Friedrich-Schiller-Universität Jena

seit 1558

universiteit hasselt

Chemical and biological factors influencing heavy metal mobilisation in the rhizosphere implications for phytoremediation

Dissertation – Doctoraatsproefschrift

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)
~
tot het behalen van de graad van
Doctor in de Wetenschappen, richting biologie

vorgelegt dem Rat der Chemisch-Geologischen Fakultät
der Friedrich-Schiller-Universität Jena
Voorgelegt aan de Faculteit der Wetenschappen van de Universiteit Hasselt

Von / door

Dipl.-Ing. Tsilla Boisselet

geboren am 24. August 1983 in Périgueux, Frankreich
Geboren op 24 Augustus 1983 in Périgueux, Frankrijk

ISBN:

Examiners / Gutachter:

1.

2.

Date of the public defence / Tag der öffentlichen Verteidigung: 15.06.2012

Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig angefertigt habe und nur unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, den 08. Mai 2012

The following work was developed in the context of the PhD Program carried on at the institute of Earth Sciences of the Friedrich Schiller Universtity in Jena, Germany, under the supervision of Prof. Dr. Georg Büchel, together with the Centre of Environmental Sciences of the University of Hasselt, Belgium, under the supervision of Prof. Dr. Jaco Vangronsveld.

Das Forschungs-Projekt in dieser Dissertation wurde am Lehrstuhl für Angewandte Geologie der Chemisch Geowissenschaftlichen Fakultät der Friedrich-Schiller-Universität Jena unter der Leitung von Prof. Dr. Georg Büchel angefertigt, gemeinsam mit dem Zentrum für Umweltkunde der Biologischen Fakultät der Universität.Hasselt unter der Leitung von Prof. Dr. Jaco Vangronsveld.

The author of this work received a PhD fellowship from the Jena School of Microbial Communication (JSMC).
Der Autor dieser Arbeit erhielt ein Stipendium der Jena School of Microbial Communication (JSMC).

Financial support was given by the German Research Foundation through the program of the Excellence Initiative.
Die finanzielle Förderung erfolgte durch die Deutsche Forschungsgemeinschaft im Rahmen der Exzellenzinitiative.

Aknowledgments - Danksagung

Completing this thesis would not have been possible without the contribution of many people.
First, I would like to thank Georg Büchel for accepting me as PhD student in his group and for guiding my studies in these challenging years. I am especially thankful for giving me the freedom to pursue my research ideas and for introducing me to the teaching activities. More importantly, I am grateful for his sincerity, his confidence and his encouraging discussions.
To Dirk Merten, for taking the time to read all manuscripts, having an open ear, and especially for leading me at the beginning when I still needed orientation. My appreciation words include also all my (former) colleagues of the group of Applied Earth Sciences, Andreas, Christian, Martin, Franziska, Anika, Anja, Delphine, Daniel, Sisay and Anahita for making my integration in Jena and in the working group easier. I express my gratitude to my collegues for their useful comments about my presentations and on the provisional results, and to all the co-authors of the manuscripts where I have been involved in, for sharing their experience with me. My special thanks to Rochus Merker for his cure of my computer and my laptop!
I wish to express my great acknowledgments to Jaco Vangronsveld for accepting me at his Institute and offering good working conditions and facilities. I would like to thank for suggesting me the cotutelle agreement, and for his valuable comments and suggestions for my research his has given. I want to thank at this place also Nele Weyens for her advises during the experimental trials, and the working group at the center for environmental sciences for their help and friendliness at any time. Bedankt Michiel, Sarah, Els, Bram, Sophie, An, Jan en Sasha!
I wish to acknowledge the members of the JSMC, especially Ulrike Schleier and Carsten Thoms, for giving me the opportunities of the JSMC network and all seminars and courses offered, and for giving me the chance and the flexibility to go abroad to continue my project.
I am deeply indebted to the persons working at the Thüringer Landesanstalt für Landwirtschaft, especially Hr. Schröter and his co-workers for the practical and useful advice to conceive pot experiments and for the cure during the growth.
I owe a special thanks to the Hydrology lab team at the Institute of Applied Earth Sciences for their advice and support for all analytical work, Dirk Merten, Ulrike Buhler, Ines Kamp and Gerit Weinzierl.
I thank also all co-workers at the Institute for Microbiology in the group of Erika Kothe, especially Götz, Eleen, Francesca, Martin, for sharing their experience and lab space with me.
I would also like to thank Björn Grübler from the Institute of Botanics, Plant Physiology for the sharing his knowledge in chlorophyll fluorescence measurements, and Elke-Martina Jung for kindly sharing her expertise for the microscopy. I would like to acknowledge the contribution of Sandra Studenik from Institute of applied and ecological microbiology for helping and letting me performing one experiment in her lab.
I would like to acknowledge the students Sebastian Wagner, and Marcus Meyer for their contribution and especially Claudia Rebentisch for her enthusiastic participation during the last experiment in Jena.
I would also like to thank Jan Meiling from the Wageningen University for his help in providing me ideas for cooperation institutes, and through whom I got to know Hasselt University.
I would like to address my gratitude to the German Research Foundation (DFG) and the JSMC for providing the financial support to accomplish this scientific investigation.

Finally, I am deeply grateful to my family for being close to me even from the distance and for following enthusiastically the experiences I shared with them. Thank you to my parents and to Christiane and Agnès for reviewing the translation of the abstract!
A very special thank goes to all my friends I met here in Jena, for sharing my life during this years, for your precious presence and the joyful moments that enriched my soul and softer the difficult moments. Thank you, Danke, Hvala lepa, Motehshakeram, Xie xie, Grazie, Dziękuję, Dhanyabad, Amesegënallô, Merci!
I cannot end without mentioning Raffaele, the linking point that provoked the deep association and tight link between private life, travelling, linguistics and scientific research from the very first day, strongly contributing to make my past three and a half years my most challenging and exciting… till now!

Thanks God, Thank you, Merci, Danke,

Tsilla Boisselet

Jena, im Mai 2012

À ceux que j'aime, à ceux qui m'ont appris la valeur de la solidarité

"L'UNION FAIT LA FORCE"

CONCORDIA PARVAE RES CRESCUNT,
DISCORDIA MAXIMAE DILABUNTUR,
Sallust (*Jugurtha*, 10)

ABSTRACT

The thesis aims at investigating the potential of phytoremediation on a heavy metal contaminated soil with very low nutrient content, low organic carbon and acidic pH. The soil originates from the Ronneburg mining district in Thuringia (Germany) which was the third largest uranium-producing area worldwide. The mining activities strongly altered the hydrogeology of the area. The acidic and highly mineralised solutions caused by leaching of waste heaps infiltrated into the soil and underlying sediments and polluted the water-soil system with high concentrations of Mn, Al, Ni, U, and Rare Earth Elements (REE). Despite remediation activities since the 1990s, contamination is still measurable. Since the soil pH is quite low (pH 4-4.5), the mobility and bioavailability of trace elements is high and so the amounts taken up by plants are significant. So, to study the interaction between soil trace elements and plants, in particular via root exudates, four plant species were chosen: Triticale (× *Triticosecale*), sunflower (*Helianthus annuus*), red fescue (*Festuca rubra*) and red clover (*Trifolium pratense*), grown as monoculture and polyculture. The last two were used for microbial studies, including the isolation and characterisation of endophytes potentially useful for remediation enhancement.

The substrate at the study area has been extensively characterised, and sequential extraction already allowed predictions about possible bioavailability of metals. However, the active influence of plants and their root exudates were not taken into consideration. Therefore, in a first part, REE will be used as a way to study root impact on element mobilisation, by comparing leaching by different organic and inorganic solutions. REE form a consistent group of so called metals, whose pattern, resulting from normalisation to a standard, can be used to describe different processes of dissolution and preferential precipitation. Our study shows that metals (REE) are mobilised in a different way by acidic solutions of different origin, and that organic acids lead to a different fractionation than inorganic ones. REE pattern changes were also observed in plants and their rhizosphere. The amounts of soluble trace elements decreased in the rhizosphere zone, while pH increased. Based on the analysis of REE patterns, it seemed that organic substances, like organic acids were an important factor that mobilises metals in the rhizosphere and allows their uptake into the plant. Furthermore, combined cultivation generally had a beneficial effect on plant growth; plants showed later necrosis and had a higher biomass production in relation to the initial seed quantity. Plants also had a clear effect on the soil structure: especially clover and red fescue were producing extended root networks, holding the soil. *Festuca* especially retained water. These features were considered to be interesting for remediation in sites with an erosion risk.

Microorganisms living around and inside the plants also influence their growth and mineral uptake. If more is known about these, they can be used, if chosen well, to enhance phytoremediation processes, especially on soil poor in nutrients. In the present study we concentrated on bacteria living inside the plant tissues, and isolated, characterised and finally identified the cultivable ones. 78 stable, morphologically distinct isolates were obtained, belonging to 32 genera, although 12 isolates could not be identified. The identified endophytic community was different for the 2 studied plants, so it seems that a selection took place. The endophytic bacteria showed additionally clear spatial compartmentalisation within the plant, suggesting that they can form specific associations with plant tissue. Furthermore, the specificity of some strains for some compartments suggests that different uptake

mechanisms for different plant tissues exist. They were found to be more diverse in the upper parts of the plants. Nevertheless, several strains isolated from roots could not be identified. Many of the isolated genera are very similar to known plant endophytes, and a large number of them are also related to strains used to support phytoremediation, mostly on sites contaminated with organic pollutants.

A number of isolates demonstrated the capacity to produce plant growth promoting substances and resistance to the trace elements enriched in the contaminant soil. As a consequence, some of these strains were used to promote growth of *Festuca rubra* and *Trifolium pratense*, and were inoculated separately to each plant and also as bacterial consortia of 2 or 3 strains. The inoculated plants showed better growth, higher plant density, healthier appearance, better and denser developed root network which, by consequence, was leading to a better soil structure. Moreover, the inoculated plants showed a higher photosynthetic efficiency, which can be interpreted as an improved fitness due to a better stress resistance. Further, the positive effect of the bacteria is enhanced in case consortia of strains are used. The effect of bacteria on trace element mobility and metal uptake depends mainly on the element itself: for instance Al was less present in the soluble fraction of the soil, and Mn more mobile in the soil after the combined action of plants and microbial consortia. Zn on the other hand was not influenced.

As the studied plants have a clear influence on metal mobility and pH, it is useful to use wisely their properties for remediation purposes. There is a large number of symbiotic bacteria described, which are living inside their tissue, and a notable part of them show promising properties for the support of plant growth and remediation. We suggest therefore using *Festuca* and *Trifolium* as complement to extracting, hyper-accumulating plants or to stabilising plants, in order to increase soil fertility and protection of soil erosion via the dense root network. *Festuca* is more influenced by bacteria concerning its root development, so should therefore get particular attention when it comes to choosing plant populations for remediation. It is also of importance to combine plants of different species to ensure long-term system stability.

ZUSAMMENFASSUNG

Diese Arbeit befasst sich mit der Untersuchung des Potenzials für Phytoremediation auf einem mit Schwermetallen kontaminierten Boden mit sehr geringem Nährstoffgehalt, niedrigem organischem Kohlenstoffgehalt und saurem pH-Wert. Der Boden stammt aus dem ehemaligen Bergbaugebiet Ronneburg in Thüringen (Deutschland), das der drittgrößte Uranproduzent weltweit war. Die Bergbauaktivitäten veränderten tiefgehend die Hydrogeologie des Gebietes. Die sauren und stark mineralhaltigen Abwässer, die durch Auslaugen der Halden entstanden, sickerten in den Boden und kontaminierten das Wasser-Boden-System mit hohen Mengen an Mangan, Aluminium, Nickel, Uran sowie Seltenen Erdelementen (SEE). Trotz umfangreicher Sanierungsaktivitäten seit den 1990er Jahren xx ist die Kontamination noch an vielen Stellen messbar. Da der Boden-pH ziemlich sauer ist (pH 4-4,5), ist die Mobilität und Bioverfügbarkeit von Spurenelementen hoch und die von Pflanzen aufgenommen Mengen signifikant. Um die Wechselwirkung zwischen Bodenelemente und Pflanzen zu untersuchen, insbesondere durch die Wurzelexudate, wurden vier Pflanzenarten ausgewählt: Triticale (× Triticosecale), Sonnenblumen (*Helianthus annuus*), Rotschwingel (*Festuca rubra*) und Rotklee (*Trifolium pratense*), die als Monokutur sowie als Polykultur kultiviert wurden. Nur die beiden letzten Pflanzenarten wurden für spätere mikrobiologische Untersuchungen verwendet, insbesondere für die Isolierung und Charakterisierung von potentiell nützlichen Endophyten in Hinblick auf Sanierungszwecke.

Das Substrat wurde umfassend charakterisiert und mithilfe sequentieller Extraktion konnten bereits Aussagen über den bioverfügbaren Metallanteil getroffen werden. Allerdings wurde der aktive Einfluss von Pflanzen und deren Wurzelausscheidungen nicht berücksichtigt. Daher werden im ersten Teil SEE zur Hilfe gezogen, um den Einfluss von Wurzelexudaten zu untersuchen, indem Elutionen mittels verschiedener organischer sowie anorganischer Lösungen und die daraus entstandenen SEE Muster verglichen werden. SEE bilden eine konsistente Gruppe von so genannten Metallen, deren Muster, das sich aus der Normalisierung zu einem Standard ergeben, für die Beschreibung unterschiedlicher Lösungs- und Präzipitationsprozesse benutzt werden kann. Unsere Studie zeigt, dass Metalle (inkl. SEE) in unterschiedlicher Weise durch verschiedene saure Lösungen mobilisiert werden und dass organischen Säuren zu einer anderen Fraktionierung führen als anorganische. SEE-Muster Veränderungen wurden auch in den Pflanzen und in ihrer Rhizosphäre beobachtet. Die Menge löslicher Spurenelemente nahm in der Rhizosphärenzone ab, während der pH-Wert zunahm. Basierend auf der Analyse von SEE-Mustern scheint es, dass organische Substanzen wie organische Säuren ein wichtiger Faktor sind, der Metalle in der Rhizosphäre mobilisiert und deren Aufnahme in die Pflanze ermöglicht.Weiters ergab sich, dass die Polykultur einen positiven Effekt auf die Pflanzen hatte; sie zeigten später Nekrosen und hatten eine höhere Biomasseproduktion in Bezug auf die ursprüngliche Menge Samen. Die Pflanzen hatten auch eine deutliche Wirkung auf die Bodenstruktur: vor allem Klee und Rotschwingel produzierten ein stark ausgebildetes Wurzelnetzwerk, das den Boden festigt; insbesondere *Festuca* konnte dadurch viel Wasser zurückhalten. Diese Eigenschaften sind vor allem für die Sanierung von Standorten mit Erosionsgefährdung wichtig.

Die Pflanzen und ihre Spurenelement-Aufnahme können durch viele andere Faktoren beeinflusst werden, da sie einen eigenen Mikrokosmos in ihrer Rhizosphäre bilden.

Mikroorganismen, die um und in der Pflanze leben, beeinflussen ebenfalls deren Wachstum und Mineralstoff-Aufnahme. Wenn mehr über diese bekannt ist, können sorgfältig ausgewählte unter ihnen verwendet werden, um Phytosanierungsprozesse, inbesondere auf nährstoffarmen Böden zu verbessern. In der vorliegenden Studie wurde der Fokus auf Bakterien, die innerhalb des Pflanzengewebes leben, gerichtet und die kultivierbaren unter ihnen wurden isoliert, charakterisiert und schließlich identifiziert. Es ergaben sich 78 stabile, morphologisch unterschiedliche Isolate, aus 32 Gattungen; 12 Isolate konnten aber nicht identifiziert werden. Die identifizierte endophytische Population war unterschiedlich für die 2 untersuchten Pflanzen, anscheinend fand eine Selektion statt. Die endophytischen Bakterien zeigten außerdem eine klare räumliche Trennung innerhalb der Pflanze, was darauf hindeutet, dass sie charakteristische Assoziationen mit bestimmten pflanzlichen Geweben bildeten. Darüber hinaus deutete die Spezifität einiger Stämme für bestimmte Kompartimente auf unterschiedliche Aufnahmemechanismen für unterschiedliche Pflanzengewebe hin. Die Diversität war größer in den oberen Pflanzenteilen. Allerdings konnten mehrere Stämme aus den Wurzeln nicht identifiziert werden. Viele der gefundenen Gattungen sind bekannten Pflanzenendophyten ähnlich und viele von ihnen werden auch verwendet, um Phytosanierung zu unterstützen, vor allem auf Standorten, die mit organischen Kontaminanten belastet sind.

Eine beachliche Anzahl der Isolaten zeigte Resistenz gegen toxische Metalle, die in dem Substrat vorhanden sind, sowie die Fähigkeit, Pflanzenwachstum fördernde Substanzen zu bilden. Daher wurden einige dieser Stämme verwendet, um das Wachstum von *Festuca rubra* und *Trifolium pratense* zu fördern; sie wurden einzeln sowie als Konsortien von 2 oder 3 Stämmen inokuliert. Die inokulierten Pflanzen zeigten ein besseres Wachstum, eine höhere Pflanzendichte, gesünderes Aussehen, ein besser und dichter entwickeltes Wurzelsystem, das zu einer besseren Bodenstruktur führte. Die inokulierten Pflanzen zeigten außerdem eine höhere photosynthetische Effizienz, die als eine verbesserte Stressresistenz interpretiert werden kann. Weiters ist die positive Wirkung der Bakterien erhöht, wenn mikrobielle Konsortien verwendet werden. Die Wirkung von Bakterien auf Spurenelement-Mobilität und Metallaufnahme hängt vor allem von dem Element selbst ab: zum Beispiel war Aluminium nach der kombinierten Wirkung von Pflanzen und mikrobiellen Konsortien in geringeren Mengen in der löslichen Fraktion des Bodens vorhanden und Mangan im Gegenteil mobiler im Boden. Zink andererseits wurde nicht beeinflusst.

Da die verwendeten Pflanzen eine klare Wirkung auf die Metallmobilität und den pH-Wert zeigen, ist es von Vorteil diese Eigenschaften gezielt zu nutzen. Eine große Anzahl an endosymbiotischen Bakterien wurde beschrieben und ein großer Anteil davon zeigt vielversprechende Eigenschaften für die Verbesserung von Pflanzenwachstum und Phytoremediation. Wir empfehlen daher die Verwendung von *Festuca* und *Trifolium* als Ergänzung zu extrahierenden, Metall-Hyperakkumulator Pflanzen, oder zu stabilisierenden Pflanzen, um einerseits die Bodenfruchtbarkeit zu erhöhen und andererseits als Erosionsschutz wegen des dichten Wurzelwerks. Am meisten wird die Wurzelentwicklung von *Festuca* durch Bakterien beeinflusst, daher sollte bei der Auswahl von Pflanzenarten für die Sanierung dieser Pflanze besondere Aufmerksamkeit gewidmet werden. Es könnte auch hilfreich sein, Pflanzen verschiedener Arten zu kombinieren, um eine langfristige Stabilität des Systems zu gewährleisten.

RÉSUMÉ

La thèse vise à étudier le potentiel de la dépollution par les plantes (phyto-assainissement) sur un sol contaminé par des métaux lourds, pauvre en éléments nutritifs, avec une faible teneur en carbone organique et un pH acide. Le sol provient de l'ancienne exploitation minière du district de Ronneburg, en Thuringe (Allemagne), qui était le troisième producteur mondial d'uranium. Les activités minières ont profondément altéré l'hydrogéologie de la région. Les solutions acides et fortement minéralisées, produites par la lixiviation des terrils, se sont infiltrées dans le sol, et ont pollué le système eau-sol avec des quantités élevées d'uranium, Terres Rares et autres éléments toxiques, principalement Mn, Al et Ni. Malgré les activités d'assainissement entreprises depuis les années 1990, le niveau de contamination est toujours mesurable dans plusieurs endroits du site. Le pH du sol étant assez bas (pH 4-4,5), la mobilité et la biodisponibilité des métaux sont élevées et les quantités absorbées par les plantes sont importantes. Afin d'étudier l'interaction entre les oligo-éléments du sol et les plantes, en particulier à travers les exsudats racinaires, quatre espèces de plantes ont été choisies: le triticale (× Triticosecale), le tournesol (*Helianthus annuus*), la fétuque rouge (*Festuca rubra*) et le trèfle violet (*Trifolium pratense*), cultivées en monoculture et polyculture. Seules les deux dernières ont été utilisées pour les examens microbiologiques, incluant l'isolation et la caractérisation de bactéries endophytes potentiellement utiles pour l'amélioration de l'assainissement.

Le sol de la zone d'étude a été largement caractérisé, et l'extraction séquentielle a été utilisée pour estimer la biodisponibilité potentielle de certains éléments toxiques. Cependant, l'influence active des plantes et leurs exsudats racinaires n'ont pas été pris en considération. Par conséquent, dans une première partie, les Terres Rares sont utilisés comme outil pour étudier l'impact des racines sur la mobilisation des métaux, en comparant la lixiviation du sol avec différentes solutions organiques et anorganiques. Les Terres Rares forment un groupe cohérent de métaux, et sont généralement graphiquement représentés sur un diagramme de distribution obtenu après la normalisation à un standard. Le motif varie en fonction des conditions physico-chimiques du système, et peut être utilisé pour décrire différents procédés notamment de dissolution et de précipitation.

Notre étude montre que les métaux (dont les terres rares) sont mobilisés d'une manière distincte par différentes solutions acides, et que les acides organiques conduisent à un fractionnement spécifique de celui causé par les acides inorganiques. Les terres rares ont également montré des changements de leur motif dans les plantes et leur rhizosphère. La part de métaux solubles a été diminuée dans la zone rhizosphère, tandis que le pH a été augmenté. En se basant sur l'analyse des motifs de terres rares, il semble que les substances organiques comme par exemple des acides organiques ont été un facteur important pour la mobilisation des métaux dans la rhizosphère et par conséquent pour leur absorption dans la plante. De plus, la polyculture s'est montrée bénéfique pour les plantes: elles montrent une nécrose plus tardive, et ont une production supérieure de biomasse par rapport à la quantité de semis initiale. Cela est particulièrement visible pour le trèfle. Les plantes ont aussi eu un effet manifeste sur la structure du sol: en particulier le trèfle et la fétuque rouge ont produit un réseau de racines longues qui tient le sol. Particulièrement avec *Festuca* la retenue d'eau était importante. Ces caractéristiques sont intéressantes pour l'assainissement dans les sites présentant un risque d'érosion.

De nombreux organismes vivant autour des plantes peuvent influencer leur croissance et l'assimilation de minéraux. Une connaissance plus approfondie de ces micro-organismes et de leurs propriétés permettrait de les utiliser, choisis judicieusement, pour améliorer les techniques d'assainissement par les plantes, en particulier sur des sols pauvres en nutriments. Nous nous sommes concentrés dans la présente étude sur les bactéries vivant à l'intérieur des tissus végétaux (endophytes), et avons isolé, caractérisé et enfin identifié les endophytes cultivables. 78 isolats stables, morphologiquement distincts ont été obtenus, appartenant à 32 genres; 12 isolats n'ont pas pu être identifiés. La communauté endophyte identifiée était différente pour les 2 plantes étudiées, il semble donc qu'une sélection spécifique pour chaque espèce ait eu lieu. Les bactéries endophytes ont montré en outre, clairement la compartimentation spatiale au sein de la plante, ce qui suggère qu'elles peuvent former des associations caractéristiques avec certains tissus végétaux. Ensuite, la spécificité de certaines souches pour certains compartiments suggère qu'il existe des mécanismes d'assimilation divergents pour différents tissus végétaux. La diversité était plus élevée dans les parties supérieures des plantes. Néanmoins, plusieurs souches isolées des racines n'ont pas pu être identifiées. La plupart des genres sont connus pour être des endophytes de plantes, et beaucoup d'entre eux sont également utilisés pour améliorer le phyto-assainissement, surtout sur les sites contaminés par des polluants organiques.

Un certain nombre d'isolats ont démontré la capacité de produire des substances favorisant la croissance végétale et la résistance aux métaux toxiques présents dans le sol. En conséquence, certaines de ces souches ont été utilisées pour promouvoir la croissance de *Festuca rubra* et *Trifolium pratense*, et ont été inoculées séparément pour chaque plante ainsi que par consortiums bactériens de 2 ou 3 souches. Les plants inoculés ont montré une meilleure croissance, une densité de plants par pot supérieure, une apparence plus saine, un réseau de racines mieux développé et plus dense, qui a, par conséquent, conduit à une meilleure structure du sol. Les plantes inoculées ont montré une plus grande efficacité photosynthétique, qui peut être interprétée comme une meilleure santé en raison d'une résistance supérieure au stress. En outre, l'action positive des bactéries est renforcée dans le cas d'inoculation de consortiums de souches. L'effet des bactéries sur la mobilité l'absorption des métaux dépend surtout de l'élément lui-même: par exemple, l'aluminium était moins présent dans la fraction soluble du sol, et le manganèse plus mobile dans le sol après l'action combinée des plantes et des consortiums microbiens. La solubilité du zinc, d'autre part n'a pas changé.

Comme les plantes étudiées ont une influence évidente sur la mobilité des métaux et le pH, il est utile d'utiliser judicieusement leurs propriétés pour la dépollution. Un grand nombre de bactéries symbiotiques vivant à l'intérieur de leurs tissus ont été décrites, et une partie importante d'entre elles présente des propriétés prometteuses pour le soutien de la croissance des plantes et l'assainissement. Nous suggérons donc d'employer *Festuca* et *Trifolium* en tant que complément à des plantes utilisées pour l'extraction, notamment des plantes hyper-accumulatrices, ou bien des plantes stabilisatrices, afin d'accroître la fertilité du sol et comme protection contre l'érosion, en raison de leur réseau racinaire dense. *Festuca* est plus influencée par des bactéries concernant le développement de ses racines, et devrait donc faire l'objet d'une attention particulière dans l'avenir quand il s'agira de choisir les populations de plantes pour l'assainissement. Il est également important de combiner des plantes de différentes espèces, pour assurer la stabilité du système à long terme.

TABLE OF CONTENTS

GENERAL INTRODUCTION -

PRESENTATION OF THE PRESENT WORK

Motivation of the research and summary of the main results

1 BACKGROUND AND MOTIVATION

Soil and water pollution by heavy metals is a major concern in many areas of the world, influencing the health of local populations, the use of the natural resources and the environmental equilibrium. Furthermore, the increasing need for raw material for diverse technological applications tend to multiply the mining sites even those with lower ore contents, causing noticeable changes for the environment despite of ameliorated mining methods. In particular, soil, surface water and groundwater are likely to get an important input in different of these persistent pollutants, compromising the biosphere including humans on large areas. Additionally, areal pollution through industry results in sites of a large area with diffuse contamination. Aside of that, the essential soil function is disturbed or destroyed first through the contaminants, but also by the significant changes in its structure and its biosphere, due to relocation activities. Bare soil or heaps are furthermore more likely to erode through the action of wind and precipitation, causing an eluviation of soil parallel to a spreading of contaminants in the air and water phase (Davies and White, 1981; Razo et al., 2004). So, the increasing industrialisation connected with increased use of land for urbanisation reveals the necessity of remediation of these former mining sites and industrially contaminated sites in order to make possible the (re-)use of these areas, if not for agricultural production, at least for energy crop production. Indeed, the potential of these vast more or less heavily polluted areas is huge, which explains the rising interest for remediation techniques. Especially techniques adapted to large surfaces, allowing a treatment without extensive work and investment are important to develop. Therefore, phytoremediation was one of the possibilities on which effort were focused on, combining land cover and soil stabilisation, use of the growing crops, and possibly cleaning of the soils.

In this context, the present thesis aims at investigating the potential of phytoremediation on a heavy metal contaminated soil with very low nutrient content, low organic carbon and acidic pH. In particular, the important impact for the metal contamination and the soil structure, of specific actors of the root zone of plants will be shown. Indeed, the local processes influencing metal mobility taking place in the rhizosphere will be considered, including the chemical action of root secretes on one hand, and on the other hand the effect of symbiotic bacteria on plants and soil. Particular attention is laid on the beneficial effect of these microorganisms on plant health and biomass production, these features being important for ecological equilibrium and remediation. It should show the importance of understanding the complex interaction between different biota, and its use to improve bioremediation and restore soil function.

1.1 History of the study area

The study was performed on a soil originating from the former Ronneburg mining district in Thuringia in Germany, one of the most important in the former GDR. During the over 40 years of mining activity 231,000 t uranium have been collected in total in the area, the highest yearly production was around 7000 t in the 60s. The former GDR was so the third world producer after US and Canada. (Jakubick et al., 2002; Kahlert, 1992; Lange, 1995).

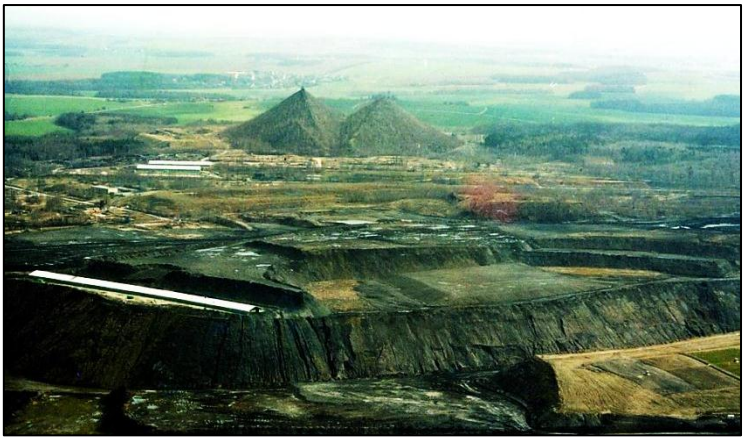


Figure 1: Ronneburg mining area during active mining time. In the front the open pit, in the back the leaching heaps. (in Chem. Erde - Geochem. Vol. 65, 2005)

The mining activities strongly altered the hydrogeology of the area. The excavation activities introduced the rock to oxidising conditions. In the 1950s and 1960s big quantities of ore have been dumped as they were considered as low-grade because their low content of uranium oxide. Hence, under the influence of oxygen, rainwater and bacterial reactions, sulphuric acid was produced. This sulphuric acid as well as pyrite oxidation lead to high sulphate concentrations in the drainage water of the heaps. (Geletneky, 2002). The resulting acid solution, rich in sulphate, also strong enriched with heavy metals is called acid mine drainage (AMD) or ARD (Acid Rock Drainage); or because of its yellowish reddish colour due to iron hydroxides also „yellow boy“ (Geletneky, 2002; Kahlert, 1992).

Among a lot of heaps in the area, the Gessen heap (Figure 1) was the only leaching heap built up by Ordovician and Silurian shales with a low grade ore mineralisation (< 300 U g/t; Rüger and Dietel 1998). To mobilise the uranium, the material was leached in the 1970s with AMD (pH 2.8) from underground mining and later on with sulphuric acid (10 g/L). The leach pad was sealed with 0.6 m of loam and was compacted in order to prevent infiltration. This seal was covered by a one-meter-thick layer of coarse waste rock containing low grade of uranium mineralisation from Lichtenberg, as a drainage layer during the leaching process. The leachate was collected over drainage hills into collection basins dug for that purpose. It is probable that these were not completely sealed, and some contamination infiltrated the under-lying soil to a great depth. In 1989, leaching was stopped (Wismut, 1994a, 1994b).

In the 1990s, after German reunification, mining activity was ceased. Then a German state company was founded, Wismut GmbH, whose main goal was the remediation of the area. The heaps were transferred into the underground part of the mine, the former underground mines were flooded in order to lower the redox potential (and so the sulphide oxidation). The area was left uncovered, which led to form puddles before Gessen heap before 10 m upper layer of the underlying Quaternary sediments were excavated, and a layer of allochtonic top soil was added as a last remediation step (Eißmann, 1997). A few years later, in 2003 and 2004, the evidence of residual heavy metal contamination was measured in water and in that upper soil layer as reported by Carlsson and Büchel (2005). It showed that the remediation was not completed despite remediation activity. The AMD, which infiltrated into the soil, polluted the water-soil system with high concentrations of uranium, Rare Earth Elements (REE) and other heavy metals, mainly Mn, Al and Ni (Figure 2). Still, in many locations at the site it is visible that plants are affected by high metal concentration indicating that contamination is present in the root zone, the upper 30 cm of soil.


[a]

[b]

Figure 2: AMD and ron precipitates in the Gessen creek near Gessenwiese [a]; Fe-, Al-, Ni-precipitates near Northern heap [b] ***(2009, own photo)***

In 2004 the test site "Gessenwiese" was created (Figure 3) in the northern part of the base area of the former leaching heap, Gessenhalde, with the aim of monitoring the groundwater and soil parameters (elements' concentration and physicochemical parameters) and improving remediation strategies for low heavy metals contaminated areas (Büchel, et al., 2005; Neagoe et al., 2009). Different plots within the test site were amended dig to 20 cm, by adding allochtonic non-contaminated soil material. The plots were amended by addition of top soil or compost soil; a non-amended plot was used as a control.

Figure 3: Test field "Gessenwiese" on the former Gessen-heap area ***(2005, photo by G. Büchel)***

1.2 Characterisation of the studied substrate

Figure 4: Hardpan layer enriched in Mn, Fe and other metals. This showed a recent mineral layer formation connected to a strong interaction with the groundwater. (in Chem. Erde - Geochem. Vol. 65, 2005)

The soil that was used to test remediation strategies was homogenised on the top 100cm. It is described as a silty sandy soil. The soil pH is quite low (pH 4-4.5). The content of organic carbon and inorganic nitrogen (both nitrate and ammonium) are very low (respectively 0.1%, 0.1% and 0.01% of the soil dry mass) (Mirgorodsky et al., 2010), compared e.g. to a fertile Renzina soil (Table 1). Phosphorus is also present in low amounts (1mg/100g), most of it

being insoluble. Similarly, Fe is poorly present in a plant available form, most of it being found as an oxide, and in the residential fraction. The soil is very rich in S, in form of sulphate; with total values of about 90 mg total mineral S for 100 g soil. The soil is further characterised by the presence of different metals (Table 3) including Rare Earth Elements (REE, La-Lu). The average total amounts of ∑REE reach values of about 180 µg/g. It is known that REE have been used as fertilisers in agriculture, especially in China, since very low doses are able to enhance plant growth - *hormesis effect* (Grawunder and Merten, 2012) - and find use in many branches of industry. However, the toxicity of these elements has to be considered. In fact, maximal permissible concentrations of REE have been established only recently for surface water and soil in the Netherlands (Sneller et al., 2000). Wang et al. (2009) demonstrated the toxic effect of Tb(III) on the plant photosynthesis through damaging of chloroplast ultrastructure. Other important contaminants are Al, Mn, Ni, Zn and Cu, and remaining U (total amounts of about 5 µg/g) from the mining. So, although the estimation of critical values is controverted because of the difficult estimation of the actual toxic effects, it can be stated that Ni concentrations found in the substrate are over the average concentrations found in soils and the threshold of 40mg/kg dry soil (Sipos and Póka, 2002). Arsenic is also more elevated compared to the average soil values (up to 15 mg/kg). Further, Copper and Zinc total amounts are above the background values given by different institutions (Table 2) although there values are not considered as a risk for human health. According to the Austrian Federal Forest Office (Bundesministerium für Land- und Forstwirtschaft, 2012), the quantities of Co and Cu present in the substrate are in a range that can affect soil microorganisms' survival and metabolic efficiency. However, the threshold values often apply for single contamination, the metals may have a different effect if present together.

As a component of the earth crust, Mn is present in all soils, with background values in the range of 40-900 mg/kg, with a mean background estimated to be about 330 mg/kg , accumulation occurring mostly in the subsoil, and in the sand fraction of soils (Šarić and Lucchini, 2007). The values of our study site are therefore in the upper range of the reported mean values. Microbial activity is one important factor influencing the oxidation state of Mn. Mean Cu concentrations in uncontaminated soils vary from 6-80 mg/kg. Total Co in most soils ranges from 0.1-50 µg/kg.

During drilling for groundwater samples, Fe precipitation was observed generally in the loam. The dark colour of some layers was also show high Fe contents compared to 'normal' soil; in this case the average Fe content calculated as Fe_2O_3 was 6%, versus over 65% for a hardpan layer (Carlsson and Büchel, 2005).

Table 1: Substrate characterisation and comparison with a soil adapted to plant growth

	pH_{aqua}	**pH_{KCl}**	**EC**	**C_{org}**	**N**	**$CaCO_3$**	**NO_3_N**	**NH_4_N**	**N_{min}**	**S_{min}**	**P**	**K**
			µS/cm	%			mg/100g					
Renzina	7.53	6.98	285	6.89	0.75	15.5	1.4	0.1	1.5	0.7	4.6	21
Substrate	**5.17**	**4.44**	**749**	**0.1**	**0.04**	**0.5**	**0.1**	**0.01**	**0.2**	**89.2**	**1**	**4**

Table 2: Metal natural values in the environment and threshold values according to different regulations

Unit	Elements												Method – Remarks	Ref.
	Al	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Mn**	**Ni**	**Pb**	**Zn**	**U**	**REE**		
mg/kg				0.1-50		6-80	40-900						Background total content soil	[1]
mg/kg								40						[2]
µg/kg		100	40		100	800		-	300	-			NH_4NO_3 extraction	[3]
mg/kg			1-3		*	50-140		30-75	50-300	150-300			Soil pH 6-7 Total soil content	[4]
												(a)	MPC	[5]
µg/g			3.5-7		> 30[a]	20 [b]			≥ 500	600			Activity soil enzymes	[6, a]
µg/g			3.5-7		20	≥ 500			600	> 30 [b]			Soil respiration	[6, a]
µg/g			7		20-35	≥500[c]			300?	-			Microflora survival	[6, a]
mg/kg					75 - 100	60		95	100-400	170			Forest soils, Microorganisms survival rate	[6, b]
µg/g			3.5						500				Microbionta survival in Humus layer	[6, c]
µg/g			3-8						100-400				Critical toxic threshold	[6, d]

(a)	**Y**	**La**	**Ce**	**Pr**	**Nd**	**Sm**	**Gd**	**Dy**					
µg/L	6.2	10	22	9	1.4	7.6	6.8	9.1					MPC in fresh water
µg/L	0.94	1.01	0.28	1.00	0.86	0.42	0.85	3.8					MPC in salt water
µg/gDW			53										MPC in soil

MPC = Maximal permissible concentration

[1] Šarić and Lucchini, 2007

[2] (Sipos and Póka, 2002)

[3] (Reichenauer et al., 2010)

[4] (European-Council, 2009 (1986, amended))

[5] (Sneller et al., 2000)

[6] http://bfw.ac.at/400/smilex/grenzwerte_schwermetalle_boden.pdf

[a]Tyler G. (1992), [b] Witter(1992) & Kabata-Pendias and Pendias (1984), [c] De Vries & Bakker (1996), [d] Dosskey & Adriano (1991)

[a] For Cr(III)

[b] Zn+Cu=200 µg/g

[c] Zn+Cu=200-300µg/g

Table 3: Amounts of selected elements (nutrients [a] and potentially toxic trace elements [b]) in µg/g, different fractions of the sequential extraction (Fractions I to VI and total extraction)

[a]	Al	Ca	Fe	K	Mg	Mn	Na	P	S
F I	3.7	1242	ND	56	529	88.4	15	ND	767
F II	2.22	71	ND	ND	29.3	9.02	ND	4	71
F III	28.6	14.3	101	ND	3.19	499	ND	5	ND
F IV	35.0	16.4	104	ND	0.63	4.03	ND	13	ND
F V	258	25.1	1386	ND	5.17	9.58	ND	122	ND
F VI	812	7.3	11079	ND	65	22.5	ND	185	ND
Total	59608	1952	37861	21208	4520	830	3669	627	

[b]	Zn	Cr	Co	Ni	Cu	As	Cd	Ti	Cs	Pb	U	∑REE
F I	2.20	ND	1.046	6.7	0.078	ND	0,08	ND	0.024	0.0132	0.0040	0.849
F II	0.56	ND	0.109	0.87	0.26	ND	0.029	ND	0.0057	0.084	0.825	1.63
F III	2.31	ND	10.36	6.5	1.05	ND	0.13	0.92	ND	0.977	0.262	1.96
F IV	0.72	ND	0.458	0.78	1.13	0.16	ND	0.65	ND	1.260	0.0549	3.09
F V	2.20	1.28	0.68	1.75	1.72	3.3	0.021	20.0	ND	1.383	0.621	9.00
F VI	12.6	6.66	2.33	10.6	15.5	7.83	0.045	45.6	0.017	2.22	0.5035	7.03
Total	76.9	34.8	21.66	**52.5**	35.2	**19.1**	0.61	5956	5.79	16.2	5.3	178.7

The soil is therefore not heavily contaminated in general, which allows plant growth, but shows also local so-called “hot spots” of elevated contamination and lower pH (personal communication, M. Reinicke). However, it was shown that the contaminant concentrations were increasing if no remediation technique was applied (Mirgorodsky et al., 2011), due to capillary rise from groundwater (Pourjabbar, 2012). Since the soil pH is quite low (pH 4-4.5), the mobility and bioavailability of metals is high and so the up taken amounts of metals by plants are significant. Therefore, this substrate is adequate for monitoring of groundwater chemistry and soil parameter and improving remediation strategies for slightly heavy metals contaminated areas.

Thus, the combination of elevated toxic metal concentrations and low nutrient availability constitute a challenge for plant growth in general, and especially for plant-based bioremediation.

1.3 Phytoremediation- principles and challenges

Strategies that are usually applied to remediate such sites include on one hand the removal and relocation of the soil itself (Eißmann, 1997) especially if the quantity of concerned substrate is small, to allow a treatment or a storage elsewhere; or soil washing, i.e. the removal of metals by leaching with acids and chelators (Abumaizar and Smith, 1999). On the other hand, metal stabilisation by surrounding the tales with an appropriate barrier (clay, composite or capillary barrier) or by using soil amendments is another strategy. Chemical remediation strategies for U in particular consist most often of injection of Fenton reagent into the soil, provoking an unspecific oxidation reaction leading to the dissolution of metals (Keith et al., 2007). Those can be removed by pumping the solution. In the case of the Ronneburg site, the most contaminated waste rock material was replaced into the underground mining

site and open pit mine; the groundwater level was allowed to rise again to install anoxic condition again and so prevent further oxidation processes and AMD formation. Carlsson and Büchel (2005) described elevated residual contamination levels in the underlying sediments, which lead to the creation of a test site to study the possibilities of alternative remediation strategies for diffuse contaminated sites.

Stabilisation is recommended when contamination is quite high on a large area, especially in the case of mixed (multi-element) contamination. Typical soil amendments are: iron oxides, liming agents, apatites, Fe-, Al or Mn-hydroxides, zero-valent iron grit, zeolites, organic matter, red muds and clays, phosphates, industrial waste (cycloning ashes) (Vangronsveld et al., 2009). The aim is to reduce the solubility by forming of insoluble trace element species, and favour absorption. AMD is often treated by chemical oxidation, increasing the pH with addition of clay or sodium carbonate, or oxidation of sulphides.
Nevertheless, conventional clean-up technologies are costly and feasible only for small but heavily polluted sites where fast and complete decontamination is required, chemical AMD treatment is time-consuming and not very efficient, and the resulting solution needs further treatment afterwards. Further, some of those methods, such as soil washing, can cause contamination of water ways through seepage waters, a negative impact on biological activity, soil structure and fertility, and generate important engineering costs (Pulford and Watson, 2003; Vangronsveld and Cunningham, 1998). Moreover, disturbing the soil structure can lead to higher metal out washing (Neagoe et al., 2009); this aspect should not be forgotten when moving soil material. The establishment of a vegetation cover on the contrary would stabilise the structure and biological activity, and so avoid erosion and spreading of contaminant into the air and the water. Therefore, sustainable *in situ* techniques for remediation of contaminated sites, as bioremediation, need to be applied and improved.
Bioremediation using bacteria, algae, fungi, plants, or combinations of those, has been studied for many different types of contaminations, and successfully applied in numerous cases, ranging from metal precipitation on cell walls to uptake by plants (Beveridge and Fyfe, 1985). Phytoremediation, i.e. the use of plants to remediate polluted areas, includes several techniques, as phytoextraction, phytostabilisation, phytovolatilisation or rhizofiltration.

Nevertheless, there are some problems connected to poor growth conditions of plants on contaminated sides, because of the toxicity and often additionally encountered difficulties as poor nutrients, bare soil, erosion or water stress. Hence, improving of phytoremediation strategies for heavy metal contaminated soils is necessary. In this context, it is useful to focus on the interaction between the soil and the plant-influenced and –influencing biosphere, in particular with soil and plant-symbiotic microorganisms, and their possible use for remediation.

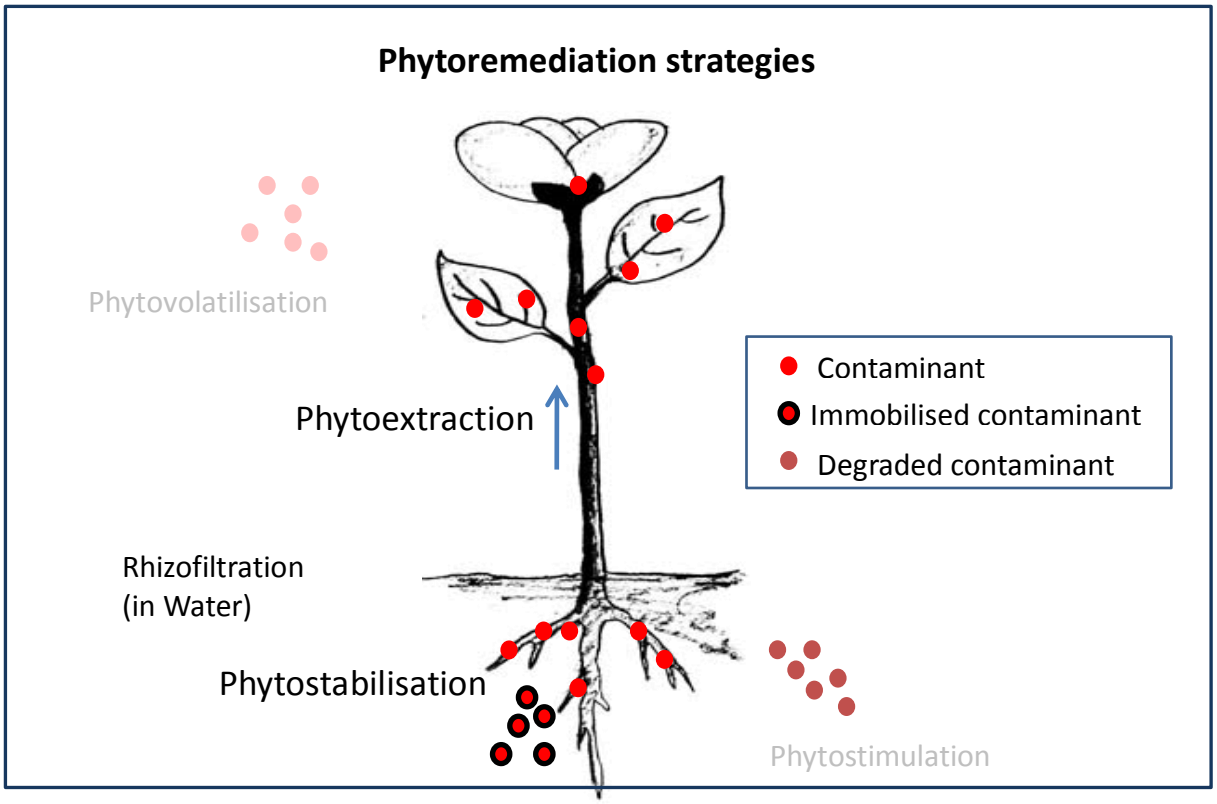


Figure 5: Schematic representation of phytoremediation strategies

Phytoextraction

Phytoextraction aims at removing contaminants through uptake and accumulation in plants, and is followed by plant biomass harvest and treatment of the biomass. This technique is best suitable for diffusely, low polluted areas, where contaminants occur on the surface and is successfully used on many sites (Mirgorodsky et al., 2010; Pulford and Watson, 2003; Raskin et al., 1997; Vangronsveld et al., 2009).

Important factors for a good phytoextraction are tolerance to metals, fast growth, accumulating trace elements in above ground biomass, easy to harvest (Vangronsveld et al., 2009). The choice of the right plant is important, since some plants are better for some contaminants than others are. In general, plants known as hyperaccumulators are chosen, since as they name indicates they can accumulate high amounts of heavy metals in their above ground biomass. The mechanism of metal solubilisation by the hyperaccumulator plant does not involve either the reduction of pH in the rhizosphere, or the release of reductants from roots (Kidd et al., 2009). However, only 400 plant species are classified as such, and their slow growth, low biomass production limit their use. That is why the trend is going towards the use of metal tolerant high biomass producing plants. For phytoextraction, a simple increase of biomass production leads to a greater total uptake of metal. That is a reason for using *Salix* sp. (Willow) and *Populus* sp. (Poplar) since they are fast growing trees (not hyperaccumulator, but have deep roots and high biomass production).

Most of the time, hyperaccumulators are metal specific. For instance, *T. caerulescens* is known to accumulate high amounts of Zn, Cd, or Co; Brassicaceae (*Alyssum*, *Thlaspi*) and Euphorbiaceae (*Phyllanthus*, *Leucocroton*) tend to be specific for Ni and Zn; Lamiaceae, Scrophulariaceae for Cu, Co; and *Pelargonium* in acidic and calcareous soils accumulate Pb.

Plants were also considered for remediation of Uranium, although biota in general are not known to accumulate specifically U. Phytoextraction and rhizofiltration were the most common used strategies, with plants as indian mustard (*Brassica juncea*), russian thistle (*Salsola tragus*), purple amaranth (*Amaranthus blitum*). The uptake was enhanced by the supply of citric acid, chelating agents or by increasing the transpiration rate (Keith et al., 2007).

Different strategies exist to use the harvested biomass: extraction and re-use of the metals, fermentation of biomass as energy crop. One of the problems for phytoextraction is the bioavailability of contaminants, which should often be enhanced by treatment of chelating agents (EDTA), although they represent themselves an environmental concern (Kidd et al., 2009; Vangronsveld et al., 2009).

The big advantage of this technique are the low costs, but many limitations make its use restricted: it is suitable only for low contaminated areas, only for surface soils; furthermore long treatment durations, as well as no full decontamination are another problem (Vangronsveld et al., 2009).

Phytostabilisation

Phytostabilisation consists in establishing a vegetation cover and inactivating toxic metals *in situ*, by combining the effect of metal tolerant vegetation and soil amendment in order to minimise the mobility and so the toxicity of metals, and if possible improve soil fertility. The expected property of plants should be the capacity to retain the contaminants in the roots or rhizosphere (excluder mechanism) to limit the spreading through the food chain. Soil rich in clay minerals or organic matter offer better starting conditions. This option is recommended when contamination is quite high on a large area, especially in the case of mixed (multi-element) contamination. Typical amendments are:, iron oxides, liming agents, apatites, Fe-, Al or Mn-hydroxides, zero-valent iron grit, zeolites, organic matter, red muds and clays, phosphates, industrial waste (cycloning ashes) (Vangronsveld et al., 2009). The aim is to reduce the solubility by forming of insoluble trace element species, and favour absorption. Further, vegetation helps to avoid erosion by precipitation and wind and stabilise the soil, reducing the percolation and thereby avoiding a spreading of pollution through water and air. The choice of the right vegetation cover is decisive, and depending on the site conditions. Grasses have been suggested for example for Cu tailings (Teng et al., 2008). To achieve a stable persistent cover it is important to use a mixed culture, and combine grasses, legumes and trees (Kidd et al., 2009).

Another suggestion for phytoremediation is to grow plants who produce sufficiently stable natural chelators to improve bioavailability and so the extraction, as a succession after excluder plants. Mycorrhizal colonisation is an important factor in phytostabilisation, while its role in phytoextraction is more ambiguous.

2 ROOT EXUDATES: THE ACTIVE CONTRIBUTION OF PLANTS TO METAL MOBILITY

Soil is a complex biogeochemical material whose physical, chemical and biological characteristics and properties differ strongly from the underlying parent rock. Soil is composed of mineral particles and organic material, populated by several microorganisms,

soil fauna and a more or less dense root net. The space between the particles forms a system of pores, filled by gases and water, allowing material exchange between solids and aqueous phases and plants (Harrison, 1999). Trace elements play a role in this dynamic system, ranging from a mobile state in soil solution and biota, to precipitates in the organic phase or complexes with organic components. In the soil solution elements are present as free ions, ion pairs, ions complexed with organic anions, and ions complexed with organic macromolecules and inorganic colloids. The most important metal pools in the solid phase include the exchange complex, metals complexed by organic matter, sorbed onto or occluded within oxides and clay minerals, co-precipitated with secondary minerals (e.g. Al-, Fe-, Mn-oxides, carbonates and phosphates, sulphides) or as part of the crystal lattices of primary minerals (Kidd et al., 2009).

Figure 6: Naturally growing flora on the study area: scare soil cover, *Trifolium sp., Festuca sp. (2009, own photo)*

Due to these properties, soil, unlike the other environmental compartments water and air, has the capacity to retain pollutants and so can act as a sink for pollutants and as a filter before pollutants reach groundwater. Non-degradable contaminants as are the metals cannot be diluted by fluxes as in liquid or gas, but accumulate and can also become a sink for secondary pollution. On the other hand, soil is in close contact with the water phase, by precipitation and also in zones with high groundwater level, and can therefore act as a reaction matrix. Thus, availability of trace elements to plants is governed by the dynamic equilibrium between those aqueous and solid soil phases, rather than by the total metal content. The knowledge about of the occurrence forms of metals and their changes are therefore essential for the comprehension of the dynamics of pollution and for risk assessment, toxicity studies or remediation techniques [*see chapter 1*]. Commonly, a sequential extraction procedure is used to estimate the proportion of metals that are soluble, and the part of those bound more or less strongly to different phases of the soil. However, one of the questions remaining is if the repartition of metals given by the chemical extraction is always representative for the processes taking place in this ground.

In fact, the active influence of plants by their root exudates was not taken enough in consideration. Several authors (Díaz-Barrientos et al., 1999; Lin et al., 2004; Puschenreiter et

al., 2005) suggest that root exudates control the replenishment of soluble metal from immobile metal fractions of the soil [*see chapter 1 §2*]. To satisfy physiological needs for nutrients or to avoid metal toxicity, plants are able to modify clearly the mobility of metals. Plant-induced modification of trace element speciation and bioavailability in the rhizosphere are the result of the interactions between soil components, organic chelators released, soil gas and soil water composition and the active microbial community (Kidd et al., 2009). Roots can excrete a wide range of different substances as acids, protons, CO_2, chelators, or organic signal molecules, in order to modify the availability and uptake of mineral nutriments (Marschner, 2005), or to interact with the other biota in the rhizosphere (Grayston et al., 1996; López-Bucio et al., 2000; van Hees et al., 2003). Plant roots release a wide range of substances that are involved in attracting beneficial microorganisms and forming mutualistic associations in the rhizosphere (Marschner, 2012) allowing a rich and diverse community to develop in the rhizosphere. These compounds include sugars, polysaccharides, amino acids, aromatic acids, aliphatic acids, fatty acids, sterols, phenolics, enzymes, proteins, plant growth regulators and secondary metabolites. The complex interactions in the soil are the result of the chemical interaction between the different organic compounds excreted by plant roots and the different microscopic actors of the soil, each interacting specifically with different compounds. Hence, each plant species have a different rhizosphere micro-flora in terms of abundance and physiological characteristics, which can be further modified by the properties of the soil, plant age and plant nutritional status (Marschner, 2012). [*Cf. chapter 1 §2 for more details]*

Root exudates play a role in the weathering of soil, for the mobilisation of nutrients as P, NH_4^+ or Fe, especially the organic acids, phytosiderophores, and phenolic compounds. Moreover they are important for the protection of plants against uptake of heavy metals into the roots; thereby the main agents are citrate, malate, or small peptides. Additionally phenolic compounds, organic acids, sugars play a role for attraction of useful microorganisms. Root exudates can also act as signal molecules or as precursors for hormones. One of the important aspects that should be stressed in this study is their influence on trace metals. Indeed, exudates are known to enhance with great efficiency their amounts in the bioavailable phase of the soil and therefore in the plant, but also to modify their speciation to avoid toxic effects by an excess of them. Very low concentrations are sufficient for their biological effect (Marschner, 2005). The sort, composition, amounts, proportion are influenced by many factors, as plant species, age, soil composition and so on. Since the secretion is motivated by physiological needs of the plants, nutrients present in the soil have a major impact on exudation, usually enhancing the process, particularly with regard to the supply of N, P and K. Unlike their secretion to attract or sustain microorganisms, the secretion for the uptake of nutrients is irregular and rather occurring as pulses of substances release in high locally concentrations within a short period of time (Marschner, 2012). Further, the distance to the root plays an important role. The high organic acid concentrations can be found in the very close rhizosphere zone, and

Important factors influencing the solubilisation of metals by plants through the quantity and composition of root exudates:

(1) root-induced changes in pH of the rhizosphere;
(2) complexing capacity of organic compounds released
(3) reducing capacity of the roots
(4) need for nutrients in particular essential trace elements

almost not at all in the bulk soil, giving to the rhizosphere very different properties. On the other hand, there can be a parallel mobilisation and immobilisation of metals by the same procedure, depending on the conditions. Organic acids are known to complex metals in the same way as EDTA (Díaz-Barrientos et al., 1999), these complexes are very stable and can enhance the availability of metals. They have also a buffer effect, that increases with the quantity of acid (Yuan et al., 2007). This leads to a complex interaction between heavy metal mixture in the soil, plant exudates, soil minerals and organic content in the soil.

The spectrum is quite broad, but the most common acids are malonate, citrate, malate, oxalate and fumarate. The first four were chosen and applied as a mixture in leaching experiments to represent the leaching occurring in the root zone. Citrate, malate and oxalate are very efficient to dissolve metals, because they can form stable 5 or 6 membered ring structures with trivalent ions like Fe^{III} and Al. That is why the most common response to Al stress, recognizable at the characteristic inhibited root growth, is complexation through organic acids (Barceló and Poschenrieder, 2002), most commonly citrate, malate (López-Bucio et al., 2000), or oxalate (Barceló and Poschenrieder, 2002). The efficiency for the detoxification of plants decreases from citrate over oxalate to malate. These phenomena are also observed for Zn-resistant plants (Barceló and Poschenrieder, 2002). An excess of Al can inhibit the uptake of other elements, like Ca, Mg, Zn and Mn. Some acids have a higher affinity to some specific metals (Yuan et al., 2007): citric acid for instance is better for Cu mobilisation, oxalic acid better for Cd mobilisation.

The capacity of dissolution of inorganic P (Pi) is thus highly correlated with the number of OH- and COOH- functional groups and their position in the chain (high affinity to divalent and trivalent acids). That is the reason why citrate has the highest P dissolving capacity among common organic acids. This way of phosphate acquisition is important for plants adapted to acid mineral soils with very low Pi availability (Grayston et al., 1996; López-Bucio

Stress by lack of nutriments or metal toxicity leads to a changes behaviour of the plant concerning the secretion of substances through the roots.

et al., 2000; Oburger et al., 2009; Shen et al., 2002). In alkaline soil and low availability of P and Fe, many dicotyledonous plants react to the iron stress by secretion of H^+ by the roots, reduction of Fe^{III} to Fe^{II}, production of root exudates, mainly malate, citrate. In the meantime, a decrease of the pH was observed.

Low concentrations, locally delimited make it difficult to detect them, especially if they are quickly degraded by the microorganisms interacting with the plants. The quantification of organic acids and in general of root exudation under natural conditions is difficult due to binding of exudates to soil components, assimilation and the degradation by microorganisms (turnover rate) under non-sterile conditions, and the lower production under sterile conditions. The presence of microorganisms is one factor which can lead to modifications in the quality and quantity of root exudates (Grayston et al., 1996). The stimulation of exudation occurs in both herbaceous plants and trees. Organic acids can also be produced by microbial activity, stimulated by the production of organic carbon and CO_2 from the roots, making it difficult to discriminate between the action of the flora and the microorganisms.

One method used to investigate the process of metal mobilisation in situ *and to overcome the analysis difficulties was to follow the signature given by the pattern of Rare Earth Elements (REE).*

3 RARE EARTH ELEMENTS – A TOOL FOR TRACING

3.1 Why REE?

Rare Earth Elements (REE) are elements of the lanthanide (La-Lu) group, often Yttrium and Scandium are also counted as REE. They occur as pure or mixed oxides, that are not found is great amounts, so their name. Though, more recent analysis showed that for instance Cerium is four times more common than Lead in the earth crust (Ferreira da Silva et al., 2009). These metals are very similar to each other's because of their similarity in the electron structure. They show smooth, but continuous variations in chemical behaviour as a function of their atomic number. They are strongly electropositive, and occur in oxidation number 3, either as stable oxides, carbides or borides (Spedding, 2009), therefore they are used as chemical analogues to the trivalent actinides (Am, Cm, Cf) which are difficult to study because of their toxicity, radioactivity and variety of oxidation states (Ding et al., 2006). Only Ce und Eu can be found in other valences (Ce^{4+} or Eu^{2+}) giving them other properties depending on the redox potential. The ion radius decreases from La^{3+} to Lu^{3+}, this phenomenon is called lanthanides contraction. Rare Earth Elements are often separated in light (LREE; La-Sm), middle (MREE; Sm−Dy) and heavy REE (HREE; Ho-Lu).

REE form a consistent group of metals, whose pattern, resulting from normalisation to a standard, can be used to describe different processes of dissolution and preferential precipitation.

The pattern obtained through normalisation with a standard (here PAAS, Post Archean Australian Shale, McLennan, 1989) is a tool to study water-rock-interactions, as tracer to find out the erosion and formation of sediments, or to follow the flow of water and in case of AMD influenced areas to follow contamination by heavy metals or radionuclides (Merten et al., 2005). To follow erosion the initial rock is often taken as the standard, for instance Basalt, or Granite (Aubert et al., 2001; Steinmann and Stille, 2008).

Different factors can influence the pattern of REE. Not only the source material causes a typical pattern, but also pH (precipitation), redox-conditions and ligands can alter this (Aström, 2001; Semhi et al., 2009; Shan et al., 2002). Cao et al. (2001) shows further how lower pH and lower Redox potential lead to release of La, Ce, Gd and Y by changing their speciation from Fe-Mn oxides In particular organic substances excreted by plants to optimise their nutrient input can play a role in the regulation of the soil pH and as well complex different metals (van Hees et al., 2003). The quantity of REE in solution therefore does not depend only on the pH, but also on the presence and amount of Al, Fe and Mn. Generally, the adsorption of REE is connected to the cation exchange capacity of the soil.

Anomalies are another aspect of the pattern. They appear mainly because of changes in the redox conditions, which change the oxidation state of some REE, like Ce or Eu. These behave then differently, being more easily precipitated, or at the contrary being dissolved more easily, or showing a higher affinity for adsorption on some phases like hydroxides. Hence, Eu occurs as Eu^{2+} under reducing conditions, and can be incorporated in minerals instead of Ca^{2+} or

Sr^{2+}. Positive Ce anomaly is said to be typical for REE fixed in oxy-hydroxides. The Ce(III) oxidising capacity of Fe oxyhydroxide-precipitating systems is considerably higher than that of systems in which dissolved REE interact with preformed Fe oxy-hydroxides (Bau, 1999). Organic content plays an important role also for the fractionation of Ce. Indeed, alkaline water with positive Ce anomaly is often poor in organics, whereas organic-rich waters show a negative Ce anomaly, showing that Ce is bound by humic acids presents in the water (Pourret et al., 2008). The critical pH for Ce anomaly is pH 5 (Lei et al., 2008). Ce is oxidised to Ce^{IV} and sediments as CeO_2. This leads to negative Ce anomaly in water. Gd anomalies are most often of anthropological origin due to the use of this element in medical imaging techniques (Möller et al., 2002; Rabiet et al., 2009).

Rare Earth Elements (REE) were chosen as a tool for several reasons. Their occurrence in all parts of the studied system in detectable concentration was one significant advantage and basic condition for their use as a tracer. Additionally, the availability of several data from other studies allowed making comparisons and drawing conclusions about the causes of some pattern characteristics. Finally, the patterns formed as a result of the different environmental parameters can be found even after the direct cause is not detectable anymore. So, some enrichment of certain REE in the soluble phase can be found, even if the solid precipitates retaining the other REE are not found at the place of study, or if the substances specifically dissolving and therefore provoking the enrichment of some REE are already degraded. Hence, it is a way to overcome analytical difficulties.

3.2 Heavy metal uptake and REE fractionation by plants on this specific contaminated soil

Because of the relatively high content of metal including REE and quite low pH, the mobility and bioavailability of metals is high and so the up taken amounts of metals by plants are significant. Therefore, this substrate is adequate for monitoring soil parameter and metal behaviour [*See chapter 2*].

3.2.1 Metal leaching and REE fractionation

Therefore, in a first part, with a focus on REE, the effect of different leaching solutions was tested; in order to describe and differentiate different metal mobilising effects of the studied system, in particular plant exudates. REE fractionation by the studied plants

In order to come closer to the natural conditions and understand on-going processes in the study area, two autochthonous plants (Dietrich and Berger) were chosen (Figure 6). *Festuca rubra* is a very resistant grass found in many heavy metal contaminated areas, and known as a pioneer plant. Clover (*Trifolium pratense*) was chosen because of its ability to fix air nitrogen due to its symbiosis with bacteria, and so to overcome partially the nitrogen-poorness of the soil. Additionally, reference plants, sunflower (*Helianthus annuus*) and Triticale (hybrid of wheat (*Triticum*) and rye (*Secale*)), enable comparison with previous studies, especially regarding REE fractionation (Kidd et al., 2009; Lonschinski, 2009). None of the plants are known to be hyperaccumulators, even though they have been studied for phytoremediation purposes. The plants were grown in single culture as well as a polyculture.

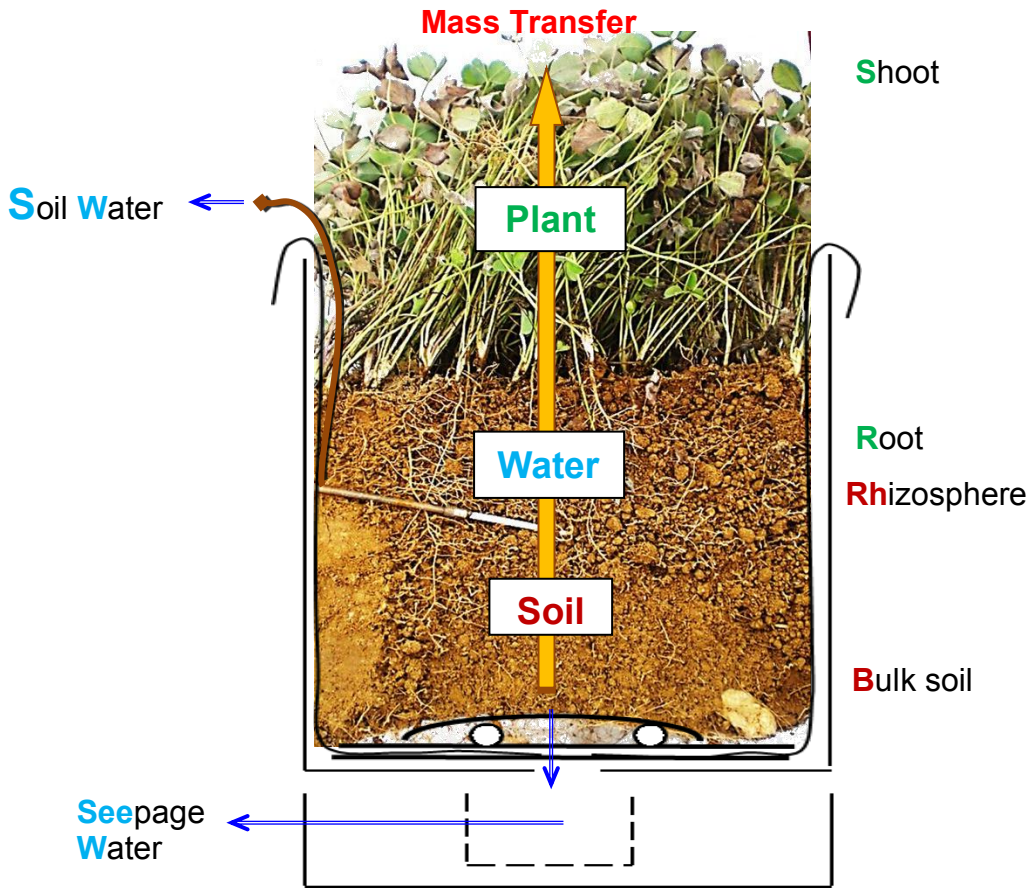


Figure 7: Overview over the experimental settings of the pot experiment and the compartments of the studied system.

The pattern obtained through normalisation of the REE to a standard (PAAS or control) was used as a tool to follow contamination by heavy metals and radionuclides.

Our study shows that metals (REE) are mobilised in a different way by acidic solutions of different origin, and that organic acids lead to a different fractionation than inorganic ones.
Indeed, the pattern of REE leached by sulphuric acids was qualitatively similar to the one obtained by water, with much higher amounts leached. A MREE enrichment was noticeable, and a positive Ce anomaly. It is recording a typical AMD influenced pattern: AMD until a pH of 4 is characterised by high concentrations in REEs and LREE depletion, Ce-enrichment, slight MREE enrichment (Grawunder and Merten, 2012; Lei et al., 2008).
In case of leaching with organic acids, the HREE were enriched compared to the LREE. Since it is not an effect of the pH, it must be due to the specific properties of organic substances used for leaching, as their complexing properties. This feature was considered as an indicator for organic substances involved in the leaching of metals.

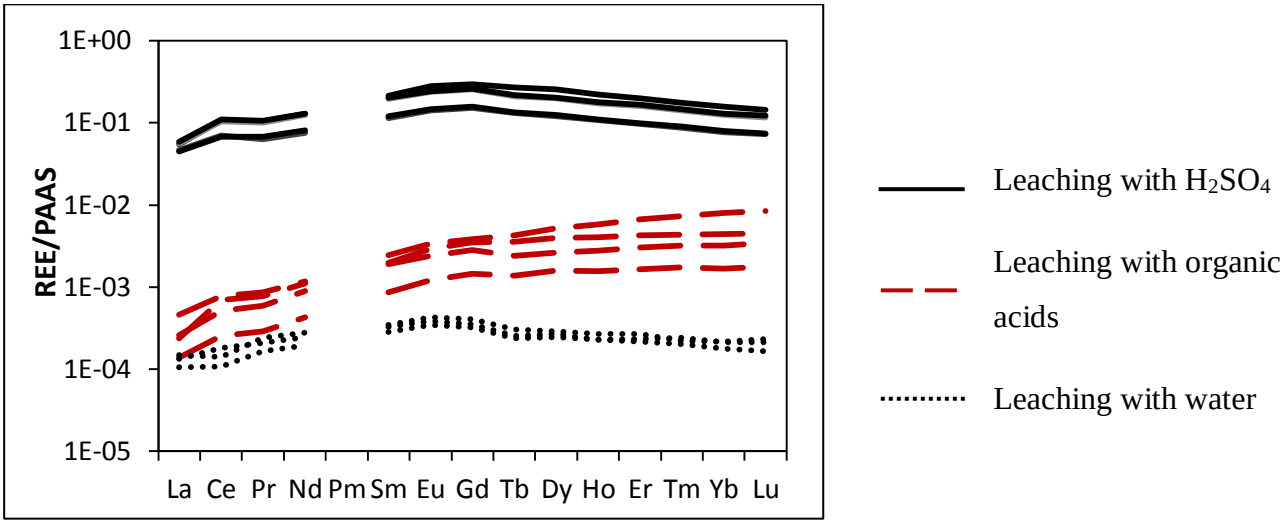


Figure 8: PAAS normalised REE pattern of soil from the test field eluted with water, sulphuric acid, and organic acids.

The REE patterns of soil, water and plants were compared to controls and to soil eluted with different solutions as water, inorganic and organic acids in order to define the factors influencing the metal behaviour.

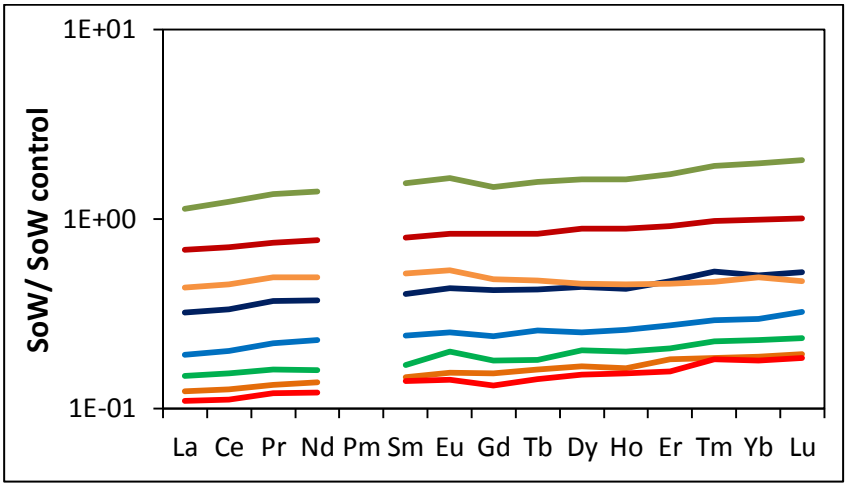


Figure 9: REE pattern of soil water (in pots with plant growth) relative to soil water of the control pot. A clear enrichment of HREE compared to LREE is visible in all samples.
SoW: Soil water

A REE fractionation from soil into the soil water and further to roots, and finally from roots to shoots was observed. The rhizosphere increased locally the soil pH and decreased locally the amounts of soluble metals in soil and soil water. The characteristic heavy REE enrichment of the soil water compared to the control soil water was a hint to the influence of organic acids (cf. Figure 8), although it could not be discriminated if there were no other organics involved, such as other exudates or microorganisms.

4 THE ROLE OF MICROORGANISMS IN SYMBIOSIS WITH PLANTS IN THE CONTEXT OF METAL CONTAMINATION

The plants and their metal uptake can be influenced by many other factors, since they form an own microcosm in their rhizosphere. Indeed, many organisms living around the plants influence their growth and mineral uptake. If more is known about these, they can be used, if chosen well, to enhance phytoremediation procedures, especially on soil poor in nutrients.

Microorganisms are known to react with metals present in their environment therefore many are used to treat wastewaters containing high amounts of metals (AMD) by precipitating them as sulphides, so concentrating or immobilising them, and increasing the pH. These biogeochemical processes are catalysed mainly by sulphate reducing bacteria like *desulfovibrio* or *desulfotomaculum* (Cohen, 2006). Compost is added to ensure an organic carbon source for sulphate. Besides there are also Mn-oxidiser and Fe-oxidiser, that precipitate metal oxides or co-precipitate them as hydroxides. However, even though these processes are of great interest in liquid media, they play a smaller role in soil. There, on the other hand, other specific microorganisms interact with plants in the rhizosphere zone. Those are the interactions on which will be lead the focus on in this study.

Plant roots release a wide range of substances, which, especially the easily decomposed low molecular weight ones, are involved in attracting beneficial microorganisms and forming mutualistic associations in the rhizosphere (Marschner, 2012). The most important mutualisms exist between plants and mycorrhizae or rhizobacteria (Badri et al., 2009), which can also exist simultaneously and influence each other's. Relations in nature are more complex in the rhizosphere and involve a rich and diverse community composed of several bacteria (endobacteria, pathogens), fungi including Arbuscular Mycorrhizal ones (AM), micro-fauna (i.e. nematodes), resulting from the specific chemical interactions between the different organic compounds excreted by plant roots and the different microscopic actors of the soil. However, the colonisation of roots is not limited to the root surface, but can occur further inside the root tissues.

4.1 Description of the endophytic population in two plants grown on this specific contaminated substrate

Bacteria have been known to exist within plant tissue since many years (Tervet and Hollis, 1948); although the pathogenicity was believed to be their main function. Later, some studies showed that bacteria living within the tissues had no negative and even beneficial effects on the host (Davison, 1988). From then endophytic bacteria are defined as bacteria residing within living plant roots without causing substantive damages to their host. While some of them are very specifically associated to one host, others are more flexible. Root colonisation is a complex procedure involving several steps (Badri et al., 2009) and establishment in plant tissue includes several complex mechanisms (Reinhold-Hurek and Hurek, 2011).
Endophytes are distributed in several bacterial phyla, including Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes, represented in 82 genera (Lodewyckx et al., 2002). They are found in various plant tissues, ranging from roots, stems, leaves and seeds (Madmony et al., 2005; Rajkumar et al., 2009; Reinhold-Hurek and Hurek, 2011). Some are known to be obligate endophytes and are transmitted over the seeds to the next generation (Majewska-Sawka and Nakashima, 2004; Mastretta et al., 2009).
Endophytes were found in numerous plant species, and studied mostly in agricultural relevant plant, although an increased interest is now given to phytoremediation plants, due to the rising interest to find ways to promote plant survival and growth under these challenging growth conditions.

4.1.1 Identification of the strains

Endophytic bacteria were isolated from the root, stem and leaf of cultivars of *Trifolium* plants, and further from shoots and roots of *Festuca* plants growing on the studied substrate in a pot experiment. 78 stable, morphologically distinct isolates were obtained belonging to the several genera of Proteobacteria, Firmicutes, Actinobacteria were found. Many of their most similar database matches were first isolated from soil and plant, and a relatively big proportion of them are described in the context of metal contamination or remediation, especially of organic contaminants. Some endophytic species can fix nitrogen *(Sphingomonas azotifigens* isolated from the roots of rice plants, Kamnev et al., 2005), and many are aromatic-degrading bacteria. The isolates were characterised and tested for properties useful for plant promotion on metal contaminated soil. So, numerous strains were able to produce IAA, organic acids, siderophores, and many were resistant to metals as Zn, Ni, Mn Cd or Al [*see Chapter 4*]. However, it is important to note that this is a result based on cultivation, and so non- or not easily cultivable strains are not detected.

4.1.2 Spatial distribution of the strains and bacterial community

The identified endophytic community was different for the two studied plants (Figure 10), so it seems that a selection takes place. It seems that generally the community is more influenced by the plant species than the substrate. The main reason is believed to be the active attraction of bacteria through different organic secretes from the roots, the resulting cocktail being plant specific, even though influenced by the surrounding conditions [*cf. chapters 1&3*]. Hence, each plant species have a different rhizosphere micro-flora in terms of abundance and physiological characteristics, which can be further modified by the properties of the soil, plant age and plant nutritional status (Marschner, 2012). Indeed, plants are able to select specifically from the bacterial pool in the soil which bacterial community would form in their tissue, probably through the production of different root exudates. (Wang et al., 2008) showed how the community changed from soil to the roots of different plants, with in particular a shift from gram positive majority in the soil and gram negative in the plants, and how some genera were found in grass *(Festuca*) and not in tree (*Betula*).

The endophytic bacteria showed clear spatial compartmentalisation within the plant, suggesting that they can be specific associations with plant tissues and also the possibility of different uptake mechanisms for different plant tissues.

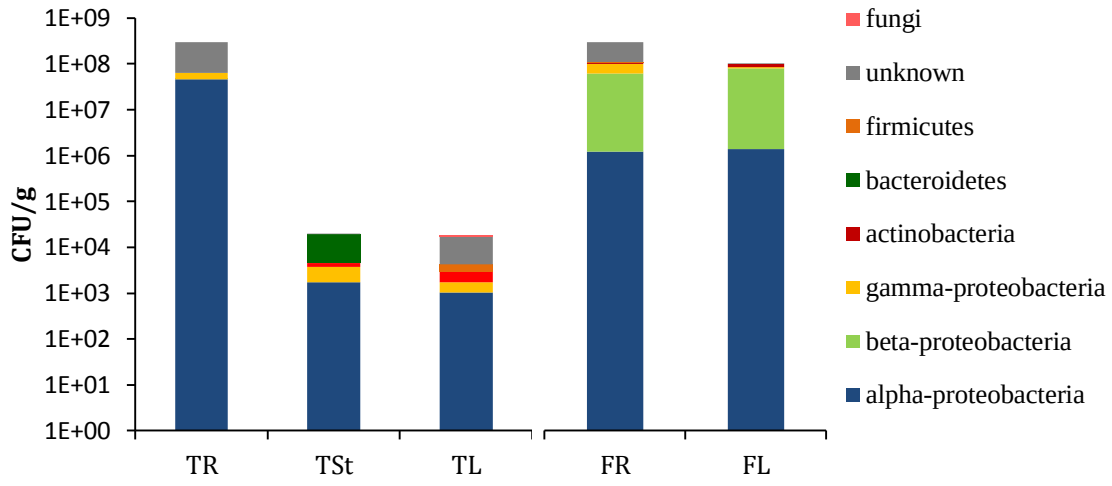


Figure 10: Diversity assessment of isolated endophytic microorganisms for different compartments (roots R, Stems St and leaves L) of *Trifolium pratense* (T) and *Festuca rubra* (F), calculated based on the isolated CFU /g

4.2 Improving plant growth on heavy metal contaminated soil using selected endophytic microorganisms

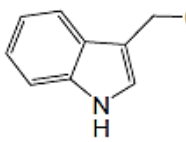
The use of beneficial bacteria to promote plant growth and health has been suggested already over 20 years ago for agricultural crops (Davison, 1988), and studied later on for microbial bioremediation of metals and also suggested as inocula to enhance re-vegetation of contaminated sites, phytoremediation, as reviewed by several authors (Beolchini et al., 2008; Kidd et al., 2009; Rajkumar et al., 2009; Shetty et al., 1994; Vangronsveld et al., 2009; Weyens et al., 2009b; Zhuang et al., 2007). Beneficial arbuscular mycorrhiza, yeasts or various soil bacteria, also called generally PGPR (Plant Growth Promoting Rhizobacteria) have been used. However, it is crucial to understand more about the plants naturally present in poor or polluted environments, in order to allow re-vegetation of sites with scarce vegetation cover, thus being subject to soil leaching and eventually to contamination spreading. In particular, the development of roots plays a decisive role in the aspect of soil stability. Vegetation can help to avoid erosion by precipitation and wind and stabilise the soil, reducing the percolation (Kidd et al., 2009). In the present study, the focus is put on their endophytic bacteria. A number of isolates demonstrated the ability to produce plant growth promoting substances and resistance to the present metallic contaminant. These plant fitness-enhancing properties are of interest for the use of this mutualism for the enhancement of plant fitness and further for remediation purposes. All these properties are helpful for the survival on metal contaminated soils. Indeed, growth-promoting properties can be seriously affected by metal contamination. On the other hand, inoculation of effective N_2-fixating strains *Rhizobium leguminosarum* bv. *trifolii* lead to a revival of the nitrogen fixation capacity if the inoculated cell number was large enough (Giller, 1989). This shows the importance of the good choice of inocula, especially in specifically contaminated sites.

4.2.1 Plant microorganism partnerships for a better biomass production

Endophytic bacteria can improve plant growth using different processes. Plant associated bacteria can improve plant nutrition by fixing N_2 and solubilising macronutrients as poorly soluble (P)-minerals, thus delivering nutrients normally unavailable for plants and fulfil so the role of natural fertilisers (Badri et al., 2009; Weyens et al., 2009a; Yanni et al., 1997). Nitrogen fixers were found in Archea (*Methanosarcina*) and many bacterial genera, mainly proteobacteria as *Sphingomonas* (*S. azotifigens*), *Burkholderia*, *Pseudomonas*, *Azotobacter, Devosia*, *Bradyrhizobium, Rhodobacter*, *Agrobacterium*, *Rhizobium*, *Frankia*, *Rhodococcus, Alcaligenes, Ralstonia,* some firmicutes *(Paenibacillus)*, cyanobacteria as *Nostoc* sp. *Rhizobium, Rhodococcus, Paenibacillus and Pseudomonas* (Franche et al., 2009; Wang et al., 2008).

Bacteria which can solubilise phosphorus are for example: *Azotobacter chroococcum, Bacillus spp., Enterobacter agglomerans, Pseudomonas chlororaphis, Pseudomonas putida, and Rhizobium and Bradyrhizobium spp.* (Weyens et al., 2009a). These bacteria can either solubilise inorganic phosphates by releasing organic acids, such as gluconic acid and 2-ketogluconic acid, or mineralise organic phosphates by secreting extracellular phosphatases. It has also been reported that endophytic bacteria can solubilise immobilised mineral phosphate.

The growth of plants is promoted by the production of plant growth regulators, phytohormones such as auxins, cytokinins and gibberellins. Indole-3-acetic acid (IAA) is the



Indole-3-acetic acid (IAA)

major auxin involved in many of the physiological processes in plants, mainly cell elongation of stem cells. IAA is produced by several bacteria such as *Azospirillum brasilense, Aeromonas veronii, Agrobacterium spp., Bradyrhizobium spp., Comamonas acidovorans, in particular by some* endophytic strains from the present study as *Agrobacterium* spp., *Alcaligenes sp, Burkholderia* sp., *Bacillus* spp., *Pseudomonas* spp., *Stenotrophomonas* sp., *Pantoea* sp., *Enterobacter* spp. and *Rhizobium leguminosarum* (Tsakelova et al., 2006). This way it is possible to regulate and stimulate plant growth without the use of hazardous herbicides and fertilisers. Not all bacteria produce auxin, but they are able to use tryptophan contained in the root exudates (Dimkpa, 2009). Cytokinin plays a role in drought resistance of plants.

Furthermore, endophytic bacteria can diminish plant stress by inhibiting ethylene production. Ethylene is also a phytohormone, whose amounts increase in plants under abiotic/biotic stress conditions. The most commonly observed mechanism that reduces levels of ethylene production is via the activity of bacterial 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) ACC being a precursor of ethylene (Kidd et al., 2009; Weyens et al., 2009a). Bacteria originating from different soils and expressing ACC deaminase activity (leading to a decrease in ACC levels and thus in ethylene production) can stimulate plant growth even in soils containing phyto-toxic concentrations of cadmium; some strains, like *Pseudomonas tolaasii* ACC23 and *P. fluorescens* ACC9, produced IAA and siderophores even more actively under Cd stress (Kidd et al., 2009). It was showed that most of the PGPR isolated from Graminaceae grasses growing in a meadow polluted with heavy metals exhibited ACC deaminase activity, which resulted in plant growth promotion. Some of the here isolated bacteria were genetically similar to strains displaying such properties.

Endophytic bacteria can also indirectly benefit plant growth by preventing the growth or activity of plant pathogens (Badri et al., 2009; Weyens et al., 2009a) through competition for space and nutrients, antibiosis, production of hydrolytic enzymes *such as gluconases or chitinases,* inhibition of pathogen-produced enzymes or toxins, and through induction of plant defence mechanisms (Kloepper and Ryu, 2006; Pavlo et al., 2011; Shiomi et al., 2006).
Indirectly, *Pseudomonas* can act by starving pathogens of iron through production of siderophores (Dimkpa, 2009; Kloepper et al., 1980), some strains of *Pseudomonas fluorescens* are already described and employed as biocontrol agents (Moënne-Loccoz et al., 1998).

Additionally, microorganisms have an influence on the uptake of metals by plants. For example, The plants inoculated with bacteria (like *Pseudomonas aeruginosa* on corn) had more leave, less roots and higher metal content; the more cells inoculated, the more uptake (Aouad et al., 2006). *Pseudomonas aeruginosa* forms biofilms and allows complexation of REE; further it is able to extract Fe and Mg. These properties are of interest for the use of this mutualisms and metal tolerance properties for remediation purposes. The main aspect of metal uptake is given by the production of siderophores, which can make Iron(III)-hydroxide available for reduction to Fe^{II}; this is crucial especially in alkaline (calcareous) soils with decreased Fe availability (Kidd et al., 2009; Weyens et al., 2009a). Siderophores take up Fe^{II}, which is then reduced intra-cellular. This process is repressed if there is sufficient Fe supply, but is stimulated by other metals. Siderophores can both enhance and prevent uptake of

metals by plants, depending on the present metals. They bind free metals, and so changing the available metal concentration and protecting the plants from metal stress. Metals inhibit auxin production, but siderophore presence alleviates it. Siderophores are generally only for Fe, but also Al and other metals (Cd, Ni; or micronutrients such as Mn, Co, Zn) can be transported in some cases (Dimkpa, 2009; Kidd et al., 2009).

The ability of siderophore production is one of the key factors that allow plants to cope with toxic metal concentrations (Dimkpa et al., 2009; Meda et al., 2007). However, Römheld and Marschner (1986) pointed out that bacterial siderophores are not taken up in the same way as phytosiderophores. This could still be a factor for protection against metal stress, metals bound to ferrichromes being possibly taken up less easily. Phytosiderophores (from monocotyledonous plants) are reported to be less efficient for iron mobilisation (Rroço et al., 2003).

Thus, the increase in the solubility of metals in the soil can be also linked to the properties of the bacteria, able to produce siderophores, or other metal-chelating substances. Metallophores are for instance produced by strains of *Pseudomonas* and *Enterobacter* (Whiting et al., 2001). *P. aeruginosa* can also allow complexation of REE; further it is able to extract Fe and Mg (Aouad et al., 2006). Sheng et al. (2008) showed the influence of some bacteria on the solubilisation of Pb in soil and water by *P. fluorescens* G10 and *Microbacterium sp*. G16.

Indeed, rhizosphere microbes play an important role for the water-soluble metals pool in soil by altering the solubility, availability and transport of trace elements and nutrients. This happens through modifying soil pH, secretion of chelators and siderophores or redox changes. (Usman and Mohamed, 2009). The influence of the bacteria on the metal uptake by plants is controverted and discussed by Rajkumar et al. (2009).

In order to select microorganisms that can promote plant growth and metal uptake, it is important that their survival in the medium be given. Those bacteria should be able to resist to the environmental constraints of the rhizosphere. Therefore, in the case of an application in a metal contaminated soil, their metal resistance (and multiple metal resistance) should be evaluated. Microorganisms dispose of different mechanisms for protection against heavy metals: exclusion, precipitation or bioaccumulation both intra- or extracellular by chelating compounds, biosorption, active efflux transport or enzymatic detoxification (Guo et al., 2010; Nies, 1999; Rönkkö et al., 1993) and many of these mechanisms were discussed already for strains found at the study area (Schmidt et al., 2009; Schmidt et al., 2005). These processes are of great importance in the context of phytoremediation since microorganisms with a natural good resistance to metals will be more able to support plant growth in a difficult environment.

4.2.2 Selection of strains for phytoremediation support

If more has been studied about endophytes in agricultural plants (Davison, 1988), less is known about the associated endophytic populations from meadow plants. In particular, although some endophytes have been described for species of the same genera, especially about N_2-fixers (Moënne-Loccoz et al., 1998; Rolfe et al., 1980) little is known about the diversity and spatial distribution of endophytes found in autochthonous plants, *Trifolium pratense* and *Festuca rubra*. As a consequence, the first step to consider for the success of plant growth promotion, especially with regard to phytoremediation of sites contaminated

with heavy metals is assessing the diversity and distribution of natural endophytic population, followed by their characterisation and selection of suitable endophytic bacteria in candidate plants appropriate for phytoremediation and the verification of their efficiency. (Porteous Moore et al., 2006). The population found at the site is likely to be better adapted to the specific conditions; therefore, it is important to select autochthonous organisms. It is particularly true for contaminants that need to be degraded, as organics, but also for metals, both affecting the physiology and ecology of microorganisms.

A high number of endophytic bacteria were isolated from the root, stem and leaf of cultivars of *Trifolium* plants, and from shoots and roots of *Festuca* plants, growing on the studied substrate. They were further characterised phenotypically by their tolerance to a range of relevant heavy metals and their capacity of producing plant-influencing substances. A number of isolates demonstrated the ability to produce plant growth promoting substances and resistance to the present metallic contaminant. Further many showed that they could produce organic acids and siderophores. These properties are of great interest for the use of these mutualisms for remediation purposes.

Out of the numerous strains with promising properties found in autochthonous *Trifolium* and *Festuca* plants, two of each plant compartment were retained (only one for the leaves of *Trifolium*) which showed the best combination of characteristics. Two of them were not identified, and the others belonged to the genera *Enterobacter, Pantoea, Rhizobium, Curtobacterium/Bacillus, Xanthomonas /Stenotrophomonas /Pseudomonas.* It is interesting to note that *Pantoea agglomerans* (also called *Erwinia herbicola* or *Enterobacter agglomerans*) is an ubiquitous plant epiphyte known to have strains used for biocontrol of fire blight caused by *Erwinia amylovora* on fruit trees, and is commercially available in USA, Canada and New Zealand (US.EPA, 2011).

However, one should be cautious about making ecological inferences from experiments conducted under typical laboratory conditions and of the additional roles that well-characterised microbial products may play in microbial interactions (Peterson et al., 2006). The knowledge about some bacterial physiological properties is helpful but does give only a little idea about the mechanisms and possibilities *in vivo*. Therefore, the selected microorganisms were used for inoculation in pot experiments. Further, consortia of these strains with complementary properties were also used as an inoculum. Plant health and growth, metal contents in soil and plants, and photosynthetic activity were analysed and compared to un-remediated soil.

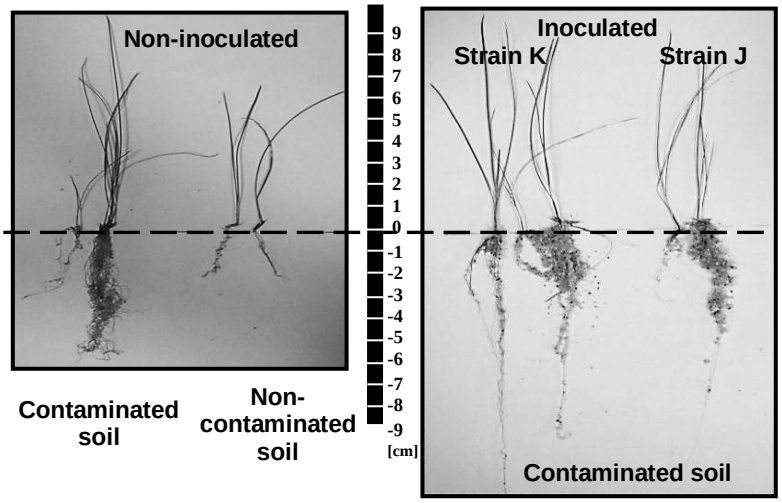


Figure 11: Root growth differences (a): root and shoot length of *Festuca rubra* after 5 weeks of growth in contaminated soil (left) and uncontaminated soil (right). Metal contamination inhibits root growth. (b): roots and shoots of *Festuca* after 5 weeks in contaminated soil inoculated with strains K and J (K: *Curtobacterium sp.*; J: *Rhizobium radiobacter*). Root growth is enhanced by inocula, comparable to growth without contamination.

The experiments showed that inoculation of some bacterial strains improved plant growth on contaminated soil to a level comparable to growth on non-contaminated soil. Further, root length was observed to be increased due to the presence of specific microorganisms (Figure 11) thus stabilising the soil by the formation of a dense root net.

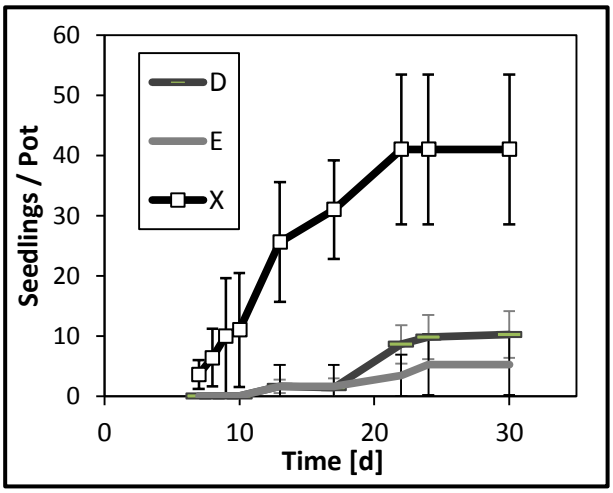


Figure 12: **Growth density for *Festuca rubra* on contaminated substrate over time. Comparison of plants inoculates with single strains D and E with plants inoculated with the consortium X=D+E**

Inoculation by a combination of strains also lead to very significant improvement of plant growth, suggesting a synergetic effect of the different strains. Based on these considerations, it is important to consider possible synergetic effects (or antagonistic) with natural soil microorganism community when adding bacteria for *in situ* phytoremediation. This study shows clearly that the simultaneous inoculation of more than one strain changes the effect on the plant. Especially with regard to the root biomass and the density of plants grown under metal stress, the combination of strains shows a synergetic effect (Figure 12). Strains who were not very efficient growth promoters had a strong positive effect on plant growth when combined with each other's. This aspect of plant growth promotion has not been extensively considered in the past, although it has been already mentioned by (Kozyrovska et al., 1996), who studied simultaneous inoculation of 2 endophytes in the context of growth promotion of agricultural crops on radionuclide contaminated soil. It was suggested that endophytes could help crops to grow in unfriendly environment, to avoid radionuclide uptake, and be a good alternative to agrochemicals. Some experiments (Whiting et al., 2001) further suggest that the positive effects on plants are not necessarily specific to the strains of bacteria added, but that also native bacterial population can have a strong influence. In our case, it is probable that native population is already present in the plants and sustains their growth; however the main effect was given by the freshly inoculated and recovered strain, or by the beneficial interaction between those and the native strains.

The promoting effect was confirmed by measurements of chlorophyll fluorescence, monitoring changes in the photosynthetic system and thereby estimating the stress of the plant. It showed that inoculated plants treated are less stressed compared to non-inoculated plants. This study provided a new insight into the opportunities given by the interaction between plants and associated microorganisms in the soil containing heavy metals. It clearly demonstrates the utility of using inoculations of endophytic bacteria to increase phytoremediation potential, and the enhanced effects of bacterial consortia. We want to emphasise in this context the importance to consider synergetic effects or possibly antagonistic effects with natural soil microorganism communities during *in situ* remediation processes.

The mobile fraction of metals was lower with plants than for un-vegetated soil, indicating a stabilising effect of plants. Some bacteria could reduce the solubility of specific metals in the

soil. Microbes and microbial consortia alone and in combination with their plant host, could influence the availability of some metals, with Al and REE behaving opposite to Mn, for which three inoculated strains caused a decrease of the soluble fraction compared to un-inoculated plants. Similarly, the uptake of metals by plant aerial parts depended on the plant species and the metal itself. Further understanding of how plant roots modify locally the chemical properties of the soil, leading to an enhanced metal mobility and availability for plant uptake will enable greater plant metal yields (one of the current limitations of phytoextraction processes). Rhizosphere processes continue to be poorly understood in field conditions.

It is also of importance to combine plants of different phyla with each other's, to ensure long-term system stability. In the past, the application of endophytes has been mostly limited to one bacterial species per host. On the other hand some studies focused on the effect of plant diversity on soil properties, as to achieve a stable persistent cover it is important to use a mixed culture, and combine grasses, legumes and trees (Inal and Gunes, 2008; Kidd et al., 2009; Tessema, 2011). To improve the efficiency of phytoremediation of toxic metal-contaminated soils, one of the suggested ways is to equip plant-associated bacteria with pathways for the synthesis of natural metal chelators, such as citric acid, to increase metal availability for plant uptake or, alternatively, with metal sequestration systems to reduce phytotoxicity and increase metal translocation to aerial plant parts. However it has already been shown that bacteria can be introduced as vectors into the plant ecosystem and that this will result in natural horizontal gene transfer to the endogenous endophytic population (Weyens et al., 2009a). The fate of inoculum over time is in fact an important question to take into account (van der Lelie et al., 2005) suggests in a reply to (Newman and Reynolds, 2005), that horizontal gene transfer to the other present bacteria is more probable than an establishment of a new strain in an already stable community. Another positive interaction between bacteria was mentioned by (Schmidt et al., 2005) concerning the better survival of microorganisms with natural good resistance to metals. The authors observed that some strains, which were not resistant, could grow near resistant ones, due to the fact that they produce substances that protect them against heavy metals. Further, if local bacteria get better adapted than the inoculated strain, we would observe a shift in the bacterial population, and could consider the inoculum rather as a starter allowing the establishment of the vegetation.
Impact of plant growth on soil and hydrological balance, advantages for phytoremediation

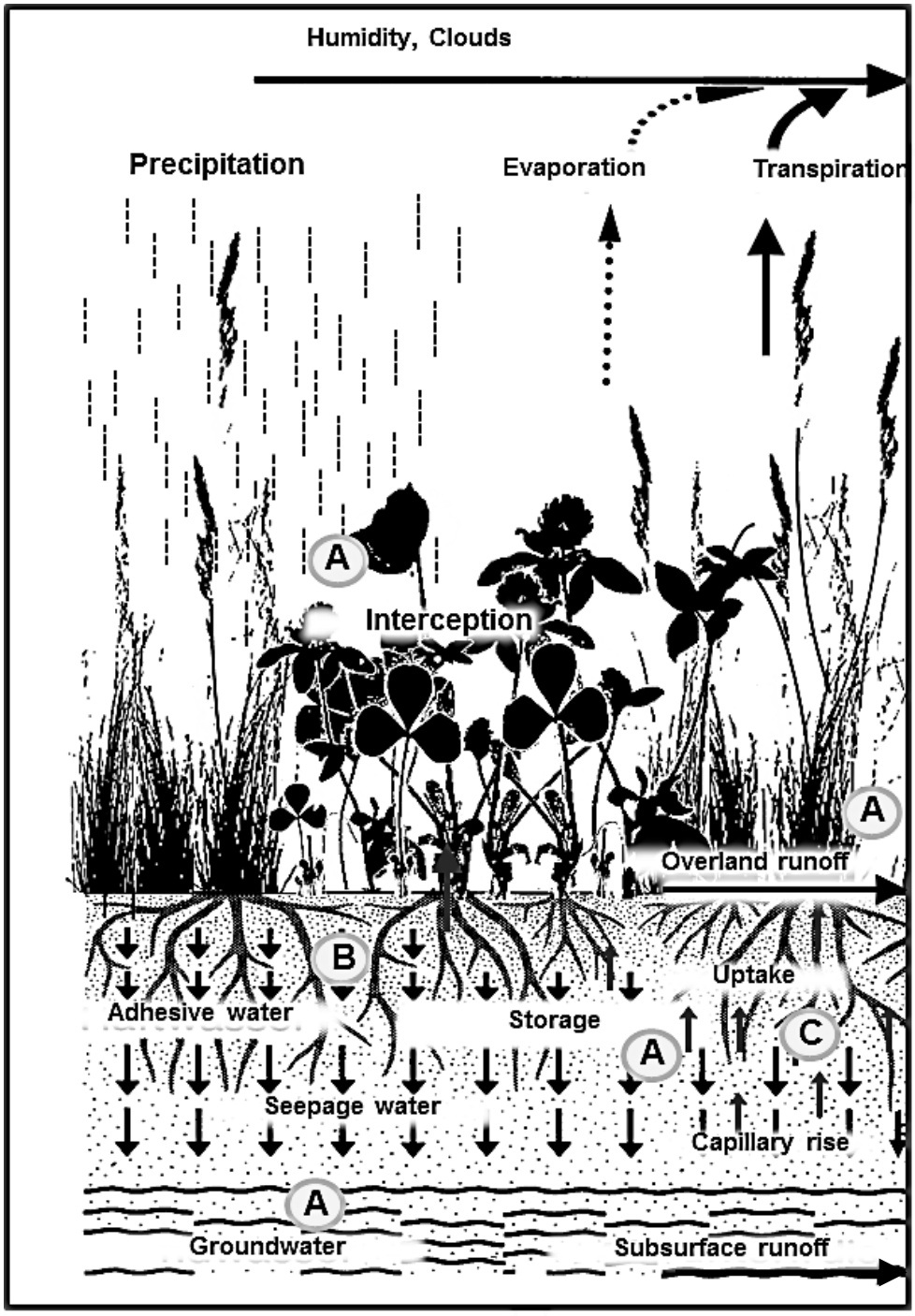


Figure 13: Plants organisms are some of the many factors that can influence element mobility, by being an active part both of the hydrologic balance and the chemical interaction with metals. Soil microorganisms and symbiotic microorganisms can support the plants' action by enhancing nutrition and stimulating growth, and protecting them against soil toxicity.

A - Protection against erosion and groundwater contamination

B - Influence of the rhizosphere pH on metal mobility

C – Microbial influence on plants and metals

5 CONCLUSIONS

This work showed the major impact of plants and their associated biosphere on the dynamics of metal mobility in soil, and how REE are useful to overcome some analytical challenges. Furthermore, it describes the present endophytic population in autochthonous plants growing on contaminated substrate. A number of endophytic bacteria showed a great influence on plant survival and development under sub-optimal growth conditions. They help influenced positively their height, density, health and root biomass, especially of *Festuca rubra*, which was also colonized easier and by a more diverse community. Metal uptake was also be altered, since the soluble part of the present metals was altered. Finally, inoculation showed protection against stress through a restauration of the photosynthesis efficiency of stressed plants. The most efficient strains were identified as belonging to the genera *Enterobacter/Pantoea*, *Rhizobium* and *Curtobacterium*. Inocula consisting of bacterial consortia were even more powerful than single bacteria inocula, showing existing synergetic interactions, and the importance of considering synergetic or possibly antagonistic effects with the natural soil microorganism community. The fate of inoculum over time is in fact an important question to take into account, horizontal gene transfer i.e. for example metal resistance, to the other present bacteria being a possibility. The inoculum could be considered rather as a starter allowing the establishment of the vegetation.

The relevance of the results for remediation and re-vegetation of sparsely vegetated sites was revealed. Indeed, possible metal transfer into deeper soil layers and groundwater by out-washing is more controlled if a sustainable plant cover is given. In particular root development is an important factor for the interaction with the hydrologic balance of the system, important equally for the supply of the plant in water, for the soil stability and fate of contamination. Furthermore, the observed growth enhancement gives the possibility for the use of areas not adapted for agricultural use, due to contamination or difficult growth conditions, and opens a way to escape conflicts between agricultural cultures and others, as energetic crops.

There is still research to be done, in particular on consortia, which carry great potential in the combination of symbiotic and complementary organisms. We would suggest using of *Festuca* and *Trifolium* as complement to extracting or hyper-accumulating plants, in order to increase soil fertility and as erosion protection (dense root network). *Festuca* is more influenced by bacteria concerning its root development, so should therefore retain particular attention when it comes to choosing plant communities for remediation.

REFERENCES

- Abumaizar, R.J., Smith, E.H., 1999. Heavy metal contaminants removal by soil washing. J. Hazard. Mater. B70, 71-86.
- Aouad, G., Stille, P., Crovisier, J.-L., Geoffroy, V.A., Meyer, J.-M., Lahd-Gaegea, M., 2006. Influence of bacteria on lathanide and actinide transfer from specific soil components (humus, soil minerals and vitrified municipal waste incinerator bottom ash) to corn plants: Sr-Nd isotope evidence. Sci. Total Environ. 370, 545-551.
- Aström, M., 2001. Abundance and fractionation patterns of rare earth elements in streams affected by acid sulphate soils. Chem. Geol. 175, 249-258.
- Aubert, D., Stille, P., Probst, A., 2001. REE fractionation during granite weathering and removal by waters and suspended loads: Sr and Nd isotopic evidence. Geochim. Cosmochim. Ac. 65, 387-406.
- Badri, D.V., Weir, T.L., van der Lelie, D., Vivanco, J.M., 2009. Rhizosphere chemical dialogues: plant-microbe interactions. Curr.Opin. Biotech. 20, 642-650.
- Barceló, J., Poschenrieder, C., 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ. Exp. Bot. 48, 75-92.
- Bau, M., 1999. Scavenging of dissolved yttrium and rare earth by precipitating iron oxyhydroxide: Experimental evidence for Ce oxidation, Y-Ho fractionation, and lanthanide tetrad effect. Geochim. Cosmochim. Ac. 63, 67-77.
- Beolchini, F., Dell'Anno, A., De Propris, L., Ubaldini, S., Cerrone, F., Danovaro, R., 2008. Auto- and heterotrophic acidophilic bacteria enhance the bioremediation efficiency of sediments contaminated by heavy metals. Chemosphere 74, 1321-1326.
- Beveridge, T.J., Fyfe, W.S., 1985. Metal fixation by bacterial cell walls. Can. J Earth Sci. 22, 1893-1898.
- Bundesministerium für Land- und Forstwirtschaft, U.u.W., 2012. http://www.lebensministerium.at/.
- Cao, X., Chen, Y., Wang, X., Deng, X., 2001. Effects of redox potential and pH value on the release of rare earth elements from soil. Chemosphere 44, 655-661.
- Carlsson, E., Büchel, G., 2005. Screening of residual contamination at a former uranium heap leaching site, Thuringia, Germany. Chem. Erde - Geochem. 65, 75-95.
- Cohen, R.R.H., 2006. Use of microbes for cost reduction of metal removal from metals and mining industry waste streams. J. Clean. Prod. 14, 1146-1157.
- Davies, B.E., White, H.M., 1981. Environmental pollution by wind blown lead mine waste: a case study in wales, U.K. Sci. Total Environ. 20, 57-74.
- Davison, J., 1988. Plant beneficial bacteria. Biotechnology 6, 282-286.
- Díaz-Barrientos, E., Madrid, L., Cardo, I., 1999. Effect of flood with mine wastes on metal extractability of some soils of the Guardiamar river basin (SW Spain). Sci. Total Environ. 242, 149-165.
- Dietrich, H., Berger, D., Vorläufige Artenliste im Bereich des Untersuchungsgebietes der ehemaligen Gessenhalde (Ronneburg/Thüringen), 2008.
- Dimkpa, C.O., 2009. Microbial Siderophores in Rhizosphere Interactions in Heavy Metal-containing Environments, Biologisch-Pharmazeutische Fakultät. Friedrich-Schiller University, Jena, p. 135, Doctoral Thesis.

- Dimkpa, C.O., Merten, D., Svatoš, A., Büchel, G., Kothe, E., 2009. Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. Soil Biol. Biochem. 41, 154-162.
- Ding, S., Liang, T., Zhang, C., Yan, J., Zhang, Z., 2006. Effects of organic ligands on fractionation of rare earth elements (REEs) in hydroponic plants: an application to the determination of binding capacities by humic acid for modelling. Chemosphere 65, 1942-1948.
- Eißmann, L., 1997. Das quartäre Eiszeitalter in Sachsen und Nordostthüringen: Landschaftswandel am Südrand des skandinavischen Vereisungsgebietes, Altenburger Naturwissenschaftliche Forschungen, Leipzig.
- European-Council, 2009 (1986, amended). Council Directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture, in: European-Union (Ed.).
- Ferreira da Silva, E., Bobos, I., Xavier Matos, J., Patinha, C., Reis, A.P., Cardoso Fonseca, E., 2009. Mineralogy and geochemistry of trace metals and REE in volcanic massive sulfide host trocks, stream sediments, stream waters and acid mine drainage from Lousal mine area (Iberian Pyrite Belt, Portugal). Appl. Geochem. 24, 383-401.
- Franche, C., Lindström, K., Elmerich, C., 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321, 35-59.
- Geletneky, J.W., 2002. Hydrogeologische/Hydrologische Untersuchung einer Prä-Flutungssituation am Beispiel des Gessentals im ehemaligen ostthüringischen Uranbergbaugebiet, Chemisch-Geowissenschaftliche Fakultät. Friedrich-Schiller-Universität, Jena, Doctoral Thesis.
- Giller, K.E., 1989. Absence of nitrogen fixation in clover grown on soil subject to long-term contamination with heavy metals is due to survival of only ineffective *Rhizobium*. Soil Biol. Biochem. 21, 841-848.
- Grawunder, A., Merten, D., 2012. Rare Earth Elements in Acidic Systems - Biotic and Abiotic Impacts, in: Varma, E.K.a.A. (Ed.), Bio-Geo Interactions in Metal-contaminated Soils, Soil Biology. Springer-Verlag, Berlin Heidelberg, pp. 81-97.
- Grayston, S.J., Vaughan, D., Jones, D., 1996. Rhizosphere carbon flow in tees, in comparison with annual plants: the importance of root exudation and its impacts on microbial activity and nutrient availability. Appl. Soil Ecol. 5, 29-56.
- Guo, H., Luo, S., Chen, L., Xiao, X., Xi, Q., Wei, W., Zeng, G., Liu, C., Wan, Y., Chen, J., He, Y., 2010. Bioremediation of heavy metals by growing hyperaccumulator endophytic bacterium *Bacillus* sp. L14. Bioresource Technol. 101, 8599-8605.
- Harrison, R.M., 1999. Understanding our environment - An introduction to environmental chemistry and pollution, 3rd ed. The Royal Society of Chemistry, Cambridge, UK, p. 445.
- Inal, A., Gunes, A., 2008. Interspecific root interactions and rhizosphere effects on salt ions and nutrient uptake between mixed grown peanut/maize and peanut/barley in original saline-sodic-boron toxic soil. J. Plant Physiol. 165, 490-503.
- Jakubick, A., Jenk, U., Kahnt, R., 2002. Modelling of mine flooding and consequences in the mine hydrogeological environment: Flooding of the Koenigstein mine, Germany. Environ. Geol. Water. S. 42, 222-234.
- Kahlert, J., 1992. Wismut und die Folgen des Uranbergbaus : eine Tagung der Friedrich-Ebert-Stiftung am 19. Juni 1992 in Gera, Wirtschaftspolitische Diskurse, Electronic edition ed, Bonn, p. 44.

- Kamnev, A.A., Tugarova, A.V., Antonyuk, L.P., Tarantilis, P.A., Polissiou, M.G., Gardiner, P.H.E., 2005. Effects of heavy metals on plant-associated rhizobacteria: Comparison of endophytic and non-endophytic strains of *Azospirillum brasilense*. J. Trace Elem. Med. Bio. 19, 91-95.
- Keith, S.I., Faroon, O.M., Fowler, B.A., 2007. Uranium, in: Nordberg, G.F., Fowler, B.A., Nordberg, M., Friberg, L.T. (Eds.), Handbook on the Toxicology of Metals, 3rd Edition ed. Academic Press, Burlington, San Diego, USA.
- Kidd, P., Juan, B., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monteroso, C., 2009. Trace element behaviour at the root-soil interface: implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.
- Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N., 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286, 885-886.
- Kloepper, J.W., Ryu, C.-M., 2006. Bacterial endophytes as elicitors of induced systemic resistance, in: Schulz, B., Boyle, C., Sieber, T. (Eds.), Microbial root endophytes. Springer, Heidelberg.
- Kozyrovska, N., Kovtunovych, G., Gromosova, E., Kuharchuk, P., Kordyum, V., 1996. Novel inoculants for an environmentally-friendly crop production. Resour. Conserv. Recy. 18, 79-85.
- Lange, G., 1995. Die Uranlagerstätte Ronneburg. Z. Geol. Wissenschaft. 23, 517-526.
- Lei, L.-q., Song, C.-a., Xie, X.-l., Li, Y.-h., 2008. REE behavior and effect factors in AMD-type acidic groundwater at sulfide tailings pond, BS nickel mine, W.A. T. Nonferr. Metal Soc. 18, 955-961.
- Lin, Q., Xu, C.Y., He, Y.F., Tian, G.M., 2004. Root-induced changes of lead availability in the rhizosphere of Oryza sativa L. Agr. Ecosyst. Environ. 104, 605-613.
- Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M., van der Lelie, D., 2002. Endophytic bacteria and their potential applications. Crit. Rev. Plant Sci. 21, 583-606.
- Lonschinski, M., 2009. Schwermetalle im System Boden-Wasser-Pflanze mit Hinblick auf Fraktionierungsprozesse der Seltenen Erden Elemente auf der Aufstandsfläche der Gessenhalde im ehemaligen Uranbergbaurevier Ronneburg (Ostthüringen), Chemisch-Geowissenschaltliche Fakultät. Friedrich-Schiller-Universität, Jena, Doctoral Thesis, p. 205.
- López-Bucio, J., Nieto-Jacobo, M.F., Ramírez-Rodríguez, V., Herrera-Estrella, L.R., 2000. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci. 160, 1-13.
- Madmony, A., Chernin, L., Pleban, S., Peleg, E., Riov, J., 2005. Enterobacter cloacae, an obligatory endophyte of pollen grains of mediterrean pines. Folia Microbiol. 50, 209-216.
- Majewska-Sawka, A., Nakashima, H., 2004. Endophyte transmission via seeds of *Lolium perenne* L.: immunodetection of fungal antigens. Fungal Genet. Biol. 41, 534-541.
- Marschner, H., 2005. Mineral Nutrition of Higher Plants, second ed. Academic Press.
- Marschner, P., 2012. Chapter 15 - Rhizosphere Biology, in: Petra, M. (Ed.), Marschner's Mineral Nutrition of Higher Plants (Third Edition). Academic Press, San Diego, pp. 369-388.
- Mastretta, C., Taghavi, S., van der Lelie, D., Mengoni, A., Galardi, F., Gonnelli, C., Barac, T., Boulet, J., Weyens, N., Vangronsveld, J., 2009. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. Int. J. Phytoremediat. 11, 251-267.
- McLennan, S.M., 1989. Rare earth elements in sedimentary rocks; influence of provenance and sedimentary processes, Reviews in Mineralogy and Geochemistry: Geochemistry and mineralogy of rare earth elements, pp. 169-200.

- Meda, A.R., Scheuermann, E.B., Prechsl, U.E., Erenoglu, B., Schaaf, G., Hayen, H., Weber, G., Wirén, N.v., 2007. Iron acquisition by phytosiderophores contributes to cadmium tolerance. Plant Physiol. 143, 1761-1773.
- Merten, D., Geletneky, J., Bergmann, H., Haferburg, G., Kothe, E., Büchel, G., 2005. Rare earth element patterns: A tool for understanding processes in remediation of acid mine drainage. Chem. Erde - Geochem. 65, 97-114.
- Mirgorodsky, D., Ollivier, D., Merten, D., Büchel, G., 2011. Quantification of heavy metal/radionuclide loads in a phytoremediation field experiment 10th symposium on remediation / 10. Sanierungskolloquium Dornburg, Germany.
- Mirgorodsky, D., Ollivier, D., Merten, D., Büchel, G., Willscher, S., Wittig, J., Jablonski, L., Werner, P., 2010. Phytoremediation experiments on a slightly contaminated test field of a former uranium mining site "Mine Water & Innovative Thinking", IMWA 2010, September 05.-09., 2010, Sydney/Canada, pp. 587-590.
- Moënne-Loccoz, Y., Powell, J., Higgins, P., McCarthy, J., O'Gara, F., 1998. An investigation of the impact of biocontrol *Pseudomonas fluorescens* F113 on the growth of sugarbeet and the performance of subsequent clover-Rhizobium symbiosis. Appl. Soil Ecol. 7, 225-237.
- Möller, P., Paces, T., Dulski, P., Morteami, G., 2002. Antropogenetic Gd in surface water drainage system, and the water supply of the city of Prague, Czech Republic. Environ. Sci. Technology 36, 2387-2394.
- Neagoe, A., Merten, D., Iordache, V., Büchel, G., 2009. The effect of bioremediation methods involving different degrees of soil disturbance on the export of metals by leaching and by plant uptake. Chem. Erde - Geochem. 69, 57-73.
- Newman, L.A., Reynolds, C.M., 2005. Bacteria and phytoremediation: new uses for endophytic bacteria in plants. Trends Biotechnol. 23, 6-8.
- Nies, D.H., 1999. Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. 51, 730-750.
- Oburger, E., Kirk, G.J.D., Wenzel, W.W., Puschenreiter, M., Jones, D.L., 2009. Interactive effects of organic acids in the rhizosphere. Soil Biol. Biochem. 41, 449-457.
- Pavlo, A., Leonid, O., Iryna, Z., Natalia, K., Maria, P.A., 2011. Endophytic bacteria enhancing growth and disease resistance of potato (*Solanum tuberosum L.*). Biol. Control 56, 43-49.
- Peterson, S.B., Dunn, A.K., Klimowicz, A.K., Handelsman, J., 2006. Peptidoglycan from *Bacillus cereus* mediates commensalism with rhizosphere bacteria from the *Cytophaga-Flavobacterium* Group. Appl. Environ. Microb. 72, 5421-5427.
- Porteous Moore, F., Barac, T., Borremans, B., Oeyen, L., Vangronsveld, J., van der Lelie, D., Campbella, C.D., Moore, E.R.B., 2006. Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: The characterisation of isolates with potential to enhance phytoremediation. Syst. Appl. Microbiol. 29, 539-556.
- Pourjabbar, A., 2012. Statistical Analysis of the groundwater and slate samples with special emphasis on rare earth elements. Friedrich Schiller University, Jena Germany, Doctoral Thesis
- Pourret, O., Davranche, M., Gruau, G., Dia, A., 2008. New insights into cerium anomalies in organic-rich alkaline waters. Chem. Geol. 251, 120-127.
- Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal-contaminated land by trees - a review. Environ. Int. 29, 529-540.
- Puschenreiter, M., Schnepf, A., Millán, I.M., Fitz, W.J., Horak, O., Klepp, J., Schrefl, T., Lombi, E., Wenzel, W.W., 2005. Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. Plant Soil 271, 205-218.

- Rabiet, M., Brissauda, F., Seidela, J.L., Pistrea, S., Elbaz-Poulicheta, F., 2009. Positive gadolinium anomalies in wastewater treatment plant effluents and aquatic environment in the Hérault watershed (South France) Chemosphere 75, 1057-1064.
- Rajkumar, M., Ae, N., Freitas, H., 2009. Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77, 153-160.
- Raskin, I., Smith, R.D., Salt, D.E., 1997. Phytoremediation of metals: using plants to remove pollutants from the environment. Curr. Opin. Biotech. 8, 221-226.
- Razo, I., Carrizales, L., Castro, J., Barriga, F.D., Monroy, M., 2004. Arsenic and heavy metal pollution of soil, water and sediments in a semi-arid climate mining area in Mexico Water Air Soil Pollut. 152, 129-152.
- Reichenauer, T.G., Friesl-Hanl, W., Edelmann, E., Hofer, H., Weisgram, M., Müller, D., Dörrie, T., 2010. Arbeitshilfe zur Expositionsabschätzung und Risikoanalyse an kontaminierten Standorten - Endbericht zum Arbeitspaket 2 des Projektes „Altlastenmanagement 2010“ (Neuausrichtung der Beurteilung und Sanierung von kontaminierten Standorten). Österreichisches Umweltbundesamt.
- Reinhold-Hurek, B., Hurek, T., 2011. Living inside plants: bacterial endophytes. Curr. Opin. Plant Biol. 14, 435-443.
- Rolfe, B.G., Gresshoff, P.M., Shine, J., 1980. Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci. Lett. 19, 277-284.
- Römheld, V., Marschner, H., 1986. Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. Plant Physiol. 80, 175-180.
- Rönkkö, R., Smolander, A., Nurmiaho-Lassila, E.-L., Haahtela, K., 1993. *Frankia* in the rhizosphere of non-host plants: A comparison with root-associated N_2-fixing *Enterobacter*, *Klebsiella* and *Pseudomonas*. Plant Soil 153, 85-95.
- Rroço, E., Kosegarten, H., Harizaj, F., Imani, J., Mengel, K., 2003. The importance of soil microbial activity for the supply of iron to sorghum and rape. Eur. J. Agron. 19, 487-493.
- Rüger, F., Dietel, W., 1998. Vier Jahrzehnte Uranerzbergbau in Ronneburg. Lapis 7/8, 14-18.
- Šarić, M., Lucchini, R., 2007. Manganese, in: Nordberg, G.F., Fowler, B.A., Nordberg, M., Friberg, L.T. (Eds.), Handbook on the Toxicology of Metals, 3rd Edition ed. Academic Press, Burlington, San Diego, USA.
- Schmidt, A., Haferburg, G., Schmidt, A., Lischke, U., Merten, D., Ghergel, F., Büchel, G., Kothe, E., 2009. Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area. Chem. Erde - Geochem. 69, 35-44.
- Schmidt, A., Haferburg, G., Sineriz, M., Merten, D., Büchel, G., Kothe, E., 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. Chem. Erde - Geochem. 65, 131-144.
- Semhi, K., Chaudhuri, S., Clauer, N., 2009. Fractionation of rare-earth elements in plants during experimental growth in vaired clay substrates. Appl. Geochem. 24, 447-453.
- Shan, X.-q., Lian, J., Wen, B., 2002. Effect of organic acids on adsorption and desorption of rare earth elements. Chemosphere 47, 701-710.
- Shen, H., Yan, X., Zhao, M., Zheng, S., Wang, X., 2002. Exudation of organic acids in common bean as related to mobilization of aluminum- and iron-bound phosphates. Environ. Exp. Bot. 48, 1-9.
- Sheng, X.-F., Xia, J.-J., Jiang, C.-Y., He, L.-Y., Qian, M., 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. Environ. Pol. 156, 1164-1170.

- Shetty, K.G., Hetrick, B.A.D., Figge, D.A.H., Schwab, A.P., 1994. Effects of mycorrhizae and other soil microbes on revegetation of heavy metal contaminated mine spoil. Environ. Pol. 86, 181-188.
- Shiomi, H.F., Silva, H.S.A., Melo, I.S.d., Nunes, F.V., Bettiol, W., 2006. Bioprospecting endophytic bacteria for biological control of coffee leaf rust. Sci. Agric. 63.
- Sipos, P., Póka, T., 2002. Threshold limit values for heavy metals in soils in the function of spacial and temporal variation of geochemical factors, XVII. Congress of CBGA. Special Issue of Geologica Carpathica (CD), Bratislava.
- Sneller, F.E.C., Kalf, D.F., Weltje, L., van Wezel, A.P., 2000. Maximum permissible concentrations and negligible concentrations for Rare Earth Elements (REEs).
- Spedding, F.H., 2009. Rare Earth Elements Encyclopaedia Britannica, electronic edition ed.
- Steinmann, M., Stille, P., 2008. Controls on transport and fractionation of the rare earth elements in stream water of a mixed basaltic-granitic catchment basin (Massif Central, France). Chem. Geol. 254, 1-18.
- Teng, Y., Luo, Y.-M., Huang, C.-Y., Long, J., Li, Z.-G., Christie, P., 2008. Tolerance of grasses to heavy metals and microbial functional diversity in soils contaminated with copper mine tailings. Pedosphere 18, 363-370.
- Tervet, I.W., Hollis, J.P., 1948. Bacteria in the storage organs of healthy plants. Phytopathology 38, 960–967.
- Tessema, S.G., 2011. Influence of plant water relation on ecosystem function under water stress. Doctoral Thesis, Friedrich-Schiller-University of Jena, Jena, Doctoral Thesis.
- Tsakelova, E.A., Cherdyntseva, T.A., Botina, S.G., Netrusov, A.I., 2006. Bacteria associated with orchid roots and microbial production of auxin. Microbiol. Res. 162, 69-76.
- US.EPA, 2011. Pesticides: Regulating Pesticides - *Pantoea agglomerans* strain E325 (006511) Fact Sheet. U.S. Environmental Protection Agency.
- Usman, A.R.A., Mohamed, H.M., 2009. Effect of microbial inoculation and EDTA on the uptake and translocation of heavy metal by corn and sunflower. Chemosphere 76, 893-899.
- van der Lelie, D., Barac, T., Taghavi, S., Vangronsveld, J., 2005. Response to Newman: New uses of endophytic bacteria to improve phytoremediation. Trends Biotechnol. 23.
- van Hees, P.A.W., Vinogradoff, S.I., Edwards, A.C., Godbold, D.L., Jones, D.L., 2003. Low molecular weight organic acid adsorption in forest soils: effects on soil solution concentrations and biodegradation rates. Soil Biol. Biochem. 35, 1015-1026.
- Vangronsveld, J., Cunningham, S.D., 1998. Introduction to the concepts, in: Cunningham, J.V.a.S.D. (Ed.), Metal-Contaminated Soils: In-situ Inactivation and Phytorestoration. Springer Verlag, Berlin Heidelberg, pp. 1-15.
- Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, a., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M., 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ. Sci. Pollut. Res. 16, 765-794.
- Wang, L., Ten, L.N., Lee, H.-G., Im, W.-T., Lee, S.-T., 2008. *Olivibacter soli* sp. nov., *Olivibacter ginsengisoli* sp. nov. and *Olivibacter terrae* sp. nov., from soil of a ginseng field and compost in South Korea. Int. J. Syst. Evol. Micr. 58, 1123–1127.
- Wang, L., Zhou, Q., Huang, X., 2009. Photosynthetic responses to heavy metal terbium stress in horseradish leaves. Chemosphere 77, 1019-1025.
- Weyens, N., van der Lelie, D., Taghavi, S., Newman, L., Vangronsveld, J., 2009a. Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends Biotechnol. 27, 591-598.

- Weyens, N., van der Lelie, D., Taghavi, S., Vangronsveld, J., 2009b. Phytoremediation: plant-endophyte partnerships take the challenge. Curr. Opin. Biotech. 20, 248-254.
- Whiting, S.N., Souza, M.P.D., Terry, N., 2001. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ. Sci. Technol. 35, 3144-3150.
- Wismut, 1994a. Sanierungskonzept Standort Ronneburg.-Stand März 1994, Internal Report. Wismut GmbH, Chemnitz
- Wismut, 1994b. Sanierungskonzept Standort Ronneburg.-Stand Dezember 1994, Internal Report, in: GmbH, W. (Ed.). Wismut GmbH, Chemnitz.
- Yanni, Y.G., Rizk, R.Y., Corich, V., Squartini, A., Ninke, K., Philip-Hollingsworth, S., Orgambide, G., Bruijn, F.d., Stoltzfus, J., Buckley, D., Schmidt, T.M., Mateos, P.F., Ladha, J.K., Dazzo, F.B., 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. Plant Soil 194, 99-114.
- Yuan, S., Xi, Z., Jiang, Y., Wan, J., Wu, C., Zheng, Z., Lu, X., 2007. Desorption of copper and cadmium from soils enhanced by organic acids. Chemosphere 68, 1289-1297.
- Zhuang, X., Chen, J., Shim, H., Bai, Z., 2007. New advances in plant growth-promoting rhizobacteria for bioremediation. Environ. Int. 33, 406-413.

Chapter 1

THE ROLE OF ROOT EXUDATES FOR THE METAL MOBILITY AND PLANT BIOAVAILABILITY

The present chapter is a review and is written to be published as a book chapter.

Chapter 1: The role of root exudates for the metal mobility and plant bioavailability

In order to understand the on-going processes in the contaminated soil system, and to conceive remediation strategies, knowing the factors that influence metal behaviour and especially its mobility is of high importance. Soil is a complex biogeochemical material whose physical, chemical and biological characteristics and properties differ strongly from the underlying parent rock. Soil comprises mineral particles and organic material, populated by a variety of microorganisms, soil fauna and a more or less dense root network. The space between the particles forms a system of pores, filled by gases and water, allowing material exchange between solids and aqueous phases and plants (Harrison, 1999; Sipos and Póka, 2002).

Due to these properties, soil has the capacity to retain pollutants and thus can act as a sink for pollutants and as a filter before pollutants reach the groundwater. Metals, which are non-degradable contaminants, cannot be diluted by fluxes as in liquid or gas, but accumulate in the solid phase, becoming possibly a source of secondary contamination. On the other hand, soil is in close contact with the water phase, by precipitation and also in zones with high groundwater level, and can therefore act as a reaction matrix.

1 METAL MOBILITY – INFLUENCE OF THE SOIL PARAMETERS

Many different factors have an influence on the mobilisation and immobilisation of metals in soil and water. Important ones are soil pH, soil composition, soil weathering as well as the type of metal and its chemical form, or heavy metal competition (Bradl, 2004). The description of the occurrence form of metals and its changes are essential for risk assessment, toxicity studies or remediation options.

1.1 pH

The pH plays a key role for the mobility of metals in the soil; therefore the buffer capacity of the soil is an important parameter for the evaluation of heavy metal hazard. In general, the mobilisation is proportional to sinking of pH values; around pH 2-3 almost all of the metals are dissolved (Karim and Khan, 2001). Thus, the pH influences some metals (as Zn and Cd) more than others (Pb, Cu or Hg) regarding their dissolution and precipitation (Blume and Brümmer, 1991). Further, pH influences also the sorption of metals (Paas, 1997). Sorbed metals can be remobilised depending on the pH, but this release is possible only at higher values than the sorption.

So for example the mobility of aluminium is strongly dependent on pH, since under a pH of 4.5 to 5 it is mainly present as a ion $Al^{3+}_{(aq)}$, and precipitates as hydroxide with increasing pH. However, at pH values over 7.5 it forms $Al(OH)^-$ which remains in solution. Al forms also complexes with F^- and organic matter (at pH 4.3-7). Al is naturally immobilised in soils with no acid pH by reaction with silicates, which leads to the formation of stable hydroxyl-aluminosilicates (Harrison, 1999).

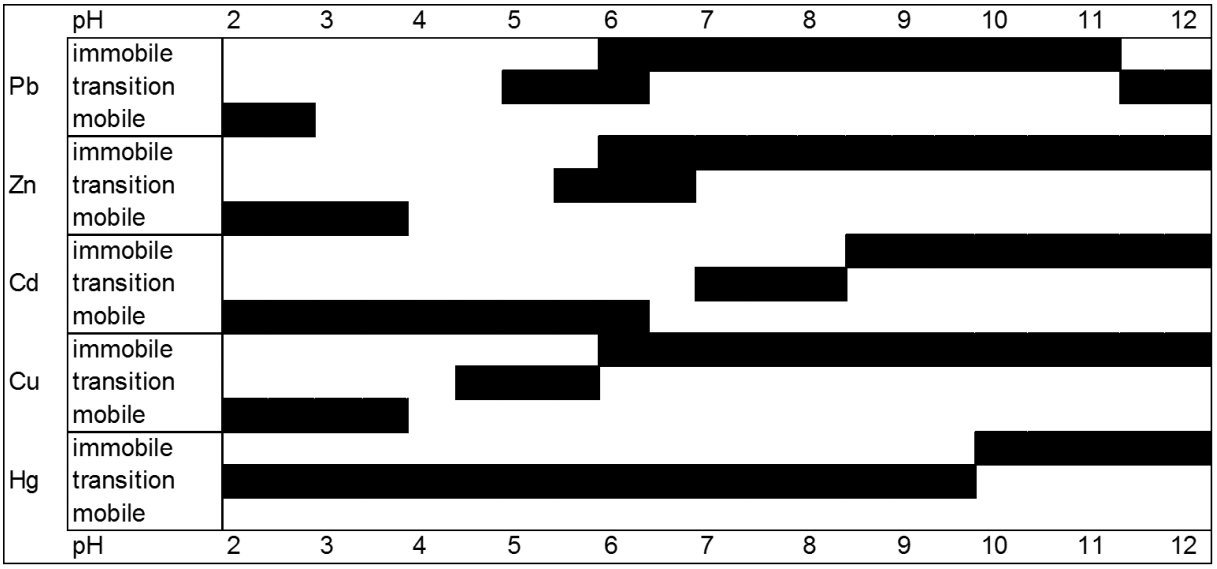


Figure 1: pH range, in which heavy metals Pb, Zn, Cd, Cu and Hg (under strongly salinary conditions) are mostly immobile, in which the mobilisation takes place (transition), as well as range in which they are mostly mobile (Paas, 1997)

1.2 Redox potential

The redox potential influences obviously the state of oxidation of the metals, and if their different forms show different behaviour in solubility, it influences also their solubility (Zehl, 2005). For example, Iron is soluble if present as Fe^{II}, but easily precipitated as a hydroxide as Fe^{III} under oxic conditions. If generally anaerobic conditions are given in deeper parts of the soil, which are saturated, the most important actors for these reactions are microorganisms, which can use redox reactions for energy production (anaerobic metabolism, *see section 3*).

pH and redox potential themselves influence the following interactions with the soil components by shifting the sorption capacity or acting on the stability of complexes.

1.3 Sorption on minerals

Definition: Sorption processes (=Absorption, Adsorption and surface precipitation)

Sorption mechanisms in soils

As the retention mechanism of metal ions at soil surfaces is often unknown, the term "sorption" is preferred, which in general involves the loss of a metal ion from an aqueous to a contiguous solid phase and consists of three important processes: adsorption, surface precipitation, and fixation (Bradl, 2004).

According to Bradl (2004) there are two mechanisms for sorption. On one hand, there is the specific sorption, characterised by specific, hardly reversible reactions, like chemisorption, where inner electrons play a role. On the other hand, there is the non-specific sorption (ion exchange) that involves rather weak complexes of the outer sphere of electrons. The specific sorption signifies a strong and irreversible bound of the metals onto organic material or different minerals, unlike the unspecific sorption, which is an electrostatic process, exchanging cations from the water with cations from the surface. Cation exchange is a form of outer electron bound with weak bound between metals and charged surface. The process is reversible, quick and typical for reactions, which are electrostatical or controlled by diffusion.

Adsorption followed by surface precipitation and fixation in one of the main processes responsible for the immobilisation and consecutive accumulation of heavy metals. The most common surfaces involved are inorganic colloids, like clay minerals, metal oxides, but also organic colloids like humic acids. The functional groups on the surface are essential (Blume and Brümmer, 1991; Bradl, 2004). Colloids influence also the transport of trace elements (Ryan and Elimelech, 1996). Generally, trace elements adsorb onto colloids or form themselves colloidal structures (Hofmann, 2004). Arsenic for instance strongly associates with ferrihydrite colloids and cause As mobility increase in liquid phase due to this association (Fritzsche et al., 2011).

In layer-silicates for example there are pH–independent charges that can function as ion-exchanger (isomorphic replacement) called permanent charge. On the contrary, the charge of functional group depends on the pH and exists in Fe- and Mn-oxides and –hydroxides. Minerals have negative charge especially at high pH, so that positive metals can be sorbed (Paas, 1997). The sorption can influence the pH if the heavy metal content is very high, because these can be exchanged against H^+, and so lower the pH. In acidic soils (pH<5) the majority of the cation exchanges places are occupied by aluminium, it replaces especially polyvalent ions like Mg, Ca, and acts as a strong absorber for phosphate and molybdate. The proportion of aluminium in the soil correlates with the pH, and also with the inhibition of the growth of plant roots.

Manganese content (exchangeable, as Mn^{2+}) increases when pH decreases, but depending as well on the redox potential, i.e. there is much free Mn in acidic soils, with much easily reducible Mn, much organic material, high microbial activity and permanent or temporal anaerobic conditions (Marschner, 2005). Different metals (Cd, Cr, Pb, Cu, Mn, Zn, and Co) can be discriminated based on their chemical behaviour, depending on the sorption, and strength of the bond (Bradl, 2004).

Complexing of metals by different components of the solution (carbonate, chloride, sulphate, fluoride) has an influence on their sorption. The sorption of metals increases with their tendency to form hydroxo-complexes. Metals with a high stability of chloro-complexes will be sorbed at higher pH than un-complexed. For instance, Pb is better sorbed if the pH is higher and the salt concentration lower.

Table 1: Summary of the factors influencing the sorption of metals (Paas, 1997)

Grain surface	Basically little influence; slower diffusion if pieces are bigger
Salt content of the water	Strong influence: especially Cd and Hg are hardly sorbed at high salt concentrations because of the high stability of chloro-complexes. Influence of sulphate in Cl-dominated waters is little
pH	For Pb, Cd, Zn, Cu important role: within 3 pH units, all can be sorbed or desorbed. Hg is pH independent. [cf. Figure]
Oxygen content	O_2 presence provokes the oxidation of Fe-containing rocks to hydroxides. Then sorption through Fe-hydroxides. Hg is completely immobile under anaerobic conditions.
Temperature	In the presence of O_2 influences the kinetics of oxidation reactions; under anaerobic conditions weak influence
Mineralogical and chemical composition of the sorbent	Very important: K_{Fr}-value variates Sorption depends strongly on the material In general, Pb, Zn, Cr, Cu are better sorbed than As und Hg, the most difficult to immobilise is Cd

Further influence of the composition of the soil: Precipitation and co-precipitation

Also single elements can influence the mobility: (Cui et al., 2004) describes the effect of elementary sulphur on the solubility of metals. This depends on the metal: Pb is much less mobilised and taken up by plants than Cd or Zn for instance.

The solubility product K_L can be exceeded if there is contact with specific mineral phases. For example, $Mg(OH)_2$ precipitates if Mg containing water comes in contact with material which reacts basic. There new phases can close pores of the carrier material and inhibit the transport of water. Further is it possible that dissolved substances, with a concentration far under their solubility limit, can co precipitated through isomorphic substitution or crystallisation. That happens with heavy metals in iron oxides (Paas, 1997).

The soil structure is also important (Paas, 1997): the space of the pores influences the quantity of metals in the senses that solution flows slower, so that metals are retained (temporary reservoir) and adsorbed, possibly irreversibly bound. The molecular mobility is influenced by different factors like geometry and dimensions of the pores, physico-chemical properties of the solid phase, ion strength of the aqueous phase, viscosity, size and charge (ion potential) and hydratation energy of the solved particles.

Influence of the weather

Climate (i.e. in this case precipitation and alternating dry and wet periods) and temperature also influence metal mobility. Indeed, Fe and Mn are particularly mobile if dry and wet periods are alternating; Ni, Zn, Cd, Cu, Pb, Fe are preferentially bound to sulphides under water excess and reducing conditions (Blume and Brümmer, 1991). Further, it was reported that the hazard through cadmium is particularly high with sandy, acid soils with low humus content, high groundwater level and humid climate. On the contrary, the risk is low with calcareous soils, low groundwater level and semi dry to arid climate.

Influence of organic material

Different kinds of organic chemicals can influence the dissolution and sorption of heavy metals (Khan et al., 1991). It can be observed in the context of contaminants of anthropogenic origin, like pesticides (Incorvia Mattina et al., 2003), or in the context of remediation, for example to enhance leaching of metals out of the soil with the use of saponin (Hong et al., 2002) for soils with low organic content**.** Saponin in one of the numerous organic components that can form complexes with metals, especially with its carboxyl groups, it can be adsorbed into the soil at relatively low pH, although at these values the metals are better leached. In that study, pH values around 5-5.5 were a good balance between both effects.

Differences between the metals

It should be kept in mind that the reactivity to the previous described factors differs for different metals. Which metals are mobilised preferentially has been already described in several articles (Bradl, 2004; Lors et al., 2004; Paas, 1997): Pb is known to be almost not mobilised, and for instance Cu much less than Zn and Cd. The reason for that is that Cu is mobilised for a short time, but immediately adsorbed by other substances formed during the process by organic material or iron hydroxides. Like Cu, Pb is immediately adsorbed, also by co-precipitation with Fe-, Al-, or Mn-hydroxides. Like for other factors, the sorption is different for different metals: Pb, Zn and Hg are better retained, Cd almost not. Additionally, the efficiency of the mobilisation of metals through addition of hydrophobic substances is higher for Cd and Zn than for Cu and Pb, because of the electronegativity and the

chemisorption on oxides, humus, clay, loam (Hong et al., 2002). Similar results were found by Khan et al. (1991), who studied the influence of organic substances on the mobility of heavy metals using thin-layer chromatography. The mobility of the metals was without any amendment decreasing from Cu to Zn in the following order: Cu >Cd >Hg >C o>Ni >Zn. The mobility was enhanced for Co, Ni, Zn, Cd, and decreased for Cu and Hg after addition of alcohol or aldehyde. Hargitai (1989) separated in the context of the humus quality control metals according to hydrophobic properties into organophiles (Pb, Ni, Cd), characterised by their facility to bind to humus and similar substances, and non-organophiles. It is also possible to classify elements according to their relative binding strength (Paas, 1997), so different metals would be ordered as follows on clay minerals: Cu>Pb>Ni>Zn>Hg>Cd and slightly differently on Fe-oxides and hydroxides: Pb >C r= Cu >Zn >Ni >Cd. Indeed, the adsorption strength depends on the charge and hydration energy of the ion. It increases to the right and to the top within the periodic table.

Table2: Summary about preferential binding (Bradl, 2004)

Element	Bounds mainly to	Influencing factors
Cd		Strong: presence of cations competing for binding sites (Ca^{2+}, Zn^{2+}), can desorb Cd
Cr	Fe-oxides; Al_2O_3, kaolinit and montmorillonit have a great affinity to Cr^{VI} Cr^{III} is adsorbed faster by Fe- and Mn-oxides and clay minerals	Redox potential, oxidations stage, pH, soil minerals, competing ions, complexing agents Adsorption increases with the pH and the content of organic matter, decreases with competing cations or dissolved ligands
Pb		Carbonate in the soil
Cu	Organic matter (strong) / Fe- and Mn-oxides /sulphides / carbonates	Cu exist in solution mainly as complex with soluble organic substances
Mn	Clay minerals	Adsorption stronger with increasing pH Stronger retention by carbonate containing soils (Precipitation as $MnCO_3$)
Zn	Clay minerals	Non-available in calcareous and alkaline soils because of sorption on carbonates as Zn-oxide, Zn-carbonate or Ca-zincate
Co	Fe- and Mn-Oxides	

Since the speciation of metals is essential for their behaviour in the soil-water system and for their interaction with the biosphere, different methods have been developed to estimate the proportion of metals that are soluble, and those bound to different phases of the soil, more or less easily released. Commonly, a sequential extraction is used. It means that soil samples are treated with solutions of increasing extraction potential. Different methods of simple or sequential extraction have been proposed and compared (Beolchini et al., 2008; Doelsch et al., 2008; Krasnodebska-Ostrega et al., 2009; Lewandowski J., 1997; Ma and Uren, 1997; Martin et al., 1987; Sauerbeck and Lübben, 1991), to describe this solubilisation and repartition of heavy metals, and the one according to Zeien and Brümmer (1989) has been mainly used for the present soil (Figure 2).

	Cd	Mn	Co	Pb	Cu	Ni	Fe	Zn	Al
Mobile									
Exchangeable									
Mn-Oxides									
Organics									
amorphous Fe-Oxides									
crystalline Fe-Oxides									
Residual fraction									

mainly occurring as

partly or in certain soil types occurring as

Figure 2: Main fraction according to Zeien and Brümmer's sequential extraction, in which metals are mainly found to bind to (Paas, 1997).

However, despite the detailed description of binding phases for metals, the active influence of plants by their root exudates was not taken enough in consideration. One question rising concerning the availability of metals in the soil (Allen and Janssen, 2006), especially regarding the sequential extraction is how exudation influences the fraction of easily extractable metals. In other words, the available fraction could be higher than the easily dissolved one found following the classical sequential extraction method. In particular Puschenreiter et al. (2005) suggest that root activities, such as the exudation of organic acids, triggered the replenishment of soluble metal from immobile metal fractions of the soil.

Another example is given about the bioavailability of Pb near the roots of rice plants (Lin et al., 2004). It is well known that the organic acids produced by roots can complex metals - Díaz-Barrientos et al. (1999) compare them in their study about sequential extraction with the properties of EDTA - and so modify their availability. This has been noticed by different authors and is an invitation to consider this aspect when estimating bioavailability of metals in the environment (Haoliang et al., 2007; Mucha et al., 2005).

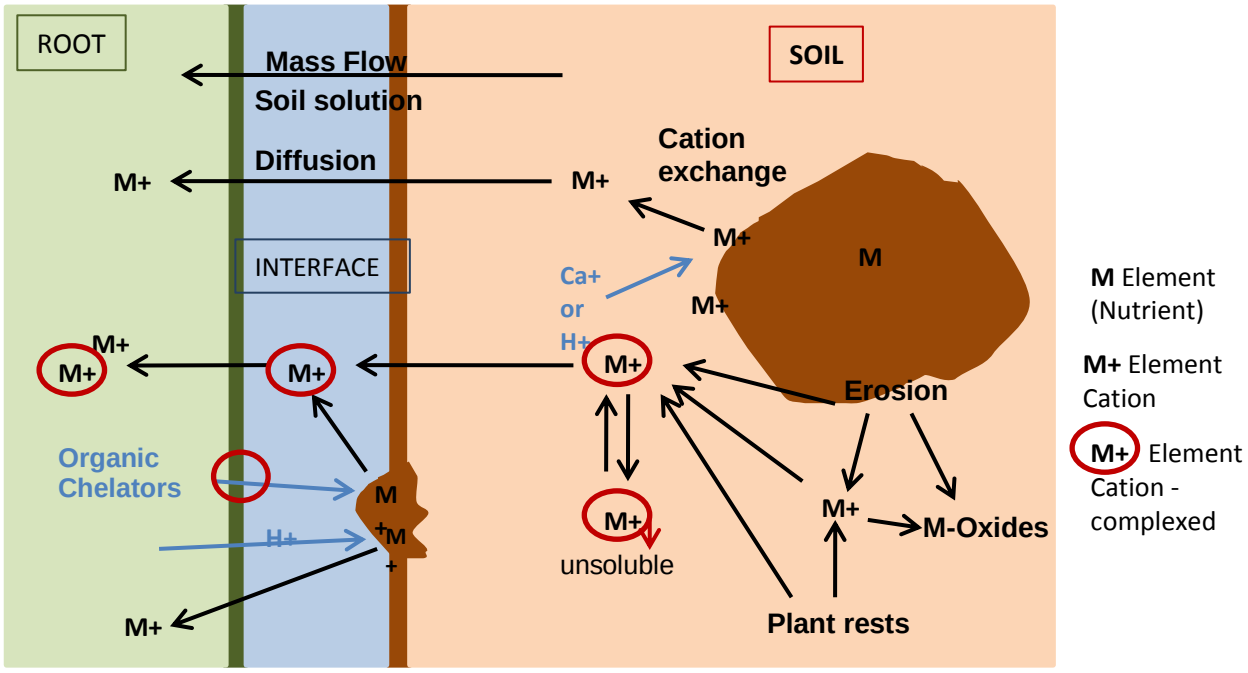


Figure 3: Important abiotic processes for the uptake of trace elements (M+). Trace elements present in form free ions or soluble chelates can be taken up by plant roots. Metals bound to soil particles or present as insoluble compounds can be mobilised by the action of root exudates, i.e. acidic substances which dissolve metallic compounds, by cation exchange, or by secretion of chelating components. (after Mitchell, 1972)

2 ROOT EXUDATES: THE ACTIVE CONTRIBUTION OF PLANTS TO METAL MOBILITY

Availability of trace elements to plants is governed by the dynamic equilibrium between aqueous and solid soil phases, rather than by the total metal content. To satisfy physiological needs for nutrients or to avoid metal toxicity, plants are able to modify clearly the mobility of metals.

In the soil solution, elements are present as free ions, ion pairs, ions complexed with organic anions, and ions complexed with organic macromolecules and inorganic colloids. The most important metal pools in the solid phase include the exchange complex, metals complexed by organic matter, sorbed onto or occluded within oxides and clay minerals, co-precipitated with secondary minerals (e.g. Al-, Fe-, Mn-oxides, carbonates and phosphates, sulphides) or as part of the crystal lattices of primary minerals (Kidd et al., 2009). Not all metal ions necessarily occur as cations; for example many elements occur as oxyanions like arsenate, selenate, selenite, chromate, chromite, which is also important among others for their behaviour in biological systems (Clarkson, 1993).

Plant-induced modifications of trace element speciation and bioavailability in the rhizosphere are the result of sharp biogeochemical gradients in elemental concentrations, pH, pCO_2, pO_2, redox potential and organic ligand concentrations, and microbial biomass (Dakora and Phillips, 2002; Kidd et al., 2009). The potential changes depend on the air content of the soil; the roots can influence this values by excreting different substances as acids, protons or chelators, and so influence the availability and uptake of mineral nutriments (Marschner, 2005). Although plants are able to influence their environment by acidification with protons or by secreting CO_2 (Dakora and Phillips, 2002), the main influencing factor is given by the secretion of diverse organic components.

Rhizodeposits include a wide spectrum of components ranging from simple chemical exudate compounds to entire root fragments, originating from dead cells. They can be grouped into five general classes: exudates (amino acids, low-molecular-weight carboxylic acids, sugars, and simple and flavonoid-type phenolics), secretions , plant mucilages, mucigel, and root lysates (Curl and Truelove, 1986 in (Kidd et al., 2009). Among the different substances secreted by plants, organic acids compose 1-3% of the dissolved organic carbon (DOC) in the soil. Root exudates and dead root material may comprise 30-40% of the total organic matter input to soils. This is released into the rhizosphere, which constitutes only 2-3% of the total soil volume (Grayston et al., 1996).

Important factors influencing the solubilisation of metals by plants through the quantity and composition of root exudates:

(5) root-induced changes in pH of the rhizosphere
(6) complexing capacity of organic compounds released
(7) reducing capacity of the roots
(8) need for nutrients in particular essential trace elements

Root exudates play a role in the weathering of soil, for the mobilisation of nutrients as P, NH_4^+ or Fe, especially through the action of organic acids, phytosiderophores, phenolic compounds. They are further important for the protection of plants against uptake of heavy

metals into the roots - mainly citrate, malate, small peptides are important in this case, or for attraction of beneficial microorganisms through phenolic compounds, organic acids, sugars (Grayston et al., 1996; López-Bucio et al., 2000; van Hees et al., 2003). Root exudates can also act as signal molecules or as precursors for hormones. Very low concentrations are sufficient for their biological effect (Marschner, 2005).

Table 3: Chemical classes of roots secretes (Modified from Bolton et al. 1992)

Class of compounds	Components
Carbohydrates	Arabinose, fructose, galactose, maltose, raffinose, rhamnose, ribose, xylose
Amino acids and amides	All 20 protein amino acids, aminobutyric acid, homoserine, cystathionine
Aliphatic acids	Acetic, butyric, citric, fumaric, glycolic, malic, malonic, oxalic, propionic, succinic, tartaric, valeric
Aromatic acids	*p*-Hydroxybenzoic, caffeic, *p*-coumaric, ferulic, gallic, gentistic, protocatechuic, salicylic, sinapic, syriagic, vanilic
	Linoleic, linolenic, oleic, palmitic, stearic
Sterols	Campesterol, cholesterol, sitosterol, stigmasterol
Proteins (Enzymes)	Amylase, deoxyribonuclease, invertase, peroxidase, phosphatase, ribonuclease
Miscellaneous	Vitamins, plant growth regulators, auxins, cytokinins, gibberelins, unidentified microbial growth stimulators and inhibitors

The type, composition, amounts, proportion are influenced by many factors. Most of the time, plant secrete a cocktail of different acids, whose amounts and composition in the soil depend on the species of the plant, the cultivation time, the constitution of the soil, as well as the age of the plant and the distance to the root. So, seedlings produce greater quantities and more diverse carbohydrates than mature trees, but the mature trees exude larger amounts of amino acids, amides and organic acids (Grayston et al., 1996). It is still unclear what are the single functions of each of them, and if they modify each other's reactions.

These exudates can influence the behaviour of nutrients and trace metals by complexing metals, in that way making them soluble and hence enhancing their bioavailability, by or precipitating them. They can on the other hand get themselves adsorbed on minerals or humus and so play a role in the pH regulation of the soil (van Hees et al., 2003), or often very fast get degraded by soil microorganisms to be used as a carbon or nitrogen source, and so influence their activity. The significance of the sorption and degradation rate depends strongly on the properties of the soil and the chemical properties of the acid or the acid mixture itself (Shan et al., 2002), but also on the activity of the microbial population in the rhizosphere (Yuan et al., 2007).

Furthermore, totally different conditions can prevail in the micro zone around the roots than in the bulk soil; for example aerobic conditions through O_2 excreting, or acidic conditions. Fe can be precipitated as Fe^{III} (Cohen, 2006). The pH can be influenced also through CO_2-production from respiration, or secretion of protons by the roots (Semhi et al., 2009).
This leads to a complex interaction between the mixture of heavy metals in the soil, plant exudates, soil minerals and organic content in the soil.

Under similar conditions, the organic acids secreted by the plant depend on the plant species (Table 4). Some data suggest that the composition of tree exudates may be as diverse as that

of herbaceous plants. Different studies reported those secreted by white lupine (*Lupinus albus*) and wheat (Weisskopf et al., 2008) or the chick pea (Veneklaas et al., 2003). Further different acids produced by legumes (Oburger et al., 2009; Shen et al., 2002), as well as trees (Haoliang et al., 2007; Sandnes et al., 2005) were analysed. For example, birch tended to have higher concentrations of oxalic, lactic and butyric acids, but lower concentrations of formic and phthalic acids than spruce when considering the differences in root density (Sandnes et al., 2005). Further factors can also play a role, as shown by Rao et al. (2002) the acid production seems to be dependent also on the amount and quality of light available to the leaves.

The spectrum is quite broad, but the most common acids are malonate, citrate, malate, fumarate and oxalate [cf. Table 4 about secreted organic acids].

2.1 Local dependence of the acid concentration

It is complicated to define precisely the root-influenced zone (rhizosphere), since the rhizocylinder volume reaches from several micrometres up to a few centimetres away from the root, as a function of plant species and of the physical, chemical and mineralogical properties of the soil. Nevertheless, even if the comparison of results between different studies and laboratories must be considered with care, because of the diversity of methods employed to separate rhizosphere soil, or by differences between research groups in what is considered to represent rhizosphere soil, it is clear through several studies that soil close to the roots differs from the one not influenced by them (bulk soil).

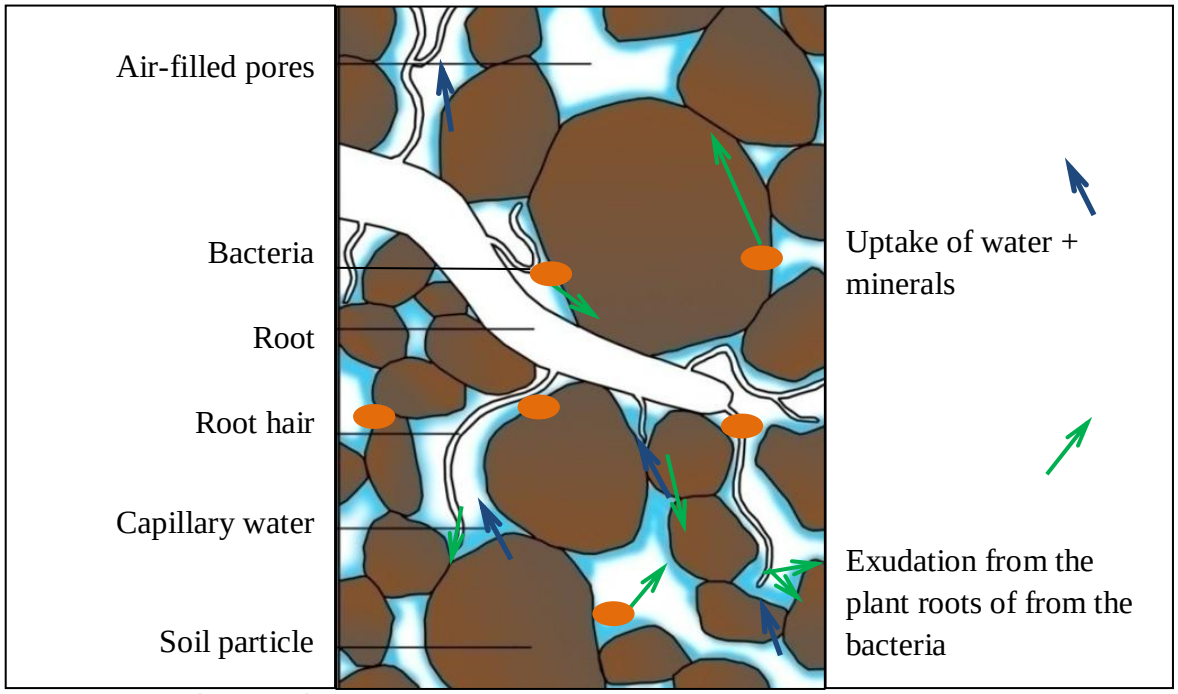


Figure 4: Uptake of metals influenced by root and microorganisms secretions.

The highest organic acid concentrations can be found in the very close rhizosphere zone, and almost not at all in the bulk soil. The bulk soil showed no difference between the different plants (Weisskopf et al., 2008). Only oxalic acid (< 55 mM) was found in the non-rhizosphere soil of some trees (Sandnes et al., 2005). Consequently, huge differences in pH and metal mobility are possible between rhizosphere and bulk soil. For instance, a difference of until 2 pH units can be measured between rhizosphere pH and bulk soil pH, depending on soil and plant sort (Marschner, 2005; Semhi et al., 2009). A decrease in redox potential, increases in

pH and microbial activity, and an increase followed by a decrease in dissolved organic carbon in the maize rhizosphere were observed by Tao et al. (2005).

The exudates production is further not the same along the roots of plants. Indeed, there are preferential locations where exudates are released, as showed schematically by (Marschner, 2012), hence influencing the whole rhizosphere around. [*see chap. 1 , §2.4 and Chap. 2&3*]

The most important factor for root induced changes of the rhizosphere area is the disequilibrium for the anion/cation uptake rate and so the corresponding differences for the release of H^+ und HCO_3^- (or OH^-), as well as the release of organic acids (Dakora and Phillips, 2002). In aerated soils, the CO_2 production has little influence on the pH of the rhizosphere, because it can diffuse away through the pores. Nevertheless, if it is dissolved as (H^+, HCO_3^-) in the soil solution, it can influence the pH, because these ions are not very mobile in the soil.

The buffer capacity of the soil as well as the initial pH of the bulk soil are the main factors deciding how much the plant can change the pH in the rhizosphere soil. The buffer capacity is not so much dependent on the content of loam, but rather from the initial pH and the content of organic matter; also, a calcareous soil has a higher buffer capacity than a sandy soil. The buffer capacity is lowest at pH 6, and rises with increasing or decreasing pH (Kidd et al., 2009; Marschner, 2005).

The quantity of root exudates produced depends also on the density and the composition of the substrate: it is higher, if the soil is more dense and poorer in nutrients. The plant species also influences it: for instance, in experimental systems higher numbers and higher concentrations of identified organic acids were found in the rhizosphere of birch compared to spruce. It should be mainly due to root exudation from the threefold higher root density in the birch rhizoboxes (Sandnes et al., 2005).

Under natural conditions, the quantification of organic acids and in general of root exudation is difficult due to binding of exudates to soil components, assimilation and the degradation by microorganisms (turnover rate) under non-sterile conditions; under sterile conditions the production is lower. Organic acids can also be produced by microbial activity, stimulated by the production of organic carbon and CO_2 from the roots. Further, microorganisms using the acids as carbon source may produce themselves some substances that have a similar effect on the metal mobility (Marschner, 2005); it is not possible to distinguish between root and microbial derived exudates. The mineralisation (degradation) of organic acids has been studied, and depends among others on the acid itself. The mineralisation rate decreases in following order: oxalate>citrate>malonate>shikimate>malate. This is important since not all acids have the same effect on minerals, shikimate for instance could not mobilise any phosphorus, but malonate does (Oburger et al., 2009).

Hence, most exudate studies have been carried out in sand or solution cultures that exclude microorganisms. However, the presence of both free-living and symbiotic organisms have been shown to affect root exudation both qualitatively and quantitatively (Grayston et al., 1996). *(See next section)*

Stress by lack of nutrients or metal toxicity leads to a changed behaviour of the plant concerning the secretion of substances through the roots.

2.2 Impact of nutrients

Since the secretion is driven by physiological needs of the plants, nutrients present in the soil have a major impact on exudation, usually enhancing the process, particularly with regard to the supply of N, P and K. Unlike their secretion to attract or sustain microorganisms, the secretion for the uptake of nutrients is irregular and rather occurring as pulses of substances released in locally high concentrations within a short period of time (Marschner, 2012).

The influence of organic acids on metal dissolution can be very important. Some authors stated that the efficiency in leaching was equivalent to the one of sulphuric acid, or even stronger (Köhler and Völsgen, 1998). Köhler and Völsgen (1998) studied leaching of copper minerals by different organic acids. Citric acid was the most efficient, but others, such as lactic acid or tartaric acid are efficient too, depending on the pH. At pH 2, there is even 100% Cu recovery. So, it is possible that the amount of metals accumulated inside the plant material exceed the initial quantity of the exchangeable metals in the soil, indicating a transformation from less bioavailable to more bioavailable forms (Tao et al., 2005). Similarly, for a set pH, 10-50 times more Mn is dissolved with organic acids than with a buffer of same pH (Marschner, 2005).

The influence of P-deficiency and P-availability is well known (López-Bucio et al., 2000; Oburger et al., 2009; Shen et al., 2002; Shen et al., 2005; Veneklaas et al., 2003; Weisskopf et al., 2008). Plants secrete higher amounts of acids (whose composition depends on the species) so that insoluble P (as Fe-, Al-, Ca-phosphate) becomes available for plants (Shen et al., 2002). If the local supply is sufficient, acid (or proton) release is inhibited (Shen et al., 2005). Plant species capable of releasing more acids were found to have a better supply in phosphorus (Zhou et al., 2009). In the case of pea, citrate, tartrate and acetate were the main acids. Malonate is exudated by legumes and non-leguminous plants, and it is also the main exudates from chickpea and non mycorrhized pine under P-deficiency (Oburger et al., 2009).

Organic acids increase the mobilisation of several metals such as Mn. Citric and malic acids form relatively stable chelates with Fe^{III} and Al, thereby increasing the solubility and rate of inorganic P (Pi) uptake. The capacity of dissolution of P is highly correlated with the number of OH- and COOH- functional groups and their position in the chain (high affinity to divalent and trivalent acids). Citrate und oxalate are therefore very efficient to dissolve metals, because they can form stable 5 or 6 membered ring structures with trivalent ions like Fe or Al. Furthermore, citrate has a high affinity to these metals, and has the highest P dissolving capacity among the tested organic acids. This method of phosphate acquisition is important for plants adapted to acid mineral soils with very low Pi availability (Grayston et al., 1996; López-Bucio et al., 2000; Oburger et al., 2009; Shen et al., 2002).

Arsenic in soil is associated with Fe-hydroxides, so that any process used by plants to mobilise Fe (siderophores or other ligands) will also lead to the release of As. Proton excretion and release of carboxylates (citric, malic, oxalic acid) increase P mobility in the soil, and due to its similarity to As, may as well mobilise As too (Kidd et al., 2009).

Organic acid are also reported to stimulate the nitrate uptake. Citrate for example can increase the activity of the nitrogenase which catalyses the reaction from N_2 to NH_3 (López-Bucio et al., 2000).

In alkaline soil and low availability of P and Fe, many dicotyledonous plants respond to the Fe stress by secretion of H^+ by the roots, reduction of Fe^{III} to Fe^{II}, production of root exudates, mainly malate and citrate. In the meantime, a decrease of the pH was observed. Phytosiderophores (monocotyledonous plants) are less efficient for Fe mobilisation.

The higher excretion can also be an answer to stress. Roots can indeed secrete different substances, to protect themselves against too high levels of trace elements. For example polygalacturonic acid, that fixes metals inside or outside of the root cells. Polychelators (glutathion derivate) bind on metals and get stored in vacuoles or on the cell membrane (Kabata-Pendias, 2001).

Al toxicity is recognizable by the characteristic inhibited root growth. The most common response to Al stress is complexation through organic acids, depending on the species there are different kinds dominating (Table) (Barceló and Poschenrieder, 2002); mostly found are citrate, malate (López-Bucio et al., 2000), also oxalate (Barceló and Poschenrieder, 2002). The efficiency for the detoxification of plants decreases in the following order: citrate>oxalate>malate. Another mechanism is the exudation of phenolic compounds like flavonoids by roots, which can bind heavy metals. These can enhance the Al-organic acid-complex stability. These phenomena are also observed for Zn-resistant plants (Barceló and Poschenrieder, 2002). An excess of Al can inhibit the uptake of other metals, like Ca, Mg, Zn and Mn.

The organic acids can cause mobilisation as well as immobilisation

2.3 Complexation by organic acids

On the other hand, metals can be bound by organic acids through complexation. The fact is that there can be a parallel mobilisation and immobilisation of metals by the same process, depending on the conditions. Organic acids are known to complex metals in a way comparable to EDTA (Díaz-Barrientos et al., 1999); these complexes are very stable and can enhance the availability of metals. They have also a buffer effect, that increases with the quantity of acid (Yuan et al., 2007).
Further, the effect depends also on the metals themselves. For instance, the mobilisation of Cd depends on organic acids produced by trees, on the example of mangrove (Haoliang et al., 2007). On the contrary, the kind of acid and quantity depends on the Cd present in the environment.
However, the detection of root exudates is difficult due to the short lifetime of them in the soil. Therefore, in a first part, concentrating on the metal mobility of soil, with a focus on REE, the effect of different leaching solutions is tested in order to describe and distinguish different metal mobilising effects of the studied system, in particular plant exudates.

2.4 Microbial influence – symbiotic microorganisms

[See also chapters 3&5]

Since root exudates, especially the easily decomposable low molecular weight ones are very attractive for microorganisms, a rich and diverse community is developing in the rhizosphere. Reciprocally, the presence of microorganisms is one factor which can lead to modifications in the quality and quantity of root exudates (Dakora and Phillips, 2002; Grayston et al., 1996).

The presence of microorganisms in the rhizosphere of plants increases root exudation. This stimulation of exudation occurs in the presence of free-living organisms, e.g. *Azospirillum* and *Azotobacter* in both herbaceous plants and trees. Trees in symbiotic association also exude more organic substances than non-mycorrhized plants. Mycorrhizal beeches exuded different organic acids from non-mycorrhized beeches. So, for example strigolactones (sesquiterpene lactones) are involved in chemical attraction of arbuscular mycorrhizal (AM) fungi, flavonoids play a role in early signalling of legume-rhizobia interactions (Badri et al., 2009). *Bacillus subtilis* is recruited by L-malic acid but not other *Bacillus* sp. Beneficial effects on plants by bacteria like *Pseudomonas*, *Burkholderia*, and fungi as *Trichoderma*, *Gliocladium* are well documented. AM fungi can interact with bacteria; indeed AM fungi's influence of the bacterial community is possible, as the bacteria's influence on fungal colonisation, root branching, among others by their antifungal properties. However, relations are more complex in nature and include several bacteria (endobacteria, pathogens), fungi, micro-fauna (i.e. nematodes). They are the result of the chemical interaction between the different organic compounds excreted by plant roots and the different microscopic actors of the soil, each interacting specifically with different compounds. Hence, each plant species has a different rhizosphere micro-flora in terms of abundance and physiological characteristics, which can be further modified by the properties of the soil, plant age and plant nutritional status (Marschner, 2012). However, the colonisation of roots is not limited to the root surface, but can occur further inside the root tissues, as in the apoplasm.

2.5 Other factors

The soil pH can be influenced by the N source of the plants: Ammonium, unlike nitrate make the zone around the roots become acidic (Marschner, 2005). According to (Shen and Yang, 2008; Tomlin et al., 1993) earthworms have a great influence on the mobilisation, spreading and fixation of heavy metals, since they play an essential role concerning the availability of Ca, Mg, K, N, C and P, and also increase the soil permeability. Further, microorganisms have also an influence. The study showed that soils amended with earthworms had greater amounts of metals in the EDTA and water extractable fraction. Microorganisms influence the availability by fixating the metals on their cell wall. The aim of that study was to choose an optimal combination of microbes, small terrestrial animals and plants to mobilise and remove metals from the soil.

Lack of oxygen (anoxia) enhances root exudation. Root exudation is affected both qualitatively and quantitatively by temperature, with increases and decreases in temperature increasing exudation (Grayston et al., 1996).

The influence of organic acids on sorption and desorption of metals has been studied, and one of the results was that the reaction depends on the metal (Cu or Cd) (Yuan et al., 2007). It appeared that some acids were better for some metals, in this case, citric acid was better for Cu mobilisation, oxalic acid better for Cd mobilisation, and other acids have no noticeable effect on them. Ion strength is also a factor influencing mobility (Shan et al., 2002). The binding to acids dropped with increasing pH between 2 and 4.5, but not over. Differences were noticed between different soils.

Table 4: Organic acids exudates: review over different experiments

Organic acid	Exudate from		described for	Remarks	Reference
Fumarate (Malate, Citrate)	Wheat			Very low concentration	(Weisskopf et al., 2008)
Fumarate Malate Citrate	Lupine (*Lupinus* sp.)			Concentration mg/g (in roots) 50-150 µg/g (rhizosphere)	(Veneklaas et al., 2003; Weisskopf et al., 2008)
Malonic acid	Chickpea				(Veneklaas et al., 2003)
formic, acetic, butyric, malic, lactic, fumaric, maleic, citric and l-tartaric acids	Mangrove		Cd		(Haoliang et al., 2007)
	Rice (*Oryza sativa*)		Pb		
Formic, Lactic, Butyric, Propionic*, Acetic acid* Oxalic, (Phtalic) Succinic*, Adipic* Malonic+, Malic acid+ Citric acid*	Birch (*Betula*)	Field-root window *Indoor rhizobox +Indoor microcosm	-	Concentration in the order of magnitude of µM	(Sandnes et al., 2005)
	Pine (*Pinus*)				(Sandnes et al., 2005)
Malic acid	Wavy hairgrass (*Deschampsia flexuosa)*		Al	more acid if Al	(Barceló and Poschenrieder, 2002)
Oxalic acid	Sheep's sorrel *(Rumex acetosella*)		Al		
Oxalic acid	*Viscaria vulgaris*		Al		
Citric acid	Heath bedstraw (*Galium saxatile*, *Galium harcynicum*)		Al		
Citric acid	Common Speedwell (*Veronica officinalis*)		Al		
Citric acid, Malic, Oxalic acid, Shikimic acid or Malonic acid	Simulation		P	0.5 mL for 5gDW soil, 35% moisture: 0,5 mM	
Citric acid, Malic acid	Rape (*Brassica napus*)		P		(López-Bucio et al., 2000)
Shikimate	Pine, Tomato, Wheat and Rice		P		(Oburger et al., 2009)
citrate, tartrate and acetate	Common bean (*Phaseolus vulgaris*)		P	Under P-deficiency	(Shen et al., 2002)
Citric acid	Lupine (*Lupinus albus*)		P		(Shen et al., 2005)
	faba bean, soybean and maize				(Zhou et al., 2009)
Malonate	Legumes and non-leguminous plants: chickpea, pine (non-mycorrhizal)		under P-deficiency		(Oburger et al., 2009)

Tricarboxylic acids, dicarboxylic acids, monocarboxylic acids

3 MICROBIAL INFLUENCE ON METAL MOBILITY

Many different factors influence the mobilisation and immobilisation of metals in soil and water. pH and oxygen content, soil composition allowing cation exchange or precipitation, and organic fraction of the soil are some of the most important ones. They can be completed and modified by plant growth, through exudates. Additionally, microorganisms can change these physico-chemical parameters, and provoke themselves further reactions. The investigation of the speciation of metals and its changes are essential for risk assessment, toxicity studies or remediation techniques. Even though fungi are capable of changing metal solubility by dissolving immobile elements (Sayer et al., 1995), the focus of this study lays on bacteria. Their role is especially important at very high metal concentrations, because of the higher adaptation capacity of microbes compared to higher organisms.

Despite the fact that many locations, especially metal contaminated areas, are generally not beneficial for life, a wide range of different microorganisms are found to live under extreme conditions (Xiao et al., 2009). Obviously, there are many parameters influencing the action of microorganisms. As before, the pH plays the decisive role for bioleaching processes, but temperature, initial bacterial concentration, oxygen conditions, and heavy metal concentration and composition are also important parameters. Furthermore particle size and nutrients also influence the process i.e. C-, O-, N-, S-sources should be present.

Microorganisms can interact directly with the trace metals to reduce their toxicity and/or influence their bioavailability: strong acids such as H_2SO_4 (i.e. *Thiobacillus*) lead to metal dissolution (Abhilash et al., 2009; Natarajan, 2008; Pathak et al., 2009); organic acids chelate metals to form metal-organic molecules; ammonia or organic bases precipitate metal hydroxide. Further, other processes such as extracellular metal precipitation (e.g. with sulphate reducing bacteria), production of extracellular polysaccharides that can chelate the heavy metals, fixation of Fe and Mn on the cell surface in the form of hydroxides or some other insoluble metal salts change the availability. Last but not least, biotransformation via bio-methylation, volatilisation, oxidation or reduction is possible.

The transformations are different depending on the metals, may it be via enzymatic catalysis, or indirect through acidification, since some are essential or useful for the microorganisms and directly used in the metabolism as energy source or as coenzymes, and some are toxic in any concentration.

The reactions taking place are very often some that occur under abiotic conditions, but they are faster, and also more complex, since microorganisms themselves influence their environment by changing pH, precipitation, oxygen consumption, formation of new substances, and succession of population can occur.

As a summary, complex processes and interactions take place between chemistry, geology and microbiology. To understand them better and get an overview, we can consider two processes used to precipitate or to dissolve heavy metals.

3.1 Bioleaching – dissolving metals from rocks and ore

Bioleaching leads to a reduced metal content in the solid phase. It is important to characterise the heavy metal content and distribution in the original rocks as well as the rock itself, since many properties especially regarding the heavy metal mobility depend on that. The metal fraction associated with sulphides depends on the metabolism of heterotrophic sulphate reducing bacteria under anaerobic conditions. On the other side, Fe/Mn oxides are formed under totally different conditions, as for example oxidation of sediments in water or re-suspension (Beolchini et al., 2008). The key point of these processes is the use of the concerned minerals by microorganisms for energy production, thus leading to redox reactions and possibly to high turnover rates.

Conditions for acid production (Natarajan, 2008)

- Presence of sulphide minerals
- Enough water (water or humid atmosphere)
- Presence of an oxidant (mostly O_2), in the water or as gas
- pH has to be acceptable for the organisms
- Temperature
- Chemical activity of Fe^{II}
- Surface of sulphide minerals should be free
- Chemical activation energy to start acid production
- Biological activity

Many indications lead to the conclusion that just a small part of the sulphides are mobilised by chemical reactions, compared with the amount mobilised by biochemical processes (Lors et al., 2004). It could be shown that Zn and Cd amounts are lower without influence of living organisms. Without microorganisms, there is no effect of an initial acidification possibly due to the buffer capacity of the rocks. Acid is produced from sulphide minerals as pyrite, sphalerite, galena, arsenopyrite, cobaltite, bornite, pyrrothite (Natarajan, 2008).

The most common microorganisms causing acidification and used for leaching are *Acidithiobacillus ferrooxidans* (Fe and S oxidiser), *Leptospirillum ferrooxidans* (Fe oxidiser), *Acidithiobacillus thiooxidans* (S–oxidiser, cannot oxidise pyrite), *Thiobacillus thioparus* (sulphide-oxidiser, neutrophilic), sulphate reducing bacteria (metal precipitation as sulphide). Further used microorganisms according to literature: *Sulfolobus* sp. as well as thermophile bacteria like *Sulfobacillus thermosulfidoxidans* and *Acidianus brierleyi* (Abhilash et al., 2009; Hallberg and Johnson, 2005; Natarajan, 2008).

Table 5: Summary about the conditions necessary for bioleaching

Organisms	Remarks	References
Thiobacillus ferrooxidans and *Thiobacillus thiooxidans*,	7 is optimal, 4 is also acceptable	(Sreekrishnan and Tyagi, 1995)
Leptospirillum ferrooxidans + *Thiobacillus organoparus*	leaching is only possible by combining two species	(Köhler and Völsgen, 1998)
Acidithiobacillus ferrooxidans	leaching worked without initial acidification, sometimes even better if soil pH was initially between 5 and 7	(Kumar and Nagendran, 2007)
Acidithiobacillus ferrooxidans	Bioleaching of U Initial pH 1.7 influence of pH and particle size was observed	(Abhilash et al., 2009)
A. thiooxidans, A. ferrooxidans. + heterotrophic bacteria (*P. fluorescens, B. cereus, B. thuringiensis)*	Sequential bioleaching process (heterotrophic strains first, then autotrophic) The combination of both strains lead to a synergetic effect, and it did not depend which species was dominant for inoculation	(Krasnodebska-Ostrega et al., 2009; Lors et al., 2004)

Further reactions and transformations can take place with other elements, indirectly caused by pyrite-like minerals. Therefore, because of the galvanic effect, arsenopyrite could be dissolved in the presence of *A. ferrooxidans*, if also pyrite or chalcopyrite were present, because these are nobler. Furthermore, Iron-III-Sulphate can be precipitate as jarosite if potassium or ammonium ions are present (Köhler and Völsgen, 1998). Jarosite is produced by *A. ferrooxidans* and can absorb metals like As^{III} (Natarajan, 2008).

3.1.1 Description of the process

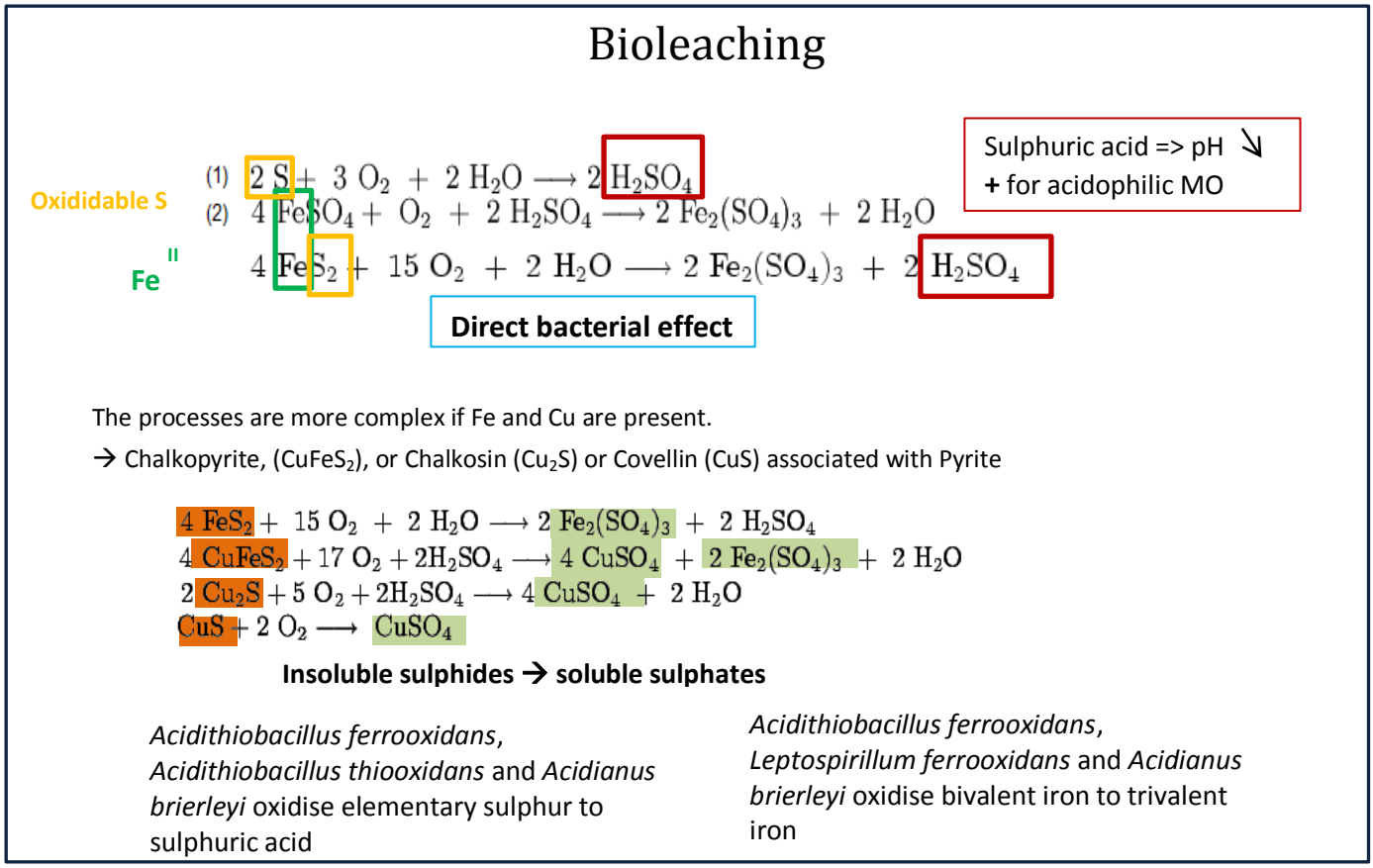


Figure 5: Main microbial reactions involved in the process of bioleaching

The dissolving is a mixed effect of, on one hand direct action of bacteria that are adsorbed on the rock, and an indirect effect of some produced substances (i.e. Fe^{3+} ion). Precipitation of Fe^{III} increases with the temperature, which means also that if the temperature is lower, more metals get solubilised (Halinen et al., 2009a; Halinen et al., 2009b). The ventilation appears to be an essential parameter for bioleaching, if the process should remain in the mesophilic zone (Mousavi et al., 2006).

The dominant microbial species depends on the conditions. Most of the time the leaching with combinations of strains appeared to be more efficient than with a single culture (like *T. ferrooxidans* + *T. thiooxidans*). Also in other studies the importance of several-step-procedures showed to be a better way. For instance, a cascade of different treatments, partly with adsorption on organic matter (peat), and influence of sulphate reducing bacteria has been described as a bioremediation method (Champagne et al., 2005). Also several authors (Krasnodebska-Ostrega et al., 2009; Lors et al., 2004; Rehman et al., 2009) used a sequential bioleaching process involving heterotrophic bacteria. The Fe^{II}-^{III} regeneration appears to be the limiting step in the procedure of bioleaching, therefore cooperation between use heterotrophic bacteria to consume iron oxides with Fe/S-oxidisers is an important point. The sample was first treated with heterotrophic bacteria (*P. fluorescens, B. cereus, B. thuringiensis*), and then leached with autotrophic strains.

This is therefore a reason to be careful while storing heaps consisting of such reactive material, which can be leached very easily by rain and consequently contaminate the area (Schippers and Bosecker, 2002). The material should be washed with water before storage. Sediments are a problem as well, since they store all kind of toxic substances, like heavy metals or organic contaminants, and can form in that way a secondary source of pollution.

3.2 Precipitation of metals by microorganisms

Some microorganisms are able to influence the availability of metals by fixing them on their cell wall (Beveridge and Fyfe, 1985; Pollmann et al., 2006). Moreover, microorganisms catalyse chemical reactions, like *Streptomyces thermocarboxydus* that transforms Cr^{VI} into Cr^{III}, which decreases its toxicity (Shen and Yang, 2008). If the reasons of the precipitation reactions are not always fully understood, it is possible that detoxification mechanisms play a role. Microorganisms are also involved in diverse processes of bio-mineralisation, i.e. the formation of minerals catalysed by biochemical reactions taking place in the cells or in their vicinity (Tebo et al., 2005). The formed minerals, for instance Mn-oxides, can also scavenge other toxic elements as As, Pb or Ce (Miyata et al., 2007).

3.3 Dissolving of nutrients and interaction with the flora

Some microbes are known to produce a broad range of substances in order to get access to nutrients, which occur as insoluble minerals in the soil. This leads to a mobilisation of essential elements as Fe or P through active action of microorganisms. These can be also used by plants if the microbes happen to be located in their rhizosphere. In contrast to the bioleaching processes described before, these processes aim to maintain a level of the element in the cells corresponding to the physiological needs. So, for example some fungi are producing acidic compounds to mobilise nutrients as phosphate (Cunningham and Kuiack,

1992). Phosphate solubilisation is also known to be achieved by bacteria, which acidify their environment (Nahas, 1996).

The plants and their metal uptake can be influenced by many other factors, since they form an own microcosm in their rhizosphere. So, many organisms living around the plants influence their growth and mineral uptake. If more is known about these, they can be used, if chosen well, to enhance phytoremediation procedures, especially on soil poor in nutrients. The most important mutualisms exist between plants and mycorrhizae or rhizobacteria (Badri et al., 2009). Arbuscular Mycorrhizal (AM) fungi can interact with bacteria by influencing the bacterial community, while bacteria can influence the colonisation, root branching and can show antifungal properties. Nevertheless, relations are more complex in nature and include several bacteria (endobacteria, pathogens), fungi, micro-fauna (i.e. nematodes). They are the result of the chemical interaction between the different organic compounds excreted by plant roots and the different microscopic actors of the soil, each interacting specifically with different compounds. Hence, each plant species possesses a different rhizosphere micro-flora in terms of abundance and physiological characteristics, which can be further modified by the properties of the soil, plant age and plant nutritional status (Marschner, 2012).

4 Bioremediation: application of natural processes

The knowledge gained about the parameters influencing mobilisation and immobilisation of metals is essential in order to use it among other things to develop remediation strategies for metal contaminated soils. In other words, remediation strategies consist in either increasing the solubility of the targeted elements in order to remove them from a solid environment, or decrease it in order to precipitate them from solutions, or to stabilise them in the soil matrix and avoid spreading.

This can for instance be achieved by a combination of mechanical procedures and chemical amendments. Strategies that are usually applied to remediate such sites include on one hand the removal and relocation of either the soil itself, (Eißmann, 1997) especially if the quantity of concerned substrate is small, to allow a treatment or a storage elsewhere; soil washing, i.e. the removal of metals by leaching with acids and/or chelators (Abumaizar and Smith, 1999) or on the other hand metal stabilisation by surrounding the tales with an appropriate barrier (clay, composite or capillary barrier) or by using soil amendments. Usually AMD is treated with chemical oxidation, increasing the pH with addition of clay or sodium carbonate, or oxidation of sulphides. These methods are time-consuming and not very efficient, and the resulting solution needs further treatment afterwards. Chemical remediation strategies for U in particular consist most often of injection of Fenton reagent into the soil, provoking an unspecific oxidation reaction leading to the dissolution of metals (Šarić and Lucchini, 2007). Those can be removed by pumping the solution. In the case of the Ronneburg site, the most contaminated waste rock material was replaced into the underground mining site and open pit mine; the groundwater level was allowed to rise again to restore anoxic conditions and so prevent further oxidation processes and AMD formation. Carlsson and Büchel (2005) described elevated residual contamination levels in the underlying sediments, which lead to

the creation of a test site to study the possibilities of alternative remediation strategies for diffuse contaminated sites.
Stabilisation is recommended when contamination is quite high on a large area, especially in the case of multi-element contamination. Typical soil amendments include iron oxides, liming agents, apatites, Fe-, Al or Mn-hydroxides, zero-valent iron grit, zeolites, organic matter, red muds and clays, phosphates, industrial by-products (cycloning ashes) (Vangronsveld et al., 2009) The aim is to reduce the solubility by forming of insoluble trace element species, and favour absorption.
However, conventional clean-up technologies are costly and feasible only for small but heavily polluted sites where fast and complete decontamination is required. Further, some of those methods, such as soil washing, can cause contamination of water ways through seepage waters, a negative impact on biological activity, soil structure and fertility, and generate important engineering costs (Pulford and Watson, 2003). Moreover, disturbing the soil structure can lead to higher metal out-washing (Neagoe et al., 2009); this aspect should not be forgotten when moving soil material. Therefore, sustainable *in situ* techniques for remediation of contaminated sites, as bioremediation, need to be applied and improved. In particular, for vast areas with a relatively low contamination level, located in the upper soil layer, or for in-situ remediation of AMD, the use of biota is of interest. Bioremediation using bacteria, algae, fungi, plants, or combinations of those, has been studied for many different types of contaminations, and successfully applied in numerous cases, ranging from metal precipitation on cell walls to uptake by plants (Baker and Herson, 1994; Cohen, 2006).

4.1 Bioremediation strategies involving microorganisms or plants

In some cases, microbes are used as a main remediation agent. For example, Cohen (2006) proposed „passive mine drainage treatment system“, with the aim to use biogeochemical processes to precipitate heavy metals as sulphides near to AMD sources, to concentrate or immobilise them, as well as to increase the pH. This reaction is catalysed by sulphate reducing bacteria like *Desulfovibrio* or *Desulfotomaculum*; besides there are also Mn-oxidisers and Fe-oxidisers involved which precipitate metal oxides or co-precipitate them as hydroxides.
More commonly, plants are the actors of bioremediation. Phytostabilisation consists in establishing a vegetation cover and inactivating toxic metals *in situ*, by combining the effect of metal tolerant vegetation and metal-immobilising soil amendments in order to minimise the mobility and thus the toxicity of metals. An expected characteristic of the used plants should be the capacity to retain the contaminants in the roots or rhizosphere (excluder mechanism) to limit the spreading through the natural food chains. This option is recommended when contamination is quite high on a large area, especially in the case of mixed (multi-element) contamination. The aim is to reduce the solubility of the trace elements by forming of insoluble trace element species, and favour adsorption, therefore soils rich in clay minerals or organic matter offer better starting conditions.

On the other hand, phytoextraction is a strategy that aims to remove contaminants through uptake and accumulation in plants, and is followed by plant biomass harvest and its treatment. This technique is best suitable for diffusely, low polluted areas, where contaminants occur on the surface, and is successfully used on many sites (Mirgorodsky et al., 2010; Pulford and

Watson, 2003; Raskin et al., 1997; Vangronsveld et al., 2009). This approach is of interest particularly if the mobility or bioavailability of the toxic elements is high. Since the biomass production is a key factor, fast growing plants are chosen, which further produce high biomass, as for example poplar or willow, even though the choice remains dependent on the kind of contaminant present. One concern is often the bioavailability of contaminants, which should often be enhanced by treatment of chelating agents (EDTA), although they represent themselves an environmental concern (Kidd et al., 2009; Vangronsveld et al., 2009). The uptake can be otherwise enhanced by the supply of citric acid or by increasing the transpiration rate (Keith et al., 2007). Another suggestion for phytoremediation is to grow plants who produce sufficiently stable natural chelators to improve bioavailability and so the extraction, as a succession after excluder plants.

4.2 Plant-microorganism partnerships for an improved bioremediation strategy and a modified metal uptake

Even if phytoremediation presents several ecological and economic advantages, its low efficiency or limited application is one of the weak points of this strategy. Therefore, it is clear that improving phytoremediation for heavy metal contaminated soils is necessary. The plants and their metal uptake can be influenced by many other factors, since they form an own microcosm in their rhizosphere. So, many organisms living around the plants influence their growth and mineral uptake. Mycorrhizal colonisation is an important factor in phytostabilisation, while its role in phytoextraction is more ambiguous. By the choice of adapted microorganisms, one or the other strategy can be enhanced. The increase in the solubility of metals in the soil can be linked to the properties of bacteria able to produce siderophores or other metal-chelating substances (Aouad et al., 2006; Sheng et al., 2008; Whiting et al., 2001).

Microorganisms have been used for microbial bioremediation of metals and also suggested as inocula to enhance re-vegetation of contaminated sites, phytoremediation, as reviewed by several authors (Beolchini et al., 2008; Glick, 2010; Kidd et al., 2009; Rajkumar et al., 2009; Shetty et al., 1994; Weyens et al., 2009b; Zhuang et al., 2007). Beneficial arbuscular mycorrhiza, yeasts or various soil and rhizosphere bacteria, generally termed PGPR (Plant Growth Promoting Rhizobacteria) have been investigated. In the present study, the focus is put on endophytic bacteria.

Endophytic bacteria can improve plant growth using different processes. Beside the effects on growth due to the *production of plant growth regulators*, phytohormones such as auxins, cytokinins and gibberellins, endophytes can cause the inhibition of the production of the stress hormone ethylene (by *ACC-deaminase activity*) or prevent the growth or activity of plant pathogens (Badri et al., 2009; Weyens et al., 2009a). Plant associated bacteria can furthermore improve plant nutrition by *fixing N_2 and solubilising macronutrients as poorly soluble (P)-minerals*, thus delivering nutrients normally unavailable for plants and fulfil so the role of natural fertilisers (Badri et al., 2009; Weyens et al., 2009a; Yanni et al., 1997).

Additionally, microorganisms have an influence on the uptake of metals by plants. For example, plants provided with bacteria had more leaves, less roots and higher metal content; the more cells inoculated, the more uptake (Aouad et al., 2006). *Pseudomonas aeruginosa*

forms biofilms and allows complexation of REE; further, it is able to extract Fe and Mg. These properties are of interest for the use of these mutualisms and metal tolerance properties for remediation purposes.

The main aspect of metal uptake is driven by the *production of siderophores*, which can make Iron(III)-hydroxide available for reduction to Fe^{II}; this is crucial especially in alkaline (calcareous) soils with decreased Fe availability (Kidd et al., 2009; Weyens et al., 2009a). Siderophores take up Fe^{III}, which is then reduced intra-cellularly. This process is repressed if there is sufficient Fe supply, but is stimulated by other metals. Siderophores can both enhance and prevent uptake of metals by plants, depending on the present metals. They bind free metals, and so changing the available metal concentration and protecting the plants from metal stress. Metals can inhibit auxin production, but siderophore presence can alleviate this. Siderophores are generally only for Fe, but also Al and other metals as Cd, Ni or micronutrients such as Mn, Co, Zn can be transported in some cases (Dimkpa, 2009; Kidd et al., 2009).

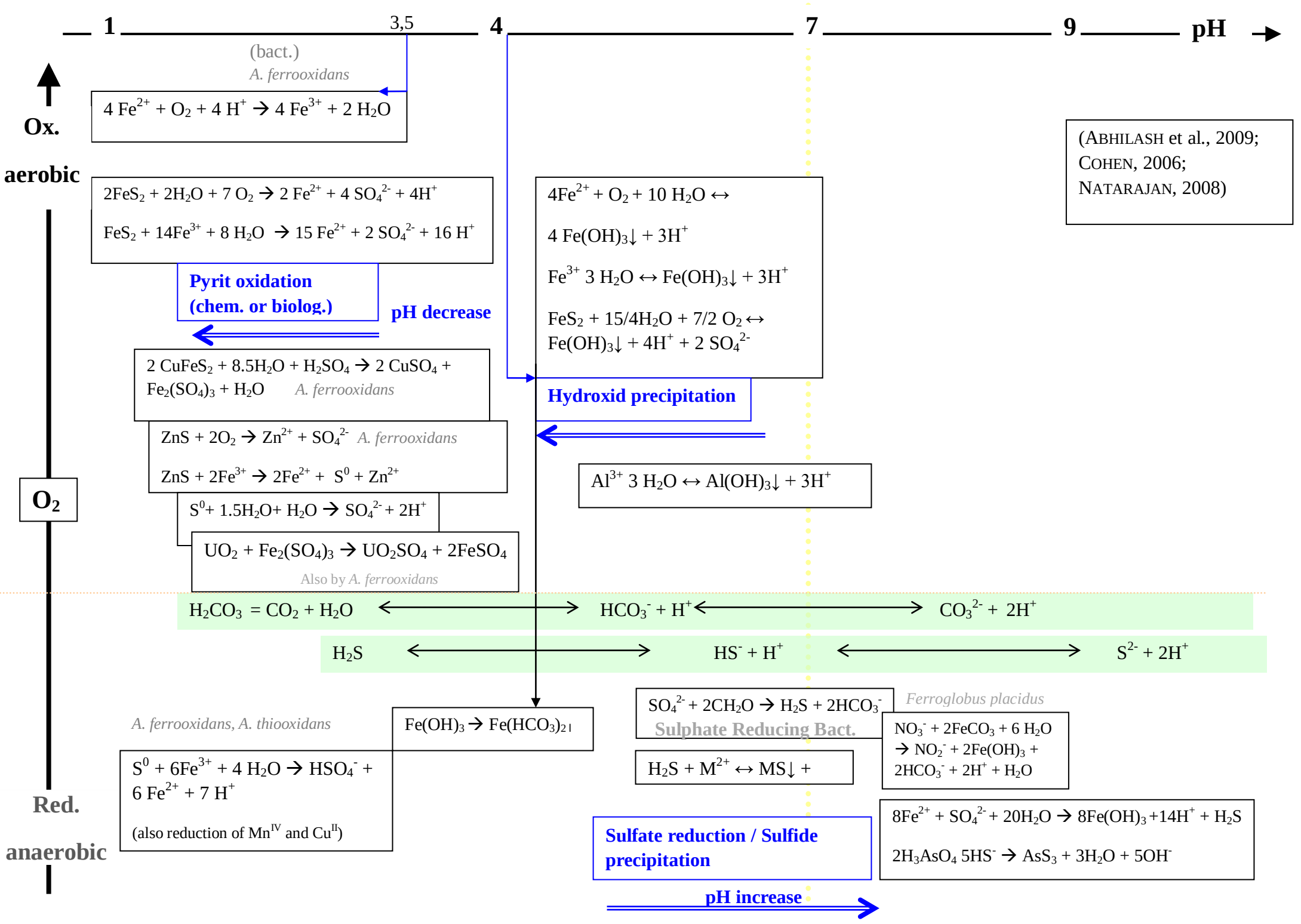
The increase in the solubility of other metals in the soil can be also linked to the properties of the bacteria, able to produce siderophores or other metal-chelating substances. Metallophores are for instance produced by strains of *Pseudomonas* and *Enterobacter* (Whiting et al., 2001). *P. aeruginosa* can also allow complexation of REE; further it is able to extract Fe and Mg (Aouad et al., 2006). Sheng et al. (2008) reported the effect of some bacteria on the solubilisation of Pb in soil and water by *P. fluorescens* G10 and *Microbacterium sp*. G16.

Indeed, rhizosphere microbes play an important role for the soluble metal pool in soils by altering the solubility, availability and transport of trace elements and nutrients by reducing soil pH, secretion of chelators and siderophores or redox changes but can also reduce the extent of contaminant uptake or translocation to aerial parts of plants by decreasing the bioavailability of metals. It has been shown that different microbial inocula can enhance metal uptake and plant growth at the same time: mycorrhizal fungi and followed by yeast treatment were shown to be highly effective in enhancement of uptake of Zn, Cu, and Cd by corn and sunflower plants (Usman and Mohamed, 2009). The increase was even stronger than in case EDTA was added to the soil, except for Pb, suggesting that microbes can be a good alternative to not-degradable artificial chelators. The influence of the bacteria on the metal uptake by plants is discussed by Rajkumar et al. (2009).

In order to select microorganisms that can promote plant growth and metal uptake, it is important that they can survive in the specific niche. In other words, those bacteria should be able to resist the environmental constraints of the rhizosphere. Therefore, in the case of an application in a metal contaminated soil, their resistance to the occurring metal concentrations (and multiple metal resistance) is of high importance. Microorganisms dispose of different mechanisms for protection against heavy metals: intra- or extracellular sequestration by chelating compounds, sorption, active efflux transport or enzymatic detoxification, and many of these mechanisms were discussed already for strains isolated from our study area (Schmidt et al., 2009; Schmidt et al., 2005). Microorganisms with a natural good resistance to metals will be more able to help the plants to grow in a difficult environment. Chaudhary et al. (2004) observed that inoculated *Rhizobium* sp. into pea or Egyptian clover showed reduced nodulation activity when the host was grown on heavy metal contaminated soil, but that other

native endophytes did not seem affected by the pollution. Nevertheless, it is interesting to notice that some strains, which are not resistant, can grow near resistant ones, due to the fact that the latter ones produce substances that protect the first ones against heavy metals. The population found at the site is better adapted to the specific conditions, therefore it is important to select autochthonous organisms. It is particularly true for contaminants that need to be degraded, as organics, but also for metals, both affecting the physiology and ecology of microorganisms. Abou-Shanab et al. 2003a, 2006 in (Kidd et al., 2009) demonstrated that the bacterial-induced enhancing effect on metal extraction effect was dependent upon the metal concentration of soils, emphasising the need for a site-specific evaluation. To improve the efficiency of phytoremediation of toxic metal-contaminated soils, one of the suggested options is to equip plant-associated bacteria with pathways for the synthesis of natural metal chelators, such as citric acid, to increase metal availability for plant uptake or, alternatively, with metal sequestration systems to reduce phytotoxicity and increase metal translocation to aerial plant parts. However, for organic contaminants, it has already been shown that bacteria can be introduced as vectors into the plant ecosystem and that this will result in natural horizontal gene transfer to the endogenous endophytic population (Weyens et al., 2009a).

Further understanding of how plant roots modify locally the chemical properties of the soil, leading to an enhanced metal mobility and availability for plant uptake will enable greater plant metal yields, one of the current limitations of phytoextraction processes. Rhizosphere processes are still poorly understood in field conditions.


1
3,5
4
7
9
pH
Ox.
aerobic
O2
Red.
anaerobic
(bact.)
A. ferrooxidans
4 Fe2+ + O2 + 4 H+ → 4 Fe3+ + 2 H2O
(ABHILASH et al., 2009; COHEN, 2006; NATARAJAN, 2008)
2FeS2 + 2H2O + 7 O2 → 2 Fe2+ + 4 SO42- + 4H+
FeS2 + 14Fe3+ + 8 H2O → 15 Fe2+ + 2 SO42- + 16 H+
Pyrit oxidation (chem. or biolog.)
pH decrease
4Fe2+ + O2 + 10 H2O ↔
4 Fe(OH)3↓ + 3H+
Fe3+ 3 H2O ↔ Fe(OH)3↓ + 3H+
FeS2 + 15/4H2O + 7/2 O2 ↔ Fe(OH)3↓ + 4H+ + 2 SO42-
Hydroxid precipitation
2 CuFeS2 + 8.5H2O + H2SO4 → 2 CuSO4 + Fe2(SO4)3 + H2O A. ferrooxidans
ZnS + 2O2 → Zn2+ + SO42- A. ferrooxidans
ZnS + 2Fe3+ → 2Fe2+ + S0 + Zn2+
S0+ 1.5H2O+ H2O → SO42- + 2H+
UO2 + Fe2(SO4)3 → UO2SO4 + 2FeSO4
Also by A. ferrooxidans
Al3+ 3 H2O ↔ Al(OH)3↓ + 3H+
H2CO3 = CO2 + H2O ↔ HCO3- + H+ ↔ CO32- + 2H+
H2S ↔ HS- + H+ ↔ S2- + 2H+
A. ferrooxidans, A. thiooxidans
Fe(OH)3 → Fe(HCO3)2↓
SO42- + 2CH2O → H2S + 2HCO3-
Sulphate Reducing Bact.
Ferroglobus placidus
NO3- + 2FeCO3 + 6 H2O → NO2- + 2Fe(OH)3 + 2HCO3- + 2H+ + H2O
S0 + 6Fe3+ + 4 H2O → HSO4- + 6 Fe2+ + 7 H+
(also reduction of MnIV and CuII)
H2S + M2+ ↔ MS↓ +
8Fe2+ + SO42- + 20H2O → 8Fe(OH)3 +14H+ + H2S
2H3AsO4 5HS- → AsS3 + 3H2O + 5OH-
Sulfate reduction / Sulfide precipitation
pH increase

REFERENCES

- Abhilash, Singh, S., Mehta, K.D., Kumar, V., Pandey, B.D., Pandey, V.M., 2009. Dissolution of uranium from silicate-apatite ore by *Acidithiobacillus ferrooxidans*. Hydrometallurgy 95, 70-75.
- Abumaizar, R.J., Smith, E.H., 1999. Heavy metal contaminants removal by soil washing. J. Hazard. Mater. B70, 71-86.
- Allen, H.E., Janssen, C.R., 2006. Incorporating bioavailability into criteria for metals, in: Twadowska, I. (Ed.), Soil and Water Pollution Monitoring, Protetion and Remediation, pp. 93-105.
- Aouad, G., Stille, P., Crovisier, J.-L., Geoffroy, V.A., Meyer, J.-M., Lahd-Gaegea, M., 2006. Influence of bacteria on lathanide and actinide transfer from specific soil components (humus, soil minerals and vitrified municipal waste incinerator bottom ash) to corn plants: Sr-Nd isotope evidence. Sci. Total Environ. 370, 545-551.
- Badri, D.V., Weir, T.L., van der Lelie, D., Vivanco, J.M., 2009. Rhizosphere chemical dialogues: plant-microbe interactions. Curr.Opin. Biotech. 20, 642-650.
- Baker, K.H., Herson, D.S., 1994. Bioremediation.
- Barceló, J., Poschenrieder, C., 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ. Exp. Bot. 48, 75-92.
- Beolchini, F., Dell'Anno, A., De Propris, L., Ubaldini, S., Cerrone, F., Danovaro, R., 2008. Auto- and heterotrophic acidophilic bacteria enhance the bioremediation efficiency of sediments contaminated by heavy metals. Chemosphere 74, 1321-1326.
- Beveridge, T.J., Fyfe, W.S., 1985. Metal fixation by bacterial cell walls. Can. J Earth Sci. 22, 1893-1898.
- Blume, H.-P., Brümmer, G., 1991. Prediction of heavy metal behaviour in soil by means of simple field tests. Ecotox. Eviron. Safe. 22, 164-174.
- Bradl, H.B., 2004. Adsorption of heavy metal ions on soils and soils constituents. J. Colloid Interf. Sci. 277, 1-18.
- Carlsson, E., Büchel, G., 2005. Screening of residual contamination at a former uranium heap leaching site, Thuringia, Germany. Chem. Erde - Geochem. 65, 75-95.
- Champagne, P., Van Geel, P., Parker, W., 2005. A bench-scale assessment of a combined passive system to reduce concentrations of metals and sulphate in acid mine drainage. Mine Water. Env 24, 124-433.
- Chaudhary, P., Dudeja, S.S., Kapoor, K.K., 2004. Effectivity of host-*Rhizobium leguminosarum* symbiosis in soils receiving sewage water containing heavy metals. Microbiol. Res. 159, 121-127.
- Clarkson, T.W., 1993. Molecular and ionic mimicry of toxic metals Annu. Rev. Pharmacol. Toxicol. 32, 545-571.
- Cohen, R.R.H., 2006. Use of microbes for cost reduction of metal removal from metals and mining industry waste streams. J. Clean. Prod. 14, 1146-1157.
- Cui, Y., Dong, Y., Li, H., Wang, Q., 2004. Effect of elemental sulphur on solubility of soil heavy metals and their uptake by maize. Environ. Int. 30, 323-328.
- Cunningham, J.E., Kuiack, C., 1992. Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. Appl. Environ. Microb. 58, 1451-1458.
- Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245, 35-47.
- Díaz-Barrientos, E., Madrid, L., Cardo, I., 1999. Effect of flood with mine wastes on metal extractability of some soils of the Guardiamar river basin (SW Spain). Sci. Total Environ. 242, 149-165.
- Dimkpa, C.O., 2009. Microbial Siderophores in Rhizosphere Interactions in Heavy Metal-containing Environments, Biologisch-Pharmazeutische Fakultät. Friedrich-Schiller University, Jena, p. 135.
- Doelsch, E., Moussard, G., Saint Macary, H., 2008. Fractionation of tropical soilborne heavy metals - Comparison of two sequential extraction procedures. Geoderma 143, 168-179.

- Eißmann, L., 1997. Das quartäre Eiszeitalter in Sachsen und Nordostthüringen: Landschaftswandel am Südrand des skandinavischen Vereisungsgebietes, Altenburger Naturwissenschaftliche Forschungen, Leipzig.
- Fritzsche, A., Rennert, T., Totsche, K.U., 2011. Arsenic strongly associates with ferrihydrite colloids formed in a soil effluent Environ. Pollut. 159, 1398-1405.
- Glick, B.R., 2010. Using soil bacteria to facilitate phytoremediation. Biotechnol. Adv. 28, 367-374.
- Grayston, S.J., Vaughan, D., Jones, D., 1996. Rhizosphere carbon flow in tees, in comparison with annual plants: the imporance of root exudation and its impacts on microbial activity and nutrient availability. Appl. Soil Ecol. 5, 29-56.
- Halinen, A.-K., Rahunen, N., Kaksonen, A.H., Phuhakka, J.A., 2009a. Heap bioleaching of a complex sulfide ore Part 2: Effect of temperature on base metal extraction and bacterial compositions. Hydrometallurgy 98, 101-107.
- Halinen, A.-K., Rahunen, N., Kaksonen, A.H., Puhakka, J.A., 2009b. Heap bioleaching of a complex ore Part 1: Effect of pH on metal extraction and microbial composition in pH controlled columns. Hydrometallurgy 98, 92-100.
- Hallberg, K.B., Johnson, D.B., 2005. Mine Water Microbiology. Mine Water. Env 24, 60-64.
- Haoliang, L., Chongling, Y., Jingchun, L., 2007. Low-molecular-weight organic acids exuded by Mangrove (*Kandelia candel* (L.) Druce) roots and their effect on cadmium species change in the rhizosphere. Environ. Exp. Bot. 61, 159-166.
- Hargitai, L., 1989. The role of humus status of soils in binding toxic elements and compouds. Sci. Total Environ. 81/82, 643-651.
- Harrison, R.M., 1999. Understanding our environment - An introduction to environmental chemistry and pollution, 3rd ed. The Royal Society of Chemistry, Cambridge, UK, p. 445.
- Hofmann, T., 2004. Die Welt der vernachlässigten Dimensionen - Kolloide. Chem. Unserer Zeit 38, 24 – 35.
- Hong, K.-J., Tokunaga, S., Kajiuchi, T., 2002. Evaluation of remediation process with plant-derived biosurfactant for recovery of heavy metals from contaminated soils. Chemosphere 49, 379-387.
- Incorvia Mattina, M., Lannucci-Berger, W., Musante, C., White, J.C., 2003. Concurrent plant uptake of heavy metals and persistent organic pollutants from soil. Environ. Pollut. 124, 375-378.
- Kabata-Pendias, A., 2001. Trace Elements in Soil and Plants, 3rd Edition ed.
- Karim, M.A., Khan, L.I., 2001. Removal of heavy metals from sandy soil using CEHIXM process. J. Hazard. Mater. 81, 83-102.
- Keith, S.I., Faroon, O.M., Fowler, B.A., 2007. Uranium, in: Nordberg, G.F., Fowler, B.A., Nordberg, M., Friberg, L.T. (Eds.), Handbook on the Toxicology of Metals, 3rd Edition ed. Academic Press, Burlington, San Diego, USA.
- Khan, S., Khan, N.N., Iqbal, N., 1991. Studies on the effect of some organic pollutants on the heavy metal transport in an indian soil. Environ. Pollut. 70, 109-115.
- Kidd, P., Juan, B., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monteroso, C., 2009. Trace element behaviour at the root-soil interface: implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.
- Köhler, M., Völsgen, F., 1998. Geomikrobiologie - Grundlagen und Anwendungen, Weinheim.
- Krasnodebska-Ostrega, B., Paldyna, J., Kowalska, J., Jedynak, L., Golimowski, J., 2009. Fractionation study in bioleached metallurgy wastes using six-step sequential extraction. J. Hazard. Mater. 167, 128-135.
- Kumar, R.N., Nagendran, R., 2007. Influence of initial pH on bioleaching of heavy metals from contaminated soil employing indigenous *Acidithiobacillus thiooxidans* Chemosphere 66, 1775-1781.
- Lewandowski J., L.s., Koß V., 1997. Schadstoffe im Boden - Eine Einführung in Analytik und Bewertung, in: Springer (Ed.).
- Lin, Q., Xu, C.Y., He, Y.F., Tian, G.M., 2004. Root-induced changes of lead availability in the rhizosphere of *Oryza sativa* L. Agr. Ecosyst. Environ. 104, 605-613.

- López-Bucio, J., Nieto-Jacobo, M.F., Ramírez-Rodríguez, V., Herrera-Estrella, L.R., 2000. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci. 160, 1-13.
- Lors, C., Tiffreau, C., Laboudigue, A., 2004. Effects of bacterial activities on the release of heavy metals from contaminated dredged sediments. Chemosphere 56, 619-630.
- Ma, Y.B., Uren, N.C., 1997. Transformations of heavy metals added to soil - application of a new sequential extraction procedure. Geoderma 84, 157-168.
- Marschner, H., 2005. Mineral Nutrition of Higher Plants, second ed. Academic Press.
- Marschner, P., 2012. Chapter 15 - Rhizosphere Biology, in: Petra, M. (Ed.), Marschner's Mineral Nutrition of Higher Plants (Third Edition). Academic Press, San Diego, pp. 369-388.
- Martin, J.M., Nirel, P., Thomas, A.J., 1987. Sequential extraction techniques: promises and problems. Mar. Chem. 22, 313-341.
- Mirgorodsky, D., Ollivier, D., Merten, D., Büchel, G., Willscher, S., Wittig, J., Jablonski, L., Werner, P., 2010. Phytoremediation experiments on a slightly contaminated test field of a former uranium mining site "Mine Water & Innovative Thinking", IMWA 2010, September 05.-09., 2010, Sydney/Canada, pp. 587-590.
- Miyata, N., Tani, Y., Sakata, M., Iwahori, K., 2007. Microbial manganese oxide formation and interaction with toxic metal ions. J. Biosci. Bioeng. 104, 1-8.
- Mousavi, S.M., Jafari, A., Yaghmaei, S., Vossoughi, M., Roostaazad, R., 2006. Bioleaching of low-grade sphalerite using a column reactor. Hydrometallurgy 82, 75-82.
- Mucha, A.P., Almeida, C.M.R., Bordalo, A.A., Vasconcelos, M.T.S.D., 2005. Exudation of organic acids by a marsh plant and implications on trace metal availability in the rhizosphere of estuarine sediments. Estuar. Coast. Shelf S. 65, 191-198.
- Nahas, E., 1996. Factors determining rock phosphate solubilization by microorganisms isolated from soil. World J. Microb. Biot. 12, 567-572.
- Natarajan, K.A., 2008. Microbial aspects of acid mine drainage and its bioremediation. T. Nonferr. Metal Soc. 18, 1352-1360.
- Neagoe, A., Merten, D., Iordache, V., Büchel, G., 2009. The effect of bioremediation methods involving different degrees of soil disturbance on the export of metals by leaching and by plant uptake. Chem. Erde - Geochem. 69, 57-73.
- Oburger, E., Kirk, G.J.D., Wenzel, W.W., Puschenreiter, M., Jones, D.L., 2009. Interactive effects of organic acids in the rhizosphere. Soil Biol. Biochem. 41, 449-457.
- Paas, N., 1997. Untersuchungen zur Ermittlung der geochemischen Barriere von Gesteinen aus dem Umfeld untertägiger Versatzräume im Steinkohlenbergbau des Ruhrkarbons. Rheinische Friedrich-Wilhelms-Universität, Bonn, p. 234.
- Pathak, A., Dastidar, M.G., Seekrishnan, T.R., 2009. Bioleaching of heavy metals from sewage sludge by indigenous iron oxidizing microorganisms using ammonium ferrous sulfate and ferrous sulfate as energy source: a comparative study. J. Hazard. Mater.
- Pollmann, K., Raff, J., Merroun, M., Fahmy, K., Selenska-Pobell, S., 2006. Metal binding by bacteria from uranium mining waste piles and its technological applications. Biotechnol. Adv. 24, 58- 68.
- Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal-contaminated land by trees - a review. Environ. Int. 29, 529-540.
- Puschenreiter, M., Schnepf, A., Millán, I.M., Fitz, W.J., Horak, O., Klepp, J., Schrefl, T., Lombi, E., Wenzel, W.W., 2005. Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. Plant Soil 271, 205-218.
- Rajkumar, M., Ae, N., Freitas, H., 2009. Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77, 153-160.
- Rao, T.P., Yano, K., Iijima, M., Yamauchi, A., Tatsumi, J., 2002. Regulation of rhizosphere acidification by photosynthetic activity in cowpea (*Vigna unguiculata* L. Walp.) seedlings Ann. Bot. - London 89, 213-220.
- Raskin, I., Smith, R.D., Salt, D.E., 1997. Phytoremediation of metals: using plants to remove pollutants from the environment. Curr.Opin. Biotech. 8, 221-226.
- Rehman, M., Anwar, M.A., Iqbal, M., Akhtar, K., Khalid, A.M., Ghauri, M.A., 2009. Bioleaching of high grade Pb-Zn ore by mesophilic and moderately thermophilic iron and sulphur oxidizers. Hydrometallurgy 97, 1-7.

- Ryan, J.N., Elimelech, M., 1996. Colloid mobilization and transport in groundwater. Colloids Surfaces A: Physicochem. Eng. Aspects 107, 1-56.
- Sandnes, A., Eldhuset, T.D., Wollebaek, G., 2005. Organic acids in root exudates and soil solution of Norway spruce and silver birch. Soil Biol. Biochem. 37, 259-269.
- Šarić, M., Lucchini, R., 2007. Manganese, in: Nordberg, G.F., Fowler, B.A., Nordberg, M., Friberg, L.T. (Eds.), Handbook on the Toxicology of Metals, 3rd Edition ed. Academic Press, Burlington, San Diego, USA.
- Sauerbeck, D., Lübben, S., 1991. Chemische Extraktionen zur Bestimmung der Bindungsformen von Schwermetallen in Böden. Berichte aus der Ökologischen Forschung 6, 62-89.
- Sayer, J.A., Raggett, S.L., Gadd, G.M., 1995. Solubilization of insoluble metal compounds by soil fungi: development of a screening method for solubilizing ability and metal tolerance. Mycol. Res. 99, 987-993.
- Schippers, A., Bosecker, K., 2002. Bakterien in Bergbauhalden gefährden die Umwelt - Bildung von schwermetallhaltigen Sauerwässern und Maßnahmen zu deren Hemmung. Z. Angew. Geol. 1, 38 - 43.
- Schmidt, A., Haferburg, G., Schmidt, A., Lischke, U., Merten, D., Ghergel, F., Büchel, G., Kothe, E., 2009. Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area. Chem. Erde - Geochem. 69, 35-44.
- Schmidt, A., Haferburg, G., Sineriz, M., Merten, D., Büchel, G., Kothe, E., 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. Chem. Erde - Geochem. 65, 131-144.
- Semhi, K., Chaudhuri, S., Clauer, N., 2009. Fractionation of rare-earth elements in plants during experimental growth in vaired clay substrates. Appl. Geochem. 24, 447-453.
- Shan, X.-q., Lian, J., Wen, B., 2002. Effect of organic acids on adsorption and desorption of rare earth elements. Chemosphere 47, 701-710.
- Shen, H., Yan, X., Zhao, M., Zheng, S., Wang, X., 2002. Exudation of organic acids in common bean as related to mobilization of aluminum- and iron-bound phosphates. Environ. Exp. Bot. 48, 1-9.
- Shen, J., Li, H., Neumann, G., Zhang, F., 2005. Nutrient uptake, cluster root formation and exudation of protons and citrate in *Lupinus albus* as affected by localized supply of phosphorus in a split-root system. Plant Sci. 168, 837-845.
- Shen, W.-b., Yang, H.-q., 2008. Effects of earthworm and microbe on soil nutrinients and heavy metals. Agr. Sci. China 7, 599-605.
- Sheng, X.-F., Xia, J.-J., Jiang, C.-Y., He, L.-Y., Qian, M., 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. Environ. Pol. 156, 1164-1170.
- Shetty, K.G., Hetrick, B.A.D., Figge, D.A.H., Schwab, A.P., 1994. Effects of mycorrhizae and other soil microbes on revegetation of heavy metal contaminated mine spoil. Environ. Pol. 86, 181-188.
- Sipos, P., Póka, T., 2002. Threshold limit values for heavy metals in soils in the function of spacial and temporal variation of geochemical factors, XVII. Congress of CBGA. Special Issue of Geologica Carpathica (CD), Bratislava.
- Sreekrishnan, T.R., Tyagi, R.D., 1995. Sensitivity of metal-bioleaching operation to process variables. Process Biochem. 30, 69-80.
- Tao, S., Liu, W.X., Chen, Y.J., Cao, J., Li, B.G., Xu, F.L., 2005. Fractionation and bioavailability of copper, cadmium and lead in rhizospere soil, Biochemistry of Trace Elements in the Rhizosphere, pp. 313-336.
- Tebo, B.M., Johnson, H.A., McCarthy, J.K., Templeton, A.S., 2005. Geomicrobiology of manganese (II) oxidation. Trends Microbiol. 13, 421-428.
- Tomlin, A.D., Protz, R., Martin, R.R., McCabe, D.C., Lagace, R.J., 1993. Relationships amongst organic matter content, heavy metal concentrations, earthworm activity, and soil microfabric on a sewage sludge disposal site Geoderma 57, 89-103.
- Usman, A.R.A., Mohamed, H.M., 2009. Effect of microbial inoculation and EDTA on the uptake and translocation of heavy metal by corn and sunflower. Chemosphere 76, 893-899.

- van Hees, P.A.W., Vinogradoff, S.I., Edwards, A.C., Godbold, D.L., Jones, D.L., 2003. Low molecular weight organic acid adsorption in forest soils: effects on soil solution concentrations and biodegradation rates. Soil Biol. Biochem. 35, 1015-1026.
- Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, a., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M., 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ. Sci. Pollut. Res. 16, 765-794.
- Veneklaas, E.J., Stevens, J., Cawthray, G.R., Turner, S., Grigg, A.M., Lambers, H., 2003. Chickpea and white lupin rhizosphere carboxylates vary with soil properties and enhance phosphorus uptake. Plant Soil 248, 187-197.
- Weisskopf, L., Le Bayon, R.-C., Kohler, F., Page, V., Jossi, M., Gobat, J.-M., Martinoia, E., Aragno, M., 2008. Spatio-temporal dynamics of bacterial communities associated with two plant species differing in organic acid secretion: A one-year microcosm study on lupin and wheat Soil Biol. Biochem. 40, 1772-1780.
- Weyens, N., van der Lelie, D., Taghavi, S., Newman, L., Vangronsveld, J., 2009a. Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends Biotechnol. 27, 591-598.
- Weyens, N., van der Lelie, D., Taghavi, S., Vangronsveld, J., 2009b. Phytoremediation: plant-endophyte partnerships take the challenge. Curr.Opin. Biotech. 20, 248-254.
- Whiting, S.N., Souza, M.P.D., Terry, N., 2001. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ. Sci. Technol. 35, 3144-3150.
- Xiao, S., Xie, X., Liu, J., 2009. Microbial communities in acid water environments of two mines, China. Environ. Pollut. 157, 1045-1050.
- Yanni, Y.G., Rizk, R.Y., Corich, V., Squartini, A., Ninke, K., Philip-Hollingsworth, S., Orgambide, G., Bruijn, F.d., Stoltzfus, J., Buckley, D., Schmidt, T.M., Mateos, P.F., Ladha, J.K., Dazzo, F.B., 1997. Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. Plant Soil 194, 99-114.
- Yuan, S., Xi, Z., Jiang, Y., Wan, J., Wu, C., Zheng, Z., Lu, X., 2007. Desorption of copper and cadmium from soils enhanced by organic acids. Chemosphere 68, 1289-1297.
- Zehl, K., 2005. Schwermetalle in Sediment und Böden unter besonderer Berücksichtigung der Mobilität und deren Beeinflussung durch Sauerstoff, Chemisch-Geowissenschaftliche Fakultät. Friedrich Schiller Universität Jena, Jena.
- Zhou, L.L., Cao, J., Zhang, F.S., Li, L., 2009. Rhizosphere acidification of faba bean, soybean and maize. Sci. Total Environ. 407, 4356-4362.
- Zhuang, X., Chen, J., Shim, H., Bai, Z., 2007. New advances in plant growth-promoting rhizobacteria for bioremediation. Environ. Int. 33, 406-413.

Chapter 2

STUDY OF THE INFLUENCE OF PLANT ROOT EXUDATES ON HEAVY METAL MOBILITY BY MEANS OF ANALYSIS OF RARE EARTH ELEMENT FRACTIONATION PATTERNS

Tsilla Boisselet[*] , Dirk Merten, Georg Büchel

[1] Institute for Geosciences, Friedrich Schiller University, Burgweg 11, 07743 Jena, Germany

The present chapter is the manuscript of an article to be submitted.

Chapter 2: Study of the influence of plant root exudates on heavy metal mobility by means of analysis of rare earth element fractionation patterns

ABSTRACT

Root exudates play a key role for the bioavailability of trace metals and their toxic effects on the biosphere. A pot experiment was performed in order to study the effect of plants on metal mobilization and uptake on a soil from a former uranium mining site contaminated by several trace metals, overall Mn, Al, Ni, Zn, U and Rare Earth Element (REE). Plants used were clover (*Trifolium pratense*), red fescue (*Festuca rubra),* sunflower (*Helianthus annuus)* and Triticale. The REE patterns of soil, water and plants were compared to controls and to soil eluted with different solutions as water, inorganic and organic acids in order to define the factors influencing the metal behaviour. It was observed that a heavy REE (HREE) enrichment compared to MREE (middle REE) and LREE (light REE) occurred in the solution only after leaching soil with organic acids. The root REE were 10-fold concentrated and showed a fractionation from soil solution to the roots. A consistent LREE enrichment of the shoots relatively to the roots was observed for all plants. For sunflower also MREE enrichment was present.

After a balance calculation, simultaneously to an increased locally the soil pH, a local decrease of the amounts of soluble metals in soil and soil water was observed in the rhizosphere. The characteristic HREE enrichment compared to previous elution experiments, of the soil water compared to the control soil water was a hint to the influence of organic acids, although some further factors could be involved too, such as other exudates or microorganisms.

1 INTRODUCTION

Many different factors have an influence on the mobilisation and immobilisation of metals in soil and water. These are mainly soil pH and oxygen content, redox conditions, soil composition allowing cation exchange or precipitation, soil weathering as well as the type of metal and its chemical form, or heavy metal competition (Blume and Brümmer, 1991; Bradl, 2004; Karim and Khan, 2001; Marschner, 2005; Paas, 1997). Additionally, microorganisms and plants are able to change these physicochemical parameters, and provoke themselves further mineralisation or mobilisation reactions, by acidification of the soil through exudates, active redox reactions for energy production, precipitation of minerals and complexation of metals, among others. The investigation of the speciation of metals and its changes are essential for risk assessment, toxicity studies or remediation techniques (Díaz-Barrientos et al., 1999; Kidd et al., 2009).

Among the different substances secreted by plants, organic acids compose 1-3% of the dissolved organic carbon (DOC) in the soil. Root exudates and dead root material is supposed to comprise 30-40% of the total organic matter input to soils (Grayston et al., 1996). They are released into the rhizosphere, which consists of the root-influenced soil volume up to few millimetre from the root surface, and constitutes only 2-3% of the total soil volume (Grayston

et al., 1996). Root exudates play a role in the weathering of soil, for the mobilisation of nutrients as phosphorus or iron; especially organic acids, phytosiderophores, phenolic compounds are involved in these processes. Further they are important for the protection of plants against uptake of heavy metals into the roots through the action of citrate, malate, small peptides, or for attraction of useful microorganisms by phenolic compounds, organic acids, sugars (Grayston et al., 1996; López-Bucio et al., 2000; van Hees et al., 2003). Root exudates can also act as signal molecules or as precursors for hormones; very low concentrations are sufficient for their biological effect (Marschner, 2005). In the past, some methods have been used to quantify the metals bound to different fractions of the soil, especially the sequential extraction (Zeien and Brümmer, 1989). In particular, ammonium nitrate extraction was found to be a good estimation for the plant available fraction and used as a DIN norm in soil analysis. However, on one hand this estimation was not valid for all soil and all metals (Gryschko et al., 2004), and on the other side the amount and the proportion of the different acids in the soil depend on the plant species, the cultivation time, the constitution of the soil, as well as the age of the plant and the distance to the root (Grayston et al., 1996). Furthermore, huge differences in pH and metal mobility are possible between rhizosphere and bulk soil. These processes can strongly influence the mobility of heavy metals in the surrounding soil. To study the effect of the rhizosphere on metal mobility a pot experiment was designed.

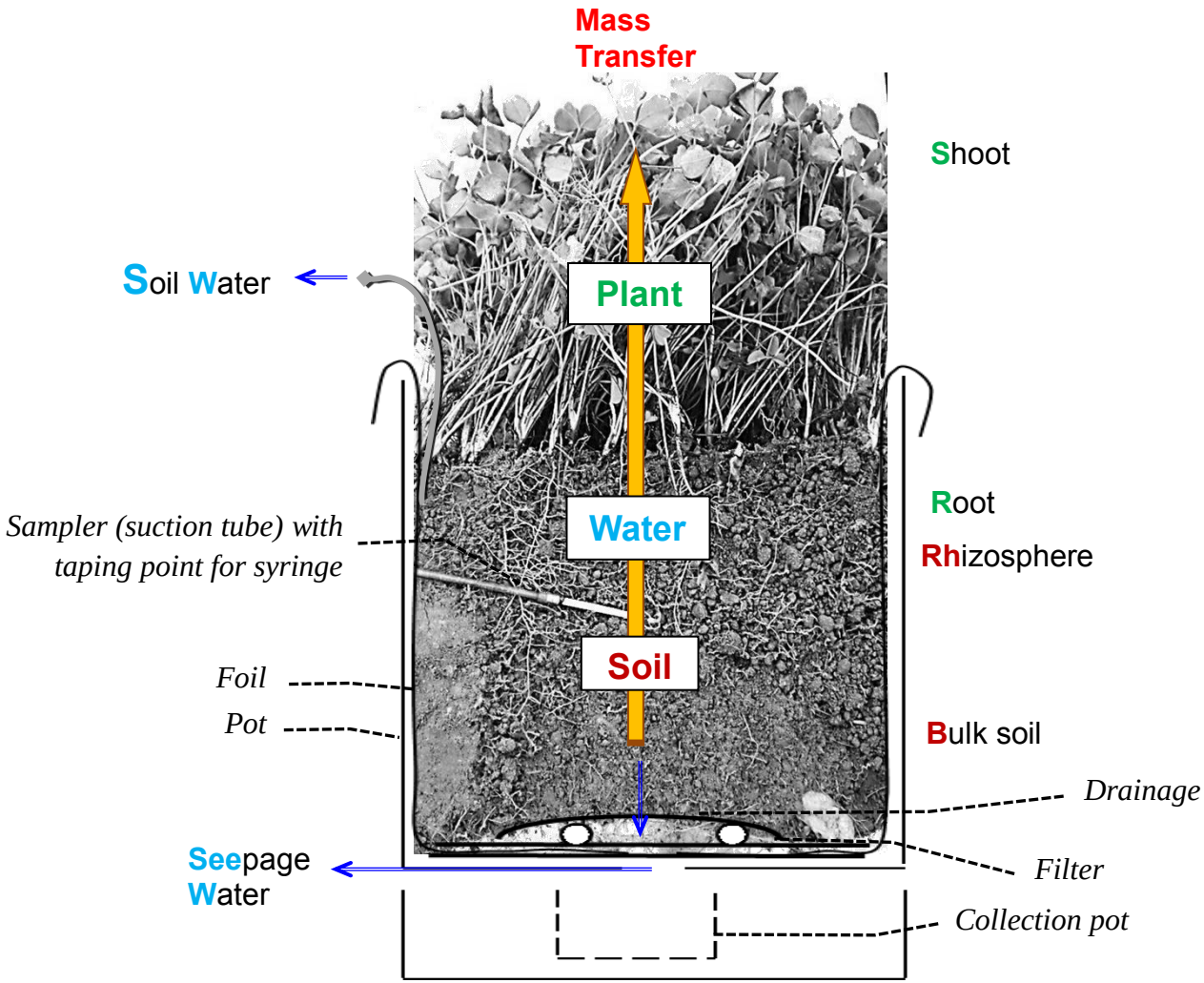
The soil originated from the Ronneburg mining district in Thuringia (Germany) which was a large uranium producing area until 1990 (Jakubick et al., 2002; Kahlert, 1992; Lange, 1995). The mining activities strongly altered the hydrogeology of the area. During mining, exhumation of sulphide minerals as well as acid-leaching (10g/L sulphuric acid) of waste heap led to metal dissolution, due to pyrite oxidation. Later, flooding and precipitation formed so-called acid mine drainage that infiltrated into the soil. These acidic and highly mineralised solutions infiltrated into the soil, and polluted the water-soil system with high concentrations of uranium, Rare Earth Elements (REE) and other metals. In the 1990s the heap and 10 m of underlying Quaternary sediments were filled into the nearby open pit Lichtenberg and the basement area was remediated. Despite remediation activity, contamination is still measurable.

Since the soil pH is quite low (pH 4-4.5), the mobility and bioavailability of metals is likely to be high and so the up-taken amounts of metals by plants would be significant, as monitored before. Therefore, this area is adequate for monitoring of groundwater chemistry and soil parameter and improving remediation strategies for slightly heavy metals contaminated areas. Four different plants were used in the present study. In order to reconstruct the natural conditions and understand on-going processes in the study area, two autochthones plants were chosen. *Festuca rubra* is a very resistant grass found in many heavy metal contaminated areas, and known as a pioneer plant. Clover was chosen because of its ability to fix air nitrogen due to its symbiosis with bacteria, and so to overcome partially the nitrogen-poorness of the soil. Additionally, reference plants (sunflower and Triticale) enable comparison with previous studies, especially regarding REE fractionation (Kidd et al., 2009; Lonschinski, 2009). None of the plants is known to be hyperaccumulators, even though they have been studied for phytoremediation purposes, mostly for phytostabilisation. The plants were grown in single culture as well as a mixed culture.

REE are elements of the lanthanide group (La-Lu), including in some cases also Y and Sc. These metals are very similar to each other because of their similarity in the electron structure. They are strongly electropositive, and occur mostly in oxidation state 3. They are considered as chemical analogues to trivalent actinides (Ding et al., 2006). Only Ce und Eu can be found in other valences (Ce^{+4} or Eu^{+2}), which causes a different chemical behaviour depending on the redox potential. Rare Earths Elements are often separated in light (LREE), middle (MREE) and heavy REE (HREE). The pattern obtained through normalisation of the REE with a standard (in this case PAAS, Post Archean Australian Shale, (McLennan, 1989) is a tool to study water-rock-interactions, as tracer for erosion processes, or to follow the flow of water and in case of AMD influenced areas to follow contamination by heavy metals or radionuclides (Aubert et al., 2001; Merten et al., 2005). Possible host phases of REE in nature are Fe-hydroxides, Mn oxides, clay minerals, and humic substances. Different factors can influence the pattern of REE. Not only the source material causes a typical pattern, but also pH (precipitation, sorption and desorption, dissolution), redox-conditions and ligands can alter it. In particular, organic substances excreted by plants to optimize their nutrient input can play a role in the regulation of the soil pH and as well complex different metals (Pourret et al., 2008; van Hees et al., 2003).

This study aims at investigating the influence of rhizosphere processes on metal mobility, using REE pattern as a tool. Therefore, four different plants (clover and red fescue as autochthonous plants and sunflower and triticale as model plants, as monocultures and polyculture) were grown on contaminated substrate, and the total metal contents of soil, soil water, seepage water and plants, as well as the soluble metal content of the soil in the rhizosphere and in the bulk soil were measured. The REE patterns of all compartments were compared to those obtained by leaching the soil with different reagents, in order to find an explanation for the mechanisms involved.

Indeed, it is important to understand how plants and their rhizosphere influence metal mobility, in particular to estimate the bioavailable fraction beyond sequential extraction, in order to optimize and use these processes for phytoremediation purposes.



Analytical parameters

Plant (Shoot + Root)
Biomass [g]
Total metal content [µg/g]
Soil (Rhizosphere + Bulk)
Elution – soluble metal content [µg/g]
Water content [%]
Total metal content [µg/g]
pH
Electrical conductivity
Water (Soil Water + Seepage Water)
Metal content [µg/L]
pH
Electrical conductivity

Figure 1: Overview over the experimental settings of the pot experiment and the compartments of the studied system, including the analytical parameters

2 MATERIALS AND METHODS

2.1 Substrate

The substrate sampled at the remediated northern part of the base area of the former leaching heap called Gessenhalde at the former Uranium mining site. The soil that was used to test remediation strategies was homogenized on the top 100 cm. It was air dried and sieved to a grain size of < 2 mm. It consists in silty sand (Mirgorodsky et al., 2010) with a high content in clay minerals. The soil pH is quite low (pH 4-4.5). The nutritive quality of the soil is weak, at least concerning the available part of nutrients. So, the contents of organic carbon and inorganic nitrogen are very low (Table 1) compared to an optimal soil as Rendzina soil. Phosphorus is also present in low amounts, most of it being insoluble. Similarly, Fe is poorly present in a plant available form, most of it being found as iron oxides, and in the residential fraction (Table 2). Mg is the only macroelement present in sufficient amounts, whereas S is present in high amounts. The soil was furthermore characterised by a moderate contamination with metals (Table 2) including REE (La-Lu), with average total amounts of ∑REE of about 180 µg/g. Other important contaminants are Al, Ni, Zn and Cu, and remaining U from the mining.

Table 1: Substrate characterisation and comparison with a soil adapted to plant growth (Mirgorodsky et al., 2010)

	pH_{KCl}	EC	C_{org}	N	$CaCO_3$	NO_3_N	NH_4_N	N_{min}	S_{min}	P	K
		µS/cm	%	%	%	----------mg/100g----------					
Rendzina	6.98	285	6.89	0.75	15.5	1.4	0.1	1.5	0.7	4.6	21
Substrate	**4.44**	**749**	**0.1**	**0.04**	**0.5**	**0.1**	**0.01**	**0.2**	**89.2**	**1**	**4**

Table 2: Amounts of selected elements in µg/g, in different fractions of the sequential extraction (Fractions I to VI and total extraction) (Mirgorodsky et al., 2010)

	Al	Ca	Fe	Mg	Mn	Ni	P	Zn	Co	Cu	Cd	Pb	U	∑REE
F I	3.7	1242	ND	529	88.4	6.7	ND	2.20	1.046	0.078	0,08	0.013	0.004	0.849
F II	2.22	71	ND	29.3	9.02	0.87	4	0.56	0.109	0.26	0.029	0.084	0.825	1.63
F III	28.6	14.3	101	3.19	499	6.5	5	2.31	10.36	1.05	0.13	0.977	0.262	1.96
F IV	35.0	16.4	104	0.63	4.03	0.78	13	0.72	0.458	1.13	ND	1.260	0.055	3.09
F V	258	25.1	1386	5.17	9.58	1.75	122	2.20	0.68	1.72	0.021	1.383	0.621	9.00
F VI	812	7.3	11079	65	22.5	10.6	185	12.6	2.33	15.5	0.045	2.22	0.503	7.03
Total	59608	1952	37861	4520	830	*52.5*	627	76.9	*21.66*	*35.2*	*0.61*	16.2	5.3	178.7
				Possible contamination		*40*		*150*	*20*	*50*	*0,5*	*50*		
				Threshold value		*60*		*300*	*50*	*100*	*1*	*100*		

ND: not detected

So, although the estimation of critical values is controverted because of the difficult estimation of the actual toxic effects, it can be stated that Ni concentrations found in the substrate are over the average concentrations found in soils and the threshold of 40 mg/kg dry soil (Sipos and Póka, 2002), Arsenic is also more elevated compared to the average soil values (up to 15 mg/kg). Further, Copper and Zinc total amounts are above the background values given by the cornwell waste management institute, USA, although there values are not considered as a risk for human health. According to the Austrian Federal Forest Office (Table 2), the quantities of Co and Cu present in the substrate are in a range that can affect soil microorganisms' survival and metabolic efficiency. However, the threshold values often apply for single contamination, the metals may have a different effect if present together.

2.2 Pot experiment

Trifolium pratense var. *kvarta* (Red clover), *Festuca rubra rubra* (red fescue), Sunflower (*Helianthus annuus*) and Triticale (hybrid of wheat (*Triticum*) and rye (*Secale*)) were used for pot experiments. 5 kg dry, sieved soil were mixed with 600 mL deionized water (corresponding to the field capacity), and filled in the pots (h = 25 cm, diameter ca. 20 cm) until 10 cm under the top. A moisture sampler (Rhizon CSS 19.21.23 F, Eijkelkamp) was placed horizontally over the first half of the substrate before all the soil was filled into the pot (Figure 1). During pouring, the soil was agitated by putting the pot on a vibrating surface to compact it. For plants with big seeds, holes were made on the plain surface with an adapted tool, and then the seeds (respectively 13 for sunflower and 25 for triticale) were put into and recovered with soil. For the finer seeds, a defined amount was weighted (2 g for clover and 1.5 g for red fescue) and distributed homogeneously over the surface, and recovered by quartz sand (about 2-3 mm). For the polyculture, all plants were sowed in the same pot, with 10 sunflower, 15 triticale, and clover and fescue each 1.5 g. The sand was acid-washed to estimate the maximum release of metals, the trace elements contained in the leachate were low, they were noticeable only for Fe, with values around 50 µg/g leachable with acid. REE were present but negligible considering the dilution factor by the little amount of sand added. At the end, all pots were watered with deionised water and covered until germination. All experiments were done in duplicates; two pots were left without plants as a control.

Since the conditions are very different in the narrow rhizosphere compared to the bulk soil, water sampling was designed in order to allow local sampling in the root zone without destroying its structure (Figure 1). Both soil water and seepage water were taken at the same time point, seepage water right after the soil water. The soil water was collected by suction cups and 100 µL thymole were added to a sample volume of about 10 mL at a final concentration of 0.5 µg/L, to avoid microbial degradation of the organic compounds. For seepage water, the soil was flushed and the water running out was collected in clean containers, and treated as the soil water. The quantity of water used for flushing was chosen to just be enough to get about 50-100 mL sample. Since the water retention was different depending on the plant growth, the amount of water added was different and noted for all pots. Furthermore, the pots were weighted before and after flushing to calculate the water content. All water samples were stored at -18°C until analysis. Before analysis of the cations, the samples were filtrated and acidified with HNO_3. The experiment was run for 14 weeks. The collected soil samples were dried and stored for further analysis.

2.3 Elution

The soil of all samples was dried after harvest in porcelain plates either at 40°C in the drying oven or at room temperature at the air until weight constancy. The water content was calculated from the loss of weight. About 4 g of dry sieved soil were weighted exactly in a 50 mL plastic tube, and 40mL of elution solution were added, so that the liquid to solid rate was 10:1 according to the DIN method S4. Different solutions were used for elution of original control soil: pure deionised water, sulphuric acid (10 g/L, Merck) and a mixture of organic acids i.e. citric acid, malonic acid, malic acid, oxalic acid, with an end-concentration of 0.5 mM each. The pot soil samples were eluted with pure deionized water in order to compare with the samples without plants and with the original soil. Soil pH (pH 320, WTW) and electrical conductivity (LF320, WTW) were measured. All soil-leaching solution suspensions were shaken 24 h overhead at about 20 rpm (Overhead shaker- ELU safety lock, Edmund

Bühler). For each experiment, tubes with only elution solution were taken as a blank. The samples were centrifuged for 15 min at 2500 rpm (multifuge 3L, Heraeus, Thermo Electron Corporation, Germany); only a few samples were left longer, and at 3000rpm, because the supernatant was still turbid. 15mL of each sample with a pH over 3.5 were filtrated through a 0.45 µm-celluloseacetate filter (Sartorius). The samples were acidified with suprapure HNO_3 (63%) and kept at 4°C until analysis. From the remaining solution pH and the electrical conductivity were measured using pH meter (pH 320, WTW) and electrical conductivity (LF320, WTW).

2.4 Analysis

The plants were carefully cleaned with deionized water in order to remove any soil from the surface, and separated into shoots and roots about a few millimetres above the underground part. They were weighted before and after drying, and then milled at 1500 rpm (Retsch mill, MM400) to an about 0.5 µm powder. Then their total metal content was extracted by a microwave assisted pressure digestion (MARS 5, from CEM) with 65% HNO_3 (Merck, p.a., subboiled) and the obtained solution was diluted and centrifuged to be ready for analysis.

The pH of the soil was determined with deionized water and with $CaCl_2$ (0,01 M) in parallel. Therefore, 10 g of dry soil was mixed with 25 mL of solution with a glass stick. Then, the sample was left to sedimentate over night and the pH of the supernatant was measured with a pH-meter (pH 320, WTW).

All soil samples were also analysed for their total metal content. For this, they were milled and 100 mg of it were filled into TFM vessels. Then, 4 ml 40% HF and 4 ml 70%$HClO_4$ (both suprapur, Merck) were added. After the mixture stood overnight in closed vessels, the vessels were tightened and heated up to 180 °C within 4 h. The temperature was maintained for 12 h and then the samples were allowed to cool down. In order to evaporate acids, the system again was heated up to 180°C for a period of 4 h, this time using a special evaporation hood. This temperature was maintained for 12 h. Then, to the remaining solid sample 2ml HNO_3 (65%, subboiled), 0.6 ml HCl (30%, Suprapur, Merck) and 7 ml of pure water (Pure Lab Plus, USF) were added and the mixture was dissolved by heating at 150°C for 10 h. The cooled samples were then transferred to calibrated 25 ml PMP flasks (Vitlab). Finally, the solution was replenished to 25 ml by the addition of pure water for analysis.

The metal contents in the samples were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES, Spectroflame, Spectro) for the main elements (Al, Ca, Fe, K, Mg, Mn, Na, P) and inductively coupled plasma mass spectrometry (ICP-MS, XSeriesII, Thermo Fisher Scientific) for the trace elements (Co, Cu, Cd, REE (La-Lu), U, Zn). Organic acids were analysed in samples previously filtrated with 0.45 µm cellulose acetate filters by ion chromatography DIONEX:, IC20 Ion Chromatograph, EG50 Eluent , CD20 Conductivity Detector, with a DionexIonPac AS11-HC column using elution with a KOH gradient (1-60 mM).

Gd and Ce anomalies were calculated respectively according to Eq. 1 and Eq. 2+3 (Rabiet et al., 2009). These equations are used to measure the divergence or anomaly of an element to its expected value relative to the other REE.

$$\mathbf{Gd/Gd^{*}= Gd_N/ (Sm_N)^{0.33} * (Tb_N)^{0.67}} \quad [1]$$

$$\mathbf{Ce/Ce^{*}} = \frac{\mathbf{CeN}}{\sqrt{\mathbf{La_N}} * \sqrt{\mathbf{Pr_N}}} \quad [2]$$

(Pourret et al., 2008)

$$\mathbf{Ce/Ce^{*}} = \frac{\mathbf{Ce_N}}{(\mathbf{Pr_N}+(\mathbf{Pr_N}-\mathbf{Nd_N})} \quad [3]$$

Where N is the measured amount of the element normalized on PAAS and (*) is the expected value. Equation 3 was selected to avoid any La anomaly interference during Ce/Ce* calculation, or in case La values where below detection limit. Anomaly values greater than or less than 1 represent preferential enrichment or removal of Ce, respectively. The Lu/La coefficient was used as quantification for HREE enrichment.

3 RESULTS

3.1 Plant growth

The biomass of all plants after 14 weeks growth showed huge differences (Table). Clover grew very well and reached a dry weight of about 25 g leaves per pot. The biomass of other plants was much lower (about 7 g for sunflower, 2 g for *Titricale* and 9 g for *Festuca rubra*). The flower and seed production was early, the growth reduced and the colour yellowish. Some plants were colonized by parasites after 8 weeks of growth.

Mixed crop cultivation had a positive influence on some plants. Relatively to the number of sowed seeds, clover had a much higher biomass production than if grown as a monoculture (Table 3 and Figure 2). Polyculture changed also the appearance of plants: clover had bigger leaves; sunflower had shorter shoot growth but slightly healthier colour. So polyculture seems to have a positive impact on each plant. In fact, even if the biomass production of *Festuca* and *Helianthus* is higher if grown as monoculture (Table 4 and Figure 2), it seems that their health is ameliorated by the neighbour plants.

Table 3: Plant dry biomass at harvest after 14 weeks growth

			dry biomass [g]	*SD*
monoculture	*Helianthus*	shoot	7	*±0.14*
	Triticale		3.9	*±0.00*
	Trifolium		24.8	*±2.55*
	Festuca		3.5	*±0.14*
	Helianthus	root	1	*±0.28*
	Triticale		0.5	*±0.00*
	Trifolium		8.5	*±1.84*
	Festuca		2.85	*±0.07*
polyculture	*Helianthus*	shoot	4.95	*±0.07*
	Triticale		2.75	*±0.78*
	Trifolium		20.85	*±1.77*
	Festuca		0.3	*±0.07*
	Helianthus	root	0.75	*±0.07*
	Triticale		0.15	*±0.07*
	Trifolium		5.3	*±0.42*
	Festuca		-	-

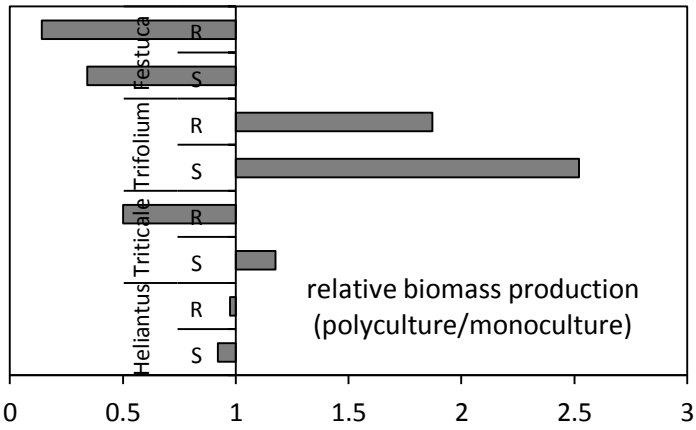


Figure 2: comparison of the effect of growing plants as a polyculture: relatively to the number of seeds sowed, the biomass production is compared between the monoculture and the polyculture. If the ratio is over 1, the biomass production is higher as a polyculture.

3.2 Soil

Table 4: soil pH and amounts of soluble metals leached with water, in rhizosphere and bulk soil

	pH	Al	Ca	Fe	K	Mg	Mn	Ni	S	Zn	Co	Cu	Cd	∑REE	Ce/Ce*
control	4.8	2.60	2614	-0.122	171	1315	76.8	12.4	3891	2.17	0.284	0.101	0.110	0.037	-3.87
***Helianthus* B**	4.6	4.31	3234	0.012	119	1846	101.9	15.2	4929	3.04	0.389	0.168	0.132	0.045	-3.67
***Helianthus* Rh**	4.7	3.54	969	1.884	205	641	62.9	6.0	1709	1.36	0.483	0.147	0.068	0.020	-4.71
***Triticale* B**	4.8	1.68	1321	-0.049	150	890	22.0	6.6	2315	1.32	0.043	0.071	0.057	0.008	
***Triticale* Rh**	4.9	1.61	1599	-0.078	153	940	31.8	7.1	2598	1.44	0.099	0.085	0.070	0.015	-4.72
***Trifolium* Rh**	4.4	4.58	1471	0.082	112	749	235.4	10.6	2352	3.55	2.871	0.396	0.139	0.027	-2.98
***Trifolium* B**	4.7	10.47	3483	-0.159	124	1776	420.9	23.9	5478	6.48	4.702	0.503	0.282	0.115	-4.00
Festuca	4.8	1.54	1308	-0.049	158	765	45.2	6.2	2140	1.36	0.244	0.093	0.065	0.010	-6.65
Poly B	4.4	18.34	3766	0.695	139	1954	620.3	27.6	6090	8.69	7.330	0.534	0.368	0.128	-4.13
Poly Rh	4.9	11.56	2044	0.869	134	1007	379.0	14.9	3281	5.60	4.783	0.419	0.214	0.053	-3.22
SD															
		Al	**Ca**	**Fe**	**K**	**Mg**	**Mn**	**Ni**	**S**	**Zn**	**Co**	**Cu**	**Cd**	**∑REE**	
control		0.98	1205	0.04	28	404	45	4.4	1498	0.73	0.234	0.032	0.040	0.028	0.43
***Helianthus* B**		1.24	926	0.07	32	430	38	4.8	1166	1.53	0.208	0.067	0.056	0.024	0.43
***Helianthus* Rh**		3.91	218	3.94	44	109	19	1.1	327	0.29	0.208	0.027	0.011	0.026	0.43
***Triticale* B**		0.39	71	0.06	4.6	57	4	0.7	124	0.07	0.006	0.008	0.007	0.001	0.41
***Triticale* Rh**		0.31	576	0.02	9.6	147	29	4.7	303	1.91	0.198	0.070	0.065	0.026	0.14
***Trifolium* Rh**		1.54	403	0.19	6.6	196	31	2.5	613	0.38	0.443	0.108	0.029	0.012	0.29
***Trifolium* B**		0.45	321	0.02	1.0	20	13	0.6	272	0.19	0.117	0.034	0.005	0.013	0.29
Festuca		0.44	401	0.07	29.6	232	14	1.8	650	0.39	0.100	0.012	0.020	0.010	0.41
Poly B		4.77	139	0.62	10.9	119	43	1.7	320	1.06	0.381	0.137	0.011	0.007	
Poly Rh		0.76	353	0.01	4.1	106	28	2.1	464	0.75	0.182	0.000	0.031	0.009	

B: Bulk soil, Rh: Rhizosphere soil; Poly: Polyculture ; SD: standard deviation

Table 5: Essential element content (a) and potentially toxic element content (b) of plant compartments in µg/g of dry mass

	Ca	Fe	K	Mg	Mn	Na	P	S	Si	Sr
Monoculture										
Sunflower shoots	8681	110	14458	5120	930	27	1820	3558	238	14
***Triticale* shoots**	2814	158	9817	3749	691	87	1864	5222	180	7
***Festuca* shoots**	3627	154	13676	2547	495	31	1977	1851	197	24
Sunflower roots	3787	2449	28884	4734	402	1820	1816	10459	254	20
***Triticale* roots**	2663	4117	2381	1352	340	114	539	2095	100	9
***Trifolium* roots**	3863	5897	6430	7477	970	660	1225	4913	148	26
***Festuca* roots**	2778	5054	3699	1197	374	132	966	1034	125	26
Polyculture										
Sunflower shoots	6579	125	10452	2925	811	17	1209	1873	232	13
***Trifolium* shoots**	11932	1234	10541	10631	1182	48	1224	4107	296	33
***Festuca* shoots**	4810	1998	17783	3168	518	178	2547	2217	177	29
***Festuca* all plant**	4509	4408	12731	3084	515	195	1987	1768	116	27
Sunflower roots	2895	2493	18658	3987	410	635	1255	5470	262	22
***Triticale* roots**	1424	2511	1633	604	139	87	375	996	407	8
***Trifolium* roots**	2159	2941	4846	5727	265	191	920	3480	180	19
***Festuca* roots**	3344	4749	6651	2375	525	203	1662	1826	232	21

	Al	Mn	Ni	P	S	Ti	Zn	Cr	Co	Cu	Cd	ΣREE
Monoculture												
Sunflower shoots	80	930	42	1820	3558	2	61	3	2	5	2	2.4
***Triticale* shoots**	97	691	22	1864	5222	2	50	10	1	4	1	1.1
***Festuca* shoots**	95	495	21	1977	1851	2	26	9	0	3	0	0.7
Sunflower roots	1716	402	106	1816	10459	24	120	10	14	41	3	19.4
***Triticale* roots**	2637	340	65	539	2095	45	39	16	9	19	1	18.2
***Trifolium* roots**	2907	970	163	1225	4913	40	49	22	24	29	4	26.4
***Festuca* roots**	4250	374	66	966	1034	43	54	53	6	21	1	28.6
Polyculture												
Sunflower shoots	83	811	27	1209	1873	2	60	2	2	4	2	2.2
***Trifolium* shoots**	884	1182	58	1224	4107	19	67	23	5	9	1	7.1
***Festuca* shoots**	1736	518	35	2547	2217	30	70	12	3	12	1	8.7
***Festuca* all plant**	2878	515	46	1987	1768	45	100	31	5	19	1	12.6
Sunflower roots	1750	410	60	1255	5470	29	100	13	9	11	2	12.6
***Triticale* roots**	1657	139	42	375	996	32	26	11	4	9	1	8.6
***Trifolium* roots**	2125	265	73	920	3480	37	27	12	6	15	2	13.0
***Festuca* roots**	4361	525	68	1662	1826	80	71	14	8	20	1	24.9

Soil pH of all samples does vary within a small range (pH_{water} 4.3-4.9, pH_{CaCl2} 3.9-4.7), though the trend between treatments is similar independently of the method used. Indeed, soil pH does change when plants are growing. In our study, the pH was more acidic in soil without plant influence compared to the rhizosphere zone. It is particularly visible for *Trifolium* (pH measured with water 4.4 in the bulk soil vs. 4.7 in the rhizosphere) and in the plant mixture (respectively 4.4 and 4.8). However, it is not significant for Triticale. *Festuca* soil could not be separated into bulk and rhizosphere, since the root net was too dense, therefore the whole soil has to be considered as rhizosphere. Indeed, the pH of soil planted with *Festuca* showed a pH of the upper part of the pH range (around 4.9 when measured with water, 4.6 with $CaCl_2$). These differences in pH were not significant for the soil water, the values for all pots varied between 4 and 5 (water has a pH of about 6).

However, in all experiments the electrical conductivity (130 - 420 µS/cm) was inversely correlated to the pH, which shows that pH is a main factor determining the amounts of dissolved elements in soil and water.
The amounts of metals collected through leaching with water are very low and in some cases below detection limit, except for the pots without plants and at less extend those planted with sunflower. Despite this, it was still possible to notice a trend: the electrical conductivity was lower in the rhizosphere compared to controls or bulk soil, showing that the amounts of soluble metals were lower.

Essential plant nutrients as Mg, K, Na and S are present in the water-soluble fraction of the soil (Table 5). The amounts of K and Na are not strongly affected by plant growth, only K is found in higher amounts in the soluble fraction of the rhizosphere soil of sunflower. S and Mg are found in high amounts (over 5 mg S/g) in the soluble bulk soil soluble fraction of pots planted with sunflower, *Trifolium* and plant combination. Phosphorus is below detection limit in the water-soluble fraction of the soil (Table 5). Manganese content is less than 100 µg/g in the water-soluble fraction for control and most of the samples, only *Trifolium* and the plant combination show amounts between 250 and 600 µg/g. The higher contents are found in the bulk soil (for instance *Trifolium* bulk soil has 420±12 µg/g Mn in the soluble fraction, the corresponding rhizosphere 235±31 µg/g). This trend is found for many other metals such as Al (about 2 µg/g vs. 5-15 µg/g for *Trifolium* and plant mixture), Cd, Zn and Co. For Ni and Cu it was similar, except that the rhizosphere of all plants and *Festuca* soil had lower amounts than unplanted soil. Values for Fe were mostly under detection limit or very low. The decrease of soluble metal content in the rhizosphere soil was in particular true for REE, which were used as a tracer to explain the metal uptake and show the active action of plant exudates (Table 5).

The amounts of REE for each sample of the elutions were normalized to the PAAS standard (Post Archean Australian Shale) (McLennan, 1989) and are plotted in Figure. The REE patterns show a slight positive Ce anomaly, except for the water elution (see Table 6, Figure 3), and an enrichment in MREE. Generally, LREE are depleted compared to HREE. The pattern is not much different comparing elution with water and elution with sulphuric acid (pH 0.9). It could be observed that the decrease of pH of the solution increased the amounts of leached REE: for sulphuric acid leaching (see Figure 3) the leached amounts are at least one order of magnitude higher than for the other solutions. The presence of organic acids compared to inorganic acids lead to a fractionation of the REE present in the soil, resulting in a significant HREE enrichment (see also Figure 3). Indeed, Lu/La values are much higher for organic leaching (18.9 ± 14) than for other leaching with water or sulphuric acid (respectively 1.6 ± 0 and 2.0 ± 0.4).

Table 6: Ce anomalies and HREE enrichment of patterns after leaching the studied substrate with three different solutions

	Ce/Ce* (1)	*SD*	**Ce/Ce* (2)**	*SD*	**Lu/La**	*SD*
H_2O	**0.25**	*±0.97*	**0.87**	±0.14	**1.57**	_
Org	**1.64**	*±0.20*	**1.40**	±0.23	**18.94**	±14.41
H_2SO_4	**1.36**	*±0.01*	**1.37**	±0.05	**1.96**	±0.42

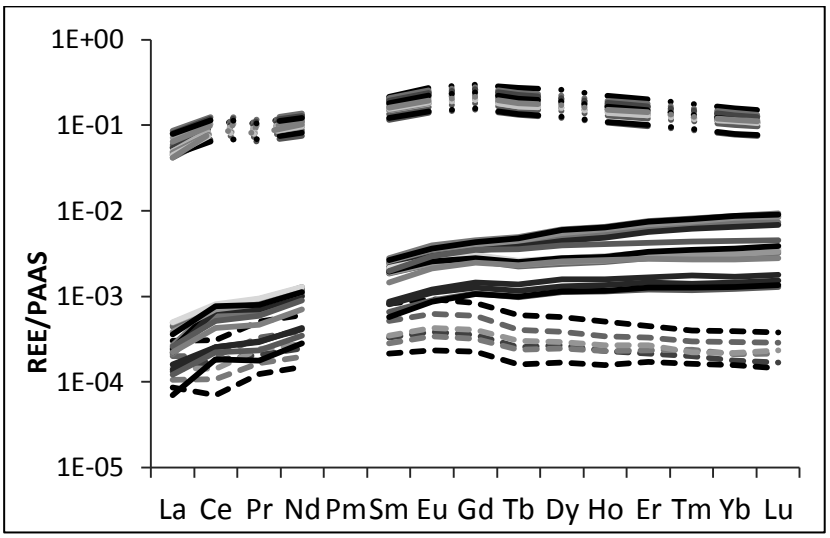


Figure 3: PAAS normalized REE pattern of soil from the test field eluted with water [dash lines], sulphuric acid [point-dash lines], organic acids [plain lines].

If REE patterns are used regarding to this result, a different pattern in soil water sampled near by the roots compared to seepage water can be expected.

Considering the water phase, the REE patterns normalized to PAAS do not show a visible HREE enrichment. On the other hand, if the water is normalized to the control pot, the effect is much more visible (Figure 4). The pattern of the soil waters normalised to the soil water from control pot (Figure 4) has this enrichment observed after eluting soil material with organic acids; this could be a hint for the influence of present organic acids in the root zone. Figure 5 shows that the HREE enrichment in the soil water corresponds to a HREE depletion in the soil total amounts.

REE can also be traced within the plant from the roots to the shoots. We could observe that here also REE behave in a different way.

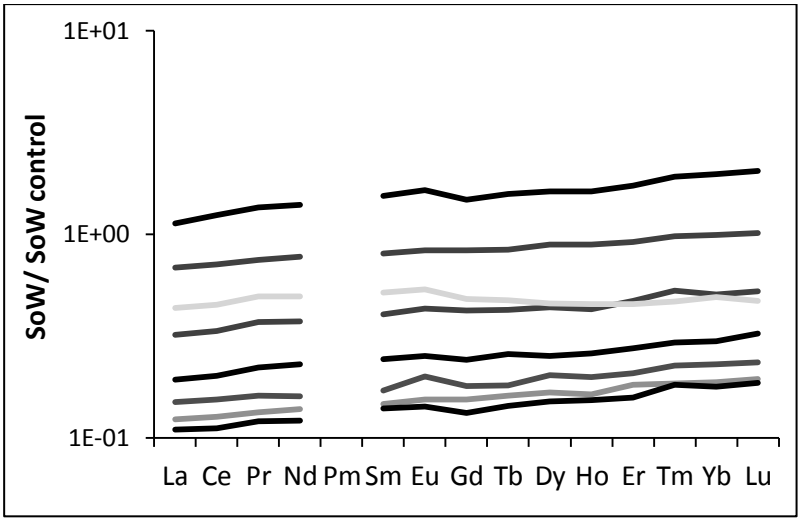


Figure 4: REE pattern of soil water (in pots with plant growth) relative to soil water of the control pot. A clear enrichment of HREE compared to LREE is visible in all samples.

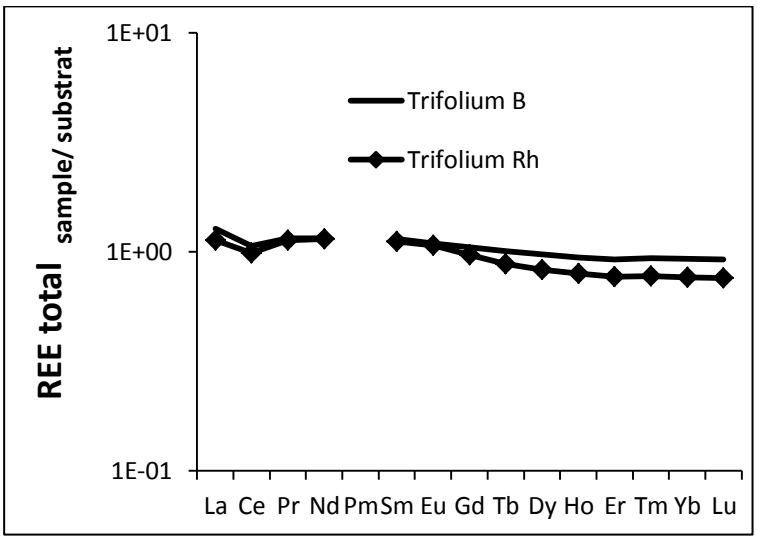


Figure 5: REE pattern of soil total contents of sample relative to control on the example of *Trifolium*: it is clear that the depletion of HREE is located in the rhizosphere. Diamonds stand for the rhizosphere, full line for bulk soil.

The amounts of REE are 10 fold higher for the roots than for the shoots, except for clover where the ratio between shoot and roots is smaller (Table). It is also noticeable that the total REE amounts per g dry biomass are lower at mixed crop cultivation than at single crop cultivation (Figure 7, Table 7), except for *Festuca*, which is taking up more metals as polyculture. Further the differences between patterns are more marked between shoots and roots than between plants. The REE patterns are similar for all plants when normalized to PAAS (see Figure 7): MREE enrichment and a slight positive Ce anomaly (Ce/Ce* = 1.2-1.4); the LREE to HREE ratio is variable (Lu/La 0.7-3.4). It was also observed that generally HREE are less translocated than LREE. The same was observed for many metals, as Al, Fe, and most of the contaminants. Only Mn had a translocation factor of over 1, and additionally Cr and Zn in the case of *Trifolium* (*results not shown*).

Table 7: Translocation factor for REE from root to shoots ; TF=metal content in shoots/metal content in roots

TF	La	Ce	Pr	Nd	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
Monoculture														
Sunflower	0.149	0.143	0.124	0.121	0.104	0.110	0.124	0.115	0.100	0.099	0.087	0.079	0.054	0.055
Triticale	0.075	0.065	0.059	0.053	0.048	0.054	0.056	0.063	0.053	0.060	0.052	0.071	0.043	0.061
Festuca	0.026	0.024	0.023	0.022	0.020	0.019	0.021	0.021	0.021	0.023	0.021	0.022	0.020	0.020
Polyculture														
Sunflower	0.157	0.187	0.158	0.169	0.157	0.176	0.209	0.190	0.173	0.168	0.151	0.127	0.100	0.088
Festuca	0.381	0.362	0.356	0.344	0.320	0.312	0.307	0.299	0.300	0.299	0.298	0.289	0.289	0.295
Trifolium	0.670	0.577	0.557	0.534	0.471	0.429	0.446	0.413	0.398	0.395	0.389	0.345	0.311	0.293

To visualise the REE fractionation from the environment to the plants, the REE amounts of the roots are normalised to the soil water (Figure 8), since it is assumed to be the fraction taken up by the plants. The pattern shows a fractionation in a wavy shape: elements from Pr to Eu on one hand and Yb and Lu on the other hand are clearly enriched.

For the fractionation within the plant, shoots total digestion are normalised to root total digestion (Figure 9). There is little fractionation from shoots to roots, though a clear LREE enrichment is observable (La/Lu ranging from 1.3 to 3), except for triticale. The patterns look similar to those shown by (Lonschinski, 2009) with similar plants on a similar substrate. In that study, sunflowers show a clear depletion of HREE to LREE, with a slight MREE

enrichment, and a slight positive Gd anomaly. *Festuca rubra* showed an almost continuous depletion in REE from light to heavy.

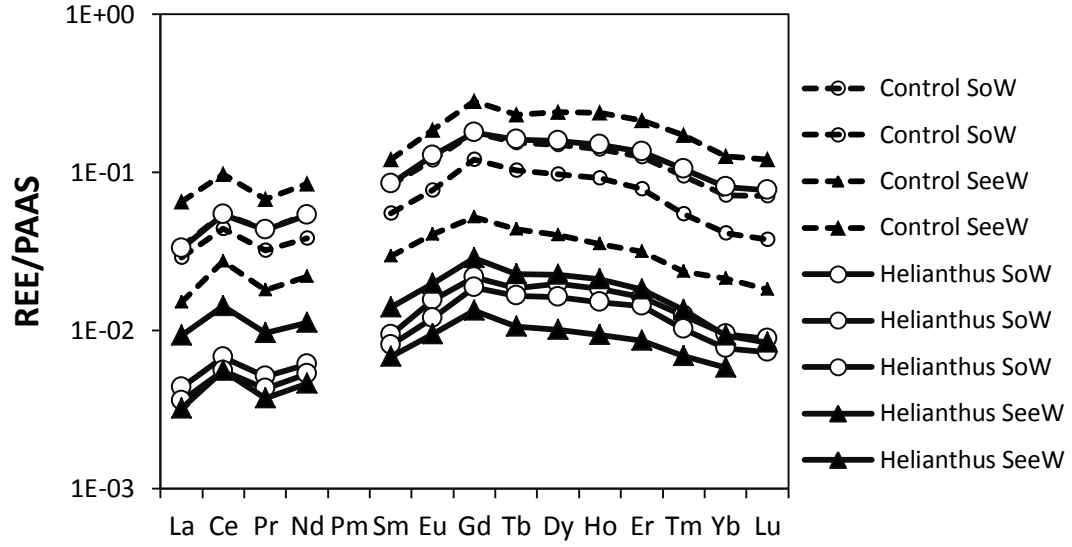


Figure 6: PAAS-normalized REE patterns of the soil water (SoW) and seepage water (SeeW) for pots planted with sunflower

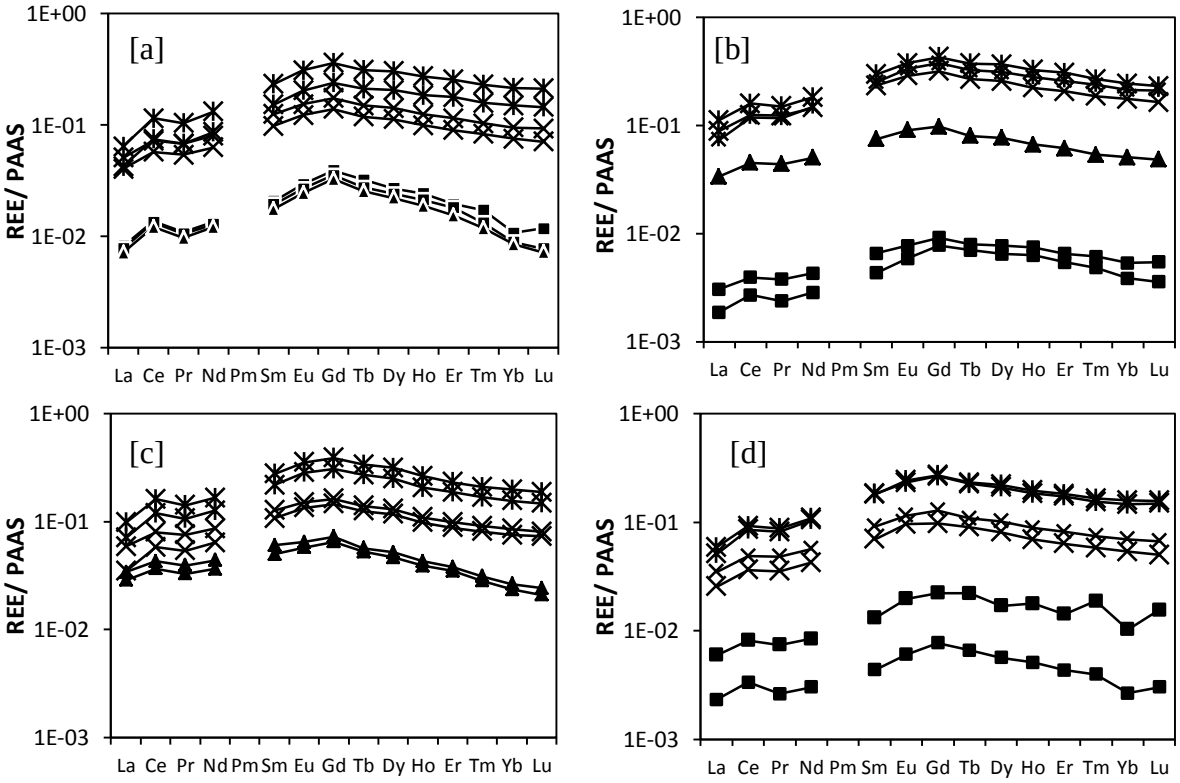
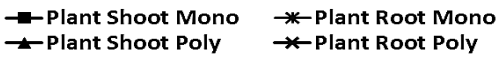


Figure 7: PAAS normalized REE patterns of all plants (roots and shoots, total digestion) normalized to PAAS. [a] Sunflower, [b] *Festuca,* [c] *Trifolium*, [d] Triticale



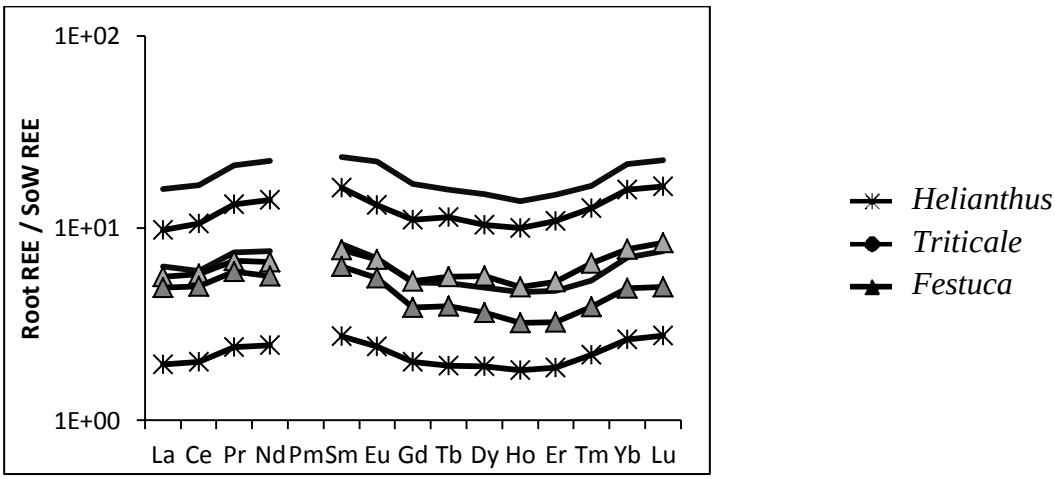


Figure 8: Plant REE patterns normalized on the corresponding soil water: fractionation from soil water to plant root

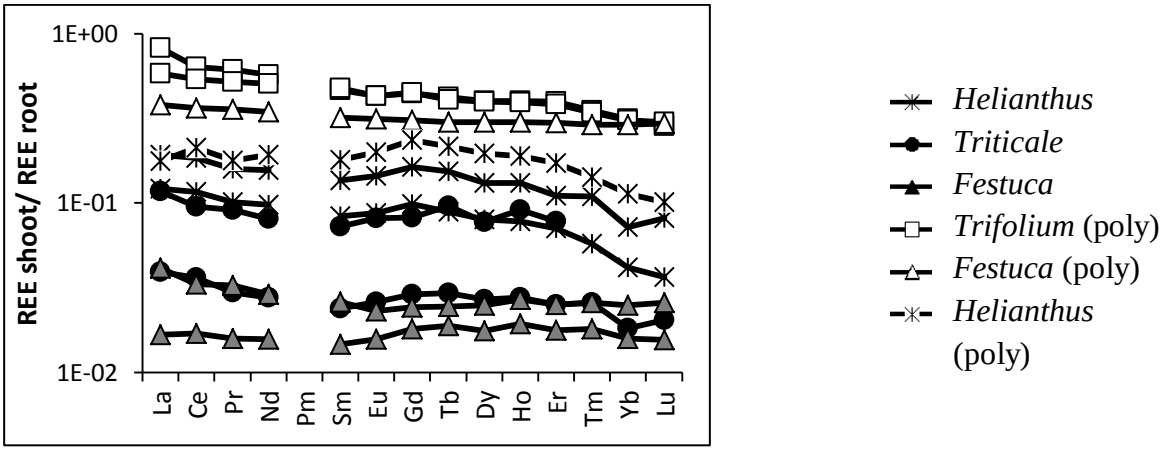


Figure 9: REE patterns of shoots (total digestion) standardized on roots

Stars stand for sunflower, circles for triticale, triangles for *Festuca rubra* and squares for clover. Plain symbols are for monoculture, empty ones for mixed crops.

4 DISCUSSION

Plants grown on the contaminated soil poor in nutrients reacted in different ways to their environment depending on the plant species and on if they were grown as monocultures or in combination with each other's. Their biomass production was affected by the soil conditions, and vice versa the plants influenced the mobility of elements in their rhizosphere soil and water, by excreting organic substances and changing the pH. In particular, REE were dissolved, taken up and fractionated on their way from the soil solid phase over the soluble phase in the soil water to the plant roots and shoots. This particular fractionation is a clue for the specific action of plant roots in the soil.

4.1 Plant growth and biomass production

There were strong differences in the biomass of different plants. Clover grew very well, possibly due to its capacity to use nitrogen from the air in association with N_2-fixing bacteria. The biomass of other plants was much lower. They seemed to try to produce seeds as fast as possible (Wierzbicka and Panufnik, 1998) and showed much reduced growth, and also unhealthy colour in general, as the symptom of the conjugated effect of lack of some nutrients

and toxic doses of some metals. Clover did not show signs of disease until at least nine weeks after germination, where diverse parasites as fungi colonized the leaves. It could be that the poor soil but probably more the heavy metals present affected the resistance of the plant.

4.2 Plant influence on element mobility in soil and soil water

Plant growth influences soil pH, one important soil parameter for metal mobility and bioavailability. In the present study, the pH was more acidic in non-planted soil than in the rhizosphere soil. This trend was not expected, since the production of acids should decrease the pH. However, the production of organic acids was not detected, and other organic components may result in the same REE pattern without decreasing the pH, for instance by complexing effects. Since organic acids have a short life time in the rhizosphere - 6 to 12 h according to Marschner (2012), it could be that there were degraded despite of the precautions taken during sampling. The amounts should be locally, if a ratio solid to liquid of 1:10 is given, in the order of magnitude of 10 mg/L or more in order to be able to influence the REE pattern according to some previous elution experiments with organic acids. Other studies suggested that under a pH value of 5.5, to avoid possible toxic effects by high metal mobility, plants would stop producing acids (Dakora and Phillips, 2002). It is to remember that metals can be mobilized actively by plants, not only through the action of excreted organic acids, but also by the release of organic chelating agents as siderophores (Römheld and Marschner, 1986). These do not influence the pH of the medium.

It was particularly noticeable for *Trifolium* and in the polyculture. The dense root net of *Festuca* did not allow the soil to be separated into bulk and rhizosphere, therefore all soil was considered as rhizosphere. Indeed, the pH of soil planted with *Festuca* showed a higher pH. On the contrary, no significant pH differences were observed in the case of triticale. This could be due to the fact that there was very little soil adhering to the roots, which were poorly developed, so that the rhizosphere soil consisted in realty of a much smaller volume as really sampled.

The leaching with water showed the highest electrical conductivity and amounts of leached metals in the control samples. The mobile fraction is in fact more easily taken up by the plants and it is likely to be the first to decrease locally after plant growth, even if it is possible that root exudates mobilise less mobile fractions. The same difference could be observed between bulk soil and rhizosphere: there are less easily available metals in the rhizosphere. Among all plants, Triticale seemed to have to least effect on soil. This is quite expectable, since its root system was poorly developed. *Trifolium* had a big influence on the amounts of soluble metals in soil, and this effect was also visible and even stronger when it was planted together with other plants. *Festuca*, generally known to be an excluder plant, showed the lowest amounts in soluble metals; it could be that they become immobile in the rhizosphere of the plant; in fact, the amounts of soluble elements were equivalent or lower than those found in the control. So, for instance Al, Zn and especially Ni and Cd were found in lower amounts in the soluble fraction of the soil influenced by *Festuca* than in the control. Generally, the plant consortium showed higher amounts of soluble metals than monocultures. Phosphorus is not detectable in the water soluble fraction of the soil. Though, some is found in the leaves and roots. This should be due to some active mechanism of the plants, which can mobilize P from less mobile fractions of the soil. One possibility is that some acids mobilise P from the soil, as suggested by Cheng et al. (2004) and Shen et al. (2005). However, we found a slightly elevated pH in

the rhizosphere, so that an enzyme activity of phosphatase by the plant or possible associated microorganisms seems to be more probable (van Aarle and Plassard, 2010).

Even though depletion of labile (i.e. the easily dissolved part) metal pools in the rhizosphere of hyperaccumulator plants often has been found to be associated with sustained or even enhanced solubility (i.e. soil solution concentration) direct evidence for mobilization of metals, either due to acidification (Bernal et al., 1994; Li et al., 2003; McGrath et al., 1997) or induced by root exudation (Salt et al., 2000; Zhao et al., 2001) has not been really put in evidence so far. Nevertheless, the decrease of Ni in the rhizosphere of the hyperaccumulator plant *Thaspi goesingense*is for instance was clearly related to excessive Ni uptake and consistent with previous field observations (Wenzel et al., 2003). The interactions of organic acids released by roots with the soil solid phase appeared to be among the key processes (Puschenreiter et al., 2005). In particular, the authors suggest that root activities of accumulators such as the exudation of organic acids triggered the replenishment of soluble Ni from immobile metal fractions of the soil.

4.3 REE fractionation and specificity of the HREE enrichment

The use of the REE fractionation could lead to an explanation for the on-going processes. However, many processes can modify REE patterns. Generally, the chemical behaviour of REE is known to be strongly related to the one of Al and Fe especially as Al- and /or Fe(hydr)oxides. In acid environment, Ln (Lanthanides) occur as $LnSO_4^+$ or as Ln^{3+}. If there are no ligands in the solution, that keep HREE dissolved, they are mostly bound by oxy-hydroxides (Aström, 2001). Indeed, in the present case REE seem to follow the same trend as Al, being depleted in the soluble fraction in the rhizosphere. The REE patterns of the rhizosphere soil compared to bulk soil show a HREE enrichment. The same is for the soil water sampled near the roots if normalised to the control pot. There are different processes that can cause this fractionation: for instance, Fe-oxyhydroxides precipitating scavenge preferably LREE (Steinmann and Stille, 2008) and result in a relative HREE enrichment compared to LREE in the remaining Al and Fe oxide particle in the colloidal fraction of the soil solution. In particular, compared to the other REE, the elements La, Gd, and possibly Lu show a significantly lower affinity for the Fe-oxy-hydroxides (Bau, 1999). HREE on the other side tend to bind to Al (Lei et al., 2008). If we relate this to the leaching experiment with organic acid mixture, and given that that this enrichment is not present in bulk soil if normalised to PAAS, we can assume that this is an indication for the presence of organic acids in the close proximity of the roots. HREE enrichment can furthermore come from binding to carbonates (Pourret et al., 2008). In our case though, since the pH is acidic, it is not likely that this is the reason for the HREE enrichment. Further, Ce (in case of alkaline water) and LREE bind preferentially to humic acids, the more alkaline the medium the stronger the fractionation. The presence of organic acids as citric, malic, tartaric acid increased desorption of REE. High concentrations of humic acids in the solution increases the adsorption of LREE on kaolin (Wan and Liu, 2006). This results in a negative Ce anomaly and HREE enrichment in the liquid dissolved phase. Nevertheless, this effect is limited and probably not visible in an acid environment. In other studies, it was found that HREE enrichment in biofilms in natural waters is possibly due to the presence of phosphate sites, and that generally HREE enrichment can possibly be found in any phases containing phosphate sites (Takahashi et al.,

2010). However, on the present site phosphorus plays a very little role and so cannot explain the observed HREE enrichment in the soil.

Therefore it is reasonable to connect the HREE enrichment to an effect of organic components excreted by plant roots. In effect, adsorption by organic substrates can produce heavy REE enrichments in water relative to the LREE, Gd solution enrichments relative to Eu, and, at relatively low carbonate ion concentrations, enrichments of very light REE compared to their immediate neighbours according to the work of (Stanley and Byrne, 1990). Stern et al. (2007) further reported that REE binding to humic substances may display a regular increase from La to Lu. This enrichment can therefore be considered as characteristic for mobilisation of REE by organic acids or other organic components not be detected by ion chromatography, since other parameters as soil composition are equal, and the decrease of pH between water and very acid sulphuric acid did not change in this way the qualitative appearance of the pattern. As a consequence, the signature given by the REE shows the importance of some organic components present in the rhizosphere even though these cannot be detected anymore, thereby overcoming the analytical short comes.

4.4 Metal uptake and REE patterns in plants

The major part of the REE which are taken up by the plant remains in the roots, only 10% is translocated to the shoots, which is expectable for non-accumulator plants. It was also observed that generally HREE are less translocated than LREE. The same was observed for many metals, as Al, Fe, and most of the contaminants. Only Mn had a translocation factor of over 1, and additionally Cr and Zn in the case of *Trifolium* (results not shown).

The REE pattern of root normalized to PAAS reflects the general pattern found in soil again, with a clear Ce positive anomaly and an enrichment of MREE. The difference for the REE patterns is more marked between shoots and roots than between plants, with for instance a systematic HREE depletion in shoots compare to roots, due to a different transport in the plant. There is no comparison possible with previous studies for the fractionation from soil water to roots, because of the lack of data on normalisation to water. Since the pattern shoed a wavy structure, it showed that even if the REE coming into the plant were not corresponding exactly to those present in the soil solution, there was no preferential uptake to HREE or LREE.

REE can be also be traced within the plant from the roots to the shoots. We could observed that here also REE behave in a different way. There are too many effects to consider when studying the fractionation to and within plants, what explains the variety of different effects described in various articles, and their apparent contradiction. In some studies HREE were depleted in some cases, in other enriched. Enrichment of MREE has also been reported. Many effects depend on the plant part considered i.e. if all areal parts, or if the stems are separated from grains and leaves. Nevertheless, according to previous studies (Lonschinski, M., 2009), the fractionation of REE within the plant are comparable for same plants on a similar soil, the pattern still being dependent on the plant species. Few studies deal with the REE fractionation within plants, so there a few comparisons possible. Nevertheless, despite differences, the most common reported trend was LREE enrichment in the shoots, as well as the tetrad effect, which is not visible in our study. Further, Eu anomaly or HREE enrichment in leaves are

reported to occur, but were not observed here (Aouad et al., 2006; Liang et al., 2008; Semhi et al., 2009; Stille et al., 2006).

4.5 Effect of plant consortium

Mixed crop cultivation had a positive influence on the appearance of some plants: clover had bigger leaves; sunflower had shorter shoot growth but slightly healthier colour. So polyculture seems to have a positive impact on each plant. Furthermore, the concentrations of metals were lower at mixed crop cultivation than at single crop cultivation. This is especially the case for *Festuca*, *Trifolium* and Triticale. It was also noticeable that the total REE amounts per g dry biomass were lower at mixed crop cultivation (Figure 2, Tableb), except for *Festuca*, which is taking up more metals as polyculture.

Plants seem to take up less metal if grown in a community. Plant can profit from protection mechanisms of another plant, or of their better nutrient uptake system. For instance, sunflower can profit from the denser root net given by red fescue that would hold water and retain metals, and so diminish toxic effects of metals and also its access to nutrients and consequently reduce growth. Similarly, clover can grow better and produce bigger leaves if the combination of plants can protect it against metal stress. So, even if the biomass production of *Festuca* and *Helianthus* is better if grown as monoculture, it seems that their health is still affect by the neighbour plants. It has been reported in effect that plants can influence each other's nutrient uptake, as for example peanut facilitates P nutrition of maize and barley, while maize and barley improve K, Fe, Zn and Mn nutrition (Inal and Gunes, 2008).

5 CONCLUSION

Plants influence the rhizosphere zone by changing its pH and the amounts of soluble trace elements, i.e. their mobility in soil and their uptake. Plants are able to change the soil properties and many other influencing factors concerning metal mobility. Here we consider one of the possible mechanisms, the exudation of organic compounds, especially organic acids. The interaction between plants grown as a consortium is also influencing the amount of metals taken up, mainly by protecting each other's from toxic effects of excessive metal uptake. In particular, REE are mobilised and taken up. The present plants do translocate only a tenth of the up taken metal into the shoots, with a preference for LREE. The fractionation observed between soil and soil water with a preferential dissolution of HREE is a hint for the action of organic substances excreted by plant roots in the rhizosphere zone. Other influences like the action of microorganisms cannot be excluded. Therefore, the REE signature is a method to be considered to detect the influence of some organic components in the rhizosphere, overcoming the analytical limitations.

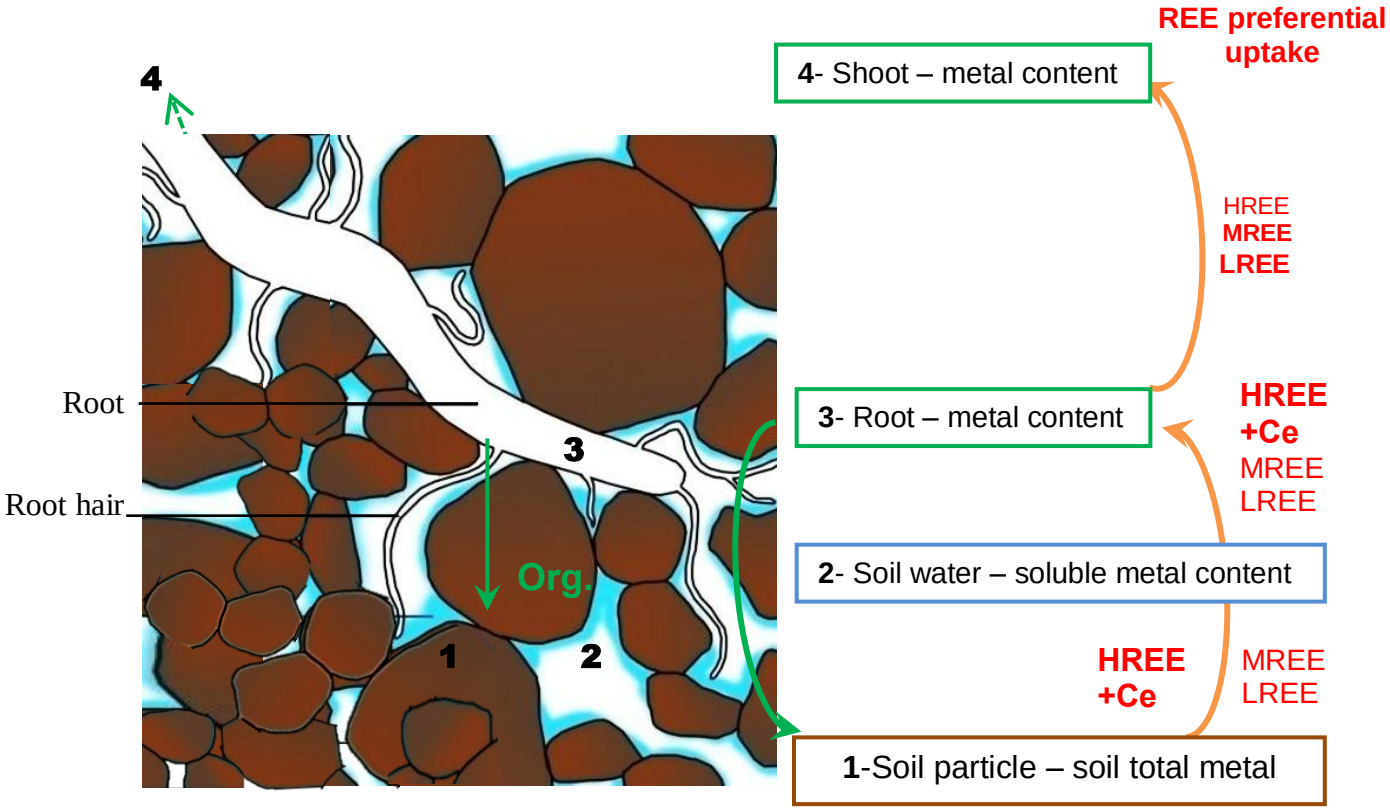


Figure 10: Overview over the mechanisms influencing REE fractionation and metal uptake in the rhizosphere

REFERENCES

- Aouad, G., Stille, P., Crovisier, J.-L., Geoffroy, V.A., Meyer, J.-M., Lahd-Gaegea, M., 2006. Influence of bacteria on lathanide and actinide transfer from specific soil components (humus, soil minerals and vitrified municipal waste incinerator bottom ash) to corn plants: Sr-Nd isotope evidence. Sci. Total Environ. 370, 545-551.
- Aubert, D., Stille, P., Probst, A., 2001. REE fractionation during granite weathering and removal by waters and suspended loads: Sr and Nd isotopic evidence. Geochim. Cosmochim. Ac. 65, 387-406.
- Bau, M., 1999. Scavenging of dissolved yttrium and rare earth by precipitating iron oxyhydroxide: Experimental evidence for Ce oxidation, Y-Ho fractionation, and lanthanide tetrad effect. Geochim. Cosmochim. Ac. 63, 67-77.
- Blume, H.-P., Brümmer, G., 1991. Prediction of heavy metal behaviour in soil by means of simple field tests. Ecotox. Eviron. Safe. 22, 164-174.
- Bradl, H.B., 2004. Adsorption of heavy metal ions on soils and soils constituents. J. Colloid Interf. Sci. 277, 1-18.
- Cheng, Y., Howieson, J.G., O'Hara, G.W., Watkin, E.L.J., Souche, G., Jaillard, B., Hinsinger, P., 2004. Proton release by roots of Medicago murex and Medicago sativa growing in acidic conditions, and implications for rhizosphere pH changes and nodulation at low pH. Soil Biol. Biochem. 36, 1357-1365.
- Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245, 35-47.
- Díaz-Barrientos, E., Madrid, L., Cardo, I., 1999. Effect of flood with mine wastes on metal extractability of some soils of the Guardiamar river basin (SW Spain). Sci. Total Environ. 242, 149-165.
- Ding, S., Liang, T., Zhang, C., Yan, J., Zhang, Z., 2006. Effects of organic ligants on fractionation of rare earth elements (REEs) in hydroponic plants: an application to the determination of binding capacities by humic acid for modeling. Chemosphere 65, 1942-1948.
- Grayston, S.J., Vaughan, D., Jones, D., 1996. Rhizosphere carbon flow in tees, in comparison with annual plants: the imporance of root exudation and its impacts on microbial activity and nutrient availability. Appl. Soil Ecol. 5, 29-56.
- Gryschko, R., Kuhnle, R., Terytze, K., Breuer, J., Stahr, K., 2004. Soil extraction of readily soluble heavy metals and As with 1 M NH4NO3-solution - Evaluation of DIN 19730. J. Soils and Sediments.
- Inal, A., Gunes, A., 2008. Interspecific root interactions and rhizosphere effects on salt ions and nutrient uptake between mixed grown peanut/maize and peanut/barley in original saline-sodic-boron toxic soil. J. Plant Physiol. 165, 490-503.
- Jakubick, A., Jenk, U., Kahnt, R., 2002. Modelling of mine flooding and consequences in the mine hydrogeological environment: Flooding of the Koenigstein mine, Germany. Environ. Geol. Water. S. 42, 222-234.
- Kahlert, J., 1992. Wismut und die Folgen des Uranbergbaus : eine Tagung der Friedrich-Ebert-Stiftung am 19. Juni 1992 in Gera, Wirtschaftspolitische Diskurse, Electronic edition ed, Bonn, p. 44.
- Karim, M.A., Khan, L.I., 2001. Removal of heavy metals from sandy soil using CEHIXM process. J. Hazard. Mater. 81, 83-102.
- Kidd, P., Juan, B., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monteroso, C., 2009. Trace element behaviour at the root-soil interface: implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.
- Lange, G., 1995. Die Uranlagerstätte Ronneburg. Z. Geol. Wissenschaft. 23, 517-526.
- Lei, L.-q., Song, C.-a., Xie, X.-l., Li, Y.-h., 2008. REE behavior and effect factors in AMD-type acidic groundwater at sulfide tailings pond, BS nickel mine, W.A. T. Nonferr. Metal Soc. 18, 955-961.
- Liang, T., Ding, S., Song, W., Chong, Z., Zhang, C., Li, H., 2008. A review of fractionations of rare earth elements in plants. J. Rare Earths 26, 7-15.
- Lonschinski, M., 2009. Schwermetalle im System Boden-Wasser-Pfl anze mit Hinblick auf Fraktionierungsprozesse der Seltenen Erden Elemente auf der Aufstandsfläche der

Gessenhalde im ehemaligen Uranbergbaurevier Ronneburg (Ostthüringen), Chemisch-Geowissenschaltliche Fakultät. Friedrich-Schiller-Universität, Jena, p. 205.
- López-Bucio, J., Nieto-Jacobo, M.F., Ramírez-Rodríguez, V., Herrera-Estrella, L.R., 2000. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci. 160, 1-13.
- Marschner, H., 2005. Mineral Nutrition of Higher Plants, second ed. Academic Press.
- Marschner, P., 2012. Chapter 15 - Rhizosphere Biology, in: Petra, M. (Ed.), Marschner's Mineral Nutrition of Higher Plants (Third Edition). Academic Press, San Diego, pp. 369-388.
- McLennan, S.M., 1989. Rare earth elements in sedimentary rocks; influence of provenance and sedimentary processes, Reviews in Mineralogy and Geochemistry: Geochemistry and mineralogy of rare earth elements, pp. 169-200.
- Merten, D., Geletneky, J., Bergmann, H., Haferburg, G., Kothe, E., Büchel, G., 2005. Rare earth element patterns: A tool for understanding processes in remediation of acid mine drainage. Chem. Erde - Geochem. 65, 97-114.
- Mirgorodsky, D., Ollivier, D., Merten, D., Büchel, G., Willscher, S., Wittig, J., Jablonski, L., Werner, P., 2010. Phytoremediation experiments on a slightly contaminated test field of a former uranium mining site "Mine Water & Innovative Thinking", IMWA 2010, September 05.-09., 2010, Sydney/Canada, pp. 587-590.
- Paas, N., 1997. Untersuchungen zur Ermittlung der geochemischen Barriere von Gesteinen aus dem Umfeld untertägiger Versatzräume im Steinkohlenbergbau des Ruhrkarbons. Rheinische Friedrich-Wilhelms-Universität, Bonn, p. 234.
- Pourret, O., Davranche, M., Gruau, G., Dia, A., 2008. New insights into cerium anomalies in organic-rich alkaline waters. Chem. Geol. 251, 120-127.
- Puschenreiter, M., Schnepf, A., Millán, I.M., Fitz, W.J., Horak, O., Klepp, J., Schrefl, T., Lombi, E., Wenzel, W.W., 2005. Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. Plant Soil 271, 205-218.
- Rabiet, M., Brissauda, F., Seidela, J.L., Pistrea, S., Elbaz-Poulicheta, F., 2009. Positive gadolinium anomalies in wastewater treatment plant effluents and aquatic environment in the Hérault watershed (South France) Chemosphere 75, 1057-1064.
- Römheld, V., Marschner, H., 1986. Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. Plant Physiol. 80, 175-180.
- Semhi, K., Chaudhuri, S., Clauer, N., 2009. Fractionation of rare-earth elements in plants during experimental growth in vaired clay substrates. Appl. Geochem. 24, 447-453.
- Shen, J., Li, H., Neumann, G., Zhang, F., 2005. Nutrient uptake, cluster root formation and exudation of protons and citrate in *Lupinus albus* as affected by localized supply of phosphorus in a split-root system. Plant Sci. 168, 837-845.
- Sipos, P., Póka, T., 2002. Threshold limit values for heavy metals in soils in the function of spacial and temporal variation of geochemical factors, XVII. Congress of CBGA. Special Issue of Geologica Carpathica (CD), Bratislava.
- Stanley, J.K.J., Byrne, R.H., 1990. The influence of solution chemistry on REE next term uptake by *Ulva lactuca L.* in seawater. Geochim. Cosmochim. Ac. 54, 1587-1595.
- Steinmann, M., Stille, P., 2008. Controls on transport and fractionation of the rare earth elements in stream water of a mixed basaltic-granitic catchment basin (Massif Central, France). Chem. Geol. 254, 1-18.
- Stern, J.C., Sonke, J.E., Salters, V.J.M., 2007. A capillary electrophoresis-ICP-MS study of rare earth element complexation by humic acids. Chem. Geol. 246, 170-180.
- Stille, P., Steinmann, M., Pierret, M.-C., Gauthier-Lafaye, F., Aubert, D., Probst, A., Viville, D., Chabaux, F., 2006. The impact of vegetation on fractionation of rare earth elements (REE) during water-rock interaction. J. Geochem. Explor. 88, 341-344.
- Takahashi, Y., Yamamoto, M., Yamamoto, Y., Tanaka, K., 2010. EXAFS study on the cause of enrichment of heavy REEs on bacterial cell surfaces. Geochim. Cosmochim. Ac. 74, 5443-5462.
- van Aarle, I.M., Plassard, C., 2010. Spatial distribution of phosphatase activity associated with ectomycorrhizal plants is related to soil type. Soil Biol. Biochem. 42, 324-330.

- van Hees, P.A.W., Vinogradoff, S.I., Edwards, A.C., Godbold, D.L., Jones, D.L., 2003. Low molecular weight organic acid adsorption in forest soils: effects on soil solution concentrations and biodegradation rates. Soil Biol. Biochem. 35, 1015-1026.
- Wan, Y., Liu, C., 2006. The effect of humic acid on the adsorption of REEs on kaolin. Colloid Surface A 209, 112-117.
- Wierzbicka, M., Panufnik, D., 1998. The adaptation of *Silene vulgaris* to growth in a calamine waste heap (S. Poland). Environ. Pol. 101, 415-426
- Zeien, H., Brümmer, G.W., 1989. Chemische Extraktion zur Bestimmung von Schwermetallbindungsformen in Böden. Mitt. Dt. Bodenkundl. Ges., 505-510.

Chapter 3

CHARACTERISATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM *FESTUCA RUBRA* AND *TRIFOLIUM PRATENSE* GROWN ON HEAVY METAL CONTAMINATED SOIL

Tsilla Boisselet[1,2], Jaco Vangronsveld[2], Nele Weyens[2], Georg Büchel[1]

[1] Institute for Geosciences, Friedrich Schiller University, Burgweg 11, 07743 Jena, Germany
[2] Centre for Environmental Sciences, Hasselt University, Agoralaan 1, 3590 Hasselt, Belgium

The present chapter is the manuscript of an article to be submitted.

Chapter 3: Characterisation and identification of endophytic bacteria from *festuca rubra* and *trifolium pratense* grown on heavy metal contaminated soil

ABSTRACT

The diversity of endophytic bacteria found in association with two plant species, red fescue (*Festuca rubra*) and red clover (*Trifolium pratense*) was investigated as part of a project to estimate the possibility of using endophytic bacteria to improve in situ phytoremediation of heavy metal contaminated soils. Endophytic bacteria were isolated from roots, stems and leaves of *Trifolium* plants and from shoots and roots of *Festuca* plants growing on a site contaminated with heavy metals.

They were further characterised genotypically by comparative sequence analysis of partial 16S rRNA genes genomic DNA fingerprinting, and phenotypically for their tolerance to a range of relevant heavy metals and their capacity of producing plant growth influencing substances. 78 stable, morphologically distinct isolates were obtained, belonging to 32 genera. 12 isolates could not be identified.

The endophytic bacteria showed clear spatial compartmentalisation within the plant, suggesting that specific associations with plant organs exist and also that the endophytes can be taken up following a different mechanism for each compartment.

A number of the isolated strains showed characteristics that can potentially promote plant growth and resistance to the metals present in the soil. These properties might be of interest for exploiting these mutualisms for remediation purposes. This study demonstrates that within the diverse bacterial communities found in autochthonous *Trifolium* and *Festuca* plants, several endophytic strains occur that have the potential to support phytoremediation strategies.

1 INTRODUCTION

Since many years, bacteria were shown to occur within plant tissues (Tervet and Hollis, 1948) although pathogenicity was believed to be their main role. Later, studies revealed that certain bacteria living within these tissues had no negative and even beneficial effects for their host. (Bashan, 1998; Davison, 1988). These endophytic bacteria are defined as bacteria residing within living plant tissues without causing substantial harm to their host. Some of them are very host-specific; others are more flexible. Root colonisation is a complex procedure involving several steps (Badri et al., 2009), and establishment in plant tissues includes several complex mechanisms (Reinhold-Hurek and Hurek, 2011).

Endophytes can be subdivided in fungi and several bacterial phyla, including Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes, further subdivided in at least 82 genera (Lodewyckx et al., 2002). They are found in various plant tissues, inside or outside the cell membrane, ranging from roots, stems, leaves and seeds (Madmony et al., 2005; Rajkumar et al., 2009; Reinhold-Hurek and Hurek, 2011; Schulz and Boyle, 2005). Some are known to be obligate endophytes and are transmitted from one generation to the next through the seeds (Majewska-Sawka and Nakashima, 2004; Mastretta et al., 2009).

Endophytes were found in almost all plant species, and typically investigated in agricultural relevant plant species, although an increased interest is now given to phytoremediation plants, as metal hyperaccumulators (Mengoni et al., 2010; Weyens et al., 2009b).
Endophytic bacteria can improve plant growth using different processes: they can improve plant nutrition by nitrogen fixation (Badri et al., 2009; Weyens et al., 2009a), and by mobilisation of low soluble phosphates through the production of organic acids and iron through the release of siderophores.
Bacteria can also enhance the growth of plants by the production of specific plant growth regulators such as auxins, cytokinins and gibberellins, suppression of the production of stress ethylene by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, and alteration of sugar sensing mechanisms in plants (Weyens et al., 2009b).
Furthermore, microorganisms have an effect on the metal uptake by plants. For example, *Pseudomonas aeruginosa* forms biofilms and allows complexation of REE; furthermore, this species is able to extract Fe and Mg (Aouad et al., 2006). These properties together with metal resistance mechanisms are of interest for the use of these mutualisms for remediation purposes. An important aspect of metal uptake is driven by the production of siderophores, which can make Iron(III)-hydroxide available for reduction to Fe^{II}; this is crucial especially in alkaline soils with low Fe availability (Kidd et al., 2009; Weyens et al., 2009a). Siderophores can both enhance and prevent uptake of metals by plants, depending on the present metals. They bind free metals, and consequently change the available metal concentration and protect the plants from metal stress. Siderophores are generally quite specific for Fe, but also Al and other metals (Cd, Ni, Mn, Co, Zn) can be transported in some cases (Dimkpa, 2009; Kidd et al., 2009). Endophytic bacteria can also indirectly benefit plant growth by preventing the growth or activity of plant pathogens (Badri et al., 2009; Weyens et al., 2009a) through competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins, and through induction of plant defence mechanisms (Hardoim et al., 2008; Pavlo et al., 2011). Indirectly, bacteria like *Pseudomonas* can act as biocontrol agent by starving pathogens of iron through production of siderophores (Dimkpa, 2009; Kloepper et al., 1980).
The first step to consider for the success of plant growth promotion, especially with regard to phytoremediation of sites contaminated with heavy metals, is assessing the diversity and distribution of the natural endophytic population, followed by their phenotypic and genotypic characterisation and selection of suitable endophytic bacteria in candidate plants appropriate for phytoremediation purposes (Porteous Moore et al., 2006).
If more has been studied about endophytes in agricultural production plants (Davison, 1988), less is known about the associated endophytic populations from grassland plants.
This paper describes the diversity and spatial distribution of endophytes found in the autochthonous plant species, *Trifolium pratense* and *Festuca rubra* growing at a phytoremediation field trial site contaminated with a range of different heavy metals as contaminants. The aim was to select some potential candidates, which can promote growth of plants on contaminated soil, and possibly also influence the metal uptake in order to enhance phytoremediation.
In view of the large number of bacterial species reported as endophytes in the literature, a likewise broad diversity was supposed to be present in the plants and also that different compartments of the plant could be inhabited by different bacterial species/communities.

The isolated endophytic bacteria were characterised by comparative sequence analysis of partial 16S RNA genes, physiological characterisation, heavy metal resistance and potential plant growth promoting properties (production of IAA, organic acids and siderophores).

2 MATERIALS AND METHODS

2.1 Collection and handling of samples

Red clover (*Trifolium pratense*) and red fescue (*Festuca rubra*) were grown during 8 weeks on a contaminated soil taken from the test field "Gessenwiese" in the former uranium mining site in Ronneburg, Thuringia, Germany (*see introduction and chapter 2*).

Isolation protocol

The plants were separated into roots and shoots, and for clover the shoots were additionally separated into leaves and stems. About 5 plants were pooled to compose 1 sample. Each of these fractions was treated as follows: (1) the plant material was put into 1% hypochlorite solution for counted time, optimised for each plant organ (Table 1), in order to sterilise the surface; (2) next it was washed by putting it in three successive petri dishes containing sterile deionised water; (3) subsequently, the excess water was removed by disposing the plant parts onto sterile filter paper.

The plants were cut into 2 mm pieces, and put into 5 mL of a sterile 10 mM $MgSO_4$ solution. The fresh weight was determined in order to allow an estimation of the number of endophytes in the plant per unit of weight. The suspension was mixed for 1min. Different dilutions were made from this suspension depending on the plant fraction (Table 1)

100 µL of the last washing water were plated onto a 869 rich medium, in order to verify if the surface sterilisation was sufficient, and 100 µL of all dilutions were plated onto 1:10 869 medium and grown for 7 days at 28 °C.

The procedure was done in 5 independent replicates.

Table 1: Overview over the plant fraction and the corresponding chosen surface sterilisation conditions and plating conditions

Plant fraction	Optimised sterilisation time	Dilutions
Trifolium root (TR)	6 min	10^0 till 10^{-4}
Trifolium stem (TSt)	5 min	10^0 till 10^{-3}
Trifolium leaf (TL)	1 min	10^0 till 10^{-2}
Festuca root (FR)	5 min	10^0 till 10^{-4}
Festuca leaf (FL)	4.5 min	10^0 till 10^{-3}

2.2 Purification

From all plates, the ones with the most distinguishable single colonies were used, about four per plant organ. The colonies were counted and separated into visibly different strains. Each strain was numbered, described and if possible five colonies of the same strain were chosen to be grown as pure culture on 1:10 rich medium agar. From each pure culture a glycerol stock (2 tubes) was made from a liquid re-cultivation of the strain. The glycerol stocks were frozen at -70°C for permanent storage, or -20°C for regular re-use. This stock was used for all following steps.

2.3 DNA extraction and PCR amplification

For the 16S rDNA analysis, genomic DNA was extracted using the Qiagen DNA extraction kit and 16S rDNA was amplified in a PCR using the genomic DNA as template and bacterial universal primers, 25f (5'-AGAGTTTGATCCCTGGCTC-3') and 1392r (5'-ACGGGCGGTGTGTRC-3') (Lane, 1991). The PCR mixture (50 µl) contained 1 µl template, 5 µl of 10xHigh fidelity PCR buffer, 2 µl of 50 mM $MgCl_2$, 1 µl of dNTP at 10 mM, 0.2 µl of Platinum Taq High fidelity DNA polymerase, and 1 µL of 10 mM primers, each.
The PCR was performed in a Mastercycler gradient (Eppendorf) with a hot start performed at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 0.5 min, and 72°C for 3 min, followed by a final extension performed at 72°C for 10 min.
The amplification products were purified using a DNA purification kit (Qiagen). The DNA content was measured on NanoDrop® Spectrophotometer ND-100 (Isogen-Lifescience).

2.4 DNA sequencing

Sequencing was performed by Macrogen Europe Laboratory (Amsterdam, The Netherlands). The 16S rDNA sequence was compared against the GenBank database using the NCBI Blast program (Zhang et al., 2000).

2.5 Genomic DNA fingerprinting

The amplified 16S rDNA was digested with the restriction enzyme HpyCH4 IV (5 units/reaction) to obtain a profile used for fingerprinting and comparing the strains.
20 µL of the PCR product were taken, and added to a reaction mixture composed of 3.6 µL buffer 1 (10x conc), 0.5 µL of HpyCH4 IV, 1.5 µL RNAse (1%) and 5 µL of DNA free water. The samples were run on a 1.5% agarose gel with GelRed.

2.6 Phenotypic characterisation

Organic acid production was tested according to the method of (Cunningham and Kuiack, 1992). The bacteria to be tested were taken from the glycerol stock (20 µL) and grown in 10 mL liquid rich medium. 20 µL of the microbial suspension were introduced in microplate wells each containing 800 µL of Sucrose Tryptone (ST) medium (which stimulates organic acid production). The microplates were incubated at 28°C and 130 rpm for 7 days. After incubation, 100 µL of alizarine red S (Sigma) reagent (0.1 %) were added. After 15 minutes, the yellow wells were considered as positive for organic acid production. Control wells (not inoculated) were pink after reagent supply.
The test for Siderophore Production was done after the method of (Schwyn and Neilands, 1987). A chrome azurol S (CAS) shuttle solution was used for routine testing of siderophore production in liquid media. This test was carried out using liquid medium 284 in microplates.
The bacteria to be tested were taken from the glycerol stock (20 µL) and grown in 10 mL liquid rich medium. 20 µL of the microbial suspension were introduced in microplate wells each containing 800 µL of 284 medium (minimal medium which stimulates siderophore production). The test was carried out using both minimal medium without iron and with minimal medium with 0.25 pM Fe(III) citrate. The microplates were incubated at 28°C and 130 rpm for 7 days. After incubation, 100 µL of the blue Chromium-Azurol S (CAS) reagent were added. After 4 h the orange wells are considered positive. The plates with 284 Medium + Fe were taken as controls. One sample was orange even if Iron was present in the medium; the siderophore production can be so high that it removes even iron present in large amounts.

The production of Indole-3-Acetic acid (IAA) was induced by the presence of tryptophan in the liquid medium. The presence of IAA was tested in the supernatant of the 6 days old bacterial culture with Salkowski reagent.

Tests for metal resistance

The strains were plated from the liquid culture in rich medium onto the metal-containing plates, and incubated at 28°C for 10 days. As a control, the microorganisms were also plated on medium without metal contamination. Tested metals, their chemical form and their concentrations are listed in Table 2.

Table 2: Elements tested for the metal resistance tests with their chemical form and their concentrations.

Element	Chemical form	Test 1	Test 2
		$mMol_{element}/L$	
Ni	$NiCl_2.6H_2O$	1	5
Zn	$ZnSO_4.7H_2O$	1	5
Al	$Al_2(SO_4)_3.18H_2O$	2	-
Cd	$CdSO_4.8H_2O$	2	5
Mn	$Mn(NO_3)_2.4H_2O$	20	40

For test 1 all colonies were tested separately for Ni, Zn, Cd, Mn and Al. For Ni, Zn, Cd and Mn, only the colonies growing at lower concentration were taken for the higher concentration. The so called lower concentrations were corresponding more or less to the MIC values of *E.Coli.*

3 RESULTS

3.1 Isolation of endophytic bacteria

From the selection of morphologically different colonies from cultures inoculated with plant tissue material, 42 isolates were obtained from *T. pratense* (root–13, stem–14, leaves–15), and 35 from *F. rubra* (root–18, leaves–17). A total of 276 colonies including the replicates were used for further study.

Some strains showed to be difficult to cultivate on media and were therefore removed from further study. The reason is that the strains with potential for plant growth promoting effect and further biotechnology applications need to be easy to handle and therefore cultivable.

3.2 Phenotypic characterisation

Many of the endophytic bacterial strains showed the capacity to produce siderophores, organic acids and IAA in culture when the medium was supplemented with L-tryptophan.

One sample (isolate 75) turned the reagent orange even if Fe was present in the medium; the siderophore production could be so high that it removes even Fe present in large amounts.

We found that 140 out of the 280 (50%) tested isolates showed potential for siderophore production, while 174 (62%) had potential for IAA production and 78 (28%) isolates could produce organic acids (see annex). If this potential is expressed under environmental conditions (pH 3.7-5.0 in the 'Gessenwiese' soil, vs. pH 7 in test medium), siderophore production may be of importance for plants in contaminated soils.

Heavy metal resistance of the strains

Several strains were found to possess resistance to multiple heavy metals. The strain 29 from *Festuca* roots showed to be resistant to 5 mM Ni, 5 mM Zn and 40 mM Mn, strains 36b and 47b from *Trifolium* stems were resistant to all metals at lower concentrations (1 mN Ni, 2 mN Al, 2 mM Cd), and 5 mM Zn and 40 mM Mn. However, the isolates possessing the broadest range of resistances were identified to be fungi, isolated from leaves of both plant species.

Strains resistant to higher concentrations of Zn were found spread in al tissues, except in stems of *Trifolium*.

Strains resistant to 2 mM Cd were found in all plant tissues except of roots of *Trifolium*, though the strains resistant to 5 mM Cd were found only in leaves of both plants.

Therefore, it seems that the most resistant strains were not found in the roots, although the exposure could be expected in this organ of the plant. Exposure depends on ‚internal availability' and thus speciation and subcellular localisation of the metals.

3.3 Genomic DNA fingerprinting

The profiling showed that visibly similar strains can have different DNA fingerprints, and vice versa. In total, we found that based on their digestion profile there were 80 different strains isolated from the different tissues of the two investigated plant species. These were sent for sequencing.

3.4 16S rDNA sequence analysis

The identified strains are listed in Table and their amplified DNA sequences represented according to their similarity with each other's and with selected type strains as a phylogenetic tree (Figure 1). Some strains were amplified only with the reverse primer, and the reverse complement sequence of the amplicon was used for the tree. The bacterial populations associated to *Festuca* and *Trifolium* identified in this study is dominated by the genera *Stenotrophomonas, Pseudomonas, Cellulomonas, Frateuria/Dyella, Burkholderia* sp., *Enterobacter/Pantoea, Agrobacterium/Rhizobium, Olivibacter, Dyella, Curtobacterium, Sphingomonas* and *Curtobacterium* (Table 3).

Figure 1: Phylogenetic tree of all sequenced strains compared to reference strains

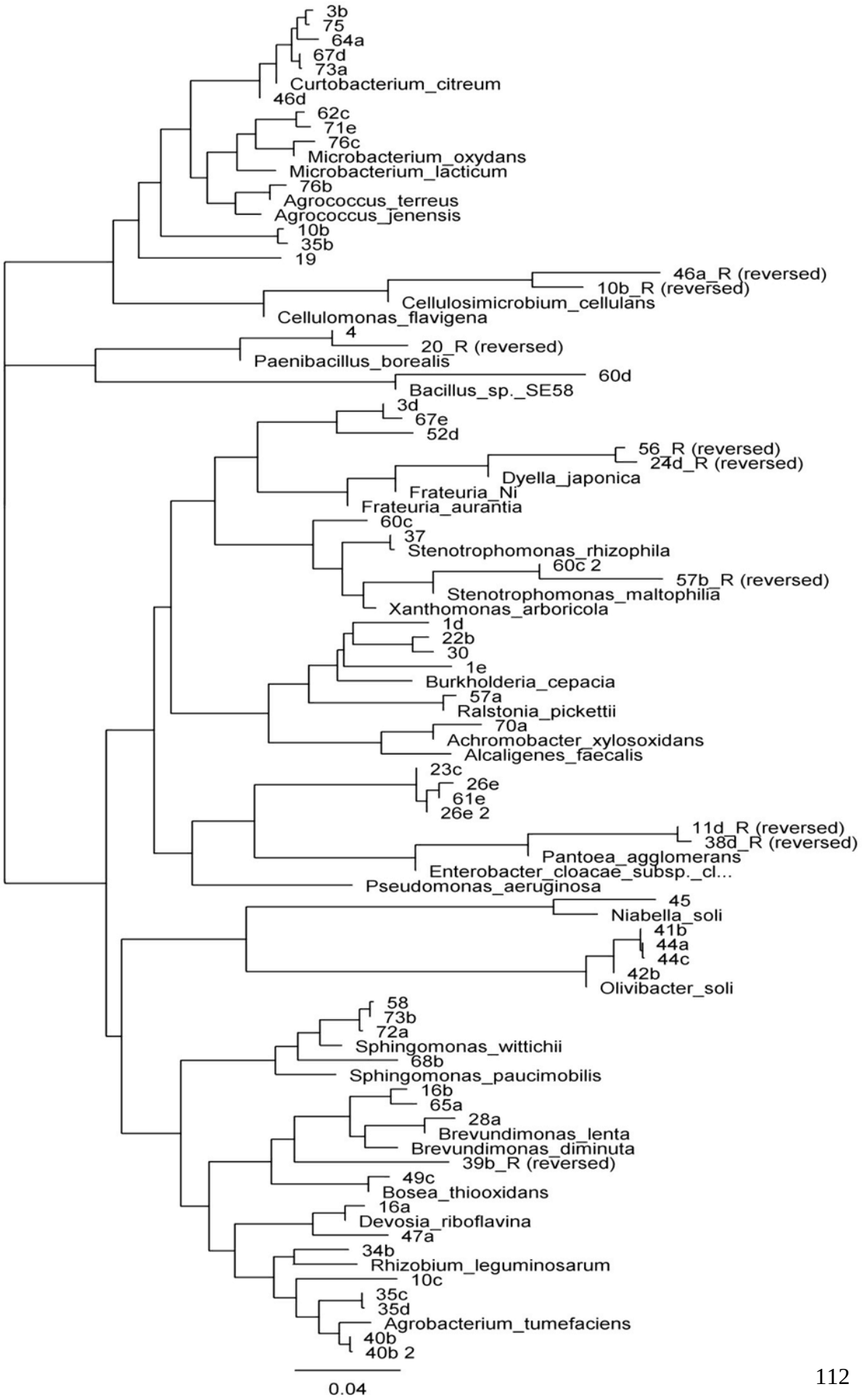


Table 3: Partial 16S rDNA sequence identification of bacterial isolates from *Trifolium pratense* and *Festuca rubra*

Isolate no	NCBI most similar strains	Accession no
1d	*Burkholderia* sp.	GU731239.1
1e	Uncultured bacterium, related to *Pandoraea*	HQ015293.1, AY268174.1
3d	*Frateuria*	EU170476.1
3b	*Curtobacterium*	JQ638288.1, HE575940.1, HE613377.1
4	*Bacillus* or *Paenibacillus*	AM745263.1
6d	*Enterobacter (cloacae)*	HQ231214.1
10b	*Cellulosimicrobium / Cellulomonas*	HQ730482.1, JN257084.1, JQ659856.1
10c	*Aurantimonas?*	AB600138.1
11d	*Pantoea (agglomerans?)*	JQ614013.1
16a	*Devosia*	JQ291598.1
16b	*Brevundimonas*	JN863452.1
19	Uncult. bacterium, isolate BF0001B026 or HelTree1-170, related to *Nakamurella*, *Humicoccus*, *Frankinea*	AM697000.1, JF345516.1
20	*Paenibacillus*	HQ423410.1
22b	*Burkholderia* sp.	DQ490307.1
23c	*Enterobacter (cloacae, ludwigii) or Pantoea agglomerans*	JQ640581.1, JQ308612.1, HQ236088.1
24d	*Dyella / Frateuria / Rhodanobacter*	GQ369135.1, EU170476.1, FJ938157.1
26e	*Enterobacter*, *Klebsiella*, *Pantoea*, *Yokenella*	JQ389636.1
26e	*Enterobacter (*or *Pantoea)*	JQ640581.1
28a	*Brevundimonas*	GU188941.1
30	Uncultured clone, related to *Burkholderia* sp.	JF809163.1, DQ490307.1
34b	*Shinella /Rhizobium*	JQ659575.1, AY972354.1
35b	*Cellulosimicrobium (cellulans) / Cellulomonas*	X79456.1
35c	*Rhizobium / (Agrobacterium)*	AB461672.1, AM403584.1
35d	*Rhizobium / (Agrobacterium)*	AB461672.1, AM403584.1
37	*Stenotrophomonas (rhizophila or maltophila)*	JQ410475.1
38d	*Pantoea (agglomerans?) / Enterobacter?*	JN392855.1
39b	Uncultured bacterium clone, related to *Caulobacter*	JF189221.1, NR_041964.1
40b	*Rhizobium / Agrobacterium*	JQ419490.1, JQ072056.1
41b	*Olivibacter*	JF262931.1, NR_041503.1
42b*	*Olivibacter*	JF262931.1, NR_041503.1
44a	*Olivibacter*	JF262931.1, NR_041503.1
44c	*Olivibacter*	JF262931.1, NR_041503.1
45	*Niabella* sp.	FJ457040.1
46a	*Curtobacterium?*	JQ660320.1
46d	*Curtobacterium (herbarum?)*	JF460761.1
47a	*Devosia*	JN863525.1
49c	*Bosea*	FR749828.1
50d	*Burkholderia*	AB438046.1
52d	*Dyella*	FJ386567.1

56	*Dyella*	GQ181058.1
57a	*Ralstonia*	JQ073896.1
57b	*Stenotrophomonas/(Pseudomonas, Xanthomonas)*	JN705917.1
58	*Sphingomonas*	EU730907.1
60c	*Pseudomonas / Stenotrophomonas / Xanthomonas*	FJ380128.1, JF460769.1
60c	*Pseudomonas / Stenotrophomonas / Xanthomonas*	FJ380128.1, JF460769.1, HQ335356.1
60d*	*Bacillus*	JQ622632.1
61e	*Enterobacter / Pantoea*	JF772064.1, HQ236088.1
62c	*Microbacterium*	HM629344.1
64a	*Curtobacterium*	DQ086779.1
65a	*Brevundimonas*	GU003879.1
67d	*Curtobacterium*	JQ660320.1
67e	*Frateuria*	EU170476.1
68b	*Sphingomonas*	EU332828.1
70a	*Achromobacter*	JQ650538.1
71e	*Microbacterium*	JQ660092.1
72a	*Sphingomonas*	FJ938158.1
73a	*Curtobacterium*	JQ660320.1
73b	*Sphingomonas*	EU730907.1
75	*Curtobacterium*	JQ638297.1
76b	*Agrococcus (terreus?)*	JN585726.1
76c	*Microbacterium*	JN627994.1

(*short sequence, < 500 bp)

4 DISCUSSION

4.1 Phenotypic characterisation

Iron acquisition by phytosiderophores was reported to be one of the important factors that allows plants to cope with toxic metal concentrations (Meda et al., 2007). However, Römheld and Marschner (1986) pointed out that bacterial siderophores are not taken up in the same way as phytosiderophores. This could still be a factor for protection against metal toxicity, metals bound to ferrichromes being possibly taken up less easily.
It is difficult to estimate the metal resistance of microorganisms, since the effectively available amounts should be considered. These are significantly different in soil and water, but also in agar and liquid culture, even if the added amounts and chemical form are identical. Hence, it is not easy to estimate the amounts of metals to be used for testing. Therefore, metal contents in groundwater from the isolation site were compared to literature values. Depending on that, the values to be tested were evaluated, considering that resistance in agar is higher than in liquid environment.

Bacteria can be equipped by different heavy metal tolerance mechanisms, which involve exclusion, active efflux transport, enzymatic detoxification, biosorption, precipitation or bioaccumulation both intra- or extracellular (Guo et al., 2010; Nies, 1999; Rönkkö et al., 1993); and many of these mechanisms were discussed already for strains found at the study area (Schmidt et al., 2009; Schmidt et al., 2005). Nevertheless, it is interesting to note that some strains, which are not resistant, can grow near resistant ones, due to the fact that they produce substances that protect them against heavy metals. Chaudhary et al. (2004) observed that inoculated *Rhizobium* sp. into pea or egyptian clover showed reduced nodulation activity when the host was grown on heavy metal contaminated soil, but that other native endophytes did not seem affected by the pollution. For instance, endophytic *Bacillus* spp. have reduced the lead toxicity in *Alnus firma* plants because of the bacteria sequestered Pb extracellularly, increasing consequently the growth rate of plants in the presence of Pb (Shin et al., 2012). These processes are of great importance in the context of phytoremediation, since they can change the solubility and the internal availability of metals to the plant, thus influencing possible toxic effects of the metals.

Resistance to metals is essential for the survival and dispersion of bacteria in contaminated environments and also to potentially protect their plant host, especially because metal stress can also affect the plant-bacteria interactions by reducing the IAA (indole-3-acetic acid)-production as suggested by Kamnev et al. (2005). However, endophytic strains showed, in that study, despite a reduction of the number of cells due to metal toxicity, still higher total IAA production than non-endophytic, so that the symbiosis proves to be a good solution under stressful environmental conditions. IAA is produced by many endophytic strains as *Agrobacterium* spp., *Alcaligenes piechaudii, Burkholderia* sp., *Bacillus* spp., *Pseudomonas* spp., *Stenotrophomonas* sp., *Pantoea* sp., *Enterobacter* spp., *Rhizobium leguminosarum, Staphylococcus, Azotobacter, and Azospirillum* (Rajkumar et al., 2009; Tsakelova et al., 2006). For some of these species, as for *Agrobacterium tumefaciens*, it is considered as a pathogenicity factor, as it is one of the causal agents for plant tumours.

The production of organic acids is an important factor which can promote the assimilation of soil phosphorus by plants. Bacteria as *Azotobacter chroococcum, Bacillus* spp., *Enterobacter agglomerans, Pseudomonas chlororaphis, Pseudomonas putida, Pantoea* sp.,

Enterobacteriaceae, Burkholderia, Ralstonia pickettii, Erwinia sp., *Agrobacterium* sp., *Rhizobium* sp., *Stenotrophomonas maltophilia* and *Caulobacter* are known to be able to efficiently solubilise phosphorus from the soil (Kozyrovska et al., 1996; Park et al., 2011; Rajkumar et al., 2009; Yu et al., 2012). These bacteria can solubilise immobilised mineral phosphate by releasing organic acids, such as gluconic acid and 2- ketogluconic acid.

Other properties are known from some of the found bacteria; for instance, the reduction of stress caused by the phytohormone ethylene, which increases in plants under abiotic or biotic stress conditions. A frequently observed mechanism that reduces levels of ethylene production is through the activity of bacterial 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) (Kidd et al., 2009; Weyens et al., 2009a), ACC being the immediate precursor of ethylene. Bacteria originating from different soils and expressing ACC deaminase activity can improve plant growth even in soils containing phytotoxic concentrations of Cd; some strains, like *Pseudomonas tolaasii* ACC23 and *P. fluorescens* ACC9, produced IAA and siderophores even more actively under Cd stress (Kidd et al., 2009). It appeared that most of the PGPR strains isolated from grasses growing in a metal contaminated meadow exhibited ACC deaminase activity, which resulted in plant growth promotion.

4.2 Diversity assessment

Table 4: Proportion of Gram negative identified isolates [in %]

	Trifolium	***Festuca***
Root	100	75
Leaf	67	54
Stem	80	-
Total	**82**	**64**

Most of the identified isolates were found to be gram negative in both plants, with a gradient from the roots to the shoots, the shoots hosting more gram positive isolates than the roots. The cultivable strains from *Trifolium* root were exclusively gram negative (Table 4). There is little known about the endophytic bacteria present in Festuca, however, (Elo et al., 2000) found that 58% of the isolates from *Festuca* were gram-negative strains, less than in the present study. Compared to literature, this is particular; indeed, Barzanti et al. (2007) reported that the major part of the ARDRA types isolated from different organs of the nickel accumulator plant *Alyssum bertolonii* were represented by Gram-positive bacteria. The most common ones were *Bacillus, Paenibacillus, Leifsonia, Curtobacterium, Microbacterium, Micrococcus,* and *Staphylococcus*, only two groups were represented by proteobacteria similar to *Pseudomonas*. Other studies concerning different plants showed that the distribution of endophytes within the host depends mainly upon the plant species and plant compartment (Lodewyckx et al., 2002; Mastretta et al., 2009; Sheng et al., 2008).

The distribution of phylotypes of the cultivable isolates was calculated based on the estimation of the number of isolates per g fresh plant tissue. With this approach, the numerical influence of isolates which are present in large number gets visible, and so it should be more representative for the proportions present in the plant (Figure 2).
One numerically dominant strain was found for the shoots of *Festuca* and the stems of *Trifolium*. On the other hand, the leaves of *Trifolium* contain many different strains but due to

their low numbers they do not appear as important. Further, due to the large number of unidentified isolates, it is difficult to estimate the overall diversity and compare the endophytic colonisation of each plant compartment. However, it is important to mention that this is a result based on cultivation, and therefore non- or not easily cultivable strains are not considered.

The *Festuca* and *Trifolium*-associated bacterial populations characterised in this study are dominated by *Stenotrophomonas, Pseudomonas, Cellulomicrobium, Frateuria/Dyella, and Burkholderia* sp., *Enterobacter/Pantoea, Agrobacterium/Rhizobium, Olivibacter, Dyella, Curtobacterium, Sphingomonas* and *Curtobacterium* (Table 3). To our knowledge, there exist no reported studies about the endophytic population of these plant species on metal contaminated soil; moreover, in general very little is known about endophytic population in these species. According to one study (Elo et al., 2000), the diversity in *Festuca* is high - about 100 isolates were found. *Pseudomonas* was the prevalent taxon, with *Alcaligenes* and *Comamonas*, which was not found in humus and might be an obligate endophyte. Further *Arthrobacter, Nocardia; Bacillus* and *Paenibacillus* were specific to this plant according to the authors. The roots of seem to be also inhabited by spore-forming bacilli and nitrogen fixers, from the genera *Rhodococcus, Paenibacillus* and *Pseudomonas* (Elo et al., 2000).

However, compared with reported rape-, hyperaccumulator plant- or trees-associated bacterial populations on metal contaminated substrates (Aouad et al., 2006; Porteous Moore et al., 2006; Sheng et al., 2008; Shin et al., 2012; Ulrich et al., 2008), we can conclude that most of the genera were isolated before for other plants, whereas the genera *Cellulosimicrobium* and *Curtobacterium* are less common among the endophytes isolated from plants; *Olivibacter* sp., known as a soil bacterium (Wang et al., 2008), was only in our work reported as a plant endophyte. The differences in the observed endophytic populations suggest that besides possible variations due to changes in isolation techniques, isolation media and identification procedures, the environmental conditions in which the plants are growing and most of all the plant species are determining factors for the composition of the community. Indeed, plants are described to select specifically which bacterial community would develop in their tissues; (Wang et al., 2008) showed how the community changed from soil to the roots of different plants, with in particular a shift from a majority of gram positive strains in the soil to a dominance of gram negative ones in the plants, and how some genera were found in *Festuca* and not in *Betula*. About 100 isolates were found in *Festuca*, which is a higher diversity than in humus (~90). *Pseudomonas* was the prevalent taxon, with *Alcaligenes* and *Comamonas* (not found in humus). Further strains found belonged to the genera *Arthrobacter, Nocardia*; *Bacillus* and *Paenibacillus*. The authors suggest that plants species select bacteria associated with the roots from the bacterial pool in the soil, probably through the production of different root exudates.

Trifolium roots seem predominantly colonised by proteobacteria (Figure 2), although a large majority of strains remained unidentified. The stems were also colonised by proteobacteria and by bacteroidetes in high numbers. Also a large proportion of strains isolated from the leaves remained unidentified, while the other isolates show higher phylotypic diversity than in the rest of the plant. Isolates found in leaves were quite equally distributed through the different phyla, except of bacteroidetes, that were only observed in the stem.

In *Festuca*, as for *Trifolium*, a great number of isolates from roots could not be identified; the identified strains were in majority Proteobacteria with a dominant beta-proteobacteria population, although firmicutes and actinobacteria were also found.

Shoots are largely dominated by one isolate belonging to the ß-proteobacteria. Further, actinobacteria were found in a high proportion.

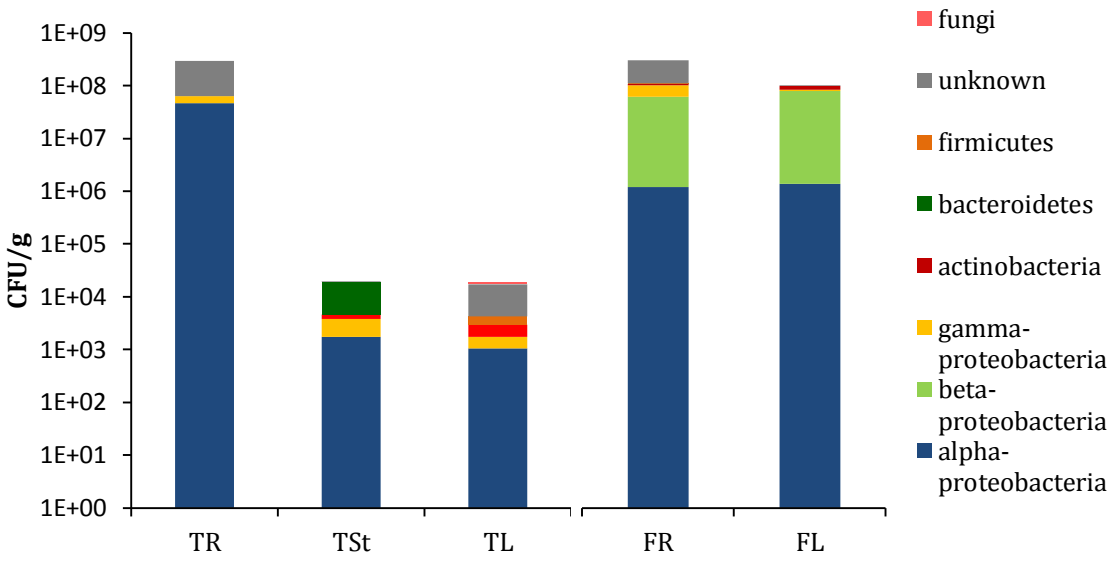


Figure 2: Diversity assessment of isolated endophytic microorganisms for different compartments (roots R, Stems St and leaves L) of *Trifolium pratense* (T) and *Festuca rubra* (F), calculated based on the isolated CFU /g

4.3 Compartmentalisation

An overview of the extent of compartmentalisation of bacteria residing within *Trifolium* and *Festuca* is given Figure 2. A good knowledge about the localisation of a strain within the plant is important especially in function of phytoremediation purposes since a successful bacterially enhanced phytoremediation strategy requires a bacterium located in the plant tissue where the pollutant's residence time is longest, in particular if, as in case of organic pollutants, it needs to be degraded. There seems to be a strong tendency for compartmentalisation in the various plant tissues, illustrated by Figure 3 (a) and (b), although this seems to be more pronounced in *Trifolium* than in *Festuca.*

Some genera are found in more than one compartment, but for example in *Trifolium* there was no common genus observed within roots and leaves; in general, endophytes of roots seem to be clearly different from those of the aerial parts of the plant. We presume that plants are capable of favouring the dominance of some specific seed endophytes as obligate endophytes and that the isolated facultative endophytes systemically colonised the inside the plant via the rhizosphere soil. Similarly the cultivation-dependent analysis showed that shoots of *T. goesingense* hosted different microbial populations, although the genera *Sphingomonas* were exclusively found in association with the interior of shoots (Rajkumar et al., 2009). This was the case in the present study for *Trifolium*; in the case of *Festuca*, only roots contained isolates related to *Sphingomonas*.

The hypothesis, that strains would primarily colonise roots and then further move up towards the leaves (Lodewyckx et al., 2002), does not seem to be confirmed, since the bacterial diversity seems to be higher in the aerial parts of the plants, and additionally the composition of the endophytes is different, another argument in favour of the presence of seed endophytes. In other studies, (Barzanti et al., 2007) higher numbers of ARDRA types were observed in roots compared to stems and leaves in the Ni-accumulator plant *A. bertolonii*. Therefore, it is probable that leaf endophytic populations are a combination of some bacteria translocated from the stem, but the majority entering through leaf wounds or stomata, the second

colonisation route supposed for endophytic bacteria originating from the outside of the plant (McCully, 2001). Additionally, an important portion of endophytes, the so-called obligate endophytes, are transferred from one generation to the next through the seeds (Mastretta et al., 2009), which is one further explanation why some strains are found only in the areal parts of the plants and not in the roots. On-going analysis about the native soil population at the study site will deliver better insights into the dynamics of endophytic colonisation.

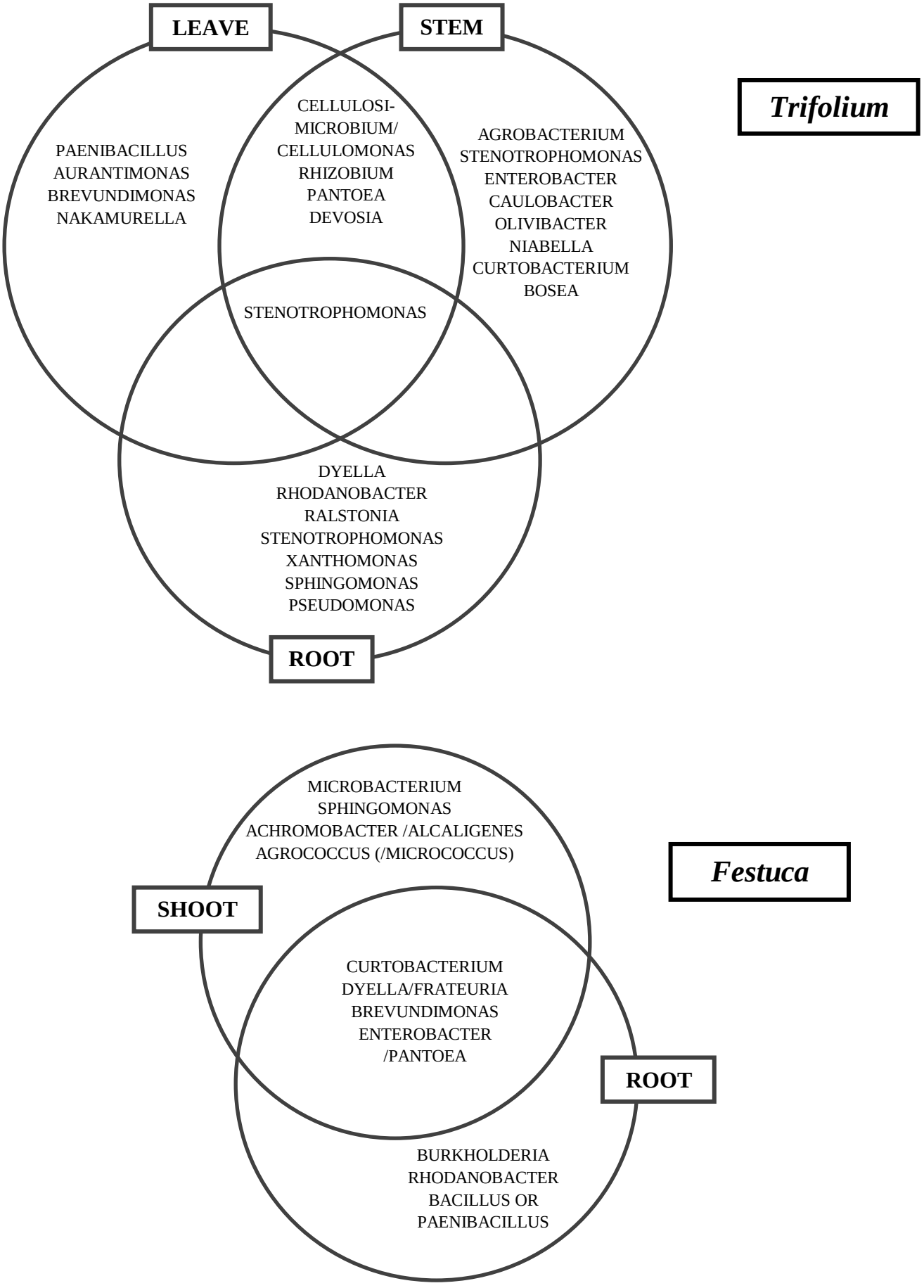


Figure 3: Schematic representation of endophyte isolate locations, with respect of genera, within the compartments of *Trifolium pratense* (a), and of *Festuca rubra* (b)

4.4 Application potential

Some of the isolated genera appear to be related to interesting strains described in the past by several authors. For instance, *Pantoea agglomerans* (also called *Erwinia herbicola* or *Enterobacter agglomerans*) is an ubiquitous plant epiphyte with known strains applied for biocontrol of fire blight caused by *Erwinia amylovora* on fruit trees, and commercially available in USA, Canada and New Zealand (US.EPA, 2011). Moreover, *Sphingomonas* are gram negative bacteria which have been isolated from diverse sources, as mineral water, sea water, wastewater, sludge, soil, plants, or as airborne bacteria. Many *Sphingomonas* species are associated with plants and produce yellow or orange pigments (Takeuchi et al., 1995). Further, *S. molluscorum* was shown to possess antagonistic effects against some gram positive bacteria (*Staphylococcus aureus, Enterococcus faecium, Bacillus subtilis, B. cereus, B. firmus, B. circulans, B. brevis, B. coagulans* and *B. licheniformis), Stenotrophomonas* species have an important ecological role in the element cycles in nature; in particular *Stenotrophomonas rhizophila* strains are plant associated and have been isolated from the rhizosphere of different plants; also endophytic colonisation was found (Wolf et al., 2002). *S. maltophilia* has biotechnological importance because of its potential plant growth promoting effects (Wolf et al., 2002). Further it is used for degradation of xenobiotic compounds. Both strains find applications in the biological control of fungal diseases, due to their antagonistic activity against plant pathogenic fungi (e.g. *Verticillium dahliae, Rhizoctonia solani, Sclerotinia sclerotiorum* and *Candida albicans)*; however, they seem not active against bacteria. Furthermore, *Burkholderia cepacia* is known as a plant pathogen in particular for onion or rice, but is also known to produce antibacterial and antifungal substances (Euzéby, 1997) and was renamed *Burkholderia xenovorans* (former *Pseudomonas cepacia*, or *Burkholderia cepacia*, *Burkholderia fungorum*) because of its capacity to degrade pesticides containing Cl, or polychlorinated biphenols (Parnell et al., 2006).

Many endophytic species like *Sphingomonas azotifigens* isolated from the roots of rice plants can fix nitrogen (Kamnev et al., 2005), and many are aromatic-degrading bacteria (Weyens et al., 2009). Nitrogen fixers were found in Archea (*Methanosarcina*) and many bacterial genera, mainly proteobacteria as *Sphingomonas* (*S. azotifigens*), *Burkholderia, Pseudomonas, Azotobacter, Devosia, Bradyrhizobium, Rhodobacter, Agrobacterium, Rhizobium, Frankia, Rhodococcus, Alcaligenes, Ralstonia,* some firmicutes *(Paenibacillus),* cyanobacteria as *Nostoc* sp. (Franche et al., 2009; Wang et al., 2008). So it is likely that numerous isolates show this capacity, even though nitrogen fixation has not been tested in our study. This is of great importance when using the isolated bacteria for biotechnological purposes. In fact, additionally to the fact that they are related to biotechnologically interesting strains, the majority of the isolates found in both autochthonous plants was found to be able to produce auxin, organic acids and siderophores *in vitro*. Many were also resistant to different heavy metals.

As a consequence, these strains are likely to (1) survive in a metal contaminates environment and (2) improve plant survival and growth under these sub-optimal conditions. However, a study of (Peterson et al., 2006) notify - with endophytic bacteria from soybean as an example - about making ecological implications from experiments conducted under typical laboratory conditions and of the additional roles that well-characterised microbial products may play in microbial interactions. Furthermore, Sturz and Christie (1996) showed that beneficial strains

for one plant turned out to be damaging for another species, showing the "clover-maize" syndrome; therefore, one should carefully choose the adapted inoculants. So the knowledge about some bacterial physiological properties is helpful but does give only a limited idea about what is going on *in plantae*.

We could select some strains which combined many of these promising properties, as potential plant growth promoters for bioremediation of sites contaminated with heavy metals. They are listed in Table 5. They should be tested in future experiments for their actual growth promoting potential on contaminated soil.

Table 5: Endophytic isolates showing potential for use as plant growth promoter on heavy metal contaminated soil. Production of OA: Organic Acids; Sid: Siderophores; Aux: Auxin; Me: Metal resistance; () little; + much

Plant	Compartment	Properties	Strain	Isolated CFU / g plant	Identification based on 16S RDNA sequence comparison
Festuca rubra	Roots	OA/Sid/Aux/Me	6d	$1.6.10^5$	*Enterobacter (cloacae?)*
		(OA)/Sid/Aux/Me+	23c	$3.5.10^5$	*Enterobacter or Pantoea agglomerans*
		Sid/Aux/Me+	26e	$1.4.10^6$	*Enterobacter*
	Shoots	OA/Sid/Aux/Me	61e	$4.2.10^5$	*Enterobacter (or Pantoea)*
		OA/Sid/Aux/Me	62c	$1.7.10^5$	*Microbacterium*
Trifolium pratense	Leaves	OA/Sid/Aux/(Me)	11d	$1.8.10^3$	*Pantoea (agglomerans?)*
	Roots	Sid/Aux/Me+	50d	$1.9.10^6$	*Burkholderia* sp.
		OA/Sid/(Aux)/Me	60d	$1.4.10^7$	*Bacillus* sp.
	Stems	OA/Sid/Aux/Me	38d	$8.4.10^2$	*Pantoea (agglomerans?) /Enterobacter?*
		Sid/(Aux)/Me	40b	$4.3.10^2$	*Rhizobium/Agrobacterium*
		OA/Aux/Me	46a	$7.0.10^2$	*Curtobacterium (herbarum?)*

5 CONCLUSIONS

Many bacteria were isolated from different plant tissues, belonging to several genera of gram positive and gram negative bacteria. There seems to occur a strong compartmentalisation in the various plant tissues; this seems to be more obvious in *Trifolium* than in *Festuca*.
From the isolated endophytic bacteria found in both autochthonous plant species, the majority was found to be able to produce IAA, organic acids and siderophores *in vitro*. Many were also resistant to different heavy metals. A number of isolates demonstrated the capacity to produce plant growth promoting substances and resistance to the metallic contaminant enriched in the soil. These plant fitness enhancing properties suggest that exploiting this mutualism for remediation purposes may be promising.

REFERENCES

- Aouad, G., Stille, P., Crovisier, J.-L., Geoffroy, V.A., Meyer, J.-M., Lahd-Gaegea, M., 2006. Influence of bacteria on lathanide and actinide transfer from specific soil components (humus, soil minerals and vitrified municipal waste incinerator bottom ash) to corn plants: Sr-Nd isotope evidence. Sci. Total Environ. 370, 545-551.
- Badri, D.V., Weir, T.L., van der Lelie, D., Vivanco, J.M., 2009. Rhizosphere chemical dialogues: plant-microbe interactions. Curr.Opin. Biotech. 20, 642-650.
- Barzanti, R., Ozino, F., Bazzicalupo, M., Gabbrielli, R., Galardi, F., Gonnelli, C., Mengoni, A., 2007. Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant *Alyssum bertolonii*. Microb. Ecol. 53, 306-316.
- Bashan, Y., 1998. Inoculants of plants growth-promoting bacteria for use in agriculture. Biotechnol. Adv. 16, 729-770.
- Chaudhary, P., Dudeja, S.S., Kapoor, K.K., 2004. Effectivity of host-*Rhizobium leguminosarum* symbiosis in soils receiving sewage water containing heavy metals. Microbiol. Res. 159, 121-127.
- Cunningham, J.E., Kuiack, C., 1992. Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. Appl. Environ. Microb. 58, 1451-1458.
- Davison, J., 1988. Plant beneficial bacteria. Biotechnology 6, 282-286.
- Dimkpa, C.O., 2009. Microbial Siderophores in Rhizosphere Interactions in Heavy Metal-containing Environments, Biologisch-Pharmazeutische Fakultät. Friedrich-Schiller University, Jena, p. 135.
- Elo, S., Maunuksela, L., Salkinoja-Salonen, M., Smolander, A., Haahtela, K., 2000. Humus bacteria of Norway spruce stands: plant growth promoting properties and birch, red fescue and alder colonizing capacity. FEMS Microbiol. Ecol. 31, 143-152.
- Euzéby, J.P., 1997. List of Prokaryotic names with Standing in Nomenclature.
- Franche, C., Lindström, K., Elmerich, C., 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321, 35-59.
- Guo, H., Luo, S., Chen, L., Xiao, X., Xi, Q., Wei, W., Zeng, G., Liu, C., Wan, Y., Chen, J., He, Y., 2010. Bioremediation of heavy metals by growing hyperaccumulator endophytic bacterium *Bacillus* sp. L14. Bioresource Technol. 101, 8599-8605.
- Hardoim, P.R., Overbeek, L.S.v., Elsas, J.D.v., 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16, 463-471.
- Kamnev, A.A., Tugarova, A.V., Antonyuk, L.P., Tarantilis, P.A., Polissiou, M.G., Gardiner, P.H.E., 2005. Effects of heavy metals on plant-associated rhizobacteria: Comparison of endophytic and non-endophytic strains of *Azospirillum brasilense*. J. Trace Elem. Med. Bio. 19, 91-95.
- Kidd, P., Juan, B., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monteroso, C., 2009. Trace element behaviour at the root-soil interface: implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.
- Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N., 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286, 885-886.
- Kozyrovska, N., Kovtunovych, G., Gromosova, E., Kuharchuk, P., Kordyum, V., 1996. Novel inoculants for an environmentally-friendly crop production. Resour. Conserv. Recy. 18, 79-85.
- Lane, D., 1991. 16S/23S rRNA sequencing, in: Goodfellow, E.S.a.M. (Ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, NY, pp. 115-175.
- Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M., van der Lelie, D., 2002. Endophytic bacteria and their potential applications. Crit. Rev. Plant Sci. 21, 583-606.
- Madmony, A., Chernin, L., Pleban, S., Peleg, E., Riov, J., 2005. *Enterobacter cloacae*, an obligatory endophyte of pollen grains of mediterrean pines. Folia Microbiol. 50, 209-216.
- Majewska-Sawka, A., Nakashima, H., 2004. Endophyte transmission via seeds of *Lolium perenne* L.: immunodetection of fungal antigens. Fungal Genet. Biol. 41, 534-541.
- Mastretta, C., Taghavi, S., van der Lelie, D., Mengoni, A., Galardi, F., Gonnelli, C., Barac, T., Boulet, J., Weyens, N., Vangronsveld, J., 2009. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. Int. J. Phytoremediat. 11, 251-267.

- McCully, M.E., 2001. Niches for bacterial endophytes in crop plants: a plant biologist's view. Aust. J. Plant Physiol. 28, 983-990.
- Meda, A.R., Scheuermann, E.B., Prechsl, U.E., Erenoglu, B., Schaaf, G., Hayen, H., Weber, G., Wirén, N.v., 2007. Iron acquisition by phytosiderophores contributes to cadmium tolerance. Plant Physiol. 143, 1761-1773.
- Mengoni, A., Schat, H., Vangronsveld, J., 2010. Plants as extreme environments? Ni-resistant bacteria and Ni-hyperaccumulators of serpentine flora. Plant Soil 331, 5–16.
- Nies, D.H., 1999. Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. 51, 730-750.
- Park, J.H., Bolan, N., Megharaj, M., Naidu, R., 2011. Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil. J.Hazard. Mater. 185, 829-836.
- Parnell, J.J., Park, J., Denef, V., Tsoi, T., Hashsham, S., III, J.Q., Tiedje, J.M., 2006. Coping with polychlorinated biphenyl (PCB) toxicity: physiological and genome-wide responses of *Burkholderia xenovorans* LB400 to PCB-mediated stress. Appl. Environ. Microbiol. 72, 6607-6614.
- Pavlo, A., Leonid, O., Iryna, Z., Natalia, K., Maria, P.A., 2011. Endophytic bacteria enhancing growth and disease resistance of potato (*Solanum tuberosum* L.). Biol. Control 56, 43-49.
- Peterson, S.B., Dunn, A.K., Klimowicz, A.K., Handelsman, J., 2006. Peptidoglycan from *Bacillus cereus* mediates commensalism with rhizosphere bacteria from the *Cytophaga-Flavobacterium* Group. Appl. Environ. Microb. 72, 5421-5427.
- Porteous Moore, F., Barac, T., Borremans, B., Oeyen, L., Vangronsveld, J., van der Lelie, D., Campbella, C.D., Moore, E.R.B., 2006. Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: The characterisation of isolates with potential to enhance phytoremediation. Syst. Appl. Microbiol. 29, 539-556.
- Rajkumar, M., Ae, N., Freitas, H., 2009. Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77, 153-160.
- Reinhold-Hurek, B., Hurek, T., 2011. Living inside plants: bacterial endophytes. Curr. Opin. Plant Biol. 14, 435-443.
- Römheld, V., Marschner, H., 1986. Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. Plant Physiol. 80, 175-180.
- Rönkkö, R., Smolander, A., Nurmiaho-Lassila, E.-L., Haahtela, K., 1993. *Frankia* in the rhizosphere of non-host plants: A comparison with root-associated N_2-fixing *Enterobacter, Klebsiella* and *Pseudomonas*. Plant Soil 153, 85-95.
- Schulz, B., Boyle, C., 2005. The endophytic continuum. Mycol. Res. 109, 661-686.
- Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47-56.
- Sheng, X.-F., Xia, J.-J., Jiang, C.-Y., He, L.-Y., Qian, M., 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. Environ. Pol. 156, 1164-1170.
- Shin, M.-N., Shim, J., You, Y., Myung, H., Bang, K.-S., Cho, M., Kamala-Kannan, S., Oh, B.-T., 2012. Characterization of lead resistant endophytic *Bacillus* sp. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator *Alnus firma*. J. Hazard. Mater. 199–200, 314-320.
- Sturz, A.V., Christie, B.R., 1996. Endophytic bacteria of red clover as agents of allelopathic clover-maize syndromes. Soil Biol. Biochem. 28, 583-588.
- Takeuchi, M., Sakane, T., Ynagi, M., Yamasato, K., Hamana, K., Yokota, A., 1995. Taxonomic Study of Bacteria Isolated from Plants: Proposal of *Sphingomonas rosa* sp. nov., *Sphingomonas pruni* sp. nov., *Sphingomonas asaccharolytica* sp. nov., and *Sphingomonas mali* sp. nov. Int. J. Syst. Bacteriol. 45, 334-341.
- Tervet, I.W., Hollis, J.P., 1948. Bacteria in the storage organs of healthy plants. Phytopathology 38, 960–967.
- Tsakelova, E.A., Cherdyntseva, T.A., Botina, S.G., Netrusov, A.I., 2006. Bacteria associated with orchid roots and microbial production of auxin. Microbiol. Res. 162, 69-76.
- Ulrich, K., Ulrich, A., Ewald, D., 2008. Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiol. Ecol. 63, 169-180.

- US.EPA, 2011. Pesticides: Regulating Pesticides - *Pantoea agglomerans* strain E325 (006511) Fact Sheet. U.S. Environmental Protection Agency.
- Wang, L., Ten, L.N., Lee, H.-G., Im, W.-T., Lee, S.-T., 2008. *Olivibacter soli* sp. nov., *Olivibacter ginsengisoli* sp. nov. and *Olivibacter terrae* sp. nov., from soil of a ginseng field and compost in South Korea. Int. J. Syst. Evol. Micr. 58, 1123–1127.
- Weyens, N., van der Lelie, D., Taghavi, S., Newman, L., Vangronsveld, J., 2009a. Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends Biotechnol. 27, 591-598.
- Weyens, N., van der Lelie, D., Taghavi, S., Vangronsveld, J., 2009b. Phytoremediation: plant-endophyte partnerships take the challenge. Curr.Opin. Biotech. 20, 248-254.
- Wolf, A., Fritze, A., Hagemann, M., Berg, G., 2002. *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. Int. J. Syst. Evol. Micr. 52, 1937-1944.
- Yu, X., Liu, X., Zhu, T.-H., Liu, G.-H., Mao, C., 2012. Co-inoculation with phosphate-solubilzing and nitrogen-fixing bacteria on solubilization of rock phosphate and their effect on growth promotion and nutrient uptake by walnut. Eur. J. Soil Biol. 50, 112-117.
- Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7, 203-214.

Chapter 4

CHARACTERISATION OF BACTERIA USED FOR INOCULATION

The present chapter is composed of the work protocols and the results of additional characterisation tests.

Chapter 4: Characterisation of bacteria used for inoculation

The selected strains were further characterised physiologically. They were therefore grown on different media in order to test their ability to degrade certain compounds and produce specific products.

Table 1: List of the 11 selected strains, and their characteristics, that were used to choose them
IAA=Auxin Indole-3 acetic acid; Metal resistance I&II: concentration levels, see previous chapter; 0-2: relative metal resistance capacity (0 no growth, 1 poor growth, 2 normal growth)

	Org Acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
		-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
A=6d	+	+	-	+++	1	0	2	2	0	0	0	0	2
F=11d	+	+	-	++++	0	0	2	2	0	0	0	0	2
B=23c	(+)	+	-	+++	2	2	2	2	0	0	0	0	2
C=26e	-	+	-	+++	2	2	2	2	0	0	0	0	2
I=38d	+	+	-	+++	0	2	2	2	0	0	0	0	2
J=40b	-	+	-	+++	0	0	0	2	0	0	0	0	2
G=50d	-	+	-	+++	2	0	1	2	0	0	0	0	2
H=60d	+	+	-	++	0	0	2	2	0	0	0	0	2
D=61e	+	+	-	+++	2	0	2	2	0	0	0	0	2
E=62c	+	+	-	+++	2	0	2	2	0	0.5	0	0	2
K=46a	+	-	-	+++	0	0	2	2	0	0	0	0.5	2

1 MATERIALS AND METHODS

The collection of these tests is taken from the test series of the *Bunte Reihe*, after (Schlegel, 1992). Some fast test with young cultures grown 24 h on standard 1 medium, were done.

1.1 KOH fast test for Gram-positive and –negative distinction

A simple, rapid method utilising a 3% solution of potassium hydroxide to distinguish between gram-positive and gram- negative bacteria was applied:

Two drops of a 3% solution of potassium hydroxide were placed on a glass slide. A 2-mm loop full of bacterial growth, obtained from a 24 h culture on standard I agar, was stirred in a circular motion in the KOH solution. The loop was occasionally raised 1 to 2 cm from the surface of the slide. The KOH solution characteristically became very viscous and mucoid with gram-negative bacteria. A string of the mixture would follow the loop when it was raised. The KOH test was only considered positive if stringing occurred within the first 30 s of mixing the bacteria in the KOH solution.

In case gram-positive bacteria are suspended in the KOH solution, no slime should be formed. Several species of anaerobic bacteria display variable Gram stain reactions which often make identification difficult. Some strains of *Clostridia*, *Eubacteria*, and *Bifidobacteria* stained gram negative or gram variable; the KOH test correctly classified these strains as gram-positive. The KOH test incorrectly grouped some strains of *Bacteroides* sp., *Fusobacterium* sp., *Leptotrichia buccalis*, and *Veillonella parvula*, but all Gram stain results for these strains were consistent for gram-negative bacteria (Halebian et al., 1981).

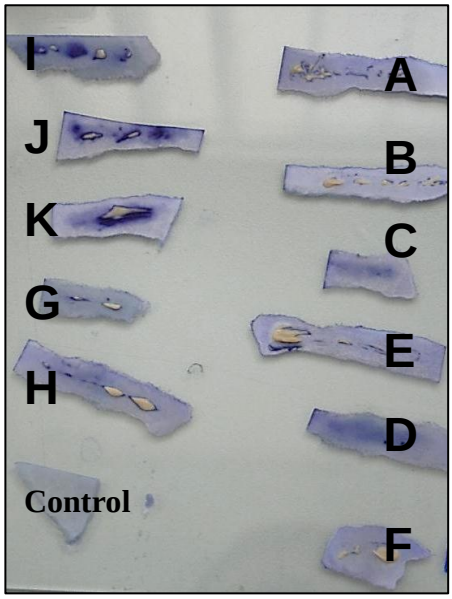
1.2 Catalase

Two drops of a 3% solution of H_2O_2 were placed on a glass slide. A 2-mm loop full of bacterial growth, obtained from a 24-h culture on standard I agar, was stirred in a circular motion in the solution. Bubbles formation around the needle after rubbing bacterial material into 3% H_2O_2 show O_2 formation, and so the activity of the enzyme catalase.

Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen. Important catalase-negative genera are *Streptococcus, Leuconostoc, Lactobacillus, Clostridium,* and *Mycoplasma.* Enterococci, Staphylococci and Micrococci are catalase-positive. Other catalase positive organisms include *Listeria, Corynebacterium diphtheriae, Burkholderia cepacia*, *Nocardia*, the family Enterobacteriaceae (*Citrobacter, E.Coli, Enterobacter, Klebsiella, Shigella, Yersinia, Proteus, Salmonella, Serratia, Pseudomonas*), *Mycobacterium tuberculosis*, *Aspergillus,* and *Cryptococcus*.

1.3 Oxidase

The oxidase test determines if a bacterium produces cytochrome c oxidase. The reagent plays the role of an electron donator, the electron being transferred if cytochrome oxidase is active, thereby oxidising the reagent which is then blue. The reaction is positive if the reaction occurs in less than 10 s, and weak positive if it takes up to 1min.



Cytochrome C oxidase is the last enzyme in the respiratory electron transport chain of mitochondria and many bacteria, located in the mitochondrial or bacterial membrane. It receives an electron from each of four cytochrome c molecules, and transfers them to one oxygen molecule, converting molecular oxygen to two molecules of water. The cytochrome system is usually only present in aerobic organisms which are capable of utilising oxygen as the final hydrogen receptor (i.e. utilize oxygen for energy production with an electron transfer chain). The end product of this metabolism is either water or hydrogen peroxide (broken down by catalase).

Figure 1: Results of the oxidase test for all selected strains

Typically the Pseudomonadaceae are OX+, since they are obligate aerobic bacteria. Another example is the preliminary identification of *Neisseria* and *Moraxella* genera, which are both oxidase positive, Gram-negative diplococci. Many Gram-negative spiral curved rods are also oxidase positive, which includes *Helicobacter pylori, Vibrio cholera, and Campylobacter jejuni*. Also *Legionella pneumophila* is oxidase positive.

OX- normally means that the bacterium does not contain cytochrome c oxidase and therefore cannot utilize oxygen for energy production with an electron transfer chain. Typically Enterobacteriaceae are OX-. *Enterobacter* has a shorter respiration chain, ending with a chinoloxidase. They possess cytochrome d and o instead of cytochrome c. Therefore the result is negative, because the coloured agent cannot transfer electrons.

1.4 Nitrate test: NO_3^- → NO_2^- (→ Ammonium ions → N_2)

After incubation, the tubes are inspected for the presence of gas. In the case of non-fermenters, this is a first hint for reduction of nitrate to nitrogen gas. The actual testing is done

through the addition of the reagents sulphanilic acid and dimethyl-α-napthalamine. If nitrite is present in the media, then it will react to form a red compound. This is considered a positive result. If no red color forms upon addition of the reagents, it indicates that either the nitrate has not been converted to nitrite (a negative result), or that nitrate was converted to nitrite and then immediately further converted into another, not-detected form of nitrogen (also a positive result). In order to know whether nitrate was reduced or not, elemental zinc is added to the broth. Zinc will convert any remaining nitrate to nitrite, thus allowing the reagents to react with the nitrite and form the red pigment (a verified negative result). If no color change occurs upon addition of zinc then this means that the nitrate was converted to nitrite and then was converted to some other not detected form of nitrogen (a positive result).

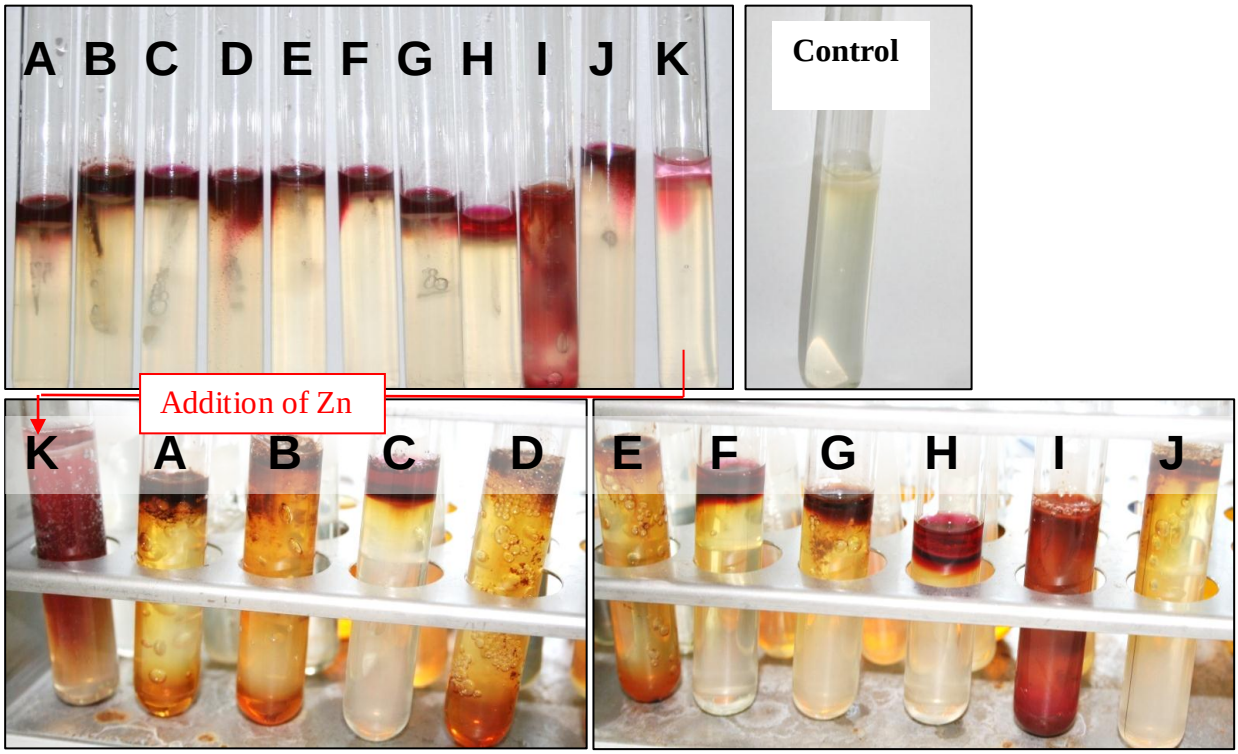


Figure 2: Photograph of the samples for the nitrate reduction test, before and after addition of the Zn reagent. Some samples (A, B, D, E, G, J) showed gas formation after 1h.

Enterobacteriaceae can reduce nitrate to nitrite. *Pseudomonas* strains are known to reduce nitrate all the way to N_2.

1.5 Methyl test

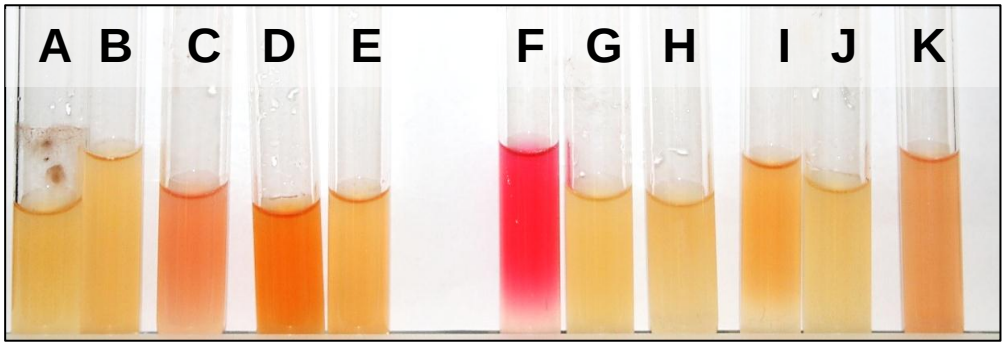


Figure 3: Photograph of all selected isolates for the methyl test

During the fermentation of glucose acidic products among other are formed, such as lactate, acetate, succinate and formate. They are evidenced by methylred, whose transition point lies between pH 4.4 and 6.2, turning from yellow into red when getting acidic.

Enterobacter transforms 2 pyruvate molecules to acetoin, under the formation of 2 CO_2. Acetoin can be further transformed into 2,3-Butandiol, and 2 $NADH+H^+$ are regenerated. The acidic molecules are produced in low amounts since pyruvate is used to produce acetoin and not for the acidification of the medium; therefore the test will be negative. *Pseudomonas* cannot achieve fermentation, so no acidic products can be formed, and the test is negative. *E.coli* and *Citrobacter freundii* produce acids through different metabolic pathways (i.e.: KDPG-pathway, mixed acidic fermentation), so the test is positive for those organisms.

1.6 Voges-Proskauer

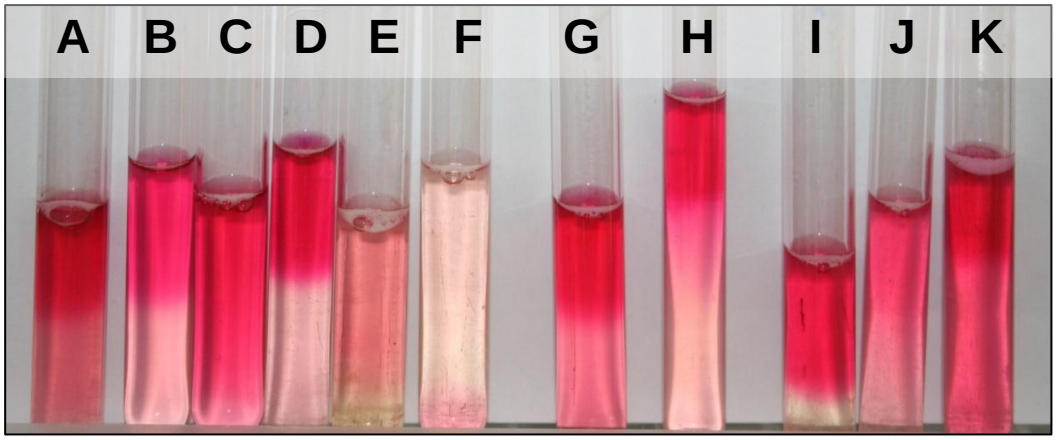


Figure 4: Photograph of the results for all isolates for the Voges-Proskauer test after addition of the reagent

The Voges-Proskauer test is positive when the bacteria are able to form acetoin. The test is positive for *Enterobacter,* but not for *E. Coli, C. freundii, Bacillus subtilis,* and *Pseudomonas aeruginosa*. The acetoin formed is oxidized to diacetyl in alkaline and forms with arginine, creatine and guanidine a red dye that is enhanced by alpha-naphthol. Shaking the samples strongly is important, since oxygen is needed to oxidize 2,3-butanediol into acetoin! The other microorganisms are not able to produce acetoin. Therefore, the test is negative.

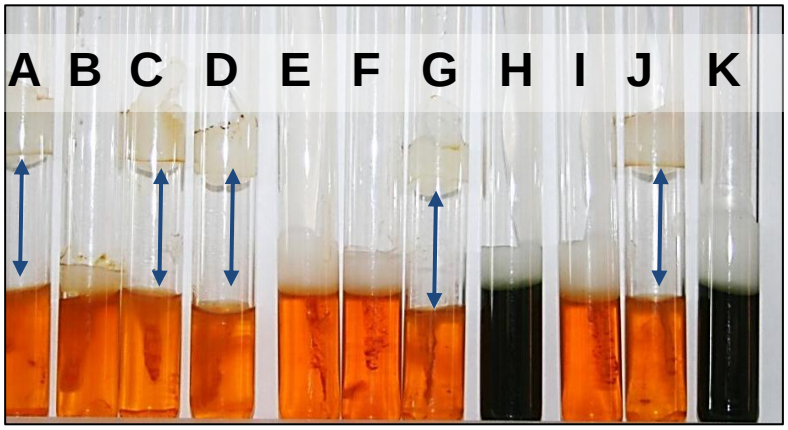
1.7 Hugh-Leifson-Test or Oxidation-Fermentation-Test

The Hugh-Leifson-Test, also called Oxidation-Fermentation-Test (OF-Test), is used to test the ability of bacteria to produce organic acids from carbohydrates under aerobic and anaerobic conditions, in order to know if the metabolism is oxidative or fermentative.

The pH indicator bromothymol blue turns yellow if an acidification of the medium occurs, indicating bacterial activity and therefore a positive result. Paraffin ensures anaerobic conditions.

Escherichia coli, Enterobacter aerogenes, Citrobacter freundii can fermentate glucose to ethanol, acetate, lactate, succinate, fumarate, formiate. Under anaerobic conditions, *Pseudomonas aeruginosa* cannot fermentate glucose, which is visible by a green colour of the medium.

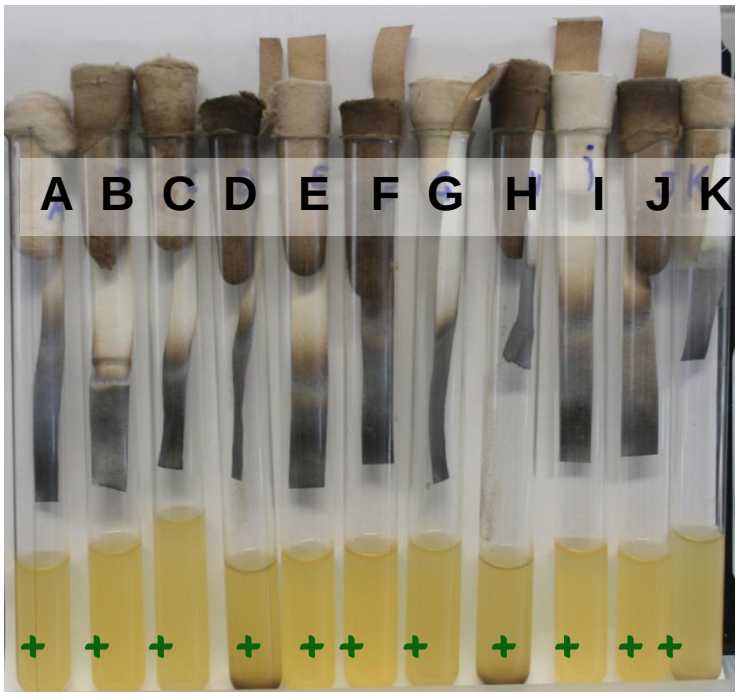
Aerobic



Anaerobic

Figure 5: Photograph of the result of the O/F testing, aerobic (top) and anaerobic (bottom), for all isolates.

1.8 H_2S



The reduction of sulphur-containing proteins / peptides or thiosulfate by the desulfurase enzyme ultimately leads to the formation of sulphide. The sulphide is reduced over several steps to H_2S, which can react with lead or iron acetate present in the medium to black iron or lead sulphide. *Pseudomonas* is one of few genera which show no blackening i.e. are not able to form hydrogen sulphide.

Figure 6: result of the H_2S-production test for all selected isolates.

1.9 Urease

Urea is degraded by the enzyme urease into ammoniac, changing the medium to an alkaline pH. The phenol red present in the medium turns red.

Figure 7: Photo of the results of the urease test of all isolates.

1.10 Citrate

If citrate can be used as only carbon source by the strain, the pH of the medium increases, due to degradation of the acid. The colour of bromothymol blue present in the medium turns blue as an indication of the pH increase. The citrate test is negative for *E. Coli*, since *E. Coli* is not able to take up citrate as a symport with protons and so to metabolise it.

2 RESULTS AND DISCUSSION

2.1 Summary of the additional physiological characterisation

Table 2: Summary of the additional physiological characterization of the selected isolates. Darkened results are remarkable, show differences between strains from same species or unexpected results, and discussed for each strain.

	O/F $+O_2$	O/F $-O_2$	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
A	+	+	(+)	+	+	+	+	+	-	(+)	+	-
B	+	+	-	+	+	+	+	+	-	(+)	-	-
C	+	+	-	+	+	+	+	+	(+)	(+)	+	+
D	+	+	-	+	+	+	+	+	-	(+)	+	-
E	-	-	(+)	+	+	+	+	(+)	-	(+)	+	-
F	+	+	-	+	-	+	+	-	+	-	+	-
G	+	+	-	+	+	+	+	+	-	(+)	+	-
H	-	-	+	+	-	+	+	+	-	(+)	+	-
I	+	+	-	+	+	++	++	+	-	++	+	-
J	+	+	-	+	+	+	+	+	-	+	+	-
K	-	-	-	+	+	-	-	+	(+)	+	+	-

2.2 Strains A, B, C, D: *Enterobacter cloacae / Pantoea* sp.

The species *Pantoea agglomerans* (Ewing and Fife 1972) is a synonym of *Enterobacter agglomerans, Erwinia herbicola* or *Erwinia milletiae*.

Strains A, B, C and D form beige to colourless colonies on standard 1 medium. On 1:10 rich medium agar, strain A forms big colonies in form of 2 concentric circles, of nacre colour, and irregular form similar to star growth. Strain B is yellowish, shiny and smooth, middle size, and shows irregular edges. Strain C shows a fair, lemon-like yellow colour, while D forms white colonies with bluish edges, and an irregular form. The cells are short rods, of about 1.5 µm length and 1 µm width, and are mobile. According to the authors first describing the species *Enterobacter agglomerans* (Ewing and Fife, 1972), most of the isolates produce yellow pigment on ordinary nutritive media and are motile.

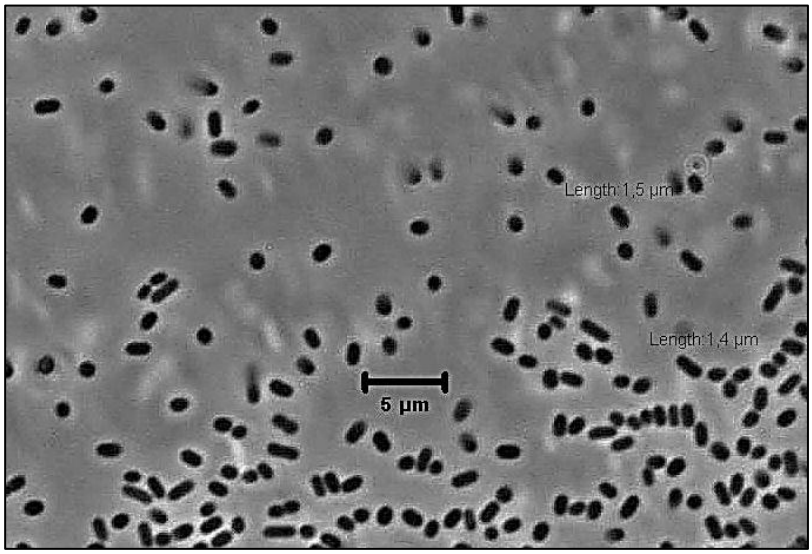


Figure 8: Strain A (6d), using a phase contrast microscope, and 100x objective

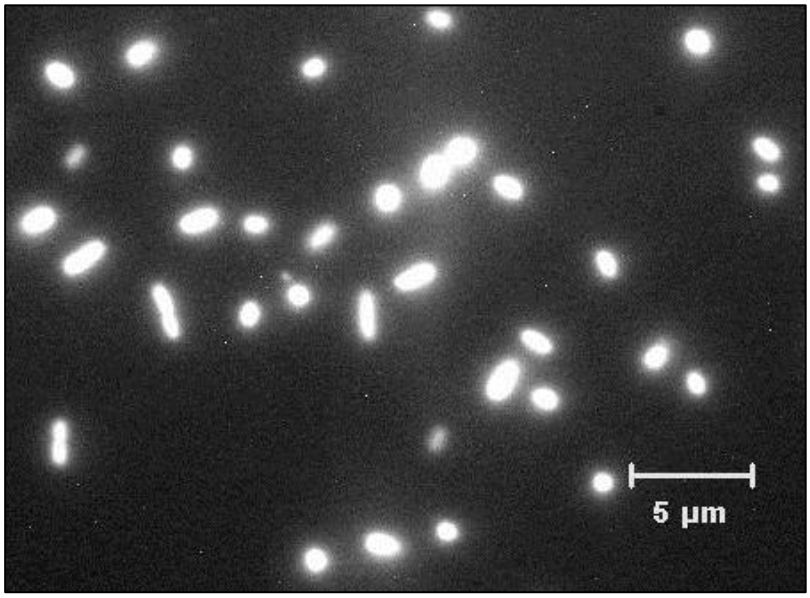


Figure 9: Strain B (23c) using a phase contrast fluorescence microscope, and 100x objective, stained with the DNA staining agent DAPI

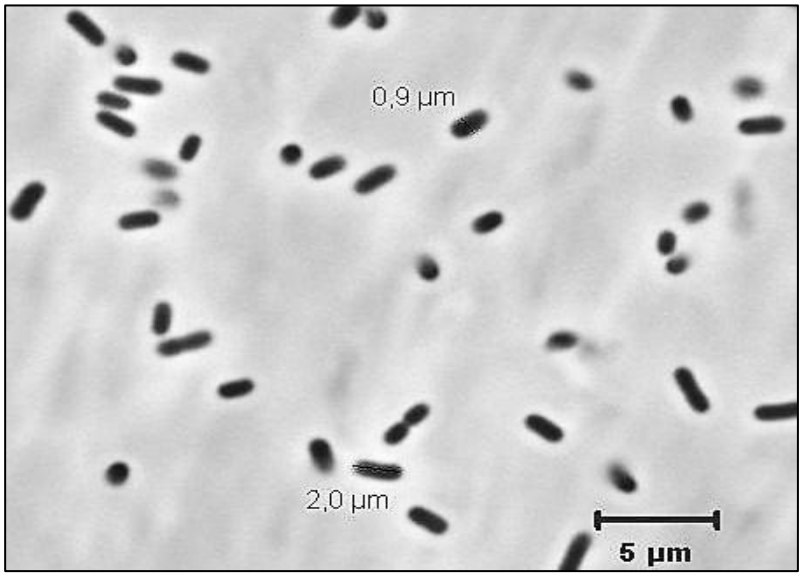


Figure 10: Strain C (26e) using a phase contrast microscope, and 100x objective

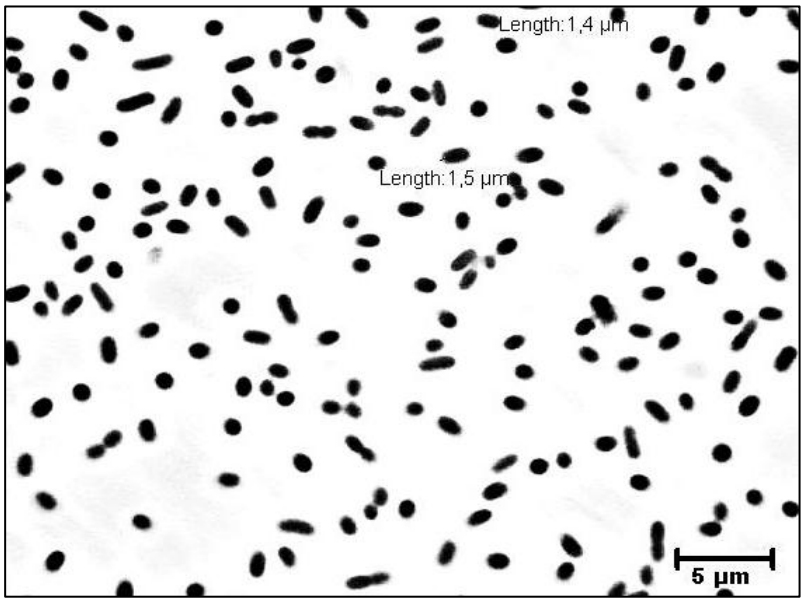


Figure 11: Strain D (61e), using a phase contrast microscope, and 100x objective

	O/F +O_2	O/F -O_2	Urease	H_2S	Citrate	Nitrate: Gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
A	+	+	(+)	+	+	+	+	+	-	(+)	+	-
B	+	+	-	+	+	+	+	+	-	(+)	-	-
C	+	+	-	+	+	+	+	+	(+)	(+)	+	+
D	+	+	-	+	+	+	+	+	-	(+)	+	-

	Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
		-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
A	+	+	-	+++	+	0	++	++	0	0	0	0	++
B	(+)	+	-	+++	++	++	++	++	0	0	0	0	++
C	-	+	-	+++	++	++	++	++	0	0	0	0	++
D	+	+	-	+++	++	0	++	++	0	0	0	0	++

Isolates A, B, C and D present similar physiological features. They are Gram- negative, are able to function oxidative and fermentative. Furthermore, all are able to form H_2S through the action of the enzyme desulfurase, use citrate as unique carbon source, reduce nitrate to nitrite, possibly, and can produce acetoin (positive VP-test). It seems that they are slightly positive for oxidase, although it is not certain. All except of A do not possess the enzyme urease, and only C is possibly able to form acidic products after fermentation of glucose, even though it is the contrary for the organic acid testing. All strains except B are catalase-positive. Furthermore, they are able to produce siderophores under Fe-deficiency, and show an intensive production of the auxin IAA. Their metal resistance is highest among all selected strains. They are all very resistant to Mn, and resistant to lesser extend to Zn and Ni, and not to Al. Only strains B and C were resistant to Cd.

The Gram-positive result for C is not realistic; all BLAST results are consistent to *Enterobacter* or related genera. However, the KOH test is not very reliable, and is positive only if the slime forms after short time. It can be that too long time passed and the result is falsely positive, or that the genetical result was wrong.

However, most of the tests are in accordance with the ones known about Enterobacter. Catalase-positive organisms include the family of Enterobacteriaceae: *Citrobacter, E.Coli, Enterobacter, Klebsiella, Shigella, Yersinia, Proteus, Salmonella, Serratia, and Pseudomonas; this resultis one hint for the confirmation of the genetical 16S-based identification of the isolates. Enterobacter* transforms 2 pyruvate to Acetoin, under the formation of 2 CO_2. Acetoin can be further transformed into 2,3-Butandiol, and 2 $NADH+H^+$ are regenerated. The acidic molecules are produced in low amounts since pyruvate is used to produce acetoin and not for the acidification of the medium; therefore the test is usually negative. Oxidase negative normally means that the bacterium does not contain cytochrome c oxidase and therefore cannot utilize oxygen for energy production with an electron transfer chain. Typically, Enterobacteriaceae are oxidase negative, which seems to be consistent with the BLAST results.

According to the authors first describing the species *Enterobacter agglomerans* (Ewing and Fife, 1972), most of the isolates reduce nitrate to nitrite, fail to form indole, grow on Simmons' citrate-agar medium, and are fermentative. Not all strains of the species show consistent reactions to Voges-Proskauer Test, citrate or urease, even if in our case all isolates

behave the same for these tests. However, *E. agglomerans* was found to be negative for the H_2S test, whereas all our isolates are positive.

2.3 Strains E: Microbacteriaceae?

E is yellow large, flat, warm yellowish and smooth on Standard I Agar. The cells are long and thin; less than 1 µm width (0.6 µm) and generally more than 2 µm length. The cells are very mobile and swim fast in all directions.

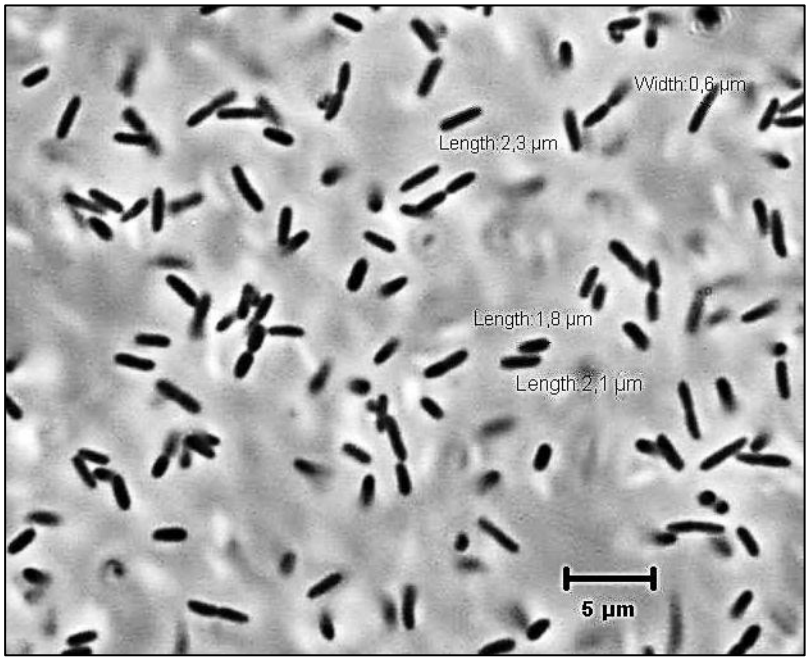


Figure 12: Microscopic picture of strain E (62c) using a phase contrast microscope, and 100x objective

O/F $+O_2$	O/F $-O_2$	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
-	-	(+)	+	+	+	+	(+)	-	(+)	+	-

Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
	-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
+	+	-	+++	++	0	++	++	0	+	0	0	++

Isolate E is gram negative and is oxidative, i.e. strictly aerobic. Furthermore, it is able to form H_2S through the action of the enzyme desulfurase, is positive for catalase, possibly possesses the enzyme urease and shows the capacity to reduce nitrate. It is not able to form acidic products after fermentation of glucose (negative methyl test), even though they are positive for the organic acid production test. Further, it use citrate as unique carbon source and seems that it can produce acetoin (positive VP-test). Additionally, it produces siderophores under Fe-deficiency and is an efficient producer of the auxin IAA. It is more resistant to metals compared to the other selected isolates; indeed it is very resistant to Mn and to Ni; it is resistant to lesser extend to Zn, and not to Al and Cd.

Microbacteriaceae are Gram-positive organisms, which is not corresponding to the test. However, the KOH test is not very reliable, and is positive only if the slime forms after short time. It can be that too long time passed and the result is falsely positive.

2.4 Strain F: *Pantoea (agglomerans?)*

Strain F forms smooth yellow on 869 medium and yellow to orange coloured colonies on standard 1 agar. The cells are rods, of 2 µm length and 1µm width. Mobility was not observed.

Pantoea is described as rods measuring 0.5 to 1.0 by 1.0 to 3.0 µm. Most are motile (Gavini et al., 1989). Colonies on nutrient agar are smooth, translucent, and more or less convex with entire margins. Colonies may or may not be yellow pigmented. So the cells of isolate F are longer than those of the type strain, and lack its motility.

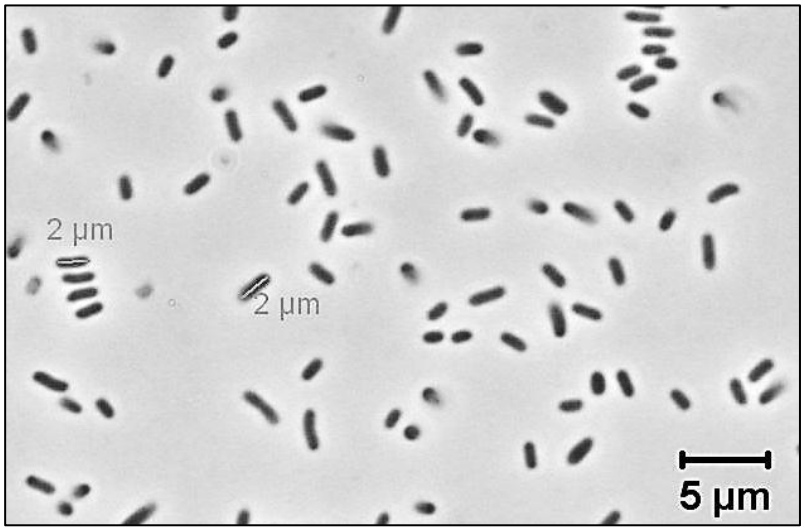


Figure 13: Microscopic picture of strain F (11d) using a phase contrast microscope, and 100x objective

O/F $+O_2$	O/F $-O_2$	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
+	+	-	+	-	+	+	-	+	-	+	-

Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
	-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
+	+	-	++++	0	0	++	++	0	0	0	0	++

Isolate F presents different physiological features from A-D. As them, it is Gram- negative and is able to function oxidative and fermentative. Furthermore, it is able to form H_2S through the action of the enzyme desulfurase, is positive for catalase, does not have the enzyme urease and reduces nitrate.

However, it is clearly able to form acidic products after fermentation of glucose (positive methyl test, production of organic acids). However, it cannot produce acetoin (negative VP-test) or use citrate as unique carbon source. Furthermore, it produces siderophores under Fe-deficiency, and it is the highest IAA producer of all our isolates. Its metal resistance is limited compared to other isolates; it is very resistant to Mn, and resistant to lesser extend to Zn, and not to Al, Ni and Cd.

Pantoea is described to be facultatively anaerobic; oxidase negative. Acid is produced from many carbon sources (Gavini et al., 1989). Even if the BLAST result shows that the strain F is related to *Pantoea*, many physiological features are contradicting this result. On one hand, catalase positive organisms include the family Enterobacteriaceae, and so *Pantoea*. Similarly, strain F is oxidase-negative, and urease negative, two typical characteristics of the family of Enterobacteriaceae, which is in accordance with the genetical identification. However, the Voges-Proskauer reaction is known to be negative for *Pantoea agglomerans*; other species of

the *Pantoea* -complex, as *Erwinia stewartii*, *Escherichia* sp, and *Leclercia adecarboxylata* are positive. Further, none of the strains of this group is known to be positive for H_2S production, or for the use of citrate as unique carbon source.
So it is likely that strain F is, if it is part of the Enterobacteriaceae, is only related to a less extend to them.

2.5 Strain G: *Burkholderia* sp.?

The colonies are white with bluish edges, and of irregular form, when grown on 1:10 869, and beige when grown on standard 1 agar. The cells are rods of 2 µm length. According to the literature, *Burkholderia* (former part of the genus *Pseudomonas*) is a group of ubiquitous gram negative, generally motile (except of *Burkholderia mallei*) bacteria. The cells are straight rods (Euzéby, 1997a).

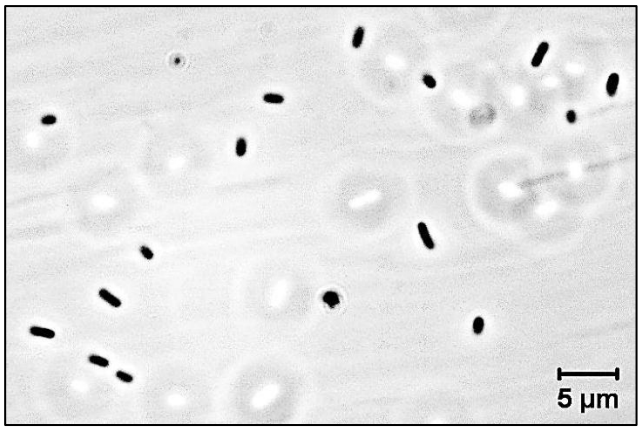


Figure 14: Microscopic picture of strain G (50d), using a phase contrast microscope, and 100x objective

O/F +O_2	O/F -O_2	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
+	+	-	+	+	+	+	+	-	(+)	+	-

Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
	-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
-	+	-	+++	++	0	+	++	0	0	0	0	++

Isolate G is Gram- negative and is oxidative and fermentative. Furthermore, it is able to form H_2S through the action of the enzyme desulfurase, is positive for catalase, does not have the enzyme urease and shows the capacity to reduce nitrate. It is not able to form acidic products after fermentation of glucose (negative methyl test, no organic acids produced). Further, it can produce acetoin (positive VP-test) and use citrate as unique carbon source. Additionally, it produces siderophores under Fe-deficiency and is an efficient producer of the auxin IAA. As the other selected isolates it is very resistant to Mn; it is resistant to lesser extend to Zn and Ni, and does not resist to Al and Cd. *Burkholderia* are indeed gram negative bacteria. They are catalase positive; oxidase is variable depending on the species. They are capable to use following carbohydrates as unique carbon sources: glucose, glycerol, inositol, galactose, sorbitol, mannose (in contrast to *Ralstonia* sp.) and mannitol (Euzéby, 1997b). They are reported to be strictly aerobic, even though in our experiment they are able to use glucose in a fermentative way.

2.6 Strain H: *Pseudomonas/ Stenotrophomonas/Xanthomonas*

Strain H forms intensive white colonies on 1:10 rich 869 medium and beige to colourless colonies on standard 1 agar. The cells are long rods of about 4 to 5 µm, mostly linked together as chains. Some very long chains were observed (up to more than 100 µm), with very long cells (Figure 15: Microscopic picture of strain H (60d), using a phase contrast microscope, and 100x objective). No mobility was observed.

We could not find similarly shaped bacteria in the literature, since filamentous bacteria form much longer chains, and *Pseudomonas, Stenotrophomonas* and *Xanthomonas* form rods, *Stenotrophomonas rhizophila* cells are straight or slightly curved rods, and its colonies are yellowish (Wolf et al., 2002). *Pseudomonas* is usually motile, although isolate H was not.

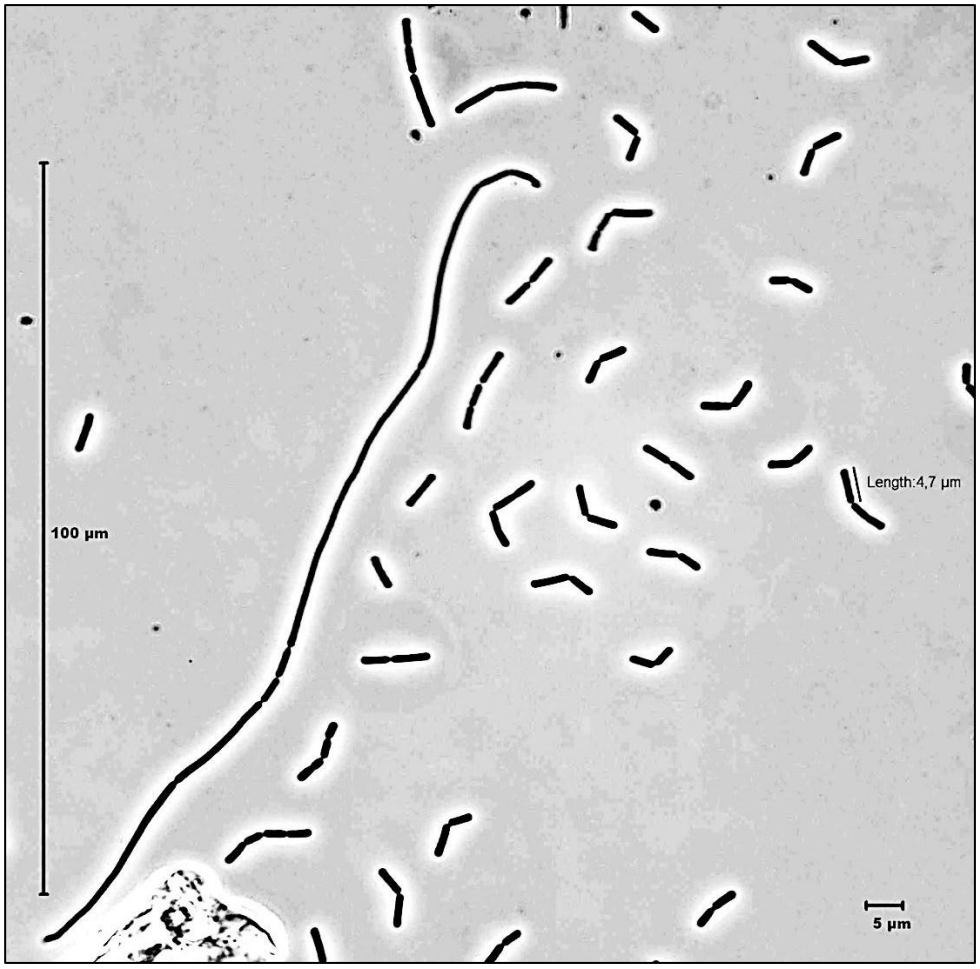


Figure 15: Microscopic picture of strain H (60d), using a phase contrast microscope, and 100x objective

O/F +O_2	O/F -O_2	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
-	-	+	+	-	+	+	+	-	(+)	+	-

Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
	-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
+	+	-	++	0	0	++	++	0	0	0	0	++

Isolate H is Gram- negative and is oxidative, i.e. obligate aerobic. Furthermore, it is able to form H_2S through the action of the enzyme desulfurase, is positive for catalase and shows the capacity to reduce nitrate. It is not able to form acidic products after fermentation of glucose

(negative methyl test) but is positive for the organic acid test. Further, it can produce acetoin (positive VP-test) and cannot use citrate as unique carbon source. It is the only one of the selected isolates that shows clearly the enzyme urease. Additionally, it produces siderophores under Fe-deficiency and is able to produce auxins (IAA). Generally its metal resistance is limited; as the other selected isolates it is very resistant to Mn; to lesser extend to Zn, and not to Al, Ni and Cd.

Some characteristics are corresponding to the typical features of the three genera suggested by the BLAST results, even though many of them do not correspond. Indeed, catalase positive organisms include *Pseudomonas*, typically the *Pseudomonadaceae* are oxidase positive, able to degrade glucose oxidatively. They are able to use citrate as unique carbon source. They are negative for the H_2S formation, VP test or methyl red reaction. Further they can reduce nitrate until nitrogen gas. *Sphingomonas* are gram negative, catalase positive bacteria, which can degrade oxidatively glucose in OF medium. They further are negative for nitrate reduction to gas, do not show urease activity (Yabuuchi et al., 1990).

2.7 Strain I: *Pantoea* (*agglomerans*?) /*Enterobacter*?

Strain I forms intensive yellow (or orange) smooth colonies, and cells are non-mobile rods of 1.9 µm length and 1 µm width.

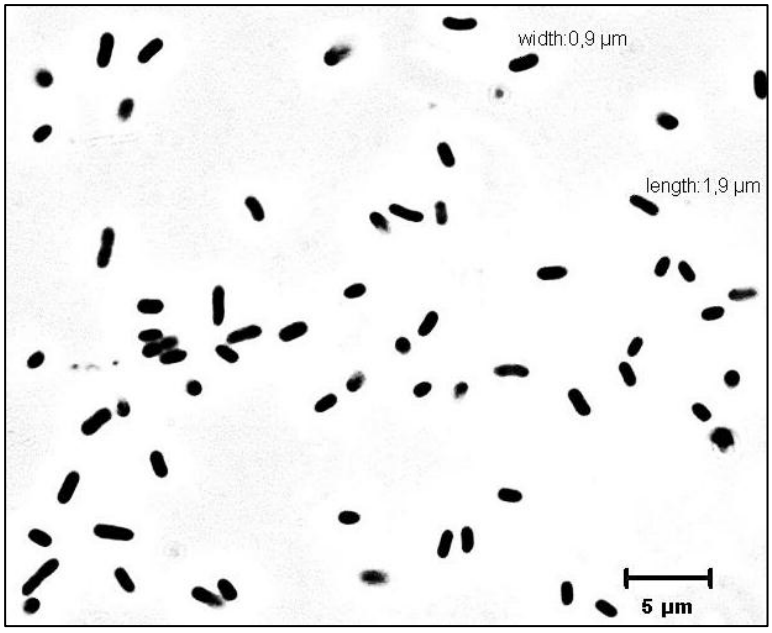


Figure 16: Microscopic picture of strain I (38d), using a phase contrast microscope, and 100x objective

O/F $+O_2$	O/F $-O_2$	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
+	+	-	+	+	+	+	+	-	++	+	-

Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
	-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
+	+	-	+++	0	++	++	++	0	0	0	0	++

Isolate I shows similar physiological features to Strains A-D, its most similar strains based on the 16S DNA comparison. It is Gram- negative, able to function in oxidative and fermentative way. Furthermore, it is able to form H_2S through the action of the enzyme desulfurase, to use citrate as unique carbon source, to reduce nitrate, and can produce acetoin (positive VP-test).

As B, C and D it does not have the enzyme urease, and is not able to form acidic products after fermentation of glucose. It is catalase-positive, a typical feature of the family of Enterobacteriaceae. Furthermore, it is able to produce siderophores under Fe-deficiency, and show an intensive production of the auxin IAA. Its metal resistance is better than many other isolates, as it shows - unlike most of the strains but like strain B and C - Cd resistance. It is very resistant to Mn, and resistant to lesser extend to Zn, Cd and Ni, and not to Al.
Typically Enterobacteriaceae are oxidase negative, however strain I is clearly positive for oxidase.

2.8 Strain J: *Rhizobium/Agrobacterium*

J forms white colonies, with 2 concentric circles visible on 1:10 rich medium agar, and beige smooth colonies on standard 1 agar; the cells are rods of irregular shape, up to 2.6 µm length.

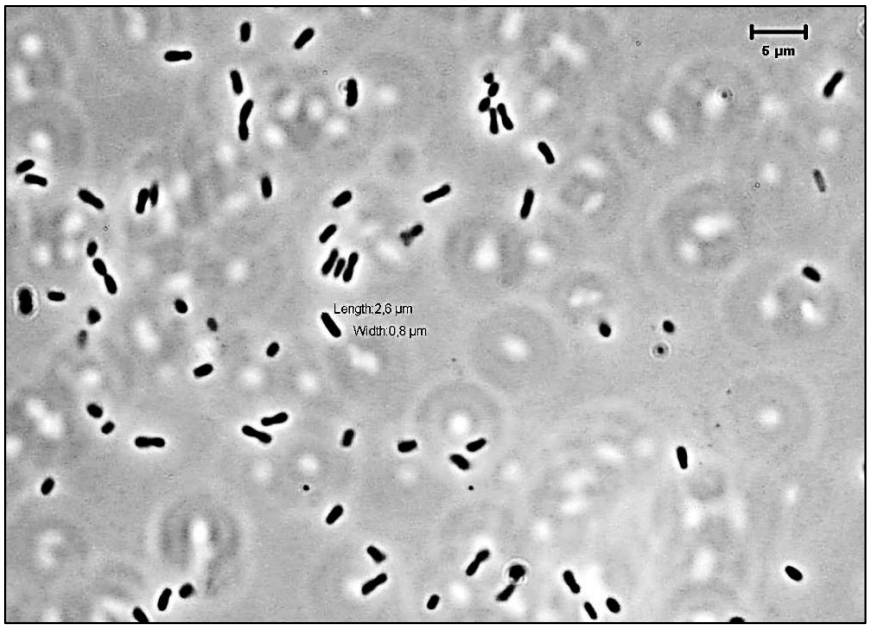


Figure 17: Microscopic picture of cells from strain J (40b), using a phase contrast microscope, and 100x objective

	O/F +O_2	O/F -O_2	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
J	+	+	-	+	+	+	+	+	-	+	+	-

Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
	-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
-	+	-	+++	0	0	0	++	0	0	0	0	++

Isolate J is Gram- negative, able to function in oxidative and fermentative way. Furthermore, it is able to form H_2S through the action of the enzyme desulfurase, to use citrate as unique carbon source, to reduce nitrate, and can produce acetoin (positive VP-test). As most of the isolates, it does not have the enzyme urease, and is not able to form acidic products after fermentation of glucose. It is catalase-positive. Furthermore, it is able to produce siderophores under Fe-deficiency, and show an intensive production of the auxin IAA. Its metal resistance is limited to Mn.

2.9 Strain K: *Curtobacterium* (*herbarum*?) or *Bacillus*

Strain K forms orange slightly shiny colonies on 1:10 rich medium and yellow to orange colonies on standard I agar; the cells are small rods, moving very little. *Curtobacterium ammoniigenes* for example are non-motile rods of irregular shape. Colonies are pale yellow, smooth, convex and round with entire margins (Ventura et al., 2007).

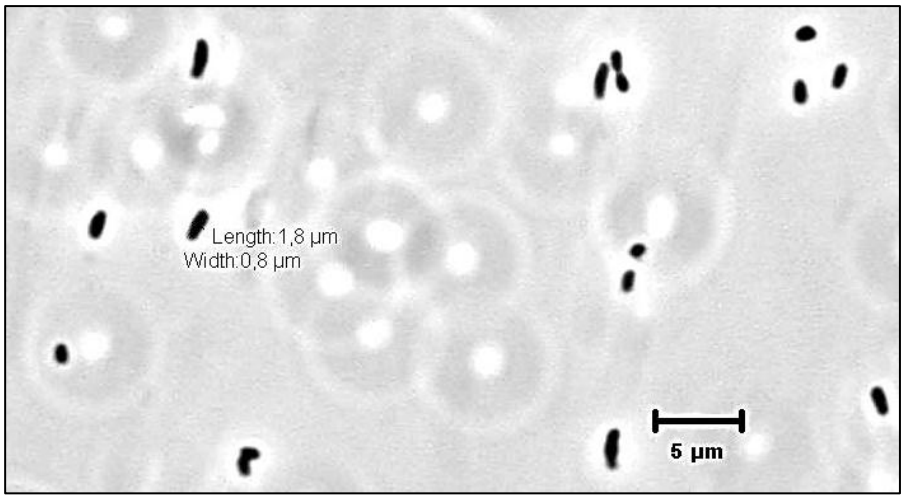


Figure 18: Microscopic picture of cells from strain K (46a), using a phase contrast microscope, and 100x objective

	O/F $+O_2$	O/F $-O_2$	Urease	H_2S	Citrate	Nitrate gas?	Nitrate	VP	Methyl	Oxidase	Catalase	Gram
K	-	-	-	+	+	-	-	+	(+)	+	+	-

Org. acids	Siderophores		IAA	Metal resistance I					Metal resistance II			
	-Fe	+Fe		Ni	Cd	Zn	Mn	Al	Ni	Cd	Zn	Mn
+	-	-	+++	0	0	++	++	0	0	0	(+)	++

Isolate K is Gram- negative, able to function only in oxidative way, i.e. is an obligate aerobic bacterium. Furthermore, it is able to form H_2S through the action of the enzyme desulfurase, to use citrate as unique carbon source, and can produce acetoin (positive VP-test). As most of the isolates, it does not have the enzyme urease. It seems that it is able to form acidic products after fermentation of glucose (slightly positive methyl test). It is not able to reduce nitrate. It is catalase-positive. Furthermore, it is able to produce siderophores under Fe-deficiency, and shows an intensive production of the auxin IAA. Its metal resistance is limited to Mn and Zn; however it has the highest Zn resistance among all selected endophytes.

The Gram-negative result is surprising and not corresponding to the genetic identification of the strain. *Curtobacterium* is a genus of bacteria of the order Actinomycetales. They are Gram-positive soil organisms. They are related to *Leifsonia*, known to be plant commensals (also Microbacteriaceae) (Ventura et al., 2007). Bacillus, the second suggested match, is also a gram positive organism. However, bacteria of the genus *Curtobacterium* were reported to show weak gram positive to gram negative result at the test (Yamada and Komagata, 1972).

REFERENCES

- Euzéby, J.P., 1997a. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int. J. Syst. Bacteriol. 47, 590-592.
- Euzéby, J.P., 1997b. List of Prokaryotic names with Standing in Nomenclature.
- Ewing, W.H., Fife, M.A., 1972. *Enterobacter agglomerans*. Int. J. Syst. Evol. Micr. 22, 4-11.
- Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K., Ley, J.d., 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. Int. J. Syst. Bacteriol. 39, 337-345.
- Halebian, S., Harris, B., Finegold, S.M., Rolfei, R.D., 1981. Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. J.Clin. Microbiol. 13, 444-448.
- Schlegel, H.G., 1992. Allgemeine Mikrobiologie, 7th edition ed. Thieme Verlag, 7th edition.
- Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F., Sinderen, D.v., 2007. Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. Microbiol. Mol. Biol R. 71, 495-548.
- Wolf, A., Fritze, A., Hagemann, M., Berg, G., 2002. *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. Int. J. Syst. Evol. Micr. 52, 1937-1944.
- Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T., Yamamoto, H., 1990. Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the Genus *Sphingomonas*. Microbiol. Immunol. 34, 99-119.
- Yamada, K., Komagata, K., 1972. Taxonomic studies on coryneform bacteria - V. Classification of coryneform bacteira. J. Gen. Appl. Microbiol. 18, 417-431.

Chapter 5

IMPROVING PLANT GROWTH ON HEAVY METAL CONTAMINATED SOIL USING SELECTED ENDOPHYTIC MICROORGANISMS

Tsilla Boisselet[1,2*], Jaco Vangronsveld[2], Nele Weyens[2], Dirk Merten[1], Georg Büchel[1]

[1] Institute for Geosciences, Friedrich Schiller University, Burgweg 11, 07743 Jena, Germany
[2] Centre for Environmental Sciences, Hasselt University, Agoralaan 1, 3590 Hasselt, Belgium

The present chapter is the manuscript of an article in progress to be submitted.

Chapter 5: Improving plant growth on heavy metal contaminated soil using selected endophytic microorganisms

ABSTRACT

In this study, we focus on the improvement of plant health and growth on heavy metal contaminated and nutrient depleted soil exploiting symbiotic endophytic bacteria. For this purpose, autochthonous microorganisms were isolated from two plant species (*Trifolium pratense* and *Festuca rubra*) grown on the heavy metal contaminated soil of the former uranium mining site located in eastern Thuringia, Germany. The microorganisms were characterised and tested for their growth promoting properties and metal resistance, and the best ones were used for inoculation in pot experiments with the mentioned plant species. Further, consortia of these strains with complementary characteristics were also used as an inoculum. Plant health and growth, metal contents in soil and plants, and photosynthetic activity were analysed and compared to un-remediated soil.

The results showed that inoculation of some bacterial strains improved plant growth on contaminated soil to a level comparable to that on non-contaminated soil. Further, root length increased due to the presence of specific microorganisms hence better stabilising the soil by the formation of a denser root system. Inoculation by consortia also led to very significant improvements of plant growth, suggesting a synergetic effect of the different strains. This was confirmed by chlorophyll fluorescence measurements which showed that, on a contaminated soil, inoculated plants were less stressed compared to non-inoculated plants. The mobile fraction of metals was lower with plants than for un-vegetated soil, indicating a stabilising effect of plants. Some bacteria could reduce the solubility of specific metals in the soil. Microbes and microbial consortia alone and in combination with their plant host, could influence the availability of some metals, with Al and REE behaving opposite to Mn, for which three inoculated strains caused a decrease of the soluble fraction compared to un-inoculated plants. Similarly, the uptake of metals by plant aerial parts depended on the plant species and the metal itself.

Keywords

metal contamination, phytoremediation, growth promotion, endophytic bacteria

1 INTRODUCTION

Soil and water pollution by heavy metals is a major concern in many areas of the world, influencing the health of local populations, the use of natural resources and the environmental equilibrium (Bridge, 2004; Gibson and Klinck, 2005; Nilsson and Randhem, 2008) Furthermore, the increasing need for raw material for diverse technological applications tend to multiply the mining sites, even those with lower ore contents, causing evident effects on large areas despite of improved mining techniques (Hester and Harrison, 1994). In particular, surface and ground water are likely to receive an important input of different of these persistent pollutants. Additionally, atmospheric deposition from industry results in large areas with diffuse contamination (Vangronsveld et al., 1995). Moreover, due to wind and water erosion, bare soils or waste heaps are important sources (re-)distribution of pollution (Davies

and White, 1981; Razo et al., 2004). So, the increased industrialisation connected with increased use of land for urbanisation lead to the necessity for remediation of these former mining sites and industrially contaminated areas in order to make the (re-)use of this land possible, if not for agricultural food or feed production, at least for growing energy crops. Indeed, the potential of these vast more or less polluted areas is huge, which explains the rising interest for new remediation strategies. Especially techniques adapted to vast areas, allowing treatment without intensive work and high investment, are important to develop. Therefore, phytoremediation was one of the possibilities on which efforts were focused on, combining land cover and soil stabilisation, valorisation of the cultivated crops, and eventually also clean-up of the soils (Vangronsveld et al., 2009).

One area which faces such problems is the former uranium mining area of Ronneburg in Thuringia, Germany (Geletneky, 2002). This mine has been the third-largest uranium producer in the world (Wismut, 1994a). In 1990, the mining activity was finished and the Wismut GmbH, financed by the German Federal Government, was established to remediate the site.

Strategies that are usually applied to remediate such sites include on the one hand removal of either the soil itself or the removal of metals by leaching with acids and chelators (Rajkumar et al., 2009) and on the other hand metal stabilisation using soil amendments. Typical amendments are: iron oxides, liming agents, apatites, Fe-, Al or Mn-hydroxides, zero-valent iron grit, zeolites, organic matter, red muds and clays, phosphates, industrial by-products (cyclonic ashes) (Vangronsveld et al., 2009). The aim of the amendment addition is to reduce the solubility by forming of insoluble trace element species, and favour adsorption.

In case of the Ronneburg site, the most contaminated waste rock material was transferred into the underground mine galeries and the open pit mine; the groundwater level was allowed to rise again to restore anoxic conditions and thus prevent further oxidation processes and acid mine drainage (AMD) formation. Carlsson and Büchel (2005) described elevated residual contamination levels in the underlying sediments. This location was chosen for the establishing a test site to study the possibilities of alternative remediation strategies for diffuse contaminations.

Nevertheless, conventional clean-up technologies are costly and feasible only for small but heavily polluted sites where fast and complete decontamination is required. Further, some of those methods, such as soil washing, can cause secondary contamination of water ways through seepage waters, and exert negative impacts on biological activity, soil structure and fertility, and generate important engineering costs (Pulford and Watson, 2003; Vangronsveld and Cunningham, 1998). Moreover, disturbing the soil structure can lead to higher metal out washing (Neagoe et al., 2009); this aspect should not be forgotten when moving soil material. The establishment of a vegetation cover on the contrary would stabilise the structure and conserve biological activity, and so avoid erosion and spreading of contaminant to air and water. Therefore, sustainable *in situ* techniques for remediation of contaminated sites, as bioremediation, need to be applied and improved.

Phytoextraction aims to remove contaminants through uptake and accumulation in plants. This technique is suitable for diffusely, slightly polluted areas, where contaminants occur in the upper soil layer, and was tested on several sites (Lebeau et al., 2008; Rajkumar et al., 2009). Phytostabilisation on the other hand consists in establishing a vegetation cover and inactivating toxic metals *in situ*, by combining the effect of metal tolerant vegetation and

metal immobilising soil amendments in order to minimise the mobility and thus spreading of the metals, and if possible improve soil fertility (Vangronsveld et al., 1996; Vangronsveld et al., 2009). It is recommended when the contamination is quite high and covers a large area, especially in the case of mixed (multi-element) contamination. However, there are some problems connected to poor growth conditions of plants on contaminated sites, because of the toxicity and often additional stress factors such as low levels of nutrients and organic matter, erosion or water stress.

Improvement of phytoremediation strategies for heavy metal contaminated soils is needed. Besides conventional soil amendments (as fertilisers, organic matter or alkaline soil) to increase plant growth, it is possible to exploit the natural beneficial effects of associated biota. In this context, it is useful to focus on the interaction between the soil and the plant-influenced and –influencing organisms in particular with soil and plant-symbiotic microorganisms, and their possible use for remediation. Indeed, it is important to ensure survival of plants on the area to remediate, providing sufficient access to nutrients and protection from stress due to toxic elements, pathogens; a high biodiversity is an advantage for a sustainable growth over longer periods (Vangronsveld et al., 1996). Further, it is a benefit if the biomass production is increased, especially in case this can be valorised for energy production or other industrial processes.

Endophytic bacteria can improve plant growth in different ways: they are able to fix atmospheric nitrogen, produce plant growth regulators such as auxins, cytokinins and gibberellins, or suppress the production of stress ethylene by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Badri et al., 2009; Weyens et al., 2009a). In addition, plant-associated bacteria can enhance nutrient uptake by producing acids that solubilise phosphate or siderophores that make micronutrients as iron or other essential metals more plant-available (Dimkpa, 2009; Kidd et al., 2009). Finally, plant growth can be indirectly supported due to competition with and biocontrol of pathogenic bacteria (Dimkpa, 2009).

These mechanisms are of particular interest on metal contaminated soils, the alleviation of heavy metal toxicity by endophytes being a known effect (Weyens et al., 2009a,b).

Indeed, bacteria can affect the solubility, availability and transport of trace elements and nutrients by the above-mentioned mechanisms. On the other hand, they can also reduce the extent of contaminant uptake or translocation to aerial parts of plants by decreasing the bioavailability of metals. The influence of the bacteria on the metal uptake by plants is controverted and is discussed by Rajkumar et al. (2009).

As a consequence, an important aspect to consider for the success of phytoremediation of soils contaminated with heavy metals is the characterisation and selection of suitable rhizospheric and endophytic bacteria in candidate plants appropriate for phytoremediation, and the verification of their efficiency (Porteous Moore et al., 2006). It is further important to test the effect of the bacterial inoculation, to verify if the bacteria possibly decrease the stress experienced by the plant. For this purpose, the chlorophyll fluorescence analysis is used. This technique is based on the property of chlorophyll to release the received light energy not used to drive photosynthesis as heat and light whose fluorescence is measurable. Since these three paths of energy are in competition, the increase of the efficiency of one means the decrease of the other two. The emitted fluorescence gives information about the ability of the plant's photosynthetic system, more precisely the first step of the photosystem II photochemistry, the electron transfer to the quinone in the photosystem II and its maximal capacity and yield. The quantum yield of the photosystem II (ΦPSII) can be correlated to the stress experienced by

plants (Lichtenthaler and Miehé, 1997), as we assume that stressed plants would have a lower capacity to use light.

In our study previously isolated endophytes were inoculated onto the seeds of plants growing on our contaminated study site, and monitored with regard to their biomass, growth parameters and the estimation of the stress of the shoots. The beneficial effect of inoculates on the development of plants on metal contaminated soil, and their possible use for improvement of phytoremediation efficiency should be verified. The role of the bacteria was confirmed by testing for their presence in the tissues at the time of harvest.

2 MATERIALS AND METHODS

2.1 Pot experiment - experimental settings

The pots were filled with 90g quartz sand. To each pot 20 mL of nutrient solution (half concentrated Hoagland solution (Hoagland and Arnon, 1950), was added, corresponding to the field capacity. To test the effect of bacteria on plants in a contaminated soil, a heavy metal mixture was added to the solution. It was composed of $MnCl_2$ x $4H_2O$, $NiCl_2$ x $6H_2O$; $ZnSO_4$ x $7H_2O$; $Al(SO_4)_3$ x $18H_2O$; $NiCl_2$ x $6H_2O$ and $CdSO_4$ x $8H_2O$ added to obtain respectively 30; 3; 1.5; 1 and 0.4 µg element per g substrate.

In a previous experiment, several endophytes were isolated from different organs of *Trifolium pratense* and *Festuca rubra* grown on the contaminated substrate of the study area. A screening was performed in order to test them for their growth promoting potential in such an environment. The chosen strains originated from all 5 plant parts (Shoot and root for *Festuca*; Shoot, Stem and Root for *Trifolium*) and were selected according to their tested phenotypic characteristics, with emphasis on siderophore production, as well as indole-3-acetic acid (IAA) and organic acid (Table 1).

For inoculation of the seeds, the optical density (OD; i.e. absorbance at 600 nm) of the suspension of actively growing bacteria suspended in 1 mM $MgSO_4$ should be similar for all strains; an OD of about 0.65 was taken. To the controls, the same volume of sterile 1mM $MgSO_4$ was added instead of the inoculum suspension.

Table 1: Bacterial strains used for inoculation of the seeds in the pot experiment. Name, origin, tested beneficial properties and original abundance at the moment of isolation of the selected endophytic strains and consortia are given.

Strain	Code Letter		Isolated from	Properties	Isolated CFU / g plant
Not identified	A	W	FR	OA/Sid/Aux/Me	$1.6x10^5$
Enterobacter or Pantoea	B			(OA)/Sid/Aux/Me+	$3.5x10^5$
Enterobacter or Pantoea	C			Sid/Aux/Me+	$1.4x10^6$
Enterobacter or Pantoea	D	X	FL	OA/Sid/Aux/Me	$4.2x10^5$
Not identified	E			OA/Sid/Aux/Me	$1.7x10^5$
Pantoea	F		TL	OA/Sid/Aux/(Me)	$1.8x10^3$
Not identified	G	Y	TR	Sid/Aux/Me+	$1.9x10^6$
Pseudomonas/ Stenotrophomonas/Xanthomonas	H			OA/Sid/(Aux)/Me	$1.4x10^7$
Pantoea /Enterobacter	I	Z	TSt	OA/Sid/Aux/Me	$8.4x10^2$
Rhizobium/Agrobacterium	J			Sid/(Aux)/Me	$4.3x10^2$
Curtobacterium OR Bacillus	K			OA/Aux/Me	$7.0x10^2$

(FR, FL: Festuca Roots and Leaves; TL, TR, TSt: Trifolium Leaves, Roots, Stems)

(Production of OA: Organic Acids; Sid: Siderophores; Aux: Auxin; Me: Metal resistance; () little; + strong)

Also, consortia of the strains were prepared, keeping into account the relative proportions of the strains as isolated. One consortium was composed for each plant and plant organ, combining strengths and weaknesses of their characteristics; for instance, particularly metal resistant but low IAA producing strains were combined with high IAA producers. They were referred as consortia W, X, Y and Z. All treatments were performed in 5 independent replicates.

2.1.1 Survey of plant growth

The plants were grown for 5 weeks in a growth chamber with a 12 h day/night cycle (T: 22°C day / 18°C night; light conditions: photosynthetic active radiation at plant level 173 µmol/m^2. s^{-1}). Germination and growth were controlled daily during the first week and every 3 days later on. The pots were watered when necessary by spraying distilled water on them till germination, and afterwards by pouring water into the tray. The general health of the plants, their height and growth density were monitored regularly over the duration of the experiment.

2.1.2 Pot experiment 2

The pot experiment was repeated with homogenised soil from the contaminated study area in order to study the metal uptake and changes in solubility.

For this, 1 kg (dry weight) soil was used. For each pot, 120 ml deionised water and 12 mL inoculum were added as described for experiment 1. The OD of the strains at time of inoculation was about 0.9. On each pot 0.6 g of seeds were sown. No nutrients were added. As for the first experiment, *Trifolium pratense* and *Festuca rubra* were chosen as test plants, but they were inoculated with only the bacterial strains, that showed to be promising in experiment 1, i. e. for clover I, J, C and red fescue I, J, C, W, X, Y, Z. Additionally, the soil was also inoculated with bacteria without plant seeds, to verify the effect of bacteria alone. Common garden soil was used as a control grown under optimal soil conditions. The plants were grown for 2 months in the greenhouse and monitored as described for experiment 1. All treatments were performed in 5 independent replicates, except the inoculation of bacteria without plants, which were done in triplicate.

2.1.3 Chlorophyll fluorescence measurements

The plants of the second experiment were transported until the measurement place, and the leaves to measure were removed from the plant and kept humid during dark adaptation of the photosystem (30 min) before measurement. The fluorescence was measured with fluorcam (Photon System instruments, Brno, Czech Republic) and the data analysed with the corresponding software. 6 *Trifolium* leaves and 6 *Festuca* shoots were taken of each treatments from 2 or 3 different pots, at 3 times points and compared to plants grown in uncontaminated soil.

2.2 Statistical testing

Trace element concentrations (Zn, Cu, Fe, Ca, K, Na, Mg, Cd, Pb) in soils and plant parts (root, stem and leaf) on one hand, and chlorophyll fluorescence on the other hand were statistically compared for the plants treated with different bacterial inocula. The significance of the differences between treatments was tested and confirmed by a one-way ANOVA test and LSD *post hoc* analysis, with a confidence of 95%.

2.3 BOX PCR genomic DNA profiling/fingerprinting

For the BOX PCR, genomic DNA was extracted using Qiagen DNA extraction kit and DNA was amplified in PCR using the genomic DNA as template and one primer 5'-CTACGGCAAGGCGACGCTGACG -3' (Murry et al., 1995). The PCR mixture (50 µl) contained 1 µl template, 5 µl of 10x High fidelity PCR buffer, 2 µl of 50 mM $MgCl_2$, 1 µl of dNTP at 10 mM, 0.2 µl of Platinum Taq High fidelity DNA polymerase, 2 µL of 10mM primer.

The PCR was performed in a Mastercycler gradient (Eppendorf) with a hot start performed at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1.5 min, and 68°C for 8 min, followed by a final extension performed at 68°C for 8 min.

2.4 Plant and soil material: sample preparation and analysis

After 5 weeks of growth, selected plants were harvested. Evaluation parameters used were the height of the plants (growth promoting effect of bacteria) as well as the estimated growth density and health ('colour') of the plant. Two pots were taken for isolation, and the controls for comparison.

The plants were taken out of the sand (5 to 10 *Festuca* seedlings and 1 to 2 clover for each isolation), and after measuring root length and evaluating general health, separated into leaves and roots (and also stems for *Trifolium*). From these organs, endophytic microorganisms were isolated.

Additionally, the plant biomass was harvested, carefully cleaned with deionised and suprapure water in order to remove any soil from the surface, dried, weighted and milled to powder using a mixer mill (type MM400, from Retsch®). Subsequently, metals were extracted by a microwave assisted pressure digestion (MARS 5, from CEM corporation, USA) with 65% HNO_3 (Merck, p.a., subboiled); the obtained solution was diluted and centrifuged to be ready for analysis. The soil samples were dried in porcelain plates at room temperature at the air until constant weight. The water content was calculated from the loss of weight.

Four g of dry sieved soil were eluted with 40mL of selective extraction solution. Both pure deionised water and a 1 M ammonium nitrate solution (Merck) were used as selective extractants. The suspensions were shaken 24h overhead at about 20 rpm (Overhead shaker-ELU safety lock, Edmund Bühler). For each experiment, blanks (tubes with only elution solution) were prepared and treated in the same way.

The samples were centrifuged 15 min at 2500 rpm. 15 mL of each sample were filtered through a 0.45 µm-celluloseacetate filter. From the remaining solution pH (pH 320, WTW) and electrical conductivity (LF320, WTW) were measured. The samples were acidified with suprapure HNO_3 (63%) and kept at 4°C until analysis.

Soil samples were also analysed for their total metal content. For this, they were milled and 100 mg were putted into TFM vessels. Subsequently, 4 ml 40% HF and 4 ml 70% $HClO_4$ (both suprapur, Merck) were added. After the mixture stood overnight in closed vessels, the vessels were tightened and heated up to 180°C within 4 h. The temperature was maintained for 12 h and then the samples were allowed to cool down. In order to evaporate acids, the system again was heated up to 180°C for a period of 4 h, this time using a special evaporation hood. This temperature was kept for 12 h. Then, to the remaining solid sample 2ml HNO_3 (65%, subboiled), 0.6 ml HCl (30%, Suprapur, Merck) and 7ml of pure water (Pure Lab Plus,

USF) were added and the mixture was dissolved by heating at 150°C for 10 h. The cooled samples were then transferred to calibrated 25 ml PMP flasks (Vitlab). Finally, the solution was replenished to 25 ml by the addition of pure water for analysis.
The elemental contents in the samples were analysed by ICP-OES (Spectroflame, Spectro) for the main elements (Al, Ca, Fe, K, Mg, Mn, Na, P, S) and ICP-MS (X Series II, Thermo Fisher Scientific) for the trace elements (As, Co, Cu, Cd, Mn, Ni, REE (La-Lu), U, Zn).

3 RESULTS

3.1 Plant growth promoting effects: macroscopic observation of roots and growth density

Obvious differences were noticed between the *Festuca* seedlings grown in contaminated and uncontaminated artificial soils. On the uncontaminated soil seedlings showed a three-fold longer root growth and a much higher density of roots (Figure 1a). Further, a clear improvement of root length was obtained after inoculation of specific strains (I, J, K) (Figure 1b). The effect of the combination of strains was also strong.

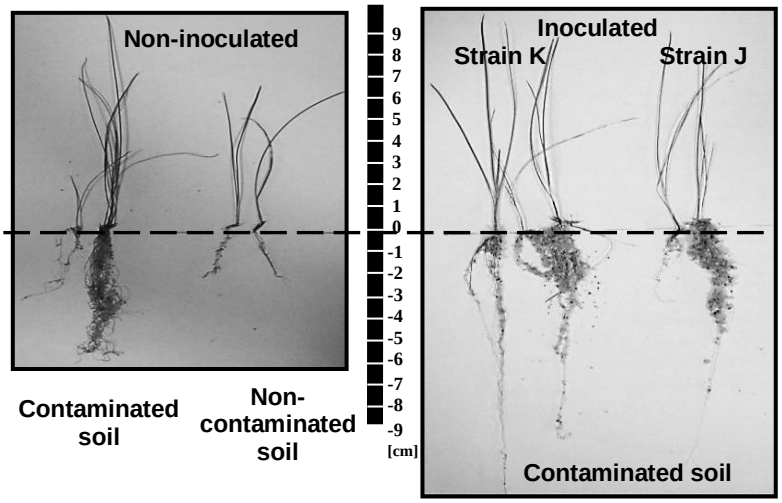


Figure 1: Root growth differences
(a): root and shoot length of *Festuca rubra* after 5 weeks of growth in contaminated soil (left) and uncontaminated soil (right). Metal contamination inhibits root growth.
(b): roots and shoots of *Festuca* after 5 weeks in contaminated soil inoculated with strains K and J (K: *Curtobacterium sp.*; J: *Rhizobium radiobacter*). Root growth is enhanced by inocula, comparable to growth without contamination.

Strains I, J and K showed to be the most promising for both species, leading to increased plant height (not shown) and plant density (Figure 2). The effect was particularly obvious for *Trifolium*, since many seedlings did not germinate or died at very early growth stages with other inocula.
For *Trifolium*, the consortia were not very efficient in promoting growth, except of Z, still showing growth below the density and height achieved after inoculation with strains I and J.

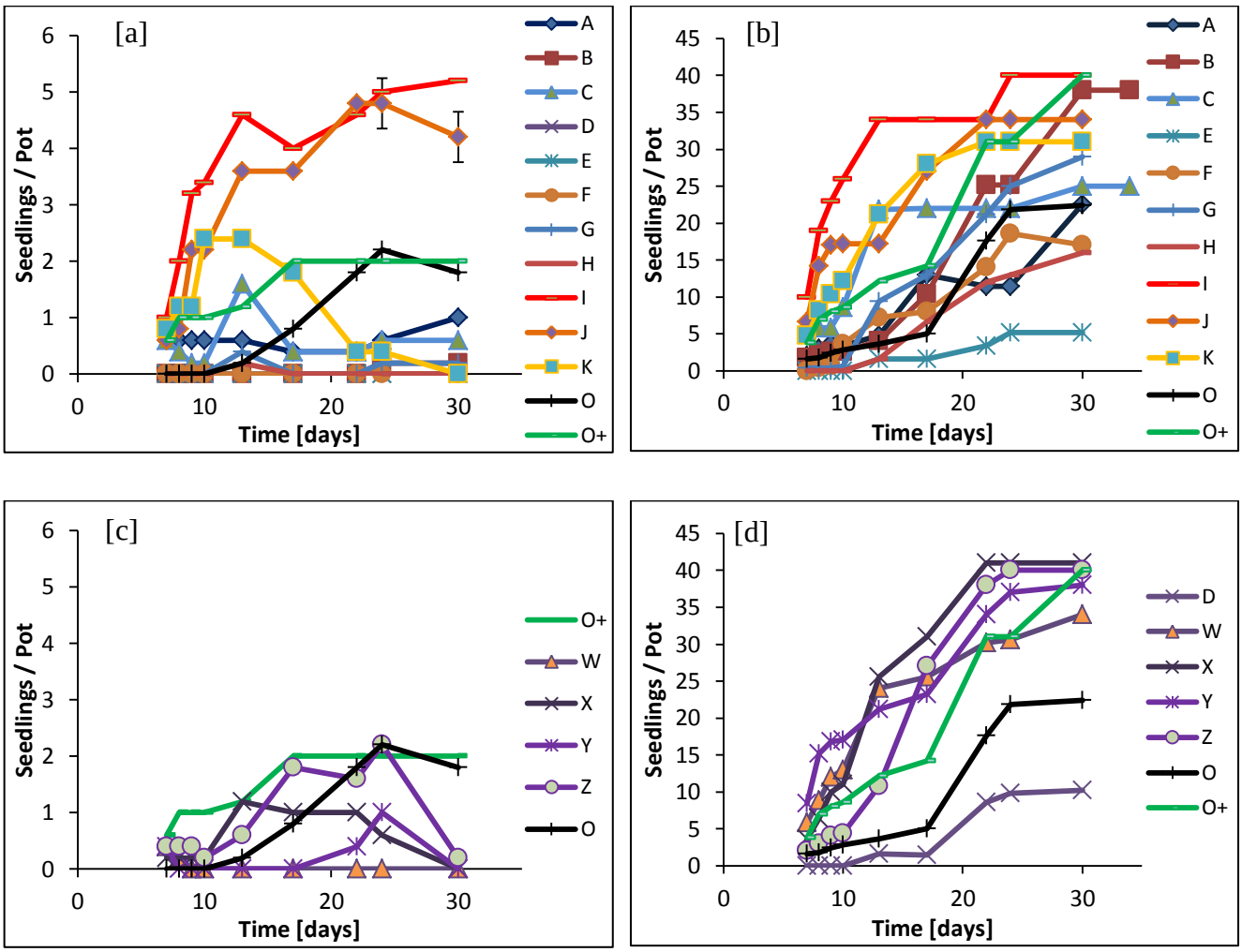


Figure 2: Growth density for *Festuca rubra* and *Trifolium pratense* on contaminated and un-contaminated substrate over time. Comparison between single inocula (A-K) with contaminated (O) and un-contaminated (O+) control ([a] and [b]); comparison between consortia (W-Z) with contaminated (O) and un-contaminated (O+)([c] and [d].

For *Festuca*, the consortia, X, Y and Z seemed to have greater effects (more seedlings) than W (Figure 2d). At that point, also some samples showed chlorotic leaves, probably due to metal toxicity. *Festuca* plants inoculated with consortia showed even a better development than plants grown on uncontaminated substrate.

The effect of inoculation seems better if inoculated as a consortium: the development of the plants is better than what could be expected by addition of the effect of the two single strains composing the consortium; the effect on plant density is even more obvious for strains without particularly high growth promoting effect (Figure 3).

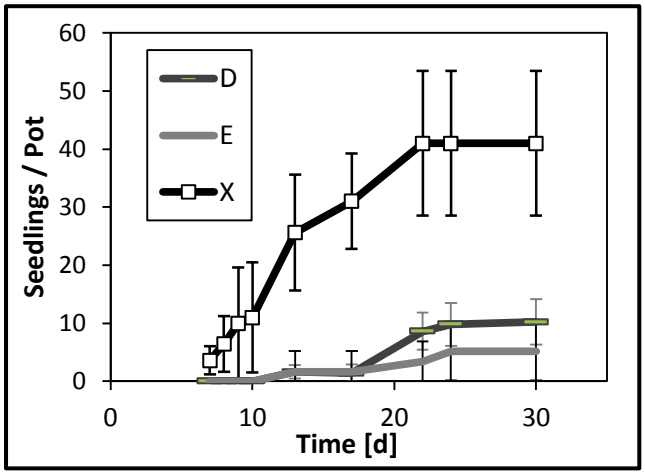


Figure 3: **Growth density for *Festuca rubra* on contaminated substrate over time. Comparison of plants inoculates with single strains D and E with plants inoculated with the consortium X=D+E**

Significant increases of root and shoot dry weights were observed when the soil was inoculated with bacteria, especially with consortia W, X and Y for *Festuca* (Figure 4a). The root to shoot ratio shifts from a ratio of about 0.5 on contaminated un-inoculated soil to over 1.5 for inoculated soils, indicating that the root biomass is even more improved than that of the shoots. Shoot biomass was more than doubled for *Trifolium* compared to un-amended plants, and for *Festuca* it was more than tripled for strain mixtures W, X and Y. For roots the trend is similar, hence it is even more pronounced for *Festuca*, root biomass being 10-fold increased with consortia W, X and Y. However, despite inoculation, on contaminated soil the biomass never reached that obtained on an uncontaminated soil with optimal nutrient supply; nevertheless, for *Trifolium* almost 80% of the control biomass could be obtained after inoculation of strains I and C.

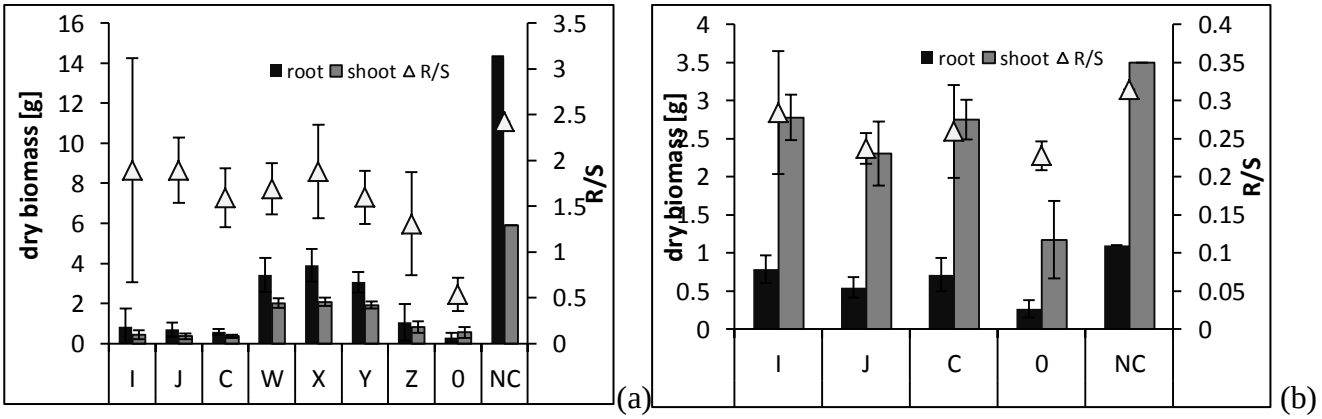


Figure 4: Effect of inoculation on plant biomass. Root and shoot biomass and root to shoot ratio (R/S) of *Festuca* (a) and *Trifolium* (b)

3.2 Recovery of inoculated strains in plants

Many bacterial strains could be isolated from the tissues of the inoculated plants. More cultivable strains were isolated from *Festuca* than *Trifolium* (there were about three orders of magnitude more); furthermore, strain diversity in *Festuca* was higher compared to *Trifolium.* The number of CFU in roots was higher than in shoots for *Festuca* (x10-100), CFU till ~5.10^8 CFU/g plant. For *Trifolium*, it was the other way around: more CFU were isolated from leaves and stems than from roots (x10). The samples with highest CFU were those inoculated with the strains C, I, J, K, W, Y (on contaminated substrate) and A, I (on uncontaminated one)

The bacterial diversity and CFU were similar if the soil was contaminated or uncontaminated, except for I (more diversity in plants grown on uncontaminated soil).

The isolated strains were compared to the inoculated one(s) based on their BOX PCR patterns (Figure 5).

The inoculations were successful since strains I, J, A, B, C, D, G could be recovered (see Table 2) from 9 samples out of 13 inoculated.

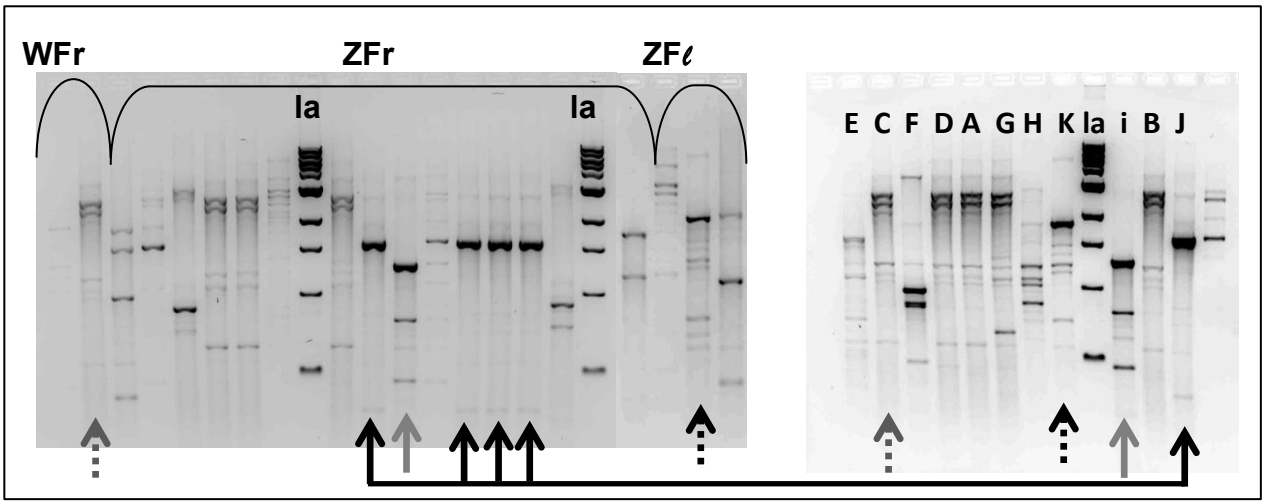


Figure 5: Example of BOX PCR patterns of some samples (on the left) compared to the inoculated strains (A till K, on the right); la stands for the 100bp DNA ladder.
Strain C is recovered from the *Festuca* root (Fr) sample inoculated with consortium W, and I, J and K are all recovered in the *Festuca* root or shoot sample (Fr or Fℓ) inoculated with consortium Z.

Table 2: Recovery of inoculated in the different plant organs

Inoculum	F	*r*	*l*
B	✓	B	-/B
C	✓	-/C	-
I	-	-	-
J	-	-	-
K	✓	-	-
W	✓	B(A,C?)/C	-
X	✓	D	D
Y	✓	G	G?
Z	✓	J, I	K
A*	✓	A	-
I*	✓	I	I

Inoculum	T	*r*	*st*	*l*
I	✓	-	-	I
J	-	-	-	-

r =roots; *l*= leaves; *st*= stems; F= Festuca; T= Trifolium

3.3 Chlorophyll fluorescence: plant stress

The quantum yield of the photosystem II (ΦPSII) is higher when the PSII is working more efficient. ΦPSII can be correlated to the stress experienced by plants (Lichtenthaler and Miehé, 1997). A lower efficiency corresponds to higher stress, as we assume that stressed plants would have a lower capacity to use light. In our experiment, ΦPSII was higher in *Trifolium* plants grown on non-contaminated soil than in those grown on contaminated soil (Figure 6), which means that PSII functioned better. Moreover, bacterial inoculation of plants grown on contaminated soil lead to a quantum yield comparable to that of plant grown on the uncontaminated soil. This effect was significant and stable over the duration of the experiment.

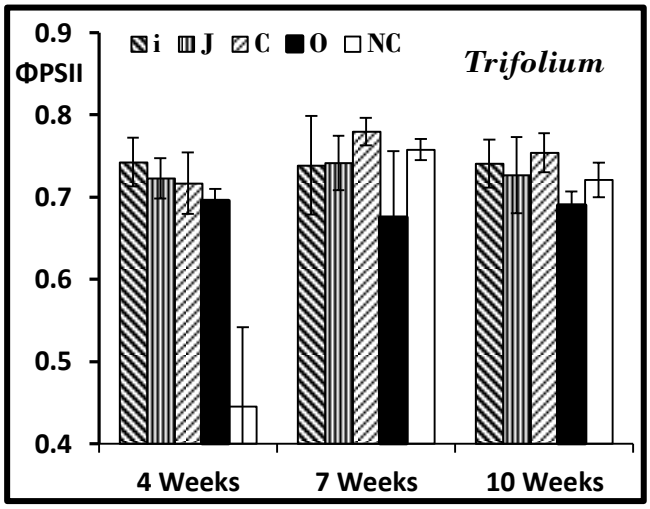
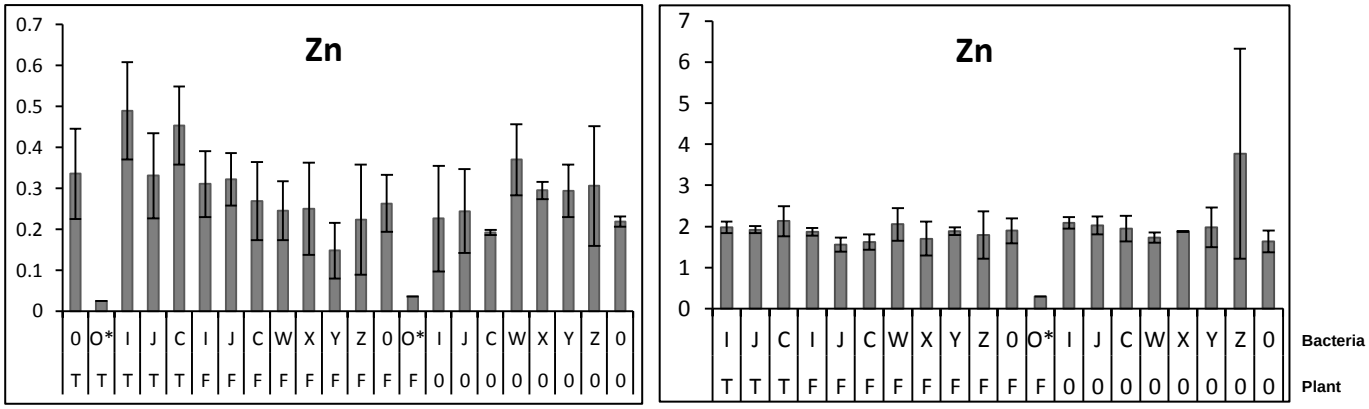


Figure 6: Quantum yield of Photosystem II over time in *Trifolium* inoculated with different bacterial endophytes. NC = uncontaminated

3.4 Analysis of soils: water and ammonium nitrate extractable fractions of metals

Generally the amounts of elements extracted with water are about a factor of 10 lower than with ammonium nitrate (Figure 7); however, the trends observed between the treatments do not depend on the extracting agent, but on the considered chemical element. *Trifolium* shows generally a higher amount of extractable metals than *Festuca* for the same treatment (i.e. strains I, J, C), even in the case there is no significant difference in the control. The ammonium-nitrate extractable fraction of some metals (Ni, Al, REE) is lower with *Festuca* than for bare soil, however only if the plants are inoculated with the strain consortia (Figure 7). Some metals as Zn or Pb do not show any differences in the amount of ammonium-nitrate and water extractable metals (Figure 7). Some metals as the REE and also Al show a significant influence of the plant and bacterial treatment (Figure 7). The ammonium-nitrate extractability and the water extractability of certain metals as REE were reduced by particular strains (I, J, C), and increased if the bacteria were inoculated to plants. Mn behaves in opposite way to REE, as for example strains C, I, and J inoculated to plants cause a decrease of soluble available metals compared to un-inoculated plants.



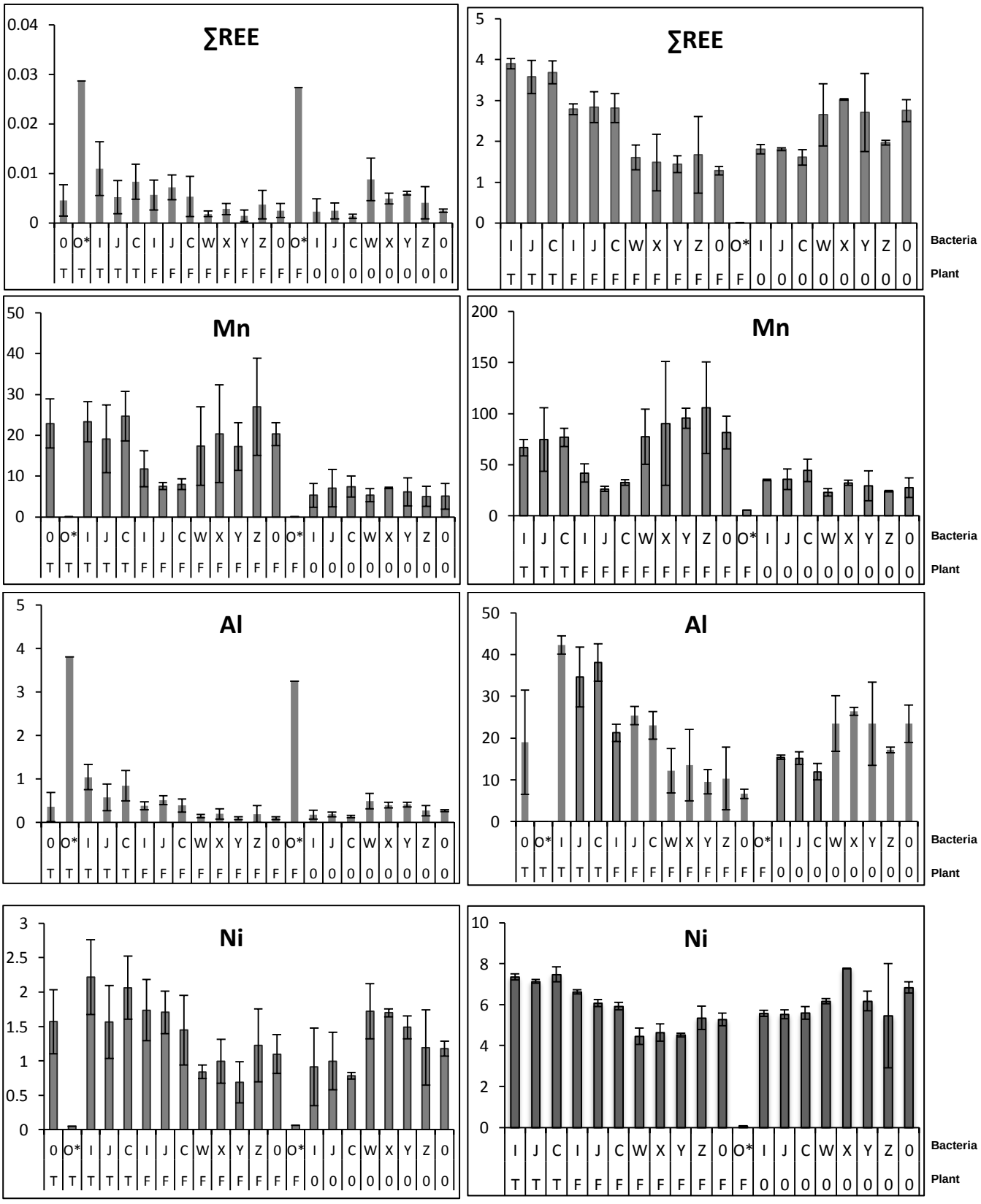


Figure 7: **Water (left) and NH_4NO_3 (right) extrable metal contents of the soil in µg/g**

I, J, C: bacterial inocula; W, X, Y, Z combinations of 2-3 bacterial inocula;

F: *Festuca rubra* , *T: Trifolium*, 0: no inoculum or no plant; O* un-contaminated control

3.5 Analysis of plants: total metal content

The uptake of metals by plant aerial parts depends on the plant species, the soil and the speciation of the metal itself.

In *Festuca*, REE, Cr and Al were taken up more if the plant was inoculated with the strains I, J and Z; Mn was behaving opposite, being taken up in lower amounts in case of inoculation with I, J, C (Figure 8).

The consortia W, X, and Y, resulted in significantly lower uptake of Ni and Cd (results not shown). Cu and Co were taken up in lower concentrations for all inoculates while Fe and Zn showed no significant change at this concentration (results not shown).

Similarly, for *Trifolium* higher concentrations of REE were found in the shoots in case they were inoculated with the strains J and C (results not shown); for Fe a slightly increased concentration was found after inoculation with I (results not shown). For U no significant differences were noticed, except a slight decrease in case the plant was inoculated with strain I (results not shown). The other relevant metals (Cd, Al, Ni, Zn, Cu, Co, Mn) were not influenced by inoculation at the present concentration (results not shown).

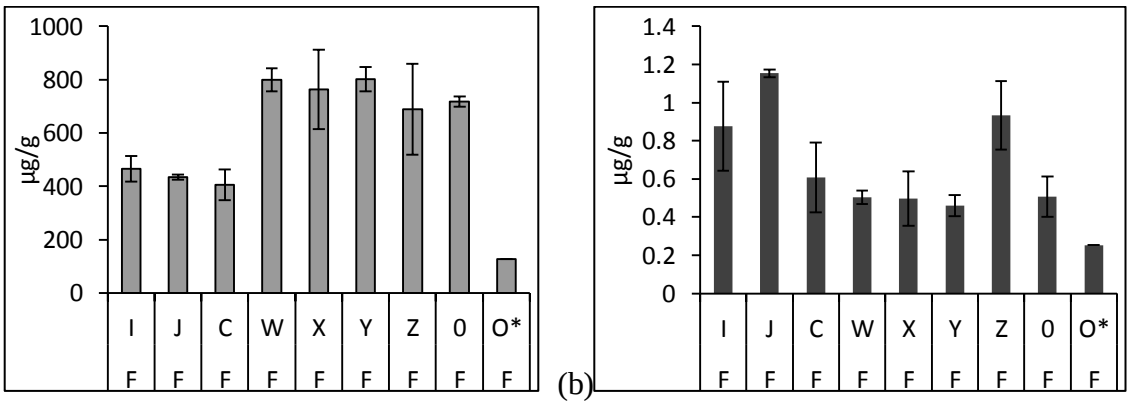


Figure 8: Metal content of shoots of *Festuca rubra*. (a) Mn; (b) ∑REE

4 DISCUSSION

For both species, *Festuca rubra* and *Trifolium rubra*, increased metal contents in the growing substrate lead to significantly decreased germination rate, plant survival and plant growth.

4.1 Root growth

The root length was clearly different between the treatments (Figure 1). This can be explained by the fact that several heavy metals are known to affect especially root development (Barceló and Poschenrieder, 2002). On the other hand, since many of the bacterial strains possess the capacity to produce plant growth promoting auxins (see Table 1), the inoculated endophytes should be able to improve germination and cell division of plant tissues, and enhance in particular root growth. This is one of the factors which may explain the strong differences we observed between un-inoculated plants grown on contaminated soil and those inoculated with the selected endophytes. Other properties of endophytes, as ACC deaminase activity may also be involved. These properties have in fact been exploited in many studies (Sheng et al., 2008) to increase the biomass of plants grown under stress.

However, the changes of plant growth parameters seemed to be typically dependent on the plant species as the changes in the root biomass were essentially noticed for *Festuca* and to a lesser extend for *Trifolium* (Figure 4). The root/shoot ratio calculated by Rönkkö et al. (1993),

showed also a great increase for *F. rubra* through inoculation of root associated N_2-fixers or free N_2 fixers as *Frankia*, whereas other tested plants did not show this effect, despite noticeable changes in the total biomass.

4.2 Bacterial colonisation of *Festuca* and *Trifolium*

Although the endophytic bacteria isolated from *Festuca* and *Trifolium* possess various plant growth promoting features, the use of microbial inoculations for growth promotion requires a sufficient level of re-colonisation of the introduced microbes.
The presence of the inoculated strains in the inoculated plants shows that colonisation of the plant occurred; comparison of the BOX PCR patterns of re-isolated strains with those of the inoculated ones shows the success of the inoculation and suggests a causal relationship between the inoculations and the growth promoting effect (Figure 5).

Strains A, B, G originate from roots, and were observed to be more recovered from the roots of the inoculated plants. This is not surprising since many endophytes indeed do not only show a host specificity but also an organ specificity. Strains D, G, I, J, K were only recovered if inoculated in combination with others, hence it seems that these strains have increased rates of inoculation success if inoculated as a consortium. Not recovered strains (E, H) were or (1) not able to colonise or (2) no real endophytes. More bacteria were found after harvest than at inoculation (for example J, in *Trifolium*, Table 2) illustrating the success of colonisation by the introduced strains. The poorer recovery of inocula in *Trifolium* is also probable causing the poor growth of the plant on the contaminated soil (cf. Figure 2 and Table 2).
The fate of the inoculated strains over time is an important question to take into account; some experiments (Whiting et al., 2001) suggest that the positive effects on plants are not necessarily specific to the strains of bacteria added, but that also native bacterial population can have a strong impact. (van der Lelie et al., 2005) suggest that horizontal gene transfer to the other present bacteria is a feature that can even be of use to help plant promoting bacteria with specific properties to establish in an already existing bacterial community. This was confirmed in a field experiment on a TCE-contaminated site by Weyens et al. (2009).

4.3 Protection against stress (Photosynthesis)

Chlorophyll fluorescence as a tool to estimate plants' susceptibility to environmental changes has been used by several authors since many years (Atlassi Pak et al., 2009; Lichtenthaler and Miehé, 1997). The quantum yield is the only parameter which is significantly affected by metal stress over the entire period of the experiment (Figure 6). Other typical parameters like the F_v/F_m show also a tendency to support the conclusion that inoculation protects plants from stress, although this effect is really visible only during the first month to 7 weeks (Figure 6). This can be also due to the fact that the method uses the blue-green fluorescence, which is only representative for a part of the photosynthesis process. (Lichtenthaler and Miehé, 1997) explain that blue-green fluorescence emission can lead to misinterpretation about the photosystem efficiency, since long term stress events reduce eventually the carotenoid content of leaves and as a consequence increase the proportion of blue light emission by the plants. To verify this effect, the fluorescence should be measured at different wavelengths (690 and 735 nm). If this should be confirmed, we can assume that our treatments reduced the blue-green fluorescence emission and compensated the loss of chlorophyll and carotenoids of the leaves during long-term stress.

4.4 Inoculation with consortia has more positive effects than single strain inocula

This study shows that the simultaneous inoculation of 2 or 3 strains changes the effect on the plant. Especially with regard to the root biomass and the density of plants grown under metal stress, the combination of strains causes a synergetic effect (Figure 3). Strains that were not very efficient growth promoters in case they were inoculated separately had a strongly positive effect on plant growth when combined with each other. This could be due to the combination of different complementary properties. Indeed, not all inocula showed for example high metal resistance, and so growth together with a resistant one could increase their survival and consequently their growth promoting effect on the host. Similarly, a strain may produce other hormones that would increase the growth of the bacterial partner, or provide nutrients with a system not available for the other bacteria. So, by combining two or more strains, the likelihood of having good survival mechanisms under difficult conditions is increased.

Indeed, Schmidt et al. (2005) reported that some not metal resistant strains, could grow near resistant ones, due to the fact that they produce substances that protect them against heavy metals. Moreover, it has already been shown that bacteria introduced as vectors into the plant ecosystem can be responsible for natural horizontal gene transfer to the endogenous endophytic population (Weyens et al., 2009a). Van der Lelie et al. (2005) even suggested that horizontal gene transfer to the other present bacteria is more probable than an establishment of a new strain in an already existing stable community.

This aspect of plant growth promotion by consortia has not yet been extensively considered in the past, even though it was already mentioned by Kozyrovska et al. (1996), who investigated simultaneous inoculation of 2 endophytes in the context of growth promotion of agricultural crops on radionuclide contaminated soil. It was suggested that endophytes could help crops to grow in unfavourable environments, to avoid radionuclide uptake, and be a good alternative to agrochemicals. In fact, the use of beneficial bacteria to promote plant growth and health has been suggested already over 20 years ago for agricultural crops (Davison, 1988), and studied for several plants in phytoremediation later on (Doty, 2008; Guo et al., 2010; Lodewyckx et al., 2002; Mastretta et al., 2006; Mastretta et al., 2009; Weyens et al., 2010; Weyens et al., 2009a; Weyens et al., 2009b) the application being mostly limited to one bacterial species per host.

On the other hand some studies focused on the effect of plant diversity on soil properties, as to achieve a stable persistent cover it is important to use a mixed vegetation, and combine grasses, legumes and trees (Kidd et al., 2009; Tessema, 2011; Vangronsveld et al., 1996). Based on these considerations, it is important to consider possible synergetic effects (or antagonistic) with the natural community of soil microorganisms when adding bacteria for *in situ* phytoremediation. In particular, the action of mycorrhiza is crucial, these fungi being studied extensively for remediation improvement on heavy metal contaminated site They are known to protect physically the roots from intrusion and physiologically from stress due to too high metal concentrations or nutrient depletion (Adriaensen et al., 2003; Adriaensen et al., 2005; Krznaric et al., 2009; Schützendübel and Polle, 2002).

It is clear that more investigations are needed to better understand the interactions in these complex systems. Their potential impact for both agriculture and in bioremediation is very high.

4.5 Mobilisation of metals in the soil

The interactions of organic acids released by roots with the soil solid phase appear to be among the key processes (Puschenreiter et al., 2005). In particular, these authors suggest that root activities of accumulators as *Thlaspi goesingense,* such as the exudation of organic acids triggered the replenishment of soluble Ni from immobile metal fractions of the soil. Different substances, like organic acids, siderophores, and other complexing agents, are known to influence the solubility of metals and their uptake by plants.

The increase in the solubility of metals in the soil can be also linked to the properties of the bacteria, since they are also able to produce siderophores and other metal-chelating substances. Metallophores are for instance produced by strains of *Pseudomonas* and *Enterobacter* (Whiting et al., 2001). *P. aeruginosa* can also allow complexation of REE; further it is able to extract Fe and Mg (Aouad et al., 2006). Sheng et al. (2008) showed the influence of some bacteria on the solubilisation of Pb in soil and water by *P. fluorescens* G10 and *Microbacterium sp*. G16.

Sheng et al. (2008) noticed that some bacteria facilitate the release of the poorly soluble Pb, thereby enhancing its uptake by plants. Certain metal resistant bacteria have been shown to possess several properties than can affect both the toxicity and plant availability of metals through the production of several complexing agents as siderophores or organic acids (Sheng et al. 2008; Rajkumar et al. 2009).

4.6 Metal uptake

Microbes and microbial consortia alone and in combination with their plant host, can influence the plant availability of some metals. In the past, even if bacteria were used to enhance plant resistance to toxic amounts of metals and by consequence also biomass production, it was often not clear if the improved phytoextraction was attributable to the plant itself or to a combination of plant and microbes (Rajkumar et al., 2009). Our study shows that bacteria alone can lead to a decrease in the soluble phase for some metals as REE on one hand, but on another hand to an increase if those same bacteria were inoculated into plants.

Metal uptake was influenced by the presence of bacteria (Figure 8), but in different ways depending on the strain and on the metal itself. Indeed, the increased biomass production after inoculation could in some cases be due to the metal immobilising effect of the endophytic bacteria, thereby lowering the internal metal availability and by consequence its toxicity for the host (Shin et al., 2012).

Although we observed a slight increase in pH in the rhizosphere compared to bare soil, the changes in mobility of metals do not seem to be necessarily correlated with pH changes. Many bacterial endophytes which are metal resistant are known to enhance metal uptake by plants and support their growth at the same time (Rajkumar et al., 2009). Whiting et al. (2001) for instance reported that the increase in the solubility of Zn in the soil was not due to changes in pH or was not a function of increased root hair growth. Their study indicates that the bacteria facilitated the release of Zn from the non-labile phase in the soil, thus enhancing Zn accumulation by *T. caerulescens*.

The fact that the samples with a higher content of metals in the soluble (i.e. water and ammonium nitrate extractable fractions) soil fraction were the same with higher metal content in plant shoots (Figures 7 and 8) suggests that the treatment influences the solubility of metals in a sustained process. In particular root exudates are known to play an important role for continuous plant availability of metals out of the soil (Puschenreiter et al., 2005).

High amounts of soluble metals in the soil result in high amounts in the plant, and vice versa. However, this is dependent on the element and on the plant species. The property of certain plants to take up preferentially specific metals above others has been described already by numerous authors (Krämer, 2010) and used for remediation purposes in the case of hyperaccumulators (Sarma, 2011). The choice of the right plant is important, since some plants are accumulating some contaminants more than others.
Abou-Shanab et al. 2003a, 2006 in (Kidd et al., 2009) demonstrated that the bacterial-induced enhancing effect on metal extraction effect was dependent upon the metal concentration of soils, emphasising thereby the need for site-specific evaluation.

5 CONCLUSION

This study provides new insights into the opportunities given by the interaction between plants and their associated microorganisms when growing on a soil containing heavy metals. It demonstrates the effectiveness of using inoculations of endophytic bacteria to increase phytoremediation potential, and the enhanced effects of bacterial consortia. We want to emphasise in this context the importance to consider synergetic effects or possibly antagonistic effects with natural soil microorganism communities during *in situ* remediation processes.
Microbes and microbial consortia alone and in combination with their plant host, can influence the solubility of some metals and therefore their availability to plants. Some bacteria can reduce the soluble (i.e. mobile) metal fraction. The mobile fraction of metals is lower with plants than for bare soil, indicating the stabilising effect of plants.
We suggest using *Festuca* and *Trifolium* in addition to metal extracting plants, in order to improve soil fertility and as protection against wind and water erosion (dense root network). *Festuca* is more influenced by bacteria concerning its root development, so should therefore get particular attention when it comes to choosing plant communities for remediation.

- **REFERENCES**

- Adriaensen, K., Lelie, D.v.d., Van Laere, A., Vagronsveld, J., Colpaert, J.V., 2003. A zinc-adapted fungus protects pines from zinc stress. New Phytologist 161, 549-555.
- Adriaensen, K., Vralstad, T., Noben, J.-P., Vangronsveld, J., Colpaert, J.V., 2005. Copper-adapted *Suillus luteus*, a symbiotic solution for pines colonizing Cu mine spoils. Appl. Environ. Microb. 71, 7279-7284.
- Aouad, G., Stille, P., Crovisier, J.-L., Geoffroy, V.A., Meyer, J.-M., Lahd-Gaegea, M., 2006. Influence of bacteria on lathanide and actinide transfer from specific soil components (humus, soil minerals and vitrified municipal waste incinerator bottom ash) to corn plants: Sr-Nd isotope evidence. Sci. Total Environ. 370, 545-551.
- Atlassi Pak, V., Nabipour, M., Meskarbashee, M., 2009. Effect of salt stress on chlorophyll content, fluorescence, Na+ and K+ Ions content in rape plants (*Brassica napus L.*). Asian Journal of Agricultural Research 3, 28-37.
- Badri, D.V., Weir, T.L., van der Lelie, D., Vivanco, J.M., 2009. Rhizosphere chemical dialogues: plant-microbe interactions. Curr.Opin. Biotech. 20, 642-650.
- Barceló, J., Poschenrieder, C., 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ. Exp. Bot. 48, 75-92.
- Bridge, G., 2004. CONTESTED TERRAIN: Mining and the Environment. Annu. Rev. Env. Resour. 29, 205-259.
- Carlsson, E., Büchel, G., 2005. Screening of residual contamination at a former uranium heap leaching site, Thuringia, Germany. Chem. Erde - Geochem. 65, 75-95.
- Davies, B.E., White, H.M., 1981. Environmental pollution by wind blown lead mine waste: a case study in wales, U.K. Sci. Total Environ. 20, 57-74.
- Davison, J., 1988. Plant beneficial bacteria. Biotechnology 6, 282-286.
- Dimkpa, C.O., 2009. Microbial Siderophores in Rhizosphere Interactions in Heavy Metal-containing Environments, Biologisch-Pharmazeutische Fakultät. Friedrich-Schiller University, Jena, p. 135.
- Doty, S.L., 2008. Enhancing phytoremediation through the use of transgenic and endophytes. New Phytologist 179, 318-333.
- Geletneky, J.W., 2002. Hydrogeologische/Hydrologische Untersuchung einer Prä-Flutungssituation am Beispiel des Gessentals im ehemaligen ostthüringischen Uranbergbaugebiet, Chemisch-Geowissenschaftliche Fakultät. Friedrich-Schiller-Universität, Jena.
- Gibson, G., Klinck, J., 2005. Canada's Resilient North: The Impact of Mining on Aboriginal Communities. Pimatisiwin: A Journal of Aboriginal and Indigenous Community Health 3, 115-140.
- Guo, H., Luo, S., Chen, L., Xiao, X., Xi, Q., Wei, W., Zeng, G., Liu, C., Wan, Y., Chen, J., He, Y., 2010. Bioremediation of heavy metals by growing hyperaccumulator endophytic bacterium *Bacillus* sp. L14. Bioresource Technol. 101, 8599-8605.
- Hester, R.E., Harrison, R.M., 1994. Mining and its environmental impact. Royal Society of Chemistry, Cambridge.
- Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. Calif. AES Circular 347, 1-32.
- Kidd, P., Juan, B., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monteroso, C., 2009. Trace element behaviour at the root-soil interface: implications in phytoremediation. Environ. Exp. Bot. 67, 243-259.
- Kozyrovska, N., Kovtunovych, G., Gromosova, E., Kuharchuk, P., Kordyum, V., 1996. Novel inoculants for an environmentally-friendly crop production. Resour. Conserv. Recy. 18, 79-85.
- Krämer, U., 2010. Metal hyperaccumulation in plants. Annu. Rev. Plant Biol. 61, 517-534.
- Krznaric, E., Verbruggen, N., Wevers, J.H.L., Carleer, R., Vagronsveld, J., Colpaert, J.V., 2009. Cd-tolerant *Suillus luteus*: a fungal insurance for pines exposed to Cd. Environ. Pol. 157, 1581-1588.

- Lebeau, T., Braud, A., Jézékel, K., 2008. Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. Environ. Pol. 153, 497-522.
- Lichtenthaler, H.K., Miehé, J.A., 1997. Fluorescence imaging as a diagnostic tool for plant stress. Trends Plant Sci. 2.
- Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M., van der Lelie, D., 2002. Endophytic bacteria and their potential applications. Crit. Rev. Plant Sci. 21, 583-606.
- Mastretta, C., Barac, T., Vangronsveld, J., Newman, L., Taghavi, S., van der Lelie, D., 2006. Endophytic bacteria and their potential application to improve the phytoremediation of contaminated environments. Biotech. Gen. Eng. Rev. 23, 175-207.
- Mastretta, C., Taghavi, S., van der Lelie, D., Mengoni, A., Galardi, F., Gonnelli, C., Barac, T., Boulet, J., Weyens, N., Vangronsveld, J., 2009. Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. Int. J. Phytoremediat. 11, 251-267.
- Murry, M.A., Zhang, D., Schneider, M., deBruijn, F.J., 1995. Use of repetitive sequences and the polymerase chain reaction (rep-PCR) to fingerprint the genomes of *Frankia* isolates. Symbiosis 19, 223-240.
- Neagoe, A., Merten, D., Iordache, V., Büchel, G., 2009. The effect of bioremediation methods involving different degrees of soil disturbance on the export of metals by leaching and by plant uptake. Chem. Erde - Geochem. 69, 57-73.
- Nilsson, J.-A., Randhem, J., 2008. Environmental Impacts and Health Aspects in the Mining Industry - A Comparative Study of the Mining and Extraction of Uranium, Copper and Gold. Chalmers University of Technology, Göteborg, Sweden.
- Porteous Moore, F., Barac, T., Borremans, B., Oeyen, L., Vangronsveld, J., van der Lelie, D., Campbella, C.D., Moore, E.R.B., 2006. Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: The characterisation of isolates with potential to enhance phytoremediation. Syst. Appl. Microbiol. 29, 539-556.
- Pulford, I.D., Watson, C., 2003. Phytoremediation of heavy metal-contaminated land by trees - a review. Environ. Int. 29, 529-540.
- Puschenreiter, M., Schnepf, A., Millán, I.M., Fitz, W.J., Horak, O., Klepp, J., Schrefl, T., Lombi, E., Wenzel, W.W., 2005. Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. Plant Soil 271, 205-218.
- Rajkumar, M., Ae, N., Freitas, H., 2009. Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77, 153-160.
- Razo, I., Carrizales, L., Castro, J., Barriga, F.D., Monroy, M., 2004. Arsenic and heavy metal pollution of soil, water and sediments in a semi-arid climate mining area in Mexico Water Air Soil Pollut. 152, 129-152.
- Rönkkö, R., Smolander, A., Nurmiaho-Lassila, E.-L., Haahtela, K., 1993. *Frankia* in the rhizosphere of non-host plants: A comparison with root-associated N_2-fixing *Enterobacter*, *Klebsiella* and *Pseudomonas*. Plant Soil 153, 85-95.
- Sarma, H., 2011. Metal hyperaccumulation in plants: a review focusing on phytoremediation technology. J. Environ. Sci. Technol. 4, 118-138.
- Schmidt, A., Haferburg, G., Sineriz, M., Merten, D., Büchel, G., Kothe, E., 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. Chem. Erde - Geochem. 65, 131-144.
- Schützendübel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53, 1351-1365.
- Sheng, X.-F., Xia, J.-J., Jiang, C.-Y., He, L.-Y., Qian, M., 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. Environ. Pol. 156, 1164-1170.
- Shin, M.-N., Shim, J., You, Y., Myung, H., Bang, K.-S., Cho, M., Kamala-Kannan, S., Oh, B.-T., 2012. Characterization of lead resistant endophytic *Bacillus* sp. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator *Alnus firma*. J. Hazard. Mater. 199–200, 314-320.
- Tessema, S.G., 2011. Influence of plant water relation on ecosystem function under water stress. Friedrich-Schiller-University of Jena, Jena.

- van der Lelie, D., Barac, T., Taghavi, S., Vangronsveld, J., 2005. Response to Newman: New uses of endophytic bacteria to improve phytoremediation. Trends Biotechnol. 23.
- Vangronsveld, J., Colpaert, J., Van Tichelen, K., 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environ. Pollut. 94, 131-140.
- Vangronsveld, J., Cunningham, S.D., 1998. Introduction to the concepts, in: Cunningham, J.V.a.S.D. (Ed.), Metal-Contaminated Soils: In-situ Inactivation and Phytorestoration. Springer Verlag, Berlin Heidelberg, pp. 1-15.
- Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, a., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M., 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ. Sci. Pollut. Res. 16, 765-794.
- Vangronsveld, J., Van Assche, F., Clijsters, H., 1995. Reclamation of a bare industrial area contaminated by non-ferrous metals: In situ metal immobilization and revegetation. Environmental Pollution 87, 51-59.
- Weyens, N., Croes, S., Dupae, J., Newman, L., van der Lelie, D., Carleer, R., Vangronsveld, J., 2010. Endophytic bacteria improve phytoremediation of Ni and TCE co-contamination. Environ. Pol. 158, 2422-2427.
- Weyens, N., van der Lelie, D., Taghavi, S., Newman, L., Vangronsveld, J., 2009a. Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends Biotechnol. 27, 591-598.
- Weyens, N., van der Lelie, D., Taghavi, S., Vangronsveld, J., 2009b. Phytoremediation: plant-endophyte partnerships take the challenge. Curr.Opin. Biotech. 20, 248-254.
- Whiting, S.N., Souza, M.P.D., Terry, N., 2001. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ. Sci. Technol. 35, 3144-3150.
- Wismut, 1994a. Sanierungskonzept Standort Ronneburg.-Stand März 1994, Internal Report. Wismut GmbH, Chemnitz

LIST OF FIGURES

LIST OF TABLES

List of publications and presentations

Publications

Grawunder, A., Lonschinski, M., Boisselet, T., Merten, D., Büchel, G., "Hydrogeochemistry of rare earth elements in an AMD-influenced environment", IMWA 2010, Sydney/Canada, Sep 5 - 9, 2010, conference proceedings

Anahita Pourjabbar, Tsilla Boisselet, Anja Grawunder, Dirk Merten, Jürgen W. Einax, Georg Büchel, "Groundwater and Soil contamination by Heavy Metals in a Former Uranium Mining Site, Eastern Thuringia, Germany", "International Mining congress and Expo", Tehran (Iran), October 2010, conference proceedings

Cornelia Gradinger, Tsilla Boisselet, Daniel Stratev, Thomas Ters, Kurt Messner, Karin Fackler, " Biological control of sapstain fungi: From laboratory experiments to field trials", Holzforschung, vol.63 no.6, 751-759

K. Krizova, L. Matlova, A. Horvathova, M. Moravkova, V. Beran, T. Boisselet, V. Babak, I. Slana, I. Pavlik, " Mycobacteria in the environment of pig farms in the Czech Republic between 2003 and 2007", Veterinarni Medicina, 55, 2010 (2): 55–69

Tsilla Boisselet, Jaco Vangronsveld, Nele Weyens, Dirk Merten, Georg Büchel, ”Improving plant growth on heavy metal contaminated soil using selected endophytic microorganisms”, PRE XI conference, Thessaloniki (Greece), July 3rd -6th 2012, conference proceedings, submitted, accepted

Presentations

Poster: Tsilla Boisselet, Dirk Merten, Georg Büchel, "Chemical and biological factors influencing heavy metal mobilisation by analysis of REE fractionation", Jenaer Sanierungskolloquium (Jena remediation symposium), Jena (Germany), Sept 28th – 29th, 2009

Poster: MiCom 2010, Jena, Sep 28th - Oct 1st, 2010

Talk: Tsilla Boisselet, Delphine Ollivier, Nele Weyens, Dirk Merten, Jaco Vangronsveld, Georg Büchel,“Improving plant growth on heavy metal contaminated soil using selected endophytic microorganisms”, Jena remediation symposium, Dornburg (Germany), October 3rd-6th 2011

Poster: Tsilla Boisselet, Dirk Merten, Jaco Vangronsveld, Georg Büchel, “Biosphere helps Hydrosphere plants and microbes as bioremediation agents in the context of heavy metal contamination”, NBV Symposium, Wageningen (Netherlands) 1st of Dec.2011

To come (accepted):

Talk: Tsilla Boisselet, Jaco Vangronsveld, Nele Weyens, Dirk Merten, Georg Büchel, ”Improving plant growth on heavy metal contaminated soil using selected endophytic microorganisms” PRE XI conference, Thessaloniki (Greece), July 3rd -6th 2012

Curriculum Vitae

Tsilla Boisselet

Nationality	French
Date of birth	24.08.1983
Place of birth	Périgueux
Gender	female

February 2009- present
PhD position, supported by JSMC
Institute for Earth Sciences
Burgweg 11, 07743 Jena
Friedrich Schiller Universität Jena, Germany
in cotutelle with
Center for environmental Sciences
Universiteit Hasselt, Campus Diepenbeek
Agoralaan, building D, 3590 Diepenbeek, Belgium

September 2007- June 2008
Internship, Microbiology
Department for Food and Feed Safety, Microbiology Laboratory ,
isolation, detection and cultivation of mycobacteria
Veterinary Research Institute (V.R.I) Hudcova 70 62100 Brno (Czech Republic)

October 2006- August 2007
Master thesis for the degree of Master of science, Grade: 1
"Characterisation and Biocontrol of Blue Sapstain Fungi"
Vienna University of Technology (*TU Wien*)
Supervisor : Prof. Kurt Messner

October 2001- December 2007
Master of science (*Diplomingenieur*)
Chemical engineering
specialization in Biochemistry, Microbiology and Molecular Biology
Vienna University of Technology (*TU Wien*)

August 2006
BEST-course at Lund University (Sweden) about renewable resources for sustainable production

September 2005
internship
Laboratory, Analysis of water and sludge quality
Hauptkläranlage Wien Simmering : Main Water treatment plant, Vienna (Austria)

August 2004
internship
Institute for Chemistry, Cellulose analysis
University of natural resources and applied sciences in Vienna (Austria)

June 2001
A-Level specialized in sciences, passed with distinction
Baccalauréat Général Scientifique avec Mention Très Bien

September 1994-June 2001
Secondary school : Collège Anne Frank, Périgueux, France / Lycée Jay de Beaufort, Périgueux, France