

Phytoremediation of Heavy Metals: An Overview

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Introduction

Among the many elements that occur in the natural environment, about 60% are referred to as heavy metals (with densities over 5 g/cm^3), most of which are d- and f-transition elements that may form compounds and complexes with various non-metal donor atoms, often at different oxidation states of a metal ion. The latter property accounts for their involvement in a variety of redox processes which occur in the environment either abiotically (chemical and/or photochemical processes) or as a result of biochemical transformations involving plants and microorganisms (Williams 2001). Some heavy metals, primarily d-elements, in trace and ultra-trace amounts are essential to many organisms, which is due to their participation mainly in a diverse range of enzymatically driven processes (Williams and Fraústo da Silva 2000, Williams 2001). However, their accumulation in the environment in somewhat noticeable amounts leads to toxic effects (for reviews see Paasivirta 2000, Zitko 2000). Some other elements, like Hg, Cd, Pb, etc., have no known physiological functions and may be toxic even at relatively low concentrations to any organism, especially at a long-term or systematic exposure with a possibility of their accumulation (Paasivirta 2000).

Heavy metal pollution, being mainly of anthropogenic origin, results from mining, smelting, burning of fossil fuels, application of fertilizers, pesticides, as well as from sewage and municipal wastes. Accumulation of heavy metals in the environment and particularly in aquifers and soil, from which they can be transferred into living organisms either via ground water or via the food chain (crops, cultivated food plants) and feed chain (fish, animals), is a matter of continuously growing environmental concern. Such heavy metals and metalloids as arsenic (As, 1st place), lead (Pb, 2nd

place), mercury (Hg, 3rd place), cadmium (Cd, 7th place), and hexavalent chromium (Cr^{VI}, 18th place) were present in the 2001 Top Twenty List of the priority hazardous substances as provided annually by the US Agency for Toxic Substances and Disease Registry (ATSDR 2001).

In contrast to organic contaminants, which can undergo biodegradation as a result of the activity of plants, soil microorganisms, or abiotic factors (for recent reviews see Fass *et al.* (Eds.) 1999, Meagher 2000, Bosma *et al.* 2001, Christensen *et al.* 2001, Margesin and Schinner 2001), giving either less toxic or, at least, less mobile and/or less bioavailable products strongly adsorbed onto fine particles, organic matter and in micropores of various soils (Angehrn *et al.* 1998, Löser *et al.* 1999) or minerals (Bertsch and Seaman 1999), heavy metals, being nondegradable, remain in the environment, and their speciation and bioavailability might virtually unpredictably change over time in the course of abiotic (chemical) and biogeochemical processes (Dumestre *et al.* 1999, Gobran *et al.* (Eds.) 2001). Moreover, in many cases organic contaminants can be largely or completely mineralized to CO₂, H₂O, as well as to inorganic N-, P-, S-, and halogen-containing products (*ultimate biodegradation*; for review see Aboul-Kassim and Simoneit 2001) in the course of oxidative biodegradation (Muratova and Turkovskaya 2001), though certain substituted organic contaminants require anaerobic or combined anaerobic plus aerobic degradation (Fass *et al.* (Eds.) 1999, Anderson and Lovley 2000, Berestovskaya *et al.* 2000, Goltz *et al.* 2001, Reineke 2001). Besides mineralization or incomplete metabolization of organics, some hydrocarbon-degrading bacteria can incorporate hydrocarbons into constituent fatty acids of their membrane phospholipids, as was, for instance, demonstrated for C₁₆ and C₁₈ alkanes utilized by *Rhodococcus rhodochrous* (Rodgers *et al.* 2000). In some cases, it is possible to enhance bioremediation of various organic contaminants using microbial enrichment cultures or mineral additives acting as electron acceptors (Kamnev *et al.* 1999b, and references therein, Anderson and Lovley 2000, Ellis *et al.* 2000, Cunningham *et al.* 2001). In higher plants, various detoxication pathways for organic xenobiotics have been observed, including their oxidative degradation and consequently possible complete oxidation, or with the final participation of their carbon atoms in regular cell metabolism (Siciliano and Germida 1999, Meagher 2000, Korte *et al.* 2000, Alkorta and Garbisu 2001, Dietz and Schnoor 2001, Hong *et al.* 2001).

However, in order to solve the environmental problem with toxic heavy metals, the latter always have either to be completely removed from the contaminated soil or to be reliably immobilized by (co)precipitation, adsorption, or an appropriate change in their chemical state (e.g., oxidation, coordination, etc.), ideally yielding much less or non-toxic species (for reviews see Lovley and Coates 1997, White *et al.* 1997, Stephen and

Macnaughton 1999). On the other hand, in contrast to, e.g. cesium, an alkali metal which has also been a cause of concern owing to its radioactive isotopes released into the environment [^{137}Cs (Entry and Watrud 1998, Lasat *et al.* 1998, Willey *et al.* 2001, for review see Zhu and Smolders 2000) and, to a lesser extent, ^{134}Cs (Avery 1995)], or strontium, an alkali earth element with its mobile radionuclide ^{90}Sr (Shevchenko *et al.* 2001), heavy metals commonly have much higher charge-to-radius ratios, are stronger Lewis acids and demonstrate much more pronounced tendencies to interact with a variety of both organic and inorganic ligands with different donor atoms (Sigel and Sigel 1996, 1998, Williams 2001).

Over a few recent years, a growing number of original papers on particular subjects, as well as a variety of monographs and journal reviews have appeared on diverse topics either directly related to plant-based bioremediation of heavy metals or relevant to this truly interdisciplinary field providing a wealth of additional information for analysis. Among the monographs, the following are of essential interest providing an expert-level consideration of a wide range of relevant problems: Kruger *et al.* (Eds.) 1997, Brooks (Ed.) 1998, Vangronsveld and Cunningham (Eds.) 1998, Raskin and Ensley (Eds.) 2000, Terry and Bañuelos (Eds.) 2000. A number of questions on trace elements in soil concerning their bioavailability, transfer, extraction, and accumulation (in particular, in plants), as well as chemical speciation and sorption kinetics are addressed in 14 chapters in Iskandar and Kirkham (Eds.) (2001). The interactions of trace elements with soil constituents, plants, and microorganisms in the rhizosphere as well as the applicability of these studies to plant-based bioremediation technologies are discussed in Gobran *et al.* (Eds.) (2001). An excellent coverage of molecular to ecological aspects of heavy metal toxicity to plants is presented in the monograph by Prasad and Hagemeyer (Eds.) (1999). For recent journal reviews of various particular aspects of heavy metal phytoremediation and their practical applications, the reader may refer to Chaney *et al.* 1997, Raskin *et al.* 1997, Boyd and Martens 1998, Ernst 1998, Anderson *et al.* 1999, Gleba *et al.* 1999, Kamnev and van der Lelie 2000, Khan *et al.* 2000, Kopriva and Rennenberg 2000, Meagher 2000, Garbisu and Alkorta 2001, McGrath *et al.* 2001, Mejáre and Bülow 2001, Krämer and Chardonens 2001.

The aim of the present overview is to draw attention to the importance of a wide range of physicochemical and biological factors which control the chemical speciation of heavy metals and their biological availability both to microbiota and to higher plants, as well as to emphasize the important role played by rhizosphere microorganisms in the processes underlying plant-based methods of remediation (phytoremediation) of heavy metal contaminations, including their phytostabilization (in combination with soil additives) and phytoextraction which can also

include soil amendment with metal chelating agents. Many physicochemical and biochemical aspects of these processes as well as their mechanisms still remain to be understood in more detail, which could pave the way to a more efficient application of the methods considered to various heavy metal-contaminated sites differing in the chemical composition of pollutants, conditions, and intrinsic properties of the environment.

Bioremediation of Heavy Metal Contaminations: General Strategies

Heavy Metal Immobilization in Soil: Pluses and Minuses

Heavy metal immobilization, despite its increasing application as a solution to decrease heavy metal toxicity in the environment, in principle resembles a kind of a time bomb (the "Time Bomb Hypothesis"; see Brown *et al.* 1998, and references therein). In this case, the potentially toxic metals still remain in the environment and under some unexpected or extreme (often virtually unpredictable) conditions, can be partly or gradually released with their possible migration via groundwater. Many reports have addressed various aspects of heavy metal liability (Liang and Schoenau 1995, Esnaola and Millán 1998), leaching capability in various soils (Lebourg *et al.* 1998, Chirenje and Ma 1999), bioleaching (Löser *et al.* 2001), and sediment-water fluxes of heavy metals (Shine *et al.* 1998, and references therein). However, little is known about the long-term fate of heavy metals and the sustainability of their immobilization in both biosolids-amended (McGrath *et al.* 1995, Sloan *et al.* 1998) and industrially affected soils (Egli *et al.* 1999), as such studies are extremely laborious and time-consuming.

Vangronsveld *et al.* (1995, 1996) studied the sustainability of heavy metal immobilization, using the combinations of compost and the strong metal-immobilizing additive beringite, at the site of a former pyrometallurgical zinc smelter. Chemical analyses, phytotoxicity tests and biodiversity studies, carried out five years after the treatment of the contaminated site, demonstrated the durability of the treatment. The water-extractable metal fraction of the treated soil was 70 times lower as compared to the non-treated soil. Phytotoxicity was maintained at the low levels observed immediately after soil treatment. Biodiversity studies showed that in contrast to the untreated soils, where the biodiversity of higher plant species and saprophytic fungi was extremely low owing to high soil toxicity, the richness of higher plant species and their associated mycorrhizal fungi was much higher on the treated soil (Vangronsveld

1998a). This biodiversity continued to increase, even ten years after the addition of the soil additives (Vangronsveld, personal communication), demonstrating the durability of the treatment. The sustainability of the immobilization was confirmed by measuring the amount of Cd and Zn percolated from untreated and beringite-treated soil columns during a 30-year simulation period (Vangronsveld 1998b). The applications of Mg and Ca carbonates added to soil were shown to significantly decrease Ni and Co uptake by the South African nickel hyperaccumulator *Berkheya coddii* as well as their solubility in the soil (Robinson *et al.* 1999); however, the long-term stability of the effect of such carbonate additions seems doubtful.

The ecology of heavy metal resistant microorganisms, which is directly affected by the bioavailability of the heavy metals, can be used as a tool to predict the efficacy of *in situ* heavy metal immobilization. A representative recent study (Pawlik-Skowronska 2002) showed that the level of production of Pb-induced thiol peptides (phytochelatins) by the green alga *Stichococcus bacillaris* reflected Pb availability (and, consequently, toxicity) to algal cells. The level of Pb availability was thus shown to be pH-dependent (within pH 5–8.5) and limited in the presence of Ca²⁺, orthophosphate, chloride, and citrate ions, whereas it did not decrease in the presence of Mg²⁺ or humic acids. Also some other reports demonstrate that the size and diversity of soil microbiota can be significantly affected in metal-contaminated soils (Dumestre *et al.* 1999, Sandaa *et al.* 1999), even when the metal concentrations do not exceed the limits accepted by the EC for agricultural soils (McGrath *et al.* 1995, Del Val *et al.* 1999). Besides the toxicity of heavy metals to soil microorganisms leading to damaging effects (Ostrovskii *et al.* 2000, Lundy *et al.* 2001, Ignatov *et al.* 2001) and/or essential metabolic changes (Kamnev *et al.* 1999c, 2001b), high concentrations of heavy metals have an impact also on plant yield, animals grazing on such lands and, consequently, on metal exposure of humans via the food chain (Giller *et al.* 1998).

The Role of Indigenous Microbiota and Bioaugmentation

In view of the "Time Bomb Hypothesis", the most appropriate and satisfactory solution to the problem of heavy metal pollution would be an essentially complete or at least significant removal of metals from the contaminated site. However, for such extremely complex biosystems as soil or aquifers this task is not at all easy and implies taking into account a great number of physicochemical and biological factors, many of which are largely interrelated.

It is quite clear that such "straightforward" methods as excavation and physicochemical treatment of contaminated soils *ex situ* (for recent review

see Mulligan *et al.* 2001) are extremely expensive, ca. US\$3M/ha (McGrath *et al.* 2001) as compared to plant-based bioremediation estimated to cost about US\$0.25M/ha (Cunningham and Berti 2000), as well as highly laborious and therefore inappropriate for large polluted territories. Moreover, such treatment would inevitably affect dramatically the natural soil biota, which is essential for high productivity of soil. Note that the most relevant approach to bioremediation has been considered to be based on optimization and stimulation of the indigenous microbiota (i.e. intrinsic bioremediation with minimal intervention) rather than on altering the microbial composition of the soil (Sadowsky 1999, Verstraete and Top 1999). Only if indigenous degraders are not capable of essential remediation or cannot be enriched using appropriate soil amendments (Stephen and Macnaughton 1999), soil inoculation with other effective degrader organisms may be a promising alternative (Timmis and Pieper 1999). However, some problems connected with this *bioaugmentation* strategy still have to be faced (Vogel 1996, Crowley *et al.* 1999). Besides its generally better applicability in case of organic contaminations, the major problem is poor predictability of the advantages of this approach for soil bioremediation owing to the diversity of interrelated biotic and abiotic factors and lack of detailed knowledge on them. Still more problems have to be solved in connection with the diverse role of microorganisms in plant-based bioremediation of heavy metals (see below) and, therefore, with possibilities of its improvement using the *bioaugmentation* approach.

Various methods involving bioreactors for treatment of metal-containing soil leachate, effluents, or wastewaters exploiting the bioprecipitation phenomenon (Diels *et al.* 1995b, White *et al.* 1998), though quite applicable and useful for bioremediation in a number of cases (and, in particular, for accompanying recovery of metals; Peys *et al.* 1999), are still limited, first of all, by size of the contaminated site to be remediated. Nevertheless, the use of readily decomposable plant wastes has recently been reported (Harris and Ragusa 2000, 2001) as a substrate for bacterially driven mitigation of heavy metal-containing acid mine drainage water involving sulfate-reducing bacteria which utilize plant wastes as a cheap and effective energy source.

Chemical and Biological Availability of Heavy Metals in the Environment

Heavy metal speciation studies correlated to microbial activity tests in long-term soil toxicity experiments clearly demonstrate the importance of physicochemical properties of the soils, such as cation exchange capacity

depending on the organic matter, clay minerals and hydrous metal oxides, pH and buffering capacity, redox potential and extent of aeration, water content and temperature. These parameters, along with a variety of biotic processes involving plants, fungi, and microbes, determine heavy metal bioavailability (Brown *et al.* 1999, Traina and Laperche 1999) and therefore should be considered together with the selected biological test (Welp and Brummer 1997, Wuertz and Mergeay 1997, Dumestre *et al.* 1999).

The rapid assessment of heavy metal bioavailability and uptake by plants is an important parameter in several respects. Firstly, it allows a rapid, cost-effective, and early assessment of the potential risk of heavy metal transfer linked to crop species grown on heavy metal enriched soils. The monitoring of this risk commonly involves the growth of crops on such soils. Secondly, a correlation between heavy metal bioavailability and plant uptake data can be used for modeling and in predicting the applicability of different plant-based remediation techniques as efficient remediation strategies for heavy metal polluted soils. This is of particular relevance for both *in situ* immobilization, aiming at reducing the plant-available heavy metal fraction and its uptake by plants, and extraction, where information on the plant-available fraction is a prerequisite to predict the efficiency of the remediation (Robinson *et al.* 1999b).

Chemical measurements alone do not provide adequate information about bioavailability of heavy metals and their adverse effects on living organisms, and modeling approaches are time-consuming, incomplete and not feasible for the monitoring where *in situ* remediation is carried out. Therefore alternative methods are needed to directly measure the effects of heavy metal pollution, and thus a number of biological assays have been developed and standardized. These assays can be classified as soil microbial tests, soil invertebrate tests and terrestrial plant tests. A panel of independent specialists recently evaluated these methods (SETAC 1999) and concluded that soil microbial biotests offer the best perspective for use as fast and efficient monitoring systems to assess heavy metal pollution. These tests include carbon-driven process assessment, such as substrate-induced respiration and basal respiration, nitrogen-driven process assessment based on mineralization of organic nitrogen, and *ex-situ* assays with specific organisms, such as *lux*-based systems and the Microtox® assay. The advantages of these bioassays are summarized in Table 1 (Kamnev and van der Lelie 2000).

The most promising microbial bioassays were proved to be the *lux*-based systems for the detection of the bioavailable fraction of specific pollutants in environmental samples, such as specific organic xenobiotics or specific heavy metals. An example of the latter is the BIOMET concept (Corbisier *et al.* 1999), which is based on transcriptional fusions between specific heavy metal-inducible promoters of the soil bacterium *Ralstonia*

Table 1. Recommended soil microorganism ecotoxicological bioassays (SETAC 1999)

Test	Reliability	Ease	Relevance	Sensitivity
Carbon-driven process assessment				
Substrate-induced respiration	High	High	High	Medium
Basal respiration	High	High	High	Low
Nitrogen-driven process assessment				
Mineralisation of organic nitrogen	High	High	High	Low
Ex-situ assays				
<i>Lux</i> -based systems	High	High	High	High
Microtox®	High	High	Medium	High

eutropha CH34 (former *Alcaligenes eutrophus*) and the *lux* operon of *Vibrio fischeri*, whose transcription can be measured by quantitative light production. The transcription level depends on the concentration of bioavailable metal, and the emitted bioluminescence can be recorded and compared to an internal standard. Bacterial sensors responding specifically to Zn, Cd, Cu, Ni, Cr, Pb, and Hg have been fully characterized in terms of specificity, detection limits and selectivity.

Tibazarwa *et al.* (2001) used a nickel specific biosensor to quantify the metal bioavailability in various nickel-enriched soils which had been treated with additives for *in situ* metal immobilization. The data obtained confirmed that the bioavailability of nickel was greatly reduced following treatment of the soils with the additives beringite and steelshots. Furthermore, the data were found to correlate linearly with data on the biological accumulation of nickel in specific parts of important agricultural crops, such as maize and potato. The test can thus be used to assess the potential transfer of nickel to organisms of higher trophic levels, in this case maize and potato plants grown on nickel-enriched soils. Therefore, the BIOMET-test and similar bacterial biosensors are very promising concepts to be further developed as efficient warning and monitoring systems for sites polluted with heavy metals, including chemically amended metal-contaminated soils (Chen *et al.* 2000), and as tools to predict the potential risk of transfer of heavy metals to the food chain.

It is noteworthy that determination of chemical availability of heavy metals aimed at predicting their bioavailability may give results contradictory to plant uptake studies. For example, soil analyses and plant uptake studies for two heavily contaminated sites showed (Pichtel *et al.* 2000) that while Pb accumulated in plant tissue slightly correlated with exchangeable and soluble soil Pb, for Cd such correlation was poor. Knight *et al.* (1998) found that concentrations of free Cd²⁺ ions (commonly

regarded as more bioavailable as compared to its complexed forms) in extracts from CdSO_4 -amended soils were smaller than from the same soil contaminated to the same level by 36 years of sewage sludge additions. (Interestingly, the results for Zn^{2+} reported by the same authors were directly opposite.) However, Brown *et al.* (1998) found that significantly less Cd was taken up by lettuce grown just on long-term biosolids-amended soil than by lettuce grown on soil amended with equivalent rates of Cd salt. Certainly, these environmentally essential discrepancies might probably be largely explained by differences in the physicochemical properties of both the soils (including differences in their microbiota) and the biosolids used. Nevertheless, it is clear that caution should be taken when interpreting the results of model leaching or metal mobility experiments with regard to certain plant species. In view of that, concerns among scientists have recently been raised regarding food safety and long-term soil productivity with regard to the use of sewage sludges as farm fertilizers, encouraged in recent years by the U.S. Environmental Protection Agency as well as a similar EU policy promoting recycling of organic wastes, just owing to the accumulation of heavy metals in soils fertilized repeatedly with sewage sludges over prolonged periods (McBride 1998, McGrath *et al.* 2001).

Plant-Based Bioremediation of Heavy Metals

Phytoremediation of Heavy Metals as a General Strategy

A very promising, environmentally friendly, and cost-effective alternative is plant-based bioremediation (*phytoremediation*) of heavy metal contaminations, which has already been used for years (Baker *et al.* 1994, Cunningham *et al.* 1997, Chaney *et al.* 1997, Raskin *et al.* 1997, Gleba *et al.* 1999) since it was first proposed by R.L. Chaney in 1983 (see Anderson *et al.* 1999, Chaney *et al.* 2000, and references therein). As a general strategy, it includes several subsets. Among these is the use of metal-accumulating (or hyperaccumulating) plants to remove heavy metals from soil, i.e. *phytoextraction*, which commonly implies accumulation of metals, taken up by the plant root system, in top parts of the plant (Ebbs and Kochian 1998). This method applied to precious metals plant-accumulated over a mineralized soil, followed by harvesting and using the biomass for producing bio-ores, can also be used for *phytomining* (Anderson *et al.* 1999), which has recently been patented (Chaney *et al.* 1998). Note also that certain plant species capable of accumulating large amounts of metals, including heavy metals, can be used as biogeochemical indicators of hidden mineral ore deposits (see, e.g., Nagaraju and Karimulla 2001, and references therein).

Removal of metals from contaminated waters may often be referred to as *rhizofiltration* (Dushenkov *et al.* 1995) which implies high root uptake of (or affinity for) metals and does not commonly require efficient translocation of metals from roots to shoots (which is evidently necessary for phytoextraction). In this case, plants with high-surface "hairy root" systems are particularly promising, considering also their high growth rates as well as biochemical stability (Shanks and Morgan 1999, Nedelkoska and Doran 2000a,b). Nevertheless, mechanisms of this stability still have to be understood in more detail. Recently, Wu *et al.* (2001) comparatively studied the cadmium response of a weakly environmentally resistant and therefore weakly adaptable species of the hairy-root herbaceous plant, *Adenophora lobophylla*, which has the endangered status, and a morphologically very similar but well-proliferating in the same region and widely distributed species, *A. potaninii*. Different accumulation kinetics of phytochelatin (PCs), content of reduced glutathione (GSH) and cysteine (Cys), and their different changes under Cd stress have been found for the two species. It has been suggested on the basis of the results obtained that, while both the species are capable of synthesizing high levels of PCs (see below), the enhanced resistance of *A. potaninii* might in addition involve also a Cd exclusion system and a tighter homeostasis mechanism(s) to maintain the cellular level of GSH.

It should be noted that, besides growing plants, plant biomass can also be used as a biofilter for removing trace heavy metals from both wastewaters and natural waters, which may be referred to as *phytosorption*. An evident advantage of this technology is the possibility to clean up highly contaminated waters simultaneously containing a range of heavy metals, which would be too toxic for growing plants. Some examples of biomass used as adsorbents, including bacteria, fungi, algae, and higher plants (agricultural and forestry wastes) have been listed by Prasad and Freitas (2000). They also showed that heavy metals (Ni, Cd, Pb, Cu, and, to the least extent, Cr) adsorbed by phytomass of *Quercus ilex* L. (holly oak, an evergreen tree playing an important role in western Mediterranean ecosystems) can be desorbed with 10 mM Na₄EDTA up to 55–90% and that the recycled phytomass can be re-usable.

For mercury and selenium, phytoextraction of their compounds from soil accompanied by their enzymatic reduction to volatile species (e.g., Hg⁰ and dimethylselenide) with further emission to the atmosphere (*phytovolatilization*) offers great promise for commercial development and application (Chaney *et al.* 1997, Leonard *et al.* 1998a,b). Note that mechanisms of bacterial resistance to mercury are also based on enzymatic conversion of Hg²⁺ to Hg⁰ in various bacteria both containing and lacking known *mer* genes (van der Lelie 1998, Reyes *et al.* 1999), which contributes to bioremediation of mercury in contaminated soil and water.

Note that selenium, not being a heavy metal and even not a metal, is nonetheless often considered from the same standpoint related to bio- or phytoremediation. Selenium contaminations, that occur in arid and semi-arid regions with alkaline soils derived from marine sediments, if present in high concentrations, would require complicated and highly expensive conventional (physical and chemical) methods for site remediation. The use of selenium-volatilizing plants (also see below) in association with microorganisms is proposed for reducing selenium (Se) toxicity in soil, while the harvested crop containing part of uptaken selenium can be used as an additive to animal forage in Se-deficient regions (Bañuelos 2001).

Finally, plant-based methods of elimination of metal toxicity and bioavailability (particularly for Pb and Cr⁶⁺) in soil without their extraction are commonly referred to as *phytostabilization* (Salt *et al.* 1995, Chaney *et al.* 1997). However, the evident disadvantages of this approach resulting from the ever remaining potential risk of heavy metal leakage or mobilization have already been mentioned above.

The growing importance of the rapidly developing interdisciplinary field of plant-based methods for environmental clean-up is represented by a specialized scientific periodical, the *International Journal of Phytoremediation* (CRC Press LLC), launched in 1999 (Lanza 1999), which publishes related basic, applied, and case studies.

Heavy Metal Hyperaccumulation in Plants

Some very interesting examples of metal-hyperaccumulating plants, among over 400 species of metal hyperaccumulators known up to now, have been described both for terrestrial and wetland plants (Chandra *et al.* 1997, Cunningham *et al.* 1997, Huang *et al.* 1997b, Zayed *et al.* 1998a, Nedelkoska and Doran 2000b, Baker *et al.* 2000, Zhao *et al.* 2001). Among hyperaccumulator plant species reported so far, there are over 300 accumulating Ni, 37 Cu, 28 Co, 19 Se, 16 Zn, 14 Pb, 11 Mn, 2 Tl, and 1 Cd (Anderson *et al.* 1999, Baker *et al.* 2000); some of them are capable of accumulating different metals, although at different levels (Chaney *et al.* 2000, Reeves *et al.* 2001). A number of species endemic to metalliferous soils have developed specific physiological and biochemical mechanisms of adaptation and exhibit both hyperaccumulating properties and hypertolerance toward heavy metals (McGrath *et al.* 2000). However, some of them also have a high physiological requirement for Zn and even do not grow at its low soil levels (Küpper *et al.* 1999, McGrath *et al.* 2000). Examples of hyperaccumulator plants which could be used for phytomining are listed in Table 2, with average metal concentrations found in their dry biomass as compared to metal content in non-accumulator plants, as well as typical biomass yields per hectare (Anderson *et al.* 1999, Chaney *et al.*

2000). In these cases, the recovery of metals from the ash of the hyperaccumulator plant shoots can be considered as a value to offset the cost of phytoremediation. Indeed, when the plant ash contains over 10% of metals (e.g., Zn, Cu, Ni, or Co), the standard metallurgical recovery of the metals may be expected to be more readily achieved as compared to their ores, where additional difficulties arise owing to the presence of high levels of accompanying Fe, Mn, and/or Si (Chaney *et al.* 2000).

For efficient phytoextraction, the main requirement is the availability of metal-hyperaccumulating fast growing plants producing large amounts of biomass (Khan *et al.* 2000). The most common drawbacks, which hinder their wide-range application, are the following. First, the method is evidently limited to relatively shallow depths of contamination that might in principle be remediated using plants (Mulligan *et al.* 2001). Most of the known metal-hyperaccumulating plant species are metal-selective (thus providing only partial solution to the more common problem of multiple heavy metal contamination), and, as indicated above, about three fourths

Table 2. Examples of specific hyperaccumulator plants (Anderson *et al.* 1999, Chaney *et al.* 2000)

Element	Hyperaccumulator plant species	Metal concentration (mg/kg dry weight)	Usual level of metal in non-accumulator plants (mg/kg dry weight)	Yield of the hyperaccumulator plant biomass (t/ha)
Au ^a	<i>Brassica juncea</i>	10	0.001	20
Cd	<i>Thlaspi caerulescens</i>	3,000	1	4
Co	<i>Haumaniastrum robertii</i>	10,200	1	4
Cu	<i>Haumaniastrum katangense</i>	8,400	1	5
	<i>Aeollanthus biformifolius</i>	13,700	-	-
Mn	<i>Macadamia neurophylla</i>	55,000	400	30
	<i>Alyxia rubicaulis</i>	11,500	-	-
Ni	<i>Alyssum bertolonii</i>	13,400	2	9
	<i>Berkheya coddii</i>	17,000	2	22
	<i>Phyllanthus serpentinus</i>	38,100	-	-
Pb	<i>Thlaspi rotundifolium</i> subsp.	8,200	5	4
Se	<i>Astragalus pattersoni</i>	6,000	1	5
	<i>Astragalus racemosus</i>	14,900	-	-
Tl	<i>Biscutella laevigata</i>	13,800	1	4
	<i>Iberis intermedia</i>	4,000	1	10
U	<i>Atriplex confertifolia</i>	100	0.5	10
Zn	<i>Thlaspi calaminare</i>	10,000	100	4
	<i>Thlaspi calaminare</i> ^b	39,600	-	-

^aHyperaccumulation induced using ammonium thiocyanate (NH₄SCN).

^bSpecies located in Germany (cited from Chaney *et al.* 2000).

of all known metal hyperaccumulators "specialize" on nickel (Krämer *et al.* 1996, Anderson *et al.* 1999, Baker *et al.* 2000). Their growth rate is usually low, and they produce relatively small amounts of biomass. As indicated by Chaney *et al.* (2000), for high value land or for small areas of contamination the overall time required for phytoremediation may appear so expensive that replacement of the contaminated soil would be more cost effective. In addition, virtually no agricultural knowledge for metal hyperaccumulator plants is available; moreover, most of their genotypes, being endemic to contaminated environments, can be used in their natural habitats only. Therefore, a need exists to either develop new plants with improved phytoextraction properties or to modify other factors affecting heavy metal uptake, translocation, and toxicity (Harkey and Pradhan 1998, Steinkellner *et al.* 1998, Briat and Lebrun 1999, Chaney *et al.* 2000) in suitable agricultural plant species (crops).

It should be noted that hyperaccumulator plants have been shown to protect themselves from both chewing insects and plant pathogens owing to high tissue concentrations of accumulated heavy metals (Boyd and Martens 1998, de Vries 2000). However, a recent test with a viral pathogen has shown (Davis *et al.* 2001) that elevated Ni concentrations in tissues of the Ni hyperaccumulator milk-wort jewelflower (*Streptanthus polygaloides* Gray) enhanced its infection with turnip mosaic virus instead of protecting the plant, which may impose the risk of infection to agricultural crops grown near the site and has to be taken into account.

Heavy Metal Tolerance in Plants

Several current theories of metal tolerance in plants implicate *in vivo* metal complexation by metallothioneins (MT), which include cysteine-rich proteins and low-molecular-weight polypeptides – phytochelatins (PCs) (Prasad 1998, Vašák 1998, Briat and Lebrun 1999, Mounicou *et al.* 2001, for recent reviews see Cobbett 2001, Mejáre and Bülow 2001). The latter, forming a family of peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ referred to as $(\text{PC})_n$, where n is generally 2 to 5, up to 11 (Rausser 1999, Leopold *et al.* 1999), have been identified in a variety of plant species and some microorganisms and are structurally related to glutathione (GSH; $\gamma\text{-Glu-Cys-Gly}$) which is the substrate for PC biosynthesis. In some plant species, a number of structural variants of PCs have been identified differing in their terminal member, e.g. $(\gamma\text{-Glu-Cys})_n\text{-}\beta\text{-Ala}$, $(\gamma\text{-Glu-Cys})_n\text{-Glu}$, or $(\gamma\text{-Glu-Cys})_n\text{-Ser}$ (Cobbett 2000).

It is interesting to note that the activity of PC synthase (PCS), an enzyme that polymerizes GSH into PCs, has until recently been found exclusively in plants, some fungi and marine diatoms. Only in 2001, the expression of the CePCS1 gene encoding a functional PC synthase was reported to have

been identified in a nematode worm, *Caenorhabditis elegans* (Vatamaniuk *et al.* 2001, Clemens *et al.* 2001). This finding shows that animals, similar to plants, might also use PCs for heavy-metal detoxification, in addition to GSH- and MT-based mechanisms only, which have until now been described in animals (Rinaldi 2001, Cobbett 2001). Some plant and fungal genes encoding PCS have recently been cloned; however, *Arabidopsis* has been reported to be the first organism in which *two* functional PCS genes were described (Cazalé and Clemens 2001, and references therein) showing apparent non-redundancy of function.

For PCs, which are rapidly induced *in vivo* by a wide range of heavy metal ions, their important role in detoxification of heavy metal ions in plants is well documented (for reviews see Cobbett 2000, 2001). Nevertheless, it still remains unclear whether this is the main function of PCs for which they evolved or an incidental role, besides their involvement in essential heavy-metal homeostasis as well as iron and sulfur metabolism (Rausser 1999, Cobbett 2000). Moreover, the formation of PC complexes with heavy metals *in vivo* seems to play only a transient role in their detoxification. It was found (Leopold *et al.* 1999) that Cd-PC and Cu-PC complexes formed in water cultures of the heavy metal-tolerant plant *Silene vulgaris* disappeared in the roots one to two weeks after heavy metal exposure. This investigation shows that PCs are not responsible for the development of heavy metal-tolerant phenotypes.

However, histidine (a first example of a free amino or organic acid produced in plants as a specific and proportional response to metal treatment) was shown to be involved both in the mechanism of nickel (and, in lower concentrations, cobalt) tolerance and in the high rates of nickel transport into the xylem (Krämer *et al.* 1996) required for hyperaccumulation in the shoot of *Alyssum lesbiacum*. It is noteworthy that exogenously applied histidine essentially reduced Ni toxicity and, when supplied in the root medium, greatly increased Ni flux through the xylem in the non-tolerant species of this genus, *A. montanum* (Krämer *et al.* 1996). Also, plant ferritin (iron-storage protein) has been shown to be capable of chelating heavy metals, thus providing for an additional possible defense mechanism at low toxic levels (Kumar and Prasad 1999) prior to phytochelatin synthesis (Patra and Panda 1998, Briat and Lebrun 1999).

Note that different heavy metals may induce disparate effects on plants. In duckweed, an aquatic vascular plant, cadmium treatment was reported (Prasad *et al.* 2001) to induce a dose-dependent accumulation of four polypeptides (7 to 18 kDa), two of which (8 and 7 kDa, supposedly metallothioneins) appeared anew, whereas copper treatment of the plant did not result in either accumulation or appearance of any protein. However, the plant could tolerate up to 10 mM of Cd without significant changes in the concentration of photosynthetic pigments, whereas Cu in

concentrations above 0.025 mM promoted significant pigment degradation, which might have been caused by an acute oxidative stress induced by Cu ions accumulating in plant cells from contaminated water. The results on different effects of different heavy metals (Cu, Zn) on the enzymes involved in the ascorbate-GSH pathway in primary leaves of bean seedlings (Cuypers *et al.* 1999) suggest that the chemical behavior of a metal plays a determining role in the induction of oxidative stress and in the relevant defense mechanism of the affected plant. Copper ions were also suggested to alter the equilibrium between synthesis and utilization of glutathione in *Scenedesmus bijugatus* (Nagalakshmi and Prasad 2001), which may be due to its antioxidant role or by serving as a precursor in PC synthesis. Nevertheless, excess of Zn was also shown to cause oxidative stress in *Phaseolus vulgaris* (Cuypers *et al.* 2001); the authors suggested that the mechanism of action is different in roots and primary leaves of the Zn-stressed plant, with translocation and/or complexation being responsible for coping with high Zn levels in roots. Note, however, that internalized speciation studies using electron microscopy and X-ray absorption spectroscopy have shown (Sarret *et al.* 2001) that Zn^{2+} was present largely as its phosphate dihydrate in both the roots and leaves of *P. vulgaris* regardless of its form in solution (i.e. sulfate or EDTA complex). For the same plant grown in Pb nitrate solution, the metal was predominantly found in the leaves in the form of carbonate (cerussite), while in the PbEDTA-treated plant a mixture of PbEDTA and an undetermined Pb species was found. These results indicate (Sarret *et al.* 2001) that in *P. vulgaris* both uptaken Zn (totally) and Pb (partly) appear to have been transformed from their EDTA complexes into other chemically bound forms.

Genetic engineering of transgenic plants expressing MTs has been shown to be a promising strategy helping to increase heavy metal tolerance in plants (Liu *et al.* 2000). Expression of heterologous metallothioneins was used to improve metal accumulation and tolerance in plants. Various MT genes – mouse MTI, human MTIA (alpha domain), human MTII, Chinese hamster MTII, yeast CUP1, pea PsMTA – have been transferred to tobacco, cauliflower or *Arabidopsis thaliana* (for review see Kärenlampi *et al.* 2000). As a result, varying degrees of enhanced Cd tolerance have been achieved, being maximally 20-fold compared with the control. Metal uptake levels were not dramatically changed; in some cases there were no differences, in others maximally 70% less or 60% more Cd was taken up by the shoots or leaves. The development of transgenic plants with heavy metal accumulation and/or tolerance enhanced by overexpressing natural or modified bacterial genes, which encode the enzymes responsible, e.g., for biosynthesis of glutathione and phytochelatin (Zhu *et al.* 1999 a,b) or for the protonolysis of the Hg-C bond in organomercurials (Bizily *et al.* 1999),

has recently been demonstrated to be a promising strategy for the production of plants with superior phytoremediation capacity. Also, the transfer of heavy metal-hyperaccumulator traits has been successfully achieved using somatic hybridization by electrofusion of protoplasts isolated from *Thlaspi caerulescens* and *Brassica napus* (Brewer *et al.* 1999), which may be useful in producing heavy metal hyperaccumulator plants with larger biomass yields, higher growth rates, and better properties facilitating their mechanical harvesting. Screening of a large number of plants, aimed at isolating mutants capable of accumulating large amounts of heavy metals, may be intensified using their radioisotopes and thus visualizing their accumulation in plant tissue, as was proposed (Schulman *et al.* 1999) for *Brassica juncea* used primarily for rhizofiltration.

Some biochemical and genetic aspects of plant adaptive responses to cadmium toxicity, as well as physiological aspects of Cd and Pb toxicity to higher plants have recently been reviewed (Sanità di Toppi and Gabbrielli 1999, Seregin and Ivanov 2001). It should also be noted that the fact of accumulation of heavy metals (Cu, Pb) in pectins (plant cell wall carboxypolysaccharides of wide industrial, nutritional, and medical importance) upon their chemical or enzymatic extraction from the plant sources (Kamnev *et al.* 1998) may indicate that metal binding to specific functional groups of pectic substances also plays a role in plant tolerance to heavy metals.

Phytoavailability of Heavy Metals and Selenium

For heavy metal phytoremediation (and for phytoextraction in particular), bioavailability of metals in contaminated soils is a crucial factor regulating heavy metal uptake by plant roots. This aspect has received much attention in the recent literature (Brown *et al.* 1998, Echevarria *et al.* 1998, Krebs *et al.* 1998, Li *et al.* 1998, Brennan and Shelley 1999, Robinson *et al.* 1999a,b, Khan *et al.* 2000). Note that the fraction of a heavy metal unavailable to plants may be essential: for Ni it was shown to be from 20 up to 87% of the total Ni in soil, and the Ni concentration in hyperaccumulator plants was found to be related to the soluble Ni fraction in soil (Robinson *et al.* 1999b).

Apart from differences among various plant species, the overall combination of soil physical and chemical properties controls both the rate and extent of metal uptake. In this respect, various types of soils and soil components have been under thorough investigation (see, e.g. Venditti *et al.* 2000, and references therein), including the effects of various conditions and additives of both organic and inorganic composition (see, e.g. Dahmani-Muller *et al.* 2001, and references therein). For instance, waste water irrigation was found to be effective in increasing biomass yield of two varieties of *Populus trichocarpa*, whereas sludge application was not;

nevertheless, modest sludge application rates (up to $100 \text{ m}^3 \text{ ha}^{-1} \text{ yr}^{-1}$) were found to be environmentally acceptable (Moffat *et al.* 2001). Screening of 10 plant species including wheat, mustard, and weed for their efficiency to take up heavy metals from soils irrigated with mixed industrial effluents suggested (Barman *et al.* 2000) that *Alternanthera sessilis* and *Cyanodon dactylon* could be used for phytoextraction. A comparative study of the uptake of several heavy metals by *Arabidopsis halleri* from an unpolluted soil (control) amended with various metal-bearing solids (such as pure zinc sulfate, fresh and decomposing organic soil residues, a soil clay fraction, or two waste slags) has shown (Dahmani-Muller *et al.* 2001) that, though the dry biomass yield of the plant was independent (except for Cu toxicity) from the nature of the metal-bearing amendments, the latter has to be considered in order to improve the predictability of phytoextraction efficiency. Liming of sludge-amended soils was shown to reduce soluble fractions of heavy metals (Krebs *et al.* 1998, Fang and Wong 1999) which correlates with their reduced plant uptake (Krebs *et al.* 1998). Chen *et al.* (2000) found that CaCO_3 , steel sludge, and furnace slag added separately to a Cd-contaminated red soil decreased Cd uptake by wetland rice, Chinese cabbage, and wheat by 23 to 95% compared with the unamended soil, furnace slag being the most efficient at suppressing Cd uptake by the plants.

On the contrary, soil acidification was shown to increase concentrations of Cd, Ni, Se, and As in column leaching studies as a result of CaCO_3 dissolution and the release of metals adsorbed to it, whereas Cu concentrations were reduced owing to reduced dissolved organic carbon (Chirenje and Ma 1999) to which Cu is known to bind more strongly and irreversibly than many other metals (Breault *et al.* 1996). Nevertheless, the presence and amount of dissolved organic matter is still regarded as a more generally important feature which has been well documented to increase the solubility and, as a consequence, the mobility and phytoavailability of heavy metals (Römken *et al.* 1999). Also, the abundance and potential solubility of carbonates (calcite, siderite) and iron oxides, though containing low amounts of heavy metals, under acidic conditions may result in an increased mobility of the contaminants, whereas heavy metals in small metal-rich particles of tin, lead, or zinc oxides are more chemically stable and poorly mobile (Venditti *et al.* 2000).

In a field study specifically aimed at assessing the possibilities of molybdenum (Mo) decontamination (Neunhäuserer *et al.* 2001), it was found that phosphate fertilization and vermiculite amendment increased plant Mo content by 88% and 84%, whereas manganese humate and sewage sludge decreased it by 25% and 40%, respectively. The results suggest that for highly Mo-contaminated sites, phosphate and vermiculite may be recommended to enhance Mo phytoextraction, and for lower

contaminations the application of Mn humate and sewage sludge may be used to lower the content of Mo in forage thus making it suitable for feeding animals.

Organic acids were recently shown to significantly enhance Cr^{III} uptake from sand and soil by maize (*Zea mays*) plants, whereas such amendments did not appear to markedly affect chromium accumulation from Cr^{VI} treatment (Srivastava *et al.* 1999). This finding, evidently based on the potential of various carboxylic and amino acids to form complexes with chromium(III), clearly shows the potential risk remaining when well water-soluble chromate(VI) in soil is reduced to chromium(III) which is commonly regarded as less mobile. Note also that in comparative studies of chromium uptake by three tree species, *Dalbergia sissoo*, *Acacia arabica*, and *Populus euroamericana*, no correlation was found (Khan 2001) between the content of Cr in the dried plant tissue and the total Cr in soil extractable by DTPA (diethylenetriaminopentaacetate).

Chelating agents such as a siderophore (see below), nitrilotriacetate (NTA), and citrate, were comparatively tested for their metal-mobilizing capability as potential soil amendments (Neubauer *et al.* 2000). Copper, zinc, and cadmium bound to clay minerals (Na-montmorillonite and kaolinite) were shown to be most efficiently mobilized with NTA, which was also the most efficient metal mobilizer in batch experiments with heavy metal-contaminated soils from field sites. However, for the metals bound to goethite (α -FeOOH) the effect of the siderophore (desferrioxamine B) was found to be more pronounced, probably because of the concomitant dissolution of the ferric mineral together with the sorbed cations. Among the three metals, Cu was the most affected by the chelating agents. For Pb, Ni, and Zn in contaminated soils (Barona *et al.* 2001), treatment of the latter with EDTA solution resulted in a considerably increased acid-extractable Pb fraction and the removal of certain amounts of the three metals from the silicate matrix, thus increasing their leachability (especially for Pb) and potentially favoring EDTA application for further phytoextraction. For a single application, the extraction recoveries were found to be 33–37% for Pb, and only 5–11% for Ni and 14–19% for Zn. These results clearly indicate that for mobilization of heavy metals aimed at improving their phytoavailability, a range of factors have to be taken into account in order to optimize the process of metal solubilization, which should then be coupled to studying metal availability to and uptake by plants.

Stanhope *et al.* (2000) assessed the EDTA-assisted increase in the phytoavailability of several heavy metals in soil historically contaminated (up to high metal concentrations) with sewage sludge using Indian mustard as a test crop. EDTA-extractable metal fractions were found to be 61% for Cd, 73% for Cu, 76% for Ni, 50% for Pb, and 72% for Zn (i.e. essentially higher than those reported by Barona *et al.* 2001) as compared to total

metal concentrations in the soil under study. EDTA addition was found to increase plant uptake of all the five metals. However, it was shown using ^{109}Cd isotopic dilution (Stanhope *et al.* 2000) that EDTA did not facilitate access to the nonlabile fraction of Cd in the soil; the values for both chemically and biologically labile Cd were similar across the whole range of EDTA applications (0, 0.1, 1.0, 5.0, and 10 mmol/kg). In the study by Chen and Cutright (2001), the application of EDTA (0.5 g per kg of soil) was also shown to significantly increase the concentrations of Cd (3.4-fold) and Ni (7.8-fold) in *Helianthus annuus* shoots; the total metal removal efficiency per plant for *N*-(2-hydroxyethyl)-ethylenediaminetriacetic acid (HEDTA) was about 71% of that for EDTA. However, the authors' comparative study on other plants and different heavy metals have shown that the chelator enhancement is both plant- and metal-specific and can be inhibited in the case of multiple heavy metal contamination; a reduction in the plant biomass yield as a result of chelator toxicity was also noted. It should also be stressed that in the case of the nickel hyperaccumulator *Berkheya coddii* the applications of NTA, EDTA, or DTPA were all found to reduce significantly plant uptake of Ni, despite increasing its solubility in the soil, whereas cobalt uptake was unaffected (Robinson *et al.* 1999). Instead, sulfur was found to greatly increase plant uptake of both Ni and Co and could therefore be used as a low-cost soil amendment for phytoremediation of these metals.

As a soil amendment specifically aimed at gold phytomining, the application of ammonium thiocyanate (over 0.16 g per kg of soil) was shown to result in Au hyperaccumulation by *Brassica juncea* (see Table 2), though, despite its low toxicity, use of SCN^- has to be strictly controlled to prevent leaching of this chemical and associated metals into ground waters (Anderson *et al.* 1999).

In studying heavy metal phytoavailability, the mechanisms of the processes can hardly be understood without chemical speciation studies (Gadd 1999, Szpunar and Eobiński 1999, Eobiński 2001) which allow the nature of the absorbed, translocated, and stored heavy metal species to be compared. Such studies which are rapidly evolving in the past several years definitely deserve special attention and analysis, even despite the fact that they often raise more questions than solve.

A very interesting mechanistic study on the role of inorganic (selenate, SeO_4^{2-} and selenite, SeO_3^{2-}) and organic (L-selenomethionine; SeMeth) selenium species and sulfate on Se uptake and volatilization (as dimethyl selenide) by plants (broccoli, Indian mustard, sugar beet, and rice), as well as on selenium speciation in plant tissues was reported by Zayed *et al.* (1998b). Selenium accumulation in shoots of Se-treated plants (1 week, 0.02 mM for each selenium species) was found to follow the order selenate > SeMeth > selenite, whereas for roots the order was SeMeth > selenite >

selenate. The rate of Se volatilization by plants followed the latter pattern with greater differences. X-ray absorption spectroscopic *in vivo* speciation analyses of treated plants showed that most of the uptaken SeO_4^{2-} remained unchanged with only a small portion being transformed into SeMeth-like species (in roots larger than in leaves), whereas plants treated with SeO_3^{2-} or SeMeth contained SeMeth-like species only. Sulfate (0.25 to 10 mM SO_4^{2-}) was found to inhibit SeMeth, SeO_3^{2-} , and SeO_4^{2-} uptake by 15–25%, 33%, and 90%, respectively; similar results were obtained with regard to sulfate effects on Se volatilization. Thus, in the latter process, the reduction step from selenate to selenite (i.e., $\text{Se}^{\text{VI}} \rightarrow \text{Se}^{\text{IV}}$) appears to be rate-limiting (Zayed *et al.* 1998b), and the inhibitory effect of sulfate on Se uptake and volatilization is evidently much lower for Se compounds with Se oxidation states lower than +6. Note that low soil moisture and the resulting plant water stress were shown to enhance the uptake of Se as a contaminant and several nutrient metals (Tennant and Wu 2000); however, reduction of plant growth was found to overcompensate this effect, so that water-stress conditions seem to be inappropriate for selenium phytoremediation in this case.

A similar speciation study of the location and nature of Cu species after Cu uptake by creosote bush (Polette *et al.* 2000) has shown that Cu^{II} taken up from soil is partly reduced to Cu^{I} upon its transport to the leaves by a phytochelatin; the plant was also found to be capable of immobilizing Cu in vacuoles and excreting it to the leaf surface into a sticky resinous substance. For water hyacinth (*Eichhornia crassipes*), a plant widely used for rhizofiltration of toxic metals from water, the large quantities of europium(III) accumulated from solution predominantly in its roots, after extraction by ethyl acetate from dried plant biomass, were studied using ^1H NMR and infrared spectroscopy (Kelley *et al.* 2000). The dried extract was shown to consist of Eu^{III} carboxylate complexes, suggesting that organic acids are responsible for binding the extractable (probably intracellular) Eu^{III} which could be used as a model for trivalent radionuclides.

In the presence of a mixture of different metals, which is more realistic in natural contaminated environment (Dahmani-Muller *et al.* 2000), the bioavailability of a certain metal may often be altered as compared to its individual behavior (Penttinen *et al.* 1998, Stewart 1999) as a result of their competitive sorption and/or binding to soil particles, humic substances and dissolved organic complexing agents (Yu *et al.* 1996, Echeverría *et al.* 1998). In this respect, the effect of complexation kinetics on the toxicity of metals in the presence of organic matter has recently been stressed (Ma *et al.* 1999). Note also that the genetic mechanism of resistance to (and uptake of) a particular heavy metal can be essentially affected by a combination of other metals (Okuyama *et al.* 1999). In the presence of multiple-metal

pollution, different heavy metal-uptake strategies of absolute metallophytes (plants growing on metal-rich soils only) and pseudometallophytes (plants capable of growing on both contaminated and non-contaminated soils) have recently been discussed (Dahmani-Muller *et al.* 2000). The authors proposed that for phytoremediation of soils polluted with multiple heavy metals, two absolute metallophytes could be used, the hyperaccumulator *Cardaminopsis halleri* (for Zn and Cd extraction) and *Armeria maritima* ssp. *halleri* (for Pb and Cu extraction), with harvesting at the end of the vegetative cycle.

It should specially be mentioned that one of the questions poorly understood so far is the role of plant root exudates in altering heavy metal mobilization and phytoavailability. In this respect, of interest is the recent comparative study of metal-mobilization capacities of root exudates from ecotypes of the Zn/Cd hyperaccumulator *Thlaspi caerulescens* and from two non-accumulators, wheat and canola (Zhao *et al.* 2001). The authors have shown that the root exudates from the hyperaccumulator plants do not significantly enhance mobilization of Zn and Cd (e.g., much less than wheat root exudates) and therefore are evidently not involved in heavy metal hyperaccumulation. A comparative physiological study of two ecotypes of *T. caerulescens*, significantly differing in terms of Cd accumulation (Lombi *et al.* 2000), using radioactive isotopes showed their difference only in Cd influx across the root plasma membrane, but not in other characteristics involved in Cd and Zn hyperaccumulation, such as sequestration of the metals in root cell vacuoles or in translocation of the metals from roots to shoots (Lombi *et al.* 2001). This result shows that screening for and selection of different ecotypes with higher phytoextraction efficiency for multiple heavy metals may be useful. From the mechanistic viewpoint, this result suggests the presence of one or more high-affinity Cd transporter system(s), different from that involved in Zn uptake, in the more efficient ecotype.

However, biological mechanisms of the underlying processes are still largely unknown. The majority of applications have so far been based on empirical observations of heavy metal uptake by certain plant species and their different parts (see, e.g., Zayed *et al.* 1998a, Angelova *et al.* 1999, Barman *et al.* 2000, Raghu 2001), though such detailed studies, if compared with concentrations of elements in the natural substrates, provide essential information on biological absorption coefficients (Raghu 2001) which reflect accumulation properties of different plant tissues towards a range of elements including heavy metals.

One largely unexplored option involves the effects of soil microbiota, and plant-associated bacteria in particular, on heavy metal speciation, phytoavailability, uptake, and toxicity to their host plants (for relevant reviews see White *et al.* 1997, 1998, van der Lelie *et al.* 2000).

The Role of Rhizosphere Microorganisms in Phytoremediation of Heavy Metals

Plant Growth-promoting Rhizobacteria (PGPR)

In soil and aquifers, the bacterial community is an integral natural active part of the biota. It has long been recognized that rhizosphere microorganisms, and plant root-associated bacteria in particular, are essentially stimulated by plant root exudates including a wide range of organic molecules which are used by microorganisms as nutrients. It is essential for bioremediation that planting of metal-contaminated and/or acidic soils increases their biological activity (Boon *et al.* 1998), especially for mineral soils with a low content of organic matter (Priha *et al.* 1999).

Plants, as well as their root exudates applied to unplanted soil, can foster degradative microorganisms (Shann 1995). In their turn, plant growth-promoting rhizobacteria (PGPR) stimulate plant growth and development owing to a range of factors (Glick *et al.* 1999). The latter include fixation of atmospheric nitrogen (Elmerich *et al.* 1998, James 2000), production of phytohormones (Costacurta and Vanderleyden 1995, Patten and Glick 1996), specific enzymatic activity (Shah *et al.* 1998, Belimov *et al.* 2001, Penrose *et al.* 2001); solubilization of inorganic phosphate (Vazquez *et al.* 2000, Rojas *et al.* 2001); plant protection from diseases owing to the synthesis of anti-fungal metabolites and other plant pathogen-depressing substances (Lugtenberg *et al.* 1991, Lugtenberg and Dekkers 1999). The latter include siderophores, specific low-molecular-weight iron(III)-chelating agents bearing largely catecholate or hydroxamate ligands (for review see Sigel and Sigel 1998). Siderophores, along with their crucial role in iron(III) sequestration from its hydroxo compounds virtually insoluble under physiological conditions, can also be involved in a relatively long-known biological defense mechanism making the chelated iron unavailable to pathogenic microorganisms (see, e.g. Emery 1980) owing to the absence of special cell surface receptor proteins in the latter (Braun 1997, Sigel and Sigel 1998). On the other hand, in such cases plant growth may additionally be stimulated owing to soil iron chelated by PGPR-produced siderophores (Kloepper *et al.* 1980) which is largely bioavailable to plants (Burd *et al.* 2000). There are quite a few reported cases of bacterial siderophore-mediated inhibition of iron uptake by plants (Becker *et al.* 1985) or bacterial degradation of siderophores (Winkelmann *et al.* 1999).

The overall result of plant-microbe interactions is a generally higher population density of bacteria and their higher metabolic activity in the rhizosphere (Lugtenberg and Dekkers 1999), even in polluted sites (van der Lelie 1998, Siciliano and Germida 1999, van der Lelie *et al.* 2000,

Kunito *et al.* 2001), as compared to the non-planted soil. Elevated concentrations of heavy metals can be generally toxic to many biological processes (for review see Wuertz and Mergeay 1997) including those catalyzed by soil microorganisms reducing their growth (Kelly and Tate III 1998), in particular, affecting their enzymic activity (Tam 1998, Bepalova *et al.* 1999), with the exception of metal-tolerant species (Stephen *et al.* 1999, Ivanova *et al.* 2001). Nevertheless, there has been certain evidence that heavy metals at lower concentrations, or simultaneous presence of organic matter (e.g., from sewage sludge), either exert no harmful effect on the bacteria (Chander and Brookes 1991, Tam 1998) or even stimulate soil microbiological activity. This might be due to an overcompensating effect of added organics and nutrients (Boon *et al.* 1998, Kelly and Tate III 1998) and thus be valid in the rhizosphere being induced by plant root exudates. Rather, bacterial growth might be more strongly affected by simply lowering the soil pH, and then this effect could be further enhanced in the presence of heavy metals (Boon *et al.* 1998).

Note, however, that a number of PGPR and other microorganisms have been reported to be resistant to relatively high concentrations of heavy metals (Nies 1999, 2000, Kunito *et al.* 2001) and remain active in moderately acidic soils (Belimov *et al.* 1998, Ivanov *et al.* 1999) which comprise over 30% of only arable territories (Von Uexkull and Mutert 1995). Such rhizobacteria could contribute to phytoremediation both indirectly, by increasing the overall fertility of the contaminated soil and facilitating plant growth, and, in certain cases of complex pollution, also directly, catabolizing certain organics and/or intermediate partly oxidized biodegradation products (Kamnev *et al.* 1999b, and references therein). Note also that, as shown by Ratti *et al.* (2001), joint inoculation of plants with arbuscular mycorrhizal fungi and phosphate-solubilizing and nitrogen-fixing bacteria may be beneficial in low-phosphate alkaline soils amended with insoluble $\text{Ca}_3(\text{PO}_4)_2$ which is otherwise not used by the plant.

The Role of Exogenous Indole-3-acetic Acid (IAA) in Plant Stimulation

As an example of an alternative role of a well-known plant growth regulator, indole-3-acetic acid (IAA), this phytohormone of the auxin series produced and excreted also by many soil bacteria (Patten and Glick 1996), as well as its metabolically related precursor, anthranilic acid, are capable of reductive solubilization of soil iron(III), thus increasing its bioavailability via a mechanism different from that involving siderophores (Kamnev 1998, Kamnev *et al.* 1999d, 2001a) and contributing to the overall pool of generally more bioavailable and reactive iron(II).

It has long been known that IAA in low concentrations is one of the best plant growth stimulators. In plant-microbe interactions, IAA has recently been proposed to serve as a reciprocal signaling molecule between micro- and macro-partners of the association (Lambrecht *et al.* 2000). Its stimulating effect includes, in particular, an enhanced development of the plant root system and an increased uptake of nutrients and other soil components.

Thus, according to the experimental data reported by Leinhos and Bergmann (1995) and Lippmann *et al.* (1995), addition of IAA to soil, in a way similar to soil inoculation with some PGPR producing this auxin, resulted in an enhanced, virtually non-selective uptake of a number of elements (Zn, Fe, Mg, Ca, K, and P) in plant roots. This simple method of non-selective plant uptake stimulation by this phytohormone, applied as a dilute solution (10^{-4} to 10^{-2} mg/L), has recently been used (Zakharova *et al.* 2000) for increasing the uptake of sulfur-containing breakdown products of yperite, $S(CH_2CH_2Cl)_2$ (sulfur mustard, or mustard gas, a chemical warfare agent), in oat (*Avena sativa* L.) from soil. Whereas the uptake level of S-containing substances (SCS) in plant biomass was indeed reported to be noticeably (2.7 to 2.9-fold) higher after IAA application, the percent of SCS removed from the contaminated soil even for untreated plants was already ca. 86%, and it further increased up to ca. 96% after treatment with IAA solution. Owing to the evident simplicity and inexpensiveness of IAA application and its availability as a reagent, stimulation of plant growth and non-selective uptake using this phytohormone may give better results in phytoremediation of heavy metal-contaminated sites by phytoextraction and is therefore definitely worth studying. In addition, it should also be mentioned that IAA may be involved in plant resistance to phytopathogenesis (Noël *et al.* 2001), so that its controlled application could be beneficial also in this respect.

The Role of 1-aminocyclopropane-1-carboxylate (ACC) Deaminase Activity in PGPR

Under stress conditions, including stress induced by heavy metal toxicity, the concentration of endogenous ethylene, an essential plant hormone, in plant tissues significantly increases (Solano and Ecker 1998, Grichko and Glick 2001a,b). This usually results in the inhibition of seed germination and root growth, especially in plant species with an enhanced ethylene sensitivity (mainly dicot plants). It has been found that application of certain chemical inhibitors of ethylene biosynthesis (e.g., aminoethoxyvinylglycine, AVG; silver (Ag^+) ions) can suppress the negative effects of stress-induced ethylene, thus reducing the expression of stress symptoms in plants, eliminating the wounding effect and even decreasing disease development (see, e.g., Belimov *et al.* 2001, and references therein).

Some soil bacteria are capable of reducing the level of stress ethylene in plants as a result of cleaving its immediate precursor, 1-aminocyclopropane-1-carboxylate (ACC), which is exuded from plant roots or seeds (Glick *et al.* 2001, Penrose and Glick 2001, Penrose *et al.* 2001), to α -ketobutyrate and ammonium, using the enzyme ACC deaminase. An interesting example of a PGPR, *Kluyvera ascorbata* SUD165, resistant to a range of heavy metals was reported (Burd *et al.* 1998) to protect plants from nickel toxicity without affecting nickel uptake by seedlings or its accumulation in roots and shoots. Rather, the plant growth-promoting effect in the presence of Ni may be attributed to the ACC deaminase activity (Shah *et al.* 1998) of this bacterium lowering the level of stress ethylene in plants induced by the uptaken Ni (Burd *et al.* 1998). At the same time, ACC deaminase-deficient mutant bacterial strains do not stimulate plant root elongation (Li *et al.* 2000, Belimov *et al.* 2001, and references therein).

A spontaneous siderophore-overproducing mutant of the aforementioned bacterium, *K. ascorbata* SUD165/26 (Burd *et al.* 2000), was shown to be even more efficient in enhancing plant growth in the presence of high levels of heavy metals by combining the ACC deaminase activity and a high level of siderophores. Considering the colonization patterns of the bacterium which was shown to tightly attach to the surface of roots and seeds forming aggregates (Ma *et al.* 2001), *K. ascorbata* SUD165/26 additionally provides an improved immediate supply of iron to the plant regardless of the presence of other heavy metals which otherwise induce iron deficiency in plants.

A number of bacterial strains assigned to various species of the genera *Alcaligenes*, *Bacillus*, *Pseudomonas*, *Rhodococcus*, and *Variovorax*, which exhibited ACC deaminase activity in cell-free extracts of their ultrasonically disrupted cells, have recently been isolated from the rhizosphere of Indian mustard (*Brassica juncea* L.) and pea (*Pisum sativum* L.) grown in different soils, as well as in a long-standing sewage sludge, contaminated with a range of heavy metals including Zn, Cu, Ni, Cr, Cd, and Pb (Belimov *et al.* 2001). Some of the strains, that were all tolerant to Cd toxicity, were also found to produce indoles, exhibit phosphate-solubilizing activity and stimulate root elongation of Indian mustard and/or rape (*Brassica napus* var. *oleifera* L.) upon inoculation (Table 3). It should be noted that even those ACC deaminase-containing strains which produced virtually no indoles and did not solubilize inorganic phosphates (e.g., *Variovorax paradoxus*) still promoted plant root elongation.

Lack of ACC deaminase activity in some plant-stimulating rhizosphere bacteria has prompted a way to improve their plant growth promoting activity by transferring ACC deaminase genes from other bacteria. The expression of ACC deaminase in the PGPR *Azospirillum brasilense* (after transformation with a plasmid containing an ACC deaminase gene from

Table 3. Examples and characterization of PGP/R strains, isolated from different heavy metal-contaminated soils, exhibiting ACC deaminase activity and plant root elongation-promoting (PREP) activity (Belimov *et al.* 2001)

Bacterial strain ^a	ACC deaminase activity (µmol NH ₄ ⁺ g ⁻¹ h ⁻¹)	PREP activity ^b (%)		Indole production ^c (mg IAA/L)	Phosphate solubilization activity ^d (mm)	Tolerance to cadmium ^e (mM CdCl ₂)
		Indian mustard	Rape			
<i>Alcaligenes</i> sp. Cm1	1.17	-2	+21*	12	4, 1, 5	0.21/0.45
<i>A. xylosoxidans</i> Cm3	0.56	+22*	+44*	60	0, 0, 0	0.21/0.50
<i>Bacillus pumilus</i> Pp1	0.76	+7	+22*	0	3, 0, 5	0.60/1.00
<i>Pseudomonas</i> sp. Dp2	0.96	+17*	+29*	0	11, 1, 12	0.21/0.45
<i>Pseudomonas</i> sp. Ep2	0.54	-13	+20*	14	7, 1, 20	0.24/0.75
<i>P. brassicacearum</i> Am3	0.97	+20*	+21*	0	6, 2, 10	0.24/0.50
<i>P. marginalis</i> Dp1	1.07	-2	+30*	0	3, 4, 2	0.21/0.50
<i>P. oryzae</i> Ep4	0.89	+16*	+22*	65	3, 1, 7	0.21/0.45
<i>P. putida</i> Am2	0.54	-2	+28*	9	11, 0, 15	0.21/0.45
<i>Variovorax paradoxus</i> Bm2	0.21	-3	+26*	0	0, 0, 0	0.50/0.75
<i>V. paradoxus</i> Ep1	1.80	+36*	+32*	0	0, 0, 0	0.40/0.95

^aDesignation of strains: the capital letter identifies the soil type (A, peaty-gleyic, pH_{KCl} 5.3; B, sod-podzolic sandy, 4.6; C, sod-podzolic gleyic loamy, 8.2; D, sandy podzol, 7.0; E, sewage sludge, 7.2; F, post-glacial clay, pH_{KCl} 5.3) and the small letter – the plant species (m, mixture of pea and Indian mustard; p, pea) used for isolation of the strain. ^bPercent increase (+) or decrease (-) in the root length upon inoculation (100% for the uninoculated controls is 85 mm for Indian mustard and 78 mm for rape); asterisk denotes a statistically significant difference from the control. ^cSpectrophotometric analysis with Salkovsky's reagent. ^dDiameters (mm) of the PO₄³⁻-solubilization zone [for Ca₃(PO₄)₂, FePO₄, Ca phytate] developed around bacterial colonies. ^eThreshold growth-inhibitory / minimum lethal concentration of Cd in the nutrient medium.

Enterobacter cloacae under the control of the *lac* promoter from *E. coli*) has been shown to result in a significant elongation of seedling roots of tomato and canola plants inoculated with the transformed bacteria (Holguin and Glick 2001). A much weaker effect of the transformed *A. brasilense* inoculants found in wheat was ascribed to its weaker sensitivity to ethylene as compared to canola or tomato plants. A similar genetic transformation in *Pseudomonas fluorescens*, a root-colonizing bacterium with a broad spectrum of biocontrol activity against plant diseases, resulted not only in increasing plant root length, but also showed a partly improved disease-suppressive capacity in some of the tested plants (Wang *et al.* 2000). Nevertheless, it has been concluded that the role of stress-induced ethylene in plant susceptibility or resistance depends on the host-pathogen system as well as on the experimental conditions applied. The authors also noted that the genetically transformed strains of *P. fluorescens* expressing ACC deaminase activity could be developed for assessing the role of ethylene in plant diseases.

A promising alternative might be the expression of bacterial ACC deaminase genes in heavy metal-accumulating plants, in addition to other novel traits that can be introduced into plants in a transgenic approach (Krämer and Chardonnens 2001). As was reported recently for transgenic non-hyperaccumulator tomato plants (Grichko *et al.* 2000), the expression of this enzyme (from *E. cloacae*) resulted in constant stimulation of plant growth (with a higher total amount of accumulated metals), in many cases in an increased metal uptake, and in some instances in an increased shoot-to-root metal ratio. In addition, the transgenic tomato plants were found to be protected to some extent against the inhibitory effects of fungal pathogens and flooding stress, being less subject to deleterious effects of root hypoxia (Grichko *et al.* 2000, Grichko and Glick 2001c). This approach may appear especially successful with other ethylene-sensitive plants that have been used for phytoremediation, e.g. Indian mustard (*Brassica juncea*).

Dissimilatory Redox Processes Involving Heavy Metals

In addition to biotic (enzymatic) reduction by bacteria-reducers, humic substances, as well as their lower-MW precursors, are able to abiotically (chemically) reduce e.g., Cr^{VI} to Cr^{III} (Nakayasu *et al.* 1999) which is regarded as much less toxic and less mobile (however, see the above discussed recent data of Srivastava *et al.* 1999, as well as the impact of Cr^{III} on the fluidity of cell membranes reported by Pesti *et al.* 2000). Just owing to the presence of quinone/hydroquinone moieties, humic substances can also serve as an electron shuttle between the humics-reducing microorganisms and iron(III) oxyhydroxides or other environmentally significant electron acceptors in soil (Lovley *et al.* 1999) essential under

limited availability of oxygen. Note that microbial reduction of soil ferric oxyhydroxides to Fe^{2+} ions may be used as a parameter of soil microbial activity to monitor microbial toxicity of heavy metals in different soils (Welp and Brummer 1997). The value of the specific rate (on the molar basis) of dissimilatory Fe^{III} reduction was found (Chidthaisong and Conrad 2000) to be ca. 3.6-fold and ca. 7.2-fold higher than that of nitrate and sulfate, respectively. Considering the number of electrons required for the reduction, these data demonstrate that the $\text{Fe}^{\text{III}} \rightarrow \text{Fe}^{\text{II}}$ reaction ($1e^-$) can be ca. twice as efficient as $\text{NO}_3^- \rightarrow \text{NO}_2^-$ ($2e^-$) or approximately equal to any of the overall processes $\text{SO}_4^{2-} \rightarrow \text{S}^0$ (total $6e^-$) or $\text{SO}_4^{2-} \rightarrow \text{S}^{2-}$ (total $8e^-$).

In clay minerals, structural ferric iron available for dissimilatory microbial reduction is very reactive and capable of extensive *in situ* redox cycling (Ernstsen *et al.* 1998) different from that in free Fe oxides which involves reductive dissolution and further Fe relocation. In certain cases, iron(III) reduction coupled to oxidation of organics can be identified in a coculture of syntrophic bacteria-partners, neither of which taken separately are capable of any redox activity (Daniel *et al.* 1999). It is essential that the resulting pool of iron(II) can abiotically reduce toxic and mobile selenate(VI) to less bioavailable and less toxic species (Brown *et al.* 1999), as well as contribute to reductive degradation of chlorinated and nitroaromatic organics (Kamnev *et al.* 1999b).

Dissimilatory bacterial reduction of gold in a catalytic cycle coupled to methane oxidation has been reported recently for *Micrococcus luteus* (Levchenko *et al.* 2001) which involves the $\text{Au}^{\text{III}} \rightarrow \text{Au}^{\text{I}}$ step with subsequent oxidation of Au^{I} by iron(III) with dioxygen back to Au^{III} . Nevertheless, the formation of colloidal gold particles after its complete reduction to Au^0 by a number of microorganisms, including dissimilatory Fe^{III} -reducing bacteria and archaea (Karamushka *et al.* 1990, Langley and Beveridge 1999, Kashefi *et al.* 2001), has long been known since it was predicted by the Russian academician V.I. Vernadsky (see, e.g. Marakushev 1991) as a way of the formation of gold metal deposits.

In contrast, the first evidence for culturable Fe^{II} -oxidizing bacteria, both acidophilic and neutrophilic (pH 3.5 to 6.8), associated with Fe^{III} oxyhydroxide precipitates (Fe-plaque) coating plant root surfaces in the rhizosphere of wetland plants has recently been reported (Emerson *et al.* 1999). The activity of such microbes contributing to the precipitation of Fe-plaque may result in immobilization of other heavy metals, which readily coprecipitate with ferric oxides.

The role of bacterial siderophores as potential environmentally friendly iron(III) carriers (instead of synthetic chelators like EDTA, NTA, etc.) for other dissimilatory iron(III)-reducing bacteria, which couple Fe^{III} reduction to oxidation of organic contaminants, has been proposed (Kamnev *et al.* 1999b). In this respect, it should be noted that, as mentioned above,

synthetic chelating agents (e.g., EDTA, HEDTA, NTA) may often be highly effective in increasing the solubility and phytoavailability of a range of heavy metals (Blaylock *et al.* 1997, Huang *et al.* 1997, Raskin *et al.* 1997, Ebbs and Kochian 1998, Khan *et al.* 2000, Stanhope *et al.* 2000, Chen and Cutright 2001, Sarret *et al.* 2001), reducing also the toxicity of the latter (and selenium) to microorganisms (Lee *et al.* 1999, and references therein). The role of phytochelators in the above processes has also been emphasized (Khan *et al.* 2000). These effects may be useful for phytoextraction and/or phytomining but evidently inappropriate for planting crops.

The Role of Microorganisms in Altering Heavy Metal Bioavailability and Phytoavailability

Since the microbial soil community can alter the soil composition and properties in the course of metabolic processes, in particular by biodegrading and mineralizing organic matter (Fass *et al.* (Eds.) 1999, Muratova and Turkovskaya 2001), it is clear that heavy metal phytoavailability can largely be affected by soil microbiota as well (Gobran *et al.* (Eds.) 2001). Both directions of this effect may in principle be useful. Increasing heavy metal phytoavailability may be beneficial for phytoextraction and recovery (phytomining) using (hyper)accumulating plants, whereas an essential reduction in toxic metal phytoavailability (phytostabilization) may be promising for planting cultural crops.

Production of organic acids by soil fungi (Gadd 1999) and bacteria, including rhizobacteria (Goldstein *et al.* 1999, Nautiyal *et al.* 2000), may promote solubilization, mobility, and bioavailability of metals and accompanying anions (e.g. phosphate) by lowering the pH and supplying metal-complexing organic acid ligands. As a result, the heavy metal contamination may be essentially affected in the rhizosphere. For instance, for ditch reed (*Phragmites*) grown in a highly Cu-contaminated soil near a copper mine with the total copper content ca. 5.8 mg per gram of soil, in the rhizosphere the Cu content was found to be 8 times lower, which was also accompanied by an increased production of bacterial exopolymers (Kunito *et al.* 2001).

Such microbially driven processes are essential also for mineral weathering (Barker *et al.* 1998, Banfield *et al.* 1999), which results in the formation of sediments and soils, supplying both nutrients and, on the other hand, toxic heavy metal constituents released from the dissolving minerals, increasing their bio- and phytoavailability and thus affecting the fertility of soils. For instance, the formation of the poorly soluble and geochemically stable lead phosphate minerals pyromorphite $Pb_5(PO_4)_3OH$ or chloropyromorphite $Pb_5(PO_4)_3Cl$ has been proposed upon application of more soluble non-toxic metal phosphates as an *in situ* remediation

strategy (Traina and Laperche 1999, Stanforth and Qiu 2001). However, pyromorphite has recently been shown to be solubilized by organic-acid-producing fungi (e.g., *Aspergillus niger*), so that plants grown with pyromorphite as a single P source took up both P and Pb (Sayer *et al.* 1999). Such processes caused by soil microbiota which can enhance the low bioavailability of Pb phosphates and other insoluble minerals should not be underestimated in model phytoextraction studies that put emphasis on plant and soil parameters (Brennan and Shelley 1999). Moreover, Pb solubility, which was found to be much higher in the alkaline pH range even in the presence of soluble phosphate additions, in heavily contaminated soil appeared to be controlled by desorption reactions from hydrous metal oxides rather than by dissolution of a separate individual lead mineral (Stanforth and Qiu 2001).

Note that some phosphate-solubilizing bacteria have been shown (Nautiyal *et al.* 2000) to be capable of solubilizing fixed (Ca-bound) forms of phosphorus in alkaline soils even under stress conditions (10% salt, pH 12, or 45°C). Similarly, naturally occurring rhizobacteria from the rhizosphere of wetland plants were found to promote Se and Hg accumulation in plant tissues (de Souza *et al.* 1999), which could be utilized owing to the increased efficiency of phytoextraction.

In mineral weathering, a complex role is played by microbial polysaccharides and proteinaceous structures (Banfield *et al.* 1999, Welch *et al.* 1999), which can either inhibit mineral dissolution under some conditions by forming biofilms or promote chemical weathering by producing extracellular polymers of acidic nature. Capsule polysaccharides or slimes, known to play an antidesiccant role in microorganisms, can also help retain water at the mineral surface facilitating hydrolysis reactions and maintaining diffusion pathways, which is essential at a reduced water potential (Barker *et al.* 1998, Welch *et al.* 1999). Langley and Beveridge (1999) showed using *Pseudomonas aeruginosa* PAO1 and its three isogenic lipopolysaccharide (LPS) mutants that certain combinations of the two different LPSs (A and B), including the acidic B-type, both of which were produced by the parent strain (A⁺B⁺), controlled the precipitation of essential amounts of Fe (ca. 9.1% of dry cells; A⁺B⁺ mutant) and La (ca. 3.2% of dry cells; A⁺B⁻ mutant) on the cell surface, whereas Cu was bound at the cell surface of all the four strains (ca. 1.4% of dry cells), assuming common surface functional groups are responsible for Cu binding. It was also shown for the first time for Gram-negative cells that gold metal was precipitated as intracellular elemental crystals in all the four strains suggesting that in *P. aeruginosa* gold binding and reduction are not surface-mediated events, in contrast to, e.g. membrane-bound processes occurring in *Bacillus subtilis* (Karamushka *et al.* 1990) or *Micrococcus luteus* (Levchenko *et al.* 2001).

An interesting process leading to cadmium immobilization in CdS crystallites, which could be formed involving cysteine-containing proteins, and further reduction to Cd metal when exposed to light, was reported for a cyanobacterium (Bekasova *et al.* 1999). It is assumed that CdS, being a photochemically active semiconductor, participates in the formation of Cd⁰ as a photosensibilizer absorbing light and forming e^- -donor and e^- -acceptor centers which can be involved in redox reactions with components of the environment.

On the other hand, it was recently found that certain rhizobacteria promoted precipitation of Cd compounds on the plant root surface, thus reducing the amount of bioavailable Cd uptaken by roots and enhancing their growth (Salt *et al.* 1999). For two endophytic bacteria (colonizing the interior of the host plant roots), *Bulkholderia cepacia* and *Herbaspirillum seropedicae*, the heterologous expression of the *ncc-nre* encoded Ni resistance system (from heavy metal-resistant bacterium *Ralstonia metallidurans*) resulted in their capability to remove 35% and 15% Ni, respectively, from the culture medium (Lodewyckx *et al.* 2001). As a consequence, the inoculation of *Lupinus luteus* L. with the recombinant *B. cepacia* strain resulted in a 30% increase of Ni concentration in the roots, while Ni concentration in the shoots remained similar to that of the control plants. This result is of interest demonstrating the capability of the recombinant endophytic bacteria to sequester Ni, e.g. by bio-precipitation, thus lowering its bioavailability to the host plant.

Moreover, excretion of siderophores by soil bacteria, owing to their lower but still significant affinity to heavy metal ions other than iron(III), can affect heavy metal bioavailability both to bacteria and to plants (van der Lelie 1998). In the metal-tolerant bacterium *Ralstonia eutropha* CH34, a novel siderophore (named alcaligin E) was found to bind Cd and, via internalization of the resulting complex by outer membrane receptor proteins, to induce *czc*-mediated proton antiporter efflux of Cd, which is then precipitated in the form of phosphate (Gilis *et al.* 1998) or carbonate (Diels *et al.* 1995, Nies 2000) owing to a gradual alkalization of the medium caused by proton influx, thus immobilizing and excluding Cd from metabolism. The microbially controlled formation of phosphate minerals, e.g., of a struvite modification associated with slime in the culture medium of a PGPR *Azospirillum brasilense* (Kamnev *et al.* 1999a), as well as other minerals in natural microbial communities (Douglas and Beveridge 1998, Lins and Farina 1999) can similarly lead to the immobilization of heavy metals entrapped within the insoluble crystalline and/or amorphous phases. It is clear, however, that this situation can be stable until the soil properties and/or its metal-immobilizing microbial community are essentially altered. Moreover, though amorphous phosphorus-rich minerals of polyphosphate nature, which can be present as intracellular granules in

bacteria, tend to incorporate much more foreign metal ions (including heavy metals) than crystalline materials do (Lins and Farina 1999), they are usually less stable for dissolution after cell death, thus releasing the foreign cations.

As an example of a non-bacterial microorganism, *Euglena mutabilis*, a unicellular protozoan (commonly referred to as an alga) highly tolerant to elevated total dissolved solids (up to 18 g/L) and acid conditions (to pH 1.7), has recently been reported to be capable of strongly influencing environmental conditions in acid mine drainage (Brake *et al.* 2001). In areas of its prolific growth it was shown to contribute to up to 3-fold oversaturation of dissolved oxygen; also it was suggested to sequester iron, and possibly other metals, in intracellular crystalline-like structures similar in color to iron oxyhydroxides.

As a practical approach in developing a novel plant-bacterium remediation system for heavy metals, we note a recent communication by Murooka *et al.* (1999) who genetically modified a well-known rhizobium-legume symbiosis by designing the genes for human metallothioneins successfully expressed in bacteria.

General Conclusions and Outlook

"A single bracelet does not jingle" (an old African proverb)

Despite some evident progress, the mechanisms of heavy metal uptake by plants (McGrath *et al.* 2001) and speciation of metal compounds in the environment (Szpunar and Łobiński 1999, Łobiński 2001), which is closely related to metal bioavailability (including phytoavailability), are still largely unknown, and the essential role of soil bacteria in altering heavy metal phytoavailability remains to be investigated in detail (Lanza 1999, Mulligan *et al.* 2001). The need for a deeper basic research in various branches of this interdisciplinary field, along with applied projects, has been specially emphasized in the recent years (Bouma 1998, Glass 1999, Kamnev and van der Lelie 2000, McGrath *et al.* 2001). Among the recommendations directed toward further basic research, aimed at understanding the mechanisms that underlie the biological processes central to phytoremediation, the following should be primarily addressed (Glass 1999, McGrath *et al.* 2001):

- mechanisms of heavy metal uptake, transport and accumulation in plant tissues;
- the role of both natural and artificial metal chelating agents and their metal complexes, their dynamics and decomposition both in the rhizosphere and in plant tissues;
- approach to a better understanding of interactions in the rhizosphere among plant roots, microorganisms, and other biota.

The aforementioned approaches, aimed at understanding the underlying mechanisms both on the molecular and cellular levels, require the application of modern powerful physicochemical techniques, along with biochemical and microbiological methods. This implies close collaboration between a wide range of specialists in plant physiology, microbiology, biochemistry, and chemistry – those “bracelets” which could “jingle” only in concert providing for sound results in the multidisciplinary fields of bio- and phytoremediation.

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Summary

In this chapter, an overview is given of the recent approaches considered or used for plant-assisted bioremediation of heavy metal contaminated soils and aquifers. Besides metal-tolerant hyperaccumulator plants, both those occurring in nature and genetically engineered (transgenic or mutant) plant species, special fast-growing crop plants giving high biomass yields can be used for phytoextraction. In the latter case, soil amendment with metal chelating agents can often be useful. A range of interrelated chemical and biological parameters are also discussed that strongly influence the chemical speciation of heavy metals, the level of their biological availability to both plants and plant-associated rhizosphere microorganisms, and, consequently, the possibilities to use plant-based bioremediation (phytoremediation) as an eco-friendly, relatively cheap and sustainable clean-up technology for heavy metal polluted sites. First, in order to assess heavy metal bioavailability, rapid and cost-effective systems are necessary that can reliably predict this parameter and, based on this, the feasibility

of using certain biological remediation techniques and strategies for site management and restoration. Second, in order to enhance the efficacy of phytoremediation of heavy metal polluted sites, a range of important parameters relevant to the process have to be taken into account. Besides the chemical forms of heavy metals and their bioavailability within the polluted site, the role of plant-associated rhizosphere bacteria and fungi in phytoremediation can in many cases be of special importance. On the one hand, they can directly influence the aforementioned parameters of heavy metal species and, on the other hand, they are capable of stimulating plant growth and development, thus indirectly contributing to the phytoremediation process.

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