie, 2010; Tedersoo et al., 2010). The taxonomic assignment of OTUs was performed using the UNITE (Kõljalg et al., 2013) database at a 97% similarity threshold.

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.

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. 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. Indicator species analysis was performed using the *multipat* function of the *indicspecies* package in R (version 1.7.1) (De Cáceres and Legendre, 2009).

4.3.2.5 Results

Root bacterial and fungal species richness and diversity across seven four different plant species

Following total genomic DNA extraction from root samples, amplicons of the 16S region were generated and a total of 5,262,333 paired-end reads were obtained through Illumina MiSeq sequencing (Supplementary Table 4.2-3.). Among the samples from each plant, those exhibiting a low sequence count were eliminated from the rest of the analysis. Thus, a total of 907,006 filtered and non-chimeric bacterial sequences constituted our final processed dataset, spread among 1,325 non-singleton OTUs defined by representative DNA sequences with sizes of 231 to 257 bp (mean = 237 bp). After subsampling, dataset was rarefied to 6,972 reads per sample, which were distributed in 1,322 non-singletons OTUs. These reads were found to be associated with 16 bacterial phyla, 38 classes and 79 bacterial families. Amplicons of the ITS region were generated and a total of 5,581,697 paired-end reads were obtained (Supplementary Table 10.). Again, plant samples exhibiting a low sequence count were eliminated and a total of 5,148,296 filtered and non-chimeric fungal sequences constituted the final processed dataset, spread among 613 non-singleton OTUs defined DNA sequences with sizes above to 100 bp. After subsampling, dataset contained 28,129 reads per sample, distributed in 577 non-singletons OTUs. These reads were found to be associated with 5 fungal phyla, 21 classes and 98 fungal families.

Rarefaction curve analysis, which assesses OTUs richness as a result of sampling, showed that all samples approached an asymptote either in bacterial and also fungal dataset, revealing that the overall bacterial diversity was well represented (Supplementary Fig. 4.). Moreover, the measured Good's coverage values (an estimator of completeness of sampling) were greater than 98% for each sample type in the case of bacterial dataset, and 100% for the fungal dataset, highlighting good overall sampling (Supplementary Table 9. and 10. respectively).

Richness, and diversity estimates were calculated for each dataset (Table 6. and Table 7.). Both bacterial richness estimations (based on Chao1 estimator and number of observed OTUs) and bacterial diversity indexes (estimated through the Shannon Index, Inverse Simpson Index and Evenness) were generally lower in the roots of the *B. pendula* plants growing at the centre of the dump than in the other plants (both, other species such as *Salix* or *Populus* or the other *B. pendula* growing at the edges of the dump). These differences were more marked (and in general only significant) in the richness indexes and between roots of *B. pendula* growing at the centre and roots of *Salix* and *Populus*. The highest bacterial richness and diversity indexes were found associated with *P. tremula* (Table 6.). Richness and diversity fungal indexes (Table 7.) followed the same tendency as in bacteria and were generally lower in the roots of the *B. pendula* growing at the centre of the dump. Both richness indexes were highest in the roots of all the birch growing at the edges.

Effects of plant species and growing location in microbial structure and composition

The bacterial dataset was composed by a total of 17 phylum, 151 families, and 246 genera (17, 79 and 100 identified respectively). The bacterial community was dominated by Proteobacteria (38%), Actinobacteria (35%) and Bacteroidetes (20%). Only 26 (20 identified) families and 31 (14 identified) genera presented an abundance higher to 0.5%. The three most abundant identified families were Streptomycetaceae (16%), Cytophagaceae (19%) and Hyphomicrobiaceae (15%) (Fig.28.) and the three most abundant identified genera were *Lentzea* (7.11%), *Devosia* (5.52%) and *Rhodoplanes* (3.33%). The roots of *B. pendula* showed a slightly enrichment in members belonging to the genera *Kribella*, *Rhodoplanes* and *Methylibium* compared to the other plants (data not shown). The fungal dataset was composed by a total of 7 phylum, 138 families, and 211 genera (5, 98 and 128 identified respectively). The fungal community was dominated by Ascomycota (60%) followed by Basidiomycota (30%). Only 18 (4 identified) families and 19 (5 identified) genera presented an abundance higher to 0.5%. The most abundant identified family was Pyronemataceae (33%), followed by Russulaceae (15.5%). The next identified family was Thelephoraceae, although present in a much lower abundance (2.0%) (Fig. 29.). The most abundant genera were *Geopora* (33.1%), *Lactarius* (15.1%) and *Tomentella* (2.0%). Roots of *B. pendula* showed a slight increase in members of the genera *Oidiiodendron* and *Geopora* (data not shown).

Table 6. Richness and diversity indexes for the bacterial communities of the different plants.

	<i>B. pendula</i>	<i>P. tremula</i>	<i>B. pendula</i> (P.t)	<i>P. nigra</i>	<i>B. pendula</i> (P.n)	<i>S. purpurea</i>	<i>B. pendula</i> (S.p)
Mean number of sequences per sample	13,317	9,912	13,024	13,907	12,163	11,973	16,405
Subsample size	6,972	6,972	6,972	6,972	6,972	6,972	6,972
N° observed OTUs	257 ± 42 d	345 ± 31 a	310 ± 32 bc	329 ± 26 ab	292 ± 36 cd	316 ± 59 abc	287 ± 59 cd
Chao estimation	377 ± 70 d	505 ± 46 a	464 ± 62 abc	483 ± 42 ab	422 ± 48 cd	450 ± 94 bcd	443 ± 107 bcd
Shannon Index (H)	3.19 ± 0.31 c	3.73 ± 0.33 a	3.47 ± 0.38 abc	3.54 ± 0.24 ab	3.44 ± 0.31 abc	3.46 ± 0.49 abc	3.36 ± 0.42 bc
Inverse Simpson Index (1/D)	10.5 ± 2.8 b	16.2 ± 7.3 a	12.2 ± 5.0 ab	14.1 ± 3.6 ab	13.5 ± 5.3 ab	12.6 ± 5.4 ab	11.9 ± 4.5 ab
Shannon Index Evenness E	0.58 ± 0.04 b	0.64 ± 0.05 a	0.61 ± 0.06 ab	0.61 ± 0.04 ab	0.61 ± 0.04 ab	0.60 ± 0.07 ab	0.59 ± 0.06 ab

All diversity indexes were calculated using an OTU threshold of ≥ 97% sequence similarity on randomly sub-sampled data at the lower sample size (6,972 reads). Richness was calculated using the number of OTUs and Chao1 estimators. Diversity was estimated using the Shannon-Wiener (H), Inverse Simpson (1/D), and Shannon Index Evenness (E) indexes. Mean values and standard deviations (mean ± SD) are provided for the root samples. Values designated with the same letters were not significantly different (Kruskal Wallis test, p<0,05).

Table 7. Richness and diversity indexes for the fungal communities of the different plants.

	<i>B. pendula</i>	<i>P. tremula</i>	<i>B. pendula</i> (Pt)	<i>P. nigra</i>	<i>B. pendula</i> (P.n)	<i>S. purpurea</i>	<i>B. pendula</i> (S.p)
Mean number of sequences per sample	81,989	65,652	64,469	74,027	74,192	70,200	84,301
Subsample size	28,129	28,129	28,129	28,129	28,129	28,129	28,129
N° observed OTUs	52 ± 13 d	79 ± 16 ab	84 ± 14 a	58 ± 14 cd	70 ± 20 bc	70 ± 18 bc	79 ± 14 ab
Chao estimation	72 ± 24 b	103 ± 18 a	105 ± 23 a	81 ± 25 b	89 ± 25 ab	94 ± 30 ab	102 ± 18 a
Shannon Index (H)	1.33 ± 0.37 c	1.80 ± 0.30 ab	1.97 ± 0.36 a	1.56 ± 0.30 bc	1.56 ± 0.56 bc	1.52 ± 0.34 bc	1.66 ± 0.55 abc
Inverse Simpson Index (1/D)	2.96 ± 1.05 b	3.78 ± 1.15 ab	4.33 ± 1.93 a	3.31 ± 0.93 ab	3.74 ± 2.50 ab	3.18 ± 0.98 ab	3.88 ± 2.16 ab
Shannon Index Evenness E	0.34 ± 0.09 b	0.41 ± 0.06 ab	0.44 ± 0.07 a	0.39 ± 0.07 ab	0.37 ± 0.11 b	0.36 ± 0.07 b	0.38 ± 0.11 ab

All diversity indexes were calculated using an OTU threshold of $\geq 97\%$ sequence similarity on randomly sub-sampled data at the lower sample size (28,129reads). Richness was calculated using the number of OTUs and Chao1 estimators. Diversity was estimated using the Shannon-Wiener (H), Inverse Simpson (1/D), and Shannon Index Evenness (E) indexes. Mean values and standard deviations (mean \pm SD) are provided for the root samples. Values designated with the same letters were not significantly different (Kruskal Wallis test, $p < 0,05$). Figure 28. and Figure 29. shows a clear similar distribution of families (both bacterial and fungal) between the couples of neighbouring plants (*P. tremula* - *B. pendula* (Pt), *P. nigra* - *B. pendula* (Pn) and *S. purpurea* - *B. pendula* (Sp)). However, due to the high data scattering few of those results were significant. In the case of bacterial dataset the statistical differences were mainly associated to the plants of *P. nigra* and the associated birch (*B. pendula* (Pn)). These plants harboured a higher number of members of the families Actinosynnemataceae (Kruskal Wallis $\chi^2=37.9$, $p=1.2 \times 10^{-6}$), Glycomycetaceae (Kruskal Wallis $\chi^2=34.61$, $p=5.2 \times 10^{-6}$) and Comamonadaceae (Kruskal Wallis $\chi^2=20.6$, $p=2.1 \times 10^{-3}$) and lower members of the families Pseudonocardiaceae (Kruskal Wallis $\chi^2=32.4$, $p=1.4 \times 10^{-5}$) and Methylocystaceae (Kruskal Wallis $\chi^2=39.2$, $p=6.6 \times 10^{-7}$) than all the other plants. In the case of the fungal dataset the statistical differences were observed only for two families and in this case the plants that showed a higher difference on this community in comparison with the others were *P. tremula* and its associated birch (*B. pendula* (Pt)). Those plants showed a lower proportion of the fungal family Pyronemataceae (Kruskal Wallis $\chi^2=24.4$, $p=4.4 \times 10^{-4}$) and a higher number of associated Sebacinaceae (Kruskal Wallis $\chi^2=20.5$, $p=2.3 \times 10^{-3}$).

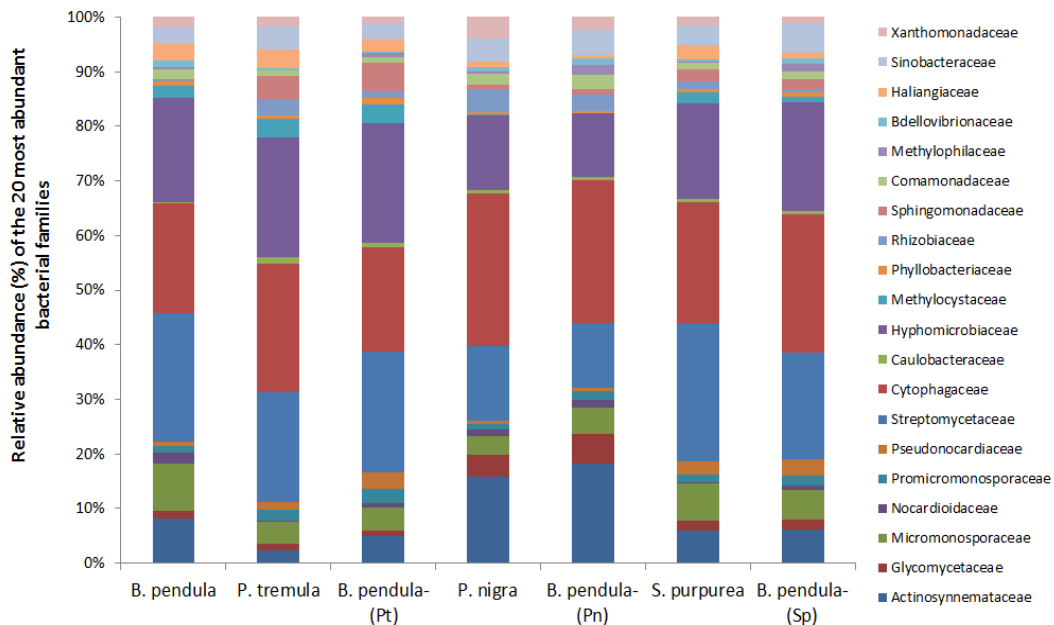


Figure 27. Composition of the bacterial communities from the different roots at the family level.

Only the 20 most abundant identified families are presented.

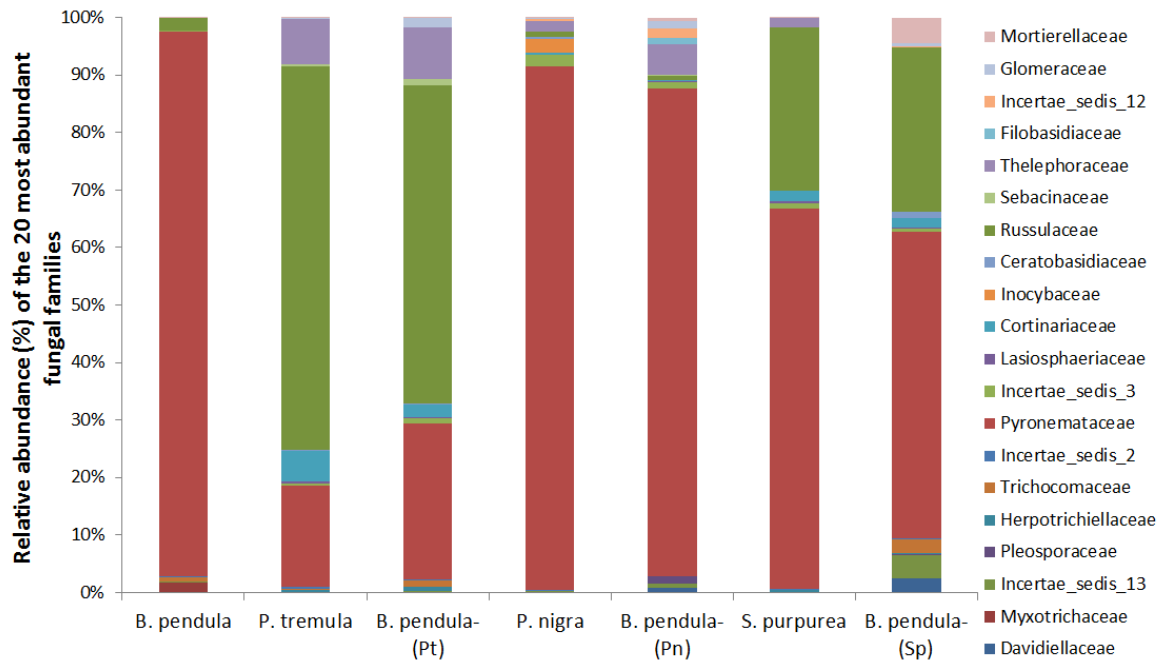


Figure 28. Composition of the fungal communities from the different roots at the family level.

Only the 20 most abundant identified families are presented.

Table 3 shows the result of the ANOSIM test which analyses the values of dissimilarity of bacterial communities between samples. A significant dissimilarity ($p < 0.05$) is observed between all the plant species growing at the dump. Both, when comparing *B. pendula* from the centre with the other plant species (*Salix* and *Populus*) or with the other *B. pendula* growing at the different locations a strong and significant dissimilarity in their bacterial communities were observed. However, plants of *B. pendula* growing at the edges of the dump *B. pendula* (Pt), *B. pendula* (Pn) and *B. pendula* (Sp) did not show a significant dissimilarity in their root bacterial communities to those of their neighbour plants (*P. tremula*, *P. nigra* and *S. purpurea* respectively). Contrary, the dissimilarity analysis between different root fungal communities (ANOSIM, Table 8.) did not show a clear pattern between the different plant species and/or locations. The highest dissimilarity was found between *B. pendula* in the centre with either *B. pendula* (Pt) ($R^2=0.621$, $p=0.001$) or with *P. tremula* ($R^2=0.804$, $p=0.001$). Also a high and significant dissimilarity was found when comparing all the *B. pendula* together ($R^2=0.327$, $p=0.001$) or the *B. pendula* at the centre and the other plant species ($R^2=0.471$, $p=0.001$). No significant dissimilarity was observed when comparing *B. pendula* with either *B. pendula* (Pn) or with *P. nigra*.

Table 8. ANOSIM of the bacterial and fungal communities associated with the different plants and their interactions.

An ANOSIM R value of 1 indicates complete dissimilarity between groups. Significant levels were estimated (p -value < 0.05).

Habitats	Bacteria		Fungi	
	R ²	p-value	R ²	p-value
Global	0.384	0.001**	0.311	0.001**
Intra-species				
<i>B. pendula</i> - <i>B. pendula</i> (Pt) - <i>B. pendula</i> (Pn) - <i>B. pendula</i> (Sp)	0.379	0.001**	0.327	0.001**
<i>B. pendula</i> - <i>B. pendula</i> (Pt)	0.284	0.001**	0.621	0.001**
<i>B. pendula</i> - <i>B. pendula</i> (Pn)	0.617	0.001**	0.058	0.134
<i>B. pendula</i> - <i>B. pendula</i> (Sp)	0.174	0.028*	0.206	0.015*
Inter-species				
<i>B. pendula</i> - <i>P. tremula</i> - <i>P. nigra</i> - <i>S. purpurea</i>	0.48	0.001**	0.471	0.001**
<i>B. pendula</i> - <i>P. tremula</i>	0.539	0.001**	0.804	0.001**
<i>B. pendula</i> - <i>P. nigra</i>	0.607	0.001**	0.145	0.038*
<i>B. pendula</i> - <i>S. purpurea</i>	0.178	0.028*	0.12	0.041*
<i>B. pendula</i> (Pt) - <i>P. tremula</i>	-0.028	0.607	0.133	0.02*
<i>B. pendula</i> (Pn) - <i>P. nigra</i>	-0.031	0.613	-0.018	0.552
<i>B. pendula</i> (Sp) - <i>S. purpurea</i>	0.014	0.338	0.122	0.04*

0 '***' 0.001 '**' 0.05 '*'

Supplementary Table 11 shows the most abundant OTU found at the bacterial collection (abundance >0.5%). Only 33 OTUs (from the 1,322) presented a higher abundance of 0.5%, highlighting the presence of very rare OTUs dominating the community. The most abundant OTU belong to the family Streptomycetaceae (OTU00001, 14.9%). This OTU presented the highest abundance in roots of *B. pendula* and *S. purpurea*, although it was uniformly distributed at the roots of all the plant species and locations. Within the rest of abundant OTUs, OTU00005_f: Micromonosporaceae, OTU00022_g: Kribella, OTU00030_f: Methylocystaceae and OTU00034_g: Methylibium tended to be more abundant in roots of *B. pendula* growing at the centre of the dump. The abundant OTUs in the fungal dataset are shown in Supplementary Table 12. Similarly to what was observed in the bacterial dataset, only few OTUs presented abundance >0.5% (24 from a total of 577). Fungal community was clearly dominated by the OTU 25_92637 belonging to *Geopora arenicola* species (31.8% of the total dataset). This OTU was present in high abundance in roots of all.

The studied plants however; its abundance was slightly higher in the roots of *B. pendula* at the centre. There was an extreme variability between the plant replicates of a same plant species or growing location (which is reflected by the high standard errors in Supplementary Table 12.) leading to the lack of important statistical differences. The other most abundant OTUs belonged to the genera *Lactarius* (15.7%) and the order Pezizales (7.2%), Agaricales (5.7%) and Helotiales (3.6%).

Supplementary Fig 5 shows the distribution of unique and shared bacterial and

fungal OTUs between the *B. pendula* growing at the centre and the other plant species growing at the edges. Looking the bacterial OTU distribution (Supplementary Fig 5.a and .b), the Venn diagram between *B. pendula* growing in the centre at the other plant species (*Salix* and *Populus*), showed that *B. pendula* hosted 44 exclusive OTU and the highest number of exclusive OTUs was found in *P. nigra* (114). The four plant species shared a total number of 115 OTUs. When looking at the distribution of these bacterial OTUs between the plants of *Betula pendula* across the whole dump (Supplementary Fig. 5.b), 60 OTUs were exclusive of the plants growing at the centre while the highest number of specific OTUs (106) was found in *B. pendula* (Pn). The number of specific OTUs in the *B. pendula* growing at the edges (compared to the *B. pendula* at the centre) was similar to that found in their neighbour plants. Supplementary Figure 5. c and .d show the distribution of fungal shared and unique OTUs between different plants and locations. It can be observed that the *B. pendula* from the centre presented 5 specific OTUs when compared to the other *B. pendula* but also when compared to the other plant species. The highest number of unique OTUs was found in roots of *P. tremula* and of *B. pendula* (Pt) (19 and 14 respectively).

Supplementary Table 13 is a detailed list of bacterial and fungal which are either, exclusively present or exclusively absent in the roots of the central *B. pendula*. There were 28 OTUs exclusive from the *B. pendula* from the centre while 19 OTUs while present in roots of plants growing at the edges but not in the central birch. In the case of fungal OTUs, a much lower amount of specific OTUs was observed. Only 5 fungal OTUs were found exclusively in roots of the central birch while there were 7 OTUs, which were in the roots of all the plants growing at the edges but not in the birch at the centre.

Supplementary Table 14. is a summary of unique and shared bacterial and fungal OTUs (based on Venn diagram analysis) between different plant pairs. This data clearly illustrate again the share of an important part of microbial communities between the neighbour plant and a higher degree of specificity of the birch growing at the centre: a higher number of shared than unique OTUs were observed when comparing plants growing together at the same sampling points while a similar number of unique and shared OTUs were observed when comparing roots of different plant species growing at the same soil sampled spot.

A Heatmap was carried out using the most abundant bacterial and fungal OTUs (>0.5% abundance) (Fig. 30.). *B. pendula* growing at the centre was clustered separately from the other plants, while the other plants were clustered in couples according to the site of growth. The birch at the centre of the dump was more closely associated to plants growing at the locations of *P. tremula* and *S. purpurea*. When a heatmap was realised separately from both the bacterial and fungal communities (data not shown) the same pattern was found for bacterial communities. Similarly, plants of birch also presented a different fungal OTUs distribution but in this case, plants of *B. pendula* were associated to the cluster made of *P. nigra* and *B. pendula* (Pn).

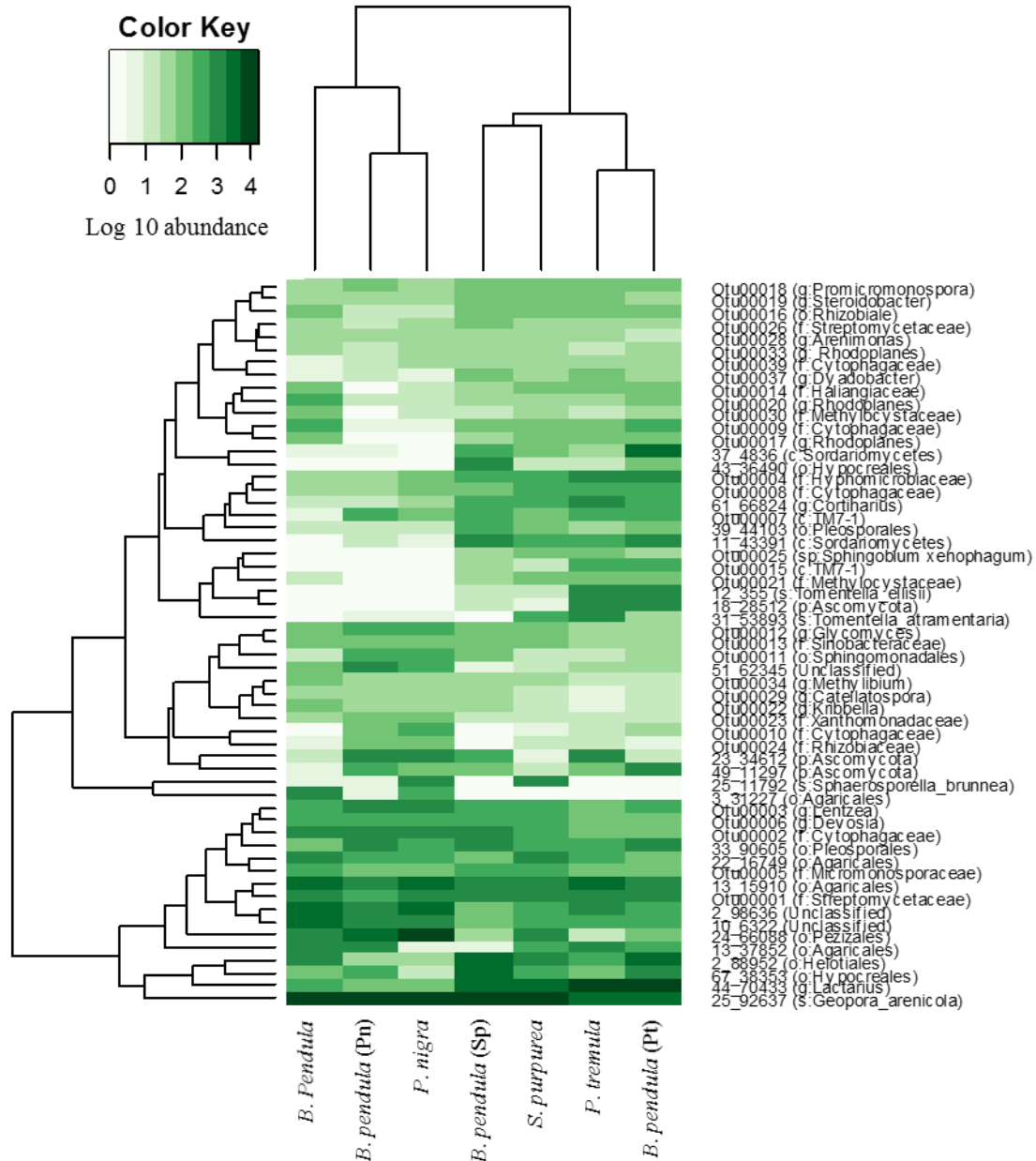


Figure 29. Heat map and hierarchical cluster analysis of the most abundant bacterial and fungal.

OTUs (>0.5%) from the various roots. The dendrogram represents linkage clustering using Euclidean distance measures. OTU delineation was based on a threshold of <97% sequence similarity. Assignments between brackets show the lowest taxonomic level associated with the OTU using the UNITE database, k kingdom, p phylum, o order, c class, f family, s genus_species.

An analysis for the study of indicator OTUs associated to each plant species and growth locations was carried out. Table 9. shows only indicator OTUs associated to *B. pendula* from the centre. Fourteen indicator bacterial OTUs and 4 indicator fungal OTUs were found. The bacterial OTUs include one belonging to the class Gammaproteobacteria, one belonging to the family Cytophagales, 3 to the family Rhizobiales, one belonging to the family Streptomycetaceae and one belonging to the family Hyphomicrobiaceae, one to the genus Rhodoplanes and one to the genus

Catellatospora. In the case of fungi, OTUs belonged to the species *Oidiodendron maius*, the genus *Russula*, and the order Agaricales and Hypocreales.

Table 9. Indicator fungal and bacterial OTUs.

	stat	p.value	Phylum	Class	Order	
BACTERIA	Otu01003	0.775	0.001 ***	Proteobacteria	Gammaproteobacteria	unclass
	Otu00390	0.721	0.004 **	Bacteroidetes	Cytophagia	Cytophagales
	Otu00260	0.655	0.001 ***	Proteobacteria	Alphaproteobacteria	Rhizobiales
	Otu01224	0.636	0.006 **	Proteobacteria	Alphaproteobacteria	Rhizobiales
	Otu00475	0.618	0.004 **	Proteobacteria	unclass	unclass
	Otu00331	0.612	0.005 **	Actinobacteria	Actinobacteria	Actinomycetales
	Otu00799	0.604	0.006 **	Proteobacteria	Alphaproteobacteria	Rhizobiales
	Otu00429	0.566	0.01 **	Proteobacteria	Alphaproteobacteria	Rhizobiales
	Otu00480	0.566	0.012 *	unclass	unclass	unclass
	Otu00416	0.555	0.008 **	Proteobacteria	Alphaproteobacteria	Rhizobiales
	Otu00574	0.53	0.017 *	Proteobacteria	Alphaproteobacteria	unclass
	Otu01267	0.516	0.036 *	Proteobacteria	Alphaproteobacteria	unclass
	Otu00716	0.5	0.038 *	Actinobacteria	Actinobacteria	Actinomycetales
Otu01261	0.474	0.041 *	unclass	unclass	unclass	
FUNGI	3_5515	0.993	0.001 ***	Ascomycota	Dothideomycetes	Incertae_sedis_8
	41_79123	0.735	0.005 **	Basidiomycota	Agaricomycetes	Agaricales
	1_99723	0.577	0.002 **	Ascomycota	Sordariomycetes	Hypocreales
	4_35502	0.572	0.007 **	Basidiomycota	Agaricomycetes	Russulales

Associations were calculated with the Dufrene–Legendre indicator species analysis routine (Indval, indicator value) in R. Significance levels: * $P \leq 0.05$; ** $P \leq 0.01$.

Relationships between microbial communities and physicochemical soil properties

Table 10 shows the correlation found between the most abundant bacterial OTUs (>0.5%), soil physicochemical parameters and leaf metal and nutrient concentrations. On the whole, a correlation of several OTUs with CaCl₂-extractable Fe and Mn concentrations (either positive correlation in the case of OTU00003_g:Lentzea, OTU00006_g:Devosia, OTU00012_g:Glycomyces, and OTU00029_g:Catellatospora or negative correlations in OTU00001 and OTU00026 both belonging to the Streptomycetaceae family) was found. Also strong positive correlations were found with CaCl₂-extractable P (OTU00002_f:Cytophagaceae, OTU00026_f: Streptomycetaceae and OTU00028_g:Arenimonas), CaCl₂-extractable K (OTU00005_f:Micromonosporaceae, OTU00016_o:Rhizobiales or OTU00037_g:Dyadobacter). However, these correlations were not observed in the correspondent leaf nutrient concentrations (except in the case of OTU00002_f:Cytophagaceae where a correlation with soil CaCl₂-extractable P

and leaf P concentration was observed). Moreover, a positive correlation with both leaf K and P concentrations was found with OTU00003_g:Lentzea and OTU00010_f:Cytophagaceae. Finally, OTU00012_g:Glycomyces showed a positive correlation with leaf P and OTU00029 with leaf K concentrations. Similarly, in the case of fungal community, a strong correlation with both, CaCl₂-extractable Fe and Mn was observed for the OTUs 24_66088_o:Pezizales, 23_37852_o:Agaricales and 51_62345_Unclassified. The OTU 24_66088 also showed a correlation with leaf K and P concentrations while the OTU 67_38353_o:Hypocreales was correlated with Mn leaf concentrations and OTU 51_62345_Unclassified with leaf P concentrations (Table 11.)

Considering an interspecies analysis based on the bacterial and fungal composition using the most abundant OTUs (>0.5%), (NMDS plots (Fig. 31.) it can be observed that roots of *B. pendula*, *P. tremula*, *P. nigra* and *S. purpurea* were clustered separately for each tree. The distribution of the central *B. pendula* at the site was mainly linked to the presence of 5 specific bacterial OTUs (Otu00001 (f:Streptomycetaceae), Otu00017 (g:Rhodoplanes), Otu00009 (f:Cytophagaceae), Otu00030 (f:Methylocystaceae) and Otu00014 (f:Haliangiaceae). The distribution was also linked to leaf Mn concentration.

Table 10. Soil- and plant- abundant bacterial OTUs relationships.

Spearman correlation coefficient values computed for OTU and soil parameters using abundance data of the OTU >0.5% abundance. Red cells indicate negative correlation and green cells positive correlation. The intensity of the color indicates the intensity of correlation.

	CaCl ₂ -extractable						Leaf metal and nutrient concentrations									
	Cu	Fe	K	Mg	Mn	P	pH_H ₂ O	Ca	Co	Cu	Fe	K	Mg	Mn	P	Zn
Otu00001..f.Streptomycetaceae.		Red			Red										Green	Green
Otu00002..f.Cytophagaceae.	Green					Green									Green	Green
Otu00003..g.Lentzea.		Green										Green				
Otu00004..f.Hyphomicrobiaceae.															Red	Red
Otu00006..g.Devosia.		Green	Red		Green											
Otu00005..f.Micromonosporaceae.			Green													
Otu00007..c.TM7.1.																
Otu00008..f.Cytophagaceae.																
Otu00011..o.Sphingomonadales.															Green	
Otu00012..g.Glycomyces.		Green			Green										Green	
Otu00009..f.Cytophagaceae.		Green			Green										Green	
Otu00013..f.Sinobacteraceae.												Green			Green	
Otu00010..f.Cytophagaceae.												Green			Green	
Otu00018..g.Promicromonospora.															Red	
Otu00017..g.Rhodoplanes.															Red	
Otu00015..c.TM7.1.															Red	
Otu00014..f.Haliangiaceae.															Red	
Otu00016..o.Rhizobiale.			Green													
Otu00020..g.Rhodoplanes.								Red			Green					
Otu00019..g.Steroidobacter.							Green									
Otu00021..f.Methylocystaceae.						Red		Green							Red	
Otu00025..sp.Sphingobium.xenophagum.							Green	Green	Green	Green						
Otu00023..f.Xanthomonadaceae.																
Otu00026..f.Streptomycetaceae.	Red	Red			Red	Green										
Otu00022..g.Kribbella.																
Otu00024..f.Rhizobiaceae.						Green										
Otu00028..g.Arenimonas.						Green										
Otu00030..f.Methylocystaceae.																
Otu00037..g.Dyadobacter.			Green													
Otu00034..g.Methylbium.			Red													
Otu00033..g.Rhodoplanes.				Green												
Otu00029..g.Catellatospora.		Green			Green							Green				
Otu00039..f.Cytophagaceae.															Red	Red

Finally, a spearman correlation was also carried out to study the potential relationships taking place between the bacterial and fungal communities. Table 12., shows the significant correlation between the main bacterial and fungal OTUs (abundance >0.5%). The fungal OTUs 44_70433_g:*Lactarius*, 24_66088_o:Pezizales and 18_28512_p:Ascomycota showed the highest number of bacterial relationships (either positive or negative). On the case of bacteria, the OTU00003_g:Lentzea, OTU00004_f:Hyphomicrobiaceae and OTU00021_f:Methylocystaceae presented the highest numbers of fungal relationships. On the contrary, there were 3 fungal OTUs (13_15910_o:Agaricales, 25_11792_s:*Sphaerospora brunnea* and 51_62345:Unclassified) and 4 bacterial OTUs (Otu00005_f:Micromonosporaceae, Otu00009_f:Cytophagaceae, Otu00030_f:Methylocystaceae and Otu00033_g:*Rhodoplanes*) did not show any significant correlation with either bacterial or fungal OTUs respectively. The most abundant fungal OTU 25_92637_s:*Geopora arenicola* only presented significant correlation with 5 bacterial OTUs and moreover 4 of those were negative. The only positive correlation was found with Otu00003_g:Lentzea. Similarly, the main bacterial OTU, Otu00001_f:Streptomycetaceae only was found to be significantly related with three fungal OTUs (from those positively correlated with 37_4836_c:Sordariomycetes and 3_31227_o:Agaricales). The strongest correlations (>0.5) were found between fungal OTU 24_66088_o:Pezizales and bacterial Otu00012_g:*Glycomyces* ($\rho=0.55$), the fungal OTU 10_6322_Unclassified and the bacterial Otu00034_g:*Methylibium* ($\rho=0.53$), the fungal OTU 37_4836_c:Sordariomycetes and the bacterial Otu00039_f:Cytophagaceae ($\rho=0.58$) and finally between the 12_355_s:*Tomentella ellisii* and the bacterial Otu00015_c:TM7_1 ($\rho=0.64$). In a more general point of view, a correlation between the most abundant fungal and bacterial families was also carried out (Supplementary Table 15.). In general a higher number of positive than negative correlations was observed. It is important to highlight there were three bacterial genera which showed a positive correlation with the abundance of *Myrothecium* but at the same time a negative abundance with the abundance of *Lactarius*. The genus *Lactarius* was found to have the highest number of correlations, and those were both, negative or positive. The bacterial genera which presented a highest number of relations was *Lentzea* and those were mainly negative although two (*Geopora* and *Myrothecium*) were positive.

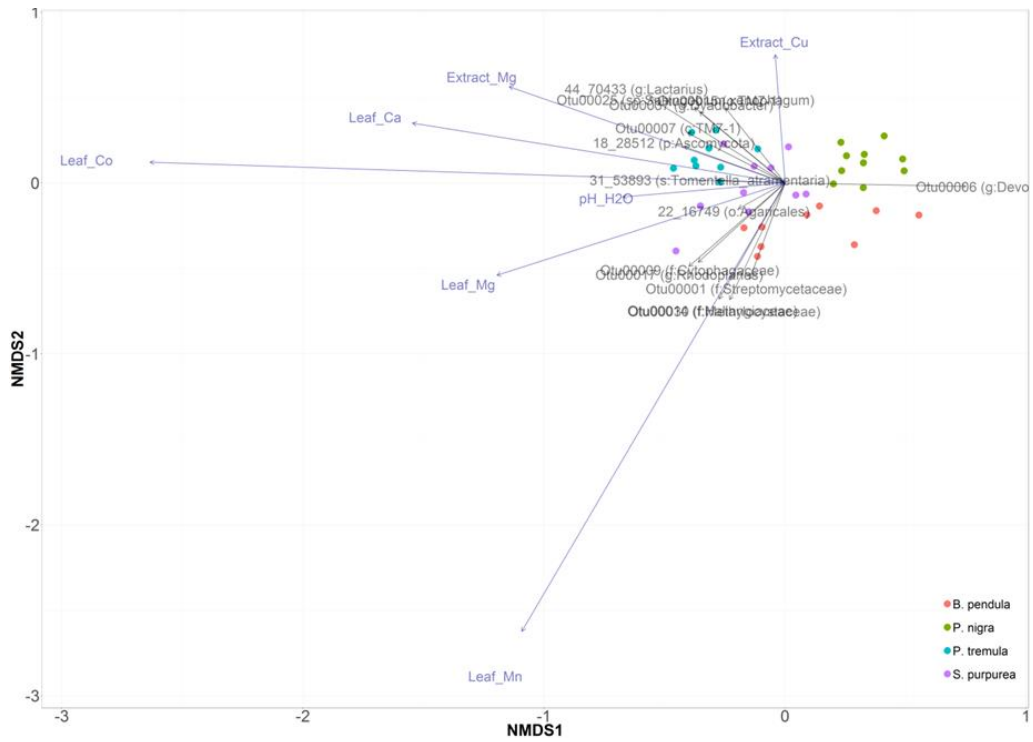


Figure 30. Non-parametric multidimensional scaling (NMDS) plot of fungal and bacterial abundant OTUs (>0.5%).

Spearman correlation coefficient values computed for OTU and soil parameters using abundance data of the OTU >0.5% abundance. Red cells indicate negative correlation and green cells positive correlation. The intensity of the color indicates the intensity of correlation. Communities associated with the roots of the B. pendula, P. tremula, P. nigra and S. purpurea. Each point represents the bacterial community of a given root. Each color represents one of the four trees.

Table 11. Soil- and plant- abundant fungal OTUs relationships.

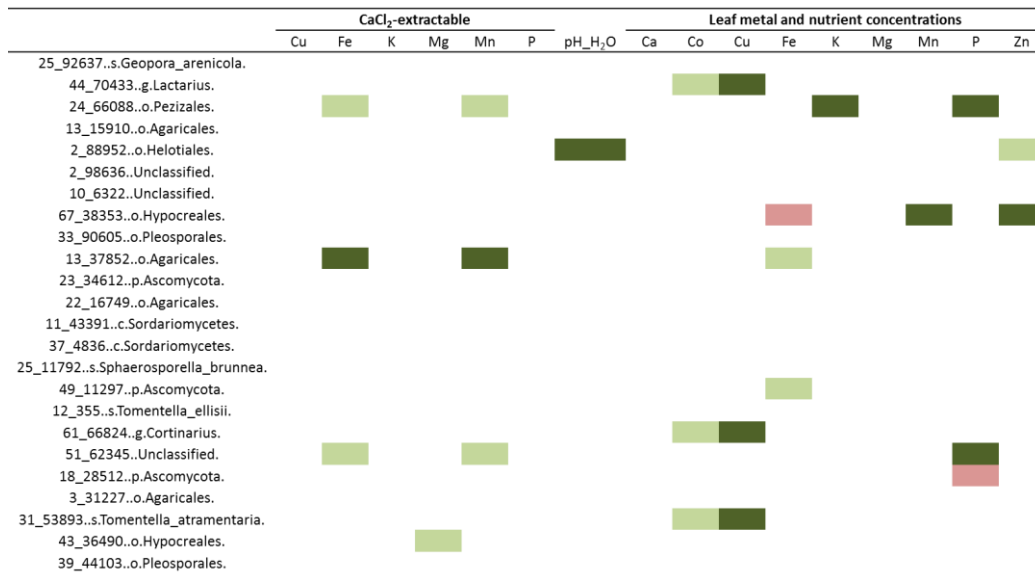
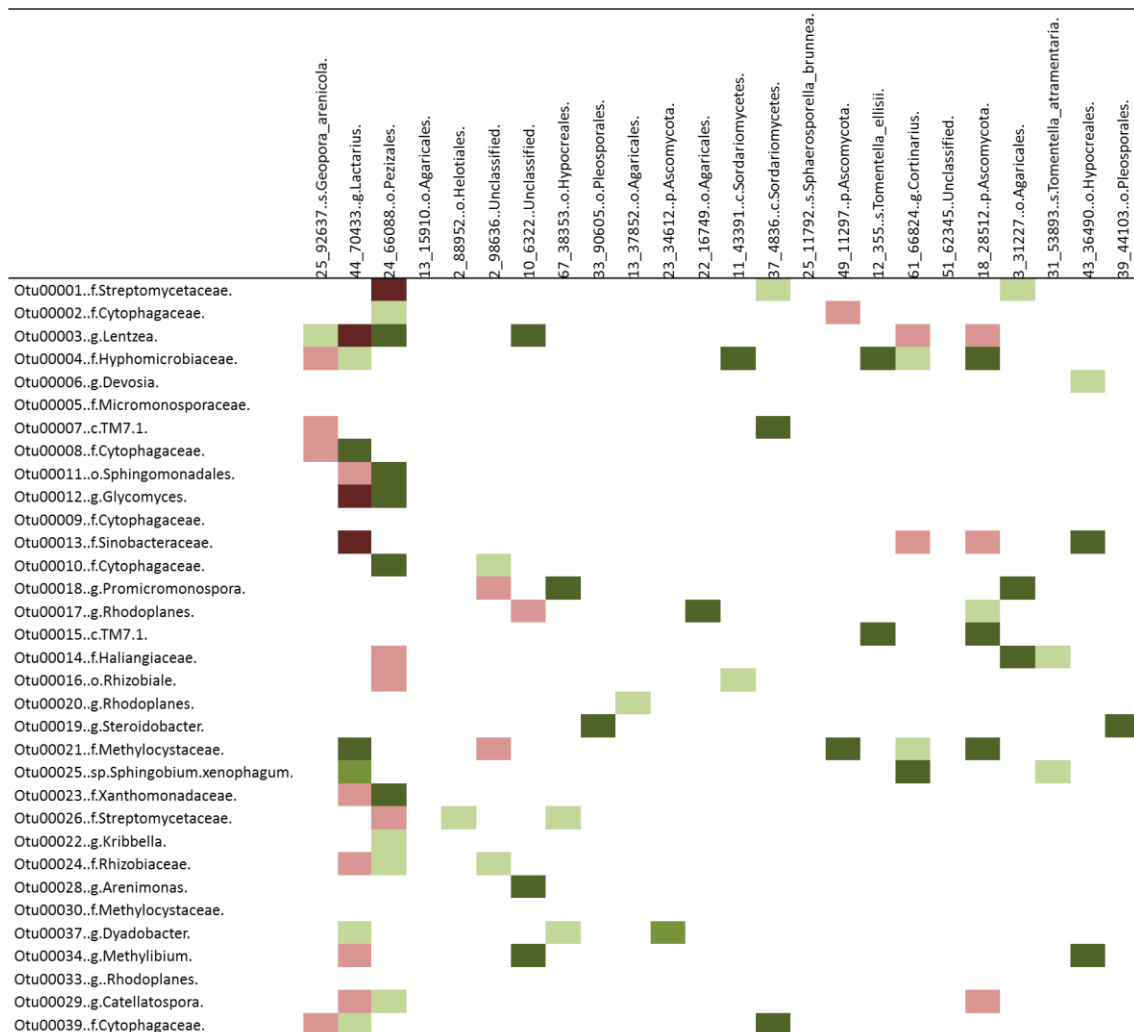


Table 12. Fungal and bacterial relationships.



Spearman correlation coefficient values computed for fungal and bacterial abundant OTU using abundance data of the OTUs >0.5% abundance.

4.3.2.6 Discussion

This study aimed to unravel the root-microbial interactions involved in the successful colonising capacity of *B. pendula* at a red-gypsum mine dump. It is well known that birch is a pioneer plant species which is able to establish at poor nutrient and degraded soils (Eltrop et al., 1991; Kopponen et al., 2001). Part of this success is related to their well-developed radical system. Compared to other tree species, *B. pendula* is characterized by thin and densely branched roots that provide an efficient foraging system for nutrient uptake (Curt and Prévosto, 2003; Ostonen, 2007; Priha et al., 1999). The structure of the EcM fungal community affects morphology and functioning of ectomycorrhizal short roots of trees (Heijden and Kuyper, 2003; Ostonen et al., 2009). Birch is a high biomass producer and fast growing tree which can provide high economic return with end-use applications such as the production of biofuels, pulp, and paper and other bio-based products such as biocatalyst (Asad et al., 2017; Kidd et al., 2015). Furthermore, birch can be grown on marginal land evading the food versus fuel debate (Searchinger et al., 2008).

It is already well known that plants harbour multiple microbial taxa, which influence a number of plant traits such as biomass production, improve nutrient availability and transport from the soil (Sugiyama et al., 2012), metabolite production (Badri et al., 2013), drought tolerance (Lau and Lennon, 2012) and flowering time (Wagner et al., 2014). In addition, the composition of the plant microbiota can enhance host resistance to pathogens (Busby et al., 2016; Mendes et al., 2011). Plant-microbe interactions are of specific interest, not only to get a better understanding of their role during plant growth and development but also to allow exploitation of their relationships in phytoremediation applications, sustainable crop production, and the production of secondary metabolites (Brader et al., 2014; Weyens et al., 2009). Members of the plant microbiota can be transmitted either horizontally (acquired from the surrounding environment) or vertically (acquired directly from the parent) (Gundel et al., 2011). Seed transmission of microorganisms may play an important role on plant productivity through influencing the primary composition of the plant microbiota and for example, fungal vertical inheritance is recognized as common mechanism of transfer across generations (Hardoim et al., 2015) and transmitted fungal symbionts have been implicated in providing direct benefit to their host plant.

Little information about root microbial communities associated to birch growing at contaminated sites using metabarcoding approaches is available for comparison. However, some studies such as Mesa et al (2017b) studied the rhizospheric and endospheric bacterial communities associated to *Betula celtiberica* growing a contaminated abandoned fertilizer industrial site at the NO of Spain. Data of number of observed OTUs and Chao index found in roots of *B. celtiberica* by these authors are comparable with those found at the present study. In general *B. pendula* growing at the centre of the mine showed lower richness and diversity (either in bacterial and fungal

communities) than the other plants growing at the edges. This could be attributed (i) to the presence of harder physicochemical soil properties (e.g. lack of organic matter) at the centre of the dump (although this could not being demonstrated using a CaCl_2 -extraction) which negatively would impact root microbial communities or (ii) to the lower growing time of this plants at the centre which could make that the microbial communities did not still had time to develop a higher maturity and complexity.

In the present study, the bacterial community was dominated by *Proteobacteria* and *Actinobacteria*. It is important to note that in the present no distinction between endophytes and epiphytes was done, and therefore one only habitat was considered. However, roots were exhaustively washed and we can consider that only strength root-associated microorganisms are being here discussed. Interestingly, *Proteobacteria* and *Actinobacteria* have been suggested to be associated with disease suppression in the rhizosphere of sugar beet plants in a study using DNA metagenomics with Phylochip (Mendes et al., 2011).

Mesa et al (2017b), also found that in *B. celtiberica*'s endosphere, *Proteobacteria* was the most abundant phylum (accounting for 64% of the total reads), (followed by *Bacteroidetes* (17%), *Actinobacteria* (7.9%), *Firmicutes* (1.8%), and *Chlamydiae* (1.3%)). It is generally accepted that the Alpha- and Beta-*Proteobacteria* are copiotrophic members (Fierer et al., 2007; Zhao et al., 2014), since they are usually associated with habitats having relatively low soil C/N ratio, high $\text{NO}^{-3}\text{-N}$ (Nugroho et al., 2005), and enriched nutrients (Leff et al., 2015; Li et al., 2016). Roots are nutrient-rich niches since they receive high amounts of available C and N as exudates, which could promote the growth of these bacterial inside and at the rhizoplane tissues.

Mesa et al (2017b) also found that root microbiome was dominated by taxa related to *Flavobacteriales*, *Burkholderiales*, and *Pseudomonadales*, especially the *Pseudomonas* and *Flavobacterium* genera. However, in the present study, the phylum *Proteobacteria* was mostly represented by the family *Hyphomicrobiaceae* (order *Rhizobiales*) and the genera *Devosia* and *Rhodoplanes*. Mesa et al (2017b) have also found members of the *Rhizobiales* present in both, rhizosphere and endosphere of *B. celtiberica*. Moreover, in the same study cultivable strains from the endosphere belonged to 19 genera; and among the most predominant were found members of the genus *Rhizobium* (11%). Several members of *rhizobiales* (such as *Ensifer*, *Rhizobium*, *Phyllobacterium*) have been shown to reduce plant stress response and improve plant growth in different hosts and soils (Guo and Chi, 2014; Lafuente et al., 2015; Larcher et al., 2008; Mesa et al., 2017b). On the other side, in the present study, genera such as *Pseudomonas*, which are frequently described in contaminated soils, where unusual. Only three OTUs belonging to the *Pseudomonas* genera were observed (Otu00824 Otu01204 and Otu00214) although with abundance ranging from 0.001% to 0.064%.

Moreover OTUs belonging to the order *Rhizobiales* (including members of the

family Hyphomicrobiaceae and the genera Rhodoplanes) were found to be indicator of roots of *B. pendula*. There is little information about the role of Rhodoplanes, and several studies related their presence preferably to the rhizospheric soil than to the endospheric habitats (both root or shoot) (Gkarmiri et al., 2017; Touceda-González et al., 2015). However, these bacteria were abundant in the root DNA-based community of *Arabidopsis thaliana* (Haichar et al., 2012) and *Panax notoginseng* (Tan et al., 2017). A study which used ¹⁵N-DNA-SIP to investigate soil microorganisms responsible for N fixation, identified, among others, bacteria of the genus Rhodoplanes as being potential N fixers (Buckley et al., 2007). It seems that microorganisms involved in N cycling could be of crucial importance for the establishment of this plant at the dump.

Actinobacteria was the second most represented phylum and the main OTU (Otu00001) of the dataset belonged to the Streptomycetaceae family. The predominance of this phylum was also found in several other studies involving either soil or plant compartments (Lopez et al., 2017). The predominance of this phylum can be explained by the high adaptability of these Gram positive bacteria to toxic concentrations of metals in soils. Several other studies have confirmed the high adaptability of these bacteria to toxic concentrations of metals (DeGroot et al., 2005; Schmidt et al., 2005) and it has been shown that their strong secondary metabolism enables them to cope with stress factors including toxic levels of heavy metals (So et al., 2001). Yang et al (2017) found that seeds growing in soils showed enrichment in Actinobacteria in roots of barley compared to seeds growing in axenic substrate suggesting that the soil had a major impact driving the root bacterial communities. Actinobacteria exhibit diverse physiological and metabolic properties, such as the production of extracellular enzymes and the formation of a wide variety of secondary metabolites (Goodfellow et al., 2012). In fact, members of the order Actinomycetales, notably the *Streptomyces* genus, remain the richest source of natural products, including clinically useful antibiotics, antimetabolites, and antitumor agents (Bérdy, 2005; Olano et al., 2009). Actinomycetes produce about 45% of all microbial bioactive secondary metabolites, with 80% of these compounds being produced by the *Streptomyces* genus (Bérdy, 2005). Actinobacteria also produce non-antibiotic molecules that exhibit bioactivity, such as enzyme inhibitors, immunosuppressors, phytotoxins, biopesticides, biosurfactants, nano-particles, probiotics and enzymes involved in the degradation of complex polymers (Lam, 2006). This versatility in secondary metabolite production makes them important tools for pharmaceutical, medical, and biotechnological applications such as bioremediation. These endophytic actinobacteria as biological control agents of plant disease is also of interest given their ability to colonize healthy plant tissues and produce antibiotics *in situ* (Shimizu, 2011). For example, endophytic Streptomycetes isolated from healthy banana plants (*Musa* sp.), were studied for the ability to produce antifungal molecules that inhibited the growth of the pathogen *Fusarium oxysporum* (Cao et al., 2005). An enrichment of

Streptomyces in roots compared to rhizospheric soil is frequently described in the literature (Durand et al., 2018; Foulon et al., 2016a). Moreover, (Gkarmiri et al., 2017) suggested that within the active bacterial community that *Streptomyces* were highly active in the root compartment but not in the rhizosphere. Otu00716 belonging to *Catellatospora* sp (family Micromonosporaceae, phylum Actinobacteria) was found to be an Indicator species of the central birch plants. Some authors have considered *Catellatospora* as a rare or a highly-specific niche genus (Saracchi et al., 2004). From the study of endophytic root actinomycete population from 252 leaf litter and 205 plant root samples (Petrolini, 1992; Sardi et al., 1992). Most of the 2950 isolates obtained belonged to *Streptomyces* (Sardi et al., 1992) and 215 to the genus *Micromonospora* (Williams et al., 1993). However, representatives of the “rare” genera were also found (Petrolini, 1992; Petrolini et al., 1995), and among these, 53 strains of *Catellatospora* spp. However, in our study, this was not a residual genus, and we have found 6 OTUs, which belonged to this genus. Moreover, one of these (Otu00029) was one of the abundant OTU (abundance >0.5%), although this was not the OTU with higher abundance in the birch roots. The genus *Catellatospora* is considered one out of the rarely occurring genera of actinomycetes: only few papers concern these microorganisms and most of all regards the descriptions of its few species and their taxonomic position (Saracchi et al., 2004).

The results showed that *B. pendula* harboured a specific microbial community unique and different not only from other plant species growing at the mine (*Populus* or *Salix* spp.) but also different from other *B. pendula* growing at the edges (Analysis of similarities ANOSIM, Heat map and hierarchical cluster analysis of the relative abundance of bacterial and fungal OTUs). Moreover, this plant-location specific community was more marked in the case of the bacterial community. However, regarding the distribution of unique and shared OTUs, it was found that although a high and consistent number of OTUs were unique of each plant, most of them were, however, shared independently of the plant species or site of growing. These results suggest that systematic plant colonization by certain soil indigenous bacterial species is occurring and that the specificity of microbial communities is related mainly to the differences between OTUs abundance. Yang et al (2017) found that the differences in root active microbiome associated to root of different barley cultivars were mainly quantitative and not as much qualitative. For example, the variation between the genotypes was manifested in the abundance of many OTUs from diverse taxa, rather than by the presence/absence of single OTUs in the given genotypes.

Data obtained from fungal sequencing showed a much higher replicate variability than those obtained in bacterial sequencing. This same pattern was found in several other studies when comparing fungal communities from soil and root habitats (Beckers et al., 2017; Durand et al., 2017; Kolaříková et al., 2017). This result suggests that root endophytic colonization and formation of stable communities appears to be a more

variable process than bacterial colonization (Beckers et al., 2017).

Although in our study most taxa (of both bacterial and fungal communities but especially in the case of fungi sequencing) were not sequenced at a high enough read depth to enable powerful comparisons, an enrichment of the genus *Geopora* (specifically *G. arenicola*) at the roots of all the plant species was observed and tended to be slightly higher at roots of *B. pendula*. Similar results were found by Kolaříková et al (2017). These authors investigated the root-associated fungal communities of two dominant trees *Salix caprea* and *Betula pendula* along a primary successional chronosequence on a mine spoil bank in the Czech Republic. This OTU was specifically associated to the younger plants in the chronosequence independently of the plant species. *Geopora arenicola* is an ectomycorrhizal fungus which within others has as characteristic to promote plant growth in stressful environments (Hryniewicz et al., 2009). Within the fungal Indicator OTU, the genera *Oidiodendron* and *Russula* (OTU 3_5515 s:*Oidiodendron_maius*|SH217755.06FU and OTU 4_35502 g:*Russula*). *Russula* is an ECM fungus frequently found in soils and also associated to birch cultures (Bent et al., 2011; Parker et al., 2017). Moreover, Parker et al (2017) found that after a screening of plant/soil associated fungi to (*Picea mariana*, *Populus tremuloides* and *Betula papyrifera*) after a wildfire each plant species seemed to be affected by different groups of fungi: a greater birch shoot mass was associated with ribotype 732 to 734 bp belonging to *Russula* spp. It is interesting to note, however, that the Russulaceae family was not well represented in roots of the central *B. pendula*. This results suggests that there are not the most abundant taxa which are behind the successful establishment of these plants. Moreover, Durand et al (2017) found that the less abundant fungal OTUs present a higher number of networks with different OTUs than the most abundant OTUs. In the present study can be also observed that the most abundant OTUs, (both fungal and bacterial) showed low number of bacterial and fungal correlations. Interestingly, the second indicator OTU belonged to *Oidiodendron maius* which is ericoid mycorrhiza (Chambers et al., 2008; Lukešová et al., 2015) but however, no Ericaceae plants are described at this dump. Although less frequently, but these mycorrhiza have been also found in association with other plant species (e.g. Chambers et al., 2008).

Similarly to what was observed in bacterial dataset (although not clearly reflected by the ANOSIM analysis) plants of *B. pendula* growing at the centre hosted a specific fungal community while plants growing at the edges shared an important part of either the structure as the composition of their root communities. Bogar and Kennedy (2013) studied the relative importance of host and neighbourhood effects on the ECM fungal communities associated with *Alnus rhombifolia* (a host of specific ECM fungi) and *Betula occidentalis* (a host of generalist ECM fungi). Authors found that while the host identity acts as a primary filter on the composition and diversity of ECM fungal communities, proximity to a closely related host can mediate significant changes in community structure. In the same way, Hausmann and Hawkes (2009) found that the

structure of arbuscular mycorrhizal (AM) fungal communities on neighbouring grass plants depended upon which pairs of host species were near each other. The first plant host to form mycorrhizal relationships could play an important role in determining which fungal species are able to colonize later hosts, providing a substantial competitive advantage to the fungi with which it associates. Neighbourhood effects can also be driven by changes in abiotic conditions that favour the growth of certain ECM fungal species, as demonstrated by Meinhardt and Gehring (2012). In that study, the authors examined the influence of invasive *Tamarix* spp. on the mycorrhizal associations of native *Populus fremontii* in an early-invasion field site and in the greenhouse and laboratory. They found that the ECM fungal community structure on *P. fremontii* shifted significantly in the presence of *Tamarix* spp., likely as a result of increases in soil nitrate concentrations and electrical conductivity associated with soils affected by *Tamarix*. Some ECM fungi, viz., *Russula luteolus*, *Suillus bovinus*, and *Hebeloma crustuliniforme*, form highly branched mycelial strands up to 40 cm (Finlay and Read, 1986; Skinner and Bowen, 1974). Moreover to the beneficial effects on the plant nutritive status by scavenging large proportions of soil this can lead also to colonization of other plants found at the site.

Fungi and bacteria share the same habitats and are therefore almost certain to frequently interact in soil and plants (Boer et al., 2005; Zhang et al., 2014). For example, changes in the gene expression and metabolism of both *Bacillus* and *Aspergillus* as interacting partners have been demonstrated (Benoit et al., 2015). Interactions between co-occurring bacteria and fungi play a considerable part in determining their respective roles, and investigation of these interactions will lead to a more complete understanding of microbial ecology (de Menezes et al., 2017).

Although significant correlations were found with both, different bacterial and fungi OTUs, those were generally very weak (rho value usually below than 0.6). This could be due to the lack of a strong gradient in soil properties and rather a slight heterogeneity frequently found in constructed wastes dumps. Moreover, although this study involves the characterisation of ephispERIC root microorganisms (which therefore would be directly influenced by the soil properties) most of the analysed community belonged to the endophytic community and therefore are not directly influenced by the soil physicochemical properties

4.3.2.7 **Conclusions**

For successful implementation of a phytomanagement strategy is of vital importance to previously characterise the plant-soil-microbial system. In this study we characterised the microbial community associated to the roots of *B. pendula* which is the more successful plant species colonising a red gypsum mine tailing. The results showed that the microbial community (mainly the bacterial community) could be involved in the establishment of these pioneer plants in the centre of the dump. These

results showed that the bacterial community of plants of birch growing at the centre were highly dissimilar to those of other plant species studied at the mine but also to other plants of *B. pendula* growing at the edges of the dump. The most abundant bacterial OTU (Out 00001) belonged to the family Streptomycetaceae and was widely spread through all the studied roots. At the same time, according to the NMDS analysis, this OTU seems also to explain the distribution of birch at the centre of the dump. Other bacterial OTUs associated to the presence of birch (such as members of the order Rhizobiales) are known to be involved in the N cycling, suggesting that the lack of this element in the soil could be an essential factor for the effective colonisation of the plants at the site.

Data obtained from fungal sequencing showed a much higher replicate variability than those obtained in bacterial sequencing. In the case of fungi, a less clear pattern was observed although it seems also that birch selected some specific OTUs (e.g. OTUs belonging to the genera *Oidiodendron* and *Russula*, and the order Agaricales and Hypocreales were found to be indicators of the presence of *B. pendula*)

The differences of communities (both bacterial and fungal communities) were based on the microbial structure but also on microbial composition and distribution: e.g. the Venn diagram showed that the unique bacterial and fungal OTUs associated to each plant were always present in a very low proportion in the total community (< 0.5% of abundance). However, analysis carried out using the most abundant OTUs, which were usually shared by all plants (e.g. heatmap or NMDS) showed also a specificity of the communities associated to *B. pendula*. Finally it is also important to remember that assemblies of host-associated microbial communities are frequently based on function rather than only on its community structure (Burke et al., 2011; Mendes et al., 2014). Therefore, other studies focusing on the active part of the microbial communities (e.g. rDNA sequencing) or on the microbial ecosystem functionality (for example based on commercial chips (e.g. GeoChip) or on the *in vitro* characterization of the culturable community) can give a complete vision of these primary plant-soil-microorganisms systems.

4.3.2.8 Supplementary data

Supplementary Table 7. CaCl₂-extractable and quantifiable metals and nutrients and pH from soils samples.

	CaCl ₂ -extractable concentration (mg kg ⁻¹)						pH H2O
	Cu	Fe	K	Mg	Mn	P	
<i>B. pendula</i>	12.47 ±0.53 a	1286 ±188 ab	2066 ±111 a	17863 ±4249 a	120.8 ±15.5 ab	51.7 ±3.5 a	7.79 ±0.02
<i>P. tremula</i> - <i>B.pendula</i> (Pt)	12.73 ±0.82 a	1353 ±208 ab	2368 ±174 a	27242 ±3724 a	130.3 ±15.9 ab	48.3 ±3.9 a	7.74 ±0.02
<i>P. nigra</i> - <i>B.pendula</i> (Pn)	14.63 ±0.44 ab	2052 ±509 b	2276 ±121 a	24703 ±3659 a	195.0 ±48.4 b	48.4 ±2.8 a	7.62 ±0.04
<i>S. purpurea</i> - <i>B.pendula</i> (Sp)	19.07 ±2.50 b	464 ±165 a	2868 ±349 a	23249 ±6007 a	50.6 ±16.2 a	56.8 ±5.0 a	9.99 ±0.69

Due to that only one sample soil was taken for each pair of plants (*B. pendula* (Pt) and *P. tremula*, *B. pendula* (Pn) and *P. nigra* and *B. pendula* (Sp) and *S. purpurea* values are the same in these samples.

Supplementary Table 8. Leaf metal and nutrient concentration in the different studied plants (mg kg⁻¹).

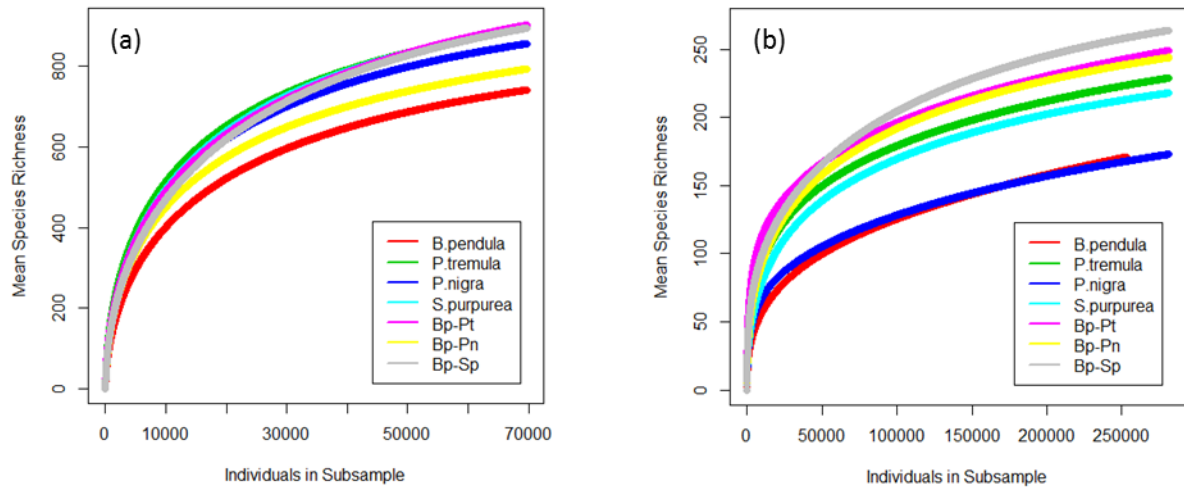
	Leaf concentration (mg kg ⁻¹)								
	Ca	Co	Cu	Fe	K	Mg	Mn	P	Zn
<i>B. pendula</i>	13412 ±720 a	0.75 ±0.06 ab	7.27 ±0.34 ab	1466 ±133 b	4560 ±449 a	4673 ±252 a	371.8 ±25.4 b	454.1 ±44.7 a	280.1 ±28.4 ab
<i>P. tremula</i>	15961 ±1447 a	1.05 ±0.12 b	9.21 ±1.72 b	1340 ±155 b	3688 ±366 a	4852 ±527 a	304.7 ±24.4 ab	368.3 ±27.0 a	170.2 ±22.1 a
<i>B. pendula</i> (Pt)	15046 ±1110 a	0.65 ±0.06 a	6.27 ±0.44 ab	1021 ±138 b	4194 ±319 a	4709 ±324 a	479.2 ±66.8 bc	530.2 ±57.4 ab	267.3 ±37.5 ab
<i>P. nigra</i>	15853 ±937 a	0.63 ±0.06 a	4.77 ±0.48 a	292 ±48 a	4115 ±580 a	4839 ±282 a	124.4 ±9.2 a	635.4 ±75.2 ab	193.2 ±22.4 a
<i>B. pendula</i> (Pn)	16762 ±944 a	0.75 ±0.10 ab	6.20 ±0.31 ab	1112 ±153 b	5046 ±477 a	4972 ±320 a	570.5 ±72.4 c	790.1 ±99.4 b	384.2 ±23.9 bc
<i>S. purpurea</i>	21790 ±1838 b	0.70 ±0.09 ab	5.73 ±0.27 b	180 ±33 a	4023 ±372 a	6310 ±679 a	144.4 ±19.6 a	480.5 ±30.3 a	283.2 ±54.7 ab
<i>B. pendula</i> (Sp)	14415 ±603 a	0.63 ±0.06 a	5.29 ±0.24 a	346 ±51 a	3991 ±266 a	5244 ±385 a	495.9 ±56.2 bc	566.1 ±71.5 ab	448.2 ±34.3 c

Supplementary Table 9. Description of the bacterial dataset before subsampling. Numbers under brackets are the percentage of total effective sequences.

Plant	Number of samples	Effective sequences	Phylum assigned sequences	Class assigned sequences	Species assigned sequences	Good Coverage
Combined dataset	70	907,006	896,518 (98.8%)	895,458 (98.7%)	12742 (1.4%)	98.30%
<i>B. pendula</i>	10	133,173	131,304 (98.6%)	131,159 (98.5%)	655 (0.5%)	98.60%
<i>P. tremula</i>	10	99,122	98,058 (98.9%)	97,936 (98.8%)	3176 (3.2%)	98.10%
<i>B. pendula</i> (Pt)	10	130,239	129,078 (99.1%)	128,908 (99.0%)	1936 (1.5%)	98.30%
<i>P. nigra</i>	10	139,071	136,882 (98.4%)	136,733 (98.3)	997 (0.7%)	98.20%
<i>B. pendula</i> (Pn)	10	121,625	120,464 (99.0%)	120,247 (98.9%)	1288 (1.1%)	98.40%
<i>S. purpurea</i>	10	119,727	118,188 (98.7%)	118,098 (98.6%)	2122 (1.8%)	98.30%
<i>B. pendula</i> (Sp)	10	164,049	162,544 (99.1%)	162,377 (99.0%)	2568 (1.6%)	98.40%

Supplementary Table 10. Description of the fungal dataset before subsampling. Numbers under brackets are the percentage of total effective sequences.

Plant	Number of samples	Effective sequences	Phylum assigned sequences	Class assigned sequences	Species assigned sequences	Good Coverage
Combined dataset	70	5,148,296	4,706,239 (91%)	4,483,534 (87%)	2,002,191 (39%)	100%
<i>B. pendula</i>	10	819,893	712,133 (87%)	710,626 (87%)	448,872 (55%)	100%
<i>P. tremula</i>	10	656,516	614,311 (94%)	552,301 (84%)	120,767 (18%)	100%
<i>B.pendula</i> (Pt)	10	644,694	625,387 (97%)	562,815 (87%)	151,871 (24%)	100%
<i>P. nigra</i>	10	740,274	655,931 (89%)	621,270 (84%)	350,677 (47%)	100%
<i>B. pendula</i> (Pn)	10	741,916	611,362 (82%)	573,117 (77%)	287,230 (39%)	100%
<i>S. purpurea</i>	10	701,998	676,105 (96%)	665,163 (95%)	336,432 (48%)	100%
<i>B. pendula</i> (Sp)	10	843,005	811,010 (96%)	798,242 (95%)	306,342 (36%)	100%



Supplementary Fig 4. Rarefaction analysis of 16S rDNA and ITS regions sequence data for estimating bacterial (a) and fungal (b).

diversity based on a threshold of < 97% sequence similarity for the delineation of operational taxonomic units (OTUs).

Supplementary Table 11. Distribution of the most abundant bacterial OTUs (abundance >0.5%) between the roots of the different plant species/locations studied.

	<i>B. pendula</i>	<i>P. tremula</i>	<i>P. nigra</i>	<i>S. purpurea</i>	<i>B. pendula</i> (Pt)	<i>B. pendula</i> (Pn)	<i>B. pendula</i> (Sp)
Otu00001 (f:Streptomycetaceae)	1359.1 ± 164.7	1005.4 ± 174.7	746.6 ± 130.8	1392.8 ± 160.7	1139.1 ± 203.3	635.1 ± 164.3	994.0 ± 150.9
Otu00002 (f:Cytophagaceae)	797.9 ± 214.3	216.2 ± 46.2	704.7 ± 162.9	606.7 ± 255.2	176.9 ± 69.9	887.8 ± 225.4	868.9 ± 254.4
Otu00003 (g:Lentzea)	491.1 ± 124.6	119.9 ± 59.4	887.5 ± 108.5	346.3 ± 87.2	266.4 ± 86.2	984.4 ± 116.6	340.6 ± 60.5
Otu00004 (f:Hyphomicrobiaceae)	71.3 ± 39.5	699.1 ± 192.2	83.8 ± 56.8	355.6 ± 113.5	646.2 ± 210.6	37.7 ± 18.3	526.1 ± 270.6
Otu00006 (g:Devesia)	357.9 ± 101.7	123.8 ± 50.4	510.6 ± 66.4	344.0 ± 126.8	163.2 ± 51.9	424.7 ± 69.6	361.0 ± 107.0
Otu00005 (f:Micromonosporaceae)	481.2 ± 108.9	170.9 ± 26.4	131.0 ± 34.5	340.6 ± 152.6	187.9 ± 33.1	175.3 ± 43.3	241.8 ± 100.1
Otu00007 (c:TM7-1)	6.0 ± 3.7	304.8 ± 111.0	86.3 ± 23.5	215.3 ± 89.7	278.9 ± 129.5	226.4 ± 71.8	335.1 ± 109.2
Otu00008 (f:Cytophagaceae)	47.1 ± 32.6	304.0 ± 73.6	90.9 ± 50.7	261.8 ± 81.1	253.3 ± 154.5	27.3 ± 16.2	204.7 ± 80.8
Otu00011 (o:Sphingomonadales)	17.5 ± 5.4	21.0 ± 4.9	365.7 ± 88.8	25.1 ± 4.8	46.0 ± 18.6	481.6 ± 84.4	79.2 ± 27.4
Otu00012 (g:Glycomyces)	83.1 ± 15.2	61.7 ± 17.0	230.2 ± 24.9	92.6 ± 20.2	51.4 ± 12.9	295.1 ± 40.5	92.6 ± 23.6
Otu00009 (f:Cytophagaceae)	231.1 ± 129.3	129.1 ± 82.2	3.3 ± 1.8	146.8 ± 78.1	247.6 ± 153.5	3.3 ± 1.3	94.7 ± 48.9
Otu00013 (f:Sinobacteraceae)	80.6 ± 20.5	46.7 ± 3.7	156.0 ± 32.1	97.4 ± 17.1	42.6 ± 11.1	197.2 ± 28.0	159.1 ± 38.9
Otu00010 (f:Cytophagaceae)	0.1 ± 0.1	17.6 ± 10.1	463.5 ± 163.5	3.8 ± 3.0	37.6 ± 36.3	199.3 ± 99.3	1.0 ± 0.5
Otu00018 (g:Promicromonospora)	64.8 ± 35.6	106.9 ± 26.8	57.5 ± 31.2	80.7 ± 16.4	136.2 ± 28.8	83.3 ± 20.8	103.5 ± 34.0
Otu00017 (g:Rhodoplanes)	195.3 ± 88.4	162.3 ± 64.5	1.5 ± 1.0	105.5 ± 77.4	113.6 ± 37.4	1.9 ± 0.9	36.0 ± 15.2
Otu00015 (c:TM7-1)	0.6 ± 0.4	321.3 ± 204.4	0.3 ± 0.2	17.4 ± 15.4	236.1 ± 227.5	0.2 ± 0.2	47.1 ± 46.9
Otu00014 (f:Haliangiaceae)	194.4 ± 36.6	119.3 ± 44.9	24.1 ± 19.4	109.3 ± 28.9	92.9 ± 24.0	2.3 ± 0.8	48.8 ± 13.9
Otu00016 (o:Rhizobiales)	93.3 ± 32.9	138.9 ± 28.6	18.7 ± 4.5	90.8 ± 15.0	80.5 ± 17.9	22.3 ± 3.0	133.5 ± 47.1
Otu00020 (g:Rhodoplanes)	277.6 ± 82.9	47.4 ± 11.6	10.9 ± 3.5	42.9 ± 18.3	95.2 ± 46.5	14.8 ± 3.9	34.8 ± 10.2
Otu00019 (g:Stteroidobacter)	53.1 ± 15.4	86.3 ± 25.5	56.9 ± 8.4	83.0 ± 26.7	70.7 ± 13.0	45.9 ± 13.9	121.4 ± 84.2
Otu00021 (f:Methyllocystaceae)	9.5 ± 4.6	153.6 ± 45.6	0.2 ± 0.1	77.3 ± 17.4	139.7 ± 46.4	0.0 ± 0.0	39.9 ± 29.9
Otu00025 (sp:Sphingobium xenophagum)	0.7 ± 0.3	179.8 ± 65.8	0.6 ± 0.3	93.2 ± 40.5	55.1 ± 41.3	1.6 ± 0.9	61.0 ± 31.9
Otu00023 (f:Xanthomonadaceae)	42.9 ± 14.2	17.0 ± 5.1	168.5 ± 67.9	15.7 ± 7.5	11.5 ± 4.1	79.6 ± 23.6	8.7 ± 3.2
Otu00026 (f:Streptomycetaceae)	53.2 ± 13.1	38.6 ± 13.7	36.6 ± 13.5	60.9 ± 11.5	48.9 ± 11.4	16.6 ± 6.5	79.6 ± 19.1
Otu00022 (g:Kribbella)	124.0 ± 97.3	3.2 ± 1.2	66.0 ± 18.8	12.2 ± 7.6	9.9 ± 3.8	68.4 ± 22.7	35.7 ± 12.9
Otu00024 (f:Rhizobiaceae)	5.5 ± 4.6	9.2 ± 4.0	158.0 ± 43.2	9.6 ± 8.3	3.5 ± 2.5	128.9 ± 47.6	1.7 ± 1.3
Otu00028 (g:Arenimonas)	59.3 ± 24.8	36.0 ± 11.8	38.9 ± 6.8	55.1 ± 19.8	22.9 ± 6.6	32.1 ± 7.0	37.8 ± 10.4
Otu00030 (f:Methyllocystaceae)	131.2 ± 43.5	22.6 ± 7.7	11.6 ± 5.6	49.4 ± 17.6	40.3 ± 19.3	0.4 ± 0.2	22.9 ± 12.5
Otu00037 (g:Dyadobacter)	3.0 ± 2.1	80.1 ± 30.6	5.5 ± 3.6	30.9 ± 17.7	69.8 ± 18.6	9.1 ± 6.3	80.5 ± 33.5
Otu00034 (g:Methylbium)	82.8 ± 33.8	9.4 ± 3.3	43.7 ± 6.4	28.8 ± 10.0	15.9 ± 4.6	44.7 ± 8.1	42.4 ± 19.7
Otu00033 (g: Rhodoplanes)	55.0 ± 12.5	18.6 ± 3.6	45.8 ± 23.9	48.2 ± 25.5	50.1 ± 10.0	17.2 ± 2.6	26.6 ± 11.5
Otu00029 (g:Catellatospora)	39.9 ± 16.4	8.2 ± 2.9	45.8 ± 5.8	24.0 ± 6.5	15.9 ± 5.2	68.8 ± 19.4	49.3 ± 12.5
Otu00039 (f:Cytophagaceae)	3.2 ± 2.0	65.4 ± 13.4	26.4 ± 7.9	41.1 ± 18.5	70.3 ± 35.0	17.8 ± 6.7	26.6 ± 12.5

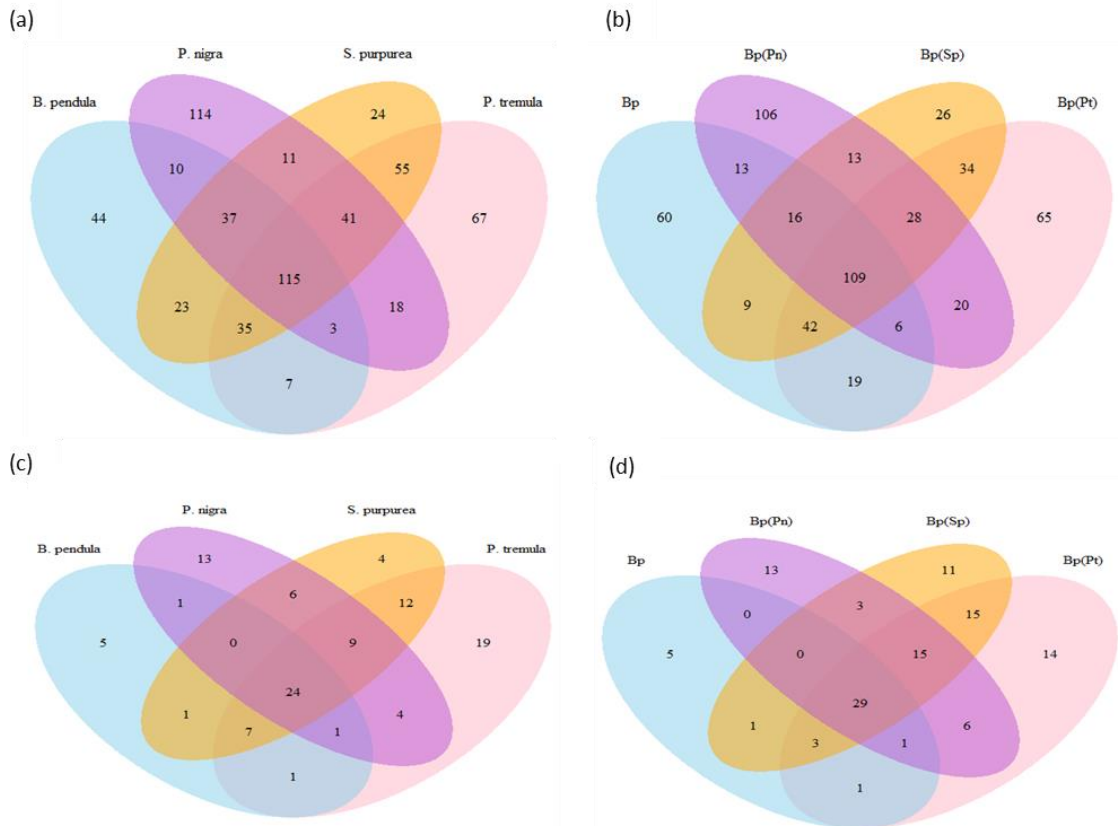
Supplementary Table 12. Distribution of the most abundant fungal OTUs (abundance >0.5%)

between the roots of the different plant species/locations studied.

	<i>B. pendula</i>	<i>P. tremula</i>	<i>P. nigra</i>	<i>S. purpurea</i>	<i>B. pendula</i> (Pt)	<i>B. pendula</i> (Pn)	<i>B. pendula</i> (Sp)
25_92637 (s:Geopora_arenicola)	14138.9 ± 1697.0	3186.4 ± 1178.1	12019.7 ± 1611.7	11688.3 ± 1832.5	4025.8 ± 1594.5	10177.8 ± 2119.8	8014.5 ± 2280.0
44_70433 (g:Lactarius)	313.1 ± 260.2	11997.8 ± 1138.5	135.8 ± 80.7	5480.7 ± 1587.0	8233.2 ± 1763.8	95.0 ± 63.9	4268.9 ± 2814.8
24_66088 (o:Pezizales)	1087.7 ± 899.7	16.7 ± 8.3	5973.7 ± 1523.9	1561.5 ± 1259.4	180.1 ± 116.9	5243.7 ± 2044.0	41.6 ± 34.8
13_15910 (o:Agaricales)	2937.8 ± 1196.0	2500.6 ± 580.7	1836.8 ± 665.8	1294.7 ± 401.7	835.2 ± 396.5	965.4 ± 651.7	993.9 ± 886.3
2_88952 (o:Helotiales)	1275.2 ± 1039.2	282.1 ± 110.4	42.4 ± 26.7	1289.8 ± 907.6	1932.2 ± 1405.2	56.6 ± 28.7	2351.6 ± 1244.0
2_98636 (Unclassified)	1815.6 ± 709.4	877.1 ± 235.0	1821.4 ± 532.7	322.8 ± 188.1	342.1 ± 164.7	1603.2 ± 527.3	186.6 ± 134.2
10_6322 (Unclassified)	1839.9 ± 913.2	591.8 ± 209.7	992.7 ± 253.4	611.5 ± 333.6	243.1 ± 134.6	1600.0 ± 537.7	156.5 ± 83.0
67_38353 (o:Hypocreales)	94.9 ± 48.7	134.3 ± 62.6	27.3 ± 25.5	495.5 ± 229.2	1400.7 ± 426.8	601.6 ± 587.8	2597.9 ± 854.0
33_90605 (o:Pleosporales)	205.1 ± 64.1	350.5 ± 68.9	621.9 ± 176.0	331.9 ± 97.0	724.1 ± 196.1	719.4 ± 259.2	1710.4 ± 601.7
13_37852 (o:Agaricales)	1385.1 ± 851.4	808.0 ± 674.3	6.2 ± 3.4	530.6 ± 405.8	232.8 ± 164.2	1522.5 ± 1444.0	7.6 ± 2.9
23_34612 (p:Ascomycota)	19.2 ± 12.9	1355.7 ± 1259.0	1010.7 ± 516.4	4.4 ± 1.4	22.3 ± 14.7	1068.0 ± 579.6	229.0 ± 226.9
22_16749 (o:Agaricales)	810.6 ± 316.3	610.1 ± 149.2	305.1 ± 127.5	797.7 ± 552.2	102.6 ± 44.1	246.9 ± 144.6	214.2 ± 180.1
11_43391 (c:Sordariomycetes)	1.1 ± 0.5	450.1 ± 420.8	6.9 ± 5.1	345.8 ± 312.1	927.7 ± 503.0	10.4 ± 8.8	914.0 ± 565.6
37_4836 (c:Sordariomycetes)	4.4 ± 4.1	47.7 ± 25.1	0.2 ± 0.1	122.1 ± 89.0	1944.7 ± 998.1	5.1 ± 1.7	409.4 ± 210.6
25_11792 (s:Sphaerosporella_brunnea)	3.8 ± 3.4	0.7 ± 0.4	1142.6 ± 1131.2	1052.8 ± 1045.4	0.4 ± 0.4	4.7 ± 1.9	0.1 ± 0.1
49_11297 (p:Ascomycota)	3.4 ± 3.0	151.8 ± 57.5	144.0 ± 57.4	12.6 ± 4.6	1190.3 ± 345.4	461.6 ± 150.2	135.7 ± 80.9
12_355 (s:Tomentella_ellisii)	0.7 ± 0.7	721.6 ± 712.6	2.2 ± 0.6	9.3 ± 7.7	1263.9 ± 1261.2	0.1 ± 0.1	10.1 ± 5.9
61_66824 (g:Cortinarius)	11.4 ± 6.9	944.9 ± 302.9	50.4 ± 44.0	332.8 ± 215.6	345.7 ± 199.8	24.3 ± 14.1	259.0 ± 179.8
51_62345 (Unclassified)	96.2 ± 39.0	57.2 ± 15.6	348.4 ± 138.5	14.1 ± 8.6	29.6 ± 8.0	1328.5 ± 1232.1	8.0 ± 5.0
18_28512 (p:Ascomycota)	1.8 ± 1.8	989.5 ± 478.1	0.1 ± 0.1	6.2 ± 3.7	750.5 ± 504.0	1.8 ± 1.3	10.4 ± 5.8
3_31227 (o:Agaricales)	1065.2 ± 1062.2	0.1 ± 0.1	237.2 ± 203.9	0.0 ± 0.0	0.2 ± 0.2	6.1 ± 4.6	0.7 ± 0.5
31_53893 (s:Tomentella_atramentaria)	0.1 ± 0.1	715.6 ± 480.4	6.7 ± 4.8	303.2 ± 271.0	58.4 ± 54.5	5.9 ± 4.9	1.2 ± 1.2
43_36490 (o:Hypocreales)	2.7 ± 0.5	23.4 ± 10.5	0.0 ± 0.0	19.9 ± 8.3	195.8 ± 91.7	0.9 ± 0.3	841.3 ± 786.3
39_44103 (o:Pleosporales)	20.7 ± 10.3	52.3 ± 14.2	14.6 ± 6.9	182.6 ± 107.4	142.2 ± 40.1	21.7 ± 12.2	588.1 ± 341.7

Supplementary Fig 5. Venn diagram showing the overlap of the bacterial communities (a; b) and fungal communities (c; d).

The different plants species (a), and for the different growth locations of *B. pendula* (b) and fungal communities (c and d) from the different plants species (c), and for the different growth locations of *B. pendula* (d) based on OTU. OTU delineation was based on a threshold < 97% of similarity. Only OTUs present in at least 3 of the 10 roots samples were considered for this analysis.



Supplementary Table 13. List of bacterial and fungal OTUs and assignment to the lowest

taxonomic.

Taxonomic level associated with the OTU using the Silva database, k kingdom, p phylum, o order, c class, f family, g genus, s genus_species present exclusively in roots of B. pendula growing at the centre of the dump or exclusively absent in these roots.

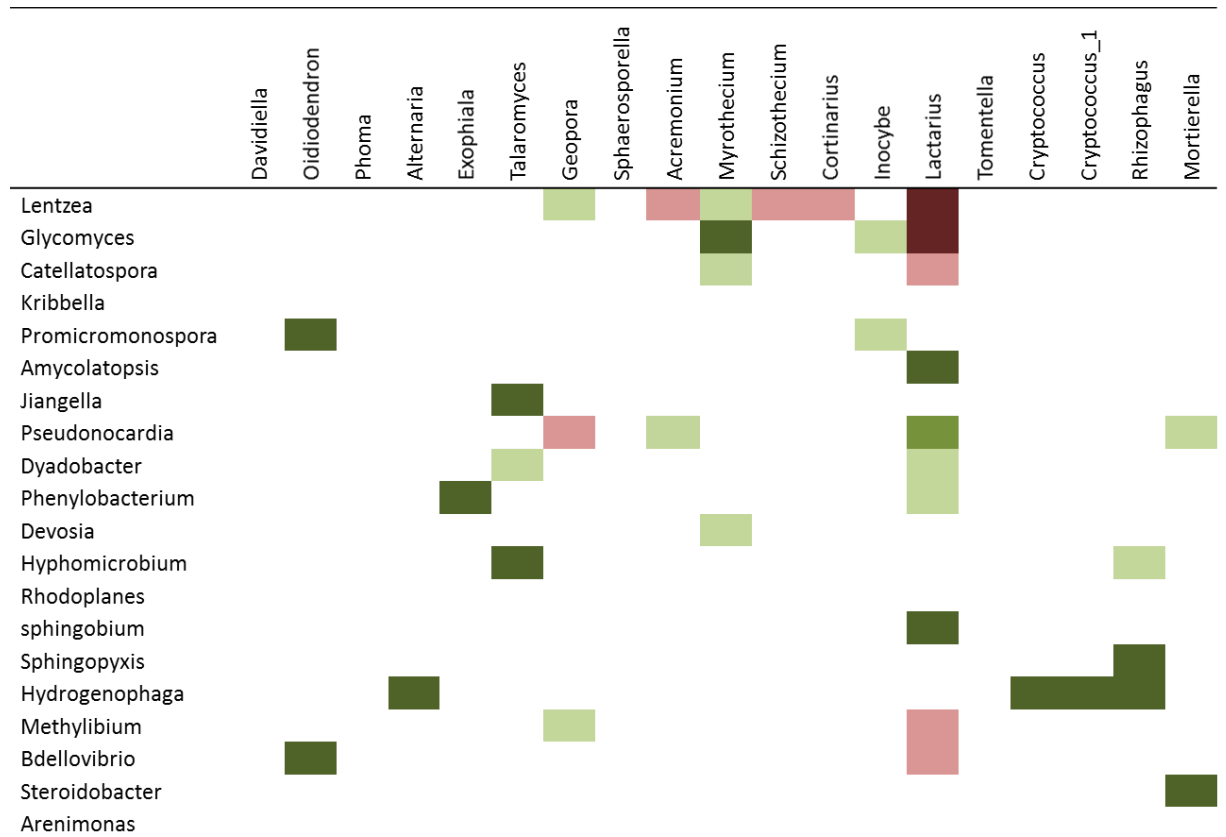
BACTERIAL DATASET		FUNGAL DATASET	
Exclusive <i>B. pendula</i>	Absent in <i>B. pendula</i>	Exclusive <i>B. pendula</i>	Absent in <i>B. pendula</i>
Otu00615 g__Lentzea	Otu00295 f__Ellin6075	41_79123 o:Agaricales	26_62835 s:Alternaria_metachromatica
Otu00331 f__Streptomyetaceae	Otu00135 f__Pseudonocardiaceae	61_75096 f:Ceratobasidiaceae	53_87848 o:Pleosporales
Otu00978 g__Sporocytophaga	Otu00147 f__Cytophagaceae	4_35502 g:Russula	36_45167 f:Incertae_sedis_3
Otu01056 f__Pirellulaceae	Otu00144 f__Kouleothrixaceae		70_59095 g:Myrothecium
Otu00260 o__Rhizobiales	Otu01311 g__Gemmata		61_72800 g:Rhizophagus
Otu00216 o__Rhizobiales	Otu00138 o__Rhizobiales		41_5715 f:Glomeraceae
Otu00386 o__Rhizobiales	Otu00073 f__Rhizobiaceae		56_45748 f:Glomeraceae
Otu00416 o__Rhizobiales	Otu00122 g__Kaistobacter		
Otu01236 o__Rhizobiales	Otu00151 g__Sphingomonas		
Otu00405 o__Rickettsiales	Otu00353 s__Sphingopyxis alaskensis		
Otu00482 c__Alphaproteobacteria	Otu00375 g__Janthinobacterium		
Otu00574 c__Alphaproteobacteria	Otu00050 o__Burkholderiales		
Otu00613 c__Betaproteobacteria	Otu00345 o__Myxococcales		
Otu00803 c__Betaproteobacteria	Otu00343 f__Coxiellaceae		
Otu00904 g__Bdellovibrio	Otu00075 g__Steroidobacter		
Otu00399 g__Aquicella	Otu00337 c__TM7-1		
Otu01003 c__Gammaproteobacteria	Otu00149 (unclassified)		
Otu00475 p__Proteobacteria	Otu00162 (unclassified)		
Otu00286 (unclassified)	Otu00083 (unclassified)		
Otu00291 (unclassified)			
Otu00393 (unclassified)			
Otu00480 (unclassified)			
Otu00551 (unclassified)			
Otu00580 (unclassified)			
Otu01044 (unclassified)			
Otu01269 (unclassified)			
Otu01284 (unclassified)			
Otu01319 (unclassified)			

Supplementary Table 14. Summary of number of unique and shared bacterial and fungal OTUs between the different plant species growing at the same sample spots.

	BACTERIA		FUNGI	
	Unique	Shared	Unique	Shared
<i>B. pendula</i> (Pn)	59	252	23	44
<i>P. nigra</i>	97		14	
<i>B. pendula</i> (Pt)	65	258	19	65
<i>P. tremula</i>	83		12	
<i>B. pendula</i> (Sp)	54	223	27	50
<i>S. purpurea</i>	118		13	
<i>B. pendula</i> (Pn)	167	144	37	30
<i>B. pendula</i>	130		10	
<i>B. pendula</i> (Pt)	147	176	50	34
<i>B. pendula</i>	98		6	
<i>B. pendula</i> (Sp)	101	176	44	33
<i>B. pendula</i>	98		7	
<i>P. nigra</i>	184	165	32	26
<i>B. pendula</i>	109		14	
<i>P. tremula</i>	181	160	44	33
<i>B. pendula</i>	114		7	
<i>S. purpurea</i>	131	210	31	32
<i>B. pendula</i>	64		8	

Supplementary Table 15. Fungal and bacterial relationships.

Spearman correlation coefficient values computed for fungal and bacterial genera using abundance data of the 20 most abundant genera.



Chapitre 5. Conclusions générales et perspectives

Objectif général	Caractériser les communautés microbiennes rhizosphériques de ligneux indigènes d'environnements exposés à des activités anthropiques génératrices d'ETMs.	
Sites étudiés	 <p style="text-align: center; color: yellow;">L'Aillon</p>	 <p style="text-align: center; color: yellow;">L'Ochsenfeld</p>
Problématique	Substrat enrichi en Hg (et As), issu de l'activité électrolytique de l'extraction du Cl (Solvay)	Substrat enrichi en Cr, Fe, Mn, As issu de l'activité d'extraction du Ti (Cristal)
Principal enjeu écologique	Développer le potentiel microbien afin d'améliorer la revégétalisation, par des espèces ligneuses, de sol contaminé. Limiter l'impact environnemental des sols anthropogéniques.	
Faits scientifiques marquants	Un terril de déchets industriels qui s'est abondamment revégétalisé depuis une quinzaine d'année. Cette revégétalisation s'est accompagnée d'une réapparition des communautés microbiennes, se traduisant par une composition et une structuration des microorganismes proches de celles du site contrôle. Un genre bactérien domine cependant : <i>Pseudomonas</i> .	Un sol très peu propice à la revégétalisation naturelle, se traduisant par le succès de quelques espèces (bouleau) pionnières avec une composition en communauté microbienne spécifique à ce type de sol. Le couvert végétal néanmoins confère une diversité microbienne supérieure à celle d'un sol non végétalisé. Un phylum bactérien domine : <i>Actinomycètes</i> .

Figure 31. Schéma explicatif de l'objectif général de la thèse, des deux sites d'études et de leur problématique, et des faits marquants de l'ensemble de ces travaux.

L'objectif principal de la thèse était l'analyse des communautés de microorganismes inféodées aux racines de ligneux indigènes d'anthrosols issus d'effluents industriels contaminés aux ETMs. Mon travail a reposé sur les deux sites expérimentaux présentés dans la Figure 31. L'objectif commun est, à terme, de limiter l'impact environnemental de ces sols anthropogéniques, en particulier l'érosion et la dispersion de poussières potentiellement contaminées, en améliorant le couvert végétal. Dans ce contexte de réhabilitation des sols, le travail présenté dans cette thèse reposait sur deux objectifs spécifiques :

Le premier objectif visait à caractériser par une approche de barcoding environnemental la structure des communautés de microorganismes de résidus revégétalisés :

- d'un terril d'effluents de l'entreprise Inovyn, issus de l'activité électrolytique de l'usine Solvay (Tavaux) et principalement contaminé par le Hg et l'As.
- d'un terril de gypse rouge de l'usine CRISTAL (site de l'Ochsenfeld) issu de l'activité d'extraction du Ti (à partir d'Ilménite) et principalement contaminé par le Ti, le S, le Fe et le Cr.

Le deuxième objectif visait à :

- réaliser une analyse physico-chimique comparative entre la zone d'échantillonnage des sols contaminés du terril où se trouve la végétation (*Betula pendula*) et la zone non végétalisée.
- isoler, caractériser et cultiver des inoculum microbiens à partir des prélèvements de sols de la zone végétalisée afin d'évaluer leurs propriétés de bioremédiation, en améliorant la reprise et la croissance du bouleau sur sols pollués.

Au travers ces objectifs, nous avons pu :

✓ **Caractériser les deux terrils de stockage de déchets industriels et préciser les objectifs du phytomanagement** : le sol du terril de Thann est très peu propice à la revégétalisation naturelle, en raison de la présence de contaminants, de la faible teneur en éléments nutritifs, et en azote (en dessous de la limite de détection) et du pH légèrement alcalin. La présence en grande quantité de Fe et de Mn, principalement sous forme d'oxyde (Carbonell, données non publiées), peut également limiter la disponibilité des nutriments sur l'ensemble du site. Le site de Tavaux en revanche a été abondamment recolonisé par les espèces ligneuses et herbacées depuis l'abandon des dépôts en 2010. Les deux sites présentent donc des caractères spécifiques et les opérations de phytomanagement devraient répondre à des objectifs différents :

- Sur Thann, l'absence de végétation naturelle imposerait une reconstruction de sol préalable et/ou une amélioration des conditions de croissance (biofertilisation) des végétaux qui s'y développent naturellement (le bouleau par exemple). La biofertilisation pourrait reposer sur l'utilisation de microorganismes indigènes. Cela passe par une meilleure connaissance des communautés microbiennes et une meilleure compréhension du rôle des microorganismes comme décrits ci-après.
 - Sur Tavaux, l'objectif serait essentiellement de rendre au terril une fonction de production de biomasse. D'ailleurs, la plantation BIOFILTREE s'était inscrite initialement dans un projet plus global de production de biomasse à valorisation énergétique. La encore, la biofertilisation pourrait contribuer à une meilleure productivité des espèces à planter. L'inoculation des peupliers par un consortium de champignons symbiotiques (non sélectionnés) pour la plantation de 2011 (ANR-BIFILTREE) a permis d'accroître la production de biomasse 18% (données 2017, non publiées).
- ✓ **Caractériser les communautés microbiennes** : les deux sols présentent des caractéristiques microbiologiques très contrastées.
- Les indices de diversité calculés sur les données issues du séquençage MiSeq sur le sol de Thann (Álvarez-López et al., 2018) et de Tavaux (Durand et al., 2017) révèlent en effet des situations contrastées : les indices de Shannon (H) sont de 1,3-1,9 et de 3,1-4,4 pour les communautés fongiques de Thann et de Tavaux, respectivement ; de 3,2-3,7 et de 4,8-5,5 pour les communautés bactériennes de Thann et de Tavaux, respectivement.
 - Le sol de Thann présente donc des communautés bactériennes et fongiques très peu diversifiées. La présence d'un couvert végétal augmente notablement la diversité microbienne sur ce type de substrat. Le sol de Tavaux, en revanche, présente des caractéristiques proches de celle de la zone contrôle, ce qui est plutôt encourageant étant donnée la nature très différente des deux sols. Ils partagent environ 2/3 de leurs communautés microbiennes, ce qui constitue un résultat peu attendu.
 - Les deux terrils sont caractérisés par la dominance d'un genre bactérien, *Pseudomonas* pour le terril de l'Aillon et *Streptomyces* pour le terril de l'Ochsenfeld.
- ✓ **Isoler et identifier les bactéries sur la base des caractéristiques phénotypiques et moléculaires** : les deux sols présentent des caractéristiques microbiologiques très contrastées, comme décrit précédemment. Les méthodes traditionnelles appliquées aux deux sites révèlent :

- La dominance du phylum des *Actinobacteria* dans la communauté microbienne du site de Thann. Ces bactéries présentes en fortes concentrations dans les sols jouent un rôle écologique important dans la métabolisation des composés xénobiotiques tels que les pesticides et les ETMs (Kieser et al., 2000). Les Actinobactéries peuvent favoriser la croissance des plantes de façon : i) directe, lorsque la plante reçoit un composé qui est synthétisé par la bactérie, ou lorsque cette dernière facilite l'assimilation des nutriments du sol par les plantes (Barka et al., 2016), ii) indirecte, en limitant les effets nocifs d'un ou plusieurs microorganismes délétères.
 - La combinaison de différents outils analytiques et statistiques (analyse ROC, Sparce-PCA, qPCR, barcoding environnemental) a permis de démontrer que le sol du terril de Tavaux est dominé par les *Pseudomonas*.
 - L'approche traditionnelle apparaît incontournable puisqu'elle permet ensuite de travailler sur les souches isolées. En revanche, l'approche par barcoding environnemental permet d'orienter l'approche traditionnelle, en sélectionnant par exemple des milieux appropriés (milieu spécifique aux *Pseudomonas* pour Tavaux, milieu spécifique aux actinobactéries voire *Streptomyces* sur Thann).
 - L'étude de l'impact de la revégétalisation naturelle sur une plus grande profondeur serait intéressante. Que ce soit sur le terril de Thann (20 m d'épaisseur) ou celui de Tavaux (5 m d'épaisseur), les végétaux qui ont pu s'installer naturellement développent des systèmes racinaires auxquels sont inféodées une multitude de microorganismes. Les activités combinées des molécules secrétées par les racines et les microorganismes peuvent ainsi avoir un impact sur les caractéristiques du sol au delà de la zone rhizosphérique notamment au travers des phénomènes de lessivages. Toutes nos études se sont en effet restreintes aux premiers 20 ou 30 cm de sol.
- ✓ **Etudier les traits fonctionnels des isolats sélectionnés *in vivo* et test d'inoculation :**
- Les tests fonctionnels utilisés ont montré la supériorité de certaines souches isolées (*Pseudomonas fluorescens* isolée du site de Tavaux, *Phyllobacterium* isolée du site de Thann) à produire des composés essentiels dans les interactions avec le milieu et les plantes hôtes. D'autres traits fonctionnels pourraient être pertinents à renseigner (production de métabolites à caractère antibiotique, bactéries inféodées aux champignons mycorhiziens) et utiles à des fins appliquées.
 - Cependant, ces tests *in vitro* renseignent sur les capacités d'une souche isolée à produire tel ou tel composé (auxine, sidérophore) mais ne s'avèrent pas toujours corrélés à une meilleure croissance des végétaux qui auraient été inoculés par ces souches (essais sur sol de Thann). Les tests en pots ont aussi

leurs limites, et il peut être difficile de transposer ces résultats aux situations de terrain.

- L'utilisation de souches microbiennes isolées et caractérisées (voir ci-dessus) à des fins de biofertilisation serait sans doute plus appropriée que l'utilisation de souches commerciales (projet BIOFILTREE). Cependant, elles nécessitent des essais sur le terrain afin de confirmer que les caractères PGP sont maintenus et s'expriment dans les conditions naturelles. Des essais de biofertilisation par des inocula mixtes (champignons mycorhiziens et bactéries) sur le terrain sont en cours.

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NOTES:

Abstract

My thesis subject includes one of the global projects of the UMR UFC/CNRS 6249 Chrono-Environnement entitled "phytoremediation strategies based on the use of trees and associated microorganisms", which is based, among other things, on 2 research projects:

- the PROLIPHYT project (Eco-Industry programme, 2013-2018, ADEME) entitled "Production of woody phytoremediants",
- the PHYTOCHEM project (ANR CD2i, 2013-2018) entitled "Development of eco-innovative chemical processes to exploit biomasses from phytotechnologies".

The general objectives are to improve the phytoremediation potential of a panel of woody species and to develop the microbial potential for assisted phytoremediation on contaminated soil. In addition to limiting the impact of pollutants, this strategy aims to promote the production of biomass on land abandoned and not exploitable by agriculture, while ensuring the biodiversity needed to restore an anthropogenic ecosystem.

My thesis work is financed through a ministerial doctoral contract for disability (dyslexia). It is based on the rehabilitation of two industrial sediment storage areas, used until the 2000s. These two experimental sites (INOVYN site of Saint-Symphorien-sur-Saône in Côte-d'Or, CRISTAL site of Ochsenfeld in Alsace) present very particular physico-chemical characteristics which make them privileged places of study. The first is a former settling lagoon whose sediments enriched in Hg, Ba and As come from the treatment of wastewater from SOLVAY's Hg electrolysis process. The second is a lagoon consisting of a backfill in which the titanium dioxide extraction residues from the CRISTAL Thann Plant have been stored since the 1930s. In contrast to the first experimental site, there is a low abundance of flora which results in heterogeneous development of a main woody species, the birch.

The natural and spontaneous recolonisation of plants, more particularly woody species on both sites, is undoubtedly the result of close collaboration with telluric microorganisms located near their root systems. We have thus chosen to work on 3 pioneer species that have naturally relocated to the two study sites: willow and poplar for the industrial wasteland of Tavaux and birch for the effluent treatment unit at the Ochsenfeld site.

Keywords:

Tailings (anthropogenic soil), natural environment (undisturbed soil), *Salicaceae* (poplar, willow), *Betulaceae* (birch), rhizobacteria, metabarcoding, diversity, identification, characterization, Plant Growth Promoting Rhizobacteria, environmental microbiology, INOVYN site of Tavaux, Ochsenfeld site.