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Editors

SOIL BIOLOGY

Soil Heavy Metals

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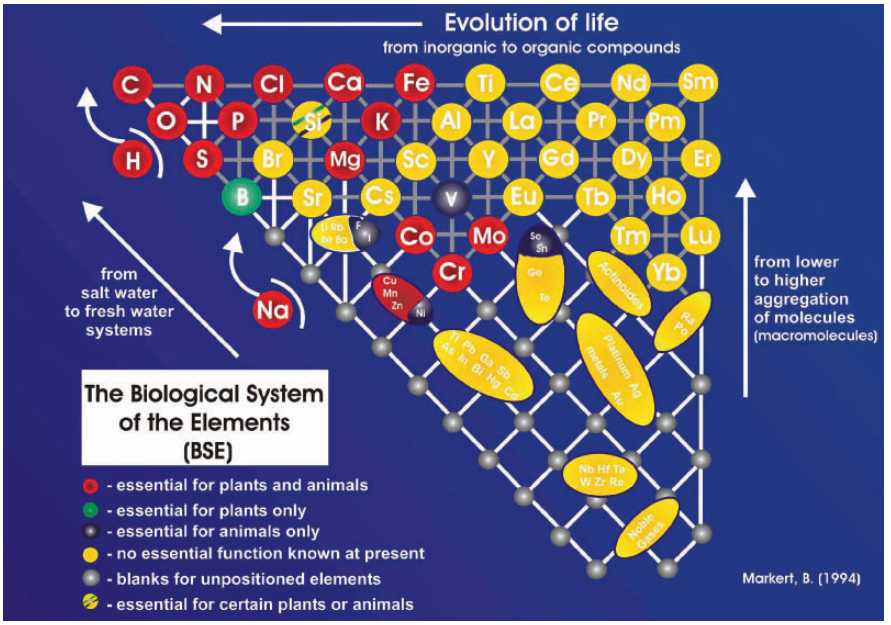
Foreword

Increases in the production of chemical substances and their release into the environment have reached a stage where individuals and society in general are no longer able to control their impact. The use and transformation of over 100,000 individual chemicals whose current locations are largely unknown has resulted in intensive basic and applied research in this area. Some of these chemicals are elements, especially heavy metals, which are found in various forms in the environment.

The positions and classification of the chemical elements in the classical periodic system (table) of the elements (PSE) do not enable us to infer how necessary these elements are to organism function, or whether they are acutely or chronically toxic to living organisms. This is due to the fact that the PSE is based purely on the physiochemical properties of the elements. However, in the past few years a "biological system of the elements" (BSE) has been established that primarily considers aspects of basic biochemical and physiological research. These include:

- The relationships between elements within individual organisms, expressed as linear correlation coefficients
- The physiological functions of individual elements, paying attention to evolutionary development during the emergence of organic life from the inorganic environment
- The forms of individual elements and their compounds taken up by living organisms..

With respect to their effect on the flow of matter and of energy in the food chain, plants represent an important link between the atmosphere and the soil on the one hand and between consumers from the first to the highest order (animals and humans) on the other. Frequently, pollutants are introduced into the food chain via plants that have taken them up from the soil or the atmosphere, and these pollutants often cause irreversible damage to individual organisms or to entire communities as a result of accumulation and exclusion processes. Therefore highest priority on the control of the influence on soil chemistry and microbial activities has to be given in the future.



The biological system of the elements (BSE) compiled from data on correlation analysis, the physiological functions of the individual elements in living organisms, evolutionary development from the inorganic environment, and the forms of the elements taken up by plants as neutral molecules or charged ions. The elements H and Na perform various functions in biological systems, so they are not conclusively fixed in the BSE. The ringed elements can only be summarized as groups of elements with similar physiological function at present, since there is a lack of correlation data on them or these data are currently too imprecise. [From Markert B (1994) The biological system of the elements (BSE) for terrestrial plants (glycophytes). Sci Total Environ 155:221–228].

Quantitatively, the uptake of substances is adequately characterized by the intensity and scale of the uptake up to a particular point in time. For a defined nutrient, the uptake by the plant is dependent on the amount of the nutrient in the medium taken up and its availability. As a rule, the plant has no positive influence on the supply, but it does have an effect on the material and spatial availability of the nutrients. For example, from a material aspect, the nutrient availability can be changed by modifying the pH of the soil solution (elimination of H_3O^+ or HCO_3^- ions by the root), by the liberation of organic acids with chelating-like activity from the root, or via the participation of microorganisms (mycorrhiza), as well as by the effect of the release of H_3O^+ and O_2 at the root surface on the redox potential in the soil. The most readily available elements are present in the soil solution as ions or as soluble soil complexes. The least readily available are tightly bound to the soil structure, for example as a secondary component of the crystal structure of primary minerals. The most important sources of elements between these two extremes are small particles that are loaded with metals and have large surface areas, such as clay, sludge, and organic material. Taken together, all of this can be termed an

"exchange complex". Ion exchange, such as that between calcium and magnesium, potassium, or hydrogen, can occur at the surface.

Thus, the intensity and the range of the uptake both influence the actual amount of a specific element in the plant. Depending on the type of plant being studied, the element species, and the specific location, one can differentiate between roughly three kinds of uptake. In the ideal situation, there is a direct proportionality between the amount of nutrients available and the amount taken up by the plant. In this case, the specific elemental content of the plant reflects the concentration ratios in the nutrient substrate. Thus, the chemical composition of the plant has an indicative character. This association, which has been observed in a series of plants and for a wide variety of elements, both in experiments and in the field, is being taken into account more and more in practical applications, such as when prospecting for ore, or when (usually low-level) plants are used for biomonitoring. Because of unfavourable locations, many plants have developed the ability to enrich themselves with high concentrations of individual elements, often regardless whether these elements are physiologically useful or not. These plants are called accumulators. For example, most Ericaceae have high concentrations of manganese, and beeches have high levels of zinc. This accumulative behaviour, which may have genetically predetermined origins rather than ones determined by location, makes it possible to chemically fingerprint a very wide variety of plant types. The rejection or a reduced uptake of individual elements occurs less frequently than the accumulation of elements, but rejection behaviour has also been demonstrated for numerous plant species. The reduction in concentration of an element in an organism can be the result of complete or partial exclusion.

To advance the abovementioned scientific fields, a more integrative approach to student education appears to be necessary. International exchanges and dialogue about complex problems related to the toxicological effects of heavy metals from the atmosphere and soils on organisms are of tremendous importance. The financial resources needed to achieve these goals must be contributed by various national and international governments.

International Graduate School
Zittau, Germany
April 2009

Prof. Dr. Bernd Markert

Preface

The volume *Soil Heavy Metals* was conceived during the summer of 2007 at an informal Indian–German get-together at Jena. We believe that brilliant ideas crop up over either a cup of Indian tea or a jar of German beer!

All life on Earth depends on the photosynthetic activity of plants, which produce oxygen and reduced carbon for all autotrophic and heterotrophic life. Most of the nutrients needed by plants come from the soil, which is the outermost solid layer of the Earth and is a combination of inorganic and organic materials. The quality of the soil has a strong influence on the overall health of plants and their existence, and the plant ecosystem controls our planet. Soils are certainly not static substrates; they are dynamic biological systems that support microbe, plant and animal life.

The innumerable developments that have taken place in recent years in the field covered by this book make a complete review impossible within the scope of a single volume. Some of the more detailed points have been omitted for brevity; yet, where conflicts do exist, contrasting viewpoints are presented. Time may change these views, but it is the very nature of science to be in a continual state of flux and for the errors of one generation to be amended by the next.

Human activities have dramatically changed the composition and organisation of the soil on Earth. Industrial and urban wastes, in particular the uncontrolled disposal of waste and the application of various substances to agricultural soils, have resulted in the contamination of our ecosystem. Another oft-cited example is mining activity, which has resulted in the deposition of unusually high concentrations of heavy metals onto the soil surface. Plant and soil microorganisms must cope with the resulting elevated levels of heavy metals in the soil, and so they have developed sophisticated techniques for surviving and coexisting in such environments.

Soils are both an important reservoir of chemical elements and a living matrix, as clearly described in Chap. 1 by Helwig Hohl and Ajit Varma. A definition of heavy metals and their role in biological systems is provided by Klaus-J. Appenroth in the following chapter. Soil microbial diversity in relation to heavy metals is expressed in detail by Shwet Kamal, Ram Prasad and Ajit Varma in Chap. 3. The uptake and effects of heavy metals on the plant detoxification cascade in the presence and absence of organic pollutants is then discussed by Ljudmila Ljubenova and Peter Schröder, who show that there is a clear-cut interrelationship between

inorganic and organic pollution. In the next chapter, Hermann Bothe, Marjana Regvar, and Katarzyna Turnau introduce the biology of arbuscular mycorrhizal fungi, and biochemical and molecular aspects of heavy metals and salt tolerance.

Analytical options and (im)possibilities relating to the trace element determination of environmental samples, placing special focus on different X-ray methods, are critically reviewed by Katarina Vogel-Mikuš, Peter Kump, Marijan Nečemer, Primož Pelicon, Iztok Arčon, Paula Pongrac, Bogdan Povh and Marjana Rengvar in Chap. 6.

In subsequent chapters, special attention is devoted to physiological and biochemical behaviour of different microbiological species, populations and communities.

The relationship between metal hyperaccumulation and glucosinolates is presented by Paula Pongrac, Roser Tolrà, Katarina Vogel-Mikuš, Charlotte Poschenrieder, Juan Barceló and Marjana Regvar. The combined effects of heavy metals and salinity on plants from various ecological groups are the focus of Chap. 8, provided by Valentina Kholodova, Kirill Volkov and Vladimir Kuznetsov. The use of the structure and functionality of the microbiological community as indicators to evaluate the health of heavy metal polluted soils is then presented by M. Belén Hinjosa, Roberto Garcia-Ruiz and José Carreira. Extra- and intracellular mechanisms of heavy metal resistance by streptomycetes are explained by Erika Kothe, Christian Dimpka, Götz Haferburg, Andre and Astrid Schmidt, and Eileen Schütze. Chapter 11 gives an assessment of the relationship between soil enzymes and heavy metals, as provided by Ayten Karaca, Sema Camci Cetin, Ozun Can Turgay and Ridvan Kizilkaya. Effects of heavy metals on saprophytic soil fungi are then discussed by Petr Baldrian, followed by a description of copper-containing oxidases, their occurrence in soil microorganisms, and related properties and applications, by Harald Claus.

The analytical detection of the biomethylation of heavy metals in soil and terrestrial invertebrates is presented in Chap. 14 by Burkhard Knopf and Helmut König, with special reference to Hg, Se, As and Bi. Andrea Zanuzzi and Angel Faz Cano then describe the possibility of phytostabilizing lead-polluted sites using native plants. The next chapter describes the impact of heavy metals on sugarcane, and is presented by D.V. Yadav, Radha Jain and R.K. Rai. In Chap. 17, the effects of the activities of earthworms on the availability and removal of heavy metal in soils is discussed by Ayten Karaca, Ridvan Kizilkaya, Oguz Can Turgay and Sema Camci Cetin. Then the phytoremediation of heavy metal contaminated soils is presented by T.J. Purakayastha and P.K. Chhonkar. Finally, Preeti Saxena and Neelam Misra focus their attention on the remediation possibilities associated with heavy metal contaminated tropical land.

In this volume, we have made great efforts to throw light on some aspects and mechanisms of how microorganisms interact with biological systems and allow them to survive in contaminated soil. An attempt has been made to highlight the mechanisms that prevent uptake or allow the detoxification of heavy metals from contaminated soil.

We would like to express our deep appreciation to each contributor for his/her work, patience and attention to detail during the entire production process. It is

hoped that the reviews, interpretations and concepts proposed by the authors will stimulate further high-quality teaching and research, as the information presented tends to highlight both the need for further work in this field and the lack of agreement on some fundamental issues.

It has been a pleasure to edit this book, primarily due to the stimulating cooperation of the contributors. We wish to thank Dr. Dieter Czeschlik and Dr. Jutta Lindenborn, at Springer Heidelberg, for their generous assistance and patience in finalizing the volume. Finally, we give specific thanks to our families – immediate and extended – for their kind support and their incentives to put everything together.

Ajit Varma in particular is very thankful to Dr. Ashok K. Chauhan, Founder President of the Ritnand Balved Education Foundation (an umbrella organization of Amity Institution), New Delhi, for his kind support and constant encouragement.

Special thanks are also due to his Ph.D. student, Mr. Neeraj Srivastava, and the technical educative secretary Anil Chandra Bahukhandi.

Jena, Germany — Irena Sherameti
New Delhi, India — Ajit Varma
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Chapter 1
Soil: The Living Matrix

Helwig Hohl and Ajit Varma

1.1 Introduction

Around the world, farmers are very intelligent and know the characteristics of soil. They know many things about the soil that scientists do not, and scientists know many things that farmers do not, so these two groups of workers must work together. This is true of North American, European, and Asian countries. Farming practices are based on empirical experience; some of these practices may not stand up to scientific testing, but others obviously must do.

The importance of soil structure as a factor in soil fertility is becoming increasingly clear. If a plant is to grow, its roots must spread so that their delicate structures of root hairs can get access to plant nutrients. They also only thrive if there is an adequate supply of water and air. In several countries with plantations of sugarcane, the continuous high yields obtained through irrigation and the extensive application of manure and fertilizers have created problems. Chemical analyzes of the soils from such areas show that common plant nutrients are still present, but that something has happened to the soil that is interfering with its productivity. At first it was thought that the cane itself is deteriorating, but this is not likely, as it propagates vegetatively. Instead, unfavorable conditions for beneficial soil microorganisms may have been produced. The deterioration of the soil structure seems to play a direct part in this, because soil microorganisms have an important influence on the soil structure. Soil organic matter – the formation, decomposition, and transformation of which are caused by microorganisms – is of great importance to sustainable soil fertility and soil structure.

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More experiments on soil structure and other physical properties of soils, such as permeability, porosity, and moisture retention capacity, are desirable. Soil fertility depends on a large number of complex factors, not all of which are known. Physical properties of the soil are no less important than chemical properties. The clay fraction determines many physical and chemical properties of soils. The properties of clays are determined by their mineralogical compositions. X-ray studies and differential thermal analyses of clays have now become necessities in soil laboratories. The electrochemical properties of clays are fundamentally important to understanding soil behavior. This chapter introduces the various types of soil and their functions, as well as the pollution of the soil with heavy metals, which is detrimental to the health of the soil.

1.2 Soil Taxonomy and Classification

A soil taxonomist distinguishes soils partly on the basis of the kind of diagnostic horizon(s) present in each soil. The current soil taxonomy (classification) was adopted in 1965; a simplified account of this classification system follows below (see US Soil Survey Report 1972, 1975).

Order. This is the most general category. All soils fit into one of ten orders.

Suborder. Suborders within a soil order are differentiated largely on the basis of soil properties and horizons resulting from differences in soil moisture and soil temperature. Forty-seven suborders are presently recognized.

Great group. Soil great groups are subdivisions of suborders. The 185 great groups found in the US, and 225 worldwide, have been established largely on the basis of differentiating soil horizons and soil features. The soil horizons include those that have accumulated clay, iron, and/or humus, and those that have pans (hardened or cemented soil layers) that interfere with water movement or root penetration.

Subgroup. Each soil great group is divided into three kinds of subgroups: one representing the central (typic) segment of the soil group; a second that has properties that tend toward other orders, suborders, or other great groups (intergrade group); and a third that has properties that prevent its classification as typic or intergrade. About 970 subgroups are known in the United States.

Family. Subgroups contain soil families, which are distinguished primarily on the basis of soil properties important to the growth of plants or the behavior of soils when used for engineering purposes. These soil properties used include texture, mineral reactions (pH), soil temperature, precipitation pattern of the area, permeability, horizon thickness, structure, and consistency. About 4,500 families have been identified in the United States.

Series. Each family contains several (similar) soil series. The 10,500 or more soil series in the United States have narrower ranges of characteristics than a soil family. The name of the soil series has no pedogenic (i.e., related to soil formation) significance; instead, it represents a prominent geographic name of a river, town,

or area near where the series was first recognized. Soil series are differentiated on the basis of observable and mappable soil characteristics, such as color, texture, structure, consistency, thickness, reactions (pH), and the number and arrangement of horizons in the soil pedon as well as their chemical and mineralization properties. Terms describing surface soil texture, percentage slope, stoniness, saltiness, erosion, and other conditions are called *phases*. Mapping units are created by adding phase names to series names. All mapping units are polypedons. Prior to 1971, soil type was a mapping unit that was used to denote a subdivision of a series indicating the series name and surface texture. Soil type is no longer official nomenclature; it has been replaced by series phase.

The *prime land* means the best land. The definition of prime land will change depending on the use of the land, and full agreement as to exactly how "prime" should be defined is unlikely, even for a specific land use. For farmland use, it is proposed that prime land should meet all the following requirements: adequate natural rainfall or adequate and good-quality irrigation water for intended use; mean annual temperature >32°F (0°C) and mean summer temperature >46°F (8°C); lack of excessive moisture – flooding should not occur more often than once every two years; water table should be below the rooting zone; soil should not be excessively acidic, alkaline, or saline; soil permeability should be at least 0.38″ h^{-1} (1.0 cm h^{-1}) in the upper 20″ (51 cm); the amount of gravel, cobbles, or stones should not be excessive enough to seriously interfere with power machinery; any restricting layer in the soil should be deep enough to permit adequate moisture storage and unhampered root growth, and; the soil should not be excessively erodible.

The objectives of soil surveys and taxonomy are to facilitate growth on soils that have never been grown on before, and to learn enough about certain soils to predict how they would respond when irrigated with a specific quantity of irrigation water of known quality. This objective also emphasizes the inclusion of a rational means of transferring technology from one soil to another, interpretations that allow the prediction of land use for every soil mapped, and that the survey should serve as a scientific database. Soil surveying has developed into a specialized subject. A survey report contains information on not only the characteristics of the soil and its profile, but also the existing and potential uses of the land, the yields obtained by the farmer or by experimental stations under different systems of management, erosion and drainage conditions, and the potential for reclamation or its suitability for irrigation, where these are necessary. Soil maps and survey reports form the basis for planning the utilization of the land, and they have also been found useful in road and building projects.

1.3 Soils of the Humid Tropics and Subtropics

The term "tropics" generally refers to areas of the world with high rainfall, dense forests, and many infertile soils. The tropics occur at low elevations within the equatorial zone, while the subtropics extend to the latitudes of 30°N and 30°S. Fifty-one percent of the world's soils occur in the tropics and subtropics. Lowlands

in the tropics have a mean annual temperature of greater than 75°F (24°C), and the mean monthly temperature of the coldest month is more than 65°F (18°C). In the low-lying subtropics, the mean annual temperature is between 62 and 75°F (17 and 24°C), and the coldest month averages between 50 and 65°F (10 and 18°C).

Freshly deposited alluvium and soils that are stony, shallow, eroded, poorly drained, or deep and sandy can be found in all humid regions throughout the world. Soils of minimal soil development can be found in temperate, subtropical and tropical regions. The primary differences between the characteristics of soil in the tropics and subtropics and their characteristics in temperate regions result from differences in temperature and major geological events such as glaciations. In the tropics and subtropics, soil temperatures are high every day of the year, whereas in most temperature regions, freezing of the soil interrupts the chemical weathering of minerals and soil profile development, even though there is some physical weathering by freeze–thaw effects. In general, for every 18°F (−10°C) rise in temperature above freezing, the chemical reaction rate is doubled, which means that tropical soils weather much faster than temperate or arctic ones. Weathering in the tropics can be at least eight times faster than in the Arctic and Antarctic regions, and four times faster than in temperate regions. Weathering rates in subtropical regions average about half those of the tropics. Products that remain from the decomposition – iron, aluminum, and some silica – recrystallize to form hydrous oxides of iron, aluminum, and sometimes titanium, plus some kaolinite clay. In soils of the tropics, many composite particles the size of sand granules consist of altered minerals cemented together by iron, in contrast with the composition and structure of sand particles in temperate and arctic regions, which are mostly primary minerals such as quartz and feldspars. Predominant soil orders that develop only in the tropics and subtropics are Oxisols, Ultisols and Vertisols.

Organic matter is rapidly lost from tropical soils following the clearing and cultivation of land because mixing and aeration increase the rate of decomposition; organic matter loss is a primary cause of decreasing crop yields in the tropics. A decrease in soil organic matter results in soil structure deterioration, lower plant nutrient reserves from organic matter, and a reduced cation exchange capacity. Ninety percent of all soils in Western Africa, Latin America and India are deficient in available phosphorus. Particularly large amounts of phosphorus fertilizers are needed on Oxisols, Ultisols, and Vertisols, as well as on tropical soils that have developed from volcanic and parent materials. Rates of phosphorus as high as 143–1,069 pounds per acre (160–1,197 kg ha^{-1}) are needed to increase food crop yields.

Oxisols have a total cation exchange capacity of less than 10 meq per 100 grams of soil; when compared with other soils containing the same levels of clay and organic matter, this is lower than soils from any of the other nine soil orders. Since plant-available potassium is stored as part of the overall soil exchange system, levels of potassium are often deficient for the satisfactory growth of many tropical crops. Oxisols and Ultisols may require treatment with lime to correct the soil pH or to reduce the toxic effects of aluminum. Vertisols do not need lime because they usually develop from calcareous materials in a wet–dry climate, and the high clay content and the subhumid climate retard the loss of lime by leaching. Vertisols are not acid enough to develop toxic levels of aluminum. Oxisols and Ultisols have

more kaolinite, more gibbsite and more goethite than soils from the other soil orders. These clay minerals do not absorb lime cations, calcium or magnesium as strongly as montmorillonite, which predominates in soils from the drier temperate regions. Tropical soils with pH values of less than 5 are not productive because of deficient levels of nitrogen, phosphorus and frequently potassium, as well as some micronutrients; high soil-solution aluminum and high exchangeable aluminum, which are toxic to the roots of many plants, such as cotton, tomato, alfalfa, celery, barley, corn, grain sorghum, and sugar beets; high exchangeable manganese, which is toxic to many crops; as well as serious calcium, magnesium and molybdenum deficiencies. Shifting cultivation is the pragmatic solution to the problems of cropping tropical soils used in primitive conditions: land is cleared and burned, planted with crops until the soil fertility is exhausted, then abandoned to return to native woody vegetation and rejuvenation, while a new forested site for planting is sought.

In arid and semiarid areas, crop production depends on the conservation of soil moisture. Data on soil water available for plant growth during the growing season form the scientific basis for deciding upon improved cropping systems.

1.4 Chemical and Colloidal Properties

Soil pH is one of the most indicative measurements of the chemical properties of a soil. All (bio)chemical reactions in soils are influenced by proton (H^+) activity, which is measured by the soil pH. The pH values of most natural soils vary between <3.00 (extremely acid) and 8.00 (weakly alkaline). The solubilities of various compounds (e.g., heavy metals) in soils are influenced by soil pH, as well as by microbial activity and the microbial degradation of pollutants. Optimum pH values for pollutant-degrading microorganisms range from 6.5 to 7.5. Soil pH is influenced by various factors: the nature and type of the inorganic and organic constituents that contribute to the soil's acidity, the soil/solution ratio, the salt or electrolyte content, and the CO_2 partial pressure.

The term "heavy metal" refers to any metallic chemical element that has a relatively high density and is toxic or poisonous. Heavy metals are at least five times as dense as water, and light metals have densities that are lower than this. Examples of light metals include sodium, magnesium, and potassium. Examples of heavy metals include mercury, cadmium, thallium, lead, copper, aluminum, arsenic, chromium, and mercury. Fertilizers contain lead and arsenic. Pesticides contain lead, arsenic and mercury. Sewage sludge contains cadmium, arsenic and lead. Irrigation water can transport dissolved metals to agricultural fields, where metals such as cadmium can be incorporated into plants. Copper occurs naturally in soil and plants. It occurs in rocks, soil, water, sediments, and air. Its average concentration in the soil is about 50 parts copper per million parts (ppm) soil. Lead is by far the most the most common contaminant of soils. Lead is virtually a permanent resident in soil. Organic matter, can bind to metals very effectively; for example, the number one source of lead contamination is lead-based paint, which chipped or scraped are off building exteriors over periods of decades or

centuries. Other sources are gasoline, exhaust, motor oil, automobiles, tires, industrial activities, coal combustion, and pesticides. Mercury occurs in two forms: organic and inorganic. Inorganic forms most often occur when mercury is combined with chlorine, sulfur or oxygen. Organic forms occur when mercury combines with carbon. Metallic forms of mercury are not absorbed by plants, but are converted by microorganisms to organic forms such as methyl mercury that are taken up by plants. Aluminum toxicity is one of the most common factors that limit plant growth and development in many acid soils. Aluminum is found in clay soils, in aluminum silicates and aluminum oxides, and plays a role in soil acidity.

1.5 Soil Water

All soils contain water under natural conditions. The amount of water can be very low in air-dried soils. The optimum water content for microbial processes is 40–60% of the maximum water-holding capacity, or corresponds to the water content that is held in soil at suction pressures of −0.01 to −0.031 MPa. The spaces between soil particles are known as the soil pores; these are filled either with air or water (resulting in a soil solution) depending on the pore size and the water saturation of the soil. Depending on their equivalent diameters, soil pores can be divided into wide coarse (<50 μm), tight coarse (10–50 μm), medium (0.2–10 μm), and fine (<0.2 μm) pores. Pore sizes are assigned in accordance with adaptation to the water content at characteristic metric pressures. Equivalent diameters of 50 and 10 m correspond to water contents of the soil at field capacity 96 and 30 kPa, respectively, while an equivalent diameter of 0.2 m corresponds to the water content at the permanent wilting point (1,500 kPa). The amount of water available to plants and microorganisms lies between the field capacity and the permanent wilting point. Water stored at metric pressures of >1,500 kPa is accessible to neither fine plant roots nor microorganisms. Before undertaking an irrigation project, a soil survey is carried out. The history of irrigation shows that many soils have been damaged or ruined due to a rise in the water table and salinity or alkalinity. The main purpose of a soil survey, however, is to provide an inventory of soil resources. The scope of a soil survey is determined by the purpose in mind (Wilke 2005).

1.6 The Living Matrix

The ground is filled with life. Soils are dynamic biological systems and are certainly not static substrates; they support the lives of microbes, plants, and animals. Although microorganisms are invisible to the naked eye, they are very important and useful to the plant world. In fact, life on Earth would cease without their existence.

In the golden era of microbiology, the study of soil organisms soon became an area of interest to a large number of early bacteriologists, and the pioneering investigations of Winogradsky, Omeliansky, and Beijerinck still stand as major contributions to our knowledge of the bacterial population (Hilgard 1911). At the same

time, it became apparent to soil chemists that the surface crust of the Earth is not merely a static phytochemical matrix upon which green plants grow; it is also a biological system that is in continuous dynamic equilibrium. In the realm of pure science, information on the ecology, function, and biochemistry of microflora has grown considerably, so that a clear picture of soil biology is beginning to emerge. Microflora are important to the ability of humans to feed themselves. For its microbial inhabitants, the soil functions as a unique ecosystem to which the organism must adapt and from which it must obtain sustenance. The rhizosphere is the compartment from which plants acquire their water and nutrients and a hot spot of microbial and animal activity. This compartment can only be understood in the context of whole soil functioning, from soil genesis to nutrient cycling, including exchanges with water and the atmosphere. In order to emphasize the diversity that results from combining the interactions of very diverse and complex communities of organisms in different types of rock material under various climatic and topographic conditions over timescales that can vary from decades to thousands or even millions of years, many soil scientists prefer to speak of "soils" rather than "the soil." The fate of the soil is linked to five important and relevant questions:

- What are the functions of microorganisms in soil genesis?
- What are the roles played by microorganisms in energy and matter fluxes and their transformations within functioning soils?
- As soil genesis and functioning involve a complex and tightly integrated biocoenosis, which kinds of biotic interactions do soil microorganisms participate in?
- What are the functions of microorganisms in specific domains of soils that are highly influenced by biotic or abiotic factors?

The more complex microorganisms, including algae, fungi and protists, are eukaryotes (i.e., they have a true nucleus). Evolutionary studies have revealed that there is a great diversity of eukaryotic organisms as compared to prokaryotic microorganisms. These organisms show characteristic features and are beneficial in many ways to mankind (see Table 1.1). Based on their nutritional requirements, prokaryotes can be categorized as photoautotrophs, photoheterotrophs, chemolithoautotrophs and chemolithoheterotrophs (Table 1.2).

Finally, considering that soils are difficult media to work on, especially for microbiologists, which approaches to study the microorganisms in soil that take the wide structural and functional diversity of soil microbes into account, but avoid diving too far into details that do not provide explanations for emergent properties and processes that are characteristic of soils, can be employed by soil microbiologists (Buscot and Varma 2005)? Soil formed soon after a volcanic eruption or the retreat of a glacier or water, the initial mother substrate, generally exhibits a reduced capacity to carry an abundant plant and animal biocoenosis. Microorganisms such as bacteria, algae, and their associations with fungi in biofilms of lichen are early colonizers. If the basic substrate is loose, the microbial community, in the form of biological crusts, will provide stabilization and suppress erosion. When the mother substrate consists of a hard rocky material such as granite or limestone, the initial process of soil formation consists of weathering. Both of the basic mechanisms of weathering, – the fractionation of the substrate and its gradual chemical

Table 1.1 Comparison of the main types of microorganisms

Microorganisms	Characteristics	Beneficial roles
Prokaryotes		
Bacteria	Rigid cell wall, divided by binary fission, some capable of photosynthesis	Recycle biomass, control atmospheric composition, components of phytoplankton and soil microbial populations
Archaea	Rigid cell wall, unusual membrane structure, photosynthetic membrane, lack chlorophyll	Produce and consume low molecular weight compounds, aid bacteria in recycling dead biomass, some are extremophiles
Eukaryotes		
Fungi	Rigid cell wall, single-cell forms (yeast), reproducing by budding, multicellular forms (hyphae, mycelium), no photosynthetic members	Recycling biomass, stimulate plant growth
Algae	Rigid cell wall, photosynthetic	Important component of phytoplankton

Table 1.2 Nutritional aspects of microbial diversity

Nutritional type	Energy source	Carbon source	Examples
Photoautotroph	Light	Carbon dioxide(CO_2)	Photosynthetic bacteria (green sulfur and purple sulfur bacteria), cynobacteria, extreme halophiles
Photoheterotroph	Light	Organic compounds	Purple nonsulfur and green nonsulfur bacteria
Chemolithoautotroph	Inorganic compounds	Carbon dioxide(CO_2)	*Nitrosomonas*, *Nitrobacter*
Chemolithoheterotroph	Organic compounds	Organic compounds	Most bacteria, fungi, and all animals

transformation – are bound together. Fractionation enhances the contact surface between the substrate and the environment, which in turn increases the chemical activity and transformation rate. As microorganisms represent the largest biotic fraction in the soil in terms of both biomass and number of organisms, and as they are tightly associated with this huge fractal surface, they play a key role in biogeochemical cycles, including those of climate-relevant gases.

1.7 Soils and Plant Nutrition

Analyzing the total N, the C/N ratio, and inorganic N (ammonium, nitrate) provides an insight into the nitrogen supply to soil microflora and plants. The total N content ranges from <0.02% (subsoils) to > 2.5% (peats). A-horizons of mineral soils

contain 0.06–0.5% N. Nitrogen, phosphorus, and/or potassium deficiency may limit the microbial decomposition of pollutants in soil. Optimum conditions are achieved at a C:N:P ratio of 100:10:2. Nitrogen is an important nutrient for plants and soil microorganisms. Ammonium and nitrate in the soil are the N sources that are immediately available to plants. These are produced by the mineralization of organic compounds or are added to the soil as fertilizer. Besides ammonium and nitrate, nitrite may also be present, although usually at very minor levels except in neutral and alkaline soils that have recently been treated with ammonium salts or ammonium-forming fertilizers.

Soil phosphorus is, like nitrogen, potassium, calcium, and magnesium, an important nutrient for soil organisms and plants. It exists as inorganic and organic fractions (the proportions of each fraction can vary between 5 and 95%). The soil organic P fraction is derived from plant residues, soil flora, and soil fauna tissues and residues that resist rapid hydrolysis. Inorganic fractions consist of Ca, Al, and Fe phosphates. The most prominent phosphate mineral in soils is apatite. The total concentration in soil is generally in the range from 200 to 800 mg kg^{-1}. A considerable amount of P is also bound in the amorphous mineral fraction. Soil microbes are involved in the mineralization of P from organic debris. Extracellular phosphatases are produced by microorganisms and roots and contribute to the mineralization of organic P. Phosphorus deficiency can limit the growth of plants and the microbial decomposition of pollutants in soil.

Without using very large quantities of fertilizers, it would not be possible to maintain agricultural production at the levels that are currently required. Because of this, Europe, America, and Japan have been using fertilizers for a long time. In Japan, roughly half of the plant food comes from bulky organic manures and half from fertilizers. Most of their straw is used to prepare manures and composts, and the Japanese have one of the highest consumptions of fertilizers per unit area of arable land. Bulky organic manures are also a major source of plant food in Europe and America. All practicable measures should be adopted to increase their supply in India too, but fertilizers are required to supplement them. Farmyard manure and composts have their virtues, but we cannot afford to make a fetish of them.

Certain chemical elements known as micronutrients or trace elements are crucial to the growth and health of plants in very small quantities, but are toxic to them at higher levels. When these elements are not taken up by the plants symptoms of diseases appear. Deficiencies of manganese, zinc, and copper are widespread in citrus trees. Such diseases are cured by applying the deficient element to the soil and spraying the trees. Some of these trace elements are now being incorporated in fertilizer mixtures.

1.8 Soil Organic Matter

Soil organic matter is one of the most important indicators of soil quality. It influences many soil properties, including nutrient supply (N, P, S), cation exchange capacity, adsorption of pollutants, infiltration and retention of water, soil structure, and soil

color, most of which in turn affect soil temperature. Soil organic matter consists of microbial cells, plant and animal residues at various stages of decomposition, stable humus (humic acids, humins) synthesized from residues by microorganisms, and highly carbonized compounds (e.g., charcoal, graphite, coal). Soil organic matter is thus a complex mixture of heterogeneous organic compounds (including sugar, starch, protein, carbohydrates, lignin, waxes, resins, and organic acids) derived from plants, microorganisms and animal residues that are formed through the decomposition, synthetic, and polymerization reactions. The process of organic matter decay in the soil begins with the decomposition of sugars and starch from carbohydrates, which quickly break down as saprophytes initially invade the dead plant. Proteins decompose into amino acids. Organic matter is an essential source of nutrients for all heterotrophic soil organisms, which in turn hold a key position in the humification and mineralization of humic substrates that lead to the production of stable humus, degradable organic compounds, and carbon dioxide.

1.9 Soil Texture

Fine earth can be split into three particle size fractions: the sand fraction, with an equivalent diameter of 50 or 63–2,000 μm; the slit fraction (2–50 or 63 μm); and the previously mentioned clay fraction (<2 μm). The proportion of each fraction in fine earth defines the texture of the soil, which is a crucial property as it determines the volume available for the two other soil phases, the gaseous (soil–air) and aqueous (soil–water or soil solution) phases. Sandy soils not only have a higher total volume of water and air, they allow better water percolation and evaporation, resulting in rapid shifts in soil moisture versus soil aeration. Breaking down clay and sand still further leads to the synthesis of nanomaterials, which in turn has a considerable impact on the plant and animal life. The soil texture and soil pore size are also important as they determine the distributions of soil organisms. The classification of organisms used by soil biologists refers to the sizes of soil particles and soil pores (Fig. 1.1).

1.10 Permafrost Soils

Permafrost, which is defined as a subsurface frozen layer that remains frozen for more than two years, makes up more than 20% of the land surface of the earth, including 82% of Alaska, 50% of Russia and Canada, 20% of China, and most of the surface of Antarctica (Williams and Smith 1989; Storad 1990). Permafrost poses unique challenges to its resident biota because of the permanently cold temperature of the soils, averaging 10–12°C, and the length of time over which the soils were frozen, which ranges from a few thousand to even 2–3 million years. Antarctica has an area of 14 million km^2; however, exposed permafrost soils cover a mere 49,000 km^2, or about 0.35% of the entire continent (Campbell and Claridge 2008). In Antarctica, the soil climate and permafrost properties are strongly influenced by the surface radiation balance, since the thermal regime of the soil is dependent upon the gains and

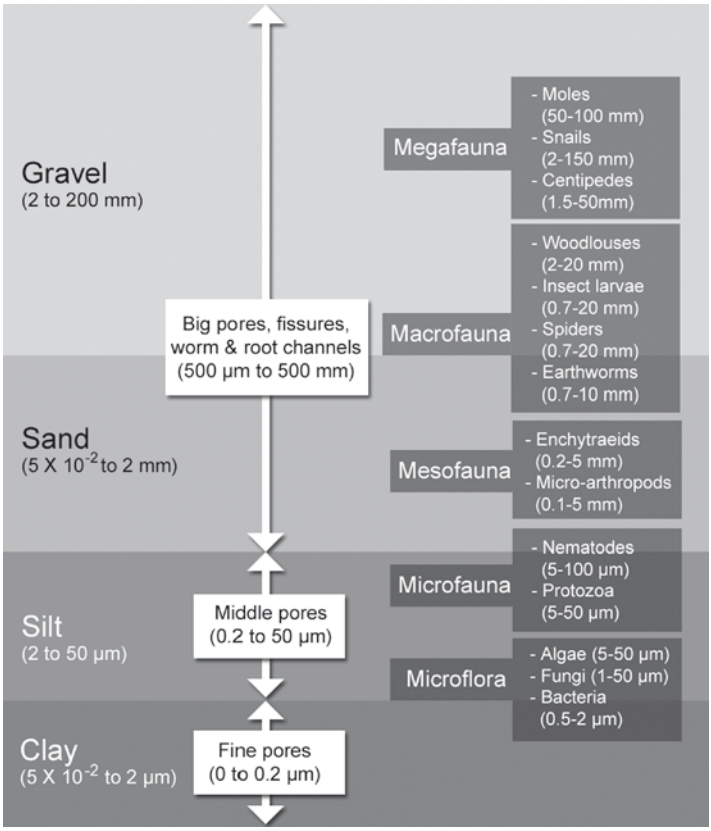

Fig. 1.1 Classification of soil biota in relation to soil pore and particle size, as used in soil biology (modified from Buscot and Varma 2005)

losses of radiation from the soil surface. Soils with dark-colored surfaces have low albedo values (approximately 5% at Scott Base), while soils with light-colored surfaces have much higher albedo values (26% at Northwind Valley) (Balks et al. 1995; Macculloch 1996). These soils are formed mainly from Precambrian to Lower Paleozoic basement rocks, intruded by granites and peneplained by weathering and glacial erosion, with overlying sediments of sandstones, siltstones, coal measures and tillites. Biodiversity is extremely low, and diminishes with increasing severity of climatic conditions. Primary producers are bryophytes, lichens, cyanobacteria and algae, and terrestrial fauna include collembolans, mites, and groups of microscopic organisms. Two important pedological processes that operate in Antarctica soils are oxidation and salinization. Coarse particle reduction takes place mainly at the soil surface, with particle size decreasing through granular disintegration and abrasion. Within the soil, coarse particles are nearly always angular and unstained, indicating low cryoturbic activity. The organic regime is significant everywhere in Antarctica, owing to the paucity of biological communities. For soil morphological properties,

see Campbell and Claridge (2008). The soils of Antarctica are mostly formed in the absence of biological processes and, as a consequence of the prevailing low temperatures, are underlain everywhere by permafrost, with the active layer varying in thickness from about one meter in northern areas to a few centimeters or less in the soils of the inland edge of the Transantarctic Mountains. The permafrost is generally ice-cemented, but in aged and dehydrated soils may be loose. Because of extreme aridity, chemical weathering processes are assisted by salts, which allow unfrozen saline solutions to be present on grain surfaces and cracks in rock particles, even at very low temperatures. Weathering comprises the breakdown of ferromagnesian minerals, releasing iron and cations into the soil matrix. The iron oxidizes and is precipitated on grain surfaces, giving rise to the red coloring of aged sols. The cations, especially calcium and magnesium, combine with nitric and sulfuric acids that arrive in precipitation to form part of the thick salt horizons found in older soils. The concentrated salt solutions react with silica, which is also released by weathering to form secondary clay minerals and in some cases zeolites.

Culture-dependent and culture-independent methods have revealed that permafrost harbors diverse and novel microbial communities. The future challenge for studies of permafrost microbiology is to begin to address the ecology of these unique microbial ecosystems. The knowledge gained from culture-independent surveys of microbial diversity can be used to design targeted strategies in order to determine if phylogenetic groups detected by molecular strategies are part of the viable microbial community. The application of technologies such as stable isotope probing and FISH–microautoradiography could identify active microorganisms and better define the functioning and maintenance of permafrost microbial ecosystems at ambient subzero temperatures (Jeewon and Hyde 2008; Solaiman 2008; Solaiman and Marschner 2008; Marschner 2008). As microbial activities in situ are expected to be minute and extremely slow, new methods and techniques specific to the permafrost environment will be required. Developing methods for detecting and characterizing the active bacteria and archaea in permafrost will lead to the differentiation of the active microbial populations that are presumed to exist in permafrost from cryopreserved microbial fossils that may have remained frozen for geological timescales.

A database on non-lichenized fungi from Antarctica has been created in the United Kingdom (see http://www.antarctica.ac.uk/bas_research/data/access/fungi/, as well as Gilichinsky et al. 2007; Ruisi et al. 2007; Somjak et al. 2007; Ozerskaya et al. 2008). The mycobiota of arctic permafrost have been studied over the last decade (Panikov and Sizova 2007). The most common fungi belong to the genera *Acremonium*, *Alternaria*, *Arthrinium*, *Aspergillus*, *Aureobasidium*, *Bispora*, *Botrytis*, *Chaetophoma*, *Chrysosporium*, *Cladosporium*, *Fusarium*, *Geomyces*, *Geotrichum*, *Gliocladium*, *Lecythophora*, *Malbranchea*, *Monodictys*, *Mucor*, *Paecilomyces*, *Penicillium*, *Phoma*, *Rhinocladiella*, *Scopulariopsis*, *Stachybotrys*, *Sphaeronaemella*, *Sporotrichum*, *Thysanophora*, *Trichoderma*, *Ulocladium*, *Valsa*, *Verticillium*, *Xylohypha*, as well as sterile mycelia with sclerotia. Permafrost fungal microfauna provide evidence of the existence of extremotolerant organisms that are capable of retaining their viability and developing under the conditions present in extreme ecological niches and that show high adoptive potential.

Microorganisms are usually found in a dormant state under frozen and permafrost conditions (endo-and exospores, cysts, non-spore antibiotic cells, etc.). High numbers of viable microorganisms have been counted. The detected phylotypes form eleven established lines of descent for bacteria and one entirely new sequence that was not assigned to any of the known groups. Most of the clones belonged to the alpha (20%) and delta (25.6%) subdivisions of the *Proteobacteria*, with fewer from the beta (9.3%) and gamma (4.7%) subdivisions, groups that are typically isolated from soil by culture methods. Most of the permafrost-derived clones (77%) had sequence similarities of less than 95–80% with those in the database, indicating a predominance of new genera and families (Panikov 2008). It is true that the anammox process has not been investigated in permafrost soils, however, "marine" anammox 16 rRNA sequences have been identified in Siberian frozen alluvial sandy loam from the Middle Pleistocene epoch 300,000–400,000 years ago (Penton and Tiedje 2006). The anammox process was found to be responsible for up to 19% of the total nitrogen production in Greenland sea ice, but was not detectable in annual sea ice, perhaps due to increased stability (Rysgard and Glud 2004). A novel cold-adapted nitrite-oxidizing bacterium was isolated from a Siberian permafrost sample (Alawi et al. 2007). The detection of anammox activity in sea ice suggests that this may be an active process in permafrost, where anammox bacteria have also been identified. In the context of current warming trends, a thorough characterization of the nitrogen cycle in permafrost soils is needed in order to quantify effects on organic matter mineralization and ultimately carbon dioxide release as a positive feedback mechanism for global warning.

Long-term survival strategies in permafrost are thought to fall into two main categories. In the first, microbes maintain viability by entering a dormant state in which they can resist damage to cellular insults; in the second, microbes maintain viability by metabolizing and repairing damage at rates sufficient to equal or exceed the rate of death due to environmentally induced damage. In situ permafrost bacteria, which are further characterized by thickened cell walls, altered structure of cytoplasm, and compact nucleoids, showed similarities to cyst-like resting forms of non-spore-forming bacteria. The survival mechanisms may include reducing the polar polysaccharide capsular layer, decreasing the fractional volume of cellular water, increasing the fraction of ordered cellular water, or extracting energy by catalyzing the redox reactions of ions in thin aqueous films in the permafrost (Gilichinsky 2002). Those that fall into latter category, such as the observed changes in the genome and in gene expression, are primarily directed toward the maintenance of molecular motion and resource efficiency for continued growth in frozen conditions. Long-term survival is closely tied to cellular metabolic activity and DNA repair, which over time proves to be superior to dormancy as a mechanism for sustaining bacterial viability (Johnson et al. 2007). Specific sets of cold-induced proteins (CIPs) are considered to facilitate and allow cell growth at low temperatures. CIPs can be classified into cold-shock proteins (CSPs) and cold-acclimation proteins (CAPs). Bacteria that contain these proteins include *Psychrobacter* and *Exiguobacterium* (Bakermans et al. 2007). The adaptive nature of permafrost bacteria at near-freezing temperatures is governed by cellular physiological processes through the regulation of certain cellular proteins. It is possible that proteins synthesized at low temperatures may support temperature

homeostasis, protect other proteins from denaturation and damage, and enable the cells to adapt to near- or below-freezing temperatures.

Most planets of the solar system, as well as their moons, asteroids and comets, are cryogenic in nature, and so the cryosphere is a common phenomenon in the cosmos. This is why the cells found in the Earth's cryosphere, as well as their metabolic by-products and biosignatures (biominerals, biomolecules and biogases), provide a range of analogs that could be used in the search for possible ecosystems and potential inhabitants of extraterrestrial cryogenic bodies. If life ever existed on other planets during their early stages of development, then it may have consisted of primitive cell forms. Similar to life on Earth, such primitive life may have been preserved on other cosmic bodies deep within their ice or permafrost layers. The orbits of both Earth and Mars lie between those of Mercury and Venus (which are close to the Sun and therefore dehydrated) and the bodies of the Jupiter system (which mostly consist of volatile hydrogen, methane, and water). Biota from the Greenland ice sheet (120,000 years old) and the Antarctic ice sheet (<400,000 years old) have been widely studied to depths of more than 3 km (Miteva et al. 2004; Miteva and Brenchley 2005; Mitrofanov et al. 2007).

The age of the oldest glacial ice, as well as immured bacteria, is still under discussion: >500,000 years old in the Guliya ice cap on the Tibetan Plateau; >2 million years old at the bottom of the Vostok ice core; or even >8.1 million years old (Bidle et al. 2007). The surface conditions in the Antarctic desert – intense levels of solar radiation, an absence of snow and vegetation cover, and ultralow temperatures, which can be as low as −60°C – share similarities with those on Mars.

On Earth, most volcanoes are located in areas where oceanic and continental plates are colliding. Despite active volcanism, permafrost often exists on slopes of high-elevation or high-latitude volcanoes (Palacios et al. 2007). The fundamental question is: do ecological niches such as volcanoes and associated environments contain microbial communities? The task is to find thermophilic microorganisms associated with volcanoes that were deposited with the products of eruption and then survived in permafrost after the scoria and ash froze. Cores extracted from a borehole into young volcanic deposits contained biogenic CH_4 and viable bacteria, including thermophilic anaerobes. Among these were methanogens growing on CO_2 plus H_2. Thermophiles may survive in permafrost and even produce biogenic gases.

1.11 Soil Pollution

Mining, manufacturing, and the use of synthetic products (e.g., pesticides, paints, batteries, industrial waste, and applications of industrial or domestic sludge to the land) can result in heavy metal contamination of urban and agricultural soils. Heavy metals also occur naturally, but rarely at toxic levels. Potentially contaminated soils can occur at old landfill sites (particularly those that accepted industrial wastes), old orchards that used insecticides containing arsenic as an active ingredient, fields that previously had wastewater or municipal sludge applied to them, areas in or around

mining waste piles and tailings, industrial areas where chemicals may have been dumped on the ground, or areas downwind from industrial sites (Table 1.3).

Excess heavy metal accumulation in soils is toxic to humans and other animals. Chronic exposure (exposure over a longer period of time) to heavy metals is normally due to food chain transfer. Acute (immediate) poisoning from heavy metals is rare through ingestion or dermal contact, but is possible. Chronic problems associated with long-term heavy metal exposures include:

- Lead: mental lapses
- Cadmium: affects kidney, liver, and the GI tract
- Arsenic: skin poisoning, affects the kidneys and the central nervous system

The most common problems from cationic metals (metallic elements whose 2+ forms in soil are positively charged cations) come from mercury, cadmium, lead, nickel, copper, zinc, chromium, and manganese. The most common problems from anionic compounds (elements whose forms in soil are combined with oxygen and are negatively charged) come from arsenic, molybdenum, selenium, and boron (see Tables 1.4 and 1.5).

Table 1.3 Heavy metal pollutants in sewage

Heavy metal	Maximum concentration in sludge (mg/kg or ppm)	Annual pollutant loading rates (kg/ha/yr)	Annual pollutant loading rates (lb/A/yr)	Cumulative pollutant loading rates (kg/ha)	Cumulative pollutant loading rates (lb/A)
Arsenic	75	2	1.8	41	36.6
Cadmium	85	1.0	1.7	39	34.8
Chromium	3,000	150	134	3,000	2,679
Copper	4,300	75	67	1,500	1,340
Lead	420	21	14	420	375
Mercury	840	15	13.4	300	268
Molybdenum	57	0.85	0.80	17	15
Nickel	75	0.90	0.80	18	16
Selenium	100	5	4	100	89
Zinc	7,500	140	125	2,800	2,500

Table 1.4 Environmental quality standards for soil pollution

Item	Environmental quality standards
Cadmium	0.0l mg l^{-1} in sample solution, and less* than 1mg kg^{-1} in soil for agricultural land
Lead	0.0l mg l^{-1} or less* in sample solution
Chromium (VI)	0.05 mg l^{-1} or less* in sample solution
Arsenic	0.0l mg l^{-1} or less* in sample solution, and less than 15 mg kg^{-1} in soil for agricultural land (paddy field only)
Total mercury	0.0005 mg l^{-1} or less* in sample solution
Alkyl mercury	Not detectable in sample solution

*The above standards are not applicable to (1) the soil in those places where natural toxic substances exist, such as the vicinities of mineral veins, and (2) the soil in those places designated for the storage of toxic materials, such as waste disposal sites

Table 1.5 Environmental quality standards for soil pollution

Item	Environmental quality standards
Cadmium	0.0l mg l^{-1} in sample solution and less[a] than 1 mg kg^{-1} in soil for agricultural land
Total cyanide	Not detectable in sample solution
Organic phosphorus[b]	Not detectable in sample solution
Lead	0.0l mg l^{-1} or less[a] in sample solution
Chromium (VI)	0.05 mg l^{-1} or less[a] in sample solution
Arsenic	0.0l mg l^{-1} or less[a] in sample solution, and less than 15 mg kg^{-1} in soil for agricultural land (paddy field only)
Total mercury	0.0005 mg l^{-1} or less[a] in sample solution
Alkyl mercury	Not detectable in sample solution
PCB	Not detectable in sample solution
Copper	Less than 125 mg kg^{-1} in soil for agricultural land (paddy field only)
Dichloromethane	0.02 mg l^{-1} or less in sample solution
Carbon tetrachloride	0.002 mg l^{-1} or less in sample solution
1,2-Dichloroethane	0.004 mg l^{-1} or less in sample solution
1,1-Dichloroethylene	0.02 mg l^{-1} or less in sample solution
cis-1,2-Dichloroethylene	0.04 mg l^{-1} or less in sample solution
1,1,1-Trichloroethane	l mg l^{-1} or less in sample solution
1,1,2-Trichloroethane	0.006 mg l^{-1} or less in sample solution
Trichloroethylene	0.03 mg l^{-1} or less in sample solution
Tetrachloroethylene	0.0l mg l^{-1} or less in sample solution
1,3-Dichloropropene	0.002 mg l^{-1} or less in sample solution
Thiram	0.006 mg l^{-1} or less in sample solution
Simazine	0.003 mg l^{-1} or less in sample solution
Thiobencarb	0.02 mg l^{-1} or less in sample solution
Benzene	0.0l mg l^{-1} or less in sample solution
Selenium	0.0l mg l^{-1} or less[a] in sample solution
Fluorine	0.8 mg l^{-1} or less[a] in sample solution
Boron	1 mg l^{-1} or less[a] in sample solution

[a]For environmental limits concerning the concentrations of cadmium, lead, chromium(VI), arsenic, total mercury, selenium, fluorine, or boron in liquid samples, when the soil contamination occurs away from the groundwater level and the concentrations of the substances do not exceed 0.0l mg, 0.0l mg, 0.05 mg, 0.0l mg, 0.0005 mg, 0.0l mg, 0.8 mg, and 1 mg, respectively, under the original conditions, then the limits per liter of liquid sample are 0.03 mg, 0.03 mg, 0.15 mg, 0.03 mg, 0.0015 mg, 0.03 mg, 2.4 mg, and 3 mg, respectively

[b]"Organic phosphorus" refers to parathion, methyl parathion, methyl demeton, and EPN

The above standards are not applicable to (1) soil in those places where natural toxic substances exist, such as the vicinities of mineral veins, and (2) soil in those places designated for the storage of toxic materials, such as waste disposal sites

Polycyclic aromatic hydrocarbons (PAHs, a group of more than 100 different compounds) are often found at contaminated sites, particularly in connection with tar contaminations at former gasworks. They also exist as diffuse contamination in urban areas and alongside roads, where they arise from the impregnation of wood with creosote and the incomplete combustion of hydrocarbons.

1.12 Conclusion

Cultivators (agriculturists/horticulturists, and foresters) know that there are many kinds of soils. Soil surveys enable us to determine their characteristics, types, and distributions, and to classify them. Soil surveys are very important for the planned utilization of land. They give valuable information on the possible uses of the land and their comparative advantages and disadvantages. The results of experiments carried out with one variety of soil have little significance with respect to another variety.

The scientific study of the soil as a natural body originated with a Russian, V.V. Dokuchaiev. Previously, two philosophical approaches were employed in soil studies. One originated with a German chemist, Liebig, who considered the soil to be merely a reservoir of plant nutrients. Plants withdraw nutrients from the soil, which must then be replenished. Maps were prepared showing the contents of the total and available plant nutrients in surface soil. Such chemical estimations are still performed and are very useful, but they do not provide a scientific basis for classification. According to the other point of view, soils were classified on the assumption that each geological formation gives rise to a characteristic soil, and so a map of the surface geology can be translated (with due interpretation) into a soil map. It has, however, since been found that different soils develop from the same geological formation, and that climate, vegetation, and relief all have important effects on soil formation.

The scientific classification of soils now centers round the characteristics of the soil profile – a vertical section of the soil in which a sequence of genetically related horizons that reflect the processes that form the soil can be seen. Many of the alluvial soils of India have genetically related horizons, but alluvial soils that do not have them are also classified in soil surveys.

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Chapter 2
Definition of "Heavy Metals" and Their Role in Biological Systems

Klaus-J. Appenroth

2.1 Introduction

At first glance, it would appear to be a rather simple matter to define a "heavy metal" – it is a metal that is "heavy". Unfortunately, a more in-depth consideration reveals a huge amount of problems with this simple definition. This definition is meant to suggest that the density of a heavy metal is high, but this physical property is quite meaningless in the context of plants and other living organisms. Plants do not deal with metals in their elemental (valence state of 0) forms; they are not accessible to plants. Metals are only available to them in solution, and it is necessary for metals to react with other elements and form compounds before they can be solubilised. Once such a chemical compound is formed (e.g. a salt), the density of the metal does not play any role. We do not know of any correlation between the density of a metal and its physiological or toxicological effects, or even the chemical properties of its compounds. Therefore, let us leave the question of how to define a "heavy metal" until later, and first consider the definition of a "metal".

2.2 The Definition of Heavy Metals in Plant Science

2.2.1 Metals

Metals are often characterised and distinguished from nonmetals by their physical properties – the ability to conduct heat, and an electrical resistance that is directly proportional to temperature, malleability, ductility and even lustre (Housecroft and Sharpe 2008; Müller 2007). These properties, especially that of a temperature-dependent conductivity, at least allow us to define what a metal is in contrast to

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nonmetals and metalloids. However as mentioned above, all of these physical properties are lost after the metal has been chemically transformed into a chemical compound that can be taken up by plants (Shaw et al. 2004). It is well known that the properties of chemical elements can be determined from their positions in the periodic table of the elements (Fig. 2.1). In general, the chemical elements become more metallic as we move towards the lower left corner of the table and nonmetallic towards the upper right corner. In other words, metallic character decreases from left to right and from the bottom to the top of the table. Metalloids (elements with properties intermediate between metals and nonmetals) occur close to the diagonal border between metals and nonmetals in the table. A metal can be categorised according to the last electronic subshell in its atom. There are s-elements, which can be subdivided into alkaline elements (first main group) and alkaline earth elements (second main group). All s-elements are metals except for H (the first element in the first main group). The first element in the second main group, Be, is also somewhat special (its oxides are amphoteric), but it is still considered to be a metal. Among the other groups of the periodic table, d-group elements (transition elements) are all metals. Many of them form compounds with different valence states, which is an important factor in their toxicity. Some of the oxides of transition elements have slightly amphoteric properties, but they are still all considered to be metals. Then there are the f-group elements, also known as the rare earth elements, which are subdivided into the lanthanide series (including La) and the actinide series (including Ac). All of these rare earth elements are also metals and so are sometimes called rare earth metals. The next group, the p-group, occurs towards the right hand side of the periodic table and thus represents a mixed group of

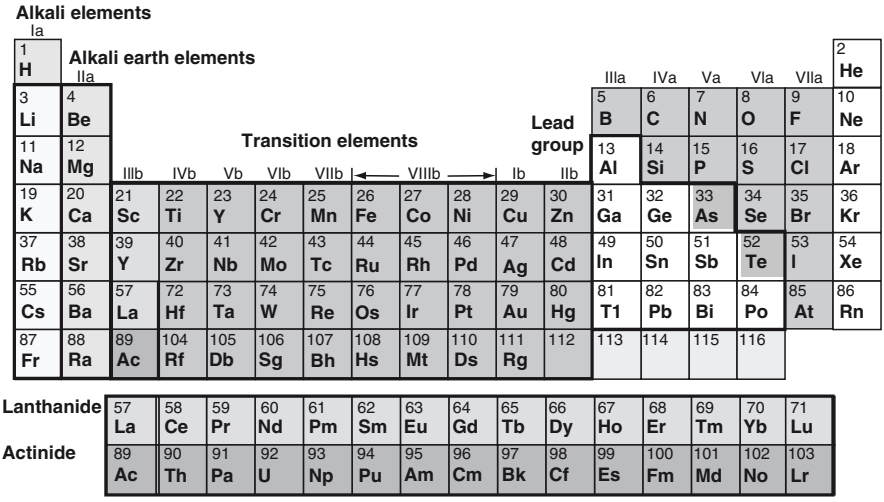

Fig. 2.1 Periodic table of the elements. Metals and some metalloids are indicated. The transition elements, the rare earth elements (lanthanide series, actinide series) and the lead-group elements on the right hand side of the table are relevant to the definition of "heavy metals" provided in this chapter

metals, metalloids and nonmetals. This includes the elements of the third to seventh main groups of the periodic table, but excludes the rare gases (the eighth main group). Metallic members of this group include Al, Ga, In, Tl, Sn, Pb, Cb, Bi, Te and Po. All of them (except Bi) form amphoteric oxides. Si, Ge, As and Te are considered to be metalloids; sometimes B and Sb are included too (Fig. 2.1). Since there is no common name for the metal/metalloid members of the p-group, we suggest that these metals and metalloids should be termed "lead-group elements", as lead is the representative of this group that has been studied in the greatest depth in plant science.

As plant scientists, we should stress at this point that we never talk about the elemental forms of these elements. We usually only deal with their salts. There are, of course, special cases where the properties of a compound formed from elements from any of the groups defined above are modified (e.g. by organic ligands or substituents). This should then be treated as a special case and does not necessarily have an impact on the divisions and subdivisions of elements. Classifying metals according to their positions in the periodic table of the elements makes sense because the chemical properties of their compounds are related to it.

2.2.2 *Heavy Metals*

In the fundamental review paper written by Duffus (2002), 13 different works were cited that used lower limits on the density of a "heavy" metal ranging from 3.5 to 7 $g\,cm^{-3}$. The author stated that the threshold varied depending on the author, and that "it is impossible to come up with a consensus". Moreover, he concluded that "any idea of defining "heavy metals" on the basis of density must be abandoned as yielding nothing but confusion". However, this is beside the point; although half of the works cited suggested similar lower limits of 4.5 or 5 $g\,cm^{-3}$, plants do not have the ability to detect the density of a metal. Thus, "heavy metal" remains an obscure term in the life sciences. It should also be noted that the review paper of Duffus (2002) was commissioned by the International Union of Pure and Applied Chemistry, and certainly represents a chemical point of view that is often neglected by biologists. Apart from the specific weight, the atomic weight, the atomic number, specific chemical properties, and the toxicity were all mentioned as a possible basis for classification – and then rejected for good reasons. So what should we base our definition of "heavy metals" upon? Indeed, is it necessary to use the term at all? Let us now consider what defining "heavy metals" according to the chemical properties of compounds can offer us.

2.2.3 *Lewis Acid Strength and Ionic Indices*

Any positively charged ion is able to accept electrons, thus defining it as a Lewis acid. In contrast to the physical properties of a metal in its elemental form, the chemical properties of a metal ion determines its ability to form complexes (Pearson 1968),

Table 2.1 Classification of some metals and metalloids according to covalent index (Nieboer and Richardson 1980; Shaw et al. 2004)

Class A elements	Borderline elements	Class B elements
Li^{+}, Na^{+}, K^{+}, Cs^{+}, Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Al^{3+}, Ga^{3+}, Sc^{3+}, Y^{3+}	Ga^{3+}, In^{3+}, Sn^{4+}, Pb^{2+}, As^{3+},Sb^{3+}, Ti^{2+}, V^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}	Tl^{+}, Tl^{3+}, Pb^{4+}, Bi^{3+}, Pd^{2+}, Pt^{2+}, Cu^{+}, Ag^{+}, Au^{+}, Hg^{2+}

both in vitro and in living systems. Depending on the stability constants of the complexes formed, metal acceptors can be classified in relation to suitable reaction partners as "hard" or "soft", with some being intermediate between the two. In this way, all metals can be classified as either hard, soft or intermediate. Based on so-called ionic indices and covalent indices, Nieboer and Richardson (1980) classified metals as well as metalloids into three classes: A, B, and borderline (see Table 2.1). Aside from some differences between the categories of "hard" and "soft" on the one side and "A" and "B" on the other side, this categorisation has consequences for possible reaction partners. It follows that "hard" acceptors, or class A group ions (e.g. Mg^{2+}, Ca^{2+}, Al^{3+}, As^{3+}), are expected to interact with oxygen-containing ligands, whereas many of the class B group ions that are often very toxic (e.g. Ag^{+}, Tl^{+}, Hg^{2+}, Cd^{2+}) form stable bonds with S- and N-containing ligands. The latter are present in the form of SH or imidazo groups in proteins, which could have immediate consequences with respect to toxicity. There is no doubt that quantitative data on the Lewis acid strength are very important for explaining the interactions of different metal ions with specific interaction partners within toxified cells. However, can we explain the toxic effects of specific ions in specific plants on the basis of Lewis acid strengths?

2.2.4 Toxicity

The term "heavy metal" is linked in many people's minds to metals (or their compounds) that are toxic. However, this is a feeling rather than a conclusion based on scientific evidence. Two facts should be kept in mind. (1) The effect of any substance on a living system is always dependent on the concentration of it available to cells. Thus, there are no substances that are always toxic. What we need to evaluate toxicity are dose–response data; i.e. quantitative dose–response relationships. (2) Several metal ions are crucial to the metabolism of cells at low concentrations but are toxic at high concentrations, resulting in bell-shaped dose–response relationships (Marschner 1995). These metals are sometimes called micronutrients.

We applied ten different heavy metals (eight transition elements and two lead-group elements) to a system established to biomonitor (based on the ISO 20079 protocol) the higher plant *Lemna minor*, clone St (Naumann et al. 2007). The growth inhibition was quantitatively measured (effective dose required for the inhibition of growth rates by 50%, ErC50) on the basis of multiplication rate, fresh

weight, dry weight, chlorophyll *a*, chlorophyll *b* and total carotenoid content. Based on the averages of all tested parameters, the following phytotoxicity series was obtained:

$$Ag^{+} > Cd^{2+} > Hg^{2+} > T1^{+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{6+} > Cr^{6+} > As^{3+} > As^{5+}.$$

This toxicity series was compared with the classification of the elements into classes A or B (van Assche and Clijsters 1990). No correlation was found. In this project, the biological objective did not change, and all tests were carried out with exactly the same plant (moreover, under the same standardised environmental conditions). Thus, we would expect the same set of potential ligands for each metal ion applied, i.e. proteins, nucleic acids and low molecular compounds. The situation would be even more complex had we used different biological objects (biomonitoring must be done for algae, fishes, water fleas and higher plants) or different environmental conditions. This was quite a disappointment considering the hope that the toxic effects could be explained, at least in part, by the chemical properties of the salts of these heavy metals. However, on closer inspection this result is less surprising. In a living plant cell there are a large number of different possible target proteins for a specific heavy metal – more precisely for ions of transition elements and lead-group elements (Naumann et al. 2007). These ions belonged to class B or borderline elements. The most sensitive responses were observed for either the water balance of the plants or their chlorophyll contents. In the future, it may be possible to discover whether a certain reaction partner responds in a specific way to a certain ion. However, under in vivo experimental conditions, changing the heavy metals is a much more complex task. Class B element ions are able to form complexes with sulfur-containing ligands. This means that all proteins are potential reaction partners. In plants there are approximately 26,000 genes (this is the number in *Arabidopsis thaliana*; there are more in other plant genomes). The number of proteins may be slightly smaller than this, but it can easily reach 10,000 at any given time and under a given set of conditions. If the heavy metal applied is changed (or perhaps only its concentration is modified) in order to compare toxic effects, there is a good chance that the dominant target protein will change as well, e.g. due to an increased or decreased Lewis acid strength. If another target protein becomes the main target, a different metabolic pathway within the complicated network of protein interactions can be affected. Thus, our initial hope, that toxic effects of heavy metal ions in living organisms can be predicted by their chemical properties (e.g. via their Lewis acid properties), is very naive and inadequate. It would be rather a big surprise if quantitative relationships between the chemical properties of heavy metal ions and toxicity held under the complex conditions present in living cells. While the Lewis acid concept remains the best basis for explaining the interactions of metal ions with organic ligands such as proteins, our present underdeveloped knowledge of the network of protein interactions in a living cell means that we cannot predict what will happen when a specific protein is blocked by a toxic concentration of a specific metal ion. A tiny difference in the chemical

properties (Lewis acid strengths) of two different heavy metals may result in a change in the preferred target of interaction. This could easily result in the inhibition of the physiological functions of different proteins. The two proteins involved (in reality it may be different sets of proteins) could have different physiological functions. Nobody can predict the consequences of toxicity in plant cells. Thus, at the moment it is quite impossible to predict the toxicity of a specific heavy metal ion on the basic of its chemical properties (its position within the periodic table of the elements or its Lewis acid properties).

The intention to use chemical properties that are relevant to complex formation in living cells is a good one, but we cannot at present expect this to allow us to make any predictions about the quality or degree of heavy metal toxicity (Duffus 2002; Shaw et al. 2004).

2.3 Toxicity of Heavy Metals in Biological Systems

Before we can describe the toxic effects of heavy metals (given the definition provided in the first part of this chapter), it is necessary to recall two well-known facts. First, a heavy metal is not toxic per se; it is only toxic when its concentration in the plant exceeds a certain threshold ("it is the dose that makes the effect"). This is especially important to the second fact: that some elements, called micronutrients, have essential functions in plant cells. This has been shown for Co, Cu, Fe, Mn, Mo, Ni and Zn. Only when the internal concentration exceeds a certain threshold do they demonstrate toxic effects, and then they are commonly termed "heavy metals". As far as we know, all of these plant micronutrients are transition elements. No lead-group elements or rare earth elements have been found to be essential for higher plants. Micronutrients are essential for biosynthesis, growth, nucleic acids, growth substances, chlorophyll and secondary metabolites, carbohydrates and lipids, as well as for stress resistance. A supply of micronutrients is also essential for the integrity of membranes (Rengel 2004). The dose–response curves for essential heavy metals have been described by Berry and Wallace (1981), and show deficiency at suboptimal concentrations, tolerance at optimal concentrations (including the potential of the plant to maintain homeostasis) and toxicity at high concentrations (cf. Hagemeyer 2004). There is another not so well-known fact to be considered too. Some of the nonessential heavy metals have a stimulating or inducing effect when they are applied at very low concentrations (these are termed "low concentration stressors"). As an example, Cd produces some stimulating effects at 5×10^{-8} M in barley seedlings, as do Pb and Ti at low levels in detached barley leaves (Kovacs et al. 2009; Nyitrai et al. 2007).

However, let us now consider the toxic effects of heavy metals. Remember that this means the toxic effects of transition element ions, rare-earth (that are not only rare in the environment but are also rarely investigated) element ions and lead-group element ions on plants.

2.3.1 General Effects

Sharma and Agrawal (2005) described the general effects of heavy metals on plant physiological processes. Because it can be easily measured, plant growth is commonly used as a general parameter to study the influence of stressors, with growth rate inhibition often being the most obvious plant reaction (Fodor 2002; Hagemeyer 2004). This is especially true of the root system, which is the first plant system to come into direct contact with toxic ions. Leaf chlorosis, disturbed water balance and reduced stomatal opening are characteristic effects of toxic Ni concentrations (Clemens 2006), but they are also caused by many heavy metals (as part of heavy metal toxicity syndrome) and even occur more generally as a stress response.

Fodor (2002) suggested an interesting stepwise model for the action of heavy metals in plants. Initially, there are interactions with other ionic components present at the locus of entry into the plant rhizosphere that subsequently have consequences for the metabolism. This is followed by an impact on the formation of reactive oxygen species (ROS) in the cell wall and an influence on the plasmalemma membrane system (stage 1). At stage 2, the metal ion reacts with all possible interaction partners within the cytoplasm, including proteins, other macromolecules and metabolites. Stage 3 is mainly related to the factors that influence homeostatic events, including water uptake, transport and transpiration. At this stage, symptoms start to develop, and they become visible at stage 4 according to Fodor's model. As an example, the chlorophyll and, usually to a smaller degree, carotenoid contents decrease, which have obvious consequences for photosynthesis and plant growth (Barcelo and Poschenrieder 2004). The death of the plant cell occurs at stage 5. This model has the advantage that visible effects are linked to metabolic events that are influenced by the metal ion of interest.

2.3.2 Primary Targets of Heavy Metal Toxicity

Many of the toxic responses induced by heavy metals that have been identified to date have to be classified as being general stress responses, rather than ones that are specific to heavy metals. The question then arises as to whether a specific metal ion actually induces a sensing mechanism in the plant cells for the presence of the toxin at all, or whether it just the damage caused by a heavy metal that induces a signal. According to Clemens (2006), the data that are available to answer this question are "rudimentary at best". To give an example, proline accumulates under Cd^{2+} stress. However, the accumulation does not occur directly in response to the presence of Cd^{2+} but because of the disturbance to the water balance caused by the excess of Cd^{2+}. One way to investigate the specificity of the stress caused by an excess of a heavy metal ion is to apply the microarray strategy to mRNA-related cDNAs in order to compare the effects of different heavy metals with those of other stress signals, e.g. water deficiency stress. Some data are already available, but we are at a very early stage in this type of research (Clemens 2006; Zimmermann et al. 2004). There is one exception: metal-induced

synthesis of phytochelatins (cf. Clemens 2006). In a posttranslational process, the activity of phytochelatin synthase is upregulated by the heavy metal or a metal–glutathione complex. This response does not need much of a signal transduction chain.

2.3.3 Water Relations

Water relations in higher plants under stress have mainly been investigated in connection with high NaCl concentrations in the submolar or even molar range (Ernst 2004). Concentrations of heavy metals are relevant in the submillimolar or even submicromolar range. Thus, direct osmotic effects can be excluded. Some of the effects mentioned here are common to many heavy metals, such as an influence on membrane transport and an inhibition of root growth and enzyme activities. Early effects may be weakly or strongly connected with water relations. It is relevant to focus here solely on the role of stomata and possible effects of heavy metals. In contrast to earlier results, it is now assumed that the primary effects of heavy metals in whole plants are not directly connected with the induction of stomata closure, but that early effects in roots (e.g. disturbed nutrient absorbance) may be responsible for changes in transpiration (Poschenrieder and Barcelo 2004). In some experiments with both high and low concentrations of heavy metals, enhanced transpiration has been measured. The reason for this is not clear, but it may be caused by damage to the cuticula. In most investigations, however, the application of heavy metals increased the stomatal resistance and in this way decreased the rate of transpiration. It is assumed that stomatal closure after the application of heavy metals is a consequence of stress due to water deficiency. This was concluded from the increased levels of proline and abscisic acid that have been measured, since both are known indicators of drought stress. Proline is evidently not involved in metal detoxification but in membrane stabilisation: K^+ loss and lipid peroxidation were reduced after pretreatment with proline.

New experimental results (see references in Poschenrieder and Barcelo 2004) have revised the old concepts that (i) heavy metals have a direct effect on stomata closure and (ii) that the roots simply act as an osmometer, producing a hydraulic signal. Instead, roots can influence the water content via chemical signals, especially abscisic acid. Moreover, water transport appears to be modulated by an impairment of aquaporins, which is one of the earliest responses to heavy metals in plants. Many well-described physiological (or toxicological) responses may not be direct effects but consequences of fast responses in the roots.

2.3.4 Formation of Reactive Oxygen Species

The bleaching effects of many heavy metals in light have been known for a long time and are connected with the formation of reactive oxygen species (ROS; Asada 1999). The main important ROS are singlet oxygen ($^1O_2$) and the hydroxy radical (HO*),

because both are highly reactive, carrying out oxidation reactions with many organic molecules at their sites of formation during their short lives. The generation of ROS is a general phenomenon; higher plants developed a highly sophisticated antioxidant system during the course of evolution. This consists of several enzymes (superoxide dismutases, catalases, ascorbate oxidases, glutathione peroxidases and glutathione reductases) and antioxidant substrates (ascorbate, glutathione and α-tocopherol). The main sources of ROS under control conditions (an absence of toxic concentrations of heavy metals) are photosynthetic and respiratory electron transport processes. Only when the capacity of cells to suppress the concentrations of ROS is exceeded do these species then damage cells over a long period. Heavy metals play many roles in this respect (Sharma and Agrawal 2005):

- They directly disturb electron transport, causing electrons to be transferred to oxygen instead of the natural electron acceptors in chloroplasts and mitochondria
- Disturbances to metabolic reactions feed back to electron transport, as just described
- Redox-active metals in different oxidation states under physiological conditions can participate in the Fenton and Haber–Weiss reaction (c.f. Shaw et al. 2004), producing hydroxyl radicals
- Inactivation and downregulation of enzymes of the antioxidant defence system
- Depletion of antioxidant substrates.

It has been shown on several occasions that lipid peroxidation (Bertrand and Poirier 2005) is just a consequence of oxidative stress, such as that caused by glutathione depletion (Schützendübel and Polle 2002).

As long as the stress is not too high, plants often respond by inducing antioxidant enzymes together with rather unspecific stress proteins, such as heat-shock proteins (Clemens 2006).

2.3.5 Photosynthesis

As already mentioned above, inhibition of photosynthesis is one effect that most of the heavy metals have in common when present at toxic concentrations. It is a very sensitive response. Measuring the photosynthetic activity is a good screening method for detecting possible stress situations, perhaps including those involving heavy metals. Direct effects of heavy metals on light and dark reactions and indirect effects caused by them decreasing the photosynthetic pigment content are involved, as well as changes in stomata function (Mysliwa-Kurdziel et al. 2004). It seems that nearly all of the components of the photosynthetic apparatus are influenced by almost all heavy metals, including chlorophyll and carotenoid content, chloroplast membrane structure, light-harvesting and oxygen-evolving complexes, photosystems and constituents of the photosynthetic electron transport chain (Barcelo and Poschenrieder 2004). Several enzymes involved in the Calvin cycle are also inhibited, especially Rubisco and PEPcarboxylase (Mysliwa-Kurdziel et al. 2004).

2.3.6 Mitochondrial Respiration

Some scientists consider that respiration increases under stress. However, this is more than an oversimplification. It is true that in some plant species the presence of some heavy metals at lower concentrations increases respiration. This is the case, for example, when 1 μM Cd^{2+} was applied to *Vicia faba* (Lee et al. 1976). This, however, is rather the exception to the rule. At toxic concentrations of heavy metals, respiration is usually inhibited (Lösch 2004).

2.4 Conclusion

There seems to be a consensus in the literature that the term "heavy metal" is badly defined and is best avoided (e.g. Duffus 2002; Nieboer and Richardson 1980). However, considering how commonly this term is used in plant science (see the title of this book for example!), it seems hopeless to expect plant scientists to suddenly abandon it. Therefore, we suggest that this term should not be avoided but defined in a better way. Its definition should certainly not be based on the density of the metal in elemental form because it is not relevant to the effects of the metal in plants, whether the defined lower limit on the density of a heavy metal is 3.5 or 7 $g\,cm^{-3}$. The term "heavy metal" should be defined in relation to the position of the element in the period table, because this position is related to the chemical properties of compounds that include the element. Although alkali metals and alkaline earth metals are clearly metals, they are not – as common sense already suggests – "heavy metals". We suggest that three groups from the periodic table should be considered heavy metals: (1) transition elements, all of which are metals, even though some of them form slightly amphoteric oxides (i.e. Ti, Zr, Hf, Rf, V, Nb, Ta, Cr, Mo, W, Mn, Tc, Re, Fe, Ru, Os and Zn); (2) rare earth elements, which are subdivided into the lanthanide series (including La itself) and the actinide series (including Ac); (3) some elements from the p-group that are either metals (Al, Ga, In, Tl, Sn, Pb, Sb, Bi and Po) or metalloids/borderline elements. To keep this definition in line with common sense, we suggest that Ge, As and Te should be included, but not B and Si. Since there is a common name for this third group of heavy metals, we suggest calling them the "lead group", after its most prominent and deeply investigated member. This limitation on the term "heavy metal" clarifies its definition.

The primary targets for the toxicity of heavy metals are still not clear yet. It has been suggested that microarrays could be used to address this issue, but conclusive data is still lacking. Most of the physiological responses are a consequence of heavy metal-induced stress rather than direct effects. An exception is the induction of the synthesis of phytochelatins, which is an example of a direct effect of either heavy metals or glutathione–heavy metal complexes.

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Chapter 3
Soil Microbial Diversity in Relation to Heavy Metals

Shwet Kamal, Ram Prasad, and Ajit Varma

3.1 Introduction

Soil has been defined as the upper weathered layer of the Earth's crust. It consists of a complex mixture of particulate materials derived from abiotic parent minerals, living biota and particulate organic detritus and humic substances. Soil formation is influenced by climate (temperature and moisture), parental material, time, topography, and organisms (Jenny 1994), and involves complex interactions between physical, chemical and biological processes. Soil texture (the relative proportion of particles of different sizes) and mineral constituents depend on the parent material (rocks) and transportation by water, ice, and wind. The soil structure is the distribution of pores of various sizes that occur between soil particles. The pore sizes depend on the level of aggregation of the particulate material in the soil, and the pores contain gases and water.

Soil is also defined as the surface layer of the Earth that is exploited by roots. This kind of definition is not the most appropriate one for introducing a chapter on soil microorganisms, as they are also found in soil compartments that are not colonized by roots. Microorganisms have been observed far below the rooting depth, and numerous bacteria and fungi colonize small pores and microaggregates that are not accessible to roots or even root hairs. According to another definition, soil genesis is a microbiologically driven process. In order to highlight the diversity that results from combining the interactions of very diverse and complex organism communities on different types of rock materials under variable climatic and topographic conditions and over different timescales, many soil scientists avoid using the term "soil," but prefer to speak of "soils."

Basically, soil is a combined mixture of organic matter (derived from living organisms) and unconsolidated minerals (composed of varying proportions of sand,

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silt and clay), and it provides habitats for thousands of soil-specific species. The habitat is the direct soil environment with which a particular species interacts. The soil habitat has edaphic properties; i.e., properties pertaining to the soil or determined by factors inherent to the soil. These properties are the result of interactions between the soil mineral composition, living organisms and their decomposition. The soil provides a porous three-dimensional matrix, with a large surface area, for soil-dwelling species. Thus, species that reside within the soil matrix are interstitial species.

The vegetation and soil biota affect soil development by weathering and controlling organic matter accumulation and mineralization. The recognition of close interactions between soils and vegetation is reflected in the division of soils into major types, which are associated with climatic vegetation zones. Microorganisms are able to modify and shape their physical and chemical environment. They dissolve and alter minerals derived from the parental material, contribute to and mineralize soil organic matter, and recycle nutrients. Microbes produce biopolymers (polysaccharides) as cell envelopes. Such polymers facilitate the formation and stabilization of soil aggregates, and thereby improve the soil's water-holding capacity. Together with colloidal clay particles and humus, the polymers create complex structures with extensive surfaces that adsorb minerals and organic molecules. Adsorption of proteins and nucleic acids to surfaces protect them from biodegradation and denaturation. Adsorbed DNA remains available for horizontal gene transfer through the transformation of competent cells (Lorenz and Wackernagel 1994). The activities of extracellular enzymes are maintained or even increased by adsorption on minerals, whereas adsorption to humic substances can either maintain or decrease their activities (Nannipieri et al. 1990; Allison 2006). Adsorption to soil colloids can strongly reduce the availability of organic molecules as nutrients for microorganisms; such soils can be oligotrophic environments.

Clay colloids of soil minerals serve as catalysts for abiotic chemical reactions. Due to their coatings of metal oxides and hydroxides and electronegative charges, they can mediate electron transfer reactions and catalyze the oxidation of phenols and polyphenols. In this way, they also contribute to humus formation through reactions like deamination, polymerization and condensation of organic molecules. It has been suggested that microbial processes like decomposition and the mineralization of organic substances prevail under moderate conditions, whereas abiotic reactions are more dominant under harsh conditions where microbial activities are hampered (Huang 1990; Ruggiero et al. 1996).

Soil habitats are different from aquatic habitats in that they are much more complex and heterogeneous, resulting in the formation of habitats that can support high microorganism abundance and diversity. A characteristic feature of soil habitats is their wide range of steep physicochemical gradients (e.g., of substrate concentrations, redox potential, pH, available water), which depend upon the size of the soil aggregate. Even small soil aggregate a few mm in size can offer many different microenvironments, resulting in different types of microorganism

colonization (Standing and Killham 2006). Microhabitats are typically a few micrometers in size for unicellular prokaryotes, but may be much larger for filamentous actinomycetes and fungi. Microhabitats for prokaryotes exist either within or between aggregates. Intra-aggregate habitats typically have small pores that are often filled with water and anaerobic, whereas interaggregate habitats are more frequently aerobic. However, the living conditions in these habitats can show considerable changes across both space and time, and so soils are highly dynamic systems.

The distributions, activities and interactions of soil biota depend on soil properties such as texture, structure, available nutrients, and water. The best growth conditions are normally found on surfaces, and so most (80–90%) soil microorganisms attach themselves to surfaces (Hattori et al. 1997) using extracellular biopolymers that stick to particles. Specific soil habitats such as organic litter aggregates, biofilms, the rhizosphere, and animal droppings are rich in readily available organic nutrients and can have very high microbial activities. Hence, the distribution of soil microbes is generally localized, and the volume occupied by microorganisms may be less than 5% of the soil (Nannipieri et al. 2003). Microorganisms are by far the most active and functionally diverse part of the soil biota. It has been estimated that 90% of the soil processes are mediated by the microbiota, including prokaryotes and fungi. Generally, about one-third of the organic carbon of temperate soils is transformed into humus and microbial biomass, whereas about two-thirds of the carbon is respired by microorganisms to CO_2 (Stotzky 1997).

Interestingly, deep subsurface terrestrial environments, which can extend for several hundreds of meters below the soil surface, have been shown to sustain ample microbial biomasses. Although the cell numbers are much lower than those found in the surface soil, a variety of microorganisms – primarily bacteria – are present in deep subsurface soils. These organisms most likely have access to organic nutrients present in the groundwater that percolates down the subsurface material and flows through their habitat. Studies on the microbial ecology of deep basalt aquifers have shown that both chemoorganotrophic and chemolithotrophic prokaryotes (see “Types of Microorganisms”) are present, but that the chemolithotrophs dominate in these environments (Stevens and McKinley 1995).

3.2 Microbial Diversity in Soils

Soil is a natural medium in which microbes live, multiply and die. Increasing attention is being directed towards microorganisms because the fertility of soil depends not only on its chemical composition but also on the qualitative and quantitative nature of the microorganisms inhabiting it. The maintenance of viable, diverse populations and functioning microbial communities in the soil is essential for sustainable agriculture (Beare et al. 1995; Benizri et al. 2002). Thus, interest in

microbial diversity has grown rapidly in the scientific community (Wilson 1988; Franklin 1993; Benizri et al. 2002).

Microorganisms are generally divided into five major taxonomic categories: algae, bacteria, fungi, protists, and viruses. In soil, they are closely associated with soil particles, mainly clay–organic matter complexes (Foster 1988). Microbes in soil can be found as single cells or as biofilms embedded in a matrix of polysaccharides. Their activities and interactions with other microbes and organisms and with soil particles depend on conditions at the microhabitat level, which may even differ over very small distances (Wieland et al. 2001). Soil can therefore be regarded as being highly heterogeneous with respect to the distribution of soil matter and organisms (Beare et al. 1995).

The most primitive organism on this earth was very similar to modern-day prokaryotes – bacteria and archaea. Microorganisms have been observed in diverse environments; consider, for example, the hot spring sulfur bacteria, *Deinococcus radiodurans* (which can survive radiation levels that are 3000 times greater than those lethal to humans).

With the production of oxygen in significant amounts in the Earth's atmosphere as a result of a microbial metabolic process called oxygenic photosynthesis, a new type of photosynthetic bacteria evolved: cyanobacteria. Algae presumably appeared after cyanobacteria because their chloroplasts were derived from cyanobacteria. Fungi appeared only comparatively recently. It is thought that terrestrial fungi may have coevolved with plants because they are closely associated with them. Fungi are often thought to be exclusively terrestrial. However, they have also been reported in marine and other locations far from land (Salyers and Whitt 2001).

3.2.1 Types of Microorganisms

There are two different kinds of organisms that coexist in the contemporary living world: the eukaryotes (more complex organisms with a true nucleus, which include algae, fungi, and protists), and the prokaryotes (simpler organisms without a defined nucleus). Prokaryotes include two microbial groups: the eubacteria (including cyanobacteria: blue-green algae) and the archaebacteria (a heterogeneous group with prokaryotic structure).

If we consider the cell structure and function as criteria, there are three groups of cellular organisms: eukaryotes, eubacteria, and the archaebacteria. The eukaryotes can be subdivided into three groups: the plants, animals, and fungi. The eubacteria can be subdivided into purple, green, Gram-positive and Gram-negative eubacteria on the basis of the cell wall. Based on their nutritional requirements, prokaryotes have been categorized as photoautotrophs, photoheterotrophs, chemolithoautotrophs, and chemolithoheterotrophs (see Table 1.2). Bacteria can also be classified as being aerobic or anaerobic on the basis of their oxygen metabolism.

Prokaryote diversity, however, is not restricted to relationships to molecular oxygen or their ability to capture radiation. Optimal diversity also depends on soil pH, temperatures (cold, ambient, hot), inorganic salts, etc. (Herman et al. 1993; Hurst 2002).

3.2.1.1 Eubacteria

Eubacteria are prokaryotic microorganisms that are known to be the dominant group of microorganisms in the various kinds of soil (Visscher et al. 1992; Borneman et al. 1996). They are present in all types of soil, but their population decreases as the depth of soil increases (Wieland et al. 2001). In general, horizon A (soil with organic matter) of a soil profile contains more microorganisms than horizon B (silicate clay minerals plus organic matter) and C (weathered parent material; Bruns and Slatar 1982; Subba Roa 1997).

The bacterial forms presents in soil are generally cocci (spheres, 0.5 μm), bacilli (rods, 0.5–0.3 μm) or spirilli (spirals; see Fig. 3.1). Bacilli are common in soil, whereas spirilli are very rare in natural environments (Baudoin et al. 2001, 2002). Bacteria have been classified into two broad categories, autochthonous and zymogenous organisms. Autochthonous or indigenous populations are more uniform and constant in soil, since their nutrition is derived from native soil organic or mineral matter (*Arthrobacter* and *Nocardia*; Herman et al. 1993). Zymogenous bacteria require an external substrate, and their activity in soils is variable. They often produce resting propagules (*Pseudomonas* and *Bacillus*). When specific substrates are added to the soil, the number of zymogenous bacteria increases and gradually declines when the added substrate is exhausted (cellulose decomposers, nitrogen-utilizing bacteria, *Nitrosomonas, Nitrobacter*).

Among the ten orders in the class Schizomycetes, three orders, *Pseudomonas*, *Eubacteria* and *Actinomycetes*, contain the species of bacteria that are predominantly reported in the soil (Liesack and Stackebrandt 1992; Benizri et al. 2001). The most common bacteria belong to the genera *Pseudomonas*, *Arthrobacter*, *Clostridium*, *Achromobacter*, *Bacillus*, *Micrococcus*, *Flavobacterium*, *Corynebacterium*, *Sarcina*, *Azosprillium*, and *Mycobacteria* (Loper et al. 1985; Lynch 1987a,b). *Escherichia* is rarely encountered in soils except as a contaminant from sewage, whereas *Aerobacter* is frequently encountered and is probably a normal inhabitant of certain soils (Subba Roa 1997). Another group of bacteria common in soil is the Myxobacteria belonging to the genera *Myxococcus*, *Chondrococcus*, *Archangium*, *Polyangium*, *Cytophaga*, and *Sporocytophaga*. The latter two genera are cellulolytic and so are dominant in cellulose-rich environments (Slater 1988; Benizri et al. 2001).

Although temperature and moisture influence bacterial populations, they can withstand extreme climates (Woese 1987; Benizri et al. 2002). Bacteria can thrive luxuriantly in arctic zones where the temperature is below freezing point, and also in arid desert soils, where temperatures are very high (Moreno et al. 1986). They

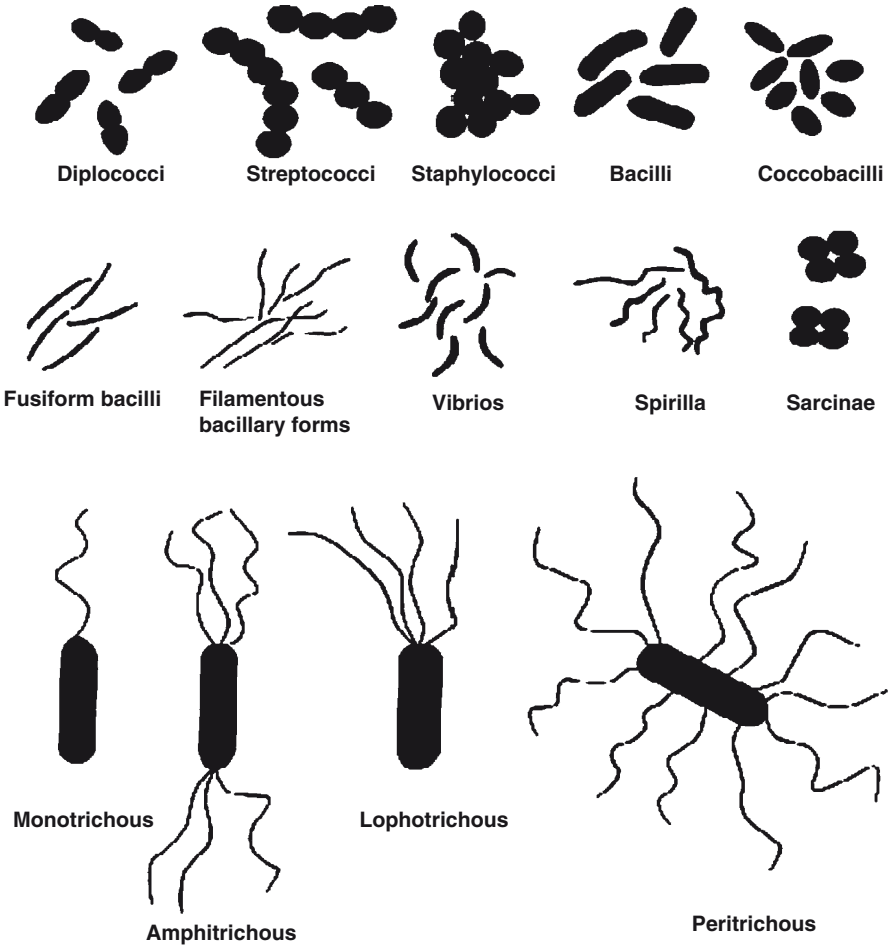

Fig. 3.1 Diversity in bacterial forms

form spores that possess a tough outer covering, facilitating the survival of bacteria in all adverse environments. Based on their temperature tolerance, bacteria can be grouped into mesophiles (15–45°C), psychrophiles (below 10°C) and thermophiles (45–65°C). However, mesophile bacteria constitute the bulk of soil bacteria (Barber and Lynch 1997). Other factors affecting bacterial populations in soils are pH, farm practices, fertilizer and pesticide application, and organic matter amendments (Tate 1987).

Autotrophic and heterotrophic bacteria are present in a wide range of soils (Tate 1995). Autotrophic bacteria (purple and green bacteria) synthesize their own organic matter from CO_2 or inorganic carbon sources, whereas heterotrophic bacteria depend on preformed organic matter for their nutrition and energy support.

Photoautotrophs derive their energy from sunlight, which they catch and transform into chemical energy through bacteriochlorophyll pigment. Chemoautotrophs oxidize inorganic materials in order to derive energy, and obtain carbon from CO_2 (Tate 1995). There is a group of bacteria known as the obligate chemoautotrophs. Within this group, *Nitrobacter* utilizes nitrite and *Nitrosomonas* ammonium, while *Thiobacillus* converts inorganic sulfur compounds to sulfate and *Ferrobacillus* converts ferrous ions to ferric ions (Alexander and Clark 1965; Baudoin et al. 2002).

The cyanobacteria are a structurally diverse assembly of Gram-negative eubacteria characterized by their ability to perform oxygenic photosynthesis. They are considered true prokaryotic microorganisms (Stanier et al. 1986). They have characteristics common to bacteria and algae and are therefore often named "blue-green algae." Cyanobacteria contain a pigment known as phycocyanin, in addition to chlorophyll, which gives these organisms their special blue-green color. The dominant cyanobacteria belong to the genera *Chrococcus*, *Aphanocapsa*, *Lyngbya*, *Oscillatoria*, *Phormidium*, *Microcoleus*, *Cylindrospermum*, *Anabaena*, *Nostoc*, *Scytonema*, and *Fischerella* (Subba Roa 1997; Benizri et al. 2002). Some cyanobacteria also possess heterocysts, which are implicated in nitrogen fixation. Rice fields are a good habitat for the development of certain cyanobacteria that fix atmospheric nitrogen (Prescott et al. 1996).

3.2.1.2 Actinomycetes

Actinomycetes are soil microorganisms that have sufficient distinctive features to delimit them into a distinct group within the prokaryotes. *Actinomycetes* are grouped with other bacteria in the class Schizomycetes, but are confined to the order Actinomycetales. They bear certain similarities to Fungi Imperfecti in the branching of the aerial mycelium, which profusely sporulates, and in the formation of distinct clumps or pellets in liquid cultures (Benson 1988).

The number of actinomycetes increases in the presence of decomposing organic matter. They are intolerant of acidity and their numbers decline below pH 5.0. Waterlogged soil is unfavorable to the growth of actinomycetes, whereas desert soils of arid and semiarid zones sustain sizeable populations, probably due to the resistance of spores to desiccation. The percentage of actinomycetes in the total microbial population increases with the depth of soil. Actinomycetes can even be isolated in sufficient numbers from the C horizons (weathered parent material) of soil profiles.

The commonest genus of actinomycetes is *Streptomyces* (nearly 70%). In contrast, *Nocardia* and *Micromonspora*, and in particular *Actinomyces*, *Actinoplanes* and *Streptosporangium*, are only encountered occasionally (Prescott et al. 1996; Subba Roa 1997). Temperatures of between 25 and 30°C are conducive to the growth of actinomycetes, although thermophilic cultures growing at 55 and 65°C are common in compost heaps, where they are present in large numbers, and belong mostly to the genera *Thermoactinomyces* and *Streptomyces*.

3.2.1.3 Archaebacteria

The term archaebacteria refers to a group of primitive prokaryotes that are considered to be the first organisms to appear on the Earth, so they are also called the ancient bacteria. They even live in extreme hostile environments, like salt pans, salt marshes, hot sulfur springs, etc. Archaebacteria is a heterogeneous group that is phylogenetically very distant from Eubacteria and possesses distinct characteristics (Table 3.1). These bacteria are characterized by the absence of peptidoglycan in their walls. Instead, their walls contain proteins and noncellulosic polysaccharides. Their cell membranes contain branched chain lipids that enable them to tolerate extreme temperatures and pHs. Their rRNA are also quite different from those of other organisms (DeLong and Pace 2001; Huber et al. 2002).

Archaebacteria comprise two subgroups, obligate and facultative anoxybionts. Obligate anoxybionts live exclusively in the absence of oxygen and die in the presence of O_2. They include methanogenic and halophilic species. Facultative anoxybionts are found in the presence of oxygen but can live under anaerobic conditions. They are represented by thermoacidophiles (Tate 1995; Barns et al. 1996; Kyrpides and Olsen 1999).

Methanogens

Methanogens occupy a narrow ecological niche. They mediate the formation of methane from simple substrates (e.g., H_2–CO_2, formate, methanol, and acetate) in highly reducing and anaerobic environments (Garcia et al. 2000). They have the

Table 3.1 Diversity of archebacteria in soil

Archaebacteria	Characteristic
Methanogens (*Methanococcus, Methanosprillum*)	Generate methane when they oxidize hydrogen gas as an energy source using CO_2 as a terminal electron acceptor.
Extreme halophiles (*Halobacterium, Halorubrum, Natrinobacterium, Natronococcus*)	Found near salt lakes, soda lakes, and brines. They produce pigments and can be seen as pink blooms in concentrated saltwater ponds.
Methane-generating thermophiles (*Methanothermus*)	Found near hydrothermal vents; can grow at temperatures near to 100°C.
Sulfur- and sulfate-reducing hyperthermophiles (*Thermococcus, Archaeoglobus, Thermoproteus, Pyrodictium, Pyrolobus*)	Obligate anaerobes that use sulfur or sulfate as a terminal electron acceptor, generating hydrogen sulfide. *Thermococcus* and *Archaeoglobus* oxidize organic compounds as an energy source; *Thermoproteus, Pyrodictium,* and *Pyrolobus* oxidize H_2 as an energy source.
Sulfur oxidizers (*Sulfolobus*)	Oxidize sulfur as a source of energy using O_2 as a terminal electron acceptor to generate sulfuric acid.
Thermophilic extreme acidophiles (*Thermophilus*, *Picrophilus*)	Grow only in extremely hot, acidic environments.

most stringent requirements of any anaerobes for the absence of oxygen (<2 ppm), and they require a redox potential of less than −330 mV for growth. Methanogenic bacteria metabolize best at near-neutral pH values, 6.7–8.0. Very low rates of methanogenesis, however, have been observed at slightly lower pH values. The cultivation of a methanogen, *Methanobacillus*, at pH 4 has been reported by a Russian worker, Kuzneceorii (Wolfe and Higgins 1979). Some of the methanogens (*Methanopyrus kandleri*) are also known to be hyperthermophiles that can grow at 98°C as well as at high salt concentrations, as the formyltransferase from *M. kandleri* was characterized extensively with respect to thermo- and halophilicity (Shima et al. 2004).

Methanogens are ubiquitous in highly reducing habitats. Some of them live as symbionts in the rumen or the first chamber of the stomach in ruminant animals. The most common species of methanogens are *Methanobacterium*, *Methanobrevibacter*, *Methanococcus*, *Methanospirillum*, and *Methanosarcina*. Methanogenesis has now been attributed to more than 50 species of bacteria (Jones 1991).

Methanogenesis occurs in three steps. First, the decomposition of organic matter in anaerobic environments is performed by bacteria via hydrolysis. This is followed by fermentation, performed by bacteria and some archaea, and methanogenesis, performed exclusively by members of the domain Archaea. Methanogens produce methane by reducing carbon dioxide using hydrogen as an electron donor, or through the cleavage of acetate into methane and carbon dioxide.

Halophiles

Large populations of a small and distinctive group of halophiles inhabit highly saline environments (*Halococcus* and *Halobacterium*). These archaebacteria live in extremely strong brine or salt solutions, salt beds, and salt marshes. Some halophiles occur in deep-sea volcanic vents at 100°C; the extreme hydrostatic pressures in these vents mean that water remains liquid at this temperature. In strong light, halophiles develop a purple-pigmented membrane that can absorb solar radiation. The absorbed light is utilized in the synthesis of ATP. These archaebacteria are unique because they carry out their metabolic processes directly using the ATP produced by their pigmented membrane. They cannot convert CO_2 to sugar (unlike in photosynthesis). Halophiles growing in salt beds give off an offensive smell and make the salt an undesirable color (Beare et al. 1995; Barns et al. 1996).

Thermoacidophiles

Thermoacidophiles occur in high-temperature environments, such as hot sulfur springs, where the temperature may be as high as 80°C and the pH as low as 2. These archaebacteria are chemoautotrophic and obtain energy and carbon by oxidizing sulfur while consuming CO_2. Under aerobic conditions they oxidize sulfur to sulfuric acid. Some archaebacteria can also reduce sulfur to hydrogen sulfide in the absence of oxygen (Tate 1995; Prescott et al. 1996).

3.2.1.4 Fungi

Fungi possess the greatest diversity among soil microorganisms (Table 3.2). They possess a filamentous mycelium consisting of individual hyphae, which can be uni-, bi- or multinucleate and nonseptate or septate (Hawksworth 1991b). The quality and quantity of organic matter have a direct impact on fungal flora and populations in soils since fungi are heterotrophic organisms. Fungi can grow in acidic, neutral, or alkaline soils, giving them an advantage over populations of bacteria and actinomyetes, which do not grow in acid soils. Arable soils contain abundant fungi, since they are strictly aerobic and so excess soil moisture decreases their numbers. Fungi exhibit a selective preference for various soil depths. Species common at lower depths are rarely found on the surface. This specific distribution is determined by the availability of organic matter and by the ratio between oxygen and carbon dioxide in the soil's atmosphere at various depths.

Fungi are classified into phycomycetes, ascomycetes, basidiomycetes and Fungi Imperfecti (Table 3.2). Most fungi that are commonly isolated from soils derive from the class Fungi Imperfecti by virtue of the fact that they produce abundant asexual spores but lack sexual stages. Members of this class are distinguished by their septate mycelium and a structure called a conidiophore from which conidia or spores are continuously produced. The other three classes of fungi employ both

Table 3.2 Major groups of soil fungi

Group and representative members	Distinguishing characteristics	Asexual reproduction	Sexual reproduction
Zygomycetes *Rhizopus stolonifer* (black bread mold)	Multicellular, coenocytic mycelia.	Asexual spores develop in sporangia on the tips of aerial hyphae.	Sexual spores known as zygospores can remain dominant in adverse environments.
Basidiomycetes *Agaricus campestris* (meadow mushroom), *Cryptococcus neoformans*	Multicellular, uninucleate mycelia. Group includes mushrooms, smuts, rusts that affect the food supply.	Commonly absent.	Produce basidiospores that are born on club-shaped structures at the tips of the hyphae.
Ascomycetes *Neurospora, Saccharomyces cerevisiae* (baker's yeast)	Unicellular and multicellular with septate hyphae.	Common by budding, conidiophores.	Involves the formation of an ascus on specialized hyphae.
Deuteromycetes (Fungi Imperfecti), *Aspergillus, Penicillium*	A number of these are human pathogens.	Budding.	Absent or unknown.

sexual and asexual methods of reproduction. Phycomycetes possess nonseptate mycelia and produce an undefined number of specialized spore cells called sporangia. In ascomycetes, the sporangium produces a species-specific number of meiotic spores (often four or eight), and different types of active or passive spore extrusion mechanisms are encountered.

A higher degree of specialization of the sporangium, known as the basidia, is realized in basidiomycetes. Here, the number of meiotic spores produced is constant (generally four). Fungi, especially asco- and basidimycetes, are able to degrade very complex organic compounds such as cellulose or lignin, but many of them also live as root symbionts (mycorrhizas) and obtain simple sugars from their plant partners (Lynch and Hobbie 1988). Some of the genera of fungi present in soils include *Acrostalagmus*, *Aspergillus*, *Botrytis*, *Cephalosporium*, *Gliocladium*, *Monilia*, *Penicillium*, *Scopulariopsis*, *Spicaria*, *Trichoderma*, *Trichothecium*, *Verticillium*, *Alternaria*, *Cladosporium*, *Pillularia*, *Cylindrocarpon* and *Fusarium*, *Absidia*, *Cunninghamella*, *Mortierella*, *Mucor*, *Rhizopus*, *Zygorynchus*, *Pythium*, *Chaetomium*, and *Rhizoctonia* (Newman 1985; Hawksworth 1991a; Subba Roa 1997).

Filamentous fungi in soil degrade organic matter and help in soil aggregation. Certain fungi, including *Alternaria*, *Aspergillus*, *Cladosporium*, *Dematium*, *Gliocladium*, *Helminthosporium*, *Humicola*, and *Metarhizium*, produce substances similar to humic substance in soil and thus may be important in the maintenance of soil organic matter (Hawksworth 1991b).

3.2.1.5 Rhizospheric Microorganisms

The rhizosphere is the region of soil that is immediately adjacent to and affected by plant roots. It is a very dynamic environment where plants, soil, microorganisms, nutrients, and water meet and interact. The rhizosphere differs from the bulk soil because of the activities of plant roots and their effect on soil organisms. A major characteristic of the rhizosphere is the release of organic compounds into the soil by plant roots. These compounds, called exudates, make the environment of the rhizosphere very different from the environment in the bulk soil. The exudates increase the availability of nutrients in the rhizosphere, and also provide a carbon source for heterotrophic microorganisms. The exudates cause the number of microorganisms to be far greater in the rhizosphere than in the bulk soil. Their presence attracts larger soil organisms that feed on microorganisms, and the concentration of organisms in the rhizosphere can be up to 500 times higher than in the bulk soil.

Bacteria are the most numerous organisms in the soil, averaging 10^6–10^9 organisms per gram of rhizosphere soil. Due to their small mass, they only account for a small amount of the biomass of soil. Nonsporulating rods, pseudomonads, and actinomycetes are the most common bacteria in rhizosphere soil.

Both pathogenic and symbiotic fungi associate with the rhizosphere. They average 10^5–10^6 organisms per gram of rhizosphere soil. Zygomycetes and hyphomycetes readily inhabit the rhizosphere because they metabolize simple sugars (Sylvia et al. 2005).

3.2.1.6 Rhizoplane Microorganisms

The rhizoplane is the root epidermis and outer cortex to which soil particles, bacteria, and fungal hyphae adhere (Singer and Munns 2006; Sylvia et al. 2005). The functional definition of the rhizoplane is the microorganisms and soil particles that remain after the roots have been shaken vigorously in water. There are more microbes in the rhizoplane than in the more loosely associated rhizosphere. This was found by counting the number of colony forming units (CFUs), as determined by spreading extracted soil microorganisms across agar and counting the number of independent clusters of microorganisms. Microbes are most abundant where the integrity of the root is compromised. For this reason, rhizoplane microorganisms tend to be found on older rather than younger roots. Bacteria and fungi that live within the cells of the root are not considered part of the rhizoplane, but are instead called endophytes (Sylvia et al. 2005).

3.2.1.7 Mycorrhiza

Many species of basidiomycetes and ascomycetes as well as most glomeromycetes form tight symbiotic associations with roots of vascular plants and rhizoids of non-vascular plants. This association between the fungal hyphal network and the plant tissues is called a mycorrhizal association (Fig. 3.2). The role of this association is to mediate competition for nutrients between plant species and fungal species. Given the continuously fluctuating nature of the soil environment, the fungus will at times support plant nutrition, while the plant will support the fungus on other occasions. This symbiotic advantage is most evident in times of water or nutrient stress, when a more competitive plant species will act as a source for another individual through the mycorrhizal hyphae.

Types of Mycorrhizae

Woody perennial species tend to form ectomycorrhizae with basidiomycetes (and a smaller number of Ascomycetes species). These include beech, birch, larch, oak and pine genera in symbiosis with a variety of Agaricales and Boletales species. Of the 6,000 or more fungal species that form ectomycorrhizae, about 4,500 are epigeous, while the remainder are hypogeous. It is estimated that about 3% of seed plants form ectomycorrhizae (Fig. 3.3). Ectomycorrhizal association involves the penetration of hyphae between plant cells without intracellular invasion. This network of hyphae within the plant tissues is called the Hartig net. At the periphery of the root, hyphae grow transverse to the root axis in a dense mycelium called the mantle. In mycorrhizae with a reduced mantle and/or more extensive Hartig net, the association is called an ectendomycorrhiza (Fig. 3.3).

Endomycorrhizae are the most common type of mycorrhizae, and they can be subdivided into several categories, as devised by Smith and Read (1997): arbuscular, arbutoid, ericoid, monotropoid, and orchid (Fig. 3.4). Arbuscular mycorrhizae

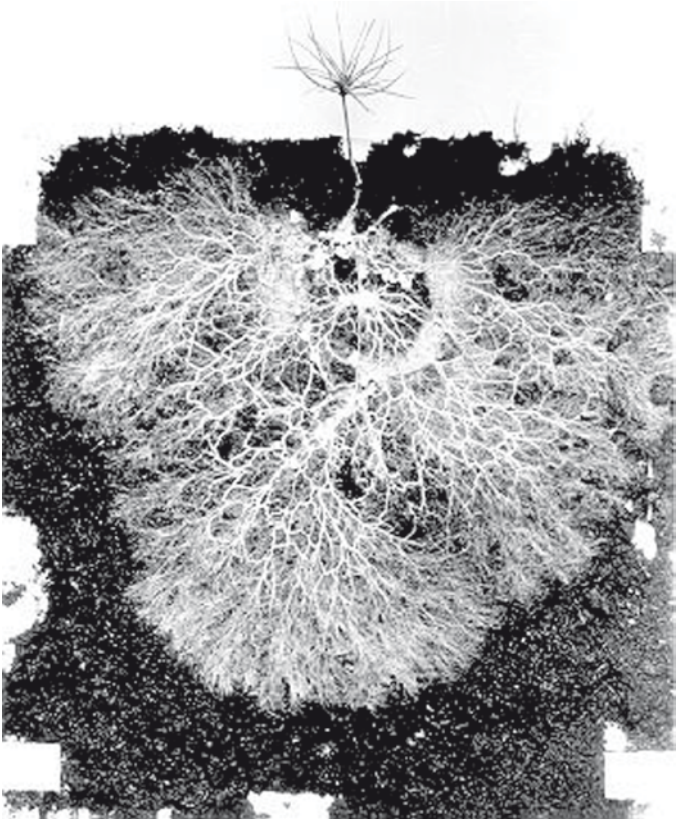

Fig. 3.2 Extensive network of mycorrhizal hyphae radiating from the roots of a larch (*Larix*) seedling grown in peat (c.f. Jim Decon; http://www.biology.ed.ac.uk/research/groups/jdeacon/mrhizas/ecbmycor.htm; used with permission)

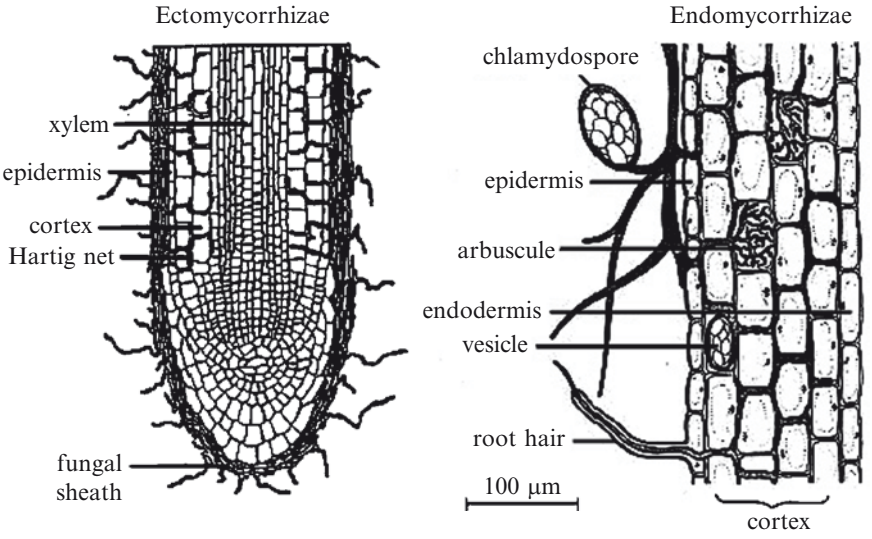

Fig. 3.3 Diagram showing ecto- and endomycorrhiza

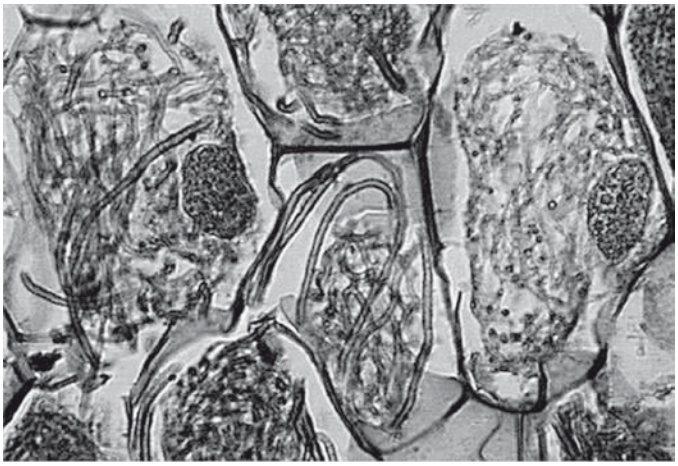

Fig. 3.4 Orchid mycorrhiza, showing coils of fungal hyphae in cells of the protocorm (c.f. Jim Decon; http://www.biology.ed.ac.uk/research/groups/jdeacon/mrhizas/ecbmycor.htm; used with permission)

occur in most vascular and nonvascular plant families. Indeed, most plants are capable of forming arbuscular mycorrhizae. The fungal species responsible are obligate symbionts that cannot be grown without the plant host. They occur in the Archemycota taxa Acaulosporacea (*Acaulospora* and *Entrophosphora*), Gigasporacea (*Gigaspora* and *Scutellospra*), and Glomacea (*Glomus* and *Sclerocystis*). These types of mycorrhizae typically consist of sparse and loose hyphae on the root or rhizoid surface, with most of the mycelium inside the plant tissue or in the soil. The hyphae inside the plant grow between the cell walls, and plant cells are also penetrated. The hyphal cell membrane does not break through the plant cell membrane. Both membranes remain in close apposition even when highly invaginated or ramified.

The hyphal extensions into plant cells branch profusely and repeatedly, and resemble a small tree – thus the term "arbuscular." In some cases, the intracellular hyphae grow into a tight coil. In about 80% of cases, the symbiosis is accompanied by the formation of intracellular "vesicles" inside some plant cells. The vesicle is a terminal hypha that is an intracellular propagule, like an intracellular spore. It contains a reinforced cell wall and storage material (lipid droplets, glycogen and proteins), as do the chlamydospores formed in the soil matrix. Three other types of mycorrhizae are recognized. In the Ericales, the roots normally have a few cell layers and are colonized by dense intracellular hyphae. Epidermal cells that are colonized do not form root hairs.

Dispersal of some fungal species is by arthroconidia, whereby the hyphae break into nucleated segments by septation. A variant form is observed in some Ericales, notably in *Arbutus*, called arbutoid mycorrhizae. These are formed by Basidiomycetes species that often form ectomycorrhizae with other plant species. The last two forms of mycorrhizae involve plants that are achlorophyllous for at least a part of

their life histories, namely those in the Monotropaceae and Orchidaceae. Both of these rely to a large extent on their mycorrhizae for survival, as they are not capable of generating carbon. The fungus mediates between soil organic matter and a second plant host, transferring organic molecules into the parasitic host. The fungal species are often basidiomycetes that form ectomycorrhizae with other plants, or saprotrophic and parasitic fungal species.

Piriformospora indica

Arbuscular mycorrhizal fungi play an indispensable role in upgrading plant growth, vigor and survival by enhancing the nutritive and hydration status of the plant and soil health, by increasing reproductive potential, improving root performance, and by providing a natural defense against invaders, including pests and pathogens. However, the growth of arbuscular mycorrhizae in pure culture in the absence of living host roots is a matter of global concern. Unfortunately, their biotechnological applications cannot currently be exploited to the level they deserve due to their axenically unculturable nature.

Scientists from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, have for the first time screened a novel endophytic root-colonizing fungus that mimics the capabilities of a typical arbuscular mycorrhizal fungus, *Piriformospora indica*. Based on anatomic and genomic studies, *P. indica* has been classified as a highly evolved species of Hymenomycetes (Basidiomycetes). This fungus has been patented (Varma and Franken 1997) at the European Patent Office, Muenchen, Germany (Patent No. 97121440.8-2105, Nov. 1998), and the culture has been deposited at Braunsweich, Germany (DMS No.11827). The 18S rDNA fragment has been deposited in GenBank, Bethesda, USA. Like arbuscular mycorrhizal (AM) fungi, *P. indica* functions as bioregulator, biofertilizer and bioprotector, overcoming water stress (dehydration), delaying the wilting of leaves, and prolonging the aging of callus tissues. Interestingly, the host spectrum of *P. indica* is very much like that of AM fungi; it can colonize the roots and improve the health, vigor and survival of a wide range of mono- and dicotyledonous plants (Fig. 3.5). This fungus mediates the uptake of phosphorus from the substratum and its translocation to the host via an energy-dependent active process. It serves as a useful agent for the biological hardening of tissue culture-raised plants, protecting them from "transplantation shock," and leading to almost hundred-percent survival rates in the hosts tested. This fungus is also a potential biological agent against potent root pathogens. Thus, it displays immense potential for use as a biological tool for plant promotion, protection from pests, and for relieving stress conditions, such as those caused by acidity, desiccation, and heavy metal toxicity.

Algae

Soil algae are ubiquitous in nature when moisture and sunlight are available. The dominant algae in soils are members of the class Chlorophyceae. Diatoms have also been found in soils. These microorganisms are visible to the unaided eye in the

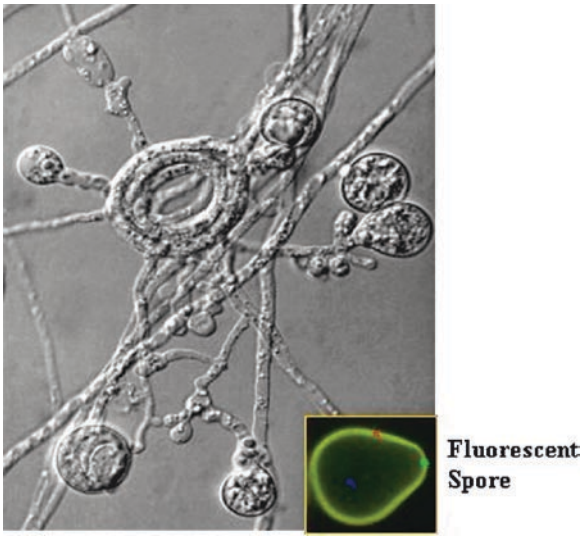

Fig. 3.5 *P. indica* mycelium and its typical pyriform spore

form of green scum on the surface of soils, whereas some algae are microscopic. In the soil, algae are not as plentiful as fungi (Metting 1988). They may be unicellular (*Chlamydomonas*) or filamentous (*Spirogyra*, *Ulothrix*). Algae are photoautotrophic organisms due to the presence of chlorophyll in their cells. They use CO_2 from the atmosphere and produce O_2. Algae have also been found below the surface of the soil and beyond the reach of sunlight. However, their numbers are low in these locations compared to those of algae that inhabit the surface of the soil (Metting 1988; Subba Roa 1997). Some of the most common green algae that occur in most soils belong to the genera *Chlorella*, *Chlamydomonas*, *Chlrococcum*, *Oedogonium*, *Chlorochytrium*, and *Protosiphone* (Metting 1988; Lynch 1990).

3.3 Soil Pollution

One form of pollution that has affected soil microbial communities and activities for many decades is acid deposition. This is caused by acid precipitation, the result of the release of nitrogen oxide (NO_x) and sulfur dioxide (SO_2) into the atmosphere, where they are oxidized to SO_4 and NO_3. Despite the efforts made to reduce the primary sources of acid input, its effects are still apparent in many regions. The effect of acid deposition on the soil ecosystem depends on the concentrations of SO_4 and NO_3, the amount of precipitation, and the buffering capacity of the soil (the cation exchange capacity via bases). The nitrogen and sulfur provided by acid rain may stimulate the growth of some soil microorganisms. On the other hand, even low-level

but prolonged acid rain will result in soil acidification, which may have adverse effects on soil bacteria, whereas its effect on fungi seems to be minor (Pennanen et al. 1998a).

The effect of acid deposition can be direct or indirect. The lower pH and reduced concentrations of divalent cations (Ca^{2+}, Mg^{2+}) can lead to the mobilization and increased bioavailability of heavy metals and other toxic compounds (Francis 1986). Acidification of soils may also reduce the solubility of organic matter and thereby reduce substrate availability for microbes. Increased soil acidity does not seem to affect prokaryotic biomass to any significant extent; instead, it reduces prokaryotic growth rates and activity (Francis 1986; Pennanen et al. 1998b).

Reduced activities of a number of soil enzymes, such as dehydrogenases, ureases and phosphatases, have been observed upon significant pH reductions (Killham et al. 1983). The reduced microbial growth observed with increased acidity may indicate that more metabolic energy is used for maintenance rather than for the biosynthesis of cell materials. It has been suggested that an increased metabolic quotient (ratio of basal respiration to microbial biomass) indicates a shift in energy use from growth to maintenance, and that this increased energy demand is a sensitive indicator of physiological adaptation to environmental stress (Post and Beeby 1996; Liao and Xie 2007).

Soil can have naturally high concentrations of heavy metals as a result of the weathering of parental material with high amounts of heavy metal minerals (e.g., mineral sulfides). Other sources include contaminations associated with mines and metal smelters, which have led to increased soil concentrations of heavy metals, such as zinc, cadmium, copper and lead. Sewage sludge may also contain heavy metals, and it has been demonstrated that the long-term application of heavy metal containing sewage sludge to agricultural soils can have profound effects on the microbial diversity and community composition (Sandaa et al. 1999; Gans et al. 2005).

The effect of heavy metal toxicity depends on soil factors such as organic matter and clay content, divalent cation concentrations (cation exchange capacity), and pH (Giller et al. 1998). These factors influence complex formation and the immobilization of heavy metals. However, the relative toxicities of different metals, namely Cd, Cu, Zn, and Pb, appear to be the same irrespective of soil type (Baath 1989). In soil contaminated for 40 years with high concentrations of Cr and Pb, the microbial biomass and activity were reduced and soil organic carbon had accumulated. These results indicated that Pb exerted a greater stress on soil microbes than Cr.

Soil microorganisms vary widely in their tolerance to heavy metal contamination, and the proportion of culturable resistant microorganisms can range from 10% to nearly 100%. The activities of enzymes in soil may serve as indicators of heavy metal contamination, as there are generally high correlations between reduced enzyme activities (of, e.g., dehydrogenases, acid phosphatases and ureases) and increased heavy metal contamination (Baath 1989). It has been reported that heavy metal contamination has different effects on soil bacteria and fungi (Rajapaksha et al. 2004). Metal addition decreased bacterial activity but increased fungal activity, and fungal activity was still higher in contaminated than in control soil after 35 days. The different effects of heavy metals were also demonstrated by an

increase in the relative fungal/bacterial ratio (estimated using phospholipid fatty acid analysis) with increased metal concentrations.

Mechanisms for metal resistance include stable complex binding (chelation) with organic ligands (extracellular or intracellular sequestration), transportation out of the cells, and biotransformation of the ions to less bioavailable or less toxic metal species. Genes for metal resistance (e.g., mercury resistance) are often present in plasmids and can easily be disseminated through a population or community in response to selection pressure associated with toxic metal exposure.

Hydrocarbon contamination of soils caused by human activities is increasing around the world. Petroleum is a rich source of carbon, and most hydrocarbon components can be biodegraded by microorganisms. The rate of degradation is normally rather low, since crude oil has low concentrations of phosphorus and nitrogen, which does not permit the extensive growth of indigenous hydrocarbon-degrading microorganisms in petroleum-contaminated soils. However, growth can be stimulated by the addition of phosphorus and nitrogen fertilizers. In many extreme environments there are hydrocarbon-polluted areas (Margesin and Schinner 2001). Bioremediation success in such environments depends on the presence of biodegrading microbes that are adapted to the prevailing environmental conditions.

Pesticides are classified according to their primary target organisms; i.e., into herbicides, fungicides, and insecticides (Johnsen et al. 2001). Normally the pesticides are very specific and restricted to a narrow range of target organisms. However, they can be modified in the environment and can become toxic to non-target organisms. For instance, triazines, which normally target photosynthetic enzymes in C3 plants, can be chlorinated in the triazine ring and thus become toxic to a wide range of organisms. The effect of pesticides on soil microbes depends on their bioavailability, which in turn is influenced by the crop being grown, as well as soil properties affecting the sorption and leaching of pesticides. Microorganisms can develop resistance to pesticides through their ability to decompose or transform them into less toxic compounds.

3.3.1 Heavy Metals

The term “heavy metal” refers to a metal or metalloid with a density exceeding 5 $g\,cm^{-3}$, and is usually associated with pollution and toxicity, although some of these elements (essential metals) are actually required by organisms at low concentrations (Adriano 2001). Several heavy metals, such as copper, zinc and iron, are essential for the physiological functioning of living organisms, but they all become toxic at high concentrations. The toxicity of a metal depends on the metal itself, its total concentration, the availability of the metal to the organism, and the organism itself. Depending on the organism and the metal, different modes of action are recognized: binding to macromolecules (proteins, DNA, RNA), disruption of enzymatic functions, catalysis of radical formation, etc. For example, zinc (Zn) is a component found in a variety of enzymes (dehydrogenases, proteinases, peptidases), but it is also involved in the metabolism of carbohydrates, proteins, phosphate, auxins, and in RNA and ribosome formation in plants (Kabata-Pendias and Pendias 2001; Mengel and Kirkby 1982).

Copper (Cu) contributes to several physiological processes in plants (photosynthesis, respiration, carbohydrate distribution, nitrogen and cell wall metabolism, seed production), including disease resistance (Kabata-Pendias and Pendias 2001). The good functioning of the metabolisms of humans and bacteria is also dependent on these two metals (Adriano 2001; Blencowe and Morby 2003; Cavet et al. 2003). However, at high concentrations, these metals exhibit toxic effects on cells (Baker and Walker 1989).

Cadmium, a nonessential, toxic metal to plants, which provides a good example of a heavy metal, can inhibit root and shoot growth, affect nutrient uptake and homeostasis, and is frequently accumulated by agriculturally important crops. Thus, diseases are caused when Cd-enriched crop products are consumed by animals and humans. It is known to disturb enzyme activities, to inhibit DNA-mediated transformation in microorganisms, to interfere in the symbiosis between microbes and plants, as well as to increase plant predisposition to fungal invasion (Kabata-Pendias and Pendias 2001). In humans, it may promote several disorders in the metabolism of Ca and vitamin D, leading to bone degeneration and kidney damage (itai-itai disease) (Adriano 2001).

The excessive uptake of heavy metals by animals and humans is the result of the successive accumulation of these elements in the food chain, with the starting point being the contamination of the soil. Assuming that the Cd pollution is cumulative, with levels increasing over time, the soil may eventually become unusable for crop production. Similarly, contamination of the soil with Cd can negatively affect biodiversity and the activity of soil microbial communities. Experiments by Burd et al. (1998) revealed that canola seeds developed normally in the presence of up to 1 mmol l^{-1} nickel chloride, but that plant root and shoot elongation were inhibited at higher levels.

3.3.2 Soil Pollution by Heavy Metals: A Highly Complex Disruption of Ecological Equilibrium

Heavy metal pollution in soils constitutes a highly complex disruption of ecological equilibrium. Soils naturally contain a broad diversity of metallic elements, and each metal may be present at variable concentrations and as different chemical species. While some metals have no biological relevance, others are essential trace elements that become toxic when present beyond a certain concentration level. As metals often occur in ionized forms in the soil, they react with negatively charged soil particles, meaning that both their concentrations and their bioavailabilities are relevant. The result of this situation is that soil biota must permanently regulate their activities in order make essential metals available and take them up in the required concentrations, as well as to exclude or detoxify detrimental forms or concentrations. In particular, soil microorganisms must display extensive physiological adaptivity. Considering the space and time variability of soils, selection pressure resulting from metal status in soils probably provides an impetus for the adaptation of physiological pathways in soil microorganisms and for their evolution. This is just one example of the complexity of soil, which may explain why the biodiversity of soil microorganisms is so high.

3.3.3 Rhizospheric Microorganisms

The rhizobacteria of metal-accumulating and hyperaccumulating plants and their roles in the heavy metal tolerance and uptake of plants have been studied. Research has also shown that many rhizobacteria are tolerant of heavy metals and play important roles in the mobilization or immobilization of heavy metals.

It is now very clear that the population of rhizobacteria is several orders of magnitude greater than the population in the bulk soil contaminated with the elevated levels of heavy metals and has significant impacts on microorganism population size, community structure, and overall activity of the soil microbial communities. Experiments have shown that the number of bacteria in the rhizosphere of *D. fusca* reached 1.0×10^7 CFU g^{-1}. This relatively low bacterial count can be attributed to the presence of heavy metals in high concentrations (Co: 39 mg kg^{-1}, Cd: 3 mg kg^{-1}, Ni: 79 mg kg^{-1}, Cu: 30 mg kg^{-1}, Zn: 4834 mg kg^{-1}, Cr: 123 mg kg^{-1} and Pb: 114 mg kg^{-1} dry soil) (Abou-Shanab et al. 2005). Chaudri et al. (1992) also found that *Rhizobium* populations were reduced for Cd concentrations of >7 mg kg^{-1} soil. Field studies of metal-contaminated soils have similarly demonstrated that elevated metal loadings can result in decreased microbial community sizes (Brookes and McGrath 1984; Chander and Brookes 1991; Konopka et al. 1999).

Aside from the microorganism community structure of the rhizosphere, population is important in the context of plant growth. This is largely attributed to the finding that microbial populations often establish some sort of positive cooperation with the host plant system. For example, soil pollution with heavy metals could lead to the appearance of heavy-metal resistant rhizobacteria in the soils of industrial regions (Aleem et al. 2003). It was revealed that a high proportion of metal-resistant bacteria persist in the rhizospheres of the hyperaccumulators *Thalaspi caerulescens* (Delorme et al. 2001) and *Alyssum bertolonii* (Mengoni et al. 2001) or *Alyssum murale* (Abou-Shanab et al. 2003a) grown in soil contaminated with Zn and Ni or Ni, respectively. The presence of rhizobacteria increased the concentrations of Zn (Whiting et al. 2001), Ni (Abou-Shanab et al. 2003b) and Se (de Souza et al. 1999) in *T. caerulescens*, *A. murale* and *A. juncea*, respectively.

Multiple metal resistances (MMR) in bacteria seem to be the rule rather than the exception. Abou-Shanab et al. (2005) tested the patterns of heavy metal tolerance in 107 rhizobacterial isolates at 1 mM concentrations, and found that all of the rhizobacterial strains were tolerant of multiple metal ions. Strains that were tolerant of hexa-, penta-, tetra-, and tri-metal ions were found to be more frequent than those with tolerance of hepta-, di- and mono-metal ions.

Notably, cadmium, copper, lead, and nickel resistance seemed to be restricted to strains that were resistant to six metals or more. Similar observations were previously reported by Sabry et al. (1997). High levels of heavy metals could decrease rhizobacterial metabolic activity, biomass, and diversity (Gremion et al. 2004; Sandaa et al. 1999). The activity of the large population of bacteria that inhabit the rhizosphere can also be expected to influence heavy metal uptake by plants. It was reported that plants showed no symptoms of iron deficiency and had fairly high iron

levels in their roots when grown in a nonsterile soil system, in contrast to plants grown in sterile system. This can be attributed to rhizosphere microbial activity, which plays an important role in iron acquisition (Masalha et al. 2000).

Some rhizobacteria can exude a class of rhizobacterial secretions such as antibiotics (including antifungals), phosphate solubilizing enzymes, hydrocyanic acid, indoleacetic acid (IAA), siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which increase bioavailability and facilitate the root absorption of heavy metals such as Fe (Crowley et al. 1991) and Mn (Barber and Lee 1974), as well as nonessential metals such as Cd (Salt et al. 1995), enhance the tolerance of host plants by improving P absorption (Davies et al. 2001; Liu et al. 2000), and promote plant growth (Budzikiewicz 1997; Duffy and Défago 1999; Burd et al. 2000; Ellis et al. 2000; Meyer 2000).

It should be mentioned that the production of IAA by rhizobacteria is believed to play an important role in plant–bacterial interactions (Lambrecht et al. 2000). Therefore, any direct influence of bacteria on IAA production may in turn affect their phytostimulating efficiency. It has been well documented that the biosynthesis and excretion of auxins into soil contributes to a large degree to the bacterial plant-growth-promoting effect (Lambrecht et al. 2000; Kamnev 2003; Steenhoudt and Vanderleyden 2000).

It was found that Cu^{2+} and Cd^{2+} significantly suppresses the production of IAA (auxin) by nonendophytic and facultatively endophytic strains of *A. brasilense*, which can directly affect the plant-growth-stimulating efficiency of associative plant–bacterial symbioses in heavy metal polluted soils (Kamnev et al. 2005).

Various N_2-fixing and auxin-producing Plant Growth Promoting Rhizobacteria (PGPR), siderophores, and antibiotics may stimulate plant growth in the presence of toxic metal concentrations. For example, Masalha et al. (2000) reported an important role of the microbial community in the iron nutrition of plants. In fact, there is evidence that at least some of the toxic effects of some heavy metals on plants results from an induced iron deficiency, and since bacterial siderophores can provide iron to various plants (Bar-Ness et al. 1991; Wang et al. 1993), siderophores produced by rhizobacteria can reduce nickel toxicity by supplying the plant with iron and hence reducing the severity of nickel toxicity (Bollard 1983; Yang et al. 1996).

3.3.4 Bioavailability of Toxic Heavy Metals

Soil rhizobacteria can also directly influence metal solubility by changing the heavy metal speciation in the rhizosphere. Studies of the roles of mycorrhizae in metal bioavailability in the rhizosphere and their ability to increase host plant tolerance of excessive levels of heavy metals in soil showed that there were different availabilities of Cu, Zn, and Pb in the rhizospheres of AM (arbuscular mycorrhiza)-infected and uninfected maize in comparison to bulk soil. The results may indicate that the mycorrhiza can protect its host plant from the phytotoxicity of excessive copper, zinc and lead by changing them from bioavailable forms into forms that are not bioavailable. The fact that copper and zinc accumulations in the roots and shoots of mycorrhiza-infected plants were significantly lower than those in the

uninfected plants may also suggest that the mycorrhiza efficiently restricted excessive copper and zinc absorption by the host plant (Huang et al. 2005).

3.4 Bacteria and Heavy Metals

3.4.1 Impact of Heavy Metals on Bacterial Community Structure and Microbial Processes

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

However, measuring these parameters is not a suitable approach for determining changes in the entire structures of soil communities exposed to pollutants. Since many of the microbiological and biochemical techniques used to study the effects of heavy metals on soil bacteria are cultivation dependent, they do not provide detailed information on noncultivable bacteria, thus neglecting the major part of the soil microbial community. Consequently, soil microbial communities are treated as a black box. These limitations have been overcome by recent advances in molecular fingerprinting methods. These fingerprinting techniques, which are based on analyses of signature biomarkers such as phospholipid fatty acids or nucleic acids, have been used in numerous studies and have indicated significant changes in the microbial community in response to heavy metal stress. Moreover, these methods have allowed the bacterial community to be monitored during the remediation process (Kelly 1998; Macnaughton et al. 1999). These studies have mainly increased our knowledge of sensitive bacterial populations that are negatively affected by heavy metals, but it should also be noted that heavy metals favor the development of tolerant species that can survive and adapt due to their genetic characteristics.

3.4.2 Influence of Soil Rhizobacteria on Heavy Metal Bioavailability

Soil rhizobacteria can directly influence metal solubility by changing the heavy metal speciation in the rhizosphere. The toxic effects of heavy metals on soil microorganisms depend on their bioavailability. Although heavy metal bioavailability is mainly dependent on soil properties (pH and organic matter), bacteria can also directly influence the solubilities of heavy metals by altering their chemical properties.

Microorganisms have developed several mechanisms that can immobilize, mobilize or transform heavy metals. These processes include: (1) extracellular precipitation; (2) intracellular accumulation; (3) oxidation and reduction reactions; (4) methylation and demethylation, and; (5) extracellular binding and complexation (Brierley 1990). The exploitation of these bacterial properties for the remediation of sites contaminated with heavy metals has been shown to be a promising bioremediation alternative (Lovely and Coates 1997; Lloyd and Lovley 2001). However, at high concentrations, bioavailable heavy metals are toxic to a great number of soil microorganisms and soil microbial processes, which in turn will result in severe ecosystem disturbance.

3.4.3 Heavy Metal Resistance Systems in Bacteria

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

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

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

3.4.4 Heavy Metal–Bacteria Interactions

Rhizobacteria have been shown to possess several traits that can alter heavy metal bioavailability (Whiting et al. 2001; Lasat 2002) through the release of chelating substances, acidification of the microenvironment, and by influencing changes in

redox potential (Smith and Read 1997). For example, Abou-Shanab et al. (2003a) reported that the addition of *Sphingomonas macrogoltabidus*, *Microbacterium liquefaciens*, and *Microbacterium arabinogalactanolyticum* to *Alyssum murale* grown in serpentine soil significantly increased the plant uptake of Ni when compared with the uninoculated controls, as a result of soil pH reduction. However, heavy metals are known to be toxic to plants and most organisms when present in soils in excessive concentrations. Giller et al. (1998) reported that there was a detrimental effect on soil microbial diversity and microbial activity (indices of microbial metabolism and soil fertility) in metal-polluted environments.

3.5 Fungi and Heavy Metals

Soil microorganisms are known to play a key role in the mobilization and immobilization of metal cations, thereby changing their availability to plants. Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that establish mutual symbioses with the majority of higher plants, providing a direct physical link between soil and plant roots (Table 3.3). AMF occur in almost all habitats and climates, including in disturbed soils, but soil degradation usually produces changes in the diversity and abundance of AMF populations. Mycorrhizal fungal populations are critical during and after soil disturbance because of their role in the establishment and survival of plants. Thus, changes in population diversity produced by the application of high amounts of metals are expected to interfere with the possible beneficial effects of this symbiotic association, since re-establishment of AMF populations is slow. However, only a few studies have been carried out involving interactions between AMF and metals as a source of soil disturbance.

Most of the results already obtained derive from laboratory and pot experiments, with metal salts used as the source of heavy metals, which are not very representative of natural field conditions, where metals usually accumulate in a less-available chemical form. Heavy metals can delay, reduce, and even completely eliminate AM colonization and AMF spore germination in the field, and a negative correlation between Zn concentrations and AM colonization has been reported in soil treated with urban industrial sludge. In other studies, however, the addition of metal-containing sludge did not significantly affect AM development under field conditions, probably because different AMF ecotypes can exhibit different degrees of metal tolerance. Thus, a relatively high rate of mycorrhizal colonization can be found in plants growing in highly polluted soils. A higher tolerance of Cu, Zn, Cd, and Pb of indigenous fungi from sludge-polluted sites was observed in comparison to those from unpolluted soils.

Species richness and diversity, as measured by the Shannon–Wiener index, increased at moderate levels of soil contamination. This increase in AM propagule diversity could be a fungal stress response whereby fungal ecotypes better adapted to unpolluted soil but affected at intermediate rates of contamination allow other fungi – probably less competitive in unstressed soils but better adapted to heavy metals – to colonize the roots and complete their life cycles. Thus, the number of fungal ecotypes in these

Table 3.3 Fungal species that have arbuscular mycorrhizal associations with plants growing on heavy metal contaminated areas (Griffioen 1994)

Arbuscular mycorrhizal fungi	Soil enriched with	Reference
Acaulospora bireticulata Rothwell and Trappe	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Acaulospora delicata Walker, Pfeiffer and Bloss	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Acaulospora nicolsonii Walker, Reed and Sanders	Pb	Walker et al. (1984)
Gigaspora gigantea (Nicol and Gerd) Gerd and Trappe	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus aggregatum Schenck and Smith emend. Koske	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus albidum Walker and Rhodes	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus deserticola Trappe, Bloss and Menge	Mn, Zn	Arines and Vilarino (1991)
Glomus deserticola Trappe, Bloss and Menge	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus fasciculatum (Thaxter) Gerd. and Trappe emend Walker and Koske	Fe, Mn, Zn	Ernst et al. (1984)
Glomus fasciculatum (Thaxter) Gerd. and Trappe emend. Walker and Koske	Fe, Mn, Zn	Dueck et al. (1986)
Glomus fasciculatum (Thaxter) Gerd. and Trappe emend. Walker and Koske	Cd, Zn	Iestwaart et al. (1992)
Glomus fasciculatum (Thaxter) Gerd. and Trappe emend. Walker and Koske	Pb, Zn	Iestwaart et al. (1992)
Glomus geosporum (Nicol and Gerd) Walker	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus intraradices Schenck and Smith	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus macrocarpum Tul and Tul	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus microcarpum Tul and Tul	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus mosseae (Nicol and Ged) Gerd and Trappe	Cd (Zn)	Gildon and Tinker (1981)
Glomus mosseae (Nicol and Ged) Gerd and Trappe	Cd (Zn)	Gildon and Tinker (1983)
Glomus mosseae (Nicol and Ged) Gerd and Trappe	Mn	Bethlenfalvay and Franson (1989)
Glomus occultum Walker	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus pubescens (Sacc and Ellis) Trappe and Gerd	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus rubiforme (Gerd. and Trappe) Almeida and Schenck	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Glomus tenue (Greenall) Hall	Zn, Cu	Christie and Kilpatrick (1992)
Glomus tortuosum Schenck and Smith	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Scutellospora hetrogama (Nicol and Gerd) Walker and Sanders	Zn (Cu, Pb, Ni, Cd)	Sambandan et al. (1992)
Scutellospora weresubiae Koske and Walker	Cu	Koske and Walker (1986)

soils can be increased. However, at the highest levels of soil pollution, both indices diminished sharply. This may have resulted from a fungi-toxic effect of metals, causing an inability of certain AMF species to colonize the root system and/or to multiply in the rhizosphere. Only the AMF species that are better adapted to the disturbance produced by the addition of metals can overcome the stress situation and complete their life cycles. However, genetic diversity studies have not yet been described for AM fungi; thus, it is not possible to relate the phenotypical changes found in the present study to changes in the genetic structure of the AMF population.

The host plant-mediated effect on the composition and diversity of the AM fungal community is noteworthy. The reasons underlying stress-related changes in the diversity of AMF populations, particularly those due to the presence of heavy metals, are not completely understood.

3.5.1 Role of Mycorrhizae in Heavy Metal Speciation

Roles of mycorrhizae in metal speciation in rhizosphere showed increased heavy metal tolerance in host plants and a significant change in the Cu, Zn and Pb speciation was observed in rhizosphere of AM infected and uninfected maize. The greatest change was exchangeable Cu, which increased by 26% and 43% in the uninfected and AM-infected rhizospheres, respectively, compared to that in bulk soil. With the exception of organically bound Cu in AM, other metal species were stable in the rhizospheres of the AM and non-AM treatments. It is understandable that Cu was activated by inducing rhizobacteria (Huang et al. 2005). The organically bound Zn and Pb increased significantly in the rhizosphere in comparison to those in the bulked soil. In contrast, carbonate and Fe-Mn oxides of Zn and Pb did not exhibit significant changes. The results may indicate that the mycorrhiza can protect its host plants from the phytotoxicity of excessive copper, zinc, and lead by changing their speciations from bioavailable to nonbioavailable forms. The fact that copper and zinc accumulations in the roots and shoots of mycorrhiza-infected plants were significantly lower than those in the uninfected plants may also suggest that the mycorrhiza efficiently restricted excessive copper and zinc absorption by the host plants (Huang et al. 2005).

It is now well known that heavy metals cannot be chemically degraded. Therefore, remediation of metal-polluted soils is limited mainly to immobilization, for example by phytostabilization, which involves promoting plant growth in order to reduce or eliminate the bioavailability of metals. In this context, AMF constitute an important functional component of the soil–plant system that is crucial to sustainable productivity in stressed soils. A better understanding of the mechanisms behind these changes in AMF diversity – and particularly of those upon which AMF adaptation to and tolerance of metals are based – is important, since such an understanding could facilitate the management of these soil microorganisms for a restoration and/or bioremediation program.

3.6 Conclusion

The soil microbial community is an essential component of terrestrial ecosystems. Microbes are the main acting agents in most soil biogeochemical processes, and they have the ability to interact with the primary productivity of ecosystems by regulating nutrient availability and the degradation pathways of soil contaminants. Contamination of the soil may alter soil processes, including the immobilization and mineralization of nutrients controlled by these microorganisms.

Soil microbial activity is often disturbed by metal contamination. Metal pollution can occur in many forms, the principal ones being mining, metallurgical and industrial waste, automobile exhausts, and land disposal of sewage sludge. The most common heavy metals include Cu, Ni, Cd, Zn, Cr, and Pb. Several heavy metals, such as Cu, Zn and Fe, are essential for the normal growth of microorganisms, but may become toxic at high concentrations.

There are many different mechanisms that organisms can use to overcome the effects of metal contamination, including avoidance, exclusion, immobilization, excretion, and those involving enzymatic changes. As just indicated, heavy metal tolerant fungi and bacteria often use an immobilization mechanism as their main defense against high concentrations of metals: the organisms bind the metals to the cell wall in order to immobilize them. Microorganisms have the ability to interact in a variety of specialized ways with metals. For example, some microorganisms are able to accumulate and immobilize trace metals, and are even capable of crystallizing them. Bacteria are able to produce extracellular polymers that can form capsules or loose aggregates around cells; their anionic properties then allow them to bind to metal cations. Fungi are also capable of accumulating metals.

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Chapter 4
Uptake and Effect of Heavy Metals on the Plant Detoxification Cascade in the Presence and Absence of Organic Pollutants

Lyudmila Lyubenova and Peter Schröder

4.1 Introduction: Which Metals are "Heavy"?

Among the 110 elements present in the periodic table of the elements, 69 possess metallic properties. Seven of the elements in the Earth's crust are also metals.

The term "metals" refers to elements with very good electrical conductance (this property declines with decreasing temperature) and that exhibit an electrical resistance that is proportional to the absolute temperature. Amongst these, heavy metals are metals that have a density of 4.5 $g\,cm^{-3}$ or more. The other metals are referred to as light metals (<4.5 $g\,cm^{-3}$).

Heavy metals are widely spread and can be found in various background concentrations in all environmental compartments. Their availabilities depend on the geology and geomorphology of the given ecological–geochemical system, and they can also be affected by anthropogenic activities.

4.2 Classification of Metals

Metals can be classified according to the HSAB (hard and soft acids and bases) concept.

$$M + L = M{:}L\ (complex)$$

Hard acceptors prefer to bind to hard donors whereas soft acceptors prefer to bind to soft donors to form stable compounds (Shaw et al. 2004). This is the so-called HSAB concept, which is very common in nature: some metals occur in the Earth's crust as ores of oxide and carbonate, whereas other metals occur as sulfides. This is because hard acids will form strong bonds with hard bases, and conversely softer acids prefer soft bases.

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For example, minerals containing Al, Ca or Mg along with oxygen and CO_3 are hard acids, while minerals with Hg or Pb and sulfur are soft acids.

4.3 Metal Toxicity

The relative toxicity of a heavy metal depends on its availability, which is determined by the properties of the soil and the plant species of interest. Once taken up by the plant, heavy metals interact with different cell components and result in disturbances to the normal metabolic processes. This can cause cell injuries and in some cases the death of the organism (Shaw et al. 2004).

The toxicity occurs because either the biological functions of the enzymes are blocked or essential metal ions in the biomolecules are replaced with nonfunctional ions (Shaw et al. 2004), so that the catalytic properties of the biomolecules change or are even lost.

According to Nieboer and Richardson (1980), metals have different ligand binding preferences, which is why they can be classified into three groups:

- Class A metals prefer ligands with available oxygen
- Class B metals bind to ligands containing sulfur or nitrogen
- Class C metals have binding properties that are intermediate between those of classes A and B.

Nieboer and Richardson (1980) hypothesise that a preference for specific ligands will lead to identical effects in different organisms.

Several enzymes rely on the presence of certain metals at their active centres; if these metal ions are shifted in position or displaced by other metals with same size and charge, the activity of the enzyme is inhibited. For example, zinc, which is crucial to the activities of several metal enzymes, can be displaced by cadmium, which is just below it in the periodic table. Despite of the chemical similarities between cadmium and zinc, enzymes that should contain zinc are not able to perform catalysis in an appropriate manner when they contain cadmium instead (Shaw et al. 2004).

The heavy metals with the highest toxicity are those included in class B of Nieboer and Richardson's system (those that bind to ligands containing sulfur and nitrogen). They also exhibit a wide spectrum of toxic mechanisms. No class B metal has ever been found to occur naturally in any enzyme. These metals bond most effectively with SH groups such as cysteine, and with nitrogen-containing groups such as lysine and the active centres of enzymes (Shaw et al. 2004).

4.4 Mixed Pollution with Organic Pollutants

Organic pollutants generate a great deal of interest in many countries due to environmental problems connected to the controlled or uncontrolled emission of organic chemicals into the environment (Schröder and Collins 2002). Frequently, these

organic chemical contaminations are accompanied by various heavy metals, yielding a complex pollution mix. Plants growing on sites that are contaminated in such a manner have many problems to contend with (Schröder et al. 2009). Again, uptake is governed by the properties of the soil, the physicochemistry of the pollutants in the mixture, and by plant-specific features. We may expect that the plants assimilate the damage as single or multiple stress signals; in any case, they must take remedial action very rapidly.

4.5 Sources of Environmental Contamination

Most known heavy metal contaminations are not natural; they have been generated by human activities. Such activities result in the transportation of heavy metals into the environment via particulates in the air, dissolution in contaminated water, or as lump ore deposits. The twentieth century, with its continuously increasing progress in industrial production and the exploitation of resources, was also a period in which the distribution of anthropogenic pollutants in the soil, ground and surface waters, and in the atmosphere increased greatly (Nriagu 1979).

Contaminants are substances that can endanger the environment, humans, animals, plants, soil or water (Hoffmann 1998). Even though the quantities of such contaminants have been reduced across much of Europe during the last decade, they are still a current major problem in many countries. The main sources of contaminants are:

- Critical spot sources, like fuelling stations, oil or metal manufacturers, military garrisons and shooting ranges, mining industry and processing ashes, open pit mining and tailings, municipal and industrial waste depots, and ore depot leaching
- Diffuse sources like burning fossil fuels, pesticides, phosphate fertilisers and communal waste waters (Kabata-Pendias and Pendias 1989).

These sources provide pathways for the release of not only organic contaminants but also heavy metals, which can be highly toxic in very small quantities (Memon et al. 2001; Memon and Schröder 2009).

Heavy metal contaminations of geogenic origin (Shaw et al. 2004) are found in areas where metalliferous veins reach close to the surface, and so metals can be washed out by rain or surface water. This can typically be seen for nickel and arsenic. However, such areas are relatively scarce in comparison to those contaminated by human activities (Pilon-Smiths 2005).

Soil and water contamination with anthropogenic burdens are a general environmental problem for which effective and affordable solutions are urgently required (Memon et al. 2001).

Most of the more frequently used technological methods, like dig and dump, soil restoration, storage or isolation of the contaminated surfaces, are very sophisticated and expensive and so are out of the reach of most of the communities with such pollution problems.

Interestingly, most contaminated sites are populated by plant species that can exist and survive undisturbed on metal-enriched soils (Memon et al. 2001; Memon and Schröder 2009). Some of these endemic plant species have attracted attention because of their high heavy metal accumulation capacities. Even more interestingly from the viewpoint of biomass productivity, some species that do not take up the metal ions and hence are classified as metal avoiders have been found. Such species obviously own detoxification mechanisms that can soften or repair the negative effects of the metals, or transform them in a chemical form that is unable to cause stress. This type of plant can be used for specific heavy metal removal from soils and waters, a task that is part of phytoremediation. The aim of phytoremediation is to remove contaminants from the environment with help of different plant species in a cost-effective, sustainable and environmentally compatible manner.

4.6 Uptake Mechanisms for Metal and Organic Xenobiotics in Plants

Hot spots for heavy metal contamination of plants are the air, water, soil and sediments, and plants make use of the opportunity to enrich themselves in metals when their growth capacities allow this (Greger 2004). Whereas higher plants can take up metals from the air via shoots and leaves, entry via roots and rhizomes from the soil substrate predominates. Of course, heavy metals can be much more concentrated in soils than in water (Förstner 1979).

In any case, metal uptake through leaves and roots depends on the concentration of the metal in the medium. However, uptake does not increase linearly with the concentration of the metal in the medium. This is because metals are often present under bound conditions. The uptake efficiency is highest at lower concentrations, because the low metal concentration also minimises competition between the metal ions at the absorption (uptake) surface (Greger et al. 1991). The larger the root surface area available, the more effective the uptake of the metal ions. Competition for the metal ions between plants at the same location can also occur, reducing uptake efficiency (Marschner 1995).

4.6.1 Factors that Influence Metal Uptake

There are different pathways associated with the entry of dissolved substances into plant cells. The cytosol is a barrier between the vacuole and the outside of the plant cell that offers high resistance to the passage of any solution that includes salts and bases (Nultsch 2001). Plants have a natural tendency to take up metals, and their passage into plant cells will probably be hampered by this barrier. The effectiveness of the metal uptake is highly dependent on the availability, which in turn depends on

many factors such as pH and content of organic matter in the soil. Solubilised metal ions enter the root via either extracellular (apoplastic) or intracellular (symplastic) pathways. The apoplast is the extracellular space into which water molecules and dissolved low molecular mass substances will diffuse. On the other hand, the symplastic compartment consists of a continuum of cells connected via plasmodesmata.

The apoplast plays an important role in the binding, transport and distribution of ions and in cellular responses to environmental stress, contributing to the total elemental content of the roots.

This space comprises about 10–25% of the capillary space of the rhizodermis and the cortex cell walls. The ions flow with the water taken up by the apoplast into free spaces, where some of them will diffuse and some of them will bind to the carboxyl groups on the cell walls or the negatively charged groups of the proteins. The specifics of this internal dissemination depend on the metal and the plant (Greger 2004). Wierzbicka (1998) reported that most of the lead taken up by *Allium cepa* remains bound in the apoplast.

The ions can reach the endodermis, which is the beginning of the "internal space", by travelling along this waterway (Nultsch 2001). To get into the xylem, the ions must pass through the endodermis and the Casparian strip. The Casparian strip (Fig. 4.1) is a waterproof lipophilic surface coating in the radial cylinder of the endodermal cells of the root that consists of suberic substances and lignin. Its role is to block the passage of soluble minerals and water from the internal symplast through the cell walls (predominantly the cells in the central cylinder).

Metal uptake generally occurs in young roots without developed Casparian strips (Marschner 1995). It is not clear how metals pass through the older parts of the root.

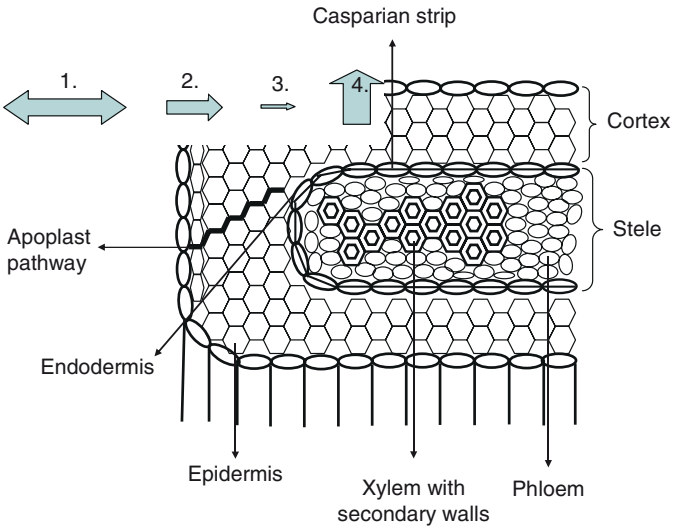

Fig. 4.1 Root uptake of solutes. *1*, Free diffusion in the rhizosphere; *2*, apoplastic diffusion in apparent free space and Donnan free space of the rhizodermis and parenchyma; *3*, transfer to the symplast in the endodermis; *4*, transpiration-stream-driven transport to the shoot

Metal uptake from the soil solution is selective and depends on specific or genetic metal ion carriers or channels located in the plasma membrane. It starts with the influx of the individual ions into the "apparent free space" (AFS) (Nultsch 2001).

Most metal ions enter the cells via an energy-dependent saturable process. Carrier systems transport cations into plant root cells. Nonessential heavy metals can compete for the same transmembrane carriers used by essential heavy metals. Heavy metals are transported acropetally to the roots.

Root uptake and transport of organic xenobiotics is determined by the so-called root concentration factor (RCF) (Schröder and Collins 2002). The RCF is heavily dependent on log $K_{o/w}$ (i.e. the lipophilicity of the compound under consideration), and this seems to be governed by the absorptive properties of the root bark. Compounds with log $K_{o/w} < 1$ cannot penetrate the lipid-containing root epidermis, while compounds with log $K_{o/w} > 2$ become increasingly retained by the lipid in the root epidermis and the mucilage surrounding the root because of their enhanced hydrophobicities (Schröder and Collins 2002).

Compounds with a log $K_{o/w}$ of about 2 are only transported in the transpiration stream, while those with a log $K_{o/w}$ of about 1 are mobile in both phloem and xylem, although these are probably the only metabolites that enter the phloem. For compounds with log $K_{o/w}$ 1.0–3.5, metabolism may occur in the leaf and stem tissue (Schröder and Collins 2002).

Once they are taken up by the roots, both organic xenobiotics and metals can be stored in underground tissues or exported to the shoot. Transport into the shoot involves loading in the xylem sap and translocation to the aerial parts.

In this case, the Casparian strip is the barrier that limits the entry of both xenobiotics and metals into the xylem. Dissociated molecules and ions are transferred relatively easy, whereas substances with higher lipophilicities or strong binding capacities are usually retained. Inside the root stele, transfer to xylem vessels follows the laws of accelerated diffusion in the water stream moving towards the plant shoot. Xylem cell walls have a high cation exchange capacity. The metal chelate complexes reduce the interior concentration of metals in the xylem and facilitate metal transfer into the transpiration stream. Organic acids (especially citrate) as well as amino acids are the main metal chelators in the xylem.

Marschner (1995) reported that the metal uptake increases when the pH increases. The opposite happens in soils. He supposed that there is a competition between the hydrogen ions and the metal ions in the root growth area.

Experiments with aquatic plants have shown that increasing the salt content decreases Cd, Cu and Zn uptake because metal–Cl_x complexes are formed (Greger et al. 1995). These types of complexes are not appropriate for plant uptake. The opposite can be expected in sediment systems containing salt; the cadmium uptake in such a system will be significantly higher. In the case of a high salt concentration, an exchange reaction occurs between sodium ions and cadmium ions bound to colloids in the soil colloids, leading to higher cadmium concentrations in the plant (Greger et al. 1995).

Most of the metal enters the plant via the roots after metal–root contact occurs. In the case of diffuse uptake, metals migrate along their concentration gradients together with other ions to the root and across the cortex tissue. Nearby, ion mass

flow can occur along the gradient in the water potential, which is held at a high level by transpiration, and this leads to enrichment in the shoot (Marschner 1995).

If the root uptake is high and the concentration of the element in the soil is low, the uptake of the element will be limited by diffusion.

4.6.2 Metal Transport in Shoots

The transport of heavy metals in phloem can be complicated because ions can easily be coupled to the phloem of living cells. Cadmium for example can be found in the stipule and in the leaf stalk of pea after the leaves have been treated, but it is not transported further (Greger et al. 1993). Stephan and Scholz (1993) suppose that nicotinamide, which is a metal chelator, influences the content of heavy metals in the phloem. Aquatic plants transport heavy metals in both vessel types. If the osmotic potential around the roots increases, the basipetal transport of zinc and cadmium will also increase; in contrast, the acropetal transport was overbalanced when the leaves were treated with osmotica (Greger 2004).

The plasma membrane acts like a barrier to toxic elements and inhibits uncontrolled uptake into the cell lumen. Metals are taken up in the form of cations and cation transport systems may be used by designated elements. Zinc is transported by specific zinc transporters (Lasat et al. 2000), and copper passes through the membranes via the ATP-dependent copper outflow (Knauer et al. 1997). Light and temperature affect the uptake of cadmium and lead (Hu et al. 1996; Chawla et al. 1991; Hooda and Alloway 1993). An increase in biomass production promotes the uptake of such elements. Accumulation in plants is reduced with dilution, which is affected by growth (Ekvall and Greger 2003).

Costa and Morel (1994) hypothesise that 30% of cadmium is taken up passively by plants; the rest passes through the membrane actively via specific H^+-ATPases. It is not yet known whether these ATPases are identical to the MDR-like tonoplast carriers responsible for the sequestration of xenobiotic glutathione conjugates. In the cytoplasm, the metal binds to negatively charged macromolecules, or to parts of bigger cell structures. Biomolecules, for example phytochelatins, can spontaneously form complexes with the cadmium; using specific transporters, these complexes can pass through the tonoplast and reach the vacuole, where the cadmium separates and complexes with organic acids. The free phytochelatin molecules exit the vacuole and again become available to act as binding partners for metals in the cytosol. The cleavage of the phytochelatin–cadmium complexes happens spontaneously under the low-pH regime of the vacuole (Steffens 1990).

4.7 What Causes Oxidative Stress?

Oxygen is essential not only for energy metabolism and respiration; it also plays a role in degenerative processes (Marx 1987). It is a biradical, which means that it has two unpaired electrons with parallel spins (see Table 4.1). While oxygen does have unpaired electrons, which would normally make it rather reactive, the fact that

Table 4.1 Nomenclature of the different oxygen species

Species	Formula
Triplet oxygen (ground state)	·O–O·
Singlet oxygen	O–O:
Superoxide	·O–O:
Perhydroxide radical	·O–O:H
Hydrogen peroxide	H:O–O:H
Hydroxy radical	H:O·
Hydroxide ion	H:O:
Water	H:O:H

these electrons have parallel spins (known as the "triplet state") actually makes it difficult for oxygen to participate in reactions with organic molecules. Such reactions become easier if the biradical is activated. Activation can occur if enough energy is absorbed by triplet oxygen to "flip" the spin of one of its unpaired electrons (i.e. making the spins antiparallel – the "singlet state"). Singlet oxygen is much more reactive towards organic molecules than triplet oxygen.

Another way to activate oxygen is through the stepwise monovalent reduction of oxygen to superoxide, superoxide to hydrogen peroxide, and hydrogen peroxide to hydroxide radical. The final product of this series of reductions is water:

$$\dot{}O\text{-}O\dot{} = \dot{}O\text{-}O\text{:} = H\text{:}O\text{-}O\text{:}H = H\text{:}O + H\text{:}O\text{:}H = H\text{:}O\text{:}H$$

Hydrogen peroxide is a very important metabolite, because it can diffuse across membranes and is not dispersed in cells. Peroxidase enzymes (POX) use hydrogen peroxide as a substrate in oxidative reactions, primarily during the complex synthesis of organic molecules.

4.7.1 Oxidative Damage to Lipids

Lipid peroxidation proceeds via three phases: activation, distribution and cleavage. The activation of one unsaturated fatty acid (linoleate) by one hydrogen radical results in the cleavage of one H^+ atom from the methyl vinyl group of the fatty acid:

$$OH + RH \rightarrow R + H_2O$$

During this reaction, the resonance structure will react with triplet oxygen, which (as discussed above) is a biradical with two unpaired electrons. These unpaired electrons make it easy for triplet oxygen to react with other radicals. This reaction produces a peroxide radical:

$$R + O_2 \rightarrow ROO$$

This peroxide radical assimilates a hydrogen atom from a second fatty acid, resulting in the formation of a lipid hydroxide. The free carbon centre can then participate in the secondary assimilation of hydrogen:

$$ROO + RH \rightarrow ROOH$$

The main reason for the high reactivity of hydroxide radicals in any given lipid system is their ability to initiate a chain reaction at even very low concentrations.

Iron is a well-known catalyst of lipid oxidation, and it is also a critical reactant in the production of OH^- via the classical Fenton reaction, leading to the formation of reactive alcohol radicals:

$$ROOH + Fe^{2+} \rightarrow OH^- + RO + Fe^{3+}$$

This is the reason for the propagation of the chain reaction in the absence of iron. The end products of the reduction of ROOH are ethylene and ethane, which derive from alkyl chains that are set free during lipid peroxidation.

4.7.2 Oxidative Damage to Proteins

Any oxidative stress that reaches the cytosol can lead to the disruption of lipid bilayers, changes in conductivity, disturbances to proton gradients, and to higher functional protein sensitivity in general. Amino acids vary considerably in terms of their binding and reactivity with radicals.

In particular, metal-containing amino acids and the thiol groups of the proteins are very sensitive. Activated oxygen can extract a hydrogen atom (H^+) from cysteine to form a thiol radical, which can then bond with other cysteines via a disulfide bond. Alternatively, oxygen can bond with methionine residues, so that methionine–sulfur derivatives are formed. The oxidation of iron–sulfur centres via superoxide disturbs enzymatic function (Gardner and Fridovich 1991). When several amino acids are modified by radicals, the protein is oxidised.

4.7.3 Where are the Products of Activated Oxygen Formed?

The reduction of oxygen to its products – superoxide, hydrogen peroxide and hydroxide radicals – is the principal oxygen activation mechanism in most biological systems (McKersie 1996). The formation of singlet oxygen in the photosystems of higher plants is their main source of radicals (Wagner 2006). Oxygen radicals are produced either as end-products of chemical reactions in order to activate certain metabolic pathways, or as an early sign of chemical or environmental stress. Plant cells will also produce active oxygen during interactions with potential pathogens (Baker and Orlandi 1995). Active oxygen species, including superoxide, hydrogen peroxide and hydroxide radicals, can potentially affect other cell processes that occur during plant–pathogen interactions.

Active oxygen formed as a response to a pathogen or elicitor probably exerts direct antimicrobial effects before it induces other defence mechanisms, such as lignin production, lipid peroxidation and oversensitive reactions (Baker and Orlandi 1995).

However, in order to protect the cell from damage, reactive oxygen is deactivated in several cell organelles.

- Chloroplasts

Elstner (1991) described chloroplasts as a source of activated oxygen. In photosystem I, oxygen can be reduced via the Mehler reaction. The monovalent reduction of oxygen is affected in cases of $NADP^+$ limitation, which can occur if the Calvin cycle cannot oxidise $NADPH^+$ as rapidly as photosystem I delivers electrons. Furthermore, photoactivated chlorophyll may transfer its energy to the reactive centre of the photosystem but electron transport may be inhibited. This has been observed in the presence of xenobiotics or herbicides, and we can expect that heavy metals will escalate this effect. Damage could be caused to the membrane transport system, to stomata movement and to nutrient acquisition. Photosystem II can fail during water cleavage and release triplet instead of normal oxygen. Last but not least, photorespiration is a fast oxidation mechanism in the chloroplast. Although RuBisCO favours CO_2 as substrate, oxygenation of RuBisCO occurs frequently, producing glycolate and glycerate. This usually happens when oxygen levels are high; i.e. when stomata are closed to prevent excessive water loss.

- Mitochondria

The largest proportion of the oxygen is consumed in cells as a substrate for cytochrome oxidase in mitochondria. Four electrons are transferred to each oxygen molecule, yielding water as a product of this reduction. Some mitochondria will also produce hydrogen peroxide and O_2 in the absence of $NADH^+$ to reduce the oxygen (Loschen et al. 1973, 1974). It is thought that Fe-S proteins and $NADH^+$ dehydrogenase are also potential sources for superoxide and hydrogen peroxide formation (Turrens et al. 1982).

- Endoplasmic Reticulum

The endoplasmic reticulum contains cytochrome P450 monooxygenases. The reaction catalysed by cytochrome P_{450} is:

$$RH + NADPH^+ + H^+ + O_2 \rightarrow ROH + NADP^+ + H_3O.$$

Hence, superoxide can be produced by the electron-dependent $NAD(P)H^+$, which includes P_{450}. After the reduction of the substrate (RH) and the addition of triplet oxygen, the complex P_{450}–ROOH is obtained. This can be degraded to P_{450}-RH by splitting off superoxide (Winston and Cederbaum 1983).

- Peroxisomes

$NAD(P)H^+$ oxidase activity was detected in the plasmalemma of peroxisomes. In roots, $NAD(P)H^+$ oxidase reduces Fe^{3+} to Fe^{2+} for iron transport. Disturbances to the function of this enzyme lead to superoxide formation (Cakmak and Marschner 1988).

Pathogens and elicitors, injuries, heat stress or xenobiotics can also stimulate superoxide formation via NADPH oxidase activation. It is thought that these reactions

cause cascades of signals in plant cells that prevent them from physical, chemical and biological stress. This could lead to hypersensitive responses and cell death (Doke et al. 1991).

- Cell Walls

Cell walls have recently been found to be regions of active metabolism and oxygen activation. This can happen during defensive reactions against pathogens or the degradation of xenobiotics. $NADH^+$, for example, is formed by extracellular malate dehydrogenase in cell walls, where it is used in hydrogen peroxide formation, probably by the $NADH^+$ oxidase of the plasmalemma (Vianello and Macri 1991). Again, this can initiate programmed cell death.

4.7.4 Defence Mechanisms Against Oxidative Stress

4.7.4.1 Superoxide Dismutase (SOD) (EC 1.15.1.11)

Superoxide dismutase was first insulated by Mann and Keilin (1938), who thought that the protein was responsible for the storage of copper. Its catalytic functions were described years later by McCord and Fridovich (1969). Since then, superoxide dismutases (Fig. 4.2) have been shown to act as catalysts for the dismutation of superoxide to hydrogen peroxide and oxygen:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Because superoxide dismutase is found in all aerobic organisms, it is generally thought to play a central role as a defence mechanism against oxidative stress (Beyer et al. 1991; Bowler et al. 1992; Scandalias 1993). A recent publication

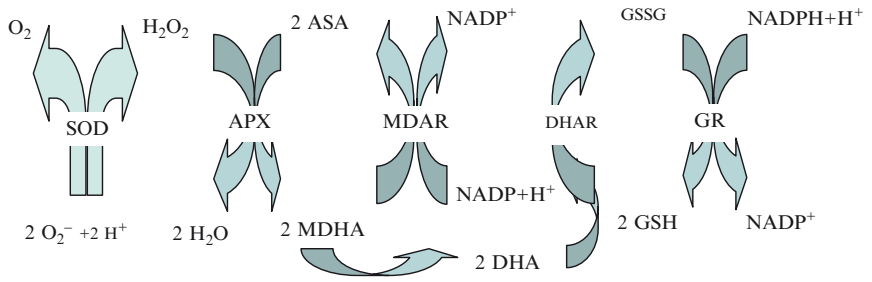

Fig. 4.2 Halliwell–Asada cycle (modified from Foyer et al. 1993). Superoxide and hydrogen peroxide detoxification with the consumption of ascorbate, and recovery of ascorbate at the expense of glutathione and NADPH. *ASA*, ascorbate; *APX*, ascorbate peroxidise; *DHA*, dehydroascorbate reductase; *GR*, glutathione reductase; *GSH*, glutathione; *GSSG*, glutathione disulfide; *MDHA*, monodehydroascorbate radical; *MDAR*, monodehydroascorbate reductase; *SOD*, superoxide dismutase

hypothesises that the level of superoxide dismutase activity may be proportional to the degree of environmental and xenobiotic stress in the cell (McKersie 1996). Three different types of superoxide dismutase are known, and they are classified according to their metal cofactors: Mn-SOD in mitochondria, Fe-SOD in chloroplasts, and CuZn-SOD in both chloroplasts and the cytosol.

The prokaryotic and eukaryotic cells of some algae possess only Mn-SOD and Fe-SOD isoenzymes, leading to the assumption that these are very old forms of superoxide dismutase.

4.7.4.2 Catalase (1.11.1.6)

Catalase mediates the dismutation of hydrogen peroxide formed by SOD and other sources:

$$H_2O_2 \rightarrow H_2O + O_2$$

This enzyme is present in all eukaryotic organisms and is primarily responsible for the breakdown of hydrogen peroxide formed in peroxisomes during the oxidation of fatty acids (Fig. 4.3). All forms of this enzyme are tetrameric. Catalase is highly photosensitive (Hertwig et al. 1992).

4.7.4.3 Ascorbic Acid

L-Ascorbic acid (vitamin C) is an omnipresent antioxidant in plants. Green leaves contain ascorbic acid and chlorophyll in equimolar concentrations. Ascorbic acid plays a very important role in many physiological processes such as growth, differentiation and metabolism (Foyer 1993). Ascorbate is also important to our discussion, as it reduces the damage caused by free radicals.

Ascorbic acid is synthesised from D-glucose and acts as an oxidant in cytosol and chloroplasts. Ascorbate binds with superoxide, hydrogen peroxide or radicals of tocopherol to yield monodehydroascorbic acid or dehydroascorbic acid. These reduced forms are then recycled to ascorbic acid, a process catalysed by

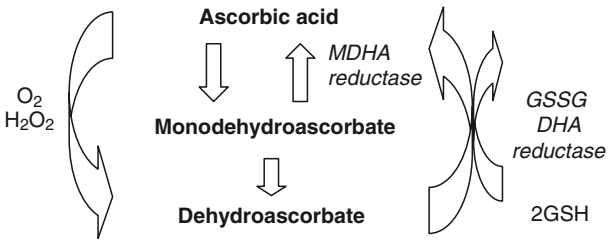

Fig. 4.3 Synthesis and degradation of ascorbic acid

monodehydroascorbate reductase (EC 1.6.5.4) and dehydroascorbate reductase (EC 1.8.5.1) using NAD(P)H^+ and GSH as sources of electrons. The dehydroascorbate can also be metabolised to tartrate or oxalate (Fig. 4.3).

4.7.4.4 Peroxidase (EC 1.11….)

The class of enzymes known as peroxidases detoxify reactive oxygen species (ROS). For many of these enzymes the optimal substrate is hydrogen peroxide (H_2O_2), but others are more active with organic hydroperoxides, such as lipid peroxides. Peroxidases can contain a heme cofactor at their active sites, or redox-active cysteine or selenocysteine residues. The nature of the electron donor is very dependent on the structure of the enzyme. Peroxidases reduce H_2O_2 with the aid of an electron donor to water. Some peroxidases are crucial to the processes of lignification (Hess 1991) and pathogen defence (Messner and Schröder 1999).

4.7.4.5 Glutathione Reductase (EC 1.6.4.2)

Glutathione reductase (Fig. 4.3) is a regenerative enzyme. With NADPH + H^+ as cosubstrate, it maintains the content of reduced glutathione in the cell at a constant level (Kiefer 2002). This means that it contributes indirectly to the detoxification of reactive oxygen. The NADPH needed for the reduction is again provided by photosynthesis, which confirms that the detoxification of ROS in the Halliwell–Asada cycle is a light-dependent process (Kiefer 2002).

Overall, in the Halliwell–Asada cycle, toxic hydrogen peroxide is reduced by glutathione reductase and GSH and subsequently by glutathione peroxidase to water. The oxidised glutathione dimer GSSG is also reduced via glutathione reductase (via the consumption of NADPH as the reductant) to GSH. This provides glutathione for various detoxification processes. Recently, we have been able to demonstrate that GR is strongly inhibited by heavy metals in vitro (Lyubenova et al. 2007). This finding indicates that the Halliwell–Asada cycle can undergo detrimental changes if its initial reactions are corrupted and free heavy metal ions flood the cytosol.

4.7.4.6 Glutathione

Glutathione (GSH) is the tripeptide Glu-Cys-Gly, which functions as an antioxidant due to the sulfhydryl group of the cysteine (Meister 1988). Glutathione is a ubiquitous molecule that performs many functions in chloroplasts and the cytosol. It is present in all of the cells of higher plants in millimolar concentrations. The glutathione level is highest under conditions of high light intensity. At the subcellular level, its concentration in chloroplasts is higher than that in the cytosol. Glutathione can function as antioxidant in different ways (Fig. 4.3). It reacts chemically with

the singlet oxygen of the superoxide and the hydroxy radical and scavenges other free radicals. On the other hand, glutathione recycles ascorbic acid from its oxidised to its reduced form with the aid of dehydroascorbate reductase (Loewus 1988).

Glutathione is also involved in the transport of reduced sulfur from the leaves to the roots (Meister 1988), and in xenobiotic detoxification, where it acts as a co-substrate for glutathione *S*-transferases.

Additionally, glutathione acts as a substrate in the formation of phytochelatins, γ-glutamylcysteine oligomers, which chelate heavy metals in plants (see below).

4.7.4.7 Phytochelatins

To avoid the toxic effects of heavy metals, plants have developed mechanisms to deactivate and scavenge metal ions that penetrate into the cytosol. Phytochelatins (PCs), which possess the structure $(NH_3)^-$-γ-Glu-Cys- γ-Glu-Cys-γ-Glu-Cys-Gly-$COO^-_{(n)}$ (Grill et al. 1985), act as chelators of Cd^{2+} and other heavy metals in such mechanisms. The length of the PC chain varies between $n=2$ to $n=11$ (γ-Glu-Cys)$_n$-Gly (Gekeler et al. 1989). Ions of cadmium have been reported to bind the most strongly to phytochelatins in vivo. The Cd–PC complex adopts the structure shown in Fig. 4.4. The dots in Fig. 4.4 represent uncoordinated carboxyl groups. They influence the transport of the PCs through the tonoplast (Strasdeit et al. 1991). The synthesis of these heavy metal–phytochelatin complexes is a vital metabolic process in higher plants. The resulting depletion of glutathione in the cytosol is compensated for by the induction of sulfur assimilation and glutathione biosynthesis (Rüegsegger et al. 1990; Rüegsegger and Brunold 1992).

The synthesis of phytochelatins is catalysed by the enzyme γ-glutamylcysteine dipeptidyl transpeptidase, also known as phytochelatin synthase (EC 2.3.2.15). This enzyme catalyses the initial reaction and couples γ-Glu-Cys-Gly to (γ-Glu-Cys)$_n$-Gly, which then results in (γ-Glu-Cys)$_{n-1}$-Gly-Gly. This reaction is strongly induced by heavy metals. A direct correlation between the properties of the respective metal and the efficacy at inducing the action the PC enzyme has been reported,

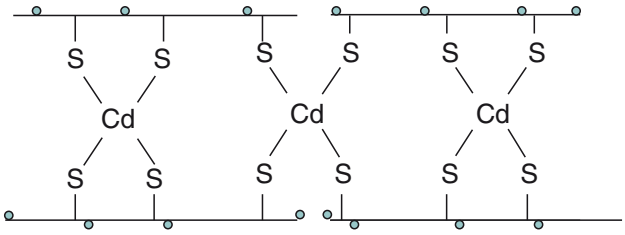

Fig. 4.4 Structure of the $[Cd_3(Pc_4)]$ complex. The *blue dots* represent uncoordinated carboxyl groups. The positions of these groups depend strongly on the negative electric charge (Strasdeit et al. 1991)

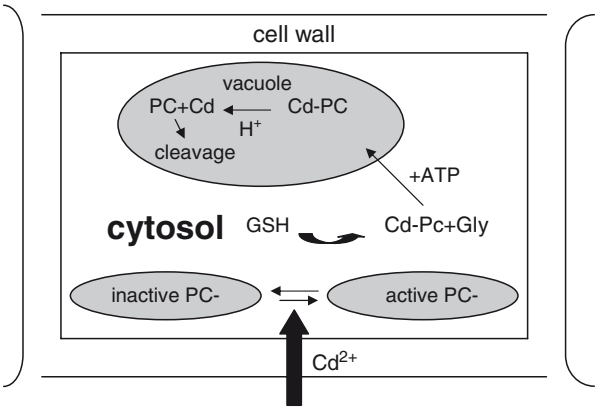

Fig. 4.5 Cd^{2+} ions penetrate into the cell and activate the synthesis of phytochelatins. Phytochelatins are synthesised from glutathione. The Cd–PC complex is actively transported into the vacuole. The metal is stored there in a different form (i.e. complexed with organic acids), while the phytochelatins are degraded and recycled to the cytosol (Zenk 1996). Hyperaccumulation in vacuoles may be the basis for a practical application, i.e. phytomining (Baker and Brooks, 1989)

according to the following sequence: Cd > Ag > Pb > Cu > Hg > Zn > Sn > Au > As > In > Tl > Ge > Bi > Ga.

Besides detoxification, phytochelatins also contribute to the regulation of metal occurrence and homeostasis in plant cells (Fig. 4.5). Metal ions like Cu and Zn play distinct roles in catalytic proteins or structural elements. Hence, phytochelatins play a double role: on the one hand they complex, detoxify and store metal ions in the vacuole, and on the other they guide essential metals to newly synthesised apoenzymes and facilitate contact (Thumann et al. 1991).

4.7.4.8 Carotenoids

Carotenoids are members of the C_{40} isoprenoid and tetraterpene groups of plant metabolites. They are mainly localised in plastids with and without photosynthetic functions. In chloroplasts, carotenoids act as light-scavenging accessory pigments, as well as inactivators of reactive oxygen species, stimulating energy dissipation within light-harvesting proteins by nonphotochemical quenching. Hence, carotenoids contribute to detoxification by reacting with the products of lipid hydroperoxidation to prevent chain reactions (Burton and Ingold 1984), reacting with triplet or excited chlorophyll molecules to inhibit the formation of singlet oxygen, and via the release of excess energy as heat through the xanthophyll cycle (Mathis and Kleo 1973; McKersie 1996). The xanthophyll cycle involves the enzymatic removal of epoxy groups from xanthophylls to create so-called de-epoxidised xanthophylls.

4.7.5 Other Detoxification Mechanisms in Plants

When organic contaminants are present at a given site along with heavy metals, other problems arise. Current research has identified the need to study other detoxification mechanisms too (Lyubenova et al. 2007). Shimabukuro was first to describe a three-phase cascade responsible for the metabolism of herbicides and organic xenobiotics that involved (1) activation of the xenobiotics, (2) detoxification and (3) excretion, a process analogous to animal hepatic metabolism (Schröder and Collins 2002). Activation may be catalysed by esterases, P450 monooxygenases (in membrane fractions of cells) and peroxidases (cytosolic). The second phase is detoxification sensu stricto, and is catalysed by glutathione and glycosyl transferases. It makes the compound under consideration less toxic through substitution and conjugation via reactions with sugars, amino acids and glutathione, which can be transferred to the activated xenobiotic according to the structure of the molecule and its active site (Schröder and Collins 2002). Available hydroxyl groups, amine groups, thiol functional groups and carboxylic acid functions on a given molecule usually trigger glycosyl transfer (Schröder and Collins 2002). When conjugated double bonds, halogen or nitro functions are present in a molecule, glutathione conjugation catalysed by glutathione *S*-transferases is the predominant reaction (Coleman et al. 1997). In this phase, reactions such as cleavage, rearrangement and secondary conjugation are also performed. The last phase can be split in two; the first part of this phase involves membrane transport and storage in the vacuole, whereas the second part includes final cell wall binding reactions or excretion (Theodolou 2000; Schröder 2006).

4.7.5.1 Glutathione *S*-Transferases

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) were first described in animals, where they catalyse the conjugation of pharmaceuticals with the tripeptide glutathione (Booth et al. 1961). Years later, they were also described in plants, where they were found to conjugate atrazine with GSH in maize (Frear and Swanson 1970). This process of conjugation leads to the cleavage of electrophilic groups from xenobiotics, and is considered to be true detoxification. Plant GSTs are found in the cytosol and in membranes (microsomal GST). Both groups include homodimeric (they have two identical subunits) or heterodimeric (different subunits) enzymes with subunit sizes ranging from 23 to 30 kDa (Schröder 2001).

GST holoenzymes possess two independent catalytic domains to create conjugates from glutathione and electrophilic substances. Each of these domains consists of a G site for GSH binding and an H site for the binding of the xenobiotic (herbicide).

The cytosolic GSTs are classified into six classes: phi (F), tau (U), theta (T), zeta (Z), lambda (L) and the dehydroascorbate reductase (DHAR) (Edwards and Dixon 2005). The phi and tau GSTs represent the largest groups, and they are plant specific.

The other classes are also present in the animal kingdom. Each of the GST classes has been found to play an important role:

- Phi: stress response, metabolism of plant hormones, drought stress
- Tau: biotic and abiotic stress, herbicide detoxification via GSH addition
- Theta: peroxidase activity
- Zeta: isomerase activity
- Lambda: reductase activity
- DHAR: ascorbate reduction.

The soluble GSTFs, GSTTs, GSTUs and GSTZs are polypeptides around 25 kDa in size that associate with other subunits of each class and form homodimers (Schröder and Collins 2002). Marrs (1996) mentions that a group of type III GSTs (due to inconsistencies in the nomenclature of animal and plant GSTs, type III GSTs were later identified as tau GSTs) regulate heat, heavy metal and pathogen stress. A list of the herbicides conjugated by plant GST is presented by Schröder (Schröder and Collins 2002), while a list of herbicide and heavy metals is presented by Lyubenova et al. (2009). Like glutathione reductase, GST enzymes are also inhibited in vitro in the presence of heavy metals, albeit at higher concentrations (Lyubenova et al. 2007). It is not yet clear whether this holds true for all isoforms or only for distinct GST classes, but it is an important finding in the context of mixed pollution.

4.7.5.2 Mixed Pollution

Contamination is an important threat to European soils, aside from loss of fertility, deterioration of the soil structure and increases in pathogens. Especially considering demographic and ecological trends, soil contamination impacts on water and food production, exerting heavy effects on ecosystems and human life. For EU countries, the estimated number of potentially contaminated sites is almost three million (EEA 2007). Around 80,000 sites have been cleaned up in the last 30 years, but treatment is urgently needed for roughly 250,000 sites in EEA member countries. Mineral oil (38%), heavy metals (37%) and PAHs (13%) are the most common soil contaminants. Contaminated soils are frequently treated as waste to be disposed of rather than as a valuable resource to be cleaned and reused. The problem is that remediation must be effective at both reducing or controlling health or environmental risks associated with the particular mixture of pollutants and also preserving and improving soil quality and function, and all at an affordable cost. Phytoremediation provides a variety of remediation techniques associated with plants and microbes that involve treatment strategies for contaminant degradation, accumulation or immobilisation. The use of plants offers efficient and environmentally friendly solutions for cleaning up contaminated sites and water, as well as food safety and the development of renewable energy sources, all of which will ultimately contribute to sustainable land use (Vangronsveld et al. 2000).

4.8 Conclusion

Most known heavy metal contaminations have been generated by human activities, and the relative heavy metal toxicity depends on its availability, which is determined by the properties of the soil and the plant species of interest. Once taken up by the plant, heavy metals interact with different cell components and disturb normal metabolic processes. The antioxidative system protects cells from immediate damage, but if exposure persists or critical doses are exceeded, the antioxidant store will be used up and so stress will occur. Antioxidative stress leads to the induction of numerous enzymes, but may also result in suppression. The latter effect is crucial under the conditions of mixed pollution, when plants must fight chemicals with various modes of action. Here, the occurrence of certain heavy metals can be detrimental to the detoxification of organic xenobiotics that would easily be detoxified if they were present alone.

Recent phytotreatments have used plants without characterising them properly beforehand. Selecting species that grew on certain local soils or in given regions was taken to be a sufficient selection parameter. However, species-specific differences seem to exist between the regulation of primary defence enzymes like SOD, catalase and peroxidases, and other species prefer to induce glutathione-dependent enzymes. As long as the pollutant mix encountered is simple and dominated by heavy metals, the defences of the plant may be sufficient. When the pollution contains heavy metals and organic xenobiotics at the same time, part of the plant's detoxification capacity – the utilisation of glutathione-conjugating reactions at the very least – is withdrawn from the heavy metal front to serve other purposes. In fact, glutathione *S*-transferases show strong reactions in stressed plants or in the presence of heavy metals. We have described in this chapter how pollution with heavy metals will interfere with both plant oxidative stress defence and the ability of plants to conjugate organic xenobiotics. Despite species-dependent differences, general reactions seem to include oxidative stress and the induction of antioxidative enzymes. Several processes seem to depend on the direct binding of heavy metals to enzyme proteins, but effects on transcription are also observed. Xenobiotic metabolism is induced at high heavy metal concentrations, when plant stress is elevated. It is becoming clear that plants intended for the phytoremediation of complex pollution mixtures must be selected according to three major issues: uptake/accumulation capacity, antioxidative stress management, and their detoxification/binding properties in relation to both trace elements and the organic xenobiotics.

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Chapter 5
Arbuscular Mycorrhiza, Heavy Metal, and Salt Tolerance

Hermann Bothe, Marjana Regvar, and Katarzyna Turnau

5.1 Introduction

Many soils around the world are polluted with heavy metals or salts and are therefore of limited value for farming purposes. Specific plants that are adapted to the adverse effects of the pollutants grow on these sites. The same appears to be true of microbes such as bacteria or fungi, although they are yet to be characterized in much detail. Arbuscular mycorrhizal fungi (AMF) could be of particular benefit to plants in relation to alleviating heavy metal and salt stress. Plants growing in both in heavy metal and saline soils can be colonized by AMF. This chapter presents current knowledge on the interactions between AMF, heavy metals or salts, and plants, and it also addresses the question of how AMF should be exploited for phytoremediation.

5.2 Heavy Metals and Their Toxicities

Heavy metals are defined as elements with a mass of ≥5.0 g cm^{-3}. Physiologically, they can be subdivided in those:

(a) Are essential for the growth of the plants, since they are irreplaceable components of the prosthetic groups of enzymes. Such metals include Fe, Cu, Mo, Mn,

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Zn, and Ni (this, however, exceptionally, in urease or hydrogenases for example) or V (again, only in this, however, exceptionally cases: in some V-peroxidases or nitrogenases). Insufficient concentrations of them may be lethal or limiting to plant growth.

(b) Have no defined or known effects in plant metabolism, but often compensate for the toxic effects of other elements. Such elements are regarded as beneficial to the growth of plants, and include Co, Cr, Sr, and Sn.

(c) Are always toxic to growing and resting cells, even in low concentrations; these metals include Cd, Hg, Ag, and Pt (Sutcliffe and Baker 1974; Marschner 1995).

At a certain threshold concentration, any heavy metal will become toxic to the organism containing it. This threshold value is species specific and varies with the heavy metal. The toxicity of a heavy metal may even vary between individuals of a given species or cultivar. Thus there is no general tolerance of plants to heavy metals. However, a plant species can be more adapted to one heavy metal than another, and so is more tolerant to Ni, for example, than the next species. Another plant species may be more tolerant to Cu than a counterpart. Figure 5.1 illustrates these impacts of metals on plants in a simplified way.

In classical plant physiology, organisms are *tolerant* when they can endure a high concentration range of a heavy metal. *Resistant* plants have developed a demand for a high concentration of a heavy metal during the course of their evolution. Examples of this are more obvious in salt resistance. The germination of few salt-resistant halophytes such as *Salicornia europaea* or *Suaeda maritima* is strictly dependent on the availability of Na^+ and Cl^- in sufficient amounts in soils or in laboratory cultures. Salt-tolerant plants grow better in unpolluted soils, but their low competitiveness compared to other plants confines their distribution to saline habitats. This difference is less obvious with heavy metal plants. Therefore, the differentiation between heavy metal resistant and tolerant plants is not always highlighted in the literature. Plants that are particularly tolerant to heavy metals are called metal(l)ophytes.

Heavy metals can exert their toxicity by binding to functionally essential SH groups of enzymes, which results in the competitive inactivation of their

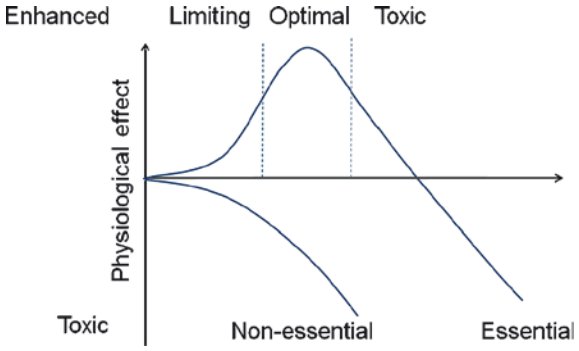

Fig. 5.1 Schematic representation of the physiological effects of increasing concentrations of essential and nonessential metals on organisms (adapted from Megharaj et al. 2002; redrawn)

catalytic capabilities. Alternatively, they may enhance the generation of ROS (reactive oxygen species) such as $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$, and $^1O_2$. These reactive oxygen species can be generated in plants by the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\cdot} + Fe^{3+},$$

or the Haber–Weiss reaction:

$$O_2^{\cdot -} + H_2O_2 + H^+ \rightarrow OH^{\cdot} + H_2O + O_2.$$

The reaction velocity of the latter reaction is enhanced by the presence of Fe or any other metal. In the Fenton reaction, iron can be replaced with other metal cations (Elstner 1990; Prasad and Hagemeyer 1999).

At the biochemical level, the following components responsible for heavy metal tolerance in organisms can be listed (Salt 2001; Hall 2002; Sanità di Toppi et al. 2002):

(a) *Siderophores*: these are generally large, complex organic molecules that are synthesized by the organism and excreted into the soil (medium). This binding prevents the heavy metals from reaching the cells of the organism. However, a role in heavy metal tolerance of plants is doubted at present.
(b) *Metallothioneins*: Small, cysteine-rich proteins where the SH groups of the cysteines bind the heavy metals in the cells.
(c) *Phytochelatins*: Compounds of $(\text{glutamate–cysteine})_n$ glycine that also bind heavy metals at the SH groups of the cysteines in the cells.
(d) *Heavy metal transporters*: Comprise a huge class of different proteins such as CPx-ATPases for Cu or Cd, ABC transporters for Cd transport into the vacuole, ZIP transporters (ZRT-, IRT-related proteins for Fe or Zn), and N*ramp* transporters. The latter were originally considered to provide the natural resistance of plants to pathogens, but were later found to serve mainly as broad-range transporters for heavy metals.

This list is far from complete. In addition, all of these components are members of multigenic families. Each transporter for a specific heavy metal is not encoded by just one gene, but by several, and this number varies from plant species to plant species. Other transporters may reside at the plasmalemma (to transport heavy metals out) rather than at the tonoplast (to transfer them into the vacuole). They may be different in the transfer of metals from the apoplast into the cells of the root hairs and root cortical cells (symplast), from the parenchyma cells to the xylem vessels within the roots, in the translocation into the leaves and flowers, and so on. Their expression patterns may show seasonal variations and can be subject to external environmental conditions. All of these variations make the studying the components responsible for heavy metal resistance of plants a rather complex task. For more detailed reviews of this subject, see Brooks (1998); Zhang and Shu (2006); Vogel-Mikuš and Regvar (2006); Regvar and Vogel-Mikuš (2008); Ernst et al. (2008).

5.3 Specific Metallophytes and Their Potential Role in Phytoremediation

A botanist can immediately recognize that a site has a heavy metal soil due to the occurrence of metallophytes. In Central Europe, 3–6 plant species typically occur at every site polluted with heavy metals. However, for some unknown reason, no heavy metal soil contains every metallophyte. In many cases, metallophytes are relicts from the glacial period, only occurring in the plain at sites polluted with heavy metals, but thriving in alpine regions above the timberline or close to arctic areas too. This is the case for *Minuartia* (= *Alsine*) *verna*, which can endure the highest concentrations of heavy metals of any Central European metallophyte. The mechanism used by this plant to cope with such high concentrations of heavy metals is not yet clear. It is said that the leaves of *M. verna* die when they are overloaded with heavy metals and that the plant frequently generates new leaves from the vegetation point. *Armeria maritima* ssp *halleri* also occurs in coastal salt marshes (ssp. *maritima*) and has a closely related species (*Armeria maritima* ssp. *alpina*) that grows in the Alps. It possesses specific glands (modified stomata) that serve to excrete toxic heavy metals taken up accidentally. *Armeria maritima* ssp. *halleri* contains 20-fold and 88-fold greater concentrations, respectively, of Pb and Cu in its roots than in its leaves, indicating that the metals are immobilized in its roots. On the other hand, high levels of Zn, Cd, Pb, and Cu in brown leaves suggest a leaf fall detoxification mechanism (Dahmani-Muller et al. 2000). The genus *Thlaspi* comprises several closely related species that thrive on heavy metal soils: *Thlaspi calaminare* in the plain; *Thlaspi goesingense* in a few places in Austria and in Eastern European countries (Fig. 5.2); and *Thlaspi praecox* and *T. caerulescens* in heavy metal soils as well as unpolluted sites. These members of the *Thlaspi montanum-alpestre* group should be differentiated from *Thlaspi cepaeifolium* (= *T. rotundifolium* ssp. *cepaeifolium*), which is next related to *Thlapsi rotundifolium*, found in alpine chalk gravel. *T. cepaeifolium* is highly endangered and currently only occurs at two sites polluted with heavy metals: Cave de Predil in Friaul in Northeastern Italy, and along the river bed of the Gailitz, close to Arnoldstein in Southern Austria. Since its gene pool (particularly the genes encoding heavy metal tolerance related proteins) may be significantly different from those of other metallophytes of the genus *Thlaspi*, *T. cepaeifolium* needs to be preserved by any means. *Thlaspi* species are generally short-living annual plants and they have developed means to keep their seeds free of toxic heavy metal concentrations. Another Brassicaceae member that occurs in almost all heavy metal soils in Central Europe (but not in the Aachen–Liège area) is *Cardaminopsis* (= *Arabidopis*) *halleri*. Since it is related to the model plant *Arabidopsis thaliana*, this plant is currently used in studies of heavy metal tolerance at the molecular level.

Zinc violets are particularly beautiful metallophytes (Fig. 5.3). Two different subspecies exist with very restricted and thus endemic occurrences. The yellow zinc violet (*Viola lutea* ssp. *calaminaria*) occupies heavy metal heaps only in the area between Aachen in Germany and Liège in Belgium, but such stands can contain thousands of these plants. The yellow zinc violet produces plenty of its striking flowers from the middle of May until about the end of September, which makes

Fig. 5.2 *Thlaspi goesingense* thrives on heavy metal heaps, particularly serpentine soils in Austria and Hungary. Due to its fairly high productivity, it is attractive for phytoremediation purposes

these sites worth visiting during this time. The blue zinc violet (*Viola lutea* ssp. *westfalica*) can only be seen in the lead ditch and its surrounding heaps at Blankenrode near Paderborn in Germany, where it covers an area of about 0.5 × 1 km^2. Recent molecular analyzes of its DNA have indicated that both of these zinc violets stem from the alpine *Viola lutea* and not from *V. tricolor* and so should be regarded as subspecies or varieties (Hildebrandt et al. 2006a). Both occur only on heavy metal soils but can also be successfully grown in unpolluted garden soils. They are therefore not obligate metallophytes or heavy metal resistant, as claimed earlier (Nauenburg 1986). Their parents currently occur (abundantly) in alpine areas such as the Vosges mountains in France in both blue and yellow forms, rarely in the Alps, in Southern England, and also in the Carpathian and Sudeten mountains (possibly as an individual subspecies there, *V. lutea* ssp. *sudetica*). *V. lutea* may have had a broad distribution following the end of the last glacial period. Afterwards

Fig. 5.3 Zinc violets are beauties on heavy metal heaps. They exist in two forms: *Viola lutea* ssp. *westfalica* (the blue form), which is only found on the lead heap in Blankenrode, Eastern Westfalia, and *Viola lutea* ssp. *calaminaria*, (the yellow form) which grows on Zn-rich soils between Aachen in Germany and Liège in Belgium

it may have wiped out in unpolluted sites by better-growing plants. It could therefore only survive at heavy metal sites, where it developed into the blue zinc violet of Blankenrode and the yellow zinc violet of Aachen–Liège due to their isolation. Alternatively, they may have been transported by medieval miners to heavy metal sites, where they subsequently developed into separate entities.

Zinc violets are unable to radiate to eastern or southern areas for some unknown reason. Heavy metal sites in these regions are occupied by *V. tricolor*, which also fascinates due to its thousands of blossoms at (for example) Bolesław heap, close to Olkusz in Southern Poland. However, *V. tricolor* can also be found at unpolluted sites. It is not yet clear whether a special ecotype of *V. tricolor* thrives on heavy metal soils, for example in Southern Poland, on the German Harz mountains, or in Southern Austria (Bad Bleiberg). *V. tricolor* is also reported to occur in the western part of Germany and in Belgium, although only with a low abundance. It is somewhat surprising that *V. tricolor* could not conquer Western European heavy metal soils, where it is replaced by zinc violets.

Other metallophytes exist in Central Europe. Shoots of *Silene vulgaris* grow curved on heavy metal heaps but straight on unpolluted sites. This specific ecotype of this member of Caryophyllaceae is often regarded as a subspecies or variety (var. *humilis*) with an enhanced tolerance to heavy metals (Wierzbicka and Panufnik 1998). *Festuca ovina* may have developed a specific ecotype on heavy metal soils (Patzke and Brown 1990). However, the *Festuca ovina* group is difficult to resolve taxonomically. *Alyssum wulfenianum* is another beauty of the alpine heavy metal soils but is now almost extinct. The need to preserve such extremely rare plants for future generations must be stressed. Sites polluted with heavy metals in South Poland possess specifically adapted ecotypes of *Biscutella laevigata* (Wierzbicka and Pielichowska 2004) and *Dianthus carthusianorum* (Zalęcka and Wierzbicka 2002),

although these have no apparent morphological differences from the individuals thriving on uncontaminated soils. Examinations of other plants that grow on unpolluted soils as well as at heavy metal sites with almost unimpaired growth rates may provide new insights into their metal exclusion strategies. Other metallophytes have also been reported around the world (Prasad and de Oliveira-Freitas 1999). Heavy metal accumulating plants occur in diverse families and genera of the plant kingdom. Thus, metal tolerance is a typical example of convergence within plant taxonomy.

In a recent review (Ernst et al. 2008), the degree of tolerance of heavy metals was divided into three categories: hypotolerance, basal tolerance, and hypertolerance. Plant species do differ in their abilities to endure heavy metal stress. Tolerance even appears to vary with the heavy metal, and from one plant species to the next. Therefore, the separation into strict categories does not seem to afford us much help when attempting to explain the physiological phenomena.

All metallophytes can be grown on unpolluted soils. Probably due to the better nutrient supply present in such soils, their productivity is even higher in garden soils than at polluted sites. This was tested for several Central European metallophytes (unpublished culture data obtained from 2000 to 2003 with *Viola lutea* ssp. *calaminaria*, *Thlaspi goesingense*, *T. calaminare*, *T. praecox*, *Armeria maritima* ssp. *halleri* in the garden of the Botanical Institute of Cologne, and with *V. lutea* ssp. *westfalica* in a private allotment at Erftstadt, Germany). Thus, there is currently no known absolute metallophyte. The inability of metallophytes to compete with other plants might restrict their occurrence to heavy metal sites and explain their failure to radiate.

Plants that thrive on heavy metal rich soils can be used in the phytoremediation of polluted areas or postflotation wastes that are created as a by-product of ore processing. After their exploitation by mining, heavy metal soils are often a harsh substratum devoid of vegetation cover (Fig. 5.4) that make any biological reclamation difficult (Turnau et al. 2006a; Turnau et al. 2006b; Strzyszcz 2003) due to their

Fig. 5.4 Heavy metal polluted sites often have no vegetation, which causes severe problems in phytoremediation projects. Photo was taken in Kalenberg, close to Mechernich, in the Eifel mountains of Germany. Further details on this site are provided in Kaldorf et al. (1999)

toxicity, problems with slope stabilization, and drought. The dust originating from the waste heap area often contains high levels of Zn, Pb, and Cd, which pose serious health hazards for plants and animals. Typical remediation practices involve covering the waste with a layer of soil or humus, transported mostly from another area, to prevent erosion. This is followed by the introduction of trees and grasses such as *Lolium perenne*. Metallophytes could be used to stabilize the soils against the erosion of the surface soil by wind and rainfall, and they can colonize such disturbed areas. However, grasses, which often develop abundant root systems, were found to be more successful at phytostabilization than metallophytes. Trees such as *Pinus sylvestris*, *Populus* spp., *Betula* spp. and shrubs such as *Hippophaë rhamnoides* are also often introduced in such areas. However, in regions where fires can be expected, trees do not recover as easily as grasses. Newly established vegetation in such an area must be watered, especially if the area is steep and erosion is taking place. In order to improve the nutrient status of the wastes, experiments concerning the use of fertilizers have been carried out (Turnau et al. 2006a, b; 2008; Ryszka and Turnau 2007). If the area is relatively flat, this can be a relatively fast way of introducing vegetation; however, the compositions of such plant communities are mostly poor, and symbiotic organisms are often poorly developed.

Sustainable remedial techniques are required for such sites. Employing the remaining pre-adapted metallophyte flora seems an especially promising approach. However, there are several obstacles to the use of this technique. In a recent phytostabilization approach, for example, the remaining vegetation around the lead mine and smelter in Northern Slovenia was screened in order to select the most suitable plant species for further phytostabilization efforts. However, the selected dominant grass species *Sesleria caerulea* and *Calamagrostis varia* at the site did not produce enough viable offspring (Regvar et al. 2006).

Metallophytes can also be used to extract heavy metals. A specific application can be envisaged in the Chernobyl area. Radionuclides can be excavated from soils and enriched in plants. The major isotopes of Chernobyl, however, behave differently in plants. Like Ca, ^{90}Sr is immobile in plants and is deposited mainly in the cell walls of the roots. ^{137}Rb, similar to K, is highly mobile in plants and can be collected in their aboveground parts. The idea is to extract radionuclides from the soils using plants and then to remove the plants to a site where the radionuclides could be concentrated.

Plants can be used for the enrichment of metals that occur in small amounts in soils; such plants are termed hyperaccumulators. Plants with the ability to accumulate high amounts of Cd, Co, Cu, Mn, Ni, and Zn include several ferns, such as *Pteris vittata* (Ma et al. 2001), and herbs (Ernst 2005). The use of such plants is not economically feasible for the enrichment of many elements. Most hyperaccumulators produce little aboveground biomass, they usually only accumulate particular metals, and they are not tolerant of other metals that may occur in such places. Neither hybridization of poorly productive hyperaccumulators with highly productive non-hyperaccumulators (Brewer et al. 1999) nor the use of genetically modified plants (Bennett et al. 2003, Ernst 2005) resulted in the production of lines that are significantly more effective. In terms of single elements, the use of the hyperaccumulating species *Alyssum murale*

(Ernst 2005), *Alyssum bertelonii* (Boominathan et al. 2004), *Berkheya codii* (Robinson et al. 2003), or of several endemic species of the serpentine flora in Zimbabwe was suggested for the enrichment of Ni (Brooks and Yang 1984).

5.4 A Short Excursion into the Biology of Arbuscular Mycorrhizal Fungi

More than 80% of all higher plants can undergo a symbiosis with arbuscular mycorrhizal fungi (AMF). Based on its activity, the arbuscular mycorrhiza is probably the most important symbiosis in nature. It appears to have developed at around the same time as the earliest land plants, in the Ordovician (Redecker et al. 2000). Scientifically, mycorrhizal all means by symbioses are not yet well understood due to the fact that the fungi cannot be grown independent of a host (Smith and Read 1997). Arbuscular mycorrhizal fungi have been found to phylogenetically belong to an own tribe, the Glomeromycota, and are therefore very different from all other fungi (Schüßler et al. 2001). Molecular studies of AMF have to encounter many difficulties due to their own, specific, codon usages. Therefore it has often been impossible to gain access to AMF genes through heterologous probing or by developing suitable primers for gene amplification by PCR. Moreover, none of the fungi has been completely sequenced as yet. The cells of AMF are multinucleate, and the different nuclei within a cell are not uniform. Sexual states of AMF do not exist or are unknown. All of these aspects explain why knowledge of AMF lags behind that of most other fields of biology.

AMF have a relatively simple life cycle (Fig. 5.5). All fungal structures stay belowground. The life cycle of AMF starts with the germination of a spore through

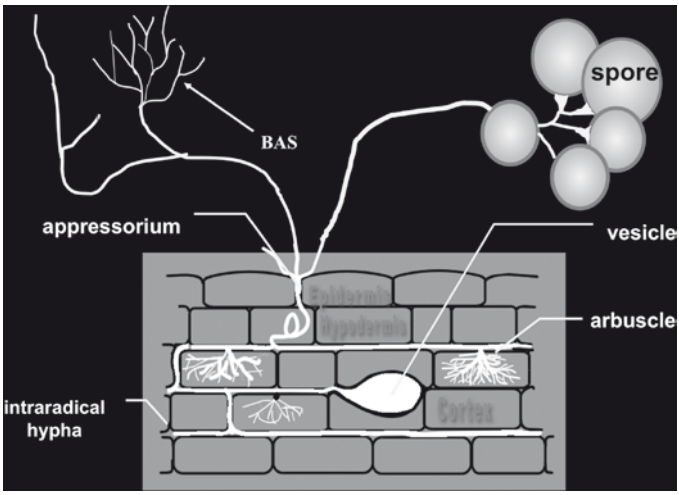

Fig. 5.5 The life cycle of an arbuscular mycorrhizal fungus. For explanations see text

the formation of hyphae. When a hypha reaches the surface of a suitable plant root, it broadens into an appressorium and then penetrates through the rhizodermis into the cortical cells of the root. Remarkably, the cell walls of the plant are dissolved where the fungus penetrates (not, however, the plasmalemma, as in a pathogenic interaction). Within the inner part of the cortex, the fungus forms arbuscules (Fig. 5.6), thus leading to the name giving structures to these fungi. An arbuscule is an extensive, tree-like folding of the intraradical hyphae. This folding is surrounded by the cytoplasmic membrane (plasmalemma) of the host, forming the so-called periarbuscular membrane. All of this results in massive increases in the surfaces of both symbiotic partners. The arbuscules and their surrounding periarbuscular membranes are the sites where most of the metabolite transfer between both symbiotic partners occurs. Most of the AMF form vesicles (Fig. 5.7) as compartments for the storage of lipids. Since not all AMF (e.g., the genus *Gigaspora*) form vesicles, most investigators do not use the former term vesicular arbuscular mycorrhiza (VAM) anymore.

Outside the roots, the fungi form extraradical hyphae that penetrate into the soil and form a wide-reaching network. The hyphae can be very fine and dense and form branched absorbing structures (= BSA, Fig. 5.5). Water and minerals are apparently more efficiently exploited from soil particles by such hyphae than by the plant roots.

AMF spores (Fig. 5.8) are formed both outside and inside the roots. Experts can distinguish AMF species by their spore morphologies. Some 150 species have been described so far. However, AMF taxonomy based on spore morphology is far from being unambiguous and definitive, since their structures can vary greatly depending on the ontogenetic state (ecological conditions). An alternative approach is to

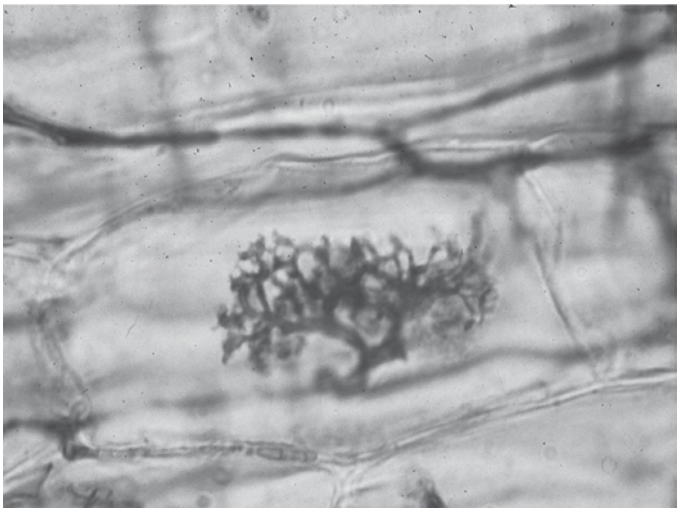

Fig. 5.6 An arbuscule of the AMF *Glomus intraradices*, detectable after staining with trypan blue

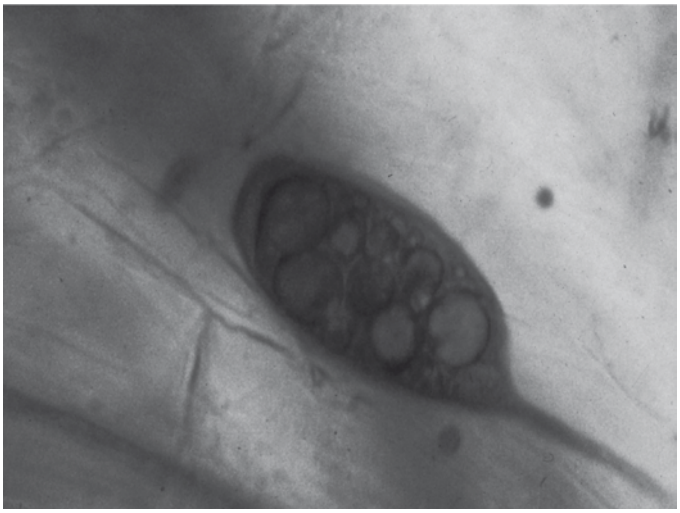

Fig. 5.7 A vesicle of *Glomus intraradices*, also after staining with trypan blue

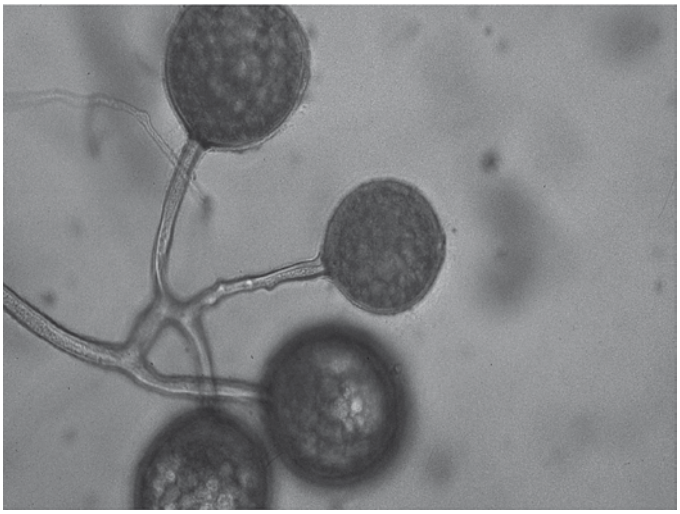

Fig. 5.8 Spores of *Glomus intraradices*

characterize AMF by DNA sequencing. Parts of the ITS1-5.8S rDNA-ITS2, 18S-rDNA or 28S-rDNA regions have been successfully amplified by performing PCR with suitable primers, and then characterized by sequencing. Such studies revealed a lot of sequences, particularly within plant roots, that were not characterized by spore morphology (Börstler et al. 2006; Wilde et al. 2009). Since the fungi cannot be grown independent of a host, it is hard to identify the spores or other

fungal structures that belong to the new AMF sequences. Moreover, recent evidence suggests that the fungal populations differ in terms of soils and intraradical fungal structures (Wilde et al. 2009) and may even differ in terms of extraradical hyphae (Börstler et al. 2006).

In the symbiosis, the fungi provide water and minerals to their plant host. The transfer of phosphorus from the soil via the fungi to the plant partner has been amply shown due to the availability of easily amenable radioactive isotopes of this element. The transfer of other substances like nitrogen, K, Zn, Cu, water, and others from the fungi to the plants has also been identified experimentally. In turn, plants provide the fungi with carbohydrates, mainly as glucose.

As previously noted, AMF cannot be grown independent of a host. However, recent experiments have shown that the plant partner can be replaced with specific bacteria. A mixture of the spore-forming bacterial genus *Paenibacillus* can promote the growth of the AMF *Glomus intraradices* until the formation of new, fertile spores (Hildebrandt et al. 2006b). It appears to be only a matter of time before the substances that are supplied by the host and are needed for the growth of the AMF are identified.

Currently, fungi are multiplied as spores or propagules by growth on suitable substrates such as peat or expanded clay, and using broad bean, leek, *Tagetes*, and others as host plants. An alternative is to in vitro culture the AMF together with Ri T-DNA-transformed carrot roots, which provide defined but costly fungal inocula (Bécard and Fortin 1988). AMF could have enormous potential for applications. This is particularly obvious for greenhouse cultures (Fig. 5.9). Inoculation with AMF helps plants such as cyclamens to grow faster, come into blossom earlier, and to be more resistant to attack by pathogenic organisms. More recently, some small companies have been started that sell AMF inocula, and some of them are said to

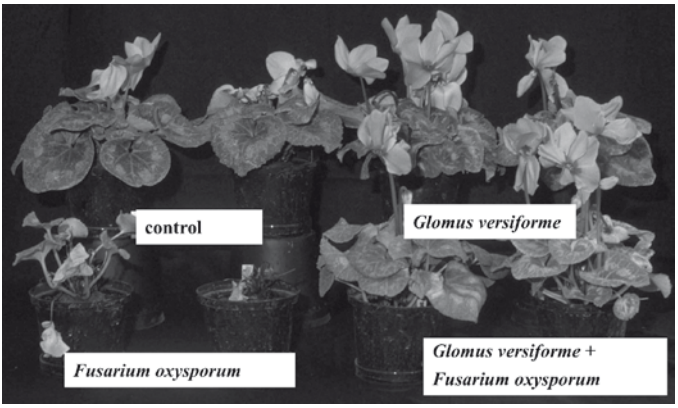

Fig. 5.9 AMF support plant growth and suppress the actions of pathogenic fungi, as shown here for the AMF *Glomus versiforme*. It enhances the growth of cyclamens (*top row in the figure*) and partially relieves the effects of the pathogenic *Fusarium oxysporum*. The experiment was performed by Dr. H. Baltruschat, Gießen, who also kindly provided the photo

be profitable. However, the difficuly with such applications is obtaining sustainable effects. Since AMF can be obtained only after growth with a symbiotic partner, inocula are somewhat variable from one multiplication step to the next.

5.5 Arbuscular Mycorrhizal Fungi in Heavy Metal Soils

Recent reviews on this subject are available thus (Leyval et al. 1997; Jeffries et al. 2003; Hildebrandt et al. 2007). The work of mainly C. Leyval and coworkers (Weissenhorn and Leyval 1993, 1995; Tonin et al. 2001) showed that polluted soils contain AMF fungi that are specifically adapted to soil pollution. It has often been stressed that specific AMF spores from heavy metal soils possess enormous potential for phytoremediation (Hildebrandt et al. 2007). However, most metallophytes—at least most European species—are nonmycorrhizal or are poorly colonized by AMF. This is the case for *Armeria maritima* ssp. *halleri*, *Minuartia verna*, *Silene vulgaris* ssp. *humilis* and *Cardaminopsis halleri*. Like the latter, other members of the Brassicaceae are also generally regarded as being nonmycorrhizal (De Mars and Boerner 1996). The genus *Thlaspi* (pennycress) of this family has been studied in more detail. *Thlaspi* contains several hyperaccumulating species. They are poorly colonized, with some 5% of all roots at most showing any fungal structure. However, despite the low colonization frequency, all fungal structure within the roots, namely intraradical hyphae, arbuscules and vesicles are discernible. Thus, the whole program for establishing AMF symbiosis has developed in *Thlaspi*. Arbuscules are particularly detectable during the flowering state (Pongrac et al. 2007), as also noted for another member of the Brassicaceae, *Biscutella laevigata* (Orłowska et al. 2002). In general, there is no direct correlation between the degree of mycorrhizal colonization of the roots and the effectiveness of the mycorrhiza; thus, a poorly colonized plant may still form an effective symbiosis. In addition, the method used to count the mycorrhizal colonization may be a crucial factor. A more thorough characterization of AMF colonization, including more details on AMF structures within the roots, as provided by Trouvelot et al. (1986), can reveal profound interactions between colonization parameters and metal content. The data obtained by the Ljubljana laboratory indicate that the AMF play a protective role against excess metals and that the fungi have nutritional value in the acquisition of essential elements that are frequently present at low concentrations compared to nonessential metals in polluted soils (Vogel-Mikuš et al. 2005, 2006; Pongrac et al. 2007). The relationship between AMF colonization in *Thlaspi praecox* collected from polluted sites and its levels of nutrients and metals indicates the significance of this symbiosis for plant fitness, and can therefore be considered an important plant trait in further phytoremediation experiments (Regvar et al. 2006, Regvar and Vogel-Mikuš 2008).

Another member of the Brassicaceae that should be discussed here is *Biscutella laevigata*, which has already been mentioned. This plant is widespread in unpolluted soil in the Alps and occasionally occurs in the plain. There, it may serve at

polluted sites as a hyperaccumulator of lead (above 1,000 mg per kg of its dry matter), cadmium and thallium (over 1.5% of its dry matter) (Anderson et al. 1999). An even better perspective on phytoremediation may be offered by the South African *Berkheya coddii* of the Asteraceae, which is strongly colonized by AMF (Turnau and Mesjasz-Przybylowicz 2003; Orłowska et al. 2008).

To our knowledge, the only strongly colonized metallophyte of Central Europe is the zinc violet. Almost every root of both the blue violet of Blankenrode and the yellow form of the Aachen–Liège area is colonized when it is isolated from its natural site (Hildebrandt et al. 1999). All typical AMF structures, arbuscules, vesicles and intraradical hyphae are visible microscopically (Fig. 5.10). The extensive yellow color of the roots, which is visible by eye and is due to the formation of a yellow pigment (mycorradicin, Klingner et al. 1995), is indicative of strong colonization of the roots of the zinc violet. The roots of the blue zinc violet are also colonized when the plants are grown in a heavily fertilized garden soil (unpublished observation). Thus, zinc violets may even be obligatory mycorrhizal plants.

An AMF fungus, *Glomus intraradices*, isolated from the soil adjacent to the roots of the yellow zinc violet at the site of Breinigerberg, close to Aachen, was shown to consistently confer heavy metal tolerance to plants (Hildebrandt et al. 1999; Kaldorf et al. 1999). A variety of plants, such as maize (Fig. 5.11), can be grown in diverse heavy metal soils when supplemented with this isolate, termed Br1, and when the trials are optimally fertilized (by supplying Hoagland solution for example). Isolates from unpolluted soils also have an effect, but are inferior to *Glomus intraradices* Br1 under such conditions (Fig. 5.11). Heavy metal soils generally contain fewer AMF fungal spores than unpolluted sites. However,

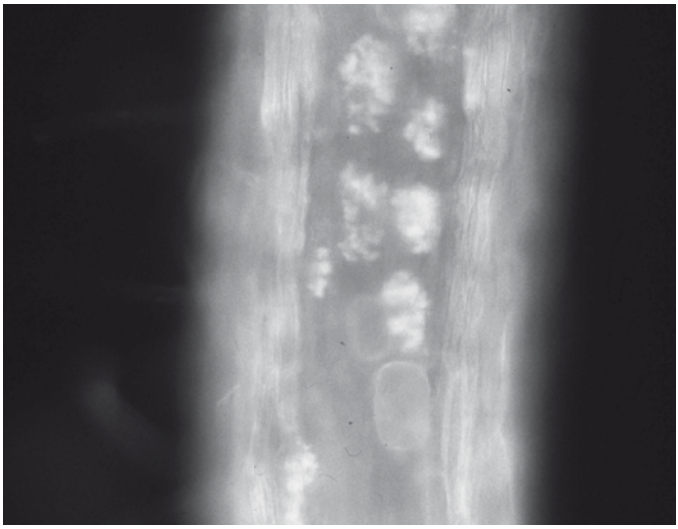

Fig. 5.10 A root from the zinc violet (*Viola lutea* ssp. *calaminaria*), showing arbuscles and vesicles

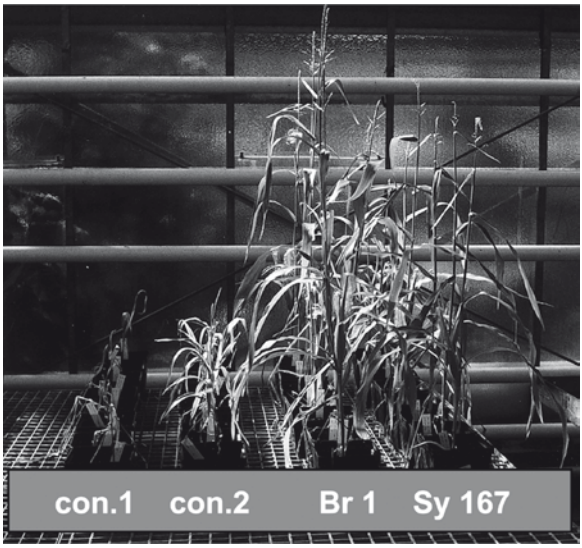

Fig. 5.11 The alleviation of heavy metal stress by the arbuscular mycorrhizal fungus *Glomus intraradices*. Maize was grown in the heavy metal soil from Breinigerberg (for its elemental composition, see Kaldorf et al. 1999) in a greenhouse experiment. The nutrient supply in this experiment was optimized by fertilizing all four samples with Hoagland's solution once a week. *Con.1*, soil sterilized; *con.2*, soil unsterilized; *Br1*, sample inoculated with the *Glomus intraradices* Br1 isolated from the zinc violet of Breinigerberg (close to Aachen); *Sys 167*, sample inoculated with *Glomus intraradices* Sys 167, an isolate from an unpolluted soil in Syria. The control mycorrhizal fungus *Glomus intraradices* Sys 167 also partially alleviates heavy metal stress, but is inferior to the Br. isolate from the heavy metal soil of Breinigerberg. In the presence of this latter isolate, the growth rate of maize is about half that observe in an unpolluted garden soil under the same conditions. Similar experiments have been performed with tomato, alfalfa (*Medicago truncatula*), rye grass, wheat, and barley, as well as with heavy metal soils from other areas, and all produce same outcome (Cologne laboratory, unpublished)

Glomus intraradices Br1 is not the only candidate for phytoremediation projects. Work by Tonin et al. (2001) showed that other AMF with potential for phytoremediation can be isolated from the rhizosphere of the yellow zinc violet (*V. lutea* ssp. *calaminaria*).

A combination of different AMF isolated from heavy metal soils may prove optimal for phytoremediation projects. AMF isolated from the site to be remediated are usually the best-adapted strains to the particular industrial waste conditions of the site (Orłowska et al. 2005). They can act by decreasing metal contents in plant roots and shoots and therefore play an important role in alleviating the toxicity (Kaldorf et al. 1999; Rivera-Becerril et al. 2002). A similar outcome was obtained in experiments with fungal strains of different origins and a model plant, *Plantago lanceolata*, cultivated on lead–zinc wastes (Orłowska et al. 2005). Different cultivars of a particular plant species may react differently in response to the particular fungal strain used for inoculation. Large differences in Zn, Pb and Cd uptake were

found among 15 cultivars of maize when inoculated with the same fungal strain (Orłowska et al. 2005). Furthermore, strains isolated from particular soil should be kept in cultures containing the potentially toxic metals from the original substratum, as the strains may lose their adaptation with time (Sudova et al. 2007). Fungi originating from unpolluted sites may also adapt to the toxicity, through unknown mechanisms, when grown in contact with heavy metals.

In addition to the typical metallophytes listed above, heavy metal soils often contain species from grasslands that are particularly adapted to drought; these are sometimes termed pseudometallophytes. Heavy metal soils often possess almost no humus layer, and the concentration of heavy metals can be highly variable over a short distance. Such fluctuations may allow drought-adapted species to thrive in spots with lower amounts of heavy metals. Such plants that occurred spontaneously on zinc–lead industrial wastes in Poland showed high or medium levels of AMF colonization (reviewed by Turnau et al. 2006a, b; Ryszka and Turnau 2007). Recently, a wide range of species from xerothermic grasslands (e.g., *Achillea millefolium*, *Agropyron intermedium*, *Agrostis capillaris*, *Anthyllis vulneraria*, *Astragalus cicer, Brachypodium pinnatum, Bromus inermis*, *Cirsium pannonicum, Convallaria majalis*, *Dianthus carthusianorum*, *Fragaria vesca*, *Fragaria viridis*, *Inula ensifolia, Libanotis montana, Onobrychis viciifolia*, *Ononis arvensis*, *Plantago media*, *Verbascum thapsus* and *Veronica spicata*) were able to survive on industrial wastes, even when the waste was not covered with a layer of humus. This experiment required the pre-adaptation of the seedlings in soil supplemented with industrial waste followed by AMF inoculum prior to their transfer to the field site. In this investigation mycorrhizal fungi were the key to successful growth, since nonmycorrhizal plants were not able to survive under these harsh conditions. At certain developmental stages during the succession, the vitality of these plants was found to be even better on this industrial waste than on unpolluted sites, as measured using a Handy PEA fluorescence spectrometer (Turnau et al. 2008). It should be stressed that most of these species could not be introduced into the waste as seeds, not even when supplemented with an AMF inoculum. Most of the plants were not able to survive as seedlings in this harsh substratum, mostly due to problem with water.

Ectomycorrhizal fungi also exist in heavy metal soils. Since several ectomycorrhizal fungi can be grown independent of a host, they may be easier to handle and may even offer better perspectives for phytoremediation. The reader is referred to a review on this subject by Jentschke and Godbold (2000). In phytoremediation experiments with ectomycorrhizal fungi, the application of a particular species/strain is rarely successful in heavy metal soils. Added fungi often cannot outcompete indigenous fungal strains. These may be less effective at plant growth promotion, but they are better adapted to forming a mycorrhizal symbiosis under the given harsh conditions. A general problem in microbiology (as well as with bacteria, for example pseudomonads, *Azospirillum*, *Azotobacter*) is that of obtaining sustainable effects in fertilization experiments with alien microorganisms. The reasons for the enhanced fitness of an indigenous microorganism in its native habitat are largely unknown. It may be easier to introduce AMF into heavily polluted sites as few indigenous AMF propagules are usually present in such places.

In heavy metal soils, plants are easy to grow when the soil pH is around 7 than in acidic conditions. At low soil pH, ericoid and ectomycorrhizal fungi play a more dominant role than AMF (Turnau et al. 2007).

5.6 Biochemical and Molecular Aspects of the Heavy Metal Tolerance Conferred on Plants by AMF

As can be seen in Table 5.1, maize colonized by *Glomus intraradices* has considerably smaller amounts of heavy metals in both its roots and its shoots than non-AMF controls. In contrast, essential elements like P, Mg^{2+} and Ca^{2+} are enriched in the roots of AMF-colonized plants, as shown by atomic absorption spectrometry. This is in contrast to Fe, Zn, Pb and also other toxic elements such as Al and As. The location of the heavy metals in plant tissues can be demonstrated by biophysical techniques such as EDXA, SIMS or LAMMA (Kaldorf et al. 1999). A more sensitive method of quantifying elements in a tissue is microPIXE, which simultaneously discerns differences in elemental content (Vogel-Mikuš et al. 2009). Within the roots, heavy metals that have been taken up are mainly found in the inner cortical cells, where most of the fungal structures (intraradical hyphae, arbuscules, vesicles) reside. The techniques employed so far do not allow us to determine whether they are located in fungal or plant cell structures. However, the idea is that the fungi lower the amounts of heavy metals in the roots to nontoxic levels for plant cells. Those heavy metals that are inevitably taken up might be mainly deposited in the fungal structures (their cell walls or their vacuoles).

Differential display approaches have been employed to identify genes that are expressed or suppressed in colonized plants as compared to non-AMF controls. The sequences deposited in GenBank were screened for conserved motifs which were then exploited for the synthesis of oligonucleotide primers in order to synthesize ~500 bp segments of target genes by PCR. This approach provided four

Table 5.1 Elemental content of 14-week-old maize grown in heavy metal contaminated soil from Breinigerberg with and without the arbuscular mycorrhizal fungus *Glomus intraradices* Sy167. The elemental concentrations in roots and shoots were measured by AAS and are given in mg/kg of dry weight. Data are from Kaldorf et al. (1999). Standard deviations are for $n = 2$

Element	Shoot (+AMF)	Shoot (–AMF)	Root (+AMF)	Root (–AMF)
Mg	3,330 ± 35	5,475 ± 30	8,750 ± 10	4,520 ± 6
P	590 ± 2	660 ± 10	1,120 ± 30	960 ± 10
Ca	7,190 ± 45	30,300±140	26,200±40	21,900±20
Al	30 ± 4	1,124 ± 3	2,720 ± 4	5,500 ±50
Fe	62 ± 2	820 ± 20	3,030 ± 150	5,670 ±150
Zn	830 ± 15	1,170 ± 120	4,600 ± 200	5,900 ±200
As	< 3	10 ± 2	< 3	40 ± 3
Pb	45 ± 2	200 ± 15	1,200 ± 90	1,800 ± 60

probes of metallothioneins (*mt*), one of phytochelatin synthase (PCS), and three of N*ramp* metal transporters, all from tomato (Ouziad et al. 2005). Transcript analyzes by Northern blotting showed that some of these genes (N*ramp* 1 and 3, *mt*2) are downregulated in AMF-colonized plants as compared to the situation in uncolonized controls, both of which were grown in a heavy metal soil. Others (PCS, *mt*1 and 3, N*ramp*2) remained unaffected in terms of their mRNA transcript levels. Such data were corroborated by real-time PCR quantifications and in situ hybridizations. The reduced expression of some of these genes in tomato grown in heavy metal soils suggests that AMF lower the concentrations of heavy metals in the roots to a level that makes the expression of these detoxifying enzymes in plants unnecessary.

Somehow we expected an upregulation in the fungi of the same genes that were downregulated in tomato. This was, however, not the case (Ouziad et al. 2005; Hildebrandt et al. 2007). Data from a suppression subtractive hybridization (SSH) cDNA library from hyphae of *Glomus intraradices* grown in the presence of high and low Zn concentrations did not reveal a sequence with homology to metallothioneins, phytochelatins or broad-range transporters. In contrast, the SSH library contained several EST sequences involved in the detoxification of reactive oxygen species (glutathione *S*-transferase, superoxide dismutase, cytochrome P450, thioredoxin). Their enhanced expression upon Zn stress was verified by reverse Northern blot analysis. Oxidative stress caused by heavy metals may therefore constitute a major problem for the fungi, and they may cope with this stress by synthesizing detoxifying enzymes in higher amounts than under conditions without heavy metal pollution. Such an interpretation was corroborated by a study of four fungal genes potentially involved in heavy metal tolerance: glutathione *S*-transferase, HSP90 as a highly stress-inducible chaperone, a metallothionein, and a Zn transporter from the ZIP family (Hildebrandt et al. 2007). The transcript levels of gluthiothione *S*-transferase and of HSP90 always increased when the fungal hyphae were exposed to either Zn, Cu, or Cd stress. The metallothionein gene was upregulated in extraradical mycelium of *G. intraradices* upon Cu stress, but barely so after the other treatments. The Zn transporter was particularly strongly expressed in intraradical hyphae. For more detail, the reader is referred to Hildebrandt et al. (2007).

Turning our attention to other data in the literature, a metallothionein gene of *Gigaspora margarita* was found to be upregulated in symbiotic mycelia upon Cu stress (Lanfranco et al. 2002). Also in *G. intraradices*, a metallothionein gene was expressed in response to oxidative stress (Gonzales-Guerrero et al. 2007). A Zn transporter gene (*GintZnT1*) of the cation diffusion facilitator family showed increased transcript levels on the mycelium of *G. intraradices* under long and short exposure to Zn (Gonzales-Guerrero et al. 2005). It is suggested that the products of such transporter genes are involved in the detoxification of specific heavy metals. In contrast, enzymes that serve in the removal of reactive oxygen species may have be more generally applicable in the fungi during AMF–plant symbiosis.

5.7 Arbuscular Mycorrhizal Fungi and Salt Tolerance

About 7% of all land is polluted with sodium chloride or other salts and is thus not amenable to agriculture. The scale of this salt problem is thus considerably higher than that of heavy metal tolerance. The role of AMF in conferring salt tolerance to plants is ambiguous. Salt has repeatedly been reported to inhibit spore formation, hyphal growth, colonization of plants, and the formation of an effective symbiosis (Juniper and Abbott 1993, 2006). Many salt-resistant plants belong to the Chenodopodiaceae, Juncaceae or Cyperaceae, and thus to nonmycorrhizal plant families. On the other hand, the sea plantains (*Plantago maritima*, *P. coronopus*) are mycorrhizal, and in the case of the salt aster, *Aster tripolium*, almost every root shows a fungal structure (Landwehr et al. 2002). The main grass of salt marshes, *Pucinellia* sp., shows a highly variable degree of AMF colonization for unknown reasons (Hildebrandt et al. 2001). An examination of the AMF spore types in diverse saline habitats showed that up to 80% of them belonged to one single species, *Glomus geosporum* (Hildebrandt et al. 2001; Landwehr et al. 2002; Carvalho et al. 2001, 2004). This fungus also occurs also outside of salt marshes, but it seemed worth testing its ability to transfer salt resistance to plants. Such experiments performed under diverse conditions in the Cologne laboratory consistently failed over a period of years. More recent molecular studies showed that molecular identification of the fungal spores from the saline soils did not match with their morphological characterization (Wilde et al. 2009). *G. geosporum* can indeed be found in the roots of halophytes, but it is far less dominant there than soil spores. In roots, *Glomus intraradices* prevailed. However, all of the sequences obtained by PCR and sequencing were new or matched with sequences that had been deposited without any further characterization and that belonged to uncultured fungi. *Glomus geosporum* is apparently forced to sporulate heavily under harsh saline conditions, even with the formation of rather small spores. However, it does not appear to be major player in salt marshes. Identifying the spores that belong to the uncultured *G. intraradices* in the roots, in order to isolate them and conduct experiments on plant salt tolerance with these fungi, appears to be a straightforward task. However, all attempts by us to perform such experiments over the last few years have been unsuccessful.

Plant distributions in salt marshes show rather similar dependences on the salt load (Fig. 5.12), regardless of the type of salt (NaCl, Na_2CO_3, Na_2SO_4, K_2CO_3) present in the soil. Since these salts are dissociated in the soils, they bind strongly water in their shells, and so plants find it very difficult to mobilize water for their purposes. Early botanists (Stocker 1928) noted that salt stress is closely related to drought stress, and that halophytes are better at coping with water deficiencies than glycophytes. The AMF of saline habitats also respond to drought stress. In a detailed study performed in two Hungarian salt marshes, Füzy et al. (2008) noted that high rainfall during the year in the area caused a reduction in AMF structures, particularly arbuscules. In contrast, the number of arbuscules in the roots was significantly higher after drought periods.

Roots of the halophyte *Aster tripolium* contain a lot of aerenchyma, and their structures are greatly altered by AMF colonization (Scheloske et al. 2004).

Fig. 5.12 Dependence of the plant distribution in a typical inland salt marsh on the salt load. Sodium chloride rises to the surface from belowground (*middle of the photo*, where the water stands after a heavy rainfall). This site with a high salt load is occupied by a monocultural stand of salt samphire (*Salicornia europaea*). Next to it is salt marsh grass (*Puccinellia distans*). The belt with plants in blossom contains salt aster (*Aster tripolium*). The photo is of the inland salt marsh of Barnstorf /Lkrs. Wolfenbüttel, Germany, and was taken in September 1966

PIXE determinations indicate gross changes in the depositions of ions and cations, particularly of Na^+ and Cl^-, in roots of *A. tripolium* colonized by AMF in comparison to nonmycorrhizal controls (Scheloske et al. 2004). However, due to the aerenchyma, roots of *A. tripolium* are fairly fragile when cut for microscopy. In addition, Na^+ and Cl^- are rather mobile and may be artificially translocated during cutting, even in freeze-dried material. Therefore, such experiments are quite difficult to perform.

Plant adaptations to salts require osmotic adjustments within the cellular compartments. This is achieved by osmolytes such as proline, glycine betaine, polyols, glycerol, or others, depending on the salt-tolerant organism. Proteins that are thought to be involved in the salt tolerance of plants include Na^+/H^+ transporters that catalyze the transfer of Na^+ from the cytoplasm to the outside or into the vacuole. Others are the aquaporins, both at the plasmalemma (the so-called PIPs) and at the tonoplast (TIPs), which transfer water into the compartments for osmotic adjustments. As with the genes of products potentially involved in heavy metal tolerance, probes were developed for tomato genes that are possibly involved in salt tolerance: two for plasma membrane aquaporins, one for a tonoplast aquaporin, and two for Na^+/H^+ transporters (Ouziad et al. 2006). Their expression was studied in tomato colonized by AMF and in controls under NaCl stress (Ouziad et al. 2006). Northern analyzes and in situ hybridizations showed that the expression of the two Na^+/H^+ transporters was not significantly affected by salt stress or AMF colonization. In contrast, transcript levels of one plasmalemma and the tonoplast aquaporin gene in roots were reduced by salt stress, and this effect was significantly enhanced by AMF colonization of the roots under salt stress. In leaves, however, the mRNA

levels of all three aquaporins were higher in AMF-colonized tomato than in the controls under salt stress.

A SSH cDNA library obtained from extraradical hyphae stressed with 0.7% NaCl for 48 h in comparison with unstressed controls provided 384 differentially expressed clones (deposited at http://www.genetik.uni-bielefeld.de/MolMyk). These ESTs contained neither aquaporin nor Na^+/H^+ transporter genes. However, genes for products involved in combating oxidative stress, such as 90 kD heat shock protein, thioredoxin peroxidase (rehydrin), glutathione reductase, glutathione *S*-transferase, Cu/Zn superoxide dismutase, DNA repair protein and peptidyl-prolyl isomerase (PPIase), were among the list of differentially expressed ESTs. In addition, two genes of ion homeostasis, vacuolar H^+-ATP synthase (V-ATPase, subunit C) and calcium-transporting ATase (P-type IIA ATPase), are worth noting in this list (unpublished data from the Cologne laboratory). Salt or drought stress may also generate reactive oxygen radicals that might cause damage to membranes and macromolecules. Therefore, the fungi may respond to salt (drought) by enhancing the formation of enzymes that serve to protect against oxidative stress, and in a similar mode of action to that employed upon exposure to toxic concentrations of heavy metals.

It was proposed that salt-tolerant plants could be engineered by overexpressing their Na^+/H^+ transporters (Yamaguchi and Blumwald 2005; Apse and Blumwald 2007). Such an approach may meet with limited success, since drought seems to be the major problem that halophytes have to cope with. AMF can help to alleviate this drought stress imposed by the salt. Besides drought, the formation of toxic oxygen radicals is seemingly a major problem faced by the AMF–plant symbiosis.

5.8 Conclusion

The present chapter has shown that AMF are of particular interest in basic research and have enormous potential applications in relation to conferring both heavy metal and salt tolerance to plants. At present, the main problem with such applications is that the fungi cannot be grown independent of another symbiotic partner. Therefore, it is difficult to produce AMF inocula that exert sustainable effects in phytoremediation projects. Most of the factors that determine the symbiotic partnership between plants and AMF will hopefully be elucidated in the nearer future. This knowledge is a prerequisite for multiplying the fungi under defined and inexpensive conditions, and will also provide great impetus to future developments in this field.

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Chapter 6
Quantitative Analyses of Trace Elements in Environmental Samples: Options and (Im)possibilities

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6.1 Introduction

Monitoring the bioavailability, toxicity and risk relationships arising from trace element contaminants in ecosystems requires the determination of the their concentrations in soil, water, microorganisms, plants and animals. Therefore, the appropriate analytical method(s) for addressing the particular question(s) of interest need to be chosen from among the broad array of analytical methods that are available. In the first part of this chapter, we will deliberately focus on the presentation and use of X-ray-fluorescence (XRF)-based methods for the analysis of "bulk" biological samples, such as standard and total-reflection energy-dispersive XRF (EDXRF, TXRF). Although EDXRF and TXRF are far less popular methods for analyses of element concentrations in biological samples than, for example, atomic absorption spectroscopy (AAS) and inductively coupled plasma atomic-emission spectroscopy (ICP-AES), there is still no particular reason why they cannot be used for this purpose. From the viewpoints of economics and environmental protection, EDXRF

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and TXRF are generally much cheaper and environmentally friendlier than the other popular methods mentioned. They also have the further advantage that a whole suite of different elements can be determined simultaneously (Nečemer et al. 2008), which is especially important for the analysis of unknown samples with unknown element concentrations. When samples are analyzed, for example, with AAS, the concentration range of the measured element must be predicted, so that the samples with high element concentrations can be appropriately diluted to bring them into the range of the reference standard curve applied. Additionally, XRF spectrometry allows the analysis of biologically important elements like phosphorus, sulfur and chlorine, which cannot be analyzed by AAS or ICP-AES.

Since the presence of elevated trace element concentrations in the environment usually raises questions about the ways that organisms interact and deal with them, the second and third parts of the chapter will be dedicated to the more specialized and highly sophisticated X-ray fluorescence/absorption-based methods. Micro-proton-induced X-ray emission (micro-PIXE) using accelerated protons is one of the most modern, sensitive, and reliable methods for the localization and quantification of different elements in biological samples at the tissue and cellular levels. On the other hand, X-ray absorption spectroscopy (extended X-ray absorption fine structure or "EXAFS", and X-ray absorption near-edge structure or "XANES") using synchrotron light offers studies of the chemical speciation and coordination of trace elements in biological samples, therefore opening up new dimensions in research into interactions between trace elements and organisms at the organ, tissue and cellular levels.

6.2 Analytical Methods for Bulk Samples

6.2.1 Basic Principles of X-Ray Fluorescence

The process of XRF represents the basis for various analytical techniques. XRF is induced by the excitation (ionization) of atoms in the tightly bound inner K (for the elements $20<Z<50$) and L ($Z>50$) atomic shells with energies that must exceed the binding energies of the K and L electrons (Markowicz 1993).

The atoms can be excited with:

- *X-rays*, by irradiation from the X-ray tube or from a radioisotope source. Ionization of an inner atomic shell is achieved through the *photoelectric effect* (Fig. 6.1a), which involves the absorption of a photon followed by the ejection of a *photoelectron* from the atom. This leaves the atom in an excited state. The kinetic energy of the photoelectron is given by the difference between the absorbed photon energy and the binding energy of the electron in the atom (Markowicz 1993).
- *Electrons or accelerated charged particles* (PIXE; protons, ^{4}He), via the electromagnetic Coulomb interaction process, in which sufficiently energetic accelerated electrons or charged particles can cause the ejection of electrons from the inner shells of the atom (Fig. 6.1b) (Small 1993, Maenhaut and Malmqvist 1993).

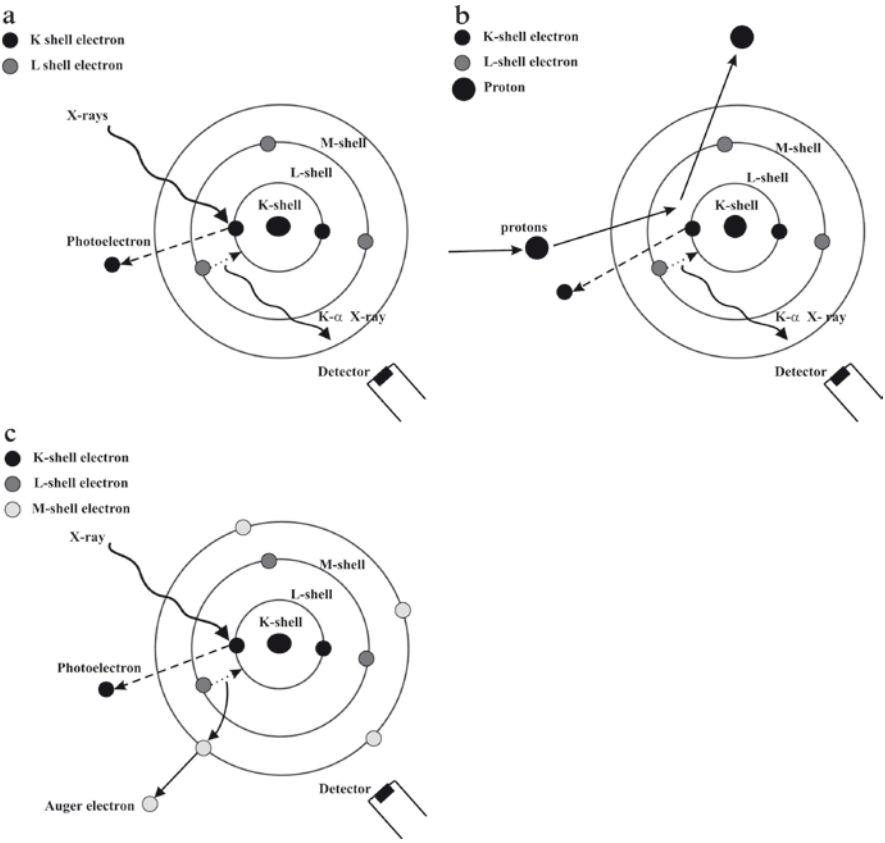

Fig. 6.1 (**a**) Interaction of an atom with X-rays: photoelectric effect; (**b**) interaction of an atom with accelerated charged particles; (**c**) Auger effect (adapted from Markowicz 1993; Maenhaut and Malmqvist 1993)

In both cases, the excitation results in the ionization of the atom, which becomes unstable because of the electron vacancy in one of its inner shells. If this inner shell vacancy in the atom is then filled by an electron from one of the outer shells (L or M), the emission of *characteristic X-ray photons (K, L, M)* can occur in this transition process, which is known as *relaxation*. Characteristic K, L and M X-ray photons are typical of particular elements, and this provides the basis for distinguishing between elements in X-ray spectrometry (Markowicz 1993).

Alternatively, however, an excited atom can return to a state of lower energy by ejecting one of its own electrons from a less tightly bound state. This radiationless transition that competes with XRF is called the *Auger effect*, and the ejected electrons are called *Auger electrons* (Fig. 6.1c). In contrast to the Auger effect, the process of XRF is relatively less probable for low-*Z* elements and rises with the atomic number. In addition, it is more probable that the atoms are excited in the K shell than in the L shells (Markowicz 1993). The relative probability for the XRF

radiation transition in the relaxation process is called the *fluorescence yield* (e.g., ω_K or ω_L), and this is lower for low-*Z* atoms (e.g., Alω_K = 0.036; Agω_K = 0.83) and for L shells (Agω_{L3} = 0.05). The value of the fluorescence yield significantly influences the sensitivity of XRF analysis (Markowicz 1993).v

6.2.2 Standard Energy-Dispersive X-Ray Fluorescence Analysis

The XRF analysis system consists of the excitation source and the X-ray spectrometer. In standard XRF, the elemental analysis is based on measurements of the XRF spectrum from the excited sample. XRF systems are classified according to the types of excitation sources and the types of detectors installed. The XRF mode in which the count rate and the energy (distribution) of characteristic X-rays are measured using an *energy-dispersive X-ray spectrometer* comprising the X-ray detector, electronic components and a multichannel analyzer (MCA) is known as EDXRF.

Excitation can be performed using almost monochromatic *radioisotope sources*, such as Fe-55 (activity, 20 mCi), Cd-109 (25 mCi) and Am-241 (25 mCi) or polychromatic radiation from *X-ray tubes*. X-ray tubes can be air cooled (low power, 50 W) or water cooled (high power, 2 kW), with different anodes, such as Ag, Mo and Cr. XRF experiments are designed according to different geometries (Figs. 6.2–6.4). When thick specimens are excited by the continuous X-ray spectrum from an X-ray tube, the sensitivity of the method is lowered because of the relatively high background from the scattered continuous radiation from the sample, or its substrate, in the spectral region of the fluorescent radiation (Fig. 6.5a). A secondary target irradiation geometry can be used to partly monochromatize the X-ray tube radiation, and thus to decrease this background scattering (Jaklevic and Giauque 1993) (Fig. 6.4).

Proportional and/or *semiconductor* X-ray detectors are available for the abovementioned EDXRF systems. The choice of detector and excitation source is very important. In cheaper spectrometers, a radioactive source and a proportional

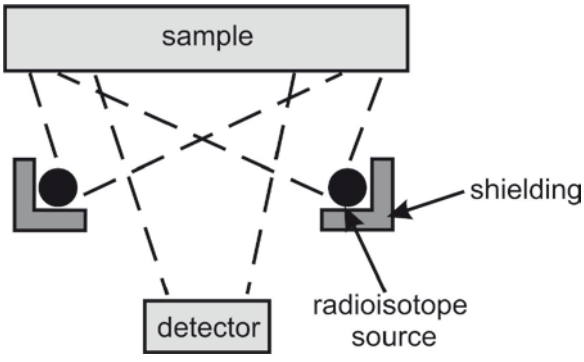

Fig. 6.2 X-ray fluorescence (XRF) geometry

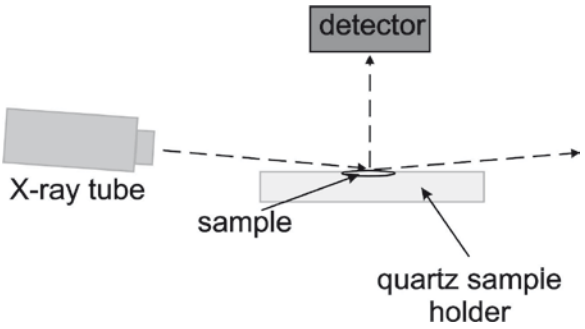

Fig. 6.3 Total reflection X-ray fluorescence (TXRF) geometry

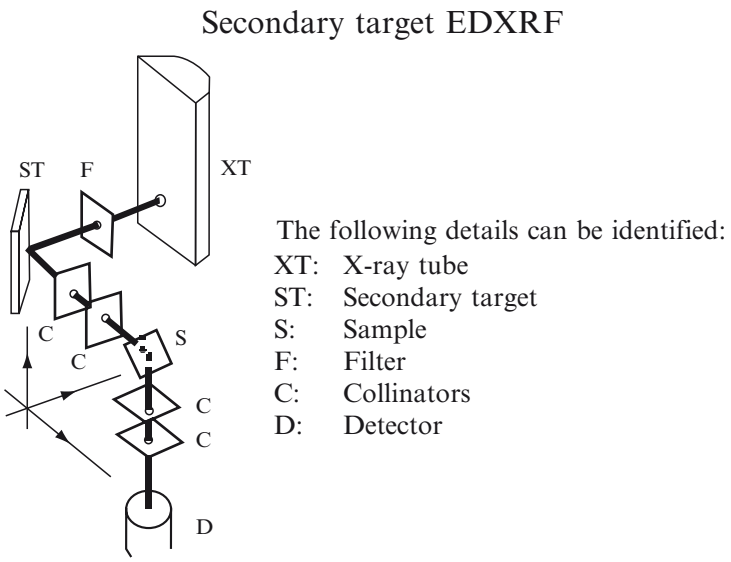

Fig. 6.4 Secondary target EDXRF geometry

detector (gas) can be used. However, one shortcoming of such a device is the poor energy resolution of the detector (800–1,000 eV at 5.9 keV), making the quantification of the insufficiently resolved characteristic X-ray lines in the measured spectrum quite difficult. This problem can be overcome by using semiconductor detectors such as Si (Li), Si PIN or Si drift (SDD) detectors, which have energy resolutions of around 140 eV at 5.9 keV, and so quantification is easier and more accurate. Si(Li) detectors are cooled by liquid nitrogen, although the newer Si PIN or SDD detectors are smaller and are cooled electrically. These electrically cooled detectors and low-power air-cooled X-ray tubes are combined in portable EDXRF analyzer systems, which have recently become very popular for in situ element analysis.

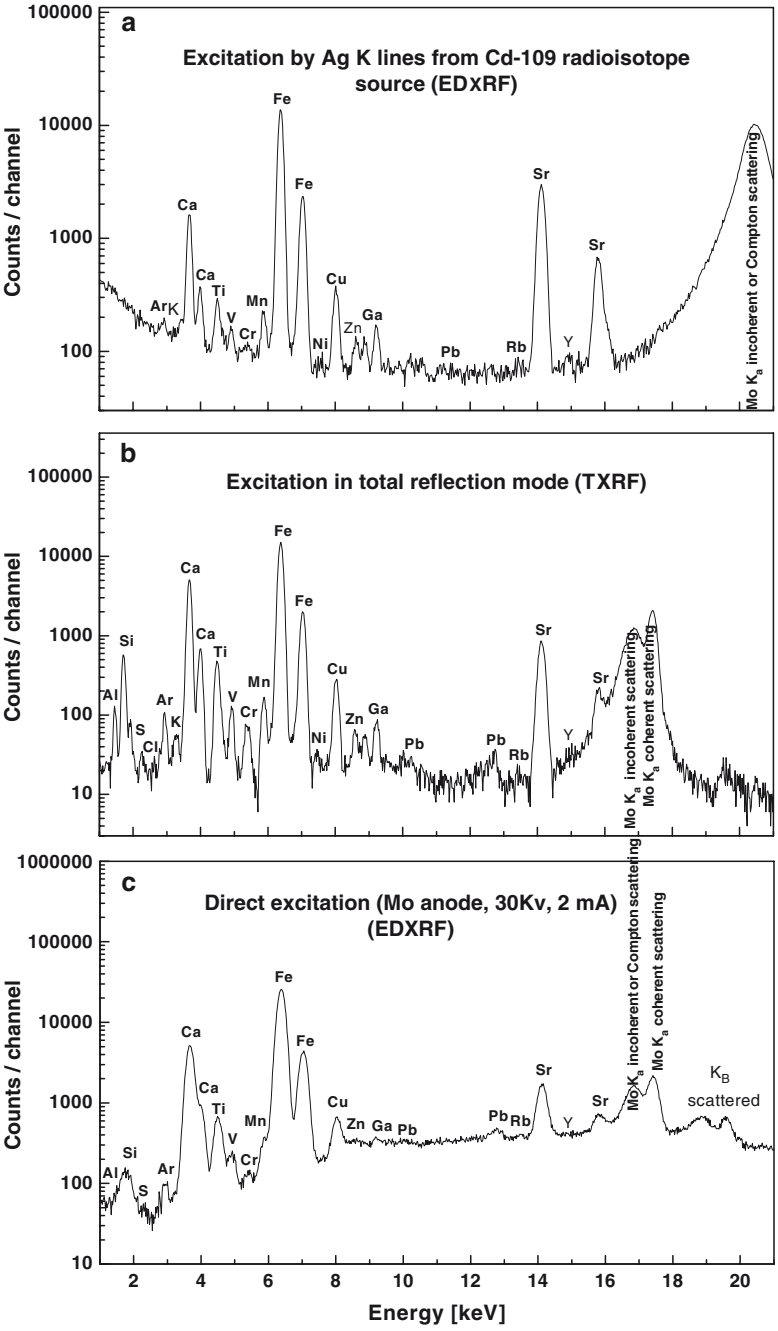

Fig. 6.5 Spectra of the same sample analyzed in different EDXRF modes a) EDXRF mode with Cd-109 radioisotope source excitation, b) TXRF mode with X-ray tube (Mo anode) excitation, and c) EDXRF mode with X-ray tube (Mo anode) excitation

Standard EDXRF usually allows the determination of elements from $Z = 13$ (Al) to $Z = 92$ (U). In some cases, depending on the installation of better detectors and the taking of measurements in a vacuum, elements with even lower Z values than 13 (F, Mg and Na) can be determined. The concentration ranges of these analyses vary from a few percent to a few μg g^{-1}. The limits of detection decrease at higher Z values, and for lighter elements these are a few percent or a few parts of a percent for F, Mg, Na, Al, Si, a few 100 μg g^{-1} for S and Cl, a few 10 μg g^{-1} for Fe, Cu, Ni and Zn, and a few μg g^{-1} for Cd, Ag and U.

Both solid and liquid samples can be analyzed by EDXRF. In the case of solid samples, no special destructive chemical treatments of the sample are necessary. Approximately 100 mg of solid sample is sufficient for analysis; however, the sample should be very well ground up and homogenized because any inhomogeneity of the pulverized solid samples can have a large influence on the accuracy of the measurements, especially in the case of lighter elements (Nečemer et al. 2008). Using the milled and homogenized sample, a pellet is pressed out by a pellet die and a hydraulic press. The pellet is then analyzed directly by the EDXRF system.

For liquid samples, 100–1,000 ml of solution is required, and the elements are extracted from the sample by precipitation. Several precipitation agents are available for this purpose. For instance, the reagent ammonium pyrrolidine dithiocarbamate (APDC) can be used for the precipitation of Cu, Fe, Ni and Pb. Note that any specific precipitating agent can selectively precipitate only some specific elements. Therefore, only certain specific elements can be determined in liquid samples using this method of sample preparation. The precipitated elements are separated from the liquid phase by filtration, and the precipitate that is gathered on the filter is measured directly by the EDXRF system. Due to the preconcentration of the precipitated elements, the limits of detection for these elements decrease to a few 10 ng g^{-1}, which cannot be achieved with the analysis of solid samples. This approach is especially suitable for monitoring contaminating elements in water.

The main advantages of EDXRF analysis are its multielement capability and its nondestructive nature, as well as the simple sample preparation required that does not involve time-consuming sample destruction and only requires personnel with a minimum of manual skills. This is undoubtedly the cheapest and the simplest analysis technique among the chemical instrumental analytical techniques discussed here. Therefore, EDXRF is particularly well suited to environmental and plant biology studies, where a large number of samples need to be analyzed. In addition, EDXRF allows the analysis of nonmetals (P, S and Cl), which can also play important roles in plant biological processes.

6.2.3 Total Reflection X-Ray Fluorescence

The basic fundamentals of total reflection XRF spectrometry (TXRF) are similar to those of EDXRF, although they have quite different excitation modes. In TXRF systems, the samples are first deposited as dry liquid residues on an optically smooth substrate,

which is usually quartz. They are then excited by a well-collimated X-ray beam at an angle smaller than the angle of total reflection for the substrate (<1.8 mrad for quartz) (Fig. 6.3) (Klockenkämper 1997; Kump et al. 1997). In this case, the majority of the incident X-ray radiation is reflected from the quartz surface, and only a minor part of it is absorbed by the deposited sample to excite the fluorescence. The penetration of the incident X-ray beam into the reflecting material is itself drastically reduced under these conditions, and thus the scattered and fluorescence radiation contributed by the carrier is negligible for this geometry. Consequently, the background radiation due to scattering on a small amount of sample is very low, significantly increasing the sensitivity of TXRF when compared to standard XRF (Schwenke and Knoth 1993; Kump et al. 1997) (Fig. 6.5b). The sensitivity level, however, still strongly depends on the atomic number of the element, although it does extend down to a few ppb (1 ng g^{-1} dry weight, "DW") in TXRF (Schwenke and Knoth 1993), compared to a few ppm (1 μg g^{-1} DW) for heavier elements in EDXRF.

To achieve the described excitation conditions, a special total reflection module is required to shape the excitation beam from the X-ray tube into a suitable form that will excite a small amount of the dried sample material placed on the quartz substrate. Although there are many commercially available TXRF spectrometers that are expensive, there are also several cheaper laboratory-built systems that exist worldwide. For EDXRF systems, these usually have a fine-focus Mo anode X-ray tube, an X-ray generator, a semiconductor X-ray detector and spectroscopy electronics that are equipped with a total reflection module that is provided by Atom Institute (Vienna). In this way, a cheap alternative to TXRF can be realized that can be used for multielement analyses of different environmental samples.

For analyses by TXRF, the samples have to be in a liquid form. The solid pulverized samples thus require destructive treatment using wet or dry digestion procedures. This process usually utilizes a decomposition procedure with a small amount of pulverized material (0.1 g), and involves the application of a mixture of mineral acids followed by microwave digestion. The resulting solutions can be analyzed directly by TXRF after the addition of an internal standard, which is usually a Ga or Y standard AAS solution. A small amount of this solution (10 μl) is pipetted onto a quartz substrate, dried, and measured.

As in the case of EDXRF, the TXRF method enables multielement analyses. Usually, with a Mo anode excitation tube, elements from $Z = 16$ (S) to $Z = 92$ (U) can be determined in the concentration range of a few percent to a few μg g^{-1}. The determination of lighter elements like Na and Mg is possible in a vacuum and with the application of a Si drift detector. Since a very small amount of dry sample residue is analyzed by TXRF, the relative sensitivity is only one to two orders of magnitude lower than for EDXRF, although the absolute sensitivity of TXRF is very good and reaches a few picograms, which should be contrasted with a few micrograms for EDXRF.

The main advantage of TXRF over EDXRF is the possibility of rapidly analyzing a larger number of samples that are prepared by a simple procedure (i.e., by the dilution of soluble samples, like bee honey, in water). TXRF is also very suitable for the analysis of very small amounts of biological samples, like plant xylem sap, where only a small amount of material is available.

It should be noted here that TXRF and EDXRF are not the most appropriate techniques for the analysis of low concentrations of light elements (e.g., Na, Mg Al, and Si). The determination of low concentrations of Cd in plant material by TXRF is also very inconvenient, because the plant material usually contains large amounts of potassium, and the fluorescent K lines strongly interfere with the Cd L lines. On the other hand, the preparation of the solid samples by wet digestion requires the application of time-consuming chemical procedures that require various highly pure and costly mineral acids and qualified personnel for sample preparation and handling.

6.3 Element Localization Analyses

6.3.1 Micro-Proton-Induced X-Ray Emission Spectroscopy

Proton-induced X-ray emission spectroscopy (PIXE) is a highly sensitive analytical X-ray detection method that is mostly used for measurements of trace elements in various types of materials. The limits of detection range from 0.1 to 1 μg g^{-1} (ppm) for mid-Z elements ($20 < Z < 40$), and are generally well below 10 ppm for other elements, from Na to U (Johansson and Campbell 1988). This sensitivity of PIXE is a result of the low continuous physical background in the X-ray spectra induced by protons. The physical background in PIXE consists of the bremsstrahlung of the proton and the bremsstrahlung of secondary electrons (Ishii et al. 2005). The standard PIXE method includes a proton beam with a diameter of several millimeters that is used to induce X-rays in the sample, and its applications extend from geology to archeometry and air aerosol particulate studies to homogenized biological materials.

The capabilities of the PIXE technique are greatly extended when it is used with a focused proton beam. High-energy focused proton beam setups are frequently referred to as nuclear microprobes (Breese et al. 1996). The ion lenses that are used can focus a proton beam down to submicrometer diameters. By scanning the focused proton beam in a raster mode, and detecting the induced X-rays, lateral element distribution maps can be obtained for samples. The acronym for this method is micro-PIXE, which shows many methodological similarities with the energy-dispersive X-ray analysis (EDX) technique that is available with scanning electron microscopes (SEMs). In contrast, micro-PIXE has enhanced (by approximately two orders of magnitude) element sensitivity (Legge and Cholewa 1994). For quantitative element analysis of thin sections of biological materials, the thickness of the slice is determined in parallel with micro-PIXE by scanning transmission ion microscopy (STIM). In cases where the light element matrix of the sample is unknown, Rutherford backscattering spectroscopy (RBS) can reveal information about the elemental composition of the tissue matrix (including C, O and N) and its thickness. STIM and RBS are usually performed simultaneously with micro-PIXE (Pallon et al. 2004).

Trace element allocation by micro-PIXE in environmental studies provides information on the uptake and localization of heavy metals in biological tissues

(Schneider et al. 1999; Orlic et al. 2003; Bhatia et al. 2004; Vogel-Mikuš et al. 2007, 2008a, b). Micro-PIXE element distribution maps for the elements that are dominant in tissue physiology (K, Ca, S and P) usually provide high-contrast morphological images that are easily correlated with images obtained by optical and electron microscopy. Where the trace element concentrations exceed 50 ppm, elemental images provide excellent allocation information. However, the trace elements are often present at concentrations on the order of 1 ppm or less. In such cases, the corresponding trace element maps may not provide useful information on the allocation due to poor spectral statistics. Using offline analysis of the micro-PIXE spectra extracted from distinctive morphological structures, the resulting data can still provide reliable information on trace element concentrations in the selected part of the tissue when the spectra are accumulated with sufficient statistics.

6.3.2 Sample Preparation

The key starting point in micro-PIXE analysis of biological materials is sample preparation. A procedure developed over the last two decades is the one most frequently used for biological tissues containing large quantities of water; this consists of tissue cryofixation, slicing and freeze drying (Schneider et al. 2002; Vogel-Mikuš et al. 2008b, c). This method provides excellent results for elemental distributions at the tissue and single-cell levels, and has been described in detail by Vogel-Mikuš et al. (2008c).

As far as elemental images of subcellular structures are concerned, even though freeze drying allows the ice formed during the rapid freezing to sublime from the samples (under vacuum, at low temperatures), the removal of water still results in intracellular morphological deformation. This can include shrinking of the cellular membranes, loosening of intracellular structures, and the sticking of these to the cellular membranes and cell walls. To avoid freeze drying, several attempts have been made to provide controlled water substitution using other agents. The resulting elemental distributions, however, indicate significant elemental redistribution from the original state (Mesjasz-Przybyłowicz and Przybyłowicz 2002; Kachenko et al. 2008). Thus, these substitution procedures are effective for morphological studies with optical or electron microscopy, but must be avoided for elemental distribution studies in tissues.

An advanced methodology for sample preparation without freeze drying is to keep the tissue frozen after cryofixation and then to transfer it to the vacuum chamber for analysis using a cryostage, where it is analyzed in the frozen hydrated state (Tylko et al. 2007). This approach is already used with several X-ray microbeams in synchrotron laboratories (Fahrni 2007). Indeed, this method of tissue preparation is the most promising approach to tissue treatment utilized in the new generation of nuclear nanoprobes, where PIXE is expected to be available with sub-500-nm beams. At the same time, it is the sample preparation technique of choice for the development of three-dimensional (3D) X-ray imaging methods with nuclear microprobes that are emerging from STIM-PIXE (Habchi et al. 2006) and from 3D PIXE with a confocal capillary setup (Karydas et al. 2007).

6.3.3 Data Processing and Evaluation

There are several remaining problems with the micro-PIXE methodology, and these need to be resolved before commercially available packages can integrate all of the required steps into the data processing. When the element map of a tissue is prepared in the form of a list mode file, the quantification needs to take into account sample thickness variations, the sample matrix composition, and a precise energy-dependent efficiency calibration of the X-ray detectors, among other aspects. Quantitative element maps should be produced by deconvolution of the background and interference peaks, and several packages do adequately cover some of the aspects that are required. The GEOPIXE package provides deconvolution mapping for biological samples (Ryan 2001) and is commercially available. The PIXEKLM (Uzonyi and Szabó 2005) and BIOPIXE (Scheloske and Schneider 2002) programs provide deconvolution mapping and handle thin samples of nonuniform thickness, but these are currently not available for the user community. In biomedical research with the nuclear microprobe of the Jožef Stefan Institute, the PIXE analysis software GUPIXWIN (Campbell et al. 2000) is used for the quantitative element analysis of selected morphological structures within a biological tissue, and it incorporates the thickness information measured by STIM.

6.3.4 Instrumentation and Examples

The required instrumentation for micro-PIXE includes an ion accelerator, ion lenses, a scanning system, detectors and acquisition software. The method is becoming readily available to external users in several laboratories as a standard technique for quantitative element mapping in biological tissue.

Although micro-PIXE is a multielement technique, where all of the elements are detected simultaneously, the duration of any single measurement depends strongly on the targeted trace element. When the interest is primarily in the distribution of light or mid-*Z* elements, the time required for a single measurement ranges from 10 min to several hours. In extreme cases, such as with cadmium distribution in plant tissue (Vogel-Mikuš et al. 2008b), a single measurement may take up to 2 days (Fig. 6.6). Figure 6.7 illustrates an example of the use of micro-PIXE for element localization in plant tissues. This includes element maps of a leaf cross-section from the Cd/Zn hyperaccumulator *Thlaspi praecox* (Wulf.) growing on soil highly polluted with Pb, Cd and Zn. The Zn map illustrates the epidermal cells that are rich in Zn, while the K map shows the regions of the vascular bundles with their abundances of K. Finally, the Cd and Pb maps demonstrate the tolerance to the uptake of both Cd and Pb of the palisade and spongy mesophyll tissue. The black-and-white maps show the selected positions of the xylem, phloem and collenchyma regions of the vascular tissues, where as the spectral data extracted provide the quantified concentrations given in Table 6.1.

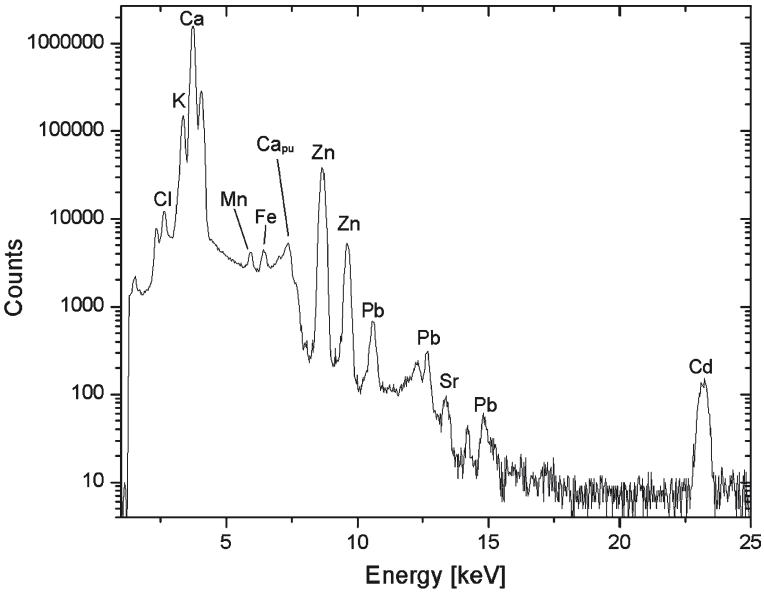

Fig. 6.6 PIXE spectrum measured in a section of a leaf of *Thlaspi praecox* (Wulf.) grown on highly polluted soil. For Cd detection, the Cd Kα line at 23 keV is used for evaluation, as the Cd L lines overlap with the strong K Kα line. This drastically increases the required detection time for Cd quantification (Vogel-Mikuš et al. 2008b)

The typical running costs of the micro-PIXE method range from ~200€ per hour to ~1,500–4,000€ per day. At several micro-PIXE facilities, access is granted after evaluating and selecting scientific proposals.

6.4 Element Complexation Analyses

6.4.1 X-Ray Absorption Spectroscopy

High-resolution X-ray absorption spectroscopy (XAS) became available with the development of synchrotron radiation sources. The advent of this form of spectroscopy has introduced powerful experimental methods for the investigation of atomic and molecular structures of materials that have enabled the identification of local structures around atoms of a selected type in the sample. In X-ray absorption near-edge structure (XANES), the valence state of the selected type of atom in the sample and the local symmetry of its unoccupied orbitals can be deduced from the information hidden in the shape and energy shift of the X-ray absorption edge. In extended X-ray absorption fine structure (EXAFS), the number and species of neighboring atoms, their distances from the selected atom, and the thermal or structural disorder in their positions can be determined from the oscillatory part of

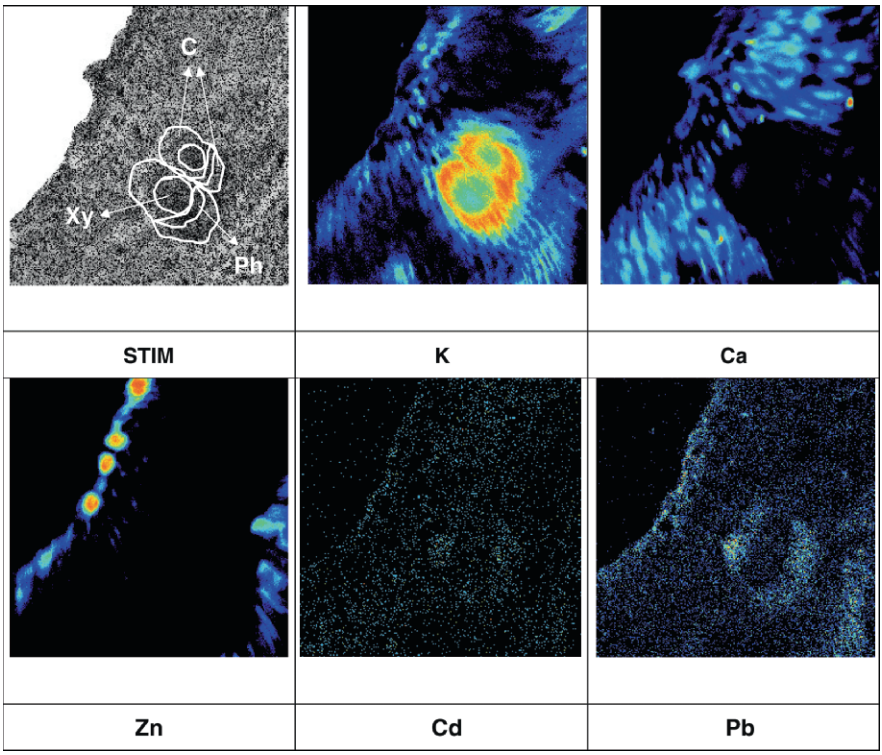

Fig. 6.7 Elemental distributions in a section of a leaf of *Thlaspi praecox* (Wulf.) grown on highly polluted soil. *C*, Collenchyma; *Xy*, xylem; *Ph*, phloem; *STIM*, scanning ion transmission microscopy image

the absorption coefficient above the K or L absorption edges (Koningsberger and Prins 1988; Rehr and Albers 2000; Wong et al. 1984). This analysis can be applied to crystalline, nanostructural or amorphous materials, as well as to biological samples, liquids and molecular gases. EXAFS is often the only practical way to study the arrangements of atoms in materials without long-range order, where traditional diffraction techniques cannot be used. In the case of biological samples, the method can provide specific structural information at the atomic level, especially about metal contaminants in ecosystems. The information on the valence states and the local structures around metal cations that are bound in different plant tissues or in the soil, or are dissolved in water, can provide direct evidence of metal complexation with organic molecules. This can help to resolve important questions about the bioavailability of toxic metals in the soil, and about soil–plant interactions and metal accumulation. The information obtained using XANES and EXAFS can explain complexation mechanisms in different plant tissues at a biochemical level, and can provide insights into tolerance mechanisms in metal (hyper)accumulating species.

A basic XAS experiment is shown in Fig. 6.8. A thin homogeneous sample of the material to be investigated is prepared with an optimal absorption thickness

Table 6.1 Elemental concentrations obtained by analyzing spectroscopic data from the xylem, collenchyma and phloem regions of the vascular tissues

	Xylem			Collenchyma			Phloem		
El.	Conc. (μg g^{-1})	Stat. err. (%)	LOD (μg g^{-1})	Conc. (μg g^{-1})	Stat. err. (%)	LOD (μg g^{-1})	Conc. (μg g^{-1})	Stat. err. (%)	LOD (μg g^{-1})
Cl	2,890	6.33	407	10,174	1.78	381	4,122	5.96	541
K	26,477	0.34	70.5	43,286	0.15	60.7	33,573	0.35	89.7
Ca	5,189	1.27	116	10,177	0.71	129	5,421	1.53	150
Mn	66.5	9.25	9.6	104	3.69	6.1	70	11	12.3
Fe	66.1	9.35	9.2	25.8	15.3	7.5	41.1	18.9	13.5
Ni	11.6	33.2	4.3	14	17.7	4.3	≤lod	/	7.5
Cu	20.5	23.5	5.9	≤lod	/	3.5	9.4	46.3	7.0
Zn	872	2.28	9.7	1,742	0.78	4.6	870	2.79	14.2
Cd	832	33.5	417	2,440	7.52	169	1,438	27.3	703
Pb	261	12.7	19.3	1,466	2.08	14.1	411	11.5	53.3

El., element; *conc.*, concentration; *LOD*, limit of detection; *stat. err.*, statistical error. Phosphorus and sulfur were below the limit of detection

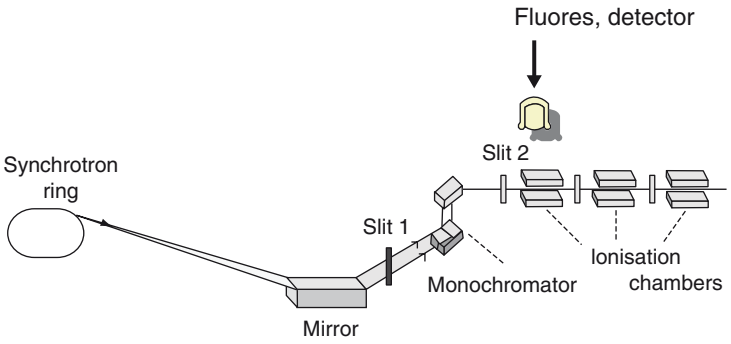

Fig. 6.8 Schematic representation of an X-ray absorption spectroscopy beamline

(μd) of about 2, and the intensities of the incident and the transmitted X-ray beams are recorded with a stepwise progression in the incident photon energy. In a typical synchrotron radiation experimental setup performed in transmission detection mode, ionization cells monitor the intensities of the incident (I_0) and transmitted (I_1) monochromatic photon beams through the sample. Since the exponential

attenuation of X-rays in a homogeneous medium is given by the well-known relation $I = I_o \exp(-\mu d)$, where d is the sample thickness, the absorption coefficient $\mu(E)$ can be obtained at a given photon energy E. The energy dependence of the absorption coefficient is obtained by a stepwise scan of the photon energy in the monochromatic beam that is provided by a double-crystal Bragg monochromator. The exact energy calibration of the monochromator is established with simultaneous absorption measurements on a reference metal foil placed between the second and third ionization chambers.

With diluted samples that contain small amounts of the element of interest, which is often the case in biological samples, and for thin films that are deposited on thick substrates, the standard transmission detection mode cannot be used. Instead, we can exploit the fluorescence detection mode, where fluorescence photons from the sample are monitored instead of the transmitted beam. The intensities of the detected fluorescence signals are linearly proportional to the absorption coefficient of the element under investigation. The investigated thickness of the sample is proportional to the penetration depth of the X-ray beam.

The dominant process in X-ray absorption at photon energies below 100 keV is the photoelectric effect, whereby the photon is completely absorbed, transferring its energy to the ejected photoelectron. The photoelectric cross-section, and hence the absorption coefficient, decreases monotonically with increasing photon energy. When the photon energy reaches one of the deep inner-shell ionization energies of the atom, there is a sharp jump (absorption edge) that marks the opening of an additional photoabsorption channel. For practical purposes, in structural analysis, the K and L absorption edges are most important. An example of an absorption spectrum for Cr metal in the energy region of the Cr K edge is shown in Fig. 6.9.

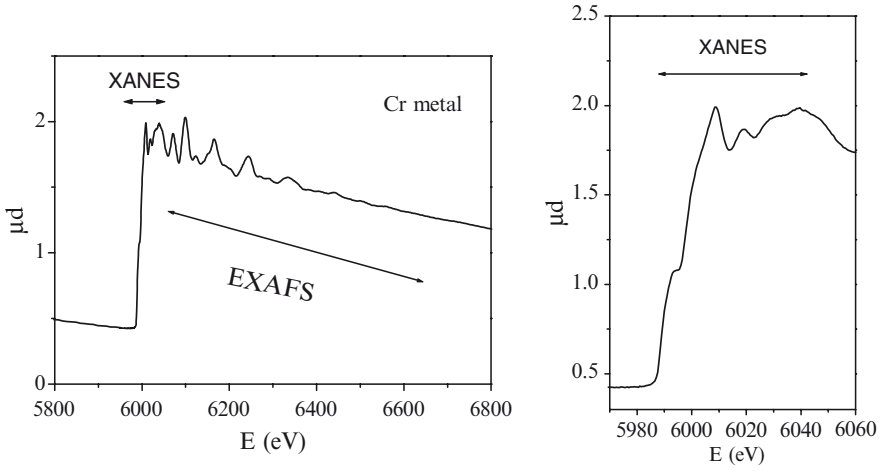

Fig. 6.9 X-ray absorption spectrum of Cr metal in the energy range of the Cr K edge (5.989 keV), showing rich EXAFS structure. The K-edge energy region, magnified in the *right graph*, reveals details of the Cr K edge XANES structure

The analysis mainly focuses on two energy regions that are usually treated separately: the energy region of the absorption edge and pre-edge structures within about 30 eV of the threshold, denoted XANES, and the energy region in a range from about 30 eV up to about 1,000 eV above the threshold, which shows oscillatory variations of the X-ray absorption coefficient, and is known as EXAFS.

6.4.2 X-Ray Absorption Near-Edge Structure

In the energy region near the absorption edge (K or L in subsequent discussions here), the slow photoelectron probes the empty electronic levels of the material. Therefore, the resulting pre-edge and edge structure within about 30 eV of the threshold (XANES) is rich in chemical and structural information. The theoretical picture that describes transitions from the inner shell (1s, 2s or 2p) to complex configurations of possible photoelectron final states (unoccupied valence bands in crystalline solids or valence and quasi-bound orbitals in molecules) is generally too complex to allow a comprehensive ab initio analysis. However, some features of the spectral shapes in XANES can be reliably used as fingerprints for identifying the symmetry of the absorbing atom.

The effects of site symmetry in the case of K-edge XANES spectra are illustrated in Fig. 6.10 with some standard Cr compounds. The tetrahedrally coordinated Cr^{6+} cations in $CaCrO_4$ and in $(CrO_4)^{2-}$ cluster in aqueous solution, and the Cr^{5+} cations in $Ca_{10}Cr_6O_{25}$ show a characteristic isolated pre-edge peak. This can be assigned to electron transition from 1*s* to an unoccupied tetrahedral state, $3t_2$. On the other hand, the octahedrally coordinated Cr^{3+} cations in Cr_2O_3 have an inversion center, and exhibit a different edge shape, with two weak resonances in the pre-edge region that can be assigned to transitions of the 1*s* electron into antibonding orbitals with octahedral symmetry (Arčon et al. 1998; Pantelouris et al. 2004). For Cr metal crystallized in body-centered cubic lattices, the XANES spectrum reflects the structure of the conducting bands in the metal.

The binding energies of the valence orbitals, and therefore the energies of the edge and pre-edge features, correlate with the valence state of the absorbing atom in the sample. This effect can be used to deduce the valence states of atoms. As the oxidation state increases, each absorption feature in the XANES spectrum is shifted to higher energies, as illustrated in Fig. 6.10 for Cr (Arčon et al. 1998), and for As K-edge XANES (Elteren et al. 2006). The largest shifts of up to a few eV per oxidation state are seen at the edge position. Shifts in the pre-edge peaks are considerably smaller, as they are on the order of a few tenths of an eV. For atoms with nearest neighbors of the same chemical species, a linear relation between the edge shift and the valence state has been established (Wong et al. 1984). This linear law is only applicable for atoms that have the same ligand type, since the energy shift also strongly depends on the electronegativities of the neighboring atoms. Small deviations from the linear empirical law have also been reported for compounds with different site symmetries of the cation under investigation (Pantelouris et al. 2004).

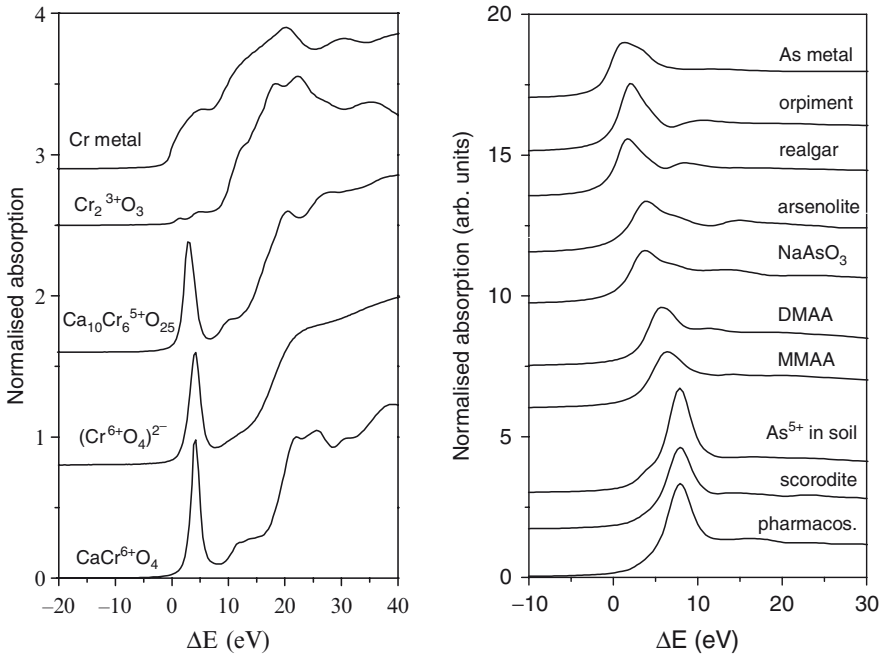

Fig. 6.10 *Left*: Cr K-edge XANES spectra for Cr compounds with different Cr valence states (0, 3+, 5+ and 6+) and site symmetries. *Right*: As K-edge XANES spectra of different As compounds and minerals and As-contaminated soil samples. The energy scales are relative to the Cr K edge in Cr metal (5.989 keV) and the As K edge in As metal (11.8667 keV), respectively. The spectra are displaced vertically for clarity. *DMAA*, dimethylarsenic acid; *MMAA*, monomethylarsenic acid

If the sample contains the same cation in two or more sites with different local structures and valence states, then the measured XANES spectrum is a linear combination of the individual XANES spectra of the different cation sites. In such cases, the relative amount of the cation at each site can be precisely determined by performing a linear combination fit of the corresponding reference XANES spectra (Arčon et al. 2007).

6.4.3 Extended X-Ray Absorption Fine Structure

EXAFS appears above the absorption edges whenever the absorbing atom is closely surrounded by other atoms, in other words in the solid state, in liquids, or in molecules in any aggregated state. It is only in the case of free atoms that there is no EXAFS component in the absorption spectrum, such as in noble gases or monatomic vapors (Rehr and Albers 2000; Kodre et al. 2002). EXAFS arises from the wavelike nature of the final photoelectron state. When an X-ray

photon is absorbed, an inner-shell electron is ejected as a photoelectron with a kinetic energy equal to the difference between the photon energy and the inner-shell binding energy.

According to quantum theory, this photoelectron can be visualized as an outgoing spherical wave centered on the excited atom. This electron wave is scattered by neighboring atoms, and the scattered waves are superposed onto the initial outgoing wave. The interference of the initial and scattered waves at the absorbing atom affects the probability of the photoeffect. With increasing photon energy, the wavevector of the photoelectron wave increases, leading to alternating constructive and destructive interference (Fig. 6.4). In the EXAFS analysis, the oscillatory part of the absorption coefficient μ above the edge is separated from the atomic absorption background μ_o (dotted middle line in Fig. 6.11), which is usually approximated by a smooth best-fit spline function, and normalized to a unit edge jump μ_K, defining the EXAFS signal: $x = (\mu - \mu_0)/\mu_K$. If we only consider the contribution from the

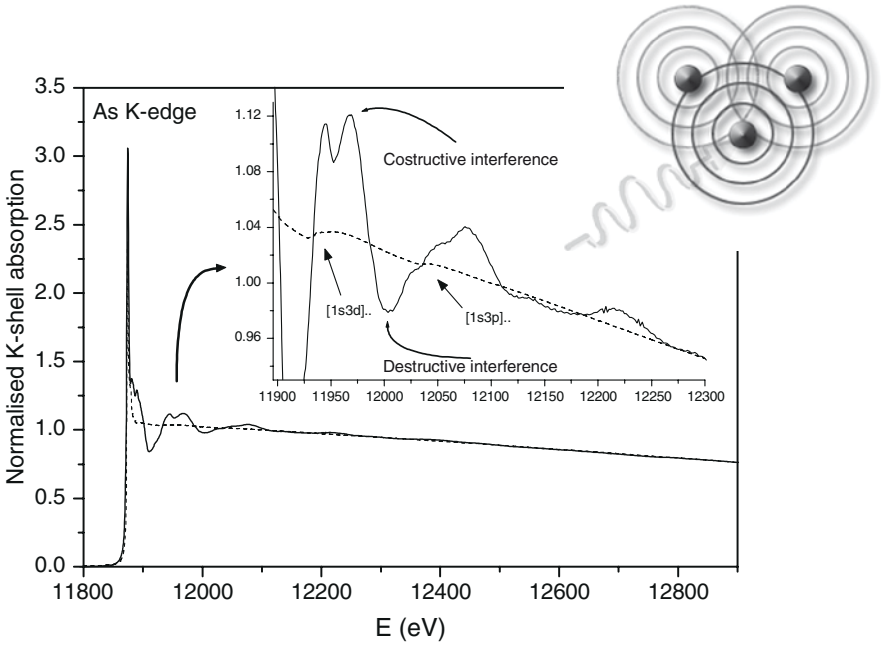

Fig. 6.11 The normalized As K-shell absorption spectrum of scorodite (*solid line*) and the normalized As K-edge atomic absorption background spectrum (*dashed middle line*) determined from absorption measurements on arsine gas AsH_3 (Prešeren et al. 2001). A schematic view of the EXAFS process illustrates the origin of EXAFS oscillations due to the interference of outgoing and backscattered photoelectron waves. The atomic absorption background incorporates all of the collective intra-atomic effects, including the sharp features of the multielectron excitations (in this case 1s3d and 1s3p, two electron excitations that are clearly visible), which in some cases cannot be adequately described by a standard "spline ansatz" and may introduce systematic errors into the EXAFS analysis if not taken into account

single scattering of the photoelectron by the surrounding atoms, then the EXAFS signal can be completely described by a sum of sine terms as the function of the wavevector k. Each term represents the contribution from a spherical shell of equivalent atoms at a distance R_i from the absorbing atom. The EXAFS spectrum measured above the absorption edge of the selected type of atom therefore contains scalar information on the local structure. The photoelectron emitted during the process of the photoelectric effect acts as a detection wave, sensing the immediate vicinity of the absorbing atom, and the information is stored in the resulting EXAFS oscillations.

Using a Fourier transformation (FT) of the measured EXAFS structure, the contributions of individual shells of atoms can be separated visually (Fig. 6.12). Each peak in the FT magnitude spectra represents one sine term in the EXAFS signal. The FT EXAFS can be regarded as the (approximate) radial distribution of the neighbors. Peaks appear at the corresponding atom positions R_i, or, more precisely, they are shifted by a few tenths of an angstroms to a lower value due to the k dependence of the photoelectron phase shift. Despite the useful visualization of the local neighborhood of the absorbing atom that is offered by the FT magnitude spectrum, the quantitative structural information on the local environment (number and species of neighboring atoms in a given shell, their distances from the absorbing atom, and their thermal or structural disorder) can only be obtained by a quantitative EXAFS analysis. Here, the model EXAFS function is fitted to the measured EXAFS spectra in real (k) or in FT (R) space (Ravel and Newville 2005; Rehr et al. 1992).

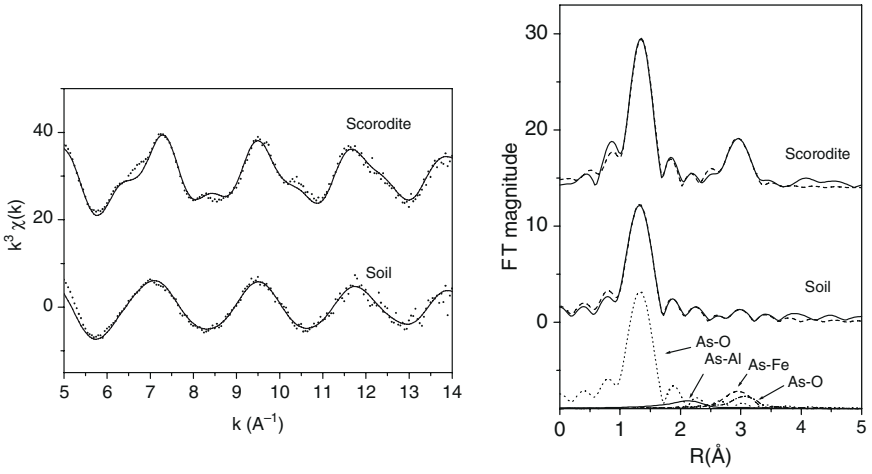

Fig. 6.12 The k^3-weighted As EXAFS spectra (*left*) and their Fourier transforms (*right*) of contaminated soil and reference scorodite samples. *Dots* are experimental values; *solid line* is the EXAFS model. The FT magnitude of consecutive neighbor shell contributions is shown for the EXAFS model of the soil sample

Several beamlines at different synchrotron radiation facilities (e.g., HASYLAB, ELETTRA, ESRF, SOLEIL) are dedicated solely to EXAFS and XANES experiments. The range of elements amenable to EXAFS analysis depends on the monochromator and on the other X-ray optical components of the beamline (e.g., mirrors, filters). The low-energy limit of the most widely used Si(111) monochromators is around 3 keV, translating to $Z = 16$ (K edge of sulfur). With special monochromators, this technique can be extended to about 2 keV (K edge of aluminum). The upper energy limit depends on the monochromator crystals available, where Si(311) is typically used to obtain a monochromatic beam of >30 keV. However, the availability of higher energies also depends on the characteristics of the synchrotron radiation source.

Within the energy range offered by the beamline, the K edge – and the L_3 absorption edge in the case of heavier elements ($Z > 50$) – of the element can be used for EXAFS analysis. The switch to L_3 is not recommended for elements that are lighter than antimony (L_3 edge, 4.132 keV; K edge, 30.491 keV), since the range of the L_3 EXAFS signal is too short due to the cut-off caused by the subsequent L_2 edge (4.381 keV), meaning that the spatial resolution of this method is seriously impaired.

In a basic EXAFS experiment, the sensitivity to the content of the element under investigation in the sample is not very high: the element of interest must contribute at least a few percent to the total absorption to produce a significant edge jump, and thus a meaningful structural EXAFS signal above the absorption edge. Weaker signals tend to be drowned out by the statistical noise of the beam. To optimize the signal-to-noise ratio, the samples should be prepared with a total absorption thickness of about 2 above the absorption edge of the element under investigation (Lee et al. 1981). In the fluorescence detection mode, the sensitivity is improved by one or two orders of magnitude (Koningsberger and Prins 1988).

A standard EXAFS spectral scan performed in transmission detection mode requires from about 30 min to 1 h with a standard XAS synchrotron beamline. In fluorescence detection mode, significantly longer detection times are needed to obtain the necessary signal-to-noise ratio in the spectrum.

In modern synchrotron X-ray sources with high brilliance, much faster detection modes are possible for studying chemical reactions in real time, including quick EXAFS (Frahm 1989) and dispersive EXAFS (Pascarelli et al. 1999). Currently, 100 ms for a scan is possible, although there is the promise of a hundredfold improvement in this figure with the next generation of coherent X-ray sources (TESLA in Hamburg). With such bright synchrotron radiation sources, XAS spectra can also be measured with a microfocusing beam (μ-XAS) to obtain the distribution of the element under investigation and a 2D map of the changes in valence state and local structure in different parts of the sample (Proost et al. 2004; Hettiarachchi et al. 2006). A lateral resolution of <10 μm can be achieved here. This method is limited though, as an excessive flux density in the microfocused X-ray beam can cause radiation damage due to the absorption of high doses of ionizing radiation. In particular, biological samples are very sensitive to radiation damage (Kanngießer et al. 2004).

6.4.4 EXAFS and XANES in Practice

To illustrate the utility of EXAFS and XANES analysis, we will examine a study of soil from Cornwall (a county in southwest England) that was polluted with very high concentrations of arsenic (1,000 μg g^{-1} or more). This occurred due to mining activities that took place in this area from Roman times up to the beginning of the twentieth century, and today it poses a health risk across a large area of Cornwall (van Elteren et al. 2006). Especially dangerous are the so-called "hot spots," where the arsenic concentrations are higher than 10% and the potential mobility in the soil could result in serious water pollution and thus the poisoning of plants, animals and people. The arsenic in the soil from former industrial sites is potentially mobile because of its association with amorphous iron oxides, which can be solubilized under reducing conditions. The fixation of arsenic in these soils has been hypothesized to occur through either precipitation, which corresponds to the formation of secondary minerals/phases (such as iron arsenates), or the sorption of arsenate (As(V)) on iron (hydr)oxides, with the arsenic oxyanion immobilized by iron (hydr)oxides. The risk of mobilization is more pronounced for arsenite (As(III)) than for arsenate, since arsenate binds more strongly to minerals. In some instances the leaching of organic arsenic compounds, such as monomethylarsenic acid (MMAA) and dimethylarsenic acid (DMAA), can occur.

The potential leaching behavior of arsenic is usually studied using sequential extraction protocols, which provide insight into its association with the soil phases. However, such an approach only yields very crude information on the binding of arsenic and gives no structural information on the surroundings of the arsenic atoms themselves, which is crucial to assessing its potential toxicity and the risk of mobility. To obtain a better understanding of the physicochemical form of arsenic in the soil and its potential mobility and uptake by plants, As K-edge XANES and EXAFS analyses were used to retrieve molecular information on the arsenic; i.e., its oxidation states and the local structure around the As atoms in the soil. In this way, the most abundant modes of As bonding were identified (X-ray absorption spectroscopy experiments described in this text were performed in various synchrotron laboratories, including ESRF in Grenoble, ELETTRA in Trieste, and HASYLAB, DESY in Hamburg, with the financial support of European Community Contract RII3-CT-2004-506008 (IA-SFS)).

Normalized As XANES spectra of the soil and of reference arsenic compounds and minerals are shown in Fig. 6.10 (right), and include metallic As, trivalent As compounds (realgar [AsS], orpiment [As_2S_3], $NaAsO_2$ and arsenolite [As_2O_3]), pentavalent organoarsenic compounds (monomethylarsenic acid [$CH_3AsO(OH)_2$] or "MMAA," and dimethylarsenic acid [$(CH_3)_2AsO(OH)_2$] or "DMAA"), and pentavalent As minerals (scorodite [$FeAsO_4$–$2H_2O$] and pharmacosiderite [$KFe_4(AsO_4)_3(OH)_4$–6–$7H_2O$]). From Fig. 6.10, it is evident that the edge position in the soil sample coincides with the position of the As(V) minerals scorodite and pharmacosiderite, which clearly indicates that the As in the soil is predominantly in a pentavalent form. In addition to the K-edge position, the shape of the As K

edge of the soil sample is very similar to the shapes of the two reference As(V) minerals scorodite and pharmacosiderite, where the As atoms are tetrahedrally coordinated to four oxygen atoms, which suggests a similar local symmetry for the As atoms in the soil.

A more detailed insight into the local structure around the As atoms in this soil can be obtained from the EXAFS analysis for the As K-edge. The k^3-weighted EXAFS spectra for the soil sample and the reference As(V) mineral scorodite are shown in Fig. 6.11. Some information about the neighborhood of the As atoms can already be obtained from the FTs. In the spectrum of scorodite, which is the most likely candidate for the As carrier in the soil, there are two distinct peaks that represent the contributions of the first two shells of the neighbors around the As atom. In the spectrum of the soil sample, only the first peak of the nearest coordination shell is similar to that in the scorodite, while the characteristic strong peak of the second shell is absent. This clearly shows that the As in the soil is predominantly not in the form of crystalline scorodite.

Quantitative EXAFS analysis is used to determine the structural parameters of the local As neighborhood: the radii and Debye–Waller factors of the nearest neighbor shells, together with the chemical species and the average number of atoms in the shell. Upon modeling the EXAFS spectrum of the soil sample, four oxygen atoms were identified in the first coordination shell at a short distance, 1.69 Å, and with a small Debye–Waller factor, which is characteristic of a tight As(V)–O bond, such as that present in scorodite. In the second coordination shell, iron atoms were clearly identified at a distance of 3.34 Å, similar to the findings for scorodite. This is not surprising considering the elemental analysis data, which showed that As and Fe concentrations in the soil are strongly correlated. However, the number of Fe neighbors for each As atom was found to be significantly lower than in the crystal structure of scorodite. This suggested that secondary arsenic compounds were formed as a result of weathering, by the (co)precipitation of arsenate, which led to the amorphous or poorly crystalline $FeAsO_4$.

In addition, one Si atom at a distance of 2.54 Å and about four oxygen atoms at 3.48 Å were observed in the local neighborhood of the As. Among the possible structures, only $As^{5+}O_4$ bonded to a SiO_4 tetrahedron in a bidentate mononuclear complex, with an As–Si distance of 2.6 Å, matched well with the short As–Si distance of 2.54 Å observed. These results strongly suggest that arsenate is partially adsorbed onto quartz or aluminosilicates, such as the abundant clay that is present in the soil.

The presence of Ca or Mg atoms as candidates for As coordination were excluded by the EXAFS analysis. Also, there was no evidence of organic As ligands, so the presence of organoarsenic compounds in the soil was also excluded.

Thus, using these As K-edge XANES and EXAFS analyses of the contaminated soil, it was possible to determine the valence state of the As and its most common modes of bonding. The results show that the arsenic in the soil takes the form of the pentavalent oxide, which is less mobile, and hence potentially less bioavailable and less dangerous to the health than the trivalent form. However, the As^{5+} occurred

predominantly in amorphous phases and sorbed species, and was not bound in the inert crystalline form, as expected, which could lead to arsenic leaching and thus a higher environmental risk.

6.5 Conclusions

The main advantages of energy-dispersive X-ray fluorescence (EDXRF) analysis are its multielement capability and its nondestructive nature, combined with the need for only simple sample preparation without time-consuming sample destruction, and which only requires personnel with minimum manual skills. This is undoubtedly the cheapest and the simplest analysis technique among the instrumental analytical techniques discussed here.

The main advantages of total-reflection X-ray fluorescence (TXRF) are the possibility of rapidly analyzing large numbers of samples, which are only required in small amounts, and which are prepared according to a simple procedure. However, this is not an appropriate analytical technique for determining low concentrations of light elements (e.g., Na, Mg Al, Si), or for determining Cd in plant material (which contains large amounts of potassium), because potassium K lines strongly interfere with the Cd L lines.

Micro-proton-induced X-ray emission (PIXE) is a highly sophisticated method that is suitable for the spatial analysis of the elemental distributions in biological samples. However, the key point in this analysis is sample preparation, during which the elemental distribution must be preserved. For now, rapid freezing followed by controlled freeze drying is the most appropriate method of sample preparation, although the development of techniques allowing the measurement of samples in a frozen hydrated state is required to reach subcellular levels.

High-resolution X-ray absorption spectroscopy (XAS) has become available with the development of synchrotron radiation sources. This has introduced powerful experimental methods for the investigation of the local structures around selected atoms in biological samples. In X-ray absorption near-edge structure (XANES), the valence state of the selected type of atom in the sample and the local symmetry of its unoccupied orbitals can be deduced from the information hidden in the shape and energy shift of the X-ray absorption edge. In extended X-ray absorption fine structure (EXAFS), the number and species of neighboring atoms, their distances from the selected atom, and the thermal or structural disorder in their positions can be determined from the oscillatory part of the absorption coefficient above the K or L absorption edges.

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Chapter 7
At the Crossroads of Metal Hyperaccumulation and Glucosinolates: Is There Anything Out There?

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7.1 Introduction

In recent years, there has been increasing awareness that multidisciplinary and integrative research is necessary to clarify the complexity of plant interactions with diverse biotic and abiotic cues. Since they are members of complex communities, plants interact with both antagonistic and beneficial organisms. Sulfur-containing compounds (including sulphate, glutathione [GSH], phytochelatins, metallothioneins, low-molecular-weight thiols, various secondary metabolites, and sulfur-rich proteins) are crucial to the survival of plants under biotic and abiotic stress (Ernst et al. 2008; Hernandez-Allica et al. 2006; Rausch and Wachter 2005). As well as pre-existing morphological and biochemical adaptations, defence strategies comprise direct and/or indirect induced synthesis of chemical compounds (mainly plant secondary metabolites) (Bezemer and van Dam 2005). Glucosinolates (GS) are natural sulfur-containing products of the Brassicaceae and other related plant families (Fahey et al. 2001), and they represent one of the best known examples of preformed defence compounds. Their biosynthesis and the expression of their related enzymes and proteins vary during development and among organs, and they can be differentially regulated upon phytohormone treatment and insect herbivory, and in response to abiotic factors (Brown et al. 2003; Kliebenstein et al. 2002; Petersen et al. 2002; Pongrac et al. 2008; Rask et al. 2000). Regulation of plant responses to such cues is complex, and acts mainly via the salicylic acid

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and jasmonic acid signalling networks (Freeman et al. 2004; Mikkelsen et al. 2003). Jasmonates contribute to signal transduction during insect herbivory, and they increase the total GS concentrations, primarily because of changes in indolyl GS concentrations (Cipollini et al. 2004; Reymond et al. 2004). Instead, salicylate, a pathogen attack signal, stimulates GS accumulation, either alone or in combination with other phytohormones, and it may counteract jasmonate-mediated GS induction (Kliebenstein et al. 2002). A model of a two-step regulation system of systemic resistance by airborne and vascular long-distance signals has been proposed, and it provides a carefully balanced systemic defence reaction to local attack by herbivores and pathogens (Heil and Ton 2008). Recent results indicate that the signals that are activated by plants in response to its "friends" and "foes" overlap. This indicates that the regulation of the adaptive response in the plant is finely balanced between protection against aggressors and acquisition of benefits (Pieterse and Dicke 2007).

Hyperaccumulation is a phenomenon that evolved in plants exposed to extreme metal concentrations (Baker 1981). Several Brassicaceae plant species are hyperaccumulators that take up exceptionally high metal concentrations in their aboveground biomass without visible toxicity symptoms (Brooks et al. 1977). Extremely high concentrations of accumulated elements have also been proposed to act in plant biotic interactions in so-called elemental allelopathy (Boyd and Martens 1998). In addition, arbuscular mycorrhizal (AM) fungi are ubiquitous soil microbes that are considered essential for the survival and growth of plants in nutrient-deficient soils (Smith and Read 1997), and have been connected to metal tolerance in plants (Hall 2002; Regvar et al. 2006; Hildebrandt et al. 2007; Vogel-Mikuš and Regvar 2006), mainly through fungal tolerance mechanisms (Joner et al. 2000). The presence of AM symbiosis in metal hyperaccumulating plants has been reviewed in detail recently (Regvar and Vogel-Mikuš 2008). The relationships between the metal and organic defences that mediate ecological interactions in plants (including symbiosis, herbivory and pathogenesis) are, however, extremely complex and have rarely been studied simultaneously under field conditions (Hartl and Baldwin 2006; Noret et al. 2007; Poschenrieder et al. 2006a).

7.2 Metal-Induced Responses in Plants

Soils with high concentrations of heavy metals can result from naturally high background levels or various anthropogenic activities. In plants, resistance to excess metals is achieved by avoidance (plants can restrict metal uptake) or tolerance (plants can cope with extreme internal metal concentrations). Thus, to survive in metal-polluted environments, plants have developed the two basic metal uptake and tolerance strategies of exclusion and accumulation (Baker 1981, 1987).

In metal excluders, low to moderate metal concentrations are maintained in the tissues over a wide range of total soil metal concentrations until critical concentrations cause damage to the exclusion mechanisms. Plants can either avoid metal uptake into their roots (resistance) or restrict metal transport to the shoots (tolerance) through several mechanisms:

- Enhanced mucus production and root cell-cap detachment, which provides a barrier to root metal uptake (Llugany et al. 2003).
- Induced cell death of root epidermal cells and production of border cells protecting deeper cells of the meristem and root elongation zone (Delisle et al. 2001).
- High affinity of some metals for the cell wall components (e.g. polygalacturonic acid) (Adriano 2001; Ernst et al. 1992).
- Secretion of strong metal chelators, such as organic acids (e.g. malic and citric acid) or phenolics (Barceló and Poschenrieder 2002; Briat and Lebrun 1999; Hall 2002; Ernst et al. 1992; Salt 2001).
- High efficiency of metal sequestration in root cell walls and vacuoles, which consequentially restricts metal xylem loading (Lasat 2002; Hall 2002).
- Mycorrhizal fungi inhibit metal uptake by binding the metals to components of the mycelium (Joner and Leyval 1997; Joner et al. 2000).

Metal-accumulating plants achieve enhanced metal uptake via the roots accompanied by successful metal loading in the xylem and transport to the shoots, where the metals concentrate (Baker 1981; Shen et al. 1997). Extreme accumulation phenotypes (i.e. hyperaccumulating plant species) can take up more than 10,000 μg g^{-1} Mn or Zn, 1,000 μg g^{-1} Ni, Cu, Pb and Se, and 100 μg g^{-1} Cd, in contrast to normal physiological requirements (if any). These levels are far in excess of those found in most other species (Baker 1981, 1987; Baker et al. 2000; Reeves 2006; Reeves and Baker 2000). Approximately 420 plant species (less than 0.2% of all angiosperms) have this character (Baker and Whiting 2002). The physiological and biochemical mechanisms of metal transport, sequestration and detoxification involve:

- Preferential spreading of the roots towards areas with higher metal concentrations in the soil (Haines 2002; Whiting et al. 2000).
- Overexpression of metal transporters in roots (Lasat et al. 1996).
- Efficient, long-distance transport of metals in the form of stable complexes bound either to free histidine, nicotinamine, organic acids or S-ligands (Krämer et al. 1996; Küpper et al. 2004).
- Accumulation of metals away from the photosynthetically active tissues within the leaves, preferentially using epidermal and vascular tissues, and, to a lesser extent, palisade and spongy mesophyll (Bhatia et al. 2004; Cosio et al. 2005 Vogel-Mikuš et al. 2008 a, b; Wójcik et al. 2005).
- High-capacity detoxification mechanisms, such as binding to organic acids, amino acids, phytochelatins and metallothioneins, and sequestration in the vacuoles that acts as a central storage for ions, thus maintaining low free metal concentrations in the symplast (Briat and Lebrun 1999; Clemens et al. 2002; Salt and Krämer 2000).
- Limited metal phloem loading and transport from the leaves to the seeds (Lasat et al. 1998; Vogel-Mikuš et al. 2007; Wójcik et al. 2005).

A number of the hyperaccumulating plants belong to the Brassicaceae family, including the *Alyssum*, *Thlaspi*, *Arabidopsis* species and *Brassica juncea* (Reeves and Baker 2000). Among these, many studies that have investigated this metal hyperaccumulation have been carried out on the Zn/Cd/Ni hyperaccumulator

Thlaspi caerulescens J. & C. Presl, which has therefore been proposed as a model metal-hyperaccumulating plant species (Assunção et al. 2003).

7.3 The Evolution of Metal Hyperaccumulation

Metal hyperaccumulation evolved in different, taxonomically unrelated groups of plants (Brooks 1987) and may fulfil multiple functions, both within and between the evolutionary lines of metal-hyperaccumulating plants. Physiological mechanisms are preserved if they have a functional basis that provides direct or consequential positive-selective value to the plant (Ernst 2006). Because of the accumulation of enormous amounts of potentially toxic metal levels, hyperaccumulation is a contradictory phenomenon. Direct and indirect advantages have nevertheless been seen. Six different hypotheses for the ecological benefits of hyperaccumulation in plants have been postulated (Boyd and Martens 1998):

- *Inadvertent uptake hypothesis*: the uptake of essential elements in nutrient-poor habitats forces plants to inadvertently take up toxic metals.
- *Tolerance hypothesis*: metal hyperaccumulation is a mechanism that allows the sequestration of metals in tissues.
- *Disposal hypothesis*: the elimination of metals from the plant body by shedding tissues containing high metal levels.
- *Elemental allelopathy hypothesis*: perennials enrich the surface soil under their canopies by producing high-metal litter, to prevent the establishment of less metal-tolerant species.
- *Drought resistance hypothesis*: hyperaccumulated metal helps the plant to withstand drought.
- *Defence hypothesis*: elevated metal concentrations in plant tissues protect plants from certain herbivores and pathogens.

A possible evolutionary pathway by which elemental hyperaccumulation may have evolved from accumulation is known as the “defensive enhancement scenario”, where stepwise increases in the element concentration may have led to further plant benefits (see Boyd 2007). Five different modes of action for metal defences have been proposed (Poschenrieder et al. 2006a):

- *Phytosanitary effects*: metal-containing compounds act to prevent herbivore and pathogen attack.
- *Elemental defence hypothesis*: the high metal concentrations that accumulate in leaf tissues either deter or intoxicate herbivores or pathogens.
- *Trade-off hypothesis*: increasing tissue concentrations of potentially toxic heavy metals in plants can replace organic defences.
- *Metal therapy*: the metal has a therapeutic effect, preventing the consequences of the metabolic disorder responsible for stress signalling failure.
- *Metal-induced fortification*: metal-stress-derived signals and metal-induced activation of pathogen-resistance-related defence genes are redundant.

The toxicity of high metal concentrations to phytopathogenic microorganisms is well established. A higher metal sensitivity of the pathogen than that of the host has been used practically in the formulation of different inorganic and organic metal compounds for phytosanitary treatments. The copper-containing Burgundy mixture is a widely used example. In these cases, the protection is achieved by the direct action of the metal ion or organometallic compound supplied, without the apparent participation of the host plant.

In the trade-off hypothesis, high metal accumulation by the plant is suggested to provide protection against herbivores, and thus energy for the synthesis of plant organic defences is saved (Boyd 1998). Nickel hyperaccumulation in the European Brassicaceae species can act in this way (Davis and Boyd 2000). However, to date, there is little evidence that confirms that the trade-off hypothesis is a general mechanism driving the evolution of metal-hyperaccumulating plants (Jhee et al. 2006; Tolrà et al. 2001). In contrast to organic defences, the metals are not degradable (Boyd and Martens 1998). Therefore, metal defences may be effective against a broad range of herbivores and pathogens, except for those that are also tolerant to the metal. A further advantage is the effectiveness of metals against specialist herbivores that are adapted to and even attracted by the GS of their specific hosts, but can be sensitive to the metal concentrations reached in hyperaccumulator species (Jhee et al. 2006). Another role of metal hyperaccumulation in plant defence is based on the possibility that organic defences can increase the efficiency of protection by metals, by either additivity or synergy, and thus magnify the benefits of each defence in the so-called joint-effect hypothesis (Boyd 2007).

Thus, it appears likely that organic defences play a role in the evolution of metal hyperaccumulation and should therefore be considered further in studies of defence hypotheses. Interactions between metal accumulation and biotic stress can, however, be much more complex than envisaged by the hypotheses listed. They exceed the remit of this chapter, and the interested reader should consult recent reviews on this topic (Boyd 2007; Poschenrieder et al. 2006a) that explain these different possibilities in detail. In all cases, it should be noted that the particular characteristic of the hyperaccumulated element, its concentration, and the particular type of feeding employed by the herbivore (phloem sucking, chewing) or pathogen (necrotrophic, biotrophic) have significant effects on the results (Boyd 2007).

7.4 Glucosinolates: Metabolism and Occurrence in Vascular Plants

Glucosinolates (GS) are natural secondary plant metabolites that derive from amino acids (Halkier and Gershenzon 2006). The general chemical structure of GS was first proposed at the end of the nineteenth century by Gadamer, and was corrected in 1956 by Ettlinger and Lundeen (1956). This general structure of GS is characterised by a –C≡N group, a sulfate and a β-D-glucopyranosyl. GS can be classified according to their precursor amino acid and the chemical structure and

modifications of the side chain (–R); aliphatic, aromatic and indolyl GS can be distinguished (Fig. 7.1).

The widely accepted model for GS biosynthesis involves three major steps: side chain elongation, glucone biosynthesis, and side chain modification (Fig. 7.2). The presence of GS in *Arabidopsis thaliana* proved very useful when attempting to clarify the biosynthetic pathway of GS (Mikkelsen et al. 2002). GS biosynthesis starts with the hydroxylation of amino acids, followed by their decarboxylation to form an aldoxime. Alanine, methionine, valine and leucine are the precursors for the aliphatic GS, while the aromatic GS derive from phenylalanine and tyrosine. Tryptophan is the source of the indolyl GS. Cytochromes P450 belonging to the CYP79 family catalyse the conversion of the amino acids to aldoximes (Wittstock and Halkier 2002). Two members of another cytochome P450 family, CYP83A1 and CYP83B1, have been identified in *Arabidopsis* as the aldoxime-metabolising enzymes involved in the fast oxidation of the aldoximes and their conjugation with a sulphur donor, possibly cysteine. The thiohydroximic acid released is glucosylated (by uridine diphosphate

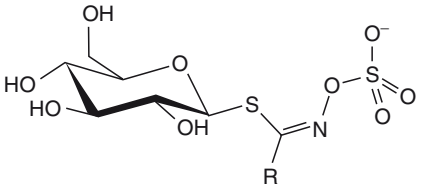

Fig. 7.1 General structure of the glucosinolates

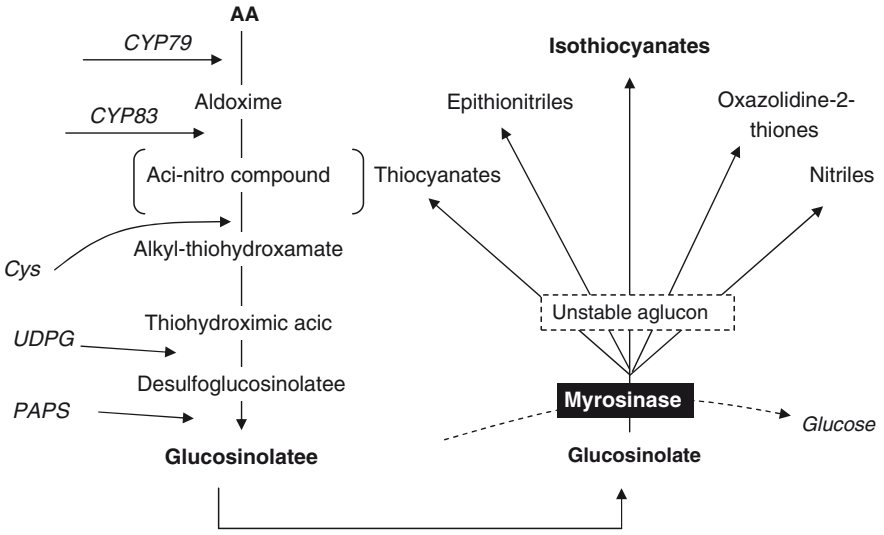

Fig. 7.2 Pathways for glucosinolate metabolism

glucose; UDPG) and sulfated (by 3-phosphoadenosine-5-phosphosulfate; PAPS) to form the GS core structure (Fig. 7.2). Several possible modifications of the R group determine the direction of GS hydrolysis and the activity of the products of hydrolysis (Halkier and Gershenzon 2006). Indeed, plant GS metabolism is regulated at multiple levels (genetic, environmental, transcriptional and metabolic).

Plants with GS also contain the enzyme β-thioglucoside hydrolase (commonly called myrosinase), which mediates GS hydrolysis. This enzyme is separated from its substrate in intact tissue, but when the tissue is damaged, the loss of cellular integrity results in the mixing of the enzyme and GS, causing the immediate hydrolysis of GS into an unstable aglycone. The aglycone rearranges spontaneously to form different products, such as isothiocyanates, oxazolidine-2-thiones, nitriles, epithionitriles and thiocyanates (Fig. 7.2). Myrosinase is highly specific, as it only uses GS as substrate and has no activity toward any *O*-glycosides or *S*-glycosides. Among the different types of GS, the myrosinase substrate range is variable, and some are highly specific. Myrosinase is encoded by a multigene family, and whereas *Arabidopsis* has four functional myrosinase genes (Xu et al. 2004), *Brassica napus* and *Sinapis alba* have 20 or more (Rask et al. 2000).

While GS are biologically inert, some of the products of their hydrolysis have important biological effects. Their nature depends mainly on the structure of the side chain, and also on the presence of epithiospecifier protein (ESP), the pH and the Fe^{2+} concentration of the medium. Usually, rearrangement of the aglycone at neutral pH will result in the formation of an isothiocyanate, while at acidic pH the nitrile derivative is the dominant product. This enzyme system has a broad pH range (Heaney and Fenwich 1993). Following the myrosinase hydrolysis of GS, nitriles and epithionitriles are generated by ESP, whereas isothiocyanates are generated in the absence of ESP (Chen and Andreasson 2001). Investigations with *A. thaliana* plants that overexpress ESP clearly support the view that isothiocyanates are more effective defences against herbivores than nitriles (Burrow et al. 2006).

Current research interest is focused mainly on the toxic and beneficial effects of plant GS in animal and humans. Antinutritional and toxic effects are of special relevance in some GS-rich fodder species, like rapeseed meal (Tripathi and Mishra 2007). Nitriles appear to be the main antinutritional factor responsible for growth depression in cattle. However, the most common GS hydrolysis products, isothiocyanates, are considered to be responsible for the protective and anticarcinogenic effects of a cruciferous-rich diet in humans. Their antifungal, antimicrobial, allelochemical and insecticidal properties contribute to the plant's defence mechanisms. While GS can defend plants against general pathogens and herbivores, they can also act as an attractant to GS-adapted specialists. For more than 30 years, GS have gained agricultural significance through the use of biofumigation (incorporation of harvested material into agricultural soil to suppress pathogens, nematodes and weeds).

A significant proportion of metal hyperaccumulators are members of Brassicaceae that typically contain GS. More than 80% of the identified GS occur in this plant family, which contains close to 3,000 species. However, GS are not restricted to Brassicaceae, as they occur throughout the order Capparales and even in *Drypetes*, a genus classically included in the Euphorbiaceae (De Craene and Haston 2006;

Rodman et al. 1996). At least 500 species from 15 other families of dicotyledonous angiosperms have been reported to contain one or more of the over 120 known natural GS. Some examples are Capparaceae, Tropaeolaceae, Moringaceae, Arabidaceae, Resedaceae and Euphorbiaceae.

Among the Brassicaeae, the genus *Brassica* contains a large number of commonly consumed vegetable species (cabbage, broccoli, radish, turnip), condiments (mustard, wasabi), oilseeds (canola, rapeseed), and forage crops (kale, forage rape). GS that have been found in all parts of these plants contribute significantly to their typical flavours. While more than 15 different GS can be found in the same plant, three to four usually predominate (Holst and Fenwick 2003). One of the best-characterised examples is the model plant *A. thaliana*, where up to 23 different GS were initially identified in the leaves and seeds (Hogge et al. 1988), with nine additional GS identified later (Wittstock et al. 2002).

The GS content in plants is highly variable and can range from less than 1% of the dry weight in some tissues of *Brassica* vegetables (Rosa et al. 1997) to 10% in the seeds of some plants, where GS may represent half of the sulfur content of the seeds. Occurrence and concentrations vary according to species, cultivar, tissue type, age, health and nutrition. Environmental factors (such as soil fertility and pathogen challenge) and plant growth regulators can affect GS levels and distribution among plant organs (Fahey et al. 2001). The accumulation of GS can also be induced after treatment with salicylic and jasmonic acids (Ludwing-Müller et al. 1997). Thus, the GS have diverse functions in plants and for plants, some of which may not have been discovered yet.

7.5 Interactions of Metals and Glucosinolates

More than 30 years ago, Mathys (1977) suggested that GS have roles in Zn tolerance mechanisms. He reported higher GS levels in Zn-resistant than in Zn-sensitive *Thlaspi alpestre*. Later on, total GS concentrations in metal hyperaccumulator/tolerant species were reported to be higher (Ernst 1990), lower (Newman et al. 1992; Mathys 1977; Sasse 1976; Davis and Boyd 2000; Noret et al. 2007), or of the same order of magnitude (Tolrà et al. 2001) as those reported for other Brassicaceae species. In addition, GS profiles have been reported to change in response to increased metal concentrations (Table 7.1).

Since individual GS hydrolysis products have different biological activities, the observed metal-induced changes in GS patterns may have complex consequences for the defence responses in these plants (Davis and Boyd 2000; Jhee et al. 2006; Noret et al. 2005; Tolrà et al. 2001, 2006). Metallicolous populations of *T. caerulescens*, for example, have lower GS levels when grown in metalliferous soils, which has been attributed to low herbivory pressure at those sites (Noret et al. 2007), and they are highly susceptible to herbivores when transferred to nonmetalliferous environments (Dechamps et al. 2008). Due to the complexity of the interactions between metals and plant metabolism, it is of no surprise that the GS levels, types and profiles

Table 7.1 Plant species, organ and developmentally specific changes in total GS, GS type, and individual GS in metal-hyperaccumulating and metal-non-hyperaccumulating (NH) plant species as a response to excess metals (↑ increase, ↓ decrease, / no change)

Plant species(hyperaccumulated element or nonhyperaccumulator (NH))	Element tested	Glucosinolates and/or *gene expression*						References
		Total		Type		Individual		
		Roots	Shoots	Roots	Shoots	Roots	Shoots	
Thlaspi caerulescens (Zn/Cd/Ni)	Zn	↑	↓	↓ Indolyl ↓ Alphatic	↓ Indolyl ↓ Aromatic	↑ Sinalbin ↓ Gluconapin	↓ Sinalbin ↓ Gluconasturtin	Tolrà et al. (2001), Noret et al. (2005, 2007)
Brassica rapa (NH)	Zn	/	↓	/	↓ Aliphatic ↑ Indolyl	/	↓ Gluconapin ↑ Glucobrassicin ↑ 4-Methoxy-glucobrassicin	Coolong et al. (2004)
Thlaspi caerulescens (Zn/Cd)	Cd	–	↑	–	↑ Aliphatic	–	↑ *CYP83A1* ↑ *MYB28*	Hirai et al. (2007), Van de Mortel et al. (2008)
Thlaspi praecox (Zn/Cd)	Cd	↑	↑	↑ Benzyl ↓ Indolyl	↑ Benzyl ↓ Indolyl	–	↑ Sinalbin	Tolrà et al. (2006)
Thlaspi arvense (NH)	Cd	/	/	/	↑ Indolyl ↓ Aliphatic	–	–	Tolrà et al. (2006)
Arabidopsis halleri (Zn/Cd)	Cd	–	↓	–	–	–	↓ *CYP79B3*	Herbette et al. (2006)
Brassica juncea (Cd)	Cd	–	↔	–	–	–	↔ *Myr*	Heiss et al. (1999)
Streptanthus polygaloides (Ni)	Ni	–	–	–	↓ Allyl-	–	↓ Sinigrin ↑4OH-3-Methylindolyl-	Jhee et al. (2006)
Streptanthus insignus (NH)	Ni	–	–	–	–	–	–	Davis and Boyd (2000)
Brassica oleracea (NH)	Se	–	↓	–	↓ Aliphatic	–	↓ 4-Methyl-sulfinylbutyl	Charron et al. (2001), Toler et al. (2007)

depend on the metal in question and its concentration, as well as the plant species, organ and developmental stage (Table 7.1) (Davis and Boyd 2000; Jhee et al. 2006; Noret et al. 2005, 2007; Pongrac et al. 2008; Tolrà et al. 2001, 2006).

The influence of metal accumulation on GS levels and GS patterns implies both direct and indirect metal-induced modifications of GS metabolism. Since GS are nitrogen- and sulfur-containing metabolites, effects of metals on nitrogen or sulfur metabolism will subsequently affect GS metabolism (Yan and Chen 2007). The complex interactions between heavy metals and sulfur metabolism have recently been reviewed by Ernst et al. (2008). Cysteine, the primary product of sulfur assimilation, is incorporated into sulfur-rich proteins, phytochelatins and GSH, and it serves as a reduced sulfur donor for the biosynthesis of GS and phytoalexins (Rausch and Wachter 2005) (Fig. 7.2). Plant tolerance and accumulation of heavy metals have been related to sulfur metabolism. Under a limiting sulfur supply, Cd, a metal with a high affinity for thiol groups, upregulates several genes that are involved in sulfate assimilation in *A. thaliana*, while this is not the case in plants with a high sulfur supply or in hypertolerant plants (Ernst et al. 2008). The regulation of sulfur assimilation may be necessary to ensure an adequate supply of the sulfur compounds that are required for heavy metal detoxification (Schiavon and Malagoli 2008). Phytochelatins and metallothioneins are involved in basal tolerance to metals and metalloids, although the metal hypertolerance of metal-hyperaccumulating plants is not due to higher phytochelatin production (Ernst et al. 2008). Glutathione accounts for only approximately 2% of the total organic sulfur in plants (Ernst et al. 2008), with up to 50% of the organic sulfur being present in the form of GS (Tolrà et al. 2006). At our present stage of knowledge, however, it is still not possible to provide a general balance of the influences of heavy metals on sulfur metabolism and the functioning of the different sulfur pools in plants. However, due to the high energy demand for sulfur assimilation, the channelling of organic sulfur into the different pools of relevance must be highly regulated. These will include GSH for antioxidant requirements, phytochelatins and metallothioneins for metal homeostasis, and GS for pathogen and herbivore defence. This regulation will be especially important in plants under metal toxicity stress, where the maintenance of both antioxidant defences and metal homeostasis is challenged.

Another crossroads in the interactions between defence against herbivores and pathogens and tolerance to metal toxicity is seen with the shikimate pathway (Fig. 7.3). Here, aromatic amino acids are essential for protein synthesis, phenolics (like salicylic acid) are directly involved in stress signalling (see above), catechol is a strong antioxidant that is relevant in Ni tolerance in the hyperaccumulator *T. goeingense* (Freeman et al. 2005), catechin is a high-affinity ligand for metal ions under neutral pH conditions (Barceló and Poschenrieder 2002), flavonoid-type phenolics exuded into the rhizosphere have been implicated in plant signals to N_2-fixing symbiotic microorganisms and in Al resistance (Stougaard 2000; Kidd et al. 2001), and phytoalexins provide suggestive links in plant responses to micro-organisms and metal ions (Fig. 7.3b) (Poschenrieder et al. 2006b). Chorismate is the branch point for the aromatic and indolyl side chains of GS compounds and

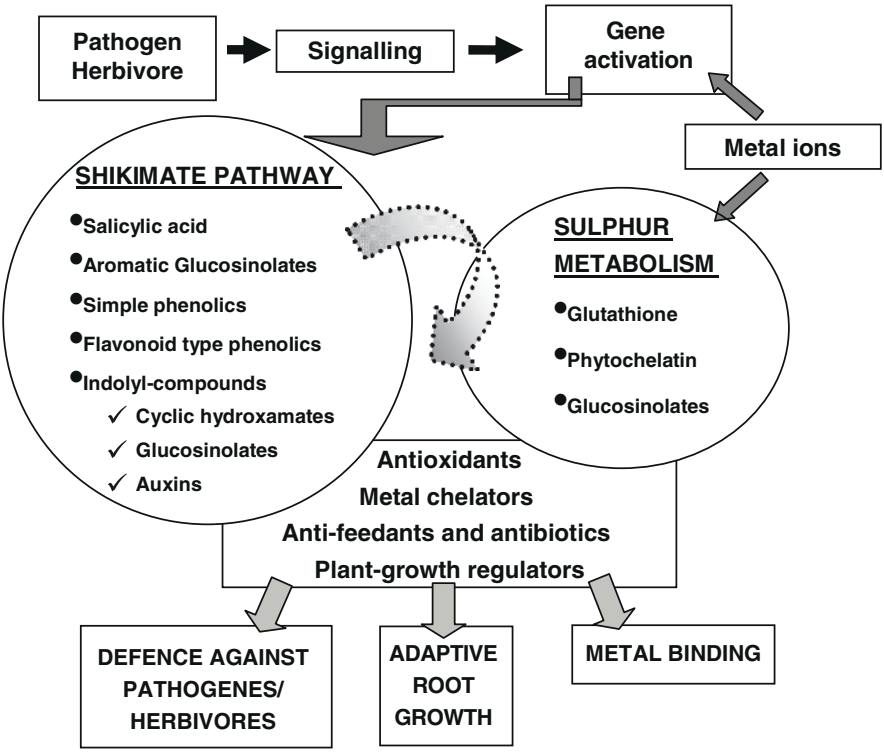

Fig. 7.3 Chemical defences against pathogens and herbivores share metabolic pathways with the biosynthetic tracks for antioxidants, metal-binding substances and growth regulators implicated in adaptive growth responses under stress conditions (adapted from Poschenrieder et al. 2006b)

provides a key point for metabolic regulation (Fig. 7.4). The indolyl pathway has an extraordinary metabolic diversity, which reflects coevolutive interactions between plants and insects, especially in metabolic reactions catalysed by cytochrome P450-type monoxygenases (Chapple 1998). As this indolyl pathway is also responsible for auxin synthesis, its regulation provides multiple possibilities for interactions among biotic stress, metal ion toxicity, and growth and developmental features. Changes in root architecture that lead to adaptive growth can be highly relevant to not only water and nutrient uptake but also in interactions with pathogenic and beneficial microorganisms in the rhizosphere (Fig. 7.3).

At present there is only fragmentary information on the influence of heavy metals in the production of total GS, and even less regarding individual GS and the regulation of their metabolism. Most of the information available is for Cd, Zn and Ni. Some investigations have also considered Se, a metalloid that has considerable influence on sulfur metabolism.

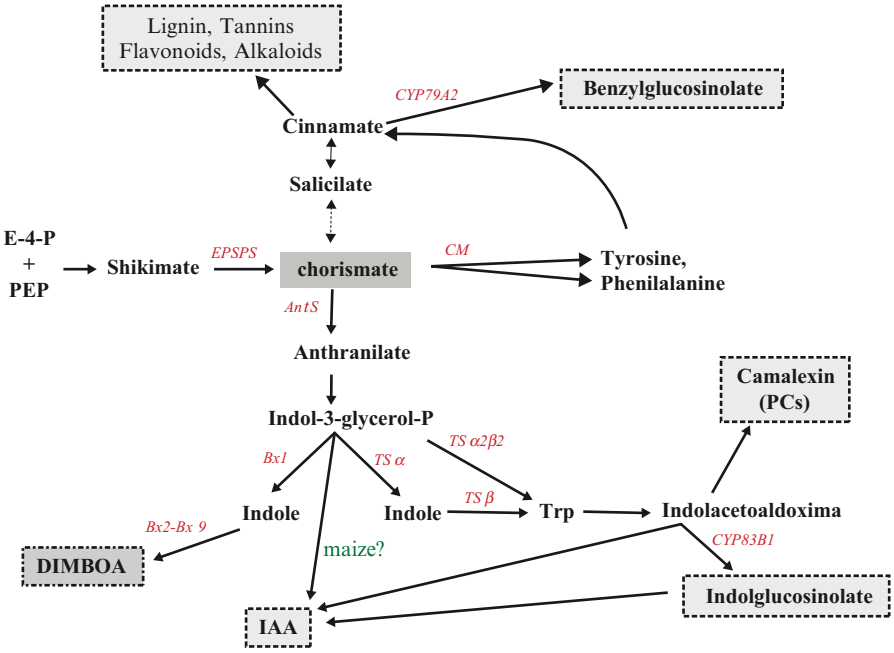

Fig. 7.4 The shikimate pathway in the synthesis of aromatic glucosinolates, indolglucosinolates, other plant-defence-related compounds, and auxins

7.5.1 Cadmium

Studies concerning the interactions of excess Cd with plant GS concentrations/content indicate their link to sulfur metabolism. Cd increases the levels of total GS in the Cd-tolerant *Thlaspi praecox* without affecting its sulfur concentrations; however, its total root sulfur content in particular (and also that of its shoot) was nevertheless increased (Tolrà et al. 2006, and unpublished). This effect may have resulted from the induction of the sulfate uptake capacity, as demonstrated for nontolerant *Zea mays* plants challenged with toxic Cd concentrations, and/or from the possible downregulation of the low-affinity sulfate transport to the shoots, as demonstrated for *B. juncea*. This latter sulfate transport pathway is needed to satisfy the increased sulfur demands for the synthesis of GSH and sulfur-chelating compounds when under Cd stress (Schiavon and Malagoli 2008). Thus, even though a changed sulfur concentration was not seen in the sensitive *Thlaspi arvense* or in the hypertolerant *T. praecox*, the proportion of sulfur used in GS synthesis increased under Cd exposure in the hypertolerant *T. praecox*, but not in the sensitive *T. arvense* (Tolrà et al. 2006).

Changes in GS type (aliphatic, benzyl, indolyl) and individual GS concentrations after Cd exposure may be mediated by plant defence signalling pathways, and may

also be related to the hyperaccumulating character of the plant, depending on the species in question. None of the benzyl-GS present in the hyperaccumulating *T. praecox* were identified in the sensitive *T. arvense*. In addition, a shift from alkenyl GS to indolyl GS occurs in Cd-treated *T. arvense* (Tolrà et al. 2006). These changes may have been jasmonate mediated, as jasmonate was shown to act in the signal transduction pathways for Cd (Xiang and Oliver 1998) and jasmonate is a strong elicitor of indolyl-GS (Mewis et al. 2005; Jost et al. 2005). In contrast, the increase in total GS in the hypertolerant *T. praecox* was matched by the enhanced levels of the aromatic GS sinalbin, which can be synthesised from tyrosine, or from phenylalanine as a secondary GS (Tolrà et al. 2006). In contrast to the wild type, in transgenic *Arabidopsis* plants that can synthesise aromatic GS, an increase in salicylic-acid-mediated defences was seen, while jasmonate-dependent defences were suppressed (Brader et al. 2006). Thus, the specific enhancement of sinalbin by Cd in *T. praecox* indicates a role for salicylic acid in Cd hypertolerance, as has already been proposed for Ni tolerance in *Thlaspi goesingense* (Freeman et al. 2004). However, further investigations need to be carried out to formally prove this hypothesis.

The involvement of plant defence signalling pathways in Cd exposure indicates both direct and indirect consequences of this metal in plant defence against herbivores and pathogens. The highest GS and Cd concentrations in leaves of field-collected *T. praecox* were found in the vegetative stage, presumably protecting the young rosette leaves. The changes seen in the total and individual GS concentrations throughout the life cycle of *T. praecox* were not matched by changes in the total sulfur concentrations, which remained constant during *T. praecox* development, and were on average 14.2 ±3.4 mg g^{-1} dry weight in roots, 8.7 ±4.7 mg g^{-1} dry weight in shoots, and 6.1 ±2.3 mg g^{-1} dry weight in stalks (Pongrac et al. 2008, and our unpublished data). Consumption of *T. caerulescens* leaves by snails, on the other hand, appears related to low GS concentrations rather than to Cd concentrations in different soil-grown ecotypes (Noret et al. 2005). The Cd-mediated changes in GS seen in *T. praecox* are not in line with the trade-off hypothesis (Boyd and Martens 1998), but support the joint effects hypothesis of Boyd (2007). In contrast, feeding deterrence of thrips in *T. caerulescens* has been attributed to Cd and not to GS, because the inhibitory effect on the thrips was correlated to shoot Cd concentrations and not to those of shoot sulfur or Zn (Jiang et al. 2005). Taking into account the large concentrations of vascular Cd in metal hyperaccumulating *T. praecox* (Vogel-Mikuš et al. 2008a, b), these results appear reasonable.

7.5.2 Zinc

Metals such as Zn, Cd and Cu have been reported to induce the absorption of sulfate to sustain the greater sulfur demand during the biosynthesis of GSH and phytochelatins, sulfur-containing compounds that are particularly involved in metal tolerance (Schiavon and Malagoli 2008). Zn can induce an increase in total GS

concentrations in roots and a decrease in shoots of *T. caerulescens* (Tolrà et al. 2001). This may result from Zn-induced changes in sulfur pools and/or responses related to defence, as high Zn concentrations have been shown to inhibit sulfation of desulfo-GS in cress seedlings (Glendering and Poulton 1988). In addition, the availability of amino acid precursors (Cakmak et al. 1989; Domingo et al. 1992) for GS synthesis and the alteration of one of the substrate-specific biosynthetic steps (e.g. aldoxime biosynthesis catalysed by cytochrome P-450, flavoproteins or peroxidases) in the synthesis of GS with different side chains may also have contributed to these results (Larsen 1981; Schnug 1990). The increase in total root GS concentrations partially resulted from an increase in the concentrations of the most abundant GS in *T. caerulescens*, the aromatic GS sinalbin, accompanied by a decrease in indolyl GS in both roots and shoots; other changes were observed to be quantitatively less important (Tolrà et al. 2001; Poschenrieder et al. 2006b). However, the extent to which Zn hyperaccumulation and/or enhanced root sinalbin concentrations contribute to defence against pathogens remains to be established. In contrast, it has already been demonstrated that organic defences (such as GS), rather than Zn, deter snails from eating the shoots of the Zn hyperaccumulator *T. caerulescens* (Noret et al. 2005).

7.5.3 Nickel

As with Zn, the nonhyperaccumulator *Streptanthus insignus* shows higher GS concentrations than those present in the Ni-hyperaccumulator *Streptanthus polygaloides*, and so the trade-off between organic and Ni-based defences has been proposed to be a constitutive rather than a substrate-induced trait in this species (Davis and Boyd 2000). In contrast, when Ni-hyperaccumulator *S. polygaloides* plants grow in low and high Ni environments, they do not differ in their total GS levels, although the low-Ni plants produce more of the dominant GS sinigrin compared to the high-Ni plants (Jhee et al. 2006). In addition, organic defences other than GS (e.g. total phenols) are higher in the Ni-hyperaccumulating *Psychotria douarrei* than in the non-hyperaccumulating *Ficus webbiana* (Davis et al. 2001).

7.5.4 Selenium

Se fertilisation can result in a modest decrease in aliphatic, indolyl and total GS, as well as glucoraphanin, although the greatly depressed sulforaphane production in *Brassica oleracea* implies that Se either upregulates or prevents the downregulation of sulfur uptake (Finley et al. 2005). In addition, the presence of Se within plants has been reported to have a negative impact on the production of certain GS, despite the adequate availability of sulfur (Toler et al. 2005).

In general, currently available data in the literature indicate that interactions of metals and biotic stress may be more complex than envisaged by the trade-off

and elemental defence hypotheses. Sulfur metabolism provides multiple points of interactions between ion toxicity and biotic stress in plants (Poschenrieder et al. 2006a, b). Phytochelatin synthesis has been implicated in the tolerance of nonmetallicolous plant species to metals, and especially to Cd, whereas maintenance of reduced GSH levels may be significant in metallicolous as well as nonmetallicolous species (Howden et al. 1995; Freeman et al. 2004). In addition, changed total GS levels and/or individual GS compositions in the plant species analysed so far indicate more general responses to excess metals, which will consequently result in changes in their interactions with herbivores and pathogens. It is necessary to bear in mind, however, that the maintenance of GS levels and composition via established complex signalling pathways is likely to be influenced by plant species, developmental stage and organ, by the metal in question, its concentrations and constitutive tolerance levels in the plant, and by the interactions of the plant with microbes, herbivores and pathogens. Clearly, more investigations are needed to decipher the full roles of the GS levels and composition in the complex biotic interactions with the metals in metal-hyperaccumulating plants.

7.6 Mycorrhizal Colonisation in Glucosinolate-Containing Plants

GS-containing plants are widely recognised as being nonmycorrhizal, or at best they show an extremely low incidence of colonisation by AM fungi (Harley and Harley 1987; Wang and Qiu 2006). The nonmycorrhizal status of these plants has been attributed to:

- In situ root GS hydrolysis products inhibiting AM fungal spore germination (Schreiner and Koide 1993).
- The structural–chemical properties of the root cell wall that hinder or inhibit fungal growth (Glenn et al. 1988).
- The differences in phosphate acquisition/ scavenging systems compared to mycorrhizal species (Murley et al. 1998).
- The lack of communication signals between the symbionts (Akiyama et al. 2005; Vierheilig et al. 2000).
- The relationship between the plant developmental stages and nutrient demands during the plant life cycle (Pongrac et al. 2007; Vogel-Mikuš et al. 2006, 2007).

Recent discoveries of AM colonisation in *Biscutella laevigata* (Orłowska et al. 2002) and *Thlaspi* sp. (Regvar et al. 2003, 2006; Vogel-Mikuš et al. 2005, 2006) have posed new questions about AM development in GS-containing species and the role of GS in AM formation. An increase in GS concentrations and a distinct change in the GS profile after inoculation are frequently seen in several GS-containing plant species, regardless of whether colonisation has occurred or not (Vierheilig et al. 2000). As the concentrations of different GS vary widely across the developmental stages and organs of plants (Rask et al. 2000), this may account

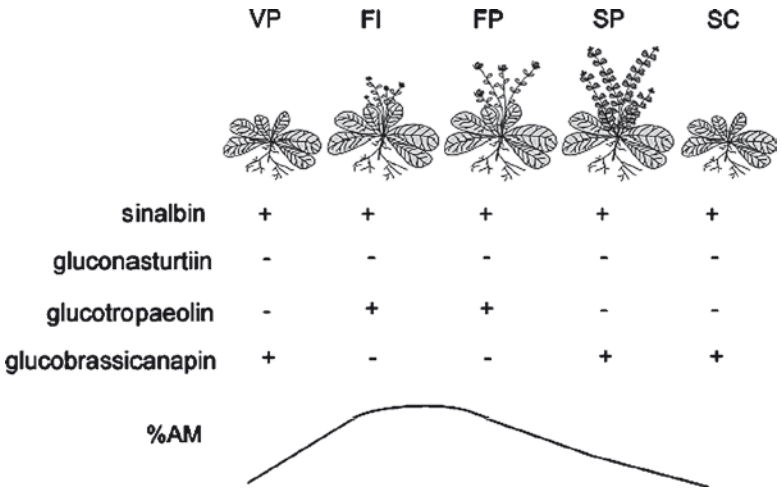

Fig. 7.5 Root glucosinolate profiles and arbuscular mycorrhiza (AM) through the different developmental phases of field-collected *Thlaspi praecox*. *VP*, vegetative; *FI*, flower induction; *FP*, flowering; *SP*, seeding; *SC*, senescence phases. Redrawn from Pongrac et al. (2008)

for AM formation during particular plant development periods. A decrease in total GS concentrations and a distinct change in GS profile were seen to coincide with the highest AM colonisation in field-collected *T. praecox*, whereas in the AM hosts *Tropaeolum majus* and *Carica papaya*, reduced AM colonisation did not correlate with the concentrations of GS induced in these plants (Fig. 7.5) (Ludwig-Müller et al. 2002; Pongrac et al. 2007, 2008).

Not only the total GS, but also the GS profile and individual GS in particular may be decisive in AM formation. Most of the non-AM plants contain gluconasturtiin in their roots, and therefore gluconasturtiin was proposed to act as a general AM inhibitory factor (Vierheilig et al. 2000). Hence gluconasturtiin was not found in *T. praecox* (Fig. 7.5) (Pongrac et al. 2008). In contrast, the main GS of the AM host *T. majus* is glucotropaeolin, and its concentration was seen to increase during AM colonisation (Vierheilig et al. 2000; Ludwig-Müller et al. 2002). In *T. praecox*, the peak glucotropaeolin concentrations are concomitant with the peak AM colonisation (Pongrac et al. 2008). This GS is known as an important metabolite for root growth regulation and a potential precursor of phenylacetic acid, another important naturally occurring auxin in plants (Ludwig-Müller 1999, Ludwig-Müller and Cohen 2002; Davies 2004). AM colonisation has also been shown to enhance auxin levels and alter auxin biosynthesis in *T. majus* during its early stages (Jentschel et al. 2007). Another individual GS disappears along with AM colonisation of *T. praecox*, namely glucobrassicanapin, which has previously been shown in the AM nonhost *B. napus* (Fig. 7.5) (Pongrac et al. 2008; Vierheilig et al. 2000). These observed changes in GS levels may also be salicylate mediated, as root colonisation by AM fungi is affected by the salicylic acid content of the plant (Herrera Medina et al. 2003). However, the effects of jasmonic acid also appear to provide a likely candidate here

(Regvar et al. 1996). Sinalbin and its hydrolysis product *p*-OH-benzylisothiocyanate were identified as the predominant antifungal compounds in *Brassica kaber* (Schreiner and Koide 1993). Sinalbin is also the predominant GS induced by inoculation with *Glomus mossae* in *Sinapis alba* (Vierheilig et al. 2000). Although it was the only GS found in all of the plant organs and all of the developmental stages of *T. praecox*, its role in AM formation remains to be established (Pongrac et al. 2008).

From the currently available literature data, we can conclude that not only total GS levels but also the GS pattern – or more likely the presence and/or absence of specific GS – appear to be decisive for AM formation in GS-containing plant species (Pongrac et al. 2008; Vierheilig et al. 2000). In addition, the factors that have previously been established as important drivers of AM formation should also be taken into account, including plant mineral demands, the seasonal dynamics of mycorrhization, pollution (Smith and Read 1997; Vogel-Mikuš et al. 2006; Pongrac et al. 2007; Regvar et al. 2006), and plant hormones and diverse signalling molecules (Gogala 1991; Akiyama et al. 2005).

7.7 Conclusion

The relationships between GS and metal hyperaccumulation are very complex, and involve metal-, species- and organ-specific GS responses to increased metal concentrations, with an obvious link to sulfur metabolism. To date, these traits have been insufficiently considered in studies of the defence hypothesis of metal hyperaccumulation evolution.

The defence signalling pathways in plant responses to metals appear to have both direct and indirect effects on plant defence systems. GS-containing plants that can hyperaccumulate heavy metals represent key species for such studies.

There is considerable evidence that AM formation in GS-containing plants is controlled via the presence and/or absence of particular GS, and/or a particular GS combination. This may be of vital importance for metallophytes, as AM formation provides an important metal barrier for plants in metal-enriched soils.

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Chapter 8
Plants Under Heavy Metal Stress in Saline Environments

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8.1 Introduction

The adaptation of plants to heavy metals (HMs) under conditions of salinity is an increasingly important problem due to the increasing pollution of salinized lands with HMs. At present, about 25% of all land is saline to some degree. The greatest degree of salinity occurs in arid and semiarid regions. In this context, recent years have seen much interest in river estuaries and salt marshes, in regions with ecological catastrophes – for example, the death of the Aral Sea and the extreme salinity of vast areas of land in countries surrounding it – and also in urban areas in northern latitudes, where salt is used in deicing technologies.

On the other hand, more than 200 years of human industrial development have resulted in much pollution of the environment – including salinized lands – with HMs. Such areas include those where fossil fuels are extracted and treated, those that have undergone active industrial development, urban territories, lands alongside major motorways, and agricultural lands polluted with HM due to the use of fertilizers and other methods of protecting plants. The total HM contents of polluted soils can exceed those of unpolluted soils 10–1,000-fold. Some HMs, such as Zn, Ni, Cu, Fe, and some others, are required for plant growth and development at low concentrations, whereas others, such as Cd and Hg, are not needed by plants and are very toxic to all living organisms.

Plants have developed various mechanisms that allow them to tolerate soils that are highly polluted with HMs. One of their main protective mechanisms is excessive HM chelation by SH-containing amino acids, proteins (metallothioneins), or peptides (phytochelatins) in the cytoplasm, with the subsequent transport of the resulting complexes into the vacuole. The systems of HM transmembrane movement,

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intracellular traffic, long-distance translocation throughout the plant, and accumulation in metabolically less active organs and tissues all play important roles. However, the functioning of plant defensive systems under saline conditions remains poorly studied, despite previous attempts to study plant responses to HMs under saline conditions (Helal et al. 1998).

In this work, we attempted to fill this gap by analyzing our own data and that found in the literature from the last decade. Our attention focused on analyzing the characteristics of the responses of plants from various groups (glycophytes, halophytes, and macrophytes) to the stress resulting from the combined effects of two damaging factors – HMs and salinity – at the level of integral physiological processes such as linear growth and biomass accumulation, as well as the abilities of plants to absorb HMs, transport them to aboveground organs, and accumulate them, which is a characteristic feature of many metallophytic species. The interactions between the effects of HMs and salt are described in terms of antagonism, additivity, and synergism. In addition, we attempted to understand the cellular and molecular mechanisms that determine plant life under heavy metal stress in saline environments.

8.2 Combined Effects of HMs and Salinity on Plants from Various Ecological Groups

The main task of this chapter is to consider the effects of salinity on plant tolerance to HMs and on HM accumulation in plants. Special attention is paid to the partitioning of HMs between underground and aboveground plant organs.

8.2.1 Halophytes of Salt Marshes

Many of the investigations of halophytes have been performed with plants from natural habitats that differ in terms of the ratios between important factors such as the degree of salinity and HM concentrations. Such differences are well manifested in river estuaries and coastal lagoons. Investigations in this direction were started some time ago and performed in several estuaries, such as the York River (USA) (Drifmeyer 1981), Ems Estuary salt marshes (Holland) (Otte et al. 1991), Suir Estuary salt marshes (Ireland) (Fitzgerald et al. 2003), and some others.

Relatively independent changes in the two parameters, HM concentration and salinity, are characteristic of such habitats, and this hampers any analysis of the action of each factor. Problems also arise due to the incomplete characterization of each of the locations where plant material was collected, and differences in plant species composition. Therefore, the most valuable reports are those where several plant species that inhabit the same region are compared.

Among the early studies, the investigation performed in salt marshes along the salinity gradient of the York River estuary (Drifmeyer 1981) seems characteristic. While a 3.5–4.5-fold salinity gradient was attained across the six locations tested, the levels of Mn, Cu, Zn, and Ni in *Spartina alterniflora* plants varied 2.5–3.5-fold, and the level of Fe by as much as ninefold. However, the authors concluded that, in plant tissues, "the levels of these metals … did not correlate strictly with the salinity of either river or sediment pore water." In the absence of direct data on the metal content of the water, and due to the high probability that there were several sources of contamination across the rather large territory studied (more than 40 km along the coast), which had a dense human population, the author's conclusion that "… trace element uptake … is not greatly affected by salinity" can only be considered a preliminary one.

The work of Otte et al. (1991) presented information on the accumulation of Zn, Cu, and Cd in the roots and shoots of four plant species in salt marshes at two locations in the Ems Estuary, upstream (Dyksterhusen) and downstream (Petkum) of Ems (a difference of 2′ N in the northerly direction). Rather representative are data for *Triglochin maritima* (Juncaginaceae) and two dicotyledonous species (*Aster tripolium* and *Spergularia maritima*, Cariophyllaceae), which grew under moderate salinity and strong HM pollution (Dyksterhusen) or heavy salinity and moderate HM pollution (Petkum). It turned out that, in the region of severe pollution (Dyksterhusen), all three HMs (Cd, Cu and Zn) accumulated to the greatest degree, whereas in the area with high salinity (Petkum), they reduced their accumulation in the roots but favored HM – especially Zn – translocation to shoots. As a result, the accumulation factor increased from 0.1 to 3.5 for Cu and from 0.4 to 6.3 for Zn (data for Cd were not significant). In contrast, the Zn and Cd contents in the roots of *T. maritima* and *A. tripolium* doubled in the region of high salinity (the effect of salinity on Cu was less pronounced or absent), but their translocation to shoots was suppressed.

More complete data were presented by Fitzgerald et al. (2003) for the Suir Estuary (Ireland). It turned out that, at four locations along the inner Suir Estuary that were analyzed in the study, Cu and Pb salt concentrations in sediment samples declined approximately twofold from the upper to lower point along the stream. However, there was no significant difference between the Cu concentrations in the roots of six tested plant species. Another pattern was observed for Pb. In two species, the highest Pb accumulation in the roots was detected mostly downstream (Checkpoint: the area with the least metal but with the highest salt concentration). In this area, the Pb content was 239.0 μmol kg^{-1} dry weight in *Aster tripolium* and 517.4 μmol kg^{-1} dry weight in *Spartina* spp., which was more than twice as high as in plants growing in other habitats. Correspondingly, the highest Pb concentrations in shoots were 360.5 and 290.5 μmol kg^{-1} dry weight, which also differed from those in plants growing in other habitats. Despite the fact that there were no significant differences between the Cu concentrations in the roots of all of the studied species at different locations in the estuary, the highest concentration in the shoots of *Schoenoplectus tabernaemontani* was observed immediately downstream of the site of pollution, significantly (threefold) exceeding the value for the unpolluted region; in *Spartina spp*., the highest Cu concentration was detected in the next region downstream

(fourfold higher than in the region located upstream). In general, the authors concluded that there was a common trend in the Cu and Pb contents of the two dicotyledonous species studied: the shoot/root ratio displayed a tendency to increase as the salinity increased. This trend was particularly evident for Pb in *A. tripolium*.

Only indirect data are available on the effects of salinity on HM accumulation by plant tissues in Tagus Estuary salt marshes (Portugal) (Reboreda and Cacador 2007; see references therein concerning the Tagus Estuary). Cu, Pb and Cd accumulation in the roots, stems and leaves of two plant species was investigated. *Spartina maritima* (Poales, Poaceae) plants inhabited the low marsh, and so were subjected to more severe salinity than *Halimione portulacoides* (Caryophyllales, Chenopodiaceae) plants, which inhabited the middle marsh. It was established that *H. portulacoides* accumulated twice as much Cd and Pb as *S. maritima* (taking into account the difference between the HM contents of the habitat sediments). Relative Cu absorption by the roots of *S. maritima* was significantly lower than that of the roots of *H. portulacoides*. However, under severe salinity, the Cu concentration in the leaves of this plant species was slightly higher than in *H. portulacoides*.

Among the studies of HM pollution in the Sheldt (Belgium) Estuary, Du Laing et al. (2008; see also references on the Sheldt Estuary therein) applied a special approach. Soil samples and sediments from four locations were flooded with waters containing different levels of salinity (0.5, 2.5, and 5 g l^{-1} NaCl for 250 days), and duckweed (*Lemna minor*) was grown (for 4 weeks) on the surface water. The salinity was found to primarily enhance the mobility of Cd, and its uptake by duckweed increased by as much as fivefold compared with the control. Moreover, the effect was also observed at a lower salinity. When the salinity of the flood water was increased from 0.5 to 5 g l^{-1} NaCl, the Cd concentrations in duckweed increased by a factor of 4. However, Zn concentrations in duckweed were only slightly enhanced by the salinity, while Ni uptake was not affected at all (excluding one treatment, where a large increase was observed). Although an effect of salinity on the total Cu concentration in surface water samples was not detected, the copper concentrations in the plants slightly (but significantly) increased.

8.2.2 Other Halophytes

In some studies, terrestrial halophytes have been used to study tolerance to HMs and as putative candidates for phytoremediation. Such works are based on the idea (Ghnaya et al. 2005) that "salt-tolerant (halophytic) plants would be better adapted to coping with environmental stresses, including HM than salt-sensitive (glycophytic) crop plants." To confirm such a possibility, the authors point to the rather high tolerance of two halophytes, *Sesuvium portulacastrum* and *Mesembryanthemum crystallinum*, to a high concentration (100 μM) of Cd, one of the most toxic HMs. After 15 days of treatment, the authors did not observe any visible damages, although the whole-plant biomass of *S. portulacastrum* decreased by 40%, and that of *M. crystallinum* dropped by 70% (Ghnaya et al. 2005, 2007a).

Most investigations of the effects of salinity on HM accumulation in plants have been performed with Cd. In work focusing on one of the aforementioned halophytes, *S. portulacastrum* (sea purslane), experiments were performed with rooted plants (Ghnaya et al. 2007b). Plant growth on a medium containing 50 or 100 μM Cd for 30 days resulted in a 50% inhibition of biomass accumulation and in the appearance of necrotic lesions on the leaves. Upon combined treatment with 100 or 400 mM NaCl and cadmium, the necrotic lesions were absent. Plant dry weight approximated and in some cases significantly exceeded control plant biomass. Salinity strongly reduced the concentration of Cd in roots and shoots; however, due to the enhanced growth of NaCl-treated plants, the Cd content per plant increased significantly.

In the work performed with rooted cuttings of a typical Mediterranean halophyte *Tamarix smyrnensis* Bunge (salt cedar) (Manousaki et al. 2008), $Cd(NO_3)_2$ was added to the soil at a concentration of 16 ppm per 1 g of soil dry weight. During ten weeks of plant growth in the presence of Cd or 0.5% NaCl, the plants did not display any signs of toxicity due to these stressors. In contrast, 3.0% NaCl strongly suppressed salt cedar growth in terms of height and biomass accumulation. The concentration of Cd in the absence of salt increased to 2.45 ppm in roots and 3.3 ppm in shoots. As a result, total Cd removal from the soil by the whole plant increased from 9.4 μg in the absence of salt to 19.7 μg at 0.5% NaCl and to 38.3 μg at 3.0% NaCl. In other words, total Cd removal increased by a factor of four when the salinity was increased from 0 to 3%.

Lopez-Chuken and Young (2005) presented data on the effect of NaCl on Cd absorption by seven species of halophytes (and also by four crops, see below). Plants were grown in soil collected from a sewage disposal farm run and containing 28 μg of Cd per 1 l of soil pore water**.** Salinity was created through the addition of 100 mM NaCl (or $NaSO_4$) to the calculated water-holding capacity (WHC) of soil. For five of the seven tested species – *Cynodon dactylon*, *Sorghum* × *drummondii*, *Paspalum vaginatum*, *Atriplex hortensis*, and *Kochia scoparia* – a significant increase in the Cd concentration in shoots was observed (not more than double the average; the highest increase, 2.8 fold, was observed for *A. hortensis*). No significant effect was detected for *Asparagus* sp., *Parthenium argentatum*. When calculating per vessel (per plant), the salinity-induced increase in the Cd content was also especially strong in *A. hortensis* (threefold), just like the absolute value (taking into account the large biomass of this plant, which exceeds those of other halophytes 15-fold).

An investigation of the effect of salinity on Cd and Zn uptake was performed with *Leucaena leucocephala* (a leguminous tree) seedlings in Egypt, where the usage of saline waters for irrigation is a common practice (Helal et al. 1999). It turned out that, after thirty years of attempting to improve poor soils where this work was performed, the soil had become almost tenfold more polluted with Cd and fourfold more polluted with Zn. It was established that, after six months of supplying 10 mM NaCl, the Cd concentration in leaves increased 2.3-fold and the Zn concentration 1.5-fold. The salinity affected neither HM concentrations in the roots and stems nor biomass production. It significantly increased the total HM content per plant (1.23–1.25-fold for each HM) and also the transfer factor of each HM

(metal concentration in plant/metal concentration in soil) by 2.36-fold for Cd and 1.46-fold for Zn.

Another work with four salt-tolerant plant species was performed in Beirut, Lebanon (Zurayk et al. 2001). These halophytes were *Hordeum vulgare*, recommended by the authors as a salt-tolerant crop, and also *Plantago coronopus* L., *Portulaca oleraceae* L. and *Inula crithmoides* L.; the two latter species "are edible and have been recommended for usage in saline agriculture" (we consider the data obtained for barley below, together with other works performed with this crop). These authors studied the effects of two levels of NaCl salinity (9 and 18 dSm^{-1} approx. 100 and 200 mM, respectively) (control plants were treated with tap water containing 0.5 dSm^{-1} NaCl) on HM accumulation in shoots. Experiments were performed with Cd, Cr, and Ni at concentrations of 2, 4, and 10 ppm, respectively. By the twenty-first day of saline treatment, Cd and Ni had caused a significant decrease in the dry biomass accumulation of *P. oleracea* but had exerted no effect on the other plant species. Metal accumulation in these four plant species was generally enhanced by the 9 dSm^{-1} but not the 18 dSm^{-1} treatment. Significant shoot metal accumulation was demonstrated for Cd and Ni in *P. oleracea.* In contrast, in *P. coronopus*, a significant decrease in the concentrations of these metals in shoots was observed at both salinity levels, and it was proportional to the degree of salinity, whereas the Cr concentration was increased. A very high degree of scatter in the data did not allow significant differences to be established for other species.

Kadukova and Kalogerakis (2007) studied the effect of salinity on Pb accumulation in roots and leaves of salt cedar (*Tamarix smyrnensis* Bunge). $Pb(NO_3)_2$ was added to the soil at the concentration of 800 ppm per dry weight of soil organic substance; salinity was created by watering the soil with tap water containing commercial edible sea salt. For ten months, the salinity did not exert any influence on dry weight accumulation, but biomass decreased significantly with Pb in the absence of salt or at a high salt concentration (3%). The highest biomass was produced by plants treated with Pb and watered with 0.5% salt solution; it exceeded the control treatment by 34%. Although Pb concentrations in roots that underwent this treatment did not differ from those in other Pb treatments, the concentrations in leaves were more than twofold lower than in those from other Pb-treated plants. However, because of a high degree of scatter in the data, no significant differences from the control treatment were recorded.

In our laboratory, the mechanisms of adaptation to salinity and HM action of another facultative halophyte, the common ice plant (*Mesembryanthemum crystallinum* L.), were investigated over many years (Kholodova et al. 2002). These plants can develop under conditions of rather severe salinity (up to 400 mM), and manifest substantial tolerance to Cu and especially Zn; they completed their life cycles at $CuSO_4$ or $CuCl_2$ concentrations of up to 50 μM and $ZnCl_2$ concentrations of up to 800 μM (Kholodova et al. 2005). Nevertheless, damage from the Cu manifested itself as a decrease in or loss of turgor, the appearance of necrotic lesions, and a sharp suppression of biomass accumulation. In the presence of excess Zn, growth suppression was less substantial.

In these studies, it was shown that 400 mM NaCl applied together with HMs partially and in some experiments almost completely neutralized the damaging

actions of copper and zinc on biomass accumulation; the leaves retained their turgor; other signs of metal toxicity were much less pronounced. At the same time, this severe salinity did not reduce HM concentrations in the leaves of the common ice plant (Fig. 8.1), which exceeded the control values by factors of 7–8 for Cu and 20–40 for Zn. These concentrations were maintained for both separate HM addition and HM addition in combination with NaCl. Taking into account the great biomass accumulation that occurred upon the combined action of the two factors, the total Cu and Zn accumulation per plant (vessel) increased by 30–80% (Fig. 8.2).

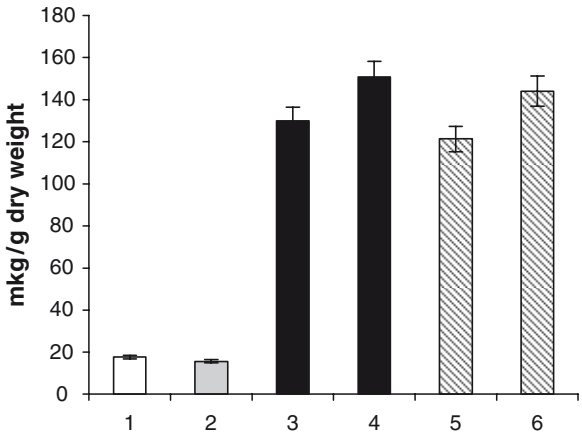

Fig. 8.1 Copper accumulation in common ice plant leaves on the seventh day of the experiment. *1*, Control; *2*, 400 mM NaCl; *3*, 25 μM $CuSO_4$; 4, 50 μM $CuSO_4$; *5*, 400 mM NaCl + 25 μM $CuSO_4$; *6*, 400 mM NaCl + 50 μM $CuSO_4$

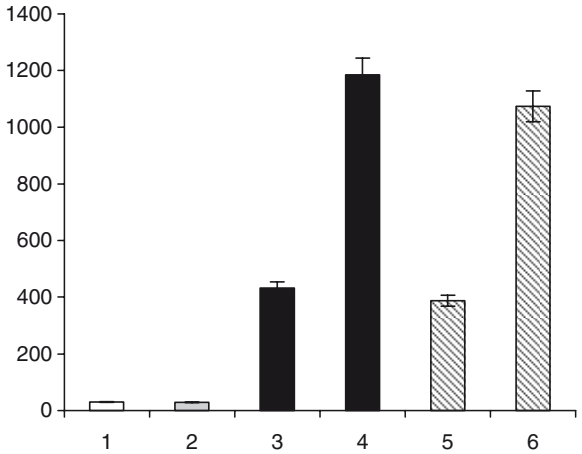

Fig. 8.2 Zinc accumulation in common ice plant leaves on the seventh day of the experiment. *1*, Control; *2*,400 mM NaCl; *3*, 250 μM $ZnSO_4$; 4, 500 μM $ZnSO_4$; *5*, 400 mM NaCl + 250 μM $ZnSO_4$; *6*, 400 mM NaCl + 500 μM $ZnSO_4$

8.2.3 Crop Plants

Most crops are glycophytes, and although it is quite evident that their initial salt tolerance is lower than that of halophytes, relatively salt-tolerant cultivars have been created by breeding. At the same time, the large aboveground green masses of particular plants from this group, or large stand densities (in the case of cereals), make crops promising candidates for use in HM phytoremediation.

8.2.3.1 Monocotyledonous Crops

Investigations of the effects of salinity on plant tolerance to HMs (Cd primarily) and the accumulation of HMs in the roots and aboveground organs of some species and cultivars of wheat (*Triticum* spp.) have been performed in various regions.

Greenhouse experiments with several wheat (*Triticum aestivum* L. and *T. durum* L.) genotypes grown on Cd-polluted soil from a field in Qom province, central Iran, have been performed (Khoshgoftar et al. 2004; Khoshgoftarmanesh et al. 2007). Different salinity levels (0, 60, 120, and 180 mM NaCl) were created by adding NaCl to irrigation water. Increasing the salinity significantly reduced the shoot weight (*T. aestivum*, cv. Rushan) (the strongest inhibition was approximately twofold); however, the concentration of Cd in shoots significantly increased (by a factor of 3.5 in comparison with the controls at the highest NaCl concentration). The highest ability to accumulate Cd was observed for *T. aestivum*, cv. Kavir and *T. durum*, cv. Durum; these accumulated about 0.1 mg Cd per kilogram dry weight at 180 mM NaCl.

Salinity exerted a different effect on Zn accumulation. Increasing the NaCl concentration actually slightly decreased the Zn concentrations in shoots (Khoshgoftar et al. 2004). However, none of the cultivars displayed a significant effect of salinity on HM accumulation in wheat shoots (Khoshgoftarmanesh et al. 2006). For the cultivars tested, a negative correlation was observed between Cu and Zn accumulation, in particular upon additional Zn fertilization (Khoshgoftar et al. 2004).

Some researchers showed that the presence of chlorine ions in the soil solution reduced the cadmium absorption of the soil, which ultimately resulted in enhanced HM accumulation in plants (Smolders et al. 1997; Weggler-Beaton et al. 2000).

Weggler (2004) performed a pot experiment with sludge application rates of 0, 20, 40, and 80 g sludge per kilogram soil and chloride concentrations in the soil solution ranging from 1 to 160 mM. The Cd concentrations in shoots and soil solution increased with sludge application rate up to 40 $g\,kg^{-1}$, but slightly decreased with the 80 $g\,kg^{-1}$ sludge treatment. The soil and plant shoot concentrations of Cd were positively correlated with the soil chloride concentration.

Muehling and Lauchli (2003) studied two wheat genotypes (*T. aestivum* and a salt-tolerant amphiploid, *T. aestivum* x *Agropyron elongatum* Host.); the plants were grown hydroponically. NaCl (75 mM) stress and Cd (10 μM) stress led to significant decreases in shoot yield in both wheat genotypes; however, combined treatment with the two stresses did not lead to further decreases in shoot and root

biomass. Upon the combined action of HM and NaCl, a significant increase in the Cd concentration was only detected in shoots of the salt-sensitive genotype.

The high toxicity of Cd makes it important to check for its possible presence in the edible parts of crops. Norvell et al. (2000) (North Dakota, USA) compared 124 samples of grain collected from a field of durum wheat (*T. turgidum* L. subsp. *durum* (Desf.) Husn.), cv. Munich, along with appropriate soil samples differing in their levels of salinity. The amount of Cd in the durum grain varied widely, from 0.025 to 0.359 mg kg^{-1}; its accumulation in the grain was strongly and positively associated with soil salinity (as represented by soluble chloride, soluble sulfate, or extractable Na). A strong association of the Cd in durum wheat grain with the soluble Cl^- in soil from the region of the root zone 0–15 cm deep was observed.

Some studies on the effects of salinity on plant tolerance to HM have been performed with barley (*Hordeum vulgare* L.) plants. Thus, Huang et al. (2006a, b) showed that adding NaCl to a water culture reduced Cd accumulation in barley plants.

Smykalova and Zamecnikova (2003) showed that biomass accumulation in barley plants grown hydroponically for seven days was equally suppressed in the presence of 10 μM Cd and 100 mM NaCl (down to 76.7% and 74.4%, respectively); the combined action of these factors only slightly enhanced the negative effect (suppressed to 61.6%). A similar inhibitory effect on Cd accumulation in the roots (61.0% of the accumulation of the control) was exerted by a combined treatment; a slightly lower effect was observed on shoots (69.3% of the accumulation of the control). This indicated that Cd translocation to shoots was slightly less inhibited than its accumulation in the roots.

The purpose of the experiment performed by Wahla and Kircham (2008) was to determine the effect of NaCl irrigation on the displacement of HMs applied to soil columns containing barley plants. The concentrations of Cd, Fe, Mn, Ni, Pb, and Zn that leached out of the columns upon irrigation with NaCl (10 g l^{-1}) were higher than those obtained with tap-water irrigation. NaCl significantly increased the concentrations of Cd and especially Mn (up to 200% of control) and Ni (up to 150% of control) in the shoots of barley plants. The presence of NaCl in the irrigation water does not affect the concentrations of Cu, Pb, or Zn in shoots or roots, whereas the Ni concentration in roots strongly (but insignificantly) decreased under saline conditions (Wahla and Kircham 2008).

Zurayk et al. (2001) demonstrated a dependence of HM accumulation in barley plants grown on perlite on the level of salinity. None of the HMs applied at the concentrations used (Cd at 2 ppm, Cr at 4 ppm and Ni at 10 ppm) in combination with NaCl (9 or 18 dSm^{-1}, i.e., ~100 or ~200 mM) inhibited biomass accumulation. It was established that 100 mM NaCl enhanced HM accumulation in shoots of barley plants; however, doubling the NaCl concentration slightly reduced HM accumulation.

The effect of salinity on the tolerance of maize (*Zea mays* L.) to HM was evaluated in pot experiments utilizing polluted desert soil (Helal et al. 1996). NaCl increased both the concentrations of the HMs studied (Zn, Cu and Cd) in soil saturation extracts and their accumulation in roots, but it accelerated root mortality.

Plants irrigated with saline water accumulated more HM than those watered with tap water. In another study with maize plants (Lopez-Chuken and Young 2005), after three weeks of plant growth on a sewage disposal farm run, root dry weight was significantly reduced for the plants undergoing 200 mM NaCl treatment, and this was accompanied by a threefold enhancement of the Cd concentration compared to that of the control.

8.2.3.2 Dicotyledonous Crops

In experiments performed with potato (McLauchlin et al. 1994; McLaughlin et al. 1997), the Cd concentrations in 89 samples of tubers from various regions of Australia were compared with some indices characterizing the soils from the exact same locations at each site. The total Cd concentrations in soils varied from 0.01 to 0.17 mg kg^{-1}, with some soil solutions having Cd concentrations of up to 222.4 nM. A wide variation in tuber Cd concentration was observed across sites, from 0.005 to 0.232 mg kg^{-1} fr wt. Potato tuber Cd concentrations were positively related to soil water-extractable Cl ($R^2 = 0.62$, $P < 0.001$) in the topsoil.

A team of researchers performed a series of studies on Swiss chard (*Beta vulgaris* L., cv. Fordhooe Giant), which is known to be a Cd-accumulating plant species (Smolders and Laughlin 1996; Smolders et al. 1998; Weggler-Beaton et al. 2000). In soil culture, NaCl (or $NaNO_3$) addition to the nutrient solution decreased plant growth significantly, starting from a concentration of 60 mM. The addition of NaCl (but not $NaNO_3$) increased the Cd concentrations in shoots: in 120 mM NaCl treatment, the Cd concentration was almost twice as high as in the zero-salt treatment (Smolders et al. 1998).

In another series of experiments, plants were grown in biosolid-amended soil. Treating the plants with moderate NaCl concentrations (not exceeding 1.6 $g\,l^{-1}$) for 30 days did not affect plant growth. Cd concentrations in shoots of Swiss chard increased linearly with increasing Cl concentration in soil solution (Weggler-Beaton et al. 2000).

Lopez-Chuken and Young (2005), along with data on several halophytes (see "Other Halophytes") and *Zea mays*, presented information on two crops, *Brassica juncea* and *Medicago sativa*, that are putative candidates for Cd phytoremediation. Salinity was created with 100 mM NaCl (and also Na_2SO_4) treatments for 3–6 weeks. These treatments did not significantly affect plant biomass but they did markedly influence Cd accumulation in them. Under saline conditions, the Cd concentrations in *B. juncea* shoots increased by factors of 3–4 compared with those of the controls, whereas the Cd concentrations in the roots increased by factors of 2.3 or less. Therefore, the Cd_{shoot}/Cd_{root} ratio strongly increased (from 1.10–1.63 in the absence of NaCl to 2.02–2.65 under saline conditions). In one of the cultivars, the transfer factor ($Cd_{shoot}/Cd_{solution}$) increased under salinity from 339 to 439. When calculated for plants in a single pot, the Cd content in shoots was 15-fold higher than in roots. NaCl treatment increased the Cd content in *M. sativa* as well, although this effect was less pronounced, and much of the (additionally) absorbed NaCl remained in the roots.

These authors also showed that NaCl treatment of *B. juncea* significantly increased Zn uptake (data not shown), but that Cu concentrations exhibited an irregular pattern among the treatments.

In an investigation of the adaptive mechanisms of another member of the *Brassica* genus, *B. napus*, which was started in our laboratory, it was demonstrated that a combined plant treatment with 100 μM Cu and 200 mM NaCl weakened the symptoms of Cu toxicity, whereas Cu concentrations in different leaves exceeded control values by factors of 3–4.

The effect of salt water irrigation (0.8 $g\,l^{-1}$ for 9 weeks) on the uptake of Cd and Ni by *Spinacea oleracea* L. (Helal et al. 1998) was investigated. Salt water irrigation, which is a commonly accepted practice in northwest Egypt, stimulated root development and enhanced the extractability of Cd and Ni from the soil as well as their concentrations in plants.

Experiments were also performed with *Sesamum indicum* L., an important oil seed crop predominantly grown on dry and salt-affected soils in India (Bharti and Singh 1994). To this end, seedlings were grown for five days on 1 mM HM (Pb^{2+}, Cu^{2+} and Cd^{2+}) solutions, which were used in pairs in the ratio 1:1. Pb + Cd and Cu + Cd induced significant and especially strong growth inhibition. The presence of NaCl largely eliminated the negative effects of the metal combinations on the fresh weight of roots, and it caused an increase in the tissue dry mass in most cases. However, a recovery in the leaf fresh weight was only observed when NaCl (2 EC or 10 EC) was added together with Cu^{2+} + Cd^{2+}.

It was established that sunflower (*Helianthus annuus* L.) plants grown on soils with increased levels of chlorides resulted in enhanced Cd accumulation in the seeds (Li et al. 1994). Thus, in one of two regions that contained very similar Cd concentrations (0.43 and 0.40 μg) but differed twofold in their chloride contents, the content of Cd in seeds was 3.5-fold higher in plants grown in an elevated chloride concentration.

Singh et al. (2003) established that NaCl enhanced the damaging effects of lead acetate on five-day-old *Vigna radiata* L. seedlings. Although these stressors (separately or together) barely affected germination, significant inhibition of root and shoot growth was observed. One millimolar Pb only slightly reduced seedling dry weight (that of shoots by 8% and of roots by 20%). Combined action of the two stressors exerted a synergistic effect that enhanced with increasing NaCl concentration. The addition of NaCl markedly reduced lead accumulation in the seedling roots, from 5 to 1.3–1.0 mg g^{-1} dry weight upon the addition of 6 EC and 12 EC NaCl, respectively. Lead concentrations in the roots exceeded those in the shoots by a factor of 20, which was evidently due to its low capacity to translocate to the aboveground organs.

8.2.4 Water Macrophytes

The results of Du Laing et al. (2008), who demonstrated a significant increase in Cd accumulation with a moderate increase in the NaCl concentration in water for a species of duckweed (*Lemna minor* L.), have already been described (see the end

of “Halophytes of Salt Marshes”). At the same time, salinity did not have any significant influence on Cu or Ni uptake.

An interesting study was performed with the same species of aquatic macrophyte (*Lemna minor* L.): it was established that the technetium (Tc) radionuclide ^{99}Tc is usually present as pertechnetate (TcO_4^-), the only form of Tc that is known to be taken up by plants (Hattink et al. 2001, The Netherlands). Earlier, these authors showed that there was no competition between anions during their uptake. In the case of Tc, absorption evidently occurs through the leaves because, under anoxic conditions, this element is reduced in the sediment to forms that are unavailable to plants.

In this study, it was established that salinity was positively correlated with accumulation because Tc mainly accumulates in the cell walls and water free spaces of plant tissues. But that the intracellular uptake of TcO_4^- by duckweed was independent of chloride concentration. This report also indicated that, in marine microalgae, increasing the salinity enhanced the rate of Tc uptake.

Demirezen (2007) studied the effects of Ni and salinity on another *Lemna* species (*Lemna gibba*, fat duckweed). The Ni concentration was 20 mg l^{-1} (~340 μM). It turned out that applying moderate salinity (125 mM NaCl, 25% of the salinity of seawater) for 10 days resulted in a substantial increase in biomass accumulation for this typical freshwater plant. However, this was accompanied by an almost twofold decrease in the Ni concentration in the plant. Greater levels of salinity (250–375 mM NaCl) suppressed Ni accumulation further and inhibited plant growth (at 500 mM NaCl, the net growth rate became negative). It appeared that there was a negative relationship between water salinity and tissue Ni concentration ($R = -0.72$, $P < 0.05$).

More comprehensive work was performed by a team of Swedish researchers with a typical water macrophyte, pondweed (Greger et al. 1995). For 2 weeks, plants of the submerged macrophyte *Potamogeton pectinatus* L. were grown either in Cd-contaminated sediment and water of varying salinity or in water with Cd and varying salinity but no sediment (Greger et al. 1995). It was shown that Cd uptake by *P. pectinatus* from water decreased with increasing salinity, but that Cd uptake increased with increasing salinity in the presence of sediment.

In one of the recent works from this team (Fritioff et al. 2005), along with *Potamogeton natans* Michx. (which has thick leaves that float on the surface of the water), *Elodea canadensis* L. (which has very thin submerged leaves) was used in experiments. These plants were grown in the presence of Cd (1 μM), Cu (1.5 μM), Zn (20 μM), and Pb (4 μM) in combination with salinities of 0, 0.5 (slightly increased salinity), and 5% (one of the highest salinity levels found in stormwater). This work permitted a comparison of the effect of salinity on the accumulation of four HMs in two plant species.

For 48 h, the salinity and HMs did not suppress plant growth significantly; no signs of their toxicity were observed either. The effect of salinity on the concentrations of the HMs tested was rather specific. Pb accumulation was generally unaffected or barely affected by (temperature and/or) salinity. At the same time, its concentration in *Elodea* was more than tenfold higher than in pondweed. This fact, and also the absence of a temperature dependence of Pb accumulation, permitted the hypothesis that the main mechanism of its uptake was its absorption on the leaf surface, which

is much more substantial in *Elodea* than in pondweed. Concentrations of Cd and Cu also were much higher in *Elodea*, by twofold or more. In both *Elodea* and pondweed, salinity resulted in a decrease in the concentrations of the HMs in the plants. Finally, the two plant species barely differed in their Zn accumulation from a 10 μM solution, in spite of the fact that Zn concentrations were 3–6 times higher in control *Elodea* plants than in pondweed. Unlike other HMs, moderate salinity induced only a slight inhibition of Zn uptake, and severe salinity was also not very efficient at producing inhibition.

Due to pondweed having a much greater biomass than *Elodea*, the total Zn accumulation (mg/container) in pondweed greatly exceeded the corresponding value for *Elodea*. However, the total accumulation of Cd and Cu at optimum temperature was roughly the same for the two plant species. In the case of Pb, this value in *Elodea* was six- to eightfold higher than in pondweed in all treatments. It seems probable that such great values are due to passive uptake via absorption on the leaf surface and in the leaf apoplast. It does not appear to be very probable that such effects could be maintained under long-term HM action. (This problem could be clarified by studying the dynamics of HM uptake by plants).

8.3 Mechanisms of the NaCl–HM Interaction

Let us now consider the basic principles that determine the responses of various plant species and ecological groups to the combined action of salinity and HMs (or how salinity modifies plant responses to HM), and what the causes of typical effects may be.

Comparative data on the effect of each factor and combinations of them on the basic integral biological indices, linear growth and biomass accumulation, have been presented in only a few works. Nevertheless, it seems evident that all three possible responses to the combined action of a HM and salinity (in comparison to the action of the HM without salinity) were observed: (1) enhanced biomass accumulation; (2) suppressed biomass accumulation, and; (3) the absence of any effect.

Significant data indicating the weakening or neutralization of HM toxicity and an improvement in the plant state are presented in only a few reports, and were obtained mainly for halophytes, such as sea purslane, salt cedar, the common ice plant, and the common duckweed (Ghnaya et al. 2007b; Kadukova and Kalogerakis 2007b; Volkov et al. 2006; Demirezen 2007).

A neutral effect, where salinity did not substantially affect the plant state, did not enhance biomass accumulation, and also did not lead to its reduction, in addition to HM-induced growth suppression, was observed more frequently (Helal et al. 1998, 1999; Weggler-Beaton et al. 2000; Weggler 2004). Such responses have not only been observed for halophytes.

However, for glycophytes, a salt-enhanced negative effect of HMs on biomass accumulation is more characteristic. Such a situation even arises at moderate salinities (75–100 mM); in some cases, a synergistic inhibitory effect was observed (wheat, barley)

(Muehling and Lauchli 2003; Khoshgoftar et al. 2004; Khoshgoftarmanesh et al. 2006; Smykalova and Zamechnikova 2003).

In some works, when the effects of varying degrees of salinity were examined, different responses were obtained for different salt concentrations: positive, neutral, or negative responses (Zurayk et al. 2001; Demirezen 2007; Lopez-Chuken and Young 2005; Kadukova and Kalogerakis 2007; Khoshgoftarmanesh et al. 2006).

Bearing in mind such observations, it is not entirely unexpected that salinity exerted a positive effect that manifested in an increased root length (up to 50%) in *Spinacea oleracea* (Helal et al. 1998), because the concentration of NaCl used was not high (~14 mM). It is more difficult to explain the results obtained by Bharti and Singh (1994) on *Sesamum indicum* seedlings. Combined treatment with two HMs at extremely high concentrations (1 mM each) strongly inhibited growth, as evaluated by the accumulation of root or leaf biomass. However, the addition of a relatively low NaCl concentration (2 or 10 EC) abolished this toxic effect or reduced it markedly.

The effect of salinity on HM accumulation in various organs of plants of various species and various ecological groups has been studied more comprehensively. Most works present data on the HM concentration per dry weight of plant organs; also, relatively frequently, data are calculated per total organ biomass or per pot (container) (when the same number of plants are used in each treatment), or (for crops) per unit of stand area.

Many researchers have reported an effect of NaCl on HM accumulation in shoots alone; only two reports have focused on this effect in underground organs. An increase in the Cd concentration under salinity was reported for potato tubers (McLauchlin et al. 1994, 1997) and the shoots of some halophytes, barley, and Swiss chard (Lopez-Chuken and Young 2005, Zurayk et al. 2001; Smolders and McLaughlin 1996; Smolders et al. 1998). In some cases, an observed increase in the HM concentration occurred with biomass reduction, and the authors consider this to be a positive effect of salinity (Lopez-Chuken and Young 2005).

In other research, there was no substantial change in the HM concentration in shoots (Cd in duckweed, Cu and Zn in the common ice plant, Cu in rapeseed; Volkov et al. 2006, and presented here). However, it was repeatedly stated that there was a significant decline in the HM concentrations in the shoots of some plant species (Cd and Cu in barley) (Smykalova and Zamechnikova 2003; Demirezen 2007; Huang et al. 2006a, b; Wahla and Kirkham 2008). The effect depended on NaCl concentration (Weggler 2004).

In a few works permitting a comparison of the effect of salinity on HM accumulation in roots and in shoots, similar, very strong increases in the Cd concentration in salt cedar were found (Manousaki et al. 2008). A most interesting result was obtained by Helal et al. (1999), when the HM concentration in *Leucaena leucocephala* shoots was found to be markedly and significantly higher than in its roots; this was considered as increase in the transfer factor (i.e., enhanced translocation from the root into aboveground organs).

Although under the combined action of salinity and HM increased biomass accumulation has never coincided with an increase in HM concentration, the HM content per plant (shoot) or area (volume of nutrient medium, container) has increased,

and this parameter is important when plants are used for HM phytoextraction (Salt et al. 1998; Lopez-Chuken and Young 2005; Manousaki et al. 2008).

In general, the information available at present reliably indicates that salinity could favor the extraction of HMs from the rooting medium (substrate, soil, sediment) and their accumulation in aboveground plant organs. Such a response to salinity has reliably been shown for Cd in several plant species at various Cd and NaCl concentrations, and at various ratios of them. It was established that this effect was (1) species and cultivar specific; (2) not reproduced in some cases, even for the same plant species, evidently due to differences in the conditions used in the experiments or some specific features of the natural populations; (3) regularly manifested for Cd but less frequently for other HMs (Zn, Cu, Ni); (4) was not usually reproduced when NaCl was replaced with Na_2SO_4; and (5) was regularly manifested only when the plants were grown on solid substrate (soil, bottom sediment, etc.). This last point makes it rather probable that the positive action of salinity (including sea water) was due to increased HM mobility, evidently due to ion exchange, with the transition of the HM from an oxidized to a reduced form (Du Laing et al. 2008). The formation of organic complexes was considered to be another possible cause, because it was demonstrated that "… NaCl treatment raised the concentration of organic carbon" (Helal et al. 1999; Kirkham 2006).

A deeper study permitted an elucidation of deeper interrelations between HMs and salinity.

Earlier, in works performed mainly on aquatic organisms, a so-called free ion activity model (FIAM) was formulated that postulates that the uptake of metal by an organism is proportional to the free ion concentration of the metal in the surrounding solution (Allen et al. 1980, according to Degryse et al. 2006). However, some contradictions with this model were soon found, even in early studies of the effect of salinity on HM accumulation (see, for example, McLaughlin et al. 1994, 1997, and references therein). A large body of information is now available which indicates that HM chlorocomplexes but not free ions play a major role at the stage of HM penetration into the plant root system. This conclusion is based on numerous works that demonstrate a tight correlation between the amount of HM accumulated by the plant (Cd mainly) and the amount of chlorocomplexes in the solution close to root surfaces. Appropriate programs have been applied for the assessment of chlorocomplex speciation and content (GEOCHEM PC, McLauchlin et al. 1997, MINTEQA2, Khoshgoftar et al. 2004; WHAM–VI, Lopez-Chuken and Young 2005); these permit the concentrations of different species of a metal to be calculated on the basis of the total concentration of the metal and some additional parameters of the system [pH and the concentrations of NaCl, some inorganic ions, dissolved organic compounds (DOC), and some others].

On this basis, it was reliably stated that the positive effect of NaCl on Cd accumulation in plant tissues of various species depends markedly on the concentrations of specific chlorocomplex species that are designated $CdCl_n^{2-n}$.

Thus, Lopez-Chuken and Young (2005) observed a strong salinity-induced activation of Cd accumulation in shoots; some data regarding the activities of Cd species in soil pore water were presented. These authors showed that, under the influence of 100 mM NaCl treatment, the total Cd concentration increased threefold

(the Zn concentration increased by a factor of 1.34); however, the concentration of Cd^{2+} increased by only by 20%, while the concentrations of $CdCl^{+}$ and $CdCl_2^0$ increased 12-fold (at 200 mM NaCl, the concentration of $CdCl^{+}$ increased 14-fold and the concentration of $CdCl_2^0$ 24-fold).

It was established that the concentrations of Cd in the shoots of *B. juncea* and *Z. mays* showed a higher correlation with Cl^{-}-dependent factors (Cl^{-}, $R^2 = 0.93$ and 0.86; $CdCl^{+}$, $R^2 = 0.91$ and 0.87; $CdCl_2^0$, $R^2 = 0.93$ and 0.89) than with Cd^{2+} ($R^2 = 0.32$ and 0.04) or even the total Cd concentration in the soil pore water ($R^2 = 0.84$ and 0.75). Correlations with SO_4^{2-}-dependent factors were negative.

Khoshgoftarmanesh et al. (2006) presented similar data regarding the effect of salinity on Cd in wheat. In 120 and 180 mM NaCl treatments, which induced the highest Cd accumulation, more than half of the total Cd was found to be in the form of chloride complexes. In contrast, free Zn^{2+} was the dominant species at all NaCl levels in soil. Increasing the soil salinity decreased the free Zn^{2+} concentration, which decreased the shoot Zn concentrations.

Similar correlations between salinity and Cd accumulation have been found for most plant species (although the opposite effect has also been obtained). This effect is usually explained by the salt-induced desorption of Cd that is tightly bound to the soil and its conversion into a bioavailable form. Therefore, this effect only manifests itself in plants growing in soil or on bottom sediment; it disappears in experiments with soil-free solutions and with free-floating plants, like *Elodea* (as distinct from pondweed), where Cd penetrates through the leaf surface and predominantly into the intercellular space.

However, it is not clear whether increased Cd bioavailability alone determines the positive effect of salinity. Some researchers propose that plants could take up Cd directly in the form of intact Cd–chloride complexes. However, this supposition is yet to find experimental support (Smolders and McLaughlin 1996; Smolders et al. 1998). Another (also hypothetical) possibility is that chlorocomplexes help to overcome diffusive resistance and, when they break down on the root surface, they increase the local Cd concentration in the vicinity of the root, in the zone of availability for membrane transporters.

Information concerning other HMs is extremely scarce. A significant effect of salinity on Zn mobility (availability) has not been strongly established. Khoshgoftarmanesh et al. (2006) and Du Laing et al. (2008) concluded that free Zn^{2+} was the dominant species at all NaCl levels in soil. Increasing the soil salinity decreased the free Zn^{2+} concentration, which caused a decrease in shoot Zn concentrations. It is also not clear how salinity influences Cu accumulation. It is supposed that one of the causes of the contradictory results could be low Cu–chloride complex stability. On the other hand, in marine water, sulfides (which exhibit low solubility) could play a decisive role in Cu and Ni accumulation in plants (Du Laing et al. 2008). In general, the results available at present do permit some conclusions.

It has repeatedly been established that, for natural habitats (estuaries, salt marshes), increased salinity is correlated with enhanced accumulation of HMs, mainly in halophytes. A stimulatory effect of salinity on the accumulation of HMs in plants and their transport to aboveground organs has also been demonstrated in laboratory

and field experiments. However, systematic investigations have not been performed so far, and only rather fragmentary data are available on the combined action of these two stressors on plant physiological processes.

It is not surprising that, in the cases where combined crop treatments resulted in a clear suppression of biomass accumulation, some important physiological functions were also disturbed. Thus, in wheat and several barley genotypes**,** the combined action of these two stressors enhanced the damaging effects of each, additionally reducing the chlorophyll content, suppressing photosynthesis, destroying transpiration, inducing membrane injuries and ionic imbalances, and suppressing the activities of the enzymes involved in nitrogen metabolism, among others (Muehling and Lauchli 2003; Smykalova and Zamecnikova 2003; Huang et al. 2006a,b; Kadukova and Kalogerakis 2007). A deeper analysis of the causes of this effect of two stressors showed that "… a significant interaction exists between Na and Cd in their influence on antioxidant enzyme activity and the accumulation of each element in the plant" (Muehling and Lauchli 2003).

In contrast, in two works performed with halophytes, an optimization of physiological parameters was demonstrated upon combined NaCl and HM treatment (both halophytes belonged to the same family, Aizoaceae). In *Sesuvium portulacastrum*, 50–100 μM Cd halved the values of the basic physiological parameters, while salinity (100 or 400 mM NaCl) completely restored biomass accumulation and even increased the relative growth rate to above control values. In spite of a salinity-induced shift of the K/Na ratio towards Na, the total optimization of the physiological state of the plant upon the combined action of both stressors was evident. This manifested itself, in particular, in the maintenance of chlorophyll content at almost control levels, even though plant treatment with Cd alone severely reduced its concentration (to 44% of the control level) (Ghnaya et al. 2007).

In the studies conducted in our laboratory with another member of the same family, the facultative halophyte *Mesembryanthemum crystallinum*, strong disturbances in plant water status by Zn and Cu attracted special attention. It was shown that supplementation of a Cu-containing medium with NaCl (400 mM) markedly increased the total water content (Fig. 8.3) and stabilized transpiration, improving the principal indices of plant water status. The combined action of the two factors (NaCl and $CuSO_4$) also resulted in sharp drops in the leaf osmotic potential (Fig. 8.4). These drops, to values (–3.0 to –4.5 MPa) that were significantly lower than the osmotic potential of the rooting medium (–1.8 MPa), resulted in an influx of water to the aboveground organs of the plants. Finally, NaCl stimulated the rapid accumulation of proline, one of the most important osmolytes found in plants (Fig. 8.5). As a result, the highest proline concentration (15–16 μmol g^{-1}) was found in plants treated with both stressors together, and this level was twice as high as those observed after treatment with each of them separately.

It was thus evident that salinity favored the adaptation of the common ice plant to the toxic action of the HM, triggering mechanisms that are specific to halophytes, such as a strong reduction in the osmotic potential and enhanced proline accumulation, a universal low molecular chaperone that protects macromolecules and cell structures against toxic HM action (Kholodova et al. 2000).

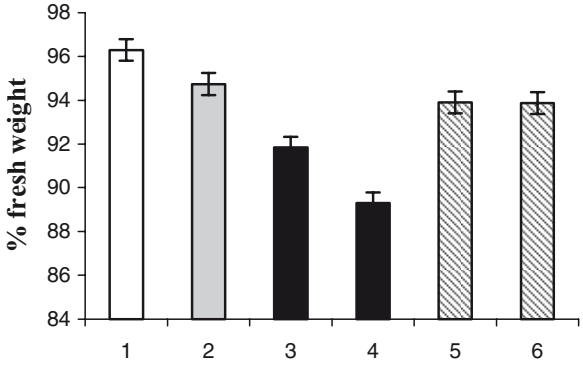

Fig. 8.3 Water content in common ice plant leaves on the seventh day of the experiment. *1*, Control; *2*, 400 mM NaCl; *3*, 25 μM $CuSO_4$; 4, 50 μM $CuSO_4$; *5*, 400 mM NaCl + 25 μM $CuSO_4$; *6*, 400 mM NaCl + 50 μM $CuSO_4$

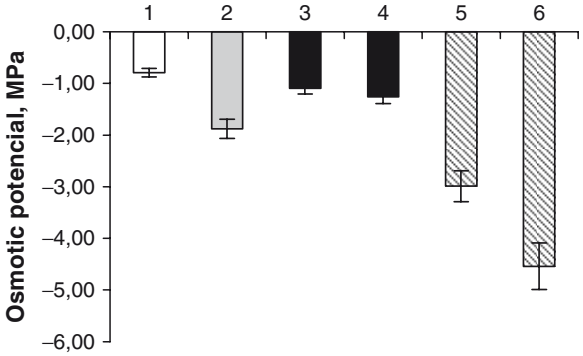

Fig. 8.4 Osmotic potential of common ice plant leaves on the seventh day of the experiment. *1*, Control; *2*, 400 mM NaCl; *3*, 25 μM $CuSO_4$; 4, 50 μM $CuSO_4$; *5*, 400 mM NaCl + 25 μM $CuSO_4$; *6*, 400 mM NaCl + 50 μM $CuSO_4$

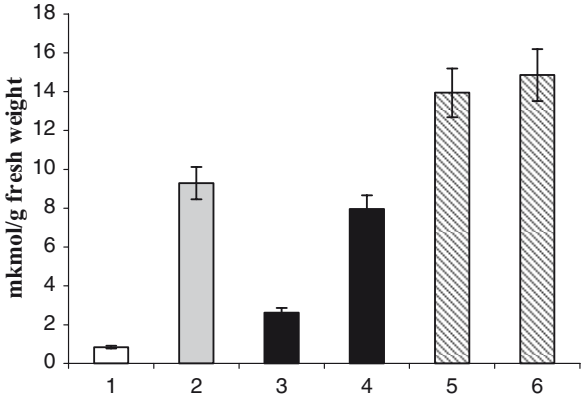

Fig. 8.5 Proline accumulation in common ice plant leaves on the seventh day of the experiment. *1*, Control; *2*, 400 mM NaCl; *3*, 25 μM $CuSO_4$; 4, 50 μM $CuSO_4$; *5*, 400 mM NaCl + 25 μM $CuSO_4$; *6*, 400 mM NaCl + 50 μM $CuSO_4$

8.4 Conclusion

It has become clear that, in some cases, moderate salinity can rather efficiently improve the HM tolerance of plants, and this should be taken into account when developing innovative phytoremediation technologies. Halophytes and some crops are especially promising for these tasks. However, so far, researchers have directed their attention to studying the effects of salinity on processes that occur in the soil before HM penetration into the plant. These studies have helped us to understand the causes of the positive influence of salinity on Cd bioavailability, whereas the interactions of NaCl with other HMs (Zn, Cu, Ni and others) still require further investigation. The joint efforts of biologists, soil scientists, and chemists are needed to this end.

Regretfully, the basic principles of the adaptation of plants to the joint action of salinity and HMs have still not been elaborated, and so we cannot make use of the additional possibilities of the adaptive potential of plants. Thus, the available information indicates that HM hyperaccumulation is not always the most efficient approach of phytoremediation. In contrast, lowering the HM concentrations in shoots – often observed under saline conditions – could lead to the optimum result. In this case, an overall improvement in the state of the plant and enhanced biomass accumulation result in increased HM extraction from the polluted substrate, despite a relatively low HM content in shoots, thus increasing the efficiency of the phytoremediation technology. Inadequate knowledge of the physiological basics of plant adaptation to the joint action of salinity and HMs also impose other limitations. In fact, when developing phytoremediation technologies, universal solutions are not expected: particular plant forms should be selected to decontaminate particular polluted territories.

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Chapter 9
Utilizing Microbial Community Structure and Function to Evaluate the Health of Heavy Metal Polluted Soils

M. Belén Hinojosa, Roberto García-Ruiz, and José A. Carreira

9.1 Introduction

The contamination of the soil with metals has become a widespread environmental problem in many industrialized countries. The fact that the Earth's surface is becoming increasingly polluted by human activities challenges society to develop strategies for sustainability that conserve nonrenewable natural resources such as soil. Soil is a dynamic, living, natural body that is vital to the functioning of terrestrial ecosystems and represents a unique balance among physical, chemical and biological factors. The concepts of soil quality and/or soil health have changed during the last decades as we have become aware of the many essential functions that the soil performs in the biosphere aside from serving as a medium for plant growth, and as social priorities have changed. However the first official definition of soil quality was proposed by the Soil Science Society of America, which described it as "the capacity of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal production, maintain or enhance water and air quality and support human health and habitation" (Allan et al. 1995). According to the committee that proposed this definition, the term "soil quality" is not synonymous with "soil health," and these terms should not be used interchangeably. Soil health was defined as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, maintain the quality of air and water environments, and promote plant, animal, and human health" (Doran et al. 1996). For the remainder of this chapter, we will

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preferentially use the term "soil health," as it more clearly portrays the idea of soil as a living dynamic organism that functions in a holistic way depending on its condition or state, rather than as an inanimate object whose value depends on its innate characteristics and intended use.

We agree with Pierce and Lal (1991) that "soil management practices in the twenty-first century must be formulated based on an understanding of the ecosystem concept." Figure 9.1 shows a modification of the framework proposed by Chapin et al. (1996) for defining the conditions that sustain the ecosystems, which has been adapted for soil processes. According to this approach, a sustainable soil is one that, over the normal cycle of disturbances, maintains its general structure, processes and interrelationships. The structure of the microbial community and the functioning of soils are determined by several factors that both affect and are affected by ecosystem processes. These factors (interactive controls) include regional climate, soil water and nutrient supply, the functional types of organisms present in the system, and the disturbance regime (Fig. 9.1). This framework could help us to understand how degraded soils can be restored through practices that enhance positive and negative feedback to return the soil processes to a desired state.

Soil health cannot be measured directly, but there are physical, chemical, and biological properties, processes, and characteristics that can be measured to monitor changes in soil health. The traditional use of physicochemical measures alone cannot completely meet this need. Therefore, soil ecology has become a hot research topic, and studies in this field aim to identify sensitive indicators that comprehensively reflect dynamic changes in soil health. In this sense, soil microbes are important components of soil ecosystems, playing an important role in the biogeochemical cycle.

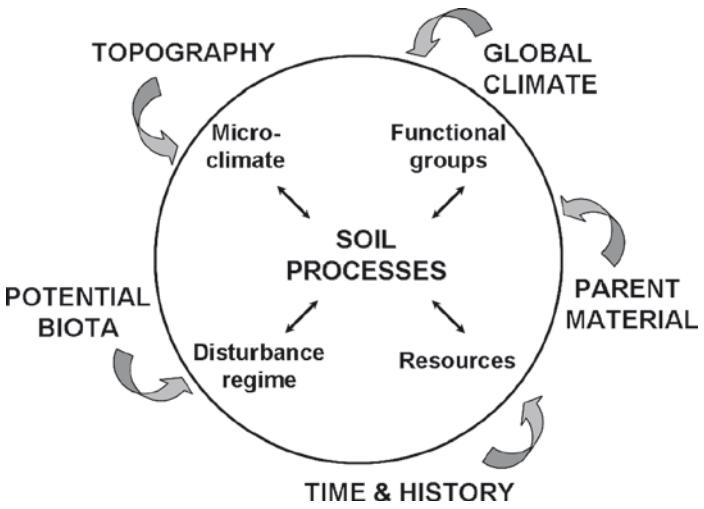

Fig. 9.1 Conceptual model of "external state factors" (global climate, geology, time, etc.) and "interactive controls" (regional climate, disturbance regime, etc.) that both affect and are affected by soil processes (modified from: Chapin et al. 1996)

In order to aid efforts to prevent soil degradation and use resources sustainably, this chapter combines perspectives developed in soil ecology, microbiology and biochemistry into a common framework for evaluating soil health using microbial indicators. Although many of these principles are applicable to soils in general, we have focused on heavy metal polluted soils here.

9.2 Microbial Properties as Indicators of Soil Health

While efforts to define and quantify soil health are hardly new, a consensus on a set of standard conditions that can be used to evaluate soil health is still lacking.

Indicators based on physicochemical soil properties are of paramount importance in soil health assessment. However, an increasingly popular view is that indicators based on biological – especially microbiological – properties are more sensitive to changes than other type of indicators, and provide a broader picture of soil health (Nannipieri et al. 1990; Yakovchenko et al. 1996; Gil-Sotres et al. 2005). In addition, microorganisms respond rapidly to perturbations, as they have intimate relationships with their surroundings due to their relatively high surface-to-volume ratios. It has been described that changes in indicators based on microbial structure and function can even precede detectable changes in soil physical and chemical properties, thereby providing an early sign of improvement or an early warning of soil degradation (Pankhurst et al. 1995).

The bioavailability of heavy metals is another important issue in soil health because of its link to microbial activity. The impact of unusually high levels of heavy metals on soil health is highly dependent on microbial activity, and microbial responses also integrate the effects of chemical mixtures and other information not provided by studying the chemical mixtures themselves.

According to authors as Elliott (1997) and Nortcliff (2002), an indicator of pollution should ideally have the ability to:

- Be sensitivity to the presence of the pollutant
- Reflect different levels of pollution
- Be reliable in terms of its response to any given pollutant
- Be sensitive to a wide variety of pollutants
- Discriminate between the effect of the pollutant and any prior degradation of the soil.

Many biological and biochemical soil properties have been proposed as pollution indicators. An overview of the works published so far shows that there are three main approaches to the use of both general and specific biochemical properties to estimate soil health: (a) the use of individual properties; (b) the use of simple indices based on a few properties; or (c) the use of complex indices resulting from combinations of several properties or that are deduced based on statistical procedures.

Management decisions are usually based on incomplete and fragmented information, but there is a great deal of opportunity to improve the quality of the information used. The challenges for monitoring (evaluating trends) and assessing (evaluating at a point in time) are more limited in scope, but still quite important.

9.3 Microbial Function and Community Structure

An ecosystem has two major attributes, structure and function, which are intimately interconnected. This framework can be used to define and illustrate the damage that ecosystems can suffer; for example after a heavy metal pollution event (Fig. 9.2). The original ecosystem may have high levels of both attributes (structure and function), but degradation might drive indicators of one or both attributes downwards (Bradshaw 2002). Thus, in terms of heavy metal polluted soils, degradations of both microbial community structure and function have been widely described in the literature over the last years. In contrast, the restoration of both of these components of the soil ecosystem has received less attention.

Ecological restoration has been defined as "the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed." According to this definition, "an ecosystem has recovered – and is restored – when it contains

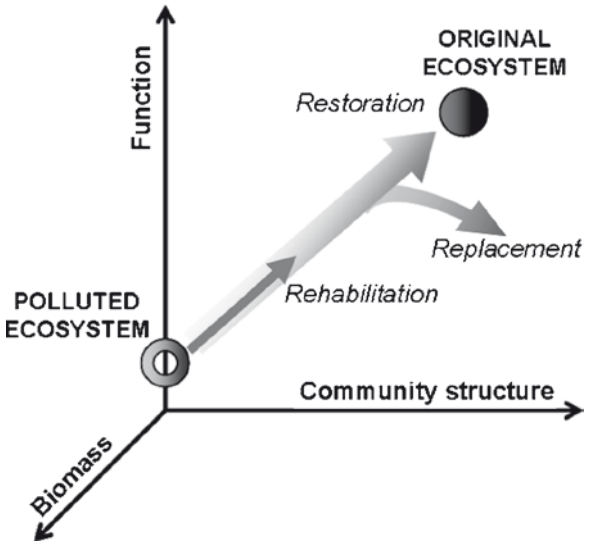

Fig. 9.2 Theoretical framework showing the movement of an ecosystem during restoration. Starting from the degraded ecosystem, a restoration plan acts to help an ecosystem to move towards some goal (often a constructed vision of the original ecosystem) by increasing the structural complexity and level of function of the ecosystem (modified from Bradshaw 2002)

sufficient biotic and abiotic resources to continue its development without further assistance or subsidy. It will sustain itself structurally and functionally. It will demonstrate resilience to normal ranges of environmental stress and disturbance. It will interact with contiguous ecosystems in terms of biotic and abiotic flows and cultural interactions" (Society for Ecological Restoration International Science & Policy Working Group 2004).

Young et al. (2005) recognized, in the context of restoration, the central role of soil microbes in the success of higher plant growth and overall ecosystem health. In this sense, two longstanding challenges in soil microbiology have been the development of effective methods for (a) determining which microorganisms are present in the soil, and (b) determining microbial function in situ.

These challenges have been exacerbated by the difficulties involved in separating microorganisms from the soil matrix, the morphological similarities of many soil microorganisms, and changing microbial taxonomies. Furthermore, the microscopic sizes of soil microorganisms have made direct visualization more difficult than for macroorganisms. However, over the past few decades, our approach to analyzing soil microbial communities has changed dramatically. Many new methods and approaches are now available that allow soil microbiologists to gain access to more of the microorganisms residing in soil and facilitate better assessments of microbial diversity. Figure 9.3 reports the most important approaches used to study soil

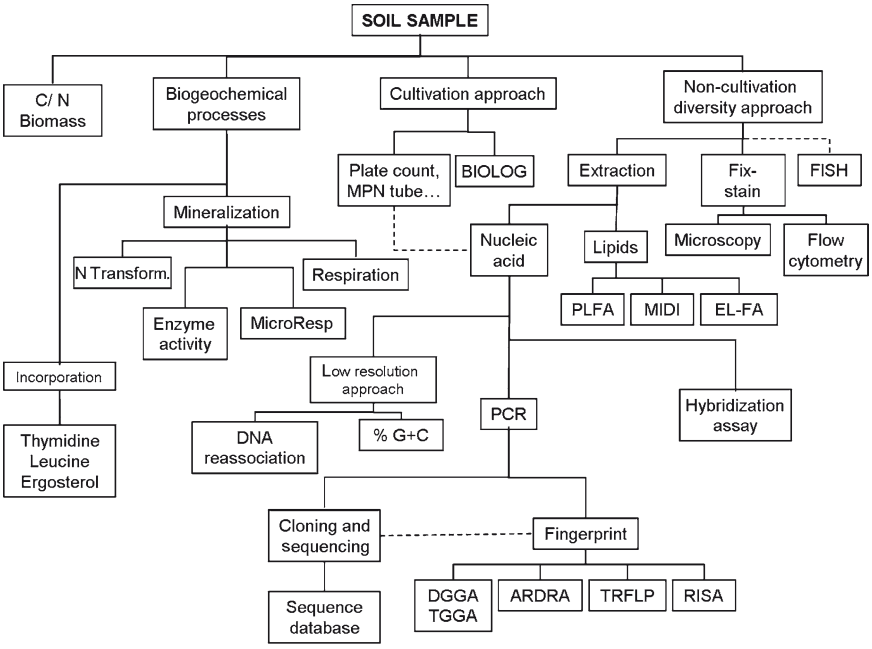

Fig. 9.3 Main methodological approaches to studying soil microbial communities that are used to evaluate the health of heavy metal polluted soils

microbial communities. These will be briefly discussed in the following sections of this chapter; their strengths and weaknesses will be described, and some examples of their use in heavy metal polluted soils will be presented.

9.4 Microbial Biomass as an Indicator

Microbial biomass is a key parameter and essential baseline in many monitoring programs (Nannipieri et al. 2002; Winding et al. 2005). However, despite the fact that microbial biomass has been proposed as an indicator for monitoring the effects of heavy metals on soil health, the quantification of total microbial biomass in a soil is a relatively difficult task due to the high diversity of the organisms involved and their different responses to heavy metals.

9.4.1 Culture-Based and Direct Methods

Bacterial and fungal abundances have traditionally been estimated as colony forming units (CFU) using the plate count technique (Bååth 1989). For example, Oliveira and Pampulha (2006) found that the total number of CFU of bacteria, fungi and actinomycetes was significantly reduced after heavy metal contamination. In this study, fungi and actinomycetes were less sensitive than culturable heterotrophic bacteria or even nonsymbiotic nitrogen fixers. However, this is now a very controversial method as far as fungi are concerned, since the plate count technique mainly determines the number of spores, and actively growing hyphae have little chance of forming a colony. It is also a doubtful method in the case of bacteria, especially when nutrient-enriched media are used, since only a fraction of the total bacterial population will be able to grow on such media (Ritz 2007; Nichols 2007).

Direct counts or biovolume estimations using conversion factors can also be used to directly estimate microbial biomass. Different soil preparation methods and staining techniques in combination with epifluorescence microscopy and automated image analysis can be used routinely in monitoring programs (Bloem and Breure 2003), as they clearly discriminate between polluted and unpolluted soils (Ellis et al. 2002).

9.4.2 Indirect Methods

Several indirect methods are now available for measuring the soil microbial biomass. Among them, the chloroform fumigation method is the most commonly used approach. Microbial biomass C is now almost invariably measured by fumigation extraction (FE) (Vance et al. 1987) rather than fumigation incubation

(FI) (Jenkinson and Powlson 1976). The FE method permits the measurement of microbial biomass when FI is invalid, such as in soils with pH values < 4.8.

Significant reductions in microbial biomass have been found in metal-contaminated soil in comparison with uncontaminated soil (Kuperman and Carreiro 1997; Yao et al. 2003). However, Chander et al. (2001) and Niklinska et al. (2006), among others, found that microbial biomass C was relatively insensitive to heavy metals, especially when the metals are present at low concentrations. This fact has been explained by the presence of resistant species that may increase in biomass after their competition has been reduced in conditions where the effects of metals on soil microbiota are not dramatic (van Beelen and Doelman 1997). Many authors have even found that microbial biomass C increases when organic wastes enriched in heavy metals are added to the soil (Leita et al. 1999; Clemente et al. 2006). These idiosyncratic results may reflect the importance of other environmental factors, such as differences in input C quantity and quality.

Because microbial biomass is strongly connected to soil carbon, the microbial quotient (ratio of microbial biomass C to total organic C, i.e., C_{mic}/C_{org}) has also been used as an indicator of the heavy metal impact and proposed as a more sensitive index of soil perturbations (Bastida et al. 2008). Thus, the microbial quotient avoids the problem of comparing trends in soils with different organic carbon contents (Sparling 1997).

The ratio C_{mic}/C_{org} generally lies in the range 1–4% (Smith and Paul 1990). A low ratio in soil with high levels of heavy metals may be explained by reduced efficiency of substrate utilization by microorganisms, as more substrate is diverted to catabolic processes at the expense of anabolic processes, leading to reduced microbial biomass in the long term (Sparling 1992). On the other hand, Kunito et al. (1999) did not find a significant negative relationship between the ratio C_{mic}/C_{org} and the levels of metals in soils. These authors explained that the discrepancy may be a consequence of: (a) additional influences of other environmental factors on the ratio; (b) large differences in the quality of soil organic matter, and; (c) significant differences in the extractabilities of organic C in the chloroform fumigation extraction.

The combination of these two measurements provides an intrinsic "internal control" for soils that are similar in terms of management practices, soil type and climate when monitoring the effects of heavy metals on soil microbial biomass. Thus, a change in the trend shown by values of microbial biomass C as percentage of soil organic C compared to that of the "internal control" could indicate a negative or positive effect of heavy metals on the biological processes of the soil ecosystem.

Several investigators have also reported that heavy metal stress can induce changes in the microbial biomass *C*/*N* ratio. Khas et al. (1998) observed a significant increase in this ratio after the application of heavy metals under laboratory conditions, due to a proportional increase of fungal biomass. On the other hand, Liao and Xie (2007) recently reported that the microbial biomass *C*/*N* ratio decreased with increasing heavy metal concentration, whereas Yao et al. (2003) found no clear change in this ratio. Consequently, this variable should be interpreted carefully, as a change in the structure of the microbial community is not always accompanied by a change in the microbial biomass *C*/*N* ratio.

The estimation of adenosine 5′-triphosphate (ATP) content provides another useful indirect indicator of the presence of microorganisms in soil, because it only occurs in living cells and exocellular ATP has a half-life of less than 1 h (Conklin and MacGregor 1972). Jenkinson and Oades (1979) and Contin et al. (2001) described a strong linear relationship between biomass C and soil ATP over a large range of concentrations from measurements taken around the world. In addition, Barajas-Aceves et al. (1999) suggested that ATP measurements provide a valid estimate of microbial biomass in both uncontaminated and metal-contaminated soils, even at very high metal concentrations. More remarkably, they also suggested that if the metals cause a shift in the microbial community structure, this is not necessarily reflected in a change in the microbial ATP concentration. Many studies have showed that direct and indirect changes in soil conditions due to heavy metal contamination have a large negative effect on the ATP content in the soil (Frostegård et al. 1993; Barajas-Aceves et al. 1999; Oliveira and Pampulha 2006). Soil ATP content is highly dynamic and depends on synthetic and degradation processes mediated by a great number of enzymes that can be directly or indirectly inhibited by heavy metals.

9.5 Microbial Function

Soil microbial activity is a quantifiable reflection of soil functioning. Soil microbial activity is a general term that is used to indicate the vast range of activities carried out by microorganisms in soil, whereas biological activity reflects not only microbial activity but also the activities of other organisms in the soil, such as the meso- and macrofauna, including plant roots (Nannipieri et al. 1990). Although the two terms are conceptually different they are often confused. Various methods have been used to determine the microbiological activity of the soil (Fig. 9.3). Some of them measure the rates of entire metabolic processes. For instance, the emission of soil CO_2 reflects the catabolic degradation of organic carbon under aerobic conditions; the net nitrification rate is the speed at which ammonia is oxidized to nitrate; thymidine incorporation represents the rate of DNA synthesis, and dehydrogenase activity measures the intracellular flux of electrons to O_2 due to the activities of several intracellular enzymes that catalyze the transfer of hydrogen and electrons from one compound to another.

9.5.1 Carbon Mineralization: Soil Respiration

Together with meso- and macrofauna, microorganisms are key players in the recycling of carbon. The extent of this ecosystem process can be measured by soil respiration or organic matter degradation, which at the same time provides an estimate of microbial activity. Therefore, soil respiration has been studied in depth

in order to evaluate the effects of metals on soil microbial activity. Soil respiration can be measured by quantifying CO_2 production, O_2 consumption, or both. However, measurements of CO_2 concentration are the most sensitive of these, due to its low atmospheric concentration compared O_2.

Soil respiration is generally positively correlated with soil organic matter content as well as with microbial biomass and activity (Alef 1995). However, this correlation is not always significant when soils are polluted with heavy metals (Yang et al. 2006). Heavy metals can reduce soil respiration by forming complexes with the substrates or killing microorganisms (Landi et al. 2000).

Responses of soil respiration to metal contamination are, however, not very consistent. Doelman and Haanstra (1984), Bååth et al. (1991), and Hattori (1992) found a significant decrease in CO_2 production in metal-contaminated soil. In contrast, other authors (Bardgett and Saggar 1994; Flieβbach et al. 1994) reported an increase in CO_2 production in metal-polluted soils. These contrasting results may be due to variations in the levels of metal contamination, in the source of the contamination (e.g., sewage sludge or mining), in the period of time over which the responses were monitored, and in the characteristics of the receiving soil. In this sense, it has been argued that high metal concentrations decrease CO_2 production, whereas moderate contamination leads to higher respiration rates (Bardgett and Saggar 1994; Flieβbach et al. 1994; Leita et al. 1995). Hattori (1992) found that the inhibition of soil respiration by heavy metals was higher in soils with lower organic matter contents and cation exchange capacities (CECs) than in soils with lower values of both. Similarly, Maliszewska et al. (1985) and Hinojosa et al. (2004a, 2008) found that metals were generally less toxic to microorganisms in fine-textured alluvial than in sandy loam soils; this finding was attributed to differences in the sorption capacities of soils with different textures.

Chander and Brookes (1991) showed that, in the presence of residues of ryegrass or glucose, more CO_2 was evolved from a soil with high levels of heavy metals than from a low-metal soil. This agrees with Killham (1985), who suggested that microorganisms in heavily polluted soils are under stress and utilize C less efficiently, resulting in more CO_2 evolved per unit of substrate. In contrast, Insam et al. (1996) reported no increase in specific microbial respiration after contamination with heavy metals.

Despite the numerous variables that influence soil respiration, it is routinely included in most soil monitoring programs and has been used as an indicator of heavy metal toxicity by many authors (Brookes 1995; Winding et al. 2005). However, preconditioning and standardization of the soil before measuring respiration is considered a necessity in order to minimize the effects of environmental variables. Another potential difficulty with interpreting the effect of metal pollution on soil respiration rate is that it is not possible to distinguish the direct effect of the metal's toxicity on the microorganism from that of substrate availability. Adding different substrates to the soil and subsequently measuring their degradations by quantifying the respiration rate is an approach that has been used to overcome this difficulty. Thus, substrate-induced respiration (SIR) has commonly also been used as an indirect measure of microbial biomass in monitoring programs.

The metabolic quotient (qCO_2, or the specific respiratory rate), defined as the microbial respiration rate per unit of microbial biomass (Anderson and Domsch 1985), has also been proposed as an alternative measure of changes in microbial biomass in response to metal stress in soils (Brookes 1995; Giller et al. 1998). A high qCO_2 is a common characteristic of the soil microbial biomass in soils that have been contaminated for long periods (Chander and Brookes 1991; Bardgett and Saggar 1994; Yao et al. 2003; Liao and Xie 2007). Increased qCO_2 has also been observed in laboratory ecotoxicological studies after the addition of metals (Leita et al. 1995). However, the similar responses observed in the short-term and long-term studies may result from different causes. It is likely that the increased qCO_2 observed in short-term manipulative studies was a response to a disturbance rather than to a stress (Wardle and Ghani 1995). Therefore, the use of qCO_2 as an indicator of general microbial activity after heavy metal pollution or after restoration practices has limitations because it might confounds the effect of disturbance (e.g., the mechanical removal of the pollutant) with the stress due to the pollutant.

9.5.2 Nitrogen Mineralization, Nitrification, Denitrification, and N_2 Fixation

Nitrogen transformations in soils are very complex processes in which the nitrogen is simultaneously reduced and oxidized under different redox conditions by different microorganisms. The major processes involved in N cycling include ammonification, nitrification, denitrification, N_2 fixation, dissimilatory reduction of nitrate to ammonium, and N immobilization.

9.5.2.1 Nitrogen Mineralization

Ammonification, the process by which organic nitrogen is oxidized to create ammonia and ammonium, is a measure of N mineralization. The gross N mineralization rate is usually higher than the net rate, as some of the NH_4^+ produced is immobilized by soil microorganisms into new biomass. Because of the difficulties involved in measuring the gross N mineralization (it is only possible using ^{15}N techniques), the net N mineralization is usually measured instead; this is estimated as the NH_4^+ that accumulates in the soil under aerobic conditions.

Investigations of the effects of metals on N mineralization have produced contradictory results. Net N mineralization under field conditions seems to be a very sensitive indicator of metal pollution, as its inhibition has generally been reported (Inubushi et al. 2000; Stuczynski et al. 2003; Hinojosa et al. 2004b). However, the results from laboratory experiments are less clear, since N mineralization has been shown to be stimulated, inhibited or unchanged due to heavy metal pollution (Bogomolov et al. 1996; Stuczynski et al. 2003). These inconsistent results are

likely due to different experimental procedures and variability in soil properties and/or organic N concentrations.

9.5.2.2 Nitrification

Nitrification is the biological oxidation of ammonia with oxygen into nitrite and the subsequent oxidation of these nitrites into nitrates. The degradation of ammonia to nitrite is usually the rate limiting step during nitrification, and so the nitrification rate is usually measured with the ammonium oxidizing assay, in which a soil slurry is incubated with an excess of ammonium and chlorate, the latter inhibiting the oxidation of nitrite to nitrate.

Nitrification is assumed to be a more sensitive measure than N mineralization because a less diverse group of bacteria, ammonia oxidizers and nitrifiers, are involved in this process (Visser and Parkinson 1992). N mineralization and nitrification are normally in a state of equilibrium. In the presence of contaminants, this balance may be disturbed; the nitrifying population is more sensitive to this due to its smaller size. Smolder et al. (2001) were among those to use the potential nitrification rate test (PNR) to identify metal toxicity in field-contaminated soils, and they concluded that the PNR is sensitive to metal stress. However, they pointed out that its power as a bioindicator is rather low because of the high variability of the endpoint for uncontaminated soils.

9.5.2.3 Denitrification

The denitrification rate is considered to be an obligate or facultative anaerobic process in which nitrate is transformed to N_2O and then to N_2. Because of its high dependence on the soil oxygen concentration, this process is very dependent on abiotic factors such as precipitation and soil compaction. Measurements of denitrification are carried out mainly by the acetylene inhibition technique (Smith and Tiedje 1979), in which the reduction of N_2O (nitrous oxide) to N_2 is inhibited by acetylene, and the accumulated nitrous oxide is measured by gas chromatography. Interpreting denitrification data is not a straightforward task, because the denitrification enzymes are only synthesized under anaerobic conditions and the enzymes are not functional under aerobic conditions (even though they persist), and so the denitrification rate may reflect historical anaerobic situations and not necessarily the number of active denitrifying microorganisms. The few studies that have focused on the effect of metal on the production of N_2O have reported a gradual decline with increasing concentrations of heavy metals (Bardgett et al. 1994; Holtan-Hartwig et al. 2002; Vásquez-Murrieta et al. 2006). Holtan-Hartwig et al. (2002) observed that N_2O reduction was more strongly affected than the N_2O production rate after the addition of a mixture of heavy metals at different concentrations. After incubating these soils for two months, a complete recovery in the N_2O production rate was observed, but the N_2O reduction capacity was still not fully restored. For this reason,

these authors claimed that the N_2O+N_2 to N_2O ratio could potentially be used to evaluate the effect of heavy metal pollution on the denitrifier community.

9.5.2.4 N_2 Fixation

N_2 fixation, a process in which atmospheric nitrogen is converted to ammonia, is carried out by specific groups of microorganisms (free-living heterotrophic bacteria, cyanobacteria, and symbiotic associations), and depends upon the enzyme nitrogenase. Measurements of N_2 fixation, or more accurately nitrogenase activity, have been made using the acetylene reduction assay (Olson et al. 1998).

Rates of N_2 fixation by free-living heterotrophs are slow and difficult to use as an indicator of soil pollution according to some authors (Rother et al. 1982). However, when soils are incubated with glucose before the test is carried out (thus enhancing the development of heterotrophic bacteria), a significant reduction in N_2 fixation due to metal pollution is reported (Brookes et al. 1984; Lorenz et al. 1992). Brookes et al. (1986), while studying potential N_2 fixation by mainly blue-green algae in sludge-amended soil, found a 50% decrease at very low levels of contamination. Martensson (1993) also evaluated the negative impact of heavy metals on heterotrophic and cyanobacterial nitrogen fixation.

Several reports have shown that heavy metals negatively affect the nodulation of plants and consequently symbiotic N_2 fixation (Giller et al. 1998). The specific mechanisms that caused the reduction in symbiotic N_2 fixation were initially unclear, but the heavy metals in the soils probably hindered the establishment of proper nodule activity. Giller et al. (1998) concluded that rhizobia are far more sensitive to the toxic effects of heavy metals than their host plants. Thus, the toxic effect of heavy metals on N_2 fixation seems to be due to their toxicity to free-living rhizobia in the soil, which results in their gradual extinction. This conclusion was confirmed by Broos et al. (2005), who found a clear negative effect on the survival of free-living rhizobia when soils had high levels of Zn. Thus, it was recommended that simply screening for the presence of rhizobia that can nodulate would provide a rapid way to test for toxic effects of heavy metals in soils.

9.5.3 Enzyme Activities

Soil enzyme activities have been proposed as suitable indicators of soil quality due to their intimate relationship with soil biology, their ease of measurement, and their rapid response to soil perturbations and/or stress, including those caused by heavy metal pollution (Dick et al. 1996). It has also been suggested that soil enzyme activities are indicators of process diversity, providing information on the biochemical potential, possible resistance and resilience, and the potential for manipulation of the soil system (Taylor et al. 2002).

However, the use of soil enzymes as indicators of microbial function is controversial because the overall enzyme activity is derived from various fractions, i.e., growing

microorganisms, dead cells, and extracellular enzymes associated with the clay–humus complex (Wallenstein and Weintraub 2008). On the other hand, although the soil enzyme activity is mainly of microbial origin, it also originates from plants and from soil meso- and macrofauna. In addition, soil enzyme assays generally provide a measure of potential activity (Speir and Ross 2002). Measurements of soil enzyme activity are usually based on the addition of an artificial substrate at a saturating concentration in order to achieve a reaction rate that is proportional to the enzyme concentration.

Many studies have used soil enzyme measurements to evaluate the effects of heavy metal pollution in soil. The types of enzymes that have been measured range from unspecific enzymes that provide information about general microbial activities, such as dehydrogenase or fluorescent fluorescein diacetate, to those involved in specific reactions related to nutrient transformations, including urease, amidases, phosphatases, phenol oxidades, β-glucosidases, cellulases, arylsulfatase, etc. For example, in our laboratory, studies of the effect of adding (heavy metal enriched) pyrite sludge to soil indicated a consistently negative effect on the activities of many soil enzymes (Hinojosa et al. 2008). Arylsulfatase activity showed the lowest ED_{50} values, agreeing with our previous results in the field (Hinojosa et al. 2004a,b). The activity of this soil enzyme was previously cited by many authors as being one of the most sensitive to trace element pollution (Dick 1997; Renella et al. 2005). On the other hand, β-glucosidase activity also showed a high sensitivity to pyrite sludge pollution, which correlates with statements by Kuperman and Carreiro (1997) and Kunito et al. (2001) suggesting that the activity of this enzyme could be a useful indicator of soil quality due to its important role in the degradation of organic matter. This conclusion do not agree with those of Effron et al. (2004) and Renella et al. (2004), who found that this enzyme was apparently insensitive to heavy metal contamination. Kizilkaya and Bayrakle (2005) reported that the activity of urease was the most strongly affected by Zn pollution. However, interestingly, in our studies this enzyme was one of the lest sensitive to pyrite sludge contamination, agreeing with the findings of Speir et al. (1995, 1999) and Karaca et al. (2002).

The wide range of sensitivities to heavy metals shown by the activities of different enzymes in different studies may be due to different experimental approaches. On the other hand, heavy metals could affect soil enzyme activities via various pathways: (a) inactivation of the produced enzyme; (b) inhibition of the biosynthesis of microbial enzymes, and; (c) changes in the specific compositions of microbial groups that produce extracellular enzymes.

In addition to these direct effects of heavy metals, other indirect effects include changes in soil pH (which heavily affect substrate–enzyme kinetics) and the availability of natural enzyme substrates.

9.5.4 Microbial DNA and Protein Synthesis

DNA synthesis (an indicator of microbial growth, mainly bacteria) has been determined by the incorporation of ^{3}H- or ^{14}C-thymidine into microbial DNA. Briefly, a soil extract is incubated with radiolabeled thymidine for a short time, and then the

amount of radiolabel in the cells is measured (Bååth 1992; Bååth et al. 2001). This method must meet with the following requirements if the results are to be interpreted correctly: (a) DNA synthesis must be linearly related with cell growth; (b) all bacteria must take up thymidine through the cell membrane, and this should not be metabolized, and; (c) the radioactive label should not be exchanged with other molecules.

The synthesis of microbial proteins is also highly correlated with microbial activity and can be determined with ^{3}H- or ^{14}C-leucine methodologies. The leucine incorporation method (Bååth 1994) is similar to the thymidine incorporation method and has similar drawbacks. However, the measurement of protein synthesis is more accurate than the measurement of DNA synthesis due to the relatively high protein content of cells. In addition, few microbial species have been found that are unable to take up leucine (Bååth 1998).

Recently, these thymidine and leucine incorporation techniques have been adapted to study the tolerance of soil microbial communities to heavy metals. Microbial growth rates determined with the thymidine and leucine incorporation techniques were found to be sensitive to heavy metal pollution in short-term experiments (Rajapaksha et al. 2004). However, this was not the case in some long-term experiments performed under laboratory conditions (Díaz-Raviña and Bååth 1996b; Rajapaksha et al. 2004) or under field conditions (Bååth et al. 2005).

The application of these techniques in soils that were experimentally polluted with metals indicates that the tolerance of the microbial community may change with time. It has been suggested that there is an immediate increase in tolerance due to the initial toxicity, followed by a more gradual increase due to the different competitive abilities of the surviving bacteria and/or their different physiological and/or genetic adaptations (Díaz-Raviña and Bååth 2001).

9.5.5 Fungal Acetate in Ergosterol Incorporation

Ergosterol is a specific component of fungal cell membranes and has been traditionally determined and used as an index of fungal biomass in soil (Djajakirana et al. 1996; Chander et al. 2001). Thus, fungal activities and growth rates in soils have been mainly estimated by the acetate-in-ergosterol incorporation technique (Bååth 2001). However, since oomycetous fungi and a number of yeast do not produce ergosterol (Stahl and Parkin 1996), and ergosterol content can vary depending on species, nutritional status and growth stage, the use of ergosterol as a fungal biomarker has become rather controversial.

This method has recently been applied to evaluate the effect of metal contamination on soil fungi. Rajapaksha et al. (2004), in a study to evaluate the different effects of heavy metals on soil fungi and bacteria, found a short-acting negative effect on bacterial activity (measured by the thymidine incorporation technique),

but an increase in the fungal activity within a few days after metal addition. These authors suggested that fungal growth, like that of many soil microorganisms, is carbon limited, and the extra carbon released from dead bacteria triggers an increase in fungal growth, overriding the possible negative effect of the presence of heavy metals. Bacteria, which were negatively affected by the metals, apparently could not take advantage of this extra carbon.

9.6 Microbial Diversity

Microbial diversity refers to the complexity and variability at different levels of biological organization. This term includes the genetic diversity – the amount and distribution of genetic information within microbial species; the diversity of bacterial and fungal species in microbial communities; and the ecological diversity – the variation in community structure, the complexity associated with interactions, the number of trophic levels, and the number of guilds. Measures of microbial diversity should include multiple methods and integrate holistic measures at the community level and partial approaches that target structural or functional subsets. Diversity can also be considered the amount and distribution of information that is directly applicable to the total genetic diversity or complexity in a community. Equations used to calculate richness and evenness and diversity indices that combine both have been discussed by Kennedy and Smith (1998).

The microbial diversity associated with soil ecosystems far exceeds the corresponding diversity of eukaryotic organisms. One gram of soil may harbor up to ten million microorganisms deriving from possibly thousands of different species (Roselló-Mora and Amann 2001). However, as less than 1% of the microorganisms observed under the microscope has been cultivated and characterized, soil ecosystems are largely uncharted.

The huge diversity of the uncultured microorganisms in soil has stimulated the development of methods for studying soil microbial communities. Broad-scale analysis of community DNA, using techniques such as DNA reassociation, provides information on the total genetic diversity of a given bacterial community. A shift in guanine + cytosine (GC) content can be used to detect changes in microbial community structure, but it does not tell us anything about the other diversity parameters: richness, evenness and composition. However, fingerprinting techniques such as phospholipid fatty acid (PLFA) analysis, denaturing gradient gel electrophoresis (DGGE), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), and ribosomal intergenic spacer analysis (RISA) provide information on the species composition and can be used to compare common species in samples. All of these methods are still being modified to improve their reproducibility, sensitivity, and relevance or to reduce the costs of analysis.

9.6.1 Membrane Lipids

Membrane lipids and their associated fatty acids are particularly useful biomarkers of the microbial community as they are essential components of every living cell and exhibit a large structural diversity coupled with a relatively high biological specificity.

The use of these compounds to identify microorganisms in situ is particularly appealing, since the same compounds are used extensively in bacterial taxonomy and hence there is a constantly growing database that can be used to interpret biomarkers. In addition, phospholipids are not found in storage products and are thought to be present only in viable microorganisms, because they are associated with the membranes of living cells and are believed to break down rapidly when the cells die. However, while Petersen et al. (1991) found a reduction in phospholipids during chloroform fumigation, this reduction was only 21–54% of their concentration before fumigation, indicating incomplete destruction of the phospholipid after cell death (supporting the findings of Tollefson and McKercher (1983)), although the authors speculated on the possibility that the chloroform did not kill all of the cells.

The PLFA technique involves extracting lipids from the soil with organic solvents based on the protocol reported by Bligh and Dyer (1959), followed by the separation of phospholipids from other lipids according to their polarities using solid phase extraction. The phospholipid fatty acids are then converted to fatty acid methyl esters (FAMEs), which are analyzed by gas chromatography in order to determine the types present and the quantities of each.

The PLFA extraction is time consuming and does not lend itself to practical environmental monitoring. In contrast to PLFA, a simpler method based on the direct extraction of fatty acids (MIDI 1995) can be used, which was originally designed to extract fatty acids and identify pure cultures of bacteria. In this method, the microbial cells in the soil are saponified by heating them in a strong aqueous alkali. Several studies have compared the MIDI and PLFA methods (Petersen et al. 2002; Steger et al. 2003; Drenovsky et al. 2004) and found that, although both extraction methods are able to differentiate between microbial communities that have undergone contrasting treatments, the MIDI method included a significant background of nonmicrobial material. Because of this concern, a less harsh method for the direct extraction of ester-linked fatty acid (ELFA) from soil has been developed, where a mildly alkaline reagent is used to lyse cells and release fatty acids as methyl esters from lipids. This method has been evaluated and successfully used to evaluate microbial communities in heavy metal polluted soils (Hinojosa et al. 2005).

Changes in fatty acid profiles are generally related to variations in the abundances of microbial groups. Thus, although analyzing fatty acids from soil does not permit detection at the species level, it can give an overall picture of the community structure and provide an estimate of overall changes.

The total amount of fatty acids has been used to assess changes in the microbial biomass in heavy metal polluted soils (Frostegård et al. 1991; Pennanen et al. 1996;

Hinojosa et al. 2005), as good correlations have generally been found between the total amount of phospholipids and the microbial biomass determined by other methods.

Species richness, evenness, and usually the Shannon–Weaver diversity index have also been calculated from fatty acid data for soil affected by metal pollution. These data can provide information on broad-scale changes in relative abundances and the dominance of certain microbial groups due to heavy metals. However, it cannot be used to measure species or genetic diversity. On the other hand, although a marker fatty acid may be abundant in one group of organisms, its concentration is often variable, making biomass calculations difficult. Moreover, some fatty acids used as specific markers for one group of organisms may occur in other groups in variable concentrations (Zelles 1997). For example, monounsaturated fatty acids (MUFAs) can occur in both Gram-negative and Gram-positive bacteria, but their relative contributions to the total fatty acid content in Gram-positive bacteria are typically very small. Thus, MUFAs can be used as general biomarkers for Gram-negative bacteria (Ratledge and Wilkinson 1988). On the other hand, since the outer membranes of Gram-negative bacteria are largely composed of lipopolysaccharides, which consist mainly of hydroxy-substituted fatty acids (OHFAs), it has been suggested that OHFAs could be used as a general indicator for Gram-negative bacteria in environmental samples. However, we should note that these fatty acids have also been found in Gram-positive bacteria, fungi, and plants (Zelles 1997). Different stress conditions, including heavy metal toxicity, have increased the abundance of Gram-negative bacterial fatty acids, and concomitantly decreased those in Gram-positive bacteria (Frostegård et al. 1993; Hinojosa et al. 2005). The enhanced survival of Gram-negative bacteria under stress conditions may be attributed to the presence of cyclo fatty acids in their membranes (also used as markers) and their outer lipopolysaccharide layers, which can resist the stress better. Furthermore, Gram-negative bacteria are considered to be fast-growing microorganisms that utilize a variety of C sources and can adapt quickly to a variety of C sources and environmental conditions (Ponder and Tadros 2002). Evidence for a similar shift in the response of the bacterial community to metal pollution has been found in studies reported by Hiroki (1992) and Frostegård et al. (1993).

However, an increase in the fatty acids commonly found in Gram-positive bacteria has also been observed in acidified soil polluted with heavy metals (Pennanen et al. 1996).

Bacterial "biomass" has been estimated by combining several fatty acid markers of both Gram-positive and Gram-negative bacteria (Frostegård and Bååth 1996), whereas polyunsaturated fatty acids (represented mainly by 18:2ω6,9c) are associated primarily with fungi (Guckert et al. 1985).

In general, fungi appear to be more tolerant to heavy metals than bacteria (Doelman 1985; Frostegård et al. 1993). Nevertheless, a fungal marker decrease has been reported in field studies such as those of Pennanen et al. (1996) and Hinojosa et al. (2005). In the study of Pennanen et al. 1996, an assumption that fungal biomass was reduced due to heavy metal pollution was supported by microscopic measurements of fungal lengths and ergosterol content. The decrease in

fungal PLFAs was partially explained by Cu, a common fungicide. Additionally, branching organisms are associated with larger pores and aggregates, which could make them more vulnerable to the soluble fractions of metals. Another factor is that the contact of hyphae with the metal pollutant likely affects the whole organism and thus a large biomass. This decrease in the amount of 18:2ω6,9 was also attributed to a decrease in ectomycorrhizal hyphae, which in turn could be linked to damage to the fine roots because of pollution, as found by Helmisaari et al. (1995). These discrepancies in the effects of metals on fungi may also be explained by differences between field and laboratory studies (Rajapaksha et al. 2004).

The fatty acid 16:1ω5c, known to be a major component of arbuscular mycorrhizal fungi (Haack et al. 1994), has also been proposed as a valuable biomarker. However, this fatty acid is also produced by a select group of bacteria that includes *Cytophaga*, *Flavobacterium*, and *Flexibacter*. This fatty acid has been reported to decrease in response to heavy metal pollution (Frostegård et al. 1993; Hinojosa et al. 2005) and to increase after performing several types of restoration method (Frostegård et al. 1993; Hinojosa et al. 2005). Thus, 16:1ω5c appears to be particularly responsive to environmental changes, and may be a good indicator of changes in microbial community structure.

The fungal-to-bacterial ratio can be determined directly from measurements of fungus-specific and bacterium-specific fatty acids, and has also been used as an index of the relative abundances of these two main groups of microbial decomposers in polluted soils (Frostegård and Bååth 1996; Hinojosa et al. 2005).

The fatty acids 10Me16:0, 10Me17:0 and 10:Me18:0 are relatively common among studied species of Actinomycetales (Kroppenstedt 1992), and have been used as a marker for this group of microorganisms. However, results in the literature indicate that different actinomycetes respond differently to elevated heavy metal levels (Hiroki 1992). Despite the branching nature of actinomycetes, which could make them more susceptible to heavy metals, their responses to heavy metal pollution can vary greatly according to the soil type and the particular type of pollution, as reflected in the mixed response of this microbial group in studies performed so far.

The PLFA composition of microorganisms is also known to vary to some degree in response to environmental conditions such as chemical stress (Vestal and White 1989; Haack et al. 1994). The isomerization of *cis*-unsaturated fatty acids (16:1ω7c, 18:1ω7c) to *trans*-unsaturated fatty acids (16:1ω7t, 18:1ω7t) is one such adaptation mechanism that is induced by environmental stress (Guckert et al. 1985). In pure culture studies, the *trans*/*cis* ratio of unsaturated fatty acids exhibited a strong increase at toxic metal concentrations (Heipieper et al. 1996). Similarly, in soil incubation studies, an increase in the *trans*/*cis* ratio of 16:1ω7 in the presence of different metals has been documented (Frostegård et al. 1993). The mode of action of heavy metals is still not understood, but they seem to interact with the microbial membrane fatty acids, disturbing their conformations. Thus, for example, the initiation of the *cis*/*trans* isomerization system in response to the heavy metal allows

microorganisms to counteract stress because the *trans*-unsaturated fatty acids are more stable than their *cis* counterparts.

Cyclopropyl fatty acids have also been shown to increase relative to their monoenoic precursors during prolonged stationary growth phases of some bacteria, and during growth under low carbon and oxygen concentrations, low pH, and high temperature (Guckert et al. 1985; Ratledge and Wilkinson 1988). Thus, increases in cy17:0 and cy19:0 relative to their respective metabolic precursors (16:1ω7c and 18:1ω7c, respectively) may indicate physiological stress due to heavy metal pollution (Frostegård et al. 1993). The transformation of *cis* double bonds into a cyclopropane ring restricts overall mobility, which helps to reduce the impact of environmental stress on membrane fluidity.

Given the caveats mentioned above regarding the interpretation of fatty acid markers, whole fatty acid profiles are generally compared among samples using multivariate statistical techniques. These comparisons reflect differences in community composition due to different types and degrees of perturbations such as elevated levels of heavy metals. Various ordination techniques (e.g., principal components analysis, correspondence analysis, nonmetric multidimensional scaling) have been used to identify structure in the data set and to test hypotheses about both the structure and the underlying environmental variables related to this structure. These multivariate statistics have discriminated shifts in microbial community structure in soils polluted with metals (e.g., Frostegård et al. 1993; Hinojosa et al. 2005). The advantages and limitations of each approach should be considered when selecting the most appropriate statistical techniques (Rencher 2002).

9.6.2 Nucleic Acid Based Methods

The genetic diversity of soil microorganisms is an indicator of genetic resources. Among the various nucleic acid techniques used to estimate microbial community composition and diversity in complex habitats, the most useful involves determining the sequences of 16 S ribosomal RNA (rRNA) genes (i.e., encoded by rDNA) in prokaryotes and 5 S or 18 S rRNA genes in eukaryotes.

These techniques are particularly well suited to such studies for a number of reasons:

- They are found universally in all three forms of life: Bacteria, Archaea, and Eucarya.
- These molecules are composed of both highly conserved regions and regions with considerable sequence variation.
- The phylogenetic information held in the SSU rDNA molecule is further enhanced by its relatively large size and the presence of many secondary structural domains.

Consequently, evolutionary changes in one domain do not affect the rates of change in other domains.

- They are easily amplified using the polymerase chain reaction (PCR), and rapidly sequenced.

Soil DNA extraction techniques mainly involve either separating the cells from the soil and then performing the DNA extraction or using a direct lysis approach for extracting DNA from cells in the soil. The direct lysis approach is now generally preferred because it gives a higher DNA recovery, the DNA is thought to be more representative of the entire community, and progress has been made in purifying the DNA of coextracted humic compounds. Though the majority of the work in this area has been done on agricultural soils, DNA extraction and purification protocols have also been developed and tested for heavy metal polluted soils (Kozdrój and van Elsas 2000; Fortin et al. 2004).

Once the DNA has been extracted from the soil, methods that can be used in relation to molecular microbial ecology include cloning and sequence analysis of rRNA genes (to yield clone ''libraries'') as well as a number of fingerprinting approaches for rapidly comparing communities. Both of these techniques rely on the use of the polymerase chain reaction (PCR) to amplify (make multiple copies of) a particular region of DNA so that enough material is available for subsequent analysis. However, the PCR amplification can be inhibited when contaminants have not been removed beforehand, and preferential or selective amplification in the presence of DNA from mixed communities can occur. PCR-based techniques also present other drawbacks; for example, the relative proportions of amplified sequences from different species may not reflect those of the original sample.

Fingerprinting techniques that provide a rapid assessment of a microbial community are particularly useful for monitoring soil health. In this case, the PCR-amplified genes of organisms within a community are separated based on length or sequence polymorphism, which produces a visual pattern – or fingerprint – of the community. PCR-based community fingerprinting techniques have several advantages: (a) they are rapid and allow parallel analyses of multiple samples; (b) they are reliable and highly reproducible, and; (c) they provide both qualitative and quantitative information on populations within a community.

Genetic fingerprinting methods include, among others, denaturing and temperature gradient gel electrophoresis (D/TGGE), ribosomal intergenic spacer analysis (RISA), and terminal restriction fragment length polymorphism (T-RFLP). The results obtained from these analyses can then be expressed as matrices of presence, intensity or similarity, and groups of samples with similar microbial structure can be identified by clustering or ordination analyses.

Clone libraries are generally used to identify soil microorganisms. This approach involves cloning (inserting DNA into a bacterial plasmid) a large number of PCR-amplified genes, determining the DNA sequences of these cloned fragments, and comparing the sequences with those of known organisms in a large public database (such as GenBank), as the number of rRNA gene sequences that can be used for comparison is constantly growing.

Applications of some of these techniques to evaluate the effects of heavy metal pollution in soil microbial communities are discussed below.

9.6.2.1 Percentage of Guanine–Cytosine and DNA Reassociation Techniques

Low-resolution methods include analyzing the base distribution of the DNA and determining the rate at which denatured single-stranded DNA reassociates (Torsvik et al. 1994). The base distribution of the DNA [mol% guanine + cytosine (%G + C)] can be determined by thermal denaturation due to the fact that single-stranded DNA has a higher absorbency than double-stranded DNA at 260 nm (Torsvik et al. 1996). Despite the fact that this is considered to be a low-resolution analysis, it has been used to indicate overall changes in microbial community structure, especially in soil samples with low diversity. The major limitation of such analyses is that there is not a clear-cut relationship between base composition and species composition; thus, two communities with similar base distributions do not necessarily have similar species compositions, since different species often have the same base composition. On the other hand, when communities have different base distributions, this method provides strong evidence that they have different species compositions. The main advantage of this approach is the possibility of detecting and analyzing microorganisms that are not identified by PCR.

The DNA reassociation technique, which measures the genetic complexity of the microbial community, has also been used to estimate microbial diversity (Torsvik et al. 1996). In this case, the total DNA is extracted from natural soil samples, purified, denatured, and allowed to reanneal. The rate of hybridization or reassociation will depend on the similarity of the sequences present. As the complexity or diversity of the DNA sequences increases, the rate at which the DNA reassociates will decrease. Under specific conditions, the time taken for half the DNA to reassociate (the half-association value $C_0t_{1/2}$) can be used as a diversity index, as it takes both the amount and the distribution of DNA reassociation into account (Torsvik et al. 1998). Determining the sequence complexity of DNA, as measured by reassociation, provides a better assessment of the total microbial diversity in soil than the %G + C technique.

Sandaa et al. (1999) studied the microbial diversity, as determined by both the %G + C and DNA reassociation techniques, of field soils amended with "uncontaminated" sewage sludge, and the results were compared with those obtained for field soils treated with metal-amended sewage sludge at two rates of application (low and high metal contamination) for several years. They found no differences in the %G + C profiles of the bacterial communities of these soils. However, DNA reassociation analysis indicated a dramatic decrease in bacterial diversity from 16,000 bacterial genomes (g soil [wet wt]) in the uncontaminated soil to 6,400 bacterial genomes (g soil [wet wt]) in soil with low metal amendments, and only 2,000 bacterial genomes (g soil [wet wt]) in soil with high metal.

Recently, Gans et al. (2005) applied sophisticated computational methods to reanalyze the reassociation kinetics for bacterial community DNA using the original data from Sandaa et al. (1999), and estimated that one million distinct genomes occurred in the pristine soil – exceeding previous estimates by two orders of magnitude. Furthermore, they found that metal pollution reduced diversity by more than 99.9%, thus revealing a highly toxic effect of heavy metals, especially on rare taxa. These results have been criticized by Volkov et al. (2006) and Bunge et al. (2006), who suggested that the studies of Gans et al. (2005) do not allow us to conclude that metal pollutants have such a devastating effect on microbial diversity.

9.6.2.2 Denaturing and Temperature Gradient Gel Electrophoresis (DGGE/TGGE)

Among the intermediate-resolution techniques, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) have been widely used to characterize microbial communities in heavy metal polluted soils (Sandaa et al. 1999; Li et al. 2006). These techniques are able to separate mixtures of PCR products that are similar in length but differ in their sequences. The separating power of each technique depends on the melting behavior of the double-stranded DNA molecule. As the DNA molecules are electrophoresed they remain double-stranded until they reach the denaturing concentration or temperature that melts the double-stranded molecule. Since the melting behavior is largely dictated by the nucleotide sequence, the separation yields individual bands, each corresponding to a unique sequence. DNA with many pairs of guanine and cytosine nucleotides melt less easily than those with many adenine and thymidine bases.

However, DGGE/TGGE techniques can underestimate the microbial diversity because bands from more than one species can appear as a single band (Heuer et al. 1997). Soil communities can easily contain several hundred bacterial strains, which contrasts with the relatively low resolution of the gel (typically less than 50 bands, and so the bands represent the predominant microbial populations). These techniques are thus highly recommended for communities with low to moderate complexity.

DGGE/TGGE techniques, when applied to heavy metal pollution, have revealed changes in community structures of and reduced numbers of bands for eubacteria, β-proteobacteria, and ammonia-oxidizing bacteria present in environments with elevated heavy metal levels (Gremion et al. 2004). However, other studies have shown that the numbers of bands are independent of the level of contamination with heavy metals (Kozdrój and van Elsas 2001). For example, Sandaa et al. (1999) revealed differences in community structure in soils with increasing heavy-metal contamination; however, while the control soils were characterized by twelve bands, low-metal soils gave patterns with six bands, and soils with a high level of heavy metals gave eleven bands. On the other hand, Anderson et al. (2008) found (using DGGE analysis) that soil fungi did not appear to be affected after the addition of heavy metal containing sewage sludge at levels up to the current UK legislated limit for Cd and Zn, and at levels well above the current legislative limit for Cu.

The main advantages of DGGE/TGGE are that it enables spatial/temporal changes in microbial community structure to be monitored and that it provides a simple view of the dominant microbial species within a sample. In addition to the simplicity and rapidity of these methodologies, the identities of bands can be investigated via hybridization with specific probes or by extraction and sequencing.

9.6.2.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) is a powerful tool for microbial identification and classification, even at species level, and it has been used to group and classify large sets of isolates and clones. Automated ARDRA has been performed with fluorescent PCR amplicons obtained by incorporating fluorescently labeled dUTP during PCR. After restriction enzyme digestion, the fragments are separated on an automated DNA sequencing gel. Different DNA sequences result in a unique profile of the analyzed community. The restriction pattern data can then be compared with the results of restriction analyses of rDNA sequences of known bacteria obtained using database sequences. ARDRA is a simple, rapid, and cost-effective technique that can be useful for detecting structural changes in simple microbial communities, but is unable to measure microbial diversity or to detect specific phylogenetic groups within a community fingerprinting profile.

This technique has been widely used to evaluate changes in microbial community structure (mainly bacteria). For example, Smit et al. (1997) and Torsvik et al. (1998) found distinct differences in microbial community structure in soil contaminated with heavy metals compared to uncontaminated soil. In a recent study, Pérez De Mora et al. (2006) evaluated the effects of the in situ remediation of a heavy metal contaminated soil on microbial structural diversity after 18 months. Results revealed differences in both bacterial and fungal community structures as a consequence of the various treatments assayed. However, different results were obtained with the two restriction enzymes employed in the bacterial and fungal fingerprinting patterns.

9.6.2.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (TRFLP) is a modification of ARDRA. TRFLP analysis is based on the restriction enzyme digestion of PCR-amplified DNA that has been fluorescently labeled at one end. Fragments are resolved by size on polyacrylamide gels using an automated analyzer with laser detection of the terminally labeled products, producing a highly reproducible fingerprint of the community.

The use of fluorescently tagged primers limits the analysis to only the terminal fragments of the digestion. This simplifies the banding patterns, thus allowing for the analysis of complex communities as well as providing information on diversity, as each visible band represents a single operational taxonomic unit or ribotype

(Tiedje et al. 1999). The banding pattern is used to measure species richness and evenness, as well as similarities between samples (Fig. 9.4).

Moreover, T-RFLP can be automated to enable the analysis of a large number of soil samples (Osborn et al. 2000). However, T-RFLP requires expensive equipment

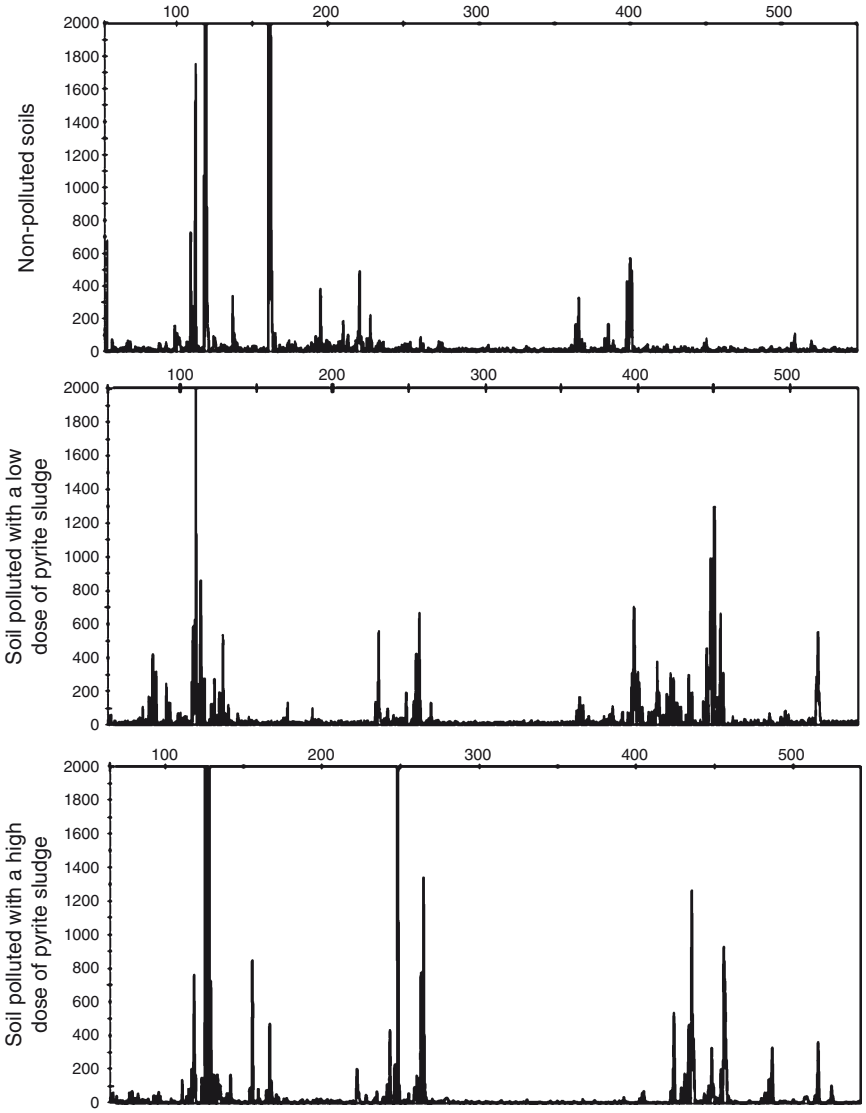

Fig. 9.4 T-RFLP profiles obtained with bacterial (16 S) amplicons from soil polluted with different doses of (heavy metal enriched) pyrite sludge. In the electropherograms, the *x*-axis marks the size of the fragment while the *y*-axis marks the fluorescence intensity of each fragment. T-RF fragment size (OTUs) and abundance change as the heavy metal concentration in the soil increases, implying gradual changes in the soil bacterial community

and has high running costs, both of which limit its routine use in ecological assessments. In addition, although the species corresponding to each profile can be inferred, it is not possible to directly clone the DNA bands of interest. Despite these limitations, T-RFLP is very useful tool for studying soil microbial diversity (Liu et al. 1997; Tiedje et al. 1999; Osborn et al. 2000). Recently, TRF patterns have been analyzed by matching the resulting profile with an existing database of restriction patterns. However, the association of sequenced clones with patterns in a database is problematic, since related organisms commonly produce TRFs of the same length, requiring several enzyme digests to distinguish among similar community members. In any case, the potential of the T-RLFP method to discriminate between soil microbial communities in polluted and unpolluted soils has been demonstrated. The T-RFLP approach has been applied to a number of marker genes and has been shown to be a powerful method for describing differences and changes in bacterial (Turpeinen et al. 2004, Hartmann et al. 2005; Lazaro et al. 2008) and arbuscular mycorrhizal fungi (Tonin et al. 2001) communities in heavy metal polluted soil.

Singh et al. (2006) developed and validated a rapid and sensitive method that could be used to simultaneously analyze communities of several microbial taxa with one PCR. They called this multiplex terminal restriction fragment length polymorphism (M-TRFLP). Recently, Macdonald et al. (2008) used M-TRFLP to assess the impact of heavy metal contamination on bacterial, fungal, and archaeal communities simultaneously, and T-RFLP to assess the impact of this contamination on actinobacterial populations. They showed that such contamination is responsible for population shifts, and that various genotypic markers (as represented by individual TRFs) within a community differ in their levels and directions of response to increasing metal contamination.

9.6.2.5 Ribosomal Intergenic Spacer Analysis (RISA)

RISA allows ribosomally based fingerprinting of the microbial community. In this method, the intergenic spacer (IGS) region that is located between the small (16 S) and large (23 S) rRNA subunit genes is amplified by PCR, denatured, and resolved by polyacrylamide gel electrophoresis under denaturing conditions. The IGS region varies in both sequence and length (50–1,500 bp) depending on the species, and this unique feature facilitates taxonomic identification of organisms (Spiegelman et al. 2005). In RISA, sequence polymorphisms are detected by silver staining, whereas the forward primer is fluorescently labeled and automatically detected using the automated RISA method (ARISA). Both methods provide highly reproducible bacterial community profiles, but ARISA increases the sensitivity of the method and reduces the time needed to perform it. RISA is a very rapid and simple rRNA fingerprinting method, but its applicability to microbial community analysis from contaminated sources is limited, partly due to the limited database for ribosomal IGS, which is not as large or as comprehensive as the 16 S sequence database (Spiegelman et al. 2005). As a result, community analysis using RISA could reduce

its effectiveness at identifying unknown or nonculturable microbial species from contaminated sources. Furthermore, RISA sequence variability may be too great for environmental applications. Its level of taxonomic resolution is greater than 16 S rRNA, which can lead to very complex community profiles.

Ranjard et al. (2006) evaluated the short-term effects of single and combined additions of copper, cadmium, and mercury at different doses on soil bacterial community structure using the bacterial automated ribosomal intergenic spacer analysis (B-ARISA) fingerprinting technique. Their results suggested that there was no simple negative correlation between pollution stresses and genetic diversity in microbial communities. Thus, it is assumed that the increase in stress reduced the innate competitive exclusion of populations and induced the enrichment and predominance of other types of populations, leading to a potential increase in diversity that was followed, when the stress reached a critical level, by the progressive extinction of organisms, leading to a loss of diversity. Gleeson et al. (2005) also demonstrated that an elevated heavy metal concentration had a profound impact on bacterial community structure, and found strong relationships between certain ribotypes and particular chemical/heavy metal elements.

9.6.2.6 Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) allows the direct identification and quantification of specific and/or general taxonomic groups of microorganisms within their natural microhabitats. In FISH, whole cells are fixed, their 16 S or 23 S rRNA is hybridized with fluorescently labeled taxon-specific oligonucleotide probes, and then the labeled cells are viewed by scanning confocal laser microscopy. This technique allows artifacts arising from biases in DNA extraction, PCR amplification, and cloning to be avoided. FISH can detect microorganisms across all phylogenetic levels, whereas immunofluorescence techniques are limited to the species and subspecies levels. In addition, scanning confocal laser microscopy surpasses epifluorescence microscopy in sensitivity and has the ability to view the distributions of several taxonomic groups simultaneously as a three-dimensional image. The community structures of soils contaminated with low and high levels of metal have been investigated by hybridization with group-specific phylogenetic probes (α-proteobacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria, *Cytophaga-Flexibacter-Bacteroides*, Gram-positive bacteria with a low mol %G+C, Gram-positive bacteria with a high mol %G+C) (Sandaa et al. 1999, 2001). The most abundant group of clones in the soil contaminated with a low level of metal was the *Cytophaga-Flexibacter Bacteroides* group. This group was twice as abundant in the low-level compared to the high-level contaminated soil. In the soil contaminated with a high level of metal, clones belonging to the α-proteobacteria were numerically dominant. With respect to the isolates, 30–37% of them belonged to Gram-positive bacteria with low mol %G+C. In the soil contaminated with a high level of metal, the abundances of isolates and clones belonging to the α-proteobacteria subclass differed markedly, as the percentage of clones was 38%

and that of isolates was only 14%. Analysis with FISH also has been extensively used in other types of studies, such as those aiming at the identification of acidophiles in bioleaching operations or AMD in metal mines (Espejo and Romero 1997; Norris et al. 1996).

9.7 Functional Diversity and Community-Level Physiological Profiles (CLPPs)

While molecular (genetic) or biochemical (phenotypic) diversity measurements have their place and generally measure the diversity of the numerically dominant members of the community (Loisel et al. 2006), functional diversity provides information on the functioning of the members of the soil microflora involved in the carbon cycle. Any loss in ability of the microbial community to maintain its wide range of functions is a warning sign of decreased soil health. Several approaches to measuring the physiological profile at the community level (CLPPs) have been devised (Biolog, Multi-SIR, and more recently the MicroResp™ method).

The original method was based upon the Biolog plate, with its range of 95 carbon substrates (Garland and Mills 1991). In this method, an extract of the soil is obtained, suitably diluted, and then inoculated into a microtiter plate (such plates are commercially available and come preloaded with buffer, substrate, and a tetrazolium dye). Incubation over 24–72 h results in the growth of the microbial population, the utilization of the carbon substrate, and the reduction of the dye to produce a red–violet coloration, which is then read on a standard laboratory microplate reader. Several variants are available containing different sets and numbers of carbon sources.

Many studies on the effects of heavy metals on the physiological profile at the community level have employed this technique. For example, Rutgers et al. (2003) recently found a significant correlation between a decaying ecosystem in a heavily contaminated (by nickel and copper) environment and the enhanced degradation of amines, amides, and amino acids in Biolog plates for both the bacterial and fungal communities.

The Biolog assay has been shown to be more sensitive to the impact of sewage sludge amendment of soil than microbial biomass and respiration measurements (Burgess et al. 2001). The utility of the assay has been extended through the use of the pollution-induced community tolerance (PICT) approach, where a range of concentrations of a specific heavy metal can be added to the plate and the tolerance of the community estimated (Rutgers and Breure 1999). The PICT concept is based on the assumption that the proportion of species tolerant to the pollutant increases at contaminated sites, resulting in increased community tolerance. This increased community tolerance indicates that the pollutant has a damaging effect. In tolerant communities, the diversity may decrease and the tolerant species are not always able to perform the same ecological functions as the sensitive ones (Almås et al. 2004).

Since the Biolog method essentially only targets the small fraction of the microbial community that can grow in the microtiter plate wells; Campbell et al. (2003)

recently developed MicroResp™, which was designed to be a "whole soil" technique. Briefly, the soil is placed within the wells of a microtiter deep-well plate, and the carbon substrate is added in solution. The plate is then closed such that it is face-to-face with a detection microtiter plate, with contact between them. The detection plate contains an indicator dye within a bicarbonate-buffered gel that responds to the pH change within the gel resulting from the evolution of carbon dioxide from the soil. Changes in color are read after incubation on a standard laboratory microplate reader.

This method was compared to the Biolog using soils treated with sludge enriched in heavy metal (Campbell et al. 2003). They found that the whole-soil method was more rapid and detected C source use earlier. In addition, the methodology discriminated well between different sludge treatments. A variant of this method uses radioactive substrates and the specific measurement of $^{14}C–CO_2$ as the detection system.

9.8 Resilience of Microbial Function and Community Structure

In sustainable ecosystems, structure and function remain within a characteristic ranges over time, even in the face of natural disturbance. If a stress or disturbance does alter the ecosystem, it should be able to bounce back quickly. In this sense, soil resilience and resistance are important components of soil quality/health, and key elements of sustainability theories (Seybold et al. 1999).

Soil resilience has been defined as the capacity of a soil to recover its functional and structural integrity after a disturbance, whereas soil resistance is the ability of a soil to continue to function without major changes when stressed (Fig. 9.5; Seybold et al. 1999). However, an accurate definition of soil resilience is difficult because of the dynamic, variable, and heterogeneous nature of the soil system. Seybold et al. (1999) pointed out that soil resilience depends on soil type (including soil biota), vegetation, climate, land use, and the temporal as well as spatial scales at which the assessment is performed, as reflected in Chapin's model, shown in Fig. 9.1.

A number of features of microorganisms, in particular bacteria and archaea, suggest that resilience is rather common. First, many microorganisms show high growth rates; thus, if their abundances are suppressed by a disturbance (e.g., heavy metal pollution), they have the potential to recover quickly. Second, many microbes have a high degree of physiological flexibility. Thus, even if the relative abundances of some taxa decrease initially, these taxa may physiologically acclimatize to the new conditions over time and return to their original abundance. Finally, if physiological adaptation is not possible, then rapid evolution (through mutations or horizontal gene exchange) could allow microbial taxa to adapt to new environmental conditions and recover from disturbances. All of these arguments assume that abundance is reduced by a disturbance, but some microbial taxa may benefit from the new conditions and increase in abundance. Allison and Martiny (2008) reviewed

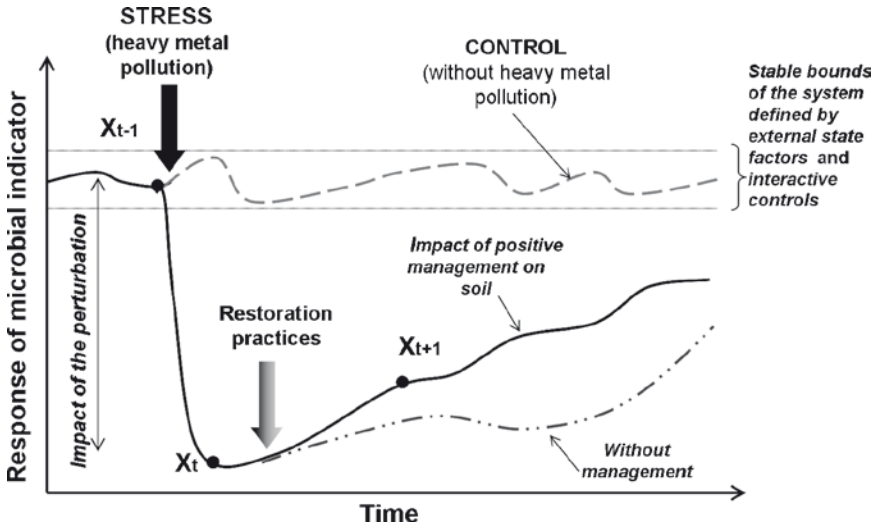

Fig. 9.5 Graph illustrating the effect of a disturbance (heavy metal pollution) on soil microbial indicators, and their abilities to recover afterwards, with and without restoration management practices

recent experiments and assessed whether microbial community composition is resistant, resilient, or functionally redundant in response to different disturbances (including heavy metal pollution). They found that the compositions of most microbial groups were sensitive and not immediately resilient to disturbance, regardless of the taxonomic breadth of the group or the type of disturbance.

With the advent of the broad set of techniques that are currently available, as described in this chapter, a whole range of new options have become available for studying microbial resilience in situ. Some resilience studies have focused on microbial communities and measured total microbial diversity or microbial community composition in relation to a specific disturbance in the soil. In some cases, such as Griffith et al. (2001), a positive link between biodiversity (PLFAs and DGGE) and functional resilience (decomposition rate, nitrification, respiration, etc.) was observed. Similarly, Tobor-Kaplan et al. (2006) studied the effect of an additional stress on the changes in microbial communities that had been exposed for more than 20 years to different levels of copper or low pH. They showed that the effect of the additional stress was highly dependent on the type of stress, and that highest resistance and/or resilience occurred in the least contaminated soils. However, these studies were conducted at the total microbial community level, whereas it has been suggested that the impact of stress is less pronounced at the functional group level than at the species level (Schindler 1990; Vinebrooke et al. 2003). For example, Philippot et al. (2008) recently showed a higher resilience of nitrate reduction activity to mercury stress in microcosms pre-exposed to copper. However, no significant effect of copper on the response of the genetic structure of the nitrate reducers to mercury contamination was observed, indicating that the

activity and structure of a given functional community may differ in stability. Similarly, Griffiths and Hallett (2005) showed that contamination with zinc increased resilience to copper stress. An increased tolerance of metals other than the metal originally added to the soil was also observed by Díaz-Raviña and Bååth (1996a), indicating the existence of multiple heavy metal tolerances at the community level. It was suggested that this increased tolerance was due to physiological and/or genetic adaptation. All of these results suggest that the effect of an initial stress on the response of the microbial community to an additional stress is complex and will depend on the relatedness of the two consecutive stresses and on the development of positive cotolerance.

These responses beg the question of whether compositional shifts will affect ecosystem processes and whether the disturbed community will be functionally similar to the original community. There are two reasons why changes in microbial composition may not affect ecosystem process rates. First, the new community may contain taxa that are functionally redundant with respect to the taxa in the old community. Second, taxa in the new community may function differently but result in similar process rates when combined at the community level.

Functional redundancy is difficult to establish because it requires detailed knowledge about the microbial populations that perform a specific process. Furthermore, organisms that are functionally redundant under one set of conditions may not be under different conditions. In general, we know little about the distribution of functional traits across microbial taxa despite years of recognition of this need. Newly emerging techniques such as stable isotope probing (SIP) and microautoradiography fluorescent in situ hybridization (MAR-FISH) or catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH), which are specifically aimed at linking identity to function, can also help to answer some of the open questions.

9.9 Conclusions: Drawbacks and Challenges for the Future

- Despite their importance to the functioning of ecosystems, microorganisms are rarely considered explicitly in individual ecosystem or global process models. In addition to methodological hurdles, one primary reason for this gap is their overwhelming diversity and a lack of knowledge about how this diversity is related to ecosystem processes. Moreover, it is unfeasible to assess and track each microbial taxon in an ecosystem, let alone include even a small fraction of these taxa in ecosystem models. Because of these obstacles, ecosystem models often ‘’blackbox’’ microbiology. In other words, microorganisms are buried within the structure of the equations as kinetic constants and response functions, and are ‘’simplified beyond recognition.’’ As a result, the abundance, diversity, and interactions of microorganisms are often assumed to be unimportant to ecosystem processes, particularly in terrestrial ecosystem models.

- The application of local, regional, national or international standards for total heavy metal concentrations of soils may not reflect the overall functional status of polluted soils. When evaluating not only the impact but also the recovery of soils after a disturbance event such as heavy metal pollution, soil microbial properties are reliable indicators of the extent of soil recovery.
- The lack of standard analytical methods accepted by all laboratories is a fundamental problem when interpreting data on microbial and biochemical properties. Differences in sample collection, storage, pretreatment, and protocols for determining microbial function and community structure (in which temperature, substrate concentration, incubation time, etc. are crucial) make it practically impossible to compare data obtained from different studies. Moreover, we should take into account the high degree of variability between biochemical properties, both seasonal and edaphic factors, as well as the lack of reference values or broad databases for high-quality soils that could be used to make comparisons. All this leads to the often contradictory conclusions reached by different researchers when describing the effects of a contaminant or a given type of soil management on soil health.
- Data on the inhibition of microbial properties in metal salt amendment studies have been used to support soil health criteria for heavy metals. Cation metal salts in solution are effective microbial inhibitors at very low concentrations. However, many metal amendment studies and field studies at contaminated sites have shown that the inhibition of soil microbial properties requires much higher heavy metal concentrations: the metal is likely rendered less available to microorganisms or soil enzymes due to interactions with soil constituents. Effectively, most of the metal is "locked up," and only a small amount is present in a soluble form. It is, however, questionable whether the approach of using metal salt amendment is appropriate, considering that metals rarely enter soils in this form or reach such high concentrations with a single application. Experiments with metal-salt amendment have generally been performed to evaluate short-term responses. However, responses in soils that have been polluted with metals for long periods are likely to be considerably different, and the effects on soil microbial properties may also include those attributable to reduced enzyme synthesis and impaired microbial growth and activity.
- There is no universal microbial assay that can be recommended for all soils under all conditions. In fact, it is highly recommended that the polluted soil should be compared with a reference soil, such as an adjacent unpolluted soil, with similar properties.
- The development of baseline information on the selected microbial indicators, including information on both spatial and temporal variations, is recommended within the first years of monitoring, in order to define reference and threshold values for repeated monitoring activities. The physical and chemical properties of the sampling sites should be characterized simultaneously.
- Despite the fact that laboratory studies are recommended to investigate the direct effects of metals, and to evaluate the sensitivities of new techniques because of

the possibility of reducing sources of variation in the data, conclusions drawn from them cannot be extrapolated to field conditions.

- We do not know whether the effect of heavy metal pollution on microbial properties is due to the effect of pH, the effect of bioavailable trace element concentrations, or to a combination of both. Establishing the relative effects of pH and bioavailable trace element concentrations on microbial properties is a difficult task, since both factors have been shown to concomitantly promote changes in microbial function and community structure.
- At present there is no consensus on an official methodology for estimating the level of soil health in heavy metal polluted soil. The current regulations only recommend the use of simple tests (e.g., soil respiration, root germination and elongation) whose generic application under the different conditions that prevail at different locations may be unreliable. The legislative interest in soil protection must give rise to a coordinated effort to search for a common methodology for the estimation of soil quality, in which both microbiological and biochemical soil properties play important roles due to their high sensitivity to distorting agents. This implies methodological standardization and the construction of databases of soil biological and biochemical properties. Unfortunately, these tasks are unattractive to most research teams as they require enormous analytical effort and are presently unsuited to financial support. Nevertheless, these basic tasks are so important that only after they have been carried out will the search for globally applicable indices for the evaluation of soil health be possible.
- To study the resilience of soil microbial properties, it is necessary to create a true full picture of the system, and this may also include its history and possible future genetic capacity. A combination of techniques that analyze the overall biogeochemical processes, the microbial activity, and the taxonomic or genetic diversity will help to link the microbial structure and diversity of a soil to its function.
- Research on microbial soil ecology may shed light on relatively unexplored ecological processes of enormous scope, and it directly responds to a need in restoration practice for practical and economical methods of site amelioration. Continued research into the ecology of soil microbes may reveal new potential pathways to increased restoration success, and may fill in the gaps in our current understanding of community development.

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Chapter 10
Streptomycete Heavy Metal Resistance: Extracellular and Intracellular Mechanisms

Erika Kothe, Christian Dimkpa, Götz Haferburg, André Schmidt, Astrid Schmidt, and Eileen Schütze

10.1 Introduction

Heavy metals in the environment influence microbial populations (Haferburg and Kothe 2007). These heavy metals mainly derive from mining activities; 240,000 km^2 of the Earth's surface are influenced by mining (Furrer et al. 2002). However, there are also some environments where high metal loads have arisen due to geogenic sources, such as serpentinite soils that have developed on nickel-rich rock substrates. Plants that are able to accumulate high nickel levels have been described, especially on serpetinites, and microbial populations that are resistant to high nickel concentrations have been found in the rhizospheres of such hyperaccumulator plants and characterized (Mengoni et al. 2001; Park et al. 2004; Schlegel et al. 1991). The evolution of metal resistance can therefore be traced back to habitats that are geogenic; the subsequent spread of microorganisms or the heterologous transfer of microbial resistance mechanisms to anthropogenic metal-rich niches can thus be envisioned.

In mining areas, heavy metal loads can reach extreme levels. This is mainly due to acid mine or rock drainage (AMD, ARD). During this process, the acidification of the environment leads to high heavy metal solubility (Singer and Stumm 1970). The AMD process is accelerated by microbial processes in which the oxidation of iron leads to the release of protons and acidification (Collins and Stotzky 1992). The exposure of sulfide ores to environmental conditions and oxygen in the air allows bacteria like *Acidithiobacillus ferrooxidans* or *Leptosprillum* species to gain energy from the oxidation of iron or manganese (Schippers et al. 1996). This leads to acidification, and the oxidation power keeps the reaction running for as long as

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reduced metal (usually present as a sulfide ore) and oxygen are available. This results in the extremely acidic and metal-rich seepage waters that can be found downstream from historic and recent mines and mining heaps. An extreme example would be the Rio Tinto region in Spain, where the high iron content and subsequent precipitation of iron hydroxides lead to waterways with orange to deep-red coloration and well-adapted biota (Zettler et al. 2003).

Bacteria cope with metals in their specific environments in different ways. The mechanisms of heavy metal resistance include extracellular and intracellular sequestration, lowering the concentration of bioavailable metal in the direct surroundings of the cell, or reducing the toxicity of the metal by changing its chemical oxidation state. Another resistance mechanism involves the expression of specific exporter proteins that keep the intracellular concentration of the metal at a tolerable level (for a recent review, see Haferburg and Kothe 2007). Other mechanisms involved in enhanced metal resistance help the cell to cope with the toxic effects of heavy metals. One specific mechanism is the formation of reactive oxygen species (ROS) via the Fenton reaction. In order to cope with the ROS, superoxide dismutases, catalases and peroxidases that relieve the cells of the adverse effects of ROS are expressed.

In aquatic environments with high metal loads, the metal concentrations around the cell are kept constant as metal is constantly delivered to the cell surface. The cells can counteract the toxic effects best if they express highly specific metal exporter proteins for use as an optimal safety guard. In soil, in contrast, the direct surroundings of the cell can be depleted of metals by excreting components that alter the bioavailability of the metal. In this way, the environment of the cell can be altered without constant resupplying the metal. Thus, mechanisms of extracellular sequestration are more likely to be found in soil microbes, while motile bacteria are more likely to have evolved export mechanisms. This hypothesis is supported by the discovery of highly specific metal exporter systems in Gram-negative bacteria (for review: Nies 2003), while filament-forming streptomycetes have been shown to possess various mechanisms for extracellular sequestration. In both environments, intracellular sequestration and mechanisms to prevent ROS damage will further enhance heavy metal resistance. Here, we summarize the mechanisms that together lead to very high heavy metal resistance in streptomycetes, an important group of soil bacteria.

10.2 Streptomycetes Are a Prominent Population in Heavy Metal Contaminated Soils

Streptomycetes are prominent in all soils. This is even evident from the smell of fresh soil – the volatile substances associated with fresh earth are geosmins, which are produced by streptomycetes. Streptomycetes have a very active secondary metabolism and can produce a wide variety of chemicals. Among these chemicals are antibiotics; up to 80% of the antibiotics used by man originated from

streptomycete metabolites (Haferburg et al. 2009; Hopwood 2006). These antibiotics are thought to provide the population that produces them with a competitive edge compared to other taxa in the soil.

In normal soils, streptomycetes – or more generally, actinobacteria – comprise about 20% of the bacterial population. Filamentous growth, the formation of hyphae, can be interpreted as an adaptation to living in soil, where nutrients may be unequally distributed spatially. The formation of a mycelium in which nutrients can be transported from one area to another enables growth under conditions where some of the nutrients needed by single-celled bacteria may be available in their immediate surroundings while others are not, and can also lead to a more constant water supply in an environment prone to drying out and rewetting. The same mechanism is utilized by fungi, which also form a high proportion of the soil biomass.

An additional adaptive advantage under dry conditions is the production of spores. This gives Gram-positives with endospore-forming bacilli and clostridia, as well as actinomycetes, a competitive edge when growing in the soil. Actinomycetes do not form endospores, but are able to differentiate spores from the aerial mycelium, either in spore chains (e.g., streptomycetes) or in sporangia (e.g., actinoplanetes). These spores are not as resistant to chemicals or heating as the endospores of Gram-positive bacteria with low genomic G+C contents (bacilli and clostridia), but they are nevertheless able to withstand heat and lack of moisture, which are conditions that can be found in natural soils. Upon the return of moist and temperate conditions, the spores germinate, forming new substrate mycelium for feeding and, subsequently, aerial mycelium for dispersing spores and spreading them to new environments.

These specific features of streptomycetes – the highly active secondary metabolism as well as the mycelial growth and spore production – appear to be mechanisms that also provide advantages under other adverse conditions, including heavy metal contamination of terrestrial environments. In a former uranium mining site near Ronneburg in Thuringia, Germany, the population of soil microbes was analyzed by cultivation-dependent and cultivation-independent DNA-based methods (Haferburg et al. 2007; Schmidt et al. 2005). It was found that, in contrast to uncontaminated soils from temperate regions, the population was highly enriched in Gram-positive bacteria, with bacilli and streptomycetes clearly dominating over Gram-negative proteobacteria, which often form large parts of the population in normal soil (Fig. 10.1). Thus, the advantages described above can be assumed to aid growth in poor soils contaminated with metals, as observed in the former mining district at Ronneburg.

10.3 Isolation of Heavy Metal Resistant Streptomycetes

The field site studied in the former uranium mining area at Ronneburg, Thuringia, Germany, is situated on a former heap leaching site (Kothe et al. 2005). The leaching process led to high infiltration of metal contaminants into the ground, and these

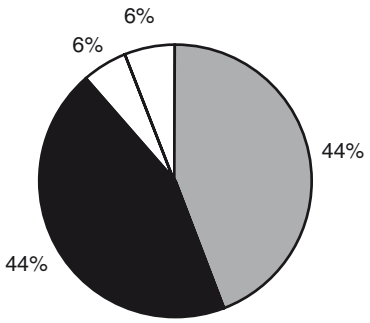

Fig. 10.1 Proportions of taxa identified in metal-contaminated soil at a former uranium mining site in Ronneburg, Germany. Actinomycetes and bacilli each comprised 44%, while both proteobacteria and non-spore-forming Gram positives with low G+C contents provided 6% of the taxa identified

contaminants are still present after removing the heap material in the 1990s (Geletneky et al. 2002). The mining activity did not start until 1949, which places an upper limit on the time available for adaptive processes to prompt the development of specialized, heavy metal resistant microbial consortia. In addition to the acidity of the leaching process, the bioavailability of heavy metals is especially high in this setting. We therefore asked whether microbial consortia that possess high heavy-metal resistance towards multiple contaminants are present in this environment. As a comparison, we chose a highly contaminated (albeit less acidic) environment in Argentina, where a ditch receiving water from a copper filter plant has operated for just 15 years in the vicinity of Tucuman City. To have a presumably uncontaminated control site, city parks at Jena, Germany were chosen. Although tolerant (defined as a tolerance of up to 0.2 mM $NiCl_2$) and even highly resistant (over 5 mM $NiCl_2$) strains were isolated from uncontaminated sites, we found only three sensitive strains. At the field site tested, 25% of the strains were resistant, while at the Argentina ditch site, as many as 50% of the strains were highly resistant to heavy metals. Thus, 15 or 50 years were sufficiently long periods to allow the establishment of a community comprising highly resistant actinobacteria.

Since highly resistant strains were isolated, we investigated the highest level of resistance exhibited by isolates obtained from the field site tested.

It is important to note that resistance levels published in the literature are hard to compare. The different media used to grow the strains make it almost impossible to compare the bioavailable fraction of heavy metals between studies, and this is the sole determinant of ecotoxicity. We used minimal media, since it has been noted that ingredients in complex media could complex metals and thus lead to artificially high estimates for the levels of resistance (Amoroso et al. 2000). In defined salt media such as those we used to grow streptomycetes, the complexation of heavy metals can only be achieved with excreted substances, and is thus linked to microbial activity. In this case, the estimates of resistance levels are more likely to reflect the actual physiological capacity of the bacteria to cope with stressors such as heavy metals in their environment.

10.4 Metal Resistance Characterization and Adaptation

In a first screening, more than 200 strains were individually tested for resistance towards different heavy metals, including nickel, cadmium, lead, mercury, zinc, copper, and chromium. Nickel was afforded special attention, since it is representative of many metals that can be found in trivalent and bivalent states in nature and is mobilized under even moderately acidic conditions. The strain with the highest resistance level was able to grow on minimal solid media containing 130 mM nickel (Schmidt et al. 2009a) This is more than 8,000 ppm – the medium appeared green due to the dissolved nickel. We tested whether this strain would be able to grow on soil agar prepared from contaminated soil (not soil extract) with 6,000 ppm nickel, and we observed clear growth on these plates, showing that strain P16-B1 has the ability to colonize the soil even under high nickel stress, even when the soil was co-contaminated with other heavy metals (Schmidt et al. 2009a). We can thus conclude that the streptomycete population at our field site had attained extremely high metal resistance levels through adaption over a limited time frame. Since it was logical to assume that such high resistance levels are only possible when different metal resistance mechanisms are exploited concomitantly, we investigated the different routes that allow microorganisms to achieve heavy metal tolerance.

10.5 Chelators and Siderophores

The bioavailability of metals can be altered by forming metal complexes with chelating molecules (or minerals, as discussed in the next section). The excretion of chelating substances has been described for bacteria and fungi, with the most active components being siderophores that are involved in iron acquisition (Crowley et al. 1984). Under oxic conditions, iron is only minimally bioavailable, and the very low solubility of Fe^{3+} hinders the uptake of iron required for electron transport chain components and in active centers of redox-active enzymes. This is especially true of aerobic, soil-dwelling organisms, including plant roots. While plants mainly acquire iron through the acidification of the rhizosphere and the excretion of organic acids such as oxalate, citrate or malate, bacteria apply another strategy. They excrete low molecular weight, high iron binding affinity substances – siderophores, which are delivered and in many cases taken up after iron loading. Many different siderophores have been described for both Gram-negative and Gram-positive bacteria. Hydroxamate siderophore production was verified in *Streptomyces acidiscabies* E13, a nickel-resistant isolate from the field test site (Dimkpa et al. 2008). The three different hydroxamate-type siderophores can be produced irrespective of the presence of nickel in the cultures, showing that their respective production mechanisms are not influenced by metals other than iron. The effects of the siderophores on the bioavailabilities of both iron and nickel were shown in plant experiments, where the addition of filtrates of hydroxamate-producing cultures of *S. acidiscabies* E13

had a plant growth promoting effect on cowpea seedlings (Dimkpa et al. 2008). The three hydroxamates found in the culture filtrates are the desferroxamines DFOE (with a circular structure) and DFOB (with an open structure), as well as coelichelin, which has an open structure with even less rigidity in the backbone. In accordance with these structural predispositions, the amount of siderophore bound to iron versus that bound to nickel varied among the three molecular types of siderophore, and with time. Similar amounts of iron and nickel were found to bind to the siderophores in the overwhelming presence of nickel, the production of all three siderophores was possible at the same time, and the production of all three siderophores changed over time (Dimkpa et al. 2008). While DFOE predominantly chelated nickel, DFOB showed a preference for iron, especially at the peak in production, while large portions of coelichelin remained unchelated, even though nickel was present at 2 mmol L^{-1} in the experiment while iron was limiting. Thus, the excretion of siderophores leads to nickel complexation in the surroundings of the bacterial hyphae, and while nickel is chelated, iron is still solubilized, aiding iron reduction and uptake into the cell. The siderophore thus plays a dual role in releasing nickel stress and allowing sufficient iron to be taken up for biosynthesis and metabolism.

Another observation made about the heavy metal resistant strains isolated was that many of them produced a dark pigment. Soluble brownish pigments are known to be formed by many streptomycetes, and it has been suggested that these melanin-like pigments are able to sequester metals from the vicinity of the growing cell. Indeed, cultures grown in media with increasing amounts of added nickel were darker, indicating the increased production of the melanin-like pigment (Fig. 10.2). In order to address these questions, mutant strains were derived from a UV-directed mutagenesis. White mutants were obtained, but did not show an unequivocally lower nickel resistance. It can thus be concluded that nickel sequestration is not driven mainly by melanin excretion, although the substance may still have a slight protective effect. Such an effect could also be due to the release of ROS stress, as melanin is also known to scavenge oxygen radicals through the

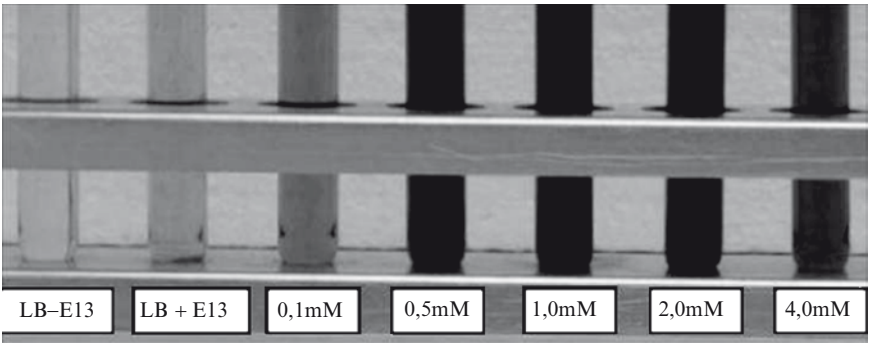

Fig. 10.2 Increased production of a melanin-like brown pigment under nickel stress by *S. acidiscabies* E13

radical-assisted formation of higher molecular weight melanins. The role of the siderophores clearly exceeded the potential function of the melanins in protecting from nickel stress.

10.6 Biomineralization

In addition to chelation, the induction of mineral formation can lead to metal depletion in the direct surroundings of a bacterial cell. Biomineralization is the process in which microorganisms aid the growth of crystals by providing either a crystallization initiator or anions for mineralization. Both of these approaches to biomineralization depend on the presence of cells, and the latter depends on the presence of actively growing, living cells. One well-studied example of biomineral formation is provided by the intracellular magnetite-containing bodies formed by magnetotactic bacteria (Bäuerlein 2003). An extracellular biomineralization process appears to be more conducive to metal resistance. The formation of hydrozincite in zinc-rich mine effluents has been described (De Giudici et al. 2007). There, the precipitates formed with bacterial inocula were the dominant species of zinc in the AMD waters. This biomineralization would also allow growth under high nickel stress. Indeed, the formation of a new biomineral with *S. acidiscabies* E13 was demonstrated (Haferburg et al. 2008). The natural mineral struvite is composed of $Mg(NH_4)(PO_4)\ 6H_2O$; nickel struvite, as we termed the biomineral observed in our cultures, is its nickel analog: $Ni(NH_4)(PO_4)\ 6H_2O$. The crystals form on top of colonies growing on solid media, but also in liquid cultures of the strain. The medium contains not ammonia but nitrate as the nitrogen source, which does not allow mineralization without active metabolism of the streptomycete. The mineral formed is extremely pure, with only minimal magnesium impurities, which is especially interesting because the natural mineral struvite could also have formed, using magnesium from the medium. However, this was not observed, which suggests a biologically controlled process. Inoculation of the media with dead biomass did not lead to nickel/struvite formation, showing that it indeed is an active, biologically controlled process that warrants the term "biomineralization." With nickel bound to a mineral phase, the cells clearly have an adaptive advantage in terms of released nickel stress.

10.7 Cell Wall Adsorption

Adsorption to extracellular surfaces also reduces the available metal concentration. In Gram-positive bacteria, the cell wall is accessible from the outside with no outer membrane protecting the surface. This leads to different binding capacities from Gram-negative bacteria. The possibility of metal binding to the cell wall or protein layers has been shown for *Bacillus sphaericus*, where attachment to S-layer

proteins allows metal sequestration (Merroun et al. 2005). This has been used in the formulation of Biocer, a ceramic with embedded *B. sphaericus* cells that is used for water treatment.

The ability of streptomycetes to bind metals to the extracellular cell wall fraction was shown by differential centrifugation. Depending on the strains used, up to 8,000 ppb nickel were found to be associated with the cell wall fraction. The strain with the highest retention capacity was *Streptomyces lincolnensis* Tosca3, while 800–1,000 ppb nickel were bound to the cell wall in *S. acidiscabies* E13, the strain used in the aforementioned studies.

In an experimental setup that was employed to show heavy metal sequestration by cells added to AMD waters, *S. acidiscabies* was shown to retain large amounts (up to 80%) of Al, Mn, Co, Ni and U, and even 90% of copper, even when dead biomass was used (Haferburg et al. 2007). This clearly indicated binding to cell wall fractions, as no active metabolism is available. In addition, rare earth elements (REEs) were retained, in the case of dead biomass, to levels of about 50%. Here, living inoculum had a greater effect, showing that metabolic activities are most likely involved in REE sequestration. The pattern of REE implied fractionation, meaning that the heavy REEs were retained better than light REEs. Such a pattern of fractionation is an indication of biological processes. In the case of the cell wall, this may be that the ionic radii of heavy REEs are better suited to binding to the cell wall components. This allows the use of the REE fractionation pattern to predict biological reactions in geohydrochemical source–sink evaluations (Merten et al. 2005).

10.8 Intracellular Storage

Potentially toxic heavy metals are usually taken up through the transporter systems that are used to acquire essential micronutrients, such as potassium for monovalent ions or calcium for bivalent ions. These low-affinity uptake systems could also take up other metals, including sodium, copper, cadmium, zinc, nickel or lead, to give just a few examples. While nickel and zinc have biological functions, only toxic effects have been reported for other metals. For the essential metals nickel, copper and zinc, intracellular homeostasis is needed to prevent damage to proteins or DNA. A highly specific efflux system could provide this homeostasis (Grass et al. 2000; for a review see Nies 2003 and references therein); nevertheless, chaperones for the metals are required to keep the intracellular stock from damaging cell components. It can therefore be assumed that metals such as nickel could be bound to chaperone molecules in the cell (Haferburg and Kothe 2007 and references therein). We could envision that phosphate, with its negative charge, could provide such a chaperone function, and that metals could be stored in volutin granules of polyphosphates. However, results from preliminary experiments indicate that a lack of polyphosphate kinase does not lead to increased sensitivity in *S. acidiscabies* E13 (Haferburg, unpublished).

In order to search for intracellular storage molecules for nickel, a fractionation by gel filtration was performed. The fractions with large amounts of nickel were of low molecular weight and contained only small amounts of protein, indicating a high binding capacity. Proteins with high contents of histidine and cysteine amino acid moieties can be detected in the genomes of the fully sequenced streptomycetes *Streptomyces coelicolor* and *Streptomyces avermitilis*. Such proteins are currently being investigated for their role in intracellular nickel storage.

One of the enzymes in streptomycetes that is known to require nickel at its active center is nickel-dependent superoxide dismutase (NiSOD). SOD is involved in ROS detoxification, making this special enzyme interesting for two reasons. Overexpression of SOD has been shown to lead to heavy metal tolerance in yeast, and the expression level of NiSOD in resistant strains was investigated to see whether NiSOD plays a protective role under in situ conditions in contaminated soil (Schmidt et al. 2007). In addition, NiSOD itself is interesting, since the enzyme is structurally different from other SODs (Schmidt et al. 2009b). The nickel-resistant strains provide a source for biochemical characterization, since nickel in the medium increases SOD expression, and so highly expressed enzymes can be purified.

Proteome analyzes have been performed to detect regulatory mechanisms induced by nickel stress (Schmidt et al. 2005). Cells grown with nickel clearly show the induction of several proteins, among them regulators of the TetR family. Analysis of the corresponding genes will allow the identification of regulatory mechanisms for cells that must cope with heavy metal stress.

10.9 Conclusion

As can be seen from the analysis of streptomycetes, bacteria can employ different mechanisms to achieve heavy metal tolerance or resistance. Especially for strains that can tolerate extreme amounts of metals, the coordinated activities of several different mechanisms of heavy metal resistance, including intracellular and extracellular sequestration and excretion of components to alter the local environment, are clearly necessary. Adaptations leading to the acquisition of several of these mechanisms may be explained by heterologous gene transfer. Indeed, most of the strains investigated contain extrachromosomal plasmids, as shown by pulse field gel electrophoresis (Schmidt et al. 2005). Whether or not these contain resistance genes, and whether or not these plasmids are from streptomycetes or elsewhere, remain to be seen. Nevertheless, it has become clear that adaptive processes, even those that occur over a limited time frame of approximately 50 years, can lead to highly resistant strains that utilize multiple resistance mechanisms, all of which contribute to heavy metal resistance in an environment influenced by AMD.

All of the above indicate that microbes could be used for bioremediation purposes (Kothe et al. 2005; Haferburg and Kothe 2007; Haferburg et al. 2007). Many of the studies investigating heavy metal resistance in microorganisms are being performed in order to find strains that are suitable for just such a purpose

(Albarracín et al. 2008; Sineriz et al. 2009). The remediation of AMD is clearly one of the major tasks to be tackled in the future (Johnson and Hallberg 2005), and in order to optimize strategies for bioremediation, a deeper knowledge of the basic biological mechanisms involved in heavy metal resistance is needed.

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Chapter 11
Effects of Heavy Metals on Soil Enzyme Activities

Ayten Karaca, Sema Camci Cetin, Oguz Can Turgay, and Ridvan Kizilkaya

11.1 Introduction

Heavy metals are considered one of the major sources of soil pollution (Huang and Shindo 2000). Heavy metal pollution of the soil is caused by various metals, especially Cu, Ni, Cd, Zn, Cr, and Pb (Effron et al. 2004). Zeng et al. (2007) reported that Pb is one of the most abundant heavy metal soil pollutants (Eick et al. 1999). Many authors have reported that heavy metals cause long-term hazardous effects on soil ecosystems and negatively influence soil biological processes (Chen et al. 2005; D'Ascoli et al. 2006; de Mora et al. 2005; Effron et al. 2004; Kunito et al. 2001; Kuperman and Carreiro 1997; Lorenz et al. 2006; Malley et al. 2006; Shen et al. 2005; Speir et al. 1999). For this reason, heavy metals need to be monitored and their concentrations in soils regulated. For example, the Commission of the European Community (CEC) has established permissible heavy metal limits in agricultural soils; for Hg, Pb, and Zn these are 1–1.5, 50–300, and 150–300 mg kg^{-1} dry soil, respectively (CEC 1986). The heavy metal contamination of soils has become a serious environmental issue around the world for various reasons, including industrial activities, solid waste disposal, fertilizer and sludge application, irrigation with wastewater, and automobile exhausts (Karaca et al. 2002; Karaca 2004; Khan et al. 2007; Yang et al. 2006). Heavy metals affect many characteristics of soils, including their biological properties (Huang and Shindo 2000). Khan et al. (2007) concluded that heavy metals have an inhibitory influence on soil enzyme

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activities and as well as microbial community structure. The strong inhibition of enzyme activity exerted by heavy metals has been well documented by many researchers (Effron et al. 2004; Kahkonen et al. 2008; Kizilkaya 2008; Kunito et al. 2001; Malley et al. 2006; Oliviera and Pampulha 2006; Shen et al. 2005; Speir et al. 1999; Wang et al. 2008). Soil enzyme activities are considered to be good bioindicators, reflecting natural and anthropogenic disturbances, and evaluating soil enzyme activities is one of the cheapest and easiest techniques that can be used to evaluate soil pollution (Hinojosa et al. 2004; Khan et al. 2007). Some researchers describe the toxicity of metals to enzymes using the ED_{50} value, which is defined as the heavy metal concentration at which the enzyme activity is half of its uninhibited level (Huang and Shindo 2000). Soil enzymes are inhibited by heavy metals to different extents depending on the characteristics of the soil, such as its clay, silt and organic matter contents and its pH value (Doelman and Haanstra 1986; Effron et al. 2004; Geiger et al. 1998). Yang et al. (2007a,b) reported that the reduction in soil microbes and the inhibition of soil enzyme activities caused by metal contamination negatively affect soil fertility.

11.2 Inhibition of Soil Enzymes

An enzyme inhibitor is an agent that reduces enzyme activity, whereas an enzyme activator is an agent that stimulates enzyme activity (Voet and Voet 1995). The effects of inhibitors and activators on enzymes are shown in Fig. 11.1. Both of these types of agents affect the parameter K_m for the enzyme reaction of interest (K_m is the substrate concentration at which the reaction rate is half of the maximum rate; Stryer 1995). As seen in Fig. 11.1, K_m values increase in the presence of an inhibitor and decrease in the presence of an activator.

The inhibition of soil enzyme activities by heavy metals is a very complex issue, as there are many factors that affect this inhibition. These factors can be divided into four main classes: metal factors, enzyme factors, soil factors, and plant factors. Metal factors include the heavy metal element in question, the concentration of the heavy metal, the chemical form of the heavy metal, the availability of the heavy

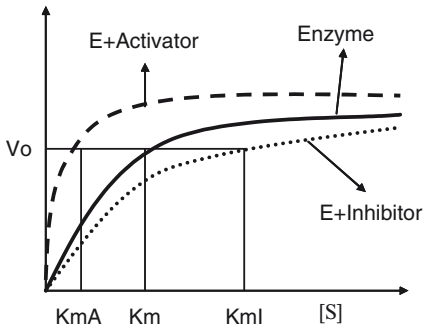

Fig. 11.1 The effects of inhibitors and activators on enzyme activity (Voet and Voet 1995)

metal, and indirect effects of the heavy metal. Enzyme factors include the enzyme sensitivity, the structural inhibition of the enzyme, and the major properties of the enzyme. Soil factors include pH, organic matter, and clay. Finally, plant factors include metal accumulation and plant community effects. We now take a closer look at these factors.

11.2.1 Metal Factors

11.2.1.1 Heavy Metal Element

Enzyme activities are influenced in different ways by different metals due to the different chemical affinities of the enzymes in the soil system. Khan et al. (2007) found that Cd was more toxic to enzymes than Pb because of its greater mobility and lower affinity for soil colloids. Shen et al. (2005) found a negative interaction between Zn and Cd resulting from competition between them for sorption sites. Zn concentrations are generally higher (by factors of 100–1,000) than Cd concentrations (Christensen 1987). Also, different metals affect soil enzymes in different ways. Geiger et al. (1998) found that copper inhibited β-glucosidase activity more than cellulase activity. Balyaeva et al. (2005) found that Pb decreased the activities of urease, catalase, invertase, and acid phosphatase significantly. Speir et al. (1999) found that phosphatase and sulfatase were inhibited by As(V) but that urease was unaffected. Lorenz et al. (2006) found that As contamination significantly affected arylsulfatase activity but not those of xylanase, invertase, protease and alkaline phosphatase; Cd contamination had a negative effect on the activities of protease, urease, alkaline phosphatase and arylsulfatase but no significant effect on that of invertase. Each soil enzyme exhibits a different sensitivity to heavy metals. Shen et al. (2005) reported that the order of inhibition of urease activity generally decreased according to the sequence Cr>Cd>Zn>Mn>Pb (Zheng et al. 1999). Effron et al. (2004) found that heavy metals inhibited the activities of arylsulfatase, acid phosphatase, protease and urease. The relative toxicities of the metals toward enzyme activity were found to be: Cd≈Cu>Pb. Acosta-Martinez and Tabatabai (2001) found that Ag(I), Hg(II) and Cd(II) were more effective inhibitors than the other 18 trace elements examined. Renella et al. (2005) found that Cd inhibited alkaline phosphatase, arylsulfatase and protease, but did not affect acid phosphatase, β-glucosidase and urease.

Vig et al. (2003) published a review of the bioavailability and toxicity of Cd towards soil microorganisms and their activities. The effects of Cd on soil enzymes are extensively summarized in their review. A summary of studies on the effects of Cd on soil enzyme activities is given in Table 11.1.

11.2.1.2 Metal Concentration

Actually, all metals, including heavy metals, are generally found in the soil at low concentrations and provide essential micronutrients for soil organisms; however,

Table 11.1 The effect of Cd on soil enzyme activity in different studies (adapted from Vig et al. 2003)

Soil type/treatment	Cd (mg kg^{-1} soil)	Inhibition (−), activation (+) or no effect (NE)	References
Field studies. Oak forest near abandoned zinc smelter: pH 5.0–6.2, 0.5–0.7% OC	Cd 26, Cu 15.0, Pb 21.6, Zn 478	+DHA 93% +UR 88%	Pancholy et al. (1975)
Lab amendments: pH 5.1–6.1, 1.5–2.9% OC, 10–21% clay	$CdCl_2$ 562	−ARA 55–82%	Acosta-Matinez and Tabatabai (2001)
Lab amendments. Soil 1: pH 6.2–7.6, 2.7–5.3% OC, 26–34% clay. Soil 2: pH 7.6, 3.2 %OC, 30% clay	2810281	−ASL 23–55% −ASL 7%	Al-Khafaji and Tabatabai (1979)
Sandy loam: pH 7.9, 0.47% OC. Loam: pH 8.1, 1.61% OC. Clay-loam: pH 7.7, 0.72% OC	$CdCl_2$ 50	−DEH, ALP	Dar (1996)
Sandy: pH 7.0, 1.6% OM. Sandy peat: pH 4.4, 12.8% OM	$CdCl_2$ 150 $CdCl_2$ 1980 $CdCl_2$ 40	−UR 10%, 6 weeks −UR 10%, 6 weeks −UR 10%, 1.5 years	Doelman and Haanstra (1986)
pH 5.6, 2.6% OC, 28% clay	562	−ADS 6%	Frankenberger and Tabatabai (1981)
Forest soil: pH 4.8, 2.3% OC, 87% sand, 8% silt, 5% clay	$CdSO_4$ 500 $CdSO_4$ 50	−DEH, ACP −ACP	Landi et al. (2000)
Montepaldi soil: pH 8.1, 1.7% TOC, 66% sand, 21% silt, 13% clay	$CdSO_4$ 3–400	−DEH, UR	Moreno et al. (2001)
Agricultural soil: 1.3% OC	$Cd(NO_3)_2$ 150	−DEH 48% −CL 29 % −AML 34%	Rogers and Li (1985)
Fir needle litter: 78% OM	$CdCl_2$ 1000	NE IN, XY, BD, PPO	Spalding (1979)
pH 4.6-7.0, 1.99–5.32% OC, 24–36% clay	$CdSO_4$ 2810	−PYP 19–50%	Stott et al. (1985)
Surface soils: pH 5.1–7.8, 2.6–5.5% OC, 17–42% clay	$CdSO_4$ 562	−UR	Tabatabai (1977).

OC organic carbon; *TOC* total organic carbon; *OM* organic matter; *ARA* arylamidase; *ASL* arylsulfatase; *DEH* dehydrogenase; *ALP* alkaline phosphatase; *ADS* amidase; *ACP* acid phosphatase; *CL* cellulase; *AML* amylase; *IN* invertase; *XY* xylanase; *BD* β-glucosidase; *PPO* polyphenoloxidase; *PYP* pyrophosphatase

their levels have increased drastically due to anthropogenic pollution (Carine et al. 2008). Zeng et al. (2007) observed a stimulating effect of Pb on soil enzyme activities at low concentrations of Pb. However, when the level of Pb was increased to

500 mg kg^{-1}, soil enzyme activities decreased. Similarly, Shah and Dubey (1998) reported that an enhancement in protease activity was observed at low Cd levels (50–100 μM); however, protease activity was inhibited above these levels. Fliessch et al. (1994) reported that sludge containing low levels of metals had a stimulating affect on soil microbial activity. Furthermore, Dar (1996) found that the addition of Cd aat 10 μg g^{-1} soil (in Sw) did not result in any significant changes in soil enzyme activity. However, the addition of Cd at 50 μg g^{-1} soil decreased the soil enzyme activity, and this effect was greater in sandy loam than in loam or clay loam soils.

Tejada et al. (2008) reported that soil enzyme activities decreased with increasing Ni concentration. Lorenz et al. (2006) found that increasing the level of Cd decreased enzyme activities. Zeng et al. (2007) stated that "it is well known that any element under specific environmental conditions would bring about the adverse effect to plants and microorganisms if its concentration is higher than a certain range." Cellulase and β-glucosidase activities were inhibited at copper concentrations above 200 μM (Geiger et al. 1998). However, it was observed that the enzyme activities were slightly reduced at 1 mM copper compared to 600 μM. Hemida et al. (1997) found that urease activity completely disappeared at 2,000 μg heavy metals (Cu^{2+} and Zn^{2+}) g^{-1} soil. Wyszkowska et al. (2006) concluded that concentration of 50 mg kg^{-1} of metals (Cu, Zn, Ni, Pb, Cd and Cr) inhibited soil enzyme activities (those of dehydrogenase, urease, acid phosphatase and alkaline phosphatase).

Mikanova (2006) studied the effects of heavy metals on the enzyme activities (arylsulfatase, invertase, urease and dehydrogenase) of heavy metal polluted alluvial soils. Increasing the heavy metal concentration inhibited all of the soil enzymes studied, but arylsulfatase and dehydrogenase were more sensitive to lower concentrations of metal than invertase and urease (Table 11.2). Hinojosa et al. (2004) conducted a study to determine enzyme sensitivity in order to find the magnitude of the heavy metal pollution (Cd, Pb, Cu and Zn) resulting from a mine spill.

Table 11.2 Effects of Cd, Pb, and Zn on soil enzyme activities in heavy metal polluted alluvial soils (adapted from Mikanova 2006)

	Heavy metal (mg kg^{-1} dry soil)			Inhibition				Activation			
Soil properties	Cd	Pb	Zn	ASL	IN	UR	DEH	ASL	IN	UR	DEH
Alluvium of Litavka River											
Unpolluted	1.9	106.0	202.5								
Low-level pollution	2.4	113.5	249.8	S		W	S		W		
Moderate	5.4	530.5	407.0	S	W	M	S				
Medium	59.0	3,450.7	6,230.8	S	M	M	S				
High	61.3	7,040.3	7,497.9	S	M	M	S				
High	113.8	6,335.9	12,557.4	S	S	S	S				
Czech standards	1	140	200								

ASL arylsulfatase; *IN* invertase; *UR* urease; *DEH* dehydrogenase; *S* strong; *M* moderate; *W* weak

Similarly, increasing the degree of pollution caused decreased soil enzyme activities. The highest enzyme activity was found in unpolluted soil and the lowest in the most polluted soil.

11.2.1.3 Chemical Form of the Heavy Metal

Different chemical forms of heavy metals can affect soil enzymes differently. Carine et al. (2008) found that phenoloxidase activity was inhibited Al chloride salt than Al sulfate salt, at higher rate and lower Al level. Yang et al. (2007a,b) found that mercury ($HgCl_2$) markedly inhibited soil urease activity, and that there was a logarithmic relationship ($P < 0.05$) between the concentration of Hg and the activity of the soil urease.

11.2.1.4 Availability of the Heavy Metal

Bioavailability is an important factor when evaluating metal toxicity. Bioavailability can be defined as "the fraction of all contaminants in the soil particles that is available to receptor organisms" (Vig et al. 2003). Bioavailability is particularly important for soil microorganisms and plants, since they are the main sources of enzymes. The bioavailability of Cd (one of the most toxic heavy metals) depends on several factors, such as soil type, Cd speciation, aging, nature of Cd applied, and the nature of the microorganisms (Vig et al. 2003). Vig et al. (2003) reported that the availability of Cd in a soil–plant system increased in the order: mineral lattices > Fe and Mn oxides > organics > metal-organic complexes > carbonates > exchangeable (Krishnamurti 2000). They also reported that the bioavailability of a heavy metal declines with the time it is in contact with the soil (Naidu et al. 2003).

The available forms of a metal are significant when attempting to understand metal toxicity, and its available forms are related to its chemical forms in the soil (Wang et al., 2007a,b). Water and NH_4NO_3 extractions can be used as methods to define the solubilities of metals, by either releasing heavy metals in a soil solution (water extraction) or by extracting soluble and exchangeable metals (NH_4NO_3 extraction). Generally, heavy metal concentrations in soil solutions decrease at neutral or alkaline pH (Munoz-Melendez et al. 2000). Soluble forms of heavy metals are considered to be most available to microorganisms and enzymes (Huang and Shindo 2000). Bhattacharyya et al. (2008) reported that water-soluble and exchangeable forms of metals showed strong inhibitory effects on soil enzyme activities. Chaperon and Sauve (2007) concluded that, since higher dissolved metal concentrations were found in agricultural soil, metals were more toxic for the studied enzymes. The metal fractions (total, soluble, or extractable) present are an important aspect of the availability of metals. Wang et al. (2007a,b) found that soil phosphatase activity was significantly negatively correlated with Cu and Zn (soil solution, NH_4NO_3-extractable, and total fractions).

11.2.2 Enzyme Factors

11.2.2.1 Enzyme Sensitivity

Shen et al. (2005) investigated the interactions of polycyclic aromatic hydrocarbons (phenanthrene, fluoranthene, benzo[*a*]pyrene) and heavy metals (cadmium, zinc and lead) with soil enzymes (urease and dehydrogenase). The results showed that dehydrogenase was more sensitive to the combined pollution than urease. Similarly, Maliszewska-Kordybach and Smreczak (2003) demonstrated that dehydrogenase activity is most sensitive to the combined effects of pollutants (heavy metals and PAHs). Shen et al. (2005) reported that urease and dehydrogenase could be suitable indicators of combined pollution (heavy metals and PAHs), particularly at the early stages of pollution (Baath 1989; Yang and Liu 2000). Renella et al. (2003) reported that alkaline phosphatase was more susceptible in acid soil, whereas acid phosphatase was more susceptible in alkaline soil. Wyszkowska et al. (2006) found that the metal sensitivities of enzymes followed the order: dehydrogenase > urease > alkaline phosphatase > acid phosphatase. The metal sensitivities of soil enzymes that have been reported in the literature are given in Table 11.3.

Table 11.3 Metal sensitivities of soil enzymes, as reported in the literature

Heavy metal	Treatment	Metal sensitivity			References
		High	Moderate	Low	
		DEH			Maliszewska-Kordybach and Smreczak (2003), Hinojosa et al. (2004) cit. Khan et al. (2007)
	Long-term pollution	UR, ACP, DEH			Aoyama and Naguma (1996) cit. Zeng et al. (2007)
Cu	Vermicomposting	DEH		PR	Malley et al. (2006)
Cu	Long-term pollution	PH			Wang et al. (2008)
Cd	Phosphate fertilizer and sewage sludge	PME	βG, ASL	UR	Karaca et al. (2002)
CdCu Pb	Incubation experiment	ASL, PR PH, PR PR			Effron et al. (2004)
Cd, Zn, Pb	Combined pollution (heavy metals and PAHs)	DEH	UR		Shen et al. (2005)
As[V]	Experiment	PH	SL, UR		Speir et al. (1999)
Hg, As	Long-term pollution	DEH			Oliveira and Pampulha (2006)
Zn	Long-term sludge-amended soil	DEH, UR, IN			Kunito et al. (2001)
Zn	Organic wastes and Zn	DEH			Kizilkaya (2008)

UR urease; *ACP* acid phosphatase; *DEH* dehydrogenase; *PH* phosphatase; *PR* protease; *SL* sulfatase; *PME* phosphomonoesterase; *ASL* arylsulfatase; *IN* invertase; *βG* β-galactosidase

11.2.2.2 Structural Inhibition of the Enzyme

Enzyme reactions are inhibited by heavy metals in three different ways: (1) complexation of the substrate; (2) combination with protein-active groups on the enzyme, and; (3) reaction with the enzyme–substrate complex (Tejada et al. 2008; Megharaj et al. 2003). D'Ascoli et al. (2006) reported that heavy metals inhibited enzyme activity in several ways: (1) by masking catalytically active groups; (2) denaturing the protein conformation, or; (3) competing with metal ions that are needed to form enzyme–substrate complexes (Gianfreda and Bollag 1996).

Khan et al. (2007) reported that extracellular enzymes were inactivated by heavy metals. Mechanisms involved the metals binding to some of the amino acids in the enzymes and indirectly reducing the number of microorganisms responsible for producing the enzymes (Doelman and Haanstra, 1986; Kuperman and Carreiro 1997; Bandick and Dick 1999; Kunito et al. 2001).

Geiger et al. (1998) reported that the interaction of a metal cation with an enzyme is largely dependent on the amino acid composition of the protein. It is assumed that the catalytic reactions of cellulases involve a hydrolysis reaction that proceeds via an acid–base mechanism involving aspartic and glutamic acid. There are two components to this mechanism: (1) acting as a catalyst (aspartic acid), (2) acting as a nucleophile (glutamic acid). Cellulose binds to cellulase in the region of the cellulose-binding domain (Esterbauer et al. 1991). Cellulose-binding domains contain plenty of glycine and cysteine, which are stabilized by two or three disulfide bridges (Wood and Garcia-Campayo 1990). In other words, the shape of the active site of cellulase is mainly provided by amino acids (glycine and cysteine) and bonds between them (disulfide bridges). The cellulose-binding domain also contains tryptophan residues (Teeri et al. 1995). Copper can form complexes with tryptophan residues in the cellulose-binding domain, resulting in the inhibition of cellulase.

Khan et al. (2007) stated that "it is well documented that heavy metals react with sulfhydryl groups of enzymes and inhibit and/or inactivate the enzymatic activities." Lorenz et al. (2006) reported that enzyme activities decreased due to the binding of Cd^{2+} to sulfhydryl groups (Sanadi 1982). Hemida et al. (1997) reported that Tabatabai (1977) stated that "there was a marked decrease in urease activity with increasing trace element ion concentrations due to the reaction of –SH groups on urease (which are involved in urease activity) with the trace element ions." Bhattacharyya et al. (2007) specified that As ions inactivate enzymes by reacting with sulfhydryl groups resulting from the formation of arsenic sulfide. They also reported that As decreases enzyme activity in three ways: (1) by interacting with the enzyme–substrate complex; (2) by denaturing the enzyme protein, or; (3) interacting with the active protein groups (Dick 1997).

Hemida et al. (1997) indicated that the amidase activity in soil to which Cu^{2+} and Zn^{2+} had been added was not strongly inhibited compared to the activities of urease and nitrate reductase, and explained this by citing the different functional groups at the active sites of amidase. Wood and Oris (1974) stated that thiol groups had no direct effect on the catalytic activity of amidase, but they were necessary to stabilize the active amidase conformation. Frankenberger and Tabatabai (1980) suggested

that α-amino groups may be effective at catalyzing amidase function, and that these groups do not react with metal ions.

Bhattacharyya et al. (2007) reported that phosphatase activity was negatively influenced by a high phosphorus content in the soil because of the structural similarity of phosphate and arsenate (Juma and Tabatabai 1977; Speir et al. 1999). Arsenic is a highly inhibitory heavy metal, even at low concentrations, due to its chemical properties (uncharged at neutral pH, can diffuse across the cell membrane). When arsenic reaches the inside of the cytoplasm, it crosslinks with sulfhydryl groups and permanently inactivates the enzyme (Dick 1997).

11.2.2.3 Seasonal Effects of Enzymes

Soil enzymes are season-dependent macromolecules because they derive from living organisms. Microorganisms, plants and animals show seasonal fluctuations in activity. Zhang et al. (2008) found that there was a seasonal difference in the effect of heavy metals on soil enzymes – the effect of the heavy metals was more obvious in spring and summer than in autumn.

11.2.3 Soil Factors

11.2.3.1 pH

Effron et al. (2004) reported that enzyme activity was sensitive to changes in pH. When a metal enters the soil, it can alter the soil pH, and usually results in acidification. Increasing the pH influences Cd sorption, reducing the concentration of Cd in the soil solution and making less Cd available in soil (Vig et al. 2003). Geiger et al. (1998) found that the effect of copper on the enzymatic decomposition of cellulose by cellulase and β-glucosidase in suspensions of montmorillonite and aluminum-treated montmorillonite was strongest in the pH range 5.0–5.5. Copper lowered the pH values corresponding to the optimal activities of cellulase and β-glucosidase. Generally, amino acids of enzymes are deprotonated at high pH involved in metal interaction. Geiger et al. (1998) reported that, in the presence of kaolinite, the optimal pH for clay-absorbed enzyme activity was shifted by one or two pH units toward alkaline values (Pflug 1982). Campbell (1988) suggested that almond β-glucosidase had a catalytic function involving two key groups, aspartic and glutamic carboxyl groups at the enzyme's active site, when they were in the appropriate protonation state. Campbell's model assumes that enzyme activity can be lost in two ways: (1) deprotonation of the aspartic carboxyl group; (2) protonation of the glutamic carboxyl group. Geiger et al. (1998) found that the effect of copper was strongest in the pH range 5.0–5.5, in which case 200 μM Cu reduced enzyme activities (of cellulase and β-glucosidase) by 25% or more. However, when the pH was close to 4, the enzyme activities were reduced by only 5% by the same level of copper.

Different enzymes can respond differently at the same pH values and metal levels. Under conditions of pH 5.5 and 600 μM copper, β-glucosidase activity was reduced by 90% whereas cellulase activity dropped by 60%.

11.2.3.2 Soil Organic Matter

D'Ascoli et al. (2006) investigated the effects of heavy metal contamination on the biological and biochemical properties (FDA hydrolase, dehydrogenase, β-glucosidase, urease, arylsulfatase, and acid phosphatase) of a soil onto which a river contaminated with Cr(III) and Cu overflowed. The results showed negative correlations between the activities of dehydrogenase, arylsulfatase, and acid phosphatase and Cr fractions (soluble, exchangeable, and carbonate-bound). Although Cu pollution negatively influenced soil biological and biochemical properties, the soil organic matter was able to mask these negative impacts of Cu on the microbial community.

Similarly, many other studies have shown that organic amendments (with municipal waste, compost, biosolid compost, leonardite, gyttja, and litter) reduce the toxicities of heavy metals to soil enzymes (de Mora et al. 2005; Karaca et al. 2006).

Karaca et al. (2002) indicated that many of the effects of Cd were reduced by sewage sludge and phosphate fertilizer amendments. Therefore, reducing the amount of fertilizer added to a contaminated agricultural site will result in an increase in the availability of Cd at that site. A positive way of reducing the impact of Cd contamination is therefore to continue phosphate and sewage sludge/organic matter amendments, which are low in pollutants, on a limited basis. For example, if 80% of the Cd added to the soil remains in the topsoil each year (Taylor 1997), the addition of phosphate or organic matter resulting in a <20% increase in the soil Cd content will eventually result in a reduction of Cd in the soil. This will also reduce the availability of Cd, resulting in less toxic soil and less Cd being sequestered by crop biomass.

Tejada et al. (2008) found that increasing Ni levels reduced soil enzyme activities, and that soil amendment with organic wastes (crushed cotton gin compost, poultry manure) reduced the toxicity of nickel to soil enzyme activities (urease, BBA-protease, alkaline phosphatase, β-glucosidase and arylsulfatase). Organic amendments enhance soil enzyme activity for the following reasons: (1) intra- and extracellular enzymes stimulate microbial activity in the added materials, (2) carboxyl, phenolic, alcohol, and carbonyl functional groups in the humic substances react with toxic ions, forming metal–humate complexes (metal chelation) and stabilizing them (Nannipieri 1994; Dick 1997; Pascual et al. 1998).

Tejada et al. (2008) summarized the following results from different studies. Carboxyl groups play an important role stabilizing toxic ions in the humic acids (McKnight et al. 2001). Although fulvic acids contain more carboxyl groups than humic acids (Stevenson 1994), studies show that metal chelation by humic acids is more effective than metal chelation by fulvic acids since humic acids provide more binding sites due to their larger molecules and more complex nature (Lobartini et al. 1994). Also, humic substances have more strongly acidic groups than fulvic

acids (Hayes 1991). Tejada et al. (2008) concluded that soil microbial biomass and soil enzyme activities are greater in humic acid (crushed cotton gin compost) than in fulvic acid-amended (poultry manure) soil, that the addition of these organic materials may be considered a good strategy for heavy metal polluted soil remediation, and also that the addition of organic materials with a higher humic acid than fulvic acid concentration is more advisable.

11.2.3.3 Clay Minerals

Zeng et al. (2007) studied the effect of lead treatment on the soil enzyme activities in a soil–lead–rice system in a greenhouse pot experiment. High inhibition was observed in sandy soil with a low organic matter content. Similarly, Renella et al. (2003) found that enzyme inhibition was greater in sandy than in fine-textured soils because the clay fraction protects soil enzyme activity.

Geiger et al. (1998) investigated the effect of copper on the enzymatic decomposition of cellulose by cellulase and β-glucosidase in suspensions of montmorillonite and aluminum-treated montmorillonite. The results showed that montmorillonite and Al-montmorillonite reduced the activities of cellulase and β-glucosidase. Also, the use of montmorillonite resulted in the largest reduction in enzyme activity due to its larger specific surface and higher surface area. Gianfreda et al. (1991) indicated that the specific surface areas of montmorillonite and Al-montmorillonite when fully dispersed were approximately 700 and 450 $m^2 g^{-1}$, respectively. There are various reasons for the different specific surface areas of these clay minerals: (1) the adsorption of enzyme molecules on both external and internal surfaces by montmorillonite (Fusi et al. 1989), and; (2) the larger net negative charge of montmorillonite (87 meq $100g^{-1}$) compared to Al-montmorillonite (15 meq $100g^{-1}$) (Lothenbach et al. 1997).

Montmorillonite and Al-montmorillonite did not reduce the toxic effect of the metal. To explain this, Geiger et al. (1998) cited the higher affinity of copper for cellulase and β-glucosidase than for montmorillonite or Al-montmorillonite, and the synergetic effects of clay minerals and copper on the inhibition of enzyme activity. Geiger et al. (1998) proposed that clay surfaces interact with both enzymes and metals and ultimately reduce the toxicity of metals.

Clay minerals can strongly affect extracellular enzyme activity in soil (Geiger et al. 1998). The adsorption of enzymes at clay surfaces caused two different responses: (1) the inactivation of enzymes due to conformational changes (Burns 1978; Boyd and Mortland 1990; Geiger et al. 1998), or; (2) enzyme activity enhancement caused by increased concentrations of enzyme and substrate at the solid–water interface (Burns 1978).

Tietjen and Wetzel (2003) investigated the effect of clay adsorption on enzyme activities (alkaline phosphatase, glucosidase, protease, and xylosidase). Montmorillonite clay (M) and clay extracted from Elledge Lake (EL) were used in enzyme–clay solutions in an adsorption experiment. While adsorption onto the EL clay decreased alkaline phosphatase activity, adsorption onto the M clay decreased the activities of

all of the studied enzymes. They also found that the adsorption of enzyme onto clay protects the enzyme from photodegradation.

Wyszkowska et al. (2006) investigated the effects of copper on soil enzymes (dehydrogenase, urease, acid phosphatase, and alkaline phosphatase) and its interactions with other heavy metals (Zn, Ni, Pb, Cd, Cr). They found that the activity of dehydrogenase was greater in heavy loamy sand, while the activities of other enzymes were higher in light silty clay. In another words, enzyme inhibition due to heavy metals was greater in heavy loamy sand than in light silty clay (except in the case of dehydrogenase).

11.2.4 Plant Factors

11.2.4.1 Metal Accumulator Plants

Wang et al. (2008) defined metal accumulator plants as those that can grow in heavy metal contaminated soils, and have evolved mechanisms to tolerate high levels of heavy metal from the soil inside their cells (Tang et al. 1999; Song et al. 2004). Mining sites, in particular, contain high heavy metal concentrations in soil and metal-tolerant plants. *Elsholtzia splendens* is a Cu-tolerant plant that is widely found at Cu mining sites and is used as a Cu-mine indicator (Wang et al. 2008). Such plants can be used in the phytoremediation of heavy metal soils because they accumulate the metals and thus reduce metal levels in the soil. Wang et al. (2008) investigated the acid phosphatase activity in the rhizospheres of a copper accumulator (*Elsholtzia splendens*) and a nonaccumulator plant (*Trifolium repens*) upon different Cu treatments (0, 200, 500, 1,000 mg kg^{-1}). The results showed that enzyme inhibition was strong in the unplanted and nonaccumulator plant rhizospheres and weak in the rhizosphere of the Cu-accumulator plant. Wang et al. (2007a,b) studied the effect of heavy metal pollution on enzyme activity near a copper smelter. They found a strong inhibition of alkaline phosphatase activity near the copper smelter (<200 m).

11.2.4.2 Plant Community Effect

Yang et al. (2007a,b) investigated the effects of coexisting plant species on soil microbes and soil enzymes in lead-contaminated soils. In a mesocosm experiment carried out in greenhouse, four different plant species (*Festuca arundinacea*: FA, *Kummerowia striata*: KS, *Echinochloa crusgalli*: EC, and *Solidago canadensis*: SC), three different species mixtures (one: FA, two: FA + KS, four: FA + KS + EC + SC), and three different lead application rates (0, 300, and 600 mg kg^{-1}) were used. Urease activity was significantly affected by plant species and Pb concentration. It was significantly greater for the four-species mixture than for the one- or two-species mixtures. Alkaline phosphatase activity was not significantly impacted

by plant species but was affected by Pb concentration. Acid phosphatase and dehydrogenase were not significantly influenced by either species mixture or Pb concentration.

11.2.5 Special Inhibition Parameters

11.2.5.1 Ecological Dose

The effect of the heavy metal on soil enzyme activity can be quantified by determining the ED_{50} (ecological dose) parameter, which is the concentration of heavy metal at which the enzyme activity, or some other biological activity, is reduced to 50% of its uninhibited value (Tejada et al. 2008). Tejada et al. (2008) reported that ED_{50} values may be more suitable indicators of the sensitivity of an ecosystem to stress, because a 50% reduction in the basic ecological process may be too extreme for its continued functioning (Babich et al. 1983). Many researchers have used this inhibition parameter to evaluate soil enzyme inhibition by heavy metals, and their results are summarized in Table 11.4.

11.2.6 Understanding the Inhibition of Soil Enzymes by Heavy Metals

11.2.6.1 Combined Effects

Heavy metals exert inhibitory effects on soil enzymes, but these effects depend on many factors in the soil.

Combined Effects of Two Metals

Khan et al. (2007) investigated soil enzyme activities (catalase, alkaline phosphatase, and dehydrogenase) when various levels of Cd and/or Pb were applied to the soil. This work thus provides a good example of the combined effects of heavy metals on soil enzyme activities (see Table 11.5). Strong inhibition was observed at high heavy metal concentrations in both the single-metal and dual-metal systems; however, the inhibition was greater in the dual-metal system than the single-metal systems; in other words, a "synergistic effect" was observed. However, some combinations of metals exhibit this synergism while others do not. Wyszkowska et al. (2006) concluded that treatment with copper alone was more inhibitory towards soil enzyme activity than copper applied in conjunction with other heavy metals (Cu with Zn, Ni, Pb, Cd, and Cr).

Table 11.4 ED_{50} values for evaluating soil enzyme inhibition by heavy metals

Soil type												
pH	OM[a]	CEC[b]	Sand[c]	Clay[c]	Treatment	ED_{50}						Reference
5.94	26.4	13.30	54	16	Pesticides + Hg	UR						Yang et al. (2007a,b)
					Hg: 0.92, 1.85, 3.69, 7.39, 14.77, 29.54	88						
6.19	20.7	15.35	62	18		5.5						
6.26	31.6	28.05	29.8	32		24						
6.71	29.4	23.0	22	36		20						
								ACP		ALP		
5.1	3.7[c]	–	77.8	2.2		Cd		558.2		15.1		Renella et al. (2003)
						Cd + Cu		46.1		9.7		
						Cd + Zn		260.7		16.1		
						Cd + Cu + Zn		159.9		7.9		
7.2	0.7[c]	–	81.9	11.4	Cd	14.3				4.1		
					Cd + Cu	3.9				2.9		
					Cd + Zn	3.5				3.1		
					Cd + Cu + Zn	3.1				2.6		
8.1	2.2[c]	–	20.5	42.2	Cd	30.0				25.5		
					Cd + Cu	15.1				15.9		
					Cd + Zn	28.1				17.1		
					Cd + Cu + Zn	10.7				14.4		
7.68	1.31[c]	6.9[d]	78.2	7.1	Pyrite sludge amended	ASL	ALP	ACP	BG	DEH	UR	Hinojosa et al. (2008)
					As 2.8, Cd 0.1, Zn 38.8, Cu 9.5,	0.61	0.55	1.89	0.68	1.71	2.74	
7.66	2.02[c]	12.3[d]	14.8	36.2	Cr 0.01, Fe 234.1, Mn 0.3,	1.36	2.69	3.65	3.38	2.12	6.27	
7.72	1.58[c]	11.3[d]	41.5	11.6	Hg 0.1, Ni 0.003, Pb 39.9	n.s.	3.15	0.88	0.49	2.71	14.59	
7.37	17.1[c]	-	4	41	Enzyme	Cr[VI]0.4		Cr[III]0.2				Samborska et al. (2004)
					Suspension soil + enzyme	0.5		0.4				
					Suspension soil + irrigated, purified wastewater	0.5		0.6				

[a]mg kg^{-1}; [b]cmol kg^{-1}; [c]%; [d]meq 100 g^{-1}; *UR*, urease; *ACP*, acid phosphatase; *ALP* alkaline phosphatase; *DEH* dehydrogenase; *ASL* arylsulfatase; *BG* β-glucosidase

Table 11.5 Combined effects of Cd and Pb on enzyme activities in soil[a] in a pot experiment performed in a greenhouse (Khan et al. 2007)

Enzyme[b]	Cd and Pb application rates[c]	Incubation time (weeks)	Inhibition of activity (%)
CATCAT	Cd1Cd3	22	5.9639.3
CAT	Cd3 + Pb3	2	39.9
CAT	Cd1 + Pb1	2	8.8
ALP	Cd1	2	7.8
ALP	Cd3	2	41.5
ALP	Pb1	2	7.8–19.3
ALP	Pb2	2	11.9–20.9
ALP	Pb3	2	13.1–24.3
ALP	Cd1 + Pb1	2	25.5
ALP	Cd2 + Pb2	2	40.5
ALP	Cd3 + Pb3	2	43.5
DEH	Cd1	2	19.3
DEH	Cd2	2	25.9
DEH	Cd3	2	32.4
DEH	Pb1	2	2.9–15.8
DEH	Pb2	2	7.2–23.7
DEH	Pb3	2	12.1–18.2
DEH	Cd1 + Pb1	2	8.9–24.1
DEH	Cd2 + Pb2	2	11.9–32.5
DEH	Cd3 + Pb3	2	15.5–41.6

[a]Soil properties: pH 8.0; OM:17.9 g kg^{-1}; 42.5% sand; 10.4% clay; total Cd: 0.14 mg kg^{-1}; total Pb: 2.57 mg kg^{-1}

[b]*CAT* catalase; *ALP* alkaline phosphatase; *DEH* dehydrogenase

[c]Cd added as $CdSO_4$, Pb as $Pb(NO_3)_2$; application rates (in mg kg^{-1}) were: *Cd1*, 1.5; *Cd2*, 3; *Cd3*, 5; *Pb1*, 150; *Pb2*, 300; *Pb3*, 500

Combined Effects of Three Metals

Yang et al. (2006) investigated the combined effects of Cd, Zn, and Pb on catalase, urease, invertase, and alkaline phosphatase in soil. The results showed that Cd significantly inhibited the activities of all of the enzymes studied, Zn only inhibited those of urease and catalase, while Pb was not significantly inhibitory compared to the other heavy metals towards the studied enzymes, and actually had a protective influence on catalase activity when all of the metals were present (Cd, Zn and Pb). Cd was the most effective enzyme inhibitor, followed by Zn. The order of the effect of Cd, Zn and Pb was Cd > Zn > Pb. There was a negative synergistic inhibitory effect of Cd and Zn on urease and catalase activity in the presence of Cd, Zn, and Pb, which can be explained by the similar ionic properties of Zn and Cd. Urease activity was enhanced by Cd and Pb at low concentration; however, it was inhibited at higher concentrations of Cd and Pb. Urease activity was reduced by 20–40% in the Cd–Zn–Pb combined metal system. Therefore, three-metal treatments had a greater inhibitory effect than the single heavy metal treatments because of a synergistic effect of the metals on enzyme activity. In this study, the enzymes showed different sensitivities to the single- and three-metal treatments. Urease was the most

sensitive of the enzymes to combined pollution (Cd, Zn and Pb). Yang et al. (2006) reported that the magnitude of enzyme inhibition or activation depends on (1) the heavy metal ion, its concentration, and the type of enzyme assayed, (2) the interaction between the heavy metals, (3) the reactions between the heavy metals in solution and the functional groups of the enzymes, (4) the chemical and physical properties of the soil (pH, organic matter content, and type and amount of clay).

Combined Effects of pH, Organic Matter (OM), Clay, and Four Metals

Irha et al. (2003) studied the effect of heavy metals and PAHs on dehydrogenase in soil. Decreasing the organic matter, clay and pH slightly inhibited the dehydrogenase (Table 11.6). Rendzina alvar and Brown pseudopodzolic soils differ only in their organic matter and amorphous mineral phase contents; their clay contents are the same. The dehydrogenase was more inhibited at lower organic matter and higher amorphous mineral phase contents (i.e, in Brown pseudopodzolic soil). Organic matter and the amorphous mineral phase may therefore mask dehydrogenase inhibition by heavy metals.

Combined Effects of pH, OM, Clay, Cation Exchange Capacity (CEC), and Chemical Form of Metal

Carine et al. (2008) evaluated the effects of different metals in different chemical forms (chloride, sulfate and acetate salt) on soil phenoloxidase activity. This study is a very good example of an investigation of soil enzyme inhibition by heavy metals because the researchers considered many factors and examined many heavy metals. The influential factors are obvious from Table 11.7. The study results lead us to conclude that soil enzyme inhibition by heavy metals depends on: (1) the heavy metal its concentration; (2) soil texture (clay content); (3) the chemical form of the heavy metal (Karaca et al. 2000).

Table 11.6 Effects of heavy metals and PAHs on soil dehydrogenase activity (adapted from Irha et al. 2003). Soils were artificially contaminated with heavy metals (as their chloride salts) at the following levels: Cr (Cr^{+3}) 3 mg L^{-1}; Pb 6 mg L^{-1}; Cu 20 mg L^{-1}; Cd 60 mg L^{-1}

Soil type	Inhibition
Rendzina alvar: pH 7.0, 22.94% OM, 30% clay, 2% amorphous phase	Weak DEH
Brown pseudopodzolic: pH 7.2, 6.64% OM, 30% clay, 1% amorphous phase	Moderate DEH
Sod podzolic: pH 6.2, 4.88% OM, 15% clay, 31% amorphous phase	Strong (no activity) DEH

Table 11.7 Combined effects of heavy metals on the enzyme activity of phenoloxidase

	Soil types					
	Sandy loam 1: pH 6.9, 11.8 g kg^{-1} OM, 7.5 cmol kg^{-1} CEC, 67% sand, 13% clay		Loam: pH 8.5, 8.8 g kg^{-1} OM, 9 cmol kg^{-1} CEC, 36% sand, 23% clay		Sandy loam 2: pH 8.2, 9.9 g kg^{-1} OM, 4 cmol kg^{-1} CEC, 67% sand, 12% clay	
	Phenoloxidase activity					
Treatments	Inhibition (−) degree	100% (−) level or activation (+) range (nM)	Inhibition (−) degree	100% (−) level or activation (+) range (nM)	Inhibition (−) degree	100% (−) level or activation (+) range (nM)
BaCl salt	Strong	150	Strong	150	Strong	50400
$CdSO_4$ salt	Weak	150	No effect	400	Weak	400
CoCl salt	Strong	150	Strong	400	Strong	200
$CoSO_4$ salt	Strong	10	Strong	10	Strong	10
CuCl salt	Moderate	5	Weak	0.5–200	Strong	50–400
$CuSO_4$ salt	Moderate		Weak	5	Moderate	5–400
$FeSO_4$ salt	Strong		Strong	150	Strong	400
MgCl salt	No effect		No effect	400	No effect	10
MnCl salt	No effect		No effect		(+) Strong	150
$MnSO_4$ salt	No effect		(+) Weak		(+) Strong	200
NiCl salt	No effect		No effect		Weak	
Pb-acetate salt	Moderate		Moderate		Strong	
SnCl salt	Strong		Strong		Strong	
ZnCl salt	(+) Weak		(+) Weak		(+) Weak	
$ZnSO_4$ salt	(+) Weak		(+) Weak		(+) Weak	
AlCl salt	Moderate		Strong		Strong	
$AlSO_4$ salt	Weak		Strong		Strong	
(0, 0.5, 5, 10, 50, 100, 150, 200, 400, 800 nM)						

Combined Effects of Metal, Metal Oxidation State, and Organic Matter

Senwo and Tabatabai (1999) conducted a study on the effects of heavy metals on aspartase activity in soils. They concluded that: (1) the most effective inhibitors of aspartase activity were Ag(I) and Hg(I); (2) aspartase activity was significantly correlated with organic carbon, total nitrogen, and clay content; (3) activity inhibition was higher in air-dried soils than in field-moist soils because the air-dried soils provided more exposure of the enzyme to heavy metals. The results of this study are shown in Table 11.8, and can be summarized as follows. (1) Higher organic matter and clay contents along with a higher soil pH results in less inhibition of aspartase activity. (2) Higher oxidation states of heavy metals are less inhibitory than lower oxidation states. (3) Ag and Hg are highly toxic elements.

11.3 Conclusion

As a result of increasing metal concentrations in the soil due to either natural or anthropogenic contamination, it has been found that soil enzyme activities are influenced by different metals in different ways, depending on the type of metal and the metal salt. However, soil characteristics such as pH, clay content, and soil organic matter, can modify the negative or positive impacts of heavy metals on soil enzymes. Therefore, in addition to monitoring changes in soil metal content, an assessment of changes in soil enzyme activities would be a useful tool for monitoring soil quality and fertility under heavy metal pollution. This definitely depends on the enzyme, the metal, and its concentration. Based on the

Table 11.8 Effects of heavy metal species on aspartase activity in soils

	Inhibition of aspartase activity (%) in following soil types:		
Heavy metal species (5 μmol g^{-1} soil)	Weller soil: pH 6.0, 12.2% OC, 235 g kg^{-1} clay, 46 g kg^{-1} sand	Webster soil: pH 6.9, 32.45 OC, 264 g kg^{-1} clay, 250 g kg^{-1} sand	Harps soil: pH 7.9, 44.0% OC, 356 g kg^{-1} clay, 188 g kg^{-1} sand
Ag(I)Cu(I)	9856	9134	8731
Cd(II)	63	54	49
Fe(II)	41	26	35
Hg(II)	97	95	87
Sn(II)	53	32	31
Fe(III)	53	32	28
Ti(IV)	48	31	25
As(V)	32	45	25
Mo(VI)	28	45	27

research findings discussed in this chapter, it can be concluded that intracellular oxidoreductases (i.e., dehydrogenase) that take part in microbial processes are more vulnerable to metal-related short-term changes than extracellular ones. Without a doubt, this is due to the linkage of the extracellular enzymes to the colloidal soil fractions, especially clay and organic matter, through adsorption and crosslinking, microencapsulation, copolymer formation, entrapment, ion exchange, and covalent attachment, and hence them becoming resistant to environmental factors. However, again, the research findings presented in this chapter reveal that different salts of a particular metal affect enzyme activities differently, and that metal solutions prepared from various metal salts cause different degrees of enzyme inhibition (Karaca et al. 2000). This fact has generally been neglected in most incubation studies that have examined the effects of heavy metals on soil enzyme activities, but it should be taken into consideration in future experimental studies.

In many laboratory studies, the application of increasing concentrations of metal nitrate or sulfate salts also results in the addition of large amounts of nitrogen and sulfur, which are nutrients for soil microflora that synthesize soil enzymes. Following the application of these metal solutions to the soil, the heavy metals would probably inhibit enzyme activity while the nutrients would support the enzyme production system. This balance between the inhibitory effects of the metals and the stimulatory effects of the nutrients in the solution may blur the actual influence of the metal on soil enzyme activities. Similarly, solutions of metal salts that do not contain microflora-activating ions (i.e., chlorides) could also result in complex effects. Therefore control treatments where only the nutrients or salt constituents are applied to the soil should also be included in laboratory incubation experiments.

This is also necessary in laboratory studies evaluating the effects of multiple heavy metals on soil enzyme activity.

On the other hand, the increase in enzyme activity resulting from the application of various metal solutions to the soil at low concentrations may be related to either the metal itself or other anions in the metal salt solution, and we need to clarify which one of these options is correct. Also, thus far, low concentrations of some heavy metals like Zn and Cu have been shown to have nutritional value, while this is not the case for other metals like Cd. The reason for the increase in enzyme activity at lower Cd concentrations, as reported in numerous research papers, needs to be clarified.

The main soil characteristics that control the influence of heavy metals on soil enzyme activities are the clay and organic matter contents and the soil pH. Since these are the primary factors that affect the binding of metals to soil colloids and their uptake by biological systems, any changes to these soil characteristics will affect the interactions between heavy metals and soil enzymes. However, most works have shown that although different soils have different physicochemical features, increasing the heavy metal concentration largely inhibits the biological activity of the soil, and so soil enzymes are highly sensitive indicators of soil degradation due to heavy metal accumulation.

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Chapter 12
Effect of Heavy Metals on Saprotrophic Soil Fungi

Petr Baldrian

12.1 Introduction

Heavy metals represent a highly abundant group of toxic compounds in the soil environment. Although their natural presence is limited to a few soil habitats, they are locally present as a consequence of human activities including mining, processing, or the extensive use of the metal. These activities typically result in soils with gradients of metal distribution where heavy metals are often present as a mixture. In contrast to organic contaminants, heavy metals are nondegradable, and although they exhibit some mobility in the environment, the contamination is usually relatively stable over time. Since the presence of heavy metals interferes with many important physiological processes, they affect the whole soil community, including soil bacteria, fungi, plants, and other organisms, and can have effects that range in scale from a single cell or spore to the community as a whole.

Some heavy metals are essential for microbial metabolism whereas others have no known biological role. In fungi, the metals necessary for growth include copper, iron, manganese, cobalt, molybdenum, zinc, and nickel. Nonessential metals that are commonly encountered include chromium, cadmium, lead, mercury, and silver. While fungi have metabolic requirements for trace metals, the same metals are often toxic at concentrations only a few times greater than those required. Both essential and nonessential heavy metals are thus toxic to fungi when present at excess levels in bioavailable forms (Baldrian 2003).

In contrast to unicellular bacteria (where heavy metal resistance is usually a plasmid-encoded property of individual strains, and is essential for their survival), mycelial fungi have a more varied array of strategies for reducing metal toxicity, including the avoidance of toxic soil domains and extracellular or cell wall associated immobilization.

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There are already several papers on fungal responses to heavy metals, both generally (Gadd 1993, 2007) and focusing on brown-rot and white-rot basidiomycetes (Jellison et al. 1997; Baldrian 2003). The interactions of heavy metals with mycorrhiza have been the subject of several reviews (Leyval et al. 1997; Dighton 2003; Colpaert 2008), and are also the topic of chapter 5 in this book. On the other hand, information on the effects of heavy metals on soil saprotrophic fungi and fungi-catalyzed processes has not been previously summarized, and is the subject of this chapter.

12.2 Interactions of Fungi with Heavy Metals in the Soil Environment

Metals may be present in soils as free metal ions, complexes with organic matter, or they may be chemically precipitated into insoluble compounds, such as oxalates, carbonates, and hydroxides. The degree of toxicity of the metal to organisms depends upon its relative availability (solubility) within the soil solution. This availability is dependent upon a number of edaphic factors, such as soil pH, Eh, organic matter, and clay content. Soil microfungi are able to tolerate higher Cd concentrations in the presence of clay or montmorillonite than in kaolinite, and are less sensitive at higher pH levels (Babich and Stotzky 1977). The biogenic factors affecting metal availability include solubilization (leaching), immobilization by co-precipitation with biogenic compounds, biosorption, and bioaccumulation (Berthelin et al. 1995; Gadd 2007).

Fungi are able to restrict the entry of toxic metal species into cells by (1) extracellular metal sequestration – binding the metal to siderophores or other fungi-derived metabolites; (2) binding it to the cell wall and wall-associated components, and; (3) reducing its uptake by intracellular chelation or sequestration. The above defense mechanisms act simultaneously; the mycorrhizal fungus *Paxillus involutus* is able to produce oxalate that binds some extracellular metals. The heavy metals that are not bound come into contact with mycelium and are localized in or near the cell wall, in the vacuoles, and in the cytoplasm. For Cd, its distribution among the three biomass components listed above was 50%, 20%, and 30%, respectively (Blaudez et al. 2000). Similar detoxification systems may act both intra- and extracellularly: in the case of nickel immobilized by *Aspergillus niger*, Ni oxalate crystals have been documented in the extracellular fraction as well as in the cell wall and cytoplasm (Magyarosy et al. 2002).

12.2.1 Mobilization and Immobilization of Heavy Metals

The availability of metals in soils is – at least in part – also dependent on fungal activity. In podzol E horizons under boreal or mountainous coniferous forests, the weathering of bedrock has been attributed to oxalic, citric, succinic, formic, and

malic acid excretion by saprotrophic and mycorrhizal fungi. Ectomycorrhizal fungi can form micropores (3–10 mm) in weatherable minerals, and hyphal tips are able to excrete micro- to millimolar concentrations of these organic acids (Jongmans et al. 1997). Heavy metals can be mobilized during this process as well as during fungal weathering of limestone, sandstone, marble or other minerals (Gadd 2007). The ability to solubilize metals from metal oxides is frequently present among soil micromycetes (e.g., *Aspergillus* and *Penicillium* spp.). One-third of 56 soil isolates were able to solubilize either ZnO, $Zn_3(PO_4)_2$, or $Co_3(PO_4)_2$, and five strains solubilized all of the compounds (Sayer et al. 1995). In addition, pyromorphite ($Pb_5(PO_4)_3Cl$) can be solubilized by several organic acid-producing fungi (Sayer et al. 1999). While acidification seems to be the most frequent mechanism of metal solubilization, there are also other mechanisms that involve metal chelation, such as MnO and Zn solubilization by *Trichoderma harzianum* (Altomare et al. 1999).

The most abundant metal chelator produced by saprotrophic as well as mycorrhizal fungi is oxalate. The production of oxalic acid by fungi provides a means of immobilizing soluble metal ions or complexes as insoluble oxalates, thus decreasing bioavailability and increasing tolerance of these metals (Dutton and Evans 1996). Recently, the inducible and concentration-dependent production of oxalate by several ectomycorrhizal fungi and two saprotrophic *Hypholoma* spp. was demonstrated in laboratory experiments (Johansson et al. 2008). Metal oxalates can be formed with Ca, Cd, Co, Cu, Mn, Sr, and Zn. In addition to metal oxalate, metal oxides (e.g., MnO or FeO, desert varnish), metal hydroxides, moolooite ($CuC_2O_4\ nH_2O$), and several other metals can be formed by fungi under certain circumstances (Gadd 2007).

Cell walls of fungi are extremely important in the reduction of metal toxicity. Cell wall binding significantly contributes to metal immobilization, for example in the cases of Cd, Cu, and Ag in *Aspergillus niger* and *Mucor rouxii* (Mullen et al. 1992). Even the intact cell walls of many fungal species exhibit high binding capacities for heavy metals (Baldrian 2003; Baldrian and Gabriel 2003b). The binding capacity of the cell wall can be further increased through the production of specific wall-associated compounds, including the polysaccharidic outer hyphal sheath and melanin. Melanins are phenolic molecules, some of which are efficient bioabsorbers of copper, cadmium, lead, zinc, or toxic tin compounds (Fogarty and Tobin 1996; Baldrian 2003). In several fungi, the presence of heavy metals induces melanin production (Caesar-Tonthat et al. 1995). The presence of melanin significantly reduces the toxicity of Cu, Zn, Cd, Pb, and probably also that of the other bivalent metal ions (Fogarty and Tobin 1996). An investigation of metal binding to the mycelial melanin of *Armillaria* spp. found that the melanized rhizomorphs concentrated Al, Zn, Fe, and Cu ions to levels up to 50–100 times higher than those found in the surrounding soil (Rizzo et al. 1992).

The uptake of metals into the cytoplasm can be caused by the low selectivity of transporters for essential metals. Metal ions with similar chemical properties (e.g., Ca, Cd, and Zn) can use the same active transport system. The intracellular accumulation of Cd in *Paxillus involutus* was shown to be metabolically mediated, although Cd is not essential for fungi (Blaudez et al. 2000). At higher concentrations, metal

uptake can result from cell wall permeabilization by metals such as Cu and Cd (Ross 1993).

In most fungi, the presence of bivalent heavy metals like Cd and Cu induces the production of intracellular binding compounds. These can be divided into two main groups: (1) metal-binding oligopeptides containing cysteine–glutathione (GSH), phytochelatins, and related compounds, and; (2) proteins, such as metallothioneins. Both types of compounds can be produced simultaneously. In strains of *Aspergillus* sp., GSH was found to be responsible for the binding of arsenic such that strains with different sensitivities to As were found to differ in their levels of GSH production (Canovas et al. 2004). In addition, other *Aspergillus* strains were found to produce copper-binding proteins (Goetghebeur et al. 1995). In *Mucor racemosus* on the other hand, Cd (but not Cu or Zn) decreased the level of GSH by the induction of phytochelatin synthesis from GSH (Miersch et al. 2001). The ascomycete *Neurospora crassa* produces both phytochelatins and metallothionein in response to copper and other heavy metals (Lerch 1980; Kneer et al. 1992).

In the basidiomycete genus *Agaricus*, a typical metallothionein is produced in response to Cu (Lerch 1980), while cadmium induces the production of a protein of another type, called mycophosphatin (Meisch and Schmitt 1986). Cd-mycophosphatin is also produced in the ectomycorrhizal *Boletus edulis*, but Cd, Cu, Hg, and Zn also induce the synthesis of phytochelatins (Collin-Hansen et al. 2003, 2007). In *Paxillus involutus*, Cd increases the levels of intracellular GSH, and both Cu and Cd (but not Zn) also induce metallothionein synthesis (Courbot et al. 2004; Bellion et al. 2007). The complexes of Cd and its binding molecules are sequestered into vacuoles (Ott et al. 2002). It seems that there is no general mechanism of response to heavy metals in basidiomycetes.

12.2.2 Accumulation of Heavy Metals by Fungi

Elevated concentrations of toxic metals can occur in the fruitbodies of basidiomycetes in polluted environments, and soil saprotrophic and mycorrhizal fungi have been frequently proposed as suitable biomonitors of metal pollution (Kalac and Svoboda 2000; Collin-Hansen et al. 2002; Baldrian 2003). The extent of accumulation of individual metals is species or strain specific, with the high levels of metal enrichment in fungal fruitbodies serving as evidence of the high metal tolerance of fungi. Also, several soil saprotrophic basidiomycetes (e.g., the members of the genera *Agaricus*, *Coprinus*, *Lepista*, *Lycoperdon*, *Marasmius*, or *Mycena*) have been found to be accumulators of heavy metals (Mejstrik and Lepšová 1993; Svoboda et al. 2006). Fruitbodies of *Armillaria mellea*, collected from metal-polluted soils near motorways, contained several parts per million of Cd and Pb and tens of parts per million of Zn. Cd accumulated with a concentration factor of 32, while Zn and Pb were excluded, with the concentration in sporophores reaching 30–40% of that in the topsoil (Cuny et al. 2001). In another study on soil saprotrophic species (*Coprinus*, *Lepiota*, *Marasmius*, and *Armillaria*), the concentration factors were found to be between 4 and 100 for Cd and Cu (Poddubny et al. 1998).

The transport of heavy metals between the substrate and fungal fruitbodies occurs in both directions. Cd and Hg tracers applied to the fruitbodies of *Agrocybe aegerita* were partially translocated into the substrate (wheat straw) and into consecutive harvests (Brunnert and Zadrazil 1979). The accumulation of heavy metals by fungi is not limited to their transport into fruitbodies. As mentioned above, the rhizomorphs of cord-forming fungi can also accumulate heavy metals, as do the fungal mycelia in soil. The saprotrophic fungi forming the so-called fairy rings, *Mycena*, *Psathyrella*, *Marasmius*, and the *Lycoperdaceae*, accumulate Ag or Cs. In a fairy ring transect, Cs concentrations correlated with ergosterol (Anderson et al. 1997).

12.3 Effects of Heavy Metals on Fungal Physiology

Metals exert toxic effects in many ways: they can (1) inhibit enzymes by the interactions with proteins; (2) displace or substitute for essential metal ions; (3) cause disruption of membranes, and; (4) cause oxidative stress or interact with systems that normally protect against the harmful effects of free radicals.

Growth reduction is a typical response of fungi to the toxicity of heavy metals (Baldrian 2003). It has been demonstrated that this reduction is dependent on nutrient availability, and that higher nutrient content can alleviate metal toxicity (Gadd et al. 2001). The growth of cord-forming saprotrophic basidiomycetes in the soil environment represents the colonization of nutrient-poor space in the search for nutrients. This growth is supported from the colonized bulky substrate, e.g., wood pieces or litter patches. This type of colonization of nutrient-limited soil regions was significantly reduced for *Pleurotus ostreatus* in the presence of Cd or Hg (Baldrian et al. 2000), and Pb exhibited the same effect on several litter-decomposing fungi (Tuomela et al. 2005; Kähkönen et al. 2008).

The morphological changes induced by heavy metals are common among all groups of fungi (Baldrian 2003). Changes in mycelial morphology were observed in *Mucor rouxii* in the presence of a high copper concentration (Gardea-Torresdey et al. 1997). In the ectomycorrhizal fungus *P. involutus*, the addition of Cd led to an increase in hyphal density caused by increasing numbers of laterals per branch point and a decrease in the distance between branch points (Darlington and Rauser 1988). Furthermore, the morphologies of whole fungal colonies are affected by heavy metals. This is the case for *Trichoderma viride* and *Rhizopus arrhizus*, which show biomass redistribution within colonies (Gadd et al. 2001).

Metal-contaminated soils usually contain a spatially heterogeneous distribution of metal concentrations and available nutritional resources, and the morphology of whole fungal colonies can reflect this heterogeneity. During the growth of fungi in metal-containing agar tiles, a wide range of morphological changes and growth responses occurred (Fomina et al. 2000, 2003). In the gap between metal-free and metal-containing tiles, the presence of copper or cadmium led to negative chemotropism in *Geotrichum candidum*, *Clonostachys rosea*, *Humicola grisea*, and *Trichoderma virens*, as well as the cessation of growth, swelling, or lysis of some hyphal tips.

In addition to growth reduction, there are detectable markers of metal toxicity in fungi. In the ectomycorrhizal fungus *Paxillus involutus*, Zn induced changes in vacuolar motility and tubularity, caused reversible mitochondrial fragmentation (Tuszynska et al. 2006), and interfered with the biosynthesis of polyamines (Zarb and Walters 1995). In fruitbodies of *Boletus edulis* collected in soils with a gradient of Cd, Zn, Cu, and Hg contamination, the extent of DNA and lipid damage was related to the metal concentration as a result of metal-induced oxidative stress (Collin-Hansen et al. 2005a).

Heavy metals in general are potent inhibitors of enzymatic reactions. Mercury exerts its toxic effect mainly by binding to sulfhydryl groups present in the active or regulatory sites of enzymes, thereby causing irreversible inactivation. Copper and cadmium – in addition to binding to aromatic amino acid residues in enzyme molecules – can also cause oxidative damage to proteins through the induction of oxidative stress associated with the production of reactive oxygen species such as hydroxyl or superoxide radicals (Baldrian 2003). In *Paxillus involutus*, cadmium stress leads to the expression of superoxide dismutase, an intracellular enzyme alleviating oxidative stress, and also increases the transcription of laccase, aconitase, and metallothionein (Jacob et al. 2001, 2004). Increased amounts of superoxide dismutase as well as catalase and HSP70 were also found in *Boletus edulis* fruitbodies exposed to higher concentrations of heavy metals in forest soils in a smelter area (Collin-Hansen et al. 2005b).

In different taxonomic groups of fungi, it was found that heavy metals are harmful to reproduction. In saprotrophic and mycorrhizal soil fungi, the reproductive stages of development (spore formation and germination) are much more sensitive to heavy metals than mycelial growth (Baldrian 2003). The litter-decomposing fungus *Agrocybe perfecta* failed to produce fruitbodies during growth on straw with 0.05–1 mM Cd or when metal-free straw was overlaid with soil containing 50 ppm of the metal; *Pleurotus ostreatus* was much less sensitive (Gabriel et al. 1996).

12.4 Effects of Heavy Metals on Soil Fungal Communities

Although different groups of microbes may show different sensitivities to heavy metals in the environment, the total microbial biomass is usually decreased in heavy metal contaminated sites; this is also true in many cases for the total fungal biomass. In oak litter polluted with Fe, Zn, Cu, Cr, Ni, and Pb, the amount of fungal biomass decreased with increasing metal content (Cotrufo et al. 1995). On the other hand, after the addition of sewage sludge containing Cu, Ni, and Zn, fungal biomass increased despite a decrease in the total microbial biomass (Khan and Scullion 2000). Soils containing more carbon are usually less affected than carbon-poor sandy soils. Several studies have shown that microbial communities respond to toxic metals by exhibiting changes in the relative abundances of bacteria and fungi. The results of these studies are summarized in Table 12.1. These findings, however, have to be treated with care: while the experimental addition of metal to soils

Table 12.1 Effect of heavy metals on soil bacterial/fungal biomass ratio

Metal(s)	Experiment[a]	Soil	F biomass	B biomass	F/B[d]	Reference
As	N (pollution)	Arable sandy loam	PLFA	PLFA	+	Lorenz et al. (2006)
Cd	A	Mor	PLFA	PLFA	–	Akerblom et al. (2007)
Cd	A	Pine forest humus	PLFA	PLFA	0	Fritze et al. (2000)
Cd	A	Arable sandy loam	PLFA	PLFA	+	Frostegard et al. (1993)
Cd	A	*Pinus* forest topsoil	PLFA	PLFA	0	Frostegard et al. (1993)
Cd	N (pollution)	Arable sandy loam	PLFA	PLFA	+	Lorenz et al. (2006)
Cd, Zn, Cu	N	Fallow paddy field	Counts	Counts	+	Hiroki (1992)
Cr	A	Mor	PLFA	PLFA	+	Akerblom et al. (2007)
Cu	A	Sandy forest soil	PLFA	PLFA	0	Frey et al. (2006)
Cu	A	Arable sandy loam	PLFA	PLFA	–	Frostegard et al. (1993)
Cu	A	*Pinus* forest topsoil	PLFA	PLFA	0	Frostegard et al. (1993)
Cu	A	Alfisol topsoil	Counts	Counts	+	Olayinka and Babalola (2001)
Cu, Cd, Zn	N (acid–metal spill)	Grassland	PLFA	PLFA	–	Hinojosa et al. (2005)
Cu, Ni, Zn, Pb, Cd	N (smelter)	*Picea* forest topsoil	PLFA	PLFA	–	Pennanen et al. (1996)
Cu, Zn	A	Forest	PLFA	PLFA	+	Rajapaksha et al. (2004)
Hg	N	Grassland	Chitinase	Counts	+	Müller et al. (2001)
Mo	A	Mor	PLFA	PLFA	–	Akerblom et al. (2007)
Multiple	N (smelter)	Coniferous forest	ML[b]	Counts	+	Nordgren et al. (1986)
Ni	A	Mor	PLFA	PLFA	–	Akerblom et al. (2007)
Ni	A	Arable sandy loam	PLFA	PLFA	+	Frostegard et al. (1993)
Ni	A	*Pinus* forest topsoil	PLFA	PLFA	0	Frostegard et al. (1993)
Pb	A	Mor	PLFA	PLFA	–	Akerblom et al. (2007)
Pb	N	Forest	PLFA	PLFA	–	Bååth et al. (2005)
Pb	A	Sandy forest soil	PLFA	PLFA	0	Frey et al. (2006)
Pb	A	Arable sandy loam	PLFA	PLFA	+	Frostegard et al. (1993)
Pb	A	*Pinus* forest topsoil	PLFA	PLFA	0	Frostegard et al. (1993)
Pb	A	*Sphagnum* litter	Counts	Counts	+	Nguyen-Viet et al. (2007)

(continued)

Table 12.1 (continued)

Metal(s)	Experiment[a]	Soil	F biomass	B biomass	F/B[d]	Reference
Pb, Cu, Zn, As, Cd, Hg	N (smelter)	*Picea* forest topsoil	PLFA	PLFA	–	Pennanen et al. (1996)
Zn	A	Mor	PLFA	PLFA	+	Akerblom et al. (2007)
Zn	A	Sandy forest soil	PLFA	PLFA	0	Frey et al. (2006)
Zn	A	Arable sandy loam	PLFA	PLFA	+	Frostegard et al. (1993)
Zn	A	*Pinus* forest topsoil	PLFA	PLFA	0	Frostegard et al. (1993)
Zn	A	Arable sandy loam	PLFA	PLFA	+	Frostegard et al. (1993)
Zn	A	*Pinus* forest topsoil	PLFA	PLFA	+	Frostegard et al. (1996)
Zn	A	Loamy sand	PLFA	PLFA	+	Kelly et al. (1999)
Zn, Cu, Ni	A (sewage sludge)	Sandy loam arable	PLFA	PLFA	+	Khan and Scullion (2000)
Zn, Cu, Ni	A (sewage sludge)	Sandy loam grassland	PLFA	PLFA	+	Khan and Scullion (2000)
Zn, Cu, Ni	A (sewage sludge)	Clay, arable	PLFA	PLFA	+	Khan and Scullion (2000)
Zn, Cu, Ni	A (sewage sludge)	Clay, grassland	PLFA	PLFA	+	Khan and Scullion (2000)
Zn, Cu, Ni	A (sewage sludge)	Clay loam	PLFA	PLFA	+	Khan and Scullion (2000)
Zn, Pb	N	Arable	Ergosterol	FE[3]	+	Chander et al. (2001a)
Zn, Pb	N	Fallow	Ergosterol	FE[c]	+	Chander et al. (2001a)
Zn, Pb	N	Heathland	Ergosterol	FE[3]	+	Chander et al. (2001a)
Zn, Pb, Cu	N	Clay marsh	Ergosterol	FE[3]	+	Chander et al. (2001b)

[a]*A*, metal addition; *N*, analysis of natural soil containing heavy metal(s); [b]*ML*, mycelial length; [c]*FE*, fumigation/extraction; [d]*F/B*, ratio of fungal to bacterial biomass (+ increase, – decrease, 0 no change)

makes it possible to study the effect of a particular metal ion in a controlled environment, this does not precisely reflect natural soil structure. In the contaminated sites that were investigated, on the other hand, it is often difficult to rule out other factors when comparing microbial communities in different soils, and heavy metals are often present in a mixture.

The level of heavy metal sensitivity among fungi varies considerably and is probably more strain specific than species specific. This is valid for both micromycetes and saprotrophic basidiomycete fungi (Gadd 1993; Baldrian and Gabriel 2002b; Baldrian 2003). In the analysis of a soil Cu gradient in a cupriferous swamp, several strains of micromycetes were found exclusively in Cu-rich samples (above 0.75% Cu) (Kendrick 1962). In a Cu soil enrichment experiment, fungal communities in higher metal concentrations shifted towards Cu-tolerant strains (Yamamoto et al. 1985). In Cu- and Zn-polluted soil, *Geomyces* and *Paecilomyces* spp. and some sterile forms increased with increasing pollution, whereas *Penicillium* and *Oidiodendron* spp. declined (Nordgren et al. 1983). In soil polluted with cadmium dust, *Strobilurus tenacellus*, *Mycena ammoniaca*, *Auriscalpium vulgare*, and *Armillaria lutea* were the most common basidiomycetes (Turnau 1991).

Based on fruitbody occurrence, mycorrhizal fungi were more tolerant of heavy metals in coniferous forest soil polluted with As, Cu, Cd, Pb, and Zn than litter-decomposing species (Ruhling and Soderstrom 1990). In Zn-supplemented loamy sand, total fungal biomass increased while the biomarker of arbuscular mycorrhiza decreased with increasing Zn level (Kelly et al. 1999). In another Zn-containing soil around a smelter, as well as in soils polluted by an acid-metal spill where Zn was accompanied by Cu and Cd, both total and arbuscular mycorrhiza-specific fungal biomass decreased at high Zn concentrations (Kelly et al. 2003; Hinojosa et al. 2005). Arbuscular mycorrhizal fungi seem to be generally more sensitive to metals than other fungal groups, as documented by the greater decrease in their biomass compared to the total fungal biomass in soils supplemented with Cr, Zn, Pb, Mo, Ni, and Cd (Akerblom et al. 2007).

In a specific study that focused on nematode-trapping fungi, no effect on their abundance was observed in soils containing up to 5000 ppm Pb, and the tolerances of strains isolated from polluted and unpolluted soils were similar (Mo et al. 2006). The fine structure of fungal communities was studied in pasture soil spiked with Zn-containing sludge. The fungal community showed a greater response to Zn addition compared to bacteria and archaea. The relative abundances of several fungal taxa increased significantly in high Zn treatments at the expense of others, some of which were lost completely (Macdonald et al. 2007; 2008). A recent study on Pb/Zn-, Ni-, and Cu-contaminated soils shows that the functional diversity of fungal communities is also affected, although to a lesser degree than that of soil bacteria (Stefanowicz et al. 2008). However, studies are lacking which describe the compositions of whole fungal communities based on culture-independent techniques in soils with contrasting metal contents.

An examination of microfungi isolated from unpolluted and copper-polluted forest soils showed that species from the polluted site were usually copper tolerant (Arnebrant et al. 1987; Baldrian and Gabriel 2002b). A comparison of isolates from

lead-enriched soil and isolates from unpolluted soils showed that *Tolypocladium inflatum* was intrinsically lead tolerant, and that the prolonged conditions with high lead had not selected for any increased tolerance (Bååth et al. 2005). Generally, there is little evidence for adaptation in isolates from sites with short or long histories of pollution (Baldrian and Gabriel 2002b; Gadd 2007). Such studies indicate that fungal survival is dependent on the intrinsic sensitivities of individual species or strains, rather than adaptive changes (Gadd 1993). Resistant fungal species are usually present at low frequencies in uncontaminated soils, but can become dominant under toxic metal stress (Gadd 2007).

12.5 Metal-Induced Changes in Fungus-Related Ecosystem Processes

In various types of soils, heavy metals were found to affect the ecosystem processes related to the cycling of C, N, and other elements. With the exception of a few studies where no effect was detectable, metals were found to inhibit general microbial processes, such as soil respiration, nitrogen transformation, or phosphatase activity (Necker and Kunze 1986; Giller et al. 1998; Macdonald et al. 2007). The effects of metals on soil processes in boreal forests were less pronounced than changes to the microbial community (Pennanen 2001). In gley soil, Cd addition decreased the mineralization of added glucose and cellulose, but did not decrease fungal counts (Hattori 1991). When different heavy metals were applied to the same type of soil, reduced mineralization of soil organic matter was observed in all cases, with Cd and Cu being the most toxic, Pb the least toxic, and Cr, Ni, and Zn showing intermediate toxicity (Hattori 1992).

Higher contents of the metals Fe, Zn, Cu, Cr, Ni, and Pb in *Quercus* litter resulted in not only reduced biomass of the soil fungi but also reduced respiration and mass loss (Cotrufo et al. 1995). Also, the decomposition of *Betula* leaf litter was retarded in soils affected by Cu, Ni, Pb, and Zn contamination (Johnson and Hale 2004). Cadmium added to straw inoculated with *Agrocybe perfecta* at concentrations from 0.01 to 1 mM significantly decreased the loss of organic matter (Gabriel et al. 1996).

Increased accumulation of litter was frequently found in areas contaminated with heavy metals (Berg and McClaugherty 2003). It is possible that the effect of heavy metals varies with the stage of decomposition and litter type. For *Pinus sylvestris* needle litter, only a weak suppression was found during the early stage of decomposition, whereas a clear trend towards considerably slower degradation was seen in the late stage requiring the degradation of lignin (Berg et al. 1991). One reason for these observations could be the retention of heavy metals in the litter structure, resulting in an increase in their concentration during litter decay. It was also previously mentioned that fungal diversity is affected by heavy metals.

Extracellular enzymes are affected by heavy metals in the same way as intracellular enzymes, but in the absence of protection by a cell wall they often act in an environment with higher metal concentrations. For most extracellular enzymes,

high concentrations of heavy metals are toxic, although not to the same extent (Frankenberger and Tabatabai 1991). The enzymes frequently produced by fungi to degrade biopolymers (e.g., ligninolytic oxidases and peroxidases, cellulases, hemicellulases, and chitinases) operate exclusively outside the fungal hyphae and represent targets for interactions with heavy metals (Baldrian 2008a, b). In a complex study on soils in a military area contaminated with different amounts of As, Cd, Cr, Cu, Ni, Pb, and Zn, samples with high metal content decreased the activity of β-glucosidase, endoglucanase, *N*-acetylglucosaminidase, and phosphomonoesterase by up to 10–50-fold. This reduction in enzyme activity was partly due to lower microbial biomass at contaminated sites (Kuperman and Carreiro 1997). Chitinase was unaffected in grassland soils with an Hg pollution gradient of up to 511 ppm (Müller et al. 2001).

It is well documented that laccase – a copper-containing enzyme – can be induced by the presence of Cu (and sometimes also Cd), while most other heavy metals inhibit its production or activity in wood-rotting and litter-decomposing fungi (Farnet et al. 1999; Baldrian and Gabriel 2002a; Baldrian 2003). In agricultural soils contaminated with Cd and Hg, Mn-peroxidase production by *Pleurotus ostreatus* was more sensitive to heavy metals than laccase. The decrease in Mn-peroxidase activity resulted in lower PAH degradation and it can be predicted that lignin degradation is also retarded in the presence of mercury and cadmium (Baldrian et al. 2000). The activities of the ligninolytic enzymes, laccase and Mn peroxidase, of the saprotrophic fungi *Collybia dryophila*, *Clitocybe nebularis*, and *Stropharia coronilla* were inhibited in Pb-contaminated soil, resulting in reduced lignin mineralization. Enzymes from different species showed different sensitivities to metals (Tuomela et al. 2005). When saprotrophic fungi were inoculated in Pb-containing soil, there was no significant change in cellulolytic enzyme production, while cellobiohydrolase and β-glucosidase were higher in control soils with fungi. This is in accord with the decrease in fungal growth observed in contaminated soil (Kähkönen et al. 2008). Manganese, the substrate of Mn-peroxidase, was demonstrated to positively affect the rate of litter degradation in the late stages of decay when lignin degradation is the predominant process (Berg et al. 2007).

Some species of saprotrophic soil micromycetes (e.g., *Torula lucifuga* and *Aspergillus ustus*) showed higher cellulase activities at high levels of Cu (Lebedeva et al. 1999). Xylanase in metal-supplemented soils was less sensitive to Zn, Cu, Cd, and Ni toxicity than arylsulfatase, phosphomonoesterase, and urease (Kandeler et al. 2000). During straw degradation by *Pleurotus ostreatus* in the presence of Cd, loss of organic matter was decreased as well as Mn-peroxidase activity, while the activities of endoglucanase, β-glucosidase, and laccase increased (Baldrian and Gabriel 2003a). In the presence of Cu, Mn, Pb, or Zn, the decrease in straw degradation was only minor. Laccase activity was increased in all metal-containing treatments, and Zn also induced higher endoglucanase and β-glucosidase activity (Baldrian et al. 2005). These data, obtained *in vitro*, confirm the fact that individual enzymes catalyzing the same reaction exhibit different sensitivities to heavy metals (Tuomela et al. 2005; Baldrian 2006). However, whether this is caused by environmental selection and what this means for the transformation of organic matter in contaminated soils remain to be clarified.

12.6 Conclusion

Heavy metals represent an important group of soil pollutants that contribute to the formation of specific "toxic" habitats characterized by alterations in the communities of soil organisms (including bacteria and fungi) and by changes in the rates of environmental processes. They also contribute to the heterogeneity of soil, forming microhabitats that are toxic to varying degrees within the soil ecosystem or soil profile. Saprotrophic fungi are especially sensitive to heavy metals since they rely heavily on extracellular enzymes for nutrient acquisition, and these enzymes are often a target of heavy metal toxicity. Although current studies show that the presence of metals can cause shifts in the relative abundances of fungi and bacteria in soils and in the compositions of fungal communities, more targeted work is required using modern molecular methods for a deeper understanding of the composition and function of fungal communities in heavy-metal affected soils.

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Chapter 13
Copper-Containing Oxidases: Occurrence in Soil Microorganisms, Properties, and Applications

Harald Claus

13.1 Introduction

Copper is an essential trace element in living systems, where it serves as a cofactor in many enzymatic redox reactions and oxygen transport (Fig. 13.1). The physiological oxidation states of copper are Cu^{1+} and Cu^{2+}, whereas Cu^{3+} is not a biologically relevant species because of the high redox potential of the Cu^{3+}/Cu^{2+} couple (Shleev et al. 2005). The copper at the active sites of redox proteins has been divided into three main classes (Table 13.1): type 1 (T1), blue copper; type 2 (T2), normal copper, and; type 3 (T3), a binuclear copper center (Malkin and Malmström 1970; Reinhammar 1984; Solomon et al. 1996, 2004; Kaim and Rall 1996).

T1 copper confers a typical blue color on the protein, which results from an intense electronic absorption band (around 600 nm) due to the covalent copper–cysteine bond. These sites are found in mononuclear copper proteins involved in intermolecular electron transfer pathways (azurin, plastocyanin, amicyanin, stellacyanin, rusticyanin), multicopper proteins (ascorbate oxidase, bilirubin oxidase, laccase, ceruloplasmin), and in a subclass of nitrite reductases, where they function in intramolecular electron transfer.

T2 copper in proteins yields positive EPR signals and only weak absorption in the visible spectrum. Type 2 sites are present in all blue multicopper oxidases, as well as in galactose oxidase, prokaryotic and eukaryotic copper amine oxidases, copper-containing superoxide dismutase, and cytochrome *c* oxidase.

The T3 binuclear copper center contains two ligand-bridged spin-coupled copper ions (Cu_A and Cu_B). T3 sites are diamagnetic and display a distinctive absorption band near 330 nm as well as a characteristic luminescence spectrum (Wynn et al. 1983; Solomon et al. 1996; Shin and Lee 2000; Shleev et al. 2005). This site is present in tyrosinase and in hemocyanin, the oxygen carrier found in molluscs and arthropods. In blue multicopper oxidases, the T2 and T3 sites form a trinuclear

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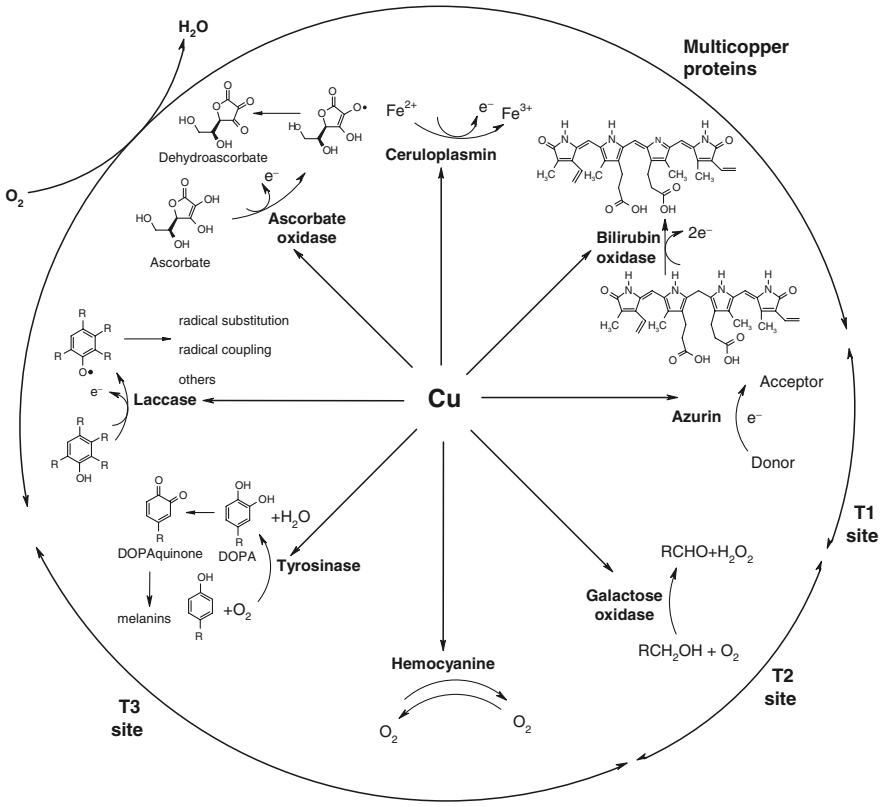

Fig. 13.1 Copper enzymes and their reactions (adapted from Shleev et al. 2005)

copper cluster (the T2/T3 cluster) (Allendorf et al. 1985; Messerschmidt and Huber 1990; Messerschmidt et al. 1992).

Tyrosinases and laccases are ubiquitously distributed in nature, and their corresponding activities can be observed intra- and/or extracellularly in soil microorganisms. A common feature is the existence of a T3 copper center, and both enzyme classes use molecular oxygen for substrate oxidation with the formation of water (Fig. 13.1).

Tyrosinases are involved in the initial steps of melanin synthesis. They catalyze the *ortho*-hydroxylation of monophenols to *ortho*-diphenols, and the latter into reactive *ortho*-quinones, which are then polymerized into dark pigments. Laccases oxidize various aromatic and nonaromatic compounds through a radical mechanism. They contribute to host defense mechanisms and the metabolic turnover of complex organic substances such as lignin and humic matter.

Both of the copper oxidases have been proposed for various biotechnological applications, such as the treatment of wastewaters or polluted soils, the removal of

Table 13.1 Some features of copper in proteins (modified from Lewis and Tolman 2004; Shleev et al. 2005)

Features	Type 1 copper	Type 2 copper	Type 3 copper
Cu atoms/ protein	1 (mononuclear)	1 (mononuclear)	2 (binuclear, spin-coupled CuA/CuB pair)
EPR signal	Paramagnetic	Paramagnetic	Diamagnetic
Light adsorption	High at 610 nm in ox. state: blue color	Low	High at 330 nm in ox. state
Coordination	Cys, 2 His, Met or Leu or Phe in multicopper proteins	3 His (2 His and 1 H_20 in the T2/T3 cluster of multicopper proteins)	6 His
Function	Electron transfer, catalysis	Electron transfer, catalysis	Binding of O_2 for transport and/or catalysis
Examples	• Multicopper proteins • Nitrite reductase • Small blue Cu proteins: Azurin Pseudoazurin Amicyanin Plastocyanin Stellacyanin Rusticyanin	• Multicopper proteins • Nitrite reductase • Amine oxidase • Cytochrome *c* oxidase (+Fe) • Galactose oxidase • Glyoxal oxidase • Quercetin 2,3-dioxygenase • Superoxide dismutase	• Multicopper proteins:Ascorbate oxidase Billirubin oxidase Ceruloplasmin[a] Fet3 protein (*Saccharomyces*)[a] Laccase • Laccase-like proteins (bacteria): Metallo oxidases (Mn, Cu, Fe) Phenoxazinone synthase • Tyrosinase • Hemocyanin[b] • Dopamine β-monooxygenase[c] • Peptidylglycine α-amidating monooxygenase[c]

[a] Also exhibits cuprous oxidase activity (Stoj and Kosman 2003)
[b] Displays tryosinase activity after specific activation (Decker et al. 2007)
[c] Contains two uncoupled Cu ions; it is not known if one or both activate oxygen

polyphenols from breweries, the synthesis of pharmaceutical drugs and new biopolymers, or for use as additives in food and cosmetic products (Couto and Herrera 2006; Halaouli et al. 2006).

This contribution provides an overview of the general biochemical and structural properties of tyrosinases and laccases, focusing on their occurrence and relevance in soil microorganisms and giving some examples of biotechnological applications of them.

13.2 Tyrosinases

13.2.1 Occurrence

The first biochemical investigations of tyrosinases were carried out with the mushroom *Russula nigricans*, the cut flesh of which turned red and then black upon exposure to air (Bourquelot and Bertrand 1895). The catalyst responsible was later found to be a copper enzyme that is widely distributed throughout the phylogenetic scale from lower to higher lifeforms, e.g., in the soil bacterium *Streptomyces*, in the common mushroom (*Agaricus bisporus*), and in human melanocytes or malignant melanoma cells (Nishioka 1978; van Gelder et al. 1997; Claus and Decker 2006; Halaouli et al. 2006). In higher plants and fungi, tyrosinases can occur in various immature, mature but latent, and active isoforms (Sánchez-Ferrer et al. 1989, 1990). Tyrosinase-like activities have been identified in the hemolymphs of insects (Lu and Jiang 2007) and as an inducible catalytic property of the hemocyanins (Decker and Tuczek 2000, Decker and Jaenicke 2004, Decker et al. 2001, 2007).

13.2.2 Relation to Melanin

Melanins are a diverse group of polymeric pigments that are widespread in a variety of organisms ranging from bacteria to humans (Plonka and Grabacka 2006). Three main types can be distinguished:

(1) *Eumelanins* (black or brown) are produced during the course of the enzymatic oxidation of tyrosine to *o*-dihydroxyphenylalanine (DOPA) and dopaquinone (Fig. 13.2). The latter spontaneously converts via the unstable leucodopachrome to red dopachrome, which can be used for the photometric determination of tyrosinase activity. Especially under alkaline conditions, dopachrome undergoes decarboxylation and further nonenzymatic polymerization reactions to become high-molecular eumelanins (Raper 1928, Mason 1948; Lerner et al. 1949). Melanogenesis in mammals is controlled by additional tyrosinase-related proteins: dopachrome tautomerase (TRP-2), which converts dopachrome into 5,6-dihydroxyindole-2-carboxylic acid, and TRP-1, which oxidizes this compound to indole-5,6-diquinone carboxylic acid. The subsequent reactions to form the dark polymers occur nonenzymatically (García-Borrón and Solano 2002). In invertebrates, additional enzymes besides tyrosinase are involved in melanogenesis, and dopamine is the preferred precursor.
(2) *Pheomelanins* (yellow-red), which initially are synthesized like eumelanins, but the DOPA undergoes the addition of cysteine or glutathione.
(3) *Allomelanins*, a heterogeneous group of polymers that arise from the oxidative polymerization of di- or tetrahydroxynaphthalene via the pentaketide pathway (*DHN-melanins*), homogentisic acid (*pyomelanins*), as well as from

γ-gluaminyl-4-hydroxybenzene, catechols and 4-hydroxyphenylacetic acid. In eu- and prokaryotes, melanins fulfill various functions such as photoprotection, photoconductivity, thermoregulation, immune defense, and chelation of metal ions (Plonka and Grabacka 2006; Wan et al. 2007).

Tyrosinase (monophenol, *o*-diphenol: oxygen oxidoreductase, EC 1.14.18.1) is the key enzyme involved in the formation of eumelanins. It catalyzes two distinct reactions: (a) the hydroxylation of monophenols to *o*-diphenols (cresolase or monophenolase activity), and (b) the (subsequent or separate) oxidation of *o*-diphenols to *o*-quinones (catechol oxidase or diphenolase activity) (Figs. 13.1 and 13.2).

The catecholoxidases (EC 1.10.3.1) frequently found in chloroplasts and fruits of higher plants (Mayer and Harel 1979, 1981; Mayer 1987, 2006) exhibit only the diphenolase activity, not the monophenolase activity, and will not be discussed further here.

It should also be pointed out that laccases (see below) can catalyze melanization when a diphenol is used as the precursor (Fig. 13.2).

Despite decades of intensive biochemical investigation, only limited information on the protein structure and the exact reaction mechanism of tyrosinase exists. Reasons for this include difficulties in purifying sufficient amounts of the enzyme from eukaryotic sources due to their intracellular localization, low enzyme concentrations, contamination with pigments, the occurrence of isoenzymes, and post-translational modifications. However, significant progress has recently been made with tyrosinases from the soil bacterium *Streptomyces*.

13.2.3 Copper Sites

The copper binding sites of tyrosinases share a high sequence homology with those of the hemocyanins, the oxygen carrier proteins of the molluscs and arthropods (Schoot-Uiterkamp and Mason 1973; van Gelder et al. 1997; Decker et al. 2007; Decker and Tuczek 2000; van Holde et al. 2001). A functional change in this protein family is proposed to have occurred during the course of evolution, from enzymatic oxygen detoxification towards oxygen transport (Jaenicke and Decker 2004).

The common feature of tyrosinases is a "type 3 copper center," a diamagnetic spin-coupled copper pair (Lerch 1995; Sánchez-Ferrer et al. 1995; García-Borrón and Solano 2002) (Table 13.1). Sequence alignments of many pro- and eukaryotic tyrosinases have shown that the copper binding regions are highly conserved. The signatures of Cu_A and Cu_B are H-x(*n*)-H-x(8)-H and H-x(3)-H-x(*n*)-H, respectively.

Each of the two metal atoms, Cu_A and Cu_B, at the active site are coordinated by three conserved histidines located in a "four α-helix bundle." During the catalytic cycle, the type 3 copper center can adopt different functional forms: the *oxy*-state [Cu(II)-O_2^{2-}-Cu(II)], the *deoxy*-state [Cu(I) Cu(I)], the *half-met* state [Cu(I) Cu(II)], and the *met* state [Cu(II)-OH^--Cu(II)]. In the latter case, the two copper atoms are bridged by hydroxo ions. The valences of the two copper atoms change from Cu(I)

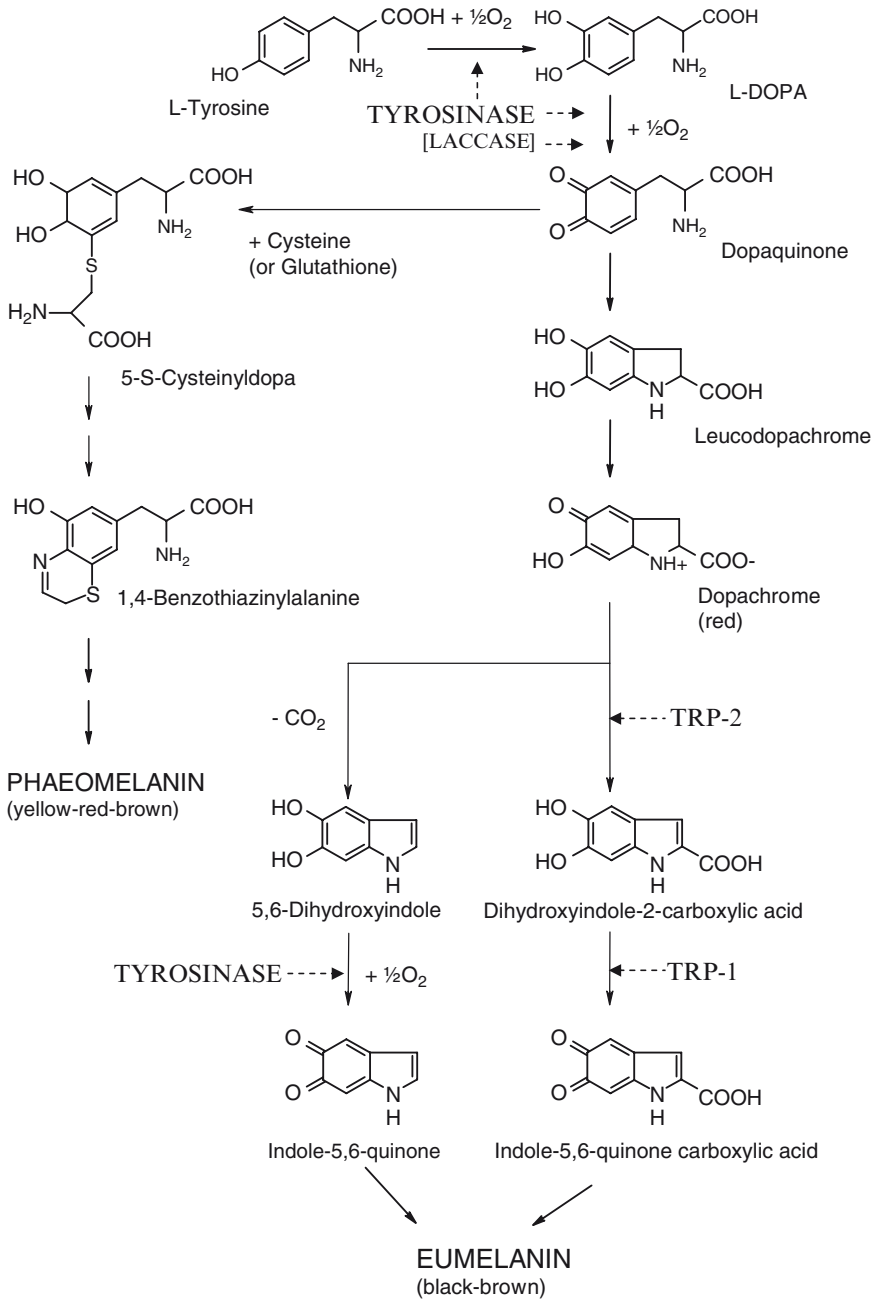

Fig. 13.2 Biosynthesis of melanin from tyrosine (modified from Kobayashi et al. 1995; Sanchez-Ferrer et al. 1995; Seo et al. 2003)

to Cu(II), which can be followed spectroscopically. In the *oxy* state, the molecular oxygen is reversibly bound as a peroxide between the two copper atoms in a "side-on" conformation. In the absence of any substrate, more than 85% of the enzyme is in the *met* state, which can be regarded as the resting form of tyrosinase. The current view is that both the *met* and the *oxy* states of tyrosinases enable diphenoloxidase activity, whereas the monohydroxylase reaction requires the *oxy* state.

13.2.4 Streptomyces Tyrosinases

Actinomycetes are Gram-positive soil bacteria with mycelial growth. Members of the genus *Streptomyces* are involved in the formation and/or degradation of complex biopolymers like lignin, melanins, and humic substances (Kutzner 1968). In addition, they are important industrial sources of bioactive compounds such as antibiotics, antitumor agents, antiparasites, immunosuppressant agents, and enzymes (Anzai et al. 2008).

About 40% of *Streptomyces* species produce melanin-like exopigments on tyrosine-containing agar media (Fig. 13.3), which usually (but not always) correlate with the appearance of tyrosinase activity (Arai and Mikami 1972; Claus and Kutzner 1985).

Fig. 13.3 Formation of melanin by a *Streptomyces* strain on a tyrosine-containing agar medium

Unlike most other tyrosinase-producing organisms, these bacteria secrete the enzyme into the environment, which facilitates isolation and biochemical characterization. Natural and recombinant tyrosinases have been purified from *Streptomyces glaucescens* (Lerch and Ettlinger 1972), *Streptomyces michiganensis* (Philipp et al. 1991), *Streptomyces castaneoglobisporus* (Kohashi et al. 2004), and *Streptomyces antibioticus* (Bernan et al. 1985). The enzyme from the latter species was the first tyrosinase for which a crystallographic structure could be elucidated (Matoba et al. 2006).

Tyrosinase genes from various *Streptomyces* species have been sequenced and translated into a protein sequence (Claus and Decker 2006). Interestingly, putative tyrosinase genes have been found in *Streptomyces* species that are phenotypically melanin negative (e.g., *Streptomyces coelicolor*), and several tyrosinase genes have been identified in some genomes (*Streptomyces avermitilis).*

Other bacterial tyrosinases have been detected and/or purified from the genera *Vibrio* (Pomerantz and Murthy 1974), *Rhizobium* (Mercado-Blanco et al. 1993; Piñero et al. 2007), *Bacillus* (Liu et al. 2004), *Thermomicrobium (*Kong et al. 2000), *Marinomonas* (López-Serrano et al. 2002, 2004), *Pseudomonas* (Wang et al. 2000), and *Ralstonia* (Hernandez-Romero et al. 2005). The presently documented molecular masses of bacterial tyrosinases range from 14 to 75 kDa; those of *Streptomyces* are about 30 kDa (Claus and Decker 2006).

13.2.4.1 Biochemical Properties

The typical double-enzymatic activity of tyrosinases has been demonstrated in melanin-positive *Streptomyces* species, whereas melanin-negative mutants lose the cresolase activity but sometimes retain some catecholase activity (Claus and Kutzner 1985). Tyrosine methylester and caffeic acid have been shown to be the best substrates for measuring both of the enzymatic activities of *Streptomyces* tyrosinase.

Electrophoretic characterizations have suggested that the intra- and extracellular tyrosinases from each *Streptomyces* species are identical, but that enzymes from different species are not (Claus and Kutzner 1985). Isoelectric focusing revealed the presence of several tyrosinase isoenzymes in some species, with their isoelectric points lying between 5.0 and 8.0. The heterogeneity of *Streptomyces* tyrosinases is also reflected in their different K_m constants and temperature stabilities.

Apart from the essential conserved copper-binding regions, significant sequence variations among bacterial tyrosinases have been detected. Among streptomycetes, the overall relationship varies between 36 and 86% (Claus and Decker 2006).

13.2.4.2 Incorporation of Copper

The melanin operons of *S. antibioticus* (Katz et al. 1983; Bernan et al. 1985; Betancourt et al. 1992), *S. glaucescens* (Hintermann et al. 1985; Huber et al. 1985),

Streptomyces lavendulae (Kawamoto et al. 1993), and *S. castaneoglobisporus* (Ikeda et al. 1996) consist of two components: *melC1*, which encodes upstream for a small chaperon-like ("caddy") protein, and the tyrosinase structure gene *melC2*. Genetic and biochemical studies, predominantly with *S. antibioticus*, have shown that the MelC1 protein is responsible for the incorporation of copper and thus the activation of the apotyrosinase (Lee et al. 1988; Chen et al. 1992). The histidine residues of the caddy protein may serve as the copper ligands: mutational exchanges of specific histidines in the MelC1 protein resulted in significant losses of tyrosinase activity (Chen et al. 1993). The MelC1 and MelC2 proteins form stable binary complexes which can be purified by chromatographic methods. Addition of copper to the binary complexes resulted in the incorporation of two copper molecules and the release of the activated tyrosinase (Chen et al. 1992).

13.2.4.3 Induction and Secretion

Tyrosinase synthesis by *S. glaucescens* is surprisingly not induced by tyrosine, but by different amino acids like phenylalanine, methionine and leucine (Baumann et al. 1976). Methionine also induces the tyrosinase from *S. antibioticus* (Katz and Betancourt 1988; Betancourt et al. 1992). The expression of the *S. castaneoglobisporus* tyrosinase is favored by methionine and copper (Ikeda et al. 1996). On the other hand, the transcription of the *S. michiganensis* tyrosinase is induced by copper and repressed by ammonium (Held and Kutzner 1990). In chemostat experiments, oxygen was found to be a negative regulator of the tyrosinase of *S. glaucescens* (Wyss and Ettlinger 1981).

Although *Streptomyces* tyrosinases are found intra- and extracellularly, they contain no signal sequences for secretion, like all bacterial tyrosinases studied so far. The TAT pathway (twin-arginine translocation pathway) allows the transport of (metallo)proteins in their native folded conformation. Proteins secreted in this way display a characteristic twin-arginine motif between the charged N-terminus and the hydrophobic core of the leader peptide. The MelC1 "caddy" proteins have this recognition signature and are most likely transported by the TAT route, which is widely used by streptomycetes (Schaerlaekens et al. 2004). A mechanism has been proposed in which the apotyrosinase forms a binary complex with the "caddy" protein, copper is incorporated, and it is then transported across the cytoplasmic membrane (Leu et al. 1992).

13.2.5 Role in Nature

Mammalian tyrosinases are located in specialized melanocytes and are responsible for the photoprotective pigmentation of hair, skin, and retina (García-Borrón and Solano 2002). Disorders in tyrosinase-catalyzed melanin synthesis are not only an aesthetic problem; they are linked with serious skin diseases, such as the well-known malignant

melanoma. Vitiligo is another such disease, characterized by hypopigmentation and total melanocyte depletion in the basal layer of the epidermis. Immunological studies of vitiligo show the generation and presence of autoantibodies directed against tyrosinase antigens in patient sera. This indicates that tyrosinase acts as an autoantigen and can serve as a marker for vitiligo (Parvez et al. 2007). Albinism, the total loss of pigmentation, is caused by different gene defects that do not primarily affect tyrosinase activity but rather transport of the enzyme into the melanosomes (Kushimoto et al. 2003).

Plant tyrosinases may be involved in biosynthetic processes and in defense against herbivores. During browning reactions, the injured tissues build up a melanin layer as protection against microbial pathogens (Mayer and Harel 1979; Mayer 2006).

In sponges and many invertebrates, tyrosinases are important components of wound healing and the primary immune response (Cerenius and Söderhäll 2004). In arthropods they are involved in sclerotization of the cuticle after molting or injury (Anderson et al. 1996). After their activation from inactive proenzymes by a cascade of serine proteases, insect phenoloxidases generate cytotoxic quinones and other reactive intermediates to immobilize and kill invading pathogens and parasites. Bacterial cell wall components are effective activators of these systems (Jiang et al 1998; Söderhäll and Cerenius 1998; Sugumaran 2002).

Fungal tyrosinases are generally associated with spore pigmentation, formation, and stability, as well as with defense and virulence mechanisms, or wound healing by melanin production (Seo et al. 2003; Halaouli et al. 2006; Mayer 2006).

The biological roles of bacterial tyrosinases are rather diverse. In soil environments, extracellular *Streptomyces* tyrosinases are probably involved in the polymerization and detoxification of plant phenolic compounds and the formation of humic matter (Kutzner 1968; Sjoblad and Bollag 1981).

Bacteria of the genus *Rhizobium* living in the root nodules of *Papillionaceae* plants carry tyrosinase genes in plasmids required for symbiosis (Mercado-Blanco et al. 1993). It was recently shown that the tyrosinase from *Rhizobium etli* plays a role in nodulation efficiency and symbiosis-associated stress resistance. Tyrosinase probably protects symbiotic microorganisms against toxic phenolic compounds in the soil environment and phytoalexins produced by plants (Piñero et al. 2007). The same mechanism is expected to be present in other plant-associated bacteria, like *Ralstonia solanacearum.*

The best-documented function of the enzyme is restricted to the formation of eumelanins. The dark pigments protect cells and spores against UV radiation, heat, enzymatic hydrolysis, antimicrobial compounds, heavy metals, or phagocytosis (Butler and Day 1998; Ruan et al. 2004; Wan et al. 2007), and contribute to microbial pathogenesis (Nosanchuk and Casadevall 2003; Plonka and Grabacka 2006)**.**

An attractive theory suggests that bacteria may use melanin as a redox polymer for adaptating to different oxygen concentrations:

- The aerobic soil bacterium *Azotobacter chroococcum* contains an active polyphenol oxidase (tyrosinase?) and forms melanin from catechol (Shivprasad and

Page 1989). This microorganism produces particularly large amounts of melanin when cultured under aerobic conditions. Although the intensity of melanogenesis does not seem to be directly correlated with the activity of nitrogenase (the key enzyme of atmospheric nitrogen fixation), it is possible that *Azotobacter* employs melanogenesis to enhance the utilization of oxygen and to maintain the reducing conditions necessary to bind atmospheric nitrogen.

- Soil bacteria can use humic acids as an electron acceptor for anaerobic respiration (Coates et al. 2002). A similar function can be assumed for the melanins.
- In *Proteus mirabilis,* an important cause of infections of the urinary tract, tyrosinase was identified as the enzyme responsible for pigmentation. The melanin decreases the level of reactive oxygen species, which probably makes the pathogen more resistant to the oxygen burst connected with the immunological response of the host (Agodi et al. 1996).

13.2.6 Applications

Tyrosinase is widely distributed in microorganisms, animals, and plants, and is a key enzyme in melanin biosynthesis and pigmentation of mammalian skin and hair. Its oxidative activities have a positive impact on the organoleptic properties of some fermentation products (raisins, cocoa, tea, coffee), but are also responsible for the undesirable enzymatic browning of fruits and vegetables, thereby causing a decrease in their nutritional quality and an inability to sell foods that have turned brown (Mayer and Harel 1979; Martinez and Whitaker 1995). Current conventional techniques of avoiding browning include heat inactivation of tyrosinase, but these processes cause undesirable losses to the quality of the product. Various chemicals such as halide salts and aromatic carboxylic acids as well as reducing compounds such as sulfite, citric acid, ascorbic acid, and cysteine are known to inhibit tyrosinase. The benefit of ascorbic acid is the focus of some discussion, and the use of sulfites is being restricted due to potential health hazards (Taylor and Bush 1986).

Widely used tyrosinase inhibitors for in vitro studies include L-mimosine, kojic acid, tropolone, phenylthiourea, and azide. However, as safety is paramount in the food industry, there is a constant search for better inhibitors from natural sources that are largely free of any harmful side effects. A number of tyrosinase inhibitors from natural sources (plants, fungi) that inhibit monophenolase and/or diphenolase have been already identified (e.g., arbutin, oxyresveratrol). Presently, 4-hexylresorcinol is considered to be safe for use in the food industry for browning control (Mayer 2006; Parvez et al. 2007).

A search for new tyrosinase inhibitors has also been launched by the cosmetic and pharmaceutical industries. Although melanin plays a crucial protective role against UV radiation and as an antioxidant, abnormal melanin pigmentation is a serious aesthetic problem in humans. Thus, tyrosinase inhibitors are important in the cosmetic industry due to their skin whitening and preventive effects (Parvez et al. 2007).

A number of tyrosinase inhibitors from natural sources have been reported, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns. For example, linoleic acid, hinokitiol, kojic acid, arbutin, naturally occurring hydroquinones, and catechols were reported to inhibit enzyme activity but have also exhibited side effects (Maeda and Fukuda 1991). Currently, arbutin (a hydroquinone glycoside) and aloesin (a glycosylated chromone) are used in the cosmetic industry as whitening agents because they are strong inhibitors of tyrosinase (Kahn 1995; Parvez et al. 2007).

Malignant melanoma is an increasingly serious clinical problem, with a high mortality rate among humans due to the failure of melanoma cells to respond to cytotoxic treatment in the form of radiation and chemotherapy. A selective strategy toward the treatment of malignant melanoma is called melanocyte-directed enzyme prodrug therapy (Jordan et al. 2001). Instead of tyrosine itself, a derivate coupled with an inactive prodrug serves as substrate in the biosynthetic pathway that converts tyrosine into melanin (Prota et al. 1994). This would allow selective conversion of inactive prodrugs into cytotoxic drugs in melanoma cells.

The substrate stereospecificity of the monophenolhydroxylase and diphenoloxidase activities of tyrosinase are the basis for many industrial applications (Halaouli et al. 2006): as biosensors for the monitoring of phenols; in the pharmaceutical industry for the production of *o*-diphenols (e.g., L-dopa, dopamine for the treatment of Parkinson's disease), and for the synthesis of biopolymers. Synthetic melanins find application as protective agents against radiation (UV, X-rays, γ-rays), cation exchangers, drug carriers, antioxidants, antiviral agents, and immunogens (Nosanchuk and Casadevall 2003; Wang et al. 2000). Their ability to crosslink proteins has opened up new application markets for tyrosinases in food industries (Thalman and Lötzbeyer 2002; Halaouli et al. 2005).

Tyrosinase has been suggested as an environmental tool for the detoxification of phenol-contaminated sites (Durán and Esposito 2000; Gianfreda and Rao 2004). However, due to their broad substrate spectrum and higher stabilities and activities under environmental conditions (such as variations in pH and temperature; presence of soil constituents), laccases appear to be much more suitable for bioremediation purposes (Claus and Filip 1988a,b, 1990a,b, 1991, Filip and Claus 1995).

13.3 Laccases

13.3.1 Distribution

Laccase [EC 1.10.3.2] belongs to the family of blue multicopper oxidases, including the eukaryotic proteins ceruloplasmin, ascorbate oxidase and bilirubin oxidase (Nakamura and Go 2005; Hoegger et al. 2006; Table 13.1). Laccase was first discovered by Yoshida (1883) in plants, based on the observation that the latex of the Japanese lacquer tree (*Rhus* sp.) hardened rapidly in the presence of air.

Subsequently, laccase enzymes have been discovered in numerous other plants (Lehman et al. 1974; Bligny and Douce 1983; de Marco and Roubelakis-Angelakis 1997; Ranocha et al. 1999). Many fungal species, including yeasts and ectomycorrhizal fungi, exhibit laccase activities (Baldrian 2006). Some laccase-like enzymes have been purified from larval and adult cuticles of insects (Kramer et al. 2001; Dittmer et al. 2004; Suderman et al. 2006). Prokaryotic laccases have been purified and investigated from the soil-inhabiting genera *Streptomyces* and *Bacillus* (Alexandre and Zhulin 2000; Claus and Filip 1997; Claus 2003, 2004; Sharma et al. 2007).

13.3.2 Properties of Fungal Laccases

Ligninolytic white-rot fungi produce high amounts of laccases and usually excrete several isoforms of the enzyme (Blaich and Esser 1975; Bollag and Leonowicz 1984; Baldrian 2006). Depending on the species, the addition of copper (Palmieri et al. 2000; Galhaup and Haltrich 2001), sugars and amino acids (Sandhu and Arora 1985), ethanol (Lomascolo et al. 2003), and phenolic compounds such as 2,5-xylidine (Sandhu and Arora 1985; Fåhreus and Reinhammar 1967) increase the production of extracellular laccases or induce the secretion of additional isoenzymes into the culture medium. Fungal laccases are glycosylated, usually in the range between 10 and 25 mol%. The glucans consist of arabinose, xylose, mannose, galactose and glucose units, which are N-linked to the polypeptide. Glycosylation may protect laccases from proteolytic degradation in the environment.

The mean optimum reaction temperature is around 55°C, although the thermostability of fungal laccases varies considerably. The half-life at 50°C ranges from minutes in *Botrytis cinerea* to over 3 h in *Lentinus edodes* and *Agaricus bisporus* and up to 70 h in *Trametes* sp. Typical fungal laccases have a molecular mass of 60–70 kDa and an acidic isoelectric point around pH 4.0. The amino acid chain contains about 520–500 amino acids, starting with an N-terminal secretion peptide (Gianfreda et al. 1999; Baldrian 2006).

Laccase is a prominent member of the blue multicopper oxidase family, which have four copper ions in their polypeptide chains (Table 13.1). The T1 copper has a trigonal coordination, with two histidines and a cysteine as conserved ligands, while one position is usually variable. It is the site of substrate oxidation and it has been widely argued that this axial ligand strongly influences the oxidation potential of the enzyme, which varies between E^0 +400 and +800 mV, depending on the individual laccase (Xu et al. 1996; Shleev et al. 2005). The T2 and T3 copper atoms form a trinuclear cluster, where the reduction of molecular oxygen to water takes place. The T2 copper is coordinated by two histidines and one water molecule, and each of the two T3 copper atoms by three histidines. Some laccase variants lack the T1 copper and are often referred to as the "yellow laccases," as they show no characteristic absorption band around 600 nm (Leontievsky et al. 1997).

The crystal structures of the fungal laccases from *Coprinus cinerius* (Ducros et al. 1998), *Melanocarpus albomyces* (Hakulinen et al. 2002), *Trametes versicolor*

(Antorini et al. 2002; Bertrand et al. 2002a, b), *Pycnoporus cinnabarinus* (Antorini et al. 2002), and *Rigidoporus lignosus* (Garavaglia et al. 2004) have been resolved. They show that the protein monomer is organized into three sequentially arranged cupredoxin domains. All three domains display a similar β-barrel type architecture that is related to those of smaller blue copper proteins such as azurin or plastocyanin. Disulfide bonds link domain one with domains two and three, while the trinuclear cluster bridges the first and third domains. The T1 copper located in domain three is the primary substrate electron acceptor site and is connected to the oxygen-reducing T2/T3 trinuclear cluster by a His–Cys–His tripeptide. Although usually active as monomeric proteins, some laccases consist of several subunits, forming hetero- (Yaver et al. 1996) or homodimers (de Souza and Peralta 2003).

Prokaryotic laccase enzymes have a similar structure (Enguita et al. 2003), although only two cupredoxin domains have been found for the bacterial laccases of *Streptomyces griseus* (Endo et al. 2003), which is active as a homotrimer, and *S. coelicolor* (Machczynski et al. 2004; Skálová et al. 2007).

13.3.3 Reaction Mechanism

Although still a matter of discussion, the following general catalytic cycle can be assumed (Messerschmidt and Huber 1990; Messerschmidt et al. 1992; Solomon et al. 1996, 2004; Tadesse et al. 2008). The reducing substrate is bound in a cleft at the enzyme surface and is oxidized by the T1 copper site in domain three, which is in close proximity. Electrons donated by four equivalents of the reducing substrate are transferred via a strongly conserved His–Cys–His tripeptide, which progressively leads to the reduction of all four Cu(II) ions in the polypeptide to the Cu(I) state. Reoxidation of the cuprous ions occurs at the trinuclear T2/T3 cluster with the concomitant reduction of molecular oxygen, resulting in the formation of two water molecules. Reduction of oxygen by laccase appears to occur in two $2e^-$ steps involving an intermediate peroxide bridging the trinuclear copper site.

The free radicals generated from laccase oxidation are very reactive and undergo further nonenzymatic reactions.

13.3.3.1 Crosslinking

The enzymatic oxidation of phenolic compounds and anilines generates radicals that react with each other to form dimers, oligomers or polymers that are covalently coupled by C–C, C–O, and C–N bonds. It should be noted that the nature of the crosslinked product is strongly influenced by the environmental pH (Leonowicz et al. 1984). In soils, natural and xenobiotic phenolics or aromatic amines can be bound to the organic humic matrix by this mechanism (Sjoblad and Bollag 1981). The oxidation of substituted compounds is accompanied by partial decarboxylations, demethylations, and dehalogenations (Dec et al. 2003). In higher plants, the

crosslinking of phenolic precursors by laccases forms part of the lignification process (O'Malley et al. 1993; Dean and Eriksson 1994). In insects, the laccase-catalyzed oxidative coupling of catechols with proteins may be involved in cuticle sclerotization (Suderman et al. 2006). In bacteria, it has been proposed that the crosslinking of protein residues (e.g., tyrosine to dityrosine) during the assembly of heat- and UV-resistant *Bacillus* spores is a function of laccases (Hullo et al. 2001; Martins et al. 2002).

13.3.3.2 Polymer Degradation

Laccase is involved in the degradation of complex natural polymers, such as lignin (Leonowicz et al. 2001) and humic acids (Claus and Filip 1998). The intermediate reactive radicals lead to the cleavage of covalent bonds and the subsequent release of monomers. The enzyme itself may not come into direct contact with the bulky polymers, and the reaction has to occur via low-molecular redox mediators.

13.3.3.3 Ring Cleavage of Aromatics

A few studies have reported laccase-catalyzed ring cleavages of aromatic compounds (Kawai et al. 1988), which are of biotechnological interest in relation to the degradation of xenobiotic compounds.

13.3.4 Substrates and Inhibitors

Laccases have low specificities for their reducing substrates but strong affinities for oxygen. Basically, any compound with characteristics similar to a diphenol will be oxidized by laccase as long as its redox potential is not too high ($E < +1{,}000$ mV). Classical substrates of laccases include various lignin-derived phenols and aromatic amines. *Ortho*-substituted compounds (e.g., guaiacol, caffeic acid, gallic acid, dihydroxyphenylalanine, pyrogallol, *o*-phenylenediamine) tend to be better laccase substrates than *para*-substituted compounds (e.g., *p*-cresol, *p*-phenylenediamine), while the lowest oxidation rates are obtained with *meta*-substituted compounds (e.g., *m*-phenylenediamine, orcinol, resorcinol). About 100 natural and artificial compounds are currently known to be oxidized by laccases, including unexpected substrates such as Mn^{2+} (Muñoz et al. 1997; Höfer and Schlosser 1999; Ridge et al. 2007) and certain lipids (Zhang et al. 2002).

Compounds commonly used for the photometric detection and measurement of laccase activity include 4-hydroxy-3,5-dimethoxy-benzaldehyde azine (syringaldazine) (Harkin and Obst 1973), 2,2′-azino-di-(-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Johannes and Majcherczyk 2000a), and 2,6-dimethoxyphenol (Solano et al. 2001). A more reliable method of measuring laccase activity is to

determine oxygen consumption, which is directly related to substrate oxidation (Claus and Filip 1990a,b, 1991; Filip and Claus 1995).

Laccases from various origins differ markedly in their substrate specificities. For ABTS, K_m ranges from 4 to 770 μM (Baldrian 2006). The optimal pH for the oxidation of ABTS is generally <4.0, while phenolic compounds like 2,6-dimethoxyphenol, guaiacol, and syringaldazine all exhibit higher values of between 4.0 and 7.0. Although a higher pH favors the phenol–phenolate interconversion of the substrate, the enzyme activity actually decreases due to the binding of OH^- to the T2/T3 copper (Muñoz et al. 1997; Xu et al. 1998).

Laccases can oxidize some low molecular compounds which in turn attack molecules that would not otherwise be appropriate substrates, such as nonphenolic chemicals (with E^0>+1,000 mV), or bulky polymers such as lignin and humic acids. Synthetic redox mediators have dramatically increased the potential use of laccases in industrial processes. Typical compounds include TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical), HBT (1-hydroxybenzotriazole), and ABTS (Bourbonnais et al. 1997; Xu et al. 2000; Baiocco et al. 2003; Riva 2006; Wells et al. 2006). There are also natural lignin-derived compounds (vanillin, acetovanillone, methylvanillate, acetosyringone, syringaldehyde, 2,4,6-trimethylphenol, *p*-coumaric acid, ferulic acid, sinapic acid, 3-hydroxyanthranilic acid) that can function as redox mediators (Cañas et al. 2007).

Laccase per se, or mediated by redox mediators, oxidizes numerous hazardous compounds, such as halogenated phenols (Bollag et al. 1988; Claus and Filip 1990a,b; Roy-Arcand and Archibald 1991; Canfora et al. 2008), aromatic amines (Claus and Filip 1990a,b), hydroxyindoles (Cai et al. 1993), the herbicide dymron (Marayuma et al. 2006), organophosphorus compounds (Amitai et al. 1998), polycyclic aromatic hydrocarbons (PAHs) (Johannes et al. 1996; Johannes and Majcherczyk 2000b; Cañas et al. 2007), chlorinated hydroxybiphenyls (Schultz et al. 2001), bisphenol A and nonylphenol (Uchida et al. 2001; Saito et al. 2004; Junghans et al. 2005), and hydroxyphenylureas (Jolivalt et al. 2006).

Fungal laccases are rather resistant to detergents like SDS, but high concentrations of heavy metals (like Fe) and NaCl can inhibit their activities. So far, no specific inhibitor has been described in addition to general inhibitors of metal-containing oxidases, like cyanide, sodium azide or fluoride. Johannes and Majcherczyk (2000a) tested a number of sulfhydryl organic compounds (dithiothreitol, thioglycolic acid, cysteine, diethyldithiocarbamic acid) that are thought to exert an inhibitory effect by interacting with the copper at the catalytic center of laccase. Only sodium azide was found to be a true laccase inhibitor and it showed no significant interference with the photometric test.

13.3.5 Role in Nature

Due to the abundance of laccase and laccase-like enzymes, there are numerous and diverse natural functions for these oxidoreductases. Although laccase is able to

polymerize lignin precursors, and its presence has been identified in xylem tissue of higher plants, there is still discussion about their involvement in lignification (Dean and Eriksson 1994; Thurston 1994; Mayer and Staples 2002). Peroxidases are regarded as the main biocatalysts in that process, but laccases operate in the absence of toxic peroxide and could play a role in the early stages of lignification in living cells (Sterjiades et al. 1992).

Evidence of laccase activity in the cuticles of larval and adult insects suggests their involvement in sclerotization (Dittmer et al. 2004; Suderman et al. 2006).

Physiological functions of laccase-like activities in bacteria include melanin production, spore coat resistance, morphogenesis, and detoxification of copper (Sharma et al. 2007). Laccase-like genes have been identified in important human pathogens such as *Escherichia coli*, *Bordetella pertusis*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Yersinia pestis*, and *Mycobacterium leprae* (Alexandre and Zhulin 2000). In all of these pathogens, the potential mechanism of virulence is suspected to be the production of melanin and laccase activity. *M. leprae* has an ability, unique among mycobacteria, to oxidize diphenols to *o*-quinones, and so the oxidation of L-DOPA has become a diagnostic feature for *M. leprae* (Prabhakaran and Harris 1985).

Fungal laccases probably play diverse roles in spore pigmentation and morphogenesis (Leatham and Stahmann 1981), fungal plant–pathogen/host interactions and stress defense (Mayer and Staples 2002), degradation of lignin (Thurston 1994; Leonowicz et al. 2001; Baldrian 2006), and turnover of humic matter (Claus and Filip 1998; Filip et al. 1998).

Similar to bacteria, laccase has been identified as a virulence factor in several human-pathogenic fungi such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Filobasidiella neoformans* due to the synthesis of melanin or the involvement of laccase in polysaccharide capsule formation (Mayer and Staples 2002).

Bollag et al. (1988) showed that the addition of laccase reversed the inhibitory effects of a number of phenolic compounds upon the growth of *Rhizoctonia praticola* inocula. They attributed the detoxification of the original phenolic compound to an ability of the laccase to transform it or cross-couple it with another phenol. This allows phytopathogenic fungi such as *B. cinerea* to detoxify phytoalexins and tannins, thereby increasing fungal virulence (Mayer and Staples 2002). It has been also demonstrated that interactions of different microorganisms, including soil fungi and bacteria, can be accompanied by strong laccase induction (Freitag and Morrell 1992; Savoie et al. 1998; Savoie 2001; Velazquez-Cedeno et al. 2004). This has been shown for laccase-producing basidiomycetes (Iakovlev and Stenlid 2000; Baldrian 2004), and also for the plant-pathogenic soil fungus *Rhizoctonia solani* when exposed to *Pseudomonas* strains producing antifungal compounds (Crowe and Olsson 2001). Laccase can probably also contribute to the degradation of phenolic antibiotics that inhibit fungal growth, like 2,4-diacetylphloroglucinol. The role of laccases in defense against heavy metals has been attributed to the production of melanins (Galhaup and Haltrich 2001; Baldrian et al. 2000; Baldrian 2003).

White-rot fungi secrete laccases and other oxidative enzymes in order to degrade complex natural polymers such as lignin (O'Malley et al. 1993; Dean and Eriksson

1994; Leonowicz et al. 2001). Laccase activity also plays an important role during composting processes, and it was isolated from both compost-specific fungi and the compost itself (Chefetz et al. 1998a,b; Chamuris et al. 2000).

Soil humic substances are considered to be the most stable part of decomposing organic matter in nature, and there is evidence that they are in a steady-state equilibrium of formation and degradation. Laccases have been shown to participate in the transformation of humic substances (Dehorter and Blondeau 1992; Chefetz et al. 1998a; Filip et al. 1998; Fakoussa and Frost 1999; Kluczek-Turpeinen et al. 2003, 2005). Laccase activity was also positively correlated with the degradation and synthesis of humic matter in experiments with *Cladosporium cladosporiodis* (Claus and Filip 1998). In vitro studies have demonstrated a 50% decolorization of humic acids by a laccase preparation from *T. versicolor* in the presence of a redox mediator (Claus and Filip 1998).

Ectomycorrhizal (EM) symbiotic fungi play a central role in the nutrition of trees by mobilizing and transporting nutrients to the roots (Smith and Read 1997). Phosphorus- and nitrogen-delivering compounds are entrapped in the complex organic macromolecules of litter and humic matter of forest soils (Ponge 2003). Acid phosphatases, proteases, and laccases are important exoenzymes that help to release matrix-bound nutrients and make them accessible to plant roots (Courty et al. 2006).

Laccase gene sequences have been identified in several EM fungi (Chen et al. 2003) and the enzymes have been purified from *Cantharellus cibarius*, *Lactarius piperatus*, *Russula delica*, *Thelephora terrestris*, and *Armillaria mellea* (Baldrian 2006). Other researchers have pointed out that tyrosinase appears to be the major phenoloxidase of EM because the oxidation of the laccase-specific substrate syringaldazine has scarcely been reported (Burke and Cairney 2002).

The seasonal dynamics of the laccase and acid phosphatase activities of EM were monitored in an oak forest. Among the most frequent and abundant EM morphotypes, those of *Lactarius quietus* and *Cortinarius anomalus* showed a peak in laccase activity in spring, while those of *Xerocomus chrysenteron* displayed their highest laccase activities in summer and fall (Courty et al. 2006).

Several authors have investigated the production of enzymes by fungi introduced into soils, and a number of protocols for laccase extraction have been proposed to optimize the extraction yield (Lang et al. 1997, 1998; Criquet et al. 1999; Baldrian et al. 2000). Laccase activities in soil extracts have been repeatedly demonstrated (Suflita and Bollag 1980; McClaughtery and Linkins 1990). An enzyme purified from a soil sample exhibited a high similarity to a laccase from *Polyporus versicolor* (Mayaudon and Sarkar 1975). A thermostable humic acid–laccase complex was isolated by Ruggiero and Radogna (1984).

Relatively high activities of laccase – compared to agricultural or meadow soils – can be detected in forest litter and soils (Rosenbrock et al. 1995; Criquet et al. 2000; Carreiro et al. 2000; Ghosh et al. 2003). The laccase activities reflect the temporal course of organic substance degradation (Fioretto et al. 2000), and their isoenzyme patterns vary during the succession (Nardo et al. 2004). Laccase activities in soil correlate with fungal biomass, which in turn is influenced by factors like temperature (Criquet et al. 2000) or nitrogen fertilization (Carreiro et al. 2000; Gallo et al. 2004).

Laccase activity in water-saturated environments (peatlands) is low due to poor oxygen availability, but increases dramatically when the oxygen concentration increases (Pind et al. 1994; Williams et al. 2000). The burst of laccase activity can lead to the depletion of phenolic compounds that inhibit organic matter degradation by oxidative and hydrolytic enzymes (Freeman et al. 2004), and it can be assumed that oxygen-regulated laccase activity plays an important role in carbon cycling in such environments (Baldrian 2006).

13.4 Applications

Due to its broad substrate spectrum, high oxidation potential (especially when combined with redox mediators), its thermal and pH stability, and its activity in organic solvents, laccase has become a powerful biocatalyst for numerous industrial applications, including delignification in the pulp and paper industries, ethanol production, solubilization of low-rank coal, textile bleaching and dyeing, bioremediation of wastewaters, and removal of polyphenols from breweries. It is also used as antioxidant and crosslinking agents in the food industry, as a catalyst in synthetic chemistry, and as a component of biosensors. These applications are not within the scope of this chapter, but they are addressed elsewhere (e.g., Yaropolov et al. 1994; Call and Mücke 1997; Smith et al. 1997; Xu 1999, 2005; Leonowicz et al. 2001; Durán et al. 2002; Minussi et al. 2002; Burton 2003; Claus et al. 2002; Wesenberg et al. 2003; Sigoillot et al. 2004; Couto and Herrera 2006; Riva 2006; Wells et al. 2006; Alcalde 2007; Camarero et al. 2005, 2007; Morozova et al. 2007; Strong and Burgess 2007; Xu et al. 2007; Kunamneni et al. 2008). One promising new application is the use of laccases in biofuel cells. Vincent et al. (2006) generated electricity from just 3% H_2 using an open fuel cell comprising an anode modified with a aerotolerant hydrogenase from *Ralstonia metallidurans* CH34, which oxidizes trace H_2 in atmospheric O_2, connected via a film of electrolyte to a cathode coupled with an O_2-reducing fungal laccase from *T. versicolor*.

The high biotechnological potential of laccases has triggered the research into new enzyme variants with appropriate features for use in industrial processes (Zumarraga et al. 2007; Festa et al. 2008) and their large-scale production in reactors (Couto and Herrera 2007).

Detoxification and bioremediation of contaminated soil environments is one of the earliest proposed and most intensively studied applications of laccases, and this will be discussed below.

13.4.1 Treatment of Polluted Soils

Many agricultural and industrial activities produce numerous xenobiotics that affect both soil and aquatic environments. Recalcitrant xenobiotics can accumulate and become harmful to these environments and their inhabitants. Enzymatic treatment

is considered an alternative method for the detoxification of contaminated aquatic and terrestrial environments (Sjoblad and Bollag 1981; Durán and Esposito 2000; Chiacchierini et al. 2004; Wesenberg et al. 2003; Claus and Filip 1991; Filip and Claus 1995; Nannipieri and Bollag 1991; Bollag 1992; Ahn et al. 2002; Gianfreda and Rao 2004; Gianfreda et al. 1999; Bollag et al. 2003). Numerous studies have shown that white-rot fungi and their ligninolytic enzymes are capable of the in vitro transformation or degradation of several xenobiotics. The underlying mechanism for laccase-induced detoxification involves oxidation of the pollutants to free radicals or quinones that subsequently undergo polymerization and partial precipitation. The pollutants are less toxic in their insoluble form and can be removed from waters by physical procedures (Bollag et al. 1988; Claus and Filip 1991; Dec and Bollag 1990; Nannipieri and Bollag 1991). In soils, detoxification occurs through the covalent coupling of the enzymatic oxidation products to the humus in the soil organic matrix (Bollag 1992). The enzymatic oxidation of substituted compounds is often accompanied by their partial dehalogenation (Claus and Filip 1990a, Dec et al. 2003; Roy-Arcand and Archibald 1991; Schultz et al. 2001), which contributes to the overall detoxification effect.

Concentrations of 2,4-dichlorophenol (2,4-DCP), a pesticide, in soils can reach up to 3,100 mg kg^{-1} (Ahn et al. 2002). Laccase enzymes from *T. versicolor* and *R. praticola* have been shown able to bind up to 65% of 2,4-DCP to humic materials in contaminated soils (Sarkar and Bollag 1987; Sarkar et al. 1988, 1989), and the transformation of phenolic derivatives has occurred after applying free and immobilized laccase (Shannon and Bartha 1988). The irreversible binding of these pollutants by laccases was shown to prevent further spread through the soil or leaching through to the water table. Dec et al. (2003) and Bollag (1988) have shown through isotope labeling that the humic–xenobiotic complexes resulting from the oxidative coupling are rather stable. Only small amounts of the xenobiotics were released over time, but these were further mineralized by soil microflora and abiotic factors. Ahn et al. (2002) compared the potential of montmorillonite-immobilized laccase and unbound laccase from *Trametes villosa* to remediate 2,4-DCP-contaminated soil. In general, immobilized laccase performed better than unbound laccase.

The presence of 2,4,6-trinitrotoluene (TNT) in soils, groundwaters, and surface waters at sites where this explosive was manufactured, loaded or demilitarized represents a serious ecological problem worldwide. Typical explosive-contaminated sites may contain up to 10,000 mg kg^{-1} of 2,4,6-trinitrotoluene (TNT) in soils and up to 100 mg L^{-1} in water. Trinitrotoluene itself is not a substrate for oxidative enzymes like laccase. However, its partial degradation results in the accumulation of reduced metabolites such as aminodinitrotoluenes (ADNT), azoxy compounds, and diaminonitrotoluenes (DANT) (Claus et al. 2007), which can be oxidized by laccase.

A number of researchers have reported the immobilization of TNT and its metabolites in complex soil organic matter and clay during composting or during anaerobic and aerobic slurry treatments. The potential of laccase for immobilizing TNT degradation metabolites in a humic matrix was recently demonstrated (Dawel et al. 1997; Thiele et al. 2002; Wang et al. 2002). During reductive transformation of TNT by *Trametes modesta* (in the presence of 200 mM ferulic acid and guaiacol),

the addition of humic monomers suppressed the accumulation of all major stable TNT metabolites by at least 92% (Nyanhongo et al. 2006).

Despite the abundance of promising experimental data in vitro, a number of limitations still restrict the use of enzymes to detoxify xenobiotics in the environment. Firstly, many cell-free enzymes are short-lived in soil environments. Enzymatic activity may be reduced or entirely eliminated through both nonbiological and biological deactivation factors, such as heavy metals, extreme acidity/alkalinity, protease degradation, and adsorption to soil constituents (Bollag 1992; Sarkar and Bollag 1987; Baldrian 2003; Gianfreda and Rao 2004). To evaluate the possible use of phenoloxidases as a tool in the remediation of chemically polluted soil and underground sites, Claus and Filip (1988a, b) investigated the behavior of laccase, tyrosinase, and peroxidase towards the most prevalent soil constituents, such as clays and humic substances. They demonstrated that laccase was strongly adsorbed to clay minerals at pH values near the isoelectric points of the enzymes. The amount of adsorbed protein correlated with the cation exchange capacities of the clays. In the presence of bentonites, laccase activity in solution was reduced at pH values below 5.0 and disappeared completely at pH 3.0. However, in the presence of kaolinites, some free laccase activity remained at pH 2.0. Adsorption of laccases to soil humic substances or inorganic soil constituents changes their temperature and activity profiles (Criquet et al. 2000). Keum and Li (2004) demonstrated that humic substances do not strongly bind laccase, and so the inactivation is not due to binding but the dissociation of copper chelated by humic substances.

Kaolinite was found to stimulate laccase production when cultures of *P. versicolor* and *Pleurotus ostreatus* were used to inoculate soil in order to replace enzyme preparations (Claus and Filip 1990b). However, in the study, the growth and enzyme production of the inocula were severely inhibited by competitive microorganisms under the nonsterile conditions used. These results indicated limitations on the in situ production of phenoloxidases.

13.5 Conclusion

The copper-containing oxidases tyrosinase and laccase have been intensively investigated for decades. For a long time, research into eukaryotic tyrosinases was hampered by low purification yields, but significant progress was recently made with those from soil microorganisms. New insights were obtained, especially in relation to structural and catalytic data. The formation of protective melanins is one well-established task of tyrosinases in eukaryotes. The physiological importance of the extracellular tyrosinases produced by the bacterium *Streptomyces* is not well understood. Tyrosine is neither an inducer of enzyme production nor a probable substrate in soil.

More assured information exists on the structural and biochemical properties of laccases from fungal sources. Many studies demonstrate their physiological role in the detoxification of phenolic compounds. Their occurrence in soils underlines their ecological importance in the metabolic turnover of complex organic polymers

such as lignin and humic matter. In ectomycorrhizal symbiotic fungi, they contribute to the nutrition of trees by mobilizing and transporting nutrients to the plant roots. The specific monohydroxylase activities of tyrosinases and the high nonspecific oxidation capacities of laccases can be exploited for numerous biotechnological processes. The screening of natural sources and genetic engineering will further expand our knowledge and applications of these old-fashioned metalloenzymes.

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Chapter 14
Biomethylation of Heavy Metals in Soil and Terrestrial Invertebrates

Burkhard Knopf and Helmut König

14.1 Introduction

Microorganisms play an essential role in the recycling of various elements. The carbon, nitrogen and sulfur cycles are well known, but biochemical cycles of heavy metals also occur in the aquatic and terrestrial environment. In the case of the microbial methylation of metals, a lot of studies have been performed, but these have largely focussed on aquatic systems. A well-studied example is the methylation of mercury. The accident in the 1950 in Minamata, Japan, prompted intense research into the influence of organic mercury in humans (Ekino et al. 2007). On the other hand, biomethylation pathways also generated special interest after the accident. In the following years, many studies provided information on alkylation mechanisms (Mason et al. 1995a–c) and the mercury cycle. These investigations led to detailed knowledge of bioaccumulation in aquatic invertebrates and in fish (Westöö 1966; Mason et al. 2000; Hightower and Moore 2003). As well as mercury, other metals such as bismuth and metalloids like arsenic or selenium have also become targets for biomethylation research. There is special interest in selenium because it is essential (in small amounts) to proper functioning in living organisms (Rotruck et al. 1973). In contrast to the aquatic pathways of alkylation, data on alkylation pathways in terrestrial ecosystems and their organisms are scarce. Just like in aquatic systems, microorganisms like bacteria and fungi are mainly responsible for the alkylation of metals and also their biochemical pathways in terrestrial habitats. Cobalamin is involved in the transfer of methyl groups to inorganic mercury (Bertilsson and Neujahr 1971; Choi and Bartha 1993). In the case of arsenic and selenium, the

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methylation is achieved by *S*-adenosylmethionine (Challenger 1945). Another possible biochemical pathway for methyl group transfer was postulated by Choi et al. 1994. In this work, it was demonstrated that the methyl group is transferred to mercury in *Desulfovibrio desulfuricans* strain LS by methyltetrahydrofolate via methylcobalamin.

Besides methylating metals, a few microorganisms are also able to reduce them (Lloyd et al. 2003). In the case of mercury, for example, some species of bacteria can reduce Hg^{2+} to nontoxic, volatile, elemental mercury. A gene called the *mer* gene is linked to this reaction. This ability exhibited by a few species of mercury-resistant bacteria is used for the bioremediation of mercury-contaminated soil, and also as biosensors that are used to monitor heavy metals (Bontidean et al. 2002; Lloyd and Lovley 2001).

Recently, the biomethylation of terrestrial microbial flora has been investigated in more depth. For example, Hirner et al. (2000) and Meyer et al. (2007) studied soil samples with different origins and degrees of contamination for their metal alkylation potentials. Recently, Raposo et al. (2008) demonstrated a relationship between the alkylation of mercury and microbiological activity in soil samples. However, there is still a lot of work to be done before we can fully comprehend the terrestrial alkylation pathways of metals.

14.2 Analysis of Methylated Heavy Metals

14.2.1 Methylation Methods

Pure reference standards are a prerequisite for any qualitative or quantitative analysis of an organometallic compound in nature. However, not every organometallic substance is available commercially. For example, different chemical methods can be applied to methylate mercury. Methylmercury derivatives can be produced using HgO and tetramethyltin (Hintelmann and Evans 1997) or by using $HgCl_2$ and MgMeCl (Snell et al. 2000). The main problem with this methylation reaction is the low yield of product. An alternative possibility for the production of methylated mercury is to use methylcobalamin, in analogy to the bacterial pathway for mercury methylation, where cobalamins are involved. The latter chemical reaction results in higher yields of methylated end-products. In addition, the conditions required for this reaction has not to be as exact as those needed for other chemical reactions.

Another example of an organometallic synthesis is the isotopic enrichment of trimethyl lead. In this case, the reaction of lead bromide with methyllithium leads to tetramethyl lead. The desired trimethyl lead iodide can then be derived by adding elementary iodine (Poperchna and Heumann 2005a,b).

The methods discussed in the following sections have also been summarised by Cornelis et al. (2003).

14.2.2 Extraction Methods

The extraction of organometallic species from complex solutions is still a difficult task, although many novel and efficient techniques are now available. In order to obtain sufficient amounts of a methyl derivative it is important to apply an appropriate extraction method to the soil sample of interest. Often a modified extraction procedure will need to be elaborated. Another important aspect that must be taken into consideration is the possible formation of artificial alkylated metals. Some selected extraction methods are described in this section.

14.2.2.1 Aqueous, Acidic and Alkaline Extractions

The easiest extraction method to perform is the application of hot water (Potin-Gautier et al. 1997). This method can be applied for the extraction of weakly bound selenium from microbial cells or soil. Biological samples can also be treated with an HCl solution and the dissolved tissue can then be analysed. Alkaline conditions are often used for the extraction of organometallics from biological samples. An appropriate reagent is tetramethylammonium hydroxide (TMAH) (Poperchna and Heumann 2005a,b). In this case, the whole biological matrix can be dissolved.

14.2.2.2 Microwave-Assisted Extraction

Another extraction method often used for metal derivatives from soil samples is an acidic microwave procedure with the addition of HNO_3 or other acids. The acidic microwave procedure also allows the solubilisation of trace elements from the soil or the total amounts of specific metals in different terrestrial samples to be determined. Rahman and Kingston (2005) developed a microwave-assisted method that applies HNO_3 in order to extract mercury species from soil specimens. This method also saves time.

14.2.2.3 Enzymatic Extraction

A alternative extraction procedure is the application of enzymes. The enzymes used should be selected according to the type of sample of interest. For example, if it is plant material then it is necessary to use cellulases and proteases in order to lyse the cell wall polymers and hydrolyse the proteins, respectively. The microwave extraction is advantageous for soil samples. If we are only interested in the organic material or the microorganisms in the soil samples, this extraction procedure is more convenient (Potin-Gautier et al. 1997, Rai et al. 2002).

14.2.2.4 Solid-Phase Extraction

Using solid-phase extraction, organic compounds can be extracted from aqueous solutions. It also allows sample concentration before detection. This procedure can also be used as a separation method, because the extraction is mainly based on ion exchange. Using this technique, an ion of interest can be isolated from a mixture. A further advantage of this method is the extraction of specific ions or compounds from soil samples. A sequential extraction is first performed, and then, in a second step, the ions are extracted using a specific SPE column (Potin-Gautier et al. 1997, Han et al. 2003).

14.2.3 Separation Methods

Many different methods are available for the separation of trace elements and organometallic species. An upstream separation is usually not required when only one trace element is of interest. In this case, the element can be directly measured by a detection system such as ICP-MS. However, if determinations of more than one trace element or alkylated metal species are desired, an appropriate separation method must be applied before detection. Some selected separation and detection methods are described below.

Several methods with special advantages are available for the separation of alkylated metals. The most common separation methods are high-performance liquid chromatography (HPLC) and gas chromatography (GC). More sophisticated methods include capillary electrophoresis (CE) and supercritical fluid chromatography (SFC), which will not be considered here.

14.2.3.1 High-Performance Liquid Chromatography

In HPLC, the analyte is introduced onto a specific chromatographic column filled with a stationary phase. The mobile phase is pumped through this column. The analyte interacts with the stationary phase and the mobile phase as it is pumped through the column. The strengths of these interactions between the analyte and the phases result in a specific retention time. Because there is a wide variety of different commercially available solid phases and mobile phases, this is a popular separation method. However, this advantage also results in a few problems, because it is therefore necessary to find a solid phase that will interacts appropriately with the analyte to give the most efficient separation. The selection of the mobile phase is an important consideration for achieving the best separation. Another advantage that makes HPLC a useful separation technique is that the extracted analytes can be injected directly into the column—derivatisation is not necessary (Bohari et al. 2001; Vidler et al. 2007; Chen et al. 2007).

14.2.3.2 Gas Chromatography

GC is an appropriate separation method if the analyte can be dissolved in organic solvents, or if it is an organic compound. After injection into the GC, the analyte is evaporated by heating. The analyte is bound onto a nonpolar solid phase (e.g. dimethyl siloxane). Helium can be used as mobile carrier gas. The separation is achieved by performing a specific temperature programme. An important advantage of GC separation is the small amount of sample required in comparison to HPLC. Of course, the metal species must be transferred into a volatile form prior to separation. This is accomplished by derivatisation with chemical reagents. Several procedures are available for this, such as hydride generation (Campbell 1992) or alkylation (Fent and Müller 1991; Honeycutt and Riddle 1961; Fernández et al. 2000). Unfortunately, these reactions can also lead to the transformation of the organometallic species, as has been shown for the ethylation of mercury with sodium tetraethylborate by Demuth and Heumann (2001). For this reason, standards are required for the organometallic species of interest. As mentioned before, not every standard is available commercially, so some have to be synthesised in house.

14.2.4 Detection Methods

Before the compound of interest can be detected, an appropriate method of determining must be chosen. Several studies have shown that it is also important to consider the chemical species of the metal and not to simply estimate the total amount of the inorganic form beforehand. The measurement of very low concentrations of organometallic species is a challenging task, so it is necessary to hyphenate the separation method of choice with the most efficient detection instrument. However, not all instruments are useful for determining organometallic species because a few of them are simply not sensitive enough. Various detection methods are available that can by connected to the appropriate separation technique. This section describes some approved detection methods, such as atomic absorbance spectrometry (AAS), the atomic fluorescence spectrometry (AFS), and inductively coupled plasma mass spectrometry (ICP-MS).

14.2.4.1 Atomic Absorbance Spectrometry

Flame AAS is an inexpensive yet effective method of determining the total amount present of a specific element. Organometallic species can be analysed by coupling flame AAS to chromatographic separation methods. The main disadvantage of this method is its relatively low detection limit. One way to improve the detection limit is to preconcentrate the sample using, for example, column absorption (Matusiewicz 1997; Pasullean et al. 1995). Another possibility is to use hydride AAS, which can be coupled to a chromatographic device. The separation can be performed by

HPLC. The eluent is transferred to a hydride generator. The reaction mixtures can be analysed by AAS. This separation and detection method is often used to investigate arsenic species (Zhang and Combs 1996), but it does not permit the analysis of metal isotopes.

14.2.4.2 Atomic Fluorescence Spectrometry

AFS is a highly sensitive method for detecting special elements such as mercury, which is often used to detect this metal. It is usually coupled with a GC in order to separate metal specimens, but other separation techniques can also be applied (Bohari et al. 2001). For example, Limper et al. (2008) used AFS coupled to cryotrapping gas chromatography for the detection and determination of mercury compounds. Gaseous analytes like dimethylmercury can also be measured with this method. Its main disadvantage is the need for thermal decomposition in order to analyse the elemental species.

14.2.4.3 Inductively Coupled Plasma Mass Spectrometry

Another detection method that is currently widely applied is ICP-MS. This method is extremely sensitive to most elements. It can also be used to differentiate between the various isotopes of an element. ICP-MS can be hyphenated with different separation techniques such as HPLC, GC or GE. The multielement capability of the ICP-MS is also a great advantage. It allows different elements to be scanned for in the same sample, which means that the interactions between different elements in a particular experimental setup can be researched. Another advantage is the use of isotopic standards for the isotope dilution method (Heumann et al. 1994). This capacity for determination has the advantage that effects of the matrix on the metal of choice can be neglected. However, spectrometric interferences can occur and are disadvantageous. This is also true of plasma instability in the presence of organic solvents. Therefore, it is necessary to decrease the sample input, which can be done using appropriate separation techniques such as GC or by avoiding the use of organic solvents.

14.3 Microbial Methylation of Heavy Metals

14.3.1 Mercury

Mercury is one of the most widely distributed and best investigated metals that can be biomethylated. The inorganic form of Hg^{2+}, but not Hg^{0}, is a highly toxic metal ion that has the ability to interact with the sulfide bonds of enzymes and thus inhibit enzyme activity. The organic form is far more toxic because of its ability to cross lipid membranes and the blood–brain barrier, leading to interactions with

the central nervous system. Therefore, the concentration of the organic form that is needed to harm an organism is about a hundredfold lower than that of the inorganic mercury ion.

Well-known examples of intoxication with mercury compounds are the accidents at Minamata in the 1950s and Iraq (Al-Tikriti and Al-Mufti 1976; Ekino et al. 2007). The incident at Minamata prompted an intense research programme into methylated mercury compounds. Consequently, the ways in which aquatic microorganisms methylate mercury and its path through the food chain from bacteria to end-consumer (humans) are now well known.

The biological methylation of metals is still not fully understood. Most probably, sulfate-reducing bacteria (SRB) are the main methylaters of mercury in aquatic systems; different SRBs are also found in soil and animal gut compartments. This may be why methylated or alkylated metals can be found in soils. Microbial pathways for the methylation of metals are not as well investigated as many other biochemical pathways. One pathway that has been investigated in detail is methylation by methylcobalamin (Bertilsson and Neujahr 1971; Choi and Bartha 1993). This cofactor, which is naturally responsible for the transfer of a methyl group, is also able to methylate the inorganic mercury ion. Other methyl group donors like *S*-adenosylmethionine and N^5-methyltetrahydrofolate can not methylate Hg^2 (Craig 1986). Another route to mercury methylation is the acetyl-CoA pathway (Choi et al. 1994). In this case, the methyl group can be transferred directly or through an enzymatic reaction.

In contrast to the methylation of mercury in aquatic systems, the corresponding terrestrial pathways and food chains have not been studied in detail. Data from a few publications on terrestrial and sedimentary ecosystems are summarised below.

14.3.1.1 Soil/Sediments

The main metal methylaters in soil have only rarely been described in the literature, but—just as in aquatic ecosystems—microorganisms, especially sulfate-reducing bacteria, appear to play the most important role in biomethylation. Raposo et al. (2008) investigated the relationship between the microbial methylation of inorganic mercury and microbial activities in sediments. Samples were collected from the estuary at Bilbao (northern Spain). These investigations showed that variations in methylmercury concentration in the samples were due to the methylation and demethylation processes that occur in the sediments. In order to elucidate whether these processes were biotic or abiotic, the samples were sterilised to eliminate the microbial activity. It was shown that, in the absence of any microbiological activity, the methylmercury demethylation is more likely to occur. Rodríguez Martín-Doimeadios et al. (2004) found that the levels of methylation of mercury in unsterilised samples were significantly higher than those in sterilised samples. Both publications showed that the main methylating processes occurred under anaerobic conditions and were caused by *Desulfovibrionaceae*, *Desulfobacteriaceae* and other sulfate-reducing bacterial groups (King et al. 2000).

Pak and Bartha (1998) described the methylation and demethylation of mercury by strictly anaerobic bacteria in anoxic lake sediments. The authors determined the methylation and demethylation potentials of pure cultures of sulfidogenic, methanogenic and acetogenic bacteria. The sulfidogenic bacteria were able to catalyse both processes, unlike the methanogenic bacteria, which only exhibited demethylation activity. In the case of the acetogenic bacteria, neither methylation nor demethylation was observed.

14.3.1.2 Sewage Sludge

In order to estimate the potential of sewage sludge for the formation of methylmercury in a wastewater treatment plant (Mainz, Germany), Limper (2006) determined the concentration of methylmercury after incubating a sewage sludge sample with inorganic mercury chloride. The results showed that, after an incubation time of 164 h, 2.6% of the total mercury was present as methylmercury. Specific inhibition experiments with molybdate indicated that sulfate-reducing bacteria were mainly involved in the formation of methylmercury. The daily methylmercury production of the plant investigated was estimated to be ca. 0.4 g.

14.3.1.3 Mastotermes darwinesis

In order to determine the metal methylation capability of the gut microbiota of a particular insect, Limper et al. (2008) fed the termite *M. darwinesis* with sawdust containing different concentrations of inorganic mercury. After incubation, termite tissue was prepared for the extraction of mercury compounds. $CuSO_4$ and KBr/H_2SO_4 were used for the extraction. Afterwards, the organic mercury compounds were alkylated with sodium tetrapropylborate, and the detection was performed by cryotrapping gas chromatography–atomic fluorescence spectrometry. The fraction of accumulated methylmercury was in the range of 0.10–0.38% of the total amount of inorganic mercury taken up by the termites. The sulfate-reducing bacterium *Desulfovibrio intestinalis* isolated from the gut of *M. darwinesis* by Fröhlich et al. (1999) was studied as a possible methylator of mercury. It was shown that this bacterium produced 0.05 ng methylmercury per gram of medium in the presence of 1 µg inorganic mercury per gram of medium with a titre of 2.1×10^8 cells per millilitre within five days.

14.3.1.4 Porcellio scaber

Nolde et al. (2005) investigated the reduction and methylation of inorganic mercury in the isopod *P. scaber* and also in its environment. In this research, $^{203}Hg^{2+}$ was used as a tracer to reconstruct the route for the mercury cycle in an experimental setup. It was shown that the methylation of mercury had already occurred on the

leaves given as food to the isopods. Surprisingly, only a small amount of the methylated mercury was assimilated by the animals. Also, a significant difference between the amount of assimilated methylmercury and the amount of extracted methylmercury was observed. To explain this, it was suggested that the demethylation process may prevail over the methylation of mercury. Also, the distribution of mercury in the tissue of the isopod differed to those found for other heavy metals (Nolde et al. 2005).

14.3.1.5 Eisenia foetida

In the work of Burton et al. (2006), the bioaccumulation of inorganic and organic mercury by the redworm *E. foetida* was observed. The model organism was incubated for a defined period of time in soils with different concentrations of inorganic and methylmercury. Burton et al. (2006) observed an accumulation of both mercury compounds in the tissue of the worm. The accumulation was higher in soils with low levels of mercury than in highly contaminated soils. It was also observed that the methylation of mercury by soil microorganisms was not responsible for the increase in methylmercury in the tissue of the worms. Possible methylation of mercury by the bacteria in the guts of the worms was not investigated.

In the study of Knopf and König (manuscript in preparation), the worm *E. foetida* was incubated in sterile and nonsterile soils artificially contaminated with inorganic mercury. After a defined period of time, the worm was prepared and its tissue was taken for mercury compound analysis. The production of methyl mercury was detectable under both conditions.

14.3.2 Selenium and Arsenic

Selenium is, in contrast to mercury, a metalloid that is essential for the proper functioning of living organisms. It is involved in enzymatic and other biochemical reactions. Rotruck et al. (1973) showed that selenium has a protective function against oxidative stress. In proteins, selenium is a component of the twenty-first amino acid (selenocysteine), where it functions as a redox-sensitive centre. Another difference from mercury is that the methylation of selenium does not lead to a more toxic product; instead, this is considered to be a detoxification reaction.

Rosenheim (1902) performed biological experiments with the fungus *Scopulariopsis brevicaulis*. He applied different arsenic species and observed a garlic smell. This smell was not released when he used pure arsenic. Challenger and North (1934) identified dimethylselenide as a reaction product. Challenger (1945) proposed a reaction mechanism for the methylation of selenium that is now known as the Challenger mechanism. It is a combination of reduction and methylation reactions that is mainly observed when *S*-adenosylmethionine is available as a donor for the methyl group. Another possible pathway is comparable to the methylation of mercury, where methylcobalamin can transfer the methyl group to selenium and

produce organic selenium species (McBride and Wolfe 1971; Thompson-Eagle et al. 1989).

Thompson-Eagle et al. (1989) investigated the volatilisation of selenium by the fungus *Alternaria alternata*. For this study, water samples from different sites were collected containing between 0.005 and 5 mg Se per litre were collected. The authors were able to isolate *A. alternata* from the samples, and they measured the production of the volatile dimethylselenide under different conditions (pH values, temperatures, Se substrates and methyl donors). The optimal conditions for methylation by *A. alternata* were 30°C and a pH value of 6.5 using Se(VI) as the source of selenium. For the methyl donors, only a small difference between L-methionine and methyl cobalamin was measured. Another work with *A. alternata* that investigated the bioavailability of inorganic selenium adsorbed to different kinds of soil samples was performed by Peitzsch (2008).

As in the case of mercury, a lot of studies have been performed on the methylation and demethylation of arsenic in ecosystems such as freshwaters (Bohari et al. 2001) and wastewaters (Segura et al. 2002). Data on the methylation of arsenic species in soil habitats are scarce. Just as it is with selenium, the methylation of arsenic is considered a detoxification reaction. The proposed pathway to the methylation of arsenic is (just like selenium) the Challenger mechanism. In the work of Pinel-Raffaitin et al. (2007), the release of inorganic and organic arsenic from landfill leachates and biogases was measured. Hirner et al. (2000) investigated the amounts of organometallic species and metalloids like arsenic in various contaminated soil samples and directly in shredder (fresh car metal and electronic waste). All methylated forms of arsenic were found in the contaminated soil, in contrast to the shredder. The importance of microorganisms in the biomethylation of arsenic was demonstrated by these authors. Duester et al. (2005) measured the concentrations of organic arsenic species in urban soils near German cities. The highest concentrations were observed in agricultural and garden soils. Soils from abandoned industrial sites showed lower concentrations. This can be explained by the increased biological activity in agricultural and garden soil. Because of their higher levels of contamination, their artificial substrates and also the destruction of the natural soil structure, the activities in the industrial soils were significant lower.

14.3.3 Bismuth

Bismuth compounds are widely used in the pharmaceutical and cosmetic industries. They can accumulate in the environment via wastewater contamination. In contrast to other heavy metals, like lead or mercury, the toxicity of bismuth is relatively low. On the other hand, methylating bismuth makes it more lipophilic and volatile, which can result in a higher toxicity. Information about the biomethylation of bismuth

is scarce. In a study by Feldmann et al. (1999), volatile bismuth compounds in landfill and sewage gas and nonvolatile derivatives in water and sediment were observed by cryotrapping gas chromatography and hydride-generation gas chromatography coupled to ICP-MS. These authors were able to identify a volatile bismuth compound, tetramethyl bismuth. Laboratory experiments with fermenters containing anaerobic cultures from sludge resulted in tetramethyl bismuth in the headspace after an incubation time of two weeks.

In the work of Meyer et al. (2008), various anaerobic archaea and bacteria were studied for their capacities to methylate metals and metalloids like arsenic, mercury and bismuth. The microorganisms observed were isolated from the human gut and cultivated under strict anaerobic conditions. The diversity of the volatile derivates and the emission rates were higher for the methanoarchaeal strains than for the eubacterial strains. It was thus concluded that methanoarchaea are largely responsible for methylation in the human gut. Another interesting observation relating to bismuth was that trimethylbismuth was the main volatile product produced in human faeces. Under these conditions, the growth of *Bacteroides thetaiotaomicron*, which is found in the human gut, was inhibited. This shows that, in addition to their direct, toxic effects on human health, interactions with organometallic species also inhibit microbial flora in the human gut.

14.4 Conclusion

In recent years, a number of efficient methods for the extraction, separation and detection of different metal species have been introduced and evaluated. The main problem is still to identify the appropriate procedure for the analysis of a certain environmental sample.

Many studies investigating the natural cycles of inorganic metals and organometallic species in terrestrial habitats, such as those done on mercury, have been performed to advance knowledge in this field. A diagram illustrating the mercury cycle is shown in Fig. 14.1. It has proven possible to identify the main methylaters of metals. In aquatic, terrestrial and intestinal ecosystems, sulfate-reducing bacteria appear to play a major role in biomethylation. Particularly in terrestrial ecosystems, the food chain from bacteria to humans via different animals needs to be studied in more detail in order to evaluate potential toxic effects and ensure that end-consumers avoid them.

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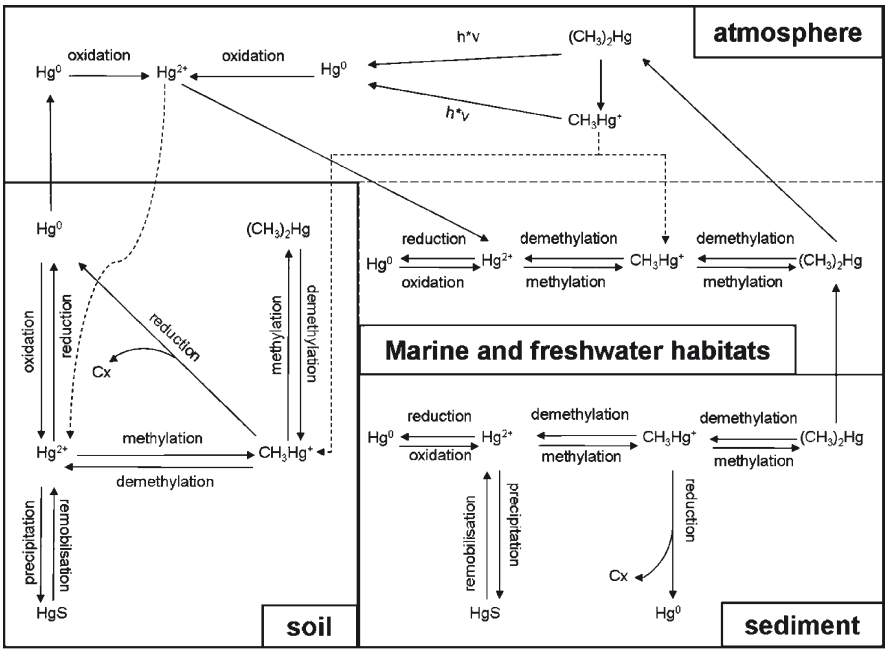

Fig. 14.1 Schematic of the mercury cycle

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Chapter 15
Phytostabilization of Lead-Polluted Sites by Native Plants

Andrea Zanuzzi and Angel Faz Cano

15.1 Introduction

Lead pollution of soils causes a wide range of environmental and health problems (Kabata-Pendias and Pendias 1992). This is the case for the Cartagena–La Unión Mining District in southeast Spain, where mining has been carried out for more than 2,500 years. This activity has resulted in many large open-pit mines and huge piles of mine wastes that contain elevated concentrations of lead, among other heavy metals, and constitute a high pollution risk for adjacent areas due to the transportation of the materials by wind and water erosion (Berrocal 2003; Arana 2004).

Remediation of heavy metal contaminated sites often involves practices based on civil engineering, such as excavation, which are environmentally invasive and expensive (Van der Lelie et al. 2001; Bert et al. 2005). In the studied area, the risks associated with any spread in the contamination due to the high concentrations of heavy metals present (especially lead) and the large surface affected made it an excellent candidate for phytostabilization (Zanuzzi et al 2008a).

Phytostabilization is a site stabilization technique that reduces the risk associated with soil contaminants through the use of soil amendments that induce the formation of insoluble contaminant species (Raskin and Ensley 2000). Converting the metal into less soluble forms will likely diminish the leaching of the metal through the soil profile, and the chances of any biological interactions with humans, animals, or plants. In this technique, the soil surface is covered with plants to prevent erosion, reduce water percolation, and to serve as a barrier to prevent direct contact with the soil (Bert et al. 2005).

Inactivating soil Pb using soil amendments and revegetation to prevent erosion is increasingly being seen as a promising soil Pb remediation technology (Chaney

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and Ryan 1994; Berti and Cunningham 1997). It is necessary to use native species in this method that are ecologically adapted to the prevailing climate (Tordoff et al. 2000; Ernst 2005). However, there are several aspects of the Sierra Minera, such as its soil chemical and physical properties, the extreme site conditions, and its warm Mediterranean semiarid climate (where rainfall is infrequent but severe), that made the task of revegetation very difficult. Some techniques for improving soil characteristics and enabling the vegetation to establish itself were needed.

This chapter deals with the chemical immobilization and phytostabilization of Pb-polluted soils that was carried out under semiarid field conditions at a very representative acidic mining pond in the Cartagena–La Unión Mining District in southeast Spain. Particular emphasis is placed on the modification of some soil characteristics, the reduction of Pb mobility, plant colonization, and the selection of the most suitable species for future activities.

15.2 Materials and Methods

15.2.1 Study Area

This research was carried out on the Cartagena–La Unión Mountain, to the east of Murcia Province in southeast Spain (Fig. 15.1). This region, which is classified as termomediterranean, has an average temperature of 18°C throughout the year and an annual precipitation that ranges between 200 and 300 mm. The study area includes one representative mine pond named Brunita, located between the cities of Cartagena and La Unión (110–0 m asl; 37°37′20″ N. 0°50′55″ W – 37°40′03″ N. 0°48′12″ W). The poorly evolved soils at the site were classified as Haplic Torriarents (US Department of Agriculture USDA 2006) and had a high concentration of lead (1,200 mg kg^{-1}). These mine soils could be classified as being polluted, considering the Dutch intervention value (530 mg kg^{-1}) (Ministry of Housing Netherlands 1994) and the generic reference level of Pb from Murcia Province (50 mg kg^{-1}) (Martínez-Sánchez and Pérez-Sirvent 2007).

15.2.2 Chemical Immobilization: Plot Experiment

The reclamation measures carried out in Brunita pond consisted of field plots amended with stabilized pig manure and lime (wastes originating from the marble extraction industry). The plots were left exposed to the semiarid climatological conditions after the amendments were added.

The pig manure dose was calculated on the basis of European and Spanish nitrogen legislation [Council Directive 91/676/EEC from the EC (1991); Real Decreto RD 261/1996 and de 16 de Febrero from Ministerio de la Presidencia

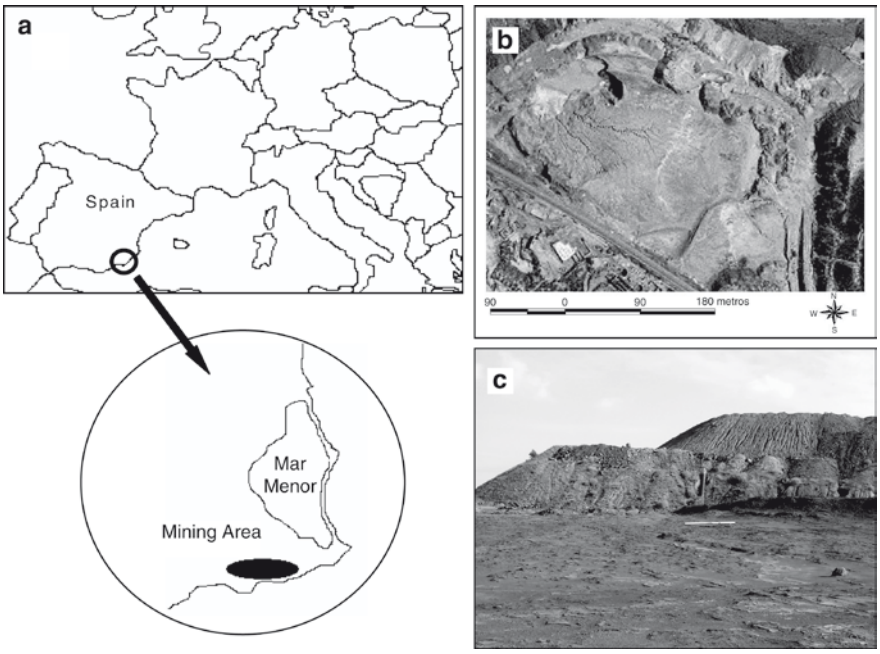

Fig. 15.1 **a** Location of the study area; **b** aerial photograph of Brunita pond; **c** general view of the pond

(1996)], and the lime dose was determined as the amount of calcium carbonate required to raise the pH to 7 (Sobek et al. 1978). The pig manure and lime doses were 12.5 t ha^{-1} and 162.5 t ha^{-1}, respectively.

15.2.3 Description of the Sampling and Analytical Methods Used

Composite samples consisting of five subsamples were taken from the plots after 24 months of adding amendments, as well as from a control plot. Soil pH was determined for a water/soil ratio of 1:1 (Peech 1965), and the electrical conductivity of each saturated extract was measured (Bower and Wilcox 1965). Total nitrogen was determined using the Kjeldahl method (Duchaufour 1970), and organic carbon was analyzed using a total organic carbon analyzer (TOC-V CSH, Shimadzu). The equivalent calcium carbonate was determined using a Bernard calcimeter.

Total Pb concentrations were determined using $HNO_3/HClO_4$ digestion at 210°C for 1.5 h (Risser and Baker 1990). DTPA-extractable Pb (considered to be the

bioavailable Pb) was determined according to Lindsay and Norvell (1978) and Norvell (1984). Metal concentrations were measured using AAS (UNICAM 969).

15.2.3.1 First Stages of Phytostabilization

Plant cover was assessed on each plot after 24 months of amendment addition, and plant species identification was also carried out. The Pb content in the aerial part of the dominant plant species, *Diplotaxis lagascana*, was analyzed in order to assess Pb accumulation and the risk of its mobilization through the food chain. Plants were harvested before the start of the senescence phase, and one representative sample per plot was obtained by combining five individual plants. Plant samples were washed with deionized water and dried at 60°C during 24 h. Afterwards, the samples were ground and incinerated at 480°C for 24 h and then diluted with 25 ml HNO_3 (65%).

Finally, a study related to the selection of the most suitable species for future activities in the phytostabilization program was carried out.

15.3 Salient Observations

15.3.1 Chemical Immobilization: Plot Experiment

Table 15.1 shows the changes in various soil parameters measured before (0 months) and after (24 months) adding amendments to the plots. pH increased from 2.7 to 7.0, electrical conductivity from 3.5 to 3.6 dS m^{-1}, equivalent calcium carbonate from 0.3 to 1.6%, total nitrogen from 0.0 to 0.1 g kg^{-1}, and organic carbon from 0.9 to 1.7 g kg^{-1}. These increases in pH, electrical conductivity, equivalent calcium carbonate, total nitrogen and organic carbon were due to the organic matter and lime additions.

However, the concentrations of DTPA-extractable Pb dropped from 16.3 to 7.6 mg kg^{-1} due to the increase in pH and the immobilization processes. It is well known that Pb mobility is reduced by adding organic matter to the soil, due to the formation of chelating agents from humic acids. Therefore, organic wastes can be successfully used in mining areas to reduce metal toxicity to plants (Bradshaw 2000; Tordoff et al. 2000).

The addition of carbonates and organic matter produced an increase in pH and thus a reduction in Pb availability. Figure 15.2 shows the reduction in Pb availability in amended plots 24 months into the experiment. Before the amendments were added the Pb availability ranged from 0.5 to 3.3%; these values reduced to 0.3–1.2% after the additions.

Chemical species of Pb in soil are usually bioavailable to some degree if the soil is ingested by children, livestock, or wildlife (Chaney and Ryan 1994), whereas Pb

Table 15.1 pH in water, electrical conductivity (EC), equivalent calcium carbonate ($EqCaCO_3$), total nitrogen (TN), organic carbon (OC), total extractable Pb, and DTPA-extractable Pb measured in plots before and after 24 months of the experiment (CA, CB, and CC are control plots, while PA, PB, and PC are pig manure-amended plots)

	0 months							24 months						
	pH water	EC (dS m^{-1})	$EqCaCO_3$ (%)	TN (g kg^{-1})	OC (g kg^{-1})	Total Pb (mg kg^{-1})	DTPA Pb (mg kg^{-1})	pH water	EC (dS m^{-1})	$EqCaCO_3$ (%)	TN (g kg^{-1})	OC (g kg^{-1})	Total Pb (mg kg^{-1})	DTPA Pb (mg kg^{-1})
CA	3.0	2.4	0.5	0.0	1.0	1,085.2	36.3	2.6	2.7	0.3	0.0	0.6	1,095.0	19.2
CB	2.5	2.6	0.3	0.0	0.7	1,415.7	10.9	2.6	2.9	0.3	0.0	0.5	1,414.3	10.5
CC	2.8	2.5	0.4	0.0	0.8	1,200.0	25.5	2.5	2.8	0.3	0.0	0.5	1,250.0	15.6
Mean C	2.8	2.5	0.4	0.0	0.8	1,233.6	24.2	2.6	2.8	0.3	0.0	0.5	1,253.1	15.1
PA	2.8	4.8	0.3	0.0	0.8	1,019.8	13.2	6.7	3.7	0.9	0.1	1.5	1,227.0	4.1
PB	2.9	2.3	0.3	0.0	0.9	1,123.3	29.2	7.3	3.5	2.9	0.1	2.0	1,134.2	9.6
PC	2.4	3.4	0.3	0.0	1.0	1,255.7	6.6	7.0	3.6	1.0	0.2	1.6	1,263.0	9.1
Mean P	2.7	3.5	0.3	0.0	0.9	1,133.0	16.3	7.0	3.6	1.6	0.1	1.7	1,208.1	7.6

*Below detection limit: 0.001 g kg^{-1}

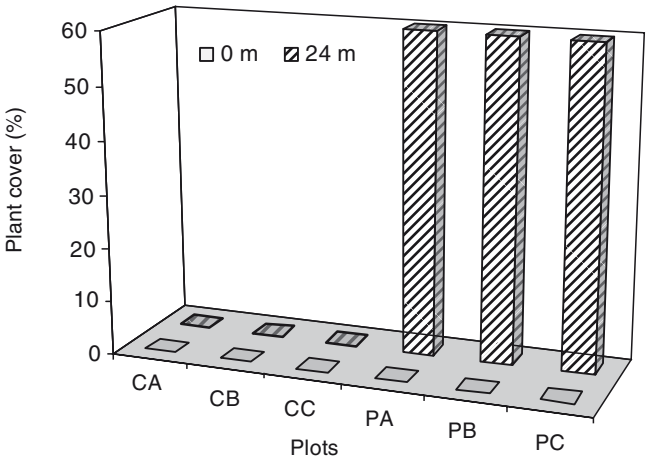

Fig. 15.2 Percentages of plant cover found in the plots before (0 m) and after (24 months of) adding amendments in control plots (*CA*, *CB*, and *CC*) and pig manure-amended plots (*PA*, *PB*, and *PC*)

phosphate minerals, such as chloropyromorphite, which is extremely insoluble and is not bioavailable (Cotter-Howells et al. 1994; Ma et al. 1995; Ruby et al. 1994; Cotter-Howells 1996; Cotter-Howells and Capom 1996), is formed slowly, apparently because the reactants have low solubility.

15.3.2 First Stages of Phytostabilization

A significant increase in plant cover was observed two years into the experiment (Fig. 15.2). No plant growth was observed in the control plot due to its extremely acidic pH, which inhibits seed germination, while amended plots exhibited 60% plant cover. The addition of the amendments had a significant and positive effect on plant cover percentages. Increasing the pH, organic carbon, total nitrogen, and micronutrient contents, as well as modifying some physical properties, had a highly positive effect on plant colonization.

Regarding the plant species that grew spontaneously in the plots, *Diplotaxis lagascana* (*Brassicaceae*) was the dominant. Other species such as *Atriplex halimus* L. (*Chenopodiaceae*), *Sisymbrium irio (Brassicaceae)*, *Sonchus tenerrimus* (*Asteraceae*), *Malva sylvestris (Malvaceae)*, *Sedum album* (*Creassulaceae*), *and Bromus fasciculatus* (*Poaceae*) were also found. Different seeds were carried by the pig manure. Moreover, seeds that originated from the surroundings of the mine pond, such as those for *Atriplex halimus*, *Dittrichia viscosa*, *Sonchus tenerrimus*, and *Sedum album*, germinated and grew on the plots.

A reduction in the heavy metal concentrations in *Diplotaxis lagascana* plants from amended plots was observed. Thus, the addition of amendments caused a

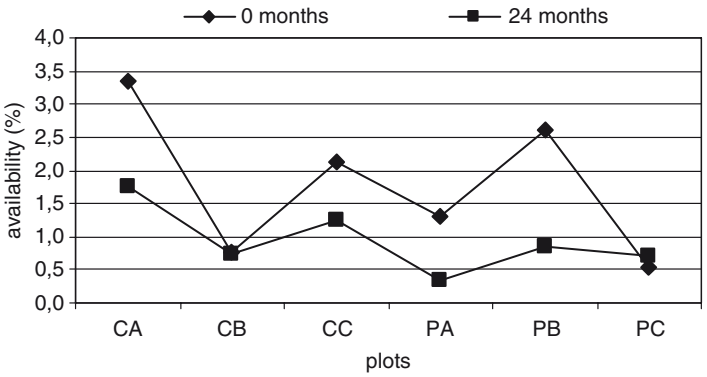

Fig. 15.3 Pb availability before and after 24 months of the experiment in control plots (*CA*, *CB*, and *CC*) and in pig manure-amended plots (*PA*, *PB*, and *PC*)

decrease in DTPA-extractable metals, which decreased the amounts of these metals taken up by the plants (Fig. 15.3). pH and organic matter content are the main factors that influence heavy metal uptake by plants.

Other studies carried out in the same study area indicate that species such as *Piptatherum miliaceum* (L.) Cosson (*Poaceae*), *Bituminaria bituminosa* (L.) Stirton (*Leguminosae*), *Lygeum spartum L.* (*Poaceae*) or *Hyparrhenia hirta* (L.) Stapt (*Poaceae*) (Walker and Correal 2004; Lefèvre et al., 2005a,b; Walker et al. 2005, 2006; Conesa et al. 2007a, b, c; Walker et al. 2007) that grow naturally in this zone could be used for phytostabilization.

Moreover, it is crucial to consider the potential of all of these plants to reduce soil erosion. Unfortunately, little information on the root systems of Mediterranean plants and their capacity to reduce soil erosion is available. De Baets et al. (2007) found that *Piptatherum miliaceum* and *Lygeum spartum* possess great potential for reducing erosion rates and constitute a good alternative for revegetation.

To continue with the phytostabilization process, the most suitable species to use may be the legume *Bituminaria bituminosa*, due to its ability to reduce erosion and fix nitrogen as well as its tolerance of heavy metals, and *Lygeum spartum* because of its ability to reduce erosion and its low rate of heavy metal accumulation. Moreover, *Zygophyllum fabago, Piptatherum miliaceum* and *Atriplex halimus* are all suitable for phytostabilization strategies, but special care should be taken with these species due to their moderate rates of heavy metal accumulation.

15.4 Conclusion

Our results show that chemical immobilization and phytostabilization of Pb-polluted soils under semiarid field conditions is a successful approach to reclaiming mining areas in southeast Spain. The most suitable species for futures activities are considered

to *Bituminaria bituminosa*, *Lygeum spartum*, *Zygophyllum fabago, Piptatherum miliaceum*, and *Atriplex halimus.*

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Chapter 16
Impact of Heavy Metals on Sugarcane

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16.1 Introduction: Extent of Sugarcane Cultivation and Cane Yield Potential

Sugarcane is one of the most important crops in the tropics, and it is also becoming an important crop in the subtropics, meaning that it is cultivated between the latitudes of 35°N and 35°S. It covers a total acreage of 20.1 million ha, yielding a global production of 1,317.9 million metric tons of cane with an average cane productivity of 65.6 t ha^{-1} (FAO 2004). On the other hand, sugarcane has the theoretical potential to yield 470 t ha^{-1} dry matter, 805 t ha^{-1} wet cane, 50–78 t ha^{-1} sucrose, and has a total solar energy harvesting efficiency of 8.5%. The highest harvestable sugarcane yield achieved so far is close to 58% of its theoretical potential. In other words, there is a 42% gap between the theoretical yield of sugarcane and the highest harvestable sugarcane yield obtained so far. Efforts are now underway to bridge this gap and thus increase the productivity of sugarcane. Such efforts include the use of high-yielding varieties, fertilizers, irrigation, different types of effluents, sewage sludge, industrial residues/by-products, spent wash, pesticides, herbicides, etc. Except for the use of high-yielding cane varieties, these methods of increasing cane productivity add heavy metals to the soils for growing sugarcane.

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16.1.1 Heavy Metal Contents in Different Materials used in Agriculture

Heavy metals are nonessential elements that do not have any known function in plants. Metals such as cadmium, chromium, copper, mercury, lead and other naturally occurring metallic elements with high molecular weights are considered important pollutants. These toxic elements either occur naturally in soils or are deposited when agricultural chemicals, urban wastes, and polluted water make their way into the soil (Dhillon and Dhillon 1996) (Table 16.1).

16.1.2 Sufficient and Phytotoxic Levels of Heavy Metals in Plants

Heavy metals present in the soil are absorbed by the plants growing in it to either sufficient or phytotoxic levels (Dhillon and Dhillon 1996) (Table 16.2).

Nevertheless, any organism has the ability to withstand the presence of certain quantities of essential and nonessential elements in its environment and include them in their growth processes. However, high levels of these elements can be toxic to the organism (Bradshaw and McNeily 1991), and can lead to characteristic stress symptoms, such as disturbances in photosynthesis and respiration as well as

Table 16.1 Heavy metal contents of different materials used in agriculture (from Dhillon and Dhillon 1996)

Element	Sewage	Sludge	City waste water	Conc SP[a]	Gypsum
	($\mu g\ l^{-1}$)			($mg\ kg^{-1}$)	
Cr	1.5–7.5	326	–	–	–
Pb	25	45	114	–	–
Cd	2.6–25	–	–	8–83	1–12
Zn	50–74	1,675	340–2,760	89–973	6–48
Mn	–	30	40–390	–	–
Fe	–	4,092	1,260–12,300	–	–
Cu	–	400	90–310	–	–
Ni	–	8,210	40–850	–	–

[a]Concentrated suspended particles

Table 16.2 Sufficient and phytotoxic levels ($mg\ kg^{-1}$) of heavy metals in plants (from Dhillon and Dhillon 1996)

Element	Sufficient	Phytotoxic	Element	Sufficient	Phytotoxic
Zn	20–100	>500	Cr	<1–5	–
Mn	15–200	>500	Co	–	25–100
Cu	5–20	>30	Mo	<1–5	>10
Ni	<1–10	>10	Se	–	>100
Pb	<1–3	–	–	–	–

reduced biomass production. The cultivation of sugarcane adjacent to industrial activity and metal-polluted landfills, and the application of municipal wastes to the soil along with the indiscriminate use of phosphatic fertilizers and pesticides can enhance the levels of toxic heavy metals in the soil, and thus in sugarcane. Therefore, the present chapter describes the effects of heavy metal pollution of the soil on the growth and juice quality of sugarcane.

16.2 Sources of Metal Contamination of Sugarcane

In this section, we consider the different sources that can contaminate sugarcane agro-ecosystems with metals/heavy metals.

16.2.1 Phosphatic Fertilizers

Recently, much attention has been directed to the entry of toxic metals into the human food chain. The application of inorganic fertilizers to plants is considered one of the routes that could allow toxic metals to enter the food chain. An analysis of 74 samples of commercial fertilizers involving 20 samples of phosphatic fertilizers (monoammonium phosphate, diammonium phosphate, and triple superphosphate), 11 samples of liquid fertilizers, 31 samples of water-soluble multiple-nutrient fertilizers, and 12 samples of solid multiple fertilizers marketed in the Kingdom of Saudi Arabia revealed that the concentrations of heavy metals varied according to the type of fertilizer (Table 16.3). In all of these types of fertilizer, the heavy metal present at the greatest concentration was Cr. The cadmium content ranged from <1 to 36.8 mg kg^{-1} of phosphatic fertilizer. However, the concentrations of heavy metals in these fertilizers were lower than the corresponding tolerance limits for Cd (100 mg kg^{-1}), Cr (100 mg kg^{-1}), and Ni (50 mg kg^{-1}), and the levels of these were found to similar to those found internationally. On average, the annual application of 80 kg P ha^{-1} in Saudi Arabia contributes 13 g of Cd ha^{-1} to the soil (Modaihsh et al. 2004).

In sugarcane-growing areas in Brazil (Ramalho et al. 1999), the Cd concentration was up to sevenfold higher than those in uncultivated sites nearby. The main reason

Table 16.3 Heavy metal contents (median values in mg kg^{-1}) of different kinds of fertilizers (from Modaihsh et al. 2004)

Heavy metal	Phosphatic fertilizers	Liquid fertilizers	Water-soluble multiple-nutrient fertilizers	Solid multiple-nutrient fertilizers
Cd	33.2	9.5	2.9	19.7
Pb	14.3	9.9	9.8	15.3
Ni	72.1	15.6	7.4	43.0
Co	11.8	11.2	5.6	12.5
Cr	249.3	64.0	–	170.7

for the high Cd levels was probably impurities in phosphatic fertilizers. The estimated input of Cd from this source varies from 38 g ha^{-1} to 340 g ha^{-1}.

16.2.2 Sewage Sludge

Sewage sludge is used as an organic manure to enrich soil fertility and thus raise crop productivity. The US produces 5.3 million metric tons (11.6 billion lb on a dry weight basis) of sewage sludge each year. About 16% of US sewage sludge is incinerated (the ash is then buried in landfills), 38% of sludge is put directly into landfills, 36% is spread onto farmland or forest land or is otherwise mixed into soils, and 10% is handled in other ways (Krauss and Page 1997). The sewage sludge contains low levels of phosphorus (Ayuso et al. 1992) and potassium (Ros et al. 1990), moderate contents of nitrogen, magnesium and sulfur, and high levels of calcium (Oliveira et al. 1995). Besides these, heavy metals are also present in the sewage sludge: 8 mg Cd, 579 mg Cr, 625 mg Cu, 44,450 mg Fe, 14 mg Mn, 346 mg Ni, 217 mg Pb, and 1,125 mg Zn per kilogram of sludge (Raij van et al. 1996). Since more and more sewage sludge is being mixed with soil, the heavy metal contents of these soils are increasing. The presence of these heavy metals in sewage sludge and their accumulation in soils raises concern about their possible toxicity to sugarcane and potential contamination of groundwater sources.

16.2.3 Industrial Residue (Vinasse)

The industrial residue or vinasse from a sugar factory contains solids, N, P, K, Ca, Mg, sulfates, carbon, organic matter, and reductors (Camhi 1979) (Table 16.4). It serves as a source of nutrients, and contains very low levels of heavy metals.

Table 16.4 The contents (kg l^{-1}) of vinasse (from Camhi 1979)

Parameter	Molasses	Cane juice	Mixture of molasses and cane juice
Total solids	81.50	23.70	52.70
Volatile solids	60.00	20.00	40.00
Fixed solids	21.50	3.70	12.70
Nitrogen	0.45–1.60	0.15–0.70	0.48–0.71
Phosphorus as P_2O_5	0.10–0.29	0.01–0.21	0.01–0.20
Potassium as K_2O	3.74–7.83	1.20–2.10	3.34–4.60
Calcium as CaO	0.45–5.18	0.13–1.54	1.33–4.57
Magnesium as MgO	0.42–1.52	0.20–0.49	0.58–0.70
Sulfates as SO_4	6.40	0.60–0.76	3.70–3.73
Carbon	11.20–22.9	5.70–13.40	8.70–12.10
Organic matter	63.40	19.50	38.00
Reductors	9.50	7.90	8.30

16.2.4 Sugar Mill By-Products

The sugar industry produces a number of by-products during the sugar production process, including bagasse, mill or filter mud, mill effluent, and trash. Of these by-products, bagasse is utilized as a source of energy, whereas trash and filter mud are used as sources of nutrients and soil ameliorants. The continued application of cane mill effluent at high rates without appropriate recognition of the soil conditions and crop requirements has raised a number of concerns about overfertilization and heavy metal concentrations (Abotal and Cabigon 2001; Liu and Chen 1991; Baruah et al. 1993) (Table 16.5), as well as offsite effects such as the impact on riverine environments. The other by-products – bagasse and mill or filter mud – are reported to have traces of heavy metals. The filter mud contains 4–10% $SiO_{2,}$ 1–4% CaO, 1–3% P_2O_5, and 0.5–1.5% MgO on a dry matter basis (Mann 1995).

16.2.5 Tannery Effluents

The disposal of tannery effluents poses a serious water pollution issue and results in unhygienic conditions. Although a waste, the utilization of tannery effluents as fertilizer does occur due to the presence of essential mineral nutrients in them. Experiments conducted at the Indian Institute of Sugarcane Research (IISR), Lucknow, on the effects of tannery effluents on sugarcane growth indicated that treated tannery effluent contains essential plant nutrients (Table 16.6). It also contains high concentrations of Cr (9 ppm) and Na (6.4 meq l^{-1}), which could be toxic to sugarcane plants (Pande et al. 1990).

Table 16.5 Concentrations (ppm) of heavy metals in cane mill effluents from different countries

Country	Cd	Cu	Pb	Cr	Zn	As	Source
Philippines	0.0004	0.18	0.003	–	–	–	Abotal and Cabigon 2001
Taiwan	–	0.025	–	–	0–0.09	–	Liu and Chen 1991
India	0–0.2	0.01–0.12	0.01–0.12	0–0.05	4.5–15	0.01–0.08	Baruah et al. 1993

Table 16.6 Contents of essential plant nutrients in tannery effluents (from Pande et al. 1990)

Essential plant nutrients								
meq l^{-1}					ppm			
Ca	K	Mg	P	S	Fe	Mn	Cu	Zn
13.3	2	13	0.5	5.1	6.4	1.0	0.2	4.7

16.2.6 Pig Effluents

In Taiwan, the concentrations of heavy metals in sugarcane fields irrigated with pig effluent have been reported. The available contents of Cd, Pb, and Zn in soils were in the ranges 0.02–0.22, 1.67–5.97, and 2.89–9.14 mg kg^{-1} soil, respectively. However, these levels of heavy metals did not affect the growth and yield of sugarcane (Liu et al. 1996a).

16.2.7 Swine Lagoon Effluents

The long-term use of swine lagoon effluents increased the extraction rates of plant-available heavy metals (Table 16.7). The mean absorptions of the heavy metals (Table 16.8) could be ordered as follows: Zn>Cu=Cr>Pb>Ni>Cd (Liu et al. 1996b).

Table 16.7 Available (extracted in 0.1 M HCl) concentrations of heavy metals in the soil following long-term application of swine lagoon effluents (from Liu et al. 1996b)

Soil depth (cm)	Distance from outlet (m)	Available content of heavy metal (mg kg^{-1} soil)					
		Cd	Ni	Cr	Cu	Pb	Zn
0–15	0–5	0.11	0.57	0.43	19.84	5.65	22.42
	5–10	0.08	0.40	0.34	13.27	5.97	14.70
	10–20	0.11	0.55	0.35	17.74	6.07	16.14
	20–40	0.13	0.69	0.33	16.31	6.65	17.85
	40–80	0.12	0.81	0.29	5.70	5.27	9.95
	Mean	0.11	0.60	0.35	14.57	5.92	16.21
	SD±	0.05	0.31	0.31	10.24	3.48	8.69
15–30	0–5	0.11	0.59	0.43	21.43	6.86	24.50
	5–10	0.08	0.41	0.32	14.24	5.18	15.68
	10–20	0.11	0.53	0.33	13.12	6.89	14.27
	20–40	0.14	0.76	0.37	20.43	7.49	18.86
	40–80	0.13	0.55	0.29	6.13	5.65	10.38
	Mean	0.11	0.57	0.35	14.51	6.41	16.74
	SD±	0.06	0.26	0.09	9.76	3.33	10.52

SD, standard deviation

Table 16.8 Concentrations of heavy metals in sugarcane tissue following the application of swine lagoon effluents to the sugarcane plot (from Liu et al. 1996b)

Distance from outlet (m)	Heavy metal concentration (mg kg^{-1})					
	Cd	Ni	Cr	Cu	Pb	Zn
0–5	0.02	0.65	2.35	6.29	0.78	31.33
5–10	ND	0.28	2.33	5.47	1.22	30.30
10–20	0.05	0.36	2.27	5.77	1.06	26.55
20–40	0.03	0.22	2.24	6.05	0.96	25.93
40–80	0.16	0.88	2.72	6.88	2.39	27.80
Mean	0.05	0.48	6.09	6.09	1.28	28.39
SD±	0.20	0.52	0.81	1.10	1.53	7.92

SD, standard deviation

16.2.8 Spent Wash/Effluents

In India, about 15,000 million liters of spent wash are produced annually from 246 distilleries. This situation has created an acute spent wash disposal problem with the expansion of distilleries in sugarcane-growing countries. This spent wash, whether treated or not, is used to both irrigate and fertilize sugarcane, as it contains essential plant nutrients (Table 16.9) required for growth (Samuels 1980). However, it also contains heavy metals (Table 16.10). As a result, large-scale applications of spent wash can have deleterious effects on sugarcane growth if it leads to the presence of heavy metals in the soil at levels exceeding their toxicity limits (Table 16.11). Spent wash also contaminates human and animal drinking water and irrigation water.

Industrial effluents containing heavy metals also pollute cultivated fields (Table 16.12) and underground water through seepage or by allowing them into fields nearby. The levels of Pb can range from 2 to 200 ppm in such agricultural lands.

When industrial effluents are diverted into streams, they pollute the water with heavy metals, resulting in Pb levels of 0.07–0.61 ppm, cadmium levels of 0.011–0.043 ppm, Ni levels of 0.22–0.83 ppm, and cobalt levels of 0.16–2.81 ppm (Anon 1992–1993). Likewise, waters from open and bore wells are also polluted with heavy metals such as Cd, Pb, Ni, and Co due to pollution from industrial effluents nearby (Table 16.13).

Table 16.9 Contents (mg l^{-1}) of essential plant nutrients in spent wash (from Jain et al. 2001)

Essential plant nutrients	Treated spent wash (pH 9.0)	Untreated spent wash (pH 4.0)
P	35.1	77.6
S	765.0	1609.0
Fe	9.52	68.5
Mn	0.72	3.0
Zn	0.72	4.01
Cu	0.38	2.66

Table 16.10 Concentrations of heavy metals in spent wash (from Jain et al. 2001)

Spent wash	Heavy metals (mg l^{-1} spent wash)			
	Cd	Cr	Ni	Pb
Treated	0.004	0.95	0.88	0.54
Untreated	0.025	0.17	0.86	1.24

Table 16.11 Toxicity limits for heavy metals (mg l^{-1}) (from SASA 2002)

Solution / water	Cu	Zn	Pb	Cd	Fe	Mn	Al
Solution for plants	0.02	1.3	1.7	2.1	9.3	0.06	0.93
Drinking water for humans	1.5	15.0	0.1	0.01	1.0	0.5	–
Drinking water for farm animals	0.5	25.0	0.1	0.05	–	–	5.01
Irrigation water	0.2	2.0	5.0	0.01	5.0	0.20	5.02

Table 16.12 Results of an analysis of soil samples collected from a commercial farm polluted with effluents (from Anon 1992–1993)

S.no.	Description	Available (DTPA extractable) heavy metals (ppm)			
		Cd	Ni	Pb	Co
1	Marshy soil near well no. 7	0.04	1.40	58.3	1.73
2	Plot no. 39	0.04	0.46	22.4	0.68
3	Plot no. 41	0.02	1.26	16.9	0.26
4	Plot no. 44	0.02	0.40	23.6	0.32
5	Noor Mohd. tank	0.05	0.60	1.76	0.39
6	Stagnant water near plot no. 27	0.05	0.49	2.65	0.66
7	Well no. 7	0.05	0.20	0.46	0.27
8	Shiva Rampally tank	0.12	0.71	1.26	1.63
9	Canal water near well no. 7	0.03	0.26	0.42	0.35
10	Water sample from well no. 7	0.03	0.88	0.37	0.73
11	Oil seed foundation bore well	0.02	0.22	0.70	0.001

Table 16.13 Heavy metal contents (ppm) in waters of open and bore wells near Naddavagu (Patancheru), India, polluted with industrial effluents (from Anon 1992–1993)

S.no.	Open well				Bore well			
	Cd	Pb	Ni	Co	Cd	Pb	Ni	Co
1	0.013	0.41	0.42	0.62	0.02	0.28	0.63	1.49
2	0.015	0.32	0.22	1.76	0.01	0.12	0.44	0.24
3	0.026	0.16	0.83	1.79	0.03	0.12	0.39	0.57
4	0.019	0.61	0.38	0.47	0.04	0.12	0.59	0.39
5	0.015	0.32	0.75	0.16	0.03	0.02	0.27	2.06
6	0.011	0.24	0.43	0.80	0.02	0.10	0.27	2.06
7	0.022	0.25	0.86	1.10	0.02	0.12	0.53	2.08
8	0.024	0.10	0.28	0.41	0.03	0.16	0.47	2.30
9	0.024	0.36	0.26	0.86	0.02	0.13	0.42	0.48
10	0.020	0.40	0.29	0.45	0.04	0.12	0.80	2.81
11	0.008	0.07	0.28	2.30	0.02	0.10	0.37	2.12
12	0.016	0.28	0.63	0.99	0.03	0.12	0.38	1.58
13	0.021	0.16	0.52	1.85	0.04	0.11	0.42	1.48

16.2.9 Fungicides

A number of fungicides are used to control diseases in sugarcane. Residues of these fungicides contain heavy metals that are added to the soil. A survey of sugarcane fields in New South Wales (Australia) indicated high concentrations of heavy metals like Cd, Cu, Hg, Mo, Ni, and Pb in the soils. The application of organomercury-based fungicides also causes high concentrations of Hg in the soil (Rayment et al. 1998).

16.2.10 Metal-Polluted Landfills

Metal-polluted landfills contain considerable amounts of heavy metals. These metals are absorbed by sugarcane growing on these landfills (Table 16.14). As a result, high levels of Zn are found in the green leaves, Cd in dried leaves, and Cu, Cr and Ni in the roots. Cane juice from such sugarcane also contains Zn, Cu, Cd, and Cr. The consumption of heavy metal containing green leaves as fodder by animals and cane juice by humans can have adverse effects on health. The release of heavy metals from dried leaves and roots upon their decomposition also pollutes the soils (Liu et al. 1994).

16.2.11 Use of Brackish Water for Irrigation

Brackish water contains heavy metals like Cd, Pb, and Cr (Table 16.15). The use of such water for irrigation also contaminates soils with heavy metals.

Table 16.14 Concentration ratios (mg kg^{-1}) for different heavy metals in different components of sugarcane grown on polluted landfills (T) and not on landfills (Ck) (from Liu et al. 1994)

Component	Treatment	Concentration ratio of heavy metal				
		Zn	Cu	Cd	Cr	Ni
Green leaf	Ck	31.06	6.29	0.33	5.62	1.85
	T	42.61	6.68	0.04	1.85	2.03
Dried leaf	Ck	3.73	0.80	1.93	ND	1.46
	T	10.28	2.46	2.56	11.05	2.58
Bagasse	Ck	8.99	2.37	ND	ND	ND
	T	11.11	2.98	ND	ND	ND
Juice	Ck	2.41	0.34	ND	ND	ND
	T	2.96	0.47	0.01	0.01	ND
Root	Ck	9.73	6.49	ND	ND	3.29
	T	10.56	149.19	1.38	22.5	14.58

ND, not detectable

Table 16.15 Heavy metal contents (ppm) in brackish water (from Anon 1992–1993)

Pond no.	Cd	Pb	Cr
63	0.11	0.64	2.02
51	0.14	0.86	2.17
20	0.13	1.22	1.92
131	0.13	1.56	2.05
45	0.14	1.17	2.30
87	0.14	0.90	2.04
17	0.14	1.32	1.93
48	0.14	0.93	2.07
130	0.07	1.04	0.61

Table 16.16 DTPA-extractable available contents of heavy metals (ppm) in cement dust polluted soils at Adilabad, Andhra Pradesh, India (from Anon 1991–1992)

S.no.	Village	Distance from factory (km)	Heavy metal contents (ppm) in soils				
			Cd	Ni	Pb	Co	Cr
1	Bellori	1	0.01	0.28	0.53	0.34	1.67
2	Chanda	2	0.03	0.56	0.65	0.16	2.13
3	Chanda	4	Nil	0.44	0.16	Nil	2.78
4	Jandapur	6	Nil	0.70	Nil	0.29	3.08
5	-do-	8	Nil	Nil	0.83	0.19	2.73
6	Bellori	2	0.04	0.59	0.24	Nil	Nil
7	Rampur	4	0.10	0.52	0.47	0.13	0.82
8	-do-	6	0.01	0.69	0.96	0.09	Nil
9	Ponnari	7	0.10	0.40	0.93	0.27	2.12
10	-do-	8	0.14	0.10	1.09	0.21	Nil
11	Adilabad (south)	1	0.04	0.76	0.18	0.34	2.41
12	Nishanghat (east)	1	0.01	0.65	0.78	0.32	Nil
13	-do-	1	0.10	0.49	0.10	0.12	Nil
14	-do-	1	0.04	0.90	0.88	0.50	0.89
15	Khanapur	1	0.02	0.38	1.47	0.05	0.80
16	Ankunt	3	0.05	0.28	0.27	0.36	0.39
17	-do-	3	0.01	0.36	0.37	0.36	5.01
18	Bangariguda	5	0.12	0.55	0.73	0.24	Nil
19	Yapulaguda	9	Nil	0.31	0.63	0.12	Nil
20	-do-	9	0.10	0.26	0.40	0.03	Nil

do, ditto

16.2.12 Soil Polluted by Industry

The pollution of cultivated areas around Adilabad District, Andhra Pradesh, India by a cement factory (Cement Corporation of India) is damaging agricultural land and standing crops. Analysis has indicated the presence of heavy metals such as Cd, Ni, Pb, and Cr in toxic amounts in soils (Table 16.16). Excess levels of lead were usually found (Anon 1991–1992).

16.3 Effects of Different Sources of Heavy Metals on Soil and Sugarcane

In the southwest of Iran, over 130,000 ha of land are devoted to sugarcane (*Saccharum officinarum*) cultivation. In these sugarcane fields, about 400 kg ha^{-1} each of diammonium phosphate (DAP) and urea are applied annually. Four sugarcane-growing sites, namely Haft-tapeh, Karoon, Shoeibieh, and Ghazali, which have been cultivated sugarcane for 36, 20, 2, and 1 year, respectively, were selected for a study. In each area, soil samples (0–30 cm) were taken from a transect of uncultivated land, and from both furrows and ridges of cultivated land. The electrical conductivity (EC), pH, and the contents of clay, calcium carbonate, organic carbon

(OC), Cl, Cd, Ni, and Zn were measured for each of the 101 soil samples taken. The cadmium distribution profile was determined to a soil depth of 300 cm. The heavy metal concentrations in sugarcane and in the associated soil samples from the three sugarcane sites were also measured (Barzegar et al. 2005).

The Cd and Ni contents among the sugarcane sites differed; Cd was related to the clay content and Ni was related to the OC content of the soil. The cadmium content in cultivated soil was lower than that in uncultivated soil, even after years of applying P fertilizers. The Ni and Cd contents of the sugarcane were much higher than the levels found in topsoils, but there was no significant relationship between either the Cd or Ni content of sugarcane and the chemical properties of the soil. The Zn content of the soil decreased as either its EC or Cl concentration increased. There were no significant differences in Zn content between different sugarcane sites or between cultivated and uncultivated soils.

16.4 Critical Limits for Heavy Metals in Soil

The heavy metal critical load depends on the acceptable total load from anthropogenic heavy metal inputs (deposition, fertilizers, other anthropogenic sources), below which ecosystem damage is unlikely.

Methods for calculating critical loads for toxic metals are currently being developed within the United Nations Economic Commission for Europe (UNECE) Convention on Long-Range Transboundary Air Pollution (LRTAP) (De Vries and Bakker 1996; Slootweg et al. 2005). In the UK, a research consortium currently contributes to the development and improvement of methods of calculating critical loads for application within the UNECE, and to the development of improved tools for assessing the effects of changing rates of atmospheric deposition on pools of metals in soils and freshwaters. Forest research is a subcontractor to this consortium and has contributed to the calculation, evaluation, and updating of heavy metal critical loads for forest ecosystems in the UK.

Both effect-based steady-state and standstill critical load approaches have been used to calculate and map heavy metal critical loads. In brief, an "effect-based" methodology identifies atmospheric depositions (critical loads) that will not lead to concentrations of heavy metals above critical limits in defined compartments at steady state.

The "critical limit" defines an acceptable maximum concentration of a metal below which long-term deleterious effects to an ecosystem should not occur. Thus, defining the critical limit is crucial to the critical load approach. For lead (Pb) and cadmium (Cd), critical limits of 8 mg Pb m^{-3} and 0.8 mg Cd m^{-3}, respectively, have been adopted.

Critical loads can be derived using:

- Critical limits of heavy metal concentrations in the soil solution that will not harm microbiota and plants, and/or
- Critical limits of (reactive) soil metal concentrations that will not lead to adverse impacts on soil functioning, such as soil invertebrates that ingest soil.

The "standstill" critical load is the atmospheric deposition that will not lead to any further accumulation of heavy metals in the soil. Standstill critical loads should also include inputs other than atmospheric deposition.

Effect-based steady-state and standstill critical load approaches are described further below.

16.4.1 Effect-Based Steady-State Critical Loads: $CL_{effb}(M)$

The steady-state equation (Slootweg et al. 2005) for the calculation of heavy metal critical loads is as follows:

$$CL(M) = M_u - M_w + M_{le(crit)}$$

where CL(M) is the critical load of heavy metal M, M_u is the removal of heavy metals by biomass harvesting or the net uptake by forest ecosystems from the mineral topsoil, M_w is the release of heavy metals from the mineral topsoil by weathering, and $M_{le(crit)}$ is the critical leaching of heavy metals from the mineral topsoil when only the vertical drainage flux is considered.

Growth uptake values are calculated from estimates of average increase in annual biomass multiplied by the content of the metal in the tree. Previous uptake values for woodland were derived from a default range of metal concentrations in trees and average yield data. However, new uptake values for heavy metals (Cu, Zn, Cd, Pb, Ni, and Cr) based on measurements from the twenty Level II Intensive Monitoring sites in the UK are now available, and will be used in future modeling.

The depth considered in the calculation is 10 cm for forest soils or the depth of the A horizon, since impacts on plants and soil organisms, which are the main target groups considered, are largely restricted to this depth.

16.4.2 Standstill Critical Loads: $CL_{stst}(M)$

The critical load equation used for the standstill approach (Slootweg et al. 2005) is the same as that used for the effect-based steady-state method, but the "critical limit" is replaced with the "concentration of metal" in the soil solution, on the premise that no further metal accumulation will be allowed.

A limitation of the stand-still approach is that data on current concentrations of heavy metals in soil solution are scarce. Leaching can therefore only be mapped on the basis of transfer functions that convert total contents of metals in the upper soil horizon into concentrations in soil solution. Transfer functions are available that describe the relationship between dissolved and adsorbed concentrations of heavy metals, accounting for the impacts of soil properties such as pH and organic carbon content.

Previously, the critical load models were only applied to upland forests. Recently, however, these models have also been applied nationally to lowland forests.

Preliminary modeling and mapping of critical loads for cadmium and lead for upland forested areas suggests that the soil solution concentrations exceed the critical limits (i.e., 8 mg Pb m^{-3}, 0.8 mg Cd m^{-3}) in some upland areas of England, Wales and Northern Ireland. The critical limit for Pb is exceeded in 41% of the grid squares; for Cd, the critical limit is exceeded in 17% of the grid squares.

However, there are still major uncertainties over the most appropriate methodological approaches to use when applying the critical load concept to metals as opposed to acid deposition. Further research will test the approaches to heavy metal critical load mapping, applying site-specific soil water chemistry, heavy metal deposition, and estimates of heavy metal uptake.

16.5 Heavy Metal Contents in Soils, Sugarcane Plants, Juice, Sugar, and Jaggery

16.5.1 In Soils

The movement of heavy metals (Pb, Fe, Cu, and As) down the soil profile was studied on two fadama crop lands devoted to sugarcane cultivation in Minna, Niger State, Nigeria. The soil type in the experimental fields was loamy sand or sandy clay loam, with a clay content of 13–34% and a pH (H_2O) of 7.04–7.68. The soil had 1.1–1.5% organic matter content and a soil cation exchange capacity of 6.32–9.45 mol kg^{-1}. The heavy metal contents were 0.04–0.15 mg Pb, 0.05–0.11 mg Fe, 0.06–0.13 mg Cu, and 0.10–0.26 mg As kg^{-1} soil. Significant proportions of these heavy metals had accumulated in the topsoil. The relatively high organic matter content in the top soil appeared to bind the metals in nonleachable forms, thereby reducing their mobility. It was evident that all of the heavy metals at the soil depths examined exceeded the acceptable standards for table water, resulting in the possibility of contaminated groundwater (Amoo et al. 2004).

16.5.2 In the Sugarcane Plant

Roots, stems and leaves of sugarcane (*Saccharum* spp.) were collected from 25 sites in an area under the direct influence of the municipal landfill site (MLS) and the medical waste treatment system (MWTS) of Ribeirao Preto, São Paulo, Brazil (Segura-Munoz 2006). The roots contained 0.22 ± 0.12 mg Cd, 64.3 ± 48.7 mg Cr, 140.6 ± 27.7 mg Cu, 0.04 ± 0.02 mg Hg, 561.6 ± 283.3 mg Mn, 7.9 ± 2.1 mg Pb, and 177.4 ± 64.9 mg Zn kg^{-1} dry weight. Metal levels in stems were 80–90% of those found in roots, while the concentrations detected in leaves were significantly lower than those in roots. The present results suggest that the activities of the MLS and MWTS may have resulted in increasing metal concentrations in edible tissues of

sugarcane grown in the area. The traditional agricultural practices used to grow the sugarcane could also be a determining factor in the current high metal levels. The results indicate that sugarcane is a crop that is able to grow in areas where metals have accumulated in soils.

In eastern Australia, 12 sugarcane (*Saccharum officinarum* L.) varieties and their different parts were screened for plant-mobile heavy metals to assess whether genetic differences were of greater significance than the soil/environment for uptake and within-plant distribution. Soil pH (1:5 soil/water) ranged from 4.5 to 6.4, and all breeding-trial sites contained relatively low levels of extractable Cd, Hg, and Pb and variable levels of Cu and Zn. Internal concentrations of Cd and Zn were more influenced by the soil/environment than by variety, while the distribution of metals in plant parts was quite consistent. About 77% of the Cd and 56% of the Zn were contained in the stem, which relocates to the mill following harvest. There was a little Hg in all plant parts (concentrations < 0.05 mg kg^{-1} dry weight). From a predictive viewpoint, correlations between extractable heavy metals such as Cd in soils and corresponding plant concentrations were inconsistent, with the narrow range of soil concentrations seen as a contributing factor. Based on this evidence, the uptake of heavy metals by sugarcane can be adequately managed by manipulating soil properties rather than by varietal selection. It is also clear that, for each 100 tons of fresh, mature cane, about 0.2 g of Cd and 110 g of Zn will relocate to the soil surface with the trash. The corresponding quantities that move to the mill are 0.54 g of Cd and 143 g of Zn, with amounts expected to be higher for cane grown in strongly acidic soils with above-average levels of heavy metals (Rayment et al. 2002). In India, the concentration of nickel (Table 16.17) and chromium (Table 16.18) in roots and leaves of sugarcane increase with increased supply of these metals.

Table 16.17 Effect of level of Ni supplied on the Ni contents (μg g^{-1} dry weight) of root and leaves of sugarcane (from Jain et al. 2004b)

Plant part	Ni supplied (ppm)			SE±
	0	10	100	
Roots	10.4	35.0	573.0	5.41
Leaves	1.39	1.75	9.58	0.18

Table 16.18 Chromium contents in leaves, roots, and juice of sugarcane following the application of various levels of Cr (from Jain et al. 2004a)

Level of Cr applied	Leaves (μg g^{-1} dry weight)	Roots (μg g^{-1} dry weight)	Cane juice (μg l^{-1} juice)
Control	ND	3.73	20
2 ppm	0.52	9.69	28
80 ppm	0.66	35.6	25

16.5.3 In Cane Juice

The heavy metal levels in the aboveground edible parts of sugarcane grown at two Zambian sites, New Farm (Mufulira) and Chilumba (Kafue), were determined. At both sites, informal crop cultivators use heavy metal contaminated wastewater to irrigate their food crops. Sources of heavy metal pollution at New Farm include mining, tailing dams and sewer ponds, and various factories at Chilumba. The "edible" portions of the sugarcane plants were collected randomly across a number of field plots at the two study sites. Sugarcane juice was extracted from the sugarcane samples. The juice extracts were then analyzed for heavy metals (Co, Cu, Cr, Ni, Pb) using an atomic absorption spectrophotometer (AAS) at three Zambian laboratories: Soil Science Laboratories, University of Zambia, Lusaka; Alfred Knight Laboratory, Kitwe; Nkana Water and Sewerage Company Laboratory, Kitwe. The results obtained at the different laboratories conflicted with each other (in some cases by orders of magnitude) in terms of the levels of heavy metal contamination. However, in a number of cases, depending on the laboratory, Cu, Pb, and Co were found at levels higher than their legislative limits, thus indicating a potential cause for concern to people eating large amounts of sugarcane in those areas (Evaristo et al. 2007). The contents of Cr (Table 16.18) and Fe and Cu (Table 16.19) in cane juice increased with the increased application of Cr up to 2 ppm and Ni up to 5 ppm (Table 16.19). However, they decreased when Ni was applied up to levels of 50 and 100 ppm. The Mn content in cane juice was not affected by the application of Ni up to 10 ppm, and it decreased as more Ni was applied. The Zn content in cane juice followed a similar trend to the Mn content when Ni was supplied. The Cd concentration in cane juice was less than 0.15 mg kg^{-1} when its concentration in the soil was less than 50 mg kg^{-1}. This should have little effect on the edible quality of sugarcane.

16.5.4 In Sugar

A very low level of metal contamination is observed in refined sugar. The contents of Zn, Cu, Ni, and Mn were about 0.75, 0.0, 1.1, and 1.3 ppm in refined sugar, whereas their contents in raw sugar were about 19, 9.3, 4, 2.0, and 1.3 ppm, respectively (Prakash et al. 1995). Most of the Pb is transferred into the bagasse (Mohamed 1999).

Table 16.19 Effect of Ni on nutrient contents (μg ml^{-1}) in cane juice (from Jain et al. 2004b)

Micronutrients	Ni supplied (ppm)						SE±
	0	1	5	10	50	100	
Fe	9.93	10.84	14.94	14.75	13.11	12.17	0.67
Mn	1.17	1.16	1.16	1.16	0.97	0.93	0.03
Zn	5.43	4.75	6.76	5.77	4.16	3.75	0.17
Cu	0.73	0.90	1.01	0.97	0.85	0.90	0.03

Further, Barzegar et al. (2005) reported that Cd is accumulated in bagasse and that Ni is primarily accumulated in bagasse and molasses. As a result, the levels of these heavy metals in white sugar are lower than their detectable values.

16.5.5 In Jaggery

Jaggery is prepared from clarified and boiled cane juice. The jaggery eaten in rural India was found to contain nickel at a concentration of 0.011 mg g^{-1} of jaggery (Patidar and Tare 2006).

16.6 Impact of Heavy Metals on Sugarcane Physiological Function

The effects of heavy metals on the growth, metabolism, and quality of juice of sugarcane have been investigated at IISR, Lucknow, India (Jain et al. 2000, 2001, 2004 a,b, 2008; Jain and Srivastava 2006; Rai et al. 2005, 2006, 2007) and in other parts of the world (Fornazier et al. 2002). The results obtained for various heavy metals are given below.

16.6.1 Nickel

Very low concentrations of Ni have beneficial effects on the growth of some higher plants, as it is an essential component of the enzyme urease, which hydrolyzes urea into CO_2 and NH_4 ions. On the other hand, nickel has become a serious pollutant, arising through anthropogenic sources such as industrial activity. In sewage sludge, Ni occurs in organic chelated forms that are readily available to plants and so it can be highly phytotoxic. High concentrations of Ni appear to reduce the plant's uptake of most other nutrients due to damaging effect on the root (Mengel 1978). Ni at very low doses (up to 5 ppm) exerts a stimulatory effect on growth (Table 16.20),

Table 16.20 Effect of Ni on the yield attributes of sugarcane (from Jain et al. 2004b)

Yield attributes	Ni supplied (ppm)						SE±
	0	1	5	10	50	100	
Millable cane (no.)	24	28	31	24	23	14	1.06
Stalk girth (cm)	2.42	2.50	2.62	2.40	2.34	2.32	0.08
Stalk height (cm)	179	183	193	182	180	144	2.14
Single cane wt (kg)	0.80	0.84	0.95	0.98	0.82	0.63	0.03
Sucrose %	19.6	19.67	19.68	19.82	19.91	19.20	0.27

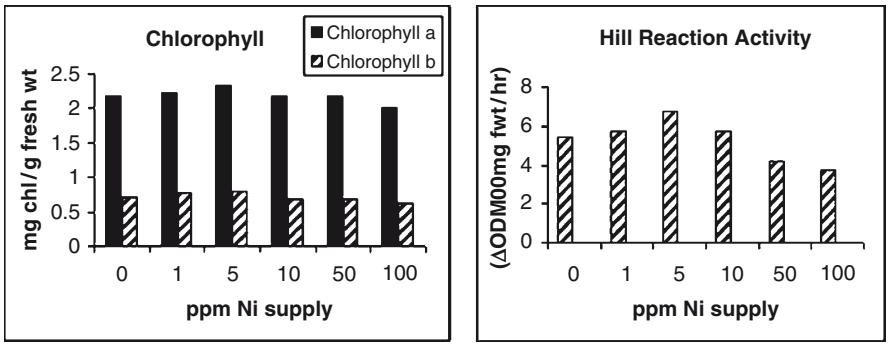

Fig. 16.1 Effect of Ni on chlorophyll content and Hill reaction activity in sugarcane (from Jain et al. 2004b)

increases the contents of chlorophyll a and b, and enhances Hill reaction activity in sugarcane (Fig. 16.1). However, a high Ni content in the growth medium exerts an inhibitory effect on root growth in the form of a reduced number of roots, reduced root length, and decreased mitotic activity of root tissue. This effect leads to alterations in mineral composition, leaf attributes, and reduced sugarcane growth.

Ni supplied in irrigation water at a level of 15 ppm was found to decrease photosynthesis by 25% in sugarcane 30 days after treatment (DAT) with Ni in March. However, 10 ppm nickel increased the net photosynthetic rate 60 DAT (Fig. 16.2) in April. The rate of transpiration and stomatal conductance had decreased 30 DAT (March), while an increase was observed with an enhanced concentration of Ni 60 DAT (April). The internal CO_2 concentration decreased in Ni-treated plants (Fig. 16.2). At 60 DAT (April), the decrease was found to be greater with the higher the concentration of metal in the irrigation water (Rai et al. 2007).

16.6.2 Chromium

Chromium enters agro-ecosystems through municipal waste-based composts and irrigation with sewage water from the chrome-plating and steel industries (Mitra and Gupta 1999). Chromium is known to cause chlorosis and leaf necrosis, and it inhibits photosynthesis. The application of 80 ppm Cr inhibits bud germination and induces chlorosis of young emerging settlings that turn necrotic at a later stage. The specific activity of catalase declines and the reducing sugar content increases in settlings supplied with Cr. Root growth is more affected than shoot growth when 80 ppm Cr is applied. There is a decrease in mitotic index and a reduction of more than 90% in metaphase and anaphase at 80 ppm Cr, indicating that Cr has a cytoxic effect (Jain et al. 2000; Rai et al. 2006). The growth-related effects on the oxidative stress response and glutathione-linked enzymes in sugarcane plants exposed to

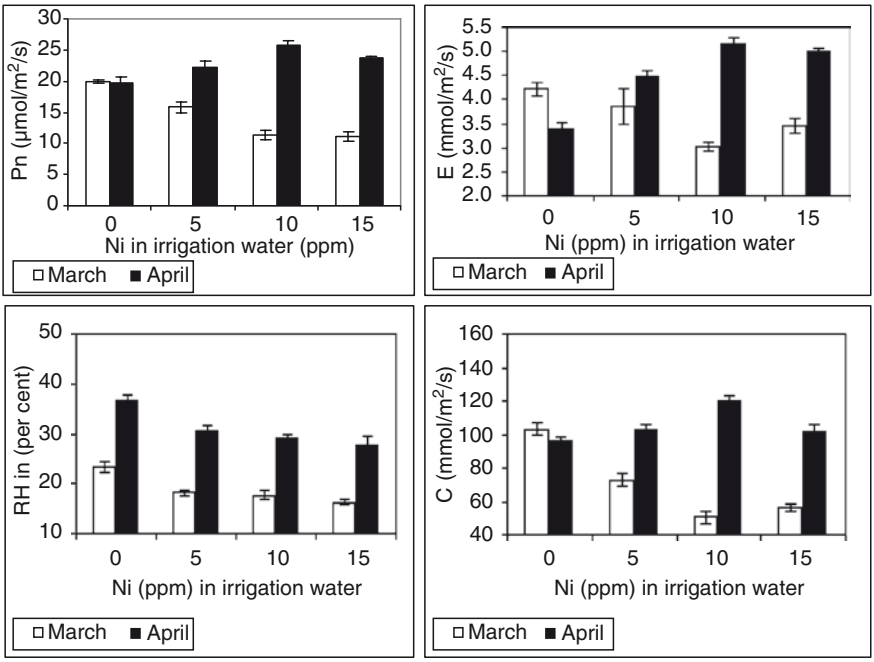

Fig. 16.2 Net photosynthetic rate (µmol/m²/s), transpiration rate (*E*), conductance (*C*), and internal relative humidity (*RH in*) in the LTM leaves of sugarcane irrigated with 0, 5, 10, and 15 ppm Ni (*bars* represent ± SEM). From Rai et al. (2006)

hexavalent chromium were investigated in sugarcane variety CoSe 92423. The inhibition of shoot and root growth due to chromium was concentration dependent. Maximum germination was obtained at 30 ppm of chromium. This was due to an increase in antioxidative enzyme activity and the operation of the glutathione–ascorbate cycle to scavenge toxic levels of H_2O_2. Despite a reduction in growth, dry matter production increased substantially as the chromium concentration was increased. Significant increases in lipid peroxidation and tissue concentrations of H_2O_2 were observed in plants exposed to 30, 60 and 90 ppm Cr. Chromium affected the glutathione reductase and ascorbate peroxidase activities in roots and leaves differently. Glutamyl cysteine synthetase activity increased for Cr levels of up to 60 ppm, but it decreased slightly at 90 ppm, although even at this level it still remained higher than that of the control. The chromium-induced suppression of sugarcane plant growth was considered to be a function of the increased cellular accumulation of chromium despite increases in the activities of antioxidative enzymes. Cr supplied through irrigation water containing 15 ppm of the metal decreased photosynthesis by 50% 30 days after treatment (DAT) during March. The Cr treated plants recorded an overall decrease in photosynthetic rate with increasing Cr concentration (Fig. 16.3). Cr treatment increased the transpiration rate at 30

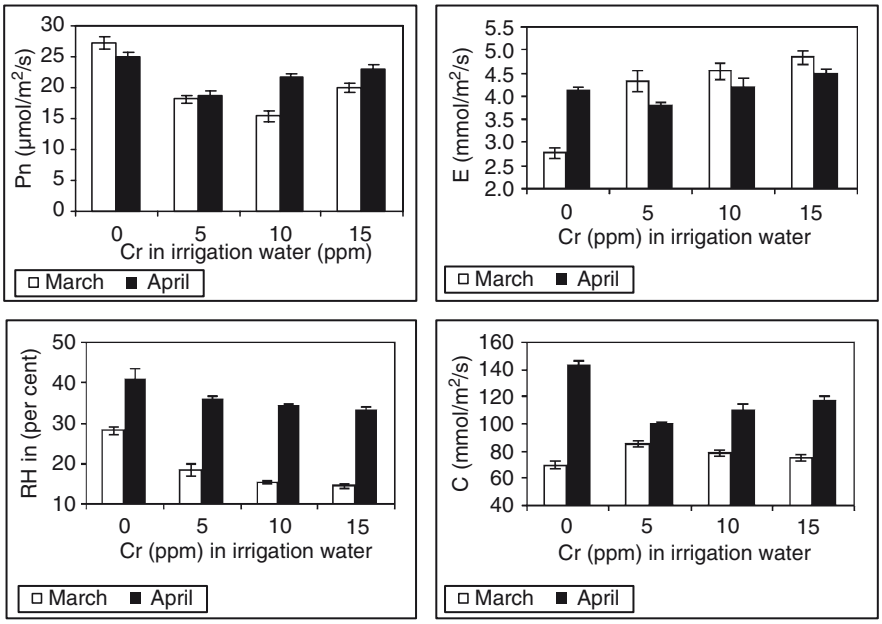

Fig. 16.3 Net photosynthetic rate (*Pn*), transpiration rate (*E*), conductance (*C*), and internal relative humidity (*RH in*) in the LTM leaves of sugarcane irrigated with 0, 5, 10, and 15 ppm Cr (*bars* represent ± SEM). From Rai et al. (2006)

DAT but decreased it at 60 DAT in April. A similar trend was recorded for stomatal conductance. The internal CO_2 concentration (Fig. 16.3) was found to be lower in Cr-treated plants (Rai et al. 2007).

16.6.3 Cadmium

Cadmium is one of the most toxic heavy metals. It enters agro-ecosystems through industrial effluents and the injudicious use of phosphatic fertilizers. Normal cadmium concentrations in plants range from 0.1 to 2.4 ppm (Alloway 1990). Cadmium can accumulate in agricultural soils through the application of soil amendments like phosphatic fertilizers and sewage sludges, which are known to contain Cd levels of 7.3–170 ppm (Lisk 1972) and <1–3410 ppm (Alloway 1990), respectively. Cadmium probably causes respiratory, photosynthetic, and structural disorders at relatively low concentrations. In the sugarcane varieties CoLk 8102 and CoJ 64, very low levels of Cd (5 and 10 ppm) decreased leaf number, leaf area, plant height, and the fresh and dry weights of different plant parts. These reductions increased as the Cd was increased to 200 ppm (Jain and Srivastava 2006). Foliar peroxidase activity was high while the catalase activity was low in

plants supplied with Cd. There were lower levels of P, Cu, and Zn in all plant parts with the exception of root P, which was high when the level of Cd supplied to the growing medium was high (100 and 200 ppm Cd). Sugarcane (*Saccharum officinarum* cv. Copersucar SP80–3280) seedlings grown in nutrient solution with varying concentrations of cadmium chloride (0.2 and 5 mM $CdCl_2$) showed lower catalase activities with increasing Cd concentration, while the glutathione reductase activity increased significantly at 2 and 5 mM $CdCl_2$ (Fornazier et al. 2002). In South China, for the purposes of agro-ecological regulation and the safe and efficient utilization of cadmium-polluted farmlands, a seven-year microplot experiment was conducted to evaluate the Cd tolerance of *Saccharum officinarum* and several other plants (Wang 2002). The study revealed that sugarcane had a strong physiological tolerance of Cd pollution. Therefore, sugarcane could be cultivated in some slightly Cd-polluted farmlands, but sugarcane bagasse was not suitable for use as a manure or stock feed due to its high Cd content, and should properly be treated as a pollutant. A pot test with paddy field soil (containing 0.26 mg Cd kg^{-1}) from Zhangzhou, Fujian, China was conducted to investigate the effects of soil Cd contents (SCCs) on the growth, sugar content (SC), sugar yield (SY) and Cd contents (CCs) in different parts of cv. Mintang 70–611 (He and Li 2002). The soil was treated with different levels of cadmium to obtain CCs of 0.53, 5.08, 50.1, 95.0, 221.0 and 0.26 mg Cd kg^{-1} soil, respectively. No negative effects of Cd on sugarcane yield, seedling emergence rate, tillering percentage, plant height, stalk diameter, weight/stalk and millable stalks/plot were found at less than 0.53 mg Cd kg^{-1} soil. However, SY and SC decreased with increasing SCC (more than 0.53 mg Cd kg^{-1} soil).

16.6.4 Aluminum

Plants growing on acid soils containing soluble Al are severely affected. In sugarcane, the toxic effect of Al is associated with higher concentrations of Al in the nodal tissue of cane stalk. A coralloid root system with very limited absorbing capacity occurs due to Al toxicity in sugarcane (Humbert 1968). Low phosphorus content in the aboveground part of the plant is often indicative of aluminum toxicity in acid soils (Bakker 1999).

16.6.5 Iron

Fe toxicity is reported as leaf freckling. Under waterlogged conditions, poor aeration can produce anaerobic conditions and acidity in the root zone. The ferrous ions (Fe^{2+}) released by the soil complex are not oxidized and poison the root system, which results in freckling of the leaves. The effects are detrimental to photosynthesis and reduce the growth rates of plants (Bakker 1999).

16.6.6 Copper

Copper is one of the most essential micronutrients. Plants require very small amounts of microelements (less than 1 ppm). Slight deficiencies in them or toxic levels of them can lead to severe yield losses or damage to standing crops. Poultry manures, sewage sludge, swine, composted refuse, fly ash, burnt tires, and copper wires often contain potentially toxic levels of metals and pose a considerable environmental risk from metals if their use is unregulated in agricultural fields (Baker 1974). The repeated use of Bordeaux sprays can cause Cu toxicity in plants (Reuther and Smith 1954). Hewitt (1953) observed that Cu consistently induces Fe chlorosis in crops. Generally, Cu toxicity has been associated with soil Cu levels of 150 and 400 ppm (Baker 1974). Copper toxicity in sugarcane was reported when the root copper content was in the range 54–375 ppm (Bakker 1999). Experiments were conducted on the influence of excess copper on the growth of and uptake of nutrients by sugarcane at IISR, Lucknow, during 2002–2003. Single bud setts of sugarcane (*Saccharum officinarum* hybrid, CoLk 8102) were planted in polyethylene pots filled with refined sand at three levels of copper (as copper sulfate): 0.065 (control), 65.0, and 130 ppm Cu (excess). The high Cu supply reduced (Table 16.21) root number and length, leaf area, leaf length, width and perimeter, plant height, and the fresh and dry weights of different plant parts (Table 16.22). The Cu concentration increases with increased Cu supply. Excess Cu caused reductions in chlorophyll a and b and carotenoid content (Table 16.23), as well as the specific activity of catalase (Table 16.24), while peroxidase activity increased with high levels of Cu in the growing medium. An excess of Cu caused significant reductions in calcium content (Table 16.25) in different plant parts. High levels of Cu depressed the uptake of Fe and Zn in leaves and shoots, and increased Mn content (Jain et al. 2008).

16.6.7 Zinc

Zinc is one of the most abundant metals in nature. The levels of Zn in plant materials are low and are generally in the range <10–100 mg kg^{-1} of dry matter.

Table 16.21 Effect of copper on the growth attributes of sugarcane (from Jain et al. 2008)

Parameter	Cu supplied (ppm)			CD at 5%
	0.065	65.0	130.0	
Root number	36.00	31.00	31.00	NS
Root length (cm)	6.50	5.43	4.58	0.49
Leaf area (cm^2)	44.50	24.91	22.86	15.5
Leaf length (cm)	33.00	26.19	24.95	7.79
Leaf width (cm)	1.40	1.16	1.25	NS
Perimeter (cm)	67.00	53.45	51.10	NS
Height (cm)	11.80	9.00	9.00	2.01

NS, not significant

Table 16.22 Effect of Cu on the root, shoot, and leaf weights of sugarcane (from Jain et al. 2008)

Parameter	Cu supplied (ppm)			CD at 5%
	0.065	65.0	130.0	
Fresh wt (g)				
Root	0.77	0.77	0.82	NS
Shoot	4.80	2.64	2.68	1.18
Leaf	4.20	1.67	1.87	0.82
Dry wt (g)				
Root	0.18	0.15	0.16	NS
Shoot	0.85	0.44	0.47	0.20
Leaf	1.00	0.45	0.44	0.15

NS, not significant

Table 16.23 Effect of Cu on the chlorophyll and carotenoid contents of sugarcane (from Jain et al. 2008)

Parameter	Cu supplied (ppm)			CD at 5%
	0.07	65.0	130.0	
Chlorophyll a (mg g^{-1} fwt)	0.83	0.60	0.49	0.04
Chlorophyll b (mg g^{-1} fwt)	0.24	0.17	0.14	0.02
Carotenoids (mg g^{-1} fwt)	0.26	0.19	0.16	0.01

fwt, fresh weight

Table 16.24 Effect of Cu on the specific activities of two enzymes in sugarcane (from Jain et al. 2008)

Enzyme	Units of specific activity	Cu supplied (ppm)			CD at 5%
		0.065	65.0	130.0	
Catalase	μmol H_2O_2 decomposed mg^{-1} protein	148	111	78	16
Peroxidase	Δ OD mg^{-1} protein	6.22	6.50	7.00	0.35

Table 16.25 Effect of Cu on calcium uptake (mg 100 mg^{-1} dry weight) by sugarcane (from Jain et al. 2008)

Plant part	Cu supplied (ppm)			CD at 5%
	0.065	65.0	130.0	
Leaf	0.57	0.49	0.37	0.04
Shoot	0.64	0.64	0.48	0.05
Root	0.33	0.10	0.07	0.04

The occurrence of Zn toxicity has been associated with Zn smelting, naturally high localized Zn concentrations, or production practices that add extremely large quantities of Zn to the soil (Staker and Cummings 1941; Lee and Page 1967). Wallace and Hewitt (1946) found Zn toxicity in several crops growing in

calcareous areas in England. Many chemicals ordinarily used in agriculture, such as chemical fertilizers, pesticides, manure, limestone, as well as urban wastes (sewage sludge, refuse, fly ash, fluidized bed material, and composted sludge or refuse) contain significant quantities of heavy metals such as Cu, Zn, Cd, Pb, Cr, and Ni (Foy et al. 1978). In sugarcane, the effects of different levels of Zn have been studied in natural soils in several areas. Reports are also available on the effect of graded doses of Zn from deficiency to excess on carbonic anhydrase activity (Chatterjee et al. 1998). An experiment was conducted at IISR, Lucknow, to study the effects of different doses of Zn (0.065, control; 65.0 and 130 ppm Zn, excess) as $ZnSO_4$ on the growth, essential nutrient availability, and biochemical attributes in sugarcane (Jain et al. 2004a). Growth depression, dark green leaves, and decreased numbers and lengths of roots resulted from higher doses of Zn (65 and 130 ppm); these effects were found to be significant at 130 ppm Zn. Plant height and leaf area decreased significantly at 65 and 130 ppm Zn. Zn concentration increased with increased Zn supply. Higher levels of Zn decreased total phosphorus content in leaf tissue. Fe and Cu contents decreased, while Mn increased in sugarcane plants.

16.6.8 Lead

Metabolic changes in sugarcane were observed after exposing it to different concentrations of lead (Rai et al. 2005). The accumulation of lead in leaf tissues increases with increasing Pb concentration in the incubation medium. The accumulation of lead increased by 14.8% after treatment with 120 ppm Pb compared with an 80 ppm treatment. The saturation of leaf tissues for further Pb uptake and accumulation was indicated by the loss of nutrients from the leaf tissues. An increase in Pb concentration above 40 ppm decreased Fe content significantly. Mn decreased more markedly at 40 ppm than at 80 and 120 ppm Pb. Lead decreased chlorophyll and total carotenoid contents in leaf lamina (Table 16.26). Lead at 40 and 120 ppm decreased chlorophyll a more markedly than chlorophyll b, as indicated by the higher Chl a/b ratios in these treatments. Pb at 40 ppm slightly increased membrane permeability, but Pb at 80 and 120 ppm decreased it by 10.8% and 18.3%, respectively. The activities of antioxidative enzymes—catalase, peroxidase, and superoxide dismutase—increased as more Pb was applied (Table 16.27).

16.6.9 Mercury

Mercury emission from the preharvest burning of sugarcane in the Florida Everglades area has been suggested as an important source of atmospheric Hg pollution. Based on a study carried out utilizing 17 soil locations for soil samples and one location for sugarcane plant samples, a total amount of 35 kg Hg was emitted

Table 16.26 Total chlorophyll, chlorophyll a, chlorophyll b, carotenoid, carotenoid/chlorophyll ratio, and chlorophyll a/b ratio in leaf laminae with increasing Pb concentrations (from Rai et al. 2005)

Treatment (Pb mg l^{-1})	Chlorophyll a (mg g^{-1} fw)	Chlorophyll b (mg g^{-1} fw)	Total chlorophyll (mg g^{-1} fw)	Carotenoids (mg g^{-1} fw)	Carotenoid/ chlorophyll ratio	Chl a/b ratio
0	2.27^{a} (±0.005)	0.61^{a} (±0.002)	2.88^{a} (±0.007)	21.61^{a} (±0.202)	7.51^{a} (±0.054)	3.73^{d} (±0.006)
40	2.25^{b} (±0.007)	0.58^{b} (±0.004)	2.83^{a} (±0.011)	20.50^{b} (±0.044)	7.25^{b} (±0.013)	3.85^{a} (±0.014)
80	2.12^{c} (±0.004)	0.57^{c} (±0.002)	2.69^{b} (±0.008)	17.43^{d} (±0.050)	7.49^{a} (±0.005)	3.73^{c} (±0.007)
120	2.06^{d} (±0.020)	0.54^{d} (±0.007)	2.60^{c} (±0.027)	17.98^{c} (±0.120)	6.93^{c} (±0.024)	3.81^{b} (±0.030)
LSD (P=0.05)	0.01	0.06	0.06	0.37	0.09	0.001
SD of mean	0.01	0.02	0.02	0.11	0.03	0.001

Data represent the mean ± S.E. of three replicates. The same letters in the same column indicate nonsignificant differences. *SD*, standard deviation

Table 16.27 Activities of antioxidative enzymes (catalase, peroxidase, and superoxide dismutase) in leaf laminae with increasing Pb concentrations (from Rai et al. 2005)

Enzyme	Pb (mg l^{-1})				LSD (P = 0.05)	SD of mean
	0	40	80	120		
Catalase (μmol H_2O_2 decomposed mg^{-1} protein)	38.32^{c} (±0.34)	38.10^{c} (±0.13)	48.84^{b} (±0.27)	52.64^{a} (±0.32)	1.18	0.34
Peroxidase (units mg^{-1} protein)	261.8^{d} (±2.38)	280.2^{c} (±1.86)	289.9^{b} (±0.77)	296.6^{a} (±0.84)	4.08	1.18
Superoxide dismutase (units mg^{-1} protein)	99.34^{c} (±11.56)	79.27^{d} (±2.55)	128.1^{b} (±2.45)	170.6^{a} (±1.09)	17.8	5.14

Data represent the mean ± S.E. of three replicates. The same letters in the same row indicate nonsignificant differences. *SD*, standard deviation

due to preharvest burning of 10% of the aboveground biomass (mostly dead leaves) of sugarcane from the entire Florida Everglades sugarcane-growing region (Patrick et al. 1994). Results indicated an average emission of 35 kg Hg from plants, including muck soils, from the entire 174,000 ha of the sugarcane-growing region in the Everglades.

16.7 Remedial Measures for Heavy Metal Pollution

Environmental pollution by toxic metals occurs through industrial, military and agricultural processes. Metal species released into the environment by various activities tend to persist indefinitely because of their nonbiodegradable nature. Once discharged into waste streams, these heavy metals are accumulated throughout the food chain, thus becoming a serious threat to the environment. Heavy metals

like Ni, Zn, Cu, Cd, Cr, and Hg are toxic, even in minute quantities. Chromium, a common pollutant, is introduced into natural waters from a variety of industrial wastewaters, including those from the textiles, leather tanning, electroplating, and metal-finishing industries. Chromium affects human physiology by accumulating in the food chain, and causes several ailments. Chromium exists in two stable oxidation states, Cr(VI) and Cr(III). The Cr(VI) state is of particular concern because of its toxicity. According to Indian standards, the permissible limit on Cr(VI) in industrial effluents to be discharged to surface water is 0.1 mg l^{-1}. Chromium-contaminated wastes are usually discharged to the environment as hexavalent chromium in the form of chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) anions that are thermodynamically stable over a wide pH range. Therefore, it is important to remove Cr(VI) from industrial effluents before discharging them into the aquatic environment or onto land.

Different metal-complexing ligands carrying synthetic and natural adsorbents have been reported in the literature for the removal of heavy metals. A new approach that gives a relatively higher adsorption capacity and utilizes modified sugarcane bagasse (a by-product of the cane sugar industry) as a natural metal adsorbent has been developed. The adsorption process parameters (pH, stirring speed, and adsorbent dose) are optimized using the Taguchi method (Garg and Sud 2005). There is almost complete adsorption (98%) of Cr(VI) by sugarcane bagasse treated with citric acid at pH 2, stirring speed 50 rpm, and adsorbent dose 2,000 mg. Thus, modified sugarcane bagasse is an effective adsorbent for the removal of Cr(VI) from aqueous solutions. The equilibrium of the solution between the liquid and solid phases is described by the Freundlich and Langmuir isotherms (Garg and Sud 2005).

16.7.1 Use of Sugarcane Bagasse Pith for Heavy Metal Removal

Another method, a batch technique (Krishnan and Anirudhan 2002), has been employed for the sorption of Pb(II), Hg(II), Cd(II), and Co(II) from aqueous solutions on sulfurized steam-activated carbon (SSAC). The SSAC is prepared from sugarcane bagasse pith by single-step steam pyrolysis in the presence of SO_2 and H_2S at 400°C. The adsorption of metal ions on SSAC depends on time, concentration, pH, and temperature. In this technique, the adsorption of Pb is 99.2%, Hg(II) 97.2%, Cd(II) 93.1%, and Co(II) 81.9% in the pH range of 4.0–8.0 compared to their initial concentrations of 100 mg l^{-1}. The order of adsorbent selectivity is: Pb(II) > Hg(II) > Cd(II) >Co(II). The maximum adsorption capacity per gram of SSAC (evaluated from fits of the Langmuir isotherm to batch adsorption data for a contact time of 4 h at 30°C) is 200 mg Pb(II), 188.68 mg Hg(II), 153.85 mg Cd(II), and 128.70 mg Co(II). The competitive adsorption capacity of the SSAC for all metal ions is lower than the capacity under noncompetitive conditions. Heavy metal adsorption from synthetic wastewaters was also studied to demonstrate its efficiency at removing metals from wastewaters containing other cations and anions. The metal ions bound to the SSAC can be stripped away by applying an acidic

solution (0.2 M HCl), meaning that the SSAC can be recycled. The surface modification of activated carbon using steam pyrolysis in the presence of SO_2 and H_2S greatly enhances metal removal and results in a product with commercial potential for wastewater treatment (Krishnan and Anirudhan 2002).

16.7.2 Sugarcane: A Phytoremediator

Sugarcane (*Saccharum* spp.) has the potential to be a phytoremediator species due to its outstanding biomass production, but its metal accumulation and tolerance have not been fully characterized. Sugarcane plantlets are able to tolerate up to 100 μM of copper in nutrient solution for 33 days with no significant reduction in fresh weight while accumulating 45 mg Cu kg^{-1} shoot dry weight. Higher levels of copper in solution (250 and 500 μM) are lethal. Sugarcane exhibits tolerance to 500 μM Cd without exhibiting any symptoms of toxicity; it accumulates 451 mg Cd kg^{-1} shoot dry weight after 33 days, indicating its potential as a Cd phytoremediator. DNA gel blot analyses yield eight fragments using a metallothionein (MT) Type I probe, ten for the MT Type II, and eight for MT Type III. The number of genes for each type of MT in sugarcane may be similar to those identified in rice, considering the inter-specific origin of sugarcane cultivars. The MT Type I gene appears to present the highest level of constitutive expression, mainly in roots, followed by MT Type II, thus corroborating the expression pattern described following large-scale expressed sequence tag sequencing. The MT Type II and III genes are more strongly expressed in shoots, where MT I is also strongly expressed. Increasing the Cu concentration has little or no effect on MT gene expression, while minor modulations of the expressions of some of the MT genes can be detected in Cd treatments. However, the level of response is too small to explain the tolerance and/or accumulation of Cd in sugarcane tissues. Thus, the tolerance and accumulation of cadmium in sugarcane may be derived from other mechanisms, although MT may be involved in oxidative responses to high levels of Cd. As a result, sugarcane should be tested as a potential candidate for Cd phytoremediation (Sereno et al. 2007).

16.8 Conclusion

In this chapter, we reviewed the sources of heavy metal contamination of sugarcane fields, focusing on the different heavy metals involved and their concentrations in different plant tissues. The high demand for cane sugar means that new areas are needed to grow sugarcane, and this demand for land can only be met by using marginal lands/landfills, thus accentuating heavy metal contamination problems. Findings to date clearly indicate the need for further research on the physiology of metal uptake and heavy metal tolerance in sugarcane plants, which will aid in the development of systems for remediating metal-contaminated soils.

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Chapter 17
Effects of Earthworms on the Availability and Removal of Heavy Metals in Soil

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17.1 Introduction

Earthworms originated in aquatic ecosystems and began to colonize terrestrial ecosystems 600 million years ago. The population dynamics and the biomasses of these invertebrates are considerably influenced by climate, soil characteristics, agricultural/industrial activities, and environmental pollution. Over the last few decades, research into earthworm life has revealed that earthworms stimulate the physical, chemical, and biological properties of soil and to contribute to soil aeration and drainage through their activities (i.e., feeding, burrowing, and casting), which also result in transformations of minerals and plant nutrients into available and accessible forms for plant and microbial uptake and thus enhance soil fertility.

Recently, another characteristic of earthworms was discovered, and is now receiving increasing attention from researchers.

Recent works have revealed that earthworms are able to direct the fates of heavy metals (availability, uptake, and accumulation) by passing and accumulating toxic metals through their body tissues, and that this distinctive feature is influenced by various factors. This chapter considers the roles of earthworms in the accumulation, availability, and uptake of heavy metals in soil. It is known that the functions and capabilities of earthworms in various soil ecosystems are closely linked to their biological characteristics and habitats, and so this chapter places special emphasis on the roles of earthworm biology (species, activities, characteristics of their diets

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and digestive systems) and surrounding environmental factors (climate, soil characteristics, human activities, and pollution) in relation to the actions of earthworms towards heavy metals.

17.2 Biology of Earthworms

Earthworm species are members of Oligochaeta, in the phylum Annelida. In particular, the Lumbricidae family includes well-known earthworms from temperate and tropical regions. So far, 20 families, 693 genera, and 3,700 species of earthworms have been identified, although there are generally considered to be around 6,000 species of earthworm (Fragoso et al. 1999; Lavelle and Spain 2001). Earthworms show a wide range of variation and their survival is usually restricted by ecological and environmental factors. On the other hand, wide earthworm diversity can be seen in small regions. For example, Turkey (area: 780,000 km^2) hosts only 66 different species of soil earthworms (Omedo and Rota 1991), and among these, *Dendrobaena veneta* predominates (Misirlioglu 2008). However, a small island country located in the eastern part of the Mediterranean Sea, Cyprus (area: 9,250 km^2), hosts 15 species of Lumbricidae that are also distributed across Turkey, Europe, the Mediterranean and the Middle East, and the Caucasus, and also one species from the Acanthodrilidae family (Pavlíček and Csuzdi 2006).

Earthworms are terrestrial, aquatic or subaquatic animals that are capable of exchanging gases in their cuticles and extracting water from their surrounding environment, and so they therefore prefer to live in humid conditions. This is the way in which they obtain water for their digestion processes (Barois and Lavelle 1986; Daniel and Anderson 1992; Trigo and Lavelle 1993), and moisture content is the main factor that limits and influences earthworm activity and distribution. In dry seasons, earthworms can survive by entering a dormant state. To avoid the heat, most earthworms evacuate their digestive systems and curl up into a ball until moisture levels become high enough for them to continue their vital activities. Species like *Polypheretima elongata* move away from the dry soil layer, burrowing to a depth of 1–2 m. Some earthworm species that live in tropical rainforests are able to store water in their body and are thus less influenced by the arrival of dry seasons (Bouché 1977).

17.2.1 Ecological Considerations

Terrestrial earthworms inhabit either the litter layer of the soil or sublayers of it, and the morphological, behavioral, and physiological characteristics of earthworms are be adapted to their surrounding environment. In general, earthworms have developed adaptation strategies for three ecological environments – epigeic, endogeic, and anecic – which are therefore also used to classify earthworms (Bouché 1977; Lavelle 1981; Lee 1985; Edwards and Bohlen 1996; Lavelle and Spain 2001). While epigeic species live above mineral soil layers, near to the soil surface, and

feed on the organic compounds of this zone, endogeic species live on organic compounds found in mineral soil layers, and inhabit the top 0–20 cm of the soil. Similar to the epigeic and endogeic species, anecic earthworm species (vertical burrowers) feed on the organic compounds in the surface layer, but they also create deep vertical galleries that may reach down three meters into the soil (Ismail 1997; Sharma et al. 2005), and they spend most of their time in these burrows. Anecic species are big worms that are darkly colored, with strong burrowing systems, long lifetimes, slow growth rates, and a low mortality. Anecic and endogeic species prefer either agricultural sites or grasslands. On the other hand, epigeic species are small, extensively inhabit forest sites, grow quickly, and have higher prolificacies and mortality rates. They may have green or red pigments, depending on their ecological environment (Lavelle and Spain 2001).

Endogeic worms (mineral dwellers) are unpigmented species that feed on organic compounds such as mineralizing plant debris, and are thus found in rhizospheric soil a few centimeters down. Various earthworm species found in Europe are outlined in Table 17.1. Epigeic species are compost producers and have no influence on soil structure. However, anecic species contribute soil structural development by facilitating the mixing of plant debris with mineral fractions and by burrowing intensively through the top few centimeters of soil. Endogeic species do not construct galleries in the soil, but similar to anecic earthworms, they excrete products on the surface, promote organic matter stabilization, and thus promote soil aggregation (Lavelle and Spain 2001).

17.2.2 Factors Effecting Earthworm Distribution and Activity in the Soil

The distribution of earthworms in soil is largely controlled by climatic factors (i.e., rain and temperature) and the plant species and populations that are growing in the soil. Moreover, earthworm distributions and activities are also affected by soil physicochemical features (i.e., texture, pH, organic matter) and agricultural activities (tillage and fertilization, etc.). Earthworms are dominant members of the soil fauna

Table 17.1 Ecological groups of relevant Central European earthworm species (from Dunger 1983)

Epigeics	Anecics	Endogeics
Lumbricus rubellus	*Lumbricus terrestris*	*Aporrectodea caliginosa*
Lumbricus castaneus	*Aporrectodea longa*	*Aporrectodea rosea*
Lumbricus festivus	*Lumbricus polyphemus*	*Allolobophora chlorotica*
Dendrodrilus rubidus	*Dendrobaena platyura*	*Aporrectodea icterica*
Dendrobaena octaedra		*Proctodrilus oculata*
Dendrobaena illyrica		*Octolasion lacteum*
Dendrobaena attemsi		*Octolasion cyaneum*
Eisenia fetida		*Octolasion tyrtaeum*

except during dry and frosty seasons. The soil can contain 100–500 individual worms or 30–100 g earthworm biomass per square meter on average. These numbers depend on plant vegetation and climate. For example, there can be up to 2,000 earthworms per square meter of soil in the temperate regions of Australia and the temperate pastures of New Zealand (Lee 1985). Moreover, grasslands in tropical regions can have 200–400 g earthworm biomass per square meter of soil (Cotton and Curry 1980). In general, soils in cold regions contain only a few earthworms, whereas soils in temperate regions show higher numbers and biomasses of earthworms and those in tropical regions usually exhibit the highest numbers but lowest biomasses of earthworms per square meter of soil. Earthworm species change according to latitude due to gradual alterations in their nutritional habits. Generally, epigeic species live at high latitudes, while anecic species inhabit temperate latitudes and endogeic species intensively colonize tropical zones (Lavelle and Spain 2001).

On a local scale, when forests and grasslands with similar climates and soil characteristics were compared, it was found that grassland soils had much more earthworm biomass than forest soils. This may be a result of the high colonization capacity of peregrine species that propagate parthenogenetically. On the other hand, rainforest soils of tropical regions have large earthworm populations due to their highly nutritional soils (Fragoso and Lavelle 1992). Similarly, it has been reported that forest soils in Europe have 400–680 kg ha^{-1} worm biomass, while grasslands in the same zone contain 500–2000 kg ha^{-1} (Lee 1985; Edwards and Bohlen 1996). However, forest soils with lower earthworm biomasses have been observed to have higher species diversity (Fragoso and Lavelle 1992). Climatic factors (i.e., temperature and precipitation) are the main influences on soil temperature and moisture, and these soil characteristics are the basic factors that control earthworm populations in the soil.

Soil moisture is the basic limiting factor on earthworm activity. Since they evolved in an aquatic environment, earthworms exhibit cutaneous respiration and so their populations and distributions in soils are mainly affected by the soil water regime. Earthworms can absorb water through their outer skins, but extreme soil desiccation in summer forces them to retreat deep into the subsoil and remain there in a dormant state until more favorable environmental conditions return to the upper soil layers. The optimal water conditions for earthworm activity depend on the species (Lee 1985; Butt 1991, 1993). If there are sufficient levels of dissolved oxygen in the water, some earthworms can even survive in water-saturated soils. However, in many cases, extremely waterlogged soils prompt them to ascend to the surface due to a lack of ventilation or the toxic compounds produced by anaerobic bacteria in saturated conditions; otherwise they can die. Many earthworms show a low tolerance of high or low temperatures (i.e., freezing temperatures). Most earthworms die in soil that remains at 85–90°F for a sustained period. Similar to their behavior under water stress, earthworms move downward through the subsoil when exposed to high soil temperatures. Ideal soil temperatures for earthworm growth and activities are 60–80°F. To some degree, earthworms live in an environment with low oxygen and high carbon dioxide concentrations.

The physicochemical characteristics of the soil are largely influenced by climatic factors (i.e., precipitation and temperature). For example, in regions with heavy rainfall, basic cations such as Ca^{2+}, Mg^{2+}, and Na^{+} are leached through the soil profile

and replaced with H^+, resulting in soil acidification. Earthworm populations and activities in acidic soils are usually lower than those in neutral or mildly alkaline soils, mainly due to this acidification and reduction in nutrients due to leaching under high precipitation. Earthworms also have fairly low population dynamics and physiological activities in alkaline and very alkaline soils. This arises from the low levels of organic matter, precipitation, and the high soil pH associated limestone soils. It was observed that earthworms were not able to survive in some West African soils with high temperatures and low rainfall (less than 800 mm annually). Their biomasses increased with increasing rainfall, and except for acid-resistant species, they declined in acidic soils of tropical rainforests that experienced over 3,000 mm annual rainfall in Africa and America (Lavelle and Spain 2001). On the other hand, earthworm biomass was found to reach 1,200 kg ha^{-1} in monsoon rainforests with 5,000 mm annual rainfall in India, which is probably related to the high pH and high levels of nutrition for earthworms in these young soils. In general, earthworms prefer neutral soil conditions, can tolerate a wide range of soil pH values – between 4.2 and 8 – but are not able to grow well below pH 5 (Edwards and Lofty 1977). Nevertheless, the application of lime in order to increase the soil pH in acidic soils has been noted to significantly increase earthworm biomass (Chan 2003).

Another soil preference of earthworms is a loamy texture. Sandy soils can easily dry out and the sand can physically damage earthworm skin, so earthworm activity may be adversely affected in coarse-textured soils (Ghilarov 1979). Some investigations have revealed that clayey soils contained fewer earthworms than medium-textured soils. The reason for this is still not clear.

Earthworms feed on organic residues in the soil, and so quality and quantity of soil organic matter are also important influences on soil earthworm populations. Organic residues with a low C:N ratio are more acceptable than those with high C:N ratios. Earthworms preferentially use animal residues which ease the consumption of plant residues. In contrast to endogeic species, anecic species prefer organic particles of a smaller size and transport them in the soil. However, epigeic species feed on the organic residues on the soil surface. Thus, the type of surface organic matter also affects the earthworm population (Ghilarov 1979). For example, conifer litter is not desirable for most species (Bernier and Ponge 1994). Instead of fresh and robust plant litter, earthworms favor plant residues that have been partially decomposed by soil microflora (Neuhauser et al. 1978). Agricultural practices also affect earthworm populations and activities in the short and long term. For example, it was noticed that soil tillage, fertilization, and direct pesticide application to the soil reduced the earthworm population (House and Parmelee 1985; Clapperton et al. 1997; Edwards and Bohlen 1992). Moreover, the removal of plant debris from agricultural soils has been found to restrict the soil nutrient pool and hence to reduce earthworm populations and activities (Haines and Uren 1990; House and Parmelee 1985; Lee 1985, MacKay and Kladivko 1985; Parmelee et al. 1990). Plant residues remaining on the soil surface serve as a nutrient source for earthworm populations and their activities. Manure application and crop rotation with legumes also stimulate earthworm populations. Unlike other plants, legume debris has a low C:N ratio and are thus attractive sources of food for earthworms. In contrast, the application of fluid manure can have a negative effect on earthworms

due to its high levels of salts and ammonia (Paoletti 1985). Other agricultural applications, such as liming and conventional chemical fertilization, can also increase the earthworm population. This is actually an indirect effect of changes in soil conditions and increasing nutrient flow on earthworms. Organic fertilization practices have been found to significantly enhance the earthworm population and earthworm biomass (Curry 1994).

17.2.3 Digestion System of the Earthworm

The digestion of organic compounds in the earthworm's body depends on a facultative symbiotic interaction between the earthworm and microorganisms. While feeding, earthworms absorb microorganisms that grow on plant litter. These microorganisms proliferate in the intestine of the earthworm and produce microbial metabolites (Barois and Lavelle 1986). Soil absorbed by the earthworm is mixed with these metabolites and other excretions (i.e., simple sugars, amino acids, and low molecular weight compounds) from the intestinal system. This material, which is rich in different organic compounds and microflora, is then discharged into the soil. The intestinal microflora of the earthworm also synthesize various enzymes (Lavelle and Spain 2001). Due to the microflora in the intestinal system, earthworm excrement usually has higher enzymatic activity than that of soil. However, this situation depends on the ecological category of the earthworm. For example, it is not yet clear whether epigeic earthworm species synthesize enzymes. However, some epigeic species have been found to include cellulose in their intestines and to digest organic compounds without requiring the microfloral symbiosis (Parle 1963; Neuhauser et al. 1978). Additionally, various members of this category are capable of using and digesting microflora just as they use other organic compounds in the soil. For example, *Lumbricus rubellus* digests microorganisms that colonize clay and polysaccharide particles (Kristufek et al. 1994). Anecic and endogeic species have been shown to increase enzyme activity through their symbioses with microflora (Nielsen and Hole 1964; Wright 1972; Hamilton and Sillman 1989; Cortez et al. 1989). However, the enzyme activities of the excrement from epigeic, endogeic, and anecic species have been found to be higher than those of soil and plant litter (Kizilkaya and Hepčan 2004, 2007; Kizilkaya 2008). The efficacy of the earthworm digestion system is also linked to the temperature. The optimal temperature for earthworm intestinal microflora is approximately 20°C, but these microorganisms are more active in the soil environment at 27°C.

17.2.4 Earthworm Excrement

An earthworm's excrement, also called its cast or casting, can be around 60% of its body weight or even several times higher than that value, depending on the species. The excrement is similar to the material initially taken in by the earthworm,

although this material undergoes a mechanical grinding step and is then digested by the microorganisms that live in the earthworm's intestinal system. This two-step digestion process means that the cast is easy to decompose and it modifies the chemical and biological properties of the original material. For example, the levels of organic C and some other nutrients in earthworm casts have been found to be higher than the original material taken in by the earthworm (Kizilkaya and Hepşen 2004, 2007). Due to the basic cations (such as Ca^{2+}, Mg^{2+}, K^{+}) in their casts, earthworms can change the acidity of the soil through their digestive discharges (Materechera 2000). Earthworm excrement has a higher enzymatic activity and a larger microbial biomass than the surrounding soil (Kizilkaya 2008). Moreover, it has a lower C:N ratio and more nutrients, and thus can be used as an alternative fertilizer or nutrient source in agriculture.

17.2.5 Effects of Earthworms on the Soil

In general, the effects of earthworms on soil properties are related to their populations and activities, such as feeding, casting, and burrowing. The quality and quantity of their casts are important influences on soil characteristics. The main features of the soil that are influenced by earthworm activities are its physical (soil aggregation and infiltration), chemical (pH, organic matter, and available nutrient content), and biological (microbial populations and enzymatic activity) characteristics. Earthworm activities and a high earthworm population density can greatly increase soil aggregate stability (Marinissen and Hillenaar 1996; Ketterings et al. 1997). The chemical composition of earthworm excrement, its freshness and its dryness play important roles in aggregate stability. Fresh casts are usually present in saturated form due to their high water content (Edwards 1983).

In soil aggregation processes supported by dry excrement, fungal hyphae and their micelles play an important role, whereas microbial polysaccharides and dead plant materials are active in fresh excrement-based aggregation. Earthworm excrement usually accumulates in the upper soil layers (0–20 cm), and so water-resistant soil aggregates are concentrated the near soil surface. The main factor in soil aggregation is the integration between earthworm excrement and soil. This integration occurs via microbial proliferation around the excrement, which results in an increase in the concentrations of carbohydrates that stick soil particles together. For this reason there are more aggregates around earthworm burrows (Haynes and Fraser 1998). Earthworms build their burrows in different depths of soil depending on the species. The diameters of these burrows and galleries range between 1–10 mm and generally reflect the body type of the earthworm (Lee 1985; Tamlin et al. 1995). Earthworm activities such as soil absorption, gallery building, and casting give rise to a macroporous structure around the burrows which allows air and water to penetrate deep into the soil (Ehlers 1975; Willoughby and Kladivko 2002).

Earthworms also affect soil structure by not only crumbling or building soil aggregates but also carrying mineral particles various distances through the soil during

their feeding and casting activities. Especially in rainy regions with high clay leaching rates, earthworms absorb clay and clay-sized soil components rather than sand and leave them as excrement near the soil surface.

Earthworms mix organic matter into the underlying mineral soil, and their excrement is remarkably rich in nutrients and easily decomposable organic compounds. Therefore, soils with earthworm burrows, galleries, and casts exhibit high microbial activity (Lee and Foster 1991) and biomass (Haynes and Fraser 1998). The influence of earthworm activities on microbial community structure is dependent on the species (Parle 1963; Daniel and Anderson 1992; Devliegher and Verstraete 1996). In addition to soil organic matter, soil microorganisms are critical to earthworm existence in the soil. Thus, their numbers and biomass may temporarily decline during the digestion process in the earthworm intestinal system, but they reach previous levels and even increase after casting (Zhang et al. 2000).

Earthworms feed by absorbing either soil particles with low nutritive value or organic matter with higher nutritive quality (MacKay and Kladivko 1985; Parmelee et al. 1990). Hence, the soil organic carbon status and carbon mineralization rate can change considerably (Hendrix et al. 1987). Earthworm activities increase the organic carbon pool in soils with medium or insufficient organic matter, whereas they enhance the content of easily mineralizable organic compounds in organic matter rich soils (Scheu and Parkinson 1994). The C:N ratio of an earthworm cast is much more lower than that of the surrounding soil, which leads to the rapid mineralization of organic compounds in casts.

Soil pH may also change due to earthworm activities. The effect on pH is mainly due to earthworm casts and their compositions. The cast may be more alkaline than the soil due to the presence of basic cations (Ca^{2+}, Mg^{2+}, K^{+}) in high amounts in the cast. Many laboratory studies have revealed that mineral N, available P, and exchangeable K, Ca, and Mg increase due to earthworm activities (Mackay et al. 1983; Lopez-Hernandez et al. 1993; Parkin and Berry 1994; Zhang et al. 2000).

More recently, it has been found that the functions of earthworms in soil ecosystems are not limited to governing the mobilities of fundamental nutrients. They are also capable of modifying the fates of various elements, such as Zn, Cu, Cd, and Pb, which are commonly known as heavy metals and can cause great damage to the whole ecosystem.

17.3 The Relationship Between Heavy Metals and Earthworms

Over the last few decades, various human agricultural activities (i.e., intensive use of chemical fertilizers and sewage sludges; Karaca et al. 2002; Kizilkaya and Bayrakli 2005) as well as industrialization (Cemek and Kizilkaya 2006) have resulted in increased levels of heavy metals in many soils, although heavy metals can also occur lithogenically in unpolluted soils (Ozdemir et al. 2007; Tarakcioglu et al. 2006). In most cases, these heavy metals are natural components of the Earth's crust (Ozdemir et al. 2007; Tarakcioglu et al. 2006). Whether they have anthropogenic or

lithogenic origins, heavy metals can accumulate in the food chain and can be accumulated by soil organisms, thus affecting their biological and biochemical activities, which leads to many environmental problems (Kizilkaya and Askin 2002; Kizilkaya et al. 2004). Since they are a major representative of soil life, earthworms are also negatively influenced by heavy metals, but in contrast to most other members of the soil fauna and flora, they can also influence heavy metal concentrations and their availability in the soil (Kizilkaya 2004, 2005). The relationship between heavy metals and earthworms involves two main processes: (1) the accumulation of metals by earthworms (the uptake of heavy metals included in organic compounds consumed by earthworms), and (2) the effects of earthworm activities on metal availability (changes in the fates of heavy metals due to the impact of the activities of earthworms on the physicochemical and biological characteristics of soil).

17.3.1 Heavy Metal Accumulation by Earthworms

Heavy metals that enter the nutritional pathway of earthworms can be delivered to the soil within earthworm excrement or can accumulate in earthworm tissues by binding to ligands. Most earthworms actually show evidence of both of these mechanisms simultaneously: some metal concentrates in the earthworm's body while the rest is discharged within defecated material (Tessier et al. 1994). Earthworm excrement can contain high concentrations of metals while the metal levels in the body of the earthworm may be lower. In such cases, the next generation of earthworms tend to move to less or uncontaminated areas.

Heavy metal accumulation by and transfer to earthworms can vary depending on the actual metal involved. For example, Zn and Cd, which function as nutrients at low concentrations, differ in terms of rates of accumulation in or defecation by earthworms compared to Cd and Pb. Another important factor that affects metal transfer through earthworm activity is excretion rate.

In general, earthworms living in Cu- and Zn-contaminated areas have higher excretion rates than those in Cd- and Pb-contaminated soils. In the presence of Cu, Zn, Pb, and Cd as their nitrate salts, Spurgeon and Hopkin (1999) noted that *Eisenia fetida* exhibited higher excretion rates and higher metal contents in Cu- and Zn-contaminated soil, while its excretion rates and metal concentrations were lower in Pb- and Cd-contaminated soils. This is due to the physiological metal control mechanisms of earthworms and is also because Cu and Zn are essential metals for proper organism functioning (Morgan and Morgan 1991; Hopkin 1995). On the other hand, Cd and Pb bind earthworm tissues through organic ligands that form complexes with metals. Heavy metals are mainly stored in chloragogenous tissue, which is a sheath of modified peritoneal cells that line the outer wall of the gut (Fischer and Molnar 1993).

X-ray microanalytical studies have shown that chloragogenous tissue contains two types of granules, called chloragosomes. The first type of granule consists of phosphate-rich complexes containing calcium and to a lesser extent zinc Morgan and

Morgan 1988) that bind to metals such as Pb by a process involving exchange with matrix-associated calcium (Morgan and Morgan 1988; Morgan et al. 1993). The second type of granule contains sulfur-donating ligands in the form of residues of metallothionein-like proteins (Suzuki et al. 1980; Morgan et al. 1989). Stürzenbaum et al. (1996) noted that metals like Cd bind to these proteins.

The amount of heavy metals accumulated in earthworm tissues is related to the metal content of the organic materials consumed by earthworms. In other words, high metal accumulation in earthworm tissues arises through a high metal concentration in the soil (Crommentuijn et al. 1997). This relationship suggests that earthworms can be used as indicators of the degree or the effect of metal pollution in soil (Wang et al. 1998; Paoletti 1999). Indeed, earthworms inhabiting metal-rich soils of industrial areas have been shown to contain more metals (Sterckeman et al. 2000; Kenette et al. 2002). Anecic and endogeic earthworm species that feed on both dead organic materials and soil accumulate dissolved metals that are found in soil pores rather than those that are insoluble in the soil's mineral structure (Kiewiet and Ma 1991).

Earthworms living in arid soils with low levels of organic matter accumulate metals from soil pores and the forms of metals that are extractable with a selected chemical agent such as DTPA, rather than all of the forms of metal in the soil (Dai et al. 2004). In addition to the heavy metal content of the soil, the capacity of earthworms to accumulate metals, and their toxicities to the earthworms, depend on the type of heavy metal (Kizilkaya 2004–2005) and the ecological category and species of earthworm (Morgan and Morgan 1992). Although endogeics (*Aporrectodea caliginora, Allolobophora chlorotica*) accumulate more Cd than epigeic (*Lumbricus rubellus)* and anecic (*Lumbricus terrestris, Allolobophora longa*) species, anecics have been found to store more Zn than endogeics and epigeics do (Morgan and Morgan 1992). Also, *L. rubellus* was found to be more tolerant of Cu pollution than *Aporrectodea caliginosa* (Paoletti et al. 1998; Ma 2005). Very high metal concentrations in the soil can have a toxic effect on earthworms. The metal toxicity also depends on the type of metal. In an experiment testing the toxicities of various heavy metals (Cu, Zn, Pb, Ni, and Cd) toward *Eisenia fetida*, Neuhauser et al. (1985) showed that Pb was least toxic.

Soil pH is one of the most important factors that control metal accumulation and toxicity in earthworms (Morgan 1985; Morgan and Morgan 1988; Ma 2004, 2005). Previous works have revealed that earthworms can accumulate more heavy metals and that these metals exert their toxic effects more rapidly on earthworms under acidic soil conditions. Metal accumulation by *L. rubellus* was shown to be higher in both in situ and in vitro studies of polluted soils with low pH values (Ma 1982, 1987; Ma et al. 1983). Similarly, *Aporrectodea tuberculata* (Beyer et al. 1987) and *Aporrectodea caliginosa* (Perämäki et al. 1992) have been found to accumulate more Cd in acidic soils. This is largely related to the fact that most of the heavy metal that accumulates in an earthworm's body originates from pools of dissolved metals in soil pores, and these are influenced by the soil pH. As the soil pH declines, the metal concentrations in the soil solution increase (Herms and Brümner 1984), and this eventually leads to increased metal accumulation by earthworms.

Liming agricultural soils may provide better conditions for earthworms (Bengtsson et al. 1986), and it also reduces metal accumulation by earthworms.

Lumbricus rubellus and *Aporrectodea tuberculata* exhibit lower metal accumulations as the level of soil organic matter increases (Ma 1982; Beyer et al. 1987), indicating that there is also a relationship between these two parameters. Many earthworm species prefer high-quality food sources in soil organic matter (i.e., leaf litter) rather than mineral soil. Therefore, metal accumulation can be influenced by the specific preferences of the earthworms with respect to organic materials and their types (Lock and Janssen 2001). For example, metal accumulation in earthworm tissues has been found to increase when organic materials such as sewage sludges containing large amounts of metals are applied to the soil (Kizilkaya 2004, 2005).

Heavy metals may have toxic effects on earthworms in soils with high salinity. This is actually due to the high salt content rather than the metal concentration. Laboratory experiments have shown that the earthworm mortality rate decreased when the saline conditions were removed by washing the soil (Chang et al. 1997). Soil moisture content and temperature are also among the factors that affect earthworm metal accumulation (Marinussen and van der Zee 1997). The highest levels of metal accumulation in earthworms occur when the moisture and temperature are such that earthworm activities are maximized.

17.3.2 Effects of Earthworms on Heavy Metal Availability in the Soil

The heavy metal content of the soil is known to be mainly dependent on the structure of the soil's parent material and its physicochemical characteristics, and it can increase due to pollution resulting from agricultural practices and industrial activities. Although the total metal concentration (amount of metal that can be extracted with strong acid) is commonly evaluated in order to test for metal pollution of the soil, this may not reflect some important aspects, such as the mobility and biological availability of the metal. The heavy metal pool in the soil actually comprises several physicochemical forms, such as easily exchangeable, weakly adsorbed, organically bound, carbonate-bound, Fe-Mn-oxide-bound, and residual metals. Fluctuations in the levels of these fractions reflect the quantitative distribution of metals in the whole metal pool, and can provide more detailed information relating to major environmental concerns, such as the origin, mode of occurrence, bioavailability, and potential mobility of the metal, as well as the impact of agricultural or industrial activity (Tessier et al. 1979; Shuman 1985; Ure et al. 1993). Many sequential extraction procedures based on the elution of metals from a sample of soil using a series of solutions have been suggested (Tessier et al. 1979; Lake et al. 1984; Pickering 1986; Beckett 1988; Ure et al. 1993), and these are commonly used in studies evaluating how common different soil–metal associations are in the soil (Kim and Fergusson 1991; Howard and Vandenbrink 1999).

As determined by sequential extraction procedures, dissolved and exchangeable metals as well as organically bound metals are the most labile metal forms, and so they are used to evaluate the mobility and biological availability of the metals, while the adsorbed, carbonate-bound, hydrous oxide-bound, and residual metal fractions are stable and constitute nonlabile metals (Zhang and Shan 1998; Wang et al. 2002).

The main factors that control the distributions of different metal species are soil characteristics such as its texture, pH and organic matter content (Leštan et al. 2003), the activities of soil organisms (Kizilkaya 2004, 2005), and – especially – agricultural practices (i.e., organic waste application and liming), which affect soil chemical processes (Shuman 1999) and thus change the flow of metals between different soil constituents. Earthworms are important members of the soil fauna and can affect soil physicochemical characteristics through their excreta and activities (Pallant and Hilster 1996; Ponder et al. 2000), which may also cause changes in soil metal fractions (Devliegher and Verstraete 1996; Wen et al. 2006).

Soil pH has a major influence on the heavy metal adsorption–desorption behavior and hence determines the biological availability of the metal in question (Cao et al. 2001; Li et al. 2001). Its effect on metal solubility and uptake depends on the metal species, but metal mobility and availability are generally higher in acidic soil conditions. For example, cadmium becomes mobile below pH 6.5, while Pb becomes active under more acidic conditions, at pH 4. Similarly, the activation thresholds for the solubility and uptake of other metals (i.e., Zn, Ni, Cu, As, and Cr) occur between pH 4.5 and 6 (Herms and Brümner 1984). Earthworm activities can increase soil pH, causing a reduction in metal solubility. This can be attributed to the characteristics of earthworm excreta. For example, an increase in nitrogen-associated excrement such as alkaline urine can increase soil pH (Parkin and Berry 1999; Salmon 2001). The calciferous glands of earthworms can also help to increase the soil pH (Lee 1985). Since most organisms take up water-soluble metal forms directly, the concentrations of soluble metals decrease as a result of earthworm activity. This decrease in the soluble metal concentration causes an increase in the pH-dependent surface-charge density, resulting in metal binding to colloids and lower concentrations of metals in the soil solution (Cao et al. 2001; Shan et al. 2002). Wen et al. (2004) previously reported that the activity of *E. fetida* resulted in increases in the water-soluble, exchangeable, and carbonate-bound fractions of Zn, Cr, Co, Cd, Cu, Ni, and Pb. On the other hand, Udovic and Lestan (2007) recently showed that earthworm excreta contained less exchangeable and water-soluble metal than the soil they feed on (Table 17.2)**.**

This contradiction can be explained by methodological differences between the analytical procedures used to measure the metal fractions, rather than the fact that the higher pH of earthworm excreta decreases metal solubility and uptake. For example, Wen et al. (2004) evaluated different metal fractions using a single extraction procedure, while other authors (Ma and Rao 1997; Kabala and Singh 2001; Leštan et al. 2003) have measured each fraction in separate extraction steps. Therefore, it is likely that the increase in carbonate-bound metals dominates over the soluble and exchangeable metal fractions.

Table 17.2 Distibutions of various Zn and Pb fractions found in the soil and the excreta of *Lumbricus rubellus* and *Eisenia fetida* (from Udovic and Lestan 2007)

	Pb			Zn		
Fractions	Soil	Cast of *L. rubellus*	Cast of *E. fetida*	Soil	Cast of *L. rubellus*	Cast of *E. fetida*
Soluble	0.02	0.04	0.23	0.01	0.01	0.01
Exchangeable	0.13	0.11	0.04	0.35	0.20	0.21
Carbonate-bound	21.8	20.4	22.5	5.08	5.30	5.78
Fe-Mn-oxide-bound	0.43	0.47	0.45	2.25	2.31	2.77
Organically bound	62.6	71.4	65.5	12.5	15.3	15.6
Residual	14.9	13.8	11.3	79.8	76.9	75.6

Dissolved organic carbon (DOC) in the soil solution may considerably affect metal adsorption and uptake through two possible mechanisms: (1) the adsorption of organic anions, increasing the negatively charged colloidal surface area, thus causing an increase in metal adsorption (Parfitt and Russell 1977; Barrow 1985), and (2) competition between the colloidal surfaces and DOC, which decreases metal adsorption to soil. Zhu and Alva (1993) showed that there is a positive relationship between the dissolved organic carbon in the soil solution and Zn solubility and uptake based on an increase in Zn chelation. Similarly, Dudley et al. (1986) noted that increasing the DOC in the soil solution also increased Cu uptake. DOC can form complexes with various metals, and these complexes are more soluble and readily taken up than free metal ions (Norvell 1972; Prasad et al. 1976). However, this enhancing effect of DOC on the uptake of metals can be restricted by adsorption to soil minerals. This is especially the case in mineral soils with low DOC (McCarthy et al. 1993). This is generally due to the strong adsorption to colloidal surfaces and a lack of DOC in the soil solution (Moore 1989; Dalva and Moore 1992). Organic matter application, clay content, and adsorbed SO_4^{2-} can also affect the adsorption of DOC and metal uptake (Moore et al. 1992; Donald et al. 1993). Natural or stimulated earthworm activity in the soil can increase DOC and hence increase the organically bound metal pool (Martin 1991).

Increases in organically bound metal species due to earthworm activity are mainly related to the high organic matter and carbon contents of earthworm excreta (Kizilkaya 2004, 2005). Many works have revealed that the excreta of earthworms in soil with or without organic matter amendment contain more organic carbon than the soil that the earthworms feed on (Martin 1991; Buck et al. 1999; Kizilkaya 2008). Furthermore, the soil surrounding an earthworm burrow contains more DOC than other soil compartments (Parkin and Berry 1999). This implies that the levels of easily decomposable organically bound metal species that readily taken up by organisms may significantly increase due to earthworm activity.

Cheng and Wong (2002) noted that organically bound metals in earthworm excreta can be released into the soil solution and thus become available for plant uptake through the rapid microbial decomposition of the organic compounds in earthworm excreta.

One of the biological uptake mechanisms for metals bound to various organic constituents in earthworm excreta is metal chelation. Chelating agents increase the

solubility of metals and facilitate metal uptake by plants and soil organisms (Blaylock et al. 1997). Soil microorganisms also play an important role in heavy metal solubility and uptake. The metallophores formed by soil microorganisms chelate metals, influencing their solubility and uptake (Whiting et al. 2001).

Common members of the soil microbiota, *Pseudomonas* and *Enterobacter*, are capable of synthesizing metallophores (Neilands and Leong 1986). Since soil microbial populations and activities increase with earthworm activity (Binet et al. 1998; Toyota and Kimura 2000; Tiunov and Scheu 2000; Kizilkaya and Hepşen 2004, 2007; Kizilkaya 2008), metallophore synthesis by microorganisms also increases, and this eventually enhances metal solubility and their uptake from the soil. In their experiments, Wen et al. (2004, 2006) investigated the effects of earthworm (Eisenia fetida) activity on a variety of soil characteristics including microbial enumeration (bacteria, actinomycetes, and fungi), and fraction distribution and bioavailability of heavy metals in earthworm inoculated Chinese soils and stated that earthworm-inoculation to soil increase the numbers of different microbial populations and mobility and bioavailability of heavy metals in soils.

17.4 Conclusion

Earthworms ensure sustainable soil fertility by improving the physical, chemical, and biological characteristics of the soil, and they play a fundamental role in the mechanisms that increase heavy metal solubility and facilitate their uptake by biological systems. This is an advantage with respect to plant production in unpolluted soils.

On the other hand, earthworm activity can lead to enhanced metal mobility and metal transfer to plants in metal-polluted soils. However, in comparison to other members of the soil fauna, earthworms are more resistant to metal toxicity; they are capable of accumulating heavy metals in their body tissues and increasing metal uptake. Therefore, their use may provide many benefits prior to initiating phytoremediation.

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Chapter 18
Phytoremediation of Heavy Metal Contaminated Soils

T.J. Purakayastha and P.K. Chhonkar

18.1 Introduction

The disposal of industrial effluents has become a serious problem due to rapid industrial development and urbanization. The application of industrial and city effluents to land has also become popular in recent years as an alternative means of treatment and disposal (Chhonkar et al. 2000a,b). Even they are useful sources of plant nutrients, these effluents often contain high amounts of various organic and inorganic materials as well as heavy metals, depending upon the industry from which they originate. The unscientific disposal of untreated or undertreated effluents has resulted in an accumulation of heavy metals in land and water bodies. Cultivated areas under peri-urban agriculture are worst affected by this problem. Heavy metals do not degrade in the environment and so can remain in soil and water bodies for long periods. Excessive metal accumulation in contaminated soils can result in decreased soil microbial activity, soil fertility, and overall soil quality, and reductions in yield (McGrath et al. 1995) and the entry of toxic materials into the food chain (Hann and Lubbers 1983). Although it is necessary to clean up contaminated sites, the application of environmental remediation strategies is often very expensive and intrusive (McGrath et al. 1995). Thus, it is important to develop low-cost and environmentally friendly strategies. Recently, the notion of using metal-accumulating plants for environmental clean-up has been vigorously pursued (Brown et al. 1995; Salt et al. 1995), giving birth to the philosophy of "phytoextraction" within the broader concept of "phytoremediation" (Kumar et al. 1995).

Using the bioaccumulation capacities of specialized group of plants may provide an effective way of removing heavy metals from contaminated soils (Grispen et al. 2006; Meers et al. 2005; Salido et al. 2003). For the last one and half decades, extensive

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research has been done on various plant species in relation to the phytoextraction of metal from contaminated soils. However, the major limitation of phytoextraction is the reduced availability of metals due to their complexation with soil clays and organic matter. Therefore, the key to the successful phytoremediation of heavy metal contaminated soils lies in the optimization of soil and nutrient management practices for enhancing the availability of heavy metals to phytoremediating plants. Nevertheless, most phytoremediation studies confine themselves to using food crops as remediating plants. This approach is acceptable to farmers who are thus compensated for cleaning up their precious land. However, in developing countries, where compensation can sometimes be a burden on the government, phytoremediating research needs to consider other non-food crops such as timber and biodiesel-producing crops as phytoremediating plants, but very few research reports are available on this topic.

18.2 Heavy Metals

Heavy metals, broadly defined as a group of toxic metals and metalloids associated with pollution and toxicity, are elements with a density of more than 6 Mg m^{-3} and atomic weights that exceed that of iron (Alloway 1990). Conventionally, all of the micronutrient cations – iron, manganese, copper, zinc, and nickel – are classified as heavy metals, and these can result in either deficiency or toxicity depending on their levels in plants/organisms. In addition, lead, cadmium, chromium, mercury, selenium, and arsenic are also classed as heavy metals, but these elements can only be toxic to animals (including human beings) and plants. The most common heavy metal contaminants are cadmium, chromium, copper, mercury, lead, and zinc.

18.2.1 Sources of Heavy Metal Contamination in Soil

Contamination of soils with heavy metals has mainly resulted from industrial activities, such as the mining and smelting of metalliferous ores, electroplating, gas exhausts, energy and fuel production, fertilizer and pesticide application, and the generation of municipal waste, fly ash, etc. As well as these anthropogenic activities, geogenic activities can also contaminate groundwater and soil with heavy metals such as arsenic and selenium. However, the human influence on heavy metals in soils is demonstrated dramatically by the highly elevated levels of metals that now characterize the soils in urban areas and around major industries (Adriano 1986). Most of these metals are delivered via the atmosphere. The median values reported for the atmospheric fallout of heavy metals in urban areas of North America are 160 g ha^{-1} yr^{-1} for copper, 910 for lead, 18 for cadmium, and 3,200 for zinc (Jeffries and Schneider 1981). The values for urban areas of Europe are 320 for copper, 400 for lead, 310 for nickel, 15 for cadmium, and 1,000 for zinc (Bergkvist et al. 1989). Based on these deposition rates, the levels of most heavy metals in surface soils will double in 2–10 years. Atmospheric fallout of metals in rural areas has also risen sharply; the rates in rural Europe have been estimated to be

150 g ha^{-1} yr^{-1} for copper, 550 for zinc, 220 for lead, 32 for nickel, and 4 for cadmium (Jeffries and Schneider 1981). For arable soils, the primary sources of metal pollution include fertilizers, agricultural chemicals, and liquid and solid wastes (Table 18.1). It has been estimated that the average cadmium input to agricultural lands in Europe is about 8 g ha^{-1} yr^{-1} from the atmosphere and 5 g ha^{-1} yr^{-1} from the application of phosphatic fertilizer (Hutton 1982). Metal contamination of agricultural soils in Belgium from fertilizers and the atmosphere has been estimated to average 16, 20, 260 and 3,800 g ha^{-1} yr^{-1} for arsenic, cadmium, lead, and zinc, respectively (Navarre et al. 1980).

Forstner (1995) reported that, of the approximately 1,000 contaminated superfund sites identified on the United States Environmental Protection Agency's National Priority list of 1986, 40% involve heavy metal contamination. The results of an experiment conducted in USA have revealed that the total concentrations of Zn and Cu in air-dry soils from certain sites were as high as 11,700 and 3,420 mg kg^{-1}, respectively (Stephen et al. 1998). In the USA, concentrations of Se in agricultural irrigation effluent increased stored soil Se to toxic levels in wetland sediments in California (Banuelos et al. 1997). Heavy metal contamination of soil and vegetation near industrial areas in Bangladesh (Kashem and Singh 1999) and China have been reported (Wang 2000).

Tannery effluent is a major source of aquatic pollution in India, possessing high chemical oxygen demand (COD), biological oxygen demand (BOD), and hexavalent chromium. There are a large number of tanneries scattered all over India, but the main areas where they are concentrated are Tamilnadu, Uttar Pradesh, and West Bengal (Yadav et al. 2005). The unscientific disposal of untreated or undertreated effluents has resulted in an accumulation of heavy metals in land and water bodies. Cultivated areas under peri-urban agriculture are worst affected by this problem. Long-term applications of sewage effluents and sludge have significantly increased the trace metals in various Indian cities (Table 18.2; Rattan et al. 2002). Rattan et al. (2005) also reported that, on average, sewage effluents emanating from sewage treatment plants in India contained 5.5, 3.6, 2.6, 6.4, and 1.3-fold higher amounts of Zn, Cu, Fe, Mn, and Ni, respectively, than ground water (Table 18.3). Recently, Purakayastha (2008a) reported that effluents contained 4, 3, 1.9, 2.1, and 1.8-fold higher amounts of Zn, Cu, Cd, Pb, and Ni than tube well water. Soils irrigated with the former effluents for more than 20 years significantly enhanced the DTPA-extractable Zn, Cu, Fe, Pb, and Ni by 186%, 158%, 144%, 19%, and 14%, respectively, compared to irrigation with tube well water (Table 18.4). The soils irrigated with the latter effluents increased the DTPA-extractable Zn, Cu, Fe, Mn, Cd, Pb, and Ni concentrations by 184, 106, 160, 117, 108, 58, and 83%, respectively.

Soil represents the ultimate sink for heavy metals in continental areas. Because metals are immobile in soil due to their high affinity for the soil matrix, they tend to accumulate there, mainly in the surface soil layers.

18.2.2 Heavy Metal Contamination in the Food Chain

Besides adversely influencing plant growth, the toxic effects of heavy metals are amplified along the food chain at each stage of the food web (Fig. 18.1).

Table 18.1 Worldwide input of heavy metals into soils (1,000 tons yr^{-1}) (from Nriagu and Pacyna 1988)

Source	Antimony	Arsenic	Cadmium	Chromium	Copper	Lead	Manganese	Mercury	Molybdenum	Nickel	Selenium	Vanadium	Zinc
Agricultural and animal wastes	4.9	5.8	2.2	82	67	26	158	0.85	34	45	4.6	19	316
Logging and wood wastes	2.8	1.7	1.1	10	28	7.4	61	1.1	1.6	13	1.6	5.5	39
Urban refuse	0.76	0.40	4.2	20	26	40	24	0.13	2.3	6.1	0.33	0.2	60
Municipal sewage and organic waste	0.18	0.25	0.18	6.5	13	7.1	8.1	0.44	0.43	15	0.11	1.3	39
Solid waste from metal fabrication	0.08	0.11	0.04	1.5	4.3	7.6	2.6	0.04	0.08	1.7	0.10	0.12	11
coal ash	12	22	7.2	289	214	144	1076	2.6	441	68	32	39	298
Fertilizers and peat	0.25	0.28	0.2	0.32	1.4	2.9	12	0.01	0.46	2.2	0.27	0.97	2.5
Discarded manufactured products[a]	2.4	38	1.2	458	592	292	300	0.68	1.9	19	0.15	1.7	465
Atmospheric fallout[b]	2.5	13	5.3	22	25	232	27	2.5	2.3	24	2.0	60	92
Total input	26	82	22	898	971	759	1669	8.3	87	294	41	128	1322

[a] Metals used for industrial installations and durable goods are assumed to have a definite lifespan and to be released into the environment at a constant rate

[b] Totals are rounded

Note that these inputs exclude mine and slags at smelter sites

Table 18.2 Accumulation of DTPA-extractable heavy metals in soils (mg kg^{-1}) of various Indian cities (from Rattan et al. 2002)

City	Zinc	Copper	Iron	Manganese	Cadmium	Lead	Nickel	Chromium	Arsenic	Mercury
Kolkata	281	36.0	115	24.0	0.45	104.3	9.45	12.5	nd	nd
Ludhiana	4.38	5.50	36.0	10.9	0.07	1.88	0.37	0.57	1.02	0.51
Jalandhar	14.7	4.20	39.7	18.9	nd[a]	nd	1.27	1.72	2.09	nd
Patna	11.4	14.5	54.9	19.4	0.21	10.2	nd	nd	nd	nd
IARI, Delhi	5.0	3.30	23.3	12.1	nd	0.40	nd	nd	nd	nd
Keshopur, Delhi	6.77	5.42	40.3	5.17	0.15	2.34	0.91	nd	nd	nd
Hyderabad	6.8	1.09	16.2	16.0	0.14	10.5	0.46	0.34	nd	nd
Madurai	5.9	5.70	32.3	39.0	0.10	3.70	4.90	2.90	nd	nd
Coimbatore	10.4	9.70	28.5	37.0	0.20	6.30	14.6	3.80	nd	nd

[a]nd–not deleted

Table 18.3 Plant nutrients and heavy metal contents in sewage effluents and tube well water from two villages in Delhi (from Purakayastha 2008a; Rattan et al. 2005)

	N	P	K	S	Fe	Mn	Zn	Cu	Cd	Pb	Ni
	(mg L^{-1})				(μg L^{-1})						
Madanpur Khadar village irrigated with sewage effluents from Okhla Sewage Treatment Plant											
Sewage effluents	4.65	2.63	9.86	10.6	1658	120	85.0	36.0	2.85	75.0	150
Tube well water	1.20	0.26	2.59	8.90	650	15.0	21.0	12.0	1.52	35.0	85.0
[a]Increase (-fold)	3.90	10.0	3.80	1.20	2.60	8.0	4.0	3.0	1.90	2.10	1.80
Bakarwala village irrigated with sewage effluents from Keshopur Sewage Treatment Plant											
Sewage effluents	–	2.57	11.7	15.9	1464	64.0	61.0	29.0	1.53	33.0	49.0
Tube well water	–	0.22	3.58	14.3	557	10.0	11.0	8.0	1.42	30.0	37.0
[a]Increase (-fold)	–	11.7	3.30	1.10	2.60	6.40	5.50	3.60	1.10	1.10	1.30

[a]Increase over tube well irrigated soils

Table 18.4 Long-term effects of sewage irrigation on available zinc, copper, iron, manganese, cadmium, lead, and nickel concentrations in soils of two villages of Delhi (from Purakayastha 2008a; Rattan et al. 2005)

	Available metal (mg kg^{-1})						
	Zinc	Copper	Iron	Manganese	Cadmium	Lead	Nickel
Madanpur Khadar village							
Sewage-irrigated	3.81**	6.44**	68.0*	22.3**	0.27**	4.09*	1.59**
Tube well irrigated	1.34	3.12	26.2	10.3	0.13	2.59	0.87
[a](%)	184	106	160	117	108	58	83
Mundka village							
Sewage-irrigated	6.38**	6.53**	62.2**	8.43**	0.22*	2.64**	1.29**
Tube well irrigated	2.23	2.53	25.4	12.7	0.19	2.22	0.54
[a](%)	247	181	341	-18	38	44	123

[a](%), increase or decrease over tube well irrigated soils,
**, * *t*-tests showing significance at the 5% and 1% levels, respectively

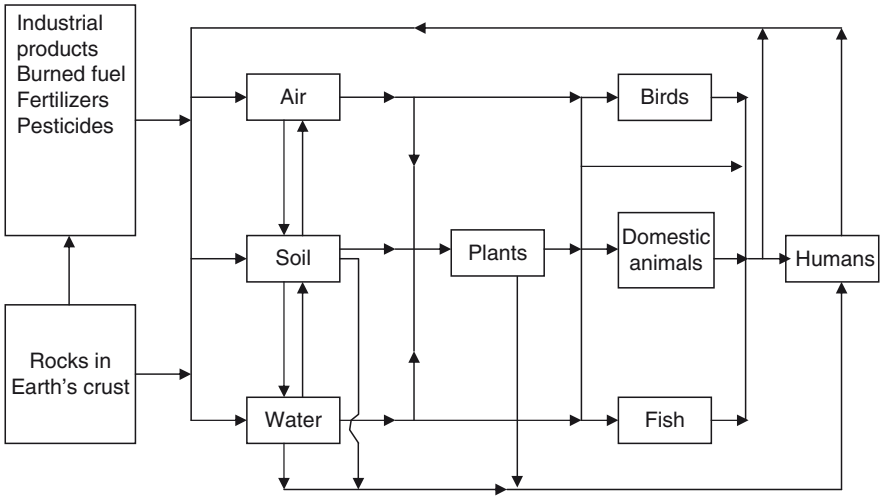

Fig. 18.1 Biomagnification of heavy metals along the food chain

The heavy metals gain entry into the human and animal food chain through crops grown on soils contaminated with them. Such soils are often used to cultivate leafy vegetables and tuber crops to meet the demands of nearby urban populations. These crops are known for their capacity to accumulate heavy metals in their edible parts. The entry of heavy metals such as Cd, Zn, Pb, Cu, Ni, Mn, and Fe into the food chain has been widely reported.

In an Indian context, Rattan et al. (2005) observed increasing accumulations of Zn, Ni, Cu, and Fe in different fields containing vegetable and fruit crops such as maize, mustard, rice, jowar, spinach, cauliflower, brinjal, radish, guavas, citrus, etc., which were grown under sewage irrigation from the Keshopur Effluent Irrigation System in Western Delhi. The agricultural sustainability of such production system depends to a large extent upon maintaining or enhancing the soil quality, which is rapidly deteriorating due to the disposal of untreated effluents onto it. About 9.5% of rice paddy soils have been rendered unsuitable for growing rice for human consumption because of excessive metal contamination.

Different doses of heavy metals can cause undetectable, therapeutic, toxic, or even lethal effects. Selenium, copper, and zinc often become toxic as the dose of the metal and exposure to it increase. These metals enter livestock as well as our own bodies through the food chain. Zinc toxicosis is manifested as gastrointestinal distress, decreased food consumption, anorexia, hemoglobinuria, anemia, poor bone mineralization, and arthritis. Lead poisoning is the most frequently diagnosed toxicological condition in veterinary medicine. Its occurrence has been reported in all domestic species.

Many trace elements are essential for normal metabolism, but most can also be toxic if the intake is much above the required level. For example, the ingestion of Zn in large amounts can cause vomiting, diarrhoea, and neurological damage. Wilson's disease is an autosomal recessive disorder in which the inherited metabolic defect is associated with the gradual and progressive accumulation of Cu in the liver. Symptoms of Cu toxicity include hemolysis, hepatic necrosis, and renal damage. A study conducted in 1990 in Bangkok, Thailand, showed that by age seven the average child had lost six points in IQ tests because of Pb poisoning from the air (Gupta and Gupta 1998). Average blood Pb levels in Thailand were 40–45 $\mu g\ dL^{-1}$, which is ten times the US standard.

18.3 Remediation of Heavy Metal Contaminated Soil

The remediation of heavy metal contaminated soils is a task of the utmost importance considering how widespread this problem is. It can be attempted through conventional remedial measures, such as landfilling and leaching, excavation and burial, or soil washing. However, these approaches are cost intensive and thus not economically viable. The remediation of soil contamination by conventional engineering techniques often costs between \$50 and \$500 ton^{-1}. Certain specialized techniques can exceed costs of \$1000 ton^{-1}. With an acre of soil (to a three-foot depth) weighing approximately 4,500 tons, this translates to a minimum cost of about a quarter of a million dollars per acre (Cunnigham et al. 1995). Besides being intrusive in nature, these methods also destroy the soil structure (McGrath et al. 1995). They are not actually decontamination measures but allow the problem to be evaded temporarily. Such treatments also destabilize the natural ecosystem and are often aesthetically unacceptable.

More recently, however, green-plant-based processes have begun to receive greater attention. The use of specially selected and engineered metal-accumulating plants for environmental clean-up is an emerging frontline technology called "phytoremediation." Phytoremediation refers to a system in which plants in association with soil organisms can remove or transform contaminants into harmless and often valuable forms. Phytoremediation takes advantage of the inherent ability of plants to take up water, soluble mineral nutrients, and their associated co-contaminants through their roots, to transpire through leaves, and to act as a transformation system to metabolize organic compounds (such as hydrocarbons and pesticides), or to absorb and bioaccumulate toxic trace elements including heavy metals. Recently, the notion of using metal-accumulating plants for environmental clean-up has been vigorously pursued (Brown et al. 1995; Salt et al. 1995), giving birth to the philosophy of "phytoextraction" within the broader concept of phytoremediation (Kumar et al. 1995).

18.3.1 Phytoremediation

Phytoremediation is an emerging technology that exploits the genetic potential of selected plant species to remove, degrade, metabolize, or immobilize a wide range of contaminants. The concept of using plants to clean up contaminated environments is not new. About 300 years ago, plants were proposed for use in the treatment of wastewater (Hartman 1975). At the end of the nineteenth century, *Thlaspi caerulescens* and *Viola calaminaria* were the first plant species that were documented to accumulate high levels of metals in their leaves (Baumann 1885). In 1935, Byers reported that plants of the genus *Astragalus* were capable of accumulating up to 0.6% selenium in dry shoot biomass. One decade later, Minguzzi and Vergnano (1948) identified plants that were able to accumulate up to 1% Ni in shoots. Later, Rascio (1977) reported tolerance and high Zn accumulation in shoots of *Thlaspi caerulescens*. In the last decade, extensive research has been conducted to investigate the biology of metal phytoextraction.

Metal hyperaccumulation is a phenomenon generally associated with species endemic to metalliferous soils, and it is found in only a very small proportion of such metallophytes. Most, but not all, hyperaccumulators are strictly endemic to metalliferous soils. The 430+ taxa described to date include representatives of many families, ranging in growth form small annual herbs to perennial shrubs and trees. They have been discovered in all continents in temperate and tropical environments. Notable centers of distribution are; for Ni: New Caledonia, Cuba, SE Asia, Brazil, Southern Europe, and Asia Minor; Zn and Pb: NW Europe; Co and Cu: Southcentral Africa. Some families and genera are particularly well represented; e.g., for Ni: Brassicaceae (*Alyssum* and *Thlaspi*), Euphorbiaceae (*Phyllanthus*, *Leucocroton* and Asteraceae (*Senecio, Pentacalia*); Zn: Brassicaceae (*Thlaspi*); Cu and Co: Lamiaceae, Scrophulariaceae.

Phytoremediation can be practiced in order to scavenge both organic and inorganic pollutants present in solid substrates (e.g., soil), liquid substrates (e.g., water), and the air. There are various phytoremediation approaches that can be employed:

- *Phytoextraction*. This involves growing plants that are selected for their capacity to concentrate one or more heavy metals on contaminated soil. The plants are then harvested, incinerated, and the ash related to a confined area or the heavy metals are extracted from it (Fig. 18.2).
- *Phytodegradation*. This approach involves the use of plants and associated microorganisms to degrade organic pollutants into less toxic forms or to render them immobilized in order to prevent their entry into the food chain or environment.
- *Rhizofiltration*. This is the use of plant roots to absorb and adsorb pollutants, mainly metals, from water bodies and aqueous waste streams. Artificially created marshes are planted with plant species capable of absorbing or adsorbing metals.

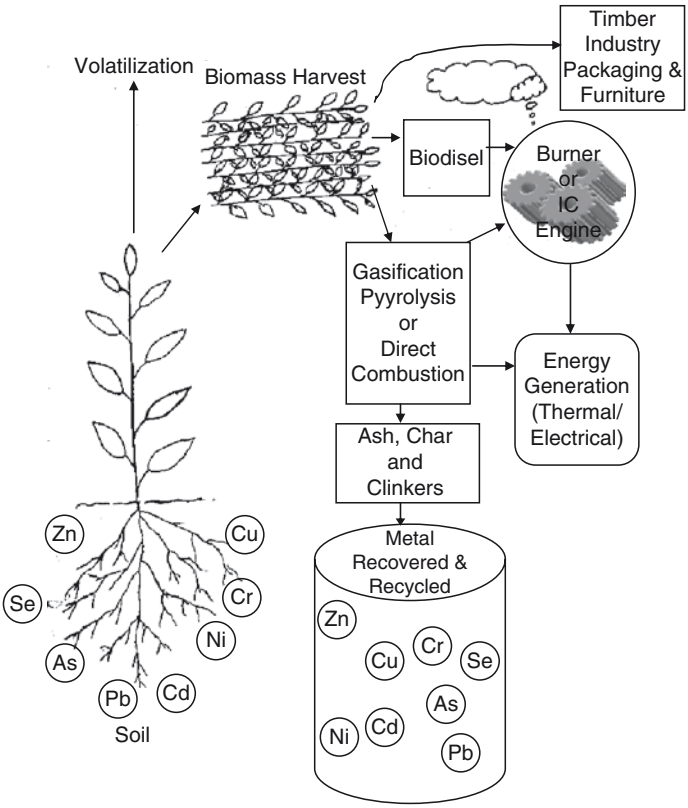

Fig. 18.2 Phytoextraction of metals from soil and their utilization

Table 18.5 Plant parameters to consider when applying different phytoremediation approaches (from Smits and Pilon 2002)

	Tolerance	Root uptake	Translocation	Shoot accumulation	Biotrans-formation	Rhizosphere microbes
Phytostabilization	X				X	
Phytoextraction	X	X	X	X		X
Phytovolatilization	X	X	X		X	X
Rhizofiltration	X	X				

Contaminated water passes through these rhizofilters, and the plants take up heavy metals. The plants are regularly harvested and incinerated. These systems can also be applied to treat sewage.

- *Phytostabilization*. This method uses plants to reduce the bioavailability of pollutants in the environment by reducing leaching, runoff, and soil erosion.
- *Phytovolatilization*. This is the use of plants to volatilize pollutants (Salt et al. 1998).

Important factors to consider when choosing a plant for specific phytoremediation approaches are given in Table 18.5 (Smits and Pilon 2002). Among these approaches, phytoextraction seems to be most attractive due to its versatility in usage. Phytodegradation can only be of use in the case of degradable wastes, and thus has limited applicability to the remediation of heavy metal contaminated soils. Rhizofiltration is specific to water treatment, while phytovolatilization is limited to certain special metals (e.g., Hg, Se, As) that are capable of forming volatile compounds. The phytostabilization process does not remove the metal from the soil system and focuses on rendering it inactive, meaning that the problem could occur again in the future. Because of these limitations, phytoextraction is a more attractive method.

18.3.1.1 Hyperaccumulators: Phytoextraction of Heavy Metals

Phytoremediation is an environmentally friendly technology that heavily depends on the efficiency of the metal hyper-accumulating plants used. A plant is classified as a hyperaccumulator when it takes up heavy metals against their concentration gradient between the soil solution and cell cytoplasm, and thus acquires the capacity to accumulate a very high metal concentration in tissues without impacting on basic growth and metabolic functions. The phenomenon is viewed as an evolutionary selection process that protects against herbivores and pathogens. The criteria for designating a plant as a hyperaccumulator for different metals are given below:

- Shoot metal concentration (oven dry basis) should be more than 1% for Mn and Zn; 0.1% for Cu, Ni and Pb; and 0.01% for Cd and As
- Should be fast growing with a high rate of biomass production

- Should be able to accumulate metals, even from low external metal concentrations
- Should be able to transfer accumulated metals from root to shoot (aboveground) quite efficiently (often with more than 90% efficiency).

This technology is still in its infancy. However, recent developments in relation to identifying or evolving high-biomass crop plants that have the capacity to accumulate heavy metals (Smith et al. 1999) suggest that the phytoremediation of metal contaminated soil will soon be a viable alternative to most conventional clean-up technologies. Initial phytoremediation research suggested that this could be achieved with hyperaccumulator plant species such as *Thlaspi caerulescens* that accumulate high levels of metals like Zn and Cd. There are many reports on the hyperaccumulating potentials of different species of plants, as mentioned in Table 18.6. Several hyperaccumulating plant species that have been identified for phytoextracting heavy metals like Zn, Cu, Ni, Pb, Cd, Cr, Se, and As are mentioned below.

Zinc

Initial phytoremediation research identified *Thlaspi caerulescens* as a high accumulator of Zn and Cd (Brown et al. 1994, 1995; Escarre et al. 2000), and some of its ecotypes can tolerate as much as 40,000 mg Zn kg^{-1} dry weight in shoots (Chaney 1983). However, the major limitations of this species for phytoremediation are its slow growth rate and small size (Black 1995; Brown et al. 1995). Recent evidence suggests that moderately accumulating high-biomass species such as Indian mustard (*Brassica juncea*) can accumulate four times more Zn than *T. caerulescens* (Kumar et al. 1995; Salt et al. 1995; Ebbs et al. 1997). This is primarily due to the fact that *B. juncea* produces ten times more biomass than *T. caerulescens.* Ebbs et al. (1997) reported Zn contents of between 500 and 600 mg kg^{-1} dry weight for all three species of *Brassica* (*B. juncea*, *B. rapa*, and *B. napus*) studied. Alternatively, sunflowers can be used for the remediation of metal-contaminated soils because of its high biomass. In a field-based sunflower screening, Nehnevajova et al. (2005) found that the "Salut" cultivar exhibited enhanced cumulative Cd, Zn, and Pb extraction efficiencies (by a factor of 4.4). The mean content of Zn in the whole *Polygonum thunbergii* was reported to be 1,507 mg kg^{-1} (Kim et al. 2003). Recently, Qiu et al. (2006) reported that *Potentilla griffithii* Hook var. Velutina Cardot could be classified as a new Zn hyperaccumulator. The fact that *P. griffithii* was able to grow in a mining soil with a Zn concentration of 193,000 mg kg^{-1} without showing any major sign of phytotoxicity demonstrated its high tolerance to Zn. For Zn phytoextraction from moderately contaminated paddy soil, Suzuyutaka soybean has been recommended (Murakami and Ae 2009).

Copper

The shoot Cu concentration in the hyperaccumulator *B. juncea* was more than three times greater than the mean Cu concentration in the shoots of the control plants

Table 18.6 Some important metal hyperaccumulators

Metal	Hyperaccumulator	Reported concentration(mg kg^{-1})	References
Zinc	*Thlaspi caerulescens*	52,000	Brown et al. (1994)
	Streptanthus polygaloides	6,000	Boyd and Davis (2001)
	Potentialla griffithii	6,250	Qiu et al. (2006)
Copper	*Ipomoea alpine*	12,300	Baker and Walker (1990)
	S. polygaloides	120	Boyd and Davis (2001)
	Medicago sativa	85	Videa-Peralta (2002)
	Brassica juncea	22	Purakayastha et al. (2008b)
Cadmium	*T. caerulescens*	1,800	Baker and Walker (1990)
	Alfa alfa	1,079	Videa-Peralta (2002)
	Nicotiana tabacum	40	Evangelou et al. (2004)
	Sinapis alba	123	Evangelou et al. (2007)
Lead	*Thlaspi rotundifolium*	8,200	Baker and Walker (1990)
	Pisum sativum	8,960	Huang et al. (1997)
	B. juncea	15,000	Blaylock et al. (1997)
	T. caerulescens	844	Robinson et al. (1998)
	Vertiberia Zizanioides	1,450	Wilde et al. (2005)
	Sonchus arvensis	3,664	Surat et al. (2008)
Nickel	*Sebertia acumunata*	25% wt. of dried sap	Jaffre et al. (1976)
	Alyssum lesbiacum	47,500	Küpper et al. (2001)
	Medicago sativa	437	Videa-Peralta (2002)
	Alyssum bracteatum	2,300	Ghaderian et al. (2007)
Chromium	*Leptospermum scoparium*	20,000	Baker and Brooks (1989)
	B. juncea	1,400	Shahandeh and Hossner (2000)
	Helianthus annus		Shahandeh and Hossner (2000)
Selenium	*Astragalus racemosus*	14,900	Beath et al. (1937)
	Astragalus pectinalus	4,000	Shrift (1969)
	Stanleyea pinnola	330	Shrift (1969)
	Actiniopteris radiate	1,028	Srivastava et al. (2005)
Arsenic	*Pteris vittata*	23,000	Ma et al. (2001)
	Pityrogramma calomenalos	8,350	Francesconi et al. (2002)
	Pteris multifida	1,977	Wang et al. (2006)

(Ebbs and Kochain 1997). Recently, *Elsholtzia splendens* has been identified as being tolerant to high Cu concentrations and to have great potential for remediating contaminated soils (Jiang et al. 2004; Wu et al. 2007). Normal growth of this plant was attained up to 80 mg kg^{-1} available soil Cu (the NH_4OAc extractable Cu) or 1,000 mg kg^{-1} total Cu, and *E. splendens* extracted 3.6-fold more Cu from paddy soil polluted by a Cu refining area than from a Cu-mined area in China (Xiao et al. 2005). The mean content of Cu in the whole *Polygonum thunbergii* was reported to be 548 mg kg^{-1} (Kim et al. 2003). There is great potential for phytoextraction of Cu by Gold Dent maize and Milyang 23 rice from paddy soils with low to moderate

contamination under aerobic soil conditions (Murakami and Ae 2009). Among various *Brassica* spp., *B. juncea* cv. Pusa Bold exhibited highest content of Cu (22 mg kg^{-1}) in its shoots, and the same species also showed a Cu uptake of 600 mg (Purakayastha et al. 2008b). In contrast, copper accumulation in *Aeolanthus biformifolius* was reported to be 9,000 mg kg^{-1} (Morrison et al. 1979), while *Haumaniustrum robertii* and *Larrea tridentate* were able to accumulate 1,000 mg kg^{-1} (McCutcheon and Schnoor 2003).

Nickel

Certain well-known hyperaccumulators of Ni are found in the genus *Alyssum* (*Brassicaceae*) (Baker et al. 2000), although the most remarkable example is perhaps *Sebertia accuminata* (*Sapotaceae*), a New Caledonian tree that can grow to a height of about 10 m. A mature tree of *Sebertia accuminata* was estimated to contain 37 kg Ni (Sagner et al. 1998). The other species that has received attention is *Berkheya coddii*, which can accumulate Ni to more than 1% of its weight and is tall and fast growing (Morrey et al. 1989). *Leptoplax emerginata* and *Bornmuellera tymphaea* are Ni hyperaccumulators of the Brassicaceae family endemic to serpentine soils in Greece (Chardot et al. 2005). They reported that *Leptoplax emerginata* produced significantly more biomass than other plants. On serpentine soil, *Bornmuellera tymphaea* showed the highest Ni concentrations in shoots. However, Ni phytoextraction was maximal with *Leptoplax emerginata. Streptanthus polygaloides* was reported to be an appropriate phytoextractor for soils contaminated with Ni or low levels of Co, but would not be useful for Cu, Zn, Mn, and Pb (Boyd and Davis 2001). Videa-Peralta (2002) demonstrated that Zn(II) reduced the toxic effects of Ni(II) to alfalfa plants, which could represent important information for the use of living alfalfa plants in the phytoremediation of nickel-contaminated soils. For example, research has shown that in *T. goesingense*, a Ni hyperacccumulator, its high tolerance is due to Ni complexation by histidine, which renders the metal inactive (Kramer et al. 1996, 1997). Recently, *Alyssum bracteatum*, which is endemic to Iran, has been reported to the first Ni hyperaccumulator from this area (Ghaderian et al. 2007).

Lead

Indian mustard (*B. juncea*) is widely reported to be a hyperaccumulator of Pb. This plant is capable of accumulating 2,675 mg Pb kg^{-1} dry biomass when grown in soil contaminated with 500 mg Pb kg^{-1} (Begonia et al. 1998). However, the accumulation of Pb in roots was almost tenfold higher than in shoots. The concentration of Pb in biomass of Indian mustard increased to 15,000 mg Pb kg^{-1} shoot dry weight upon the addition of 10 mmol kg^{-1} EDTA. As well as Indian mustard, chelating agents (1.5 mmol EDTA kg^{-1}) were also found to be effective at enhancing Pb concentrations by 8,960 and 2,410 mg Pb kg^{-1} in two-week-old pea and corn

shoots, respectively (Huang et al. 1997). However, several other species, such as hemp dogbane (*Apocynum cannabinum*), common ragweed (*Ambrosia artemisiifolia*), nodding thistle (*Carduus nutans*), and Asiatic dayflower (*Commelina communis*) have been shown to have superior Pb-accumulating properties (Berti and Cunningham 1993). Under chelate-induced conditions, maize (Huang and Cunningham 1996) and Indian mustard (Blaylock et al. 1997) have been successfully used to remove Pb from solution culture and contaminated soil. The mean content of Pb in the whole *Polygonum thunbergii* was reported to be 183.3 mg kg^{-1} (Kim et al. 2003). Krishnasamy et al. (2004) reported that *Fioria vitifolia* was the best accumulator of Pb. Vetiver grass (*Vetiveria zizanioides* L.) registered the highest rate of Pb absorption (10.16 ± 2.81 mg kg^{-1}), followed by cogon grass (*Imperata cylindrica* L.) (2.34 ± 0.52 mg kg^{-1}), and carabao grass (*Paspalum conjugatum* L.) with a mean Pb level of 0.49 ± 0.56 mg kg^{-1} (Paz-Alberto et al. 2007). The total Pb uptake by vetiver grass was much higher than for the other two grass species because of the higher biomass yield of the former species. It was the most tolerant and could grow in soil contaminated with high Pb concentrations. *Borago officinalis* and *Sinapis alba* L. have recently displayed Pb concentrations of 25 and 29 mg kg^{-1}, respectively, at the highest Pb-spiked soil concentration (Evangelou et al. 2007). In a field trial study in Thailand, *Sonchus arvensis* was reported to tolerate a total of 100,000 mg Pb kg^{-1} soil and to accumulate Pb in shoots up to a level of 3,664 mg kg^{-1} with high translocation factor (2.19) and bioaccumulation factor (2.38) values (Surat et al. 2008).

Cadmium

The ability of *T. caerulescens* to hyperaccumulate Cd (and Zn) has been known about for a long time (Ernst 1968). Robinson et al. (1998) found that Cd accumulates in the leaves of *T. caerulescens* up to levels of 1,600 mg Cd kg^{-1} dry weight without any detectable decrease in its dry biomass for up to 50 mg extractable Cd kg^{-1} soil. Lombi et al. (2000) noted that, in a hydroponics experiment, one French population of *T. caerulescens* (Ganges ecotype) was able to accumulate Cd in shoots to over 10,000 mg kg^{-1} without biomass reduction. Moreover, in a field trial, this population was able to accumulate up to 500 mg Cd kg^{-1} in the shoots at 12 mg Cd kg^{-1} soil, which is encouraging for Cd phytoextraction from agricultural soils. However, there were wide variations in Cd content and uptake within the population of *T. caerulescens*. One population of *Thlaspi* from Col du Mas de l'Air in France showed 40% more Cd uptake than the population at large (Schwartz et al. 2006). Some ecotypes of the metal hyperaccumulator species *T. caerulescens* J. & C. Presl possess extraordinary Cd accumulation and tolerance levels but have low biomass yields. Some fast-growing trees (*Salix*, *Populus*) and high biomass producing crops such as oilseed rape (*Brassica napus*), tobacco (*Nicotiana tabacum*), flax (*Linium usitatissimum*), peppermint (*Mentha piperita*), cotton (*Gossipium hirsutum*), triticale and maize (*Zea mays*), sunflower (*Helianthus annuus*), cereals, and Indian mustard (*B. juncea*) are also considered to be suitable species for phytoextraction,

as they can compensate for lower Cd accumulation levels with much higher biomass yields (Vassilev and Zaprianova 1999; Yankov et al. 2000; Griga et al. 2002). Some information on the Cd phytoextraction potentials of several plant species is presented in Table 18.1, but the availability of this kind of data is generally still limited. Indian mustard (*B. juncea*) was reported to be more tolerant of Cd than rape (*B. rapa*) and *B. napus* (Ebbs and Kochain 1997). However, Ghosh and Singh (2005) reported that *Ipomoea carnea* was more effective at removing Cd from soil than *B juncea*. *I. carnea* followed by *Dhatura innoxia* and *Phragmytes karka* were the most suitable species for phytoextracting cadmium from soil when the whole plant or aboveground biomass was harvested. In the relatively short duration of this experiment, *I. carnea* produced more than five times more biomass than *B. juncea*.

Willows (*Salix* species), woody tree species, are not actually metal hyperaccumulators, but it was shown that some clones are able to accumulate up to 70 mg Cu kg^{-1} dry weight in leaves (Landberg and Greger 1996). Due to large variations in shoot Cd concentrations (5–70 mg kg^{-1}) found in different *Salix* species and clones, very different values concerning Cd removal have been calculated in the literature: 222 g Cd $ha^{-1} yr^{-1}$ (Felix 1997), 61.7 g Cd $ha^{-1} yr^{-1}$ (Rulford et al. 2002), and about 1,060 g Cd $ha^{-1} yr^{-1}$ (Robinson et al. 2000). Some tree species like *Salix viminalis* have also been reported to remove five times more Cd than *T. caerulescens* and *Alysum murale*, which may be due to the higher biomass production of the former species (Greger 1999). In some treated poplar clones, PE 4/68, B-229, 665, and 45/51, the Cd contents in root increased from 38.57 to 511.51 mg kg^{-1}, the leaf contents from 0.91 to 7.50, while the stem contents ranged from 1.37 to 9.50 mg kg^{-1} (Andrej et al. 2005).

Most high biomass producing plants such as, maize, oats, and sunflower are plants that do not grow in cold climates or need intensive care. Therefore, three "weed" plants, *Borago officinalis*, *Sinapis alba* L., and *Phacelia boratus*, were investigated for their ability to tolerate and accumulate high amounts of Cd and Pb (Evangelou et al. 2007). Pot experiments were performed with soil containing Cd and Pb at concentrations of up to 180 and 2,400 mg kg^{-1}, respectively. All three plants showed high levels of tolerance. *Borago officinalis* and *Sinapis alba* L. accumulated 109 and 123 mg Cd kg^{-1}, respectively, at the highest Cd soil concentration (2,400 mg kg^{-1}). However, alfalfa (*Medicago sativa*) plants were able to tolerate upto 500 mg L^{-1} of Cd(II), Cu(II), and Zn(II). In these conditions, the alfalfa accumulated up to 1,079 mg Cd kg^{-1} of dry shoot tissue, which represented 26% of the Cd concentrated in root tissue (Videa-Peralta 2002).

Chromium

Very few plant species, including *Sutera fodina*, *Dicoma niccolifera*, and *Leptospermum scoparium*, have been reported to accumulate Cr to high concentrations in their tissues. Attempts are now being made to use promising aquatic plant species for the phytoextraction of Cr from contaminated tannery sludge. In this

respect, three plant species (*Scirpus lacustris*, *Phragmites karka*, and *Bacopa monnieri*) were found to absorb, translocate, and concentrate Cr in their tissues. Among the 36 plant species examined to select high Cr accumulators, it was reported that Indian mustard (*B. juncea* cv. 426308) and sunflower (*Helianthus annus* L.) accumulated the most Cr (Shahandeh and Hossner 2000). However, *Ipomoea carnea* was reported to be more effective than *B. juncea*, a widely reported hyperaccumulator for phytoextraction of Cr-contaminated soils (Ghosh and Singh 2005). This was due to two factors: (1) it was able to tolerate/accumulate levels of Cr equivalent to those in the soil; (2) though the shoot/root ratio of Cr was much lower in *I. carnea*, its shoot biomass was much higher, so a large proportion of the total Cr in the plant was sequestered in harvestable tissue. However, Bluskov et al. (2005) demonstrated the ability of *B. juncea* to detoxify more toxic Cr(VI), thus making this plant a potential candidate for phytostabilization. *Calendula arvensis* appears to absorb more Cr than *Calendula officinalis*, so Bini et al. (1999) suggested that it is suitable for the phytoremediation of Cr-affected soil. The Cr tolerance of the plant *Typha angustifolia* L. appears to be associated with enhanced superoxide dismutase and peroxidase activities and improvements in the uptake and translocation of essential microelements (Dong et al. 2007). In soil with more than 20 mg Cr kg^{-1} soil, only *Phragmytes karka* showed the potential for phytoextraction. Among tree species, hybrid willows (*Salix matsudana* Koidz x *Salix alba* L.) showed higher removal rates of both chemical forms of Cr than weeping willows (*Salix babylonica* L.) (Yu and Gu 2008).

Selenium

Two of the options available for Se phytoremediation of contaminated soils are the volatilization of methylated Se forms or the harvesting and removal of Se-enriched biomass. Many species of general *Astragalus*, *Xylorrhiza*, and *Stanleyea* are typical Se accumulators and are capable of growing on high-Se soils without any detrimental effects on growth while reaching shoot selenium contents as high as 20,000–30,000 mg kg^{-1} dry matter (Rosenfeld and Beath 1964). Indian mustard (*B. juncea*) was more efficient at accumulating Se than milk vetch (*Astragalua incanus* L.), Australian saltbush (*Atriplex semibaccata* R. Br.), old man saltbush (*Atriplex mumularia* Lindl.), or tall fescue (*Festuca arundinacea* Schreb.) Preliminary results suggest that *Stanleya pinnata* may volatilize unusually large quantities of Se when grown at high sulfate concentrations, an unexpected result not reported previously for any species (Parker et al. 2003). *Stanleya pinnata* is a perennial that responded favorably to repeated cutting in the greenhouse, a trait that could prove valuable in field-scale phytoremediation. Indian mustard (*B. juncea*), has been reported to reduce soil Se concentration to nontoxic levels (Banuelos and Meek 1990; Banuelos et al. 1993, 1997). Indian mustard (*B. juncea*) overexpressing ATP sulfurylase (APS transgenics) were previously shown to have higher shoot Se levels and enhanced Se tolerance compared to the wild type when supplied with selenate in a hydroponic system. Another transgenic Indian mustard overexpressing cystathion-

ine gamma-synthase (CGS) showed a higher Se volatilization rate, lower shoot Se levels, and higher Se tolerance than the wild type. In the present study, these APS and CGS transgenics were evaluated for their capacity to accumulate Se from soil that is naturally rich in Se. Wild-type Indian mustard and the Se hyperaccumulator *Stanleya pinnata* were included for comparison. After ten weeks on Se soil, the ATP sulfurylase transgenics contained 2.5-fold higher shoot Se levels than wild-type Indian mustard, similar to those of *S. pinnata*. The cystathionine gamma-synthase transgenics contained 40% lower shoot Se levels than the wild type. Besides *B. juncea*, *B. napus*, and *Hibiscus cannabinus* have also been reported to decrease total selenium in soil (Banuelos et al. 1997). Among 11 fern species, *Actiniopteris radiate* has been reported to be the best species for Se accumulation (Srivastava et al. 2005).

Arsenic

The arsenic hyperaccumulator Chinese brake fern (*Pteris vittata* L.) was first discovered growing on a site in Central Florida (Ma et al. 2001) contaminated with chromated copper arsenate, a commonly used wood preservative. The fern efficiently accumulates As (up to 2.3% in its fronds) and produces a large amount of aboveground biomass (up to 1.7 m in height), which makes it feasible for use for phytoremediation purposes. The fern accumulated 11.8–64.0 mg As kg^{-1} dry weight when grown in an uncontaminated soil (Ma et al. 2001). However, when the ferns were grown in an arsenic-contaminated soil, they accumulated 1442–7526 mg As kg^{-1} dry weight in their fronds. Also, this fern is capable of taking up many different forms of arsenic (Ma et al. 2001; Tu and Ma 2002). Further, Chinese brake fern was found to have a high efficiency of translocation of arsenic to its fronds (Tu et al. 2002). The arsenic concentrations in fronds of *P. vittata* ranged from 66 to 6,151 mg kg^{-1}, 110 to 3,056 mg kg^{-1}, and 162 to 2,139 mg kg^{-1} from the first, second, and third harvests, respectively (Gonzaga et al. 2008). Another silver fern (*Pityrogramma calomenalos* L.) has also been reported to hyperaccumulate As to levels of up to 8,350 mg kg^{-1} dry mass from 135 mg As kg^{-1} soil (Francesconi et al. 2002).

In a screening experiment, Wang et al. (2006) reported that although As concentrations in the fronds of *Pteris oshimensis* (789 mg kg^{-1}) were lower than those of *Pteris multifida* (1,977 mg kg^{-1}), its high aboveground biomass makes it more suitable for phytoremediating As-contaminated soils. Recently, Ampiah-Bonney et al. (2007) reported that As uptake by *Leersia oryzoides* (rice-cut grass) was comparable to that reported for duckweed (*Lemna gibba* L.) and overlaps with the range of values reported for Chinese brake fern (*P. vittata* L.).

Arsenic accumulation in shoots of the various plant species investigated ranged from 0.1 to 107 mg kg^{-1} in *Bassia scoparia* (Chenopodiaceae), *Inula viscosa* (Asteraceae), *Solanum nigrum* (Solanaceae), and *Hirschfeldia incana* (Brassicaceae) had the highest values for As accumulation (Gisbert et al. 2008). *B. scoparia* (Chenopodiaceae) survive in soil with 8,375 mg kg^{-1} As. Three floating plants

(*Eichhornia crassipes*, *Spirodela polyrhiza*, and *Azolla pinnata*) and a common wetland weed (*Monochoria vaginalis*) that grow in arsenic-contaminated soils in Bangladesh showed high bioaccumulation coefficients and transfer factor values; so, these plants may be promising candidates for cleaning up As-contaminated surface water and wetland areas. The bioconcentration factor of *Oryza sativa* obtained from As-contaminated districts was >1, which highlights possible food-chain transfer issues for As-contaminated areas in Bangladesh (Mahmud et al. 2008).

18.3.1.2 Changes in Metal Concentrations in Soil

Hyperaccumulator plant species have the capacity to take up heavy metals in excess, thus reducing the heavy metal concentration in soil. The concentration of water-extractable Zn in soils on which *T. caerulescens* was grown dropped to 7.2 mg kg^{-1} as compared to soils on which tomato was grown (9.3 mg kg^{-1}) (Brown et al. 1994). The concentrations of mobile Zn in both rhizospheric and nonrhizospheric soils decreased compared with the initial amounts before *T. caerulescens* and *T. ocheoleucum* were planted (McGrath et al. 1997). Three crops of *T. caerulescens* grown over 391 days removed more than 8 mg Cd kg^{-1} and 200 mg Zn kg^{-1} from industrially contaminated soil, representing 43 and 7% of the two metals in the soil, respectively (Lombi et al. 2001). In contrast, the high concentration of Cu in the agricultural soil severely reduced the growth of *T. caerulescens*, thus limiting its phytoextraction potential. The best M2 sunflower "giant mutant" 14/185/04 was able to produce up to 26 tons dry matter per hectare and remove 13.3 kg Zn per hectare at a sewage sludge contaminated site in Rafz, Switzerland (Nehnevajova et al. 2007). To evaluate their phytoextraction potential, maize (Gold Dent), soybean (Enrei and Suzuyutaka), and rice (Nipponbare and Milyang 23) were pot-grown under aerobic soil conditions for 60 days on Andosol or Fluvisol with low to moderate Cu, Pb, and Zn contamination (Murakami and Ae 2009). After two months of cultivation, the Gold Dent maize and Milyang 23 rice shoots took up 20.2–29.5% and 18.5–20.2% of the 0.1 mol L^{-1} HCl-extractable Cu, 10.0–37.3% and 8.5–34.3% of the DTPA-extractable Cu, and 2.4–6.5% and 2.1–5.9% of the total Cu, respectively, in the two soils. Suzuyutaka soybean shoots took up 23.0–29.4% of the 0.1 mol L^{-1} HCl-extractable Zn, 35.1–52.6% of the DTPA-extractable Zn, and 3.8–5.3% of the total Zn in the two soils. Therefore, there is great potential for Cu phytoextraction by Gold Dent maize and Milyang 23 rice, and for Zn phytoextraction by Suzuyutaka soybean from paddy soils with low to moderate contamination under aerobic soil conditions.

The NH_4OAc-extractable Cu level in a polluted soil was reduced from 78 to 55 mg kg^{-1} after phytoextraction and removal of Cu by *Elsholtzia splendens* for one growth season. A depletion of the extractable Cu level in the rhizosphere was noted; even at high Cu levels, the NH_4OAc-extractable Cu in the rhizosphere was 30% lower than that in the bulk soil. These results indicate that phytoextraction by *E. splendens* can effectively reduce the plant-available Cu level

in polluted soils (Jiang et al. 2004). Three transgenics of Indian mustard developed on the basis of overproducing γ-glutamylcysteine synthetase (ECS), glutathione synthetase (GS), or adenosine triphosphate sulfurylase (APS) removed between 6% (Zn) and 25% (Cd) of the metal in the soil (Bennett et al. 2003). Rice (*Oryza sativa* L., cv. Milyang 23) accumulated 10–15% of the total soil Cd in its shoots, and the same species is thus promising for the phytoextraction of Cd from paddy soils with low levels of contamination (Murakami et al. 2007). Results of a field experiment suggest that certain *B. napus* L. accessions are suitable for the phytoextraction of moderately heavy metal contaminated soils (Grispen et al. 2006).

It was reported that when *Scirpus lacustris, Phragmites karka*, and *Bacopa mannieri* were grown in tannery effluent and sludge containing 2.3 μg mL^{-1} and 214 mg Cr kg^{-1}, respectively, there was a significant reduction in Cr concentration (Chandra et al. 1997) and an increase in biomass; no visible phytotoxic symptoms were exhibited by the treated plants. In another study on the decontamination of water from Lake Nainital (India) (a prime source of drinking water) by plants, it was reported that water roots of *Salix babylonica* and *Salix acmophylla* were more efficient than others (Ali et al. 1999).

Leptoplax emerginata was reported to be the most efficient species for phytoextracting and decreasing the available pool of soil Ni as measured by DTPA-TEA extraction (Chardot et al. 2005). *Alysum murale*, another widely reported hyperaccumulator of Ni, was found to be the least efficient species for Ni phytoextracting and decreasing the available Ni pool.

The total Se content in soil was decreased by *B. napus* and *Hibiscus cannabinus* (Banuelos et al. 1997). They noted a successive reduction in total Se from pre-plant to subsequent harvests. The extractable Se was found to follow a reverse trend in the rhizospheres of both plants. Rape (*B. napus*, cv. Wester), kenaf (*Hibiscus cannabinus* L. cv. Indian), and tall fescue (*Festuca arundinacea* L. cv. Alta) reduced the total soil Se between preplant and the final harvest by 47, 23, and 21%, respectively.

After two years, Chinese brake fern (*Pteris vittata*) reduced surface soil arsenic levels from 190 to 140 mg kg^{-1} (Kertulis-Tartar et al. 2006). Approximately 19.3 g of arsenic were removed from the soil by Chinese brake fern. In a potting study by Tu and Ma (2002), 26% of the initial soil arsenic was depleted using Chinese brake fern after 20 weeks of growth. However, three consecutive harvests of *P. vittata* for one year significantly reduced soil arsenic by 6.4–13%, as depicted in Fig. 18.3 (Gonzaga et al. 2008). Arsenic in the soil was primarily associated with amorphous hydrous oxides (40–59%), which contributed the most to the arsenic taken up by *P. vittata* (45–72%).

Growing *B. carinata* caused the highest percent reduction in soil Zn and Pb, while it also produced the highest reduction in soil Ni along with *B. napus* (Fig. 18.4) (Purakayastha et al. 2008b). *B. juncea* caused the highest reduction in soil Cu. The scale of the reduction was in the range of 4–11% for Ni, 4–12% for Pb, 4–15% for Zn, and 5–21% for Cu.

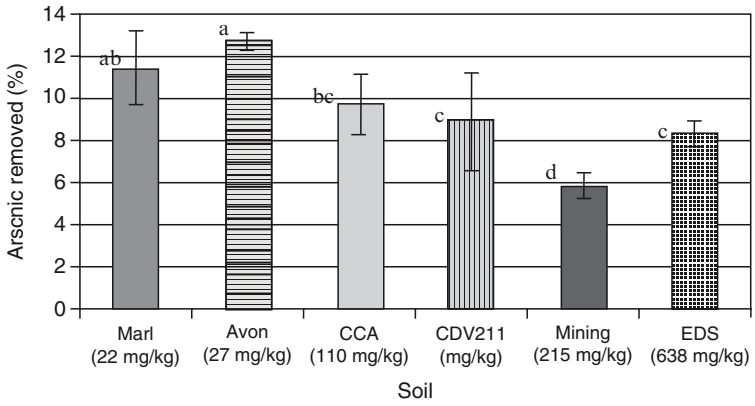

Fig. 18.3 Arsenic removed from six soils by *Pteris vittata* after three harvests. *Bars* represent mean ± standard deviation ($n = 4$) (from Gonzaga et al. 2008)

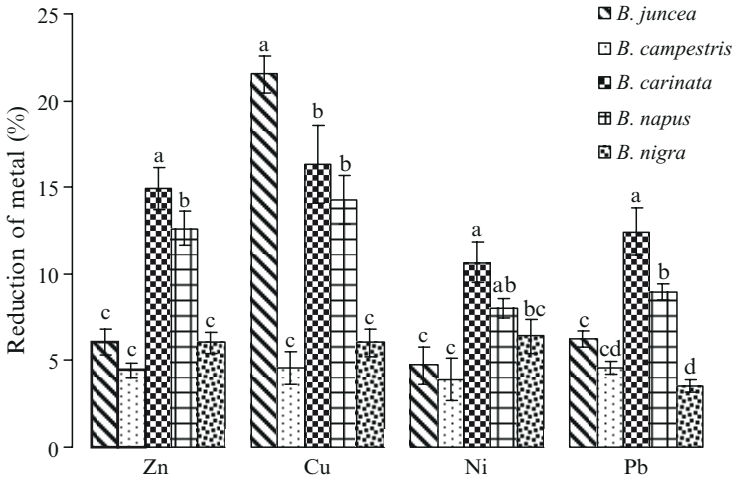

Fig. 18.4 Reduction in total heavy metal concentration in sewage-irrigated soil grown with various *Brassica* spp.; number of observations, $n = 3$. *Bars with different lower-case letters* are significantly different for a particular metal according to Duncan's multiple range test at $p = 0.05$. The *vertical line (±) in each bar* indicates the standard error of the mean (from Purakayastha et al. 2008b)

18.3.1.3 Optimization of Metal Phytoextraction

Plant Selection and Genetically Engineered Plants

Heavy metal hyperaccumulating plant species are usually slow growing and therefore unsuitable for phytoextraction purposes. The genetic modification of fast-growing crops may be a viable alternative. Genetic engineering has already been successfully

used to enhance plant metal tolerance and accumulation. This was achieved either by overproducing metal-chelating molecules such as citrate (de la Fuente et al. 1997), phytochelatins (Zhu et al. 1999), metallothioneins (Hasegawa et al. 1997), or ferritin (Goto et al. 1999), or by overexpressing metal transporter proteins (Arazi et al. 1999; Hirschi et al. 2000). Mercury volatilization and tolerance were also achieved by introducing a bacterial pathway (Rugh et al. 1996; Bizily et al. 2000).

γ-Glutamylcysteine synthetase (ECS) or glutathione synthetase (GS) transgenics accumulated significantly ($P<0.05$) more metal in their shoots than wild-type (WT) Indian mustard, while adenosine triphosphate sulfurylase (APS) transgenic plants did not (Bennett et al. 2003). They also reported that, compared to wild-type Indian mustard, ECS and GS transgenics contained higher shoot concentrations of Cd (+50%) and Zn (+45% for GS and +93% for ECS). Furthermore, the ECS transgenics had higher levels of Cr (+170%), Cu (+140%), and Pb (+200%) than in the WT (Fig. 18.5). The ECS and GS transgenics accumulated 1.5-fold more Cd

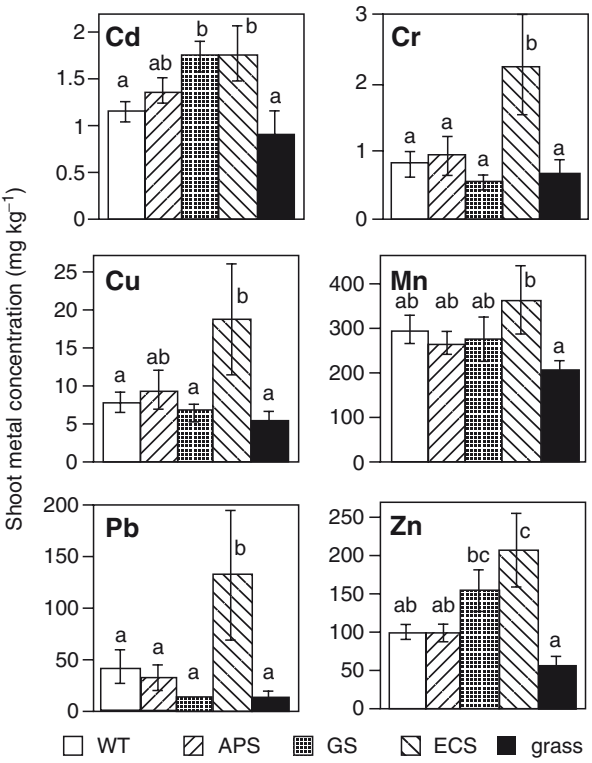

Fig. 18.5 Shoot metal concentrations for the five planted treatments at harvest. Values shown are the means and standard errors of ten replicate pots (shoot material was pooled for each pot, dried, homogenized, and analyzed for metals). *WT*, wild-type Indian mustard; *APS*, adenosine triphosphate sulfurylase overexpressing Indian mustard; *GS*, glutathione synthetase overexpressing Indian mustard; *γ-ECS*, γ-glutamylcysteine synthetase overexpressing Indian mustard. The *letters above the bars* indicate statistically significant groups (*t* test comparing each pair, $\alpha = 0.05$) (from Bennett et al. 2003)

and 1.5–2-fold more Zn compared with wild-type Indian mustard. Furthermore, the ECS transgenics accumulated 2.4–3-fold more Cr, Cu, and Pb relative to WT.

Recently, Reisinger et al. (2008) analyzed the effects of γ-ECS or GS overexpression on tolerance to and accumulation of other metal(loid)s supplied to Indian mustard (*B. juncea* L.) individually in agar medium (seedlings) or in hydroponics (mature plants). Also, as pollution in nature generally consists of mixtures of metals, glutamylcysteine synthetase (ECS) and GS seedlings were tested on combinations of metals. Compared to wild-type plants, the ECS and GS transgenics exhibited significantly higher capacities to tolerate and accumulate a variety of metal(loid)s (particularly As, Cd, and Cr) as well as mixed-metal combinations (As, Cd, Zn/As, Pb, and Zn). This enhanced metal tolerance and accumulation of the ECS and GS transgenics may be attributed to enhanced production of PCs, sustained by a greater availability of GSH as substrate, as suggested by their higher concentrations of GSH, PC2, PC3, and PC4 as compared to wild-type plants. Overexpression of GS and γ-ECS may represent a promising strategy for the development of plants with enhanced phytoremediation capacities for mixtures of metals.

Transgenic poplars showed elevated heavy metal uptake as compared to the untransformed clones (Bittsánszky et al. 2005). Treatments with Zn^{2+} strongly induced the activity of the enzyme glutathione *S*-transferase in untransformed poplar lines, but to a lesser extent in the transgenic clones. These results suggest that transgenic poplars are more suitable for the phytoremediation of soils contaminated with Zn^{2+} than wild-type plants.The transgenic poplar cyt-ECS (ggs11) clone, when stimulated by the presence of Zn, showed elevated heavy metal (Cu) uptake as compared to the untransformed clone (Gyulai et al. 2005). These results suggest that gshI-transgenic poplars may be suitable for the phytoremediation of soils contaminated with zinc and copper. The studies are carried out with grey poplar (*Populus tremula* x *P. alba*), wild-type plants, and plants overexpressing the gene for γ-glutamylcysteine synthetase (gshI) from *Escherichia coli* in the cytosol (Peuke and Rennenberg 2005). The expression of this gene in poplar leads to two- to fourfold enhanced GSH concentrations in the leaves. In greenhouse experiments performed under controlled conditions, these transgenic poplars showed high potential for taking up and detoxifying heavy metals and pesticides.

Overexpressing the gene encoding selenocysteine methyltransferase from the selenium hyperaccumulator *Astralagus bisulcatus* in Indian mustard (*B. juncea*) significantly increased selenium accumulation and volatilization (Banuelos et al. 2005). Wild-type Indian mustard and the Se hyperaccumulator *Stanleya pinnata* were included for comparison (Van Huysen et al. 2004). After ten weeks on Se soil, the APS transgenics contained 2.5-fold higher shoot Se levels than wild-type Indian mustard, similar to those of *S. pinnata*. The CGS transgenics contained 40% lower shoot Se levels than the wild type. Shoot biomass was comparable for all Indian mustard types and was higher than for *S. pinnata*.

However, the use of traditional breeding approaches to improve metal hyperaccumulator species and incorporate significant traits related to metal tolerance and uptake characteristics into plants producing high biomass has been proposed.

Partial success has been claimed in the literature. Somatic hybrids between *T. caerulescens* and the high-biomass crop oilseed rape (*B. napus*) produced a larger biomass than *T. caerulescens* and had an erect growth habit that is suitable for mechanical harvesting (Brewer et al. 1999). The hybrids were able to accumulate and tolerate Zn and Cd at levels that are toxic to *B. napus*, although their ability to accumulate metals appeared to be lower than that of *T. caerulescens.* Somatic hybrids capable of removing significant amounts of Pb were also obtained from *B. juncea* and *T. caerulescens* (Gleba et al. 1999).

Soil Fertilization

Phytoremediation is essentially an agronomic approach, and its success ultimately depends on agronomic practices applied at the site. Chaney et al. (1999) investigated the effect of soil acidification on Zn and Cd phytoextraction, and proposed the use of $(NH_4)_2SO_4$ as a soil additive to provide nutrients (N and S) that are needed for high yield and to acidify the soil for greater metal bioavailability. It should be noted that there can be some negative side effects associated with soil acidification. For example, due to increased solubility, some toxic metals may leach into the groundwater, creating an additional environmental risk. Chaney et al. (1999) indicated that, following metal phytoextraction, soil can be limed to elevate the pH so that it is close to neutral, meaning that normal farm uses or ecosystem development can resume. However, premature liming may increase the soil's capacity for metal binding and restrict the potential for phytoextraction. A similar effect can be expected following the addition of organic fertilizers. Correction of soil pH through amendments might also lead to higher metal phytoextractability. Thus, liming has a contradictory role to play in phytoextraction by hyperaccumulators. On the one hand, correcting pH has been found to increase Ni uptake by *Alyssum* species (Kukier et al. 2004) (Table 18.7), while increasing the pH in acid soil is likely to reduce solution concentrations of heavy metal cations, as dictated by the solid-solution equilibria of the respective metals, which could limit metal accessibility. For example, raising the pH may stimulate the formation of metal hydroxy ions, such as $ZnOH^+$, which is more strongly sorbed to soil solids than the uncomplexed ions.

Phosphorus is a major nutrient, and plants respond favorably to the application of P fertilizer by increasing biomass production. The addition of P fertilizer, however, can also inhibit the uptake of some major metal contaminants, such as Pb, due to metal precipitation as pyromorphite and chloropyromorphite (Chaney et al. 2000). This underlines the importance of finding new approaches for P application. Such an alternative may be foliage application. This method can lead to an improvement in plant P status without inhibiting Pb mobility in soil. Since it is a P analog, arsenic is taken up by plants via the P transport system (Meharg and Hartley-Whitaker 2002). Thus, it is expected that P and As will compete for uptake by *P. vittata.* The P contents in *P. vittata* fronds were affected by both the timing of P application and plant age. A single P addition favored arsenic

Table 18.7 Increase in phytoextractability of nickel resulting from soil amendments aimed at correcting soil pH (from Kukier et al. 2004)

		Phytoextracted nickel (mg/kg soil)	
Treatment	Soil pH	*Alyssum murale*	*Allysum corsicum*
Control	5.2	37.6[b]	26.3[b]
Limed 1	5.7	39.8[b]	50.8[b]
Limed 2	6.5	67.1[a]	93.3[a]
Calcareous	7.6	83.7[a]	110.1[a]
Calcareous + HFO	7.7	74.8[a]	89.6[a]

Values in the same column with the same *superscripts* are statistically similar
The two rates of *liming* (1 and 2) arrive at different soil pH values
Calcareous denotes the ratio $CaCO_3$:$MgCO_3$ = 5:1
HFO, hydrous ferric oxide

accumulation in the roots, while split-P addition increased frond arsenic accumulation. Young ferns (A45d) in treatment P134+66 were the most efficient at removing arsenic, reducing it to below 10 mg L^{-1} in 35 days. The results indicated that the use of young ferns coupled with the application of low initial P or split-P application increases the efficiency of arsenic removal by *P. vittata* (Santos et al. 2008). However, it was also reported that neither the addition of 50 mg P kg^{-1} soil nor liming (4.6 g $CaCO_3$ kg^{-1} soil) was found to affect the As concentration in the fronds of *P. vittata*, even though phosphate addition increased the As concentration in the soil pore water (Caille et al. 2004).

Nitrogen application had no effect on plant Cu concentrations, but the addition of P slightly decreased plant Cu concentrations, likely due to a dilution effect resulting from the increase in yield (Wu et al. 2004). Among the treatments, N and P applied at 100 and 200 mg kg^{-1}, respectively, with no K application resulted in the highest Cu uptake. Thus, a combination of low N and high P produced a yield increase in Indian mustard that was more than adequate to compensate for a slight decrease in Cu concentration, resulting in the highest Cu removal from the contaminated soil. Phytoextraction techniques (for Pb) utilizing a sterile strain of vetiver grass (*Vetiveria zizanoides*) yielded better results when the plants were fertilized with Osmocote® fertilizer in comparison to plants fertilized with 10–10–10 (NPK) fertilizer (Wilde et al. 2005).

Maize exhibited better results than poplar when extracting Pb from acidic (pH 4) and contaminated (up to 1,360 mg Pb kg^{-1}) agricultural soils originating from a smelting area (Michael et al. 2007). On the other hand, poplars proved to be more efficient when grown on near-neutral (pH 6) and less contaminated (up to 200 mg Pb kg^{-1}) agricultural soil originating from the mining area.

Enhancement of Metal Bioavailability

In soil, metals exist as a variety of chemical species in a dynamic equilibrium governed by soil physical, chemical, and biological properties (Chaney 1988). A major

factor limiting metal uptake into roots is slow transport from soil particles to root surfaces (Nye and Tinker 1977; Barber 1984). In soil, metal solubility is restricted due to adsorption to soil particles. Some of the soil-binding sites are not particularly selective. For example, they bind Cd as strongly as Ca. Nonspecific binding occurs at clay-cation exchange sites and carboxylic groups associated with soil organic matter. Other sites are more selective and bind Cd more strongly than Ca. For example, most clay particles are covered with a thin layer of hydrous Fe, Mn, and Al oxides. These selective sites maintain Cd activity in the soil solution at low levels (Chaney 1988). Lead, a major contaminant, is notorious for its lack of soil mobility, primarily due to metal precipitation as insoluble phosphates, carbonates, and (hydr)oxides (Blaylock and Huang 2000). Thus, increasing metal solubility in the soil is an important prerequisite for enhancing the potential for Pb phytoextraction. Therefore, to achieve efficient phytoextraction, the heavy metals should be made available in the soil solution for their further uptake by plants. Several approaches that are used to enhance the bioavailability of heavy metals are discussed below.

Chemically Enhanced Phytoextraction

Two approaches have been proposed for phytoextracting heavy metals, namely continuous or natural phytoextraction and chemically enhanced phytoextraction (Salt et al. 1998). The first is based on the use of natural hyperaccumulator plants with exceptional metal-accumulating capacities (Baker et al. 2000). On the other hand, many hyperaccumulator plants tend to be slow-growing and produce low biomass. With the plant materials currently available, years or decades are needed to clean up a contaminated site. For instance, McGrath et al. (1993), using field data, calculated that nine harvests of *T. caerulescens* would be required to decrease the Zn concentration in the soil from 440 to 300 mg Zn kg^{-1}. Similarly, Brown et al. (1994) estimated that 28 years of *T. caerulescens* cultivation would be necessary to remove all of the Zn from a soil containing 2,100 mg Zn kg^{-1}. Another problem with the continuous phytoextraction of metals from soils is that some metals such as Pb are largely immobile in soil, and so their extraction rate is limited by solubility and diffusion to the root surface.

The use of soil amendments such as chelators has been attempted in order to enhance the phytoextractability of metals, thus assisting in their hyperaccumulation. This approach makes use of high-biomass crops that are induced to take up large amounts of metals when their mobility in soil is enhanced by chemical treatments. Several chelating agents, such as citric acid, EDTA (ethylenediaminetetraacetic acid), CDTA (trans-1, 2-cyclohexylenedinitrilotetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), EGTA (ethyleneglycol-bis(2-aminoethylether)-*N,N,N´,N´*-tetraacetic acid), EDDHA (ethylenediamine-*N*, *N´*-bis (4-hydroxyphenyl) acetic acid), and NTA (nitrilotriacetic acid), have been studied for their ability to mobilize metals and increase metal accumulation in different plant species (Huang et al. 1997; Cooper et al. 1999). Different metals have been targeted, such as Pb (Blaylock et al. 1997; Huang et al. 1997), U (Huang et al.

1996), ^{137}Cs (Lasat et al. 1998), and Au (Anderson et al. 1998). The general order of chelate effectiveness, based on the total Pb desorbed, was HEDTA > CDTA > DTPA > EGTA > HEIDA (*N*-(2-hydroxyethyl)iminodiacetic acid) > EDDHA ~ NTA (Cooper et al. 1999). The application of EDTA, DTPA, CDTA, EGTA, and citric acid to the soil both solubilized the Pb and Cd in the soil and also increased Pb and Cd uptake and translocation to the shoots of *B. juncea* (Fig. 18.6). Among the chelating agents, EDTA and EGTA were found to be promising for enhancing the solubility of Pb and Cd, respectively.

However, at the moment, the most promising application of this technology is for the remediation of Pb-contaminated soils using Indian mustard [*Brassica juncea* (L.) Czern.] in combination with EDTA (Blaylock 2000). Blaylock et al. (1997) used three-week-old seedlings and measured more than 15,000 mg Pb kg^{-1} dry shoot weight in *B. juncea* after the addition of 10 mmol kg^{-1} EDTA. Huang et al. (1997) determined 8,960 mg kg^{-1} and 2,410 mg Pb kg^{-1} in two-week-old pea and corn shoots transplanted into a soil substrate pretreated with 1.5 mmol EDTA kg^{-1}. The EDTA treatment greatly increased the solubility of heavy metals in contaminated soil, but this did not result in a large increase in metal concentrations in the maize shoots. Phytoextraction of Cd and Zn by maize + EDTA was much less than that by *T. caerulescens* from the industrially contaminated soil, and was either less than (Cd) or similar to (Zn) that from the agricultural soil. After EDTA treatment, soluble heavy metals in soil pore water occurred mainly as metal–EDTA complexes, which were persistent for several weeks. High concentrations of heavy metals in soil pore water after EDTA treatment could pose an environmental risk in the form of groundwater contamination.

The mobility of Cu in soil was clearly improved when EDTA was added at the vigorous growth stage. Both the water-extractable and exchangeable Cu concentrations increased significantly following EDTA addition (Wu et al. 2001). Citric acid

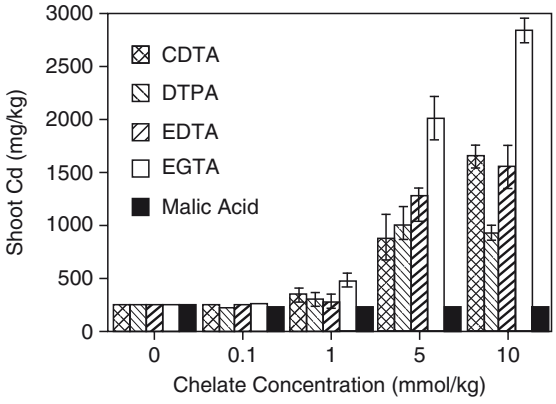

Fig. 18.6 Effect of soil-applied chelating agents on shoot Cd concentrations in *Brassica juncea* in a Sassafras Ap soil amended with cadmium carbonate (100 mg of Cd kg^{-1}) and limed to pH 7.3 (from Blaylock et al. 1997)

and malic acid only had an effect on water-extractable Cu; they had no effect on Cu uptake by the plant. EDTA significantly increased the concentrations of Cu in plant leaves and roots and the Cu uptake by *B. juncea*. EDTA was found to be better than low molecular weight organic acids (citric, oxalic, and tartaric acids) at phytoextracting Cu and Pb from soil with tobacco (*N. tabacum)* (Evangelou et al. 2006).

Humic acids added at a rate of 2 g kg^{-1} soil increased the cadmium concentration in the shoots of tobacco (*N. tabacum*) SR-1 from 30.9 to 39.9 mg kg^{-1} (Evangelou et al. 2004). A possible reason for this enhancement is the resulting decrease in pH, which led to higher cadmium availability. Another possibility is that plants may take up cadmium complexes with humic acid fragments resulting from microbiological degradation or self-dissociation.

The application of a chelating agent, EDTA, one week prior to harvesting of vetiver grass *V. Zizanioides* significantly increased the amount of Pb that was phytoextracted (Wilde et al. 2005). Lead concentrations of up to 1,390–1,450 mg kg^{-1} in tissue samples were detected. Maximum Pb levels were observed in root tissues. The study indicated that the use of vetiver grass coupled with the use of chelating soil amendments has considerable potential for use as a remedial strategy for Pb-contaminated soils such as those associated with firing ranges.

In a treatment with 10 mmol kg^{-1} EDDS, Pb, Zn, and Cd concentrations of 1053 ± 125, 211 ± 16, and 5.4 ± 0.8 mg kg^{-1}, respectively, were measured in the biomass of *Cannabis sativa*, and these were 105, 2.3, and 31.7 times higher, respectively, than in the control treatment (Kos et al. 2003). The calculated Pb phytoextraction potential of *C. sativa* amounted to 26.3 kg EDTA ha^{-1}. was more efficient than EDDS at desorbing and complexing Pb from both soils, removing as much as 60% of Pb (Michael et al. 2007). EDTA led to a significant increase in Pb content, especially in poplar leaves, proving that there was a strong translocation rate within the poplar plants. Addition of EDDS increased the amounts of soluble Cu and Zn in the soil that were taken up by *Elsholtzia splendens*, concentrated in the xylem sap, and translocated from roots to stems and leaves (Wu et al. 2007). EDDS exerted greater effects at the end of the vegetative growth stage than at the start of the flowering or reproductive stages.

Despite the success of this technology, some concerns have been expressed regarding the enhanced mobility of metals in soil and their potential risk of leaching into ground water (Cooper et al. 1999).

Microbially Enhanced Phytoextraction

It is an established fact that rhizospheric soil supports a larger microbial population than the bulk soil, and these microorganisms possess mechanisms capable of altering the environmental mobilities of metal contaminants, which has subsequent effects on the potential for root uptake. For example, microorganisms are capable of creating acidification by excreting H^+ ions in the rhizosphere, which enhances the availability of heavy metals. In addition, some microorganisms may excrete organic compounds (e.g., organic acids) that increase bioavailability and facilitate root

absorption of essential metals, such as Fe (Crowley et al. 1991) and Mn (Barber and Lee 1974), as well as nonessential metals, such as Cd (Salt et al. 1995). Certain hyperaccumulating *Brassica* species are also capable of capturing heavy metals through the excretion of low molecular organic acids like succinic and oxalic acids (Chhonkar et al. 2005). Several strains of *Pseudomonas* and *Bacillus* were capable of increasing the total amount of Cd accumulated from a hydroponic solution by two-week-old *B. juncea* seedlings. Rhizospheric microorganisms may interact symbiotically with the roots of phytoextracting plants to enhance the potential for metal uptake. In this respect, the establishment of arbuscular mycorrhizal fungi (AMF) in the roots of phytoextracting plants has the potential to enhance root surface area and stimulate the acquisition of heavy metals from contaminated soils. Examples of the mycorrhizal fungal species identified in metal-rich soils are *Glomus*, *Gigaspora*, and *Eutrophosphora*.

In maize (*Zea mays*), mycorrhizal colonization by *Glomus mosseae* accounted for up to 41% of the total Cd uptake and 19% of the total Cu uptake, while the same AMF contributed 37% of total Cd uptake and 33% of total Cu uptake by bean plants (*Phaseolus vulgaris* L.) (Guo and Marschner 1996). A microbial inoculum consisting of *Gigaspora margarita* ZJ37, *Gigaspora decipens* ZJ38, *Scutellospora gilmori* ZJ39, *Acaulospora* spp., and *Glomus* spp. was able to increase Cu, Zn, Pb, and Cd uptake into shoots and roots of maize plants (Wang et al. 2007). Sunflower (*Helianthus annuus* L.) plants associated with *Glomus intraradices* were less sensitive to Cd stress than nonmycorrhizal plants (Andrade et al. 2008). Mycorrhizal colonization significantly increased root uranium (U) concentrations at both harvests. Root colonization with *G. mosseae* or *G. intraradices* led to an increase in transfer factor (ratio of metal content in shoot to that in root) values for uranium (U) from 7 (noninoculation control) to 14 at the first harvest. The highest U concentration of 1,574 mg kg^{-1} was recorded in roots colonized by *G. mosseae* at the second harvest. Arbuscular mycorrhizal fungi increased As uptake across a range of P levels, while P uptake was generally increased only when there was no As amendment (Agely et al. 2005). These data indicate that AM fungi play an important role in arsenic accumulation by Chinese brake fern (*P. vittata*) (Fig. 18.7). Therefore, to effectively phytoremediate As-contaminated soils, the mycorrhizal status of ferns needs to be taken into account. Poplar clones have also shown variable degrees of colonization by AMF, suggesting differential host susceptibility or mycorrhizal dependency (Takács et al. 2005).

18.3.1.4 Disposal of Contaminated Plant Residues

One concern associated with the application of phytotechnology is the handling and disposal of contaminated plant waste. The need to harvest contaminated biomass, and possibly dispose of it as hazardous waste subject to RCRA standards, creates an added cost and represents a potential drawback to the technology. One option is the disposal of contaminated biomass in a regulated landfill. To decrease handling, processing, and potential landfilling costs, the waste volume can be reduced by

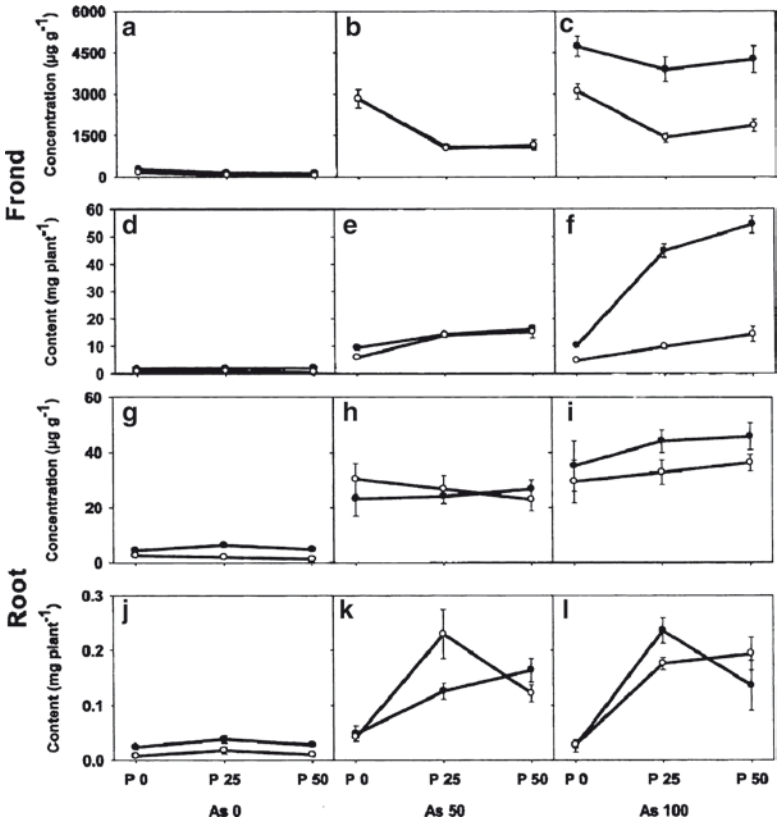

Fig. 18.7 Effect of As and P amendments (mg kg^{-1}) on As accumulation by Chinese brake fern (*P vittata* L.) grown in the presence (*closed circle*) or absence (*open circle*) of mycorrhizal inoculum: frond concentration (*A–C*) and content (*D–F*); root concentration (*G–I*) and content (*J–L*). *Data points* represent means of five replicates ± SEM (from Agely et al. 2005)

thermal, microbial, physical, or chemical means. With some metals (Ni, Zn, and Cu), the value of the reclaimed metal may provide an additional incentive for phytoextraction. Chaney et al. (1999) proposed the incineration of plant biomass to further concentrate the bio-ore. These authors showed that the value of the metal recovered in the biomass could offset the cost of the technology. Furthermore, Watanabe (1997) showed that Zn and Cd, recovered from a typically contaminated site, could have a resale value of \$1,060 ha^{-1}. Phytoextraction is typically less costly than excavation; however, actual costs depend on site-specific conditions. Current estimates range from \$16 to \$62 per cubic yard of soil treated. The harvested biomass would be analyzed and disposed of according to its composition. Disposal could involve air drying, processing for volume reduction (cutting and baling, or composting as appropriate), and, ultimately, either landfilling or incinerating in approved facilities.

18.3.1.5 Making Phytoremediation More Efficient

Major approaches to make phytoremediation more efficient include:

- Screening to identify the most suitable plant species or varieties
- Optimizing agronomic/management practices to maximize biomass production and metal uptake
- Creating more efficient hyperaccumulators from the selected species by conventional breeding or genetic engineering.

The role of the soil scientist is highly specific in the first two approaches. While the screening of species is a never-ending process, as new taxa are regularly reported, these seem to be location and soil specific. Therefore, one should depend more on local vegetation resources and not necessarily on reported species.

18.3.1.6 Prospects and Potential of Phytoremediation

Recent research and development activities have resulted in the development of novel technologies for combating pollution to make this planet a much better place to live. Many laboratories are now starting to perform research into phytoremediation, and many agriculturally important species still await the discovery of their hyperaccumulating potential so that they may be used to tackle the issue of cleaning up the environment. Phytoremediation is cost effective and can remediate a site without dramatically disturbing the landscape or causing any type of intrusion. It can fulfill the need for the physical removal of heavy metals from the contaminated site, which is impossible through other alternatives. The advantages of this technology over other approaches to decontamination are:

- Aesthetically pleasing and accepted by the public
- Relatively inexpensive
- Less disruptive to the remediation site
- Creates a beneficial habitat for wildlife
- Plant roots and shoots can take up heavy metals, thus realizing the physical removal of toxicity
- No/marginal accumulation in the edible parts of hyperaccumulating plants, thus keeping consumers safe

Although phytoremediation is time consuming and may require several years before contaminant concentrations are significantly lowered, vegetation technology can reduce the total contaminant concentration at minimal cost. The scope of this technology can be extended to phytomining in order to extract metals from soils or ores that are not economic to mine conventionally (Brooks et al. 1999). Improvements such as software for the design and implementation of phytoremediation have revolutionized this green cure technology (Fleisher et al. 1997). Moreover, it is a

fact that many of the remediation techniques currently in use will lose economic favor and public acceptance in the near future. Therefore, new technologies like phytoremediation based on eco-friendly and low-cost processes will be needed for the biosphere for ecosystem sustainability. We are now in an era where plants ranging from pennycress to popular trees are proving their worth as clean-up tools. Thus, it is clear that the remarkable potential of green plants to accumulate elements and compounds from the environment will provide a green cure technology for the agro-ecosystem in the future.

18.3.1.7 Future Opportunities

Future work will involve genetic engineering to further improve metal-uptake characteristics, assuming that the genes for metal accumulation can be identified and manipulated. The possibility of transferring genes for metal hyperaccumulation into a very productive (but inedible) sterile host plant then exists. Excellent opportunities also exist through protoplast fusion techniques. Very few hyperaccumulator plants have been discovered to date that have the capacity for multiple metal accumulation. Some, whilst primarily accumulating a single metal, do also show enhanced uptake of others. However, there is some experimental evidence to indicate that metal antagonism may limit uptake from multiply metal-contaminated soils. Increasing systematic efforts to screen plant materials for these characteristics will most certainly reveal new hyperaccumulator plants, and thus new potentials for phytoextraction, phytomining, and biorecovery.

18.4 Conclusion

Phytoremediation is a fast-emerging field. Phytoremediation, and especially phytoextraction, is becoming more popular as a method of remediating heavy metal contaminated soil. The success of a phytoextraction technique is largely dependent on the continuous availability of the metal of interest to the phytoextracting plants. A lot of research has already been initiated that is aimed at increasing the bioavailability of metals through chemical amendment. As chemical amendment is a costly input, the thrust of research should be to look for other economically efficient and locally available organic amendments. Research should focus on identifying remediating plants that are adapted to the local climate and soil conditions. As phytoremediation is a slow process, biotechnological as well as classical hybridization techniques should be used to develop more efficient metal hyperaccumulator plant species. In order to develop successful phytoremediation techniques, there is an urgent need for plant physiologists, biotechnologists, geneticists, agronomists, and soil scientists to work together.

To make phytoremediation more popular among farmers in developing countries, it is essential to look for plants that are used to produce timber or non-edible oilseeds. This approach is needed to ensure that farmers receive some income from their precious lands. Nevertheless, phytoremediation using such non-edible plant species can restrict the contaminant from being introduced into the food web. The disposal of phytoremediating plants is a serious problem. In this respect, composting and compaction can be treated as pretreatment steps for volume reduction, but care should be taken to collect leachate resulting from compaction. Among the two methods that significantly reduce contaminated biomass, incineration seems to be least time consuming and more environmentally sound than direct burning or ashing. While several methods of plant disposal have been described, data on these methods are scarce. Further, in order to make phytoremediation more attractive, an economically efficient technique for extracting metals from the ash and recycling them must be developed. There is a need to enhance research efforts on this emerging and environmentally friendly "green" technology.

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Chapter 19
Remediation of Heavy Metal Contaminated Tropical Land

Preeti Saxena and Neelam Misra

19.1 Introduction: What are Heavy Metals?

There are approximately sixty-five elements that may be termed "heavy metals," as they exhibit metallic properties and having atomic weights of between 63.54 and 200.59. Generally, heavy metals have densities above 5 $g\,cm^{-3}$ (Hawkes 1997), and cannot be degraded or destroyed, meaning that they persist in all compartments of the environment. However, some heavy metals known as "trace metals" (e.g., Cu, Zn, Fe, Ni, Mo, Co) are essential for the growth and metabolism of organisms at low concentrations, and microorganisms possess mechanisms of varying specificity for their intracellular accumulation from the external environment. In contrast, many other heavy metals have no essential biological function (e.g., Pb, Sn, Cd, Al, Hg) but can still be accumulated in biomass and are freely transferred from one organism to another through the food chain.

19.2 Contamination of Land by Heavy Metals

In nature, heavy metals are concentrated in the Earth's core. The Earth has a three-layered structure that can be compared to hard-boiled egg, with the yolk surrounded by the white and then by the shell. Deep inside the Earth is a heavy metallic sphere called the core. The Earth's core is divided into two parts, the outer core and the inner core. Lighter elements such as aluminum and silicon float to the outer core while heavier molten materials, such as the elements iron and nickel, settle in the inner core. Due to geological activity, these metals are redistributed from the core to the Earth's crust (its outer layer) and thus its surface. However, humans affect the

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natural geological and biological distributions of heavy metals by polluting the air, water, and soil. The primary anthropogenic sources of heavy metals are point sources, such as mines, foundries, smelters, and coal-burning power plants, as well as diffuse sources, such as combustion by-products and vehicle emissions. Metal pollutants can be produced through industrial processes such as mining, refining, and electroplating. Electroplating is the primary source of chromium and cadmium. Due to anthropogenic activities, heavy metals are released into the environment and bioaccumulate in the food chain, or they exert toxic effects on specific plants or animals (Goyer 1996). Cadmium has no constructive role in the human body. It and its compounds are extremely toxic, even in low concentrations, and will bioaccumulate in organisms and the ecosystem. Through precipitation or by ion exchange into soils and muds, heavy metal pollutants can localize and lay dormant. Unlike organic pollutants, heavy metals do not decay, and so they pose a different kind of challenge to remediation. Industrial solid waste can be a significant source of heavy metal pollution of the land if they are not treated properly (Saxena et al. 2006). For example, small-scale industry has caused serious environmental problems due to their disposal of heavy metal (Cr, Ni, Cu) rich, low-pH waste in the Wazirpur Industrial Area, Delhi, and the persistence of untreated waste in the open environment. Hence there is a need to protect the environment through the implementation of environmental laws while also facilitating small-scale industry. Regulatory compliance is necessary if we are to implement a sustainable environment (Saxena et al. 2006).

Heavy metal contaminants typically alter plant metabolism, most commonly reducing crop yields. This has a secondary effect upon soil conservation, since the languishing crops cannot shield the soil from erosion phenomena. Some of these chemical contaminants have long half-lives; in other cases, derivative chemicals are formed from the decay of primary soil contaminants. Heavy metals are a very significant component of the environment, and influence flora and fauna in many ways. Many heavy metals are essential for organism growth and metabolism at low concentrations, but are toxic in excess. Plants accumulate both essential as well as nonessential elements in their tissues. The toxicities of a number of heavy metals have been established by many workers (Antonovics et al.; Chaphekar and Shetye 1988; Bhowmik and Sharma 1999) based on agronomic efficiency. A reduction in *Amaranthus* plant height was observed in copper-amended soil. The maximum reduction in size was observed in plants raised on 500 ppm of Cu in soil. A similar toxic effect of copper has been reported in vegetables (Frank et al. 1976; Hara and Sonoda 1979). Elevated concentrations of copper in soils (averaging between 180 and 338 mg kg^{-1}) resulted in reduced biological activity, including microbial activity, earthworm populations, and other processes such as bioturbation and subsequent loss of fertility (Van Zwieten et al. 2004; Dumestre et al. 1999). Elevated Cu concentrations have been shown to reduce beneficial mycorrhizal associations (Liao et al. 2003), to reduce microbial activity and function (Bogomolov et al. 1996), and to impact on a range of mesofauna (Böckl et al. 1998).

Saxena et al. (2006) studied the effect of heavy metal rich sludge generated from metal-finishing industries upon the soil microbiology. Results revealed that

soil pH is the major factor that controls the composition of the microbial community, together with the maintenance demand, as reflected in qCO_2 and the C_{mic}:C_{org} ratio. When soil microflora are exposed for a long time to high metal concentrations (Cr, Ni), their qCO_2 increases, indicating a greater energy requirement for maintenance and C_{mic}:C_{org} ratio due to a reduction in microbial biomass. Brenes and Pearson (1973) established phospholipid-linked fatty acid (PLFA) and ester-linked fatty acid (ELFA) methods to study and understand the impact of heavy metal (pyrite mud) pollution on the microbial community and to assess the effectiveness of the remediation of these polluted soils. He found that a microbial stress marker, monounsaturated fatty acids, was significantly lower for reclaimed and polluted soil compared to unpolluted soils for both PLFA and ELFA extraction. The general fungal marker, the arbuscular mycorrhiza marker, and iso- and anteiso-branched PLFAs (Gram-positive bacteria) were suppressed with increasing pollution, whereas Gram-negative bacteria increased with metal pollution. PLFAs and ELFAs are major cell membrane constituents that rapidly degrade on cell death (Pinkart et al. 2002). Both of these methods have been used to characterize microbial communities from heavy metal contaminated soils (Pennanen et al. 1996; Bååth et al. 1998; Shi et al. 2002; Rajapaksha et al. 2004). However, the only studies of microbial community structure in remediated metal-polluted soils are those of Kelly and Tate (1998) and Kelly et al. (2003), in which a mixture of municipal sewage sludge and power plant fly ash was applied to remediate polluted soil.

19.3 Mobility of Metal Contaminants in Tropical Soil

19.3.1 Tropical Zone Ecology

The tropical zones are the largest of all of the world's sixteen terrestrial ecosystems. Globally, tropical regions can be divided into three major ecosystems: tropical rainforest, tropical semideciduous, and tropical scrub woodland. Tropical rainforest can be found in three major geographical areas around the world: Central America (the Amazon river basin); Africa (Zaire basin, along with a small area of West Africa and eastern Madagascar); and Indo-Malaysia (west coast of India, Assam and Southeast Asia, New Guinea, and Queensland, Australia) (Fig. 19.1).

The climate in a tropical rainforest is typically very humid because of its high rainfall: about 150 cm of rain per year. The climate of tropical rainforest is very hot and wet because it is found near to the equator and so it is exposed to more direct sunlight than most ecosystems. The main plants in this biome are trees. Rainforest is never found in climates that are exposed to temperatures of 32°F or below because the plant life cannot live in frost. All rainforest plants die at cooler temperatures. The annual average rate of net primary plant production (kcal $m^{-2}\,yr^{-1}$) higher for tropical forest compared to any other terrestrial ecosystem. Tropical rain

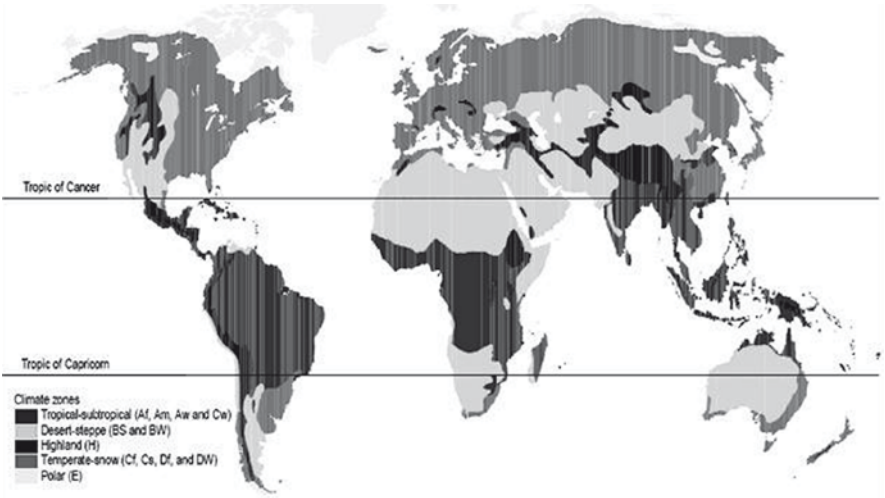

Fig. 19.1 Global ecological regions of tropical zones (from http://www.maps.com)

forests are nearly twice as productive as temperate forests. The most productive systems among the world's biomes are open ocean, tropical rainforest, savanna, and tropical seasonal forest. The total primary plant production of a tropical rainforest is 178 kcal $m^{-2}yr^{-1}$ (Sharma 2007).

19.3.2 Tropical Soil

One-third of the world's soil is located in the tropics, and more than three-quarters of the world's population inhabit this region, and yet more is known about the soil resources of temperate regions (Lal and Sanchez 1992). Tropical soil is classified into four groups according to the dominant clay mineralogy: kaolinitic soils, oxidic soils, allophanic soils, and smectite soils. Kaolinitic soils are deeply weathered, with sand, loamy sand, or sandy loam topsoil and clayey subsoils dominated by kaolinite. Oxidic soils are strongly weathered, red and yellowish, fine-textured soils that typically have low bulk density and large amounts of stable microaggregates. Low water-holding capacity, low fertility, and high P-fixation are the major constraints of such soils. Allophanic soils are dark-colored young soils derived from volcanic ash that exhibit low bulk density, high water retention, and contain predominantly allophanes, imogolite, halloysite and amorphous Al in the clay fraction. Finally, smectite soils are loamy to clayey alluvial soils that contain moderate to large amounts of smectite (Alfred and Hartemink 2004).

19.3.3 Biodiversity of Tropical Land

The tropical region is world's richest in terms of biodiversity. Over geological timescales, the tropics have had a more stable climate than the temperate zones. In the tropics, therefore, local species have continued to confine themselves to this region, whereas in temperate zones they have tended to disperse to other areas. Tropical communities are older than temperate ones and so there has been more time for them to evolve. This could have allowed them greater degree of specialization and local adaptation to occur. The warm temperatures and high humidities of most tropical areas provide favorable conditions for many species that are unable to survive in temperate areas. In the tropics there can be greater pressure from pests, parasites, and disease, which stops any single species from dominating, and so there is the opportunity for many species to coexist. Among plants, rates of outcrossing appear to be higher in the tropics, which can lead to higher levels of genetic variability. Tropical areas also receive more solar energy over the year, so tropical communities are more productive or have a greater resource base that can support a wider range of species (Michael 2001).

19.3.4 Mobility of Heavy Metals in Tropical Land

Heavy metals exist in two forms in nature. As stated above, microbes can convert contaminants to less harmful products; however, they can also immobilize contaminants (National Research Council 2003). Metal immobility is primarily achieved through reactions that cause the metal to precipitate or that keep the metal in a solid phase (Evanko Cynthia and Dzombak 1997). Chemical and physical properties affect the mobility of metals in soils and groundwater. Under acidic conditions (pH between 4.0 and 8.5) metal cations are mobile while anions tend to bind into oxide minerals. At high pH values, cations are adsorbed onto mineral surfaces and metal anions are mobilized. Hydrous metal oxides of iron, aluminum, and manganese can affect metal concentrations because these minerals can remove cations and anions.

A "biocurtain" is a term used to describe a process in which large amounts of biomass stop or slow contaminant movement. The biomass can absorb hydrophobic organic molecules (National Research Council 2003). A large biomass also can hinder the migration of a contaminant. When a microorganism oxidizes or reduces a species, this reaction causes metals to precipitate (National Research Council 2003). Mercury is an example of a metal that can be precipitated. This process begins when mercury (Hg^{2+}) is reduced to mercuric sulfide, causing mercury to transform into a precipitated form. Chromium is another metal that can be converted into a precipitated form through the use of microorganisms. This process involves the reduction of hexavalent chromium (Cr^{6+}) to trivalent chromium (Cr^{3+}), which can then be precipitated as chromium oxides, sulfides, or phosphates

(National Research Council 2003). Current research is focusing on other metal and radioactive contaminants that can undergo precipitation processes.

Soil acidity is a major problem to agriculture in the tropics. Estimates of the world's potentially arable land resources indicate that only 10.6% of the total land area of the world is cultivated while about 24.2% is considered cultivable (US President's Advisory Committee Report 1967; FAO 1991; Buringh et al. 1975). Of these 2.5 billion hectares of potentially cultivable land, 68% is located in the humid tropics (Von Uexküll and Mutert 1995). The acid soils of the tropics, especially those in the savannas, have the greatest potential for future agricultural development (Dunal 1988). On a global scale, there are two main geographical belts of acid soils: the humid northern temperate zone that is covered by coniferous forest, and the humid tropics, which are (or in some cases were) covered mainly by savanna and tropical rainforest. Soil acidification can develop naturally in humid climates when basic cations are leached from soils, but can also be considerably accelerated by certain farming practices and by acid rain (Kennedy 1986). Approximately 43% of the world's tropical land area is classified as acidic, comprising about 68% of tropical America, 38% of tropical Asia, and 27% of tropical Africa (Pandey et al. 1994; Von Uexküll and Mutert 1995). Tropical forests are invaluable with regard to their role in local, regional, and global ecosystems and to the biodiversity found within them (over 90% of plant and animal species live in forest ecosystems). Indiscriminate conversion of tropical forest into agricultural land will have far-reaching ecological consequences; in spite of these consequences, 11 million or so hectares of forest are cleared each year, of which only a small fraction is converted into productive agricultural land, and most of which becomes unproductive grassland (Von Uexküll and Mutert 1995). Policies to use acid soils for agriculture should be directed at the acid savannas of the world such as the Cerrado in Brazil, Los Llanos of Venezuela and Colombia, the savannas in Africa, and the largely anthropic savannas of tropical Asia. These acid savannas cover an area of over 700 million hectares (which is approximately 50% of the global area that is currently under cultivation), and their potential for human and animal food production could account for a large portion of that required to satisfy the needs of the growing population in the next millennium. There are good examples in Brazil and Asia of the successful development of acid savanna into productive land for the cultivation of sugarcane and soybean (Von Uexküll and Mutert 1995). The use of biotechnology could hugely facilitate the conversion of low-productivity acid savannas into productive croplands.

Aluminum toxicity, poor crop productivity, and soil fertility in acid soils are mainly caused by a combination of aluminum and manganese toxicity and nutrient deficiencies (mainly deficiencies in P, Ca, Mg, and K). Among these problems, aluminum toxicity has been identified as the most important constraint on crop production in acid soils. Aluminum toxicity problems are of enormous importance for the production of maize, sorghum, and rice in developing countries located in tropical areas of Asia, Africa, and Latin America. Most of the maize, sorghum, and rice cultivars currently in use are susceptible to toxic aluminum in the soil, and decreases in

yield of up to 80% resulting from aluminum toxicity have been extensively reported in the literature (Brenes and Pearson 1973; Lopes and Cox 1977). In tropical South America, aluminum toxicity is a problem shared by several countries, where about 850 million hectares, or 66% of the region, has acid soils. In Brazil alone, acid savannas with low cation exchange capacity and high toxic aluminum saturation cover 205 million hectares, of which 112 million are suitable for maize and sorghum production (Pandey et al. 1994). Aluminum has a clear toxic effect on roots, disturbing plant metabolism by decreasing mineral nutrition and water absorption. Therefore, crop production in acid soils is, to a great extent, limited by nutrient uptake deficiency caused by the inhibition of root growth and function that results from the toxic effects of Al (Kochian 1995). Moreover, in some acid soils, plant growth is affected not only by aluminum toxicity but also by the low availability of some essential elements, such as P, Ca, Mg, and Fe, some of which form complexes with Al and thus are not readily available for root uptake (Haug 1984). It is well documented that many plant species exhibit significant genetic variability in their ability to tolerate Al. Although it is clear that certain plant genotypes have evolved mechanisms that confer Al resistance, the cellular and molecular basis for Al resistance is still poorly understood (Kochian 1995). Two basic strategies by which plants can tolerate Al have been proposed: (1) the ability to exclude Al entry into the root apex and root hairs, and (2) the development of mechanisms that allow the plant to tolerate toxic concentrations of Al within the cell.

A major environmental concern due to the dispersal of industrial, urban and peri-urban wastes generated by anthropogenic activities is the contamination of agricultural land in tropical regions. Controlled and uncontrolled methods of disposing of waste, accidental and process spillage, mining and smelting of metalliferous ores, and the application of sewage sludge to agricultural soils are responsible for the migration of contaminants into uncontaminated sites as dust or leachate, thus contributing towards the overall contamination of the ecosystem. The tropical regions of the world have the greatest area of agricultural land. However, the population density in such regions is also very high. Hence, remediation of contaminated land is very important in this region in order to maximize the acceptable agricultural land. In the light of these severe problems, this chapter proposes several methods of remediating tropical land.

19.4 Remediation of Heavy Metal Contaminated Tropical Land

The remediation of soils contaminated with metals and radionuclides predominantly involves immobilizing these species to prevent them from spreading further. Various remediation technologies have been applied to remove heavy metals from contaminated land, which are now reviewed.

19.4.1 Physicochemical Remediation

Incineration and soil washing are typical physicochemical soil remediation processes applied to munitions-contaminated soils. In the incineration treatment, the contaminated soil material is burned at 800–1000°C, but this is should be performed at a special plant that ensures that the exhaust fumes are detoxified. Hence, the process itself is very expensive, even ignoring the additional costs of excavation and transportation of the soil material, as incineration is an offsite process. This decontamination is, however, very successful, although the soil material is biologically dead and the texture is destroyed. Soil washing, too, is mostly performed offsite. The contaminated soil material is usually crushed and then washed. To ensure that the contaminants are passed from the soil material to the washing solution, either acids, bases, tensides, organic solvents or physical methods like kinetic energy are used. The contaminants remain in the fine-grain fraction, which must be separated, dried and disposed. Advantages are its relatively low cost compared to incineration, the rapidity of the process, and the preservation of the soil texture. The applicability of the process depends on the characteristics of the toxicant as well as those of the soil material. Good conditions are provided by soluble substances and sandy and gravelly soil materials. Excavation and physical removal of the soil is perhaps the oldest remediation method for contaminated soil. The advantages of excavation include the complete removal of the contaminants and the relatively rapid clean up of a contaminated site (Wood 1997). Disadvantages include the fact that the contaminants are simply moved to a different place, where they must be monitored; the risk of spreading contaminated soil and dust particles during the removal and transportation of contaminated soil; and the relatively high cost. Excavation can be the most expensive option when large amounts of soil must be removed or disposal as hazardous or toxic waste is required.

Stabilizing the heavy metals in the soil is another useful method of minimizing the bioavailability of heavy metals onsite, and has many advantages over excavation. One way of stabilizing heavy metals consists of adding chemicals to the soil that cause the formation of minerals that contain the heavy metals in a form that is not easily absorbed by plants, animals, or people. This method is called in situ fixation or stabilization, and it does not disrupt the environment or generate hazardous wastes. Instead, the heavy metal combines with the added chemical to create a less toxic compound. The heavy metal remains in the soil, but in a form that is much less harmful. One example of the in situ fixation of heavy metals involves adding phosphate fertilizer as a soil amendment to soil that has high amounts of the heavy metal lead. Chemical reactions between the phosphate and the lead cause a mineral called lead pyromorphite to form. Lead pyromorphite and similar minerals called heavy metal phosphates are extremely insoluble in water (Lambert et al. 1997). This has two beneficial effects. The minerals (and the heavy metals) cannot be easily spread by water to pollute streams, lakes, or other groundwater. Also, the heavy metal phosphates are less likely to enter the food chain by being absorbed into plants or animals that may eat soil particles. This method is relatively rapid and takes about the same amount of time as excavation.

19.4.2 Bioremediation of Heavy Metals

Bioremediation can be defined as any process that uses microorganisms or their enzymes to return an environment altered by contaminants to its original condition. There are a number of advantages to bioremediation, which can be employed in areas that cannot be reached easily without excavation. It is well documented that the presence of metals in the soil impacts both the physiology and the ecology of microorganisms by inhibiting a broad range of microbial processes, including methane metabolism, growth, and nitrogen and sulfur conversion. It is known that toxic metal cations can substitute for essential physiological cations within enzymes in organisms, rendering them nonfunctional. Metals also tend to impose oxidative stresses on microorganisms. The extent in which metals tend to inhibit the biodegradation of organic compounds is directly related to the metal speciation – the physical or chemical form of the metal species in the soil, which also governs its toxicity to microorganisms, and therefore its impact on the remediation technique. The physical and chemical state of a metal species can also be influenced by environmental conditions such as the pH, the ionic strength of the water phase, and soil properties, which include ion exchange capacity, clay type and content, and organic matter content. Indeed, when dealing with the remediation of soil contaminated with organic compounds, one must also account for the bioavailability of heavy metals.

Generally, bioremediation technologies are performed either in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site, while ex situ bioremediation involves the removal of the contaminated material to be treated elsewhere. Some examples of in situ bioremediation technologies are composting, bioventing, bioaugmentation and biostimulation, and ex situ soil bioremediation technologies include soil biopiles, landfarming, and bioreactors (Obed and Kenneth 2002).

19.4.2.1 In Situ Bioremediation

In situ bioremediation is defined as treating a soil pollutant without removing the contaminated soil. Because these types of technologies usually do not require the excavation of the contaminated soil, they are less expensive, create less dust, and release of reduced amounts of volatile contaminants. Some in situ technologies are discussed below.

19.4.2.2 Composting

Compost is the decomposed remnants of organic materials (those with plant and animal origins). Compost is used in gardening and agriculture, where it is mixed in with the soil. It improves soil structure, increases the amount of organic matter, and provides nutrients. Compost is a common name for humus, which results from the decomposition of organic matter. In the presence of large amounts of organic matter,

heavy metals are immobilized and do not enter the food chain. Microbes perform most of the decomposition, although larger creatures such as worms and ants also contribute to the process. Decomposition occurs naturally in all but the most hostile environments, such as landfills or extremely arid deserts, which prevent the microbes and other decomposers from thriving.

Composting is the controlled decomposition of organic matter. Rather than allowing nature to take its slow course, a composter provides an optimal environment in which decomposers can thrive. To encourage the most active microbes, the compost pile needs an appropriate mix of the following ingredients: carbon, nitrogen, oxygen (air), and water. Decomposition happens even in the absence of some of these ingredients, but not nearly as quickly and not nearly as pleasantly (for example, the plastic bag of vegetables in your refrigerator is decomposed by microbes, but the absence of air encourages anaerobic microbes that produce disagreeable odors). The most effective decomposers are bacteria and other microorganisms. Also important are fungi, molds, protozoa, and actinomycetes, which are bacteria that look like fungi or mold and often appear as white filaments in decomposing organic matter. At a macroscopic level, earthworms, ants, snails, slugs, millipedes, sow bugs, springtails, and other organisms consume and break down the organic matter. Centipedes and other predators feed upon these decomposers.

The most rapid composting occurs with the ideal ratio (by dry chemical weight) of carbon to nitrogen, which ranges from 25:1 to 30:1. In other words, the ingredients placed in the pile should contain 30 times as much carbon as nitrogen. Since, grass clippings average about 19:1 and dry autumn leaves average about 55:1, mixing equal parts of these by volume approximates the ideal ratio. Commercial-grade composting operations pay strict attention to this ratio. For backyard composters, however, charts of the carbon-to-nitrogen ratios of various ingredients and the calculations required to obtain the ideal mixture can be intimidating, so many rules of thumb exist to guide composters to approximate this mixture. High-carbon sources provide the cellulose needed by the composting bacteria for conversion to sugars and heat. High-nitrogen sources provide the most concentrated protein, which allow the compost bacteria to thrive.

To perform bioremediation using composting, the compost is mixed with the contaminated soil along with a bulking agent such as straw, hay, or corncobs to make it easier to deliver optimum levels of air and water to the microorganisms. The most common designs are static pile composting, mechanically agitated composting, and window composting. In static pile composting, the contaminated soil is placed into piles and aerated with blowers or vacuum pumps. Mechanically agitated composting involves the placement of the contaminated soil in treatment vessels, where it is mixed to achieve aeration. In window composting, the soil is placed in long piles knows as windows and periodically mixed by tractors (Cunningham and Philip 2000). As stated before, the contaminated soil is mixed with a bulking agent or compost to enhance bacterial growth. A typical ratio of soil to compost is 75% contaminated soil to 25% compost. This ratio depends on the soil type and the characteristics and level of contamination. After mixing, the soil is covered to protect it from erosion and to maintain the proper moisture and temperature necessary for bacterial growth.

Compost remediation is known to give fast clean-up results, taking weeks rather than the months needed for other approaches. Allen (1992) revealed that considerable alleviation of hazardous wastes or contaminated plants, soils, and sediments was possible through composting. Compostable substrates (feedstocks) contain metabolizable carbon, which enhance microbial diversity and activity during composting and promote the degradation of xenobiotic organic compounds such as pesticides, PAHs, and PCBs. Metallic pollutants are not degraded during composting but may be converted into organic species that are less bioavailable. Recalcitrant materials, such as organochlorines, may not undergo degradation in composts or in soils, and the effects of forming organic complexes with metallic pollutants may be nonpermanent or short-lived. Ultimately, composting degrades the pollutants to innocuous levels or binds them into innocuous compounds, and has substantial potential for the remediation of polluted materials.

19.4.2.3 Bioventing

Bioventing is a remediation technique that involves the introduction of oxygen into the contaminated soil through injection wells in order to stimulate the growth of indigenous and exogenous microorganisms. This technique is mostly used at sites where contamination consists of light petroleum products. This is due to the fact that light products are more easily biodegraded than heavier petroleum products. As can be seen in Fig. 19.2, bioventing is a soil vapor extraction (SVE) technique in which oxygen is injected into the soil to stimulate the growth of indigenous bacteria as well as the aerobic biodegradation of contaminants. Although the injected airflow is carefully controlled to minimize volatilization, the resulting vapor by-products from the

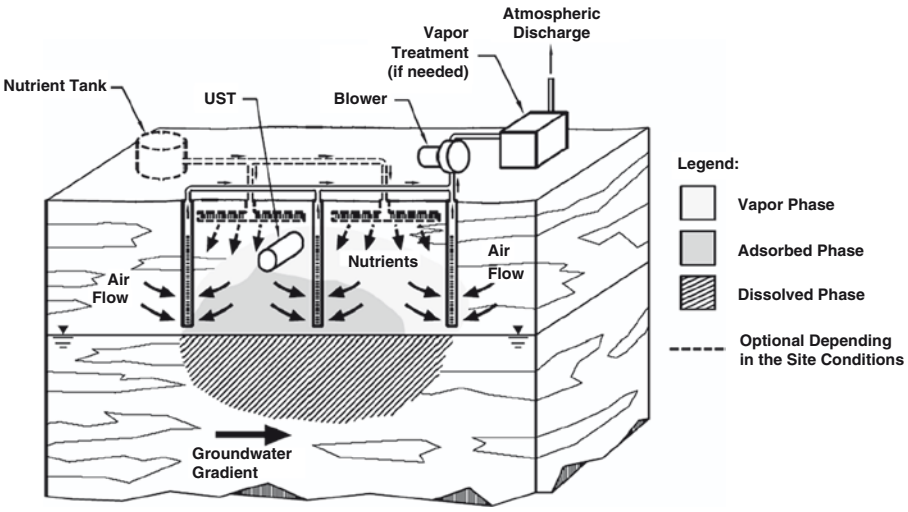

Fig. 19.2 Bioventing (from FRTR 2000)

biodegradation of pollutants are extracted by means of extraction wells. This vapor is then treated and atmospherically discharged. Nutrients can also be added to the soil to stimulate the growth and metabolism of the indigenous species. Although bioventing is an effective remediation technique, it cannot be used in sites where the depth to the groundwater table is less than three meters. This is because groundwater upwelling can occur within bioventing wells under vacuum pressures, provoking the elimination of vacuum-induced soil vapor flow (USEPA 2003).

19.4.2.4 Bioaugmentation

Bioaugmentation refers to the use of a microbial strain that occurs naturally in the contaminated soil or the introduction of a genetically engineered variant in order to achieve soil bioremediation. Usually, the first step involves studying the indigenous varieties present in the contaminated soil. If the indigenous varieties do not have the metabolic machinery to perform the remediation process, exogenous varieties (or enzymes) that do have it are introduced. This process is usually used to remove by-products of raw materials and waste. Bacteria are the agents most commonly used in this degradation process.

19.4.2.5 Biostimulation

Biostimulation involves the introduction of nutrients or substrates such as fertilizers, to stimulate the growth and metabolism of the indigenous species performing the biodegradation of the pollutant. Substrates containing nitrogen and phosphorus are the most popular of these stimulants due to their electron-accepting capabilities.

19.4.2.6 Ex Situ Bioremediation

One of the main advantages of ex situ bioremediation is that it requires less time than in situ treatment. Another advantage is the certainty over the outcome of the treatment due to the ability to uniformly screen, homogenize, and mix the soil. These factors have made this one of the most commonly used treatment approaches. However, ex situ bioremediation involves the excavation of the contaminated soil and its subsequent treatment elsewhere, which makes it less cost-effective. Ex situ treatment technologies include slurry-phase bioremediation and solid-phase bioremediation.

Slurry-Phase Bioremediation

Slurry-phase bioremediation, also known as the bioreactor method, is a controlled treatment that involves excavating the contaminated soil, mixing it with water, and

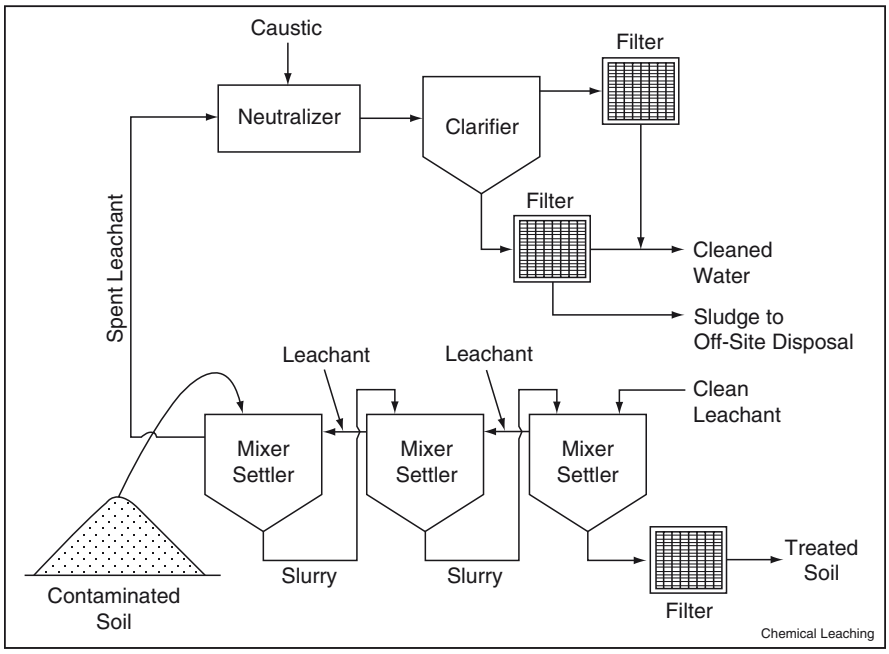

Fig. 19.3 Bioreactor system (from FRTR 2000)

placing it in a bioreactor. Figure 19.3 shows a typical bioreactor system. As shown in the figure, the method involves processing the soil to achieve a low viscosity. This processing involves the separation of stones and rubbles from the contaminated soil. Next, the soil is mixed with a predetermined amount of water to form the slurry. The concentration of water added depends on the concentration of pollutants, the rate of biodegradation, and the physical nature of the soil (USEPA 2003). When this is done, the soil is removed and dried using pressure filters, vacuum filters, or centrifuges. The final procedure is the disposition of the soil and the further treatment of the resulting fluids.

Solid-Phase Bioremediation

Solid-Phase Bioremediation is an ex situ technology in which the contaminated soil is excavated and placed in piles. Bacterial growth is stimulated through a network of pipes that are distributed throughout the piles. By pulling air through the pipes, ventilation is provided for microbial respiration. Moisture is introduced by spraying the soil with water. Solid-phase systems require a large amount of space, and the clean up requires more time than slurry-phase processes (USEPA 2001). Some solid-phase treatment processes include land farming, soil biopiles, and composting.

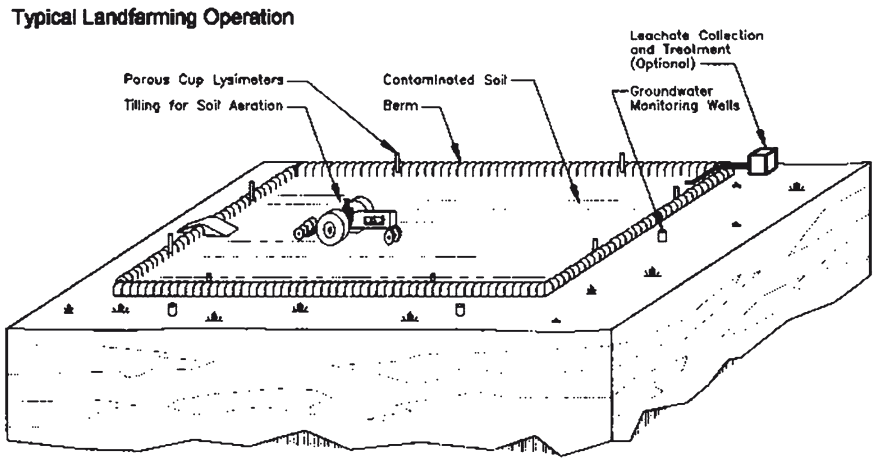

Fig. 19.4 Landfarming (from FRTR 2000)

Landfarming

Landfarming, also known as land treatment, is a bioremediation technique that involves excavating the contaminated soil and spreading it on a thin surface. Biodegradation of pollutants is stimulated aerobically by tilling or plowing the soil. Nutrients and minerals are also added to promote the growth of the indigenous species. Figure 19.4 is a schematic representation of the landfarming system. According to US Environmental Protection Agency report on underground storage tanks (USEPA 2003), before the remediation can take place, the site must be prepared by clearing and grading the soil, installing leachate collection and treatment systems, and building vapor treatment facilities. Also, the report states that if the soil is contaminated to a depth of less than three feet then there is no need for excavation. As can be seen in the figure, soil moisture is controlled by periodically sprinkling the soil with water, and erosion is controlled by erecting barriers or terraces around the contaminated soil. Sprinkling with water also minimizes the dust created while tilling the soil to promote aeration.

Soil Biopiles

Soil biopiles, also known as biocells, is a biodegradation technique used for the remediation of excavated soil contaminated with petroleum products. This technology involves the accumulation of contaminated soil into piles and the stimulation of microbial activity either aerobically or through the addition of nutrients, minerals, or moisture.

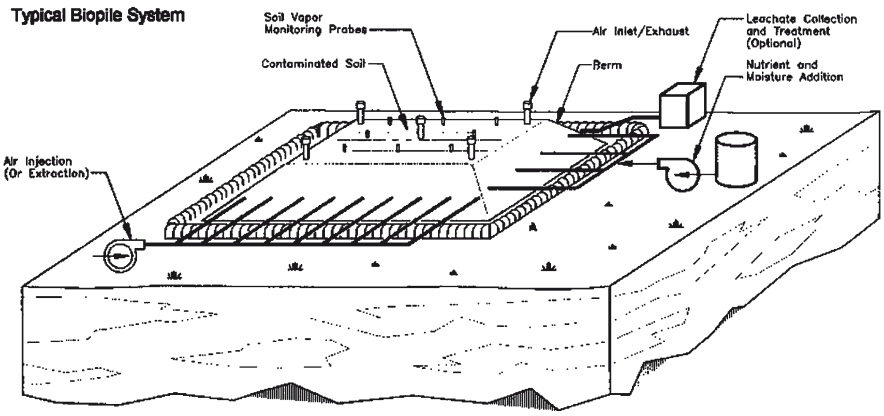

Fig. 19.5 Biopile system (from FRTR 2000)

The biopiles are typically between three and ten feet high. This technique is similar to the landfarm method due to the fact that it also uses oxygen as a way to stimulate bacterial growth. However, while tilling or plowing is used to aerate land farms, biopiles are aerated by injecting the air through perforated piping placed throughout the pile (USEPA 2003). A schematic of this technology can be seen in Fig. 19.5. As can be seen in the figure, the contaminated soil is piled up to a depth of a few feet and then the piping is laid down. The next load of contaminated soil is then added. This process continues until the desired pile height is achieved. The soil is usually mixed with a bulking agent (straw) to improve aeration and thus enhance the growth of the microbial population. Since air is also injected into the soil there is also the possibility of the evaporation or volatilization of contaminants. To counter this problem, the system also incorporates the monitoring and containment of soil vapors.

19.5 Mechanism of the Remediation of Heavy Metal Contaminated Soil Using Microbes

Land pollution in the tropics is caused by industrial activities such as mining, refining, and electroplating. Due to the acidity of the soils in this region, microbes can easily mobilize metals that cause serious ecological risks. Therefore, the methylation, complexation, and changes in valence state of heavy metals are currently being studied in order to reduce the mobility and bioavailability of metals (Natural and Accelerated Bioremediation Research NABIR 2003). There is a great interest in microorganisms that can transform and/or remove metal contaminants. The remediation of metal contaminated soils often involves five general approaches: metal

isolation, immobilization, mobilization, physical separation, or extraction (Evanko Cynthia and Dzombak 1997). Bioremediation processes involve immobilization or mobilization. A combination of these approaches is often used by industry to treat metal-contaminated sites; combining approaches can be more cost-effective than using just one.

The ability of a microorganism to survive and grow in a metal-contaminated habitat can depend on genetic and/or physiological adaptation. Such physiological changes in the cells of microbes reduce the rate of metal uptake and intracellular metal toxicity, while genetic changes result in the reduced intracellular and extracellular concentrations of the toxic metal species. Tolerance of heavy metals can result from intrinsic properties of the organism, such as the possession of extracellular mucilage or polysaccharides or an impermeable cell wall. A good example is provided by fungi that can grow in saturated $CuSO_4$ (about 1.3 M) and very high concentrations of other heavy metals. Such solutions are very acidic, and these organisms are actually sensitive to submillimolar levels at close to neutral pH. Although fungal abundance and species diversity were found to be reduced in Zn-polluted soil, there was little difference in Zn tolerance between fungi isolated from control or polluted sites, and most achieved 50% growth at 700 μM Zn^{2+}. *Bdellospora, Verticillium*, and *Paecilomyces* sp. were Zn tolerant at the control site, while *Aureobasidium* and *Penicillium* sp. were Zn tolerant at the polluted site (Gadd 1990). Similar findings were obtained from Cu, Ni, Fe, and Co-polluted soils, where fungal populations were not significantly different at polluted or control sites and tolerance was displayed by fungi towards heavy metals. *Penicillium* sp. comprised 60% of the tolerant isolates, followed by *Trichoderma*, *Rhodotorula*, *Oidiodendron*, *Mortierella*, and *Mucor* sp. (Freedman and Hutchinson 1980). Four filamentous fungi were found to be able to remove significant amounts of nickel from a 10 mM Ni solution according to the following order: *Fusarium solani* > *Papulaspora sepedonoides* > *Mucor racemosus* > *Aspergillus flavus* (Saxena et al. 2006). The interactions between microorganisms and metals can be divided into six distinct processes (Mohapatra 2006): (1) intracellular accumulation; (2) cell wall associated metal interactions; (3) metal siderophores; (4) extracellular mobilization/immobilization of metals by bacterial metabolites; (5) extracellular polymer–metal interactions, and; (6) transformation and volatilization of metals.

19.5.1 Intracellular Accumulation

The assimilation of metals by bacteria plays an important role in detoxification. Sigg (1987) presented a probable scenario for intracellular accumulation. Extracellular ligands or ligands attached to the cell wall are thought to bind toxic metals. These ligands transport the complexed metals through the cell wall in a slow transport step. The metals are released inside the cell, incorporated into biochemical pathways, or trapped in an inactive form via complexation with another high-affinity ligand.

Cadmium is accumulated by a large number of organisms. Research by Macaskie et al. (1987) on *Citrobacter* suggests that it is accumulated as cell-bound cadmium phosphate. This is presumably a detoxification mechanism and is similar to the accumulation of lead as $PbHPO_4$ by a different *Citrobacter* sp., as suggested by Aickin et al. (1979). The ability of certain bacterial cells to accumulate metals intracellularly has been exploited in mining practices, particularly in the management of effluent treatment lagoons. Uranium has been shown to accumulate rapidly in cells of *Saccharomyces cerevisiae, Pseudomonas aeruginosa*, and many other species of bacteria, and the process is used when mining low-grade uranium deposits.

19.5.2 Cell Wall Associated Metal Interactions

The binding of metals to cell surfaces plays a dominant role in the distribution of metals in natural waters. In addition, the sorption of metals to living or dead cells is considered a practical solution to many metal contamination problems. Algal surfaces contain functional groups that have been shown to bind metals competitively to many dissolved ligands. It is suggested that carboxylic, amino, thio, hydroxo, and hydroxy-carboxylic groups on the surfaces of phytoplankton cells interact coordinately with metal ions and are responsible for the complexation of many active metal species on the cell surface. Bacteria possess lipopolysaccharides (LPSs) in their outer membranes. These chemicals are extremely complex, consisting of a hydrophobic, phosphorylated section known as lipid A (a core oligosaccharide), and various O-specific side chains consisting of a number of unusual sugars. The side chains project out from the cell membrane and contain different functional groups capable of binding metals. The phosphoryl groups of LPSs and phospholipids are the most abundant electronegative sites available for metal binding. Polyvalent toxic metals are primarily bound to LPS molecules because of the presence of reactive sites nearby. It has been suggested that this may provide a mechanism for immobilizing toxic metals and preventing their entry into cells, thus protecting many other organisms from metal contamination (Ford and Mitchell 1992).

19.5.3 Siderophores

Siderophores are low molecular weight organic compounds that have the ability to concentrate iron in environments where the Fe concentration is low and to facilitate its transport into the cell. This is accomplished due to the very strong affinity of these molecules for iron. They can be classified into two major types: hydroxamate and catecholate siderophores. Siderophores are more active at the microbial cell surface under iron-deficient conditions. However, analogs can also be strongly bound by siderophores. For example, Al, Ga, and Cr form trivalent metal ions of a similar size to Fe. Al is a specific competitor to Fe when binding to catecholate

siderophores. Like Fe, Mo and Cu also form strong complexes with siderophores, resulting in the accumulation of these metals. In particular, complexes between catecholate siderophores and molybdenum may provide an important uptake mechanism for intracellular Mo, which is required for the enzyme nitrogenase. Copper complexion with both hydroxamate and catecholate siderophores has been reported, and may be important to the use of Cu in the production of tyrosinase (Ford and Mitchell 1992).

19.5.4 Extracellular Mobilization/Immobilization of Metals by Bacterial Metabolites

Mercuric iron has been well studied in many microorganisms, especially bacteria. The mechanism is generally accepted to involve intracellular reduction of Hg^{2+} to Hg^{0} by mercuric reductase, with subsequent volatilization. Mercuric reductases have been isolated from a number of microorganisms, including *E.coli*, *Thiobacillus ferrooxidans, Streptomyces*, and *Caulobacter.* Certain metal-tolerant bacteria use toxic metal species as electron acceptors. In the environment, the transformation of toxic metals is mediated by the methylation of mercury. Many bacteria, including *Clostridium*, *Pseudomonas*, *Bacillus*, *Mycobacterium*, *E. coli, Aerobacter aerogenes*, *Bacillus megaterium*, and many fungi have the ability to methylate mercury. Sulfur-reducing bacteria (SRB) are the most significant of these, and mercury methylation has been found to intensify in sulfate-enriched habitats. Acidification up to a certain limit stimulates SRB activity and hence mercury methylation. In addition to acidity, mercury methylation is enhanced by organic matter availability. The metal is methylated intracellularly by the nonenzymatic transfer of methyl groups from methylcobalamin (vitamin B_{12}).

19.5.5 Extracellular Polymer–Metal Interactions

Many bacteria produce large amounts of extracellular polysaccharides that have ionic properties and thus function as efficient biosorbents for metal cations. Interactions with metal ions are generally considered to be a direct consequence of the presence of negatively charged functional groups on these exopolymers. These groups include pyruvate, phosphate, hydroxyl, succinyl, and uronic acid. pH-dependent binding the positively charged cation to these groups can occur rapidly, with stability constants in excess of those measured for humic substances and other naturally occurring ligands. Extracellular polymers such as those produced by *Zoogloea* sp. are strongly involved in metal removal from sewage treatment processes, and the extraction and removal of polymers from these and other bacterial cultures can greatly reduce biosorption capacities and also increase metal sensitivity. The extracellular matrices act as an efficient barrier and prevent significant entry of

metal ions into the cells. Similar interactions are also likely for cyanobacteria, algae, and fungi that produce extracellular polymers. Many organic metabolites are important in metal detoxification because of their chelating or complexion properties. Organic or inorganic acids produced by microorganisms, including *Thiobacillus*, *Serratia*, *Pseudomonas*, *Penicillium*, and *Aspergillus* are able to extract metals from solid substrate. Citric acid is an efficient metal chelator, whereas oxalic acid can precipitate metals as insoluble oxalates around cell walls and in the external medium. For example, citric acid production by *Penicillium* has been used to extract Zn selectively from industrial waste. Oxalic acid producing fungi often exhibit marked metal tolerance, and this detoxification mechanism is often found in wood-rotting fungi, particularly those exposed to chromated copper arsenate wood preservatives, in *Poria*, as well as in other species such as *Aspergillus niger*, *Penicillium spinulosum*, and *Verticillium psalliotae.*

Certain organisms, particularly the sulfate-reducing bacteria of the genus *Desulfovibrio*, are involved in the formation of sulfide deposits that contain large amounts of metal. Sulfide formation thus leads to metal removal from solution, and this is associated with resistance in a variety of microbes. Metal-resistant strains of *Klebsiella aerogenes* precipitate lead, mercury, or cadmium as insoluble sulfide granules on the outer surfaces of cells, and particles of Ag_2S are deposited on *Thiobacilli* were grown in silver containing sulfide leaching system. Strains of the green algae *Gyanidium caldarium* can grow in acidic water at 45°C containing high concentrations of metal ions. Fe, Cu, Ni, Al and Cr can be removed from solution by precipitation at cell surfaces as metal sulfides. Cells can contain up to 20% metals on a dry weight basis. Yeast can also precipitate metals as sulfides in and around cell walls, and colonies may appear dark brown in the presence of Cu (Gadd 1990). Many other examples of crystallization and precipitation on microbial surfaces are known, with some of these representing resistance mechanisms. Microbes and several bacteria are implicated in the formation of ferromanganese nodules on ocean floors; for example, *Hyphomicrobium*, algae, and fungi promote Mn^{2+} oxidation in a variety of habitats and can become encrusted with manganese oxides. Other bacteria can become encrusted with oxidized iron compounds by metabolism-dependent and independent processes. In metal-resistant strains of *Citrobacter*, one major mechanism of metal uptake is the activity of cell-bound phosphatase, induced by growth in glycerol-2-phosphate, which can precipitate Cd, Pb, Cu, and U as insoluble metal phosphates on the cell surface.

19.5.6 Transformation and Volatilization of Metals

The methylation process is considered a metal mobilization process, as it not only increases the mercury toxicity but also increases the bioavailability of the metal. The volatilization of metal by microbial methylation is another form of transformation. The rates of other transformations, such as ethylation and phenylation, are unlikely to be significant because of the large sizes of the organic groups involved. The volatilization of metal, specifically methylation, increases metal bioavailability.

Methylated metals are more lipophilic. Despite the toxicity of some metals to them, many microbes volatilize metals to facilitate their removal from the environment. Therefore, the methylation of some metals has been used as a bioremediation tactic: the most famous example is the removal of Se from contaminated soil in San Joaquin, CA, USA.

19.6 Enzymatic Transformation of Metals

Microorganisms can carry out chemical transformations of heavy metals, such as oxidation and reduction, methylation and demethylation, and these are important to not only biogeochemical cycling but also many metal resistance mechanisms. Most research into metal transformation has concentrated on the involvement of bacteria in the mercury cycle, which can be represented as:

$$CH_3Hg^+ \underset{\text{Methylation}}{\overset{\text{Demethylation}}{\rightleftharpoons}} Hg^{2+} \underset{\text{Oxid}}{\overset{\text{Redu}}{\rightleftharpoons}}$$

Mercury resistance is a common property of Gram-positive and Gram-negative bacteria, and the determinants are usually plasmid encoded, particularly in Gram-negatives. The most common mercury resistance mechanism employed by bacteria is the enzymatic reduction of Hg^{2+} by cytoplasmic mercuric reductase to metallic HgO, which is less toxic than Hg^{2+}, volatile, and is rapidly lost from the environment. This enzyme has also been found in certain fungi and yeast. Organomercury compounds are enzymatically detoxified by organomercurial lyase, which cleaves the Hg–C bonds of methyl-, ethyl-, and phenylmercury (for example) to form Hg^{2+} and methane, ethane, and benzene, respectively. The Hg^{2+} can be volatilized by mercuric reductase. FAD-containing mercuric reductase is a flavoprotein and is the best-studied metal detoxification enzyme. This enzyme and organomercurial lyase both require an excess of thiol for activity. NADPH is the preferred reducing cofactor for mercuric reductase as well as organomercurial lyase, but in some bacteria NADH is effective. The thiols prevent the formation of NADPH–Hg^{2+} complexes and ensure that Hg^{2+} is present as a dimercaptide. The reaction catalyzed by the mercuric reductase in vitro can be represented as:

$$RS - Hg - SR + NADPH + H^+ \rightarrow HgO + NADP + 2RSH$$

Mercuric reductase is structurally and mechanically related to glutathione reductase and lipoamide dehydrogenase. The mechanism of mercuric reductase probable involves electrons being transferred from NADPH via FAD to reduce the active site cystine, converting it into two cysteine residues with titratable SH groups. One cysteine residue forms a charge transfer complex with FAD. The active site cysteines then reduce Hg^{2+} (which is bound to the C-terminal cysteines), forming HgO.

19.7 Chemical Processes Involved in the Bioremediation of Heavy Metal Contaminated Soils

Bioremediation involves the use of microorganisms to aid with the destruction of contaminants, a process called microbial metabolism. This process involves biochemical reactions or pathways that result in organism activity and growth, as well as the reproduction of the organism. The chemical processes involved in microbial metabolism make use of reactants, contaminants, oxygen, or other electron acceptors to convert metabolites to well-defined products. The microbial metabolism system enables organisms to retrieve carbon, electrons, and other vital components to survive. In some cases, the contaminant may be transformed while the microorganisms seek other sources of energy or carbon. This reaction is described as "cometabolism," indicating that the transformation of the contaminant yields little or no benefit to the cell. Secondary utilization is another way to describe a nonbeneficial biotransformation. This transformation of the contaminant is an incidental reaction that is catalyzed by enzymes present in the cell's metabolic system (National Research Council 2003).

Aerobic respiration is a process that involves the use of oxygen by microorganisms to oxidize sources of carbon that may also exist in the contaminant. Thus, many microorganisms use aerobic respiration as a way to destroy organic contaminants. During the process of aerobic respiration, oxygen is reduced, resulting in the formation of water. Thus, a drop in oxygen concentration occurs when aerobic microbes are active, and this is instrumental in the reproduction of some living organisms. Microorganisms that can live without oxygen use anaerobic respiration as a metabolic process. Unlike aerobic respiration, where oxygen serves as the main electron acceptor, anaerobic respiration uses inorganic compounds such as nitrate, sulfate, and iron as electron acceptors.

Inorganic molecules such as ammonium, nitrite, as well as reduced iron, can serve as electron donors. When these molecules are used as electron donors, they are oxidized and their electrons are transferred to electron acceptors (usually oxygen) to produce energy for cell synthesis. Microorganisms that use inorganic molecules as their primary electron donors must obtain their carbon from carbon dioxide – a process called carbon dioxide fixation. Nitrogen gas, hydrogen sulfide, and reduced forms of metals are other by-products of anaerobic respiration. In circumstances where metals are used as electron acceptors by anaerobic organisms, the metal precipitates, which decreases its concentration and mobility in groundwater (National Research Council 2003). In general, under anaerobic conditions, concentrations of electron acceptors (such as nitrate and sulfate) will decrease. Today, research is focusing on metal contaminants that convert into a precipitated form when exposed to microorganisms. Metals that undergo this procedure offer promising solutions concerning the removal of persistent metal contaminants in the environment.

19.7.1 Factors Affecting Biodegradation

19.7.1.1 Soil water

Soil water affects not only the moisture available to microorganisms, but also the soil aeration status, the nature and amount of soluble species, the osmotic pressure, and the pH (Subhas and Irvine 1998). Water activity in the soil is measured in terms of potential (matrix and osmotic). The matrix potential is the capacity of water to adsorb to solid surfaces. This potential is usually negative because it reduces the free energy of water. The osmotic potential, on the other hand, is related to solubility. Since water is a universal solvent, the presence of solute in the soil tends to reduce the free energy of water and create another negative potential (Subhas and Irvine 1998). The sum of the osmotic and matrix potentials describes the availability of water, and as a result will define how much energy a microorganism must use to obtain water. Microbial activity is known to peak in terms of reaction rate at a water potential of –0.01 MPa (megapascals); however, these rates tend to decrease if the soil becomes either waterlogged (i.e., the water potential approaches zero MPa) or drier (i.e., the water potential becomes more negative).

19.7.1.2 Redox potential

Bacteria obtain their energy from the oxidation and reduction of compounds present in the soil. They remove electrons from these compounds in order to obtain the energy given out during the oxidation process. This process depends largely on the presence of a compound that can accept electrons. In aerobic degradation, the final electron acceptor is oxygen.

19.7.1.3 Soil pH

Soil pH values influence the number of organisms present in the soil and the multiplicity of enzymes at the microbial level (Subhas and Irvine 1998). Bacteria favor pH values between 6.5 and 7.5, which equals the intracellular pH. The biodegradation of compounds depends on specific enzymes secreted by the organisms that perform the degradation. These enzymes are largely pH dependent.

19.7.1.4 Soil temperature

Temperature is one of the main factors that influence the biodegradation of a toxic compound. It affects not only the rates of biochemical reactions in the organisms, but also the soil moisture and redox potential. All microbial activities are dependent upon the laws of thermodynamics. When the temperature is too high, proteins are denatured and the cell membrane becomes more permeable. While microbial

metabolism tends to slow down at low temperatures, psychrophiles – bacteria that can grow at cold temperatures – are capable of degrading contaminants through osmotic regulation and using cytoplasmic constituents that prevent the cell interior from freezing (Subhas and Irvine 1998).

19.7.2 Limitations of Bioremediation

One of the biggest limitations of bioremediation involves the nature of the microorganisms being used. The degradation of pollutants is a survival strategy used by the organisms as a way to obtain the energy necessary for their metabolic reactions. As a result, it is usually necessary that certain conditions be created to enhance the development of these organisms. The introduction of oxygen or fertilizers into the contaminated soil is sometimes required when bacteria and fungi are being used in an in situ treatment. This can disrupt the diet of the pre-existing indigenous species. As stated before, the rate of degradation of a pollutant is highly dependent on the initial concentration and the toxicity of the pollutants present to microorganisms, the biodegradability of the pollutants, the properties of the contaminated soil, and the selected treatment technique. The effectiveness of bioremediation is limited at sites with high concentrations of metals, highly chlorinated organics, and inorganic organic salts. This is because these compounds tend to be toxic to microorganisms (Cunningham and Philip 2000).

In situ techniques have an important disadvantage compared to ex situ techniques: they require long periods of time to have an effect, whereas ex situ treatments give results more rapidly. In situ bioremediation is also more effective for sites with relatively permeable soil (sand) rather than sites situated on clay. Clay is known to have a low porosity, and so oxygen, a much-needed ingredient for organism development, is not easily circulated through the contaminated area. Ex situ techniques such as landfarming must be performed carefully to avoid creating a larger contamination problem in the case of treatment failure. Landfarming can also only be practiced for the bioremediation of easily biodegradable compounds. It also requires a deep groundwater table due to the possibility of contaminants leaching into the groundwater. In composting, the need to frequently turn the piles makes it a labor-intensive technique. Another drawback is that this remediation technique can only be carried out in mesophilic or thermophilic habitats; in other words, at above-freezing temperatures. One last limitation of this technique is that the byproducts left by the organism biodegrading the pollutant can be as or even more toxic than the original contaminant.

19.8 Genetic Aspects of Heavy Metal Resistance

With a few exceptions, genetic studies of metal–microbe interactions have been limited and often confined to studies of mutant strains, mainly because knowledge of the physiology and biochemistry of resistance is fragmentary in many cases.

However, certain areas of metal–microbe interactions have received specific attention from genetics researchers, and this work has contributed not only to our knowledge of microbial responses to heavy metals but also to the wider fields of molecular biology and biotechnology. The areas in which considerable genetic advances have been made to date are heavy metal resistance in bacteria and cyanobacteria, and metallothionein in *S. cerevisiae.* In several areas of metal studies, not only is genetic information limited, but basic essential physiological and biochemical studies are lacking too.

19.8.1 In Bacteria

Mercury resistance has been observed in many bacteria inhabiting metal contaminated environments. Usually the determinants of mercury resistance are plasmid encoded in both Gram-positive and Gram-negative bacteria. The specialty of Hg^{2+} positive resistant determinant of bacteria is the absence of linkage to drug-resistance markers, in contrast to clinical bacterial isolates from the environment, where Hg^{2+}resistance is generally linked to genes for antibiotic resistance. Various Hg^{2+} resistances have been reported in Gram-negative bacteria. In Gram-negative bacteria DNA sequencing strategies are the *Mer* genes, which are located on the transposons Tn501 and Tn21. These types of studies have been carried out in detail in both Gram-positive and Gram-negative bacteria by Gadd (1990). Some additional are reported to be associated with *Mer* genes viz., *Mer* C in Tn21 and *Mer* B in the operon of small pDU1358. Two mercury-resistant phenotypes have been found in Gram-negative bacteria. First narrow spectrum resistance solely expressed the reductase and other, ii), broad-spectrum resistance to Hg^{2+} and a several number of organomercurials is found in strains specify both reductase and lyase which is associated with plasmids of a very few in compatibility groups. However, in some organisms, organomercurial resistance does not involve lyase activity. Through genetic analysis of the R100 plasmid, which contains Tn21, showed that at least three genes were involved in Hg resistance. The *Mer* gene is regulatory and involved in the induction of the other genes, the *Mer T* gene encodes a protein involved with Hg^{2+} transport, whereas *Mer A* is the structural gene for mercuric reductase (Gadd 1990; Brown 1985)

19.8.2 In Fungi

The Cu-induced metallothionein of *S. cerevesiae* (MW 6573) has gained a great deal of attention, while a copper-inducible metallothionein has also been reported from *Neurospora crassa.* This yeast protein is inducible only by copper, not zinc or cadmium, and is called Cu-MT or yeast MT. Inducible cadmium-binding proteins that are structurally different from Cu-MT have been isolated from *Schizosaccharomyces*

pombe. These proteins are termed phytochelatins (sulfur-rich, metal-binding polypeptides). These phytochelators consist of three amino acids – cystine, glutamic acid, and glycine – that are analogs to similar peptides found in plant cells exposed to heavy metals such as Cd, Cu, Hg, Pb, and Zn. Multiple enzymes may be involved in phytochelatin synthesis, and so there must be mechanisms for the metal-dependent induction or activation of such enzymes. A low molecular weight Cd-binding protein has been isolated from Cd-resistant strains of *S. cerevisiae*. Yeast MT has been found in Cu-tolerant *S. cenedesmus,* and Cd induced metallothionein-like proteins in *Chlorella pyrenoidosa* and *Dunaliella*. A metallothionein protein has been identified in the ciliate *Tetrahymena pyriformis* after exposure to Cd, and this is also capable of Zn binding.

19.9 Phytoremediation of Heavy Metals

Phytoremediation has been defined as the in situ use of plants to stabilize, remediate, and reduce or restore contaminated soil. Phytoremediation relies on the plant's ability to act as a solar-driven pump and filter system, and enhances or stimulates the natural tendency of ecosystem to restore itself.

19.9.1 Ex Situ and In Situ Methods of Phytoremediation

19.9.1.1 Ex Situ Methods

Here, the contaminated soil is treated on- or offsite, and then returned to its original site. Conventional ex situ methods applied to remediate polluted soils include excavation, detoxification, and/or destruction of the contaminant physically or chemically, meaning that the contaminant undergoes stabilization, solidification, immobilization, incineration or destruction.

19.9.1.2 In Situ Methods

These methods perform remediation without the need to excavate the contaminated site. Reed et al. defined in situ remediation technologies as the destruction or transformation of the contaminant, its immobilization to reduce bioavailability, and the separation of the contaminant from the soil (Reed et al. 1992). In situ techniques are often preferable to ex situ techniques due to their low cost and reduced impact on the ecosystem. Conventional ex-situ techniques involve excavating the soil contaminated with heavy metals and burying it in a landfill site (McNeil and Waring 1992; Smith 1993). However, offsite burial merely shifts the contamination problem elsewhere (Smith 1993), and leads to hazards associated with the transport of

contaminated soil (Williams 1988). Diluting the heavy metal content up to a safe level by importing clean soil and mixing it with the contaminated soil may be an alternative for onsite management (Musgrove 1991). Onsite containment and barriers also provide an alternative; these involve covering the soil with inert material (Body et al. 1988). Immobilizing the heavy metals through the application of inorganic contaminants can also be used as a remedial method for heavy metal contaminated soils (Mench et al. 1994). This involves complexing the contaminants or increasing the soil pH by liming (Alloway and Jackson 1991), since increasing the pH decreases the solubilities of heavy metals such as Cd, Cu, Ni, and Zn in the soil. However, most of these conventional remediation technologies are very costly to implement and cause further disturbance to the already damaged ecology (Mench et al. 1994; Alloway and Jackson 1991).

Plant-based bioremediation technologies have been collectively termed "phytoremediation," and involve the use of green plants and their associated microbes for the in situ treatment of contaminated soil and groundwater (Sadowsky 1999). Metal-accumulating plants were first used to remove heavy metals from the soil in 1983, but this concept has actually been implemented for the past 300 years (Henry 2000). This technology can be applied to both organic and inorganic pollutants found in the soil (solid substrates) as well as water (liquid substrates) and the air (Salt et al. 1998; Raskin et al. 1994). The application of physicochemical techniques for soil remediation renders the land useless for plant growth, as they remove all of the biological components, such as useful microbes (nitrogen-fixing bacteria), mycorrhiza, fungi, and fauna during the process of decontamination (Burns et al. 1996). Finally, conventional methods of bioremediation can cost from \$10 to \$1,000 m^{-3}, while the cost of phytoextraction costs is estimated to be as low as \$0.05 m^{-3} (Cunningham et al. 1997).

Phytoremediation consists of five main processes, as described below.

Rhizofiltration

Rhizofiltration involves using the plants (terrestrial or aquatic) to absorb, concentrate, and precipitate low-concentration contaminants from aqueous sources in their roots. This process can be applied to partially treated industrial discharges, agricultural runoff, or acid mine drainage. It can be used for heavy metals like lead, cadmium, copper, nickel, zinc, and chromium, which are primarily held within the roots (Chaudhry et al. 1998; USEPA 2000). The advantages of this process are that it can be applied in situ or ex situ, and that species other than hyperaccumulators can also be used. Plants like sunflower, mustard, tobacco, rye, spinach, and corn have been studied for their ability to remove lead from effluent, with sunflower showing the greatest ability. Mustard has proven to be very effective at removing lead at a wide range of concentrations (4–500 mg L^{-1}) (Raskin and Ensley 2000). The technology has also been applied to water from a field contaminated with uranium. Uranium concentrations of 21–874 μg L^{-1} were treated; these concentrations

were reduced to $< 20\,\mu g\ L^{-1}$ before the water was discharged into the environment (Dushenkov et al. 1997).

Phytostabilization

This process is generally used for the remediation of soil, sediment, and sludges (USEPA 2000; Mueller et al. 1999), and it depends on the ability of plant roots to limit the mobility and bioavailability of the contaminant in the soil. This can occur through sorption, precipitation, complexation, or metal valence reduction. The primary purpose of the plants is to decrease the amount of water percolating through the soil matrix, which reduces the formation of hazardous leachate and prevents soil erosion and thus the redistribution of the toxic metal to other sites. A dense root system stabilizes the soil and prevents erosion (Beti and Cunningham 1993). Phytostabilization is considered a very effective process whenever rapid immobilization is required to preserve ground and surface waters, and the disposal of biomass is not required in this process. However, its major disadvantage is that the contaminant remains in the soil and so needs regular monitoring.

Phytoextraction

This is the best phytoremediation process. It involves first removing the contamination from the soil and then isolating it without harming the soil structure and fertility. It is also referred as phytoaccumulation (USEPA 2000). As the plants absorb, concentrate, and precipitate toxic metals and radionuclides from contaminated soils into their biomass, it is best suited for the remediation of diffusely polluted areas, where pollutants occur at relatively low concentrations (Rulkens et al. 1998). Several approaches have been used, but the two main phytoextraction methods are (1) chelate-assisted phytoextraction or induced phytoextraction, where artificial chelates are added to the soil to increase the mobility and uptake of metal the contaminant; (2) continuous phytoextraction, where the removal of the metal depends on the natural ability of the plant to remediate the soil; only the number of plant growth repetitions is controlled (Salt et al. 1995, 1997). In order to make this technology feasible, the plants must draw up large concentrations of heavy metals into their roots, translocate the heavy metals to their biomass on the surface, and produce large quantities of plant biomass. The heavy metal removed by the plant can be recycled from the contaminated plant biomass (Brooks et al. 1998). Factors such as plant growth rate (vigor), element selectivity, resistance to disease, and method of harvesting are also important (Cunningham and Ow 1996; Baker et al. 1994). However, slow growth, a shallow root system, low biomass production, and difficult final disposal of the extracted metal all limit the use of a particular plant species as a hyperaccumulator (Brooks 1994).

19.9.2 Mechanism of Phytoextraction

The metal contaminant must be mobilized into the soil solution for plants to be able to accumulate it. The bioavailability of a metal in soil can be increased in various ways (Fig. 19.6). In one mode of action, plants secrete phytosiderophores into the rhizosphere to chelate and solublize soil-bound metals (Kinnersely 1993). Acidification of the rhizosphere and exudation of carboxylates are considered potential methods of enhancing metal accumulation. In first instance, a metal mobilization has to be coped by root cells. Metals are first bound to the cell wall through ion exchange at binding sites with comparatively low-affinity and/or selectivity, and than transport systems that make use of intracellular high-affinity binding sites then mediate and drive metal uptake across the plasma membrane. Uptake of metal ions takes place via secondary transporters like channel proteins and/or H^+-coupled carrier proteins. The membrane potential is negative on the inner side of the plasma

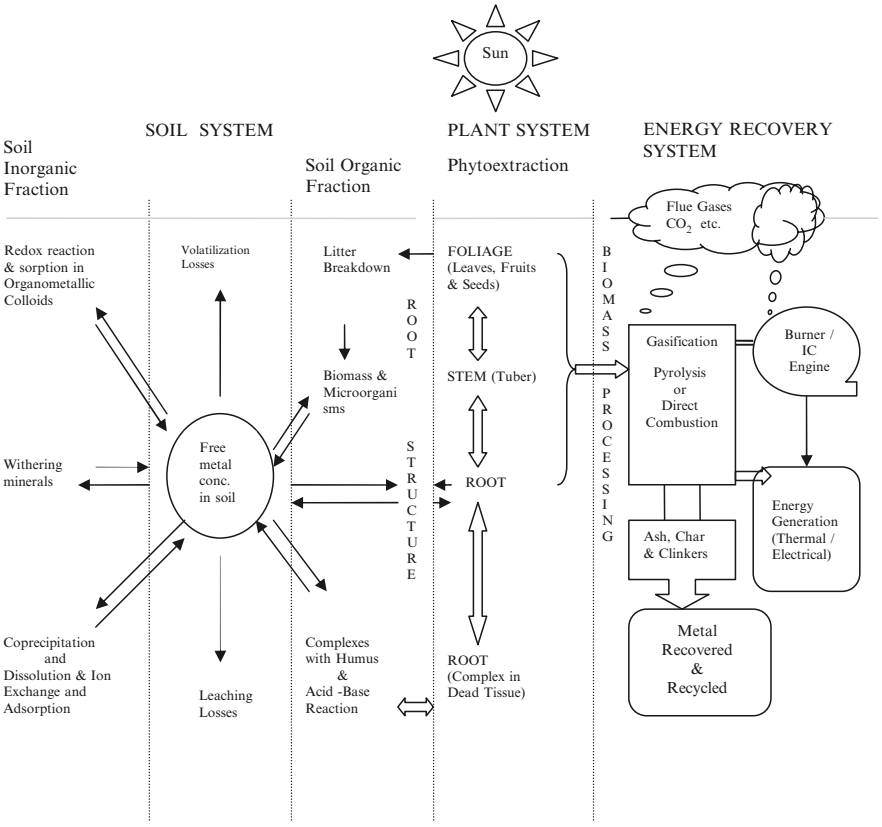

Fig. 19.6 Soil, plant, and energy recovery systems associated with phytoextraction. The figure depicts the key components involved, along with the mass transfer process and the dynamics of phytoextraction (from Singh and Ghosh 2005)

membrane and can rise to –200 mV in root epidermal cells, providing a strong driving force for the uptake of cations through secondary transporters (Hirsch 1998). Most metals are insoluble and so cannot move freely through the plant's vascular system; thus, they generally form carbonate, sulfate, and phosphate compounds that are immobilized in apoplastic (extracellular) and symplastic (intracellular) compartments (Raskin et al. 1997). Metal ions are transported as noncationic metal–chelate complexes unless apoplastic transport is limited by the high cation exchange capacity of cell walls (Raskin et al. 1997), while the apoplast continuum of root epidermis and cortex is readily permeable to solutes. The apoplastic pathway is relatively unregulated, as water and dissolved substances can flow and diffuse without having to cross the membrane. The endodermal cell wall acts as a barrier to apoplastic diffusion into the vascular system. Solutes must be taken up into the root symplasm before they can enter the xylem (Tester and Leigh 2001).

There are three processes that regulate the movement of metals from root tips to xylem (root symplasm). The sequestration of metals takes place inside cells; they then undergo symplastic transport to the stele, and are released into the xylem. The transport of metals ions into the xylem is a controlled process that is mediated by membrane transport proteins. Symplastic transport of heavy metals generally takes place in the xylem after they cross the Casparian strip. This mechanism is regulated because of the selectively permeable plasma membrane of the cells that control the symplast by specific metal ion carriers/or channels (Gaymard 1998). It also requires that metal ions move across the plasma membrane, which usually has a large negative resting potential of approximately –170 mV. This membrane potential provides a very strong electrochemical gradient that drives the movement of metal ions inside. The process is energy dependent and involves specific or generic metal ion carriers/or channels (Bubb and Lester 1991). Nonessential heavy metals compete with essential heavy metals for the same transmembrane carriers. For example, the essential metals Cu^{2+} and Zn^{2+} and the nonessential metals Ni^{2+} and Cd^{2+} compete for the same transmembrane carrier, as established kinetically (Crowley et al. 1991). Metal–chelate complexes can also be transported across the plasma membrane via specialized carriers, as in the case of Fe–phytosiderophore transport in graminaceous species (Beti and Cunningham 1993).

After heavy metals have entered the roots, they are either stored in root tissues or translocated to shoots. Heavy metal ions can also be actively transported across the tonoplast as free ions or as metal–chelate complexes (Cataldo and Wildung 1978). Water and soluble ions (salt and metal) require active transport across the Casparian strip, utilizing energy-driven forces. For example, Cd is actively transported across the tonoplast of oat roots as either a free ion or via a Cd/H^{+} antiport (Dierberg et al. 1987). The vacuole is an important organelle for storing metal ions; here, the metal ions are often chelated by organic acids or phytochelatins. Insoluble precipitates may form under certain conditions.

Precipitation, compartmentalization, and chelation are the major processes used to prevent the damaging effects of heavy metals (Cunningham 1995). Transporters mediate uptake into the symplast, and distribution within the leaf occurs via the apoplast or the symplast (Karley et al. 2000).

Plants transpire water molecules to move nutrients from the soil solution to leaves and stems, where photosynthesis occurs. The hybrid poplar (a member of the willow family) is also a phytoremediator, as it takes up and processes soil water. A single willow tree, on a hot summer day, can transpire more than 19,000 l of soil water (Hinchman and Negri 1997).

19.10 Types of Phytoextraction

19.10.1 Natural Phytoextraction

Some plants have been identified that have the potential to take up heavy metals. At least about 45 families have been identified as hyperaccumulator plants; these are *Brassicaceae*, *Fabaceae*, *Euphorbiaceae*, *Asteraceae, Lamiaceae,* and *Scrophulariaceae* (Salt et al. 1998; Dushenkov 2003). Among these, *Thlaspi caerulescens* (alpine pennyacre) is the best hyperaccumulator (Kochian 1996), since it did not show any injury upon accumulating up to 26,000 mg kg^{-1} Zn and up to 22% of soil-exchangeable Cd from a contaminated site (Brown et al. 1995; Gerard et al. 2000). *Brassica juncea* (Indian mustard) has been found to have a good ability (phytoextraction coefficient is 1.7–500 mg L^{-1}) to transport Pb from roots to shoots, the range is not phytotoxic for plants (Henry 2000). The phytoextraction coefficient is the ratio of the metal concentration found within the surface biomass of the plant to the metal concentration found in the soil. *Brassica juncea* is capable of removing 11,550 kg of Pb $acre^{-1}$ (Henry 2000). Metal concentrations of > 1,000 mg kg^{-1} have been found in more than 320 plant species for Ni, Co (30 species), Cu (34 species), Se (20 species), Pb (14 species) and Cd (one species). The species involved in hyperaccumulation have been found (Baker Reeves and Baker 2000), and substantial numbers of these species are from Congo and Zaire. Concentrations exceeding 10,000 mg kg^{-1} have been recorded for Zn (11 species) and Mn (10 species). The hyperaccumulation threshold levels for these elements have been set higher than those for the others because their normal ranges in plants (20–500 mg kg^{-1}) are much higher than those for other heavy metals (Reeves 2003).

Aquatic plants such as the floating *Eichhornia crassipes* (water hyacinth), *Lemna minor* (duckweed), and *Azolla pinnata* (water velvet) have been investigated for use in rhizofiltration, phytodegradation, and phytoextraction (Salt et al. 1997). Farago and Parsons (1994) reported the bioremoval of Pt using *Eichhorniacrassipes*. Many aquatic plants are used in the bioremoval of heavy metals, including *Azolla filliculoides*, *A. pinnata*, *Typha orientalis*, and *Salvinia molesta*. Jin-Hong et al., in their study of twelve wetland species, reported that *Polygonum hydropiperoides* Michx (smartweed) was the best for heavy metal phytoremediation due to its fast growth and high plant density (Qian et al. 1999). Recently, a fern, *Pteris vitatta*, has been shown to accumulate as much as 14,500 mg kg^{-1} As in fronds without showing any symptoms of toxicity (Ma et al. 2001).

19.10.2 Induced Phytoextraction or Chelate-Assisted Phytoextraction

Oligopeptide ligands, such as phytochelatins (PCs) and metallothioneins (MTs), are induced by the presence of or interact with heavy metals found in plant cells (Cobbett 2000). These peptides bind with the heavy metal, forming stable complexes, and thus neutralize them and minimize the toxicity of the metal ion[68]. Phytochelatins (PCs) are synthesized from glutathione, and have the structure Gly-$(\gamma\text{-Glu-Cys})_n$, where $n = 2–11$. Around a hundred phytochelating ligands have been reported in plant species exposed to heavy metals (Rauser 1999). MTs are small, gene-encoded, Cys-rich polypeptides. PCs are functionally the same as MTs (Grill et al. 1987). Chelating agents such as ethylenediaminetetraacetic acid (EDTA) have been isolated from plants that are involved in the uptake of heavy metals and their detoxification. The addition of chelators to a Pb-contaminated soil (total soil Pb 2,500 mg kg^{-1}) increased shoot Pb concentrations of *Zea mays* (corn) and *Pisun sativum* (pea) from less than 500 mg kg^{-1} to more than 10,000 mg kg^{-1}. The synthetic chelator EDTA is used to enhance Ur uptake from the soil and also to enhance or facilitate Pb transport into the xylem, as well as to increase Pb translocation from roots to shoots. Various chelators can be ordered according to their ability to increase Pb desorption from the soil as follows: EDTA > hydroxyethylethylenediaminetriacetic acid (HEDTA) > diethylenetriaminepentaacetic acid (DTPA) > ethylenediamine di(*o*-hydroxyphenylacetic acid) EDDHA (Huang et al. 1997). Vassil et al. (1998) reported that *Brassica juncea* exposed to Pb and EDTA in hydroponic solution was able to accumulate up to 55 mM kg^{-1} Pb in dry shoot tissue (1.1% w/w). This corresponds to a ~75-fold increase in the Pb concentration in its shoots compared to that found in the solution. Thus, EDTA (0.25 mM) was shown to stimulate this accumulation of Pb in shoots.

19.10.3 Limitations of Phytoextraction

Phytoextraction and plant-assisted bioremediation are most effective when the soil contamination is limited to within three feet of the surface of soil, and when the groundwater table starts within ten feet of the surface (Raskin et al. 1994; Cunningham et al. 1997). It is applicable to sites with low-to-moderate soil contamination and to sites with large volumes of low-level contaminated groundwater that have to be cleaned (Salt et al. 1995). Scientists have investigated the effect of soil acidification on Zn and Cd phytoextraction and proposed the use of $(NH_4)_2SO_4$ as a soil additive to provide the nutrients (N and S) required for high yield, and to acidify the soil for greater metal bioavailability. However, there may be some negative side effects of soil acidification. For example, due to the increased metal solubility, some toxic metals may leach into the groundwater, creating an additional environmental risk. Chaney et al. (2000) reported that the soil could be limed during

metal phytoextraction to elevate the pH to be close to neutral, aiding normal ecosystem development. However, liming may also increase the soil's capacity for metal binding and restrict the potential for phytoextraction.

Phosphorus is another major nutrient, and plant growth increases upon the application of phosphatic fertilizer (Vassil et al. 1998). The addition of such fertilizers, however, can also inhibit the uptake of some major metal contaminants, such as Pb, due to metal precipitation as pyromorphite and chloropyromorphite (Chaney et al. 2000).

Natural chelators of plant or microbial origin appear to be more promising than synthetic chemical chelators (Rauser 1999). It is not clear that the application of chemical chelators is a practical method for improving phytoextraction, since chemical chelators are also toxic to plants. Thus, they may increase the uptake of metals, but they also decrease plant biomass and so prove to be of limited benefit.

19.10.4 Phytovolatilization

Phytovolatilization involves the use of plants to take up contaminants from the soil, transform them into volatile form, and ultimately transpire them into the atmosphere. Phytovolatilization occurs as growing trees and other shrubs and herbs take up water, organic and inorganic contaminants. Some of these contaminants can pass through the plants to the leaves and volatilize into the atmosphere at comparatively low concentrations (Mueller et al. 1999). Phytovolatilization has been used primarily for the removal of mercury; here, the mercuric ion is transformed into less toxic mercury. The disadvantage of this process is that the mercury released into the atmosphere is likely to be recycled by precipitation and then redeposited into the ecosystem (Henry 2000). Phytovolatilization has been successful for tritium (^{3}H: a radioactive isotope of hydrogen), which decays to helium (which is far more stable) with a half-life of about 12 years Dushenkov (2003). Gary Banuelos of the USDS's Agricultural Research Service found that some plants that grow in high-selenium media produce volatile selenium in the form of dimethylselenide and dimethyldiselenide (Bañuelos 2000).

19.10.5 Phytodegradation

Phytodegradation is the breakdown of organic contaminants by plants into simpler molecules that are then incorporated/and or metabolized into their vascular systems (Chaudhry et al. 1998). Plants contain enzymes that can catalyze the degradation of ammunition wastes and chlorinated solvents, like trichloroethylene and other herbicides. These enzymes are generally dehalogenases, oxygenases, and reductases (Black 1995).

19.10.6 Rhizodegradation

Rhizodegradation is the breakdown of organics in the soil through the microbial activity of the root zone (the rhizosphere), and is a slower process than phytodegradation. Microbes such as yeast, fungi, bacteria, and other microorganisms consume and digest organic matter (fuels and solvents). Different types of phytoremediation can be used simultaneously, but metal extraction depends on its bioavailable fraction in soil.

19.10.7 Plant Growth on Heavy Metal Contaminated Soils

Plants employ three basic strategies for growth on metal-contaminated soils (Raskin et al. 1994).

19.10.8 Metal Excluders

Excluders act to prevent t metals from entering the aerial parts of plants, and thus help to maintain very low and constant metal concentrations in the plant. Mostly they restrict metal uptake by roots. Such plants can alter their membrane permeabilities as well as change the metal binding capacity of its cell walls and exude more chelating substances (Cunningham 1995).

19.10.9 Metal Indicators

Most plant species that actively accumulate metal in their aerial parts reflect the concentration of that metal in the soil. Such plants tolerate the high metal concentrations inside them by producing intracellular metal-binding compounds (chelators), or they alter their metal compartmentalization by accumulating metals in nonsensitive parts.

19.10.10 Metal Accumulator

These plants accumulate and concentrate metal at higher levels in their aerial parts that the metal concentration in the soil. Hyperaccumulators absorb high levels of contaminants which are then concentrated either in their roots, shoots and/or leaves (Raskin et al. 1994; Cunningham and Ow 1996; Baker et al. 1994). Baker and

Brooks reported metal hyperaccumulators that contain >0.1% (>1,000 mg g^{-1}) of Cu, Cd, Cr, Pb, Ni, or Co, or >1% (>10,000 mg g^{-1}) of Zn or Mg in their dry matter. Scientists have identified hyperaccumulator species by collecting plants from areas where the soil contains higher metal levels than normal (Gleba et al. 1999). So far, around 400 hyperaccumulator species from 22 different families have been identified. For example, the family *Brassicaceae* contains a large number of species that hyperaccumulate a wide range of metals (87 species from 11 genera) (Baker and Brooks 1989).

19.10.11 Utilization of Phytoremediation By-Products

Phytoextraction involves growing plants in heavy metal contaminated soil until the metal concentration declines to tolerable levels. The metal removed can be determined by measuring the metal concentration in the plant, multiplying it by the biomass produced, and comparing this with the reduction in the metal concentration in the soil. In commercial phytoextraction, the next step is to dispose of the contaminated plant material. After harvesting, the plant is removed from the field, and this leads to the accumulation of a large amount of hazardous biomass. This hazardous biomass should be stored or disposed of properly so that it does not create any risk to the ecosystem. Biomass is solar energy stored as plant mass; it is also considered to be the combustible organic matter from the plant. It contains C, H, and O, the elements found in oxygenated hydrocarbons. Biomass (especially wood) can be represented by the chemical formula $CH_{1.44}O_{0.66}$ (Iyer et al. 2002); its main constituents are lignin, hemicellulose, cellulose, minerals, and ash. It contains high levels of moisture and volatile matter, has a low bulk density, and calorific value. The proportions of these constituents vary from species to species. The dry weight of *Brassica juncea* for induced phytoextraction of Pb amounts to 6 tonnes/hectare, with 10,000–15,000 mg kg^{-1} of metal in dry weight (Blaylock et al. 1997). This huge amount of waste is a problem and thus the volume must be reduced (Blaylock and Huang 2000).

Composting and compaction has been proposed as a postharvest biomass treatment by some researchers (Raskin et al. 1997; Kumar et al. 1995; Garbisu and Alkorta 2001). Leaching tests for the composted material showed that soluble organic compounds enhanced metal (Pb) solubility (Hetland et al. 2001). Reduction in dry weight of contaminated plant biomass is advantageous, as it will lower the cost of transportation (Blaylock and Huang 2000). One very promising way to utilize the biomass produced by phytoremediation in an integrated manner is to use a thermochemical conversion process. If phytoextraction could be combined with biomass generation and commercially utilized as an energy source, then it can be turned into a profit-making operation, and the remaining ash could be used as bio-ore (Brooks et al. 1998). This is the basic principle of phytomining. Nicks and Chambers (1994) reported a second potential use for hyperaccumulator plants for economic gain in the mining industry. This operation, termed phytomining,

includes the generation of revenue by extracting saleable heavy metals produced by the plant biomass ash, also known as bio-ore. Combustion and gasification are the most important routes to the organized generation of electrical and thermal energy. Recovery of the energy in biomass by burning or gasification could help make phytoextraction more cost-effective. Thermochemical energy conversion best suits the phytoextraction biomass residue because it cannot be utilized in any other way as fodder and fertilizers. Combustion is a crude method of burning the biomass, but it can be done under controlled conditions, allowing the volume of biomass to be reduced to 2–5% of the original mass, and then the ash can be disposed of properly (Raskin et al. 1997; Bridgwater et al. 1999). It is not wise to burn the metal-bearing hazardous waste in the open, as the gases and particulates released into the environment may be detrimental; also, while the volume is reduced, the heat produced in the process is wasted using this approach.

Gasification is the process by which biomass material can be subjected to a series of chemical changes to yield clean and combustible gas at high thermal efficiencies. This mixture of gases is termed producer gas and/or pyro gas, and it can be combusted to generate thermal and electrical energy. The gasification of biomass in a gasifier is a complex process; it involves drying, heating, thermal decomposition (pyrolysis) and gasification, as well as combustion chemical reactions that occur simultaneously (Iyer et al. 2002).

Hetland et al. (2001) reported the possibility of co-firing plant biomass with coal. Results suggested that ashing reduced the mass of lead-contaminated plant material by over 90% and partitioned lead into ash. It may be possible to recycle the metal residue from the ash, but so far the cost or feasibility of such a process has not been estimated (Raskin et al. 1997). Future experiments should concentrate on the development of a combustion system and methods to recycle different metals from the ash. The process destroys organic matter, releasing metals as oxides. The liberated metals remain in the slag, and modern flue gas cleaning technology ensures effective capture of the metal-containing dust. Considering the other disposal technologies, this method is environmentally friendly.

Bridgwater et al. (1999) reported that pyrolysis is a novel municipal waste treatment technique that could also be used for contaminated plant material. Pyrolysis decomposes material under anaerobic conditions; there is no emission to the air. The final products are pyrolytic fluid oil and coke; heavy metals remain in the coke, which could be used in a smelter. Koppolua et al. (2003) reported that 99% of the metal recovered in the product stream was concentrated in the char formed by pyrolyzing the synthetic hyperaccumulator biomass used in the pilot scale reactor. The metal component was concentrated 3.2- to 6-fold in the char compared to the feed. The fates of the metals in various feeds during pyrolysis have been studied and addressed in the literature in different contexts, but results on the pyrolysis of phytoextraction plant biomass are limited. Helsen et al. (1997) conducted low-temperature pyrolysis experiments with chromated copper arsenate-treated wood, and it was concluded that most of the metal was retained in the pyrolysis residue. The influence of metal ions on the pyrolysis of wood has been studied extensively by many researchers (Pan and Richards 1990; Richards and Zheng 1991). The high

cost of installation and operation could be limiting factors on such a treatment if used solely for plant disposal. To avoid this, the plant material could be processed in existing facilities together with municipal waste.

Singh and Ghosh (2003) worked on high-biomass species, as they showed positive results in screening (germination) studies. The schematic shown in Fig. 19.6 describes the work performed by these authors into relation to phytoextraction. Their results showed that more Cd, Cr, and Pb was phytoextracted by *Ipomoea carnea*, *Datura innoxia*, and *Phragmytes karka* than by *Brassica juncea* and *Brassica campestris* (known to be indicator species) (Henry 2000; Ghosh and Singh 2005). The study, conducted with 10–200 mg kg^{-1} of Cd, Cr, and Pb (separately), indicated that *I. carnea* was more effective at extracting these species from soil than *B. juncea*. Among the five species, *B. juncea* accumulated maximum Cd but *I. carnea* followed by *D. innoxia* and *P. karka* were the most suitable species for phytoextracting cadmium, provided the whole plant or aboveground biomass was harvested. In a relatively short time, *I. carnea* produced more than five times more biomass than *B. juncea*. It was more effective at translocating Cr from soil to plant shoots. *P. karka* showed a much greater tolerance of chromium than other plants, though its uptake was low. *Ipomoea* extracted the most lead at 200 mg kg^{-1}; *Datura* and *Phragmytes* were the best extractors at 100 mg kg^{-1}, whereas the *Brassica* species were the best at 50 mg Pb kg^{-1} soil. *Brassica* species were difficult to cultivate, as they required pesticides to protect them from army moths, and they also cannot grow throughout the year. High-biomass species do not have these limitations and show higher potential, and their extraction capacities can be increased through the use of chelators or soil additives.

19.11 Genetic Engineering to Improve Phytoremediation

Plant productivity is controlled by many genes and is difficult to promote by single gene insertion. Genetic engineering techniques to implant more efficient accumulator genes into other plants have been suggested by many researchers (Cunningham and Ow 1996; Brown et al. 1995; Chaney et al. 2000). Implanting more efficient accumulator genes into other plants that are taller than natural plants increases the final biomass. Zhu et al. (1999) genetically engineered *Brassica juncea* to investigate rate-limiting factors for glutathione and phytochelatin production; they introduced the *Escherichia coli* gshl gene. γ-ECS transgenic seedlings showed increased tolerance to Cd and had significantly higher concentrations of phytochelatins, γ-GluCys, glutathione, and total nonprotein thiols as compared to wild-type seedlings. The potential success of genetic engineering may be limited by anatomical constraints (Ow 1996).

The effectiveness of transgenic plant varieties at increasing production and lowering production costs has been demonstrated in the cases of virus-, insect-, and herbicide-resistant plants, in which average increases in production of 5–10% and savings in relation to herbicides of up to 40% and to insecticides of between $60

and $120 per acre were reported in 1996 and 1997 (James 1997). However, these increases in total yield, impressive as they are in terms of their economic and environmental value, will have a limited impact for global food supply. In fact, most of the developments in transgenic crops are aimed either at reducing production costs in agricultural areas that already have high productivity levels, or at increasing the value added to the final product by improving, for instance, oil quality. This trend has been used by developed countries to limit the production of key products, like cereals, meat, and dairy products due to the reductions in the international prices of these products, and also to reduce the intensive use of fertilizers and pesticides because of their harmful effects on the ecosystem.

At a global level, a more effective strategy would be to increase productivity in tropical areas, where an increase in food production is required and where crop yields are significantly lower than those obtained in developed countries. In tropical areas, the losses caused by pests, diseases, and soil problems are exacerbated by climatic conditions that favor high levels of insect pests and vectors, and by a lack of the economic resources needed to purchase insecticides, fertilizers, and high-quality seeds. In addition to low productivity levels, postharvest losses in tropical areas are very high, again because of climatic conditions that favor fungal and insect infestation and because of the lack of appropriate storage facilities. Despite efforts to prevent pre- and postharvest crop losses, pests destroy over half of all crop production worldwide. Preharvest losses caused by insects, the majority of which occur in the developing world, are calculated at around 15% of the world's production.

Using biotechnology to produce transgenic plants that better withstand diseases, insect attack, or unfavorable soil conditions is not a simple task. There are an estimated 67,000 species of insects worldwide that damage crops, and a similar or even higher number of plant pathogens. For instance, in the case of *Phaseolus vulgaris*, over 200 diseases and 200–300 species of insects can affect bean productivity (Van Schoonhoven and Voysest 1980). These numbers give an idea of the complexity of the task that scientists face in increasing productivity. There are of course a certain number of diseases and insect pests that can be singled out as the most important constraints on the production of each crop. However, it is also true that when a particular disease or insect pest is controlled, others that were originally considered to be minor pests can then flourish and become major productivity constraints themselves.

One of the major advantages of plant biotechnology is that it can generate strategies for crop improvement that can be applied to many different crops. In this sense, genetically engineered virus resistance, insect resistance, and delayed ripening are good examples of strategies that can benefit many different crops. Transgenic plants of over 20 plant species that are resistant to more than 30 different viral diseases have been produced using different variations of the pathogen-derived resistance strategy. Insect-resistant plant varieties that use the D-endotoxin of *Bacillus thuringiensis* have been produced for several important plant species, including tobacco, tomato, potato, cotton, walnut, maize, sugarcane, and rice. Of these, maize, potato, and cotton are already under commercial production. It is envisaged that these strategies can be used for many other crops that are important

for developing countries. Genetically engineered delayed ripening, although tested only on a commercial scale for tomato, has an enormous potential application in tropical fruit crops, which suffer severe losses because they ripen rapidly (in many developing countries there are neither appropriate storage conditions nor adequate transportation systems to allow their efficient commercialization).

To date, most of the developments in plant gene transfer technology and the different strategies for producing improved transgenic plant varieties have been driven by the economic value of the species or the trait. These economic values are in turn mainly determined by their importance to agriculture in the developed world, particularly the United States and Western Europe. This economical emphasis is understandable, because important investments are needed to develop, field test, and commercialize new transgenic plant varieties. However, in terms of global food production, it is necessary to ensure that this technology is effectively transferred to the developing world and adapted to the local crops and/or local varieties of crops for which it was originally developed. Developing improved transgenic versions of local varieties or local crops is not a trivial issue; in most, if not all, cultures, the use of specific crops has a deep social and/or religious meaning. Cultural preservation is just as important as environmental preservation. Cultural aspects of technology transfer need to be considered because simply replacing crops to increase productivity could have an enormously negative effect on certain cultures, and new introductions may not be accepted easily for human consumption.

It is unfortunate that most developing countries do not have sufficient resources to implement the biotechnological capacity needed to solve the major problems that limit agricultural productivity, at least not in the time frame that is required to cope with the increasing demand for food. However, it is in the developing world that biotechnology could have its biggest impact in increasing crop production, especially in the areas of the world where yields are low because of the lack of technology. Plant genetic engineering could be considered a neutral technology that in principle does not require major changes in the agricultural practices of farmers in developing countries. Perhaps more importantly, it has the potential to bring about great benefits to the small farmers who lack the economic resources to purchase agrochemicals or prevent postharvest losses because of the lack of storage facilities.

Whether there is time to increase agricultural productivity in the developing world is a question with a complex answer, because there are many factors that need to be taken into account to make this happen. We need to identify and establish mechanisms of technology transfer from developed countries, from both academic institutions and the private sector, to the developing world; there is a need to create a sufficient number of research centers with the capacity to acquire this technology, adapt it to local crops, and develop their own technologies. Seed production facilities must be improved, and an effective mechanism implemented to reach subsistence farmers with this new technology.

To meet these requirements, several economic, political, and social issues must be dealt with to ensure the general application of plant biotechnology to the agriculture of developing countries. The discussion of these issues goes beyond the

scope of this chapter. However, it is our opinion that it will not be technological limitations but rather political and/or economic constraints that will determine how successful we are at supplying food to the hundreds of millions of people who will be malnourished in the next millennium.

19.12 Future Prospects for Phytoremediation

The acceptance of phytoextraction depends largely on its performance, the ultimate utilization of its by-products, and its overall economic viability. To date, commercial phytoextraction has been constrained by the expectation that site remediation should be achieved in a time comparable to other clean-up technologies. So far, most phytoremediation experiments have been performed at the lab scale, where plants grown in hydroponics setting are fed heavy metal diets. While these results are promising, scientists readily admit that solution culture is quite different from that of soil. In real soil, many metals are tied up in insoluble forms, making them less available, which is the biggest hurdle to extracting them from the soil (Kochian 1996). Phytoremediation is still in its research and development phase, and there are many technical barriers to this approach that need to be addressed. Both agronomic management practices and plant genetic abilities need to be optimized to develop commercially useful practices. Many hyperaccumulator plants remain to be discovered, and there is a need to understand their physiology in greater depth (Raskin et al. 1994). Process optimization, a proper understanding of heavy metal uptake by plants, and methods for properly disposing of the biomass produced are still needed.

19.13 Conclusion

We can conclude that in situ bioremediation is a suitable method for reclaiming the heavy metals in contaminated soils. Bacterial bioremediation is more effective than fungal remediation in tropical regions, as they experience heavy rainfall. The tropics have the highest terrestrial biomass in the world due to their favorable climatic conditions, which suggests that phytoremediation would be the most appropriate method for reclaiming heavy metals from contaminated land.

For the last 15 years, phytoremediation has been a fast-developing field around the world, and studies have included the phytoremediation of organics, inorganics, and radionuclides. This sustainable and inexpensive process is fast emerging as a viable alternative to conventional remediation methods, and should be highly suited to a developing country like India. However, most of the studies performed in this field have been done in developed countries, and knowledge of suitable plants is particularly limited in India, where the commercial application of phytoremediation of soils contaminated with heavy metals or organic compounds is in its earliest phase.

Table 19.1

S.N.	Application	Media	Mechanism	Contaminants	Typical plants
1.	Phytotransformation	Soil, groundwater, landfill leachate, application of wastewater to land	Degradation in plants	Herbicides (atrazine, alachlor)Aromatics (BTEX) Chlorinated aliphatics (TCE) Nutrients (NO_3^-, NH^{4+}, PO_4^{3-}) Ammunition wastes (TNT, RDX)	Phreatophyte trees (poplar, willow, cottonwood, aspen)Grasses (rye, Bermuda, sorghum, fescue) Legumes (clover, alfalfa, cowpeas)
2.	Rhizosphere bioremediation	Soil, sediments, application of wastewater to land	Degradation by rhizospheric microorganisms	Organic contaminants (pesticides, aromatics, and polynuclear aromatic hydrocarbons [PAHs])	Phenolics releasers (mulberry, apple, osage orange)Grasses with fibrous roots (rye, fescue, Bermuda) for contaminants 0–3 ft deep Phreatophyte trees for 0–10 ft Aquatic plants for sediments
3.	Phytostabilization	Soil, sediments	Complexation	Metals (Pb, Cd, Zn, As, Cu, Cr, Se, U)Hydrophobic organics (PAHs, PCBs, dioxins, furans, pentachlorophenol, DDT, dieldrin)	Phreatophyte trees to transpire large amounts of water for hydraulic controlGrasses with fibrous roots to stabilize soil erosion Dense root systems are needed to sorb/bind contaminants
4.	Phytoextraction	Soil, brownfields, sediments	Hyperaccumulation	Metals (Pb, Cd, Zn, Ni, Cu) with EDTA addition for Pb, selenium (volatilization)	SunflowersIndian mustard Rape seed plants Barley, hops Crucifers Serpentine plants Nettles, dandelions
5.	Rhizofiltration	Groundwater, water and wastewater in lagoons or created wetlands	Rhizospheric accumulation	Metals (Pb, Cd, Zn, Ni, Cu)Radionuclides (^{137}Cs, ^{90}Sr, U) Hydrophobic organics	Aquatic plants: emergents (bullrush, cattail, coontail, pondweed, arrowroot, duckweed) and submergents (algae, stonewort, parrot feather, Eurasian watermilfoil, *Hydrilla*)
6.	Phytovolatilization	Groundwater, soil, sediment and sludge	Volatilization by leaves	Organics and inorganics	Poplar, alfalfa, black locust, Indian mustard

Fast-growing plants with high biomass and good metal uptake abilities are required for phytoremediation. Hardy, tolerant weed species exist at most contaminated sites, and phytoremediation using these or other nonedible species could restrict the contaminant from being introduced into the food web.

Several methods of plant disposal have been described, but data on these methods are still scarce. Composting and compaction can be treated as pretreatment steps for volume reduction, but care should be taken to collect the leachate resulting from compaction. Of the two methods that can significantly reduce the contaminated biomass, incineration seems to less time consuming and environmentally sound than direct burning or ashing.

The application of genetically modified plants may help to reduce the hazardous constituents from the contaminant site. Even if this technology is successfully transferred to developing countries and transgenic varieties are developed for local crops, the problem of getting this technology into hands of the small farmer is still an important issue. The government of each country needs to implement a system for producing and distributing transgenic seeds and any other input, at low or no cost, to the small farmer. Whether technology transfer to developing countries takes place will, of course, depend on the political will of each national government and the resources required.

Local public awareness of the problem of heavy metal contaminated soils and methods for their remediation are equally important. The implementation and/or enforcement of environmental laws related to this issue among the local population are urgently required.

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