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REVIEW

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## Chemical and Biological Parameters as Tools to Evaluate and Improve Heavy Metal Phytoremediation

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In this review, chemical and biological parameters are discussed that strongly influence the speciation of heavy metals, their availability to biological systems and, consequently, the possibilities to use bioremediation as a cleanup tool for heavy metal polluted sites. In order to assess heavy metal availability, a need exists for rapid, cost-effective systems that reliably predict this parameter and, based on this, the feasibility of using biological remediation techniques for site management and restoration. Special attention is paid to phytoremediation as an emerging technology for stabilization and remediation of heavy metal pollution. In order to improve phytoremediation of heavy metal polluted sites, several important points relevant to the process have to be elucidated. These include the speciation and bioavailability of the heavy metals in the soil determined by many chemical and biological parameters, the role of plant-associated soil microorganisms and fungi in phytoremediation, and the plants. Several options are described how plant-associated soil microorganisms can be used to improve heavy metal phytoremediation.

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**KEY WORDS:** Heavy metals; phytoremediation; bioremediation; bioavailability; chemical availability; soil microorganisms; plant-microbe interactions.

### INTRODUCTION

Accumulation of heavy metals in the environment and particularly in 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 growing environmental concern. At present, such heavy metals and metalloids as arsenic (first place), lead (second place), mercury (third place) and cadmium (seventh place) are present in the top ten of the 1999 priority hazardous substances

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<sup>4</sup>I dedicate this article to my former teachers from Saratov Secondary School No. 42 of Extensive English Learning, Sofia E. Rapoport who, for 9 years up to 1975, skilfully and tactfully taught us to understand, feel and use the English language, and Gennadii V. Alexeyev who, now also 25 years ago, combined the feeling of, and the love to the language with our capability of comprehending and expressing scientific and technical ideas in English.

list as provided annually by the American Agency for Toxic Substances and Disease Registry (ATSDR). In contrast to organic contaminants, which can undergo biodegradation as a result of the activity of soil microorganisms or abiotic factors (for review see Fass *et al.*, 1999), giving less toxic, less mobile and/or less bioavailable products strongly adsorbed onto fine particles, organic matter and in micropores of various soils (Angehrn *et al.*, 1998; Loser *et al.*, 1999) or minerals (Bertsch and Seaman, 1999), heavy metals remain in the environment, and their speciation and bioavailability might change over time. Moreover, in many cases organic contaminants can be completely mineralized to CO<sub>2</sub> and H<sub>2</sub>O in the course of oxidative biodegradation, though certain substituted organic contaminants require anaerobic or combined anaerobic plus aerobic degradation. On the contrary, toxic heavy metals always have to be either 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 review see Lovley and Coates, 1997; White *et al.*, 1997; Stephen and Macnaughton, 1999). The aim of the present review is to draw attention to the role played by soil microorganisms in the processes underlying plant-based methods of remediation (phytoremediation) of heavy metal contaminations, including phytostabilization (in combination with soil additives) and phytoextraction (for recent review of various particular aspects of heavy metal phytoremediation and their practical applications see Chaney *et al.*, 1997; Raskin *et al.*, 1997; Vangronsveld and Cunningham, 1998; Gleba *et al.*, 1999; Khan *et al.*, 2000).

## GENERAL STRATEGIES FOR HEAVY METAL BIOREMEDIATION: PLUSES AND MINUSES

### Heavy Metal Immobilization in Soil: The “Time Bomb Hypothesis”

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, possibly accumulating elsewhere. 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) 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, 1998). 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, 1998a).

The effects of heavy metal immobilization on bacterial populations was examined for zinc contaminated soils (Vangronsveld *et al.*, 2000). For untreated soils, a strong increase in the metal resistant subpopulations was observed with increasing metal contamination. Twelve months after the application of the soil additives, up to a 100-fold decrease in the heavy metal resistant subpopulations was observed for beringite and beringite plus steelshots treated soils. However, treatment with other soil additives resulted in hardly any decrease of the heavy metal resistant subpopulations. It was hypothesized that the observed decrease in heavy metal resistant bacterial subpopulations was directly reflecting the efficacy of the *in situ* metal immobilization. Further physicochemical and biological evaluation confirmed the sustainability of zinc and cadmium immobilization by beringite and beringite plus steelshot treatment, while the inefficient heavy metal immobilization by the other soil additives was also confirmed. This shows that the ecology of heavy metal resistant soil bacteria, 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. 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 its effects on soil microorganisms, the toxicity of high concentrations of heavy metals has 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).

### Chemical and Biological Availability of Heavy Metals in Soil

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 phytoremediation 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 phytoextraction, where information on the plant-available fraction is a prerequisite to predict the efficiency of the remediation (Robinson *et al.*, 1999). 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 alternatives 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<sup>®</sup> assay. The advantages of these bioassays are summarized in Table 1.

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 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

**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				
Mineralization of organic nitrogen	High	High	High	Low
<i>Ex-situ</i> assays				
Lux-based systems	High	High	High	High
Microtox <sup>®</sup>	High	High	Medium	High

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.* (2000) 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 the 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, Knight *et al.* (1998) found that concentrations of free Cd<sup>2+</sup> ions (commonly regarded as more bioavailable as compared to its complexed forms) in extracts from CdSO<sub>4</sub>-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<sup>2+</sup> 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, just owing to the accumulation of heavy metals in soils fertilized repeatedly with sewage sludges over prolonged periods (McBride, 1998).

### **Phytoremediation of Heavy Metals as an Approach to soil Bioremediation**

In view of the "Time Bomb Hypothesis", the most appropriate and satisfactory solution to the problem of heavy metal pollution is an essentially complete or at least significant removal of metals from the contaminated site. However, for such an extremely complex system as soil this task is not at all easy and implies taking into account a large 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* are very

expensive and 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.

A very promising, environmentally friendly and cost-effective alternative is plant-based heavy metal bioremediation, 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). *Phytoremediation* as a general strategy includes several subsets. Among these is the use of metal-accumulating plants to remove heavy metals from soil (*phytoextraction*, which commonly implies accumulation of uptaken metals in top parts of the plant; also used for *phytomining*) or from contaminated waters (*rhizofiltration*, which implies high root uptake of (or affinity for) metals and does not require efficient translocation of metals from roots to shoots necessary for phytoextraction). In this case, plants with high-surface "hairy root" systems are promising, considering also their high growth rates and biochemical stability (Shanks and Morgan, 1999; Nedelkoska and Doran, 2000). For Hg and Se, phytoextraction of their compounds from soil accompanied by their enzymatic reduction to volatile species (e.g., Hg<sup>0</sup> and dimethylselenide) with further emission to the atmosphere (*phytovolatilization*) offers great promise for commercial development and application (Chaney *et al.*, 1997; Leonard *et al.*, 1998, 1998a). Note that mechanisms of bacterial resistance to mercury are also based on enzymatic conversion of Hg<sup>2+</sup> to Hg<sup>0</sup> 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. Plant-based methods of elimination of metal toxicity and bioavailability (particularly for Pb and Cr<sup>6+</sup>) in soil are commonly referred to as *phytostabilization* (Salt *et al.*, 1995; Chaney *et al.*, 1997). The growing importance of this rapidly developing interdisciplinary field is represented by a brand new scientific periodical, the *International Journal of Phytoremediation* (CRC Press LLC), launched in 1999 (Lanza, 1999).

### Metal Hyperaccumulation and Tolerance in Plants

Some very interesting examples of metal-hyperaccumulating plants, among ca. 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.*, 1997a; Zayed *et al.*, 1998; Nedelkoska and Doran, 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. Most of the known metal-hyperaccumulating plant species are metal-selective (thus providing only partial solution to the more common problem of complex heavy metal contamination), about three fourths of all metal hyperaccumulators "specializing" on nickel (Krämer *et al.*, 1996). Their growth rate is usually low, and they produce relatively small amounts of biomass. In addition, virtually no agricultural knowledge for them is available; moreover, most of them 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) in suitable agricultural plant species (crops).

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) (Prasad, 1998; Vařák, 1998; Briat and Lebrun, 1999). 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 *Altyssum 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 defence mechanism at low toxic levels (Kumar and Prasad, 1999) prior to phytochelatin synthesis (Patra and Panda, 1998; Briat and Lebrun, 1999).

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 phytochelatins (Zhu *et al.*, 1999, 1999a) 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.

### Phytoavailability of Heavy Metals

For heavy metal phytoremediation (and 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.*, 1999; 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.*, 1999).

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, including the effects of various conditions and additives of both organic and inorganic composition. Thus, 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  $\text{CaCO}_3$ , 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  $\text{CaCO}_3$  dissolution and 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).

Organic acids were recently shown to significantly enhance  $\text{Cr}^{\text{III}}$  uptake from sand and soil by maize (*Z. mays*) plants, whereas such amendments did not appear to markedly affect chromium accumulation from  $\text{Cr}^{\text{VI}}$  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 regarded as less mobile. 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 overcompensated this effect, so that water-stress conditions seem to be inappropriate for selenium phytoremediation in this case.

Moreover, in the presence of a mixture of different metals, which is more realistic in natural contaminated environment, the bioavailability of a certain metal may often be altered as compared to its individual behaviour (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; Echeverria *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).

However, biological mechanisms of the underlying processes are still largely unknown, and the majority of applications have so far been based on empirical observations of heavy metal uptake by certain plant species and their different parts (Zayed *et al.*, 1998; Aangelova *et al.*, 1999). 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).

## THE ROLE OF SOIL MICROORGANISMS IN PHYTOREMEDIATION

### Plant Growth-Promoting Rhizobacteria

In soil, the bacterial community is an integral natural active part of the biota. It has long been recognized that soil 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 owing to a range of factors including atmospheric N<sub>2</sub> fixation (Elmerich *et al.*, 1998; James, 2000), phytohormone production (Costacurta and Vanderleyden, 1995), specific enzymic activity (Shah *et al.*, 1998); 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). These include siderophores, specific iron(III)-chelating agents which make the chelated iron unavailable to pathogenic microorganisms owing to the absence of special cell surface receptor proteins in the latter (Emery, 1980; Briat, 1992; Braun, 1997), thus in most cases enhancing plant growth (Kloepper *et al.*, 1980), with quite a few reported cases of bacterial siderophore-mediated inhibition of iron uptake by plants (Becker *et al.*, 1985) or bacterial degradation of siderophores (Winkelman *et al.*, 1999).

As an example of plant growth regulators, indole-3-acetic acid, phytohormone of the auxin series produced also by many soil bacteria (Patten and Glick, 1996), and 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 and Kuzmann, 1997; Kamnev, 1998; Kamnev *et al.*, 1999b). According to the experimental data reported by Leinhos and Bergmann (1995) and Lippmann *et al.* (1995), addition of indole-3-acetic acid to soil, similar to soil inoculation with some PGPR producing this auxin, resulted in an enhanced uptake of iron and other elements (Zn, Mg, Ca, K and P) in plant roots.

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 Geremida, 1999), as compared to the non-planted soil. Elevated concentrations of heavy metals can be generally toxic to many biological processes including those catalyzed by soil microorganisms reducing their growth (Kelly and Tate III, 1998), in particular, affecting their enzymic activity (Tam, 1998; Bespalova *et al.*, 1999), with the exception of metal-tolerant species (Stephen *et al.*, 1999). 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 have been reported to be resistant to relatively high concentrations of heavy metals 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.*, 1999a, and references therein). 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 1-aminocyclopropane-1-carboxylate (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).

### Dissimilatory Redox Processes Involving 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.,  $\text{Cr}^{\text{VI}}$  to  $\text{Cr}^{\text{III}}$  (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  $\text{Cr}^{\text{III}}$  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  $\text{Fe}^{2+}$  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  $\text{Fe}^{\text{III}}$  reduction was found (Chidthaisong and Conrad, 2000) to be ca. 3.6-fold and ca. 7.2-fold higher than that of nitrate and sulphate, 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 about 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.*, 1999a).

In contrast, the first evidence for culturable Fe<sup>II</sup>-oxidizing bacteria, both acidophilic and neutrophilic (pH 3.5 to 6.8), associated with Fe<sup>III</sup> 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<sup>III</sup> reduction to oxidation of organic contaminants, has been proposed (Kamnev *et al.*, 1999a). In this respect, it should be noted that synthetic chelating agents (e.g., EDTA, HEDTA) have recently been shown to 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), 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 Soil 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, it is clear that heavy metal phytoavailability can largely be affected by soil microbiota as well. Both directions of such effect may in principle be useful. Increasing metal phytoavailability may be beneficial for phytoextraction and recovery 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. These 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, even such a poorly soluble and geochemically stable lead phosphate mineral as pyromorphite  $Pb_5(PO_4)_3Cl$ , the formation of which upon application of more soluble non-toxic metal phosphates was proposed as an *in situ* remediation strategy (Traina and Laperche, 1999), was recently 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). 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<sup>+</sup>B<sup>+</sup>), controlled the precipitation of essential amounts of Fe (ca. 9.1 of dry cells; A<sup>-</sup>B<sup>+</sup> mutant) and La (ca. 3.2% of dry cells; A<sup>+</sup>B<sup>-</sup> 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 responsible for Cu binding. It was also shown for the first time for Gram-negative cells that gold 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.

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). 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) 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 (Kamnev *et al.*, 1999), 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 phosphorous-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.

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 Cd<sup>0</sup> as a photosensibilizer absorbing light and forming  $e^-$ -donor and  $e^-$ -acceptor centres which can be involved in redox reactions with components of the environment.

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.

#### **“A Single Bracelet Does Not Jingle” (An Old African Proverb)**

Despite some evident progress, the mechanisms of heavy metal uptake by plants and speciation of metal compounds in soils, 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). The need for a deeper basic research in this field, along with applied projects, has been recently specially emphasized (Bouma, 1998; Glass, 1999). Among the recommendations directed toward basic research, aimed at understanding the mechanisms that underlie the biological processes central to phytoremediation, the following should be primarily addressed (Glass, 1999):

- approach to a better understanding of mechanisms of uptake, transport and accumulation;
- approach to a better understanding of interactions in the rhizosphere among plant roots, microbes and other biota.

These approaches, aimed at understanding the underlying mechanisms on the molecular and cellular levels, require the application of modern physicochemical techniques, along with biochemical and microbiological methods, which implies close collaboration between a wide range of different “bracelets”, including specialists in plant physiology, microbiology, biochemistry and chemistry, in the multidisciplinary fields of bio- and phytoremediation.

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