

Chapter 11

Metal Elements and the Diversity and Function of Ectomycorrhizal Communities

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11.1 Introduction

A few metal elements, Al, Fe, Ca, Na, Mg and K, are major components of the continental earth crust, while the majority of metals are present in traces only (Wedepohl 1995). Certain metal elements are required as essential nutrients (e.g. Na, K, Ca, Mg, Fe, Zn, Cu) while others have no known biological function (e.g. Al, Pb, Hg). All trace metals can be toxic, depending on concentration and speciation. Human activities have resulted in the release of trace metals into the environment far above natural levels. Metal and metalloid pollution is highly persistent and constitutes a major ecotoxicological concern, particularly in case of highly toxic elements such as As, Pb, Cd, Cu, Ni and Zn. The natural biogeochemical cycling of metals, that is the transformation and translocation of metal elements in the biosphere, has increasingly turned into a recycling of anthropogenic pollutants.

Soils are major sinks of metals emitted by human activities (Berthelsen et al. 1995). The largest quantities of metals are released in the northern hemisphere (Europe, North America, and, increasingly, Asia; Pacyna and Pacyna 2001). Large areas of metal-contaminated terrestrial environments are actually or potentially vegetated with ectomycorrhizal (ECM) forests. Metal-contaminated environments are dynamic systems, the speciation and compartmentation of metal elements depends on interactions of physico-chemical soil properties and biotic activities (Fomina et al. 2007). Recently, free-living and symbiotic fungi have been recognized as important agents in metal biogeochemistry, since they are involved in metal mobilization, transformation and immobilization (Gadd 2007). Ectomycorrhizal fungi (ECMF) are the dominant microbial component of ECM forest soils and key players in metal biogeochemical cycles in these environments, due to their important biomass and due to their manifold interactions with the organic and inorganic soil matrix. Increased interest in ECM–metal interactions was triggered

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by the potential applications of ECMF in phytoremediation of polluted sites. The evolution of metal tolerance in ECMF and potential host protection by ECMF and the role of ECM fungi in metal biogeochemical cycles (Gadd 2007) were covered by several reviews (Hartley et al. 1997; Leyval et al. 1997; Godbold et al. 1998; Jentschke and Godbold 2000; Meharg 2003; Bellion et al. 2006). Here I want to focus on studies that link various aspects of trace metal toxicity to population biology, community composition, biodiversity of ECMF and on the integration of ECMF into food webs.

11.2 Metal Element Cycling in ECM Forests

11.2.1 Biogeochemical Transformations of Metals in ECM Forest Soil

Transformations and transport of metals are part of natural biogeochemical cycles. Changes of metal speciation in biochemical cycles have important effects on solubility, bioavailability and toxicity. The soil pools of trace elements are distributed across different phases and are associated with different matrices: soil solution, living biomass, organic matter, organomineral complexes, clay minerals, and hydrous oxides of Al, Fe and Mn. Main processes that control the distribution, speciation and availability of metals are adsorption–desorption, precipitation–dissolution, complexation, redox reactions and volatilization (Gadd 2007; Huang 2008). Adsorption to minerals, organic matter and microbial or plant cell walls limits the transport in aqueous soil solution, reducing leaching to deeper soil horizons and water bodies. Cu, Pb and Cr are strongly retained by the forest floor, while Zn and Cd are regarded as more mobile. Fungi can bind metals to cell walls or extracellular polysaccharides. Precipitation of metals in the soil solution may occur under the influence of sulphide in reduced environments or by biotic oxalic acid exudation. Complexation reactions are highly diverse and they may involve specialized chelates such as siderophores or diverse organic acids (Gadd 2007; Huang 2008).

11.2.2 Weathering and Mineralization

Weathering of the parental rock matrix is an essential source of most plant nutrients, including biogenic metal elements (Landeweert et al. 2001). Weathering is important in replacing element losses that may occur naturally or anthropogenically through leaky nutrient cycles, leaching, removal of biomass, or as a result of forest fires and subsequent discharge of ashes.

The discovery of micrometer-scale tunnels inside mineral grains (feldspar and hornblende, which contain K, Ca and Mg) in the elution horizons of podzols led to

the hypothesis that ECM fungi can actively penetrate and dissolve minerals and transport nutrients directly from minerals to ECM host plants (Jongmans et al. 1997; Landeweert et al. 2001). The direct link between rock and trees was thought to improve tree growth by protecting trees from nutrient deficiency and Al toxicity. It was demonstrated that certain ECM fungi are able to mobilize P or cationic nutrients from minerals such as apatite, biotite, goethite and muscovite. Fungal mobilization and translocation of K, Ca, Mg via ECM hyphae appeared to be driven by reduced bioavailability of these elements (van Schöll et al. 2008). Pot experiments with muscovite as only K source demonstrated an increase of weathering rates by tree seedlings by a factor 1.7, inoculation with *Paxillus involutus* increased weathering to a factor of 3.3, compared to abiotic weathering. Inoculation with *Suillus bovinus* or *Piloderma croceum* did not change weathering rates (van Schöll et al. 2006). Fungi target hyphal growth and weathering agents (organic acids, particularly oxalic acid, and siderophores) at minerals containing nutrient elements (P, K, Ca, Mg). Fungi can dissolve minerals and metal compounds by acidolysis, complexolysis, redoxolysis and metal accumulation. Combined acidolysis and complexolysis by organic acids serving as a source of both protons and ligands was found more effective than protonation alone (Gadd 2007). The process of heterotrophic leaching had been first studied from a biotechnological perspective (Burgstaller and Schinner 1993), before it was recognized as an important ecosystem process. Many minerals contain both nutrients and potentially toxic metal elements. Weathering, whether mediated by ECMF or not, is likely to increase the bioavailability of toxic metals. Al, the most abundant metal element in the earth crust, is toxic when present as free ion. Acidity increases Al availability but may reduce Al toxicity. Dicarboxylic and tricarboxylic acids such as oxalic acid and citric acid are involved in both weathering and detoxification. Complexation with citric acid (Tahara et al. 2005) and precipitation with oxalic acid are effective means of Al detoxification (Gadd 2007).

(Fomina et al. 2005) inferred metal tolerance of ECM fungi from growth rates in metal-spiked growth media and found that tolerant strains solubilized metal-containing minerals more effectively. Opposing results were obtained in an experiment with the ericoid mycorrhizal fungus *Oidiodendron maius*. Metal-tolerant strains from polluted soils did hardly respond to ZnO and $Zn_3(PO_4)_2$ by ligand excretion, while strains from unpolluted habitats increased ligand excretion and Zn solubilization (Martino et al. 2003). The lack of responsiveness to Zn minerals in metal-tolerant *Oidiodendron* is possibly a strategy to save organic carbon, since low-molecular weight organic acid (LMWOA) excretion can constitute a major carbon sink (van Hees et al. 2005). Gibson and Mitchell (2004) confirmed the Glucose dependency of Zn phosphate solubilization in *Hymenoscyphus ericae*.

11.2.3 Litter Decomposition

Litter is a major source of nutrients in forest ecosystems and litter decomposition is essential in nutrient cycling, particularly in ecosystems that are less

herbivore-driven. Potentially toxic elements may accumulate in tree foliage and litter in several ways: through uptake from the soil solution via the xylem mass flow, through atmospheric deposition onto the tree canopies, or via translocation of elements from the soil solution to the decomposing litter. Atmospheric deposition is the main route for anthropogenically mobilized metal elements to enter forest ecosystems, with tree canopies being important scavengers of pollutants. The process of litter decomposition involving saprotrophic fungi, bacteria and soil arthropods results in changes of metal speciation, availability and concentration. ECM fungi potentially compete with saprotrophic fungi for nutrients released from decomposed litter and have been reported to partially inhibit litter decomposition (Gadgil and Gadgil 1971; Koide and Wu 2003). Lindahl et al. (2001) analysed the transfer of ^{32}P -labelled P in interactions between saprotrophic and ECM mycelia. The direction and quantity of P transfer was influenced by resource availability. Recent studies by Lindahl et al. (2007) demonstrated a spatial separation of litter decomposition and ECM nitrogen uptake. Saprotrophic fungi were essentially confined to more recently (<4 years) shed litter. Organic carbon was mineralized during decomposition, while N was retained, most likely within fungal mycelia. The dwindling source of organic C as expressed by a decreasing C:N ratio was hypothesized to favour the succession of ECM fungi.

Litter decomposition can be a long-lasting process, particularly in temperature- and nutrient-limited environments. Brun et al. (2008) measured element contents in conifer litter bags exposed on forest soils for as long as 8 years and recorded different types of evolution of mass-normalized concentrations in different groups of elements. Nutrient elements tended to decrease, along with the unessential or toxic elements Rb, Sc, Sr and Tl. The concentrations of most unessential and potentially toxic elements increased during decay. The concentrations of Cd, Hf, Hg, Ta and Zr initially increased, followed by a net decrease. The anthropogenic component of atmospheric deposition resulted in elevated concentrations of Pb, Cd and Hg.

11.2.4 Metal Element Cycling and Translocation by ECMF

ECMF are considered highly efficient recyclers of mineral nutrients, which minimize element losses through leaching. Very high affinities to certain nutrients can result in the uptake of similar non-essential elements, for example Rb and Cs that are accumulated by certain ECMF along with K. High bioconcentration factors (BCFs) for certain non-essential trace elements are a side effect of high affinity essential element capture. The efficiency of ECM element recycling can be illustrated by the observation that radioactive Cs has hardly been leached from forest soils more than 20 years after the fall out impact caused by the accident in the nuclear power plant in Chernobyl (Dighton et al. 2008). The ability of ECM fungi to capture cationic nutrient elements and to limit losses due to leaching is particularly

important when the retention capacity of the soil matrix is low and when climatic conditions enhance the leaching potential.

Fungal translocation of metal elements might explain some surprising results from both weathering and litter decomposition studies. Budget studies in boreal podzols indicated an upward transport of Al and Fe from mineral soil to the organic soil layer, a phenomenon that cannot be explained by passive leaching. Smits and Hoffland (2009) tested the hypothesis that fungal mycelia in the E horizon connected to ECM roots in the O horizon might be responsible for the uplift of Al. A two-compartment *in vitro* experiment demonstrated that *Rhizopogon roseolus* and one isolate of *Paxillus involus* transported Al, while *Piloderma croceum*, *Hebeloma longicaudum* and *Suillus bovinus* did not. Ga, an element with similar properties as Al, was used as a tracer in a pot experiment with *Pinus sylvestris* seedlings planted into a reconstructed podzol. Ga uptake by *Pinus sylvestris* seedlings was largest when naturally present ECMF mycelia but not the tree roots had access to the compartment with Ga added. Comparisons between hyphal transfers of Ga and Al suggested that Ga is a good proxy for Al.

Scheid et al. (2009) compared accumulation and solubility of metals during the decomposition of leaf litter harvested from trees grown in non-polluted and polluted sites and exposed in non-polluted and polluted soils. Decomposition rates were not changed by elevated metal contents, suggesting that the function of decomposer communities was not affected. Leaves from trees grown on polluted soil had larger initial Zn, Cd and dissolved organic carbon concentrations. Total metal contents increased during decomposition in polluted soils. The solubility of metals decreased over time, indicating that litter acted as a temporal sink for metals from the soil. The sorbed metals were strongly bound in the litter 2 years after decomposition. The significant rise of metal contents in uncontaminated litter exposed on polluted soil documents the tight communication of soil and decomposing litter in interchanging elements. The agents of decomposition and metal translocation were not identified in this study, but it is well established that saprotrophic fungi have a primordial role in the earlier phases of litter decomposition, while ECMF take over in later phases.

11.2.5 Relevance of Fungal Soil Metal Transformations

Manifold experiments have demonstrated the potential of ECMF to dissolve nutrient-containing minerals by means of mechanical and chemical attack. Weathering by ECMF is fuelled by host tree organic carbon, weathering agents such as LMWOAs are important sinks of reduced carbon (van Hees et al. 2005). The relative proportion of ECMF weathering is still a matter of controversy. Earlier estimations that ECMF contributed to mineral weathering in a podzol via tunnelling alone by 50% (van Breemen et al. 2000) were probably far too high. Smits et al. (2005) quantified the contribution of ECM mineral tunnelling to total weathering by image analysis of soil thin sections and concluded that this process accounted for less than 0.5% of total

feldspar weathering in the upper 2 cm of the mineral horizon of the investigated soil. Surface mineral weathering by ECM fungi was suggested to be quantitatively much more important than tunnelling (Smits et al. 2005). Pot experiments demonstrated that ECM weathering by exudation of LMWOAs can be upregulated under deficiency conditions, this regulatory potential might increase the relevance of ECMF weathering for forest health (van Schöll et al. 2008).

Anthropogenic trace metal contaminations are likely to be metabolized by ECM forest soils in similar ways like naturally occurring metal species, as long as the impact on the forest ecosystem does not result in disruption of ecosystem functions. Berthelsen et al. (1995) investigated a forest in southern Norway that had been submitted to long-term heavy metal emissions. They concluded that nearly all Cu in the upper humus layer might be contained in ECM structures, for Zn and Cd the respective proportions were between 30 and 40%. Only 2% of Pb was found associated with fungal biomass. Importantly, this study included morphotype-based identifications of the ECMF and linked the contribution of individual ECMF species to soil trace metal budgets. The considerable biomass and the larger surface: biomass ratio of ECMF mycelia compared to host root systems may explain the significant metal-binding capacity of ECMF. Most quantitative information about adsorption of trace metals to fungal mycelia was obtained in wastewater treatment studies. High-affinity adsorption of certain trace metals to the mycelia of fast growing saprotrophic fungi was found by Kapoor and Viraraghavan (1997). In ECMF, the adsorption potential is potentially limited by the availability of sorption sites and might be rapidly saturated in metalliferous soils (Godbold et al. 1998). Precipitation of trace metals on or near fungal hyphae, typically as oxalates, is not limited by hyphal surface area.

11.3 Metal Tolerance of ECM Associations

11.3.1 Metal Toxicity

Metal toxicity can interfere with the essential physiological and reproductive processes in fungi (Gadd 1993; Amir and Pineau 1998). Metal toxicity-based antimycotica illustrates the relevance of fungal metal sensitivity. Cu(II)sulphate, a fungicide still used in viticulture, is effective in controlling plant parasitic fungi at concentrations which are not toxic to the host plant.

Metal toxicity depends largely on speciation. Free metal ions, oxyanions and certain organic metal compounds such as methylated Hg are particularly toxic (Gadd 2007).

Mechanisms of metal toxicity comprise the elicitation of oxidative stress (even in non-redox-active metals such as Cd), depletion of antioxidant pools, competitive inhibition of the uptake of essential elements, denaturation of proteins, interference with functional groups of proteins by displacement of essential cationic cofactors, precipitation of P inducing P deficiency and membrane disruption. Cytoplasmatic

metal homeostasis is an essential cellular function given the dual role of many trace metals as nutrients and potential toxicants.

11.3.2 Mechanisms of Metal Tolerance in ECMF

Metal tolerance may be defined as the genetically conditioned ability to grow and reproduce in environments with high concentrations of potentially toxic metals (Hartley et al. 1997). A metal-tolerant organism must be able to maintain metal homeostasis in the presence of high metal concentrations, controlling the concentrations of free metal ions in the cytosol. A multitude of mechanisms were proposed to contribute to metal tolerance in ECMF (Hartley et al. 1997; Leyval et al. 1997). Avoidance mechanisms reduce the exposure of ECMF cells to toxic metals and limit metal entry into the cell (Gadd 1993; Hartley et al. 1997). Avoidance mechanisms include the extracellular biochemical transformations of metals discussed above and the regulation of metal uptake: extracellular ligation of metal ions to di- and tricarboxylic acids and chelation with siderophores; extracellular metal immobilization by adsorption to cell walls, pigments and extracellular polysaccharides (fungal slimes); extracellular precipitation as oxalates; restriction of net metal uptake by reduced influx or increased efflux, through changes in the activity or specificity of metal transport channels (Bellion et al. 2006).

Cytoplasmatic and vacuolar sequestration of metals reduces the concentration of free ions in the cytosol. Mechanism of cellular sequestration and detoxification of metals comprise cytoplasmatic chelation by thiols (metallothioneins, glutathione and similar oligopeptides) and metal sequestration in the vacuole. Metal coordination within cells of ECMF was recently analysed by Fomina et al. (2007).

Increased production of cellular redox buffers such as glutathione and of the enzyme superoxide dismutase protects the cells from metal-induced oxidative damage, adding another line of defence against metal toxicity (Ott et al. 2002; Bellion et al. 2006).

The basic mechanisms of cytoplasmatic metal homeostasis used by fungi are shared with other eukaryotes. Studies in model organisms indicate that more cellular functions and molecules are involved in fungal metal tolerance. Kennedy et al. (2008) found many genes involved in Cd tolerance through a screen of knockout mutants of *Schizosaccharomyces pombe*. Their results suggested inter alia an involvement of coenzyme Q₁₀ (ubiquinone) in Cd tolerance.

Most metal tolerance mechanisms imply metabolic costs: oxalate and exopolysaccharide production requires organic carbon and increased efflux by metal transport channels is energy dependent. Intracellular metal chelation with metallothioneins and glutathione requires considerable amounts of cysteine. Under acute metal stress, other cysteine-requiring cellular activities such as hydrophobin synthesis are down-regulated in support of intracellular thiol levels (Bellion et al. 2006).

Metal adsorption to cell walls does not require additional synthetic effort but might be of limited relevance due to rapid saturation of potential sorption sites in

highly metalliferous soils (Jentschke and Godbold 2000). Down-regulation of the expression of metal influx channels would save metabolic energy and might be the most economic adaptation to constantly increased substrate metal concentrations. The evolution of higher specificity of metal influx channels might improve the discrimination of non-essential and essential elements, but evidence for such a process in ECMF is not yet available.

Hartley et al. (1997) and Meharg (2003) pointed out that ECM and ERM fungi share a long evolutionary history of exposition to toxic metal concentrations, since ECM forests with understoreys of ERM plants cover huge areas with highly acidic soils, and soil acidity increases the concentration of free ions of Al, Fe and Mn. Mechanisms of Al detoxification and of Fe and Mn homeostasis were supposed to confer cotolerance to other metal elements. More particularly, sites with geogenically elevated levels or toxic trace metals are potential hotspots for the evolution of tolerance of certain trace metals. Serpentine soils, the most widespread type of geogenically trace metal enriched substrate, are characterized by elevated levels of Mn, Ni and Cr (Urban et al. 2008; Moser et al. 2008). Other types of metalliferous outcrops are relatively rare and more restricted in surface.

The weathering of soil minerals by certain ECM fungi is considered important for the acquisition of essential cationic elements (van Schöll et al. 2008). By fungal attack on rock, potentially toxic metal ions can be liberated too, depending on the chemical composition of the rock material. Similar mechanisms are involved in ECM weathering and in the detoxification of metal ions by fungi, most importantly the exudation of LMWOAs. Citrate is the main ligand of Al³⁺ in podzolized forest soils (Landeweert et al. 2001) and oxalic acid is the main component of mycogenic precipitates of various metals (Gadd 2007). Extracellular ligation and precipitation, two essential mechanisms of avoidance of metal toxicity, may thus be an evolutionary by-product of the involvement of ECMF in mineral transformations driven by nutrient foraging.

Hartley et al. (1997) and Meharg (2003) hypothesized that cotolerance against several metals was likely to occur and would facilitate the evolution of metal tolerance. This argument may apply at least to very common mechanisms involving simple molecules, for example the up-regulation of oxalic acid exudation. On the other hand, it was frequently observed that increased metal tolerance in ECMF is metal specific, and that specificity in metal tolerance and local metal exposition can be correlated. Metal tolerance in *Suillus* spp. was found strain and metal specific and could be linked to the respective histories of metal exposure (Adriaensen et al. 2005; Krznaric et al. 2009). Recently, it could be demonstrated that metallothioneins of the ECMF *Paxillus involutus* and *Hebeloma cylindrosporum* and the regulation of metallothionein gene expression are metal specific (Bellion et al. 2007; Ramesh et al. 2009). More information on specific mechanisms of metal homeostasis is available for model organisms. A Cd regulated Cd efflux system based on a P1B-type ATPase (PCA1) was recently reported from *Saccharomyces cerevisiae* (Adele et al. 2006). Lin et al. (2008) discovered that a single amino acid change in the vacuolar Zn transporter ZRC1 changed the substrate specificity of the transporter from Zn to Fe.

11.3.3 Protection of Host Trees

Schramm (1966) investigated plant colonization of barren coal mine spoils in Pennsylvania, a highly acidic substrate with toxic metal concentrations. He observed that both planted and spontaneously established trees (*Pinus* spp., *Quercus borealis*, *Betula populifolia*) were consistently associated with ECMF. Fruit bodies of *Pisolithus tinctorius*, *Thelephora terrestris*, *T. caryophyllea*, *Astraeus hygrometricus* and *Inocybe* sp. appeared near young trees established on barren substrate. The majority of spontaneously established pine seedlings were mycorrhized, despite apparent limitation of ECM inoculum. The non-mycorrhized seedlings were reported as stunted and chlorotic, in contrast to the mycorrhized seedlings. Ever since it was observed many times that trees mycorrhized spontaneously or inoculated with appropriate ECMF species resist much better to extreme soil conditions, including metal toxicity. Trees were considered to tolerate metal contamination by means of phenotypic plasticity and ECM symbiosis. It was hypothesized that the presumably shorter life cycles of ECM fungi would open up more opportunity for genetic adaptation. Adaptive genetic change in ECM communities was considered essential for the understanding of the survival of ECM tree species challenged with metal toxicity (Wilkinson and Dickinson 1995). This hypothesis raised numerous questions, some of them being still under discussion.

11.3.4 Are ECMF More Resistant to Toxic Metals Than Their Host Trees?

Hartley et al. (1997) reviewed available data and concluded that a wide range of metal sensitivity can be found in both trees and ECMF, but upper tolerance limits appear to be far lower in the tree species. Growth-inhibiting Cd concentrations range from 0.3 to 30 μM for non-mycorrhized trees and 0.1 to 90 μM for ECM fungi in liquid media. In case of Pb exposition, the respective values were 0.5–230 μM for trees and 125–960 μM for ECMF in liquid media. Hartley et al. (1997) attributed the higher adaptability of ECMF as expressed by higher upper limits of metal resistance to the higher diversity of ECMF compared to ECM trees, and not to the presumably shorter fungal generation cycles (Wilkinson and Dickinson 1995).

11.3.5 Can Metal-Resistant ECMF Confer Resistance to Their Host Trees?

From a co-evolutionary point of view, it seems obvious that a metal-resistant ECMF which supplies mineral nutrients to its host and which depends on organic

carbon obtained from the host would benefit more if it alleviates metal stress in its host tree, with other words, selection is likely to favour host protection by ECMF, especially in pioneer populations, where only one tree individual might be available as carbon source. However, there is some experimental evidence that the most resistant ECMF species is not necessarily the most protective one (Jones and Hutchinson 1986). Godbold et al. (1998) concluded that only in a small number of experiments, amelioration of metal toxicity could be demonstrated, and that this was the case for specific metals and certain fungi only. The statement that amelioration of metal toxicity is highly species, strain and metal specific is still valid. However, it has to be considered that many earlier experiments with negative results had used ECMF from unpolluted sites, while amelioration of metal toxicity under experimental conditions had been recorded most often when fungi from metalliferous were used. Later studies provided unequivocal evidence that certain ECMF protect their host trees highly efficiently against specific metals (Adriaensen et al. 2004, 2005, 2006; Krznanic et al. 2009). Given recent evidence that ECMF communities in highly metalliferous soils can be surprisingly diverse, the question arises, if all those metal-tolerant ECMF have a similarly beneficial effect on their host trees. (Colpaert and van Assche 1993) inferred from experimental results in a semi-hydroponic system that species with abundant mycelia have the most beneficial effect. Field observations suggest that species with abundant mycelia such as *Suillus* spp. can be highly metal tolerant and beneficial for their host trees (Colpaert 2008). However, if ECM trees are competent of rewarding the most beneficial ECMF through selective organic carbon allocation, the diversity of metal tolerant ECMF might be per se beneficial.

ECM-associated microbes are likely to be important, too. Coinoculation with the ECM-associated bacterium *Pseudomonas putida* improved the growth promoting effect of *Amanita rubescens* on *Pinus sylvestris* exposed to Cd (Kozdroj et al. 2007).

11.3.6 ECMF and Host Tree Nutrient Status and Metal Uptake

Many metal polluted sites are poor in essential nutrients, and toxic metals can interfere with the uptake of essential nutrients. It is not easy to disentangle the beneficial effects of improved access to nutrients and/or reduced metal uptake. If revegetation of a devastated area is the first goal, this distinction might seem secondary. If metal cycles and the potential contamination of food webs or applications such as phytoextraction are considered, the quantity of metal uptake is of high practical relevance. Experimental results on the transfer of metal elements to trees via ECM fungi are manifold, but in many cases the toxic element filter hypothesis (Turnau et al. 1996) might be applicable. ECM fungi can reduce levels of available metals in soil by precipitation and by binding to organic compounds (Huang 2008), they can control symplastic metal transfers and cell wall components with high-metal affinity are likely to reduce apoplastic transport to the fine root. A clear

amelioration of Cd toxicity in *Picea abies* seedlings by *Paxillus involutus* was found (Godbold et al. 1998), but Cd uptake was not decreased. Shoot metal concentrations are not necessarily reduced due to ECM colonization, effects on total shoot metal contents can be very variable. Ahonen-Jonnarth and Finlay (2001) observed a positive growth response of Ni and Cd exposed *Pinus sylvestris* seedling upon inoculation with *Laccaria bicolor*. Shoot metal concentrations were not affected, resulting in enhanced total metal uptake. In accumulating tree species, evidence for an ECM filtering effect may be lacking too (Krpata et al. 2009). Again, the metal exposition histories of both the ECMF and the host tree are essential to interpret experimental results.

11.3.7 Can ECM Symbiosis Confer Resistance to Sensitive Host Tree Genotypes?

The role of host sensitivity in the success of ECM associations was rarely investigated. Brown and Wilkins (1985) found increased Zn tolerance due to ECM inoculation in both tolerant and non-tolerant *Betula*. The translocation of Zn to the shoots of *Betula* was reduced, but Zn accumulated in the ECM. The differences of tolerance of the trees as expressed in growth rate and the respective limitations of leaf Zn concentrations were largely maintained. Adaptive tolerance of metal toxicity was suggested to occur in populations of *Pinus ponderosa* (Wright 2007) and *P. balfouriana* (Oline et al. 2000) growing in serpentine soils, but the potential role of ECMF was not assessed. Kayama et al. (2006) observed significantly reduced ECM colonization in non-tolerant *Picea abies* planted into serpentine soil, while ECM colonization was not decreased in serpentine adapted *Picea glehnii*. Two alternative hypotheses are proposed here to be tested in future studies: (a) metal uptake exceeds a critical threshold despite ECM colonization due to the low tolerance of non-adapted trees; (b) the naive, non-adapted host tree fails to select the most beneficial, toxic metal filtering ECMF via selective carbon allocation. In both cases, the metal sensitive tree will fail to grow normally despite nutrients offered by metal tolerant ECMF, the consequent reduction of photosynthesis and carbon supply will reduce colonization intensity and growth of fungal mycelia and destabilize the symbiosis.

11.4 Population Genetics of Adaptive Metal Tolerance in ECMF

Metal-contaminated soils are an attractive model system to investigate environment-driven population genetic processes, and important new insights into the microevolution of adaptive metal tolerance were reported for a few ECM model species. In subpopulations of the ECM basidiomycete *Suillus luteus* growing in Zn polluted

soils, considerable genetic diversity was found and no reduction of genetic diversity compared to control populations could be detected using AFLP (Muller et al. 2004) and microsatellite (Muller et al. 2007) population markers. In contrast to a priori expectations, there was no evidence for clustering of subpopulations from polluted vs. unpolluted sites, despite significant differences in metal tolerance. It was concluded that metal pollution had a limited effect on the genetic structure of *S. luteus* populations, and that extensive gene flow and a high frequency of sexual reproduction allowed rapid evolution of tolerance while maintaining high levels of genetic diversity (Muller et al. 2007).

Adaption to Ni toxicity in naturally metalliferous soils was demonstrated in the ECM ascomycete *Cenococcum geophilum* (Gonçalves et al. 2009). Mean in vitro 50% growth-inhibiting concentrations of Ni were about seven times higher in isolates from serpentine (23.4 µg/ml) than in control isolates (3.38 µg/ml). Furthermore, a marginally significant ($P = 0.06$) trend towards a negative correlation between Ni tolerance and growth rates in non-toxic conditions was found. This trade-off had been postulated earlier (Hartley et al. 1997) in order to explain why tolerance of metal toxicity fails to become a frequent trait in non-exposed populations. Moderate costs of metal tolerance are compatible with the observation of considerable variation in metal tolerance in non-exposed populations (Colpaert 2008). In contrast to the results of Gonçalves et al. (2009), Colpaert et al. (2005) found no reduction of growth rates at low Zn levels linked to reduced Zn uptake in Zn-tolerant strains of *Suillus* spp. However, in vitro experiments can at best partially reproduce selective forces in ECM symbioses in natural environments.

Results concerning the population genetics of serpentine colonizing *C. geophilum* are rather contradictory. Panaccione et al. (2001) detected genetic divergence between *C. geophilum* from serpentine and from control sites, while Gonçalves et al. (2007) found no differences linked to serpentine, but this result might be due to a limited sample size. Furthermore, Douhan et al. (2007) reconfirmed that *C. geophilum* s.l. is a species complex and recommended caution when conducting population genetic studies in *C. geophilum* due to the risk of comparing unrelated isolates.

The above-mentioned results suggest that in both anthropogenically and geogenically metal-contaminated soils, tolerance of very high levels of toxic metals can be acquired by adaptive evolution, with or without high rates of genetic exchange with non-exposed populations. It is not clear if adaptive evolution of metal tolerance is widespread among ECMF. Strains of *Paxillus involutus* collected from Zn polluted sites were as Zn sensitive as control strains (Colpaert 2008). In certain ECMF species, constitutive levels of metal tolerance seemingly suffice to survive and compete in contaminated sites, while in others the frequently observed variation of metal tolerance in non-exposed populations may be the base for rapid selection of highly metal-tolerant genotypes.

Adaptive evolution of metal tolerance might be compatible with different population genetic patterns, respectively, reproductive systems and life history traits. *Suillus luteus* is a panmictic, sexually reproducing ECMF, *C. geophilum* is a complex of cryptic species possibly lacking a sexual state. Population genetics

might rather be conditioned by life cycle traits than by metal stress. The probability of evolving distinct, specialized genotypes and genetically isolated populations might rather be determined by the structure of reproductive systems than by the nature and intensity of environmental selection pressure. At present, there is no evidence for serpentine driven speciation in ECMF (Urban et al. 2008) and the genetic structure of metal-tolerant *Suillus luteus* is similar like in pseudo-metallophytes (Colpaert 2008).

11.5 Distribution of Metal Elements in ECMF

Most studies on the elemental composition of ECMF were based on sporophores, motivated by the interest in nutritional value and ecotoxicological concerns, since wild edible fungi are an important component of human diets in many parts of the world. Earlier studies on metal contents in macrofungi were reviewed by Kalač and Svoboda (2000). They summarized that BCFs in wild edible mushrooms were found high for Cd (50–300), which is probably the most problematic element in mushrooms, and Hg (30–500) but low for Pb (10^{-2} – 10^{-1}). Melgar et al. (2009) confirmed that all fungal species investigated accumulated Hg (BCF > 1). Highest values were found in ECM *Boletus pinophilus* and *B. aereus* and in saprotrophic *Agaricus macrosporus* and *Lepista nuda* (mean BCF between 300 and 450 in the hymenophore). Other ECM species had generally lower BCF values than saprotrophs.

Borovička and Řanda (2007) found Fe accumulation in *Hygrophoropsis aurantiaca* and Zn accumulation in ECM *Russula atropurpurea*. Generally, lower Se accumulation was found in checked ECMF compared to saprotrophs, but some of the highest Se concentrations were recorded in ECMF (*Boletus edulis*, *Boletus pinophilus*, *Amanita strobiliformis*, *Albatrellus pes-caprae*).

Metal hyperaccumulation by fungi was rarely reported. Stijve et al. (1990) measured As hyperaccumulation in *Sarcosphaera coronaria* (100–7,000 ppm) and Borovička et al. (2007) reported Ag hyperaccumulation in *Amanita strobiliformis* (mostly 200–700 mg/kg, highest value 1,253; mg/kg; BCF 800–2,500). Ag is microbicidal at low concentrations. Very high BCFs (about 1,000) were reported for the K analogue Rb in *Suillus grevillei* (Chudzynski and Falandysz 2008).

Bioavailability, nutritional value and toxicity of metals and metalloids in ECMF depend on speciation. Slejkovec et al. (1997) found some relation between As speciation and phylogenetic relationships in mushrooms. Mutanen (1986) reported low bioavailability of fungal Se. The metalloid Se is of interest due to frequent Se deficiency of human nutrition. Serafín Muñoz et al. (2006) suggested that a major part of Se in *Pleurotus ostreatus* is bound to the cell wall component chitin. Serafín Muñoz et al. (2007) demonstrated a speciation-dependent protective role of Se against oxidative damage induced by Cd and Ag in liquid cultures of *Pleurotus ostreatus*.

Krpata et al. (2009) compared Zn and Cd concentrations in fruit bodies of ECM fungi and in leaves of their host trees, metal-accumulating *Populus tremula*, in

locations highly contaminated by Pb/Zn smelting. BCFs were based on total metal concentrations in the mor-type organic layer (BCF_{tot}) or on NH_4NO_3 -extractable metal concentrations in mineral soil (BCF_{lab}). When plotted on log–log scale, a linear model described well the decrease of BCFs with increasing soil metal concentrations. A better correlation was found for BCF_{lab} than for BCF_{tot} . The observation of decreasing BCFs with increasing substrate metal concentrations is not uncommon in fungi (Gast et al. 1988) and plants (Langer et al. 2009). Differences between fungal genera were found in Zn-BCFs but not in Cd-BCFs. *Tricholoma scalpturatum* (762 ppm), *Scleroderma verrucosum* (598–777 ppm) and *Amanita vaginata* (403–571 ppm) had highest Zn concentrations, *Laccaria laccata* (12.3–93.3 ppm) and *Amanita vaginata* (10.1–48.5 ppm) had highest Cd values. Concentrations of Zn were in the range reported for fungal fruit bodies, Cd concentrations were highly elevated. Cd levels above 10 ppm are typically found in contaminated sites (Gast et al. 1988; Svoboda et al. 2006).

Accumulation and BCFs of Zn and Cd in the host trees were of the same order of magnitude as in the ECM fungi. Studies on metal element concentrations in sporophores demonstrated differences in metal affinities between various fungal species and strains and high metal specificity in fungal BCFs. Fungi growing in substrates with excessive metal concentrations usually have drastically reduced BCFs, probably a result of physiological control of metal uptake. The relative contributions of phenotypic plasticity and population genetic factors to the control of metal uptake rates as expressed by BCFs may vary according to the species concerned. Colpaert et al. (2005) found reduced Zn uptake in Zn-tolerant strains of *Suillus* spp. at low and high Zn concentrations and concluded that partial Zn exclusion contributed most to Zn tolerance. Zn tolerance as expressed by reduced Zn accumulation was specific, the concentrations of other micronutrients were not affected. Despite reduced Zn uptake in tolerant strains, the Zn concentrations in the mycelia of all treatments were very high, reaching up to 15.57 mg/g, a concentration representative of plant hyper-accumulators. Turnau et al. (2001) used micro-proton-induced X-ray emission (PIXE) true elemental maps to quantify metals in cryo-fixed *S. luteus* mycorrhizas collected from Zn wastes. They found similarly elevated Zn concentration in *Suillus* rhizomorphs, in average 12.83 mg/g. Colpaert and van Assche (1992) detected high concentrations of Zn in *Suillus* ECM grown in Zn-spiked substrate, while transfer to the host plant remained low. Representative Zn concentrations in *Suillus* sporophores are 30–150 mg/kg (Kalač and Svoboda 2000), a value about 100 times lower than in *Suillus* mycelia. Studies on metal concentrations in environmental samples of ECM mycelia are still scarce, despite growing awareness of their ecological significance (Finlay 2008). Wallander et al. (2003) investigated the elemental composition of ECM mycelia grown in contact with wood ash or apatite in forest soil. They measured high K accumulation by mycelium of *Suillus granulatus* and high concentrations of Ca, Ti, Mn and Pb in *Paxillus involutus* rhizomorphs. *Piloderma croceum* appears to accumulate and translocate Ca, an element that is scarce in podzols (Blum et al. 2002; Hagerberg et al. 2005).

11.6 Transfer of Trace Metals to Vertebrate Food Webs via ECMF

ECM fungi are a part of terrestrial food webs and of various pathways of human exposure to soil borne contaminants. Apart from direct use of fungi as food, the consumption of partly fungivorous game may be a major source of contaminant exposure in some populations.

The knowledge about heavy metal transfers via food webs to game is still fragmentary, despite concerns about elevated heavy metal concentrations in various species of game.

Pokorny et al. (2004) found a correlation between amounts of fungal spores and Hg levels in roe deer (*Capreolus capreolus*) faeces collected in a moderately polluted area in Slovenia. Fungal spores were present in 89% of all faecal samples. Following fungal genera were reported: *Lycoperdon*, *Calvatia*, *Hypholoma*, *Coprinus*, *Russula*, *Elaphomyces*, *Xerocomus*, *Entoloma*, *Amanita*, *Cortinarius*, *Agaricus*, *Inocybe*, *Boletus*, *Macrolepiota*, *Suillus* and *Pluteus*. Analyses of heavy metal concentrations in fungal sporophores and in important food plants of roe deer showed that Hg and As concentrations were in average by about two to three orders of magnitude higher in fungi, and Cd concentrations were elevated by about one order of magnitude. Pb was accumulated in saprotrophic puffballs (*Lycoperdon perlatum*, *Calvatia utriformis*) only, but these species accounted for a major part of the spores found in roe deer faeces. These results explained well the late aestival and autumnal increase of Hg observed in roe deer kidneys. Surprisingly, no parallel increase of As levels was found. This might indicate lower bioavailability of fungal As, or higher relevance of other As sources than those covered by this study.

Cs transfer from soil to roe deer was suggested by Kiefer et al. (1996), based on circumstantial evidence such as elevated Cs concentrations in *Xerocomus badius*, annual variations of roe deer Cs contamination and correlations with precipitation. Hohmann and Huckschlag (2005) investigated the pathways of ^{137}Cs contamination in wild boars in Rhineland-Palatinate, Germany. They found deer truffles (*Elaphomyces granulatus*) in significantly higher proportions (average weight proportion 15.3%) in highly contaminated stomach contents (345–1,749 Bq/kg) and in lower proportions (average weight proportion 1.6%) in less contaminated stomach contents (≤ 20 –199 Bq/kg, median ≤ 20 Bq/kg). ^{137}Cs activity concentrations in *Elaphomyces granulatus* were in average 6,030 Bq/kg (800–18,800 Bq/kg). These results explain why ^{137}Cs concentrations in wild boar meat remain high in many regions more than 20 years after the fallout of the Chernobyl nuclear accident. ECM fungi play a paramount role in the long-term recycling and retention of highly leachable ^{137}Cs in soil organic matter and as a gateway of this radioactive element to vertebrate food webs. Top predators such as *Lynx lynx* may accumulate extremely high concentrations of ^{137}Cs , up to 125,000 Bq/kg, depending on food choice (Åhman et al. 2004).

11.7 Diversity and Structure of ECM Communities Exposed to Metal Toxicity

Decreases in species diversity of ECMF are a common result of metal pollution, suggesting a selective elimination of more sensitive fungi (Gadd 1993; Godbold et al. 1998). Field studies on sporophore production along heavy-metal gradients in north and south Sweden found strong evidence for decreasing ECM diversity and productivity at elevated toxic metal concentrations. Most ECM species were found to decrease with increasing metal (Cu, Zn) concentration, for example *Cantharellus cibarius*, *Cortinarius* spp., *Gomphidius* spp., *Lactarius* spp. and *Russula* spp. Among the more tolerant taxa were *Albatrellus ovinus*, *Amanita* spp., *Cantharellus tubaeformis*, *Laccaria laccata*, *Leccinum* spp. (Rühling et al. 1984; Rühling and Söderström 1990). Colpaert (2008) reported that only four morphotypes of *Pinus sylvestris* ECM were detected in the most polluted area along the Zn pollution gradient in northern Limburg, Belgium. A dark ascomycete from the *Rhizoscyphus ericae* aggregate was a frequent mycorrhiza former in the most polluted plots only, confirming earlier observations by Vralstad et al. (2002).

Recent studies provided deeper insights into the below-ground diversity of ECMF in geogenically and anthropogenically metal enriched soils. Mleczko (2004) described 14 ECM morphotypes associated with *Pinus sylvestris* and *Betula pendula* growing on Zn wastes in southern Poland. Staudenrausch et al. (2005) analysed the ECM community of Birch regenerating on the wastes of a uranium mine in Thuringia, Germany. Twenty-three ECM morphotypes were distinguished, 14 of them were identified by ITS sequence analysis. Total ECM colonization and ECM diversity were lowest on a mine heap without organic layer, intermediate on mine wastes with organic layer and highest in a reference site nearby. All ECMF dominating on the mine heap were present in the reference site, indicating that fungi with high resistance against U toxicity and other stress factors associated with barren substrate had been selected from the local ECM community. Urban et al. (2008) identified 18 different ECMF from eight fungal orders associated with *Pinus sylvestris* in a highly metalliferous serpentine soil. Statistic estimators of species richness indicated the presence of about 30 (Jackknife estimator) to 40 (Michaelis–Menten estimator) ECMF species. Moser et al. (2008) differentiated 18 ECM morphotypes associated with *Quercus garryana*. Fifteen of these morphotypes were found in serpentine soil and 13 in non-serpentine soil. Multivariate analysis detected no differences between the communities. In contrast to the results of Urban et al. (2008), pezizalean hypogeous fungi were frequent and diverse. Brearley (2006) found higher percentage ECM colonization and higher ECM diversity in a pot experiment with *Dryobalanops lanceolata* (Dipterocarpaceae) grown in Ni- and Mg-rich ultra-mafic soil compared to seedlings grown in tropical ultisol. Relatively high diversity of ECMF is not limited to naturally metalliferous serpentine. Hryniewicz et al. (2007) detected 14 different ECM fungi, mainly *Tomentella* spp. and *Cortinariaceae* associated with *Salix caprea* at a heavy metal polluted former ore mining site near Freiburg in Germany. Krpata et al. (2008) differentiated

as many as 54 ECMF associated with *Populus tremula* in a severely heavy metal-contaminated (Pb, Zn, Cd) site. The community was rich in Basidiomycota and dominated by *Cenococcum geophilum* and tomentelloid fungi. The study site had a very long pollution history of about 500 years, and the mining sites were not far away, hence there had been good opportunity for local microevolution of metal tolerance in many species.

The finding of phylogenetically diverse ECM communities in metal enriched soils indicates that the potential of metal tolerance is widespread among ECMF. In plants on the contrary, the potential to evolve increased metal tolerance appears to be limited to few phylogenetic groups, for example Brassicaceae or Poaceae. Plant communities on metalliferous soils such as serpentine are typically limited in diversity but rich in specialized metallophytes. ECMF communities might be highly diverse, but no metal-specific fungi have been found as yet. Patterns of ECM diversity do not seem to agree with patterns of vascular plant diversity in metalliferous soils.

Are there fungal species, genera or larger phylogenetic groups that are excluded by metal toxicity? The representation of major phylogenetic groups in ECM communities can be very different in similar edaphic conditions. Urban et al. (2008) found most major ECMF orders associated with pines and oaks on serpentine, except Pezizales and Gomphales. Moser et al. (2008) found several pezizalean ECMF associated with oaks on serpentine. Possibly, the potential of various species to colonize metalliferous soils is conditioned by the local evolutionary history rather than by genetic predispositions. At present, our knowledge about constitutive and adaptive mechanisms of tolerance is limited to a few well-studied model organisms. It is unknown whether different ECMF species use similar or diverse tolerance mechanisms, and if their population structures are similar. It appears that metal-tolerant ECMF genotypes are typically derived from local species pools (Staudenrausch et al. 2005; Colpaert 2008).

11.8 Biodiversity and Conservation

Geogenically metalliferous sites are potential evolutionary hotspots of metal tolerance traits, and they use to harbour unique organisms and communities. The essential genetic adaptations and innovations that confer metal tolerance are likely to have evolved originally in naturally metal enriched soils (Ernst 2000). The proximity of natural outcrops of ore and historic mining and smelting sites might have facilitated colonization of mine spoils by locally adapted metal-tolerant plants and fungi. Some historic mining and metal processing sites might merit protection, like naturally metalliferous soils, since they can host specialized communities of metal-tolerant plants and fungi.

The effects of widespread background contamination of soils with toxic metals on ECMF biodiversity, community composition and potential species losses are difficult to estimate. It can be inferred from the relatively rapid recovery of ECMF

diversity in historically polluted sites that many species can adapt to high levels of toxic metals. If generic, constitutive mechanisms of metal tolerance suffice to resist moderate levels of metal toxicity, the challenge might not be fatal for most ECM fungal species. Are toxic trace metals involved in the decline of certain rare ECM species such as stipitate hydroids and certain *Tricholoma* species? The enormous potential of ECMF from different phylogenetic groups to adapt to metalliferous soils might suggest that this risk is of minor concern, and that other types of pollution, particularly N emissions, are more important (Arnolds 1991). However, rare fungi with small population sizes might lack the genetic diversity necessary to adapt to metal toxicity. Given the high BCFs of certain fungal species challenged with elements to which they are not adapted, the accumulation of toxic metal concentrations cannot be excluded. The effects of experimental Ni pollution on an ECM community were assessed by Markkola et al. (2002), but the analysis of the ECM community was too coarse to allow conclusions about the effect of low pollution levels on rare fungi. Nevertheless, this study design hints at an important question: what is the fate of biodiversity, when pollution starts? Science usually enters the stage when public awareness and funding are available, for example when the results of pollution restrictions should be evaluated. Most studies on ECMF diversity have assessed effects of historic pollution events, the impact of ongoing pollution due to emerging industrialization in hitherto uncontaminated areas is rarely documented, but such studies would be required to know more about the impact of metal pollution on pristine ECMF communities. The study of sites with a very long or terminated pollution history rather informs about resilience of ECMF communities, long term impacts on nutrient cycling (Jamnická et al. 2007) and microevolutionary processes (Muller et al. 2007).

11.9 Conclusions

The effects of metal inputs to ecosystems far above natural levels are a priori unpredictable. The study of naturally or anthropogenically metal enriched sites, may assist in understanding the fundamental processes of the biogeochemical cycling of potentially toxic metals in ecosystems and in modelling the potential pathways of metal pollutants. The ECM association of trees and fungi is successful in recycling scarce essential metal elements and in colonizing soils with high levels of toxic metals. Uptake and translocation of metal elements by ECMF account for major deviations from simple models of soil metal budgets. Despite considerable conceptual and analytical progress concerning the fundamental biogeochemical and cellular processes, the role of ECM fungi in metal cycling and budgets has only started to be recognized by modellers (Rosling et al. 2009). Only a few studies attempt to link processes to budgets, and the inference of quantitative information about the role of ECMF in trace metal cycles is still largely based on microcosm experiments. New technologies for nano-scale element and isotope analysis may assist in demonstrating the role of ECMF in metal cycles in the field.

ECMF dispose of a variety of extracellular and cellular metal tolerance and homeostasis mechanisms. Some of these mechanisms can confer cotolerance of various metals, while others are metal specific. Metal-adapted ECMF generally provide better host protection than non-adapted strains.

Accumulation of certain essential and non-essential metals and metalloids (e.g. K, Rb, Cs, As, Se, Zn, Cd, Hg, Ag) is common in ECMF, while other elements tend to be excluded (e.g. Al, Pb). BCFs are species, strain and metal specific. A negative correlation between environmental metal concentrations and BCFs appears to be the rule. Metal hyperaccumulation is rare in fruit bodies of ECMF but appears to be rather common in ECM mycelia, which may accumulate a major part of the soil pools of certain trace metals. The role of ECMF as a nutrient source of rare elements (e.g. Se) and as an important gateway of toxic metals to vertebrate foodwebs in polluted areas merits further study.

ECM communities in metalliferous soils are surprisingly diverse, and the diversity of ECMF is likely to be important in alleviating metal toxicity in host trees. The potential to colonize metalliferous soils is widespread in various phylogenetic groups of ECMF. The microevolution of metal tolerance does not require populational differentiation. From a population genetic perspective, metal toxicity is just another environmental gradient exerting selection pressure. Reports on speciation in ECMF driven by metal tolerance are lacking thus far.

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