ANALYSIS OF MICROBIAL COMMUNITY STRUCTURES AND FUNCTIONS IN HEAVY METAL-CONTAMINATED SOILS USING MOLECULAR METHODS

THÈSE N^{O} 2862 (2003)

PRÉSENTÉE À LA FACULTÉ ENVIRONNEMENT NATUREL, ARCHITECTURAL ET CONSTRUIT

Institut des sciences et technologies de l'environnement

SECTION DES SCIENCES ET INGÉNIERIE DE L'ENVIRONNEMENT

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

PAR

Fabienne GREMION

biologiste diplômée de l'Université de Neuchâtel
de nationalité suisse et originaire de Gruyères (FR)

acceptée sur proposition du jury:

Prof. H. Harms, directeur de thèse
Dr A. Chatzinotas, rapporteur
Prof. A. Hartmann, rapporteur
Prof. Ch. Holliger, rapporteur
Dr F. Widmer, rapporteur

Lausanne, EPFL
2003

SUMMARY

The contamination of agricultural land and groundwater by heavy metals is essentially linked to human activities. A major problem with heavy metals is that they cannot be biodegraded and therefore reside in the environment for long periods of time if they are not removed. Thus, depending of the kind and depth of contamination, different remediation techniques were developed. One of these methods, called «phytoextraction», uses the ability of so-called hyperaccumulating plants to extract high amounts of heavy metals.

Accumulation of heavy metals in the environment is a serious concern for animal and human health. At the microscopic scale, heavy metals may have also deleterious effects on bacteria which are the key-players of the different nutrient turnovers in soils. Consequently, ecosystem functioning can be seriously perturbed and the long-term soil fertility may be threatened. The recent development of molecular biology greatly contributed to the discovery of the microbial diversity and its function in the soil. However, to date only a small number of studies used molecular methods to investigate the impacts of heavy metals on the bacterial community.

In this thesis, a pot experiment was conducted under controlled conditions with one hyperaccumulating plant (*Thlaspi caerulescens*) grown in two different soils, a long-term and an artificially heavy metal-contaminated soil. The impact of heavy metals on the microbial community was then investigated with several molecular methods. Moreover, the decrease of the bioavailable heavy metals concentrations in soil due to plant uptake allowed to study the consequences on bacterial community structure and function.

Based on the 16S ribosomal RNA and the corresponding gene (16S rDNA), four clone libraries were constructed to retrieve information on the structure of the microbial community and the potentially active part of the microbial community in the rhizosphere of *Thlaspi caerulescens* grown during three months in a long-term contaminated soil. The data obtained with the two clone libraries (rRNA and rDNA) from the rhizosphere of *Thlaspi caerulescens* were compared with the bulk soil data to identify any effect of the plant on the soil microbial community structure. Partial sequence analysis of 282 clones revealed that most of the environmental sequences in both soils affiliated with five major phylogenetic groups, the *Actinobacteria*, α-*Proteobacteria*, β-*Proteobacteria*, *Acidobacteria* and the *Planctomycetales*. The taxa dominating the bacterial community structure in the bulk soil also dominated the rhizosphere community, indicating that the plant did not exert a major influence on the overall

bacterial diversity. However, all dominant taxa, with the exception of the *Actinobacteria*, were relatively less represented in the rRNA libraries as compared to the rDNA libraries. On the contrary, sequences belonging to the *Actinobacteria* dominated both bulk and rhizosphere soil libraries derived from rRNA. Seventy per cent of these clone sequences were related to two subgroups of the *Rubrobacteria*, which was an indication that this group of bacteria was probably metabolically active in heavy metal-contaminated soils.

Fluorescence *in situ* hybridization (FISH) was used for the *in situ* detection and quantification of selected bacterial groups previously detected in the clone libraries from the rhizosphere of *Thlaspi caerulescens*. By applying the most general probe EUB338, only 20% of the total rhizosphere microbial community could be detected. Based on this result, it was difficult to conclude with certitude which were the most dominant bacteria in the rhizosphere. However, despite this low detection rate, it was possible to detect the major groups present in the rhizosphere clone libraries using group-specific oligonucleotide probes. As part of our sequences were affiliated to two emerging bacterial groups, the *Acidobacteria* and the *Rubrobacteria*, two new probes were designed, Acido228 (specific for the subgroup 1 of the *Acidobacterium* division) and Rubro198 (specific for the all *Rubrobacteria* subclass), for the detection of these microorganisms. These two probes were first checked for their specificity with pure cultures and finally applied in the rhizosphere soil allowing for the first time the detection of these organisms *in situ*.

Finally, the impact of heavy metals and a subsequent one-year phytoextraction with *Thlaspi caerulescens* on the soil microbial community was investigated in an artificially heavy metal-contaminated soil. All the different molecular and culture-dependent techniques used, denaturing gradient gel electrophoresis (DGGE), community level physiological profile (CLPP) and potential ammonium-oxidation measurement showed that the heavy metal addition induced drastic changes in the bacterial community. Moreover, the analysis of the different bacterial DGGE patterns (*Bacteria*, β-*Proteobacteria* and ammonia-oxidising bacteria) obtained during this experiment showed that one-year phytoremediation was not sufficient to recover the initial community present in the non-contaminated soil. However, with the CLPP analysis, it was possible to detect a stimulating effect of the plant on a part of the microbial community in both contaminated and non-contaminated soils. The most obvious result was obtained in the contaminated soil where the number of substrates metabolised increased significantly in the presence of the plant as compared to the unplanted contaminated samples.

The measurement of the potential ammonium-oxidation was used as a criterion for soil quality. Although this test showed that the ammonia-oxidising bacteria were significantly stimulated in the planted non-contaminated soil samples, the positive effect of the plant on these bacteria was not sufficient to overcome the inhibition induced by the presence of the heavy metals in the contaminated soil, even after one year phytoremediation.

To conclude, molecular methods in combination with culture-dependent techniques have proven in this study to be very useful for the detection of the changes induced by the heavy metals in the structure and the function of the microbial community. Moreover, the molecular techniques contributed to the identification of bacteria which could be potentially used for the bioremediation of contaminated soils thus offering new perspectives of investigation and technology development.

VERSION ABRÉGÉE

La présence de métaux lourds dans les terres agricoles et les nappes phréatiques est essentiellement liée à l'activité humaine. Les métaux lourds n'étant pas biodégradables, il a fallu développer différentes techniques pour décontaminer les sites pollués. L'une d'elles, la phytoextraction, exploite les propriétés hyperaccumulatrices de certaines plantes qui peuvent extraire de grandes quantités de métaux lourds.

L'accumulation des métaux lourds dans l'environnement peut se répercuter sur la santé des êtres humains et des animaux. A l'échelle microscopique, les métaux lourds ont aussi des effets néfastes sur les populations bactériennes ce qui n'est pas sans conséquences sur le fonctionnement de l'écosystème. En effet, les micro-organismes occupent des positions clés dans les cycles des bioéléments. Leur disparition ne permet donc plus de garantir à long terme la fertilité du sol. Ces dernières années, le développement de la biologie moléculaire a largement contribué à la découverte de la diversité microbienne et de son rôle dans le sol. Cependant, à ce jour, peu d'études ont utilisé des méthodes moléculaires pour mesurer l'impact des métaux lourds sur les populations bactériennes.

Dans le cadre de cette thèse, une expérience en pots a été menée en laboratoire avec une plante hyperaccumulatrice (*Thlaspi caerulescens*) plantée dans deux sols contaminés différemment, soit par l'ajout d'une poudre de métaux lourds ou par l'application de boues d'épuration durant plusieurs années. L'impact des métaux lourds sur les populations bactériennes a ensuite été évalué par le biais de différentes méthodes moléculaires. Dans un deuxième temps, l'utilisation de *Thlaspi caerulescens* a permis de diminuer les concentrations biodisponibles de métaux lourds dans le sol et d'en étudier les conséquences sur la structure et les fonctions de la communauté bactérienne.

La création de banques de clones, basées sur l'ADN et l'ARN ribosomal bactérien (16S), a tout d'abord permis de déterminer la structure de la communauté microbienne et d'identifier les populations actives dans la rhizosphere de *Thlaspi caerulescens* qui avait été planté durant trois mois dans le sol contaminé naturellement. Par la suite, la comparaison des banques de clones obtenues à partir de l'ADN et de l'ARN du sol rhizosphérique avec celles du sol distant a permis de vérifier si la plante influençait la composition de la communauté microbienne. Le séquençage partiel de 282 clones a révélé que la plupart des séquences obtenues à partir du sol rhizosphérique et distant étaient affiliées à 5 grands groupes phylogénétiques, les

Actinobacteria, α-*Proteobacteria*, β-*Proteobacteria*, *Acidobacteria* et les *Planctomycetales*. La prédominance de ces taxa, aussi bien dans la communauté microbienne du sol rhizosphérique que dans celle du sol distant, a montré que la plante avait une influence minime sur la structure générale de la communauté bactérienne. Cependant, à l'exception des *Actinobacteria*, les quatre autres taxa étaient généralement moins représentés dans les banques de clones basées sur l'ARN ribosomal que dans celles basées sur l'ADN. Au contraire, les séquences affiliées aux *Actinobacteria* prédominaient largement dans les banques de clones du sol distant et rhizosphérique construites à partir de l'ARN ribosomal. Plus précisément, 70% de ces séquences ont été apparentées à deux sous-groupes des *Rubrobacteria*, indiquant que ce groupe de bactéries était probablement actif dans les sols contaminés aux métaux lourds.

L'hybridation *in situ* par fluorescence (FISH) a été utilisée pour détecter et quantifier *in situ* les différents groupes bactériens présents dans la rhizosphère de *Thlaspi caerulescens* et précédemment identifiés avec les banques de clones. Seulement 20% de la communauté rhizosphérique a pu être détectée avec la sonde la plus générale (EUB338) empêchant par conséquent de conclure avec certitude quelles étaient les populations microbiennes les plus dominantes dans la rhizosphère. Malgré cette limite de détection très basse, il a quand même été possible de détecter les groupes qui dominaient auparavant dans les banques de clones grâce à l'usage de sondes spécifiques. Pour les *Acidobacteria* et les *Rubrobacteria*, deux groupes relativement récents, il a fallu développer deux nouvelles sondes, Acido228 (spécifique au sous-groupe 1 de la division) et Rubro198 (spécifique à toute la sous-classe), pour pouvoir détecter les micro-organismes dont les séquences avaient été retrouvées dans les banques de clones. Après avoir évalué leur spécificité avec des souches pures, leur utilisation dans le sol rhizosphérique a permis, pour la première fois, de détecter *in situ* ces nouveaux groupes bactériens.

Finalement, l'impact des métaux lourds sur la communauté bactérienne suivi d'une année de phytoextraction avec *Thlaspi caerulescens* a été examiné dans un sol artificiellement contaminé. Toutes les méthodes utilisées, électrophorèse sur gradient de dénaturant (DGGE), utilisation de 95 substrats pour la détermination du profil physiologique d'une communauté (CLPP) et mesure du potentiel d'oxydation de l'ammonium ont montré un effet drastique des métaux lourds sur la communauté microbienne. De plus, l'analyse des différents profils bactériens (*Bacteria*, β-*Proteobacteria* et bactéries responsables de l'oxydation de l'ammoniaque) obtenus par DGGE a permis de constater qu'une année de phytoremediation

n'était pas suffisante pour permettre le rétablissement des populations initialement présentes dans le sol non contaminé. L'utilisation des 95 substrats a cependant permis d'observer un effet stimulant de la plante sur une partie de la communauté bactérienne qui s'est traduit par une augmentation du nombre de substrats métabolisés aussi bien dans le sol non contaminé que dans le sol contaminé. La qualité du sol a pu être évaluée en mesurant le potentiel d'oxydation de l'ammonium. Ce test a montré que les bactéries responsables de l'oxydation de l'ammoniaque n'étaient toujours pas fonctionnelles après une année de phytoremediation et que l'effet stimulant de la plante sur ces bactéries, clairement visible dans le sol non contaminé, n'était pas suffisant pour lever l'inhibition engendrée par les métaux lourds dans le sol contaminé.

En conclusion, ce travail a démontré que l'utilisation de méthodes moléculaires combinée avec des techniques plus traditionnelles était très utile pour observer les changements induits par les métaux lourds au niveau de la structure et des fonctions de la communauté bactérienne. De plus, les techniques moléculaires ont permis d'identifier certaines populations qui pourraient s'avérer utiles pour la décontamination des sols pollués, offrant ainsi plusieurs perspectives de recherche pouvant déboucher sur le développement de nouvelles technologies.

TABLE OF CONTENT

CHAPTER 1

Introduction

1.1 Heavy metals

1.1.1 Biochemical roles

The term «heavy metals» refers to metals and metalloids having densities greater than 5 g cm^{-3} and is usually associated with pollution and toxicity although some of these elements (essential metals) are required by organisms at low concentrations (Adriano, 2001). For example, zinc (Zn) is the component of a variety of enzymes (dehydrogenases, proteinases, peptidases) but is also involved in the metabolism of carbohydrates, proteins, phosphate, auxins, in RNA and ribosome formation in plants (Kabata-Pendias & Pendias, 2001), (Mengel & Kirkby, 1982). Copper (Cu) contributes to several physiological processes in plants (photosynthesis, respiration, carbohydrate distribution, nitrogen and cell wall metabolism, seed production) including also disease resistance (Kabata-Pendias & Pendias, 2001). The good functioning of the metabolisms of humans and bacteria is also dependent on these two metals (Adriano, 2001; Blencowe & Morby, 2003; Cavet *et al.*, 2003). However, at high concentrations, these metals exhibit toxic effects on cells (Baker & Walker, 1989).

On the contrary, cadmium (Cd) is not involved in any known biological processes (non-essential metal) and may be quite toxic as it is accumulated by organisms. It is known to disturb enzyme activities, to inhibit the DNA-mediated transformation in microorganisms, to interfere in the symbiosis between microbes and plants, as well as to increase plant predisposition to fungal invasion (Kabata-Pendias & Pendias, 2001). In humans, it may promote several

disorders in the metabolism of Ca and vitamin D leading to bone degeneration and kidney damage (*itai-itai* disease) (Adriano, 2001).

The excessive uptake of heavy metals by animals and humans is the result of the successive accumulation of these elements in the food chain, the starting point being the contamination of the soil.

1.1.2 Origin of the contamination in soils

The main problem with heavy metals such as Cu, Zn and Cd in soils is that, unlike organic pollutants, they cannot be biodegraded and therefore reside in the environment for long periods of time. Their presence in soils may be from natural or anthropogenic origins. Natural sources include atmospheric emissions from volcanoes, the transport of continental dusts and the weathering of metal-enriched rocks (Ernst, 1998). However, the major source of contamination is from anthropogenic origin: the exploitation of mines and smelters, the application of metal-based pesticides and metal-enriched sewage sludges in agriculture, combustion of fossil fuel, metallurgical industries and electronics (manufacture, use and disposal), military training, etc. contribute to an increased input of heavy metals in soils (Alloway, 1995).

Whereas the industrial atmospheric emissions may be controlled by the installation of adequate air filters, the main source of contamination for humans remains the ingestion of plants that grew on contaminated soils. The use of intensive farming practices including addition of phosphatic fertilizers, sewage sludge input and pesticide treatment are responsible for the pollution of agricultural soils. Although these practices increase significantly the yield by protecting plants and providing them with all the nutrients necessary for a rapid and better growth, they may also introduce large amounts of heavy metals (Cu, Zn, Pb, Cd) and organic pollutants in soil which may then be accumulated by the plant. For instance, Hamon *et al.* (1998) have shown that the addition of phosphatic fertilizers increased Cd uptake of wheat. However, the risk arising from heavy metals largely depends on their bioavailability which in turn depends on their chemical speciation (Adriano, 2001).

1.1.3 Situation in Switzerland

Switzerland has implemented with the revision of the *Ordinance relating to Impacts on the Soil* (OIS, 1998) a three level evaluation scheme of soil-related hazards to man, animals and plants. It consists of guide, trigger and clean-up values of metal concentrations in soils (Table 1.1). Two

values are measured, the *pseudo*-total metal content (t) extracted with 2 M HNO_3 and the soluble fraction (s) with 0.1 M $NaNO_3$. Both values (t and s) differ with the land use (Table 1.1).

Table 1.1: Guide, trigger and clean-up values in the ordinance relating to impacts on soils (OIS, 1998).

	Use of land	Cd		Cu		Pb		Zn	
		t	s	t	s	t	s	t	s
Guide value		0.8	0.02	40	0.7	50	-	150	0.5
Trigger value	Food plant cultivation	2	0.02	-	-	200	-	-	-
	Fodder plant cultivation	2	0.02	150	0.7	200	-	-	-
	Direct cultivation	10	-	-	-	200	-	-	-
Clean-up value	Agriculture and horticulture	30	0.1	1000	4	2000	-	2000	5
	Domestic gardens and allotments	20	0.1	1000	4	1000	-	2000	5
	Children playground	20	-	-	-	1000	-	-	-

t = pseudo-total; s = soluble; all values are in mg metal kg^{-1} of soil

Guide values account for the impacts on the soil ecosystem. If either t or s exceeds the guide value, the soil is considered as contaminated because the long-term soil fertility or multifunctionality is not guaranteed any more (Vollmer *et al.*, 1997).

Soil trigger values assume harmful effects on exposed risk receptors, such as humans, higher plants and animals. Where trigger values are exceeded, further site specific investigations have to be performed to determine whether or not a hazard exists. Clean-up values indicate severe contamination and the definite need for measures (Vollmer *et al.*, 1997).

In 1997, a study of the Swiss Agency for the Environment Forests and Landscape (SAEFL) estimated that around 50'000 sites are contaminated with heavy metals (Elsenbeer *et al.*, 1997). According to this report, industrial sites account for 50%, landfills for 45% and accident sites for 5%. Five to eight percent of these sites need to be remediated.

In 2002, a second study was performed by the same institute based on the data of the NABO network (NAtionales BOdenbeobachtungsnetz) which is a national project for the monitoring of the soil pollution in 105 representative sites with respect to land use, climate, geology, soil type and geographic distribution (Desaules & Dahinden, 2000). They showed that 42% of the 105 sites of the NABO were above the guide values for Cu, Pb and Cd (SAEFL, 2002). This represents roughly 10% of the surface in Switzerland.

1.2 Remediation technologies

1.2.1 In situ and ex situ approaches

Depending on the extension, depth and kind of the contamination, different remediation approaches have been proposed (Mulligan *et al.*, 2001). In general, three strategies are possible: the containment of the contaminants, their removal from the environment or their *in situ* stabilisation. Physical containment is the least expensive approach but this leaves the contaminant in place without treatment. As *ex situ* techniques are expensive, environmentally invasive and labor intensive, *in situ* approaches are generally preferred. One of these *in situ* techniques, phytoremediation, uses plants to remove pollutants from the environment or to render them harmless (Salt *et al.*, 1995; Flathman & Lanza, 1998). This *in situ* technology can be applied to both organic and inorganic pollutants present in soil or water and is quite competitive as it costs only 10-40 per ton soil (Mulligan *et al.*, 2001). Only the phytoremediation processes applicable for heavy metal-polluted soils are described below and illustrated in Figure 1.1:

- *Phytostimulation*: Plants secrete roots exudates that may be utilised by bacteria and promote their growth and activity. This microbial stimulation in the plant rhizosphere modifies the bioaccumulation, biological oxidation/reduction and biomethylation of heavy metals.

- *Phytostabilisation*: The use of plants to reduce the bioavailability of pollutants in the environment either with or without non toxic-metal-immobilizing or fertilizing soil amendments. Revegetation stabilize pollutants in soils, thus rendering them harmless and reducing the risk of further environmental pollution by leaching of pollutants into the groundwater or by airborne spread.

- *Phytoextraction*: The use of plants to remove metals or organics from soil by concentrating them in the harvestable parts. The metals are recovered by incinerating or composting the plant biomass. If plants are incinerated, the metals are recovered through suitable air filters.

- *Phytovolatilization*: The use of plants to volatilize pollutants. Plants extract volatile pollutants (e.g.,arsenic, selenium, mercury) from soil and volatilize them from the foliage. If the process takes place in the rhizosphere, it is microbially assisted.

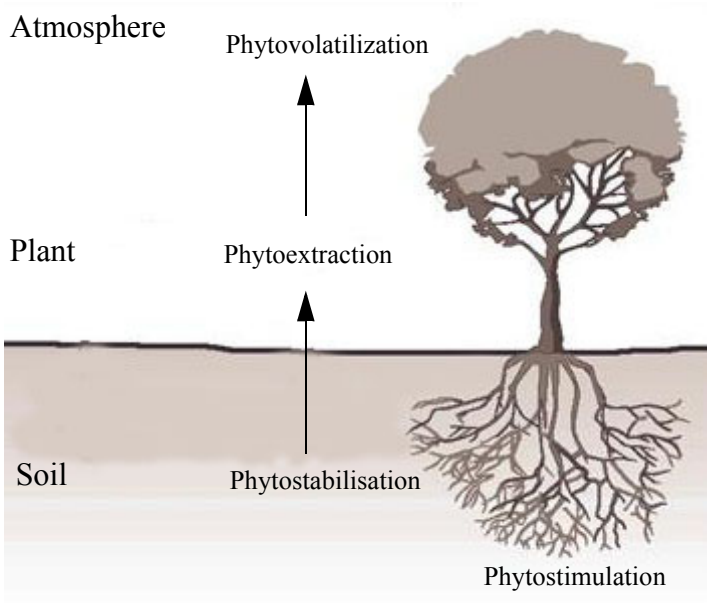

Figure 1.1: Schematic diagram showing the mechanisms of the phytoremediation process for metal uptake.

1.2.2 Phytoextraction: limits and solutions

The success of the phytoextraction process depends on three factors: the degree of metal contamination, the metal bioavailability and the capacity of the higher plants to accumulate the metal in the shoots. Soils with a high degree of metal pollution can be revegetated by metal resistant plants, but their decontamination capacity is restricted by their low biomass production so that decontamination of the soil cannot be achieved in a reasonable time. However, the revegetation of these soils avoids further dispersal of metals by water or wind erosion (phytostabilisation).

In the case of low metal availability, the use of synthetic chelators (chelate-assisted phytoextraction) has been shown to increase significantly the accumulation of Pb but also of other metals in the plant (Blaylock *et al.*, 1997; Salt *et al.*, 1998). These compounds (e.g. ethylenediaminetetraacetic acid (EDTA)) prevent Pb precipitation and keep the metal as soluble chelate-Pb complexes available for plant uptake. Because accumulation of elevated Pb levels is

highly toxic for the plant, it is recommended to apply chelators only after a maximum amount of plant biomass has been produced. The plant material is subsequently harvested within a few days. Chelators ability to facilitate phytoextraction was also shown to be directly related to its affinity for metals (Blaylock *et al.*, 1997). The major disadvantage of this technique is that many synthetic chelators, e.g. EDTA, show a low degree of biodegradability (Kari & Giger, 1996). Consequently, the *in situ* application of such chelators could pose an environmental risk of water pollution by uncontrolled metal solubilization and leaching. However, a suitable alternative is the use of easily biodegradable chelating agents such as nitrilotriacetate (NTA) or elemental sulfur (Kayser *et al.*, 2000).

Two categories of plants are suitable for phytoextraction. The first one, the hyperaccumulating plants (e.g. *Thlaspi caerulescens*), usually small, with high foliar metal concentration but with slow growth rates that do not provide a high annual biomass. The second category includes high biomass crops (e.g. *Brassica juncea*) that have a large biomass production but take up lower metal concentrations.

To overcome the limitations due to plant characteristics, different strategies have been suggested to improve the phytoextraction process. Brown *et al.* (1995) proposed to transfer the metal-removal properties of hyperaccumulator plants to high-biomass producing species. However, this approach is limited by the lack of information on the genetics of metal hyperaccumulation in plants. Particularly, the heredity of relevant plant mechanisms, such as metal transport and storage (Lasat *et al.*, 2000) and metal tolerance (Ortiz *et al.*, 1995) must be better understood.

Alternatively, Brewer *et al.* (1999) tried to increase the size of hyperaccumulating plants by generating somatic hybrids between *Thlaspi caerulescens* and *Brassica napus* followed by hybride selection for Zn tolerance. They could recover high biomass hybrids with superior Zn tolerance which survived up to 4 months and even flowered sometimes. Another promising strategy is the use of genetically modified plants (Kramer & Chardonnens, 2001). *Arabidopsis* and tobacco plants expressing the bacterial genes *merA* and *merB* have been successfully used for the remediation of mercury-contaminated soils (Heaton *et al.*, 1998). However, the use and release of genetically engineered organisms into the environment is still restricted in many countries and often criticized by scientists and the public.

1.2.3 Phytoextraction in Switzerland

Several sites in Switzerland have been investigated in order to assess their potential to be reclaimed through phytoremediation. In a first attempt, Geiger *et al.* (1993) studied the influence of different remediation methods on the heavy metal uptake by *Lactuca sativa* at Dornach (NW, Switzerland), where the soil is calcareous and heavy metal-polluted. As the different techniques used were not satisfactory regarding the normal development of the plant and its heavy metal content, he suggested to use heavy metal-tolerant plants or physiologically adapted plants to high tissue concentrations to stabilise or remove the heavy metals, respectively. In 1997, Felix (1997) followed this idea and used in this site, but also in another location, two hyperaccumulating species *Alyssum murale* and *Thlaspi caerulescens* in combination with different cultivars of tobacco, maize, rapeseed, elephant grass, Indian mustard and willows. He concluded that due to the high soil pH, the bioavailability of Cd, Zn and Cu was too low to be phytoremediated in a reasonable time span. An enhancement of the availability of the metal would be necessary to render phytoextraction suitable (Felix, 1997).

More recently, Hammer *et al.* performed a field experiment in two different heavy metal-contaminated sites, Caslano (Caslano, TI, Switzerland) and Dornach (Hammer *et al.*, 2003; Hammer & Keller, 2003). Their aim was to compare the Cd and Zn uptake of a hyperaccumulating plant (*Thlaspi caerulescens*) and a high biomass crop (*Salix vimitalis*) grown either in an acidic or a calcareous heavy metal-polluted soil . They concluded that *Thlaspi* was the most efficient plant for the decontamination of superficially contaminated soils and especially for soils moderately contaminated with Cd. However, for more deeply contaminated soils, *Salix* would perform better and moreover its fast growth would make it a good candidate for the stabilization of contaminated soils. They also showed that soil properties can affect significantly the rate of phytoextraction.

1.3 Bacteria and heavy metals

1.3.1 Influence of bacteria on heavy metal bioavailability

Overall toxic effects of heavy metals to soil microorganisms depend on their bioavailability. Although heavy metal bioavailability is mainly dependent on the soil properties (pH and organic matter), bacteria can also directly influence the solubility of heavy metals by altering their chemical properties. Microorganisms have developed several mechanisms which can immobilize, mobilize or transform heavy metals. These processes include 1) extracellular precipitation, 2) intracellular accumulation, 3) oxidation and reduction reactions, 4) methylation and demethylation, and 5) extracellular binding and complexation (Brierley, 1990). The exploitation of these bacterial properties for the remediation of heavy metal-contaminated sites has been shown to be a promising bioremediation alternative (Brierley, 1990; Lovley & Coates, 1997; Lloyd & Lovley, 2001). However, at high concentrations, bioavailable heavy metals are toxic for a great number of soil microorganisms and soil microbial processes which in turn will result in severe ecosystem disturbance.

1.3.2 Heavy metal impacts on bacterial community structure and microbial processes

The deleterious effects of heavy metals on microbe-mediated processes have been discussed in detail in several publications (Duxbury, 1985; Babich & Stotzky, 1985; Baath, 1989; Giller *et al.*, 1998). Generally, a decrease in carbon mineralization and fixation, in nitrogen transformation, soil enzyme activities and litter decomposition can be observed. Other typical effects of heavy metal contamination are a decrease in the microbial numbers (CFU), biomass, or an increase of the frequency of heavy metal resistant bacteria (Doelman *et al.*, 1994; Pennanen *et al.*, 1996; Müller *et al.*, 2001).

However, measuring these parameters is not suitable for the determination of changes in the entire structure of soil communities exposed to pollutants. Since many of the microbiological and biochemical techniques used to study the effects of heavy metals on soil bacteria are cultivation dependent, they do not provide detailed information on the non-cultivable bacteria, neglecting thus the major part of the soil microbial community. Consequently, soil microbial communities are treated as a black box. These limitations have been overcome by the recent advances in molecular fingerprinting methods. Based on the analyses of signature biomarkers such as phospholipid fatty acids or nucleic acids, the fingerprinting techniques have been used

in numerous studies and showed significant changes in the microbial community in response to a heavy metal stress (Kozdrój & van Elsas, 2001). Moreover, these methods allowed to monitor the bacterial community during the remediation process (Kelly & Tate, 1998; Macnaughton *et al.*, 1999). These studies have mainly increased our knowledge on sensitive bacterial populations which are negatively affected by heavy metals, yet it should also be considered that heavy metals favour the development of tolerant species which can survive and adapt due to their genetic characteristics.

1.3.3 Heavy metal resistance systems in bacteria

Bacteria have developed several efficient systems to detoxify the metals. These mechanisms can be grouped into five categories: 1) intracellular sequestration, 2) export, 3) reduced permeability 4) extracellular sequestration and 5) extracellular detoxification (Rough *et al.*, 1995). Almost all known bacterial resistance mechanisms are encoded on plasmids and transposons (Silver & Walderhaug, 1992) and it is probably by gene transfer or spontaneous mutation that bacteria acquire their resistance to heavy metals (Osborn *et al.*, 1997).

In gram-negative bacteria (e.g. *Ralstonia eutropha*), the *czc* system is responsible for the resistance to Cd, Zn and Co. The *czc*-genes encode for a cation-proton antiporter (CzcABC) which exports Cd, Zn and Co (Nies, 1995). A similar mechanism, called *ncc* system, has been found in *Alcaligenes xylosoxidans* which is resistant to Ni, Cd and Co. On the contrary, the Cd resistance mechanism in gram-positive bacteria (e.g. *Staphylococcus*, *Bacillus* or *Listeria)* is a Cd-efflux ATPase. The two most well-studied Cu resistance systems are *cop* from *Pseudomonas syringae* pv. *tomato* and *pco* from *Escherichia coli*. The *cop* genes encode for different Cu-binding proteins which allow the sequestration of Cu in the periplasm or in the outer membrane. In contrast, the *pco* system is expected to be an ion-dependent Cu antiporter (Kunito *et al.*, 1998).

The bacterial resistance properties can be used for different purposes: in the case of mercury pollution, the insertion of the microbial mercury reductase in a transgenic plant improved significantly the phytoextraction process (Heaton *et al.*, 1998). Another example was the inoculation of heavy metal resistant bacteria in a contaminated soil which seemed to protect the indigenous sensitive ammonia-oxidising bacteria from metal toxicity (Stephen *et al.*, 1999).

1.4 Plants and heavy metals

1.4.1 The soil-root interface

The rhizosphere is defined as the soil part directly in contact with plant roots. This region is physically and chemically modified due to the root processes induced by the plant for its nutrition. As metals remain sorbed to soil particles, plants have evolved several strategies for increasing their bioavailability. A list of the different plant-mediated processes that affect metal contaminant chemistry is given by Mclaughlin *et al.* (1998):

1. Reduction of ion activity in soil solution, desorption of contaminants from surfaces, convective flow of solution to the root.
2. Changes in solution chemistry (pH, ionic strength, macronutrients cation concentrations (e.g., Ca)) affecting sorption.
3. Excretion of organic ligands (root exudates) increasing or decreasing the total concentration of contaminant ions in solution.
4. Living or dead plant material acting as new sorbing surfaces for contaminants.
5. Stimulation of the microbial activity in the rhizosphere.

However, the mechanisms involved in the changes of metal mobility at the root interface and in the acquisition of these elements can vary widely among plant species.

1.4.2 The different strategies adopted by plants

The sensitivity or tolerance of plants towards metals is influenced by plant species and genotypes. According to Baker (1981), plants can be grouped into three categories: excluders, indicators and accumulators (Figure 1.1). Excluders survive through restriction mechanisms and are sensitive to metals over a wide range of soil concentrations. Members of the grass family (e.g., sudangrass, bromegrass, fescue, etc.) belong to this group of plants. Indicators show poor control over metal uptake and transport processes and correspondingly respond to metal concentrations in soils (Figure 1.1). This group includes the grain and cereal crops (e.g. corn, soybean, wheat, oats, etc.). As accumulators do not prevent metals from entering the roots, they have evolved specific mechanisms for detoxifying high metal levels accumulated in the cells.

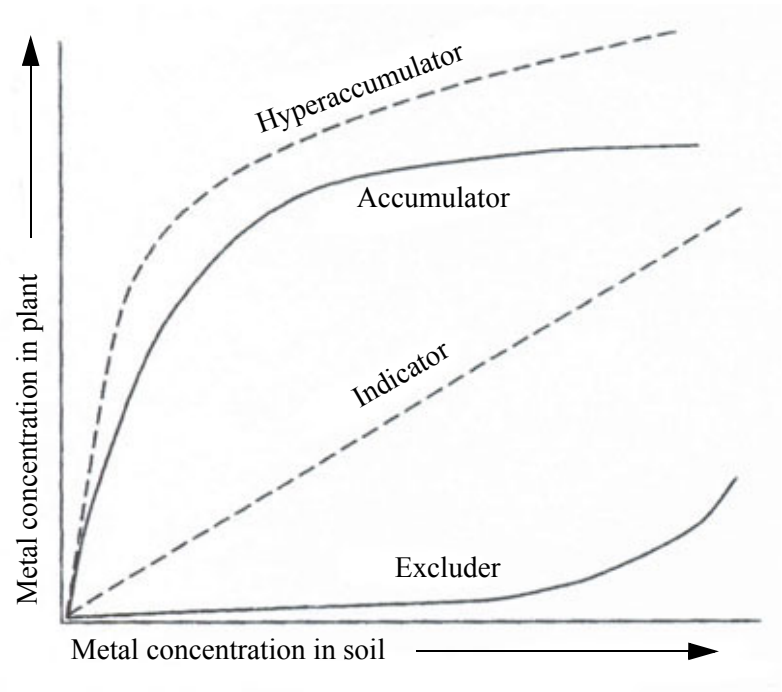

Figure 1.1: The different strategies of metal uptake by plant in relation to metal concentration in soil (adapted from Adriano, 2001).

Tobacco, the mustard and *Compositae* families (e.g. lettuce, spinach, etc.) belong to this category. Extreme accumulators, called hyperaccumulators, form a fourth category as they have exceptional metal-accumulating capacity which allows them to survive and even thrive in heavily contaminated soils (or near ore deposits) through a tolerance mechanism. The characteristics of this group are further developed in the following section.

1.4.3 Hyperaccumulating plants

Brooks *et al.* (1977) introduced the term hyperaccumulators to describe plants which in their natural habitats were capable of accumulating more than 1000 mg Ni kg^{-1} in their shoots dry weight. This criterion is also applied to other metals including Co, Cu and Pb, whereas for Cd and Zn the respective threshold is 100 and 10'000 mg kg^{-1} shoots dry weight (Brooks, 1998; Baker *et al.*, 2000). Compared to non-hyperaccumulator plants, metal concentrations in hyperaccumulator plants are 1-3 orders of magnitudes higher. Apart from these arbitrary criteria, hyperaccumulators usually have a shoot to root metal concentration ratio of >1, whereas non-hyperaccumulator plants generally have higher metal concentrations in roots than in shoots (Baker *et al.*, 1994b; Shen *et al.*, 1997).

Metal hyperaccumulation is an extreme evolutionary response to the presence of high metal concentrations in soils and is not a common characteristic among terrestrial higher plants. To date, about 400 plant species have been identified as metal hyperaccumulators, representing <0.2% of all angiosperms (Brooks, 1998; Baker *et al.*, 2000). The number of known hyperaccumulators of several elements and the families to which most of them belong are given in Table 1.2.

Table 1.2: Numbers (n) of known hyperaccumulating plants for eight heavy metals and the families in which they are most often found (from Brooks, 1998).

Element	n	Families
Cadmium	1	Brassicaceae
Cobalt	26	Lamiaceae, Scrophulariaceae
Copper	24	Cyperaceae, Lamiaceae, Poaceae, Scrophulariaceae
Manganese	11	Apocynaceae, Cunoniaceae, Proteaceae
Nickel	290	Brassicaceae, Cunoniaceae, Euphorbiaceae, Flacourtinaceae, Violaceae
Selenium	19	Fabaceae
Thallium	1	Brassicaceae
Zinc	16	Brassicaceae, Violaceae

Hyperaccumulators are mainly found in soils rich in metals, either for geochemical reasons or due to pollution. They usually have a low biomass as they need to invest more energy in the mechanisms necessary to adapt to high metal concentrations in their tissues. Since metal accumulation is an energy consuming process, one would wonder what evolutionary advantage does metal hyperaccumulation gives to these species. Recent studies have shown that metal accumulation in the foliage may allow hyperaccumulator species to evade predators including caterpillars, fungi and bacteria (Pollard & Baker, 1997; Boyd, 2000).

1.5 Bacteria and plants

1.5.1 Plant-microbial interactions in the rhizosphere

Plant-microbe interactions may be beneficial or harmful to the plant depending on the specific microorganisms and plant involved. Plant beneficial interactions can be divided into three categories (Brimecombe *et al.*, 2001). The first interaction includes microorganisms which, in association with the plant, increase the supply of mineral nutrients to the plant. This is the case of the symbiotic dinitrogen-fixing bacteria of leguminous plants (i.e. *Rhizobium*, *Bradyrhizobium* species) or of monocots (i.e. *Azospirillum brasiliense*) and the free nitrogen-fixing bacteria such as *Klebsiella pneumoniae* (Lugtenberg *et al.*, 1991). Secondly, there are microorganisms (i.e. fluorescent pseudomonads) that stimulate plant growth indirectly by the production of antibiotics, siderophores, volatile compounds or hydrolytic enzymes which prevent the growth or activity of plant pathogens. Nowadays, these bacteria are used as biocontrol agents. Thirdly, there are the plant growth-promoting rhizobacteria (i.e. *Azospirillum*, *Azotobacter*, *Pseudomonas* and *Bacillus*) that stimulate directly plant growth by the production of phytohormones (Okon, 1985). Detrimental interactions within the rhizosphere involve deleterious rhizobacteria which inhibit shoot or root growth whithout causing any other visual symptoms by the production of phytotoxins such as cyanide (Alström & Burns, 1989) or phytohormones (Schippers *et al.*, 1987).

1.5.2 Effects of root exudates on rhizosphere microbial populations

Plant roots can stimulate or inhibit microbial populations and their activities through the exudation of different compounds. Root exudates are water-soluble organic compounds, mainly carbohydrates, organic acids and amino acids, released from the root cells along concentration gradients in the rhizosphere soil (Lynch & Whipps, 1990). For microorganisms, these exudates represent a convenient source of carbon (and possibly nitrogen) since they are readily assimilated without the need to synthesize exoenzymes. Due to this large availability of substrates in the rhizosphere, microbial biomass and activity are generally much higher in the rhizosphere than in the bulk soil (Brimecombe *et al.*, 2001).

Moreover, the quantity and the chemical composition of root exudates, which vary during plant developmental stage and between plant species (Brimecombe *et al.*, 2001), may also affect the microbial community structure in the rhizosphere. This was confirmed by the results of

several studies using cultivation (Miller *et al.*, 1989; Lemanceau *et al.*, 1995; Germida *et al.*, 1998) or molecular fingerprinting techniques (Grayston *et al.*, 1998; Smalla *et al.*, 2001; Baudoin *et al.*, 2002; Kuske *et al.*, 2002).

According to the key role that root exudates seem to play in determining the composition of their associated rhizobacterial populations, they could potentially be used in the future for the remediation of contaminated sites by the selective enhancement of certain bacterial populations which might improve the heavy metal uptake by the plant (Kozdrój & van Elsas, 2000). Consequently, stimulated bacteria could change the bioavailability of heavy metals through different chemical processes (see point 1.3.1), which in turn would increase plant heavy metal uptake (see point 1.4.1). Phytovolatilization is also a concrete example of a plant-bacteria-mediated bioremediation process (see point 1.2.1).

1.6 Stress, biodiversity and ecosystem functioning

1.6.1 Microbial biodiversity - ecosystem functioning relationships

Despite the obvious ecological importance of soil microorganisms, they had a negligible influence on the development of contemporary ecological theory (Wardle & Giller, 1996). A number of hypotheses suggest how species diversity may be related to ecosystem functioning. Some authors suggested that a high diversity is beneficial to ecosystem function (Naeem *et al.*, 1994; Tilman *et al.*, 1996). In contrast, others proposed that the properties of an ecosystem depend more upon the functional abilities of particular species than on the total number of species (Hooper & Vitousek, 1997; Wardle *et al.*, 1997).

One important aspect in ecosystem functioning is stability, defined as the system's ability to avoid displacement following a pertubation (**resistance**) and to return to its previous state after a disturbance (**resilience**) (Begon *et al.*, 1996). Mooney *et al.* (1995) stated that the capacity of ecosystems to resist changing conditions was correlated positively to species numbers and that diversity provides insurance against large changes in ecosystem processes. Later on, Griffiths *et al.* (2000a) showed that this held true for soil microbial systems. The undelying theory is the redundancy in function among species, i.e. different species are able to fulfil the same function, which prevents changes in the ecosystem functioning (Gitay *et al.*, 1996). Consequently, in a diverse system any applied stress may remove some redundant species without loss of function, whereas in less diverse systems a reduction in the species richness could more easily lead to a

loss of function (Griffiths *et al.*, 2000a).

1.6.2 Effect of a stress on the ecosystem

The effect of a disturbance on microbial community function depends on its duration and specificity. By definition, a disturbance is either transient or permanent. Theoretically, after a transient disturbance (i.e. heat stress), system function may eventually return to its former state, whereas permanent disturbance (i.e. heavy metals addition) will result in a new altered state (Rykiel, 1985). Experimentally, Griffiths *et al.* (2000a) observed no resilience in soils submitted to a permanent stress (copper addition), as the reduction in microbial function was largely unchanged over two months following perturbation. On the contrary, soils exposed to a transient heat stress showed a clear trend of resilience with the most diverse soils regaining pre-stress levels of function faster than the least diverse soil.

Based on observations and as illustrated in Figure 1.2, two hypothetical models have been proposed to explain the relationships between biodiversity and disturbance (Giller *et al.*, 1998):

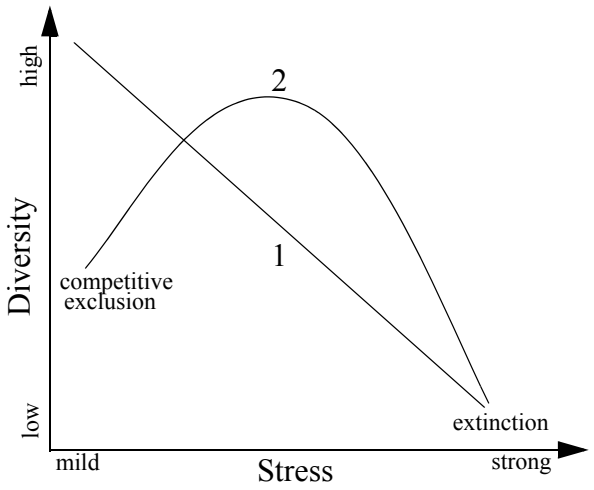

Figure 1.2: Hypothetical models of the effects of a stress on diversity (and consequently function) of a community of microorganisms (from Giller *et al.*, 1998).

In model 1, an increasingly severe stress results in a linear decline in microbial diversity (Figure 1.2). Experimentally, it has been shown that chloroform fumigation reduces gradually bacterial diversity (Griffiths *et al.*, 2000a). On the contrary, in model 2, when the stress is mild, it is hypothesized that competitive species can predominate resulting in a lack of diversity. As soon as the stress increases, these microorganisms lose their competitive advantage and more types

can proliferate (Connell, 1978). However, at high levels of stress, the progressive extinction of organisms leads to a loss of diversity in both models. Hump-backed relationships between species diversity and disturbance (see model 2) seem to be common for animal, plant and even microbial communities (Connell, 1978; Giller *et al.*, 1998). Nevertheless, the exceptionnally high levels of biodiversity in soil might prevent the development of the expected stress-diversity relationship and completely falsify the models.

1.7 Objective of this thesis

Nowadays, it is well established that microorganisms, involved in the nutrients turnovers, are essential for the good functioning of soil ecosystems. However, heavy metals can alter the microbial community in such a way that it will not be able to fulfil its tasks any more. Among the different remediation techniques available for the decontamination of heavy metal-polluted soils, one in particular, including the use of hyperaccumulating plants, seems quite promising. Although microorganisms might improve the efficiency of this approach, their potential for remediation has largely been ignored. This disinterest was certainly due to the lack of suitable methods for their detection but the recent development of molecular methods overcame these limitations and allowed to get a new insight into the bacterial diversity and in particular the interactions between the plant and its rhizosphere bacterial communities.

During this work a set of molecular methods was used to investigate the structure and the function of the bacterial community exposed to a heavy metal stress. Further, information on the consequences of the partial relief of this stress by phytoextraction for the microbial community was obtained. In this purpose, a simplified model ecosystem was established including one hyperaccumulating plant, *Thlaspi caerulescens*, and a long-term as well as an artificially heavy metal-contaminated soils. The main objectives of this study were the following:

- To characterise the bacterial diversity in a long-term heavy metal-polluted soil and in the rhizosphere of *Thlaspi caerulescens* by constructing clone libraries based on the 16S ribosomal RNA and its corresponding gene (16S rDNA). Firstly, this approach allowed to retrieve information on the structure of the microbial community, including in particular the potentially active part of the microbial community. Secondly, the data

obtained with the two clone libraries (rRNA and rDNA) from the rhizosphere of *Thlaspi caerulescens* were compared with the bulk soil data to identify any effect of the plant on the soil microbial community structure (**Chapter 2**).

- To use fluorescence *in situ* hybridisation (FISH) for the *in situ* detection and quantification of selected bacterial groups previously detected in the 16S rRNA clone library from the rhizosphere of *Thlaspi caerulescens*. New probes were designed and successfully applied for the detection of the emerging phylogenetic groups retrieved from the rhizosphere clone library (**Chapter 3**).

- To study the impact of heavy metals followed by one year phytoextraction with *Thlaspi caerulescens* on the structure and the function of the microbial community in an artificially heavy metal-contaminated soil. This microcosm experiment allowed (i) to investigate the effects of heavy metals and of the plant on the microbial community separately (ii) to check if one year remediation was sufficient to recover the initial population present in the pristine soil and (iii) to observe a possible protective and even stimulating effect of the plant on the microorganisms. For this purpose, culture-dependent and independent techniques (denaturing gradient gel electrophoresis, community-level physiological profiles, measurement of the potential ammonium-oxidation) were used to analyse the microbial community (**Chapter 4**).

CHAPTER 2

Comparative 16S rDNA- and 16S rRNA sequence analysis indicates that *Actinobacteria* might be a dominating part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil

FABIENNE GREMION, ANTONIS CHATZINOTAS AND HAUKE HARMS

Environmental Microbiology 5 (10), 896-907

2.1 Abstract

Bacterial diversity in 16S ribosomal DNA and reverse-transcribed 16S rRNA clone libraries originating from the heavy metal-contaminated rhizosphere of the metal-hyperaccumulating plant *Thlaspi caerulescens* was analyzed and compared to that of contaminated bulk soil. Partial sequence analysis of 282 clones revealed that most of the environmental sequences in both soils affiliated with five major phylogenetic groups, the *Actinobacteria*, α-*Proteobacteria*, β-*Proteobacteria*, *Acidobacteria* and the *Planctomycetales*. Only 14.7% of all phylotypes (sequences with similarities >97%), but 45% of all clones, were common in the rhizosphere and the bulk soil clone libraries. The combined use of rDNA and rRNA libraries indicated which taxa might be metabolically active in this soil. All dominant taxa, with the exception of the *Actinobacteria* were relatively less represented in the rRNA libraries as compared to the rDNA libraries. Clones belonging to the *Verrucomicrobiales*, *Firmicutes*, *Cytophaga-Flavobacterium-Bacteroides* and OP10 were found only in rDNA clone libraries indicating that they might not represent active constituents in our samples. The most remarkable result was that

sequences belonging to the *Actinobacteria* dominated both the bulk and rhizosphere soil libraries derived from rRNA (50% and 60% of all phylotypes, respectively). Seventy percent of these clone sequences were related to the *Rubrobacteria*-subgroups 2 and 3, thus providing for the first time evidence that this group of bacteria is likely metabolically active in heavy metal-contaminated soil.

2.2 Introduction

Toxic metal contamination of soils, sediments and groundwater causes major environmental problems which, in many cases, call for immediate action. The current cleanup technology involves excavation and removal of the contaminated soil and subsequent deposition in landfills. Besides its detrimental impact on the soil ecosystem, this technology is cost-intensive. An alternative approach is phytoextraction, an in situ technique relying on plants that translocate heavy metals from the soil and accumulate them in their roots and above-ground tissues (Salt *et al.*, 1995).

To date, more than 400 hyperaccumulating plant species, among them *Thlaspi caerulescens*, *Silena vulgaris*, and *Brassica juncea*, have been shown to remove Cd, Cu, Ni, Pb and Zn from metal-contaminated soils (Salt *et al.*, 1998). Successful phytoextraction may not only depend on the plant itself but also on the interaction of the plant roots with the rhizosphere bacterial community (Whiting *et al.*, 2001a). It has been suggested that soil microorganisms and in particular the active rhizosphere bacteria might improve heavy metal mobilization and uptake by plants. Although the mechanisms have yet to be understood in detail, rhizosphere bacteria were shown to promote the accumulation of selenium and mercury in wetland plants (De Souza *et al.*, 1999) and to increase the dissolution of Zn from the nonlabile phase in soil (Whiting *et al.*, 2001a). The description of the rhizosphere bacterial community and the careful characterization of the plant-bacterial interactions in heavy metal-polluted soils may therefore provide valuable information needed to increase the efficiency of phytoextraction.

Whereas microbial communities in heavy metal-polluted bulk soils have been studied in a few cases (Brim *et al.*, 1999; Sandaa *et al.*, 1999b), information on the microbial community composition in the rhizosphere of heavy metal-accumulating plants is scarce. In particular, it is unclear if heavy metal-accumulating plants selectively stimulate e.g. by providing root exudates the bacterial community composition in heavy metal-contaminated soils and thus establish

different communities in the root-zone (Kozdrój & van Elsas, 2000), which may not be dominant or active in an unplanted bulk soil. Likewise, it is important to study the effects of metal-accumulating plants on the soil organisms indigenous to the polluted sites (Pawlowska *et al.*, 2000). These organisms may be important for revegetation after a significant reduction of the soil metal concentration has been achieved. Besides, studies on heavy metal-polluted soils may provide new insight into bacterial diversity under unfavorable conditions, new isolates and likely new genetic information on heavy metal resistance, which could be exploited.

We report here on a culture-independent, 16S rRNA-based phylogenetic survey of the bacterial community inhabiting heavy metal-contaminated rhizosphere and bulk soil. Samples were taken from the rhizosphere of the heavy metal-accumulating plant *Thlaspi caerulescens*. Unplanted bulk soil was also sampled in order to investigate if there is an effect of the plant rhizosphere on the bacterial composition in a heavy metal-contaminated soil. We were in particular interested in the potentially active bacterial populations in a heavy-metal contaminated soil. Metabolically active bacterial cells are usually characterized by a higher amount of ribosomes than resting or dormant cells (Nomura *et al.*, 1984). Therefore we studied reverse-transcribed rRNA as well as rDNA clone libraries, since sequences obtained from reverse-transcribed rRNA are better indicators of the active bacterial populations at the time of sampling than sequences from rDNA templates.

2.3 Material and Methods

2.3.1 Soil characteristics and sample preparation

Soil samples were obtained from a site in Ticino/Switzerland, which had been contaminated with wastes from septic tanks from 1960 to 1980. The soil is a sandy loam with 12.3% clay, 19.8% silt and 67.9% sand. It contained 5.9% organic matter and had a pH_{H2O} of 5.15. Total metal concentrations per kg soil were 2.5 mg Cd, 227 mg Cu and 1144 mg Zn. Bioavailable metal concentrations per kg dry soil were as follows: 21.2 mg Zn in the bulk soil and 13.9 mg Zn in the planted soil; 26.5 mg Cd in the bulk soil and 2.4 mg Cd in the planted soil. Before use, topsoil samples were homogenized, sieved (< 1 cm) and stored at 10°C in the dark. Pot experiments were carried out in flower pots containing 2 kg of the contaminated soil.

2.3.2 Plant growth conditions

Dr. C. Keller (EPFL) provided *Thlaspi caerulescens* seeds. They had been obtained from a population grown near an ancient Pb/Zn mine in Saint-Laurent-le Minier, Southern France. They were stored at 4°C before use. The seeds were germinated on uncontaminated gardening soil during 25 days. Three seedlings of *T. caerulescens* were subsequently transplanted each into a different pots. The plants were grown for three months in a climate chamber at 20°C during daytime and 16°C during night-time with 16 h of light alternating with 8 h of darkness. Three additional pots containing only contaminated soil were also incubated in the climate chamber. The pots were watered with 100 ml deionized water every 4 days.

2.3.3 Sampling

After 3 months, plants and soil were removed from the pots and each plant was shaken carefully to remove the bulk soil. The soil still adhering to the roots was defined as rhizosphere soil. It was separated from the roots by moderate agitation in 50 ml of sterile 0.9% NaCl solution during 5 min and then centrifuged at 8'000 g for 10 min (Marilley *et al.*, 1998). Subsamples (0.6 g wet weight) from each of the three rhizospheres were filled in 2-ml cryotubes and stored at -20°C until further use. Bulk soil samples from unplanted pots were sampled and directly frozen without any additional resuspension in NaCl until further use.

2.3.4 DNA extraction

Nucleic acids were extracted from 0.6 g (wet weight) samples of each of the three rhizosphere soil fractions and the three unplanted pots using a modification of the bead beating protocol of Kuske *et al.* (1998). Briefly, 1 ml of TENS buffer (50 mM Tris HCl [pH 8], 20 mM EDTA, 100 mM NaCl, 1% [wt/vol] sodium dodecyl sulfate [SDS]), and 0.75 g 0.1-mm glass beads were added to each sample. Samples were vortexed briefly and incubated at 70°C for 20 min. During this time, the samples were resuspended and mixed by vortexing for 5 s every 10 min. Subsequently, 20 µl of skim milk powder solution (0.1 g of milk powder in 500 µl H_2O) was added. The mixture was three times shaken for 45 s in a FastPrep bead beater (Bio 101) at 4 m/s. Samples were stored on ice during 1 min between each run. The lysed sample mixture was then centrifuged at 13'000 x g for 4 min and the supernatant transferred into a fresh sterile 2-ml reaction tube. The supernatant was subjected to phenol-chloroform extraction. The aqueous phase (700 µl) was then incubated with 750 µl precipitation solution (20% polyethylenglycol

6000 and 2.5 M NaCl) at 37°C. DNA was pelleted by centrifugation at 16'000 x g for 20 min at 4°C, washed once with 70% ethanol and resuspended in 200 µl TE (10 mM Tris-HCl, 1mM EDTA, pH 8). One µl of a 1:10 dilution of the purified DNA-solution was used as template for PCR amplification.

2.3.5 RNA extraction and RT-PCR

Precautions were taken to prevent degradation of RNA by RNases. All glassware was treated by baking overnight at 200°C. All solutions were prepared with diethylene pyrocarbonate (DEPC) treated water (2 hours with 0.1% DEPC at 37°C, followed by autoclaving) and autoclaved at 121°C during 20 min. Since the procedure used for DNA extraction did not result in sufficient amounts of RNA, a different protocol was chosen for RNA-extraction. Total RNA was extracted from 0.6 g (wet weight) of each of the three unplanted bulk and rhizosphere soil samples following the protocol of Griffiths *et al.* (2000b) and the bead-beating protocol described above. To obtain pure RNA, the RNA-solution was digested with 3 U of RQ1 Rnase-free DNAse (Promega) according to the manufacturer's instructions. One µl of a dilution of 1:5 of the total RNA extracted from rhizosphere and bulk soil samples was mixed with 1 µM of primer 1492r, 4.95 µg/µl BSA and filled with 8.3 µl Rnase-free water. To denature the secondary structure of 16S rRNA, the template-primer mixture was incubated at 65°C for 5 min and then immediately stored on ice. Subsequently, the following agents were added: 1x reaction buffer, 0.5 mM (each) dNTP and 1 µl of Sensiscript reverse transcriptase (all from Qiagen). The reaction was performed in a total volume of 20 µl at 42°C for 1 h. One µl of a dilution of 1:200 of the cDNA was used as template for PCR amplification.

2.3.6 PCR-amplification, clone library construction and sequencing

PCR amplifications targeting bacterial 16S rDNA were performed with all DNA and cDNA solutions using the primers 27F and 1492R (Lane, 1991; Dojka *et al.*, 1998). PCR-amplifications were performed in a PTC 200 (MJ Research). The 50 µl PCR mixture contained 0.2 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 1x PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 4.95 µg/µl BSA, and 1 U of Taq Polymerase (Qiagen). PCR amplification began with a hot start of 5 min at 95°C after which the Taq Polymerase was added, followed by 30 cycles at 94°C for 1 min, 50°C annealing for 45 s, and primer extension at 72°C for 2 min. PCR was finished with a final extension at 72°C for 5 min. Replicate PCR products originating

from each nucleic acid sample (3 x bulk soil DNA and cDNA; 3 x rhizosphere soil DNA and cDNA) were pooled and purified on QIAquick PCR purification columns (Qiagen), ligated into the pGEM-T easy cloning vector and used to transform E. coli JM109 competent cells (Promega). White colonies were randomly picked for plasmid isolation using a Nucleospin plasmid kit (Macherey-Nagel), followed by PCR using the same primers and the same cycle profile as described above to select clones with the correct size insert. Plasmid templates were used for partial sequencing of the 5' end of the rRNA gene (up to 750 bp) by Microsynth GmbH (Balgach, Switzerland).

2.3.7 Phylogenetic analyses and chimera detection

Unaligned sequences were submitted to the Sequence Match program of the Ribosomal Database Program (RDP) (Maidak *et al.*, 1997) and to the Advanced BLAST search program of the National Center for Biotechnology Information (NCBI) to find closely related sequences. We tried to identify potential chimeric sequences by use of the CHECK_CHIMERA program (Maidak *et al.*, 1997). In some cases secondary structure analysis and separate phylogenetic analysis of the 3' and 5'-end of the sequences was performed. Sequences were aligned using the "Clustal W" option (Thompson *et al.*, 1997) in the BioEdit 5.0.9 sequence analysis software Hall, 1999). Sequence identities between clones and next related sequences were calculated using the "sequence identity matrix" option in BioEdit. The TREECON 1.3b software package was used to calculate distance matrices by the Kimura algorithm (Kimura, 1980) and to generate phylogenetic trees by the neighbor-joining method (Van de Peer & De Wachter, 1994). Maximum likelihood based trees were calculated using the program package PHYLIP (version 3.5; J. Felsenstein, Dept. of Genetics, Univ. of Washington, Seattle). One hundred bootstrapped replicate resampling data were generated with SEQBOOT (PHYLIP). Clone sequences with >97% sequence identity were considered to represent a provisional phylotype. It is understood that these sequences may not represent true phylotypes in the strict sense since we sequenced only up to 750bp. We used the term phylotype to simplify the comparison of the sequences and to define groups for rarefaction and diversity calculations.

2.3.8 Rarefaction analyses and diversity indexes

Rarefaction gives an estimation of the decrease in apparent species or phylotype richness of a community with decreasing subsample size. Rarefaction calculations were done using the

software Analytic Rarefaction (version 1.3; Stratigraphy Laboratory, University of Georgia [http://www.uga.edu/~strata/software/]). Phylotypes were also used to calculate the Shannon diversity index $H = -\Sigma[n_i * \ln(n_i)]$, where n_i is the relative contribution of phylotype *i* to the whole library. The Shannon evenness index *J* was calculated using the formula $J=H/\ln S$, where S is the total number of phylotypes. Coverage (C) values were calculated by the equation $C = [1 - (n/N)] \times 100$, where n is the number of unique clones and N is the total number of clones examined.

2.3.9 Nucleotide sequence accession numbers

The partial clone sequences determined in this study have been deposited in the NCBI database under accession numbers AF445085 to AF445157 and AY242608 to AY242816.

2.4 Results

2.4.1 Comparison of the overall diversity in the clone libraries from unplanted bulk and rhizosphere soil

Extracted total DNA and RNA from the rhizosphere and the unplanted bulk soil were used to create a total of four clone libraries. The number of clone sequences and phylotypes for each bacterial taxon is presented in (Table 2.1). Clone sequences with >97% sequence identity were considered to represent a provisional phylotype. Selected clone sequences representing distinct dominant phylotypes are listed in (Table 2.2) with affiliation to higher taxa, number of clones per phylotype and percentage identity to the closest related sequence.

Eleven major bacterial taxa in total were identified from the 5'-partial sequence analysis of 73 clones from the rhizosphere soil rDNA clone library, 69 clones from the rhizosphere soil rRNA clone library, 75 clones from the bulk soil rDNA clone library and 65 clones from the bulk soil rRNA clone library. Only 22 phylotypes (14.6% of the total number of phylotypes analyzed) were found in both rhizosphere and bulk soil clone libraries (Table 2.1). However, these phylotypes accounted for 45.4% of the total sequences (128 clones of a total of 282), indicating that they include the predominant phylotypes in the clone libraries. As shown in (Table 2.1), the phylotypes found in both soil fractions were the most abundant ones within the respective phylogenetic groups and represented in the case of the α-*Proteobacteria*, *Acidobacteria* and *Actinobacteria* 45-56% of all sequences in each taxon. The highest relative amount of phylotypes and clones found in both soil fractions was observed for the

Table 2.1: Number of phylotypes and clones from heavy-metal contaminated rhizosphere and bulk soil for each of the bacterial taxa observed in the four clone libraries.

Bacterial division	All libraries	Rhizosphere	Bulk soil	Found in both bulk soil and rhizosphere
Total	150[a]/282[b]	83/142	89/140	22/128
α-*Proteobacteria*	27/54	13/28	19/26	5/30
β-*Proteobacteria*	15/30	8/17	10/13	3/11
γ-*Proteobacteria*	10/11	3/3	7/8	0/0
δ- *Proteobacteria*	4/4	1/1	3/3	0/0
Acidobacteria	21/40	12/20	11/20	2/18
Verrucomicrobiales	3/12	3/10	2/2	2/11
Planctomycetales	14/18	11/13	5/5	2/4
Actinobacteria	48/102	30/47	26/55	8/54
Firmicutes	5/6	0/0	5/6	0/0
CFB	1/1	1/1	0/0	0/0
OP10	1/2	1/2	0/0	0/0
not affiliated	1/2	0/0	1/2	0/0

[a] Phylotypes: clones with sequences identities > 97%

[b] Clones

Verrucomicrobiales (66% of the phylotypes and 92% of the clones within this taxon). In contrast to that, the *Planctomycetales* represented the taxon with the lowest relative amount of phylotypes (14%) and clones (22%) common to both the rhizosphere and the bulk soil. Although sequences belonging to the γ- and δ-*Proteobacteria* were retrieved from both the bulk and the rhizosphere soil, not a single common phylotype was observed in both soil fractions. Altogether, the phylotypes belonging to the *Actinobacteria* were the predominant ones (29.2% of the total bulk soil phylotypes; 36.1% of the total rhizosphere phylotypes), followed by the α-*Proteobacteria* (21.3% and 15.7%), *Acidobacteria* (12.4% and 14.5%), β-*Proteobacteria* (11.2% and 9.6%) and *Planctomycetales* (5.6% and 13.2%) (Table 2.1).

In general, more phylotypes affiliated to the *Proteobacteria* were retrieved from the total bulk soil clone libraries whereas more phylotypes clustering within the *Planctomycetales*, the *Verrucomicrobiales* and the *Actinobacteria* were found in the rhizosphere soil clone libraries (Table 2.1). *Firmicutes*-phylotypes were only observed in the bulk soil clone library originating

from rDNA, while phylotypes affiliated to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) and OP10 where only observed in the rhizosphere.

2.4.2 Comparison of the composition of the 16S rDNA and 16S rRNA clone libraries

Only a minority of the phylotypes present in the 16S rDNA clone libraries was identical to those present in the libraries obtained from 16S rRNA: 2 α-*Proteobacteria*, 2 β-*Proteobacteria*, 1 *Acidobacteria* and 4 *Actinobacteria*. However, these phylotypes represented 29.6%, 26.6%, 27.5% and 42.2% of the clones within the respective group and included also the predominant phylotype for each of these four taxa (Table 2.2). Most of the bacterial taxa which were highly abundant in the rDNA clone libraries, such as the α-*Proteobacteria* and the *Acidobacteria*, were significantly less numerous in the rRNA clone libraries (Figure 2.1).

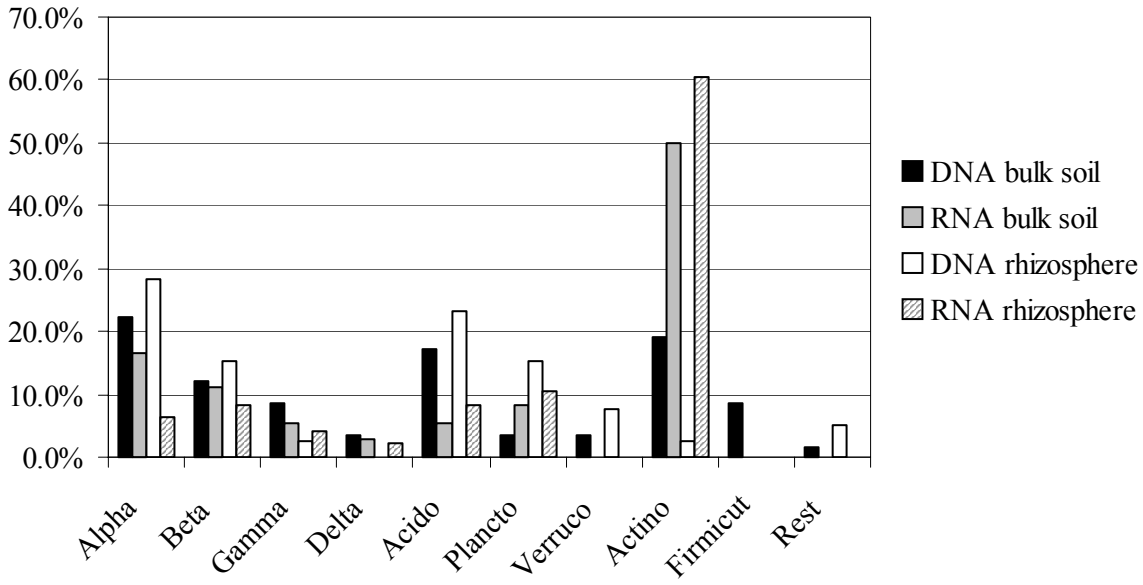

Figure 2.1: Percentage of phylotypes belonging to different bacterial taxa in the bulk soil DNA, bulk soil RNA, rhizosphere DNA and rhizosphere RNA clone libraries. Alpha = α-*Proteobacteria*; Beta = β-*Proteobacteria*; Gamma = γ-*Proteobacteria*; Delta = δ-*Proteobacteria*; Acido = *Acidobacteria*; Plancto = *Planctomycetales*; Verruco = *Verrucomicrobiales*; Actino = *Actinobacteria*; Firmicut = *Firmicutes*; Rest = CFB, OP10 and not affiliated phylotypes.

For example, α-*Proteobacteria* and *Acidobacteria* each represented more than 20% of the phylotypes in the rhizosphere rDNA clone library. However, both taxa dropped below 10% in the rhizosphere rRNA clone library. On the other hand, the number of *Actinobacteria*-phylotypes significantly increased in the 16S rRNA clone libraries as compared to the 16S rDNA clone libraries. *Actinobacteria* dominated both the 16S rRNA clone libraries from

rhizosphere (60% of the phylotypes) and bulk soil (50% of the phylotypes) (Figure 2.1). In contrast to that, 19% of the bulk DNA phylotypes were affiliated to the *Actinobacteria* and only one single *Actinobacteria* sequence was observed in the rhizosphere rDNA clone library. All other taxa each made up less than 11% of the phylotypes in both rRNA clone libraries (with the exception of the α-*Proteobacteria* in the bulk soil rRNA library). Four taxa (*Verrucomicrobiales*, *Firmicutes*, CFB and OP10) were only present in 16S rDNA clone libraries.

2.4.3 Rarefaction analysis and diversity indices

Rarefaction analysis was performed with two data sets comprising the two rhizosphere and the two bulk soil clone libraries, respectively, to estimate to what extent the diversity of the samples can be described with the number of clones analyzed. Complete coverage of a data set would be expected to result in a plateau-shaped curve. Rarefaction plots were rather similar (with overlapping 95% confidence limits; not shown) for both soil fractions and suggested that the number of clones was insufficient to reach saturation and to completely describe the diversity of bacterial phylotypes in the rhizosphere and bulk soil with the clone libraries obtained (Figure 2.2). Separate rarefaction analysis for the four libraries showed that the expected number of phylotypes was highest in the bulk DNA clone library, followed by the rhizosphere rRNA clone library. Lowest expected numbers of phylotypes were observed for the rhizosphere rDNA and the bulk soil rRNA clone libraries.

Rarefaction plots are consistent with the Shannon diversity indices calculated for the four data sets (3.908 for the bulk soil DNA library, 3.504 for the rhizosphere RNA library, 3.463 for the rhizosphere DNA library and 2.991 for the bulk soil RNA library). The maximum possible values for the Shannon diversity index (in the case that each clone represents a unique phylotype) were similar for all four clone libraries and ranged between 4.317 (bulk soil DNA library) and 4.174 (bulk soil RNA library). The Shannon evenness index was 0.905 for the bulk soil DNA clones, 0.828 and 0.807 for the rhizosphere RNA and DNA clones, respectively, and 0.716 for the bulk soil RNA clones. Another approach to assess the completeness of a clone library is to calculate the percentage coverage. Coverage was the highest for the rhizosphere DNA library (65.7%), followed by the bulk soil RNA library (58.5%), the rhizosphere RNA library (42%) and the bulk soil DNA library (33.3%).

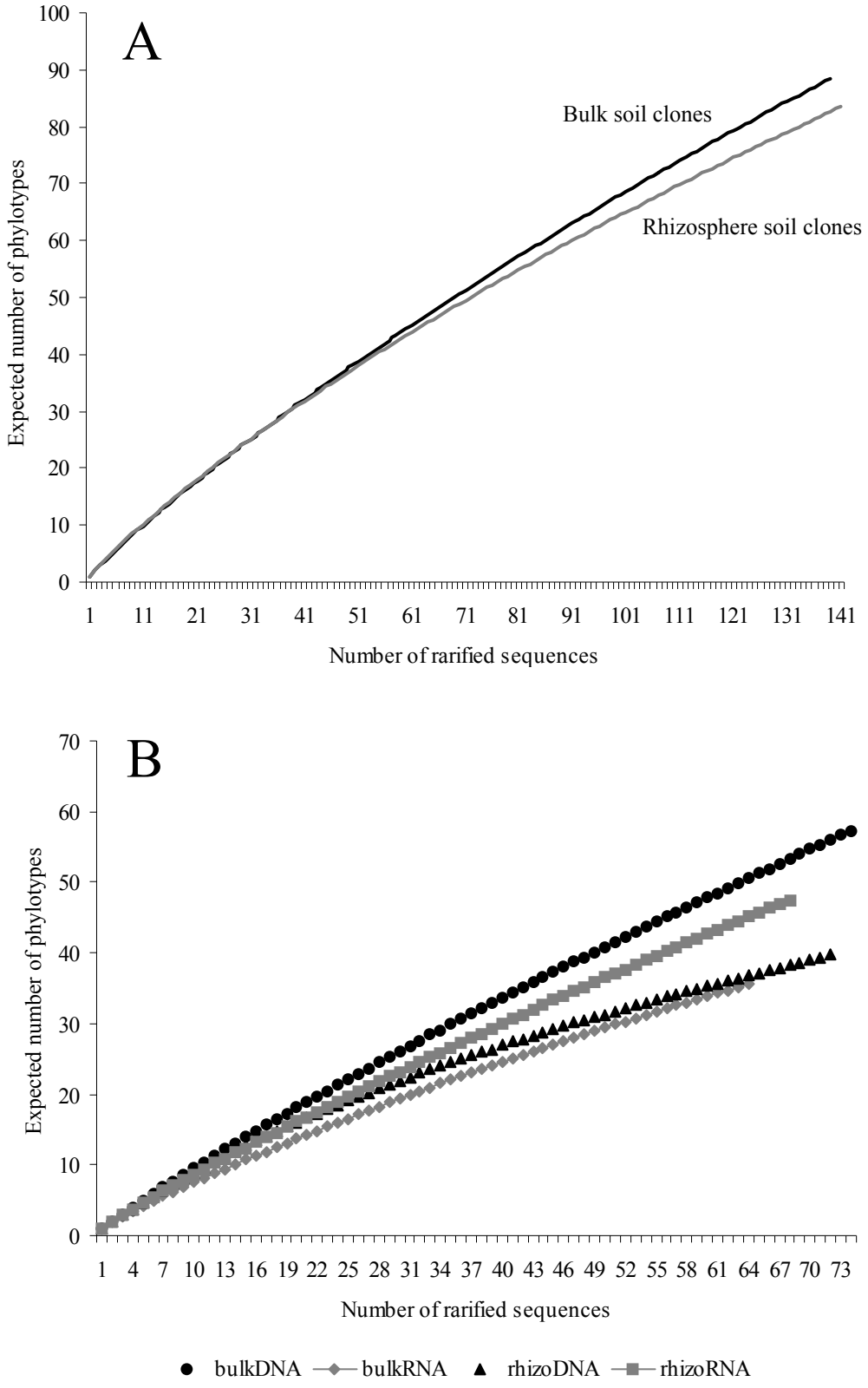

Figure 2.2: (A) Rarefaction analysis of two data sets including combined bulk soil and rhizosphere soil clone libraries (rDNA plus rRNA libraries), respectively. (B) Separate rarefaction analysis of all four clone libraries.

2.4.4 Phylogenetic assignment of 16S rDNA and 16S rRNA sequences

Only a minority of the clone sequences, belonging to the *Actinobacteria* (10 clones), the *Firmicutes* (6 clones), the α- and β-*Proteobacteria* (17 and 4 clones, respectively) were closely related (> 97% sequence identity) to 16S rDNA sequences from species commonly isolated from soil, such as *Mycobacterium*, *Actinomyces*, *Bacillus*, *Bradyrhizobium*, *Mesorhizobium*, *Sphingomonas* and *Variovorax*. Interestingly, nine sequences highly similar to *Bradyrhizobium* sp. MSDJ 5725 were found in the two rDNA clone libraries (rhizosphere and bulk soil), however, not a single one was obtained from reverse transcribed 16S rRNA (Table 2.2). On the other hand, four clones with very high sequence-similarity with the β-proteobacterium *Variovorax paradoxus* str. MBIC3839 (98-99.7% similarity), were obtained only from rhizosphere clone libraries (rDNA and rRNA) (Table 2.2).

The majority of the clones from this heavy-metal contaminated bulk and rhizosphere soil displayed relationships to a wide range of environmental sequences from various yet uncultured bacteria. For instance, the α2-subgroup of the α-*Proteobacteria*, represented e.g. by the *Rhizobiaceae*, the *Methylobacterium*- and the *Methylosinus*-group, included the majority of our α-*Proteobacteria* clones (82.1% of the rhizosphere sequences and 73.1% of the unplanted soil sequences). The most abundant phylotype within the α2-*Proteobacteria* closely affiliated with the environmental clone sequence C062 (>97% similarity; Table 2.2) recently isolated from earthworm cast (Furlong *et al.*, 2002). This phylotype was observed in both rDNA clone libraries as well as in the rRNA clone library from rhizosphere soil. Most of the sequences assigned to the β- and γ-*Proteobacteria* were related either to environmental sequences from potato rhizosphere (e.g. SC-I-66, unpublished) or from forest soils (e.g. NMW3.108WL; Axelrood *et al.*, 2002).

The *Verrucomicrobiales*-like sequences were found only in rDNA-libraries and were closely related (96-99% similarity) to environmental sequences often observed in terrestrial systems, such as MC17 (Liesack & Stackebrandt, 1992) and EA25 (Lee *et al.*, 1996) (Table 2.2). All rDNA-derived *Planctomycetales*-like clones (from rhizosphere and bulk soil) grouped together with environmental sequences obtained either from a metal-contaminated superfund site (e.g. K20-31, unpublished) or from a forest soil clone library (e.g. clone 26, unpublished) (Table 2.2). These sequences cluster within or close to the genera *Pirellula* and *Gemmata* (Figure 2.3). In contrast to that, sequences obtained from reverse transcribed rRNA (from rhizosphere and bulk soil) were either related to some *Nostocoida limicola* III strains or to the environmental clone WD287 from a PCB-polluted moorland soil (Nogales *et al.*, 2001) (Figure 2.3). The

Table 2.2: Clones present in the 16S rDNA and/or the 16S rRNA clone libraries representing phylotypes with more than one clone within higher bacterial taxa. Abbreviations in parentheses indicate in which type of clone library the phylotype was found (r: rhizosphere rDNA, b: bulk soil rDNA; R: rhizosphere rRNA; B: bulk soil rRNA).

Taxon	Clones representing distinct phylotypes	Length	No. of clones/ phylotypes	Closest phylogenetic relatives	% identity
α- *Proteobacteria*	Tc11 (r)	725	2	Uncultured rape rhizosphere bacterium wr0007 (AJ295468)	97.7
	Tc129-17 (r,R,b)	629	13	Uncultured earthworm cast bacterium C062 (AY037712)	99.5
	Tc60 (r,b)	700	9	*Bradyrhizobium* sp. MSDJ 5725 (AF363148)	99.2
	Tc81 (r,b)	567	2	Uncultured soil bacterium KF-JG30-B3 (AJ295650)	92.0
	Tc96 (r,b)	622	3	*Agrobacterium sanguineum* strain ATCC 25660 (AB062105)	98.7
	Tc133-97 (B)	609	2	Uncultured grassland soil bacterium DA122 (Y12598)	95.4
	Tc135-223 (b,R)	610	3	Uncultured peat soil bacterium L013.3 (AF358017)	98.5
β-*Proteobacteria*	Tc30 (r,b)	672	4	Uncultured agricultural soil bacterium SC-I-66 (AJ252648)	97.1
	Tc64 (r)	737	3	Uncultured agricultural soil bacterium SC-I-24 (AJ252625)	99.3
	Tc45 (r,b,R,B)	608	4	Uncultured agricultural soil bacterium SC-I-39 (AJ252633)	94.7
	Tc70 (r,R)	744	4	*Variovorax paradoxus* strain MBIC3839 (AB008000)	99.7
	Tc134-102 (B)	634	2	Uncultured wetland bacterium FW 145 (AF523975)	96.3
	Tc119-F01 (R,B)	630	3	Uncultured soil bacterium 1326-2 (AF423222)	98.0
Acidobacteria	Tc86 (r,b)	561	8	Uncultured grassland soil bacterium sl1_220 (AF078357)	99.3
	TcA3 (b)	606	3	Metal-contaminated soil clone K20-26 (AF145826)	98.7
	Tc88 (r)	657	2	Uncultured soil bacterium clone C101 (AF013528)	96.2
	Tc129-9 (r,b,R,B)	667	11	Uncultured earthworm cast bacterium C034 (AY037688)	97.5
Verrucomicrobiales	Tc4 (r;b)	709	6	Uncultured soil bacterium EA25 (U51864)	99.8
	TcA24 (r,b)	648	5	Uncultured soil bacterium MC17 (X64381)	96.4
Planctomycetales	Tc89 (r)	561	2	Uncultured forest soil bacterium clone26 (AF271321)	90.3
	Tc130-30 (r,b)	606	2	Uncultured forest soil bacterium clone23 (AF271319)	98.1
	Tc74 (r)	657	2	Metal-contaminated soil clone K20-31 (AF145829)	92.2
	Tc75 (r,b)	667	2	Metal-contaminated soil clone K20-09 (AF145812)	96.4
Actinobacteria	Tc120-D04 (R,B)	636	4	Uncultured forest soil bacterium SMS9.137WL (AY043898)	93.0
	Tc120-E04 (b,R)	650	2	Uncultured soil bacterium MC58 (X68456)	92.4
	Tc134-14 (R,B)	616	2	Uncultured rice paddy soil bacterium ARFS-13 (AJ277692)	91.5
	Tc134-105 (B)	619	2	*Mycobacterium fortuitum* (AF480581)	99.7
	Tc122-B08 (R)	620	2	Uncultured earthworm cast bacterium C136 (AY37739)	96.0
	Tc122-D08 (R)	630	2	Uncultured earthworm cast bacterium C136 (AY37739)	93.4
	Tc121-H06 (R,B)	639	2	Bacterium Elllin404 (AF432234)	94.6
	Tc133-99 (B)	646	3	Earthworm burrow bacterium B33D1 (AY039806)	94.4
	Tc119-E02 (R,B)	620	2	Uncultured thermal soil bacterium YNPFFP1 (AF391984)	92.9
	Tc128-91 (B)	620	2	Uncultured grassland soil bacterium sl1_017 (AF078323)	94.7
	Tc57 (r,b)	654	3	Uncultured grassland soil bacterium sl2_504 (AF078365)	99.2
	Tc 130-25 (b,B)	585	2	Uncultured forest soil bacterium SMS9.14WL (AF432686)	96.1
	Tc132-52 (b,B)	587	2	Uncultured forest soil bacterium SMS9.14WL (AF432686)	96.8
	Tc123-E10 (b,R,B)	600	37	Uncultured earthworm cast bacterium c238 (AY154594)	99.6
Firmicutes	TcA4 (B)	577	2	*Turicibacter sanguinis* (AF349724)	100

Acidobacteria-sequences were distributed within six of the eight subgroups described recently (Hugenholtz *et al.*, 1998a) and displayed similarities to environmental soil sequences from 93% to 99% (Table 2.2; Figure 2.4). This group of sequences was dominated by two phylotypes. The first phylotype (8 rhizosphere and bulk soil rDNA sequences) was closely related to the upland grass pasture sequence sl1_220 (McCaig *et al.*, 1999) from *Acidobacteria*-subgroup 1. The second phylotype (11 sequences from all four clone libraries) was highly similar to the earthworm cast sequence C034 (Furlong *et al.*, 2002) from *Acidobacteria*-subgroup 4 (Table 2.2; Figure 2.4). The most interesting and remarkable result is, that the predominant group within the bulk and rhizosphere rRNA clone libraries (Table 2.1), the *Actinobacteria*, was dominated by a deeply branching actinomycetes lineage, the *Rubrobacteria*, suggesting that members of this group might indeed be metabolically active in heavy metal-polluted soils.

Seventy percent of these *Actinobacteria* clone sequences were related to the *Rubrobacteria*-subgroups 2 and 3 (Holmes *et al.*, 2000) (Figure 2.5). Most of the *Rubrobacteria*-subgroup 3 clones (37 out of 43) represented one single phylotype with a sequence similarity of almost 100% to the uncultured earthworm cast bacterium clone c238 (Furlong *et al.*, 2002) (Table 2.2). Among the remaining sequences clustering within the *Actinobacteria*, fifteen sequences (2 from soil rDNA; 13 from rhizosphere and soil rRNA) appeared to be related to the *Acidimicrobium ferrooxidans* cluster, another deeply branching actinomycetes lineage, with a moderate relationship to the environmental clones MC58 (Stackebrandt *et al.*, 1993), SMS9.137WL (Axelrood *et al.*, 2002) and ARF-13 (Ludemann & Conrad, 2000) (Table 2.2).

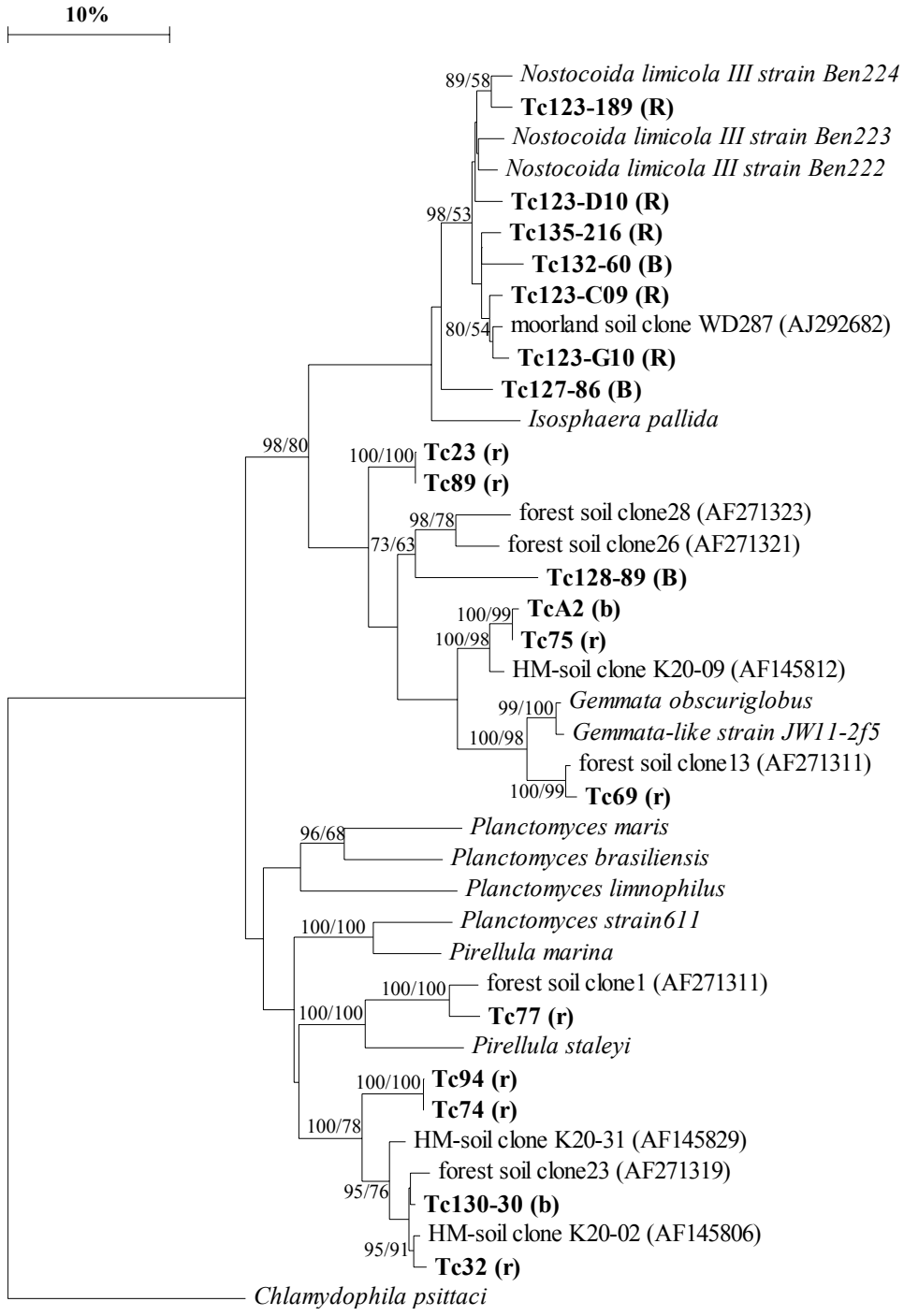

Figure 2.3: Neighbourg-joining tree showing the affiliation of 16S rRNA and 16S rDNA sequences from heavy metal-polluted bulk and rhizosphere soil to the *Planctomycetales*. Abbreviations in parentheses indicate which type of clone library the sequence was found (r, rhizosphere rDNA; b, bulk soil rDNA; R, rhizosphere rRNA; B, bulk soil rRNA). Bootstrap values are only shown for nodes that had >50% support in bootstrap analysis of 100 replicates in trees generated by both the neighbour-joining (before slash) and the maximum likelihood method (after slash). The scale bar indicates 10% estimated sequence divergence. *Chlamydophila psittaci* served as the outgroup organism.

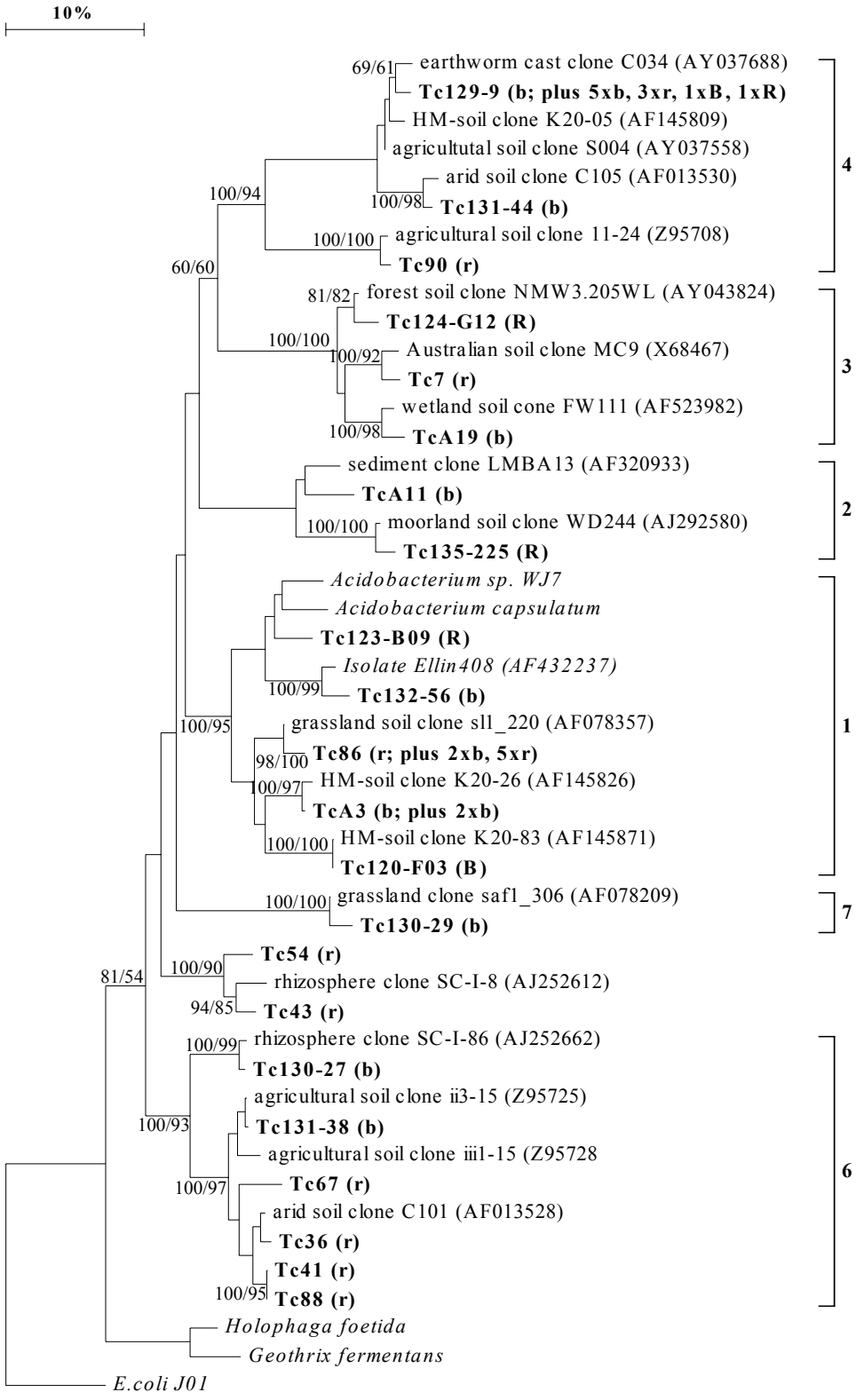

Figure 2.4: Neighbour-joining tree showing the affiliation of 16S rRNA and 16S rDNA sequences from heavy metal-polluted bulk and rhizosphere soil to the *Acidobacteria*-subgroups. Clones Tc129-9, Tc86 and TcA3 are representatives of the respective phytotype. Abbreviations in parentheses indicate in which type of clone library the sequence was found (r, rhizosphere rDNA; b, bulk soil rDNA; R, rhizosphere rRNA; B, bulk soil rRNA). Bootstrap values are only shown for nodes that had >50% support in bootstrap analysis of 100 replicates in trees generated by both the neighbour-joining (before slash) and the maximum likelihood method (after slash). The scale bar indicates 10% estimated sequence divergence. *Escherichia coli* served as the outgroup organism.

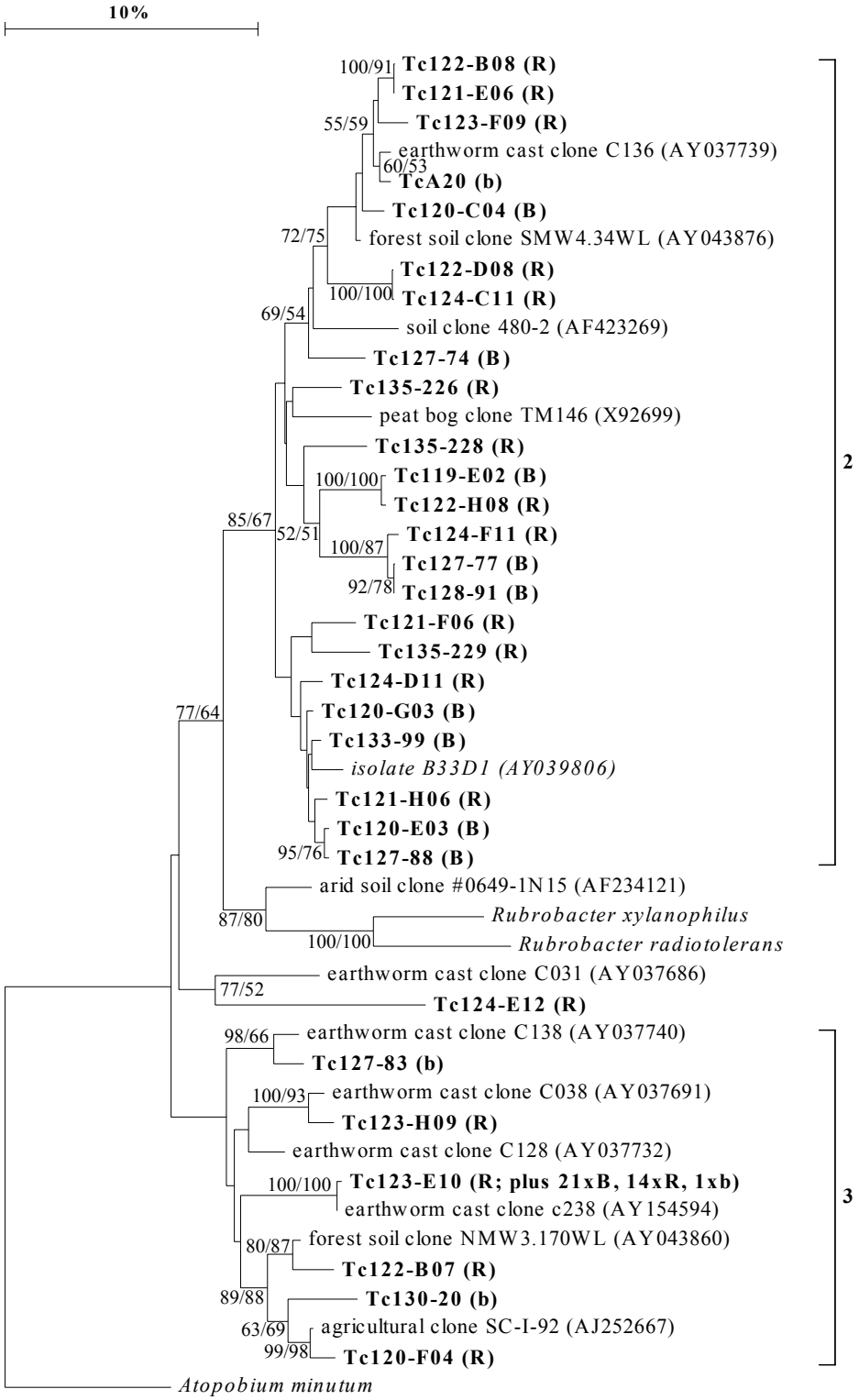

Figure 2.5: Neighbour-joining tree showing the affiliation of 16S rRNA and 16S rDNA sequences from heavy metal-polluted bulk and rhizosphere soil to the *Rubrobacteria*-subgroups. Clone Tc123-E10 is a representative of one phylotype with 37 sequences. Abbreviations in parentheses indicate in which type of clone library the sequence was found (r, rhizosphere rDNA; b, bulk soil rDNA; R, rhizosphere rRNA; B, bulk soil rRNA). Bootstrap values are only shown for nodes that had >50% support in bootstrap analysis of 100 replicates in trees generated by both the neighbour-joining (before slash) and the maximum likelihood method (after slash). The scale bar indicates 10% estimated sequence divergence. *Atopobium minutum* served as the outgroup organism.

2.5 Discussion

We have analyzed the rhizosphere and unplanted bulk soil bacterial community structures in a heavy metal-contaminated acidic soil by a culture-independent molecular survey using 16S rDNA and 16S rRNA clone libraries. Diversity studies based on clone libraries both from 16S rDNA and 16S rRNA templates are considered to better represent the bacterial community, since 16S rRNA-libraries in principal include also metabolically active members. Yet only a small number of studies have analyzed soil bacterial communities using 16S rRNA clone libraries or a combination of libraries derived from rDNA and reverse transcribed rRNA (Felske *et al.*, 1997; Nogales *et al.*, 2001). Calculation of coverage indicates that 33-65% of the total diversity was detected in the respective clone libraries. However, since coverage does not take evenness into account and is therefore calculated only relative to total richness, it represents only a rough estimate of the diversity. Using phylotypes based on partial sequences is a rather conservative approach to estimate coverage and values would probably drop if calculation was done based on truly different full-length sequences. Moreover, as shown by the rarefaction plots, our clone libraries did not cover the full phylotype diversity in this soil. The high coverage calculated for the rRNA libraries would be significantly lower and the rarefaction curves for the rRNA libraries would be even steeper if the dominant *Rubrobacter*-phylotype (21 clones in the bulk rRNA and 15 clones in the rhizosphere RNA library) was excluded from the analysis. The shallower rarefaction curve of the rhizosphere rDNA clone library might also be explained by the higher abundance of redundant phylotypes in this library.

It might be expected that root exudates result in a nutrient-enriched root zone, which could increase the bacterial diversity as compared to the otherwise oligotrophic bulk soil (Gilbert *et al.*, 1996). An effect on the bacterial diversity might be also expected due to the reduction of the heavy metal content in soil by the metal-accumulating plant. On the other hand, a solubilisation of heavy metals by the plant roots might also act as a selective pressure upon rhizosphere bacteria. However, the rather similar combined rarefaction plots for the bulk soil and the rhizosphere soil clone libraries, respectively, indicate that *Thlaspi caerulescens* did not exert a major influence on the overall bacterial diversity. The taxa dominating the bacterial community structure in this heavy metal-polluted unplanted soil also dominated the rhizosphere community. Yet, the fact that approximately 15% of the phylotypes, which were found in the clone libraries from both soil fractions, contained 45% of all clones indicates that the differences

between the rhizosphere and the bulk soil are based on rare clones, even though these clones may be quite similar. Variability in the clone libraries caused by methodological parameters, such as the efficiency of DNA and RNA-extraction, RT-PCR or preferential PCR-amplification cannot be excluded (von Wintzingerode *et al.*, 1997; Nogales *et al.*, 2001). For example, biases in the composition of rRNA-clone libraries might be due to the use of the primer 1492r for reverse transcription. As shown by Weller et al. (Weller & Ward, 1989; Weller *et al.*, 1991), using primers targeting the universally conserved 1400 region of the 16S rRNA can result in premature termination of reverse transcription. Another aspect is that clone libraries can only reflect quantitative abundances if the amplification efficiencies are the same for all templates, which is obviously an unlikely assumption for environmental samples. Taking also into account that rRNA templates are present in different concentrations at a given sampling time, our data and the calculation of diversity indices have to be treated only as semi-quantitative.

Finally, a clear correlation between relative abundance of rRNA-sequences in clone libraries and high metabolic activity of the organisms representing these sequences is probably not always given. In general, metabolically active and growing bacteria contain more ribosomes than resting or starved bacterial cells (Nomura *et al.*, 1984). However, there has been an increasing number of publications indicating that bacteria with very low reproduction rates and metabolic activities, as often found in soils, possess high amounts of rRNA per cell (Wagner *et al.*, 1995; Oda *et al.*, 2000). Despite this, our study demonstrates that bacterial taxa, which are predominant in the rDNA-clone libraries are (with the exception of the *Actinobacteria*) less dominant in the libraries derived from rRNA, indicating that only a part of the bacterial community is presumably metabolically active in this heavy metal-contaminated soil.

For instance, the high abundance of α-*Proteobacteria* in the rDNA clone libraries is in accordance with other studies investigating rhizosphere-associated bacteria (McCaig *et al.*, 1999; Kaiser *et al.*, 2001) and heavy metal-contaminated bulk soils (Sandaa *et al.*, 1999b). However, the high abundance of α-*Proteobacteria* phylotypes e.g. in the rhizosphere rDNA clone library was not reflected in the rhizosphere rRNA clone library, where the number of α-*Proteobacteria* phylotypes was drastically reduced. Many plant-symbiotic and plant-associated bacteria are affiliated to the *Proteobacteria*. The beneficial effects of these bacteria include growth promotion of the plant by e.g. synthesizing phytohormones and fixation of atmospheric nitrogen. Several studies have provided evidence that heavy metal-resistant *Proteobacteria* may protect plants or bacteria from the toxic effects of heavy metals or even enhance the metal

uptake by hyperaccumulator plants (Burd *et al.*, 1998; Stephen *et al.*, 1999; Belimov *et al.*, 2001; Whiting *et al.*, 2001a). However, it has been shown, that heavy metals may indirectly affect N_2-fixation rates, in particular in acidic soils, by reducing the number or the diversity of free-living cultivable rhizobia (Hirsch *et al.*, 1993; Chaudri *et al.*, 2000). Interestingly, a highly abundant rDNA-phylotype (9 clones) closely related to *Bradyrhizobium* sp. MSDJ 5725 was not obtained from reverse transcribed rRNA, suggesting that this *Bradyrhizobium* might not be metabolically active in this soil.

A similar discrepancy between the relative amount of rDNA- and rRNA-phylotypes in the clone libraries was observed for almost all the detected taxa, in particular for the *Acidobacteria*. It was also remarkable that all *Planctomycetales*-sequences from the rhizosphere rDNA-clone library were related to the genera *Gemmata* and *Pirellula*, which accords with the results of another study, where *Pirellula*-like sequences were considered to be typical root-associated bacteria, both in oxic and anoxic environments (Derakshani *et al.*, 2001). Some members of the *Planctomycetales* are characterized either by the excretion of holdfast substances or the formation of stalks and prosthecae, which may allow an improved attachment to the root surface (Staley *et al.*, 1991). However, only populations affiliated to the *Nostocoida limicola* III cluster seem to be metabolically active, since only sequences of this group were represented in the rRNA-clone library.

As in many other investigations on bacterial diversity in terrestrial systems, we revealed a considerable bacterial diversity in this soil, despite the long-term heavy metal contamination. Not unexpectedly we recovered sequences of bacterial taxa, which are still mainly characterized by environmental sequences rather than isolated strains, e.g. the *Planctomycetales*, *Verrucomicrobiales* and *Acidobacteria* (Liesack & Stackebrandt, 1992; Lee *et al.*, 1996; Ludwig *et al.*, 1997; Barns *et al.*, 1999; O'Farell & Janssen, 1999). However, it is unusual that *Actinobacteria*-sequences make up the majority in environmental clone libraries, and in particular in rRNA-derived clone libraries. This is in contrast to most so far conducted molecular surveys on rhizosphere and bulk soil bacterial populations (Borneman & Triplett, 1997; Macrae *et al.*, 2000; Kaiser *et al.*, 2001; Nogales *et al.*, 2001). A few other reports are in agreement with our observation that gram-positive bacteria might play a more important role in rhizosphere and bulk soil than previously assumed (McCaig *et al.*, 1999; Smalla *et al.*, 2001). Similar to the study of Nogales et al. (Nogales *et al.*, 2001), the presence and diversity of *Actinobacteria* would be underestimated if only 16S rDNA sequences were retrieved.

The most surprising result of our study is, that members of the *Rubrobacteria* dominate the clone libraries derived from reverse transcribed 16S rRNA indicating that they might in fact make up a major portion of the metabolically active bacteria in heavy metal-contaminated rhizosphere and bulk soil. The *Rubrobacteria* represent a poorly described *Actinobacteria*-subdivision. This subdivision branches deeply from the high G+C gram positive division line of descent and presently consists of two species *R. radiotolerans* (Yoshinak *et al.*, 1973) and *R. xylanophylus* (Carreto *et al.*, 1996). Both species cluster within the *Rubrobacteria*-subgroup 1, are moderate thermophylic and have been isolated from a thermally polluted effluent (Carreto *et al.*, 1996) and hot springs in Japan (Yoshinak *et al.*, 1973) and Portugal (Ferreira *et al.*, 1999). However, it has been shown that *Rubrobacteria* represent a significant part of rDNA clone libraries in Australian arid soils (Holmes *et al.*, 2000), Scottish grassland soils (McCaig *et al.*, 1999), and a acidic peatbog soil (Rheims *et al.*, 1996). It has also been reported that 16S rDNA clones affiliating to the *Rubrobacteria* dominate the clone library obtained from a Zn-polluted soil (Moffett *et al.*, 2003) and were also the most represented group among earthworm cast clones (Furlong *et al.*, 2002). Recently, new isolates have been obtained from an Australian pasture soil (Janssen *et al.*, 2002; Sait *et al.*, 2002) and earthworm burrow (Furlong *et al.*, 2002), which all group within *Rubrobacteria*-subgroup 2 and are closely related to *Rubrobacteria*-like organisms so far detected only as 16S rDNA sequences.

However, based on the available publications and databases, it is still questionable if *Rubrobacteria* are commonly abundant and ubiquitous in terrestrial systems. Holmes *et al.* (2000) assumed that the *Rubrobacteria*-subgroups 1 to 3 might indeed be specialized to thrive in different soil niches. The dominance of Rubrobacteria-phylotypes in the rRNA-libraries does not necessarily allow an estimation of their quantitative abundance in this soil, since structure analysis solely based on clone libraries does not reflect the real situation in a sample. An improved understanding of the ecology of the different *Rubrobacteria*-subgroups requires the development of tools, such as real-time PCR, dot-blot or whole cell hybridization to detect and quantify these organisms in their environment.

Identification and tracking of these potentially active, but yet largely uncultured bacteria might help to understand how phytoremediation techniques influence dominant bacterial populations and their functions, but also if soil bacteria play indeed an important role in the heavy metal-uptake by plants. Presence of rRNA sequences in the rhizosphere of heavy metal-accumulating plants alone indicates only that these organisms probably show a high resilience

against heavy metals, however this does not tell us anything about the potential role of these organisms in phytoextraction. This study gave us a picture of the bacterial communities in heavy metal-polluted soils, yet, cultivation of members of these groups remains an important goal in this respect. Isolates of these bulk and rhizosphere soil bacteria will allow to study their metal-resistance, possible metal-transforming capacities and their potential use as inocula in (phyto)remediation processes.

2.6 Acknowledgements

We thank Dr. Catherine Keller and Dr. Daniel Hammer (EPFL) for providing *Thlaspi caerulescens* seeds and many helpful instructions regarding the plant cultivation. This study was funded by EPFL-grant 599993.

CHAPTER 3

Probe design for emerging phylogenetic groups and *in situ* characterisation of the rhizosphere microbial community of *Thlaspi caerulescens* in a heavy metal-contaminated soil by fluorescence *in situ* hybridization (FISH)

3.1 Introduction

In the second chapter, we studied the bacterial diversity in the rhizosphere of the hyperaccumulating plant *Thlaspi caerulescens* grown in a heavy metal-polluted soil with clone libraries based on the 16S ribosomal RNA and the corresponding gene (16S rDNA). Five major phylogenetic groups, i.e. the *Actinobacteria*, the α- and β-*Proteobacteria*, the *Acidobacteria* and the *Planctomycetales* dominated the clone libraries from this soil. Surprisingly, the *Actinobacteria* dominated both the bulk and rhizosphere soil clone libraries derived from rRNA. A deep branching lineage of the *Actinobacteria*, the *Rubrobacteria*, made up approximately 70% of these rRNA sequences, indicating that this group of organisms might be particularly active in this heavy metal-polluted soil.

One of the most important questions to ask if one wants to study and understand microbial community structure is "how many individuals correspond to an identified group of organisms in a given sample at a given time?". The frequency of different sequences or phylotypes in a 16S rDNA or rRNA clone library does most likely not exactly reflect the *in situ* abundance of these sequences in complex microbial communities. The different methodological steps involved in the retrieval and analysis of rDNA or rRNA sequences (nucleic acids extraction, reverse

transcription, PCR amplification and cloning steps) inevitably introduce biases which affect the abundance and distribution of the sequences in the clone libraries (von Wintzingerode *et al.*, 1997; Weller & Ward, 1989; Weller *et al.*, 1991). Therefore it is important to confirm the presence and the relative abundance of the retrieved sequences with other methods.

One alternative is the use of fluorescence *in situ* hybridization (FISH) which allows the *in situ* identification and enumeration of microbial cells in their natural microhabitat (Amann *et al.*, 1995). This technique is based on fluorescently labeled oligonucleotide probes hybridizing to the 16S rRNA of bacterial cells which are subsequently viewed by fluorescence microscopy (DeLong *et al.*, 1989). The signal intensity of the probe depends on the cellular rRNA content which in some cases may be linked to the physiological status of the cell (DeLong *et al.*, 1989). However, several factors (low rRNA content, cell permeabilisation, accessibility of the rRNA target-site) may limit the use of FISH for environmental samples (see Amann *et al.*, 1995 and references therein).

Based on sequences present in the RDP (Ribosomal Database Project) and Genbank databases, a wide range of oligonucleotide probes are available specific for different taxonomic levels, such as the kingdoms, the major phylogenetic groups and several genera and species (Manz *et al.*, 1992; Neef *et al.*, 1998; Wagner *et al.*, 1995; Bourne *et al.*, 2000; Felske *et al.*, 1998).

New phylogenetic groups are emerging with the increasing number of studies applying culture-independent comparative analysis of rRNA-sequences from diverse habitats (Barns *et al.*, 1999; Holmes *et al.*, 2000; Jurgens *et al.*, 1997; Hugenholtz *et al.*, 1998b; Hugenholtz *et al.*, 2001). These studies resulted in an increasing number of new bacterial phyla, however, the majority of these bacterial phyla are poorly represented by cultured organisms (Hugenholtz *et al.*, 1998a). The challenge is to design new probes in order to detect these groups but also to re-evaluated the specificity of the existing probes with the new sequences (Amann *et al.*, 2001). The first part of this study addresses the design of new 16S rRNA targeting oligonucleotide probes specific for the *Acidobacterium* subdivision 1 and the subclass *Rubrobacteria*. Both groups were observed in our clone libraries from rhizosphere rDNA and rhizosphere rRNA, respectively (see chapter 2). In the second part of this work we applied these new probes together with some published group-specific probes to analyse the rhizosphere microbial community of *Thlaspi caerulescens* in a heavy metal-polluted soil.

3.2 Materials and Methods

3.2.1 Microbial strains and soil samples preparation

Four strains were used to check the specificity of the designed probes: *Acidobacterium capsulatum* (DSMZ 11244), *Rubrobacter radiotolerans* (DSMZ 5868), *Conexibacter woesi* (DSMZ 14684) and *Bacillus subtilis* (DSMZ 704). These strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and cultivated following the recommendations provided by the DSMZ. The bacteria were subsequently fixed in paraformaldehyde or ethanol as described previously (Zarda *et al.*, 1997). Ten μl of both ethanol- and paraformaldehyde-fixed samples were spotted onto the gelatine-coated slides (0.1% gelatine, 0.01% $KCr(SO_4)_2$) and quickly dried on a hot plate at 70°C in order to obtain a homogeneous distribution. The slides were dehydrated in 50, 80, and 96% ethanol for 3 min each. Slides with immobilized *R. radiotolerans* and *C. woesi* were subsequently pretreated with 10 μl of a 10 mg ml^{-1} lysozyme solution during 1 h at 37°C followed by a second dehydration step for 3 min.

The hyperaccumulating plant *Thlaspi caerulescens* grew during three months in a heavy metal-contaminated soil from Ticino, Switzerland (see chapter 2). 0.4 g of rhizosphere soil samples, defined as soil still adhering to the roots after gentle shaking (see chapter 2), were fixed with paraformaldehyde or ethanol (Zarda *et al.*, 1997). Sixty μl of the fixed rhizosphere soil samples were dispersed in 940 μl of 0.1% sodium pyrophosphate in distilled water and sonicated 70 s at cycle 9, power 20% in a Sonopuls HD 2070 (Bandelin Electronic GmbH & Co, Switzerland) (Zarda *et al.*, 1997). During the sonication, the sample was kept on ice. Twenty μl of the sonicated soil samples were spotted onto the gelatine-coated slides and subsequently dehydrated as described above.

3.2.2 Whole-cell hybridization

Hybridizations with 1 μl Cy3-labeled oligonucleotide probes (50 ng ml^{-1}) (Microsynth, Switzerland) and 9 μl of hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl, and 0.01% SDS (pH 7.2)] were performed in the presence of 20% (ALF968 (Neef, 1997), Rubro198 (this study)), 30% (PLA46, PLA886 (Neef *et al.*, 1998); Acido228 (this study)) or 35% formamide (EUB338–II (Daims *et al.*, 1999); EUB338 (Amann *et al.*, 1990b), BETA42a, GAM42a (Manz *et al.*, 1992); IRog 1, IRog 2 (Ludwig *et al.*, 1997)) at 42°C for 2 h (Zarda *et al.*, 1997). The two

new probes, S-Sc-Acido-0228-a-A-19 (Acido228: 5'-TAATCDGCCGCGACCYCCT-3'), specific for the phylogenetic subdivision 1 of the *Acidobacterium* division (Barns *et al.*, 1999) and S-Sc-Rubro-0198-a-A-17 (Rubro 198: 5'-GGCCGAAGCTWCCTTTY-3'), specific for the whole *Rubrobacteria* subclass (Holmes *et al.*, 2000), were designed on the basis of complete and partial 16S rRNA sequences present in the RDP- and the Genebank-databases. These two probes were checked for their specificity in these two databases and first tested with pure cultures. After hybridization, slides were washed for 15 min at 48°C in a buffer containing 20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 0.01% SDS, and either 225, 102, or 80 mM NaCl depending on the formamide concentration during hybridization (20, 30 and 35%, respectively), subsequently rinsed with distilled water and dried in the dark. Hybridization with probe EUB338 was used as a positive control.

For total cell counts, bacteria were stained 15 min in the dark at room temperature with 10 µl of SYBR-Green II ($5x10^{-3}$ dilution of the stock, Molecular Probes, Inc.), rinsed with distilled water and dried in the dark (Weinbauer *et al.*, 1998). Slides were finally mounted with Citifluor solution AF1 (Citifluor Ltd., London, UK) and the preparations were examined with an Olympus BX-60 equipped for epifluorescence with a high pressure mercury bulb (100W) and filter sets HQ-Cy3 or HQ-EGFP (AHF Analysentechnik AG, Germany). Forty (whole-cell hybridization) or forty-eight (total cell counts) fields distributed over two circular areas of 53 mm^2 were examined for the fixed rhizosphere soil samples.

3.3 Results

3.3.1 Probe design for the Acidobacterium subdivision 1

We designed a novel probe specific for the *Acidobacterium* subgroup 1. This subgroup included half of the *Acidobacteria*-sequences found in the rhizosphere of *Thlaspi caerulescens*. The new probe perfectly matched *Acidobacterium capsulatum* and more than 70 environmental sequences, i.e. more than 86% of the sequences affiliated to subdivision 1 that are currently available in the RDP- and the Genbank-databases (Hugenholtz *et al.*, 1998b). The target regions of all other prokaryotic 16S rRNA sequences exhibited at least 2 mismatches (Table 3.1).

Signal intensity of hybridized *Acidobacterium capsulatum* cells remained equally high with 20 to 45% formamide concentration in the hybridization buffer. Considering position and strength of the mismatches in non-targeted environmental sequences, a formamide

concentration of 30% in the hybridization buffer was considered as sufficiently stringent for Acido228 (Figure 3.1). It is possible and in this case even desirable that a few environmental sequences from subdivision 1 with only one mismatch in the target region might also be detected at the applied stringency (Table 3.1). No further optimisation experiments were necessary (e.g. pretreatment of the fixed cells, ethanol fixation instead of paraformaldehyde fixation) as a good hybridization signal was obtained with paraformaldehyde-fixed cells.

Table 3.1: Alignment of 16S rRNA regions homologous to the target site of probe Acido228 designed for the phylogenetic subdivision 1 of the *Acidobacterium* division. Examples of minimum numbers of mismatches are shown for the different phylogenetic *Acidobacterium* subgroups as defined by Hugenholtz *et al.* (1998b). D= A, G, T; H = A, C, T; R = G, A; Y = C, T; * = no base.

Probe sequence	5′-TAATCDGCCGCGACCYCCT-3′
Target sequence	5′-AGGRGGTCGCGGCHGATTA-3′
A. capsulatum + 73 clones from subdivision 1	-------------------
9 subdivision 1 clones with 1 mismatch	-----T------------
Subdivision 2	-----CC----CA------
Subdivision 3	-----YC-----------
Subdivision 4	-----CC-----------
Subdivision 4	--A--C------------
Subdivision 5	-----TCY----------
Subdivision 6	-----CC----Y-------
Subdivision 7	-----CC----C-------
Subdivision 7	-T---TC----T-------
Subdivision 8	----TCCT---T-T-----
Subdivision 8	T----CAT-T-***-T---
Chloroplast *Olisthodiscus luteus*	-----C------------
Uncultured haloarcheon MSP41	-T----C-----------
Veillonella criceti	-------T---T-------

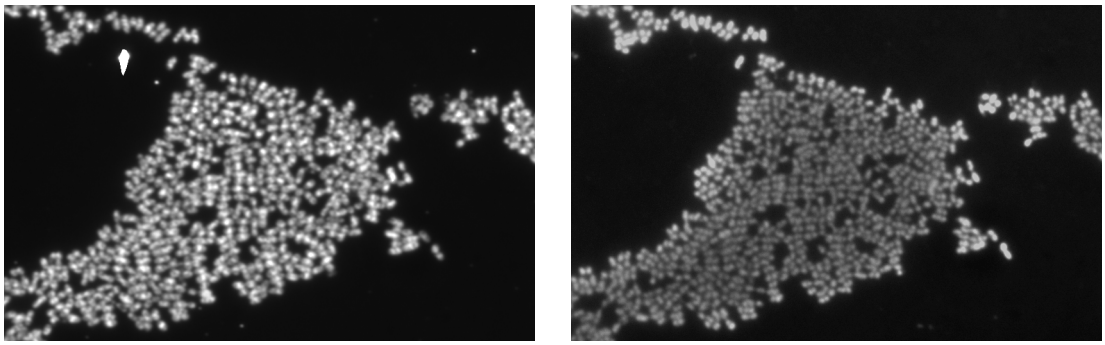

Figure 3.1: Epifluorescence micrographs of *Acidobacterium capsulatum* detected after hybridization with the probe Acido228 at 20% formamide (left) and after SYBR Green II staining (right).

3.3.2 Probe design for the Rubrobacteria subclass

As we have shown in chapter 2, sequences clustering with the *Rubrobacteria* were highly abundant in the rRNA-derived clone libraries. The new probe specifically designed for the *Rubrobacteria* perfectly matched all cultured *Rubrobacteria* isolates and more than 133 environmental sequences, i.e. more than 80% of the sequences affiliated to this subclass that are currently available in the RDP- and the Genbank-databases (Table 3.2).

Table 3.2: Alignment of 16S rRNA regions homologous to the target site of probe Rubro198 designed for the subclass *Rubrobacteria* (including subgroups 1, 2, 3 of the *Rubrobacteria* as defined by Holmes *et al.* (2000). R= G, A; Y= C, T; W= A, T.

Probe sequence	5′-GGCCGAAGCTWCCTTTY-3′
Target sequence	5′-RAAAGGWAGCTTCGGCC-3′
Rubrobacter radiotolerans + all clones from subgroup 1	----------
Conexibacter woesi + 23 clone sequences from subgroup 2	----------
97 clone sequences from subgroup 3	----------
27 clones with 1 mismatch (subgroups 2,3)	T---------
5 clones with 1 mismatch (subgroup 2)	----------T
2 clones with 2 mismatches (subgroup 2)	T---------T
Bacillus subtilis	-------G--------T

Probe Rubro198 was tested with *Rubrobacter radiotolerans* (*Rubrobacteria* subgoup 1) and *Conexibacter woesi* (*Rubrobacteria* subgoup 2). Isolates of subgroup 3 were not available at the time of this study. The influence of different fixatives on the hybridization signal was evaluated using the probe EUB338. Hybridization of paraformaldehyde-fixed *C. woesi* with EUB338 resulted in medium fluorescence intensities, whereas paraformaldehyde-fixed *R. radiotolerans* cells showed only a very weak hybridization signal, indicating limited permeabilization (Figure 3.2). However, lysozyme treatment of ethanol-fixed cells improved permeabilization of *R. radiotolerans* and resulted in higher signal intensity after hybridization with EUB338 and Rubro198, respectively (Figure 3.2). Increasing the formamide concentration from 25% to 40% in the hybridization buffer for probe Rubro198 resulted in a significant decrease of the signal intensity. For this reason, a concentration of 20% formamide was chosen (Figure 3.3)which would probably also permit to detect the clone sequences harbouring one weak mismatch at the

5' end of the target sequence (Table 3.2). Non specific binding to lysozyme-treated *Bacillus subtilis*, which has two mismatches in the target region of Rubro198 was not observed.

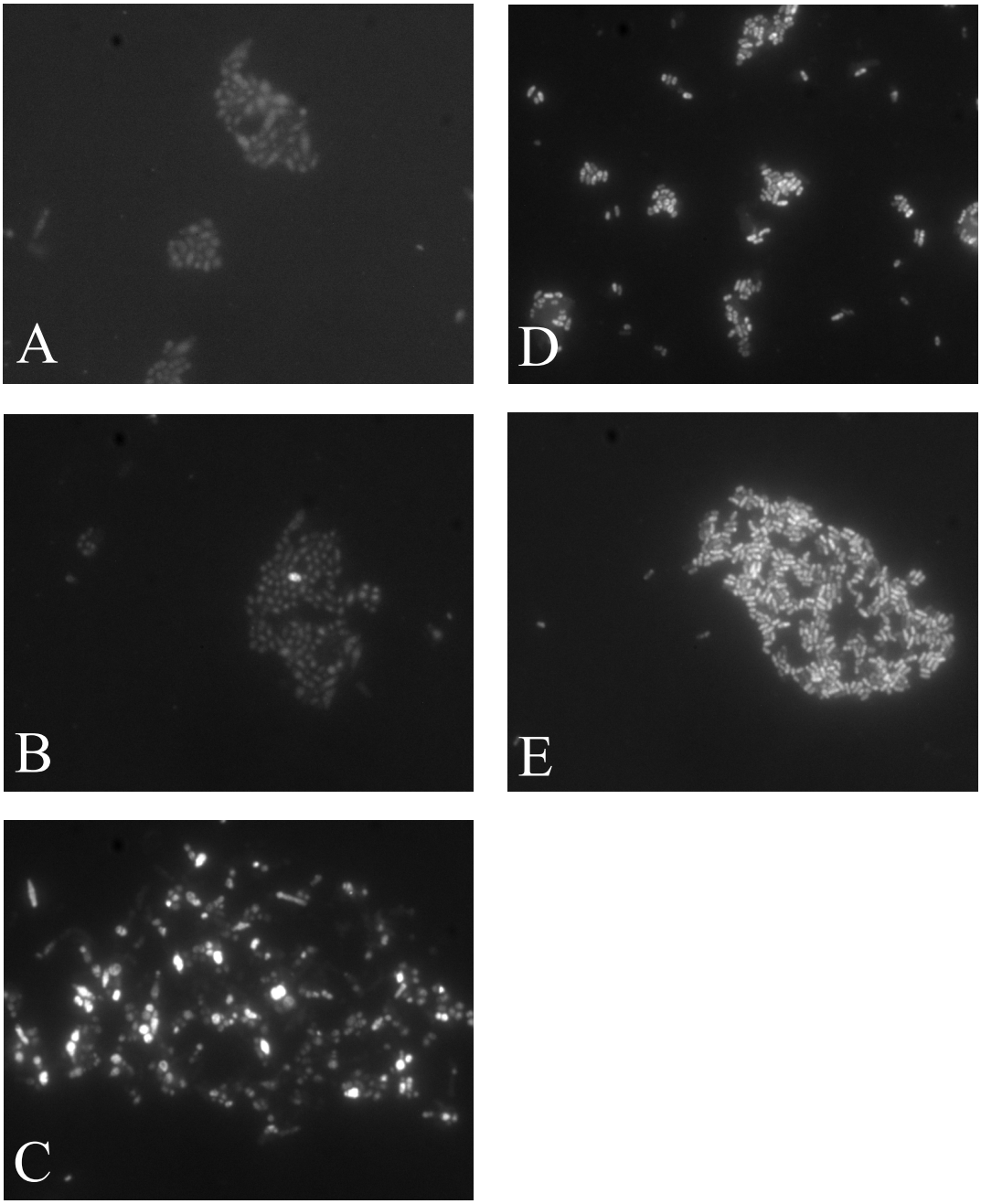

Figure 3.2: Epifluorescence micrographs of *Rubrobacter radiotolerans* (panels A, B, C) and *Conexibacter woesi* (panels D and E) cells detected with the probe EUB338.The cells are fixed with paraformaldehyde (panels A and D) or ethanol (panels B and E). In the panel C, the cells of *Rubrobacter radiotolerans* were pretreated with lysozyme.

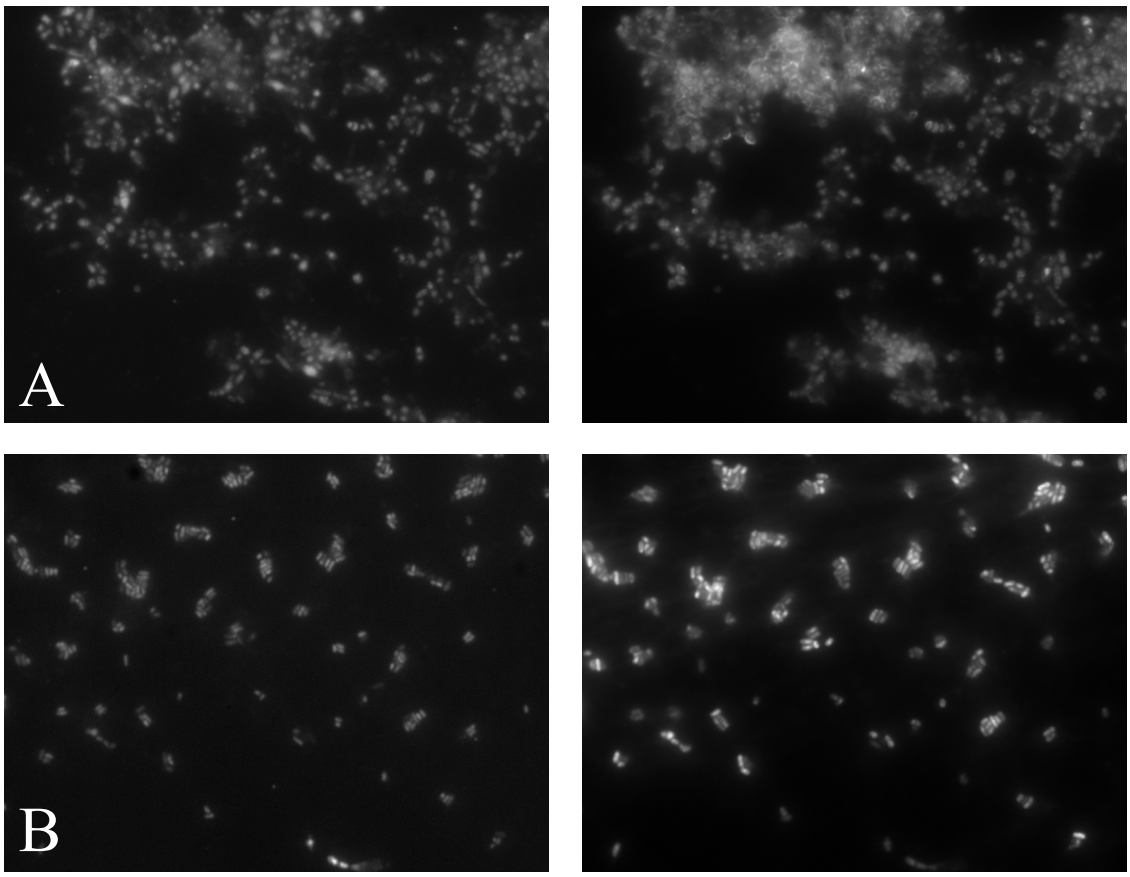

Figure 3.3: Epifluorescence micrographs of *Rubrobacter radiotolerans* (A) and *Conexibacter woesi* (B) after hybridization with the probe Rubro198 specific to the whole *Rubrobacteridae* subclass (left) and after SYBR Green II staining (right).

3.3.3 Environmental application

Fluorescence *in situ* hybridization was performed in a first step with paraformaldehyde-fixed rhizosphere soil samples to quantify some major phylogenetic groups within the kingdom *Bacteria*. Fixed rhizosphere samples were first analysed by SYBR-Green II staining and *in situ* hybridization with the Cy3-labeled bacterial probes EUB338 and EUB338-II (Amann *et al.*, 1990a, Daims *et al.*, 1999). We included EUB338-II, since it has been shown that different bacterial groups including many environmental 16S rRNA sequences contain at least one mismatch in the target site and are not detected by EUB338 (Daims *et al.*, 1999). Sequences with three mismatches in the target region of EUB338 as found in the *Planctomycetales*-genera *Planctomyces*, *Pirellula* and *Gemmata,* can be detected with EUB338-II. Application of EUB338-II under low stringency conditions (35% formamide in the hybridization buffer instead of 70%) allows to detect also 16S rRNA-sequences of organisms containing a single

mismatch at position 353 in the target sequence of EUB338-II, as found in most *Verrucomicrobia* (Daims *et al.*, 1999). Only about 20% of the total SYBR Green II-stained cells (137×10^8 g dw^{-1}) could be detected using the bacterial probes EUB338 and EUB338-II (Table 3.3).

Selected major phylogenetic groups were quantified with different group-specific 16S and 23S rRNA-targeting oligonucleotide probes. Based on the actual databases, it was possible to calculate the relative number of cells as a percentage of EUB338-detected cells as most of the cells detected with the specific probes have also a target region for the EUB338 probe. Only approximately 31% of the cells detected with the bacterial probes could be assigned to some major phylogenetic groups, i.e. the α, β and γ subdivisions of the division *Proteobacteria* and the *Acidobacterium* division (Table 3.3). Members of the order *Planctomycetales* accounted for 1% of the total SYBR Green-detected cells after hybridization with Pla46/Pla886 (Neef *et al.*, 1998), which is in the same range as the numbers obtained after hybridization with EUB338-II (detecting most of the planctomycetes and the verrucomicrobia).

Table 3.3: Relative cell numbers detected by FISH with Cy3-labeled probes as [1] percentage of total cell numbers, or as [2] percentage of EUB338/EUB338-II detectable cells.

Probe	Target group	Percentage	
EUB338	Bacteria	19±8	[1]
EUB338-II	*Planctomycetes* + *Verrucomicrobia*	1±1	
PLA886, PLA46	*Planctomycetes*	1±1	
ALF968	α-*Proteobacteria*	13±7	[2]
BET42a	β-*Proteobacteria*	5±4	
GAM42a	γ-*Proteobacteria*	5±4	
IRog1, IRog2	*Acidobacteria subdivision 6*	4±4	
Acido228	*Acidobacteria subdivision 1*	4±5	

The oligonucleotide probes Irog1 and Irog2 used in this study are specific for environmental sequences affiliated to cluster a of the *Acidobacterium* division as defined by Ludwig *et al.* (1997). Bacteria hybridizing with Acido228 corresponded to 4% of the EUB338/EUB338-II detectable cells, thus resulted in a duplication of *Acidobacterium*-cells in the rhizosphere soil

samples. In the ethanol-fixed and lysozyme-treated samples, 10^8 bacteria per g soil (dry weight) hybridized with Rubro198 (Figure 3.4) which corresponded to 3% of the total Sybr-Green-II-stained cells (36 x 10^8 g dw^{-1}) and to 15% of the EUB338/EUB338-II detectable cells (69 x 10^7 g dw^{-1}).

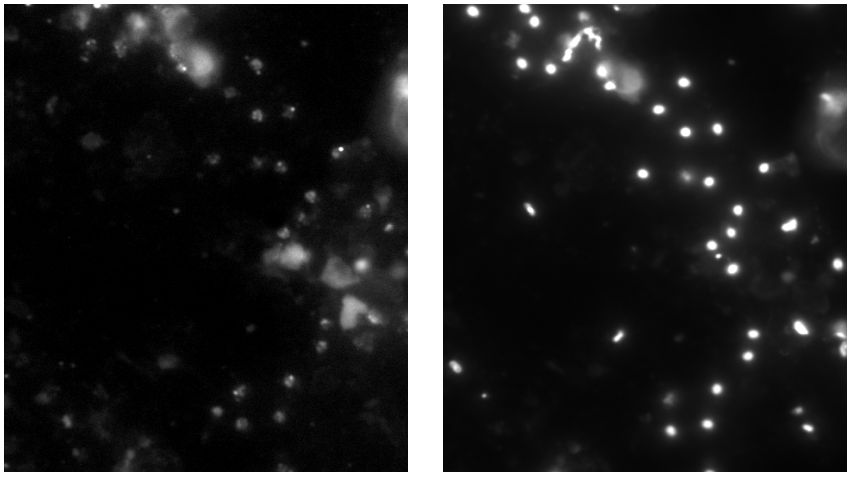

Figure 3.4: Epifluorescent micrographs of ethanol-fixed rhizosphere heavy-metal contaminated soil samples hybridized with the probe Rubro198 (left) and corresponding SYBR-Green-II-stained cells (right).

3.4 Discussion

We used fluorescence in situ hybridization (FISH) to enumerate different major bacterial groups in the rhizosphere of *Thlaspi caerulescens* grown in a heavy metal-contaminated soil. Furthermore, we were interested to compare the relative abundance of sequence types within the rhizosphere clone libraries (as determined in chapter 2) to the abundance of the corresponding bacterial groups as detected *in situ* with FISH. The initial *in situ* probing with the bacterial probes EUB338 and EUB338-II was performed to evaluate the general detectability of the microbial community in this heavy metal-contaminated rhizosphere. Only 20% of the SYBR Green II-stained cells were detected in paraformaldehyde-fixed samples with the bacterial probes. This number is lower than the values obtained in earlier studies in pristine (Zarda *et al.*, 1997; Chatzinotas *et al.*, 1998) but also contaminated soils (Sandaa *et al.*, 1999b; Nogales *et al.*, 2001). The failure to detect a higher amount of cells with the probes EUB338 and EUB338-II might be due to low probe-conferred signals and restricted permeability of cells (Hahn & Zeyer, 1994; Fischer *et al.*, 1995). Low signal intensity could be explained by low

levels of target rRNA in the cells, which may reflect reduced general metabolic activity in this perturbed systems. However, it has been shown that a clear correlation between low metabolic activities and low signal intensity per cell is not always given (Wagner *et al.*, 1995; Oda *et al.*, 2000). Although we could assume that a huge proportion of the FISH-undetectable, SYBR Green II-stained cells are dormant cells (Roszak & Colwell, 1987), any interpretation about the metabolic status of the bacterial cells in this heavy metal-polluted soil based only on probe-conferred signals is speculation.

Only a minor part of the cells detected by EUB338/EUB338-II could be affiliated to large phylogenetic groups. Apparently, the group-specific probes usually applied in FISH studies did not cover a major part of the microbial diversity in this soil. This is in contrast to e.g. activated sludge (Snaidr *et al.*, 1997) or bioreactors (Stoffels *et al.*, 1998), where these probes detect much higher fractions of the total community. Although we did not test all available probes covering large phylogenetic groups, such as the δ-*Proteobacteria*, the *Cytophaga-Bacteroides-Flexibacter* group or the gram positive bacteria with low and high G+C DNA content, we believe that there is an obvious need of designing new specific probes, in particular for terrestrial environments. If we take into account the vast number of publications on comparative analysis of rRNA sequences from soil systems, it appears more than surprising that only a few research groups develop and apply new oligonucleotide probes for soil microbial diversity studies (Stoffels *et al.*, 2001; Dedysh *et al.*, 2001).

In order to specifically detect and quantify organisms from which part of our sequences derived, we designed two novel probes detecting most members of subdivision 1 of the *Acidobacterium* division and most of the subclass *Rubrobacteria*. The reasoning for the selection of these two target groups is as follows: 16S rDNA sequences affiliating to the *Acidobacterium* division are commonly found in clone libraries of environmental DNA from terrestrial systems. Up to know only one division-specific probe (Juretschko *et al.*, 2002) and the two probes specific for subdivision 6 (Ludwig *et al.*, 1997) used in this study have been published. Taking into account the considerable phylogenetic depth of this division, we intended to specifically quantify subdivision 1 that was well represented in our clone library. The second target group, the *Rubrobacteria*, dominated the rRNA-based clone libraries (see chapter 2) in terms of sequence numbers. This group has only recently been observed in culture-independent diversity studies (Rheims *et al.*, 1996; McCaig *et al.*, 1999; Holmes *et al.*, 2000;

Moffett *et al.*, 2003). Since abundance of sequence types in clone libraries is expected to be biased, only FISH can offer some quantitative information regarding this particular group.

Fuchs *et al.* (1998) recently published the distribution of relative fluorescence intensities of oligonucleotide probes on a 16S rRNA secondary structure model. Although this systematic study facilitates the successful design of oligonucleotide probes targeting accessible sites on the 16S rRNA, every novel probe should also be tested with a reference organism. This inevitably poses a problem for many environmental systems, since many of the bacterial groups found are not represented by any cultured organism. Probe Acido228 covered (at the time of its design) the only cultured organism within the *Acidobacterium* subdivision 1, and thus allowed to evaluate the specificity of this probe and the accessibility of the respective 16S rRNA target site. Hybridization of PFA-fixed rhizosphere samples with Acido228 confirmed the presence of sequences clustering within the subdivision 1 of the *Acidobacterium* division, yet numbers detected were only a little bit higher than the detection limit (0.5-1% of all SYBR Green II-stained cells in soil).

The specificity of probe Rubro198 could be checked only for subgroups 1 and 2 as no member of subgroup 3 has been isolated yet. However, hybridization with probe Rubro198 still requires some improvement, as the signal intensity obtained with cultures of *Rubrobacter radiotolerans* and *Conexibacter woesi* was relatively weak. Some possible reasons are mentioned here: The three-dimensional structure of the ribosome may hinder the access of oligonucleotide probes to their target site, resulting in low fluorescence intensities (Fuchs *et al.*, 1998). Unlabeled oligonucleotides (helpers) may improve the application of FISH with this probe, since they may increase the accessibility of the target region by opening the structure of the rRNA (Fuchs *et al.*, 2000). Moreover, the cell walls of Gram-positive bacteria hinder in many cases the probe to enter the cell, in particular if paraformaldehyde fixation is performed. Lysozyme treatment of ethanol-fixed cells clearly improved permeabilisation of *Rubrobacteria*, however it also significantly decreased total cell numbers. We assume that a prolonged storage time in combination with lysozyme treatment were responsible for the decreased total cell counts since ethanol-fixed samples were analysed only after several months of storage at -20°C. Obviously, more work is needed to find an appropriate protocol for applying probe Rubro198 to environmental samples. A promising alternative to increase the sensitivity of FISH is the use of enzyme-labelled probes which have been shown to improve in particular the detection of *Actinobacteria* (Sekar *et al.*, 2003; Chatzinotas *et al.*, 1998). Despite the low signal intensity

observed with pure cultures, 3% of all SYBR Green II-stained cells were detected with the probe Rubro198 in ethanol-fixed samples, indicating that this group of organisms is indeed abundant in this heavy metal-polluted soil.

To conclude, the two, novel oligonucleotide probes Acido228 and Rubro198 allowed the detection and enumeration of selected bacterial groups represented in our clone libraries. However, the sensitivity of FISH for this heavy metal-polluted soil has to be substantially improved. Although FISH is supposed to be used as a quantitative approach, the obtained numbers have to be interpreted with care. As long as general detectability remains as low as in our study, a reasonable quantification of bacterial groups and eventually a comparison with abundance estimated from clone library studies is not possible. One should also not overlook an often-encountered difficulty during the design of specific oligonucleotide probes: Only few complete sequences are available for these emerging groups, consequently, the search of signature sequences is limited to only two or three variable regions in the 16S rRNA molecule. Regarding our probes (and all published probes), this means that there is a constant need for probe re-evaluation, as a probe designed now might not be specific any more in the future or might not cover all organisms intended to be targeted (Amann *et al.*, 2001). The functions of the yet unculturable bacteria in the rhizosphere of heavy metal-hyperaccumulating plants still remain unclear. Their investigation requires combinations of molecular techniques with other approaches, such as novel isolation (Zengler *et al.*, 2002) or isotope techniques (Radajewski *et al.*, 2000). We believe that the development of appropriate cultivation methods will benefit from whole-cell hybridization techniques using novel probes, which will allow to track the microorganisms in the environment and during enrichment procedures.

CHAPTER 4

Impacts of heavy metal contamination and phytoremediation on the microbial community during a twelve months microcosm experiment

FABIENNE GREMION, ANTONIS CHATZINOTAS, KARIN KAUFMANN, WILLIAM VON SIGLER AND HAUKE HARMS

Extended version of a paper submitted to FEMS Microbiology Ecology

4.1 Abstract

The effects of heavy metals and phytoextraction practices on soil microbial community structure and function were studied during twelve months using a hyperaccumulating plant (*Thlaspi caerulescens*) grown in an artificially contaminated soil. The 16S ribosomal RNA gene of the *Bacteria*, the β-*Proteobacteria* and the ammonia-oxidising bacteria (AOB), a functional group of the β-*Proteobacteria*, was amplified by polymerase chain reaction (PCR) and analysed by denaturing gradient gel electrophoresis (DGGE). Principal component analysis (PCA) of the DGGE data revealed that: (i) the heavy metals addition had the most drastic effects on the three bacterial groups targeted (ii) the presence of the plant induced minor changes which could clearly be observed in the AOB and to a lesser extent in the *Bacteria* pattern (iii) the changes observed during the twelve months experiment in the different DGGE-patterns of the planted contaminated soil did not lead to a recovery of the initial bacterial community present in the non-contaminated soil. The potential function of the microbial community was assessed recording community level physiological profiles (CLPP) and analysing them by PCA. Its

lower capability to degrade the different substrates provided in the BIOLOG microtiter plates, in particular the amino acids, amides and amines, as well as a delay in the average well color development (AWCD) differentiated the cultivable bacterial community from contaminated samples from that of the non-contaminated ones. However, the plant had a positive effect on substrate utilisation as shown by the greater number of substrates used in planted samples (both in contaminated and non-contaminated soil) as compared to unplanted ones. Finally, the measurement of the potential ammonia oxidation indicated that the AOB were completely inhibited in the contaminated soil. The stimulation of the ammonia oxidation by the plant which could clearly be observed in the non-contaminated samples was surpassed by the inhibitory effect of the heavy metals in the contaminated soil. This study emphasises the combined use of culture-independent techniques with conventional methods to investigate the ecology of bacteria in their natural habitats.

4.2 Introduction

Heavy metals contamination of soils originating from agricultural (e.g. fertilizers, sewage sludge) or industrial activities (e.g. metal mining, smelting) is one of the major environmental problems in large parts of the world. The resulting damage is difficult to cure as metals cannot be chemically degraded (Salt *et al.*, 1995). Heavy metals affect all groups of organisms and ecosystem processes, including microbial activities (Babich & Stotzky, 1985; Baath, 1989; Giller *et al.*, 1998). Remediation approaches such as excavation and landfilling, thermal treatment, electroreclamation, and soil capping have been proposed depending on the extension, depth and kind of contamination, but they are all expensive and environmentally destructive (Vangronsveld & Cunningham, 1998). Phytoextraction, the use of plants to extract metals from soil, has been reported to be very efficient for cleaning up superficially-contaminated soils (Robinson *et al.*, 1998; Baker *et al.*, 1994a; Salt *et al.*, 1995; Garbisu & Alkorta, 2001). This alternative remediation technique is promising as it is cheaper and less invasive than traditional methods (Glass, 2000; Mulligan *et al.*, 2001). In particular, the hyperaccumulator plant *Thlaspi caerulescens* is a good candidate for the phytoextraction of Zn and Cd from contaminated soils as it can accumulate more than 1% Zn and 0.1% Cd in its shoots on a dry-weight basis (Baker *et al.*, 1994b; Brown *et al.*, 1994; Reeves & Baker, 2000). The potential of microorganisms to enhance phytoremediation processes and the exact mechanism by which bacteria could enhance

heavy metal accumulation in plants has recently received some attention (De Souza *et al.*, 1999; Whiting *et al.*, 2001a). Despite the great interest to improve the efficiency of metal hyperaccumulating plants, their influence on microorganisms has been rarely investigated (Pawlowska *et al.*, 2000; Delorme *et al.*, 2001; Gremion *et al.*, 2003). Molecular fingerprinting techniques such as PCR in combination with denaturing gradient gel electrophoresis (DGGE) offer new perspectives to study microorganisms in their habitat as they account for as yet uncultured organisms (Muyzer & Smalla, 1998). They are an important complement to conventional methods that require cultivation, or measure bacterial activities.

The aim of this study was to determine the impact of heavy metals and phytoextraction practices on microbial community structure at different levels of resolution and, more specifically, on an exemplary key function of soil (ammonia oxidation). We were particularly interested in ammonia-oxidising bacteria (AOB) as these important catalysts of an essential step in the N cycle are known to be very sensitive to heavy metal pollution. For this purpose, we combined the two complementary fingerprinting techniques denaturing gradient gel electrophoresis and community level physiological profiles with the measurement of the potential ammonia oxidation rates. The resolution of the DGGE-approach was varied by targeting either the total bacterial community or more specific groups.

4.3 Materials and methods

4.3.1 Soil characteristics and sample preparation

The soil was kindly provided by the Swiss Federal Institute for Forest, Snow and Landscape Research (Birmensdorf, Switzerland). It was loamy with 15.1% clay, 49.4% silt and 35.5% sand. pH values and amounts of total and soluble heavy metals are presented in Table 4.1. Heavy metal dust (Zn [755 mg kg^{-1}], Cu [85 mg kg^{-1}], Cd [3 mg kg^{-1}]) was obtained from the air filters of a brass-smelter in Dornach (Switzerland) and mixed (3.7 g dust/kg soil) into one part of the sieved soil (<2 mm). The rest remained non-contaminated. Both soils were wetted to 80% of their water-holding capacity, stored at 10°C in the dark and mixed each once a week during three months. Pot experiments were carried out in flower pots containing 2 kg of soil.

4.3.2 Plant growth conditions

Thlaspi caerulescens seeds have been obtained from a population grown near an ancient Pb/Zn mine in Saint-Laurent-le Minier, Southern France. They were stored at 4°C before use. The

seeds were germinated on non-contaminated gardening soil during 25 days. One seedling of *T. caerulescens* was subsequently transplanted into each of three pots containing contaminated and each of three pots containing non-contaminated soil. The plants were grown for three months in a climate chamber at 20°C during daytime and 16°C during night time with 16 h of light alternating with 8 h of darkness. Three unplanted pots were filled with contaminated soil and three others with non-contaminated soil and also incubated in the climate chamber. The pots were watered with 100 ml deionized water every 4 days.

4.3.3 Heavy metal analysis

Total heavy metal content (extracted with 2M HNO_3) and the soluble heavy metal fraction (extracted with 0.1 M $NaNO_3$) of the contaminated soil were measured according to the Swiss law recommendations (OIS, 1998). Plant shoots were collected, oven-dried at 70°C, weighed and ground in a tungsten Retsch mill (Haan, Germany). They were subsequently digested according to Hammer & Keller (2002) in order to analyze their heavy metal contents. All soil and plant heavy metal concentrations were determined with Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Perkin Elmer Plasma 2000) and all samples were run together with certified reference materials.

4.3.4 Sampling

The sampling was carried out over twelve months. Every 3 months, plants and soil were removed from the pots and each plant was shaken carefully to remove the bulk soil. The soil still adhering to the roots was defined as rhizosphere soil. It was separated from the roots by moderate agitation in 50 ml of sterile 0.9% NaCl solution during 5 min and then centrifuged at 8'000 g for 10 min (Marilley *et al.*, 1998). Samples from the unplanted pots were taken as controls. Subsamples (0.6 g wet weight) were filled in 2-ml cryotubes and stored at –20°C until further use. After the sampling, the pots were refilled with the soil and fresh seedlings were subsequently transplanted into it.

4.3.5 DNA extraction

Total soil DNA was extracted from 0.6 g (wet weight) samples of each of the three rhizosphere and unplanted soil samples from contaminated or non-contaminated pots using the protocol of Griffiths *et al.* (Griffiths *et al.*, 2000b) with some modifications. Briefly, 0.5 ml of CTAB

buffer, 0.5 ml of phenol-chloroform-isoamylalcohol (25:24:1) and 0.75 g 0.1-mm glass beads were added to each sample. The mixture was two times shaken for 45 s in a Fastprep bead-beater (Bio 101) at 4 m/s. Samples were stored on ice during 1 min between each run. The lysed sample was then centrifuged (16'000 x g) for 5 min at 4°C. An equal volume of chloroform-isoamylalcohol (24:1) was added to the aqueous phase for removing the remaining phenol. The supernatant was subsequently incubated 2 h at room temperature with 2 volumes of the precipitation solution (30% polyethylene glycol 6000 in 1.6 M NaCl). DNA was pelleted by centrifugation at 18'000 x g for 10 min at 4°C, washed once with ice cold 70% ethanol, and resuspended in 50 µl TE (pH 7.4). One µl of the DNA solution was used as template for PCR amplification.

4.3.6 PCR amplification

PCR amplification of 16S ribosomal DNA (rDNA) was performed with a PTC-200 thermalcycler (MJ Research). The 50 µl PCR mixture contained 0.2 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 1x PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 4.95 µg/µl BSA, and 1 U of Taq Polymerase (Qiagen). To minimize non-specific annealing of the primers to nontargeted DNA, the Taq Polymerase was always added after an initial denaturing step at 94°C for 5 min. Bacterial 16S rDNA was amplified with the primer combination 341f-GC and 907r according to Muyzer *et al.* (1996) and the following program: 30 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. A semi-nested PCR was used for the amplification of the 16S rDNA of β-*Proteobacteria*. 16S rDNA PCR products from ammonia-oxidising bacteria (AOB) were obtained after nested-PCR. In both cases, only 20 cycles were performed in the first amplification and 1 µl of the first PCR amplification served as template for the second amplification (30 cycles). The β-*Proteobacteria* were initially specifically amplified with the primers 948f and 1492r (Lane, 1991) following the protocol of Gomes *et al.* (2001). Reamplification was performed with the primer pair 984f-GC and 1492r according to Heuer *et al.* (1997). For the AOB, the first amplification was performed with the universal primers 27f and 1492r followed by a second specific amplification using the CTO primers and the PCR conditions according to Kowalchuk *et al.* (1998). Subsequently, the PCR products were quantified in a fluorometer (Turner Designs, TD-700, Witec) using the PicoGreen nucleic acid dye (Molecular Probes, Inc.) (Stark *et al.*, 2000).

4.3.7 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE analysis was performed with the D-Code system (Bio-Rad Laboratories) using the 16 cm x 16 cm x 1 mm plates for *Bacteria* and β-*Proteobacteria* and the 20 cm x 20 cm x 1 mm plates for AOB. Four hundred ng of each PCR product was loaded onto 6% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide ratio [37.5:1]) (*Bacteria* and β-*Proteobacteria*) or onto 8% (wt/vol) polyacrylamide gels (AOB). The gels were poured at a flow rate of 14 ml/ min using a two chamber gradient mixer linked to a peristaltic pump (Ismatec). The gradient of denaturant ranged from 35 to 58% (*Bacteria*), 43 to 58% (β-*Proteobacteria*) or 38 to 50 % (AOB) (Kowalchuk *et al.*, 1997) where a 100% denaturing solution is defined as 7 M urea and 40% formamide. A 5 ml stacking gel (0% denaturant) was added to the top of the denaturing gel. The gels were run in 1x TAE buffer (pH 7.4) at 60°C, for the first 15 min at 30 V, and subsequently for 14 h at 70 V (*Bacteria*, β-*Proteobacteria*) or at 85 V (AOB). The reproducibility of the results and the analysis method was checked by loading on the gels the PCR amplifications of three different extractions per treatment but also triplicates within one extraction. To compare the patterns of all different treatments on a single denaturing gel, only one PCR product amplified from the DNA from one pot was finally loaded on the gel. Afterwards, the gels were stained for 30 min with SYBR Green I nucleic acid gel stain (Molecular Probes) as specified by the manufacturer. The stained gels were immediately photographed on a UV transillumination table with a CCD camera (Syngene, Multigenius Bioimaging System, UK) using a short wavelength filter provided by the manufacturer. Digital images of the gels were further analysed by Quantity One image analysis software version 4.0 (Bio-Rad). A band of DNA was detected if it accounted for greater than 1% of the total lane intensity. The lanes were normalized to contain the same amount of total signal after background subtraction (rolling disc) and further used for statistical comparison by principal component analysis (PCA). We also analyzed the data taking into account only the presence or absence of bands according to McCaig *et al.* (2001).

4.3.8 Community level physiological profile (CLPP)

CLPP was performed in BIOLOG-GN2 plates (Oxoid). Five g of soil (wet weight) of each treatment was mixed with 12.5 ml autoclaved water and 3 g of 3 mm-glass beads. The mixture was shaken at 180 rpm for 30 min at room temperature and subsequently centrifuged at 500 x g for 1 min to remove larger suspended soil particles.

In order to adjust the inoculum cell density in each well, total cell counts were determined. Briefly, 100 µl of the soil suspension was filtered on a polycarbonate filter and subsequently dewatered by sucking increasing ethanol concentrations through the system and finally stained 15 min in the dark with 15 µl of SYBR Green II (5×10^{-3} dilution of the stock) (Weinbauer *et al.*, 1998). The filters were mounted with Citifluor solution AF1 (Citifluor Ltd., London, UK) and the preparations were examined with an Olympus BX-60 microscope equipped for epifluorescence with a high pressure mercury bulb (100 W) and filter set HQ-EGFP (AHF Analysentechnik AG, Germany).

Each of the 96 wells of the BIOLOG-GN2 plates was inoculated with 125 µl of a 1:100 dilution of the initial soil suspension (5×10^6 cells ml^{-1}). BIOLOG-analysis was performed with three replicate plates inoculated with a dilution originating from the same initial soil suspension. Plates were incubated at 20°C in the dark and absorbance was measured at 590 nm every 12 h with a plate reader (Dynex Technologies Inc. MRX II, Chantilly, USA) for 111 h.

For further analysis, the optical density (OD) of the control well was subtracted from the OD of each of the substrate-containing wells in order to obtain blanked absorbance values. The rate of substrate utilization over time was determined by the average well colour development (AWCD) which was calculated as the mean of the blanked absorbance values for all 95 response wells per reading time (Garland, 1996). The area under the resulting curve of each substrate, using the readings between 0 and 111 h for each plate, was calculated with the trapezium rule as described in Hackett & Griffiths (1997) and subsequently used for statistical analysis (PCA). Substrate richness, defined as the number of substrate utilized, was determined as the number of background-corrected absorbance values higher than 0.25 after 111 h incubation (Garland, 1996).

4.3.9 Determination of the potential ammonia-oxidation activity (PAO)

Five g of planted and unplanted soil (each contaminated or non-contaminated) were incubated at 25°C in a test medium for 6 h according to the international standard draft ISO/DIS 15685 (ISO, 1999). Every hour, samples of the soil slurries were taken and one volume of 4 M KCl was added to stop the ammonia oxidation. Nitrite accumulated was subsequently determined by colorimetry. The ammonia oxidation rates were calculated by linear regression of accumulated nitrite over time.

4.3.10 Statistical analysis

All our experiments were carried out in triplicates. Student t-tests were carried out to determine significant differences ($p<0.01$) between planted and unplanted pots at different sampling times. The data obtained by DGGE and CLPP were interpreted by principal component analysis (PCA) using SPSS 10.0.5 for windows applying a covariance matrix and no rotation. With this method, the number of variables (16S rDNA bands and 95 substrates) is reduced to a few numbers of axes (PCs) which explain more variance than randomly regressed variables. The two first PCs were subsequently plotted to visualize the results.

4.4 Results

4.4.1 Effects of Thlaspi caerulescens on heavy metal content and soil properties

At any sampling time, the concentrations of $NaNO_3$-extractable Cd and Zn were significantly lower ($p<0.05$) in planted pots than in unplanted pots (Table 4.1). On the contrary, concentrations of soluble Cu were significantly increased in planted pots until 6 months ($p<0.05$). After 9 and 12 months soluble Cu was also increased in unplanted pots. Analysis of the plants revealed that Cu did not accumulate in the shoots. Regarding the total heavy metal concentration in soil, only Cd was significantly reduced after 12 months ($p<0.05$). The plant was able to take up between 1554-10296 μg Zn and 170-936 μg Cd per kg of soil (Table 4.1). Accordingly, the plant removed up to 7 times more Zn and up to 67 times more Cd than the difference observed between the soluble concentrations of planted and unplanted pots. An increase in available heavy metals and at the same time a decrease in the soil pH was observed in all pots of month 6 and 12 as compared to month 3 and 9. During the whole period of the experiment, the pH in the planted contaminated soil was between 0.24 and 0.30 units higher than the values measured for the unplanted contaminated soil ($p<0.01$). A similar trend was observed in the non-contaminated soil (pH 6.7-6.9 in planted soil; pH 6.5-6.8 in unplanted soil). Total C and N contents were 1.5% and 0.14%, and did not change during the 12 months.

Table 4.1: Mean values of pH and heavy metal concentrations in the contaminated soil and in the shoots of *Thlaspi caerulescens* used in the pot experiment with contaminated soil. Total concentrations in shoots were further used for the calculation of metal uptake.

		Total concentrations in soil (HNO_3-extractable)			Soluble concentrations in soil ($NaNO_3$-extractable)			Total concentrations in shoots			Metal uptake by shoots[a] per kg soil		
		Zn_{tot}	Cd_{tot}	Cu_{tot}	Zn_{sol}	Cd_{sol}	Cu_{sol}	Zn	Cd	Cu	Zn	Cd	Cu
Sampling time	pH_{H2O}	mg kg^{-1}			µg kg^{-1}			mg kg^{-1}			µg kg^{-1}		
Time 0	n.d.[b]	3438±14	10.2±0.7	552±9	4925±180	21±2	371±11						
Unplanted													
3 months	7.21±0.09	n.d.	n.d.	n.d.	5123±535	27±6	669±56						
6 months	6.83±0.06	n.d.	n.d.	n.d.	6663±305	36±4	632±48						
9 months	7.23±0.04	n.d.	n.d.	n.d.	5107±119	32±2	804±195						
12 months	6.90±0.10	3293±101	8.8±0.7	561±13	6750±565	36±3	836±74						
Planted													
3 months	7.52±0.04	n.d.	n.d.	n.d.	3607±85	13±1	985±22	6435±1112	585±77	25±1	10296±1779	936±123	40±1
6 months	7.08±0.05	n.d.	n.d.	n.d.	4500±394	15±2	875±51	6990±177	1153±53	19±2	4544±115	749±34	12±1
9 months	7.47±0.02	n.d.	n.d.	n.d.	3733±253	13±1	989±209	5934±1842	872±439	18±5	3263±1013	480±241	10±3
12 months	7.29±0.06	3385±24	7.2±0.4	548±7	5007±354	16±2	940±51	6216±2027	679±368	18±2	1554±506	170±92	5±1

[a] Plant dry weight was 3.2 g at 3 months, 1.3 g at 6 months, 1.1 g at 9 months, 0.5 g at 12 months. Pots contained 2 kg soil.

[b] not determined

4.4.2 Effects of heavy metals and Thlaspi caerulescens on the microbial communities

a) Potential ammonia-oxidation activity (PAO)

The rates of nitrite production were 335±51 ng g^{-1} dry soil h^{-1} and 190±9 ng g^{-1} dry soil h^{-1} in planted and unplanted non-contaminated soil, respectively. No nitrite was produced in planted or unplanted contaminated soil.

b) Microbial genetic diversity (DGGE)

We performed PCR-amplification using the different primer pairs on DNA-extracts from the four pot experiments (contaminated - non-contaminated, rhizospheric-unplanted soil) sampled at different times (3, 6, 9, 12 months). Amplification products were run on DGGE gels (see Appendix p.73) to analyze the bacterial communities. DGGE-patterns resulting from replicate DNA-extractions obtained from one pot at a given sampling time were highly reproducible, as were those from DNA-extractions from the rhizospheres of different plants. For all sampling times and both treatments (contaminated - non-contaminated), 43 different bands positions were observed for the *Bacteria* pattern (24-31 bands per PCR-product), 32 different bands for the β-*Proteobacteria* (13-22 bands per PCR-product) and 14 different bands for the ammonia-oxidising bacteria (10-13 bands per PCR-product). With each of the primer pairs used, the DGGE-patterns of the contaminated soil clearly differed from the corresponding non-contaminated ones. Within one treatment, the rhizospheric and the unplanted soil patterns from the different sampling times were very similar but could still be differentiated by the presence of weak bands and changes in band intensities. Subsequently, DGGE gels were interpreted using principal component analysis (PCA). For this purpose, the data were transformed in two ways taking into account either the relative intensity or the presence of bands. Using the intensity data, the first two principal components (PC1 and PC2) were sufficient to explain 65.7% of the variance for the *Bacteria*, 71.7% for the β-*Proteobacteria* and 80.3% for the AOB (Figure 4.1). Regardless of the target groups, the first axis (PC1) separated the contaminated from the non-contaminated samples. DGGE-profiles of PCR-products of β-*Proteobacteria* were most stable over the experimental period as shown by tight clustering in PCA plots. In contrast to that, DGGE profiles of the *Bacteria* and the AOB formed more open clusters that, in case of both planted and unplanted contaminated soil samples seemed to drift apart with incubation time. PCA considering only the presence or the absence of bands resulted in similar plots.

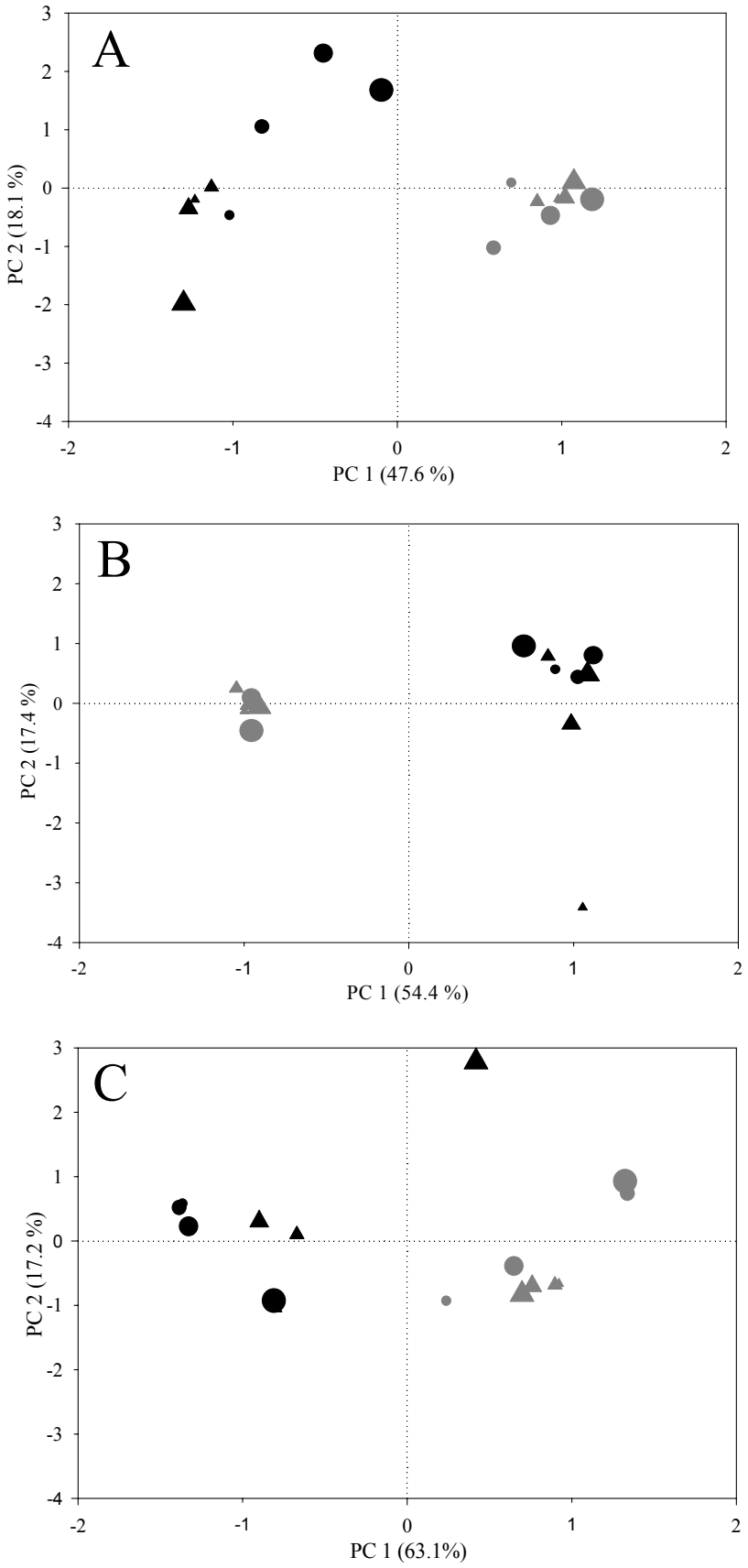

Figure 4.1: Principal component analysis (PCA) on the DGGE data of A) the *Bacteria* B) the β-*Proteobacteria* C) the ammonia-oxidising bacteria 16S rDNA PCR-products. The increase in size of the symbols represents the different sampling points (3, 6, 9 and 12 months). ▲ unplanted contaminated, ● rhizospheric contaminated, △ unplanted non-contaminated, ○ rhizospheric non-contaminated.

c) Potential microbial functional diversity (CLPP)

The potential capacity for substrate utilization of the soil microbial communities was investigated using BIOLOG-GN2 plates providing 95 different substrates. This method clearly differentiated contaminated soil samples from non-contaminated ones (Figure 4.2).

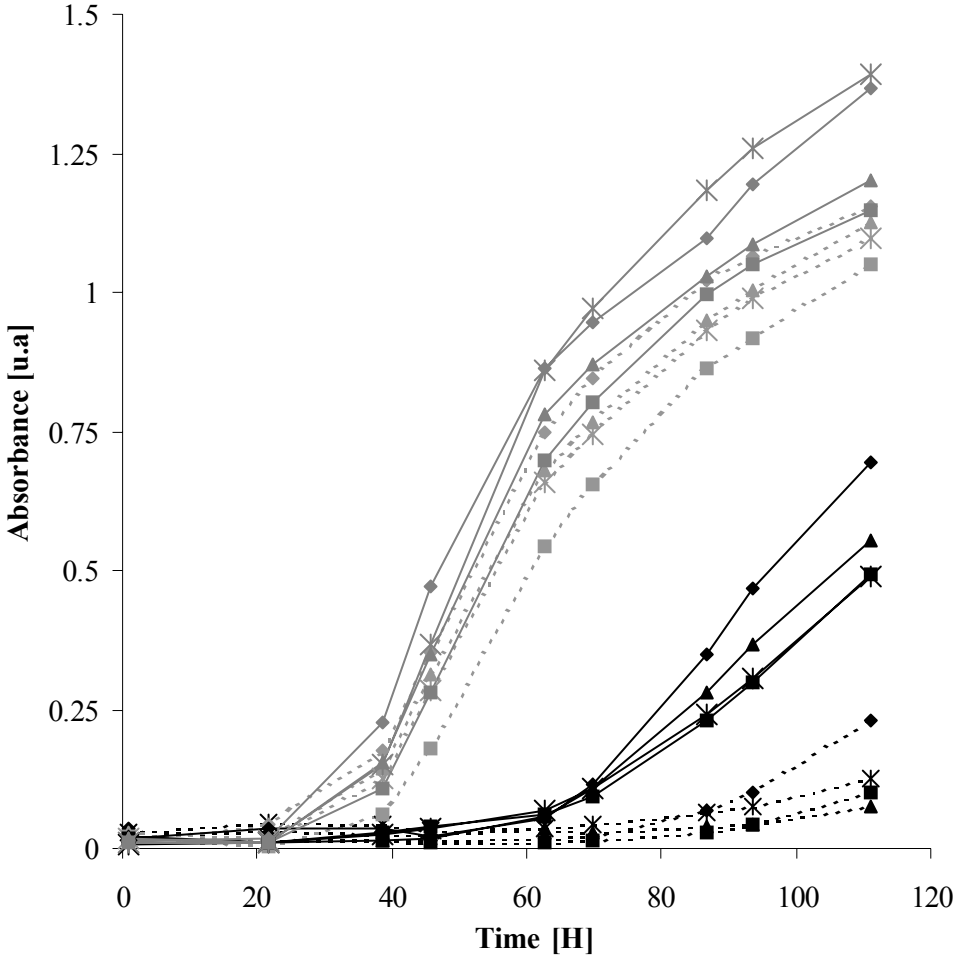

Figure 4.2: Average well colour development (AWCD) of the non-contaminated (grey) and contaminated (black) soil samples during the twelve months experiment. — planted, --- unplanted, ■ 3 months, ▲ 6 months, ✱ 9 months, ◆ 12 months.

Average well colours of plates inoculated with non-contaminated samples increased rapidly after 39 h and reached significantly higher values within 111 h than plates with inocula from contaminated soil samples. The AWCD for both pristine soils was sigmoid, the absorbance measured for the rhizosphere soil being generally higher than that of the unplanted soil. Utilization of substrates in plates with inocula from contaminated soil started only after

approximately 70 h. In particular the utilization rates for the unplanted contaminated samples remained very low. Soil samples were also compared at a fixed incubation time (111 h) with respect to the substrate richness, i.e. the number of substrates metabolized (Table 4.2). BIOLOG plates with the highest AWCD values, i.e. plates inoculated with the two pristine soils, also showed a significantly higher utilization of substrates than plates inoculated with contaminated soil (Table 4.2). Independently of the soil treatment, the number of substrates degraded increased significantly during this one-year experiment in the planted samples ($p<0.01$). As seen in Table 4.2, the effect of *Thlaspi caerulescens* was significantly more pronounced for plates with contaminated soil samples ($p<0.001$).

Table 4.2: Number of substrates metabolised by the microbial communities after 111 hours incubation. Only the mean values (background-corrected, OD>0.25) of each substrate of the three replicate plates for each soil treatment were taken into account.

Sampling time	Non-contaminated soil		Contaminated soil	
	Planted	Unplanted	Planted	Unplanted
3 months	67±4	65±5	39±4	8±3
6 months	72±3	66±3	40±6	6±2
9 months	77±3	65±5	35±2	9±2
12 months	83±4	67±1	57±4	19±4

The areas under the curves, summarizing statistically the substrate utilization during 111 h incubation, were used for principal component analysis (PCA). Using areas instead of endpoint optical density (OD) values allows to take into account different parameters such as the lag phase, the rate of increase and the maximum absorbance obtained during the incubation time (Guckert *et al.*, 1996). PCA discriminated all four soil types. 92% of the variance in the Biolog data was explained by the first two axes (PC1 and PC2) (Figure 4.3). The first principal component had the greatest power of separation as it accounted for 89.3% of the variance. This can be explained by the high number of substrates used by the non-contaminated samples mainly carbohydrates, carboxylic acids, amino acids, amides, and amines. Seventy-two per cent of these substrates were highly correlated to the first axis (Pearson coefficient >0.9; $p<0.05$). The planted samples were separated from the unplanted ones along the second axis.

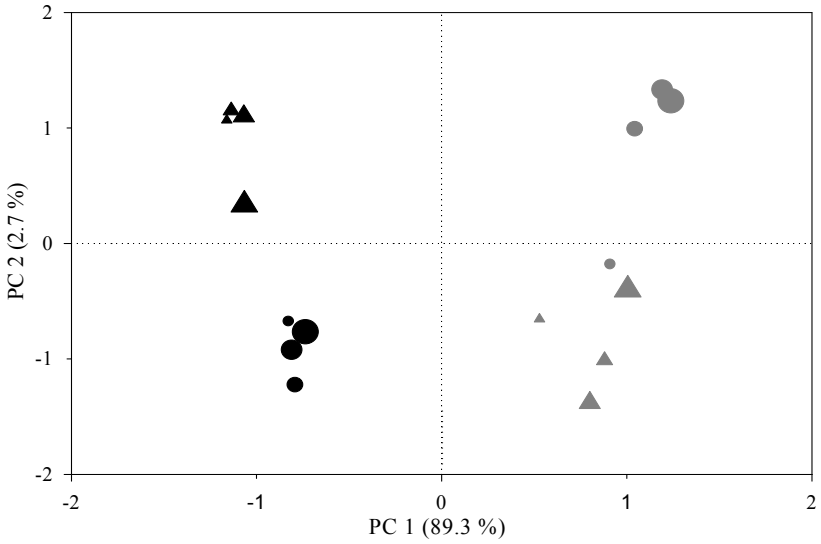

Figure 4.3: Principal component analysis (PCA) on the Biolog data after 111 hours incubation. The increase in size of the symbols represents the different sampling points (3, 6, 9 and 12 months). ▲ unplanted contaminated, ● planted contaminated, ▲ unplanted non-contaminated, ● planted non-contaminated.

4.5 Discussion

In this study, we used an approach combining various complementary methods to determine the effect of heavy metal contamination followed by phytoextraction on indigenous soil microbial populations. During one year pot experiments, four consecutive harvests of *Thlaspi caerulescens* resulted in a decrease of the soluble, bioavailable Zn- and Cd-concentration by 30% and 60%, respectively. The amounts of Zn and Cd in the shoots were higher than the depletion of the soluble heavy metal pool. It appeared that uptake of Cd and Zn from the soluble pool was partly compensated by replenishment with heavy metals from initially less available pools. It is unclear if this is due to passive re-equilibration of the heavy metal pools in soil, or actively driven by the plant, which may have e.g. access to non $NaNO_3$-extractable Zn and Cd fractions (Hammer & Keller, 2002; Knight *et al.*, 1997a; McGrath *et al.*, 1997; Whiting *et al.*, 2001b). Observed variations in the available pool of heavy metals in both planted and unplanted samples during the experiment were concomitant with pH changes of the soil solution. An increase of pH-values is usually considered to be the major factor for a decrease in the concentration of available heavy metals (Hornburg & Brümmer, 1993). However, our data indicate that the driving force for the observed decrease of extractable Zn and Cd is mainly the

heavy metal uptake by the plant.

The impact of the heavy metals could be seen in the results obtained with all the three approaches, i.e. denaturing gradient gel electrophoresis (DGGE), community level physiological profiles (CLPP) and potential ammonia-oxidation activity measurement. These approaches account for different parts of the community. DGGE analysis was done at three levels of resolution using different 16S rDNA-targeting primer pairs. This approach potentially reduces the complexity of DGGE-banding patterns usually observed after PCR-amplification with primers targeting the total bacterial community. In addition, primers specific for selected populations allow the analysis of less abundant groups that may be outcompeted in the PCR reaction using the *Bacterial* primer pair. Nested PCR has been shown to be necessary for the detection of minor but ecological significant populations which make up less than 0.01% of the total bacterial soil community (Phillips *et al.*, 2000). In a few cases, such as for the ammonia-oxidising bacteria (AOB), group-specific primers allow to draw conclusions about processes potentially carried out by the organisms targeted. The monophyletic nature of ammonia oxidisers affiliated to the β-*Proteobacteria* has facilitated the development of specific PCR-primers. AOB are important key-players in the nitrogen cycle since they are responsible for the rate-limiting step in the nitrification process (Kowalchuk & Stephen, 2001). Therefore one can assume that any 16S rDNA sequence amplified with AOB-specific primers from this soil represents most probably not only one organism belonging to a specific phylogenetic group but also one organism with the potential to aerobically oxidise ammonia. Normalised DGGE-profiles were used to perform statistical analysis by PCA based on number of bands or on number and intensity of bands, respectively. It is widely accepted that the number and intensity of bands in a DGGE-profile should be regarded as semi-quantitative information due to different PCR-biases (von Wintzingerode *et al.*, 1997) and to the possibility of similar melting behaviour of 16S rDNA fragments from non-related organisms (Muyzer & Smalla, 1998).

Independently of the phylogenetic group studied, multivariate analysis of the DGGE data clearly revealed consistent differences between contaminated and non-contaminated samples. This is in accordance with other studies which detected shifts in the bacterial community caused by heavy metal contamination using DGGE and other molecular techniques (Müller *et al.*, 2001; Sandaa *et al.*, 1999a ; Griffiths *et al.*, 1997). Therefore, we conclude that the application of group-specific primers does not offer any additional resolution power if the goal was to only distinguish a heavy metal-polluted soil from a pristine soil.

DGGE-analysis also revealed gradual changes of the microbial community structure in the contaminated, unplanted soil during 12 months in contrast to the community structure in the non-contaminated, unplanted soil, which remained quite stable. A possible explanation for the ongoing changes on all three levels of resolution in the contaminated, unplanted soil is that the microbial community was initially disturbed by the addition of the heavy metal dust and that community shifts towards a new steady state were not finished within the adaptation period of three months and the experimental period.

DGGE-profiles of AOB varied not only in the contaminated soils (planted and unplanted) during the 12 months but showed some dynamics also in the non-contaminated, planted soil. Since this tendency was not observed to that extent with DGGE-profiles of the β-*Proteobacteria* and the total bacterial community, one could assume that AOB might be a more sensitive indicator for the impact of the rhizosphere in pristine environments. Kowalchuk *et al.* (2000) have shown that shifts in dominant ammonia-oxidising populations in chalk grassland soils occurred in a background of general stability in the dominant bacterial populations as determined by DGGE using a *Bacterial* primer pair. Our observation corroborates also with the ammonia-oxidation activity measurements. In the non-contaminated soil, the potential activity of the AOB was significantly higher in planted pots than in the unplanted samples. It has been shown that plant roots may stimulate growth of AOB (Klemedtsson *et al.*, 1987; Briones *et al.*, 2003). Assuming that *T. caerulescens* took up preferentially nitrate instead of ammonium, as indicated by an increase in pH (Luo *et al.*, 2000), the AOB would be less in competition with the plant for this substrate.

Although growth of *T. caerulescens* in contaminated soil resulted in changes of the DGGE-profiles of the AOB (and the total bacterial community), the plant did not exert any positive influence on the potential ammonia oxidation activity in the contaminated soil as generally no ammonia oxidation activity was detected in contaminated soil samples, indicating complete inhibition of the AOB. Ammonia oxidation is a microbial process very sensitive to soil pollution (Lee *et al.*, 1997; Pell *et al.*, 1998; Sauve *et al.*, 1999). Although the plant removed a significant part of the soluble Zn and Cd, no recovery of the nitrification activity in the contaminated planted samples was observed. If this is due to the remaining Zn and Cd in the soil solution or the unchanged soluble Cu fraction is not clear. This result, together with the DGGE-profiling, indicates that the AOB populations present in the non-contaminated samples may have been

replaced in the contaminated soil by a heavy metal-resistant but probably ineffective population (Gong *et al.*, 2002).

Community level physiological profiles (CLPP) reflect the potential activity of that fraction of the bacterial community that is able to grow on the substrates provided on Biolog microtiter plates. CLPP is not necessarily related to the functional potential of the most abundant bacteria in soil (Smalla *et al.*, 1998), and is prone to the biases inherent to methods measuring diversity under culture conditions (Preston-Mafham *et al.*, 2002). Yet, it has been shown useful as a rapid technique in providing habitat-specific patterns. One can expect that substrates in the plant rhizosphere were present in lower concentrations than the substrates in the wells. Although the bacteria grown in the Biolog plates were not further analyzed with molecular methods, it is most likely that fast growing r-selected populations which were not necessarily dominant in the inoculum accounted for the colour development in the wells. As already shown in other studies, the potential degradation capabilities of the microbial community were drastically reduced in the heavy metal-contaminated soil (Knight *et al.*, 1997b; Kelly & Tate, 1998; Dobler *et al.*, 2000; Ellis *et al.*, 2001). The rate of colour development was lower and delayed in the contaminated samples as compared to the non-contaminated ones. As the inoculum size was previously adjusted in the wells, the differences in the average well colour development must be due to the heavy metal contamination (Kelly & Tate, 1998). In contrast to the contaminated samples, we observed a positive association of the non-contaminated samples with PC1. The C-sources which were also positively correlated to this axis should consequently be metabolized at a higher rate by the non-contaminated samples (Garland & Mills, 1991). Interestingly, the majority of the amino acids, amines and amides were correlated positively as well to PC1. The lower utilization of these compounds in the contaminated samples indicates that bacterial adaptation to heavy metals is probably maintained at the expense of specific or rare degradative abilities (Reber, 1992; Doelman *et al.*, 1994; Wenderoth & Reber, 1999; Wenderoth *et al.*, 2001). For instance, heavy metal contamination has been shown to delay the degradation of several compounds such as starch, cellulose, glutamic acid and casamino acids (Babich & Stotzky, 1985; Obbard & Jones, 1993).

The higher number of substrates used in the planted samples as compared to the unplanted samples suggests a stimulating effect of the plant, probably due to the root exudates (Heinonsalo *et al.*, 2000). Most interesting, however, is the observation that the phytoextraction practice involving *T. caerulescens* had a positive impact on some potential functional abilities of

rhizosphere microbial communities in the heavy metal-contaminated soil. Kozdrój & van Elsas (2000) showed that artificial root exudates supported the development of bacterial populations in heavy metal-contaminated soils. Campbell *et al.* (1997) suggested to use carbon sources reported as constituents of root exudates as BIOLOG substrates. The authors argued that the carbon sources present in exudates represent a more diverse set of substrates than the carbon sources used in the Biolog GN plates and consequently also select for the slower growing soil organisms. Although the commercially available microtiter plates sufficiently separated the planted from the unplanted samples, we cannot exclude that the use of plates with root exudates might provide some additional information. If we take also into account the observed changes in the DGGE-profiles of the total rhizosphere bacterial community in the contaminated soil, we could speculate that in contrast to the ammonia-oxidation other microbial-mediated processes may be stimulated as well. Since these microorganisms may be important for the recovery of the vegetation in a soil following the reduction of the pollutant, we believe that more studies have to focus on the effect of metal remediation on functional diversity in soils undergoing phytoremediation.

Although the duration of our experiment does not allow to predict the microbial genetic diversity and the potential functional abilities after a longer period of phytoextraction, we can draw the following conclusions: Firstly, exposing soil to heavy metals changed the microbial community structure as measured by DGGE analysis of 16S rDNA fragments representing dominant but also minor populations. Secondly, bacterial functional abilities were heavily affected by the heavy metal contamination. Thirdly, growth of the hyperaccumulator *T. caerulescens* in the contaminated soil did not allow the potential ammonia-oxidising activity in the rhizosphere to recover. Fourthly, phytoextraction with *T. caerulescens* did not only result in some changes in the rhizosphere microbial community structure (i.e. the AOB and the total bacterial community), but fifthly clearly improved the potential functional abilities as compared to the contaminated unplanted soil. Further studies are needed in order to be able to predict if in

particular the functional abilities of soil microbial communities will be fully re-established after the endpoint of phytoremediation has been reached.

4.6 Acknowledgements

We thank Catherine Keller and Daniel Hammer (EPFL) for providing *Thlaspi caerulescens* seeds and many helpful instructions regarding the plant cultivation, Didier Perret, Jean-David Teuscher, Valérie Rochat, Delphine Rota, for the chemical analyses. This study was funded by EPFL-grant 599993.

4.7 Appendix

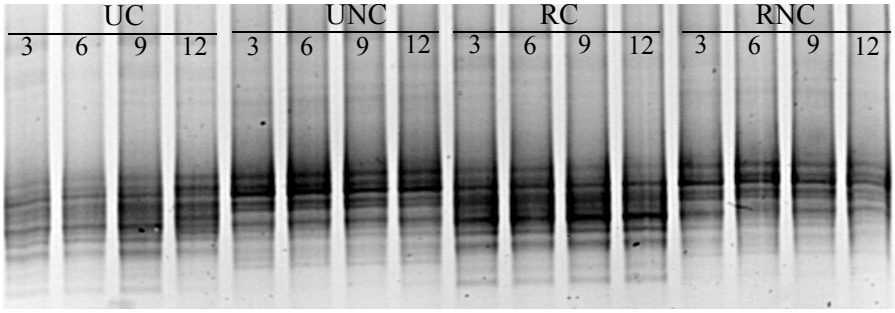

Figure 4.4: DGGE gel of the ammonia-oxidising bacteria 16S rDNA PCR products (gradient 38%-50%). The numbers represent the different sampling points (3, 6, 9, and 12 months). UC: unplanted contaminated, UNC: unplanted non-contaminated, RC: rhizospheric contaminated, RNC: rhizospheric non-contaminated.

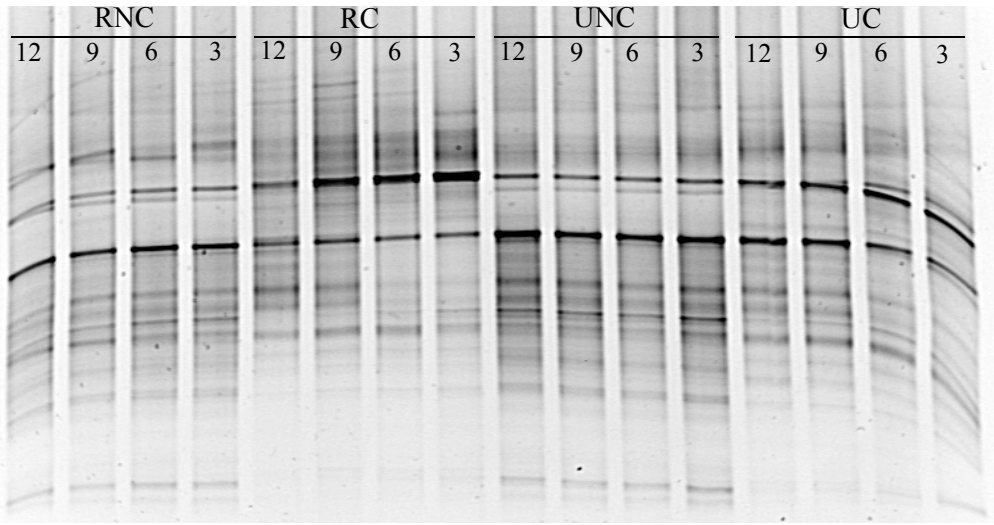

Figure 4.5: DGGE gel of the β-*Proteobacteria* 16S rDNA PCR products (gradient 47%-58%). The numbers represent the different sampling points (3, 6, 9 and 12 months). RNC: rhizospheric non-contaminated, RC: rhizospheric contaminated, UNC: unplanted non-contaminated, UC: unplanted contaminated.

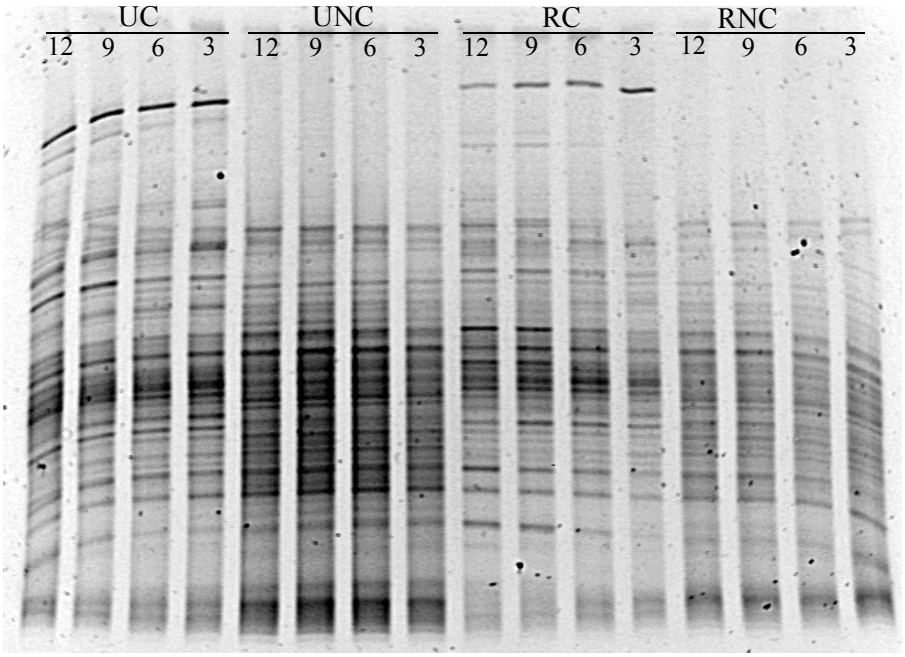

Figure 4.6: DGGE gel of the *Bacteria* 16S rDNA PCR products (gradient 35%-58%). The numbers represent the different sampling points (3, 6, 9 and 12 months). UC: unplanted contaminated, UNC: unplanted non-contaminated, RC: rhizospheric contaminated, RNC: rhizospheric non-contaminated.

CHAPTER 5

General conclusions

The main objective of this study was to characterise the microbial diversity and its function in heavy metal-polluted soils using molecular methods. In order to reduce the number of variables, that normally interact in a real ecosystem, we used a simplified model based on pot experiments including only one plant species and two soils. Although there is much literature on the behaviour of heavy metals in soils and on the phytoremediation process, their impact on bacterial communities has largely been ignored. As bacteria are the key-players of nutrient turnovers, they certainly play an important role in restoring the biological quality of perturbed ecosystems undergoing long-term remediation. This study tried to elucidate the consequences of (i) a stress on the microorganisms due to the presence of heavy metals and (ii) the partial relief of this stress by phytoremediation.

First, we investigated the bacterial diversity in the rhizosphere of the hyperaccumulating plant, *Thlaspi caerulescens*, which grew during three months in a long-term heavy metal-contaminated soil (chapter 2). Four clone libraries were constructed based on the 16S rDNA and rRNA from rhizosphere and bulk soil samples. Although this combined culture-independent approach has the advantage to give information on both the structure and the active part of the microbial community, it has rarely been used (Felske *et al.*, 1997; Miskin *et al.*, 1999; Nogales *et al.*, 2001; Duineveld *et al.*, 2001; Griffiths *et al.*, 2003). We showed that the dominant bacterial groups in the 16S rDNA clone libraries were not the dominant ones in the 16S rRNA clone libraries. Not surprisingly, the major phyla present in the 16S rDNA have often been described in both polluted and non-polluted environments, however, the predominance of

Rubrobacteria in the rRNA clone libraries was unexpected and quite intriguing. As already shown by Moffett *et al.* (2003), it seems that this group is widely represented in heavy metal-contaminated soils. Their predominance in the rRNA clone libraries indirectly suggests that they have a certain level of resistance to heavy metals. According to this observation, we can speculate that they might be of some interest for a potential application in environmental microbiology. For instance, the isolation of the genes responsible for their metal resistance and their transfer into the plant might increase the plant's heavy metal uptake, as it has already been shown with tobacco and *Arabidopsis thaliana* for mercury decontamination (Heaton *et al.*, 1998). Alternatively, these bacteria could be inoculated in the rhizosphere and possibly increase metal availability thus enhancing phytoextraction efficiency or protect plants from metal toxicity through metal immobilisation as it has been observed with other bacteria (Salt *et al.*, 1995; Valls *et al.*, 2000). Nevertheless, as our clone libraries are based on one soil only, the ubiquity and the function of this group have to be investigated in other (polluted) ecosystems. Future work including their tracking and isolation in different environments will certainly contribute to a better understanding of their ecology.

The use of clone libraries to describe the bacterial community structure does not completely account for the real microbial diversity. In fact, nucleic acids extraction, amplification and cloning steps introduce biases (von Wintzingerode *et al.*, 1997). Consequently, one should also try to include quantitative data in order to confirm the results obtained by comparative sequence analysis of clone libraries. To date, only two techniques, fluorescence *in situ* hybridization (FISH) and real-time polymerase chain reaction (RT-PCR) are able to give this kind of information.

We used FISH for the *in situ* quantification of the different groups present in the rhizosphere clone libraries (chapter 3). Although this technique has shown to be very effective in the detection of microorganisms in aquatic systems, it has rarely been used in soil due to its complex texture. By applying the most general probe EUB338 to fixed rhizosphere samples, we were able to detect only 20% of the total rhizosphere microbial community. With this low percentage, it is difficult to conclude with certitude, which are the most dominant bacteria if the remaining 80% are not detected. Despite the low detection rate, we were able to detect the major groups present in the rhizosphere rRNA clone libraries using group-specific oligonucleotide probes. For the same above mentioned reason, however, we cannot draw any conclusion regarding their true abundance in this soil.

According to these results, it seems that the sensitivity of FISH is still the major problem hampering the cell detection in soil. Even though the main limiting factors are known (see Amann *et al.*, 1995 and references therein), most improvements of this technique were applied and evaluated on aquatic environments. The recent development of new methods including the combined use of probes with enzymes or antibodies offers promising perspectives for the cell detection in complex environments (Assmus *et al.*, 1997; Pernthaler *et al.*, 2002). However, it is quite urgent now to make up for the lost time regarding the improvement of this technique for application in soil. As environmental microbiologists, we should not restrict the application of FISH to aquatic systems just because they are easier to work with. Probe design should not be neglected, too. In fact, with the increasing number of sequences in the world databases, our knowledge of the bacterial diversity is changing. Consequently, the specificity of old probes should be re-evaluated and new ones should be designed. The two newly designed probes, i.e. Acido228 targeting subgroup 1 of the *Acidobacterium* division and Rubro198 for most of the *Rubrobacteria*, were insofar important as they allowed for the first time the *in situ* detection and identification of soil microorganisms affiliated to these two groups. These probes contribute not only to the tracking of these new groups but they can also be used for the rapid screening of isolates using dot-blot hybridization.

Beside FISH, a new technique, real-time PCR, has been recently developed for fast and highly sensitive quantification of target DNA (Bustin, 2000, 2002; Mackay *et al.*, 2002). The principle of the method is to measure the increase of fluorescent signal due to PCR product accumulation throughout the amplification reaction. The fluorescent signal is obtained using either from the degradation of a dual-labeled fluorogenic probe (Taqman technology or others (Bustin, 2000; Mackay *et al.*, 2002)) or the SYBR Green dye which binds to double stranded DNA (Heid *et al.*, 1996; Stubner, 2002). It has already been applied successfully for quantification of bacteria in various environments (Becker *et al.*, 2000; Grüntzig *et al.*, 2001; Hermansson & Lindgren, 2001; Stults *et al.*, 2001). However, different factors such as a limited probe or primer specificity, differing standart line performance, or the presence of nonspecific DNA in the PCR reaction may lead to a wrong estimation of the target DNA (Becker *et al.*, 2000;Bellete *et al.*, 2003).

Secondly, we were interested in studying the impact of heavy metal contamination and a subsequent one-year phytoextraction with *Thlaspi caerulescens* on the soil microbial community (chapter 4). We hypothesized that the plant, by hyperaccumulating bioavailable

zinc and cadmium, could indirectly protect bacteria from the toxicity of heavy metals and so favor their growth. Another interesting question was if it was possible to reestablish the initial bacterial community present in the pristine soil or at least to "shift" the bacterial community structure towards its original state. For this purpose, we performed a microcosm experiment with the four combinations of polluted and non-polluted soil and with and without plants using an artificially heavy metal-contaminated soil.

First of all, independently of the technique used, denaturing gradient gel electrophoresis (DGGE), community level physiological profile (CLPP) and potential ammonium-oxidation measurement, they all showed that the heavy metal addition induced drastic changes in the bacterial community structure. By analysing the DGGE patterns obtained during this one-year experiment, we could not observe any recovery of the initial community in the planted pots. One explanation may be the remaining high concentrations of Zn and Cu in the soil. In fact, the total concentration of Zn was too high to be significantly decreased by the plant in this period and Cu was not hyperaccumulated by *Thlaspi*. The addition of only Zn or Cd, respectively, or an equal mix of both metals in our microcosm experiment may have led to different results and should be considered for future experiments. This points out a major obstacle for phytoextraction: the presence of multiple heavy metals. Too high concentrations of Zn render the decontamination of a soil impossible even by hyperaccumulators and Cu hyperaccumulation is to date unknown for plants found in the temperate zone. However, one has to keep in mind that pollution with one single heavy metal rarely occurs. Consequently, the use of simplified microcosm experiment can only provide the first information needed for refined setups designed to obtain ecologically more relevant information.

Secondly, gradual changes in the bacterial unplanted contaminated DGGE profiles during these twelve months experiment were in contrast to the stable community profiles of the unplanted non-contaminated samples. We believe that this is due to another limitation of microcosm experiments, the artificial addition of heavy metals. The bacterial changes clearly indicated that the system has not yet reached an equilibrium, although it was allowed to adapt to the contaminants for 3 months before the start of the experiment. However, the advantage of artificial contamination is that it allows to use non-contaminated control samples, which is in most cases impossible when real long-term contaminated soil is used. Consequently, we can expect that the general trends seen in these microcosms studies were due to an isolated short-term stress obtained by excluding many of the interactions occuring in a real ecosystem.

As recommended by several authors, we also used group-specific primers for increasing the resolution power of DGGE. However, the relatively few numbers of group-specific primers available limit this approach. As already stated above for the FISH-probes, there is also an urgent need to design group-specific PCR-primers. Our approach focused on two aspects: We increased twice the phylogenetic resolution by targeting smaller phylogenetic entities within an already detected larger phylogenetic group, i.e. the β-*Proteobacteria* within the *Bacteria* and subsequently the ammonia-oxidising bacteria within the β-*Proteobacteria*. At the same time, the ammonia-oxidising bacteria represented not only a monophyletic group within the β-*Proteobacteria* , but also a well-defined, ecologically important metabolic group of bacteria within the metabolically diverse β-*Proteobacteria*. We saw that targeting the β-*Proteobacteria* and the ammonia-oxidising bacteria for separating bacterial populations in pristine and polluted soils was as good as targeting the bacterial community completely. On the one hand, one possible conclusion might be that the additional use of group specific DGGE-screening is not necessary to distinguish bacterial populations from pristine and heavy metal-polluted soils. On the other hand, we believe that it might be necessary to monitor specific groups which represent only a minor part of the whole bacterial community. These groups should perferably be responsible for biochemical cycles in soil since a well-defined metabolic group may better serve as an indicator for soil health than a metabolically diverse phylogenetic group. In this regard, it seems useful to include functional genes as genetic markers for monitoring environmental impacts on soil bacteria.

Our data also suggest that methods like the potential ammonium-oxidation assay or the CLPP are probably more powerful than DGGE to reveal a stimulating effect of the plant on the microbial community in particular in the polluted soil. In fact, the measurement of the potential ammonium-oxidation showed clearly that the activity of the AOB was higher in the planted non-contaminated samples than in the unplanted samples. However, this positive effect of the plant was not sufficient to compensate for the deleterious effects of the heavy metals in the contaminated soil. Although AOB are still present in the polluted soils (as shown by the DGGE data), the absence of ammonia-oxidising activity is an indication that this function was at least temporary lost. This observation has an important ecological implication: the persistent stress induced by the heavy metal addition affected the resilience of the ecosystem as this loss of function was not recovered at the end of the time experiment (Griffiths *et al.*, 2000a). The vulnerability of a process depends on the number of microorganisms able to catalyze it when

the system is perturbed (Holling *et al.*, 1995). If this function can be ensured by several species, i.e. there is a redundancy in function within the soil microbial populations, the loss of one of these species will not have much effect on the functioning of the whole community as this function can be fulfiled by another organism (Gitay *et al.*, 1996). Logically, the redundancy in function is directly depending on species richness (Naeem & Li, 1997) and the diversity may be seen as a reservoir of compensating species which will determine the stability and the plasticity of a perturbed system (Folke *et al.*, 1996). As the first step of the nitrification is quite specific to the AOB, the inhibition of these microorganisms led to a loss of function in the ecosystem. Consequently, the AOB may be seen as keystone organisms (Folke *et al.*, 1996) as they play an exceptionnally important role for the ecosystem functioning.

The use of the community level physiological profile (CLPP) is an easy-to-use method, which provides quickly information on the potential degradation capabilities of the bacterial fraction able to grow on the Biolog microtiter plates. A delay in the average well color development (AWCD) and a reduced number of metabolised substrates characterised the contaminated samples. However, the presence of the plant significantly increased the number of metabolised substrates in the contaminated samples, indicating a positive impact of the phytoextraction practice on the microbial communities. These results confirmed our hypothesis indicating that the plant contribute in the development of bacteria by providing them a favorable niche either by excreting root exudates or by decreasing an environmental stress, i.e. the bioavailable heavy metal content. From an ecological point of view, we can see a recovery of the system, as the bacterial community got back some of its degradative capabilities during the phytoremediation process.

This study was a first step to link the effects of heavy metal contamination and a subsequent phytoremediation on the structure and the functions of the microbial communities. It raised several questions, which offer new perspectives of investigation:

Which role do the *Rubrobacteria* play in heavy metal-contaminated soils?
What will be the composition of the microbial community in two, three years if the phytoremediation process is maintained?
Will the initial bacterial community recover?
Is it at all necessary to recover the initial bacterial community in order to maintain the function of a remediated soil?

Are there indicator species that can be used to predict the endpoint of the phytoremediation process?
Is it possible to increase the phytoextraction efficiency by inoculating indigenous heavy metal-resistant bacteria?

Today, the interest for the microorganisms is increasing as we have understood that they make important contributions to ecosystem functioning. It has even been suggested to take into consideration the effects of heavy metals on soil microorganisms in legislation for soil protection (McGrath *et al.*, 1995). However, until now it remains an open and topical debate in Europe, with very different stances taken between member states of the European Union (Giller *et al.*, 1999). Since it appears that we are presently unable to completely prevent heavy metal contamination, it is necessary to pursue our efforts to diminish its deleterious effects, e.g. by employing bacterial activities. A better understanding of the microorganisms, their behaviour in contaminated environments and their interaction with higher organisms, such as plants, will probably help future generations to deal with the heavy metal problem.

REFERENCES

Adriano, D.C. (2001). Trace elements in terrestrial environments; Biochemistry, bioavailability and risks of metals. Springer-Verlag, New York.

Alloway, B.J. (1995). Soil processes and the behaviour of heavy metals. pp. 11-37. *In:* Heavy metals in soils. Alloway, B. (Eds). Chapman & Hall, New York.

Alström, S. and Burns, R.G. (1989). Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. *Biology and Fertility of Soil*, **7**: 232-238.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990a). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, **56**: 1919-1925.

Amann, R.I., Fuchs, B.M. and Behrens, S. (2001). The identification of microorganisms by fluorescence in situ hybridisation. *Current Opinion In Biotechnology*, **12**: 231-6.

Amann, R.I., Krumholz, L. and Stahl, D.A. (1990b). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, **172**: 762-770.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**: 143-169.

Assmus, B., Schloter, M., Kirchhof, G. and Hartmann, A. (1997). Improved in situ tracking of rhizosphere bacteria using dual staining with fluorescence-labeled antibodies and rRNA-targeted oligonucleotides. *Microbial Ecology*, **33**: 32-40.

Axelrood, P.E., Chow, M.L., Radomski, C.C., McDermott, J.M. and Davies, J. (2002). Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Canadian Journal of Microbiology*, **48**: 655-74.

Baath, E. (1989). Effects of heavy metals in soil microbial processes and populations (a review). *Water, Air, and Soil Pollution*, **47**: 335-379.

Babich, H. and Stotzky, G. (1985). Heavy metal toxicity to microbe-mediated ecologic processes: A review and potential application to regulatory policies. *Environmental Research*, **36**: 111-137.

Baker, A.J.M. (1981). Accumulators and excluders - Strategies in the response of plants to heavy metals. *Journal of Plant Nutrition*, **3**: 643-654.

Baker, A.J.M., McGrath, S.P., Reeves, R.D. and Smith, J.A.C. (2000). Metal hyperaccumulator plants: a review of the ecology and physiology of biochemical resource for phytoremediation of metal-polluted soils. pp. 85-107. *In:* Phytoremediation of contaminated soil and water. Terry, N., Bañuelos, G. and Vangronsveld, J. (Eds). Lewis publishers, Boca Raton, FL, USA.

Baker, A.J.M., McGrath, S.P., Sidoli, C.D.M. and Reeves, R.D. (1994a). The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. *Resources, Conservation and Recycling*, **11**: 41-49.

Baker, A.J.M., Reeves, R.D. and Hajar, A.S.M. (1994b). Heavy metal accumulation and tolerance in british populations of the metallophyte *Thlaspi caerulescens* J.&C. Presl (Brassicaceae). *New Phytologist*, **127**: 61-63.

Baker, A.J.M. and Walker, P.L. (1989). Ecophysiology of metal uptake by tolerant plants. pp. 155-177. *In:* Heavy metal tolerance in plants - Evolutionary aspects. Shaw, A. (Eds). CRC Press

Barns, S.M., Takala, S.L. and Kuske, C.R. (1999). Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Applied and Environmental Microbiology*, **65**: 1731-1737.

Baudoin, E., Benizri, E. and Guckert, A. (2002). Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. *Applied Soil Ecology*, **19**: 135-145.

Becker, S., Boger, P., Oehlmann, R. and Ernst, A. (2000). PCR bias in ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. *Applied and Environmental Microbiology*, **66**: 4945-4953.

Begon, M., Harper, J.L. and Townsend, C.R. (1996). Ecology: Individuals, populations and communities. Blackwell Science Ltd.

Belimov, A.A., Safranova, V.I., Sergeyeva, T.A., Egorova, T.N., Matveyeva, V.A. and Tsyganov, V.E. (2001). Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. *Canadian Journal of Microbiology*, **47**: 642-652.

Bellete, B., Flori, P., Hafid, J., Raberin, H. and Tran Manh Sung, R. (2003). Influence of the quantity of nonspecific DNA and repeated freezing and thawing of samples on the quantification of DNA by the Light Cycler. *Journal of Microbiological Methods*, **in press**.

Blaylock, M.J., Salt, D.E., Dushenkov, S., Zakharova, O., Gussman, C., Kapulnik, Y., Ensley, B.D. and Raskin, I. (1997). Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. *Environmental Science & Technology*, **31**: 860-865.

Blencowe, D.K. and Morby, A.P. (2003). Zn(II) metabolism in prokaryotes. *FEMS Microbiology Reviews*, **27**: 291-311.

Borneman, J. and Triplett, E.W. (1997). Molecular microbial diversity in soils from Eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Applied and Environmental Microbiology*, **63**: 2647-2653.

Bourne, D.G., Holmes, A.J., Iversen, N. and Murrell, J.C. (2000). Fluorescent oligonucleotide rDNA probes for specific detection of methane oxidising bacteria. *FEMS Microbiology Ecology*, **31**: 29-38.

Boyd, R.S. (2000). Hyperaccumulation as a plant defensive strategy. pp. 181-201. *In:* Plants that hyperaccumulate heavy metals. Brooks, R.R. (Eds). CAB International, Wallingford, UK.

Brewer, E.P., Saunders, J.A., Angle, J.S., Chaney, R.L. and McIntosh, M.S. (1999). Somatic hybridization between the zinc accumulator *Thlaspi caerulescens* and *Brassica napus*. *Theoretical and Applied Genetics*, **99**: 761-771.

Brierley, C.L. (1990). Bioremediation of metal-contaminated surface and groundwaters. *Geomicrobiology Journal*, **8**: 201-223.

Brim, H., Heuer, H., Krögerrecklenfort, E., Mergeay, M. and Smalla, K. (1999). Characterization of the bacterial community of a zinc-polluted soil. *Canadian Journal of Microbiology*, **45**: 326-338.

Brimecombe, M.J., De Leij, F.A. and Lynch, J.M. (2001). The effects of root exudates on rhizosphere microbial populations. pp. 95-137. *In:* The rhizosphere: Biochemistry and organic

substances at the soil-plant interface. Pinta, R., Varanini, Z. and Nannipieri, P. (Eds). Marcel Dekker Inc., New York.

Briones, A.M., Okabe, S., Umemiya, Y., Ramsing, N.B., Reichardt, W. and Okuyama, H. (2003). Ammonia-oxidizing bacteria on root biofilms and their possible contribution to N use efficiency of different rice cultivars. *Plant and Soil*, **250**: 335-348.

Brooks, R.R. (1998). Geobotany and hyperaccumulators. pp. 55-94. *In:* Plants that hyperaccumulate heavy metals. Brooks, R.R. (Eds). CAB International, Wallingford, UK.

Brooks, R.R., Lee, J., Reeves, R.D. and Jaffre, T. (1977). Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Journal of Geochemical Exploration*, **7**: 49-57.

Brown, S.L., Chaney, R.L., Angle, J.S. and Baker, A.J.M. (1994). Phytoremediation potential of *Thlaspi caerulescens* and Bladder Campion for zinc- and cadmium-contaminated soil. *Journal of Environmental Quality*, **23**: 1151-1157.

Brown, S.L., Chaney, R.L., Angle, J.S. and Baker, A.J.M. (1995). Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* and metal tolerant *Silene vulgaris* grown on sludge-amended soils. *Environmental Science & Technology*, **29**: 1581-1585.

Burd, G.I., Dixon, D.G. and Glick, B.R. (1998). A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. *Applied and Environmental Microbiology*, **64**: 3663-3668.

Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology*, **25**: 169-193.

Bustin, S.A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology*, **29**: 23-39.

Campbell, C.D., Grayston, S.J. and Hirst, D.J. (1997). Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. *Journal of Microbiological Methods*, **30**: 33-41.

Carreto, L., Moore, E., Nobre, M.F., Wait, R., Riley, P.W., Sharp, R.J. and da Costa, M.S., (1996). *Rubrobacter xylanophilus* sp. nov., a new thermophilic species isolated from a thermally polluted effluent. *International Journal of Systematic Bacteriology*, **46**: 460-465.

Cavet, J.S., Borrelly, G.P.M. and Robinson, N.J. (2003). Zn, Cu and Co in cyanobacteria: selective control of metal availability. *FEMS Microbiology Reviews*, **27**: 165-181.

Chatzinotas, A., Sandaa, R.A., Schönhuber, W., Amann, R.I., Daae, F.L., Torsvik, V., Zeyer, J. and Hahn, D. (1998). Analysis of broad-scale differences in microbial communitiy composition of two prisine forest soil. *Systematic and Applied Microbiology*, **21**: 579-587.

Chaudri, A.M., Allain, C.M.G., Barbosa-Jefferson, V.L., Nicholson, F.A., Chambers, B.J. and McGrath, S.P. (2000). A study of the impacts of Zn and Cu on two rhizobial species in soils of a long-term field experiment. *Plant and Soil*, **221**: 167-179.

Connell, J.H. (1978). Diversity in tropical rain forests and coral reefs. *Science*, **199**: 1302-1310.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H. and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*, **22**: 434-444.

De Souza, M.P., Huang, C.P., Chee, N. and Terry, N. (1999). Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetland plants. *Planta*, **209**: 259-263.

Dedysh, S.N., Derakshani, M. and Liesack, W. (2001). Detection and enumeration of methanotrophs in acidic *Sphagnum* peat by 16S rRNA fluorescence in situ hybridization, including the use of newly developed oligonucleotide probes for *Methylocella palustris*. *Applied and Environmental Microbiology*, **67**: 4850-4857.

DeLong, E.F., Wickham, G.S. and Pace, N.R. (1989). Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science*, **243**: 1360-3.

Delorme, T.A., Galiardi, J.V., Angle, J.S. and Chaney, R.L. (2001). Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J.&C. Presl. and the nonmetal accumulator *Trifolium pratense* L. on soil microbial populations. *Canadian Journal of Microbiology*, **47**: 773-776.

Derakshani, M., Lukow, T. and Liesack, W. (2001). Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Applied and Environmental Microbiology*, **67**: 623-631.

Desaules, A. and Dahinden, R. (2000). NABO; réseau national d'observation des sols; variations des teneurs en polluants après 5 et 10 ans de suivi. Cahier de l'environnement n°320, Berne.

Dobler, R., Saner, M. and Bachofen, R. (2000). Population changes of soil microbial communities induced by hydrocarbon and heavy metal contamination. *Bioremediation Journal*, **4**: 41-56.

Doelman, P., Jansen, E., Michels, M. and van Til, M. (1994). Effects of heavy metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. *Biology and Fertility of Soils*, **17**: 177-184.

Dojka, M.A., Hugenholtz, P., Haack, S.K. and Pace, N.R. (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Applied and Environmental Microbiology*, **64**: 3869-3877.

Duineveld, B.M., Kowalchuk, G.A., Keijzer, A., van Elsas, J.D. and van Veen, J.A. (2001). Analysis of bacterial communities in the rhizosphere of *Chrysanthemum* via denaturing gradient gel eletrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Applied and Environmental Microbiology*, **67**: 172-178.

Duxbury, T. (1985). Ecological aspects of heavy metal responses in microorganisms. *Advance in Microbial Ecology*, **6**: 185-235.

Ellis, R.J., Neish, B., Trett, M.W., Best, J.G., Weightman, A.J., Morgan, P. and Fry, J.C. (2001). Comparison of microbial and meiofaunal community analyses for determining impact of heavy metal contamination. *Journal of Microbiological Methods*, **45**: 171-185.

Elsenbeer, M.F., Haltmeyer, C., Kästli, E., Kyburz, E., Tschudin, A. and Wiener, D. (1997). The environment in Switzerland. Federal printing and supplies office (EDMZ), Bern.

Ernst, W.H.O. (1998). The origin and ecology of contaminated, stabilized and non-pristine soils (Chapter 2). pp. 17-29. *In:* Metal-contaminated soil. Vangronsveld, J. and Cunningham, S.D. (Eds). Springer, New York.

Felix, H. (1997). Field trials for in situ decontamination of heavy metal polluted soils using crops of metal-accumulating plants. *Zeitschrift Fur Pflanzenernahrung Und Bodenkunde*, **160**: 525-529.

Felske, A., Akkermans, A.D.L. and De Vos, W.M. (1998). In situ detection of an uncultured predominant *Bacillus* in Dutch Grassland soils. *Applied and Environmental Microbiology*, **64**: 4588-4590.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. and Akkermans, A.D.L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class *Actinobacteria* in grassland soils. *Microbiology*, **143**: 2983-2989.

Ferreira, A.C., Nobre, M.F., Moore, E., Rainey, F.A., Battista, J.R. and da Costa, M.S. (1999). Characterization and radiation resistance of new isolates of *Rubrobacter radiotolerans* and *Rubrobacter xylanophilus*. *Extremophiles*, **3**: 235-8.

Fischer, K., Hahn, D., Honerlage, W., Schonholzer, F. and Zeyer, J. (1995). In-situ detection of spores and vegetative cells of *Bacillus megaterium* in soil by whole-cell hybridization. *Systematic and Applied Microbiology*, **18**: 265-273.

Flathman, P.E. and Lanza, G.R. (1998). Phytoremediation: Current views on an emerging green technology. *Journal of Soil Contamination*, **7**: 415-432.

Folke, C., Holling, C.S. and Perrings, C. (1996). Biological diversity, ecosystems, and the human scale. *Ecological Applications*, **6**: 1018-1024.

Fuchs, B.M., Glockner, F.O., Wulf, J. and Amann, R. (2000). Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Applied and Environmental Microbiology*, **66**: 3603-3607.

Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W. and Amann, R. (1998). Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. *Applied and Environmental Microbiology*, **64**: 4973-4982.

Furlong, M.A., Singleton, D.R., Coleman, D.C. and Whitman, W.B. (2002). Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and aasts of the earthworm *Lumbricus rubellus*. *Applied and Environmental Microbiology*, **68**: 1265-1279.

Garbisu, C. and Alkorta, I. (2001). Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment. *Bioresource Technology*, **77**: 229-236.

Garland, J.L. (1996). Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. *Soil Biology and Biochemistry*, **28**: 213-221.

Garland, J.L. and Mills, A.L. (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology*, **57**: 2351-2359.

Geiger, G., Federer, P. and Sticher, H. (1993). Reclamation of heavy metal-contaminated soils: Field studies and germination experiments. *Journal of Environmental Quality*, **22**: 201-207.

Germida, J.J., Siciliano, S.D., de Freitas, J.R. and Seib, A.M. (1998). Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). *FEMS Microbiology Ecology*, **26**: 43-50.

Gilbert, G.S., Clayton, M.K., Handelsman, J. and Parke, J.L. (1996). Use of cluster and discriminant analyses to compare rhizosphere bacterial communities following biological perturbation. *Microbial Ecology*, **32**: 123-47.

Giller, K.E., Witter, E. and McGrath, S.P. (1998). Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biology and Biochemistry*, **30**: 1389-1414.

Giller, K.E., Witter, E. and McGrath, S.P. (1999). Assessing risks of heavy metal toxicity un agricultural soils: do microbes matter? *Human and Ecological Risk Assessment*, **5**: 683-689.

Gitay, H., Wilson, J.B. and Lee, W.G. (1996). Species redundancy: A redundant concept? *Journal of Ecology*, **84**: 121-124.

Glass, D.J. (2000). Economic potential of phytoremediation. pp. 15-31. *In:* Phytoremediation of toxic metals: Using plants to clean up the environment. Raskin, I. and Ensley, B. (Eds). Wiley John and Sons Inc., New York.

Gomes, N.C.M., Heuer, H., Schönfeld, J., Costa, R., Mendonça-Hagler, L. and Smalla, K. (2001). Bacterial diversity of the rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. *Plant and Soil*, **232**: 167-180.

Gong, P., Siciliano, S.D., Srivastava, S., Greer, C.W. and Sunahara, G.I. (2002). Assessment of pollution-induced microbial community tolerance to heavy metals in soil using ammonia-oxidizing bacteria and Biolog assay. *Human and Ecological Risk Assessment*, **8**: 1067-1081.

Grayston, S.J., Shenquiang, W., Campbell, C.D. and Edwards, A.C. (1998). Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry*, **30**: 369-378.

Gremion, F., Chatzinotas, A. and Harms, H. (2003). Comparative 16S rDNA and 16S rRNA sequence analysis indicates that *Actinobacteria* might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. *Environmental Microbiology*, **5**: 896-907.

Griffiths, B.S., Diaz-Ravina, M., Ritz, K., McNicol, J.W., Ebblewhite, N. and Baath, E. (1997). Community DNA hybridisation and %G+C profiles of microbial communities from heavy metal polluted soils. *Microbial Ecology*, **24**: 103-112.

Griffiths, B.S., Ritz, K., Bardgett, R.D., Cook, R., Christensen, S., Ekelund, F., Sorensen, S.J., Baath, E., Bloem, J., de Ruiter, P.C., Dolfing, J. and Nicolardot, B. (2000a). Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an

examination of the biodiversity-ecosystem function relationship. *Oikos*, **90**: 279-294.

Griffiths, R.I., Whiteley, A.S., O'Donnell , A.G. and Bailey, M.J. (2000b). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology*, **66**: 5488-5491.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G. and Bailey, M.J. (2003). Influence of depth and sampling time on bacterial community structure in an upland grassland soil. *FEMS Microbiology Ecology*, **43**: 35-43.

Grüntzig, V., Nold, S.C., Zhou, J. and Tiedje, J.M. (2001). *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. *Applied and Environmental Microbiology*, **67**: 760-768.

Guckert, J.B., Carr, G.J., Johnson, T.D., Hamm, B.G., Davidson, D.H. and Kumagai, Y. (1996). Community analysis by Biolog: curve integration for statistical analysis of activated sludge microbial habitats. *Journal of Microbiological Methods*, **27**: 183-197.

Hackett, C.A. and Griffiths, B.S., (1997). Statistical analysis of the time-course of Biolog substrate utilization. *Journal of Microbiological Methods*, **30**: 63-69.

Hahn, D. and Zeyer, J. (1994). *In situ* detection of bacteria in the environment. pp. 137-148. *In:* Beyond the biomass. Ritz, K., Digthon, J. and Giller, K. (Eds). John Wiley & Sons, Chichester, UK.

Hall, T.A. (1999). Bioedit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic acids Symposium Series*, **41**: 95-98.

Hammer, D., Kayser, A. and Keller, C. (2003). Phytoextraction of Cd and Zn with *Salix vimitalis* in field trials. *Soil Use and Management*, in press.

Hammer, D. and Keller, C. (2002). Changes in the rhizosphere of metal-accumulating plants evidenced by chemical extractions. *Journal of Environmental Quality*, **31**: 1561-1569.

Hammer, D. and Keller, C. (2003). Phytoextraction of Cd and Zn with *Thlaspi caerulescens* in field trials. *Soil Use and Management*, **19**: 144-149.

Hamon, R.E., Mclaughlin, M.J., Naidu, R. and Correll, R. (1998). Long-term changes in cadmium bioavailability in soil. *Envionmental Science & Technology*, **32**: 3699-3703.

Heaton, A.C.P., Rugh, C.L., Wang, N.J. and Meagher, R.B. (1998). Phytoremediation of mercury- and methylmercury-polluted soils using genetically engineered plants. *Journal of Soil Contamination*, **7**: 497-509.

Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M. (1996). Real time quantitative PCR. *Genome Research*, **6**: 986-994.

Heinonsalo, J., Jorgensen, K.S., Haahtela, K. and Sen, R. (2000). Effects of *Pinus sylvestris* rooth growth and mycorrhizosphere development on bacterial carbon source utilization and hydrocarbon oxidation in forest and petroleum-contaminated soils. *Canadian Journal of Microbiology*, **46**: 451-464.

Hermansson, A. and Lindgren, P.-E. (2001). Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. *Applied and Environmental Microbiology*, **67**: 972-976.

Heuer, H., Kresk, M., Baker, P., Smalla, K. and Wellington, E.M.H. (1997). Analysis of *Actinomycete* communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology*, **63**: 3233-3241.

Hirsch, P.R., Jones, K.C., McGrath, S.P. and Giller, K.E. (1993). Heavy metals from past applications of sewage sludge decrease the genetic diversity of *Rhizobium leguminosarum* biovar *trifolii* populations. *Soil Biology and Biochemistry*, **25**: 1485-1490.

Holling, C.S.G., Schindler, D.W., Walker, B.H. and Roughgarden, J. (1995). Biodiversity in the functioning of ecosystems: an ecological primer and synthesis. pp. 44-83. *In:* Biodiversity loss: ecological and economic issues. Perrings, C., Mäler, K., Folke, C., Holling, C. and Jansson, B. (Eds). Cambridge University Press, Cambridge, England.

Holmes, A.J., Bowyer, J., Holley, M.P., O'Donoghue, M., Montgomery, M. and Gillings, M.R. (2000). Diverse, yet-to-be cultured members of the *Rubrobacter* subdivision of the *Actinobacteria* are widespread in Australian arid soils. *FEMS Microbiology Ecology*, **33**: 111-120.

Hooper, D.U. and Vitousek, P.M. (1997). The effects of plant composition on ecosystem processes. *Science*, **277**: 1302-1305.

Hornburg, V. and Brümmer, G.W. (1993). Behaviour of heavy metals in soil 1. Investigations on heavy metal mobility (In German). *Zeitschrift für Pflanzenernahrung und Bodenkunde*, **156**: 467-477.

Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998a). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*, **180**: 4765-4774.

Hugenholtz, P., Pitulle, C., Hershberger, L. and Pace, N.R. (1998b). Novel division level bacterial diversity in a Yellowstone hot spring. *Journal of Bacteriology*, **180**: 366-376.

Hugenholtz, P., Tyson, G.W., Webb, R.I., Wagner, A.M. and Blackall, L.L. (2001). Investigation of candidate division TM7, a recently recognized major lineage of the domain *Bacteria* with no known pure-culture representatives. *Applied and Environmental Microbiology*, **67**: 411-419.

International Organization for Standardization (ISO) (1999). Ammonium oxidation, a rapid method to test potential nitrification in soil. *ISO/CD 15686*, Geneva, Switzerland.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M. and Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. *Applied and Environmental Microbiology*, **68**: 2391-2396.

Juretschko, S., Loy, A., Lehner, A. and Wagner, M. (2002). The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Systematic and Applied Microbiology*, **25**: 84-99.

Jurgens, G., Lindstrom, K. and Saano, A. (1997). Novel group within the kingdom *Crenarchaeota* from boreal forest soil. *Applied and Environmental Microbiology*, **63**: 803-805.

Kabata-Pendias, A. and Pendias, H. (2001). Trace elements in soils and plants. CRC Press, London.

Kaiser, O., Puhler, A. and Selbitschka, W. (2001). Phylogenetic analysis of microbial diversity in the rhizoplane of oilseed rape (*Brassica napus* cv. Westar) employing cultivation-dependent and cultivation-independent approaches. *Microbial Ecology*, **42**: 136-149.

Kari, F.G. and Giger, W. (1996). Speciation and fate of ethylenediaminetetraacetate (EDTA) in municipal wastewater treatment. *Water Research*, **30**: 122-134.

Kayser, A., Wenger, K., Keller, A., Attinger, W., Felix, H.R., Gupta, S.K. and Schulin, R. (2000). Enhancement of phytoextraction of Zn, Cd, and Cu from calcareous soil: The use of NTA and sulfur amendments. *Environmental Science & Technology*, **34**: 1778-1783.

Kelly, J.J. and Tate, R.L. (1998). Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. *Journal of Environmental Quality*, **27**: 609-617.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**: 111-20.

Klemedtsson, L., Berg, P., Clarholm, M., Schnurer, J. and Rosswall, T. (1987). Microbial Nitrogen Transformations in the Root Environment of Barley. *Soil Biology and Biochemistry*, **19**: 551-558.

Knight, B., Zhao, F.J. and Shen, Z.G. (1997a). Zinc and cadmium uptake by the hyperaccumulator *Thlaspi caerulescens* in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution. *Plant and Soil*, **197**: 71-78.

Knight, B.P., McGrath, S.P. and Chaudri, A.M. (1997b). Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. *Applied and Environmental Microbiology*, **63**: 39-43.

Kowalchuk, G., Stienstra, A., Heilig, G., Stephen, J. and Woldendorp, J. (2000). Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. *Environmental Microbiology*, **2**: 99-110.

Kowalchuk, G.A., Bodelier, P.L.E., Heilig, G.H.J., Stephen, J.R. and Laanbroek, H.J. (1998). Community analysis of ammonia-oxidising bacteria, in relation to oxygen, availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. *FEMS Microbiology Ecology*, **27**: 339-350.

Kowalchuk, G.A. and Stephen, J.R. (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annual Review of Microbiology*, **55**: 485-529.

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M. and Woldendorp, J.W. (1997). Analysis of ammonia-oxidising bacteria of the β-subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel eletrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Applied and Environmental Microbiology*, **63**: 1489-1497.

Kozdrój, J. and van Elsas, J.D. (2000). Response of the bacterial community to root exudates in soil polluted with heavy metal assessed by molecular and cultural approaches. *Soil Biology and Biochemistry*, **32**: 1405-1417.

Kozdrój, J. and van Elsas, J.D. (2001). Structural diversity of microorganims in chemically perturbed soil assessed by molecular and cytochemical approaches. *Journal of Microbiological Methods*, **43**: 197-212.

Kramer, U. and Chardonnens, A.N. (2001). The use of transgenic plants in the bioremediation of soils contaminated with trace elements. *Applied Microbiology and Biotechnology*, **55**: 661-672.

Kunito, T., Oyaizu, H. and Matsumoto, S. (1998). Ecology of soil heavy metal-resistant bacteria and perspective of bioremediation of heavy metal-contaminated soils. *Recent Research Development in Agricultural & Biological Chemistry*, **2**: 185-206.

Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K. and Jackson, P.J. (1998). Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Applied and Environmental Microbiology*, **64**: 2463-2472.

Kuske, C.R., Ticknor, L.O., Miller, M.E., Dunbar, J.M., Davis, J.A., Barns, S.M. and Belnap, J. (2002). Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland. *Applied and Environmental Microbiology*, **68**: 1854-63.

Lane, D.J. (1991). 16S/23S rRNA sequencing. pp. 115-175. *In:* Nucleic acid techniques in bacterial systematics. Stackebrandt, E. and Goodfellow, M. (Eds). John Wiley & Sons, Inc., New York.

Lasat, M.M., Pence, N.S., Garvin, D.F., Ebbs, S.D. and Kochian, L.V. (2000). Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany*, **51**: 71-79.

Lee, S.Y., Bollinger, J., Bedzdicek, D. and Ogram, A. (1996). Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. *Applied and Environmental Microbiology*, **62**: 3787-3793.

Lee, Y.W., Ong, S.K. and Sato, C. (1997). Effects of heavy metals on nitrifying bacteria. *Water and Science Technology*, **36**: 69-74.

Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G., Boeufgras, J.M. and Alabouvette, C. (1995). Effect of two plant species, flax (*linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. *Applied and Environmental Microbiology*, **61**: 1004-1012.

Liesack, W. and Stackebrandt, E. (1992). Occurence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an australian terrestrial environment. *Journal of Bacteriology*, **174**: 5072-5078.

Lloyd, J.R. and Lovley, D.R. (2001). Microbial detoxification of metals and radionuclides. *Current Opinion In Biotechnology*, **12**: 248-53.

Lodewyckx, C., Mergeay, M., Vangronsveld, J., Clijsters, H. and Van der Lelie, D. (2002). Isolation, characterization, and identification of bacteria associated with the zinc hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*. *International Journal of Phytoremediation*, **4**: 101-115.

Lovley, D. and Coates, J.D. (1997). Bioremediation of metal contamination. *Current Opinion In Biotechnology*, **8**: 285-289.

Ludemann, H. and Conrad, R. (2000). Molecular retrieval of large 16S rRNA gene fragments from an Italian rice paddy soil affiliated with the class *Actinobacteria*. *Systematic and Applied Microbiology*, **23**: 582-4.

Ludwig, W., Bauer, S.H., Bauer, M., Held, I., Kirchhof, G., Schulze, R., Huber, I., Spring, S., Hartmann, A. and Schleifer, K.H. (1997). Detection and in situ identification of representatives of a widely distributed new bacterial phylum. *FEMS Microbiology letters*, **153**: 181-190.

Lugtenberg, B.J.J., de Weger, L.A. and Bennett, J.W. (1991). Microbial stimulation of plant growth and protection from disease. *Current Opinion In Biotechnology*, **2**: 457-464.

Luo, Y.M., Christie, P. and Baker, A.J.M. (2000). Soil solution Zn and pH dynamics in non-rhizosphere soil and in the rhizosphere of *Thlaspi caerulescens* grown in a Zn/Cd-contaminated soil. *Chemosphere*, **41**: 161-164.

Lynch, J.M. and Whipps, J.M. (1990). Substrate flow in the rhizosphere. *Plant and Soil*, **129**: 1-10.

Mackay, I.M., Arden, K.E. and Nitsche, A. (2002). Real-time PCR in virology. *Nucleic Acids Research*, **30**: 1292-1305.

Macnaughton, S., Stephen, J., Venosa, A., Davis, G., Chang, Y. and White, D. (1999). Microbial population changes during bioremediation of an experimental oil spill. *Applied and Environmental Microbiology*, **65**: 3566-3574.

Macrae, A., Rimmer, D.L. and O'Donnell, A.G. (2000). Novel bacterial diversity recovered from the rhizosphere of oilseed rape (*Brassica napus*) determined by the analysis fo 16S ribosomal DNA. *Antonie van Leeuwenhoek*, **78**: 13-21.

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J. and Woese, C.R. (1997). The RDP (Ribosomal Database Project). *Nucleic Acids Research*, **25**: 109-11.

Manz, W., Amann, R.I., Ludwig, W., Wagner, M. and Schleifer, K.H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: Problems and solutions. *Systematic and Applied Microbiology*, **15**: 593-600.

Marilley, L., Vogt, G., Blanc, M. and Aragno, M. (1998). Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA. *Plant and Soil*, **198**: 219-224.

McCaig, A.E., Glover, A.L. and Prosser, J.I. (1999). Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. *Applied and Environmental Microbiology*, **65**: 1712-1730.

McCaig, A.E., Glover, A.L. and Prosser, J.I. (2001). Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Applied and Environmental Microbiology*, **67**: 4554-4559.

McGrath, S.P., Chaudri, A.M. and Giller, K.E. (1995). Long-term effects of metals in sewage-sludge on soils, microorganisms and plants. *Journal of Industrial Microbiology*, **14**: 94-104.

McGrath, S.P., Shen, Z.G. and Zhao, F.J. (1997). Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. *Plant and Soil*, **188**: 153-159.

Mclaughlin, M.J., Smolders, E. and Merckx, R. (1998). Soil-root interface: physicochemical processes. pp. 233-277. *In:* Soil chemistry and ecosystem health. Huang, P. (Eds). Soil Science Society of America, Madison, Winsconsin.

Mengel, K. and Kirkby, E.A. (1982). Principles of plant nutrition. International Potash Institute, Bern, Switzerland.

Miller, H.J., Henken, G. and van Veen, J.A. (1989). Variation and composition of bacterial populations in the rhizospheres of maize, wheat, and grass cultivars. *Canadian Journal of Microbiology*, **35**: 656-660.

Miskin, I.P., Farrimond, P. and Head, I.M. (1999). Identification of novel bacterial lineages as active members of microbial populations in a freshwater sediment using a rapid RNA extraction procedure and RT-PCR. *Microbiology-Uk*, **145**: 1977-1987.

Moffett, B.F., Nicholson, F.A., Uwakwe, N.C., Chambers, B.J., Harris, J.A. and Hill, T.C.J. (2003). Zinc contamination decreases the bacterial diversity of agricultural soil. *FEMS Microbiology Ecology*, **43**: 13-19.

Mooney, H.A., Lubchenco, J., Dirzo, R. and Sala, O.E. (1995). Biodiversity and ecosystem functioning: Basic principles. pp. 275-325. *In:* Global biodiversity assessment. Heywood, V. and Watson, R. (Eds). Cambridge Univ. Press

Müller, A.K., Westergaard, K., Christensen, H. and Sorensen, S.J. (2001). The effect of long-term mercury pollution on the soil microbial community. *FEMS: Microbiology Ecology*, **36**: 11-19.

Mulligan, C.N., Yong, R.N. and Gibbs, B.F. (2001). Remediation technologies for metal-contaminated soils and groundwater: an evaluation. *Engineering Geology*, **60**: 193-207.

Muyzer, G., S, H., Teske, A. and Wawer, C. (1996). Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA - a new molecular approach to analyze the genetic diversity of mixed microbial communities. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Muyzer, G. and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, **73**: 127-141.

Naeem, S. and Li, S.B. (1997). Biodiversity enhances ecosystem reliability. *Nature*, **390**: 507-509.

Naeem, S., Thompson, L.J., Lawer, S.P., Lawton, J.H. and Woodfin, R.M. (1994). Declining biodiversity can alter the performance of ecosystems. *Nature*, **368**: 734-737.

Neef, A. (1997). PhD-Thesis, Technische Universität München, Germany.

Neef, A., Amann, R.I., Schlesner, H. and Schleifer, K.H. (1998). Monitoring a widespread bacterial group: in situ detection of *Planctomycetes* with 16S rRNA-targeted probes. *Microbiology*, **144**: 3257-3266.

Nies, D.H. (1995). The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. *Journal of Bacteriology*, **177**: 2707-12.

Nogales, B., Moore, E.R.B., Llobet-Brossa, E., Rossello-Mora, R., Amann, R. and Timmis, K.N. (2001). Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Applied and Environmental Microbiology*, **67**: 1874-1884.

Nomura, M., Gourse, R. and Baughman, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. *Annual Review of Biochemistry*, **53**: 75-117.

Obbard, J.P. and Jones, K.C. (1993). The use of the cotton-strip assay to assess cellulose decomposition in heavy metal-contaminated sewage sludge-amended soils. *Environmental Pollution*, **81**: 173-178.

Oda, Y., Slagman, S.-J., Meijer, W.G., Forney, L.J. and Gottschal, J.C. (2000). Influence of growth rate and starvation on fluorescent in situ hybridization of *Rhodopseudomonas palustris*. *FEMS Microbiology Ecology*, **32**: 205-213.

O'Farell, K.A. and Janssen, P.H. (1999). Detection of *Verrucomicrobia* in a pasture soil by PCR-mediated amplification of 16S rRNA genes. *Applied and Environmental Microbiology*, **65**: 4280-4284.

OIS (1998). Swiss Ordinance relating to Impacts on the Soil ;1st July 1998. SR 814.12 Switzerland.

Okon, Y. (1985). *Azospirillum* as a potential inoculant for agriculture. *Trends in Biotechnology*, **3**: 223-228.

Ortiz, D.F., Ruscitti, T., Mccue, K.F. and Ow, D.W. (1995). Transport of metal-binding peptides by HMT1, a fission yeast ABC-type B vacuolar membrane-protein. *Journal of Biological Chemistry*, **270**: 4721-4728.

Osborn, A.M., Bruce, K.D., Strike, P. and Ritchie, D.A. (1997). Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon. *FEMS Microbiology Reviews*, **19**: 239-262.

Pawlowska, T.E., Chaney, R.L., Chin, M. and Charvat, I. (2000). Effects of metal phytoextraction practices on the indigenous community of arbuscular mycorrhizal fungi at a metal-contaminated landfill. *Applied and Environmental Microbiology*, **66**: 2526-2530.

Pell, M., Stenberg, B. and Torstensson, L. (1998). Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. *Ambio*, **27**: 24-28.

Pennanen, T., Frostegard, A., Fritze, H. and Baath, E. (1996). Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-

polluted gradients in coniferous forests. *Applied and Environmental Microbiology*, **62**: 420-428.

Pernthaler, A., Pernthaler, J. and Amann, R.I. (2002). Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Applied and Environmental Microbiology*, **68**: 3094-3101.

Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I. and Paul, E.A. (2000). Effects of agronomic treatements on structure and function of ammonia-oxidizing communities. *Applied and Environmental Microbiology*, **66**: 5410-5418.

Pollard, A.J. and Baker, A.J.M. (1997). Deterrence of herbivory by zinc hyperaccumulation in *Thlaspi caerulescens* (Brassicaceae). *New Phytologist*, **135**: 655-658.

Preston-Mafham, J., Boddy, L. and Randerson, P.F. (2002). Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles - a critique. *FEMS Microbiology Ecology*, **42**: 1-14.

Radajewski, S., Ineson, P., Parekh, N.R. and Murrell, J.C. (2000). Stable-isotope probing as a tool in microbial ecology. *Nature*, **403**: 646-649.

Reber, H.H. (1992). Simultaneous estimates of the diversity and the degradative capability of heavy-metal-affected soil bacterial communities. *Soil Biology and Biochemistry*, **13**: 181-186.

Reeves, R.D. and Baker, A.J.M. (2000). Metal-accumulating plants. pp. 193-229. *In:* Phytoremediation of toxic metals: Using plants to clean up the environment. Raskin, I. and Ensley, B.D. (Eds). Jonh Wiley and Sons Inc., New York.

Rheims, H., Sproer, C., Rainey, F.A. and Stackebrandt, E. (1996). Molecular biological evidence for the occurrence of uncultured members of the actinomycete line of descent in different environments and geographical locations. *Microbiology-Uk*, **142**: 2863-2870.

Robinson, B.H., Leblanc, M., Petit, D., Brooks, R.R., Kirkman, J.H. and Gregg, P.E.H. (1998). The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. *Plant and Soil*, **203**: 47-56.

Roszak, D.B. and Colwell, R.R. (1987). Survival strategies of bacteria in the natural-environment. *Microbiological Reviews*, **51**: 365-379.

Rough, D.A., Lee, B.T.O. and Morby, A.P. (1995). Understanding celllular responses to toxic agents: a model for mechanism-choice in bacterial metal resistance. *Journal of Industrial Microbiology*, **14**: 132-141.

Rykiel, E.J. (1985). Towards a definition of ecological disturbance. *Australian Journal of Ecology*, **10**: 361-365.

SAEFL (2002). Environnement suisse 2002 - politique et perspectives. Federal printing and supplied office (EDMZ), Bern.

Sait, M., Hugenholtz, P. and Janssen, P.H. (2002). Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environmental Microbiology*, **4**: 654-666.

Salt, D.E., Blaylock, M., Kumar, N.P.B.A., Dushenkov, V., Ensley, B.D., Chet, I. and Raskin, I. (1995). Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Biotechnology*, **13**: 468-474.

Salt, D.E., Smith, R.D. and Raskin, I. (1998). Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**: 643-668.

Sandaa, R.A., Enger, O. and Torsvik, V. (1999a). Abundance and diversity of Archaea in heavy-metal-contaminated soil. *Applied and Environmental Microbiology*, **65**: 3293-3297.

Sandaa, R.-A., Torsvik, V., Enger, O., Daae, F.L., Castberg, T. and Hahn, D. (1999b). Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. *FEMS Microbiology Ecology*, **30**: 237-251.

Sauve, S., Dumestre, A., McBride, M., Gillett, J.W., Berthelin, J. and Hendershot, W. (1999). Nitrification potential in field-collected soils contaminated with Pb or Cu. *Applied Soil Ecology*, **12**: 29-39.

Schippers, B., Bakker, A.W. and Bakker, P.A.H.M. (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annual Review of Phytopathology*, **25**: 339-358.

Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T. and Amann, R.I. (2003). An improved protocol for quantification of freshwater *Actinobacteria* by fluorescence in situ hybridization. *Applied and Environmental Microbiology*, **69**: 2928-2935.

Shen, Z.G., Zhao, F.J. and McGrath, S.P. (1997). Uptake and transport of zinc in the hyperaccumulator *Thlaspi caerulescens* and the non-hyperaccumulator *Thlaspi ochroleucum*. *Plant Cell and Environment*, **20**: 898-906.

Silver, S. and Walderhaug, M. (1992). Gene-regulation of plasmid-determined and chromosome-determined inorganic ion transport in bacteria. *Microbiological Reviews*, **56**: 195-228.

Smalla, K., Wachtendorf, U., Heuer, H., Liu, W.T. and Forney, L. (1998). Analysis of BIOLOG GN substrate utilization patterns by microbial communities. *Applied and Environmental Microbiology*, **64**: 1220-1225.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H. and Berg, G. (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology*, **67**: 4742-4751.

Snaidr, J., Amann, R.I., Huber, I., Ludwig, W. and Schleifer, K.H. (1997). Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Applied and Environmental Microbiology*, **63**: 2884-2896.

Stackebrandt, E., Liesack, W. and Goebel, B.M. (1993). Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB*, **7**: 232-236.

Staley, J.T., Fuerst, J.A., Giovannoni, S. and Schlesner, H. (1991). The order Planctomycetales and the genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphera*. Springer Verlag, New York.

Stark, P.C., Mullen, K.I., Banton, K.L., Russotti, R., Soran, D. and Kuske, C.R. (2000). Pre-PCR DNA quantification of soil and sediment samples: Method development and instrument design. *Soil Biology and Biochemistry*, **32**: 1101-1110.

Stephen, J.R., Chang, Y.L., Macnaughton, S.J., Kowalchuk, G.A., Leung, K.T., Flemming, C.A. and White, D.C. (1999). Effect of toxic metals on indigenous soil β-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resitant bacteria. *Applied and Environmental Microbiology*, **65**: 95-101.

Stoffels, M., Amann, R., Ludwig, W., Hekmat, D. and Schleifer, K.-H. (1998). Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds. *Applied and Environmental Microbiology*, **64**: 930-939.

Stoffels, M., Castellanos, T. and Hartmann, A. (2001). Design and application of new 16S rRNA-targeted oligonucleotide probes for the *Azospirillum-Skermanella-Rhodocista*-cluster. *Systematic and Applied Microbiology*, **24**: 83-97.

Stubner, S. (2002). Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen (TM) detection. *Journal of Microbiological Methods*, **50**: 155-164.

Stults, J.R., Snoeyenbos-West, O., Methe, B., Lovley, D.R. and Chandler, D.P. (2001). Application of the 5' fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. *Applied and Environmental Microbiology*, **67**: 2781-2789.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**: 4876-82.

Tilman, D., Wedin, D. and Knops, J. (1996). Productivity and sustainability influenced by biodiversity in grassland ecosystems. *Nature*, **379**: 718-720.

Valls, M., Atrian, S., de Lorenzo, V. and Fernandez, L.A. (2000). Engineering a mouse metallothionein on the cell surface of *Ralstonia eutropha* CH34 for immobilization of heavy metals in soil. *Nature Biotechnology*, **18**: 661-665.

Van de Peer, Y. and De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Bioinformatics*, **10**: 569-70.

Vangronsveld, J. and Cunningham, S.D. (1998). Introduction to the concepts. pp. 1-15. *In:* Metal-contaminated soils: in situ inactivation and phytorestoration. Vangronsveld, J. and Cunningham, S. (Eds). Springer-Verlag, New York.

Vollmer, M., Gupta, S.K. and Krebs, R. (1997). New standards on contaminated soil in Switzerland in comparison with Dutch and German quality criteria. pp. 445-457. *In:* Contaminated soils: 3rd international conference on the biochemistry of trace elements. Prost, R. (Eds). INRA, Paris.

von Wintzingerode, F., Gobel, U.B. and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews*, **21**: 213-29.

Wagner, M., Rath, G., Amann, R., Koops, H.P. and Schleifer, K.H. (1995). In-situ identification of ammonia-oxidizing bacteria. *Systematic and Applied Microbiology*, **18**: 251-264.

Wardle, D.A. and Giller, K.E. (1996). The quest for a contemporary ecological dimension to soil biology. *Soil Biology and Biochemistry*, **28**: 1549-1554.

Wardle, D.A., Zackrisson, O., Hörnberg, G. and Gallet, C. (1997). The influence of island area on ecosystem properties. *Science*, **277**: 1296-1299.

Weinbauer, M.G., Beckmann, C. and Höfle, M.G. (1998). Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. *Applied and Environmental Microbiology*, **64**: 5000-5003.

Weller, R. and Ward, D.M. (1989). Selective recovery of 16S ribosomal-RNA Sequences from natural microbial communities in the form of cDNA. *Applied and Environmental Microbiology*, **55**: 1818-1822.

Weller, R., Weller, W.J. and Ward, D.M. (1991). 16S rRNA sequences of uncultivated hot spring cyanobacterial mat inhabitants retrieved as randomly primed cDNA. *Applied and Environmental Microbiology*, **57**: 1146-1151.

Wenderoth, D.F. and Reber, H.H. (1999). Correlation between structural diversity and catabolic versatility of metal-affected prototrophic bacteria in soil. *Soil Biology and Biochemistry*, **31**: 345-352.

Wenderoth, D.F., Stackebrandt, E. and Reber, H.H. (2001). Metal stress selects for bacterial ARDRA-types with a reduced catabolic versatility. *Soil Biology and Biochemistry*, **33**: 667-670.

Whiting, S.N., de Souza, M.P. and Terry, N. (2001a). Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. *Environmental Science and Technology*.

Whiting, S.N., Leake, J.R., McGrath, S.P. and Baker, A.J.M. (2001b). Assessment of Zn mobilization in the rhizosphere of *Thlaspi caerulescens* by bioassays with non-acumulator plants and soil extraction. *Plant and Soil*, 147-156.

Yoshinak, T., Yano, K. and Yamaguch, H. (1973). Isolation of highly radioresistant bacterium, *Arthrobacter radiotolerans* nov.sp. *Agricultural and Biological Chemistry*, **37**: 2269-2275.

Zarda, B., Hahn, D., Chatzinotas, A., Schönhuber, W., Neef, A., Amann, R.I. and Zeyer, J. (1997). Analysis of bacteria community structure in bulk soil by in situ hybridization. *Archives of Microbiology*, **168**: 185-192.

Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E.J., Short, J.M. and Keller, M. (2002). Cultivating the uncultured. *Proceedings in Natural Academical Sciences of the United States of America*, **99**: 15681-15686.

REMERCIEMENTS

Cette thèse est le fruit de la collaboration de nombreuses personnes, travaillant dans différents instituts, que je tiens à remercier particulièrement:

Tout d'abord Hauke Harms pour m'avoir accueillie au sein de son groupe et donné l'opportunité de mener non seulement une recherche m'ayant permis d'acquérir des connaissances dans plusieurs domaines, mais aussi de mettre sur pied le laboratoire de biologie moléculaire. J'ai beaucoup apprécié la confiance qu'il m'a témoignée et son soutien pour résoudre les problèmes d'ordre financiers qui sont venus troubler ces 3 ans et demi de thèse.

Antonis Chatzinotas pour m'avoir supervisée directement. Tout en me laissant une grande liberté pour gérer mon projet, il a toujours été présent et disponible quand cela s'avérait nécessaire. Ton enthousiasme a créé une atmosphère de travail vraiment très agréable.

Madeleine Goerg-Günthard, responsable du projet «Zelle-Baum» au WSL, projet dont je faisais également partie. Bien que basée à Lausanne, j'ai toujours entretenu des contacts étroits avec différentes personnes de cet institut de recherche qui m'ont fourni le sol et les métaux lourds nécessaires à mes expérimentations. Je remercie aussi Philipp Egli, Jörg Luster, Bernd Nowack et David Rais pour toutes les informations qu'ils m'ont fournies concernant ce sol.

Jean-Pascal Dubois et Didier Perret pour m'avoir donné de précieux conseils en chimie et pour avoir planifié les nombreuses analyses de mes sols tels que les métaux lourds, sans oublier Jean-David Teuscher, Valérie Rochat, Delphine Rota et Elena Rossel qui ont fait toutes les analyses en question.

Catherine Keller et Daniel Hammer (dit «der Dani») pour m'avoir initiée à la culture des *Thlaspis* et délivré quelques-uns de leurs secrets (le rire fait pousser plus vite les plantes!). Merci aussi à Claudia Cosio pour avoir veillé sur mes *Thlaspis* quand j'étais en vacances.

Karin Kaufmann avec qui j'ai eu la chance de collaborer et grâce à qui les Biolog et leur analyse statistique n'ont maintenant plus de secrets pour moi.

Von Sigler for his help and valuable advices during these three years.

Ma soeur Christel, Karine, David, Karin, Edi, Mira, Enrique et Didier que j'ai sollicités et qui n'ont pas hésité à venir m'aider que ce soit pour peser du sol pendant des heures, purifier des clones à n'en plus finir ou faire des mesures toutes les minutes.

Tous les collaborateurs de pédologie qui m'ont accueillie chaleureusement.

Christof Holliger et toute l'équipe du laboratoire de biotechnologie environnementale. Les

«molecolo-meetings» ont été très instructifs et j'ai eu la chance de collaborer avec Katia Szynalski, Emmanuelle Rohrbach et Julien Maillard que je remercie infiniment pour leurs conseils et leur aide technique.
Jean-Pierre Kradolfer du même laboratoire ainsi que mon père qui, grâce à leur génie bricoleur hors du commun, ont facilité mes expériences.
Nicole Jeanneret pour m'avoir dépannée de nombreuses fois avec des souchettes.
Jean-Marc Froehlich pour sa disponibilité et son efficacité à résoudre mes problèmes informatiques.
Tous les membres de mon jury.
Pauline qui m'a rendue zen ces deux dernières années.
Lukas, avec qui j'ai eu la chance de partager mon bureau. Ta bonne humeur, ton extrême gentillesse et ton calme à tout épreuve m'ont souvent prouvé qu'un grand sage se cachait en toi.
Yves, Ben, Karin, Enrique, Mira, Dani, Nevena, Naser, Maud, Dawn, Stefan pour les nombreux moments de rigolade passés ensemble.
Katia, j'ai eu la chance non seulement de travailler avec toi mais surtout de t'avoir comme amie. Tu as largement contribué à la bonne entente qui régnait entre nos deux laboratoires et grâce à toi je me suis sentie moins seule à faire de la biologie moléculaire en pédologie.
Karine et Edi, mes deux meilleurs amis, sans lesquels cette thèse n'aurait pas été pareille. Vous m'avez montré le chemin à suivre et soutenus jusqu'au bout, c'est la plus belle preuve d'amitié que vous ayez pu me faire. Je peux vous assurer que notre trio de choc n'a pas encore fini de faire parler de lui!

Je dédie cette thèse à quatre personnes qui comptent beaucoup dans ma vie:

A David, ton amour a été le plus beau des soutiens. J'espère pouvoir t'épauler autant que tu l'as fait pour moi dans les semaines à venir.
A ma soeur Christel, j'espère que cette thèse te donnera la force et le courage nécessaires pour affronter les derniers mois de ta thèse.
A mes parents, pour leur soutien inconditionnel durant ces vingt-huit ans. Maman, papa, je vous dois tout, merci pour votre amour, votre dévouement et vos précieux conseils. Cette thèse est l'aboutissement de ce que vous m'avez toujours appris: savoir se battre et persévérer permettent de réaliser nos voeux les plus chers.

CURRICULUM VITAE

Surname: GREMION

Name: Fabienne

Date of birth: September 25th, 1975

Nationality: Swiss citizen

EDUCATION

2000- present	Ph.D. studies at the Swiss Federal Institute of Technology of Lausanne (ETH-Lausanne), Institute of Soil Sciences. Investigations in the field of microbiology.
1999	Diploma in biology, specialisation in parasitology and microbiology, University of Neuchâtel, Switzerland
1995-2000	Study of biology at the University of Neuchâtel, Switzerland
1991-1995	High school, diploma type B, Gymnase français, Bienne, Switzerland

PROFESSIONAL EXPERIENCE

1998-1999	Teaching assistant in General Microbiology, University of Neuchâtel
1994	Training at Ciba-Geigy, Marly (FR)

PUBLICATIONS

Gremion, F., Chatzinotas A. and Harms H. (2003). Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. *Environmental Microbiology* **5**: 896-907.

Gremion F., Chatzinotas A., Kaufmann K., Sigler W.V. and Harms H. (2003). Impacts of heavy metal contamination and phytoremediation on the microbial community during a twelve months microcosm experiment. *Submitted to FEMS Microbial Ecology.*